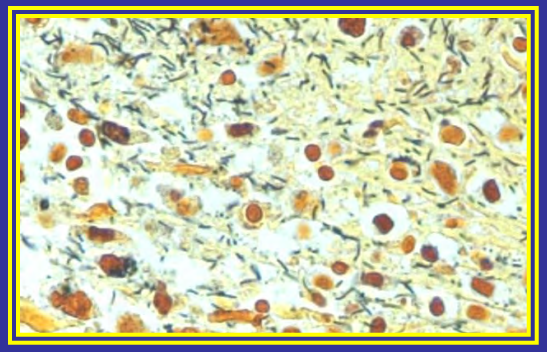
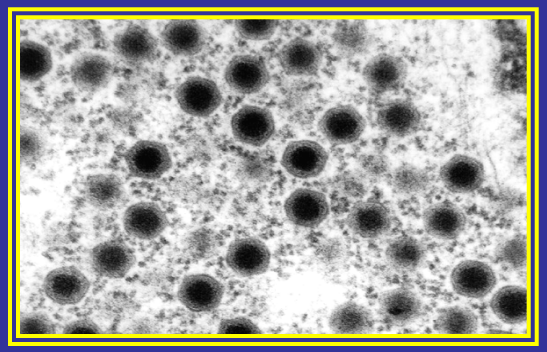








Fish Health Section

BLUE BOOK

2007

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Preface

This new 2007 edition of the American Fisheries Society, Fish Health Section Blue Book contains important updates on Viral Hemorrhagic Septicemia Virus in both the Diagnostic and the Inspection Sections. Information on susceptible species, appropriate cell lines for testing and PCR primers for confirmation have been updated. **The primer sequences for VHSV RT-PCR have been modified compared to those provided in previous editions of this manual. The new sequences now match the OIE (2006) VHSV primers. Also in this edition, the primer sequences for the IHNV RT-PCR assay have been modified from those in previous editions of this manual.** Additionally, the Diagnostic Manual chapter on Infectious Pancreatic Necrosis Virus has been updated and four new chapters including Goldfish Herpesviral Hematopoietic Necrosis Virus, Largemouth Bass Virus Disease, Necrotizing Hepatopancreatitis of Penaeid Shrimp, and Taura Syndrome Virus of Penaeid Shrimp have been added. The shrimp virus chapters are in a new portion of the Diagnostic Section entitled "Diseases of Crustaceans". The date of the most recent revision of each section is at the bottom of each page. **No 2006 Edition of the Blue Book was produced.**

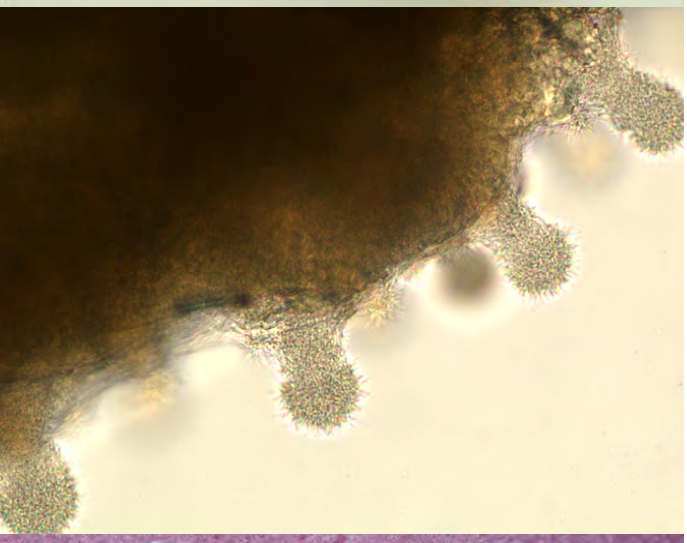
As in the 2005 edition of the AFS-FHS Blue Book, a major feature is the Standard Procedures for Aquatic Animal Health Inspections. This Section is a collaborative effort between the U. S. Fish and Wildlife Service and the Fish Health Section and identifies specific procedures recommended for the detection of certain pathogens of regulatory concern. This Section replaces Chapter 1 of the 4th Edition, and is greatly expanded for the purpose of providing information for persons performing inspections for the inter- and intra-state movement of fish. An identical document also appears in the USFWS Handbook. The USFWS and FHS are committed to an annual review and revision of these procedures according to a formal protocol detailed in the Inspection Section, Appendix 1. While some pathogens are included in both the Inspection and Diagnostic sections, the information provided and the technical approaches are different. The individual pathogen chapters in the Diagnostic Section cover topics like geographic and host range, and epizootiology, which are not included in the inspection chapter. They also describe a wider range of diagnostic methods, recognizing that these methods are used for much broader purposes than inspections, and that there are many pathogens of importance to diagnosticians but that are not often included in formal fish health inspections. You will also note that the 2007 Blue Book has no general editor. The “Citation Guidelines” section has the suggested method for citing chapters in the Blue Book. This format provides the authors with recognition for their efforts and also allows for different publication dates within a volume.

This digital edition of the Blue Book is available as a subscription, somewhat like a journal published annually. This was done because the new inspection portion is required to be updated annually and because this new format provides an opportunity to make timely additions and updates to the rest of the Blue Book. Another great advantage is that it is simple to incorporate more images and even movies, which appear now in two chapters. Subscriptions for the new Blue Book will run for five years, with the understanding that publication options are rapidly changing and we will need to re-evaluate this process during this period.

Production of this 2007 edition required hundreds of volunteer hours contributed by the members of the Inspection Section Committees, by the FHS Technical Standards Committee, and by authors of Diagnostic Chapters. We hope readers and users of this document find it useful and will participate in the process of improving it in the years to come.









Section 1
Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens

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1.1 General Procedures for Bacteriology

Diane G. Elliott

U. S. Geological Survey, Biological Resources Discipline
Western Fisheries Research Center
6505 NE 65th Street
Seattle, WA 98115
206/526-6282
diane_elliott@nbs.gov

I. Sampling and Handling of Samples

For the detection and identification of bacterial pathogens in populations of fish showing disease signs, ideal samples are multiple (five or more) moribund fish or those showing clinical signs typical of the disease outbreak. For the detection of subclinical infections in populations of asymptomatic fish, larger sample numbers may be necessary (see Section 2, 2.2.D "Sample Number"). Fish that are found dead at the time of sampling are not acceptable for bacteriological examination, unless they are known to be very fresh. Contaminating bacteria can grow quickly in dead fish, particularly in warm water. Fish usually are not pooled for testing, although this is sometimes done for certain assays when fish are very small.

The selection of tissue samples for bacteriological assays varies depending on the pathogen suspected; the reader is referred to chapters on specific bacterial diseases. Organs most commonly tested include the kidneys and portions of any organ with visible lesions, although other organs such as the spleen or brain, and fluids such as blood plasma or ovarian fluid, are used for the detection of certain bacterial infections.

Samples for attempted culture of bacteria must be taken aseptically. If samples are to be taken from internal organs, disinfection of the body surface is recommended before incisions are made to expose the organs. Sterile tools must be used for making incisions and removing samples for culture. Samples intended for Gram stains or immunological tests also should be taken aseptically to prevent contamination with bacteria or antigens from extraneous sources. Homogenization of samples prior to culture or immunological testing may enhance the detection of bacteria or their antigens.

If samples cannot be inoculated immediately onto appropriate culture media, they may be stored on ice for up to 24 hours. Samples for culture should not be frozen. For the storage of samples intended for immunological tests, refer to the chapters on specific bacterial diseases.

II. Bacteriological Media and Test Reagents

A. Media

Specialized media for the detection of certain bacteria are referenced in the appropriate chapters. The following media commonly are used for the isolation of fish pathogens:

1. **Trypticase (or Tryptic) Soy Agar (TSA)**
   This is a commonly used medium for routine isolation and culture of many fish pathogens, and it is available from commercial sources.

2. **Brain-Heart Infusion Agar (BHIA)**
   This medium is used for routine isolation and culture of many fish pathogens, and it is available from commercial sources.

3. **Blood Agar (BA)**
   This medium is used for routine isolation and culture of many fish pathogens, and is also used for the visualization of hemolysis (see below). It is available from commercial sources, or can be prepared by the addition of sterile defibrinated blood (usually sheep, horse, or rabbit) at a concentration of 5% (volume/volume) to a sterile base medium such as TSA or BHIA, when the base medium has cooled to 48 to 50^{o}C.

4. **Tryptone Yeast Extract Salts Agar (TYES) Medium (Holt et al. 1989)**
   This medium is used for growth of *Flavobacterium psychrophilum.* This medium is a modification of one reported by Fujihara and Nakatani (1971) for growth of *F. columnare*.

Tryptone (Difco)	0.4%
Yeast extract (Difco)	0.04%
$MgSO_4.7H_2O$	0.05%
$CaCl_2.2H_2O$	0.05%
Agar	1.0%

   Adjust pH to 7.1-7.3.

   TYES plus skim milk agar is a recent modification that has the advantage of observing clearing in the medium around the *F. psychrophilum* colonies. For this medium sterile skim milk is added to TYES agar after the medium is cooled to 45 to 50°C just prior to pouring the plates. One mL of a 20% sterile skim milk solution is added for every 100 mL of TYES agar. The 20% skim milk is prepared by dissolving the skim milk powder in distilled water on a hot plate, then sterilizing the solution in 8 to 10 mL quantities.

5. **Cytophaga Agar**
   This medium (Anacker and Ordal 1959) is used for the isolation of flavobacteria. The formula is:

Tryptone	0.5 g
Yeast extract	0.5 g
Sodium acetate	0.2 g
Beef extract	0.2 g
Agar	11.0 g

Distilled water to	1000.0 mL

Adjust pH to 7.2.

The growth of flavobacteria can be enhanced by the addition of 2 to 5% fetal bovine serum to this medium.

6. **Hsu-Shotts Medium.**
This medium (Bullock et al. 1986) is used for the isolation of flavobacteria; the addition of neomycin sulfate enhances the isolation of these bacteria by suppressing the growth of many other bacteria. The formula is:

Tryptone	2.0 g
Yeast extract	0.5 g
Gelatin	3.0 g
Agar	15.0 g
Distilled water to	1000.0 mL

After it is autoclaved, the medium is cooled to 45^{o}C, and filter-decontaminated neomycin sulfate is added to a final concentration of 4.0 mg/L.

Formulations for routine bacteriological media can be found in manuals on bacteriological methods such as MacFaddin (1985), Difco Laboratories (1998), Baron and Finegold (1990), and Murray (1999).

B. Test Reagents and Procedures for Phenotypic Characterization of Bacteria

The following are some tests commonly employed in the presumptive identification of bacteria isolated from fish. Commercially prepared media and reagents are available for biochemical tests. Detailed instructions on their preparation and use are also included in bacteriology manuals, and some descriptions are included in Section 2, 3.7 Reagents, Media, and Media Preparation and Section 2, 3.8 Bacterial Identification Techniques. Miniaturized bacterial identification systems that include many of the tests are also available commercially; their use for fish pathogen identification is further discussed in Section 2, 3.8.D.2 “Commercial Identification System.”

1. **Gram Stain**
This test is important for the broad classification of bacteria, and should be done on young (log-phase) cultures. Gram stain procedures are described in Section 2, 3.8.A.1 “Gram Stain.” Nonstain methods are available to aid in the determination of the true Gram stain reaction of problem organisms. One of these is the potassium hydroxide (KOH) test. The cell walls of gram-negative bacteria are broken down by KOH, and viscid chromosomal material is released. This causes the suspension to become thick and stringy. Procedures for the KOH test are described in Section 2, 3.8.A.2 “3% Potassium Hydroxide.” Fluorescent stains for differentiating gram-positive and gram-negative bacteria can also be used and are available from commercial sources.

2. Acid-Fast Stain

This test is used to distinguish gram-positive (or gram-variable) bacteria with cell walls that contain mycolic acids (long-chain, multiple cross-linked fatty acids) from other gram-positive bacteria. Mycobacteria (and to a lesser extent, *Nocardia*) stain acid fast.

3. Motility

For most bacteria (i.e., those motile by flagella), wet mounts (hanging drops) are prepared from log-phase cultures with TSB (trypticase soy broth or tryptic soy broth) as the suspending medium. If microscopic examination of wet mounts (hanging drops) gives equivocal or negative results, motility can be evaluated further by stab-inoculating tubes of commercially available semi-solid motility test medium (Difco Motility Test Medium or equivalent). The use of the hanging drop method and motility test medium for motility evaluations are described in Section 2, 3.8.C "Motility."

For flavobacteria, an agar block motility test can be done by excising a 5-mm square block of agar supporting a suspected flavobacterial colony, placing the block (colony side up) on a glass slide, and covering the block with a cover glass. The margin of the colony is examined with a microscope at about 400X magnification for evidence of gliding or creeping motility.

4. Cytochrome Oxidase Test

This test indicates the presence of the enzyme cytochrome oxidase, an iron-containing porphyrin enzyme that participates in the electron transport mechanism and in the nitrate metabolic pathways of some bacteria. The test is used to initially characterize gram-negative bacilli. Cytochrome oxidase procedures are discussed in Section 2, 3.8.B "Cytochrome Oxidase."

5. Carbohydrate Utilization Tests

Preparations of basal media to which specific carbohydrates are added are used to differentiate bacteria according to their patterns of carbohydrate utilization. Utilization of a specific carbohydrate causes a pH change, which is detected by a pH indicator in the medium. To determine if organisms metabolize carbohydrates oxidatively or fermentatively, OF medium is used. Gas production can be detected in some media. Further discussions of OF basal medium and certain carbohydrate utilization tests are included in Section 2, 3.7.B.4 "Oxidation/Fermentation (OF) Medium," Section 2, 3.8.D.1.a "Glucose Fermentation," and Section 2, 3.8.D.1.e "Carbohydrate Utilization (MacFaddin 1980)."

6. Triple Sugar Iron (TSI) Agar

Triple sugar iron agar is frequently used during the initial identification of gram-negative bacilli, particularly members of the Enterobacteriaceae. This medium can detect three primary characteristics of a bacterium: the ability to produce gas from the fermentation of sugars, the production of large amounts of hydrogen sulfide (H_2S) gas, and the ability to ferment glucose, lactose and sucrose. The interpretation of results is described in Section 2, 3.8.D.1.b "Triple Sugar Iron (TSI)." Lead acetate paper, a more sensitive indicator of H_2S production, is sometimes used in conjunction with TSI or other media containing available sulfur compounds.

7. Single Substrate Utilization Tests

Certain bacteria can be characterized by their ability to grow in the presence of a single compound. Common substrates that can fulfill this function and are useful for the differentiation of bacteria are citrate, malonate, and acetate. Growth on an agar slant containing the substrate of interest (with or without a pH indicator) is used as the end point of the test. The malonate test is described in and Section 2, 3.8.D.1.g "Malonate Test,"

respectively.

8. **Indole Test**
   This is a test for the enzyme tryptophanase, and is used in the characterization of gram-negative bacteria, particularly Enterobacteriaceae. Bacteria that produce this enzyme can degrade the amino acid tryptophan into pyruvic acid, ammonia, and indole. Indole is detected by its combination with an indicator aldehyde to form a colored product. The indole test is described in Section 2, 3.8.D.1.d "Indole Test."

9. **Decarboxylase/Dihydrolase Reactions**
   These are tests for enzymes that degrade certain amino acids by decarboxylation or dihydrolation. Common tests used in the identification of fish pathogenic bacteria include those for arginine dihydrolase/decarboxylase (ADH), lysine decarboxylase (LDC), and ornithine decarboxylase (ODC). The lysine decarboxylase test is described in Section 2, 3.8.D.1.f "Decarboxylase Test (Lysine)."

10. **Hydrolysis Tests**
    These tests are used to differentiate bacterial species according to their abilities to produce certain hydrolyzing enzymes. The products of hydrolysis are detected by some visual reaction. Substrates commonly used in the characterization of fish pathogenic bacteria are urea (for urease activity), gelatin (for gelatinase activity), esculin (for esculin hydrolysis), and starch (for starch hydrolysis). The gelatinase test and esculin test are described in Section 2, 3.8.D.1.c "Gelatinase" and Section 2, 3.8.D.1.h "Esculin Test," respectively.

11. **DNAse Test**
    Extracellular nucleases are produced primarily by the same bacterial species that produce the protease gelatinase. Commercially produced agar media are available for this test.

12. **Phenylalanine Deaminase Test**
    This test determines the ability of an organism to deaminate the amino acid phenylalanine to yield indolepyruvic acid, and is used for grouping Enterobacteriaceae.

13. **Catalase Test**
    This test is used to detect the presence of the enzyme catalase, which catalyzes the liberation of oxygen and water from hydrogen peroxide. The test reagent (3% hydrogen peroxide) is readily available. Because red blood cells in blood agar media contain catalase, weak false positive reactions can be obtained with colonies taken from these media. It is recommended that control catalase tests be performed with a small loopful of blood-containing agar on the same slide with the organism; a strong positive reaction from the organism can be distinguished from a weak positive reaction from the medium alone.

14. **Bile Solubility Test**
    Streptococcus pneumoniae can be distinguished from other alpha hemolytic streptococci (see below) by this test. *Streptococcus pneumoniae* possesses an autocatalytic enzyme that normally functions to lyse the cell wall during cell division; colonies of this organism also will autocatalyze within 30 minutes after exposure to the surfactant sodium deoxycholate, one of the major components of bile. Other alpha hemolytic streptococci lack such an active enzyme and will not dissolve in bile. The bile solubility test may not work with old colonies of *S. pneumoniae*, which may have lost their active enzyme.

15. **Nitrate Reduction Test**
    This test distinguishes between bacteria that cannot utilize nitrate (NO_3) as a nitrogen source,

and those that can reduce nitrate to nitrite (NO_2) or a product beyond nitrite.

16. Antimicrobial Sensitivity Tests

Patterns of sensitivity to various antimicrobial reagents are sometimes used in the characterization of fish pathogenic bacteria. Most commonly, sensitivity to novobiocin is used to distinguish *Vibrio* spp. from motile *Aeromonas* spp. The novobiocin disk (5 µg concentration) is applied to a TSA plate that has been surface-seeded uniformly with the organism under test. After incubation at 20 to 22°C for 16 to 24 hours, a sensitive organism shows a clear zone of inhibition around the disk. Because TSA is widely used for the primary isolation of many fish pathogenic bacteria, this is the medium generally used for novobiocin sensitivity tests performed to differentiate *Vibrio* spp. from motile *Aeromonas* spp. However, disk diffusion tests performed to determine the sensitivity patterns of bacterial isolates to a battery of compounds are done on Mueller-Hinton agar (the para-aminobenzoic acid present in TSA interferes with the action of sulfonamides). Procedures for the conduct and interpretation of disk diffusion antimicrobial susceptibility tests are described in more detail in manuals on bacteriological procedures.

17. Vibriostatic Agent 0/129 Sensitivity Test

Sensitivity to the vibriostatic agent 0/129 (2,4-diamino-6,7-diisopropylpteridine phosphate, available from Sigma Chemical Co.) is used to differentiate *Vibrio* spp. from motile *Aeromonas* spp. The sensitivity disks are prepared as follows: Dissolve 0/129 in acetone at 0.1% (weight/volume). Saturate Whatman antibiotic filter paper disks (6 mm) with the 0/129 solution, and drain off the excess. Dry the disks at 37°C, and store them in a tightly sealed bottle at 4°C. Control disks (saturated with acetone only) also should be prepared. For 0/129 sensitivity tests, TSA plates are surface-seeded with bacteria, test and control disks are applied to the plates, the plates are incubated, and results are interpreted in the same manner as for novobiocin sensitivity tests. Novobiocin sensitivity and 0/129 sensitivity tests can be conducted on the same plate.

18. P Disk Test

The P disk (optochin resistance) test is used to identify strains of *Streptococcus pneumoniae*. Disks impregnated with 5 µg of optochin (ethyl hydrocupreine) are placed on inoculated blood agar plates. *Streptococcus pneumoniae* is not optochin resistant and a zone of inhibition will develop around the disk. The isolate is resistant to optochin if the zone of inhibition is greater than 14 mm for a 6-mm disc or 16 mm for a 10-mm disk. Correct interpretation of the optochin zone requires a confluent lawn of bacterial growth. If the growth is too light, an erroneously large zone (false susceptibility) may be observed.

19. LAP Test

The LAP test detects the presence of leucine aminopeptidase (LAP). Among the LAP-positive bacteria are the lactococci, enterococci, and several streptococci, but the ß-hemolytic streptococci and aerococci are LAP-negative. The test is performed using commercially available discs that are impregnated with leucine-ß-naphthylamide. The LAP enzymatic activity releases ß-naphthylamine, which turns red on the addition of p-dimethylaminocinnamaldehyde.

20. Characterization of Hemolysis

Patterns of hemolytic action of bacteria on erythrocytes in blood-containing media (usually BA) are used in the identification of some bacteria, principally streptococci. The major types of hemolysis are: α (alpha), consisting of an indistinct zone of incomplete lysis of erythrocytes around the bacterial colony, accompanied by a greenish to brownish

discoloration of the medium; and β (beta), a clear, colorless zone around the bacterial colony, in which the erythrocytes have undergone complete destruction; and γ (gamma), no hemolysis.

21. CAMP Test

The CAMP test is used to identify Lancefield Group B streptococci (see Immunological Procedures below). Group B streptococci produce a protein-like substance called the "CAMP factor," which can act synergistically with the beta toxin produced by certain strains of *Staphylococcus aureus*, resulting in an enhanced zone of hemolysis around the streptococci. The test is performed with an appropriate strain of *S. aureus*, or with commercially available CAMP factor-impregnated filter paper disks.

22. Simmons Citrate Test

Organisms that are able to use citrate as the sole source of carbon for metabolism and growth are able to grow on simmons citrate agar. In the process of metabolizing citrate, bacteria produce alkaline conditions in the medium. Simmons citrate agar contains the pH indicator bromothymol blue, which is green under acidic conditions, and royal blue when the medium becomes alkaline.

Magnesium sulfate	0.20 g
Monoammonium phosphate (NH4H2PO4)	1.00
Dipotassium phosphate (K2HPO4)	1.00
Sodium citrate	2.00
Sodium chloride	5.00
Agar	15.00
Bromthymol blue	0.08
Final pH 6.9 ± 0.2 at 25°C	

Heat gently with occasional agitation. Boil 1 to 2 minutes until agar dissolves. Fill 13 x 100 or 16 x 150 mm screw-cap tubes 1/3 full. Autoclave 15 minutes at 121°C. Before medium solidifies, incline tubes to obtain 4 to 5 cm slants and 2 to 3 cm butts. Final pH, 6.8 ± 0.2.

III. Immunological Procedures

Confirmation of the identity of most fish pathogenic bacteria is based on serological tests. Most common among these are the fluorescent antibody test and the slide or microtiter agglutination test. These and certain other techniques developed for the identification of bacteria in culture, or for the detection and identification of bacteria directly in fish tissues or body fluids, are discussed briefly below. The specificity of these and other immunological tests for the identification of fish pathogens will depend on the specificity of the antibodies used.

A. Fluorescent Antibody Test (FAT)

Both direct and indirect FATs have been developed for the identification of bacterial fish pathogens. Antibodies for specific bacteria are available from commercial sources, or from certain research laboratories. Fluorochrome-conjugated antibodies for the indirect FAT are readily available from commercial sources. Most commonly, the FAT is used to confirm the identity of bacteria isolated in culture. For some pathogens, notably *Renibacterium salmoninarum*, the FAT is used for the detection and identification of bacteria directly in fish

tissues or body fluids. Descriptions of direct and indirect FAT procedures are included in Section 2, 3.8.E "Fluorescent Antibody Test (FAT)." The reader is also referred to chapters on specific pathogens for modifications of the FAT for use on fish tissue and body fluid samples.

B. Agglutination Tests

The identity of many bacterial fish pathogens can be confirmed by agglutination reactions with specific antisera. Certain bacterial species exhibit a tendency to autoaggregate spontaneously and are therefore difficult to examine by agglutination tests. Such bacteria may be subjected to pretreatment procedures to prevent autoaggregation, or they may be tested by alternative procedures such as latex bead agglutination or staphylococcal coagglutination. The reader is referred to chapters on specific bacterial pathogens for modifications to agglutination tests. Principles and procedures for some agglutination techniques are also discussed by Roberson (1990).

1. Slide Agglutination Test

This test is used for rapid confirmatory identification of bacteria grown in culture.

a. Clean glass microscope slides and mark circular divisions (two circles per slide) with a wax crayon.

b. Prepare a suspension of one or more colonies of the unknown bacterium in a small amount of physiological saline. To ensure a uniform suspension without clumps, it is best prepared in a small test tube rather than directly on the slide. The bacterial suspension should be standardized to a known concentration of particulate materials; i.e. a McFarland standard #3 or a spectrophotometer reading of 40% T at 645 nm. If this cannot be done, preparation of a cloudy suspension approximating the color and density of skimmed milk is generally acceptable. Suspensions of positive controls (bacteria known to be the species of interest) and negative controls (known bacteria of an unrelated species) can be prepared in the same manner.

c. Place a drop of the unknown bacterial suspension in each of the two circles on a slide. Add a drop of the appropriate specific antiserum to one circle on the slide. Add a drop of normal serum to the other circle on the slide. Mix gently with an applicator stick, or rock the slide gently. Follow the same procedure for the control bacteria.

d. Observe the reactions immediately for agglutination (clumping), then incubate the slides at room temperature and observe after five and 10 minutes. Results can be checked with a microscope at low magnification.

For positive identification of the unknown bacterium, the following reactions should be observed:

Circle with unknown bacterium plus antiserum: agglutination

Circle with unknown bacterium plus normal serum: no agglutination

Circle with positive control bacterium plus antiserum: agglutination

Circle with positive control bacterium plus normal serum: no agglutination

Circle with negative control bacterium plus antiserum: no agglutination

Circle with negative control bacterium plus normal serum: no agglutination

2. **Microtiter agglutination test.** This test gives the titer of the antiserum used, in addition to confirmatory identification of the bacterium being tested. The test is done in 96-well microtiter plates. A constant bacterial concentration, but two-fold serial dilutions of antiserum are used. The procedure is described by Roberson (1990).

C. Lancefield Grouping of Streptococci

Most beta-hemolytic streptococci and some alpha-hemolytic or nonhemolytic streptococci possess specific carbohydrate cell wall antigens. These carbohydrate antigens are called streptococcal group antigens or Lancefield group antigens. In the past, serological typing of cell wall components was used to separate streptococci into species. Although recent DNA homology studies have shown that this is not possible, serological typing is still a useful aid for the identification of isolates. Group-specific sera (precipitating, agglutinating, and fluorescent-antibody sera) are commercially available for use with extracts, cell suspensions, and spent broth media. Beta-hemolytic streptococci are characteristically tested with group A, B, C, D, and F antisera. Alpha-hemolytic or nonhemolytic streptococci are usually tested with antisera to groups B, D, and N. Procedures for streptococcal antigen extraction and serotyping are described in bacteriological procedures manuals.

D. Other Immunological Tests

In addition to the tests described above, other immunological tests have been developed for the identification of bacteria in culture, or for the detection and identification of specific bacteria in fish tissues or body fluids. Included among these are such techniques as staphylococcal coagglutination, latex bead agglutination, counterimmunoelectrophoresis, and enzyme immunoassays. Of the enzyme immunoassays, the enzyme-linked immunosorbent assay (ELISA) has perhaps gained the widest use. An ELISA is designed to detect a specific substance in a complex mixture by binding that substance to an antigen- or antibody-coated surface. After binding has occurred, an enzyme-labeled antibody specific for the bound substance is applied. With the addition of appropriate reagents, the enzyme catalyzes a reaction that yields a colored end product, thus allowing detection and quantification of the bound substance. Various ELISAs have been developed for the detection of bacteria or bacterial products as well as viruses, drugs, hormones, toxins, carcinogens, and antibodies. The reader is referred to chapters on specific pathogens for further discussions of the ELISA and other immunological techniques.

IV. Nucleic Acid-based Identification Procedures

Nucleic-acid-based tests are now gaining favor for identification of certain bacterial fish pathogens. Among the early tests developed were nucleic acid probes designed to detect specific unique segments of DNA or RNA of the target bacterium. These techniques have been largely supplanted by a plethora of molecular tools known collectively under the name polymerase chain reaction (PCR). The PCR procedures are considered to have a greater potential for improving both the sensitivity and specificity for detection and identification of a target bacterium because of the enzymatic amplification steps incorporated in the tests. The reader is referred to chapters on specific bacteria for discussions of PCR procedures.

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1.2 Flow Chart for the Presumptive Identification of Selected Bacteria from Fishes*

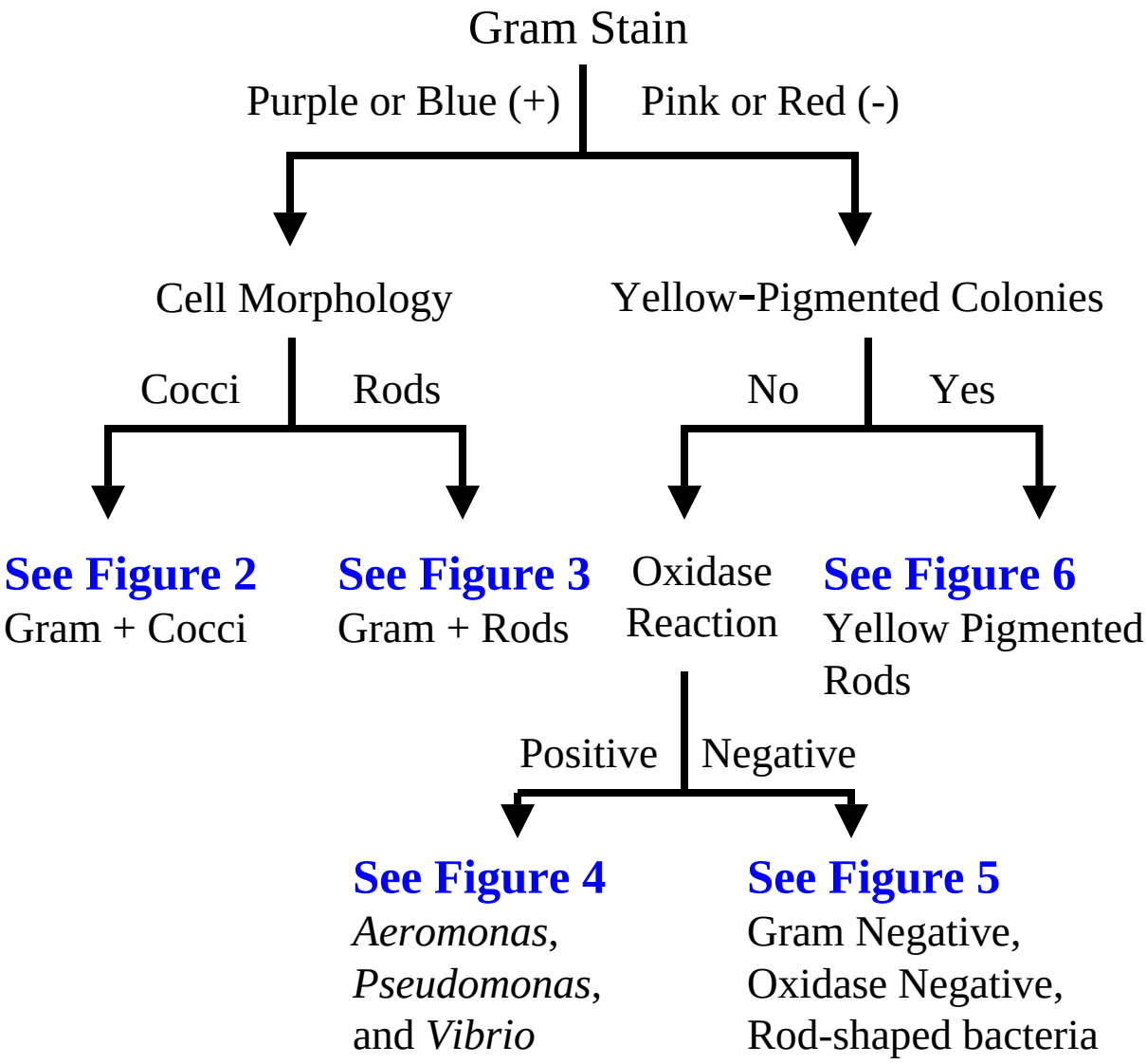
Dr. Emmett B. Shotts, Jr.

Department of Veterinary Medicine
University of Georgia
Athens, GA 30602
404/542-5811
emshotts@alltel.net

This revised key for the identification of bacterial pathogens of fish was developed by Dr. Emmett Shotts in 2001 and was designed to replace and simplify the key found in the 1994 edition of the Blue Book. This new key provides a broad perspective of strategies useful for the biochemical identification of bacterial fish pathogens and it will be helpful in both diagnostic work and in teaching. Users of these new tables must keep in mind that this is a presumptive key and thus it may lead to an incorrect identification of bacterial isolates having atypical biochemical characteristics or of species not included in this key. It is also important to note that some bacteria require special growth media or conditions for growth and that the initial isolations must be made using appropriate substrates and conditions. Diagnosticians should use this key as a helpful guide, but final diagnostic decisions should be based on more information provided in other specific bacteriology subchapters of this chapter or in Section 1, 1.1 General Procedures for Bacteriology.

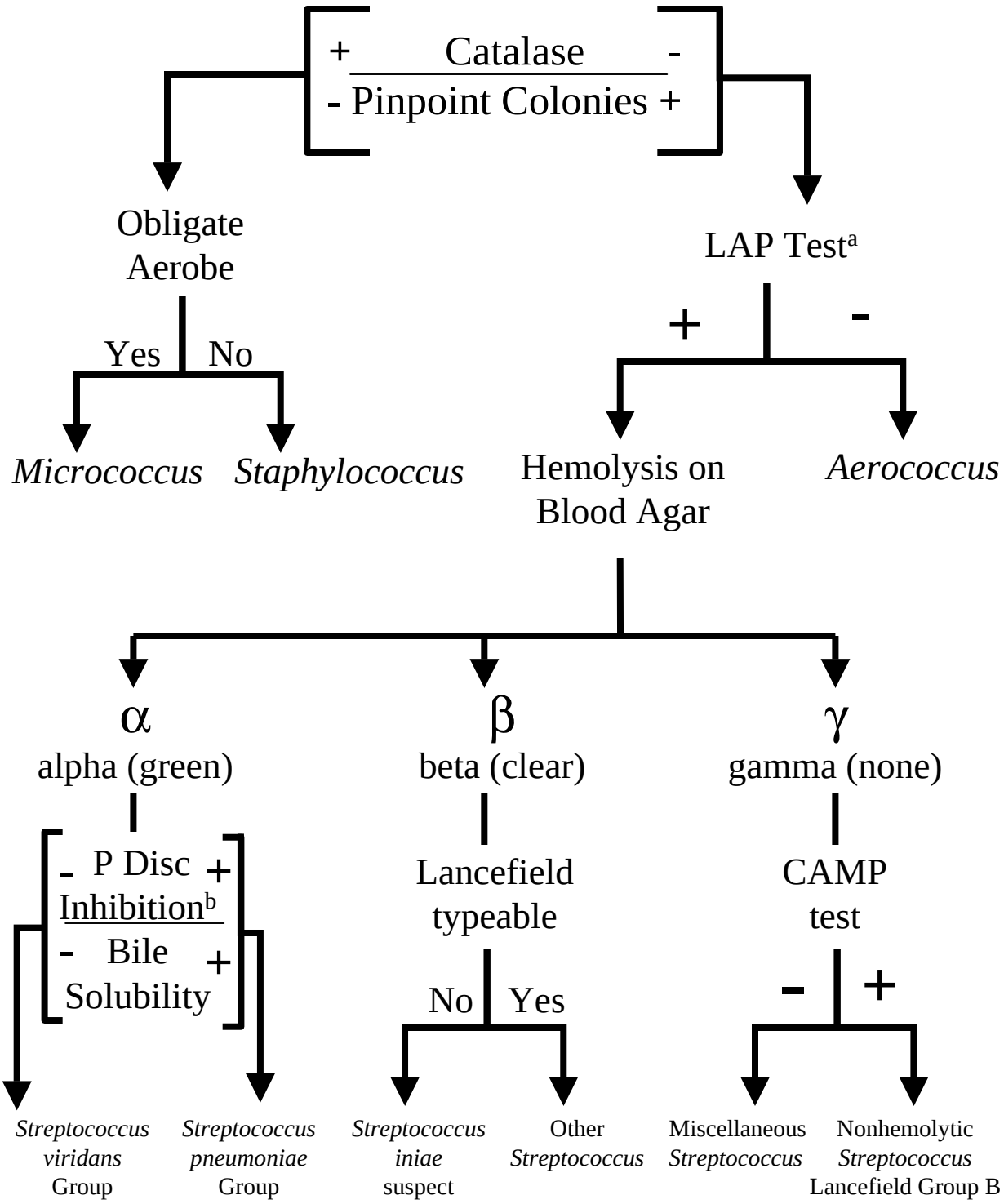
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Figure 1: Gram Stain



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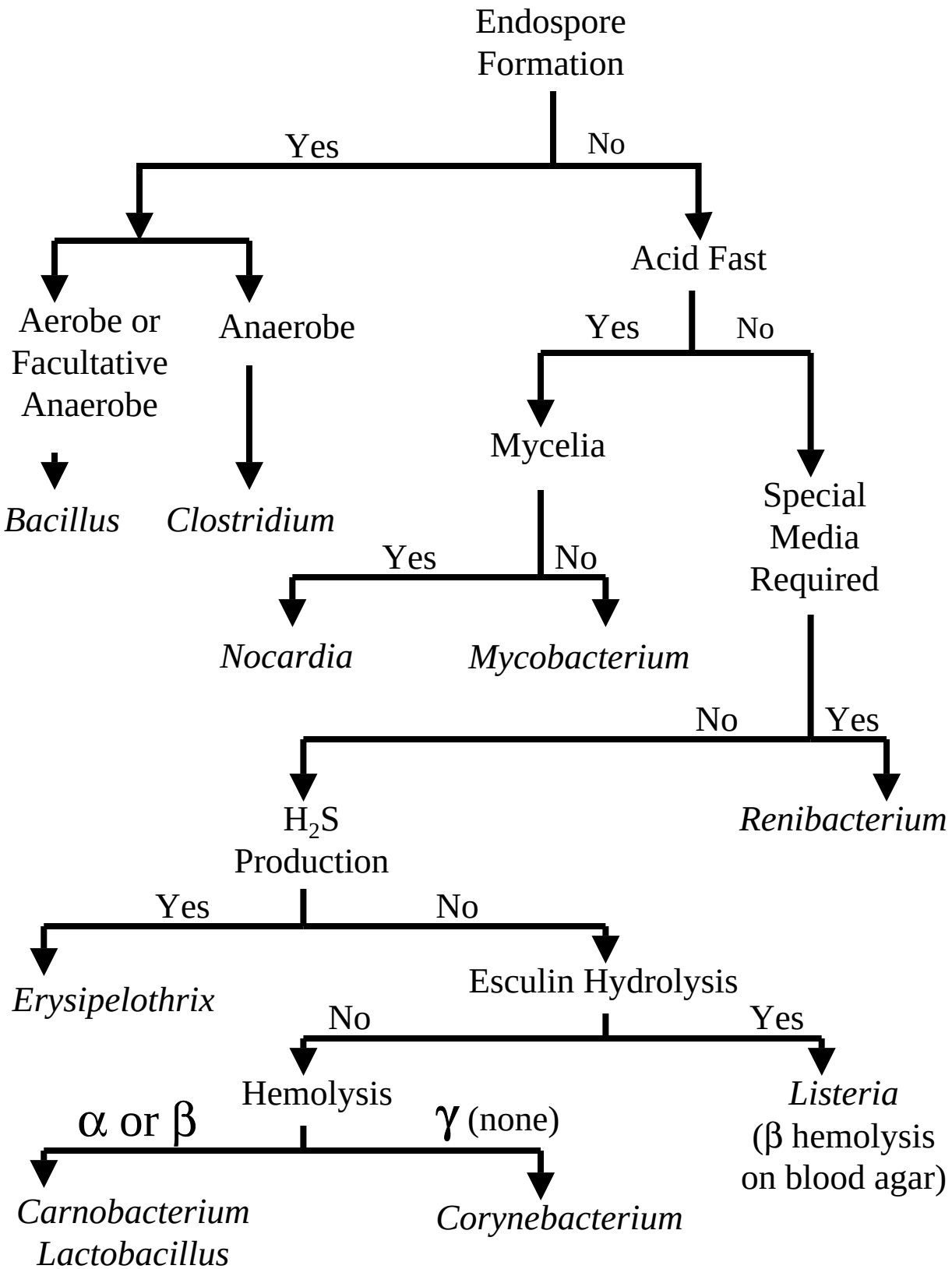
Figure 2: Gram Positive Cocci



a = leucine aminopeptidase test
b = ethyl hydrocuprein hydrochloride (5 µg) disc

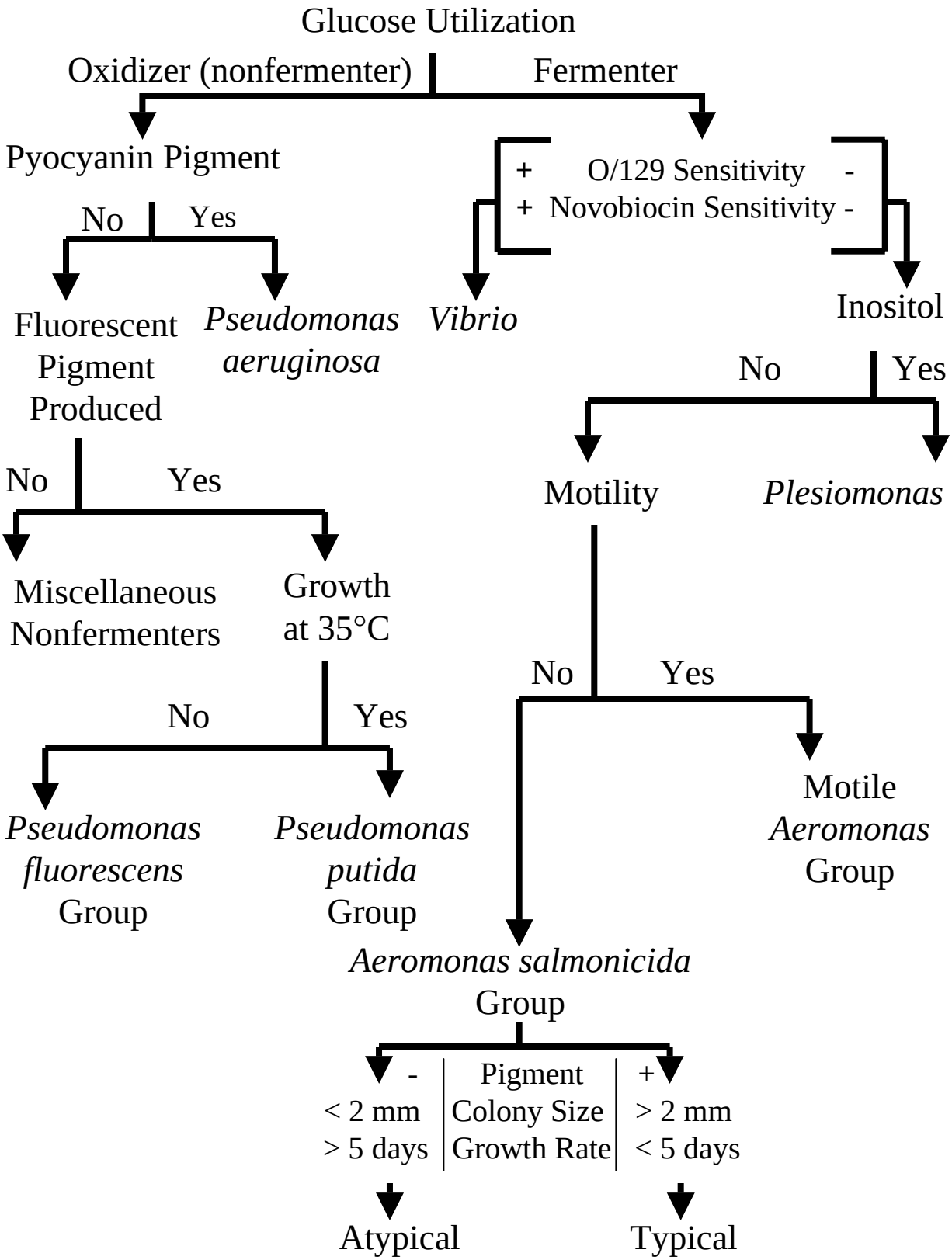
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Figure 3: Gram Positive Rods



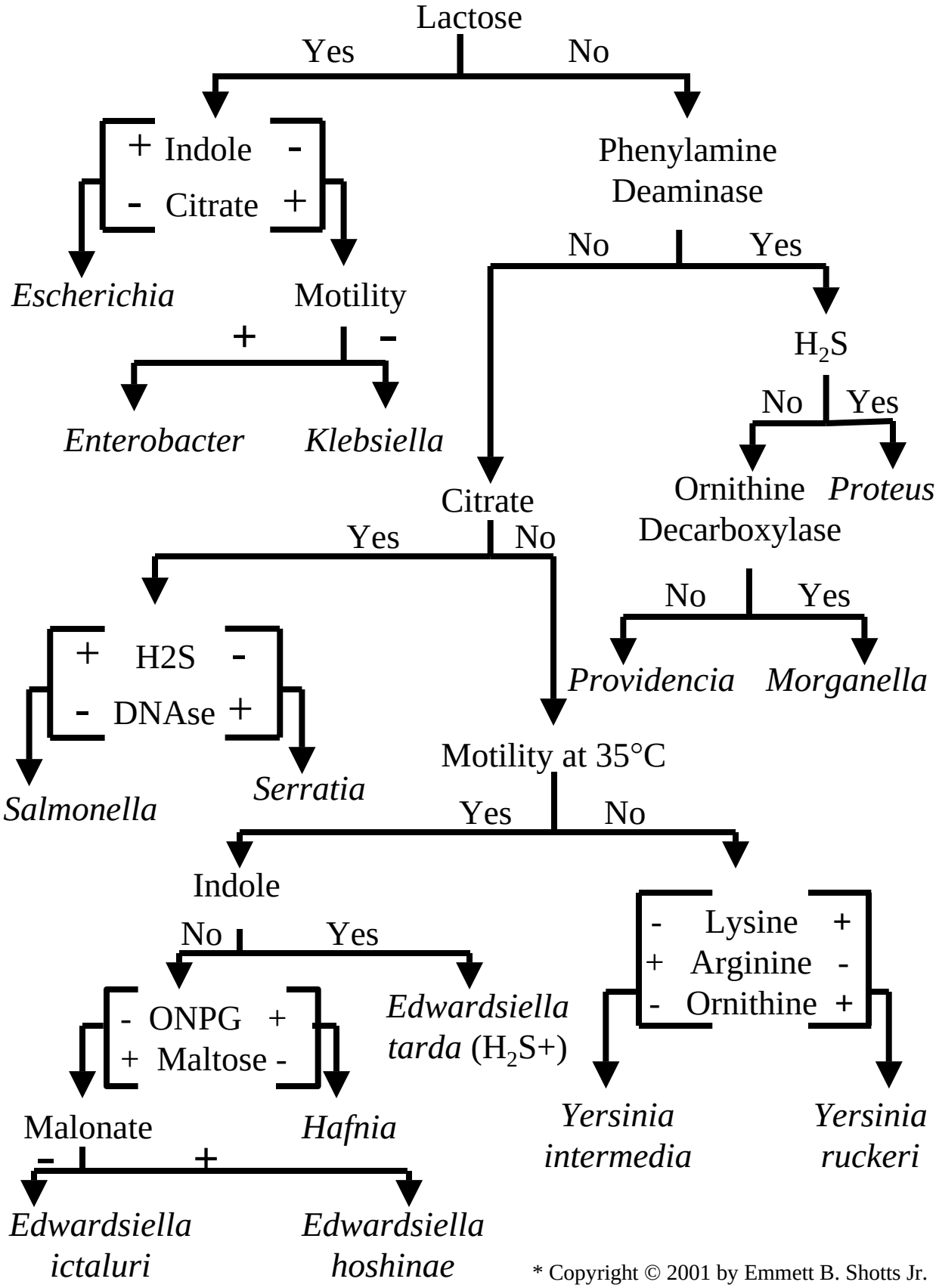
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Figure 4: *Aeromonas*, *Pseudomonas*, and *Vibrio*



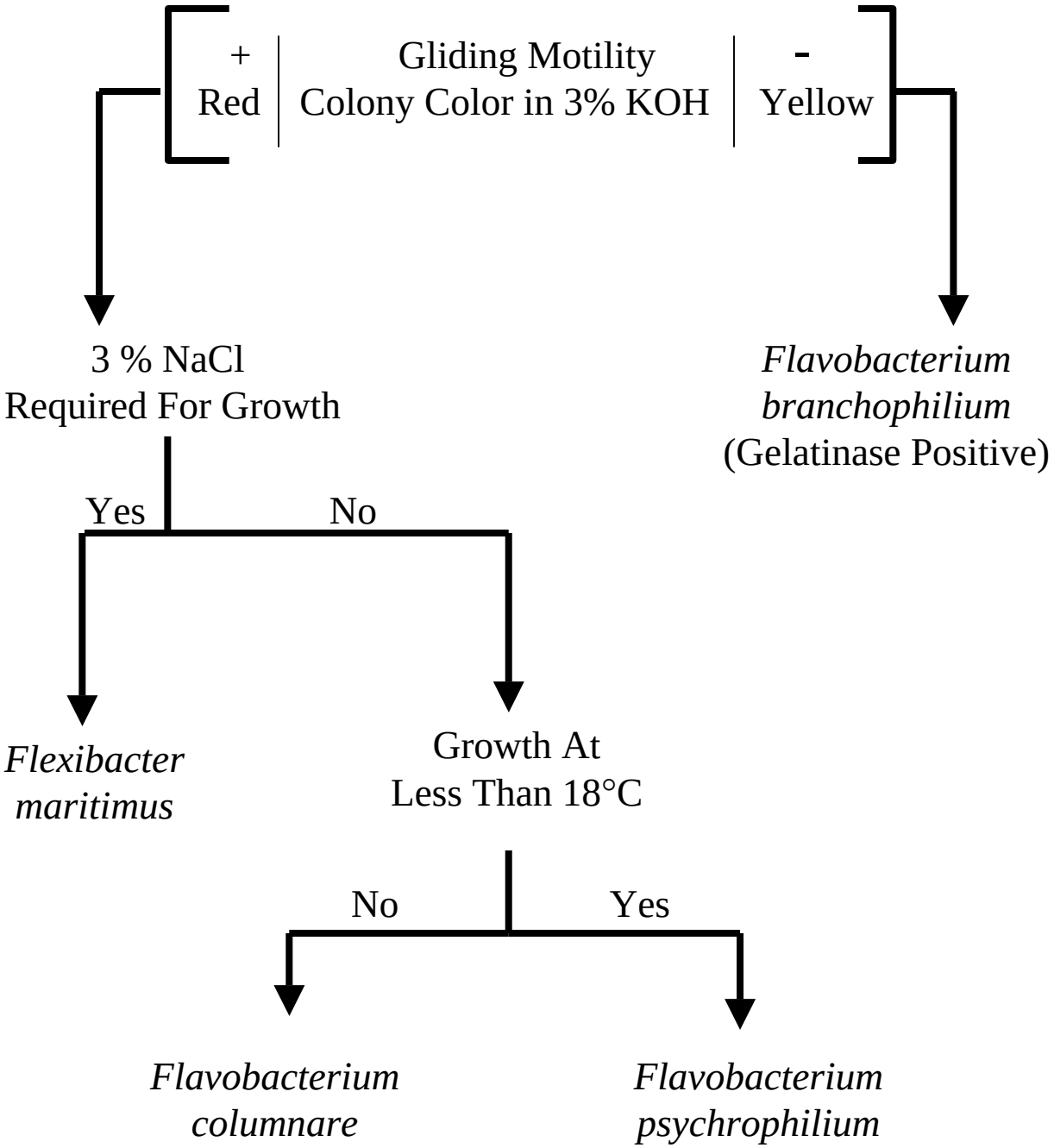
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Figure 5:Gram Negative, Oxidase Negative, Rod-shaped bacteria



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Figure 6: Yellow-Pigmented Rods



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1.3 Bacterial Gill Disease

Steven D. Roberts

Washington Department of Fish and Wildlife
122 South Mitchell Court
Liberty Lake, WA 99019
509/255-5907
robersdr@dfw.wa.gov

A. Name of Disease and Etiological Agent

Bacterial gill disease, is caused by *Flavobacterium branchiphilum.* Other species of yellow pigmented, filamentous bacteria may also be involved.

B. Known Geographical Range and Host Species of the Disease

1. **Geographical Range**
   Worldwide, usually seen in fishes in aquaculture facilities.

2. **Host Species**
   All salmonids are probably affected; may occur in other fishes.

C. Epizootiology

Outbreaks of bacterial gill disease are associated with stressors such as crowding, low dissolved oxygen, high ammonia concentrations, and high feeding rates. Bacterial gill disease has been observed in all fish life stages.

The source of *Flavobacterium branchiphilum* is not known. The bacterium may be a common resident of water and sediment.

The incubation period for bacterial gill disease is variable because epizootics are dependent on the presence of the stressor. Bacterial gill disease has been induced experimentally in rainbow trout fingerlings in 10 to 14 days when the fish were subjected to poor environmental conditions. Fish less than 4.5 g are particularly susceptible. Reinfection will occur if stressors persist. In salmonids, bacterial gill disease usually occurs in the spring and summer. In severe cases, cumulative mortality can exceed 50%.

D. Disease Signs

Clinical signs include lethargy, loss of appetite, increased gill activity, extended gill opercules, and fusion of gills filaments.

Histologically, a proliferative hyperplasia of the gill lamellae epithelium is observed. As the disease progresses, the proliferation of the epithelium causes a clubbing and fusion of the gill lamellae. Ruthenium red stain of the gill tissues reveals of bacterial cells adhered to the epithelium (Kudo and Kimura 1983a). (See Figure 1.)

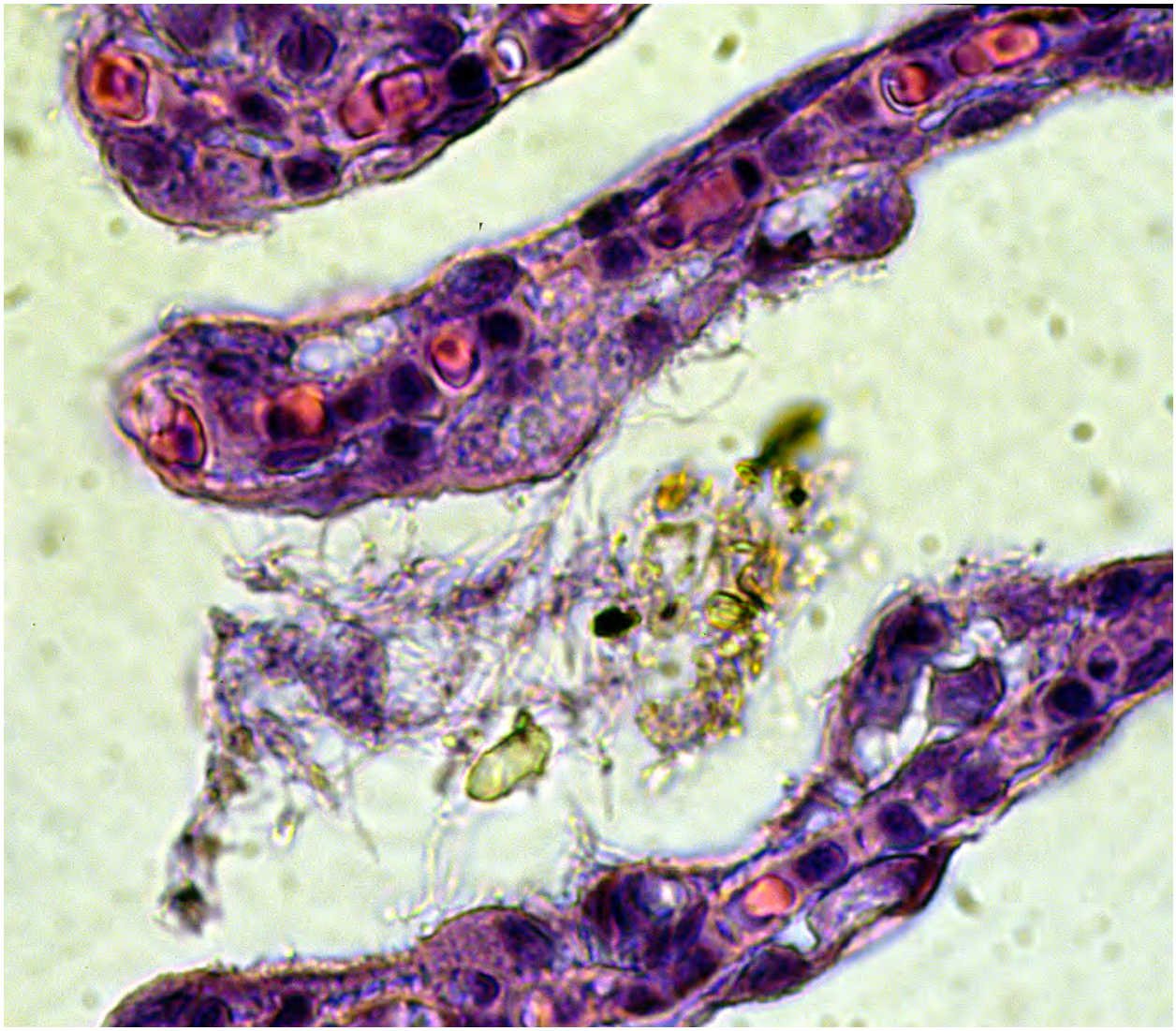


Figure 1. H&E section of gill from a rainbow trout showing large mats of flavobacterium causing bacterial gill disease. Photo courtesy of Dr. Chris Wilson.

E. Disease Diagnostic Procedures

Diagnosis is based on clinical signs along with examination of wet mounts of the gill lamellae for gill hyperplasia and the presence of bacteria. Enhanced observation of wet mount of gill filaments can be seen with phase contrast microscopy. Gram stain preparations of gill imprints can also be useful in diagnosis.

An FAT (Huh and Wakabayashi 1987) and ELISA (MacPhee et al. 1995b) test have been developed for *Flavobacterium branchiphilum* but are not in widespread use.

Diagnosis can also be based on histological examination of tissue sections stained with hematoxylin and eosin. Proliferation of the gill epithelium, and clubbing and fusion of the gill lamellae are diagnostic features. Bacteria can also be seen in tissue sections.

F. Procedures for Detecting Subclinical Infection

No procedures have been reported.

G. Procedures for Determining Prior Exposure to Etiological Agent

No procedures have been reported.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Fish can be preserved in 10% neutral buffered formalin or Bouin's fixative for later histological examination.

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1.4 Coldwater Disease

Richard A. Holt, Jim Bertolini,* and Peter W. Taylor**

Oregon Department of Fish & Wildlife
Department of Microbiology
Oregon State University
Corvallis, OR 97331-3804
503/737-1863
holtr@ucs.orst.edu

*Northwest Indian Fisheries Commission
Tribal Fish Health Laboratory
6730 Martin Way E.
Olympia, WA 98516-5540
jbertoli@nwifc.wa.gov

**Abernathy Fish Technology Center
U.S. Fish and Wildlife Service
Longview, WA 98632
pete_taylor@fws.gov

A. Name of the Disease and Etiological Agent

Coldwater disease is also referred to as bacterial coldwater disease, fry mortality syndrome, peduncle disease, or low temperature disease and is caused by *Flavobacterium psychrophilum*.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Found in the United States, Canada, France, Denmark, Sweden, Finland, Estonia, Spain, Japan, Chile, and Australia. The disease probably occurs in temperate regions worldwide.

2. Host Species

All salmonids are probably affected, but juvenile coho salmon (*Oncorhynchus kisutch) and* rainbow and steelhead trout (*Oncorhynchus mykiss)* are particularly susceptible. Also reported in chinook salmon (*Oncorhynchus tshawytscha)*, sockeye salmon (*Oncorhynchus nerka)*, chum salmon *(Oncorhynchus keta)*, and pink salmon *(Oncorhynchus gorbuscha)*. Serious losses also occur in cutthroat trout (*Oncorhynchus clarki)*, brook trout (*Salvelinus fontinalis)*, brown trout (*Salmo trutta)*, and Atlantic salmon (*Salmo salar)*. More recently, the disease has been reported in several non-salmonid species including eel (*Anguilla anguilla)*, common carp (*Cyprinus carpio)*, tench (*Tinca tinca)*, crucian carp (*Carassius carassius*), and ayu (*Pleoglossus altivelis)*.

C. Epizootiology

Juvenile fish are primarily affected; infections also occur in yearlings and smolts. *Flavobacterium psychrophilum* has been isolated from mature adult coho and chinook salmon from spleen, kidney, ovarian fluid, and milt. The possibility of egg associated transmission whether within or on the egg of this bacterium from these carrier adults to their progeny has been reported. Brown et al. (1997) isolated *F. psychrophilum* from the contents of surface-disinfected steelhead trout eggs. Also, they determined that 2% of a prepared suspension of *F. psychrophilum* cells survived after exposure to 100 ppm povidone/iodine for 30 minutes. Other researchers have reported fish egg associated *F. psychrophilum* (Cipriano et al. 1995; Rangdale et al. 1996; and Kumagai et al. 2000).

The disease occurs when water temperatures are 12^{o}C or below. However, some outbreaks occur or persist at temperatures up to 16^{o}C.

Although the reservoirs for the bacterium are unclear, it should be emphasized that *Flavobacterium sp.* are common inhabitants of aquatic ecosystems.

D. Disease Signs

Skin and muscle lesions are most common. These are frequently observed first in the peduncle area, but they may also occur on other areas of the body surface. The lesions may enlarge and the underlying tissue may be extensively eroded. If the fish survives long enough, it may suffer loss of its caudal fin and the vertebral column in the caudal peduncle may be exposed. In later stages of an epizootic, fish may darken either in the peduncle region or entirely and die with no external lesions. In trout, the bacterium can occur on the eroded caudal fin, and it may be present in internal organs. On rare occasions this bacterium is isolated from lesions on the gills of yearling rainbow trout. In coho alevins, the ventral surface of the yolk sac becomes eroded and the sac may rupture.

Following a severe epizootic, at least two disease conditions may be observed. In the first, fish appear lethargic and are found swimming near the outlet screens. Thereafter, spinal deformities (scoliosis and lordosis) and chronic mortality occur. In the other, fish display a spiral swimming behavior, a dorsal swelling posterior to skull, and dark pigmentation on one side of the body. In the latter case, *F. psychrophilum* is readily isolated from brain tissue. These two disease condtions are most commonly observed in coho salmon. However, these signs are not infrequently observed in steelhead trout and rainbow trout. *Flavobacterium psychrophilum* is often found detected as a secondary pathogen when fish populations are experiencing viral infections of erythrocytic inclusion body syndrome (EIBS), infectious hematopoietic necrosis, and infectious pancreatic necrosis. When yearling coho or chinook salmon are found to have coldwater disease infections it is advisable to test for EIBS virus.



Figure 1. Juvenile coho salmon (upper fish is uninfected) affected with coldwater disease with severe infection and erosion of the caudal fin and peduncle.





Figures 2 and 3. Juvenile coho salmon with coldwater disease lesions of the lower jaw, belly, and peduncle area.



Figure 4. Juvenile steelhead trout with hemorrhaging on the body near location of the spleen.



Figure 5. Juvenile steelhead trout with swollen hemorrhaged deep muscle lesion containing coldwater disease bacteria.





Figures 6 and 7. Coho salmon juveniles with *F. psychrophilum* infections of the brain, meninges, spinal cord area, and spinal deformed "survivors" of a coldwater disease epizootic.

E. Disease Diagnostic Procedures

Diagnosis is based on clinical signs along with isolation and identification of the etiological agent. Primary isolation should be made from lesions, kidney, or the spleen on either TYES agar (Holt et al. 1989) (see formulation for this medium in Section 1, 1.1 General Procedures for Bacteriology) or one of the other media listed below. Cultures are incubated at 15 to 20°C for 3 to 6 days. The brain is the organ of choice for culture under certain conditions as described above.

1. Presumptive Diagnosis

Skin and muscle lesions contain long, thin, gram-negative rods (0.5-0.7 x 2-7 um). In some cases, no external lesions are observed. Large numbers of rods can sometimes be observed in spleen tissue. The organism should produce moist, golden-yellow, raised, convex colonies with or without a thin, spreading, smooth to irregular edge on TYES agar in 3 to 6 days at 15 to 20°C. The following media support good growth of this bacterium: cytophaga agar (Anacker and Ordal 1959), Shieh (Shieh 1980), modified cytophaga agar (Wakabayashi and Egusa 1974), Hsu-Shotts (Baxa et al. 1986), TYE (Fujihara and Nakatani 1971), or TYES. Isolation of some strains is enhanced with addition of 1 to 5% fetal calf serum to cytophaga agar. Also, the addition of 0.2 to 0.5% sterile, skim milk (added just prior to pouring plates) or 0.5% bovine serum albumin (filter sterilized) to TYES may promote heavier and more rapid growth.

Characteristics helpful for distinguishing *F. psychrophilum* from other commonly encountered aquatic, yellow-pigmented bacteria include: failure to grow on brain heart infusion agar and failure to grow at 30°C. Also, when a portion of a *F. psychrophilum* colony is swirled in a drop of 20% potassium hydroxide, the cell material turns red or reddish-brown indicating the presence of flexirubin-type pigments.

Diagnosis is also aided by using *F. psychrophilum* antiserum for slide or microtiter agglutination test or indirect FAT.

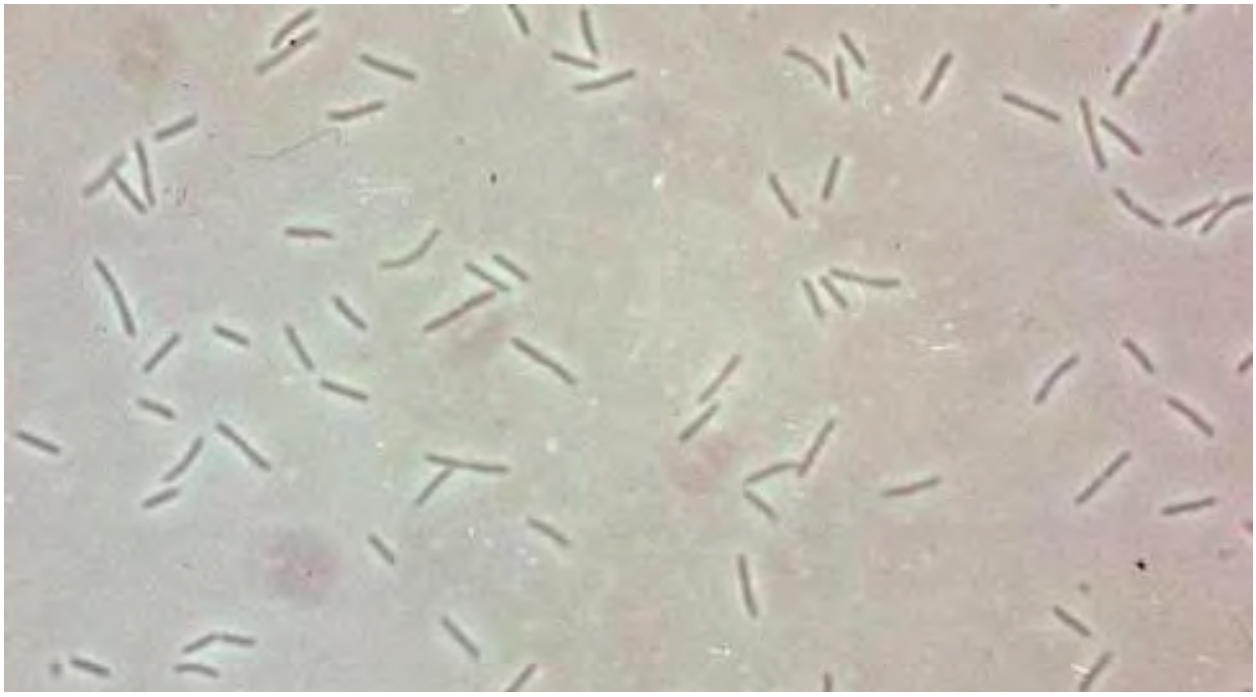


Figure 8. Cells of *F. psychrophilum* in wet mount preparation observed at 1000X.

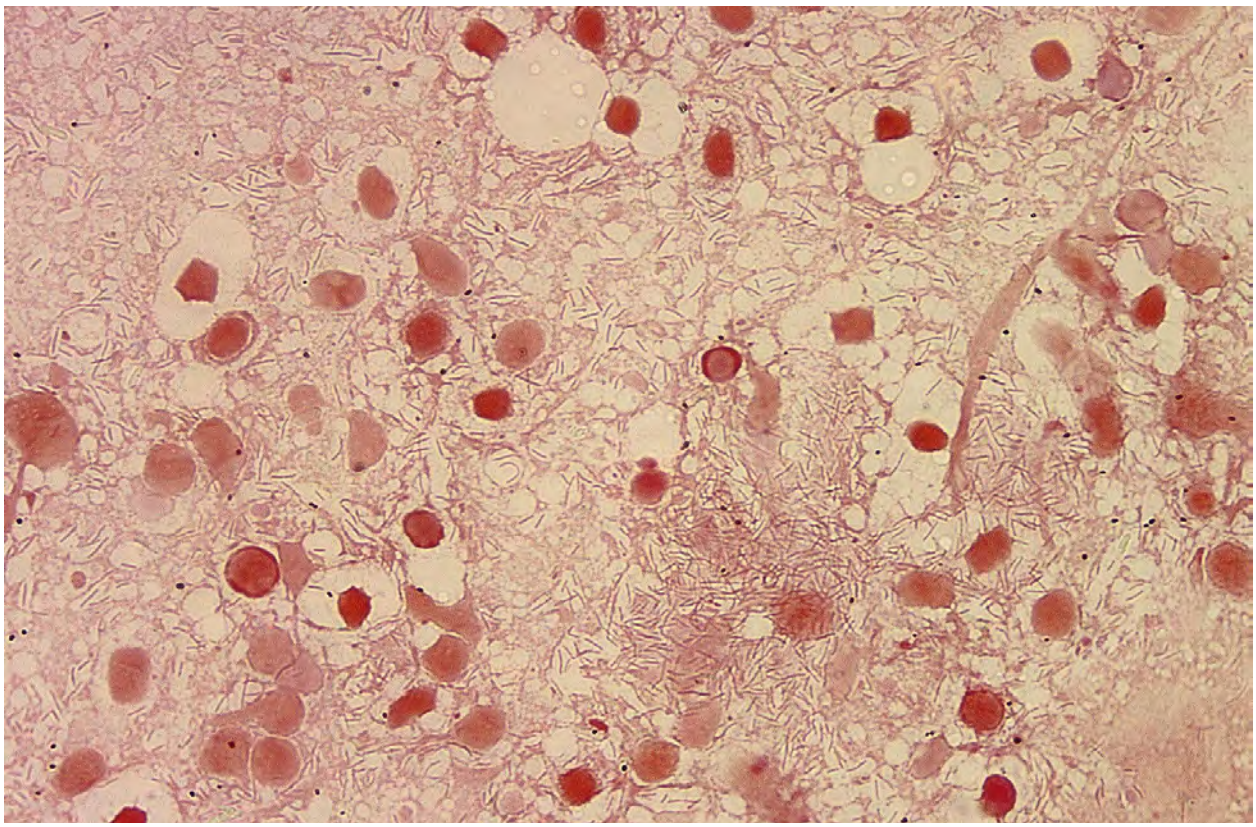


Figure 9. Gram stained *F. psychrophilum* cells. A smear prepared from the spleen of an infected fish (picture provided by C. Banner, Oregon Dept. of Fish and Wildlife).

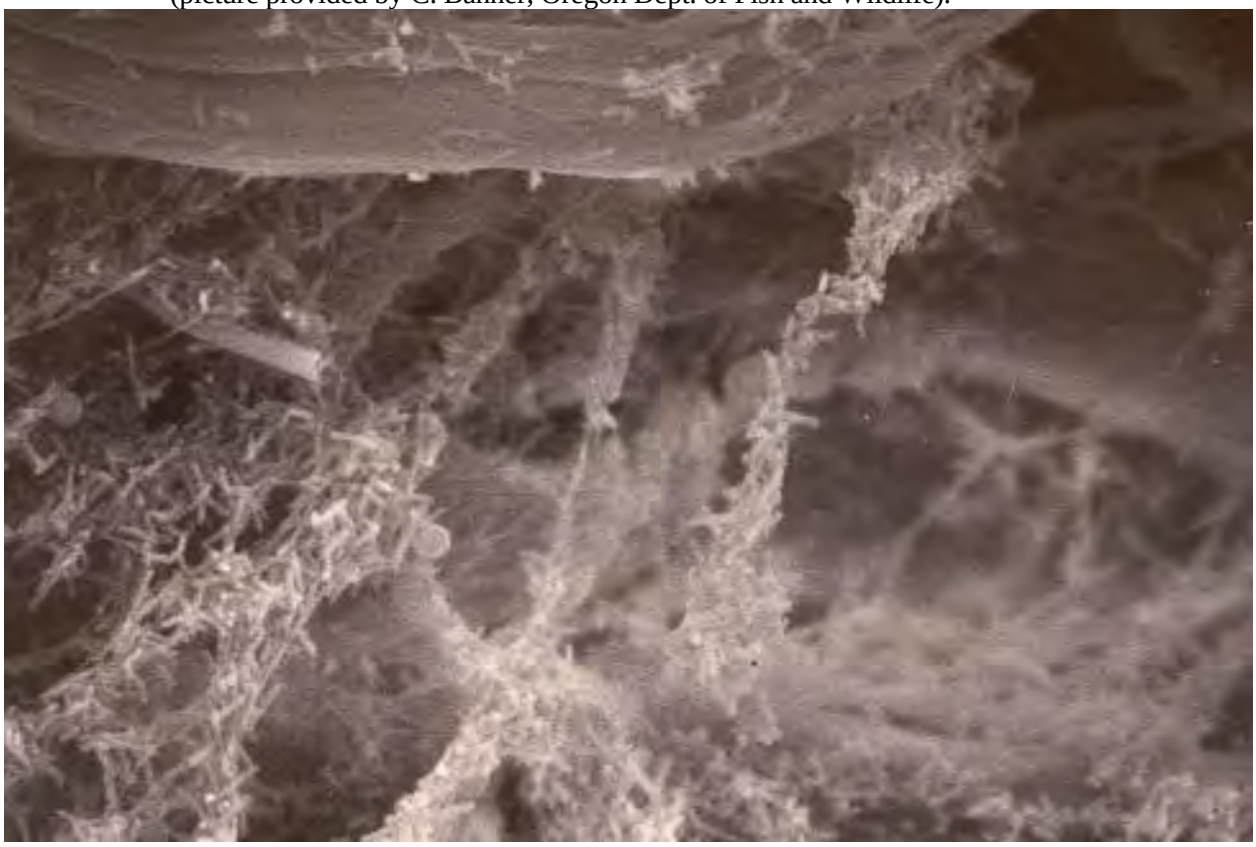


Figure 10. Scanning EM of coldwater disease cells in a muscle lesion of a juvenile coho salmon.

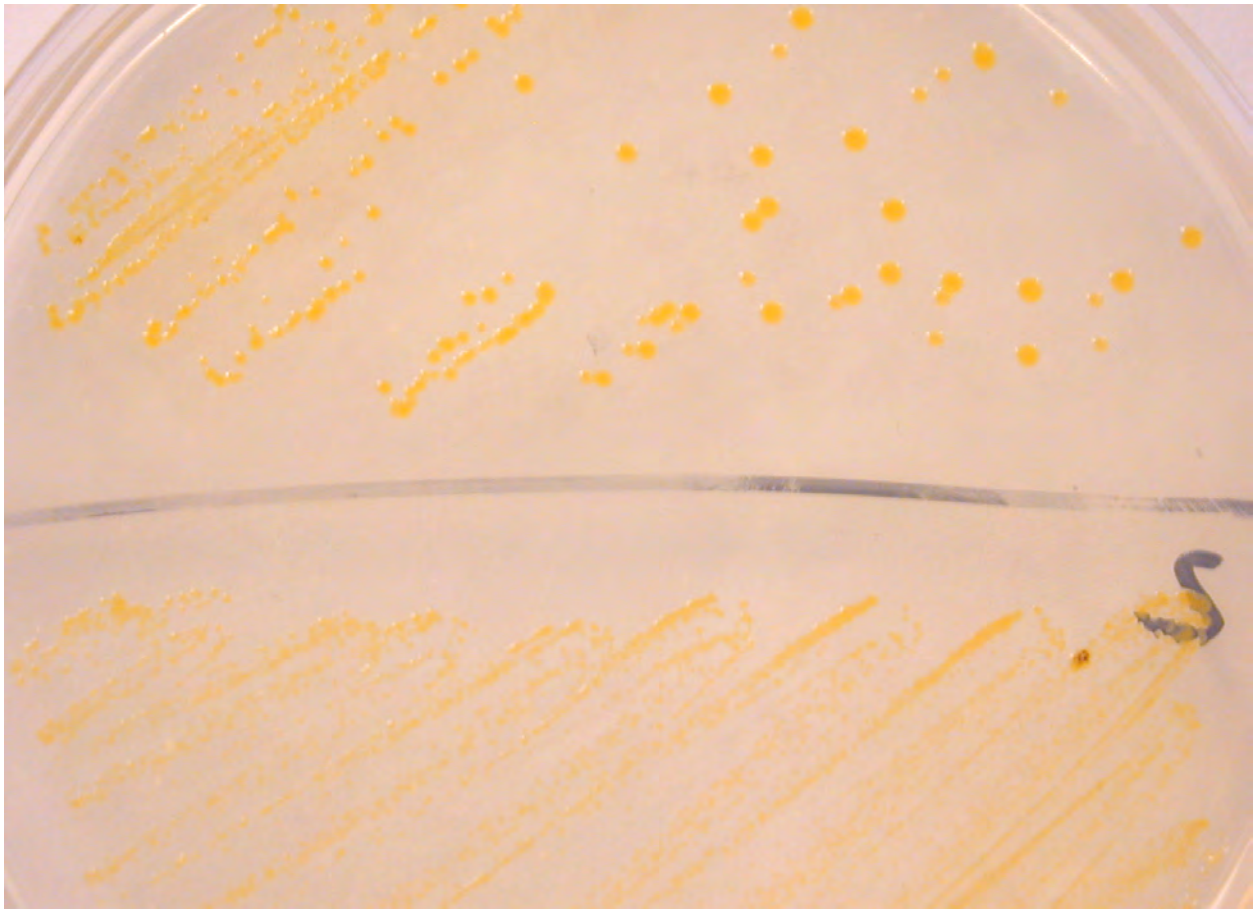


Figure 11. *Flavobacterium psychrophilum* colonies on TYES Agar.

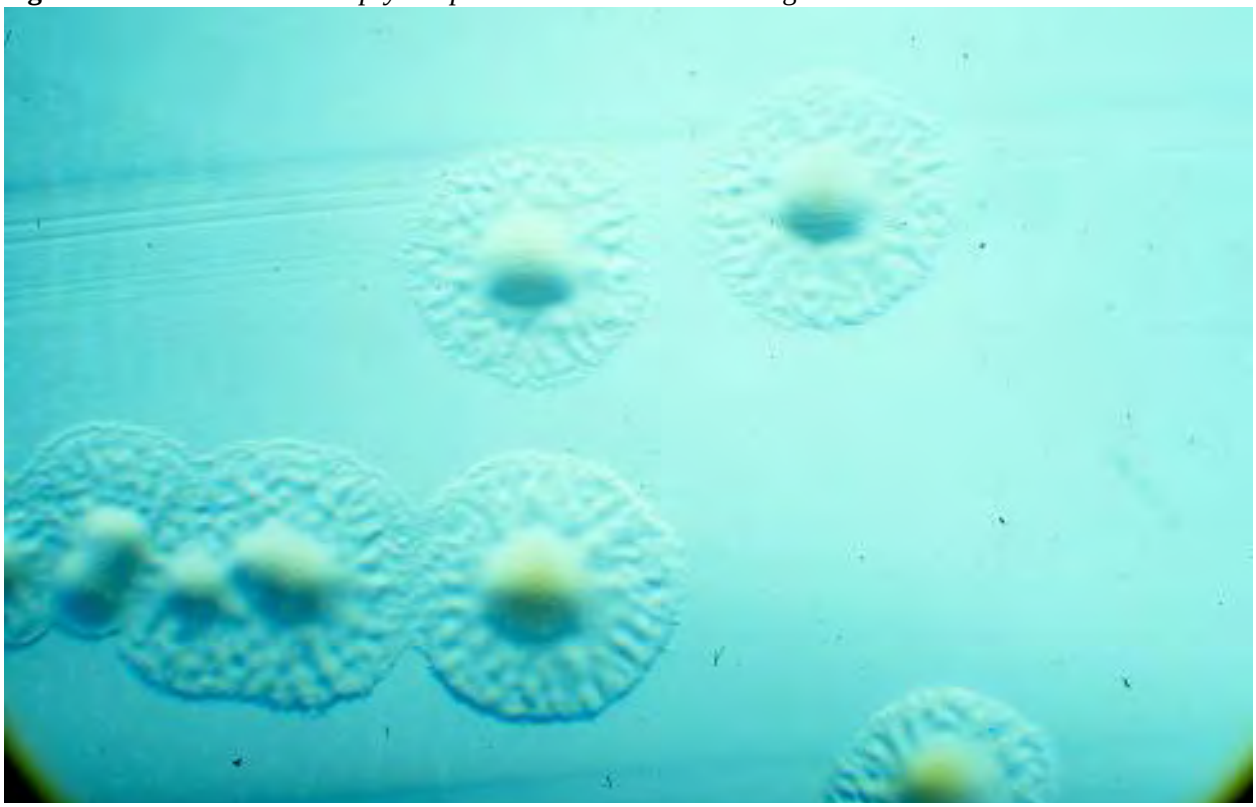


Figure 12. Typical *F. psychrophilum* "fried egg" colony morphology on cytophaga agar.

2. Confirmatory Diagnosis

The diagnosis is confirmed upon positive serological identification by either slide or microtiter agglutination test or indirect FAT. A small percentage of isolates meet all the criteria for a presumptive diagnosis, but fail to react with *F. psychrophilum* antiserum. The polymerase chain reaction assay (PCR) is recommended for confirmation of these isolates and is also available as an alternative to confirmation with serological tests. A nested PCR is available for confirmation of clinical isolations (Taylor and Winton 2002). In recent years there have been several reports of PCR diagnostic confirmation methods for this bacterium (Bader and Shotts 1998; Toyama et al. 1994; Del Cerro et al. 2002; Urdaci et al. 1998).

Current possible sources for *F. psychrophilum* antiserum*:

1. Pete Taylor
   Abernathy Salmon Culture Technology Center
   1440 Abernathy Creek Rd.
   Longview, WA 98632
   Tel (360) 425-6072

2. Kathy Clemens
   U.S.Fish & Wildlife Service
   Dworshak FHC
   PO Box 18, State Highway 7
   Ahsahka, ID 83520-0018
   Tel (208) 476-9500

3. Microtek International Ltd
   6761 Kirkpatrick Crescent,
   Saanichton, B.C.
   Canada V8M 1Z8
   Tel (250) 652-4482
   www.microtek-intl.com/antibodies/polyclonal.html

* Contact the source of the antiserum for information on specificity, sensitivity and recommended working dilution.

F. Procedures for Detecting Subclinical Infections

No procedures have been reported but we expect PCR will be utilized in the future.

G. Procedures for Determining Prior Exposure to Etiological Agent

No serological procedures have been reported.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

See Section 1, 1.1 General Procedures for Bacteriology. *Flavobacterium psychrophilum* isolates can be prepared for long-term storage by culturing in TYES medium containing 0.18% agar, after 48 to 72 hours at 16 to 18°C, homogenizing the growth with a pipet, and then freezing aliquots of the suspension at -70°C. This storage method should provide viable cultures for up to 10 years. Lyophilization in 20% sterile skim milk and then storage of the vials at -20 to -70°C has provided viable cultures after more than 30 years. Some freezer storage methods, which work well for other bacteria, do not seem to work with *F. psychrophilum*. Isolates can be stored for short periods of 1 to 2 months by inoculating the cytophaga or TYES medium containing 0.18% agar, allowing 2 to 3 days of growth at 16 to 18°C and then storing at 4°C.

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1.5 Columnaris Disease

Graham L. Bullock

The Conservation Fund's Freshwater Institute
PO Box 1889
Shepherdstown, WV 25443
304/876-2815

A. Name of Disease and Etiological Agent

Columnaris disease is caused by *Flavobacterium columnare*.

B. Known Geographical Range and Host Species of the Disease

1. **Geographical Range**
   Probably worldwide.

2. **Host Species**
   All freshwater fishes are considered susceptible.

C. Epizootiology

The disease affects fish of all ages and is favored by warm water conditions (14^{o}C). The severity of columnaris is also affected by other environmental factors; for example, the impact of the disease may increase under conditions of low dissolved oxygen or high concentrations of ammonia. Mortality rates can be extremely high, with 60 to 90% mortality common. The precise reservoirs of the pathogen are unclear, but it may occur naturally in the aquatic environment. Large numbers of *Flavobacterium columnare* cells have been isolated from water during epizootics and good survival of the bacterium occurs over a wide range of water pH and hardness.

D. Disease Signs

When highly virulent strains of the bacterium are involved, the fish may die without any gross clinical signs but the pathogen is recoverable from the gills. With strains of lower virulence, external lesions of some diagnostic value are produced (internally, gross lesions are usually absent even though the pathogen may be present). External lesions may occur on the body surface, on the gills, or on both.

On scaled fish, lesions occur initially as greyish-white cutaneous foci on the fins, head, and trunk (Figure 1). The foci may enlarge to be several centimeters in diameter, and skin in the affected area may

be eroded, resulting in shallow ulcers (Figure 2). On the gills, the lesions appear to radiate from a focal point; the affected gill tissue becomes bleached and necrotic, but fusion of the lamellae does not occur. (Figure 3 and Figure 4). Often, the pathogen's yellow-pigmented cells may be present in large enough numbers to color the lesions yellow or orange (Figure 5). On scaleless fish, the center of the lesion appears to be a dark blue area covered by a milky veil with a defined red tinge (due to a hyperemia) around the margin. Sometimes called saddleback, these lesions resemble those caused by a fungus (Figure 6). The yellow pigmentation may also be seen around the edge of the lesion. In aquarium fish species, necrotizing stomatitis is common. This condition is popularly called cotton-wool mouth, and fungi are frequent secondary invaders. The infection may involve the opercula, teeth, maxillae, mandibles, and the spongy bones of the head.



Figure 1. Focal skin lesion caused by columnaris (picture by Andy Goodwin).

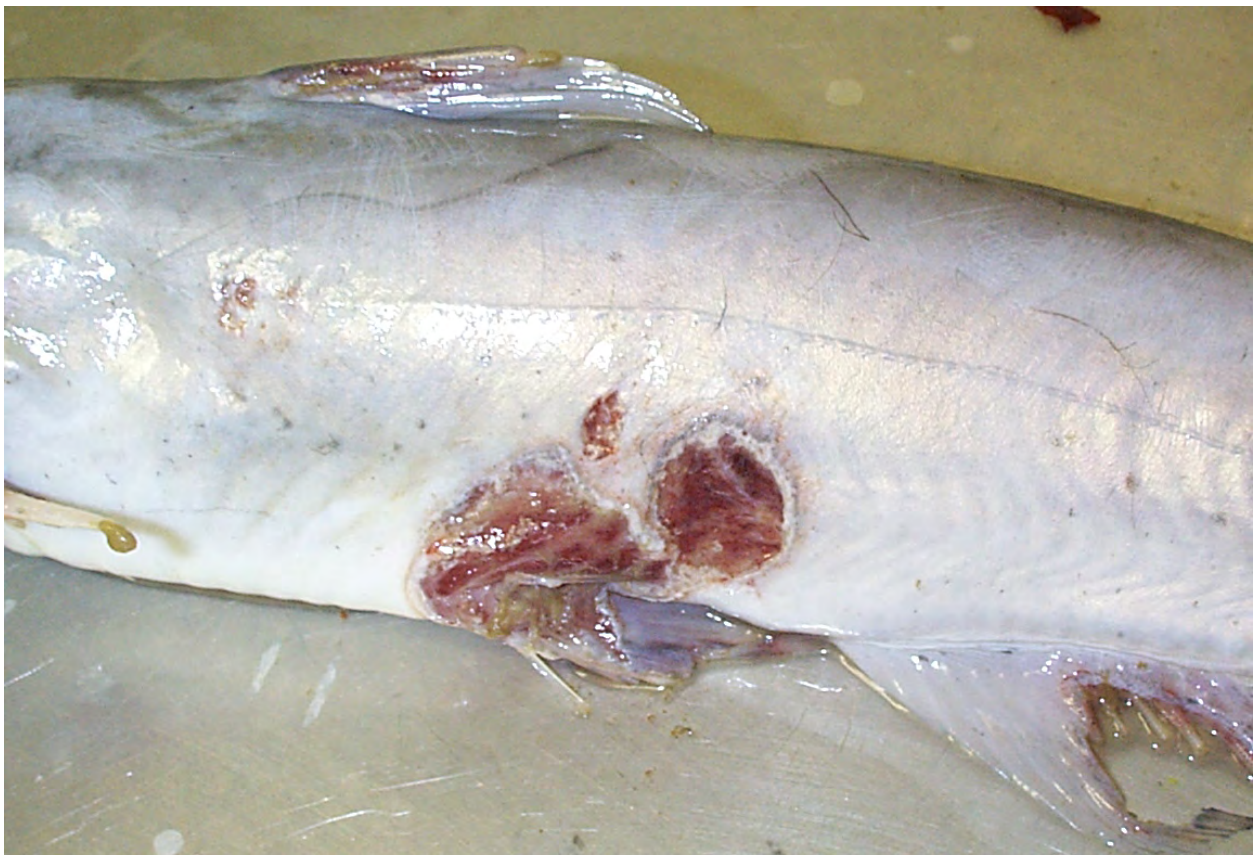


Figure 2. Shallow ulcers in channel catfish skin (picture by Andy Goodwin).



Figure 3. Columnaris gill lesions on a koi (picture by Andy Goodwin).



Figure 4. Columnaris gill lesions on a channel catfish (picture by Andy Goodwin).



Figure 5. Skin lesions on channel catfish. Sufficient columnaris are present to color the lesions yellow (picture by Andy Goodwin).



Figure 6. A bluehead chub with multiple saddleback lesions (picture by Andy Goodwin).

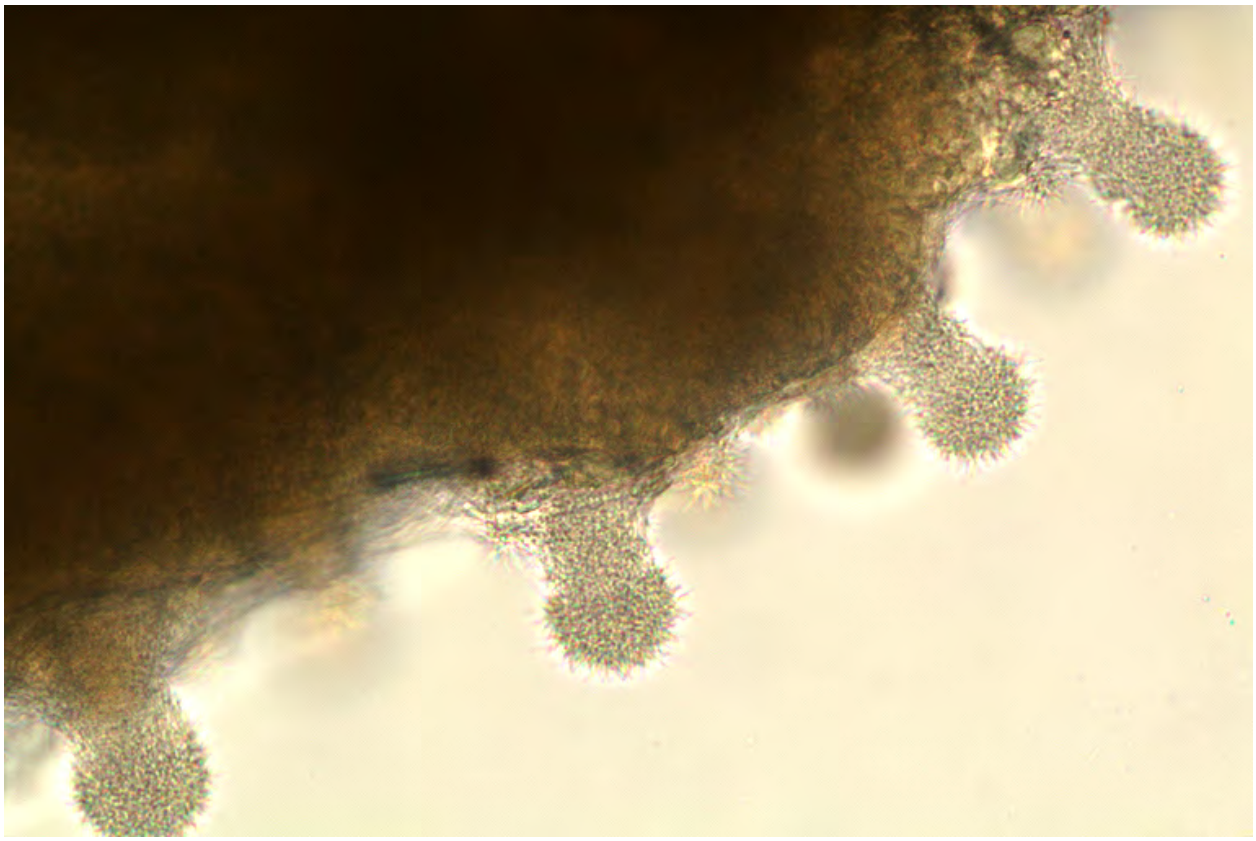


Figure 7. "Hay stacks" of *Flavobacterium columnare* on the gills of a koi(picture by Andy Goodwin).

E. Disease Diagnostic Procedures

1. **Presumptive Diagnosis**

   The following criteria provide a basis for presumptive diagnosis: observation of characteristic clinical signs and the presence of long, thin (5 to 17 x 0.7 um) gram-negative rods in lesions; dry rhizoid, yellowish colonies are produced on cytophaga agar (Anacker and Ordal 1959) after three days incubation at 20^{o}C; cells are motile by gliding or flexing, but lack flagella. The tendency of the columnaris bacterium to form mounds (haystacks) or columns, as detected in wet mounts of diseased tissue, also aids diagnosis (Figure 7). The selective medium developed by Hsu and Shotts (Bullock et al. 1986) enhances isolation of *Flavobacterium columnare* from clinical specimens.

2. Confirmatory Diagnosis

a. Identification of *F. columnare* isolates may be accomplished by screening for: adherence of colonies to agar, colorshift in colonies from yellow to pink in presence of 3% sodium hydroxide, and production of chondroitinase and gelatinase. A PCR procedure is also available.

b. If the diagnostic antiserum is unavailable, an alternate confirmatory procedure is to show that the isolate is identical in its morphological, cultural, and biochemical features with *Flavobacterium columnare*. Descriptions of *Flavobacterium columnare* are provided in several of the papers listed in the references.

F. Procedures for Detecting Subclinical Infections

Because *Flavobacterium columnare* is considered ubiquitous in fresh water, a suitably sensitive detection procedure has not been developed.

G. Procedures for Determining Prior Exposure to the Etiological Agent.

No procedures have been reported.

H. Procedures for Transportation and storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent.

See Section 1, 1.1 General Procedures for Bacteriology.

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1.6 *Edwardsiella tarda* Septicemia

John P. Hawke

Department of Pathobiological Sciences
School of Veterinary Medicine
Louisiana State University
Baton Rouge, LA 70803
225/578-9705
jhawke1@lsu.edu

A. Name of Disease and Etiological Agent

The disease Edwardsiellosis is caused by *Edwardsiella tarda* (Ewing et al. 1965). Synonyms include: emphesematous putrefactive disease of catfish, red disease of eels, and Edwardsiella septicemia of salmon, tilapia, and striped bass.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Edwardsiella tarda is found in both freshwater and brackishwater environments. It has been reported from 25 countries in North and Central America, Europe, Asia, Australia, Africa, and the Middle East (Austin and Austin 1987).

2. Host Species

The bacterium has been isolated from over 20 species of freshwater and marine aquatic animals (Plumb 1999). Natural infections have been reported in channel catfish *Ictalurus punctatus,* carp *Cyprinus carpio,* goldfish *Carassius auratus*, largemouth bass *Micropterus salmoides*, brown bullhead *Ameiurus nebulosus*, chinook salmon *Oncorhynchus tshawytscha*, striped bass *Morone saxatilis,* freshwater eel *Anguilla japonicus*, tilapia *Sarotherodon niloticus*, red sea bream *Chrysophrys major*, and flounder *Paralichthys olivaceus*. Fish species that have shown susceptibility to laboratory challenge include: rainbow trout *Oncorhynchus mykiss*, yellowtail *Seriola quinqueradiata,* and Ioach *Misgurnus anguillicaudatus*. The bacterium also has been found to cause disease in a variety of other animals including seals, sea lions, porpoises, turtles, alligators, snakes, cattle, pigs, and birds. In humans, it causes acute gastroenteritis (Bockemuhl et al. 1971).

C. Epizootiology

In Japan, edwardsiellosis in eels occurs in the warmer months at temperatures near 30°C (Wakabayashi and Egusa 1973; Kuo 1977). In Taiwan, infections in eels can occur when water temperatures are fluctuating between 10 to 18°C in the spring (Liu and Tsai 1982).

Edwardsiella septicemia in most species of fish in the U. S. appears to be favored by high water temperatures (30°C and above), and the presence of high levels of organic matter particularly in catfish ponds. The prevalence of *E. tarda* infections is relatively rare in commercial channel catfish ponds and mortality rates are usually low (~ 5%) when it does occur. However, when infected fish are moved into confined areas such as holding tanks, mortality can reach levels as high as 50%. The reservoir of infection is unclear but the bacterium has been associated with a variety of aquatic invertebrates and aquatic and terrestrial vertebrates. Snakes or fecal contamination of water from human or animal sources may be a source of the bacterium. There is also some speculation that *E. tarda* may comprise a part of the normal microflora of the surfaces of certain fishes. In the U. S., *E. tarda* was isolated from as many as 88% of domestic dressed channel catfish and was found in 30% of imported dressed fish. The bacterium was also found in 75% of catfish pond water samples, 64% of pond mud samples, and 100% of frogs, turtles, and crayfish from catfish ponds (Wyatt et al. 1979).

D. Disease Signs

In channel catfish, the disease can occur as small cutaneous lesions that progress into large abscesses within the muscle (Figure 1 and Figure 2). These abscesses become filled with malodorous gas and necrotic tissue in chronic infections (Meyer and Bullock 1973). The most common manifestation in catfish and other species is generalized septicemia. In rare cases, clinical signs may mimic those of enteric septicemia of catfish caused by *Edwardsiella ictaluri* (Schwedler and Goodwin personal communication). In naturally diseased tilapia, clinical signs include loss of pigmentation, ascitic fluid in the abdominal cavity, and corneal opacity. Small white nodules may be present in the kidney, liver, spleen, and gills (Plumb 1999). Juvenile striped bass were reported to swim listlessly at the surface and exhibited pale gills. Histologically, the trunk kidney contained numerous abscesses (Herman and Bullock 1986). Eye disease has also been observed resulting in blindness in one or both eyes. In eels, two different pathological manifestations of the disease have been described: the suppurative interstitial nephritis form, characterized by abscesses in the kidney and the suppurative hepatitis form, characterized by ulcers and abscesses in the liver (Miyazaki and Egusa 1976a and b). Edwardsiella septicemia in eels is characterized by hemorrhagic fins, petechiae on the belly, swollen protruding anus, and necrotic foci in the internal organs (Wakabayashi and Egusa 1973).

E. Disease Diagnostic Procedures

Diagnosis is based on the observation of clinical signs consistent with the disease and isolation and identification of the etiological agent. Primary isolation should be made from the kidney on either blood agar or TSA and incubated at 30 to 35°C for 24 to 48 hours (Hawke 2000).

1. Presumptive Diagnosis

For presumptive identification, the etiological agent is a short, motile, gram-negative rod that is cytochrome oxidase negative. The isolate should ferment glucose with both acid and gas production, and the TSI (triple sugar iron) reaction should be alkaline slant and acid butt, with gas and H_2S production.



Figure 1. *Edwardiella tarda* septicemia in a channel catfish. Note the large area of hemorrhage surrounding an abscess near the pectoral fin. (Original photo by Fred Meyer.)



Figure 2. Area of muscle necrosis associated with *Edwardsiella tarda* infection in a catfish. (Photo by John Hawke.)

2. Confirmatory Diagnosis

a. A confirmed diagnosis is accomplished if the isolate agglutinates in the slide or microtiter agglutination test with *Edwardsiella tarda* antiserum. The enzyme immunoassay (EIA) and FAT were shown to be fast and efficient for the confirmatory diagnosis of *Edwardsiella tarda* (Rogers 1981).

The EIA method also has the advantage of being inexpensive and adaptable for most labs. Caution should be used in these tests because there are many serotypes. Confirmatory tests may fail with the use of a monovalent antiserum.

b. If a diagnostic antiserum is not immediately available, the isolate may be identified by use of the API 20E system (Analytab Products, Inc., Plainview, New York). *Edwardsiella tarda* should produce reactions leading to the code number 4544000, although there may be some variation depending on the biotype cultured.

F. Procedures for Detecting Subclinical Infection

Culture can be attempted from the internal organs, intestine, or surface tissues of fish. It is not known if species-specific primers are available for PCR analysis.

G. Procedures for Determining Prior Exposure to Etiological Agent

No procedures have been reported.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

See Section 1, 1.1 General Procedures for Bacteriology.

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1.7 Enteric Redmouth Disease

Graham L. Bullock

The Conservation Fund's Freshwater Institute
PO Box 1889
Shepherdstown, WV 25443
304/876-2815

A. Name of Disease and Etiological Agent

Enteric redmouth, ERM, is caused by *Yersinia ruckeri*.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Probably worldwide where salmonids are cultured.

2. Host Species

Potentially all salmonids. Confirmed isolations have been made from Atlantic salmon *Salmo salar*, brown trout *Salmo trutta*, cutthroat trout *Oncorhynchus clarki*, brook trout *Salvelinus fontinalis*, lake trout *Salvelinus namaycush*, rainbow trout and steelhead *Oncorhynchus mykiss*, chinook salmon *Oncorhynchus tshawytscha*, coho salmon *Oncorhynchus kisutch*, and sockeye salmon *Oncorhynchus nerka*. Isolations have also been made in goldfish *Carassius auratus*, cisco *Coregonus* sp., largemouth bass *Micropterus salmoides*, emerald shiners *Notropis atherinoides*, sturgeon *Acipenser baeri*, fathead minnows *Pimephales promelas*, and walleye (*Stizostedion vitreum vitreum*), and channel catfish *Ictalurus punctatus* (Rafinesque). Certain aquatic invertebrates such as crayfish and even mammals, notably muskrats, have been found to harbor large numbers of *Y. ruckeri* cells.

C. Epizootiology

Typically ERM causes sustained low-level mortality that in salmonids increases dramatically if fish are stressed by handling or unfavorable environmental conditions such as crowding or low dissolved oxygen. However, even weighing or moving apparently healthy carrier salmonids can precipitate an epizootic. The most commonly affected size of rainbow trout is 7.5 cm, with chronic infections occurring in larger trout (12.5 cm and larger). The bacterium does not appear to be ubiquitous in nature. Survivors of ERM epizootics become carriers and a regular 36 to 40 days cycle of intestinal shedding of *Yersinia ruckeri* occurs. This cyclic shedding may release large numbers of *Yersinia ruckeri* in the water and precipitate mortality depending on such factors as temperature, loading density, handling, immunity, and resistance of the population. The severity of infection and mortality decrease at water temperatures below 10^{o}C.

D. Disease Signs

The disease may occur as a peracute, acute, subacute, or chronic condition. The clinical signs of acute disease are very similar to those seen in other bacterial septicemias; however, the frequent presence of a reddening (hemorrhage) in the mouth, hemorrhages in the lower intestine, a hypertrophied spleen, and a yellow discharge from the vent may be of some diagnostic value. In chronic infections, the fish are dark, lethargic, and commonly show bilateral exophthalmia, which may progress to rupture of the eye. There may be cutaneous petechiation but the skin is intact. Petechial hemorrhages occur diffusely on (and in) the viscera and musculature.

E. Disease Diagnostic Procedures

Diagnosis is based on observation of characteristic clinical signs and isolation and identification of the causative organism. Primary isolation should be made from the kidney on TSA incubated at 20 to 25^{o}C for 24 to 48 hours.

1. Presumptive Diagnosis

The organism should be a gram-negative, cytochrome oxidase-negative motile rod that ferments glucose anaerogenically. It does not produce indole in tryptone broth and produces an alkaline slant and an acid (only) butt in TSI agar. The isolate should also be negative in salicin and esculin tests to separate it from certain *Serratia* spp. Note: If ERM is strongly suspected, kidney tissue may, in addition, be inoculated onto Shotts-Waltman (SW; Waltman and Shotts 1984) medium, which should then be incubated at 20 to 25^{o}C. The SW medium facilitates the rapid identification of *Yersinia ruckeri*, which will appear as very small, slightly green colonies with an opaque halo (2% of *Aeromonas hydrophila* strains tested will grow on SW, but they will produce large, yellow colonies).

2. Confirmatory Diagnosis

a. Confirmatory testing of the sorbitol-negative Hagerman strain (Type I) is best accomplished serologically by use of the direct FAT with Type I antisera. However, the sorbitol positive, Type II bacteria are more serologically diverse than once presumed. Up to five different sorbitol-positive Type II bacteria have thus far been identified. Antigenic diversity in the lipopolysaccharide antigens of these bacteria precludes reliable serodiagnostic confirmation. However, a recently developed PCR procedure detected serotypes 01, 02, and an unknown serotype. Identification of sorbitol-positive *Yersinia ruckeri* can also be achieved by more detailed biochemical analysis.

b. If anti-*Yersinia ruckeri* serum is not available, the isolate must be morphologically, culturally, and biochemically identical to *Yersinia ruckeri*.

c. To make a diagnosis, a sample of five moribund fish from each affected holding unit is recommended.

F. Procedures for Detecting Subclinical Infections

The chances of detecting covert infections are considerably enhanced if material from the lower intestine is cultured; tissues from other organs do not appear to harbor the pathogen as regularly.

G. Procedures for Determining Prior Exposure to the Etiological Agent.

Prior exposure to *Y. ruckeri* may be achieved by examination of historical records of a facility, culture of fecal material on selective or differential media, or a PCR test on blood samples.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent.

See Section 1, 1.1 General Procedures for Bacteriology.

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1.8 Enteric Septicemia

Emmett B. Shotts, Jr. and John A. Plumb*

College of Veterinary Medicine
University of Georgia
Athens, GA 30602
404/542-5811
emshotts@alltel.net

*Department of Fisheries and Allied Aquacultures
Auburn University
Auburn, AL 36849-5419
205/826-4786
jplumb@ag.auburn.edu

A. Name of the Disease and Etiological Agent

Enteric septicemia, enteric septicemia of catfish, ESC, and "hole-in-the-head disease," is caused by *Edwardsiella ictaluri.*

B. Known Geographical Range and Host Species of the Disease

1. Geographic Range

Found in the contiguous United States, Thailand, and Australia.

2. Host Species

Natural infections have occurred in channel catfish *Ictalurus punctatus*, white catfish *Ameiurus catus*, black bullhead catfish *Ameiurus melas*, yellow bullhead catfish *Ameiurus natalis*, brown bullhead catfish *Ameiurus nebulosus*, danio *Danio devario*, walking catfish *Clarias batrachus*, green knifefish *Eigenmannia virescens*, and blue tilapia *Tilapia aurea.* Experimental infections have been established in salmonids.

C. Epizootiology

Primarily fingerling but also production and adult channel catfish may be affected by the so-called "hole-in-the-head disease," which results from the uptake of organisms from water or mud and subsequent progression of the infection along the olfactory stem to the brain. Such infections are visible as longitudinal lesions between the eyes and occur when water temperatures are 20 to 30°C. Smaller fish contract an enteric form of the disease from ingestion of contaminated tissues. The bacteria overwinter in carrier fish, in the forebrain and hindgut, at very low prevalences (1% or less of the population). The bacterium survives less than eight days in pond water.

D. Disease Signs

Channel catfish infected with *Edwardsiella ictaluri* refuse feed, and swim at the surface with a spiral movement that includes erratic bursts. External lesions include hemorrhage around the mouth, on lateral and ventral portions of the body, and the on the fins (Figure 1 and Figure 2). Pale gills, exophthalmia, and small ulcerations on the body are additional signs. Ulceration in the fontanelle of the frontal bones gives the diseases its common name, "hole-in-the-head disease" (Figure 3). Internally, petechiae are noted throughout the visceral mass and in the peritoneum and musculature. Red and pale mottling of the liver (Figure 4) due to granulomatous inflammation and hemorrhage (Figure 5) is common. Ascites and enlargement of the liver, kidney, and spleen are sometimes observed (Figure 6 and Figure 7). Fish overwintering with *E. ictaluri* may show small white ulcerations on the body surface. These are probably disease survivors and the ulcerations are probable sites of healing (Figure 8). Danios infected with *Edwardsiella ictaluri* swim erratically in a spinning motion. Gross lesions have not been described in these fish.



Figure 1. Channel catfish with petechial hemorrhages of the skin.



Figure 2. Catfish with larger red-rimmed shallow ulcers, the characteristic "buckshot" lesion (picture by Andy Goodwin).



Figure 3. Channel catfish “hole-in-the-head disease” lesion (Picture by Jon Stein).

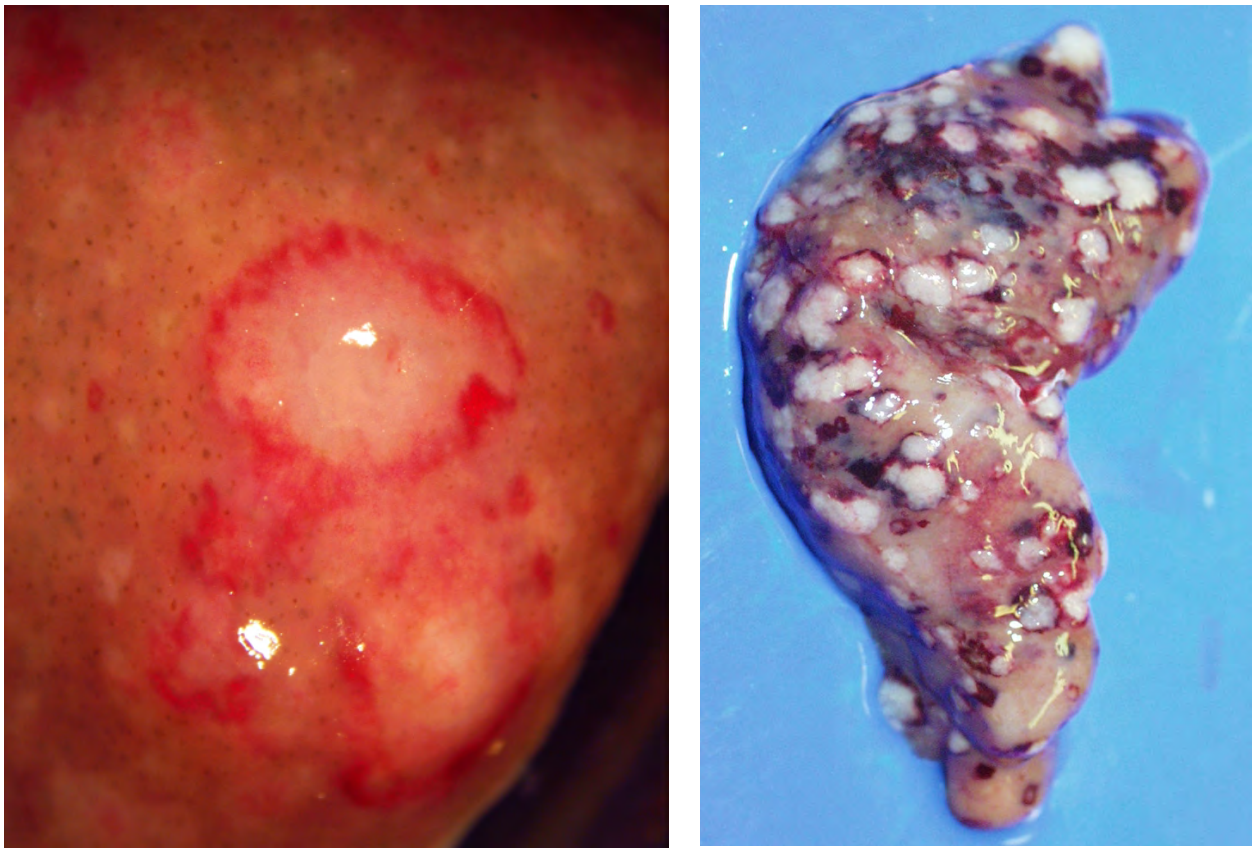


Figure 4. Typical (left) and very severe (right) red and white mottling in the livers of catfish with ESC (pictures by Andy Goodwin).

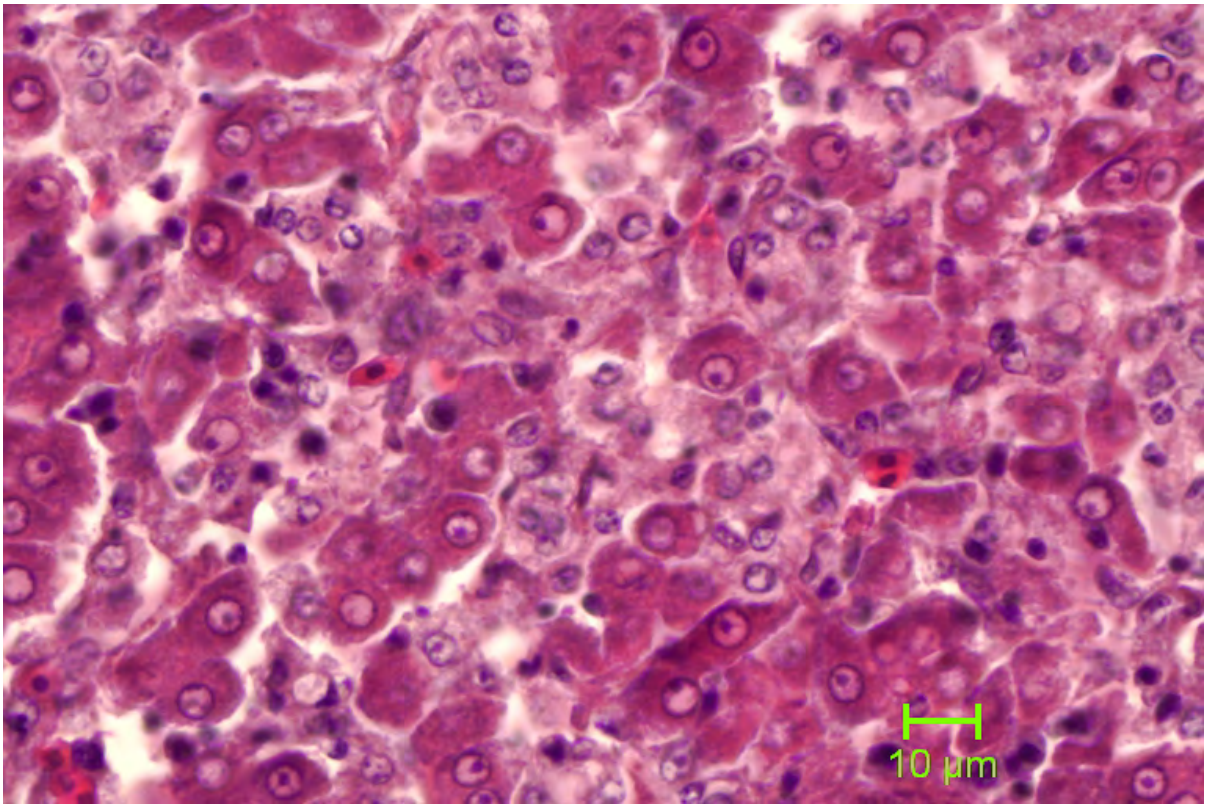


Figure 5. Granulomatous exudate (pale pink cells) in the liver of a channel catfish with ESC (picture by Andy Goodwin).



Figure 6. Channel catfish fingerlings with severe ascites (picture by Andy Goodwin).

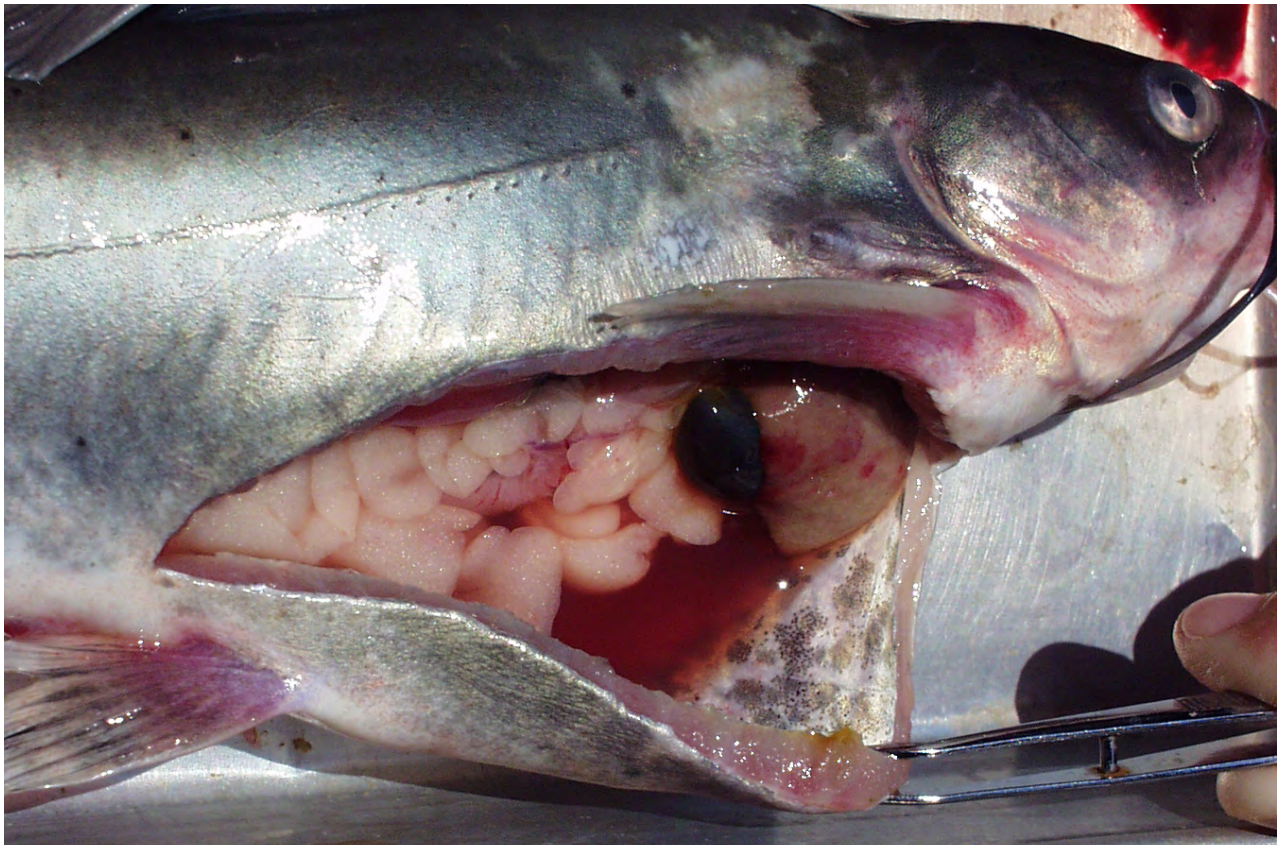


Figure 7. Hemorhagic exudate and hepatic edema in a channel catfish with ESC (picture by Andy Goodwin).



Figure 8. Shallow white ulcers on the skin if a catfish with ESC (picture by Andy Goodwin).

E. Disease Diagnostic Procedures

Diagnosis is based on the observation of characteristic clinical signs and the isolation and identification of the etiological agent. Primary isolation should be made from either kidney or head lesion (brain) inoculated onto TSA, McConkey agar, blood agar, or EIM (*Edwardsiella* isolation medium; Shotts and Waltman 1990), which is incubated at 30 to 35°C for 2 to 4 days.

1. **Presumptive Diagnosis**
   The etiological agent should be a short, gram-negative, cytochrome oxidase-negative rod. No indole is produced in tryptone broth. It grows slowly (2 to 4 days at 25°C) and sparsely at 37°C. It is nonmotile or weakly motile and does not produce H_2S.

2. **Confirmatory Diagnosis**
   A diagnosis is confirmed if the isolate is agglutinated in the slide or microtiter agglutination test with *Edwardsiella ictaluri* antiserum, or by the demonstration of specific fluorescence with the FAT. An enzyme immunoassay (EIA) has been developed (Rogers 1981).

F. Procedures for Detecting Subclinical Infection

Culture from the forebrain and hindgut can be attempted.

G. Procedures for Determining Prior Exposure to Etiological Agent

No procedures have been reported.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

See Section 1, 1.1 General Procedures for Bacteriology.

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1.9 Furunculosis

Emmett B. Shotts, Jr.

College of Veterinary Medicine
University of Georgia
Athens, GA 30602
404/542-5811
emshotts@alltel.net

A. Name of Disease and Etiological Agent

Furunculosis is caused by *Aeromonas salmonicida.*

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

This disease is known to occur in North America, South America, Europe, Asia, and Africa.

2. Host Species

Although it is usually associated with freshwater fishes, marine fishes are also considered to be susceptible.

C. Epizootiology

This organism has been known for almost 100 years, but the exact route of transmission is not completely understood. The primary points of controversy are: 1) the ability of the organism to exist in the natural environment outside the fish host, 2) the role of the natural environment in transmission, and 3) the mechanism(s) of entry into the fish. It is known that the organism can be transmitted horizontally both among and within fish populations, and is present at extremely low levels in carrier fish. However, vertical transmission of the bacterium has not yet been shown to occur in naturally infected fish populations. Studies on the ecology of the pathogen have been hampered by the lack of a suitable sensitive selective medium, although the differential Coomassie Brilliant Blue agar medium (Markwardt et al. 1989) may prove useful.

The occurrence of clinical disease is known to follow generally the seasonal temperature patterns. At water temperatures of 20^{o}C, susceptible fishes can develop furunculosis within 4 to 12 days after viable bacteria are released into their water supply. At temperatures below about 13^{o}C, chronic infections are more likely to develop, with incubation periods up to several weeks. At temperatures below 8 to 9^{o}C, overt disease signs may never develop in infected fish. Adverse conditions such as high water temperatures (within the limits tolerated by the bacterium) and low dissolved oxygen may precipitate clinical disease. There is some evidence that the organism can be transmitted in seawater.

D. Disease Signs

Typical furunculosis in salmonids is caused by *Aeromonas salmonicida* var. *salmonicida* and may occur in one of several forms:

1. **Peracute**
   Noted especially in fingerlings; the fish usually appear dark and die readily. Internally, the gross pathological changes resemble the acute disease.

2. **Acute** (not size-specific)
   Generally some indication of disease is noted 2 to 3 days before mortality (fish darken and go off feed). Internally, the viscera are hemorrhagic, the kidney tissue is very soft, the spleen is enlarged, and the liver is pale or mottled with petechiae.

3. **Subacute**
   The onset of mortality is more gradual. Internal lesions are present but the fish also commonly have skin lesions.

4. **Chronic**
   Similar to subacute, but is distinguished by evidence of healing around lesions.

5. **Latent**
   No mortality or clinical signs associated with *Aeromonas salmonicida* infections are evident.



Figure 1. Skin lesions typical of chronic furunculosis.

Histopathological examination of fish with acute infections often reveals foci of bacteria in the heart, kidneys, and spleen, and in the vasculature of other organs. Hematopoietic tissue necrosis, focal hepatic necrosis, and degeneration of myocardial and renal tubular tissues are often observed. In chronic disease, the heart and spleen are the organs most consistently affected. In fact, the presence of large colonies of small rod-shaped bacteria within the myocardial trabeculae is considered almost pathognomonic for furunculosis in salmonids in fresh water. The “furuncles” occurring in the skeletal musculature of some chronically diseased fish consist of necrotic tissue, tissue fluid exudate, and some macrophages. These lesions differ from true furuncles of homoiothermic vertebrates, which characteristically contain numerous polymorphonuclear leukocytes in addition to the necrotic debris and tissue fluid.

E. Disease Diagnostic Procedures

Diagnosis is based on the observation of clinical signs characteristic of the disease, and isolation of the causative organism. Primary isolation is best made from the kidney either on TSA or BHIA at 20 to 25^{o}C for 24 to 48 hours. There is some indication that certain strains of *Aeromonas salmonicida* cannot be readily grown on TSA.

1. Presumptive Diagnosis

When grown on the above media, the organism is a very short (1-2 x 0.8 μm), nonmotile, gram-negative rod that is oxidase positive, glucose positive, and gelatinase positive. Most strains produce a brown diffusing pigment. An oxidase-negative isolate has been described (Chapman et al. 1991).

Several immunological techniques have been used for the rapid diagnosis of furunculosis directly in the tissues of clinically diseased fish. These techniques include latex bead agglutination, staphylococcal coagglutination and the enzyme- linked immunosorbent assay (ELISA, Austin et al. 1986).

2. Confirmatory Diagnosis

Aeromonas salmonicida isolated in culture can be identified rapidly by serological procedures such as the FAT or agglutination tests (microtiter or slide). Care must be exercised in the use of agglutination tests, because many strains of *Aeromonas salmonicida* autoagglutinate in saline. Modified agglutination tests, such as latex bead agglutination (McCarthy 1975b) or staphylococcal coagglutination (Kimura and Yoshimizu 1983, 1984), have been used to avoid the problem of autoagglutination. The organism can also be identified by extensive phenotypic identification. In some cases this may be desirable because several subspecies of *Aeromonas salmonicida* are described in the current Bergey's Manual of Systematic Bacteriology (Holt et al. 1984).

F. Procedures for Detecting Subclinical Infections

Detection of latent furunculosis is significantly enhanced by the FAT and culture of intestinal material. The kidney should be used as a second organ for culture.

G. Procedures for Determining Prior Exposure to the Etiological Agent

No reliable methods are available at present.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

See Section 1, 1.1 General Procedures for Bacteriology.

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1.10 Other Diseases Caused by *Aeromonas salmonicida*

Emmett B. Shotts, Jr.

College of Veterinary Medicine
University of Georgia
Athens, GA 30602
404/542-5811
emshotts@alltel.net

Variants of *Aeromonas salmonicida* produce diseases other than furunculosis, including goldfish ulcerative disease, carp erythrodermatitis, trout ulcer disease, and systemic infections among several warmwater and marine species. Like typical strains of *Aeromonas salmonicida*, these atypical variants are nonmotile, oxidase-positive rods that ferment glucose. However, the atypical strains vary in certain other biochemical characteristics and may not produce water-soluble brown pigment.

A. Goldfish Ulcer Disease

Goldfish ulcer disease (GUD) causes serious losses on commercial goldfish *Carassius auratus* farms. Because of extensive fish transfers, there are few or no sources of goldfish in the USA that are GUD-free. Bacterial involvement is predominantly external. The infection usually starts as white tufts on the skin or a fin and develops into large, open necrotic lesions. A bacteremia is generally not caused by *Aeromonas salmonicida*; if one develops, it is usually caused by opportunistic bacteria that gain entry through the skin lesions.

B. Carp Erythrodermatitis

Carp erythrodermatitis (CE) is a subacute to chronic skin disease that occurs at 4 to 30^{o}C and was originally associated with carp dropsy syndrome. Grass carp *Ctenopharyngodon idella* and silver carp *Hypophthalmichthys molitrix* are the most sensitive of the affected species. The first sign of CE is one or more small inflamed hemorrhagic areas, which develop into ulcers. The causative bacterium is present exclusively in lesions between the dermis and epidermis.

C. Trout Ulcer Disease

Trout ulcer disease starts as epithelial thickenings that enlarge to white tufts and eventually form well-defined ulcers. In acute infections, there may be no ulcers present and internal pathological changes are similar to typical furunculosis.

Diagnostic procedures for atypical *Aeromonas salmonicida* infections are similar to those for furunculosis. However, the use of BA or some other serum-containing medium is recommended for the isolation of fastidious strains. In cases of apparent systemic disease, primary isolation should be made from the kidney. For fish exhibiting skin lesions, material from the outer edges of the lesions should be inoculated into the bacteriological medium (the central areas of open ulcers are often contaminated with opportunistic organisms). For the identification of atypical *Aeromonas salmonicida* isolates, refer to Holt et al. (1984) or one of the references below. The most common characteristic differentiating atypical *Aeromonas salmonicida* from typical strains is a lack of (or delayed) production of brown diffusible pigment in culture. Atypical isolates also differ from typical isolates in other characteristics such as the ability to ferment certain carbohydrates, but the differences observed will depend upon the atypical isolate tested (Austin and Austin 1987). Immunological procedures such as the FAT or modified agglutination tests can be used for confirmatory identification of atypical isolates as *A. salmonicida* (see Section 1, 1.9 Furunculosis).

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1.11 Motile *Aeromonas* Septicemia

Emmett B. Shotts, Jr.

Department of Veterinary Medicine
University of Georgia
Athens, GA 30602
404/542-5811
emshotts@alltel.net

A. Name of Disease and Etiological Agent

1. Name of Disease

Motile aeromonas septicemia (MAS) is known as bacterial hemorrhagic septicemia (BHS), hemorrhagic septicemia, and other synonyms.

2. Etiological Agent

Aeromonas hydrophila, Aeromonas formicans, Aeromonas liquefaciens, and *Aeromonas hydrophila* complex.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

This disease is worldwide.

2. Host Species

All species of fishes, as well as frogs, turtles, and snakes are susceptible to diseases caused by motile aeromonads. There has been increased interest in *Aeromonas* spp. as a cause of various human disease problems.

C. Epizootiology

The epizootiology has not been extensively studied. Research suggests that these organisms are present in the water column and are associated with fish and other aquatic life. The organism is prominently associated with disease in stress conditions. The disease is usually worse at water temperatures above 10^{o}C, in poor water quality situations (e.g. in water with low dissolved oxygen or a high content of organic matter), and where pollutants are present. Stress or injury from handling, parasites, or poor overwintering conditions may also result in MAS.

D. Disease Signs

The disease is usually noted as a generalized septicemia indistinguishable from that caused by other gram- negative rods. Externally, the disease may range from peracute (mortality, but no gross lesions) to acute with hemorrhaging of the gills and vent. Internally, hemorrhaging of various organs along with the presence of blood-tinged fluid in the body cavity may be noted. Chronic cases may occur where abscesses, or ulcers, or both, are evident (Figure 1 and Figure 2). Histopathologically, the disease is similar to other generalized septicemic diseases caused by gram-negative rod-shaped bacteria.



Figure 1. Ulcer and discoloration in the skin of a hybrid striped bass with MAS (picture by Andy Goodwin).



Figure 2. Ulcers in the skin of channel catfish with MAS (picture by John Plumb).

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

Diagnosis is based on observation of characteristic clinical signs and the isolation and identification of the etiological agent. Initial isolation should be made from the posterior kidney utilizing either a nonselective (e.g. TSA) or a selective medium e.g. (RS medium; Shotts and Rimler 1973). The incubation temperature and/or time may vary with the medium used; however, ideal conditions would be 20 to 25^{o}C for 24 to 48 hours unless otherwise indicated. Isolates selected for further consideration should be: (1) cytochrome oxidase-positive (when grown on media such as TSA that are free of sugars), (2) glucose fermenters, and (3) gram-negative rods.

2. Confirmatory Diagnosis

Confirmation of the identity of an isolate may be obtained by a further battery of tests. An isolate should be: arginine dihydrolase positive, ornithine decarboxylase negative, lysine decarboxylase variable, 0/129-resistant, novobiocin-resistant, and motile. Serological identification is difficult because the bacteria have a wide range of antigenic properties.

F. Procedures for Detecting Subclinical Infections

Isolation of *Aeromonas hydrophila* from kidney tissue of an asymptomatic fish would indicate a subclinical infection.

G. Procedures for Determining Prior Exposure to the Etiological Agent

Whereas previous exposure to *Aeromonas hydrophila* may result in an immune response, this group of organisms is antigenically heterogeneous, and the antibodies produced are not sufficiently characterized at this time to determine if a response can be reliably monitored.

H. Procedures for Transportation and Storage of Samples To Ensure Maximum Viability and Survival of the Etiological Agent

See Section 1, 1.1 General Procedures for Bacteriology.

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1.12 Vibriosis

Jack Ganzhorn

Peninsula College
1502 E. Lauridsen Blvd.
Port Angeles, WA 98362
206/452-9277
jackg@pcadmin.ctc.edu

A. Name of the Disease and Etiological Agent

Vibriosis is caused by bacteria of the genus *Vibrio*. This section deals with *Vibrio anguillarum* and *Vibrio ordalii* which have routinely been associated with vibriosis in North America.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Vibriosis occurs throughout the world primarily in the marine environment. While *Vibrio anguillarum* is widespread, *Vibrio ordalii* has been primarily observed in Japan and North America.

2. Host Species

With respect to fin fish, there appears to be no known limits to the host range of this genus. *Vibrio anguillarum* has been isolated from a wide array of species.

C. Epizootiology

Water-borne infection is the primary means of fish to fish transmission. Bacteria are shed from the vent and open lesions. Portal of entry is through the integument with the gills probably being a very common entry site.

D. Disease Signs

Vibriosis may take many forms depending on which *Vibrio* species is involved, the host, and the environmental circumstances. The following descriptions are of disease processes that are common in North America with *Vibrio anguillarum* and *Vibrio ordalii*.

1. *Vibrio anguillarum*

External signs often include erythema and hemorrhaging at the base of fins, the vent, and around or in the mouth. Petechiae in the musculature and hemorrhaging of the gills may be observed. Ulcerative hemorrhagic lesions often develop in later stages of the disease. Internally, hemorrhaging

and erythema can be observed in the organs.

Liquefactive necrosis of the internal organs may develop in some cases. The bacteria will typically be dispersed throughout host tissues. Leukopenia has been associated with *V. anguillarum* infections. Peracute infections may exhibit very high mortality but the moribund and dead fish may show few of these clinical signs.

2. *Vibrio ordalii*

Gross external and internal pathology is similar to that caused by *V. anguillarum*. The major difference with *V. ordalii* is histopathological. The bacteria are less dispersed in the host and primarily infect cardiac and skeletal muscle, gill tissue, and the gastro-intestinal tract including the pyloric caeca. *Vibrio ordalii* has been observed to form micro-colonies within infected tissues. Leukopenia has been associated with *V. ordalii* infections.

E. Disease Diagnostic Procedures

In addition to observing typical signs, diagnosis of vibriosis is based on identification of the causative agent. Normally, *Vibrio* species can be readily isolated on a standard bacteriological medium like tryptic soy; however, the addition of sodium chloride to a concentration of 1 to 1.5% often facilitates initial isolation. The kidney is the primary organ for bacteriological culture; however, *V. anguillarum* can also be found in the blood, loose connective tissue, spleen, gills, posterior intestinal tract, or external lesions. *Vibrio ordalii* can be found in skeletal muscle, gill tissue, gastro-intestinal tract, as well as in the kidney. *Vibrio anguillarum* and *V. ordalii* cultures are often isolated at incubation temperatures of 20 to 25^{o} C.

1. Presumptive Diagnosis

The genus, *Vibrio*, is presumptively diagnosed on the basis of the following characteristics.

a. Gram stain reaction: negative

b. Cell morphology: short (0.5 to 3.0 μm), curved rods; motile by polar flagella

c. Cytochrome oxidase reaction: positive

d. Glucose O/F medium: fermentative with no gas produced

2. Confirmatory Diagnosis

Confirmatory diagnosis is based on a sensitivity to vibriostatic agent 0/129 (2,4-diamino 6,7-diisopropyl pteridine phosphate) and novobiocin. Identification of specific species is determined serologically, for example, the slide agglutination test or the fluorescent antibody test may be used.

F. Procedures for Detecting Subclinical Infections

Bacteria of the genus *Vibrio*, including *Vibrio anguillarum*, are often observed or cultured from the gastro-intestinal tract of normal appearing marine fish. It has not been apparent whether these situations represent subclinical or latent infections. It has been demonstrated that non-pathogenic strains of *V. anguillarum* occur commensally as members of the normal intestinal bacteria.

G. Procedures for Determining Prior Exposure to the Etiological Agent

No procedures for determining prior exposure are routinely used.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Samples should be placed in individual sealed plastic bags and labeled appropriately for identification. They should be transported on ice or under refrigeration. Samples should not be stored for more than 72 hours.

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1.13 Coldwater Vibriosis

Jack Ganzhorn

Peninsula College
1502 E. Lauridsen Blvd.
Port Angeles, WA 98362
206/452-9277
jackg@pcadmin.ctc.edu

A. Name of the Disease and Etiological Agent

Coldwater vibriosis or Hitra disease is caused by *Vibrio salmonicida*.

B. Known Geographical Range and Host Species of the Disease

1. **Geographical Range**
   Vibrio salmonicida has only been observed in the marine waters of Norway, Scotland, and Eastern Canada.

2. **Host Species**
   Coldwater vibriosis has been observed in Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss*.

C. Epizootiology

Coldwater vibriosis typically occurs when water temperatures are below 10°C. The disease has been observed in smolts, juveniles, and adults. Water-borne infection is probably the primary means of fish to fish transmission.

D. Disease Signs

Typical external signs may include hermorrhaging of the skin, the area around the gills, and the vent. Internally, hemorrhaging may be evident in all the organs and at times, in the muscle. The liver may be pale. Histologically, necrosis can be observed in the kidney, muscle, gastro-intestinal tract, spleen, and gills. Gross pathological changes may be absent in some cases.

The coldwater vibriosis that occurred in Canada was in Atlantic salmon and was characterized by extensive ascites with associated visceral hemorrhaging. Significant losses due to the disease were not observed.

E. Disease Diagnostic Procedures

In addition to observing typical signs, diagnosis of coldwater vibriosis is based on identification of the causative agent. *Vibrio salmonicida* can be grown on standard bacteriological media like tryptic soy with added sodium chloride. Nutrient agar supplemented with 5% sheep blood and 1 to 1.5% sodium chloride is often used for primary isolation. Optimum growth has been observed at a medium salinity of 1.5% sodium choride and an incubation temperature of 15°C. The bacteria can be isolated from a variety of internal organs and from ascites.

1. Presumptive Diagnosis

The genus, *Vibrio*, is presumptively diagnosed on the basis of the following characteristics.

a. Gram stain reaction: negative

b. Cell morphology: short (0.5 to 3.0μm), curved rods; motile by polar flagella

c. Cytochrome oxidase reaction: positive

d. Glucose O/F medium: fermentative with no gas produced

2. Confirmatory Diagnosis

Confirmatory diagnosis is based on a sensitivity to vibriostatis agent 0/129 (2,4-diamino 6,7-diisopropyl pteridine phosphate) and novobiocin. Identification of the specific species *Vibrio salmonicida* is determined serologically, for example, the slide agglutination test or the fluorescent antibody test may be used.

F. Procedures for Detecting Subclinical Infection

No procedures for detecting latent infections are routinely used.

G. Procedures for Determining Prior Exposure to the Etiological Agent

No procedures for determining prior exposure are routinely used.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Samples should be placed in individual sealed plastic bags and labeled appropriately for identification. They should be transported on ice or under refrigeration. Samples should not be stored for more than 72 hours.

References

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1.14 Bacterial Kidney Disease

Ronald J. Pascho and Diane G. Elliott

U. S. Geological Survey, Biological Resources Discipline
Western Fisheries Research Center
6505 NE 65th Street
Seattle, WA 98115
206/526-6282
ron_pascho@usgs.gov
diane_elliott@nbs.gov

A. Name of the Disease and Etiological Agent

Bacterial kidney disease (BKD) is caused by *Renibacterium salmoninarum.*

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

This disease is found in North America, Chile, Europe (including Iceland), and Japan.

2. Host Species

All salmonids are considered susceptible. In addition, experimental infections have been established in sablefish *Anoplopoma fimbria*, and Pacific herring *Clupea pallasi*.

C. Epizootiology

Renibacterium salmoninarum can be transmitted both vertically and horizontally, and the bacterium has been detected in both wild fish and hatchery populations. Infections can occur at any life stage in a salmonid population, but clinical signs of disease are uncommon in fish less than six months old. Bacterial kidney disease occurs over a wide range of temperatures. Mortality has been reported in experimentally infected salmonids at temperatures between 4°C and 20.5°C, with the disease progressing most rapidly at the higher temperatures tested (15.0 to 20.5°C), but with the highest mortality at temperatures between the two extremes. Fish cultural practices may influence the progress of the disease in a hatchery. For example, there are indications that certain components of diets fed to hatchery salmonids may decrease their susceptibility to BKD, but further research is needed to define precisely the relation between diet and BKD susceptibility. Anadromous salmonids that are infected with *R. salmoninarum* in fresh water may continue to die of BKD after entry into seawater. Infected salmonid populations are believed to be the principal reservoir of infection.

D. Disease Signs

Acute and subacute forms of the disease occur only sporadically. More typically, the disease is a chronic one, frequently characterized internally by a large edematous kidney that may appear gray and corrugated. The kidney often exhibits off-white lesions that vary in size and number (Figure 1). These lesions sometimes occur in other organs, chiefly the liver and spleen. An opaque false membrane may cover the kidney, liver, spleen, or gonads. A turbid fluid is often present in the abdominal and pericardial cavities. Externally, the clinical signs are of less diagnostic value. Fish may appear normal or they may show one or more of the following signs: exophthalmos (Figure 2), abdominal distension (Figure 3), skin petechiation, and vesicles in the skin. Although BKD is generally considered to be a systemic infection, more localized forms may occur, such as postorbital infections in Pacific salmon (Figure 2), and the dermatitis known as "spawning rash" in rainbow trout (Figure 4).

Histologically, typical BKD is classified as a systemic, chronic granulomatous inflammatory reaction, characterized by a proliferation of macrophages and fibroblasts in areas of infection. The granulomas may be discrete or diffuse (Figure 5), but are rarely encapsulated. Central areas of necrosis are common. Gram or Giemsa staining of tissue sections (Figure 6 and Figure 7) reveals both extracellular bacteria and bacteria within macrophages.

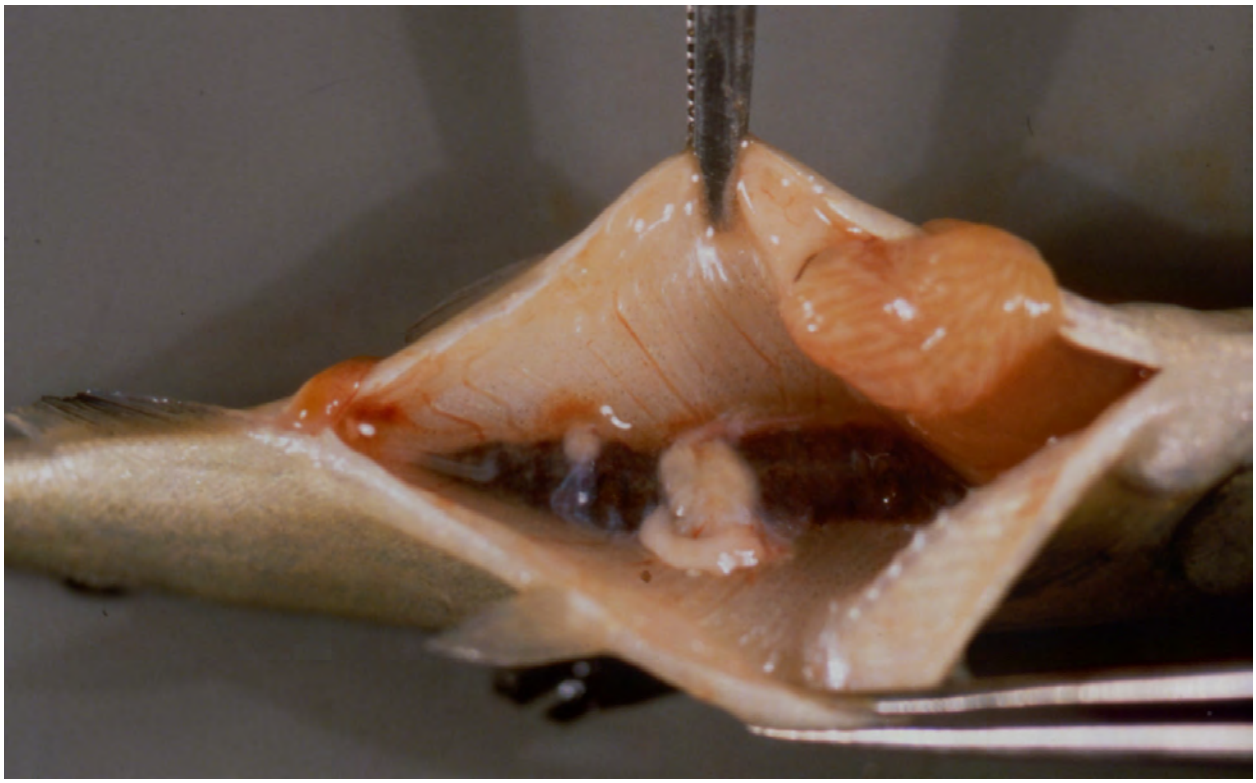


Figure 1. Kidney lesions in a juvenile chinook salmon *Oncorhynchus tshawytscha* affected by BKD. Photo courtesy of Dr. Caroline O'Farrell.



Figure 2. Exophthalmos and swollen narial tissues in juvenile chinook salmon *Oncorhynchus tshawytscha* affected by BKD. Many fish in this population had focal BKD lesions associated with coded-wire tags injected into the snouts.



Figure 3. Abdominal distension associated with ascites in a juvenile chinook salmon *Oncorhynchus tshawytscha* affected by BKD.



Figure 4. Lateral view (top) and ventral view (bottom) of an adult chinook salmon *Oncorhynchus tshawytscha* with dermatitis ("spawning rash") caused by *Renibacterium salmoninarum*.

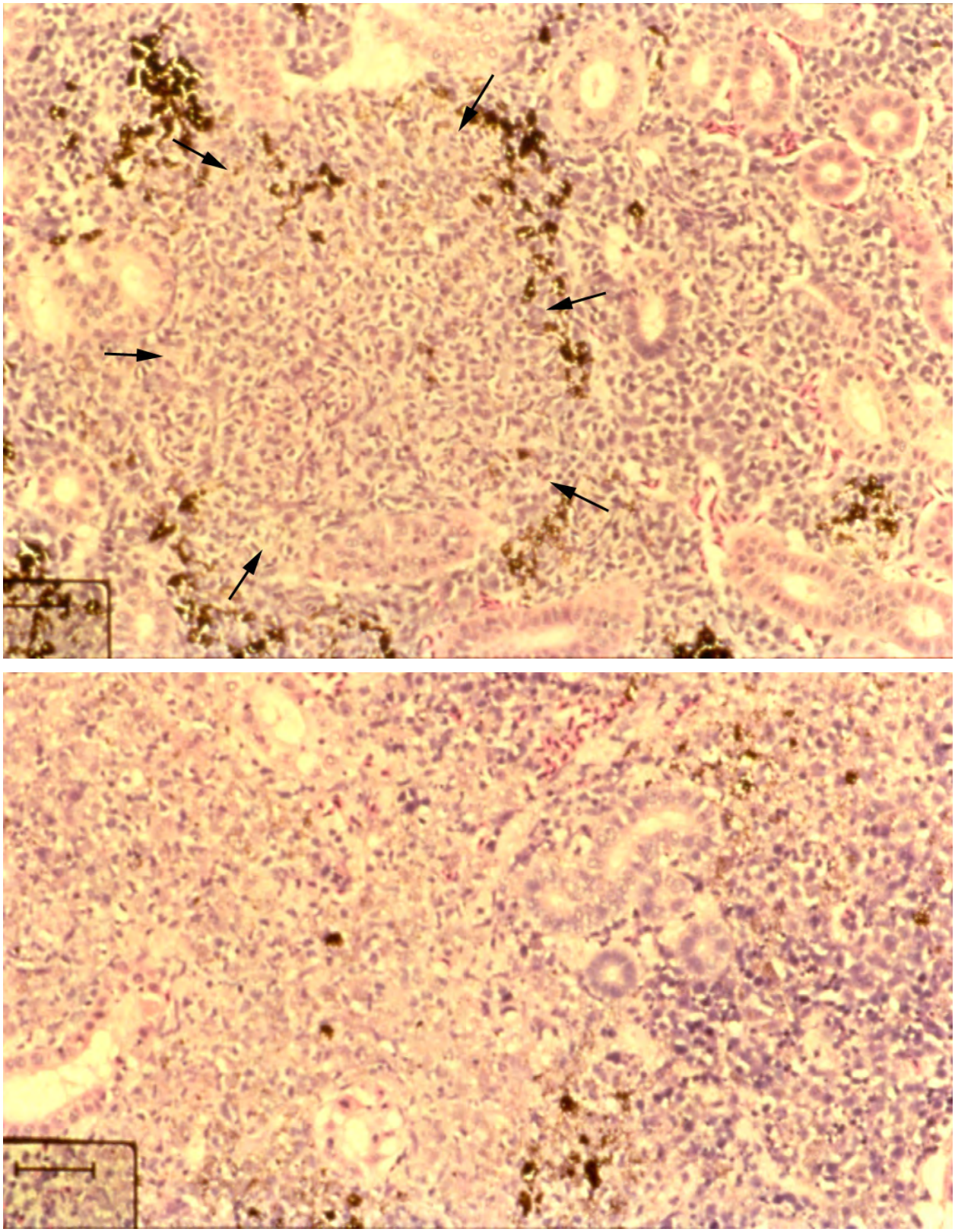


Figure 5. Focal (top photo, arrows) and diffuse (bottom photo) granulomas in the posterior kidney of juvenile chinook salmon *Oncorhynchus tshawytscha* with BKD. Hematoxylin and eosin stain; scale bars = 50 µm. Photos courtesy of Dr. Caroline O'Farrell.

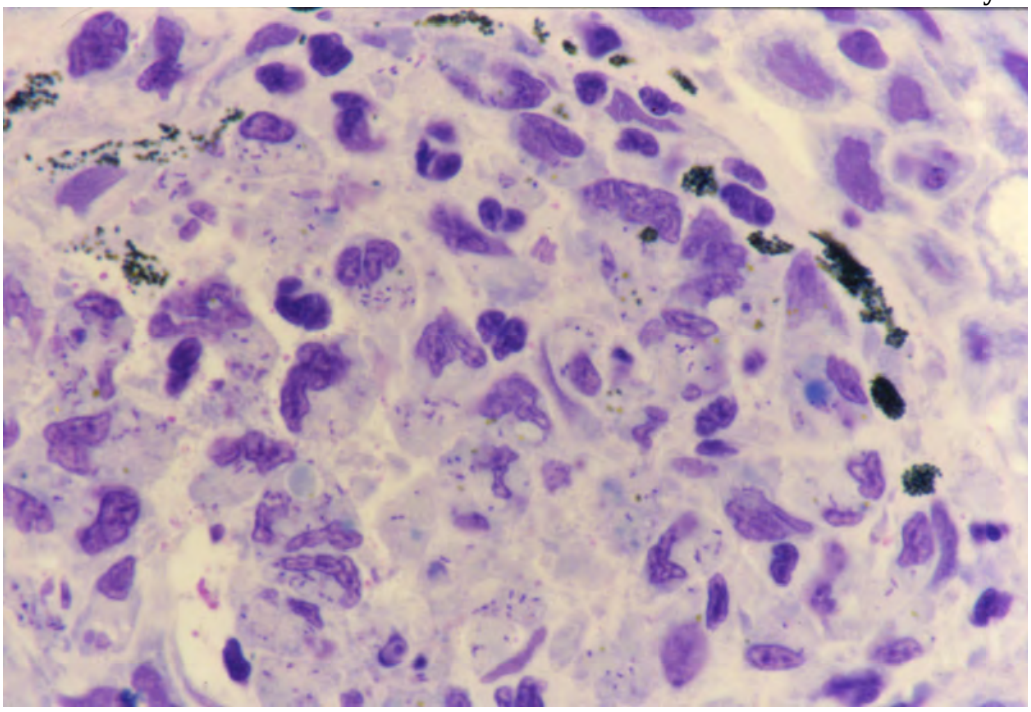


Figure 6. Histological section of a skin lesion of a juvenile chinook salmon *Oncorhynchus tshawytscha* infected with *Renibacterium salmoninarum*. Most of the small rod-shaped *R. salmoninarum* are visible within the cytoplasm of macrophages. Note that the bacteria are purple-blue in this Giemsa-stained preparation, in contrast to the black melanin granules.

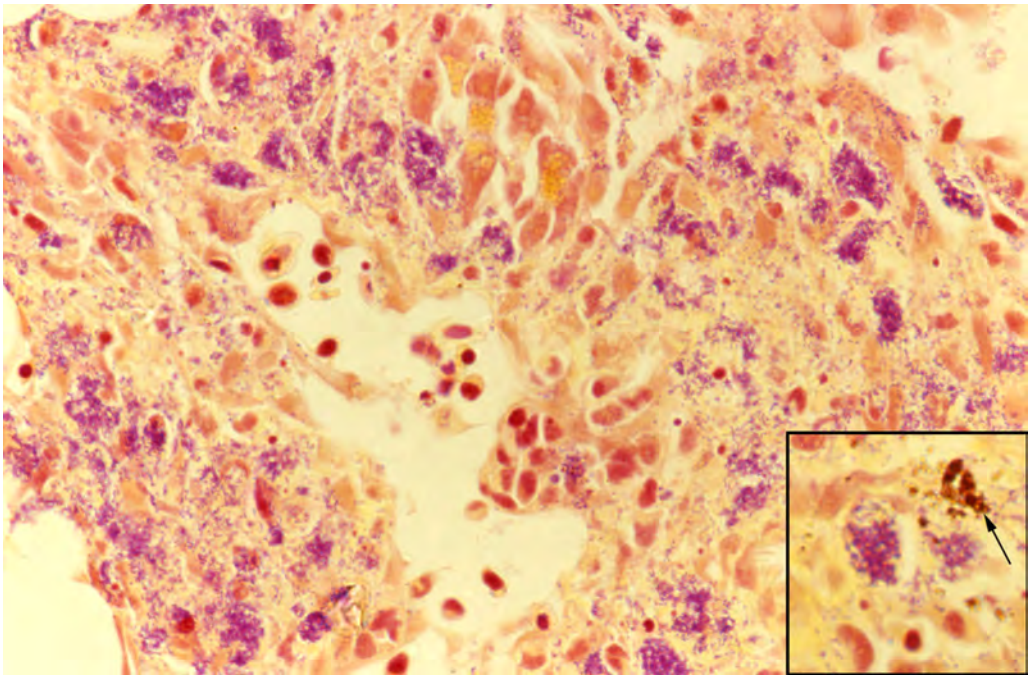


Figure 7. Gram-stained histological section of pancreatic tissue of a juvenile chinook salmon *Oncorhynchus tshawytscha* with systemic BKD. *Renibacterium salmoninarum* cells are present extracellularly and intracellularly within macrophages. Note the color difference between the gram-positive (purple-blue) bacteria and the brown-black melanin granules (arrow, inset). Procedures are available for bleaching of melanin in tissue sections to allow easier observation of bacteria (Bruno and Poppe 1996).

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

Smears or histological sections of infected tissue should contain numerous small, gram-positive non-acid-fast diplobacilli that occur both intracellularly and extracellularly (Figure 7). The organism should fail to grow on TSA at 20°C, even when extended incubation periods (e.g. two weeks) are used.

2. Confirmatory diagnosis

In addition to bacteriological culture, several immunological tests to confirm the presence of cellular or soluble antigens of *Renibacterium salmoninarum* have been reported, including immunodiffusion (Chen et al. 1974), the fluorescent antibody test (FAT; Bullock and Stuckey 1975; Bullock et al. 1980), staphylococcal coagglutination (Kimura and Yoshimizu 1981), counterimmunoelectrophoresis (Cipriano et al. 1985), immunoblot (Western blot; Turaga et al. 1987; Rockey et al. 1991), the semi-quantitative enzyme-linked immunosorbent assay (ELISA; Pascho and Mulcahy 1987; Pascho et al. 1987; Turaga et al. 1987; Pascho et al. 1991) and several qualitative enzyme immunoassays (Sakai et al. 1987a and b, 1989). One is encouraged to review several of the published reports describing comparative abilities of these tests to confirm the presence of BKD (e.g. Kimura and Yoshimizu 1981; Cipriano et al. 1985; Dixon 1987; Pascho et al. 1987, Sakai et al. 1987a and b, 1989; Pascho et al. 2002). More recently, polymerase chain reaction (PCR) techniques have been developed to confirm the presence of unique *R. salmoninarum* nucleic acid sequences; the strengths and weaknesses of PCR compared with other *R. salmoninarum* diagnostic techniques are reviewed by Pascho et al. (2002). The most widely used procedures for confirming the presence of *Renibacterium salmoninarum* in clinically diseased fish are those described below.

a. Bacterial kidney disease can be diagnosed by a direct FAT (Bullock et al. 1980) or indirect FAT (Bullock and Stuckey 1975) applied to fresh, formalin-fixed, or frozen infected tissues. Procedures for FAT staining of tissue smears are described in Section 2, 3.8.E "Fluorescent Antibody Test (FAT)." The quality of the preparations and consequent sensitivity of the test may be enhanced by homogenization of kidney tissue samples prior to smear preparation and treating smears with a solvent (xylene or acetone for 1 to 2 minutes followed by a tap water rinse) on the same day as staining (Cvitanich 1994). The use of a counterstain such as Evans blue (Cvitanich 1994), Eriochrome black T (Pascho et al. 1987), or rhodamine (Anderson 1990) can reduce background fluorescence or provide background contrast in tissue smears.

b. The ELISA also can be used to diagnose BKD. Fresh or frozen tissues or blood plasma from infected fish are used. Procedures for ELISAs using polyclonal antisera (e.g. Pascho and Mulcahy 1987; Pascho et al. 1987, 1991; Gudmundsdóttir et al. 1993; Meyers et al. 1993; Olea et al. 1993; Jansson et al. 1996) and monoclonal antibodies (e.g. Turaga et al. 1987; Rockey et al. 1991; Hsu et al. 1991) have been developed. Commercially prepared ELISA reagents are available for use in some ELISA procedures. A polyclonal antibody ELISA procedure for *R. salmoninarum* antigen detection is described in Appendices 1-5 to this chapter.

c. The kidney disease bacterium can be cultured from fresh tissues or body fluids and then shown to be biochemically (Austin et al. 1983; Goodfellow et al. 1985; Bruno and Munro 1986) or immunologically identical to *R. salmoninarum*. The culture medium for primary isolation of the bacterium should include cysteine and serum; the most commonly used media are variations of KDM2 medium (Evelyn 1977). Although it has been reported that charcoal can be substituted

for the serum component in KDM2 medium (Daly and Stevenson 1985), this may reduce the effectiveness of the medium for *R. salmoninarum* isolation from fish kidney tissues (Gudmundsdóttir et al. 1991). Antimicrobial agents can be added to KDM2 medium to inhibit the growth of other organisms present in the samples (SKDM; Austin et al. 1983). The formulation of SKDM is described on Section 2, 2.3.A.2 "Selective Kidney Disease Medium-2 (SKDM-2) (Austin, et.al.1983)." The optimal incubation temperature is 15°C and extended incubations (up to 12 weeks or more) have been recommended (Benediktsdóttir et al. 1991). The use of a nurse culture (Evelyn et al., 1989) or media supplemented with spent growth medium (Evelyn et al. 1990) has been reported to accelerate the growth of *Renibacterium salmoninarum*. Metabolite(s) released into a medium by *R. salmoninarum* might provide growth initiating factor(s) (Evelyn et al. 1990) or detoxifying factors (Teska 1994).

d. Several polymerase chain reaction (PCR) procedures, which incorporate the enzymatic amplification of specific unique nucleic acid fragments, have been developed for *R. salmoninarum* diagnosis. Various PCR procedures have been described for the detection of *R. salmoninarum* in a variety of fish samples, including: kidney tissue (Léon et al. 1994; McIntosh et al. 1996; Miriam et al. 1997; Chase and Pascho 1998; Rhodes et al. 1998; Cook and Lynch 1999), ovarian fluid (Magnússon et al. 1994; Miriam et al. 1997; Pascho et al. 1998; Rhodes et al. 1998; Cook and Lynch 1999), blood (Rhodes et al. 1998), whole fry (Rhodes et al. 1998), and eggs (Brown et al. 1994; Rhodes et al. 1998). A nested PCR procedure for *R. salmoninarum* detection is described in Section 2, 3.5.B.2 "Nested Polymerase Chain Reaction (PCR) for Confirmation of *R. salmoninarum* DNA."

F. Procedures for Detecting Subclinical Infections

Monitoring of moribund fish in seemingly healthy stocks can be done by the FAT, by the ELISA, by culture, or by PCR. In critical situations, the use of more than one technique for *R. salmoninarum* detection is recommended. Some ELISAs have proven sensitive enough to detect the presence of low levels of *R. salmoninarum* antigen in asymptomatic fish in a population (Pascho et al. 1987; 1991), and are suitable for large-scale population screening. Certain PCR procedures have been shown to have greater sensitivity than FAT or ELISA (Chase and Pascho 1998), and sensitivity equal to or greater than culture (McIntosh et al. 1996; Miriam et al. 1997; Cook and Lynch 1999), for detection of *R. salmoninarum* in fish kidney tissue. Although a lack of quantitative methods and other technical considerations limit the suitability of current PCR procedures for large-scale population screening (Pascho et al. 2002), these procedures are frequently used as confirmatory tests for population screening procedures such as the ELISA. The FAT, ELISA, and most PCR procedures cannot distinguish viable from non-viable *R. salmoninarum*. The viability of *R. salmoninarum* can be determined by culture. Enhancement techniques such as the use of nurse cultures or supplementation of a medium with spent growth medium (*R. salmoninarum* metabolite) may be necessary to detect the bacterium in asymptomatic fish. A nested reverse transcription PCR (RT-PCR) was developed by Cook and Lynch (1999) for the detection of viable or recently killed cells of *R. salmoninarum*. This PCR detects specific sequences of *R. salmoninarum* messenger RNA, which has a very short half-life.

Examination of spawning fish is recommended. The bacterium can be transmitted vertically, and the expression of infections is often enhanced at spawning. The ELISA is a useful test for detecting and quantifying *R. salmoninarum* antigen in tissues such as the kidney and spleen and in the blood plasma of fish that cannot be sacrificed (Pascho et al. 1987). However, both polyclonal antibody ELISAs (Pascho et al. 1991; 1998) and monoclonal antibody ELISAs (Griffiths et al. 1996) may be unreliable for

detecting antigens of the bacterium in ovarian fluid. In contrast, some PCR procedures are sensitive for detection of low numbers of *R. salmoninarum* in the ovarian fluid; one such procedure is described in Section 2, 3.5.B.2 "Nested Polymerase Chain Reaction (PCR) for Confirmation of *R. salmoninarum* DNA." A drawback of current PCR procedures for *R. salmoninarum* detection in ovarian fluid is that they are not quantitative. The membrane filtration-FAT (Elliott and Barila 1987; Elliott and McKibben 1997), in which bacteria are concentrated on 0.2 µm polycarbonate filters prior to FAT staining, is useful for enumeration of *R salmoninarum* in ovarian fluid. Although FAT staining of smears of material pelleted by centrifugation from ovarian fluid is frequently used instead of the MF-FAT for *R. salmoninarum* detection, the smear FAT (S-FAT) is not reliable for detection and quantification of *R. salmoninarum* in ovarian fluid when bacterial concentrations are low (Armstrong 1989; Elliott and McKibben 1997). Because of its greater sensitivity and quantitative ability, the MF-FAT is preferable to the S-FAT for use in critical situations requiring the detection of low numbers of bacteria (<2.4 x 10^3 cells/mL; Elliott and McKibben 1997).

G. Procedures for Determining Prior Exposure to the Etiological Agent

The antibody response of fish to *Renibacterium salmoninarum* infection has been reported to be variable. The measurement of antibody response is therefore not considered a reliable means of determining prior exposure to the pathogen. Conversely, the detection of *Renibacterium salmoninarum* antigen in fish by ELISA testing is not a reliable indication of an active infection, as antigens of killed *R. salmoninarum* can persist for more than three months in fish (Pascho et al. 1997).

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Samples that are to be cultured should be stored under refrigeration (10°C or lower, but not frozen) and inoculated into the appropriate culture medium within 72 hours. Samples for ELISA testing should be refrigerated if they are to be analyzed within 24 hours of collection, or frozen if longer storage is necessary. Freezing of ELISA samples at -20°C is acceptable, but freezing at colder temperatures (-70°C) is desirable for retention of the antigen during prolonged storage. Samples for the FAT should be treated in the same manner as samples for the ELISA. If tissue smears are prepared on slides and the slides are not examined within one week, they should be stored under refrigeration until FAT staining is done.

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1.14.A1 Bacterial Kidney Disease Methods Appendix 1

Ronald J. Pascho and Diane G. Elliott

U. S. Geological Survey, Biological Resources Discipline
Western Fisheries Research Center
6505 NE 65th Street
Seattle, WA 98115
206/526-6282
ron_pascho@usgs.gov
diane_elliott@nbs.gov

Reagents Required for the ELISA to Detect *Renibacterium salmoninarum*

A. Test Sample and Control Preparation Diluent

Phosphate-buffered saline (PBS), pH 7.4 supplemented with 0.05% (v/v) Tween-20 (PBS-T20), and 0.01% (w/v) Thimerosal as a preservative. For 1 liter:

NaCl	8.00 grams
KH_2PO_4	0.20 gram
Na_2HPO_4	1.09 grams
KCl	0.20 gram
Thimerosal	0.10 gram
Confirm pH=7.4	
Tween-20	0.5 mL

B. Positive Control Antigen

Renibacterium salmoninarum cells (0.5% (w/v) wet packed cells) in PBS pH 7.4 + Thimerosal. The bacterial cells can be lyophilized for long-term storage; lyophilized bacteria normally contain a carrier compound such as dextrose for stability.

1. Store lypohilized preparation at 4°C. Stable for a minimum of one year.

2. Rehydrate lyophilized bacteria with 1.0 mL Nanopure water.

3. Prepare necessary dilutions, 1:100, 1:500, 1:1,000, and 1:5,000 (v/v), in PBS-T20 and store at -70°C.

C. Coating Buffer

Sodium carbonate, pH 9.6, or a commercial coating solution.

1. Prepare fresh for each ELISA.

2. Sodium carbonate, pH 9.6. Store at room temperature and discard after 30 days. For 1 liter:

Na_2CO_3	1.59 grams
$NaHCO_3$	2.93 grams
Thimerosal	0.10 gram
Confirm pH=9.6	

3. Commercial coating solution concentrates. Store concentrate at 4°C after opening; usually stable for a minimum of one year. Dilute according to manufacturers instructions.

D. Coating Antibody

Affinity purified immunoglobulin to *R. salmoninarum*; normally each vial contains 1.0 mg of lyophilized immunoglobulin.

1. Store lypohilized preparation at 4°C. Stable for a minimum of one year.

2. Rehydrate lyophilized coating antibody by first mixing 1.0 mL glycerol + 1.0 mL Nanopure water that contains 0.2% (2x) strength thimerosal, then transferring 1 mL of the 50% glycerol solution to each product vial.

3. Rehydrate the contents of a sufficient number vials to test the anticipated number of fish that will be sampled for a given spawning season. Pool the contents of all vials, then dispense into several cryovials and store at -70°C.

E. Wash Solution

PBS-T20, or a commerical wash solution that contains Tween 20.

1. Prepare fresh for each ELISA.

2. PBS-T20. Prepare as described above.

3. Commercial wash solution concentrates. Store concentrate at 4°C after opening; usually stable for a minimum of one year. Dilute according to manufacturers instructions.

F. Conjugate Diluent

PBS-T20, or 2% (w/v) non-fat dry milk in PBS pH 7.4, or a borate buffer**.** Commercial products are available as concentrates, often marketed as diluents or blocking solutions.

1. Prepare fresh for each ELISA.

2. PBS-T20. Prepare as described above. The non-fat dry milk may be substituted for the Tween 20; 2% (w/v), or 2 grams non-fat dry milk up to 100 mL in PBS pH 7.4.

3. Commercial diluent/blocking solution concentrates. Store concentrate at 4°C after opening; usually stable for a minimum of one year. Dilute according to manufacturers instructions.

G. Goat Anti-*R. salmoninarum* Conjugate

Affinity purified immunoglobulin to *R. salmoninarum* labeled with horseradish peroxidase; normally each vial contains 0.1 mg of lyophilized conjugate.

1. Store lypohilized preparation at 4°C. Stable for a minimum of one year.

2. Rehydrate lyophilized coating antibody by first mixing 1.0 mL glycerol + 1.0 mL Nanopure water that contains 0.2% (2x) strength thimerosal, then transferring 1.0 mL of the 50% glycerol solution to each product vial.

3. Rehydrate the contents of a sufficient number vials to test the anticipated number of fish that will be sampled for a given spawning season. Pool the contents of all vials, then dispense into several cryovials and store at -70°C.

H. ABTS-Peroxidase Chromogen-Substrate

Commercial products are available and often are provided as a two-part system: the ABTS chromogen, 0.6 g/L 2.2'-azino-di[3-ethyl-] benzthiazoline sulfonate in a glycine buffer, and a hydrogen peroxide substrate, 0.02% hydrogen peroxide in a citric acid buffer.

I. Stop Solution

5% (v/v) sodium dodecyl sulfate in water.

1. Store at room temperature; stable for a minimum of one year.

2. Combine 4 parts concentrate with 1 part reagent grade water immediately before use.

1.14.A2 Bacterial Kidney Disease Methods Appendix 2

Ronald J. Pascho and Diane G. Elliott

U. S. Geological Survey, Biological Resources Discipline
Western Fisheries Research Center
6505 NE 65th Street
Seattle, WA 98115
206/526-6282
ron_pascho@usgs.gov
diane_elliott@nbs.gov

Checkerboard Titration to Determine the Working Concentrations of Coating Antibody and Conjugate used in the ELISA to Detect *Renibacterium salmoninarum*

Each time new vials of the coating antibody or conjugate are rehydrated, slight variations between lots of the manufacturer's antibody preparations, the rehydration procedure, etc. may affect the amounts of those stock preparations used in the ELISA. To determine the optimal (working) concentration of each reagent, several dilutions of both reagents must be evaluated together with a prescribed set of test samples. From these data, one can choose a coating antibody-conjugate combination that produces acceptable absorbances.

This appendix describes a checkboard titration method for determining the working concentrations of the coating antibody and conjugate preparations. It is based on the standard ELISA protocol.

A. Reagents and Test Samples

1. Coating Antibody and Conjugate

a. Rehydrate several vials of each preparation according to instructions in Section 1, 1.14.A1 BKD Methods Appendix 1.

b. Combine the contents of the antibody vials into a single pool, aliquot into several clean vials, and store at -70°C. Repeat with the vials of rehydrated conjugate.

c. Thaw one vial from each pool for the checkerboard titration.

d. It is suggested that you test the coating antibody at 1.0, 1.5, and 2.0 µg/mL, and that you test the conjugate at 1:1,000, 1:1,500, and 1:2,000 (v/v) dilutions.

2. Test Samples and Buffers

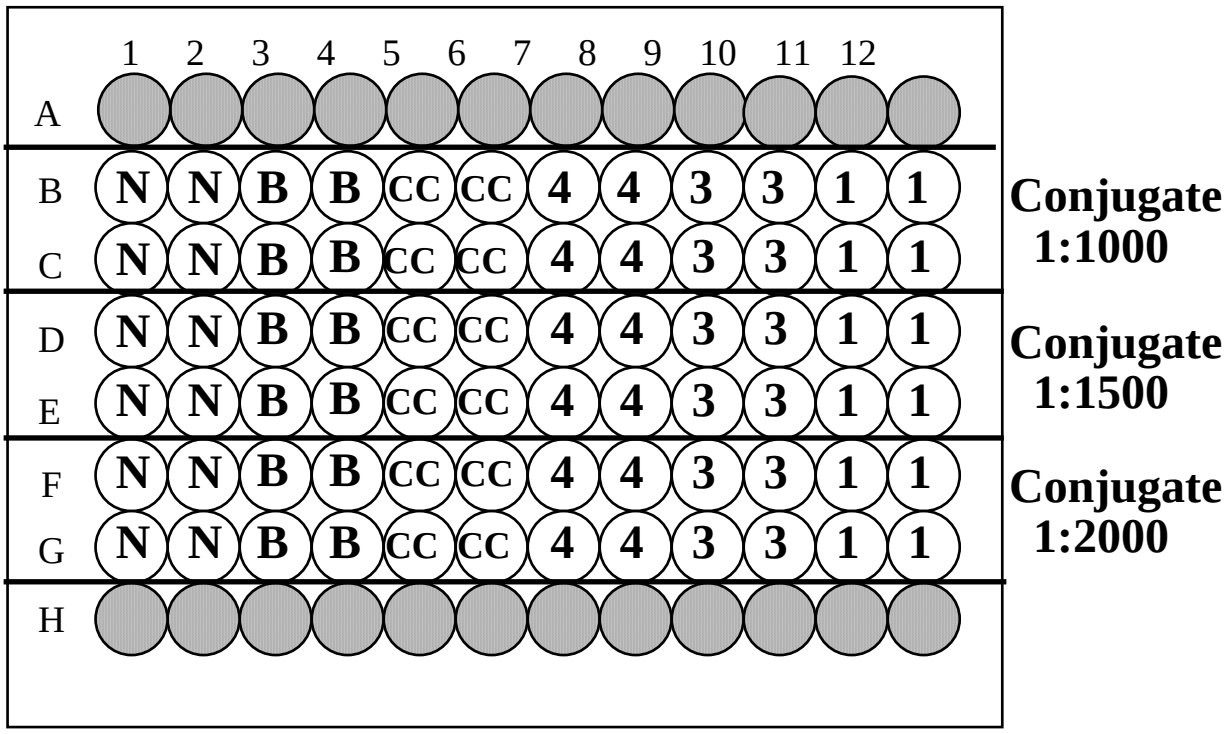
a. The test samples are the blank (B), the negative control (N), the conjugate control (CC), and the four positive controls (1:100 (1), 1:1,000 (2), 1:2,000 (3), and 1:5,000 v/v (4) dilutions) described in Section 1, 1.14.A4 BKD Methods Appendix 4.

b. The buffer formulations and their uses are described in Section 1, 1.14.A1 BKD Methods Appendix 1 and Section 1, 1.14.A4 BKD Methods Appendix 4.

B. Microplate Format

The suggested microplate format for evaluating one concentration of the coating antibody with three concentrations of conjugate is shown below. A separate microplate is used for each concentration of coating antibody tested. Rows A and H have been eliminated to because there are four replicate wells in each subgroup. The numerical and letter codes are described elsewhere.

For each coating antibody concentration



C. Checkerboard Titration Procedure

1. For each coating antibody concentration, coat the appropriate wells of a separate microplate.

a. For each coating antibody concentration group, 60 wells will receive 200 µL of antibody.

b. Calculate the amount of coating buffer and coating antibody needed to coat 60 wells. Prepare an excess volume of coating antibody to compensate for solution lost during preparation and pipetting.

Example 1. Calculation of the total volume of coating antibody at 1 µg/mL necessary to apply 200 µL of antibody to the wells of 5 microplates.

(60 wells/plate) (200 µL/well) = 12,000 µL = 12 mL
Prepare 15 mL coating antibody at 1 µg/mL

X (1,000 µg/mL) = 15,000 µL (1 µg/mL)
X = 15 µL

Combine 15 µL concentrated anti-*R. salmoninarum* IgG with 15 mL coating solution.

c. Dilute the concentrated goat antibody to *R. salmoninarum* in carbonate-bicarbonate coating buffer pH 9.6 or a commercial coating solution. When using the coating solution, make a fresh preparation for each ELISA. The carbonate-bicarbonate buffer is normally discarded after 30 days. Use water of reagent grade or equivalent.

d. The conjugate control (CC) and substrate-chromagen control wells (SC) receive no coating antibody. Place 200 µL of coating buffer or diluted coating solution in each of these wells.

e. The blanks (B), negative controls wells (N), and the positive controls wells (1:100, 1:1,000, 1:2,000, and 1:5,000 dilutions) receive 200 µL of coating antibody:

2. Seal each plate with an adhesive plate sealer after addition of the buffer or coating antibody. Place each plate in a humid chamber and incubate at 4°C for 16 hours.

3. Wash microplates to remove unbound immunoglobulin.

4. Place aliquots of controls and test samples into microplate wells.

a. The following control wells receive 200 µL of the test sample diluent (phosphate-buffer saline pH 7.4 supplemented with 0.05% (v/v) Tween 20):

Blank (B)
Conjugate Control (CC)
Substrate-Chromogen Control (SC)
Control tissue (N) wells receive the appropriate tissue or body fluid.

Seal these wells with a strip of adhesive plate sealer; then, load the positive controls.

b. Place 200 µL aliquots of each positive control in the appropriate wells.

Seal these wells with a second strip of adhesive plate sealer before loading the test samples.

c. Cover each plate with an adhesive plate sealer. Write time of completion on the sealer flap on each plate and on the template sheet.

d. Incubate for three hours at 25°C in a humid chamber.

5. Wash microplates five times as described previously.

6. Add the goat anti-*R. salmoninarum*-HRP conjugate.

   a. First, calculate amount of HRP-conjugated antibody needed for each dilution.

      Example 2. Calculation of the volume of a 1:2,000 dilution of the HRP-conjugate necessary when testing three concentrations of coating antibody; three microplates x 64 wells/plate = 192 wells.

      (192 wells) (200 µL/well) = 38,400 µL = 38.4 mL
      Prepare 45 mL.

      1/2000 = X /45 mL
      X = 22.5 µL

      Combine 22.5 µL of the stock anti-*R. salmoninarum* -HRP conjugate with 45 mL diluent.

      Repeat calculations for each conjugate dilution.

   b. Prepare the appropriate volume HRP-conjugated antibody in conjugate diluent. Apply 200 µL of the diluted conjugate to the appropriate wells. Substrate-chromogen control wells (SC) receive an equivalent amount of diluent without conjugated antibody.

   c. Seal each plate with an adhesive plate sealer and incubate in a humid chamber for two hours at 25°C.

7. Wash microplates five times as described previously.

8. Substrate-Chromogen Reaction

   a. Start the substrate-chromogen reaction.

      i. Put the required number of humid chambers (without plates) into 37°C incubator and allow to equilibrate prior to beginning substrate-chromogen reaction. Store washed plates in another humid chamber at room temperature, or leave on counter with plastic lids on if there are an insufficient number of available humid chambers.

      ii. For each plate, set a timer for 20 minutes (but don't start).

      iii. Apply 200 µL of substrate-chromogen solution to each well. Start the appropriate timer immediately when you begin to apply the solution. Fill all plates in numerical order.

      iv. Immediately after all the wells have received the substrate-chromogen solution, seal the plate and put it in the humid chamber at 37°C.

   b. Procedure to stop the substrate-chromogen reaction and to measure the absorbance.

      i. While the substrate-chromogen reaction is proceeding at 37°C, prepare the stop solution. Each well will receive 50 µL.

ii. Begin to apply the stop solution immediately after the incubation period is complete. Wells should receive the stop solution in the exact sequence used to apply the substrate-chromogen solution.

iii. Wipe any condensation off the bottom of the plate and immediately measure the absorbances at 405 nm.

1.14.A3 Bacterial Kidney Disease Methods Appendix 3

Ronald J. Pascho and Diane G. Elliott

U. S. Geological Survey, Biological Resources Discipline
Western Fisheries Research Center
6505 NE 65th Street
Seattle, WA 98115
206/526-6282
ron_pascho@usgs.gov
diane_elliott@nbs.gov

Collection and Processing of Tissue or Body Fluid Samples for the ELISA

A. Sample Collection and Handling

1. General Comments

a. Field containers should be labeled, sterile, stomacher-type bags with a top closure, affecting a minimum of handling.

b. All samples should be treated similarly, e.g., dissect fish, store on ice, freeze-thaw, add diluent and homogenize/transfer sample into microcentrifuge tube, etc.

c. Store processed tissues at -70°C.

2. Sample Buffer and Sample Tubes

a. Sample Buffer
PBS-T20.

b. Sample Tubes
Recommended sample tubes are microcentrifuge tubes from Sarstedt, Inc., 3584 Arden Road, Hayward, CA 94545. 1-800-321-5680. Catalog number 72.694.006. Microcentrifuge tube with screen-printed graduation and writing space, sterile, 2.0 mL volume.

3. Sample Collection from Adult Fish

a. Samples are usually taken following spawning and care should be exercised to avoid cross-contamination between fish and contamination of tissue sample from body fluids.

b. Collect a 2 to 5 gram sample; when sampling the kidney, it is recommended that this sample consist of a pool of small tissue pieces from the anterior, mid, and posterior kidney.

c. For ovarian fluid, collect approximately 1 mL.

d. Keep tissue and body fluid samples on ice during collection.

4. Sample Collection from Juvenile Fish

a. Juvenile fish are often collected as whole fish and dissected upon return to the laboratory.

b. Remove the entire kidney and spleen from each fish. It is preferrable to test tissues from individual fish, but a tissue pool may be made if the fish are too small. Two-fish pools are recommended when an insufficient amount of tissue is recovered from an individual fish. *If you cannot adhere to these guidelines, then the fish are too small for testing.*

c. As a minimum, one needs approximately 0.08 g of sample for testing.

d. Keep fish or tissues on ice during collection and processing.

B. Sample Processing

1. General Comments

a. All buffers should be prepared and stored in acid-washed glassware or disposable containers. Many of the antigens of *R. salmoninarum* adhere to glassware, are heat stable, and will not be removed or destroyed by detergent washing and autoclaving.

b. It is important that when tissue samples are removed, care is taken to avoid rupturing the stomach or intestine. Commercial fish food frequently contains antigens of *R. salmoninarum* which can be detected by the ELISA, resulting in a false positive reading.

c. The cleaning of instruments between fish is important. The requirements for the FAT and the ELISA may be different, because the former is detecting *R. salmoninarum* cells and the latter a soluble antigenic fraction of the bacterium. *Renibacterium salmoninarum* cells can be disrupted by soaking instruments in 100% bleach or flaming them for at least 5 seconds (Scott and Nesbitt 1995). When tissues are removed in the laboratory, instruments should be flamed between fish in a given group, and mechanically cleaned with detergent between groups of fish. For field sampling, use disposable instruments when possible to remove organs, or have a sufficient number of precleaned instruments so that there is a separate instrument for each fish.

d. Store processed tissues at -70°C before testing. Recording the storage details for tissues and processed samples will assist in maintaining uniform treatment and storage conditions.

2. Kidney or Spleen

a. Adult fish. 1:4 (w/v); 1 part tissue + 3 parts PBS-T20
Dilute and homogenize tissue, dispense adequate volume into a microcentrifuge tube. All fish taken from a group of fish must be processed identically. You cannot change the dilution of test samples within, or among, test groups. This material can then be stored at -70°C, or for

immediate testing, heated at 100°C for 15 minutes. and refrigerated overnight. Each sample must be centrifuged (8,000 to 10,000 x *g* for 10 minutes) to remove debris; centrifugation can be done the night before, or just prior to an ELISA. Test the supernatant in the ELISA.

NOTE: If you are also going to prepare a tissue smear for the FAT, homogenize the tissue *without* dilution and make a smear from this material, then dilute the sample for the ELISA.

b. Juvenile fish. Recommend 1:4 (w/v) dilution, but can use 1:8 (w/v); 1 part tissue + 7 parts PBS-T20
Place tissue from individual fish directly into a pre-weighed microcentrifuge tube and homogenize with a stirring rod (Sarstedt, Catalogue Number 81.970), make a tissue smear for the FAT if necessary, then reweigh the tube + sample to get the sample weight for dilution. Dilute and process as described above for the kidney and spleen. All fish taken from a group of fish must be processed identically. You cannot change the dilution of test samples within, or among, test groups.

3. Ovarian fluid

1:2 (v/v); 1 volume ovarian fluid + 1 volume PBS-T20. Combine 1000 µL of ovarian fluid and 1000 µL of PBS-T20 in a microcentrifuge tube, store fluid or heat at 100°C for 15 minutes, remove debris by centrifugation (again, 8,000-10,000 x *g* for 10 minutes), and test supernatant.

4. Plasma

a. Adult fish. 1:5 (w/v); 1 volume whole blood + 4 volumes PBS-T20

b. Juvenile fish. 1:10 (v/v); 1 volume whole blood + 9 volumes PBS-T20

Collect blood in a syringe or capillary tube, then dispense the correct volume of blood into the appropriate volume of PBS-T20, remove the cellular fraction by low speed centrifugation, decant the supernatant, store fluid or heat at 100°C for 15 minutes, and test supernatant. All fish taken from a group of fish must be processed identically. You cannot change the dilution of test samples within, or among, test groups.

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1.14.A4 Bacterial Kidney Disease Methods Appendix 4

Ronald J. Pascho and Diane G. Elliott

U. S. Geological Survey, Biological Resources Discipline
Western Fisheries Research Center
6505 NE 65th Street
Seattle, WA 98115
206/526-6282
ron_pascho@usgs.gov
diane_elliott@nbs.gov

Description of Controls Used in the ELISA to Detect *Renibacterium salmoninarum*

Control Group	Purpose	Step in the ELISA				
		Coating Antibody	Sample Application	Conjugate	Substrate-Chromogen	Stop Solution
Blank (B)	Determine background absorbance levels in the absence of a test sample	Yes	PBS-T20 Only	Yes	Yes	Yes
Reference or Positive Controls [1]	Internal control to insure that predictable absorbances are produced by certain levels of antigen	Yes	Yes	Yes	Yes	Yes
Negative Control (N)[2]	Measure absorbance produced by sample from a negative control fish	Yes	Yes	Yes	Yes	Yes
Conjugate Control (CC)	Insure that there was no nonspecific binding of the conjugate to well surfaces or to the coating antibody	Coating Buffer Only	PBS-T20 Only	Yes	Yes	Yes
Substrate-Chromogen Control (SC)	Test for nonenzymatic production of the color reaction	Coating Buffer Only	PBS-T20 Only	Diluent Only	Yes	Yes

[1] Sample buffer containing a known level of *R. salmoninarum*, e.g. dilutions of 1:100, 1:1,000, 1:2,000, and 1:5,000 (v/v) of control antigen.

[2] Appropriate tissues or body fluids with no or undetectable levels of *R. salmoninarum* antigen. Store at -70°C; thaw morning of use; heat at 100°C for 15 minutes; and centrifuge 8,800 g for 5 minutes.

1.14.A5 Bacterial Kidney Disease Methods Appendix 5

Ronald J. Pascho and Diane G. Elliott

U. S. Geological Survey, Biological Resources Discipline
Western Fisheries Research Center
6505 NE 65th Street
Seattle, WA 98115
206/526-6282
ron_pascho@usgs.gov
diane_elliott@nbs.gov

Method to Detect *Renibacterium salmoninarum* by the ELISA

A. Before the ELISA

1. **Reagent Preparation**
   See 1.14.A1 BKD Methods Appendix 1.

2. Determine the working concentrations of the anti-*R. salmoninarum* immunoglobulin preparations.

   a. Working concentrations for the purified goat anti-*R. salmoninarum* antibody (coating antibody), and the horseradish peroxidase (HRP)-conjugated goat anti-*R. salmoninarum* antibody must first be determined by checkerboard titration with the coating antibody and the HRP-conjugate (see 1.14.A2 BKD Methods Appendix 2).

   b. The anti-*R. salmoninarum* is typically applied to the microplate wells at 1 µg per mL; the dilution is made from a concentrated antibody preparation at 1 mg/mL, and the working dilution of the HRP-conjugated antibody is normally about 1:2,000 (v/v).

3. **Sample Collection and Preparation**
   Prepare fish tissue or body fluid samples for the ELISA as described in 1.14.A3 BKD Methods Appendix 3. Heat each sample at 100°C for 15 minutes, then centrifuge 8,000 to 10,000 *x g* for 10 minutes at 4°C. If the samples were prepared earlier and frozen, thaw before heating. Store processed and heated samples at 4°C, or freeze at -70°C for later testing.

B. ELISA Day 1

1. Coat the microplate wells with immunoglobulin (coating antibody).

   a. General Comments

i. For the ELISA you must use a microplate that is designed for use in immunoassays. They are available from several manufacturers, including Corning Glass Works, Costar, Dynatech Laboratories, Inc., and Nunc, Inc. The performance of these plates will vary, and for a given ELISA, microplates from several manufacturers should be tested to determine which is most suitable.

ii. It is advisable to dedicate a separate set of glassware for use in preparing reagents for the ELISA. This is most important in laboratories that routinely culture *R. salmoninarum*. Extracellular proteins of the bacterium, especially the p57 antigen, may remain on container surfaces after conventional detergent washing and steam sterilization.

iii. There are several controls that must be included in each ELISA. Their preparation and use are described in 1.14.A4 BKD Methods Appendix 4.

b. Procedure

i. To determine the number of microplate wells that will receive coating antibody, prepare microplate maps that show the location of each replicate of a given control or test sample. Sequentially number microplates.

ii. Calculate the amount of coating buffer and coating antibody needed to coat each of the microplates in a particular ELISA. Prepare an excess volume of coating antibody to compensate for solution lost during preparation and pipetting.

Example 1. Calculation of the total volume of coating antibody at 1 µg/mL necessary to apply 200 µL of antibody to the wells of five microplates.

(5 plates) (96 wells/plate) (200 µL/well) = 96,000 µL = 96 mL
Prepare 105 mL coating antibody at 1 µg/mL

X (1000 µg/mL) = 105 mL (1 µg/mL)
X = 0.105 mL = 105 µL

Combine 105 µL concentrated anti-*R. salmoninarum* IgG with 105 mL coating buffer (see below).

iii. Dilute the concentrated goat antibody to *R. salmoninarum* in coating buffer, carbonate-bicarbonate coating buffer pH 9.6, or a commercial coating solution. When using the coating solution, make a fresh preparation for each ELISA. The carbonate-bicarbonate buffer is normally discarded after 30 days. Use water of reagent grade or equivalent.

iv. The conjugate control (CC) and substrate-chromagen control wells (SC) receive no coating antibody. Place 200 µL of coating buffer in each of these wells.

v. The blanks (B), negative controls wells (N), positive controls wells (1:100, 1:1,000, 1:2,000, and 1:5,000 dilutions), and the wells designated for the test samples receive 200 µL of coating antibody.

vi. Seal each plate with an adhesive plate sealer after addition of the buffer or coating antibody. Place each plate in a humid chamber and incubate at 4°C for 16 hours.

C. ELISA Day 2

1. Wash microplates to remove unbound immunoglobulin.

    a. General Comments

        i. Prepare PBS-T20 wash solution or dilute a commercial wash solution concentrate in reagent grade water and store overnight at 4°C. Swirl to insure mixing before adding to washer.

        ii. The wash buffer should remain at room temperature during the ELISA.

    b. Procedure

        i. This procedure is used in all subsequent wash steps.

        ii. Wash plates in numerical order.

        iii. Remove the unbound coating antibody by washing each plate five times, with a 30 second soak each time the wells are refilled. Shake excess wash buffer out of each plate after the five washes are completed.

2. Place aliquots of controls and test samples into microplate wells.

    a. The following control wells receive 200 µL of the test sample diluent (phosphate-buffer saline pH 7.4 supplemented with 0.05% (v/v) Tween 20):

        Blank (B)
        Conjugate Control (CC)
        Substrate-Chromogen Control (SC)
        Control tissue (N) wells receive the appropriate tissue or body fluid.

        Seal these wells with a strip of adhesive plate sealer, then load the positive controls.

    b. Place 200 µL aliquots of each positive control in the appropriate wells.

        Seal these wells with a second strip of adhesive plate sealer before loading the test samples.

    c. Place 200 µL aliquots of each test sample in the appropriate wells.

    d. Cover each plate with an adhesive plate sealer. Write time of completion on the sealer flap on each plate and on the template sheet.

    e. Incubate for three hours at 25°C in a humid chamber.

3. Wash microplates five times as described previously.

4. Add the goat anti-*R. salmoninarum*-HRP conjugate.

    a. First, calculate amount of HRP-conjugated antibody needed (200µL/well).

Example 2. Calculation of the volume of a 1:2,000 dilution of the HRP-conjugate necessary for 5 microplates.

(5 plates) (96 wells/plate) (200 µL/well) = 96,000 µL = 96 mL
Again, make ~10 mL extra, or 105 mL.

1/2000 = X /105 mL
X = 0.0525 mL = 52.5 µL

Combine 52.5 µL of the stock anti-*R. salmoninarum* -HRP conjugate with 105 mL diluent (see below).

b. Prepare the diluent for the HRP-conjugated antibody; 2%(w/v) nonfat dry milk in PBS pH 7.4, or from a commercial milk diluent/blocking solution concentrate. Apply 200 µL of the diluted conjugate to the appropriate wells. Substrate-chromogen control wells (SC) receive an equivalent amount of diluent without conjugated antibody.

c. Seal each plate with an adhesive plate sealer and incubate in a humid chamber for two hours at 25°C.

5. Wash microplates five times as described previously.

6. Substrate-Chromogen Reaction

a. General Comments

i. The timing of the substrate-chromogen reaction is critical. The reaction must be stopped after exactly 20 minutes.

ii. The volume of stop solution is 50 µL to insure that the wells are not over-filled. The SDS stop solution is prepared from a 5% (v/v) concentrate as follows:

4 parts concentrate + 1 part water

b. Procedure to Start the Substrate (Hydrogen Peroxide)-Chromogen (ABTS) Reaction

i. Put the required number of humid chambers (without plates) into 37°C incubator and allow to equilibrate prior to beginning substrate-chromogen reaction. Store washed plates in another humid chamber at room temperature, or leave on counter with plastic lids on if there are an insufficient number of available humid chambers.

ii. For each plate, set a timer for 20 minutes (but don't start).

iii. Prepare the substrate-chromogen solution.

iv. Apply 200 µL of substrate-chromogen solution to each well. Start the appropriate timer immediately when you begin to apply the solution. Fill all plates in numerical order.

v. Immediately after all the wells have received the substrate-chromogen solution, seal the plate and put it in the humid chamber at 37°C.

c. Procedure to Stop the Substrate-Chromogen Reaction and to Measure the Absorbance

i. While the substrate-chromogen reaction is proceeding at 37°C, prepare the stop solution. Each well will receive 50 µL.

ii. Begin to apply the stop solution immediately after the incubation period is complete. Wells should receive the stop solution in the exact sequence used to apply the substrate-chromogen solution.

iii. Wipe any condensation off the bottom of the plate and immediately measure the absorbances at 405 nm.

1.15 Pseudokidney Disease

J. Scott Foott

U. S. Fish and Wildlife Service
California-Nevada Fish Health Center
Rt. 1, Box 2105
Anderson, CA 96007
916/365-4271
scott_foot@fws.gov

A. Name of Disease and Etiological Agent

Pseudokidney disease is caused by *Carnobacterium piscicola* (formerly classified as *Lactobacillus piscicola*).

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

It is found in the United States, Canada, and United Kingdom. Possibly worldwide distribution because lactic acid bacteria are part of the normal oral and gut flora of animals.

2. Host Species

Reported isolations from coho *Oncorhynchus kisutch* and chinook salmon *Oncorhynchus tshawytscha*, rainbow trout *Oncorhynchus mykiss*, and cutthroat trout *Oncorhynchus clarki*. Potentially all freshwater and marine fish may be susceptible under stressful conditions.

C. Epizootiology

Epizootics have occurred in fish one year and older and especially in broodstock following the stress of spawning.

The reservoirs of the infection are unknown.

D. Disease Signs

External signs include abdominal distension, erythema at the base of fins, and sub-dermal blood blisters. Internally, there may be enlargement of the liver, spleen, and kidney. Ascitic fluid in the peritoneal cavity is common. Hemorrhages may be present in the testes, intestine, and muscle. A grey pseudomembrane resembling that seen in some BKD infections has been reported.

E. Disease Diagnostic Procedures

Along with the clinical signs, diagnosis is based on isolation and identification of the etiological agent. Primary isolation should be made from kidney or lesion on either TSA or BHIA cultured aerobically at 15 to 24°C for 24 to 72 hours.

1. **Presumptive Diagnosis**
   Colonies are pinpoint, opaque, entire, circular, and nonpigmented when grown on TSA. The organism is a nonmotile, non-sporeforming, non-acid fast, facultatively anaerobic, gram-positive rods or coccobacillus of 1.1 to 1.4 x 0.5 to 0.6 μm in size. Older cultures become gram variable. Other phenotypic traits include negative results for oxidase, catalase, urease, H_2S, nitrite reduction, and lactose and xylose fermentation. The organism shows positive reactions for arginine dihydrolase and lactic acid production from glucose (no gas), maltose, mannitol, and sucrose.

2. **Confirmatory Diagnosis**
   Additional tests can be done to show that the isolate is phenotypically identical or very similar to the type strain B270, ATCC 35586 (Hiu et al. 1984).

F. Procedures for Detecting Subclinical Infection

No procedures have been reported.

G. Procedures for Determining Prior Exposure to Etiological Agent

No procedures have been reported.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

See Section 1, 1.1 General Sampling Procedures for Bacteria.

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1.16 Streptococcal Disease

Emmett B. Shotts, Jr. and John Plumb*

College of Veterinary Medicine
University of Georgia
Athens, GA 30602
404/542-55811
emshotts@alltel.net

*Department of Fisheries and Allied Aquaculture
Auburn University
Auburn, AL 36849-5419
205/826-4786
jplumb@acesag.auburn.edu

A. Name of Disease and Etiological Agent

Streptococcal disease is caused by *Streptococcus iniae* and *Streptococcus* spp.

Although nonhemolytic Lancefield Group B is most commonly reported, there are reports in the literature, particularly from Japan and South Africa, that other Lancefield groups of streptococci have caused fish mortality. Of these other Lancefield groups, group D (alpha or beta hemolytic streptococcus) is most often reported and *Streptococcus iniae* is of particular note in closed culture systems.

B. Known Geographical Range and Host Species of the Disease

1. **Geographical Range**
   It is found in the United States, South and Central America, South Africa, Japan, and Republic of China

2. **Host Species**
   There is no specific host. The disease has been reported from fishes in brackish and marine waters and from several freshwater fishes, including aquarium fish species. Tilapia cultured in closed and intensive systems are particularly vulnerable.

C. Epizootiology

The epizootiology of the Group B type is not understood at this time. It appears to spread from fish to fish (aquarium species) by cannibalism, and has been isolated primarily from the brain of affected

fish. Cases of Group D streptococcal infections have been associated with fecal contamination. Water salinity in brackish environments may exert some selectivity for specific streptococcal types.

The infection appears to be very communicable as long as infected fish are present. Fish stress and unfavorable environmental conditions enhances the probability and severity of infection.

D. Disease Signs

Disease signs are variable, but affected fish commonly have numerous raised, hemorrhagic, inflamed areas on the skin including the operculum, around the mouth, at the bases of fins, and in general along the dorsolateral portions of the body (Figures 1 and 2). The abdomen is distended and the peritoneal cavity often contains bloody fluid (Figure 3). Exophthalmia is present with hemorrhage in the eye (Figure 4). The liver is usually pale and the spleen dark red. Kidneys appear normal and are not the primary target organs. A hemorrhagic enteritis with bloody fluid present in the intestinal lumen is also seen. Infected fish are lethargic and often swim in a tail-chasing spiral (Figure 5). Histological findings vary depending on the organisms and host species involved. Infections in the head often produce a granulomatous encephalitis and meningitis.



Figure 1. Atlantic Menhaden with a streptococcal infection.



Figure 2. Severe skin hemorrhage in a tilapia with a streptococcal infection (picture by Andy Goodwin).



Figure 3. Hemorrhage from the vent of Atlantic Menhaden with a streptococcal infection.

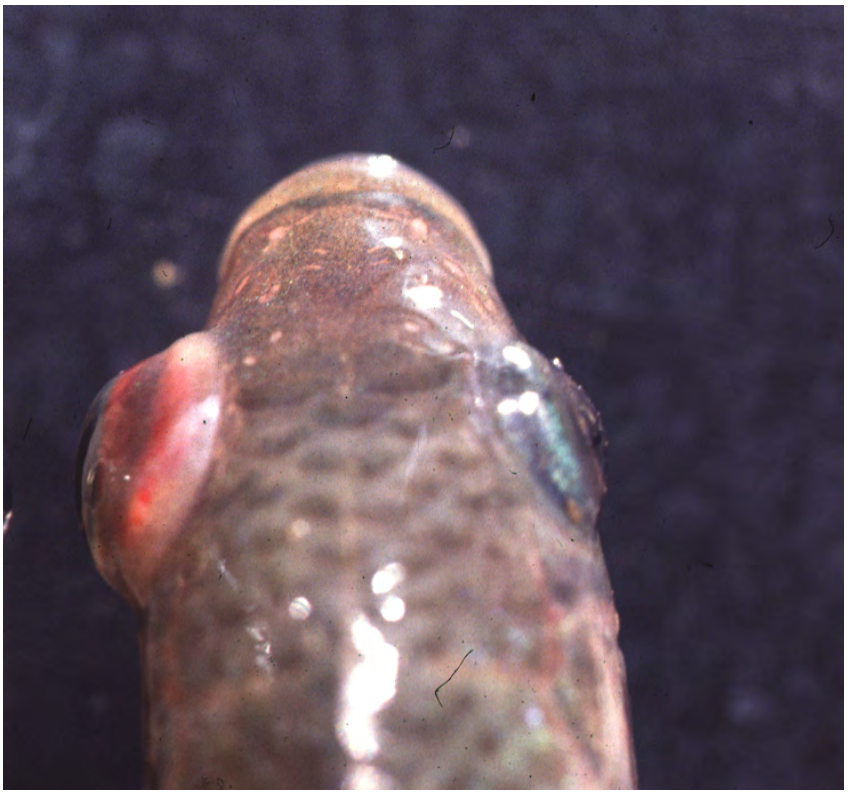


Figure 4. Exophthalmia in a tilapia with a streptococcal infection.



Figure 5. Tilapia with bent tails characteristic of some streptococcal infections.

E. Disease Diagnostic Procedures

Diagnosis is based on the observation of characteristic clinical signs and the isolation and identification of the causative organism. Primary isolation should be made from fish tissue on TSA or BA, or both, incubated at 25 to 35°C for 24 to 48 hours. The addition of whole blood increases isolation efficiency. The brain often is the best organ for bacterial isolation.

1. **Presumptive Diagnosis**
   Organisms isolated in culture are gram-positive, nonmotile, cytochrome oxidase-negative, catalase-negative cocci in chains. The colonies are pinpoint to pinhead in size and convex. In liquid culture, the broth may appear viscous. On BA, colonies of Group B streptococci are nonhemolytic and CAMP-positive, whereas colonies of Group D streptococci show alpha or beta hemolysis and are CAMP-negative.

2. **Confirmatory Diagnosis**
   Diagnosis is confirmed by the isolation of a catalase-negative, gram-positive cocci in chains which is typed by Lancefield grouping as Group B or Group D. Isolates from Asia may be serologically distinct from North American isolates (Kitao 1982).

F. Procedures for Detecting Subclinical Infections

Isolation of *Streptococcus* sp. from locations such as brain tissue of an asymptomatic fish would indicate a subclinical infection.

G. Procedures for Determining Prior Exposure to the Etiological Agent

No procedures have been reported.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

See Section 1, 1.1 General Procedures for Bacteriology.

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1.17 Piscirickettsiosis

Marcia L. House and John L. Fryer

Center for Salmon Disease Research
Department of Microbiology
Oregon State University
Corvallis, OR 97331-3804
mhouse@nwifc.org
fryerj@bcc.orst.edu

A. Name of Disease and Etiological Agent

1. **Name of Disease**
   Piscirickettsiosis, salmonid rickettsial septicemia, coho salmon septicemia, and Huito disease.

2. **Etiological Agent**
   Piscirickettsia salmonis

   This organism is an intracellular rickettsial-like pathogen of fish that replicates within membrane-bound cytoplasmic vacuoles of infected cells. The bacterium is fastidious and does not grow on any known artificial media. It is distantly related to the genera Coxiella and Francisella, and is grouped with the gamma subdivision of the proteobacteria. J. L. Fryer and C. N. Lannan have suggested this bacterium be included in a new family Piscirickettsiaceae and the proposal for the family with the formal description will appear in volume 2, second edition of Bergey's Manual of Systematic Bacteriology (expected publication date in 2002).

B. Known Geographical Range and Host Species of the Disease

1. **Geographical Range**
   Isolated from salmonid fish in Chile, Ireland, Norway, and both the west and east coasts of Canada.

2. **Host Species**
   Piscirickettsiosis has been observed in or isolated from coho salmon (*Oncorhynchus kisutch*), chinook salmon (*O. tshawytscha*), sakura salmon (*O. masou*), rainbow trout (*O. mykiss*), pink salmon (*O. gorbuscha*), and Atlantic salmon (*Salmo salar*). Coho salmon appear most susceptible. Other species of fish may also be susceptible to this bacterium.

C. Epizootiology

Piscirickettsiosis was initially described in 1989 from infected salmonids in Chile. Onset of mortality occurred 6 to 12 weeks after farmed coho salmon had been moved to seawater netpens. Since that time, the disease has been primarily reported in farmed marine fish and has also been observed in salmonids from freshwater facilities. The mechanisms of transmission are not completely understood. Horizontal transmission in salt and freshwater has been demonstrated. Transmission by vectors and the role of vertical transmission remains uncertain. Different isolates have been shown to have varying levels of virulence.

D. Disease Signs

The first evidence of disease may be the appearance of small white lesions or shallow haemorrhagic ulcers on the skin. Affected fish appear dark, lethargic, and collect along the sides of the netpen. The major gross pathological changes are gill pallor, peritonitis, ascites, enlarged spleen, swollen grey kidney, and liver with large pale necrotic lesions.

E. Disease Diagnostic Procedures

Piscirickettsiosis is diagnosed based on clinical signs, isolation of *P. salmonis* in cell culture, and detection in Giemsa stained tissue impressions or sections. It is pleomorphic, ranging from 0.5 to 1.5 µm in diameter, and frequently occurs in pairs (Figure 1). Positive identification is confirmed using *P. salmonis*-specific antibodies in the IFAT.

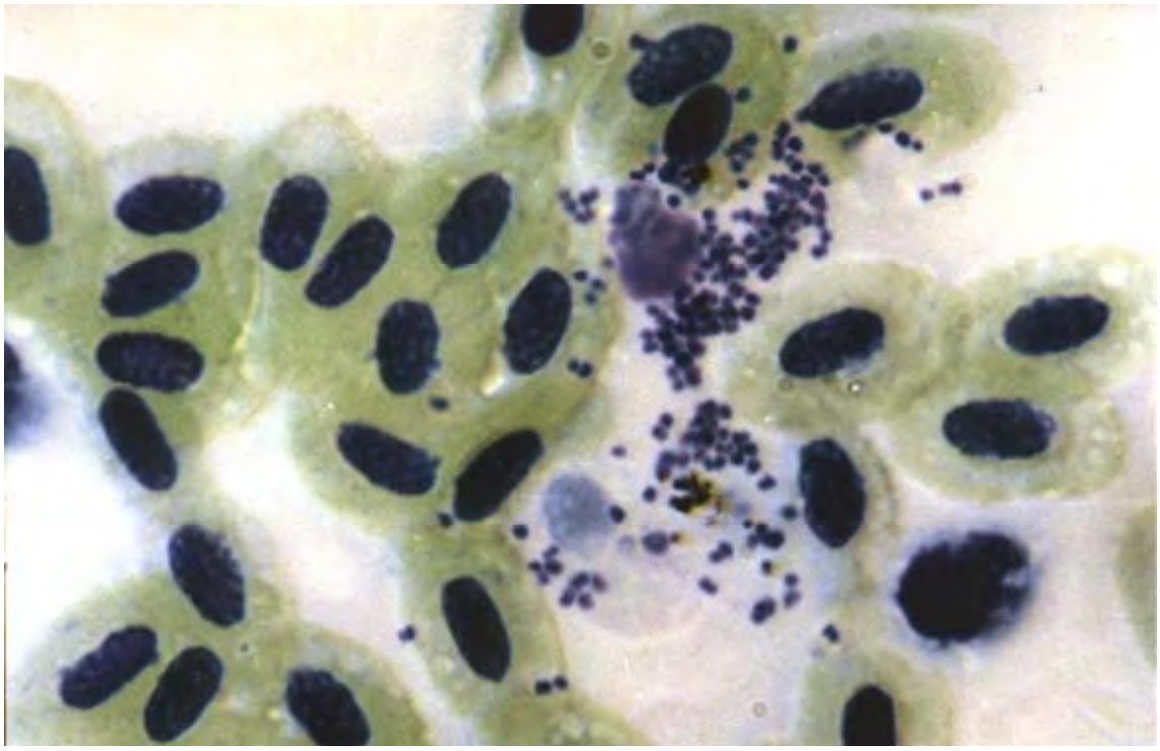


Figure 1. Blood smear from coho salmon infected with *Piscirickettsia salmonis*.

1. Presumptive Diagnosis

Piscirickettsia salmonis is a gram-negative, non-motile, highly fastidious intracellular bacterial pathogen. The organism does not grow on artificial bacteriological media and fish cell cultures are required for isolation. The techniques used to isolate and culture *P. salmonis* are more similar to those used in virology than traditional bacteriology. **DUE TO SENSITIVITY OF *P. SALMONIS* TO ANTIBIOTICS *IN VITRO*, NONE SHOULD BE USED IN MEDIA DURING COLLECTION OF TISSUE OR THE CULTURE OF CELLS.**

Samples of kidney, liver, and blood suitable for bacteriological testing are aseptically collected from diseased fish during either overt or covert infections as described in Section 1, 1.1 General Procedures for Bacteriology. Tissue should be homogenized at 1/20 in antibiotic-free balanced salt solution (BSS), and then, **WITHOUT CENTRIFUGATION**, further diluted 1/5 and 1/50 in antibiotic free BSS for inoculation onto cell cultures. *Piscirickettsia salmonis* cells are bound in membranes and centrifugation will remove the bacteria from the supernatant. Final dilutions for use are 10^{-2} and 10^{-3}. The diluted homogenate can be inoculated directly (0.1 mL/culture) into the antibiotic-free culture medium overlaying the CHSE-214 cell monolayer. The cell cultures must be incubated at 15 to 18°C for 28 days and observed for the appearance of cytopathic effect (CPE). The piscirickettsial CPE consists of plaque-like clusters or rounded cells (Figure 2). With time, the CPE progresses until the entire cell sheet is destroyed. If CPE does not occur (except in the positive controls), cultures should be incubated at 15 to 18°C for an additional 14 days.

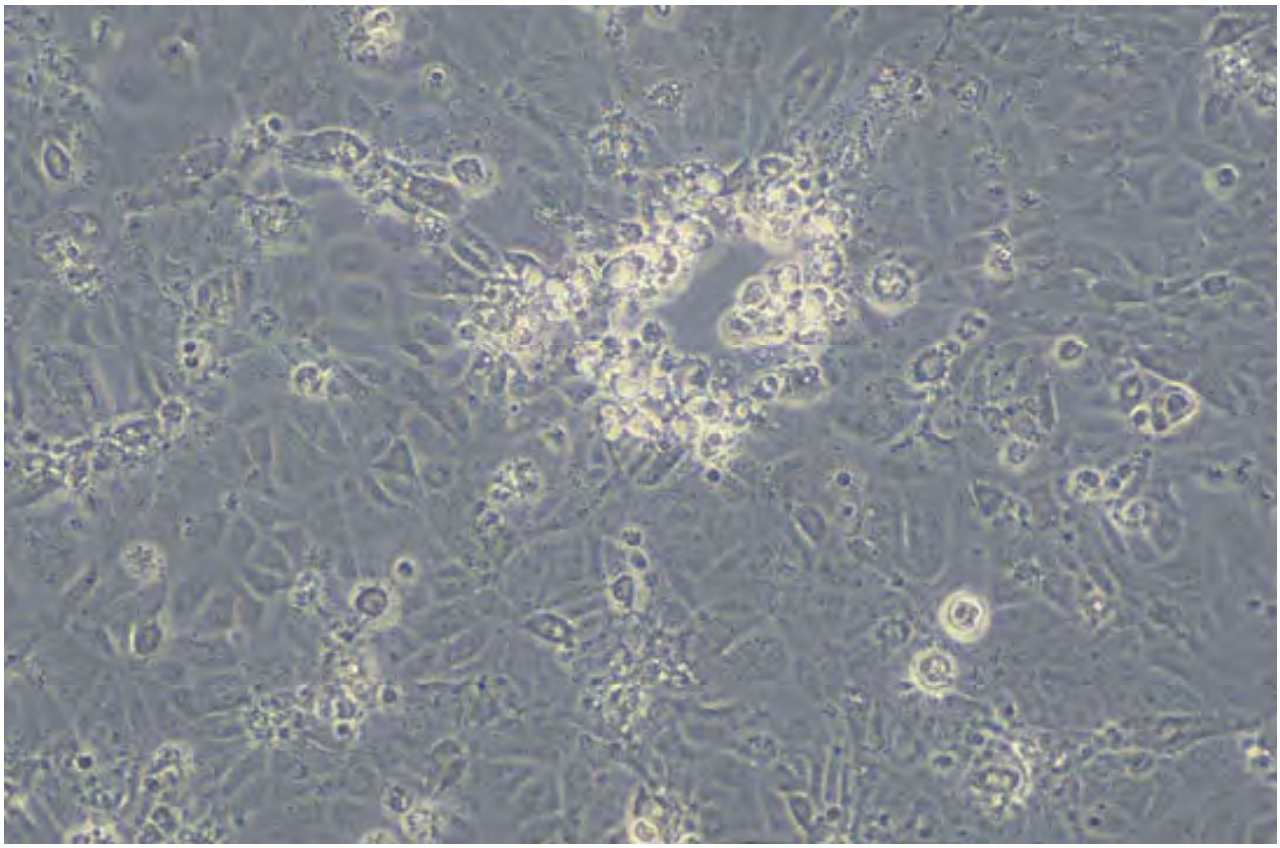


Figure 2. Cytopathic effect (CPE) of *Piscirickettsia salmonis* grown in a CHSE-214 cell culture.

2. Confirmatory Diagnosis

Positive identification using *P. salmonis*-specific antibodies in the IFAT.

F. Procedures for Detecting Subclinical Infections

No procedures have been reported.

G. Procedures for Determining Prior Exposure to the Etiological Agent

No procedures have been reported.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

DO NOT USE ANTIBIOTICS IN THE ISOLATION PROCEDURE. For isolation, the tissue, preferably the kidney, must be aseptically removed and transferred to a sterile container, and, if possible, prepared immediately. If it is not possible to process tissues immediately, they must be kept at 4°C or on ice. **DO NOT FREEZE**. Further preparation should take place as soon as possible (within 6 to 12 hours), as the risk of overgrowth by contaminants increases with time. Smears or impressions of the kidney, liver, and spleen should be prepared, air-dried, and fixed for five minutes in absolute methanol. Tissues smears to be examined by IFAT must be freshly prepared or stored at $\leq$-20°C.

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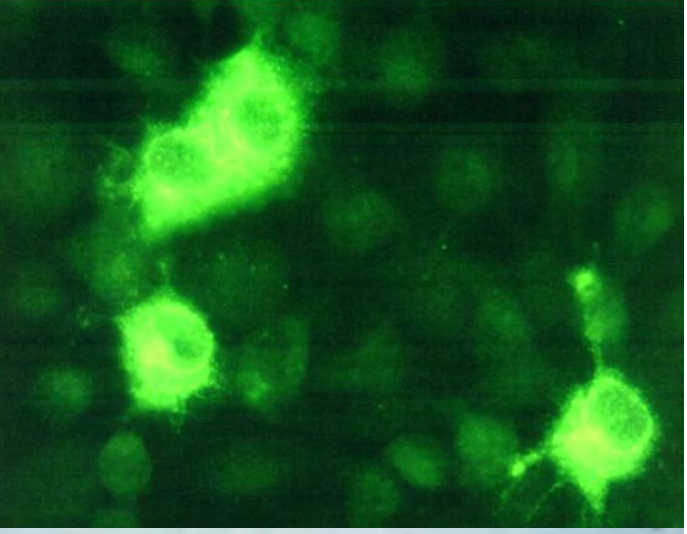
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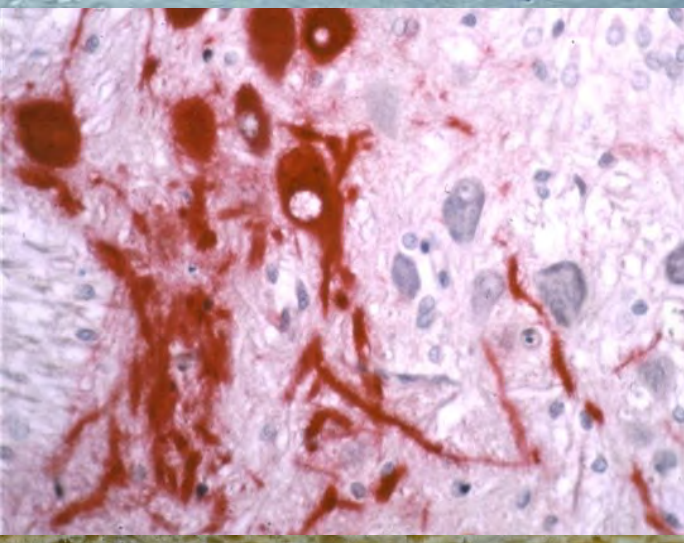
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Section 1
Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens

Chapter 2 Viral Diseases of Fish

2.1 General Procedures for Virology

2.2 Channel Catfish Virus Disease

2.3 Erythrocytic Inclusion Body Syndrome

2.4 Herpesvirus Diseases of Salmonids

2.5 Infectious Hematopoietic Necrosis

2.6 Infectious Pancreatic Necrosis

2.7 Viral Erythrocytic Necrosis

2.8 Viral Hemorrhagic Septicemia

2.9 Infectious Salmon Anemia

2.9.A1 Infectious Salmon Anemia Appendix 1

2.9.A2 Infectious Salmon Anemia Appendix 2

2.10 Spring Viremia of Carp

2.11 Koi Herpesvirus Disease

2.12 Goldfish Herpesvirus Disease

2.13 Largemouth Bass Virus Disease

2.A1 Methods for Testing for the Presence of Mycoplasma in Stock Cell Cultures Appendix 1

2.1 General Procedures for Virology

S. E. LaPatra

Clear Springs Foods, Inc.
Research Division
PO Box 712
Buhl, ID 83316
208/543-3456
scottl@clearsprings.com

A. Tissue and Fluid Sampling and Handling

The selection of tissues for virus assays varies according to the size and life stage of fish. The following tissues are the minimum that should be taken for virus assays.

Size/maturity of fish	Tissue assayed
Under 4 cm	Entire fish (remove yolk sac if present)
4-6 cm	Entire viscera (includes kidney)
Over 6 cm	Kidney and spleen
Sexually mature	Ovarian fluid, kidney, and spleen

For fish 4 to 6 cm or over 6 cm, it is recommended (OIE 2000) that brain tissue also be included. The addition of gill filaments to the sample pool may also increase the sensitivity of detection for some viruses.

After tissues and fluid are removed from the fish, they can be pooled; however, no more than five fish should be in one pooled sample of tissue or fluid. Approximately equal volume or weight proportions should be maintained for each specimen in a pool.

1. Storage of Samples

a. The samples should be maintained between 4 and 10 °C according to the virus(es) suspected. Samples should not be frozen.

b. The samples should not be stored longer than 48 hours.

c. Tissues may be stored in a buffered solution that contains antibiotics, antifungals, or both. The pH should be maintained within 7.4 to 7.8 or within the range that the suspected virus(es) are stable.

B. Preparing Samples for Virus Assays

The preparation of samples involves the homogenization of tissues and bacterial and fungal decontamination of tissues and fluids.

Homogenization can be accomplished in several ways; however, sonication is not acceptable for tissues. After homogenization, cellular material should be removed by centrifugation.

Decontamination can be accomplished either with the use of antibiotics and antifungals or by filtration of the supernatant of centrifuged tissues samples.

1. The antibiotics and antifungals that are used should be wide spectrum in their activity, and their concentrations should be effective in decontamination but not adversely affect cell cultures.

   The following compounds and concentrations should not exceed:

Gentamicin	1000 µg/mL
Penicillin	800 IU/mL
Streptomycin	800 µg/mL
Fungizone®	40 IU/mL
Mycostatin®	400 IU/mL

2. The supernatant from centrifuged tissue samples can also be decontaminated by filtration through a 0.45 µm filter. Passing tissue culture medium supplemented with serum through the filter before the sample is passed is recommended to minimize virus adherence to the filter.

C. Inoculating the Samples

1. Selection of Cell Cultures

Each virus section should be consulted to determine the most sensitive cell line(s) for a given virus. The cells should be normal appearing, rapidly dividing, and mycoplasma-free. Stock cell cultures should be routinely tested for susceptibility to specific viruses and for the presence of mycoplasma (see 2.A1 Methods for Testing for Mycoplasma Appendix 1). Penicillin (100 IU/mL) streptomycin (100 µg/mL), and antifungal agents such as Mycostatin (50 IU/mL) can be used in media for cell culture and virus assay work.

2. Inoculation

The cell cultures used for sample inoculation should be 80-90% confluent and not older than 48 hours. A minimum of 50 µL of sample should be inoculated per l.0 $cm^2$of cell sheet. Uninoculated controls must be used. Dilution of original samples should not exceed 1:10 for fluids and 1:100 for tissue samples.

3. Duration of Assay

The cell cultures should be incubated at 15°C and observed for cytopathological changes for a minimum of 14 days but 28 days incubation is recommended. Cell culture medium should be buffered or cells incubated so that a pH between 7.4 and 7.8 is maintained. A blind pass of 14 days is also recommended. The duration of the assay may need to be longer depending on which

viruses are suspected. When cytopathogical changes occur, the cultures should be subcultured or analyzed by serum neutralization or other confirmatory tests.

D. Virus Identification

Presumptive identification is based on the disease history of the fish stock, clinical signs observed at sampling, and cell culture results (CPE). However, to definitively identify a particular virus, certain confirmatory methods must be used.

1. Serum Neutralization Test

a. A dilution of neutralizing antiserum (polyclonal or monoclonal) should be used that allows neutralization of 10^2 to 10^6 plaque forming units (PFU) or 50% tissue culture infective dose ($TCID_{50}$) per mL of the homologous virus.

b. Dilute the suspect sample from 10^2 to 10^6 in a sterile balanced salts solution.

c. Combine equal volumes of each dilution of the suspect sample and diluted antiserum. Repeat the procedure for a positive control virus. Include negative controls for both the suspect and homologous virus. Incubate for one hour at 15°C with agitation.

d. Inoculate onto the cell line in which the suspect virus was isolated. Incubate at 15°C for 14 days and observe for cytopathic effect (CPE). Equivalent inhibition of CPE by a specific antiserum for both the suspect and homologous virus, but not for negative controls, provides confirmatory identification.

2. Other Confirmatory Procedures

Other procedures that are routinely used for confirmatory virus identity include the fluorescent antibody test (FAT), dot blot methods, enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR). Consult each virus section for references on alternative confirmatory methods or Section 2, 2.2 Table 2.1 Confirmatory Technique.

References

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2.2 Channel Catfish Virus Disease

J. A. Plumb

Auburn University
Department of Fisheries and Allied Aquaculture
Auburn, AL 36849
205/826-4786
jplumb@ag.auburn.edu

A. Name of Disease and Etiological Agent

Channel catfish virus disease (CCVD) is the common name of the disease. The etiological agent is channel catfish virus (CCV). This virus is a member of the *Herpesviridae* family and the name *Herpesvirus ictaluri* has been suggested.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Found in Alabama, Arkansas, California, Colorado, Georgia, Iowa, Kansas, Kentucky, Mississippi, Nebraska, Oklahoma, Texas, West Virginia, Minnesota, Idaho, and Honduras.

2. Host Species

The channel catfish *Ictalurus punctatus* is the primary host, and experimental infection of the blue catfish *Ictalurus furcatus*, suggests that this species could be infected under natural conditions. European catfish *Silurus glanis* are marginally susceptible to CCV whereas other species are refractive.

C. Epizootiology

Channel catfish virus disease is generally considered a highly communicable infection among young-of-the-year cultured catfish. The virus infects fry and fingerlings during summer months when water temperatures exceed 25^{o}C. Although CCV is sometimes seen in one-year-old fish, it is rare. Fish less than 5 cm long may suffer over 90% mortality but as they become older the mortality is reduced. After mortality stops, CCV cannot be isolated from survivors. However, there is one report of CCV isolation from stressed adult channel catfish in the winter when water temperatures were below 8^{o}C. The mortality pattern of infected young fish is characterized by a very rapid acceleration of deaths. The mortality may depend upon age and size of the fish, environmental conditions, fish density, strain of channel catfish, and invasion by secondary bacterial pathogens such as *Aeromonas hydrophila* and *Flexibacter (Cytophaga) columnaris*.

D. Disease Signs

Infected fish swim erratically, sometimes rotating about the longitudinal axis and at times holding their head up in the water. Externally, diseased fish show abdominal distention, exophthalmia, (Figure 1) pale or hemorrhagic gills, and petechiae at the base of fins and throughout the skin, particularly on the ventral surface.

The body cavity is filled with clear to yellowish fluid (ascites), and hemorrhages are evident throughout the musculature, liver, kidney, and spleen. The liver, kidney, stomach, and intestine may be pale in advanced stages of the disease. The gastrointestinal tract is filled with a mucoid secretion and is void of food.

Histopathology is characterized by an increase in lymphoid cells in the kidney. Renal tubules are necrotic and edematous. Necrosis and edema occur in hematopoietic tissue surrounding the tubules. The liver shows diffuse necrosis, edema, and hemorrhage. Hemorrhage, edema, and possibly mucosal sloughing occur in the intestine. The spleen becomes congested and edematous, and macrophages are laden with degenerated erythrocytes. Cardiac tissue may become necrotic, and focal hemorrhages may occur in the cardiac musculature.

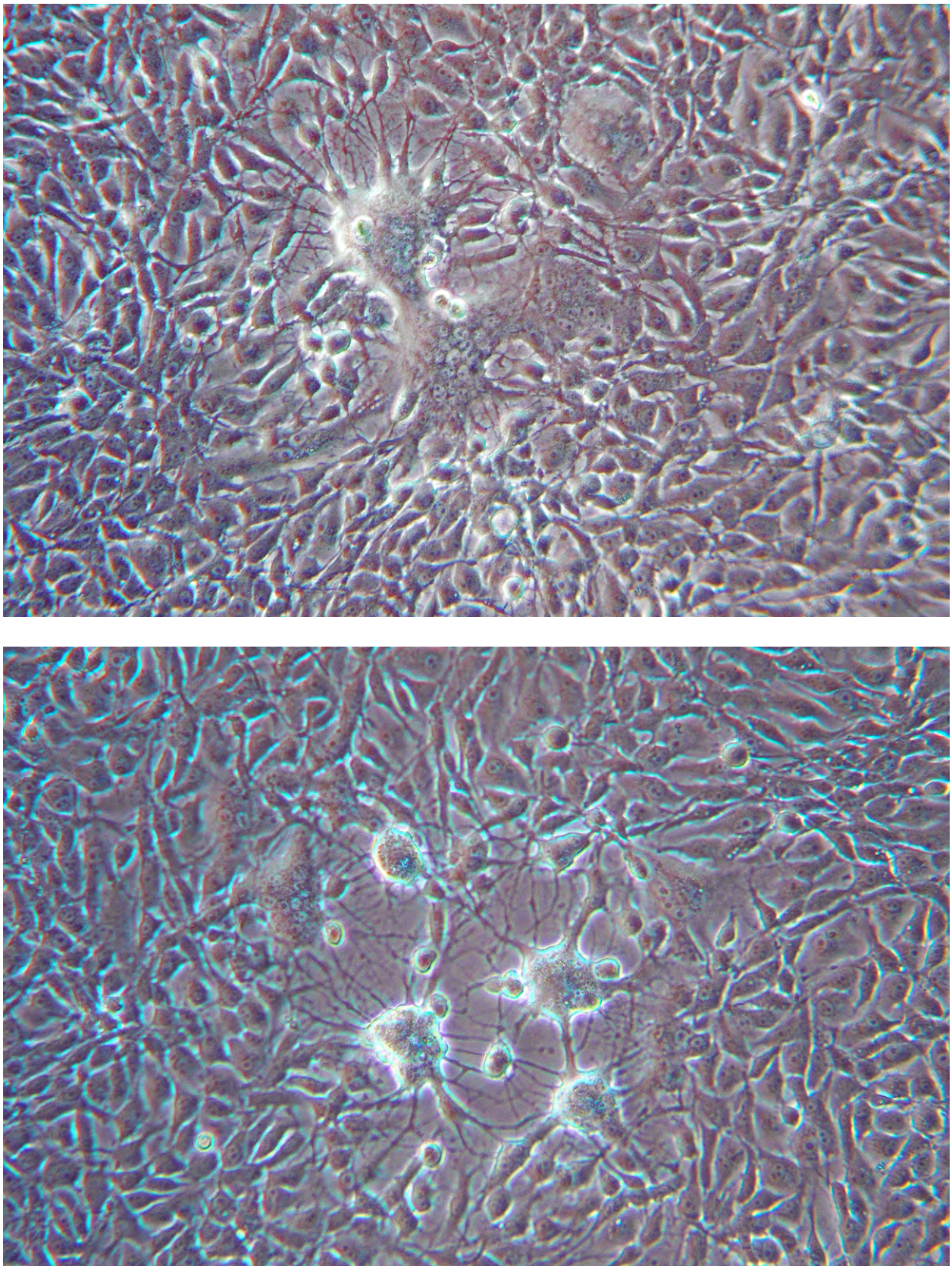


Figure 1. Channel catfish fingerling with abdominal distention and exophthalmia typical of CCV disease.

E. Disease Diagnostic Procedures

1. **Presumptive Diagnosis**
   Processed samples are inoculated onto brown bullhead (BB) cells (ATCC 59) or channel catfish ovary (CCO) cells and incubated at 25 to 30^{o}C at pH 7.2 to 7.4. Channel catfish ovary cells are approximately ten times more sensitive than BB cells. Inoculated cultures are incubated for 14 days and observed for typical CPE (cell fusion and syncytium formation). The presence of clinical signs during a period when water temperatures exceed 24^{o}C can serve as a presumptive diagnosis.

2. **Confirmatory Diagnosis**
   The virus must be isolated and its identity confirmed by serum neutralization.



Figures 2 and 3. Phase contrast micrographs showing the cytopathic effect of CCV on CCO cells. The large multi-nucleate syncytia are typical of this virus.

F. Procedures for Detecting Subclinical Infections

No procedures have been reported.

G. Procedures for Determining Prior Exposure to the Etiological Agent

Prior exposure to CCV can be determined by detection of specific antibodies (Plumb 1973b; Amend and McDowell 1984). Serum samples from adult fish are heat inactivated at 45°C for 30 minutes. The serum is diluted 1:50 and reacted with known CCV at 100 $TCID_{50}$ or PFU/0.1 mL of the serum and virus mixture. Sera demonstrating greater than 50% plaque or $TCID_{50}$ reduction are considered to be from fish previously exposed to CCV. The CCV antibody titers in fish increase as water temperatures rise.

Prior exposure to CCV may also be indicated by detection of CCV DNA (Colyer et al. 1986; Bird et al. 1988). Several reports describe the use of molecular cloning of channel catfish virus genome and the use of nucleic acid probes to detect CCV genetic material in channel catfish. These techniques are not yet sufficiently refined for practical application.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

See Section 1, 2.1.1.B General Procedures for Virology.

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2.3 Erythrocytic Inclusion Body Syndrome

J. S. Rohovec

Oregon State University
Department of Microbiology
Corvallis, OR 97331-3804

A. Name of Disease and Etiological Agent

Erythrocytic inclusion body syndrome (EIBS) is caused by EIBS virus. The disease also has been called salmon anemia or viral induced anemia. The agent that causes this disease has not been isolated nor fully characterized.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

To date the disease has been presumptively recognized in the states of California, Idaho, Oregon, and Washington. A morphologically similar virus has been observed in fish from Norway, Ireland, and Japan.

2. Host Species

Naturally occurring infections have been demonstrated in coho salmon *Oncorhynchus kisutch,* chinook salmon *Oncorhynchus tshawytscha*, and Atlantic salmon *Salmo salar*. Artificial infections have been induced in rainbow (steelhead) trout *Oncorhynchus mykiss,* cutthroat trout *Oncorhynchus clarki*, brown trout *Salmo trutta*, brook trout *Salvelinus fontinalis*, chum salmon *Oncorhynchus keta,* and kokanee salmon *Oncorhynchus nerka*. The susceptibility of other salmonid fish has not been determined.

C. Epizootiology

Erythrocytic inclusions and typical virus particles have been detected in spawned adult salmon, but in fresh water the disease seems to be limited to juvenile and smolting fish. In salt water, EIBS has been diagnosed in fish as large as 500g and has caused problems in netpen culture. The disease is more frequently diagnosed in fish during periods of cool water in the fall, winter, and spring. Diet may affect the severity of the disease. Epizootics can have long durations (up to five months) or be more acute and are most often complicated with other pathogens, i.e. *Flavobacter psychrophilus, Renibacterium salmoninarum,* and external fungi.

D. Disease Signs

Diseased fish are usually anemic and lethargic. Other pathogens are frequently observed, but fish can succumb to anemia and oxygen deprivation. Pale gills and pigmentation abnormalities may be observed. Internal tissues exhibit signs associated with anemia and splenomegaly is usually apparent. Hematocrits may range from as low as 1 to 4% to slightly below normal values (25 to 30%).

Histopathologically there is congestion in the kidney and spleen. Hemosiderin deposits can be found in the spleen. Blood smears stained with pinacyanol chloride reveal erythrocytic cytoplasmic inclusions which range in size from 0.8 to 2 μm. Occasionally multiple inclusions of varying size are observed.

E. Disease Diagnostic Procedures.

The EIBS virus has not yet been isolated in cell culture. Presumptive diagnosis is based on light microscopic observation of characteristic erythrocytic cytoplasmic inclusions in blood smears fixed with methanol and stained with pinacyanol chloride or Leishman-Giemsa (Leek 1987).

1. **Pinacyanol Chloride Staining and Light Microscopy**
   Air dried blood smears are fixed in 100% methanol for five minutes and then stained with pinacyanol chloride for two minutes. The stain can be prepared by mixing 2.5 g pinacyanol chloride powder with 367.0 mL 95% ethanol and 132.5 mL distilled water. Smears are examined at 1000X for characteristic erythrocytic inclusions.

2. **Acridine Orange Staining and Fluorescent Microscopy**
   Methanol/ethanol (1:1) fixed smears which are rehydated in ethanol series of 100%, 70%, and 50% and stained with 0.1% aqueous acridine orange, (3,6-bis[dimethylamino] acridinium chloride hemi-[zinc chloride]) then washed in phosphate buffered saline which can aid in differentiating inclusions of EIBS and those of viral erythrocytic necrosis (VEN). Under fluorescence microscopy at 1000X, inclusions of EIBS stain red; those of VEN stain green.

F. Procedures for Detecting Subclinical Infections

Often typical inclusions can be observed in fish that do not show overt anemia or disease. Conversely, at times when anemia is most severe, inclusions may be absent or difficult to find.

G. Procedures for Determining Prior Exposure to the Etiological Agent

No procedures have been reported.

H. Procedures for Transportation and Storage of Samples to Insure Maximum Viability and Survival of the Etiological Agent

Diagnosis does not depend on viability or survival of the pathogen. Blood should be collected in heparin

and could be made immediately. Within a few hours, smears should be fixed for five minutes with absolute methanol and then stained with pinacyanol chloride (Leek 1987). For electron microscopy, heparinized blood should be fixed in Hawke's fixative (Rohovec and Amandi 1981) within 24 hours of collection.

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2.4 Herpesvirus Diseases of Salmonids

W. D. Eaton and R. P. Hedrick*

Science and Technology
Malaspina College
900 5th St.
Nanaimo, BC, Canada V9R 5S5
604/753-3245

*School of Veterinary Medicine
Department of Medicine
University of California, Davis
Davis, CA 95616
916/752-3411
rphedrick@ucdavis.edu

I. *Herpesvirus salmonis* Type 1

A. Name of Disease and Etiological Agent

Herpesvirus disease of salmonids is caused by *Herpesvirus salmonis* Type 1 previously *Herpesvirus salmonis* (HPV) and steelhead herpesvirus (SHV).

Herpesvirus salmonis and steelhead herpesvirus from salmonids in western USA have been shown to be closely related strains of the same virus as demonstrated by serological and DNA homology comparisons (Hedrick et al. 1987; Eaton et al. 1991). The North American viruses have properties in common with herpesviruses found among salmonids in Japan but differ sufficiently to separate them into two respective groups, *Herpesvirus salmonis* Type 1 and Type 2 (HPV-1 and HPV-2) based on both serology and DNA homology (Hedrick et al. 1987; Eaton et al. 1991).

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Herpesvirus salmonis Type 1 has been found only in the states of Washington and California.

2. Host Range

HPV-1 was first recognized in 1971 in rainbow trout adults at the Winthrop National Fish Hatchery. However, both rainbow trout *Oncorhynchus mykiss* and chinook salmon *Oncorhynchus tshawytscha* juveniles were found to be susceptible to experimental infection by bath exposure. Atlantic salmon *Salmo salar*, brown trout *Salmo* trutta, and brook trout *Salvelinus fontinalis* were refractory to the virus following intraperitoneal (IP) injection. A second strain of HPV-1 was found in 1985 in California among hatchery-reared steelhead trout

and rainbow trout adults and later among steelhead alevins suffering mortality. The HPV from California was experimentally transmitted to rainbow trout and chinook salmon juveniles by the IP and waterborne routes but not to brown trout *Salmo trutta* or coho salmon *Oncorhynchus kisutch.*

C. Epizootiology

HPV-1 was isolated from 1971 to 1975 at the Winthrop Hatchery from the ovarian fluid of moribund adult rainbow trout that suffered up to 50% post spawning losses. HPV-1 was never isolated from alevins or other juvenile rainbow trout at the hatchery. The virus was transmitted experimentally to juvenile rainbow trout and chinook salmon but severity and prevalence of infection seemingly decreased with fish age. HPV-1 has also been isolated from ovarian fluids of several healthy rainbow and steelhead trout brood stocks in California since 1985 (W. H. Wingfield, California Department of Fish and Game, personal communication). The sole isolation of HPV-1 from juvenile steelhead trout occurred in 1991. These fish were dying from an infestation of ectoparasites and the role of the virus in the mortality remains unknown (Hedrick et al. 1992). The presence of HPV-1 in ovarian and seminal fluids indicates a potential for vertical transmission. Horizontal transmission is also suspected since experimental waterborne exposures to HPV have resulted in infections

D. Disease Signs

The role of HPV in post-spawning losses is unknown but these fish exhibit overall darkening of the body, a slightly distended abdomen, and occasional exophthalmia. A mild ascitic fluid, pink discoloration of liver and adipose tissues accompanied by a flaccid condition of visceral organs and skeletal muscle resulting from edema occurs.

Experimentally-infected fish show darkened bodies, abdominal distention, and exophthalmos with hemorrhages in the orbital regions and the base of the fins. Mucoid fecal casts and pale gills are also evident. Ascites is evident as is a mottled liver, pale kidney, and empty anterior digestive tract. The liver, spleen, kidney, and heart area are flaccid.

Microscopic pathology of natural infections include a generalized edema of visceral organs and hyperemia of the liver and adipose tissue. In experimentally infected fish, edema of cardiac and skeletal muscle, congestion and necrosis of the hematopoietic tissues of the kidney are evident. Congestion and necrosis are also observed in the gill, heart, digestive tract, liver and pancreas. Syncytium formation and small multifocal regions of inflammatory cells in the acinar pancreas and among hepatocytes is considered pathognomonic for HPV-1 infections. Syncytium formation and small multifocal regions of inflammatory cells in the liver are the only signs in rainbow trout experimentally infected with HPV-1 from California.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

Presumptive diagnosis results from the observation of typical herpesvirus CPE in inoculated salmonid tissue culture cells. Final dilution of ovarian fluid should not exceed 1:10 and final

dilution of tissue samples should not exceed 1:50 prior to inoculation onto either CHSE-214 or RTG-2 cells. Cells should be inoculated before a completed monolayer is formed (50% monolayer) because these viruses often take several weeks to induce CPE. Inoculated cells should be incubated at 10°C because HPV and SHV do not grow well at higher temperatures. Cells should be observed for typical herpesvirus CPE, which consists of cell fusion resulting in plaques and multinucleated giant cells with intranuclear Cowdry type A inclusion bodies. Samples containing very low levels of SHV or HPV will appear negative after 2 to 3 weeks on tissue culture cells, yet after one or two blind passages, the viral-induced CPE can occur. Thus, routine sampling procedures should include a three week initial incubation period followed by a blind passage and another three week incubation before a sample is considered negative.

2. Confirmatory Diagnosis

Confirmatory diagnosis requires serum neutralization assays using anti-HPV or anti-SHV antisera. This will allow differentiation between the strains of Type 1 *Herpesvirus salmonis* (HPV and SHV) and Type 2 (OMV, YTV, CSTV, NeVTA, etc.).

F. Procedures For Detecting Subclinical Infections

No procedures have been reported.

G. Procedures For Determining Prior Exposure to the Etiological Agent

No procedures have been reported.

H. Procedures For Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

See Section 1, 2.1.1.B General Procedures for Virology.

II. *Herpesvirus salmonis* Type 2

A. Name of Disease and Etiological Agent

Herpesvirus disease of salmonids is caused by *Herpesvirus salmonis* Type 2. Previously the oncogenic viruses include *Oncorhynchus masou* virus (OMV) and H-83, yamame tumor virus (YTV), coho salmon tumor viruses (CSTV, OKV, COTV, CSLV), and the nononcogenic Nerka virus from Towada Lake, Akita and Amori Prefecture (NeVTA).

The salmonid herpesviruses from Japan appear to be related strains of *Herpesvirus salmonis* Type 2 (HPV-2). NeTVA appears to be a unique isolate among HPV-2 strains because it does not show the oncogenic properties shared by other strains. A serological and DNA homology comparison of OMV, YTV, and NeTVA, with two HPV-1 strains supports their separation into a related but distinct group designated HPV-2.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Found in Japan.

2. Host Range

OMV has been isolated from masou salmon *Oncorhynchus masou*, but juvenile kokanee *Oncorhynchus nerka*, coho *Oncorhynchus kisutch* and chum salmon *Oncorhynchus keta*, and rainbow trout *Oncorhynchus mykiss* are susceptible to experimental infections via the waterborne route. YTV has been isolated from a mandibulal tumor in yamame salmon *Oncorhynchus masou* and it has been shown to be pathogenic for juvenile yamame salmon and chum salmon. CSTV and additional coho salmon tumor isolates have been obtained from fin and mandibular tumors of pen-reared coho salmon. NeVTA has been isolated from kokanee salmon fry suffering mortality as high as 80%. The virus has only been recovered from fry reared at the hatchery between 1970 to 1974 at Towasa Lake, Akita and Amori Prefecture.

C. Epizootiology

In 1978, OMV was isolated from the ovarian fluid of healthy appearing adult female masou salmon whose progeny had a history of poor fry survival rates. In the laboratory, fry were exposed to water containing OMV to determine the pathogenicity of the virus. Kokanee salmon were found to be the most susceptible to OMV, followed by the masou, chum and coho salmon, and rainbow trout. Both vertical and horizontal transmission are likely. Susceptibility decreased with increasing age; by eight months of age salmon were completely refractory to the virus; however, squamous cell epitheliomas developed in the opercula, head, mouth, eyes, caudal, fin, and kidney of chum and coho salmon surviving experimental OMV infections. OMV was isolated from some of the tumors and from primary cell cultures derived from tumor tissue.

YTV was originally isolated from mandibular tumors on juvenile yamame salmon in Japan. Five-month-old yamame salmon were more susceptible to infections of YTV than chum salmon. Some surviving yamame and chum salmon developed tumors. The tumors were classified as basal cell epitheliomas.

Only limited information is available on the pathogenesis and epizootiology of CSTV. Virus isolated from tumors of coho salmon was infectious for other coho salmon injected with the virus. The experimental challenge resulted in less than 28% mortality, and tumor formation occurred in 8% of the coho salmon.

NeVTA epizootics in sockeye salmon (kokanee) fry in Towada Lake (Honshu) between 1970 and 1974 caused mortality as high as 80%. The virus has been isolated from adult kokanee on Honshu.

D. Disease Signs

1. OMV

Fish experimentally infected with OMV became lethargic, anorexic, and congregate at the water intake. Gross signs include exophthalmia and petechiation of the body surface. Naturally infected fish only showed a darkened body color. Experimentally challenged fish have mottled

and swollen livers, swollen spleens, and empty digestive tracts. Surviving fish may develop tumors on the external surfaces of the opercula, heads, mouths, eyes, and caudal fins. Microscopic examination of tissues from fish infected with OMV show necrosis and syncytia formation in the liver and hematopoietic tissue of the kidney and edema, and less severe necrosis of the spleen, pancreas, cardiac muscle, and brain. One fish developed an epithelioma in the kidney.

Fish experimentally infected with YTV developed exophthalmia, abdominal distention, and hyperemia of the anus. Mandibular tumors have been observed in fish naturally infected with YTV. Fish infected with YTV develop basal cell epitheliomas but showed no other histopathological changes.

2. **CSTV**
   Fish naturally infected with CSTV develop fin and mandibular tumors. No other gross external signs are observed.

3. **NeVTA**
   Infected fish are lethargic clustering around water intake and are anorexic, and some swim erratically. External gross signs include darkened bodies and abdominal distention. No internal gross signs are reported. Microscopic findings include granular degeneration of the skeletal muscle, proteinaceous fluid in Bowman's spaces, kidney tubule necrosis, hypertrophy and desquamation of the gill epithelium, vacuolation of the pancreatic acinar cells and syncytia, and cytoplasmic inclusions in the kidney interstitium.

E. Disease Diagnostic Procedure

1. **Presumptive Diagnosis**
   Ovarian fluid and tissue samples should be diluted as described previously for HPV and SHV; however, CHSE-214 or RTG-2 cells should be incubated at 15^{o}C rather than 10^{o}C. OMV, YTV, and NeTVA replicate at 10 to 20^{o}C and virus replication at 15^{o}C suggests that neither HPV nor SHV is involved. Infected tissue culture cells should exhibit the typical herpesvirus CPE.

2. **Confirmatory Diagnosis**
   Serum neutralization assays using antiserum against OMV, YTV, or CSTV can be performed. However, this will only differentiate OMV, YTV, CSTV, and NeVTA from SHV and HPV. There is serological cross-reaction among OMV, YTV, CSTV, and NeVTA.

F. Procedures for Detecting Subclinical Infections

No procedures have been reported. Subclinical infections may be detected by cell culture assays.

G. Procedures for Determining Prior Exposure to the Etiological Agent

No procedures have been reported. Anti-OMV neutralizing activity has been detected in serum of experimentally infected chum salmon. Titers up to 1:320 were detected.

H. Procedures For Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

See Section 1, 2.1.1.B General Procedures for Virology.

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2.5 Infectious Hematopoietic Necrosis

S. E. LaPatra

Clear Springs Foods, Inc.
Research Division
P.O. Box 712
Buhl, ID 83316
208/543-3456
scottl@clearsprings.com

A. Name of Disease and Etiological Agent

Infectious hematopoietic necrosis (IHN) is caused by infectious hematopoietic necrosis virus (IHNV). Synonyms include: sockeye salmon virus disease, Oregon sockeye virus (OSV), Sacramento River chinook disease (SRCD), and Sacramento River chinook virus (SRCV) (see Figure 1 and Figure 2).

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Infectious hematopoietic necrosis virus is considered to be enzootic to areas of the west coast of North America including Alaska, Washington, Idaho, Oregon, and California in the United States, and British Columbia, Canada. The disease has been observed in other areas of the United States including Colorado, Minnesota, Montana, New York, South Dakota, West Virginia, and Virginia but apparently did not become established. Currently, IHNV is considered to have a wide geographic range that includes North America, Europe, and the Far East.

2. Host Species

Epizootics of IHN have commonly occurred in juvenile sockeye (kokanee) *Oncorhynchus nerka* and chinook salmon *Oncorhynchus tshawytscha* and rainbow and steelhead trout *Oncorhynchus mykiss.* The disease has also been reported in Atlantic salmon *Salmo salar*, pink salmon *Oncorhynchus gorbuscha*, chum salmon *Oncorhynchus keta*, and cutthroat trout *Oncorhynchus clarki* in North America. Brook trout *Salvelinus fontinalis* and brown trout *Salmo trutta* have also been shown to be susceptible. However, recent studies examining the IHNV susceptibility of trout and char in the genus *Salvelinus* have suggested that certain species within this group may be resistant or exhibit intermediate resistance to IHNV. In Japan, epizootics have occurred in amago salmon *Oncorhynchus rhodurus*, yamame salmon *Oncorhynchus masou*, and chum salmon. Coho salmon *Oncorhynchus kisutch* are considered to be refractory to the disease but the virus has been isolated from adults.

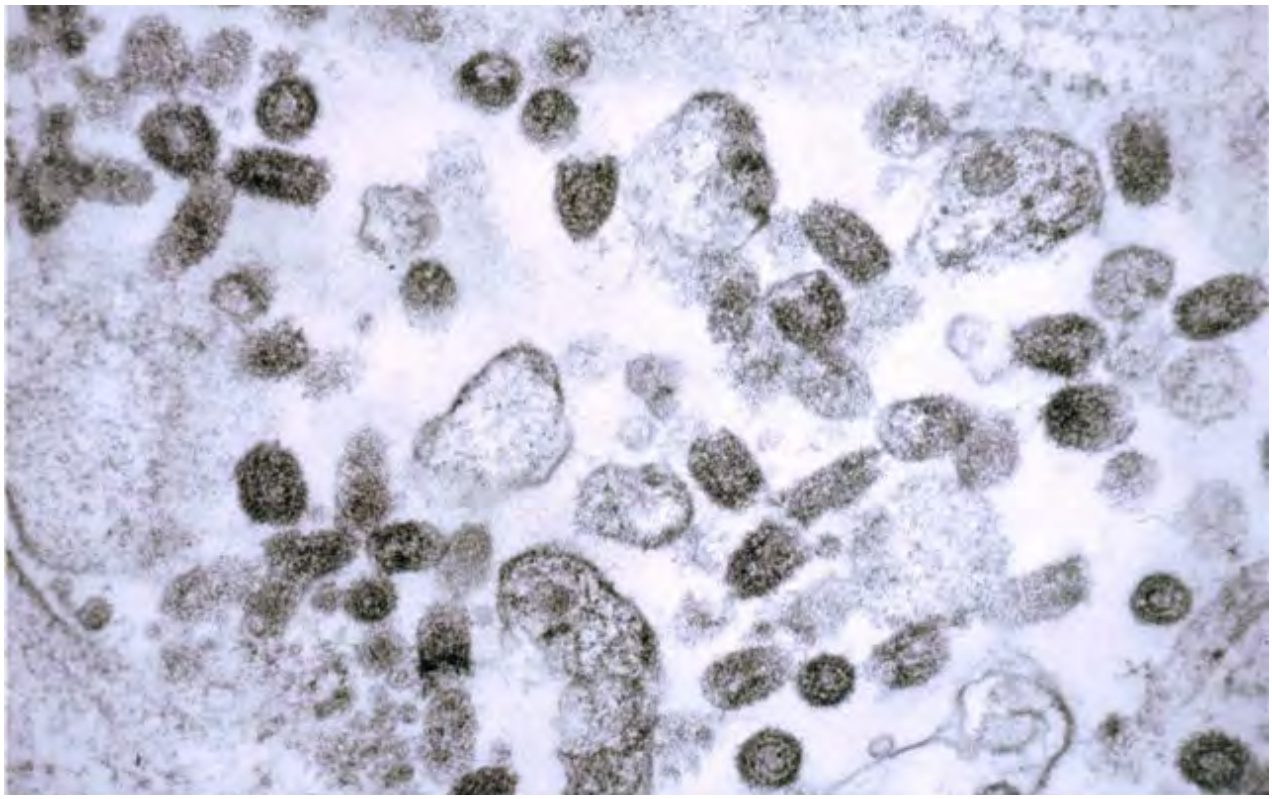


Figure 1. Electronmicrograph image of IHNV in tissue culture.



Figure 2. Electronmicrograph image of negative stained IHNV particles.

C. Epizootiology

Observation from naturally occurring disease and experimental infections indicate that fish up to two months of age are most susceptible. In recent years, epizootics have also been reported in yearling sockeye and two-year-old kokanee salmon. The disease has been reported in large rainbow trout (100-500g) and yearling chinook salmon and steelhead. The disease is still less common and more chronic in larger fish than in smaller fish. Most IHN occurs at temperatures of 15^{o}C or less in freshwater.

Beginning in 1992, IHN was diagnosed in netpen reared Atlantic salmon in the marine environment in British Columbia. By 2002 the disease had spread to numerous sites and was reported in fish that ranged in size from 60 g to 6.8 kg and was associated with high rates of mortality independent of fish size.

Horizontal transmission has been demonstrated; waterborne transmission can be accomplished in the laboratory. Clinically infected juvenile salmonids and carrier adults are the reservoirs of virus for waterborne transmission. No other reservoirs of virus have been identified. Evidence for vertical transmission in natural outbreaks is circumstantial and only one report documents such an event under laboratory conditions. A life-long carrier state in a portion of adult rainbow trout that had survived an epizootic as juveniles has been reported. Attempts to confirm this observation in sockeye salmon or steelhead have not been successful, but the numbers of animals examined may not have been sufficient.

D. Disease Signs

Moribund fish are lethargic, swim high in the water column, and are anorexic. They exhibit exophthalmia, darkening of body color, abdominal distension, pale gills, and hemorrhages at the bases of fins. Fecal casts trailing from the vent have been reported but are not always observed. Swelling of the dorsal cranium has also been observed in some salmon species. Spinal deformities among surviving sockeye salmon and rainbow trout can occur. None of the signs described are considered to be pathognomonic for the disease. Signs of the disease in older fish are different from those observed in young fish. Cutaneous lesions at the bases of fins and on the caudal peduncle often with secondary invasion by fungi and loss of the dorsal fin have been reported. In general, the disease in older fish is more subtle than IHN in smaller fish and different manifestations of the condition are known to occur. Sexually mature adults that are carriers of the virus show no clinical signs.

Gross internal signs include paleness of the viscera (because of anemia) and petechial hemorrhaging in adipose tissue and mesenteries surrounding the viscera. The stomach is devoid of food and contains a milky white exudate. Ascites can be found in the body cavity and petechiation and hemorrhaging is sometimes seen in the swim bladder and lower intestine.

Extensive degeneration and necrosis of the kidney, spleen, liver, and pancreas are observed microscopically. Granular cells of the stomach and intestine may show degenerative changes. Histological changes in the hematopoietic tissue of the anterior kidney are observed during early stages of the disease and may extend throughout the kidney. The tubules and glomeruli are not significantly affected. Necrosis of splenic hematopoietic tissue and of the endocrine and exocrine tissue of the pancreas is diffuse, but the liver can have areas of focal necrosis. Kidney imprints and blood films from infected fish show degenerating leukocytes, large quantities of cellular debris, and macrophages with vacuolated cytoplasm (see Figure 3).

Rainbow trout have also been reported with a “neurotropic” form of IHN and exhibited behavioral

abnormalities and had high virus concentrations localized in brain tissue (LaPatra et al. 1995) (see Figure 4).

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

To aid in the diagnosis of IHN, certain key features such as life stage and species of fish, water temperature, clinical signs, and disease history of the facility and stock of fish are evaluated. To isolate IHNV, tissue and reproductive fluids are examined by standard cell culture techniques. Processed specimens must be inoculated onto the *Epithelioma papillosum cyprini* (EPC), chinook salmon embryo (CHSE-214), or fathead minnow (FHM) cell lines. Pretreatment of EPC cell monolayers with a 7% (w/v) solution of polyethylene glycol (PEG; 20,000 MW) has been shown to increase sensitivity of detection and decrease incubation time (Batts and Winton 1990). Cytopathic effect includes grape-like clusters of round refractile cells which can be observed 48 to 96 hours post-inoculation depending on the concentration of virus in the inoculum (see Figure 5 and Figure 6). Plaque assay procedures similar to those of Burke and Mulcahy (1983) which use a methyl cellulose overlay are also used for isolation and enumeration of IHNV The SSN-1 cell line derived from striped snakehead has also been evaluated by plaque assay. The results of a preliminary study indicated that the SSN-1 cell line may be more sensitive to IHNV then the EPC line (LaPatra et al. 1999). Clinical signs, microscopic pathology, past history of IHNV, and observation of typical CPE provide the best evidence for a presumptive diagnosis.

2. Confirmatory Diagnosis

Confirmation of IHNV is accomplished by serum neutralization tests with polyclonal rabbit antisera or monoclonal antibodies. Use of a battery of monoclonal antibodies can relate isolates by geographic location (Winton et al. 1988; Huang et al. 1994). Hsu and Leong (1985) described techniques that could be used to detect and differentiate strains of IHNV, but these are not commonly used because radioisotopes are required. An ELISA for IHNV also has been reported (Way and Dixon 1988). A simple immunoblot assay was used to detect IHNV concentrations as low as 100 PFU/mL in cell culture supernatant (McAllister and Schill 1986; Arnzen et al. 1991). A FAT for IHNV was developed using either polyclonal or monoclonal reagents (LaPatra et al. 1989a; Arnzen et al. 1991). The FAT was specific and reacted with all isolates of IHNV tested (see Figure 7). The test was shown to be equal in sensitivity to the plaque assay method but required less time to obtain a definitive diagnosis. Ristow and Arnzen (1989) also described a FAT that was more rapid than previous methods and could be used for serological strain typing of IHNV isolates. Fixed tissue culture cells can also be serologically confirmed to be infected with IHNV by an alkaline phosphatase immunocytochemistry procedure (Drolet et al. 1993). A Polymerase Chain Reaction (PCR) method (modified from Arakawa et al. 1990) has been accepted for confirmation of IHNV (see Section 2, 4.6.A.2.a “Polymerase Chain Reaction (PCR) Method for Confirmation of IHNV (Modified from Arakawa 1990): The Reverse Transcriptase”). A PCR test has also been developed for fixed and embedded fish tissue (Chiou et al. 1995) along with a biotinylated DNA probe (Deering et al. 1991).

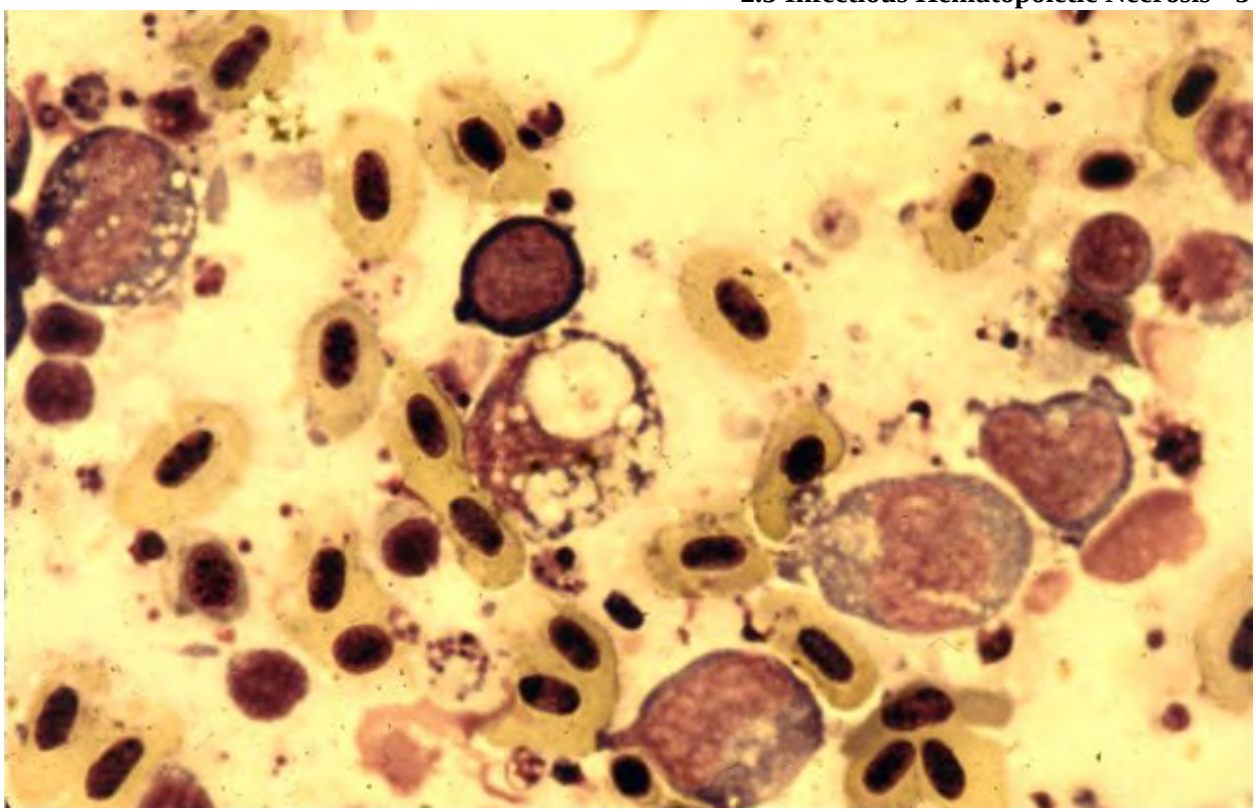


Figure 3. A kidney imprint from an IHN infected rainbow trout stained with Diff Quik®.

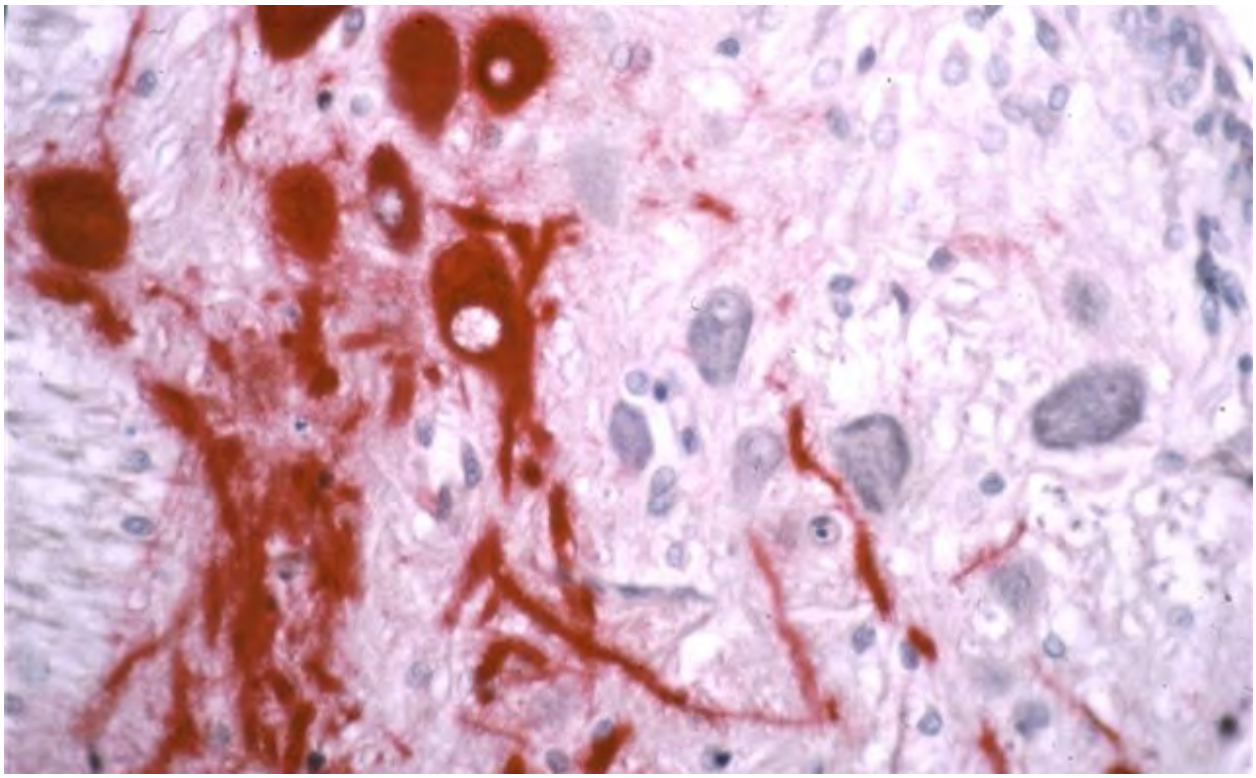


Figure 4. Alkaline phosphatase immunohistochemistry procedure (Drolet et al. 1994) on deparaffinized sections of brain tissue from an effected rainbow trout; the presence of IHNV antigen is indicated by the red staining).

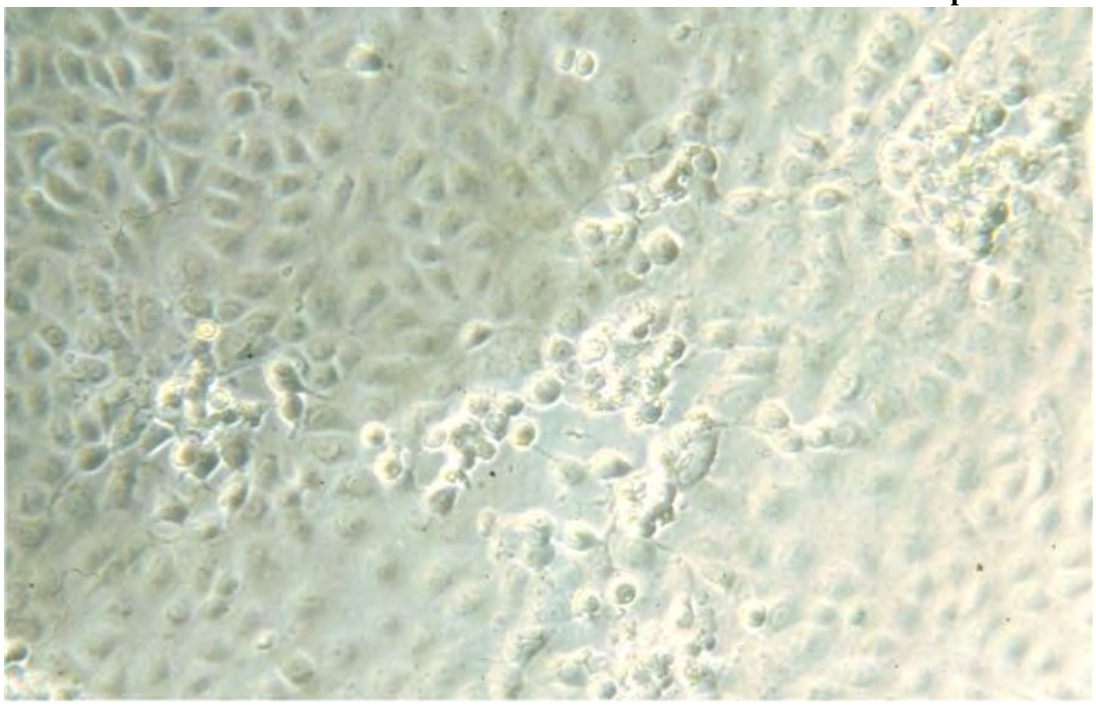


Figure 5. IHNV induced cytopathic effect on the CHSE-214 cell line 72 hours post-inoculation. The CPE also can be observed by studying plaque morphology.

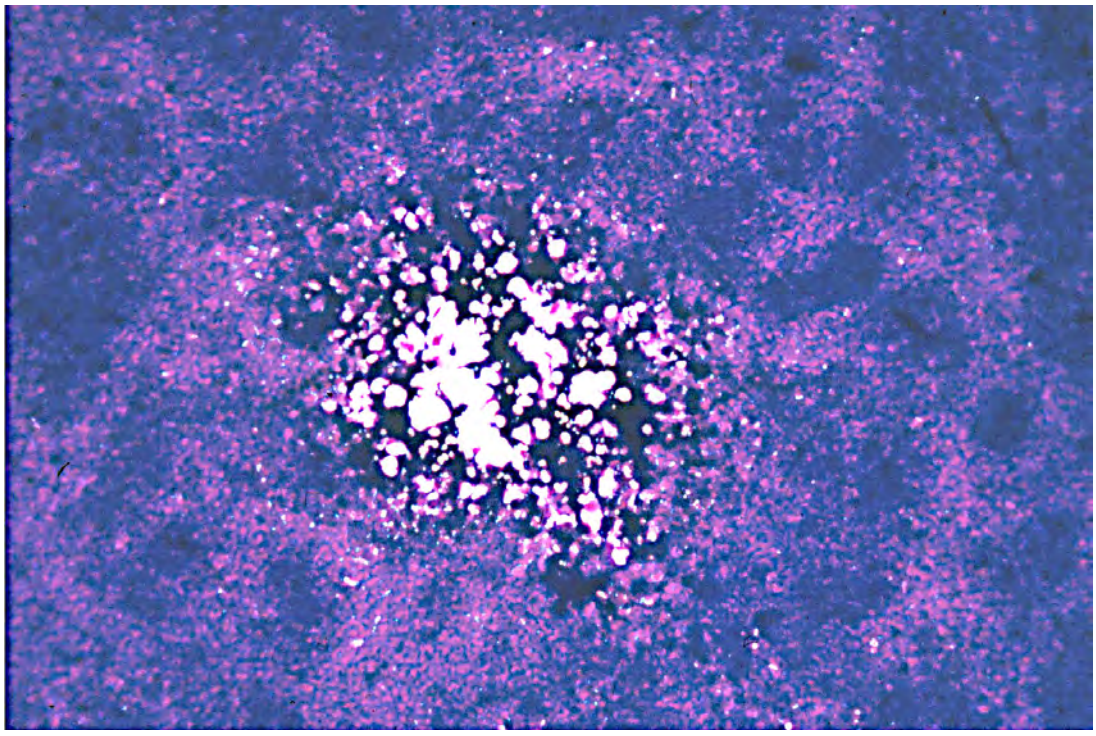


Figure 6. An IHNV plaque on the EPC cell line fixed with formalin and stain with Crystal violet.

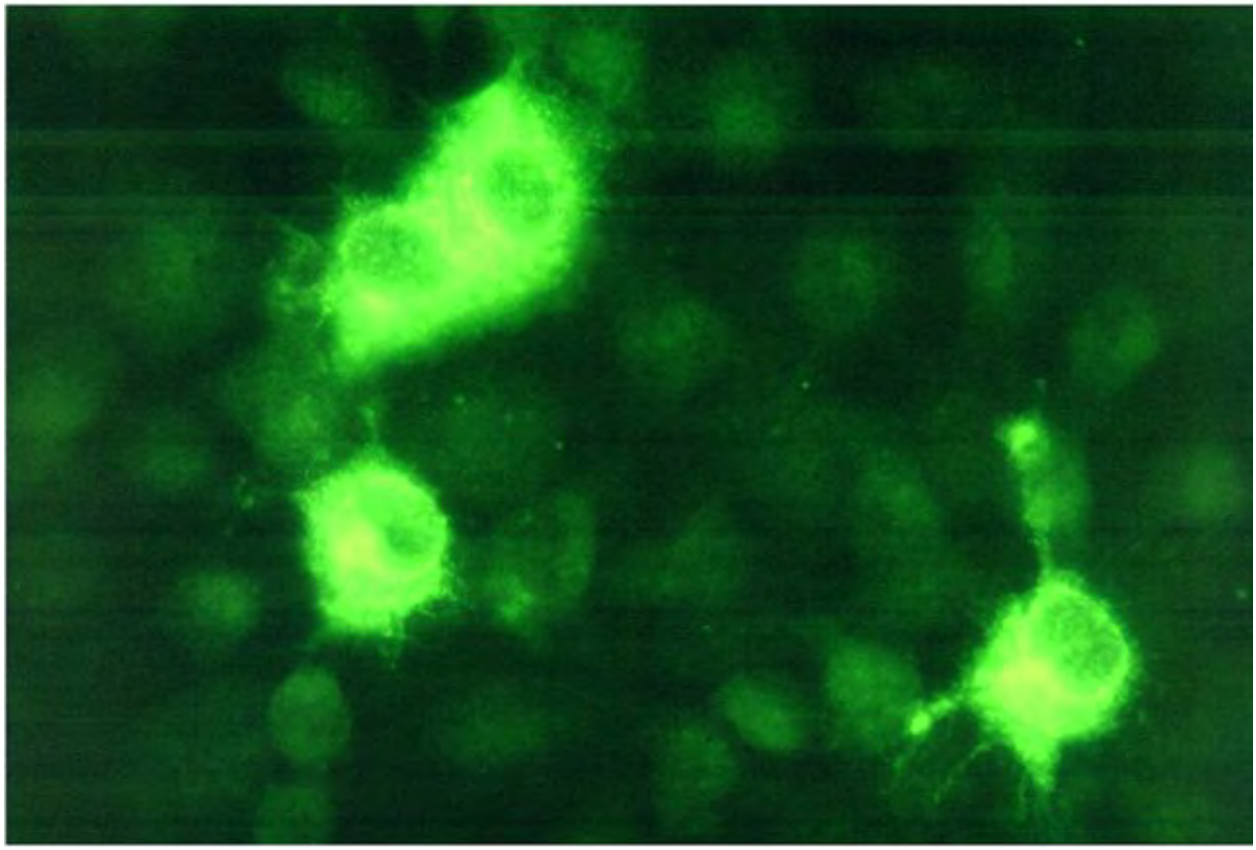


Figure 7. Confirmation of IHNV by FAT on infected cell cultures; the presence of IHNV antigen is indicated by the apple green fluorescence).

F. Procedures for Detecting Subclinical Infections

The virus can be isolated readily from specimens obtained from epizootics and can be detected in yearling and adult salmon exhibiting no clinical signs. In adult salmonids infected with IHNV, the highest prevalence of infection may be found in the latest spawning fish and in post-spawned fish. Adult fish from late in the spawning run and gill tissue from older fish should be examined to optimize chances for IHNV detection. IHNV was recently detected in external mucus from naturally and artificially infected juvenile and adult salmonids; mucus may prove to be a useful test sample (LaPatra et al. 1989b).

G. Procedures for Determining Prior Exposure to the Etiological Agent

Hattenberger-Baudouy et al. (1989) found complement dependent neutralizing antibodies in juvenile and adult rainbow trout and suggested that this could be used as a tool for fish health monitoring. Techniques that have been used to detect antibodies to IHNV in fish serum include: neutralization (Hattenberger-Baudouy et al. 1989; Jorgensen, et al. 1991; LaPatra et al. 1993), fluorescent antibody, and ELISA (Jorgensen et al. 1991). In the future, validation of serological techniques for the surveillance and diagnosis for fish virus infections could make fish serology a more acceptable diagnostic technique (LaPatra 1996).

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiologic Agent

See Section 1, 2.1.1.B General Procedures for Virology.

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2.6. Infectious Pancreatic Necrosis

Philip E. McAllister

USGS, LSC, National Fish Health Research Laboratory
11649 Leetown Road
Kearneysville, WV 25430
304.724.4441
pmcallister@usgs.gov

A. Name of Disease and Etiological Agent

The family Birnaviridae is presently composed of three type genera, *Aquabirnavirus* (aquatic and marine vertebrate and invertebrate hosts), *Avibirnavirus* (bird hosts), and *Entomobirnavirus* (insect hosts). The descriptive term "birna" highlights the bisegmented, double-stranded RNA genome that is the distinctive characteristic of this family of viruses. The birnavirion presents as a non-enveloped, single-layer capsid having icosahedral morphology and measuring about 60 nm in diameter. The complete, infectious virion has a buoyant density of 1.33 gm/cm^3 in CsCl, is stable at pH 3-9, and is resistant to heating at 60C for 1 hr. The major viral structural proteins are VP1 (minor internal protein; RNA-dependent RNA polymerase), VP2 (outer capsid protein; antigen for type-specific character and stimulator of infectivity-neutralizing antibody), and VP3 (internal protein; antigen for group-specific character and stimulator of non-neutralizing antibody) (Delmas et al. 2005).

The type species for the genus *Aquabirnavirus* is infectious pancreatic necrosis virus (IPNV). While IPNV infections were originally associated solely with salmonid fish, IPNV and IPNV-like viruses have been isolated from a variety of nonsalmonid fish and invertebrates from freshwater, estuarine, and marine environments (Wolf 1988, McAllister 1993, Reno 1999). The term "infectious pancreatic necrosis virus" is often used to specifically describe those birnavirus isolates that originate from or cause disease in salmonid fish, while the terms "IPNV-like," "aquatic birnavirus," and "marine birnavirus" are often used to encompass those *Aquabirnavirus* isolates recovered from other fishes and invertebrates and to characterize their respective environments.

The rich immunological and genomic character of the genus *Aquabirnavirus* correlates with the diversity of host species from which the virus has been isolated. Infectivity neutralization assays using polyclonal antibody led to cataloging of isolates into a format where serogroup A contains nine serotypes and serogroup B contains one serotype (Hill and Way 1995). Findings from monoclonal antibody-based epitope characterization (isolate relationships based on reactivity to a panel of monoclonal antibodies) and from genomic characterization (isolate relationships based on gene sequence of conserved and variable regions that code for viral structural proteins) show parallels to the cataloging of isolates based on infectivity neutralization assays (Caswell-Reno et al.1989, Blake et al. 2001).

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

The genus *Aquabirnavirus* is among the more geographically dispersed groups of viruses. Table 1 provides a list of locations where IPNV isolations have occurred involving cultured and feral species. Information about the distribution of IPNV in feral fishes in the United States can be obtained from the database of the U.S. Fish and Wildlife Service, National Wild Fish Survey (http://www.fws.gov/wildfishsurvey/). For a global perspective, a search on the worldwide web using a descriptor "Aquabirnavirus" or "infectious pancreatic necrosis virus" coupled with the geographical area of concern can provide citations of the worldwide geographical range of the genus.

Table 1. Locations where IPNV isolations have occurred involving natural infections in cultured or feral species.

Continent	**Country**
North America	Canada
	Mexico
	United States
South America	Chile
Europe	Belgium
	Areas of the former Czechoslovakia
	Denmark
	Finland
	France
	Germany
	Italy
	Luxembourg
	Norway
	Republic of Ireland
	Russia
	Spain
	Sweden
	United Kingdom
	Areas of the former Yugoslavia
Asia	Cambodia
	Japan
	Laos
	People's Republic of China
	South Korea
	Taiwan
	Thailand
Oceania	Australia
	New Zealand
Africa	South Africa

2. Host Species
The earliest isolations of IPNV were made from brook trout *Salvelinus fontinalis*, rainbow trout *Oncorhynchus mykiss*, and brown trout *Salmo trutta*; however, IPNV and IPN-like birnaviruses have been isolated from a variety of nonsalmonid fishes and invertebrates from freshwater, estuarine, and marine environments. Table 2 provides a list of families of organisms from which IPNV has been isolated as a naturally occurring infection. An appreciation of the increasing number of host species susceptible to Aquabirnaviruses (species found to be naturally infected or experimentally susceptible) can be accessed by a search on the worldwide web using a descriptor "Aquabirnavirus" or "infectious pancreatic necrosis virus." Information about the distribution of IPNV in wild fishes in the United States can be obtained from the database of the U.S. Fish and Wildlife Service, National Wild Fish Survey (http://www.fws.gov/wildfishsurvey/). The recovery of IPNV under natural and experimentally-induced conditions suggests that avian, mammalian, and invertebrate species might serve as passive mechanical vectors for horizontal transmission of IPNV to fishes.

C. Epidemiology

The clinical presentations in hosts from which Aquabirnaviruses have been isolated are varied. In salmonids, infection with IPNV can occur at any life stage beginning soon after hatching. Clinical signs of infection can occur from about swim up stage through about 5 months of age. Clinical epidemics occur in salmonid fish less than 6 months of age. Disease outbreaks are unusual in older fish, but if they occur, they generally involve virus carriers experiencing a precipitating environmental stressor, such as temperature shift, oxygen depletion, or handling, e.g. spawning practices and transport. Epidemic events can result in cumulative mortality approaching 100% in young fish. The apparent virulence of *Aquabirnavirus* isolates, as judged by virus-induced disease or mortality, varies between and within virus serotype, epitope type, and genetic groupings. Further, the apparent virulence paradigm of an individual isolate can be affected by undefined, intrinsic host-related factors such that an isolate may present a level of virulence in one species and a different level of virulence in another, even closely related, species of fish. Such variations in apparent virulence are evident in isolates of salmonid fish origin. In fish with acute IPNV infection, mortality develops rapidly within a temperature range of 10 to 14C, can be more protracted at temperatures less than 10C, and can develop more slowly and be limited in duration at temperatures greater than 14C (Dorson and Torchy 1981). Salmonid fish of any age, whether cultured or wild, can experience subclinical or inapparent infection with negligible mortality. Antibody response to infection is variable, and environmental conditions and stress factors can affect host immune response to infection. A variety of studies are in progress to develop subunit vaccines for immunization against IPNV infection.

Survivors of exposure to IPNV may become virus carriers, shedding virus for an extended period via feces, urine, and sex products and serving as reservoirs for infection of contemporary and subsequent generations. Horizontal transmission is the mechanism for virus dissemination. Although the virus can be isolated from sex products, IPNV has not been demonstrated to reside within the egg or the sperm. Thus, sex product transmission of IPNV represents a form of horizontal transmission rather than true vertical transmission.

Table 2. Families from which IPNV has been isolated as a naturally occurring infection.

	Family name	**Common name**
Fishes	Acipenseridae	Sturgeons
	Amiidae	Bowfins
	Anguillidae	Eels
	Atherinidae	Silversides
	Bothidaw	Lefteye Flounders
	Carangidae	Jacks
	Catostomidae	Suckers
	Centrarchidae	Sunfishes
	Cichlidae	Cichlids
	Channidae	Snakeheads
	Clupeidae	Herrings
	Cobitidae	Loaches
	Coregonidae	Whitefishes
	Cottidae	Sculpins
	Cyprinidae	Carps and Minnows
	Cyprinodontidae	Killifishes
	Embiotocidae	Surfperches
	Esocidae	Pikes
	Gadidae	Cods
	Gasterosteidae	Stickelbacks
	Gobiidae	Gobies
	Hiodontidae	Mooneyes
	Ictaluridae	Bullhead Catfish
	Lepisosteidae	Gars
	Moronidae	Temperate Basses
	Paralichthyidae	Large-toothed Flounders
	Percichthyidae	Temperate Perches
	Percidae	Perches
	Petromyzontidae	Lampreys
	Pleuronectidae	Righteye Flounders
	Poeciliidae	Livebearers
	Polyodontidae	Paddlefishes
	Salmonidae	Trouts
	Sciaenidae	Drums
	Soleidae	Soles
	Thymallidae	Graylings
	Triglidae	Grunards
	Umbridae	Mudminnows

Table 2 continued. Families from which IPNV has been isolated as a naturally occurring infection.

	Family name	**Common name**
Molluscs	Acmaeidae	Limpets
	Littorinidae	Periwinkle
	Mytilidae	Mussels
	Ostreidae	Oysters
	Pectinidae	Scallops
	Tellinidae	Tellina
	Veneridae	Clams
Crustaceans	Astacidae	European Crayfish
	Daphniidae	Daphnia
	Palaemonidae	Shrimps
	Penaeidae	Prawns
	Portunidae	Crabs
Pseudocoelomates	Branchionidae	Rotifers

Experimental studies show that distribution of IPNV within a susceptible population shows aspects of both propagative and point-source infection models (Bebak et al. 1998, Smith et al. 2000; Bebak-Williams et al. 2002), which may be more indicative of a continuous source infection model. Infection is influenced in the broad sense by the environmental concentration of virus and by host population density and in a more restrictive sense by the character of the virus, by the species, age, and nutritional status of the host, and by ambient environmental factors (McAllister and Owens 1986). The general scenario for IPNV infection in salmonid fish is for fish to become infected by contact with virus laden water. Fish become infectious and begin shedding virus after about 2 days latency and continue shedding virus for about the next 10 days. Those fish that are infectious, but do not develop clinical signs, are candidates to be virus carriers; whereas, those fish that are infectious and develop clinical signs are candidates for death due to IPN. Shedding of virus augments the virus concentration of the ambient environment. An increasing concentration of environmental virus exacerbates the probability for horizontal transmission on a population-wide basis and the probability for subsequent disease and mortality.

D. Clinical Signs

The disease description “infectious pancreatic necrosis” was derived from histopathologic findings in acutely affected young, salmonid fishes. However, animals infected with the IPNV-like aquatic and marine birnaviruses can present with varied clinical signs and pathology. Some isolates of IPN and IPN-like viruses cause acute and chronic disease; whereas, other isolations have been made from ostensibly healthy specimens. Some designations given to Aquabirnavirus

infections and diseases in nonsalmonid species are branchionephritis in eels (*Anguilla japonica),* spinning disease in menhaden (*Brevoortia tyrannus*), viral ascites in yellowtail (*Seriola quinqueradiata*), Kumura shrimp disease (*Penaeus japonicus*), and clam gill necrosis (*Meretrix lusoria*) (Reno 1999).

IPNV infection in salmonid fishes can cause a variety of clinical signs and pathologic changes, but none of the presentations truly distinguishes IPN from other fish viral diseases. In addition, some IPNV-infected salmonids develop clinical signs while others appear clinically normal. External clinical signs of IPN can include darkened color (hyperpigmentation), unilateral or bilateral swelling of the eyes (exophthalmia), abdominal (coelomic) distention, presence of a mucoid pseudo-cast extruding from the vent, and hemorrhages at the base and in the fins and on the body surface. Fish often swim erratically, rotating about the long axis or whirling violently. Internal signs can include liver and spleen that are pale compared to normal color and a stomach and intestine that are void of food, but filled with mucoid material that may extrude from the vent. Hemorrhages can occur in the visceral organs.

Marked pathological changes can accompany IPNV infection (Yasutake 1970). Pancreatic tissue can undergo severe necrosis characterized by condensation of chromatin (pyknosis), fragmentation of the nucleus (karyorrhexis), and intracytoplasmic inclusions. The pylorus, pyloric caeca, and anterior intestine can undergo extensive necrosis. Sloughing intestinal epithelium combines with mucus to form a heavy, whitish-colored exudate (catarrah) that may extrude from the vent. Degenerative changes can occur in renal hematopoietic and excretory tissues, liver tissue, and splenic tissue, and demyelinating lesions with inflammation and hemorrhage can occur in the brain. Pancreatic and hepatic tissues can be infiltrated by macrophages and polymorphonuclear leukocytes.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

Because clinical signs and pathological changes associated with IPN are variable, they cannot be used for presumptive or definitive diagnosis or to reliably distinguish IPN from other fish viral diseases. In an intensive culture setting, history of the fish populations, the culture station history, clinical signs, and gross necropsy are used to prepare a list of potential diagnoses. The absence of clinical signs does not indicate that the fish are free of IPNV. Virological examination coupled with serological or molecular identification is required for confirmation of clinical or subclinical infection with IPNV.

A variety of cell culture-, immunologic-, and molecular-based assay protocols have been described for detection of IPNV infection. Similarly, a variety of clinical materials have been used, some require sacrifice of fish (internal organs) and others are obtained by non-lethal sampling methods (reproductive fluids, blood fractions, feces, mucus, and external tissues). Although many assay formats and clinical samples have been described, few have undergone rigorous validation by comparison to cell culture assay of internal organ samples. Cell cultures of blue gill fry (BF-2), Chinook salmon embryo (CHSE-214), or rainbow trout gonad (RTG-2) are commonly used for evaluation of clinical materials for detection of IPNV in salmonid fishes. Assays of coelomic (ovarian) fluid for IPNV can provide a level of detection comparable to tissue samples if pellets obtained from centrifugation of coelomic (ovarian) fluid samples are sonicated (10 seconds at 75-100W). However, sonication should not be considered if those same clinical samples are to be used for detection of any of the enveloped fish viruses, e.g. IHNV and VHSV (McAllister et al.1987).

The USFWS/AFS specified format of virological examination for detection of IPNV consists of the assay in cell culture of life stage-appropriate clinical samples of whole fry, internal organs, or reproductive fluids.

> For USFWS/AFS specified clinical samples, see Section 2, 2.2, Table 2.1, "Sampling, Virology." (USFWS and AFS-FHS, 2007).
>
> For USFWS/AFS specified sample processing procedures, see Section 2, 4.4, "Virology, Sample Processing Procedures." (USFWS and AFS-FHS, 2007).
>
> For USFWS/AFS specified virus screening procedures, see Section 2, 4.5, "Virology, Screening Method for Viral Isolation." (USFWS and AFS-FHS, 2007).
>
> For USFWS/AFS specified procedures for identification of IPNV, see Section 2, 4.6.B, "Identification of Viruses: infectious pancreatic necrosis virus." (USFWS and AFS-FHS, 2007).

2. Confirmatory Diagnosis

Confirmatory identification of IPNV can be accomplished using immunologic-based or molecular-based assay formats (Dopazo and Barja 2002).

Immunological-based assays include: infectivity neutralization, immunoblot, enzyme-linked immunosorbent assay (ELISA), fluorescent antibody, and immunohistochemical staining. Because several serotypes of IPN virus can be distinguished by infectivity neutralization, use of either an antiserum to each serotype or a polyvalent antiserum preparation is necessary for a competent infectivity neutralization assay. Similarly, even though significant cross-reactivity is apparent using ELISA and other non-infectivity based assay formats, antiserum to each serotype or a polyvalent antiserum is needed for a competent assay.

> For USFWS/AFS specified procedures for identification of IPNV by immunologic-based assay, see Section 2, 4.6.B, "Identification of Viruses: infectious pancreatic necrosis virus." (USFWS and AFS-FHS, 2007).

Because of limitations associated with immunological-based confirmatory assays, reverse transcriptase-polymerase chain reaction (RT-PCR) assay is often used to confirm isolations of IPNV in cell culture. More extensive genomic characterization can be applied to develop refined taxonomic and epidemiologic associations.

> For USFWS/AFS specified procedures for identification of IPNV by reverse transcriptase polymerase chain reaction (RT-PCR) assay, see Section 2, 4.6.B, "Identification of Viruses: infectious pancreatic necrosis virus." (USFWS and AFS-FHS, 2007).
>
> For USFWS/AFS specified procedures for formulation of reverse transcriptase polymerase chain reaction (RT-PCR) assay, see Section 2, 4.A1.B.2, "Worksheet B.2 – Infectious Pancreatic Necrosis Virus (IPNV)." (USFWS and AFS-FHS, 2007).

F. Procedures for Detecting Subclinical Infections

Subclinical infections can be detected by cell culture assay as described in Section E. "Disease Diagnostic Procedures, Presumptive Diagnosis."

To limit the numbers of fish sacrificed, surveillance and monitoring programs are increasingly using non-lethal sample protocols for collection of clinical materials. However, because these protocols have not been validated by comparative study, the absence of virus detection should be regarded as a provisional observation.

G. Procedures for Determining Prior Exposure to the Etiological Agent

Definitive procedures have not been developed for determining prior exposure to IPNV other than assay for subclinical infection. Export and import facilities should maintain complete records of fish movement and inspection history. Monitoring for IPNV-specific antibody has limited utility because immune response to the virus can be varied and transient. In addition, some IPNV's react with components of normal salmonid serum. Such reactivity can confound assay interpretation (Larsen et al. 2004, Park et al. 2004).

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

See Section 2, 2.2, "Sampling." (USFWS and AFS-FHS, 2007).

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2.7 Viral Erythrocytic Necrosis

J. S. Rohovec

Oregon State University
Department of Microbiology
Corvallis, OR 97331-3804

A. Name of Disease and Etiological Agent

Viral erythrocytic necrosis (VEN) is caused by erythrocytic necrosis virus (ENV). This disease was originally designated as piscine erythrocytic necrosis (PEN). The viruses are tentatively placed in the Iridoviridae.

Agents of this disease have not been isolated or fully characterized. There are several morphologically distinct viral particles that cause the disease. Some of these viruses probably cause different disease signs and have distinct host specificities.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Found worldwide in the marine environment. Virus particles have been detected in some salmonid juveniles that have been reared exclusively in fresh water.

2. Host Species

The viruses have been demonstrated in many anadromous and marine species of fish.

C. Epizootiology

Little is known about the epizootiology of VEN. The numerous agents that cause the disease probably affect their hosts differently under various conditions. In salmonids, the disease is manifested as a chronic or subacute infection that may not cause overt mortality, but debilitates fish so that other pathogens (e.g. *Vibrio anguillarum*) or adverse environmental conditions (e.g. low dissolved oxygen) cause death of the fish.

D. Disease Signs

The most common and consistent disease sign among the species affected is anemia, which can be observed externally as pale gills and internally as a general pallor of visceral organs. Hematocrits may be 2 to 10%. Stained blood smears reveal cytoplasmic inclusions in the erythrocytes. The inclusions have differing morphology and can range in size from 1 to 4 μm in diameter. The percentage of infected

erythrocytes can range from less than 1 to 100%.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

The viruses that cause VEN have not yet been isolated in cell cultures. Presumptive diagnosis depends on observation of inclusion bodies in the cytoplasm of infected erythrocytes. Blood smears are made, air dried, fixed for five minutes in absolute methanol and stained with Giemsa, Wright, or other blood stains (e.g. Romanowsky). Pinacyanol chloride (Leek 1987) has been used to stain blood smears for VEN diagnosis. If blood is not available for examination, stained smears prepared from blood-rich tissues such as kidney or spleen can be used.

2. Confirmatory Diagnosis

A confirmed diagnosis of VEN depends on electron microscopic observation of iridovirus-like particles within the cytoplasm of erythrocytes. In thin sections, the classically described virion outer coat is typically hexagonal in outline and there is usually a closely apposed inner coat that surrounds a centrally-located circular nucleoid. The diameter of the virions in teleosts range from 154 nm in the Atlantic herring to 330 nm in the Atlantic cod.

Methanol/ethanol (1:1) fixed smears that are rehydrated in ethanol series of 100%, 70%, and 50% stained with 0.1% aqueous acridine orange and washed in phosphate buffered saline can aid in differentiating inclusions of EIBS and those of viral erythrocytic necrosis (VEN). Inclusions of VEN stain green: those of EIBS stain orange.

F. Procedures for Detecting Subclinical Infections

None are available; however, low-grade infections may be detected by resorting to exhaustive examinations of stained blood films.

G. Procedures for Determining Prior Exposure to the Etiological Agent

No procedures have been reported.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

It is best to make blood smears on site and fix them with absolute methanol before transporting to a laboratory. If blood is to be examined by electron microscopy, 10 to 20 units of heparin must be added per mL of blood. Samples should be transported on ice and fixed in Hawke's fixative (Rohovec and Amandi 1981) within 48 hours of collection.

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2.8 Viral Hemorrhagic Septicemia

W. N. Batts and J. R. Winton

USGS Western Fisheries Research Center
6505 NE 65th Street, Seattle, WA 98115
Phone: 206-526-6282
bill_batts@usgs.gov; jim_winton@usgs.gov

A. Name of Disease and Etiological Agent

Viral hemorrhagic septicemia (VHS) is one of the most important viral diseases of finfish worldwide. In the past, VHS was thought to affect mainly rainbow trout (*Oncorhynchus mykiss*) reared at freshwater facilities in Western Europe where it was known by various names including Egtved disease and infectious kidney swelling and liver degeneration (Wolf 1988). Today, VHS is known as an important source of mortality for cultured and wild fish in freshwater and marine environments in several regions of the northern hemisphere (Dixon 1999; Marty et al. 1998, 2003; Meyers and Winton 1995; Skall et al. 2005b; Smail 1999). Viral hemorrhagic septicemia is caused by the fish rhabdovirus, viral hemorrhagic septicemia virus (VHSV), a member of the genus *Novirhabdovirus* of the family *Rhabdoviridae*.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Viral hemorrhagic septicemia is endemic among marine and freshwater fish in Western Europe, North America and Eastern Asia. Countries or regions where VHSV has been isolated from natural infections of fish using cell culture methods and confirmed by serological or molecular assays are listed in Table 1. Isolates of VHSV from different geographic areas tend to group into distinct genotypes (Benmansour et al. 1997; Einer-Jensen et al. 2004, 2005 a, b; Elsayed et al. 2006; Kim et al. 2003; Nishizawa et al. 2002; Snow et al. 1999, 2004; Stone et al. 1997; Thiéry et al. 2002).

2. Host Species

Over 70 species of freshwater and marine fish are currently known to be natural hosts of VHSV (Table 1). Other species have been shown to be experimentally susceptible (Wolf 1988). While large outbreaks of disease associated with high mortality have occurred in aquaculture facilities and in some populations of wild fish, VHSV has also been isolated from fish that appeared normal.

C. Epizootiology

VHSV is readily transmissible to susceptible fish of all ages. The main portal of entry is believed to be the epithelial tissues of the gills or skin, especially at the base of the fins (Harmache et al. 2006). Disease outbreaks are typically seen at water temperatures from 3-12^{o}C and are most severe at lower temperatures. As temperature increases, mortality and the proportion of virus carriers decreases. At temperatures above 15^{o}C, mortality from VHS is typically low. Infected fish mount a strong interferon response, which may play a role in transiently mitigating the effects of VHS at higher temperature. The antibody response in survivors of epizootics and in fish with inapparent infections is variable. In captive fish, the disease can recur as a consequence of immunosuppression due to culture conditions and stress.

Table 1. Natural Host and Geographic Range of Viral Hemorrhagic Septicemia Virus.

Common name	Scientific name	Geographic location	Reference
Rainbow trout	*Oncorhynchus mykiss*	Europe (most countries) Inland Lakes – North America	Wolf 1988; Skall et al. 2005b; Unpublished
Brown trout	*Salmo trutta*	Western Europe Atlantic Coast - North America	deKinkelin & Le Berre 1977; Gagné et al. 2007
Northern pike	*Esox lucius*	Western Europe Great Lakes – North America	Meier & Jorgensen 1979; Unpublished
Grayling	*Thymallus thymallus*	Switzerland	Meier & Wahli 1988
Whitefish	*Coregonus sp.*	Switzerland	Meier et al. 1994
European eel	*Anguilla anguilla*	France	Castric et al. 1992
Largemouth bass	*Micropterus salmoides*	France Great Lakes - North America	deKinkelin et al. 1999; Unpublished
Turbot	*Scophthalmus maximus* *Psetta maxima*	Germany, Gigha, Ireland, Black Sea (Turkey)	Schlotfeldt et al. 1991; Ross et al. 1994; Nishizawa et al. 2006
Atlantic cod	*Gadus morhua*	Baltic Sea, North Sea, North Atlantic	Jensen et al. 1979; Mortensen et al. 1999; Smail 2000; King et al. 2001
Atlantic herring	*Clupea harengus*	Baltic Sea, English Channel, Kattegat, Skagerrak, North Sea Atlantic Coast – North America	Mortensen et al. 1999; Dixon et al. 1997; King et al. 2001; Unpublished
European sprat	*Sprattus sprattus*	Baltic Sea	Mortensen et al. 1999
Common dab	*Limanda limanda*	Kattegat; Baltic Sea	Skall et al. 2005a
European plaice	*Pleuronectes platessa*	Skagerrak; Kattegat	Skall et al. 2005a
European flounder	*Platichthys flesus*	Baltic Sea	Skall et al. 2005a
Sand goby	*Pomatoschistus minutus*	Baltic Sea	Skall et al. 2005a
Sand eel	*Ammodytes sp.*	Baltic Sea	Skall et al. 2005a
Four-bearded rockling	*Rhinonemus cimbrius*	Baltic Sea	Mortensen et al. 1999
Haddock	*Melanogrammus aeglefinus*	North Sea	Smail 2000
Norway pout	*Trisopterus esmarkii*	North Sea; North Atlantic; Skagerrak	Mortensen et al. 1999; King et al. 2001
Poor cod	*Trisopterus minutus*	North Atlantic	King et al. 2001
Whiting	*Merlangius merlangus*	North Sea	Mortensen et al. 1999; King et al. 2001
Lesser argentine	*Argentina sphyraena*	North Sea	Mortensen et al. 1999
Blue whiting	*Micromesistius poutassou*	North Sea	Mortensen et al. 1999
Greenland halibut	*Reinhardtius hippoglossoides*	Flemish Cap - North Atlantic	Dopazo et al. 2002
Chinook salmon	*Oncorhynchus tshawytscha*	Pacific Coast - North America; Great Lakes – North America	Winton et al. 1991; Unpublished
Coho salmon	*Oncorhynchus kisutch*	Pacific Coast - North America	Winton et al. 1991
Steelhead	*Oncorhynchus mykiss*	Pacific Coast - North America	Winton et al. 1991
Atlantic salmon	*Salmo salar*	Pacific Coast - North America Spain	Traxler et al. 1995; Jimenez de la Fuente et al. 1988
Pacific cod	*Gadus macrocephalus*	Pacific Coast - North America	Meyers et al. 1992
Pacific herring	*Clupea pallasi*	Pacific Coast - North America	Meyers et al. 1994; Kocan et al. 2001
Tube-snout	*Aulorhynchus flavidus*	Pacific Coast - North America	Traxler et al. 1995
Walleye pollock	*Theragra chalcogramma*	Pacific Coast - North America	Meyers et al. 1999
Pacific hake	*Merluccius productus*	Pacific Coast - North America	Meyers et al. 1999
Pacific tomcod	*Microgadus proximus*	Pacific Coast - North America	Meyers et al. 1999
English sole	*Parophrys vetula*	Pacific Coast - North America	Hershberger et al. 1999
Pacific sand lance	*Ammodytes hexapterus*	Pacific Coast - North America	Kocan et al. 2001

Pacific sardine (Pilchard)	*Sardinops sagax*	Pacific Coast - North America	Hedrick et al. 2003
Pacific mackerel	*Scomber japonicus*	Pacific Coast - North America	Hedrick et al. 2003
Sablefish (Black cod)	*Anoplopoma fimbria*	Pacific Coast - North America	Hedrick et al. 2003
Eulachon	*Thaleichthys pacificus*	Pacific Coast - North America	Hedrick et al. 2003
Surf smelt	*Hypomesus pretiosus*	Pacific Coast - North America	Hedrick et al. 2003
Shiner perch	*Cymatogaster aggregata*	Pacific Coast - North America	Hedrick et al. 2003
Japanese (Olive) flounder	*Paralichthys olivaceus*	Japan, Korea	Takano et al. 2000, 2001
Japanese sandlance	*Ammodytes personatus*	Japan	Watanabe et al. 2002
Mebaru (Black rockfish)	*Sebastes inermis*	Japan	Isshiki et al. 2003
3-spine stickleback	*Gasterosteus aculeatus*	Pacific Coast - North America Atlantic Coast - North America	Kent et al. 1998; Olivier 2002
Mummichog	*Fundulus heteroclitus*	Atlantic Coast - North America	Olivier 2002
Striped bass	*Morone saxatilis*	Atlantic Coast - North America	Gagné et al. 2007
Freshwater drum	*Aplodinotus grunniens*	Great Lakes - North America	Lumsden et al.2007
Muskellunge	*Esox masquinongy*	Great Lakes - North America	Elsayed et al. 2006
Round goby	*Neogobius melanostomus*	Great Lakes - North America	Groocock et al. 2007
Black crappie	*Pomoxis nigromaculatus*	Great Lakes - North America	Unpublished
Bluegill	*Lepomis macrochirus*	Great Lakes - North America	Unpublished
Bluntnose minnow	*Pimephales notatus*	Great Lakes - North America	Unpublished
Burbot	*Lota lota*	Great Lakes - North America	Unpublished
Channel catfish	*Ictalurus punctatus*	Great Lakes - North America	Unpublished
Common carp	*Cyprinus carpio*	Inland Lakes - North America	Unpublished
Emerald shiner	*Notropis atherinoides*	Great Lakes - North America	Unpublished
Gizzard shad	*Dorosoma cepedianum*	Great Lakes - North America	Unpublished
Lake trout	*Salvelinus namaycush*	Inland Lakes - North America	Unpublished
Lake whitefish	*Coregonus clupeaformis*	Great Lakes - North America	Unpublished
Northern shortnose redhorse	*Moxostoma macrolepidotum*	Great Lakes - North America	Unpublished
Pumpkinseed	*Lepomis gibbosus*	Great Lakes - North America	Unpublished
Rock bass	*Ambloplites rupestris*	Great Lakes - North America	Unpublished
Silver redhorse	*Moxostoma anisurum*	Great Lakes - North America	Unpublished
Smallmouth bass	*Micropterus dolomieu*	Great Lakes - North America	Unpublished
Spottail shiner	*Notropis hudsonius*	Great Lakes - North America	Unpublished
Trout-perch	*Percopsis omiscomaycus*	Great Lakes - North America	Unpublished
Walleye	*Sander vitreus*	Great Lakes - North America	Unpublished
White bass	*Morone chrysops*	Great Lakes - North America	Unpublished
White perch	*Morone americana*	Great Lakes - North America	Unpublished
Yellow perch	*Perca flavescens*	Great Lakes - North America	Unpublished

D. Disease Signs

A variety of clinical signs and histopathological changes can be apparent in infected fish (Wolf 1988; Smail 1999). Some fish can show profound clinical manifestation whereas others appear to be nearly normal. Historically, clinical and histological signs of VHS have been categorized into acute, chronic, and latent forms or stages. Such descriptions represent degrees of severity rather than progressive forms or stages of the disease.

External clinical signs of disease can include exophthalmia, abdominal distention, darkened coloration, anemia, lethargy, hyperactivity, and hemorrhages in the eyes, skin, gills, and at the base of fins (Figures 1 and 2). Internally, visceral mesenteries can show diffuse hemorrhage (Figure 3), the kidneys and liver can be hyperemic, swollen, and discolored, and hemorrhages can occur in skeletal muscle.

Histopathological changes are generally confined to the liver, kidney, spleen, and skeletal musculature. The hematopoietic areas of the kidney and spleen are the principal sites of viral replication. In the liver, kidney and spleen, extensive areas of necrosis with vacuolation, pyknosis, karyolysis and lymphocytic infiltration are typically seen in moribund fish. Pathological changes can also be focal in nature, and evidence of cellular repair is seen in recovering fish.

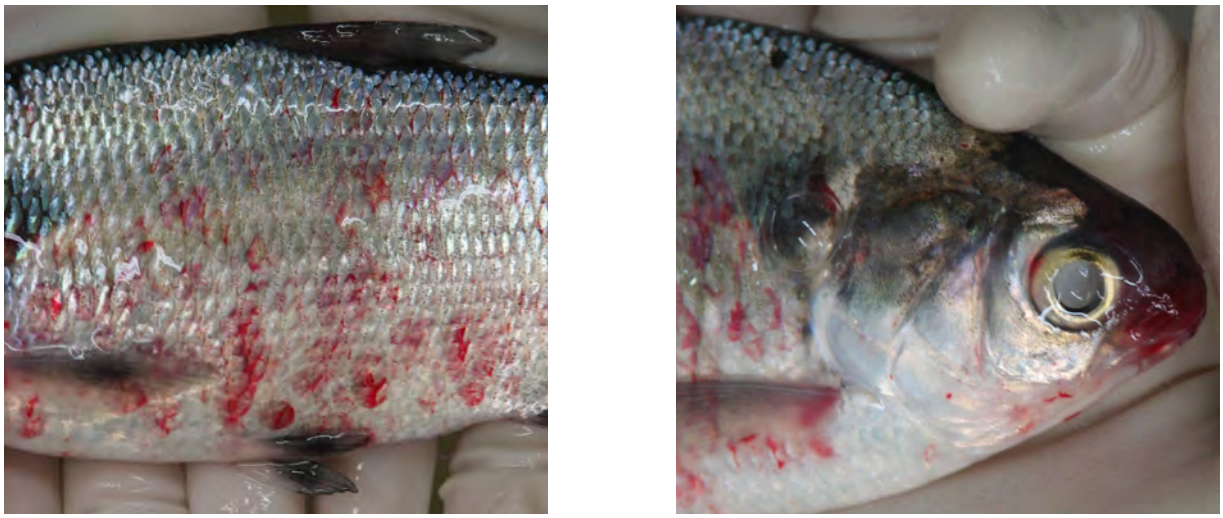


Figure 1. Widespread hemorrhages on the body of a VHSV infected gizzard shad. Photos provided by Mohamed Faisal.

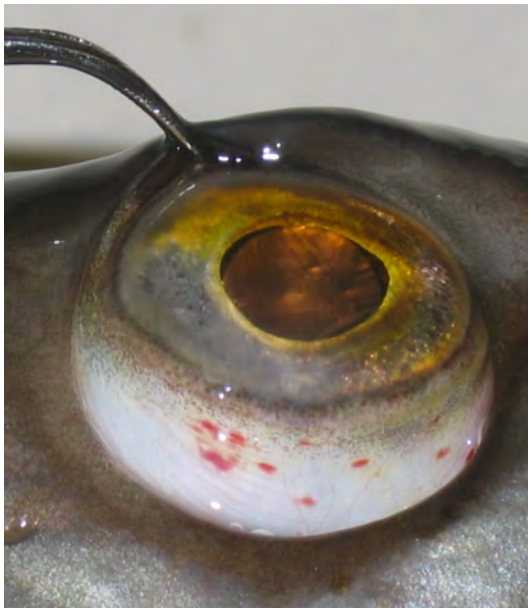


Figure 2. Petechial hemorrhages in the eye of a VHSV infected muskellunge. Photo provided by

Mohamed Faisal.

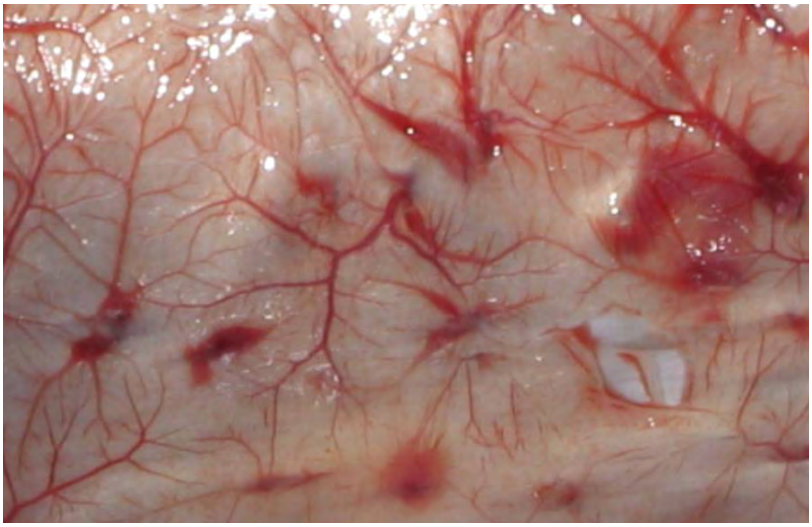


Figure 3. Hemorrhages and congestion in the swimbladder membrane of a VHSV infected gizzard shad. Photo provided by Mohamed Faisal.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

Clinical signs and histopathological changes associated with VHS are variable and cannot be used for definitive diagnosis or to distinguish VHS from the other fish viral diseases. The absence of clinical signs does not indicate that the fish are free from VHSV. Consequently, virological examination is required for diagnosis of VHS. Virological examination consists of the assay of tissue and reproductive fluid samples in cell culture or by a validated serological or molecular assay. For cell culture assays, the *Epithelioma papulosum cyprini* (EPC), fathead minnow (FHM) and bluegill fry (BF-2) cell lines are considered to be the most sensitive, although VHSV will replicate in the rainbow trout gonad (RTG-2) and Chinook salmon embryo (CHSE-214) cell lines. During incubation, it is critically important that the pH of the medium remain within the range of 7.4 - 7.8 because cytopathic effects (CPE) of VHSV will not develop in acidic cultures (Batts et al. 1991), probably due to pH-dependent conformational changes in the glycoprotein (Gaudin et al. 1999). This is especially problematic for cell lines derived from coolwater species that continue to metabolize efficiently at the incubation temperatures of the assay. In recent years, polymerase chain reaction (PCR) assays have become widely used for presumptive diagnosis of the disease (Winton and Einer-Jensen 2002).

2. Confirmatory Diagnosis

Confirmatory diagnosis of VHS and identification of VHSV can be performed using a serum neutralization assay, immunoblot assay (McAllister and Owens 1987; McAllister and Schill 1986), enzyme-linked immunosorbent assay (ELISA; Mourton et al. 1992; Olesen and Jorgensen 1991; Way and Dixon 1988), fluorescent antibody test (FAT; Lorenzen et al. 1988), DNA probe (Batts et al. 1993), PCR (Winton and Einer-Jensen 2002) and sequence analysis (Snow et al. 2004). For the serum neutralization assay, the cell cultures and the conditions of incubation and pH control must be maintained as indicated above. Antiserum specific to each serotype must be used in the serum neutralization assay because three serological types of VHSV can be distinguished by certain

neutralizing antisera. For the ELISA and FAT polyclonal or monoclonal antibodies are available that react with all VHSV serotypes. Primer sets for PCR assays that react with all genotypes of VHSV can be used for confirmatory diagnosis of the disease as well as for identification of VHSV that has been isolated in cell culture (Winton and Einer-Jensen 2002). Confirmed isolates of VHSV from a new host species or a new geographic area should be sent to a reference laboratory for additional molecular or serological analysis and the finding reported to state or national fisheries agencies and, in the United States, to USDA-APHIS.

F. Procedures for Detecting Subclinical Infections

Subclinical infections can be detected by cell culture assay and by PCR. In some instances, VHSV has only been detected by examination of certain tissues or organs such as the brain.

G. Procedures for Determining Prior Exposure to the Etiological Agent

The specific immune response among survivors of VHS epizootics and inapparent virus carriers varies with both the fish and season of the year. Nevertheless, detection of VHSV-specific antibody can be useful as part of a VHSV surveillance program for evidence of past infections and for determining the status of the protective immune response following vaccination (Bernard et al. 1983; Olesen and Jorgensen 1986).

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Tissue samples should be moistened with buffered physiological saline (pH 7.4 - 7.8) or cell culture medium (pH 7.4 - 7.8) and shipped on ice or at 4^{o}C. Samples should not be frozen nor should glycerol be used to preserve specimens because VHSV has been shown to be inactivated by these methods. It is important that the samples be transported, processed and assayed in a timely manner.

I. Procedures for Enumeration of VHSV

The EPC cell line is recommended for enumeration of VHSV via a plaque assay. Virus adsorption to EPC cells can be enhanced by pre-treating the cells with a 7% solution (final concentration) of 20,000 MW polyethylene glycol (PEG; Sigma Catalog Number P2263-500G; Batts and Winton 1989) or by adding DEAE dextran (Campbell and Wolf 1969; 50 μg/mL final concentration) or PEG to the sample diluent during adsorption. Quantitative PCR assays have been developed that can be used for enumeration of VHSV (Chico et al. 2006).

J. Procedures for Determination of Disease-free Status

Inspection procedures for determination of disease-free status rely upon virological assays using cell culture. This is because all strains of VHSV can be grown in cells and because any isolates obtained can be examined using one or more of the confirmatory tests discussed above. In addition, cell culture assays have a long track record of use and, while potentially less sensitive than some newer PCR-based methods, are considered the gold standard. In the future, it is expected that some of the newer molecular assays will be sufficiently validated against this gold standard to become accepted methods for determination of disease-free status.

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2.9 Infectious Salmon Anemia

W.R. Keleher, D. A. Bouchard, and P. L. Merrill

Micro Technologies, Inc.
41 Main Street
Richmond, ME 04357
207/737-2637
microtech@wiscassett.net
dbouchard@microtechnologies.biz

A. Name of Disease and Etiological Agent

Infectious salmon anemia (ISA) is caused by infectious salmon anemia virus (ISAV). The virus is a member of the orthomyxoviridae family. Synonyms include: hemorrhagic kidney syndrome (HKS).

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Infectious salmon anemia has been found in Norway, the Faroe Islands, and the United Kingdom (Scotland and Shetland Islands). In North America, the disease has been restricted to the east coast, with cases seen in Canada (New Brunswick) and in the United States (Maine). The virus has also been detected in Nova Scotia, Canada.

2. Host Species

ISA has been noted in only one species of salmonids: Atlantic salmon *Salmo salar*. Sea-run brown trout *Salmo trutta*, rainbow trout *Oncorhynchus mykiss*, and Atlantic herring *Clupea harengus* are potential asymptomatic carriers of the virus.

C. Epizootiology

While age does not appear to be a factor of ISA susceptibility, epizootics have almost exclusively occurred during saltwater stages of life or in fish that have been exposed to seawater. Nylund et al. (1999) reported a case of ISA in Atlantic salmon first feeding fry indicating that all stages are susceptible.

Horizontal transmission has been demonstrated using mucus, feces, urine, and blood in the laboratory (Totland et al. 1996; Nyland et al. 1994). Coprophagy and waterborne transmission have been identified as possible mechanisms for the spread of ISA virus. Experiments indicate that ectoparasites may play a role in transmission with the sea louse species *Lepeophtheirus salmonis* and *Caligus elongatus* being implicated as potential ISA virus vectors (Nylund et al. 1993). Vertical transmission has been suggested as a mode of virus transmission but remains unproven.

D. Disease Signs

Moribund fish are lethargic, anorexic, and swim high in the water column. They can have markedly pale gills, a slightly swollen abdomen, and exophthalmia and hemorrhaging of the eye. There may be ventral abdominal petechial hemorrhaging on the skin.

Internal gross signs can include swelling and reddening of the kidney, serosanguinous ascites, and petechial hemorrhaging throughout the peritoneal cavity. The liver is often enlarged and congested with a black or mottled appearance. Enlargement of the spleen has also been noted.

Anemia is highly variable but hematocrits of <10% are not uncommon in clinically affected fish.

Several pathological changes have been noted by histology in fish with ISA. The presence of renal interstitial hemorrhaging and tubular epithelial degeneration, necrosis, and casting within the posterior kidney have all been noted (Bouchard et al. 1999; Byrne et al. 1998). Liver sections show multifocal to confluent hemorrhagic hepatic necrosis, focal congestion, and dilatation of hepatic sinusoids (Speilberg et al. 1995; Evenson et al. 1991). Rupture of sinusoidal endothelium may also be observed.

E. Disease Diagnostic Procedures

1. **Presumptive Diagnosis**

   In making a preliminary diagnosis of ISA, several factors such as the presence of gross clinical pathology, time since transfer to salt water, disease history of site, water temperature, and rate of mortality should be considered. Isolation of ISA virus can be accomplished with tissue from infected fish using standard cell culture techniques.

   Kidney and spleen tissues ($1cm^3$ sample size) are collected, homogenized, and diluted 1/10 in phosphate buffered saline. Tissue homogenates are further diluted 1/10 in Minimum Essential Medium (MEM) with Hank's salts, L-glutamine, and 2% fetal bovine serum (FBS). Samples are inoculated onto either the chinook salmon embryo (CHSE-214) or salmon head kidney (SHK) cell line at a volume of 0.1 mL/well and adsorbed for one hour. CHSE-214 cells are overlaid with 1 mL of MEM with Hank's, L-glutamine, 5% FBS, and gentamicin. SHK cells should be overlaid with Leibovitz's L-15, 5% FBS, and gentamicin. Inoculated cells are incubated at 16°C for 28 days. Cytopathic effect (CPE) seen in CHSE-214 cells is characterized by areas of refractile cells which can become confluent over time (Figure 1). In SHK cells, CPE is more diffuse, with refractile and necrotic cells present in the monolayer. CPE on either cell line can occur from 5 to 26 days (Bouchard et al. 1999).

2. **Confirmatory Diagnosis**

   The routine method of confirmation of ISA virus is by RT-PCR (see Section 1, 2.9.A1 ISA Appendix 1). Monoclonal antibodies have also been used to confirm cell culture supernatants using serum neutralization assays. Cell culture monolayers can be tested for ISA virus using the indirect fluorescent antibody technique (IFAT) using both monoclonal and polyclonal antibodies (Falk and Dannevig 1995).

F. Procedures for Detecting Subclinical Infections

IFAT and RT-PCR can be used in detecting sub-clinical infections but the detection limits of these assays have not been determined. Both tests are routinely used to determine clinical and sub-clinical infections in fish populations. Specific protocols are given in Section 1, 2.9A1 ISA Appendix 1 (ISAV RT-PCR) and Section 1, 2.9.A2 ISA Appendix 2 (ISAV IFAT).

G. Procedures for Determining Prior Exposure to the Etiological Agent

Falk and Dannevig (1995b) found indirect evidence that fish exposed to ISA virus do possess neutralizing antibodies. Other more direct assays are not currently available.

H. Procedures of Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Direct tissue RT-PCR samples should be stored in RNA-later™ (Ambion, Inc.). Virus isolation samples should be stored in phosphate-buffered saline (pH 7.4). Tissue imprint slides for IFAT should be acetone-fixed as soon as possible. All samples should be transported on ice (4°C): virology samples should be processed and inoculated within 24 hours; RT-PCR and IFAT samples should be stored at –20°C if not run immediately.

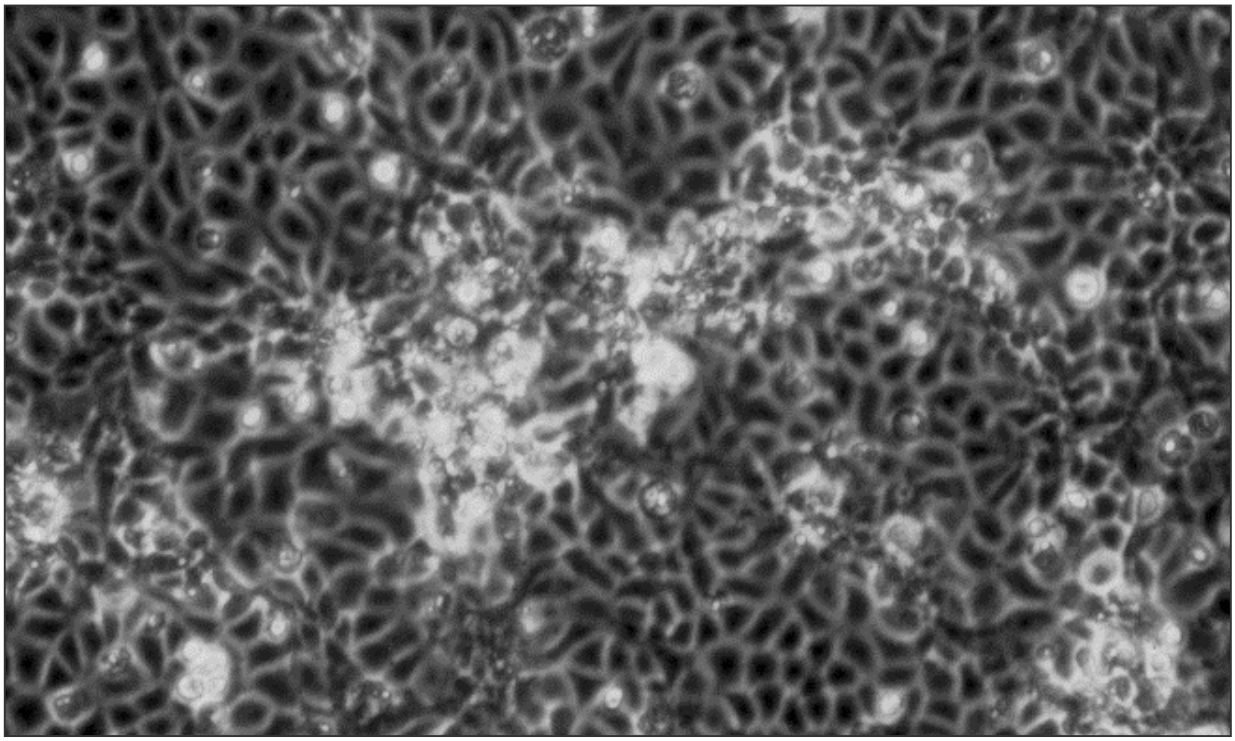


Figure 1. Cytopathic effect on CHSE-214 cells.

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2.9.A1 Infectious Salmon Anemia Appendix 1

W.R. Keleher, D. A. Bouchard, and P. L. Merrill

Micro Technologies, Inc.
41 Main Street
Richmond, ME 04357
207/737-2637
microtech@wiscassett.net
dbouchard@microtechnologies.biz

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for Infectious Salmon Anemia Virus (ISAV)

A. Principle/Purpose

To detect the presence of ISAV in kidney tissues and to confirm that cytopathic effect (CPE) observed in virology cell cultures is due to ISAV, through the amplification of cDNA fragments from the (-) sense ssRNA genome of ISAV using specific oligonucleotide primers complementary to the ISAV genome sequence.

B. Specimen

1. **Kidney tissue**
   A 30 to 50 mg size piece of middle kidney is excised from the sampled fish and blotted on a clean paper towel to remove excess blood. The scalpel should be flamed with 70 to 90% ethanol or dipped sequentially in a 10,000 ppm bleach solution and DI-H_2O between samples to prevent carryover contamination. The kidney is then submerged in five volumes (1 mL) of RNA Later™ in a 1.5 or 2.0 mL screw cap microfuge tube. The tissue can be stored in RNA Later™ for one day at 37ºC, one week at 25ºC, one month at 4ºC, or indefinitely at -20ºC without degradation. The tissue is considered compromised unless it is placed in RNA Later™ directly after sampling from the fish and stored below 37ºC before and during shipment to the laboratory.

2. **Supernatant**
   For cell culture plates that are planned to be maintained longer, the sample is harvested in a laminar flow hood by inserting a 1.0 mL sterile syringe with needle through the mylar film covering the cell culture plate. Some of the cells are scraped from the monolayer and pulled up with 0.25 mL or more of the supernatant and placed in a sterile 1.5 mL microfuge tube. If the cell culture plate is no longer needed or supernatant is being harvested from a flask, supernatant is harvested in a laminar flow hood using a sterile pipet.

C. Materials

RNA Later™ (Ambion)
1.5 or 2.0 mL screw cap microfuge tubes
Sterile 1 and 10 mL pipets
Sterile plugged nuclease free 10 µL, 100 µL, and 1000 µL pipet tips (Fisher, VWR)
Trizol LS® Reagent and Trizol® Reagent (Life Technologies)
Ethanol (Fisher Scientific)
Chloroform (Sigma)
Nuclease-free disposable pellet pestle (Fisher Scientific)
Isopropyl alcohol (Fisher Scientific)
Alcohol burner
1 mL sterile syringes with needle (Fisher Scientific)
Ready-to-Go™ RTPCR Beads 0.5mL or 0.2mL format (AmershamPharmacia Biotech)
0.5-10 µL, 10-100 µL, 200-1000 µL micro pipetors with sterile, nuclease free plugged tips (Eppendorf/Fisher Scientific)
Nuclease free water (Promega)
Tris-EDTA buffer (Sigma)
Rnasin (Promega)
ISAV specific upstream primer (1D) 5' GGC TAT CTA CCA TGA ACG AATC (Life Technologies or Sigma)
ISAV specific downstream primer (2) 5' TAG GGG CAT ACA TCT GCA TC (Life Technologies or Sigma)
1.5 mL sterile nuclease free tubes (Fisher Scientific)
0.5 mL sterile nuclease free tubes (Eppendorf)
100 base pair DNA ladder (Promega)
ϕx174 HAE III digested DNA size markers (Promega)
TAE buffer (see Section 1, 2.9.A2 ISA Appendix 2)
Molecular biology grade agarose (Life Technologies)
Ethidium bromide
Electrophoresis equipment
UV transilluminator
Mastercycler gradient thermocycler (Eppendorf/Fisher Scientific)
Microcentifuge (Eppendorf and IEC)
Photographic equipment with UV filter

1. **Preparation**
   Primers are rehydrated in Tris-EDTA buffer (final stock concentration 500 pmol/µL) and aliquoted as needed in nuclease free water (working stock concentration 50pmol/µL).

2. **Storage requirements**
   Primers, enzymes, and DNA ladders are held at –20°C. Manufacturer's requirements are followed for reagent kits and remaining materials are stored at room temperature.

D. Procedure

Listed below are procedures for the various stages of sample preparation for kidney tissue and cell culture supernatant using Trizol and Trizol LS Reagents, followed by a RT-PCR protocol.

1. Kidney Tissue Homogenization

a. Trizol Reagent

i. Remove kidney tissue to be extracted from RNA Later, place on a clean surface and trim a 20 to 30 mg piece using a scalpel flamed with 70 to 90% ethanol. Keep scalpel and forceps in 70 to 90% ethanol and flame between samples in order to prevent carryover to subsequent samples. Replace remaining kidney tissues in original microfuge tubes with RNA Later.

ii. Place trimmed tissue in a 1.5 mL microfuge tube containing 500 µL of Trizol.

iii. Grind tissue in the microfuge tube using a nuclease-free pellet pestle.

iv. Pulse-centrifuge microfuge tube for a few seconds in order to pull down any remaining large pieces of tissue.

v. Transfer 250 µL of the supernatant to another microfuge tube containing 750 µL of Trizol for a total of 1 mL.

2. RNA Extraction from Kidney Tissue

a. Trizol Reagent

i. Phase Separation
Incubate supernatant/Trizol mixture at room temperature for five minutes. Add 200 µL chloroform to each tube using a new pipet tip for each sample. Shake for 15 seconds and incubate at room temperature for five minutes. Centrifuge cold at 12000 X g for 15 minutes in order to separate phases.

ii. RNA Precipitation
Transfer clear aqueous phase to a new 1.5 mL microfuge tube (0.4 to 0.45mL). Avoid disturbing DNA in the interphase with the pipet tip. Add 500 µL of isopropyl alcohol. Incubate samples at room temperature for 10 minutes. Centrifuge cold at 12000 X g for 10 minutes.

iii. RNA Wash
Remove supernatant, leaving a small amount in the bottom of the tube so as not to disturb the pellet. Add 1 mL of 75% ethanol and vortex. Centrifuge cold at 7500 X g for five minutes.

iv. Redissolving RNA
Remove all of the supernatant. Add 20 to 40 µL Tris-EDTA buffer with Rnasin. Gently pipet up and down in order to dissolve RNA pellet.

3. RNA Extraction from Cell Culture Supernatant
Follow same procedures as described above for RNA Extraction from Kidney Tissue, except use Trizol LS instead of Trizol.

4. RT-PCR

a. Procedure for Ready-to-Go™ RT-PCR Beads

i. Prepare a master mix for the number of samples to be amplified (add one extra volume in order to ensure that the last tube to be filled is not short of the 50 µL reaction volume). Prepare master mix on ice according to manufacturer's instructions as follows:

Nuclease free water	46.8 µL
Pd $(N)_6$ random primers	1 µL
Upstream primer	50 pmol
Downstream primer	50 pmol

ii. Aliquot 49 µL of the master mix into each Ready-to-Go™ RT-PCR Beads tube.

iii. Add 1 µL of each RNA sample to a Ready to Go™ RT-PCR Beads tube.

iv. Place tubes in thermocycler and run RT-PCR59 program:
Lid 105ºC

1. 42ºC for 15 minutes.

2. 94ºC for 5 minutes.

3. 59ºC for 45 seconds.

4. 72ºC for 105 seconds.

5. 94ºC for 45 seconds.

6. 59ºC for 45 seconds.

7. 72ºC for 105 seconds.

Go to (*5*) for 36 cycles.

72ºC for 7 minutes.

Hold 4ºC.

v. Electrophorese PCR products along with ϕx174 HAE III digested DNA size markers or other appropriate DNA size markers on a 10 cm 2% agarose gel at 65V for 1 to 1.5 hours.

vi. Stain gel with ethidium bromide for 30-40 minutes. De-stain gel in DI-H_2O for 10 minutes.

vii. Photograph gel under UV illumination.

E. Reporting Results

Using the ISAV 1D/2 (Mjaaland et al. 1997) primer set, a 493 base pair fragment is amplified from ISAV-positive samples. Positive results are reported as an amplified band at the position where a 493 base pair fragment would be expected to migrate, based on the location of the positive control and appropriate DNA size marker bands.

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2.9.A2 Infectious Salmon Anemia Appendix 2

W.R. Keleher, D. A. Bouchard, and P. L. Merrill

Micro Technologies, Inc.
41 Main Street
Richmond, ME 04357
207/737-2637
microtech@wiscassett.net
dbouchard@microtechnologies.biz

Indirect Fluorescent Antibody Test for Infectious Salmon Anemia Virus (ISAV)

A. Principle/Purpose

To determine the presumptive presence/absence of ISAV in samples of fish tissue, blood, mucus, and/or body fluids.

B. Specimen

Most common samples tested consist of kidney tissue and blood. The former involves lethal sampling while the latter can be performed non-lethally. In both cases, a blot is prepared on a slide that is in turn used for the staining. Please refer below for procedures used in the preparation of blots.

C. Materials

Slides
Forceps
Scalpel
70% isopropanol
Bench paper
PAP pen [a]
5% solution of skim milk in sterile PBS
α ISAV monoclonal antibody (primary)[b]
TRITC-labeled goat α-mouse antibody (secondary)[c]
Phosphate buffered saline (PBS) with 0.1% Tween; pH 7.2

[a] Purchased from Ted Pella Inc., Redding CA
[b] Purchased from Dr. Knut Falk, National Veterinary Institute, Oslo, Norway
[c] Purchased from Sigma Chemicals, St. Louis, MO

D. Procedure

1. Sample Preparation

a. Kidney Blot
Using sterile forceps and scalpel, remove a $1cm^3$ piece of tissue from the mid-kidney. Firmly touch one surface of the kidney chunk two times onto a clean slide. Each blot should cover approximately 1/2 of the slide surface. The first blot will contain more blood than the second, however, avoid leaving a blot which is too thick or too thin.

b. Blood Sample
Place two 25 µL drops of blood onto a clean slide. Spread each blood sample, being careful to keep some separation between each drop.

c. Allow sample to dry for 30 to 60 minutes and fix by immersing in acetone for 10 minutes. Store slides at -20°C until stained.

2. Staining

a. If slides are removed from -20°C, allow to reach room temperature before staining.

b. Trace each blot with the PAP pen. Allow to dry for a few minutes. Place 2 to 3 layers of paper towels on a plastic tupperware lid and moisten thoroughly with de-ionized water. Lay slides on the moist paper towels, making sure that they are level.

c. Prepare a solution of 5% skim milk using sterile PBS (pH 7.2). The solution can be used for up to a week if stored at 2 to 8°C. Place 100 µL of this block solution per blot and spread to cover the whole blot using the edge of the pipet tip. Avoid touching the blot; change pipet tips if necessary.

d. Cover slides with bottom of tupperware container and allow incubate for 30 minutes. Do not allow slides to dry during the staining procedure.

e. Prepare a primary antibody staining solution of α ISAV monoclonal antibody by making a 1/100 dilution in sterile PBS (pH 7.2). Use laminar flow hood and aseptic technique while preparing the solution. The solution can be kept for several weeks at 2 to 8°C.

f. Tap off skim milk solution by tilting each slide. Lay slides back on the moist paper towel and place 50 µL of primary antibody (α ISAV mAb) on each blot. The antibody solution should cover the blot without any need for spreading; check by viewing the slides at an angle to light. Cover with tupperware bottom and allow to incubate for 60 minutes.

g. Tap slides on paper towels as before, dip in a solution of PBS with 0.1% Tween and soak in a second container of the same solution for six minutes. Either metal slide racks or plastic specimen cups may be used during the washes.

h. The remainder of the staining procedure must be performed away from direct light.

i. Prepare a secondary antibody staining solution of TRITC labeled goat α mouse antibody by making a 1/100 dilution in sterile PBS; prepare aseptically as before. The antibody solution can be kept for several weeks at 2 to 8°C.

j. Place slides back on the moist paper towels. Dispense 50 mL of secondary antibody onto each blot and check to make sure it is spread evenly over the blot. Cover slides with tupperware bottom and incubate for 60 minutes.

k. Repeat step g.

l. Place slides face up on dry paper towels and allow to dry at room temperature. If slides are not going to be viewed immediately, place in a slide box and keep at 2 to 8°C until analysis.

3. Resulting Stained Slides

a. View slides under fluorescence with a wide green filter after mounting a coverslip with 1 to 2 drops of immersion oil.

b. The interpretive scoring method for IFAT/ISAV is as follows:
   1+ : sparse distribution of fluorescent grains or specks throughout
   2+ : more obvious distribution of grains, (>10 field^{-1}) e.g. around cell membranes
   3+ : obvious distribution of grains, e.g. broken lines around cell membranes
   4+ : broad fluorescence; 3 to 4 fully involved cells per field of view

c. An interpretive scoring of 1+ or 2+ is considered a suspect rating result for ISAV by IFAT.

d. An interpretive scoring of 3+ or 4+ is considered a positive rating result for ISAV by IFAT.

2.10 Spring Viremia of Carp

A. E. Goodwin and J. R. Winton*

Aquaculture/Fisheries Center
University of Arkansas at Pine Bluff
1200 N. University Drive
Mail Slot 4912
Pine Bluff, AR 71601
agoodwin@uaex.edu

*Western Fisheries Research Center
U. S. Geological Survey
6505 NE 65th Street
Seattle, WA 98115
jim_winton@usgs.gov

A. Name of Disease and Etiological Agent

Spring Viremia of Carp (SVC) is caused by the fish rhabdovirus, Spring Viremia of Carp Virus (SVCV). The following names have historically been used for diseases that may have been caused by SVCV: swim-bladder inflammation (SBI), infectious dropsy, rubella, infectious ascites, hemorrhagic septicemia, and red contagious disease. For reviews of the disease and the causative virus see Ahne et al. (2002), Fijan (1999), and Wolf (1988).

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Most reports of Spring Viremia of Carp are from Europe, but the virus has been isolated in other regions of the world including the Middle East, China and, in one incomplete report, South America. The SVCV has been reported from cultured shrimp in Hawaii, but not from Hawaiian finfish (Johnson et al. 1999). The virus was first reported in North America in 2002 when it was isolated from farmed koi in North Carolina and from feral common carp in Cedar Lake, Wisconsin. The DNA sequences of these North American isolates are very closely related to those from China.

2. Host Species

Natural infections have occurred in bighead carp, common carp, crucian carp, goldfish, koi, penaeid shrimp, pike, silver carp, tench, and the wells catfish (sheatfish). Experimental infections have produced disease or demonstrated viral replication in golden shiners, grass carp, guppies, pike, pumpkinseeds, roach, zebra danios, and fruit flies. Few North American species have been tested, but it is probable that many, especially cyprinids, will prove susceptible.

C. Epizootiology

In typical outbreaks, SVCV spreads horizontally during the winter when water temperatures are low and host immune systems are less active. When spring approaches and temperatures rise toward 10°C, fish develop clinical signs of SVC and begin to die. The peak mortality for SVC occurs at 15 to 17°C. Above 20°C, most fish are able to develop immunity to the virus and mortality ceases. Fish may also develop SVC in the fall if temperatures are in the right range for virus transmission and disease. The virus has not been successfully isolated from infected populations of fish following periods of water temperatures in the 25 to 30°C range. In experimental infections, it has been shown that immunosuppression triggered by cold temperatures is critical to the development of SVC. The actual temperature required to produce that immunosuppression is apparently related to the thermal optimum of the infected fish species. It is not known if stressing coolwater fish with high temperatures (temperatures well above optimal for the fish, but still below the upper limit for virus replication in culture) will induce clinical SVC.

Most reported losses from SVC have occurred in farmed carp during their first winter and spring. Little mortality has been reported in older fish in Europe, but this may be due to immunity in older fish previously exposed to the virus. The first outbreak reported in Wisconsin occurred in large mature carp, perhaps indicating their naïve status.

While SVCV has been isolated from fish lice (*Argulus sp.*) and leeches (*Pisicola sp.*) feeding on infected fish, there is no evidence that these mechanical vectors are important in the spread of SVCV. It has not been shown that vertical transmission is a significant mechanism for the spread of SVCV. However, there is one report of the virus being found in ovarian fluid from a population of common carp broodfish tested on farms in Europe with a history of SVC.

D. Disease Signs

The clinical signs of SVC are not very significantly different from those associated with bacterial septicemias in carp. Infected fish typically have hemorrhages of the skin, exophthalmia, abdominal distension, and a discharge of bloody mucoid material from the vent (Figure 1). Internal signs include petechial hemorrhages of the swim bladder and muscle, ascites, edema of the kidney, liver and spleen, and catarrhal and hemorrhagic enteritis.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

To aid in the diagnosis of SVCV, certain key features such as life stage and species of fish, water temperature, season, clinical signs, and disease history of the facility are evaluated. To isolate SVCV, kidney and spleen homogenates are examined by standard cell culture techniques. Processed specimens may be inoculated onto the *Epithelioma papillosum cyprini* (EPC) or fathead minnow (FHM) cell lines and incubated at 22 to 25°C. Some isolates may be easier to detect on bluegill fin (BF-2) cells. Many cell lines are susceptible to SVCV and the virus will produce cytopathic effects (CPE) in fish cell lines inoculated with SVCV and incubated at a broad range of temperatures (15 to 30°C). Cytopathic effects include focal patches of round refractile cells that can be observed 24 to 96 hours post-inoculation depending on the concentration of virus in the inoculum and the temperature (Figure 2). Some clinical cases may have such high titers that focal CPE is not evident, and the entire monolayer may be destroyed. Appropriate clinical signs, fish species, season, water temperature, and observation of typical CPE provide the best evidence for a presumptive diagnosis.

Spring Viremia of Carp can easily be distinguished from Koi Herpes Virus (KHV). The KHV produces disease at temperatures above 20°C, does not cause CPE in EPC, FHM, or BF-2 cells, and commonly produces focal necrosis of the gill filaments, not the septicemia signs common with SVCV.

2. Confirmatory Diagnosis

Confirmation of SVCV has been achieved through a variety of immunological methods including serum neutralization, ELISA, and immunocytochemistry using both monoclonal and polyclonal antibodies. However, it has been shown that SVCV isolates similar to those recovered from fish imported from Asia may bind poorly to SVCV monoclonals and antisera. Additionally, care must be taken to insure that antibodies used do not cross react with Pike Fry Rhabdovirus (PFRV) and that appropriate PFRV controls are included in the assays. For these reasons, a reverse transcriptase, polymerase chain reaction (RT-PCR) assay is now regarded as the best confirmatory technique for virus isolated in cell culture. The RT-PCR works well with both European and Asian-related isolates and has the advantage that the PCR product can be sequenced and compared to other isolates for epidemiology. The RT-PCR protocol for SVCV is detailed in Section 2, 4.6.F.2.b "Polymerase Chain Reaction (PCR) Method for Confirmation of SVCV."

F. Procedures for Detecting Subclinical Infections

Carp infected with SVC at water temperatures within the range of viral replication, but below that where overt disease begins to occur (4 to 10°C), routinely test positive for SVCV by tissue culture. The virus generally cannot be isolated from survivors of previous SVC outbreaks or from fish tested during the summer when water temperatures are greater than 20°C. There is little known about asymptomatic carrier fish other than one report of detection of SVCV by tissue culture of ovarian fluids from brood fish sampled during the spring. There has been no work investigating the detection of carrier fish by PCR.



Figure 1. Common carp experimentally infected with SVCV. Note the exophthalmia and hemorrhage of the eye and petechial hemorrhages on the skin.

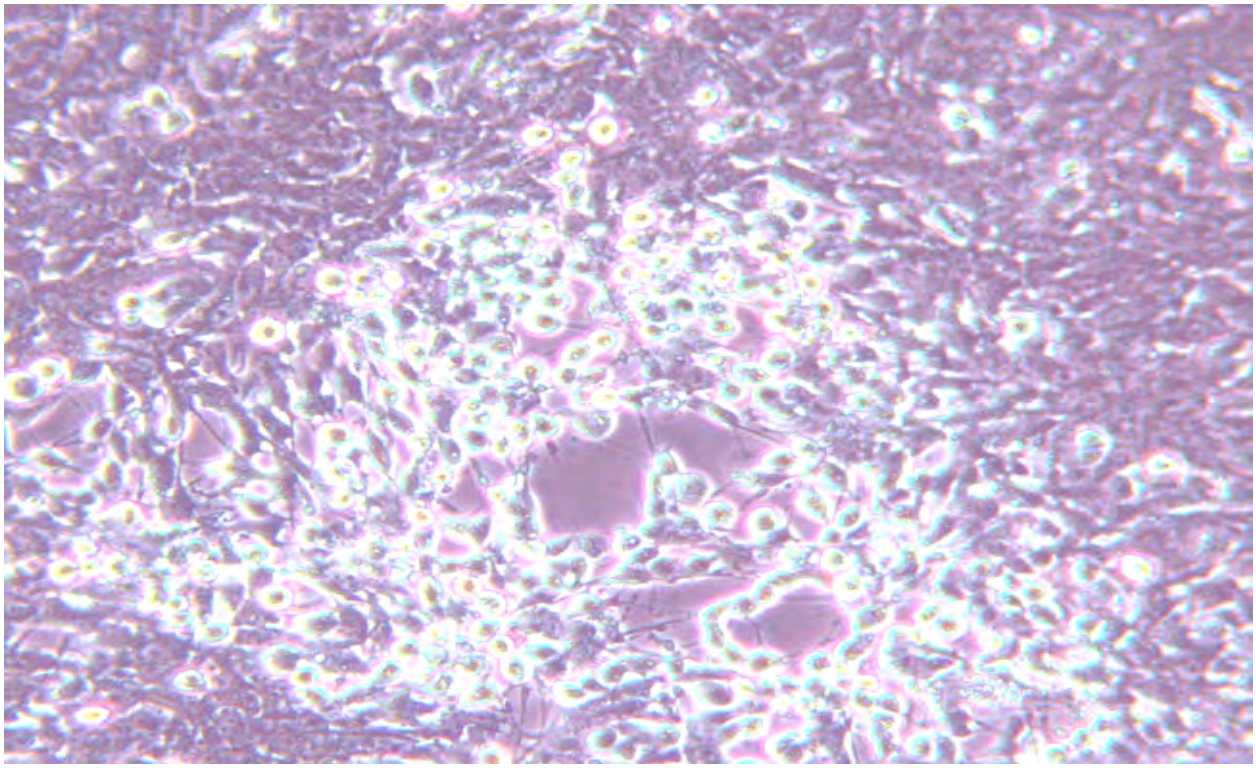


Figure 2. Cytopathic effects of SVCV in a monolayer of EPC cells. Note the foci of rounded and dead cells. Phase contrast.

G. Procedures for Determining Prior Exposure to the Etiological Agent

Prior exposure of common carp to SVCV can be detected by a competitive ELISA for antibodies to SVCV (Dixon et al. 1994). This assay is well accepted for common carp and koi but not for other species. The assay relies on polyclonal antibodies that may be difficult to produce and standardize.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiologic Agent

See Section 2, 2.2.A.2.b "Guidelines for Preparation of a Fish Health Inspection".

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2.11 Koi Herpesvirus Disease

R.P. Hedrick

Department of Medicine and Epidemiology
School of Veterinary Medicine
University of California
Davis, CA 95616
rphedrick@ucdavis.edu

A. Name of Disease and Etiological Agent

Koi herpesvirus disease (KHVD) is caused by a recently recognized herpes-like virus (KHV) also known in Israel as carp nephritis and gill necrosis virus (CNGV). While clearly different from herpesviruses found in mammals, birds and reptiles, KHV is related to a group of herpes-like viruses found in fish and amphibians currently classified in the family Herpesviridae.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

The virus has been detected in koi or common carp from USA, Israel, England, Denmark, Germany, Netherlands, Luxembourg, Belgium, France, Austria, Switzerland, Italy, Poland, China, Taiwan, Japan, Indonesia and South Africa.

2. Host Species

To date only *Cyprinus carpio* (koi and common carp) is known to be susceptible to KHV infection. Other carps and goldfish are resistant to infection and those tested appear not to be capable of carrying the virus as a subclinical infection (Perelberg et al. 2003). The virus has been detected principally among ornamental fish and koi in particular but more recently KHV has been detected in wild common carp in Europe and South Carolina, U.S.A.

C. Epizootiology

The origins of KHV are somewhat obscure but the cases in Germany in 1996–1997 maybe the first recorded observations (Bretzinger et al. 1999). Isolation of the virus and confirmation that it was the cause of the disease were completed in 1998 (Hedrick et al. 1999, 2000). Young koi and common carp are most susceptible although outbreaks and high mortality (90 %) have been observed in larger and adult fish. Mortality begins approximately 7–14 days following contact with the virus most often via a recently introduced fish to the pond (koi) with one of two common outcomes: 1) the introduced fish dies shortly after introduction and spreads the virus to the naïve population or 2) the introduced fish stays healthy yet the naïve fish succumb. The movement of the virus with suspected carriers has

been the principal means by which the virus has spread so rapidly at both the regional and global scales.

D. Disease Signs

1. Behavioral Signs

Infected fish may demonstrate a hypersecretion of mucus which may cloud the pond water early in infection. Later, fish are lethargic, may lose equilibrium and position in the water column and may demonstrate signs of suffocation (Walster 1999).

2. Gross Signs

A white patchy appearance to the epidermis and the gills may be the first observed external signs. Swelling and necrosis of gill filaments (Figure 1) are common and secondary infections with *Flavobacterium* spp. may occur. Diffuse hemorrhages on the epidermis may or may not be present. Eyes may demonstrate a sunken appearance. Internal signs include swelling of the kidney and spleen. There may or may not be hemorrhages evident in the body wall of the peritoneum.

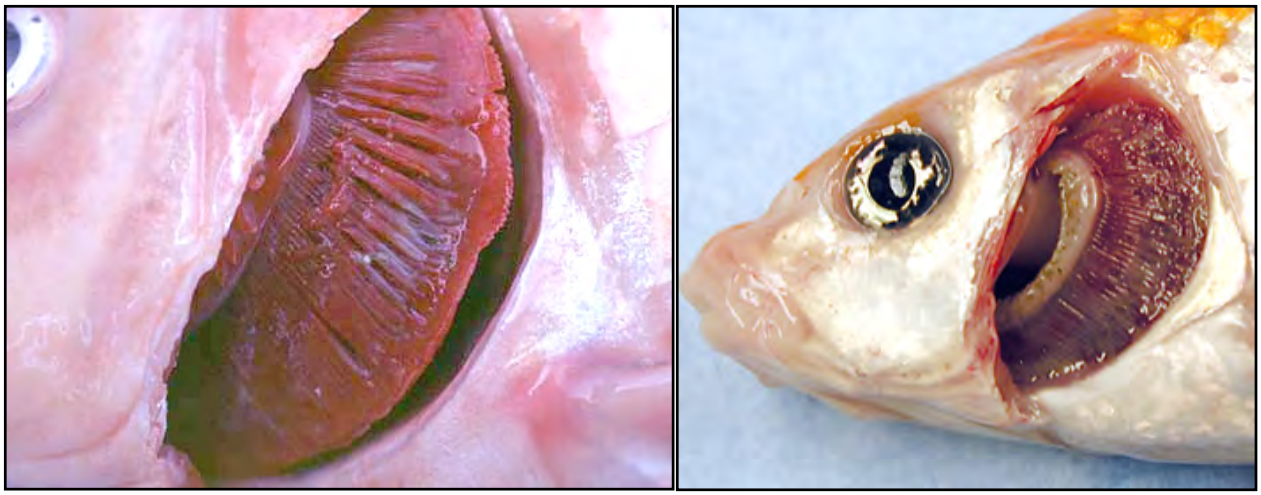


Figure 1. Gills of koi with KHVD showing (left) the swollen and fused lamellae and (right) white patchy appearance and necrosis progressing from distal to more basal portions of the lamella.

3. Microscopic Signs

There is both a hyperplasia and hypertrophy of gill epithelial cells. Inflammation and necrosis may also be evident in gill, kidney interstitium, parenchyma of the spleen and liver and the lamina propria of the intestine (Hedrick et al. 2000). Intranuclear inclusions are characterized by margination of the chromatin and a thickened nuclear membrane which surrounds a flocculent amphophilic to basophilic matrix (Figure 2). Intranuclear inclusions may not always be prominent in KHV infections.



Figure 2. Secondary gill lamellae of a koi with KHVD. Adjacent lamellae are fused in the basal regions. Hypertrophy and necrosis of cells in the interlamellar region is evident as is the enlarged nucleus with a thickened membrane surrounding an amphophilic to basophilic matrix (arrow).

E. Disease Diagnostic Procedures

The diagnosis of KHVD is dependent upon demonstration of the clinical signs typical for the disease among koi or common carp at water temperatures between 16–28°C and presence of evidence for KHV either directly by isolation of the virus in cell culture or viral DNA by PCR. Confirmation that an isolated virus is KHV is accomplished by PCR. Immunostaining or indirect fluorescent antibody confirmation of infected cell cultures with anti-KHV rabbit antibodies are known but the antibodies are not routinely available nor has the test procedure been published.

Sampling Criteria

Most outbreaks of KHVD in the northern hemisphere occur during the spring and autumn when water temperatures are between 23–26°C. Infections can occur at any time of the year when water temperatures

are between 16–28°C . The ability to routinely detect the virus at temperatures above or below these limits is difficult or impossible with current methods. Samples taken from the gills, kidney and spleen are deemed the most valuable for isolation of the virus or detection of viral DNA.

1. Presumptive Diagnosis

a. Clinical Signs

Lethargy and signs of suffocation and white patches on the gill and skin, gill necrosis, sunken eyes, enlarged kidney and spleen.

b. Virus Isolation

Two available cells lines are routinely used for virus isolation. The KF-1 cell line from koi fin (Hedrick et al. 2000) and the CCB line from common carp brain (Neukirch et al. 2000) support growth of KHV. The cells should be incubated at 20°C (Gilad et al. 2003). Cytopathic effects are characterized by the presence of small focally forming syncytia (fusing of the cytoplasm of several adjacent cells), enlarged nuclei with distinct nuclear membranes and the presence of numerous cytoplasmic vacuoles (Figure 3).

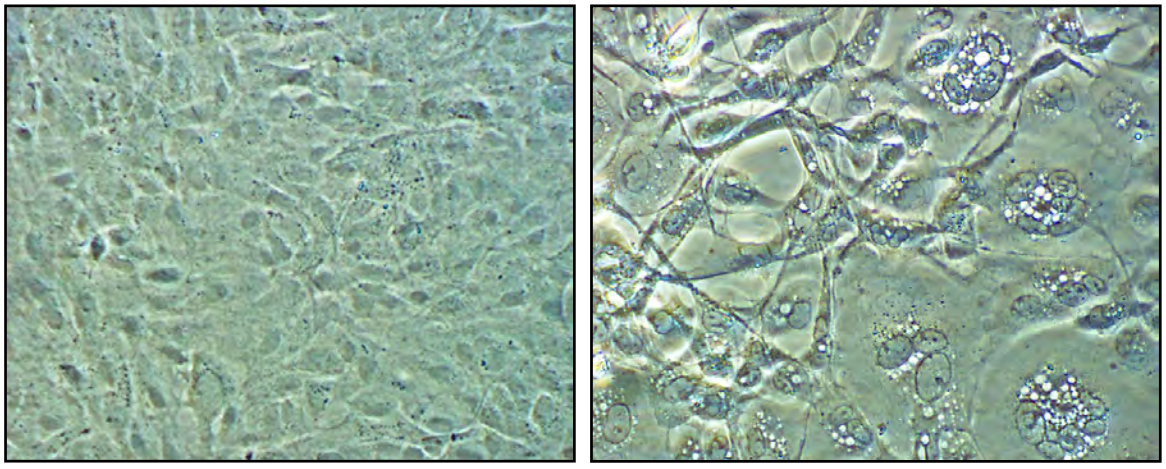


Figure 3. Cytopathic effects induced in KF-1 cells at 7 days post infection. Left panel is uninfected KF-1 cells and right panel is KHV-infected KF-1 cells. Note the presence of several enlarged nuclei within a large continuous cytoplasm (syncytium). Also evident is the intense vacuolation characteristic of KHV infected cells.

2. Confirmatory Diagnosis

a. Polymerase Chain Reaction

Confirmation that the virus present in KF-1 or CCB cells is KHV is based upon amplification of viral DNA using the polymerase chain reaction (PCR) assay. Two PCR tests are available, one described by Gilad et al. (2002) and the other by Gray et al. (2002). Primer sets and conditions for amplification can be found in these reports.

1. DNA Extraction from Fish Tissues

A pool of gill, kidney, and spleen tissues (ca. 1 g) are suggested samples for PCR analysis from suspect fish. Other suspect tissues such as gut or brain of infected fish may also be used. Tissues from moribund or freshly dead fish are preferred. The PCR can also be used on cells and supernatant from a suspect culture showing cytopathic. DNA

for PCR is obtained from fish tissues or a 0.5 ml cell suspension (cells and media from a suspect culture) using a conventional extraction protocol (chloroform isoamyl alcohol) and then eluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8) or sterile water. Alternatively, commercially available kits (Dneasy Tissue Kit, Qiagen Inc., CA, USA) can be used to extract DNA from tissues or cell suspensions following the manufacturer's protocol. The DNA concentration is then determined by spectrophotometry. A total of 70-100 ng of DNA is used in each PCR using cycling conditions and primers as described below. DNA samples for PCR can be stored temporally at 4ºC or indefinitely at –70°C until used.

2. PCR Conditions

The conditions that provide amplification of the specific 484 bp fragment are as follows:

Cocktail: A 50 µl reaction mixture contains 2 mM $MgCl_2$, 1X PCR Buffer, 400 µM deoxynucleotide triphosphate, 30 pmol of each primer, and 1U *Taq* polymerase

Cycling conditions: 95°C for 5 min and then 39 cycles of 94°C for 1 min, 68°C for 1 min, 72°C for 30 s and followed by an extension at 72°C for 7 min.

Forward primer: KHV9/5F 5'-GAC GAC GCC GGA GAC CTT GTG-3';

Reverse primer: KHV9/5R 5'- CAC AAG TTC AGT CTG TTC CTC AAC-3'.

3. PCR Product Visualization and Sequencing

The 484 bp amplicon (derived from KHV DNA) can be visualized and compared to a commercially made DNA ladder following electrophoresis using a 1.5% agarose gel. Confirmation that the product is KHV can be obtained by DNA sequencing and comparison to reference sequences available in GenBank (AF411803).

F. Procedures for Detecting Subclinical Infections

There are no currently established criteria for detection of subclinical infections. The presence of viral DNA has been detected by PCR in fish recently recovered from clinical episodes of KHVD.

G. Procedures for Detecting Prior Exposure

An ELISA described by Ronen et al. (2003) has been utilized to demonstrate that fish exposed to the virus develop serum antibodies to KHV. This assay has not been widely field tested but is presumed to be a good indicator that fish have been exposed to the virus.

H. Procedures for Transportation and Storage of Samples

Samples collected from apparently moribund or dead fish should be packed on ice for shipment for virus isolation. Tissues for PCR analyses can be stored in 95% ethanol or kept frozen at ultracold temperatures. Virus isolation is difficult on fish dead for any period of time or following freezing.

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2.12 Goldfish Herpesviral Hematopoietic Necrosis Disease

Andrew E. Goodwin

University of Arkansas at Pine Bluff
Aquaculture Fisheries Center
Pine Bluff, AR 71601
agoodwin@uaex.edu

A. Name of Disease and Etiological Agent

Goldfish Herpesviral Hematopoietic Necrosis Disease (HVHN) is caused by a herpes-like virus classified as *Cyprinid Herpesvirus 2* (CyHV-2) by the International Committee on the Taxonomy of Viruses. The DNA sequence of CyHV-2 is most similar to those of CyHV-1 (the carp pox virus) and CyHV-3 (the koi herpesvirus).

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

There have been published accounts of HVHN in goldfish from Japan, the US, Taiwan, and Australia. There are unpublished reports of CyHV-2 in Europe. Given the known distribution of the virus and the level of international commerce in goldfish, it seems likely that the virus is present in most goldfish producing countries.

2. Host Species

The CyHV-2 virus and HVHN disease have only been reported in goldfish (*Carassius auratus*), but diagnostic tools sufficient to detect carrier states in other species have only recently been developed. In experimental infections, goldfish X koi (*Cyprinus carpio*) hybrids carry the virus but do not develop the disease. Koi did not carry the virus or develop disease.

C. Epizootiology

Despite the wide geographic distribution of CyHV-2 and the global commerce in goldfish, reports of HVHN are quite rare. This may have been partially due to technical challenges that made definitive diagnosis difficult prior to 2006. The virus does produce very high mortality during epizootics and some small breeders and private pond owners have experienced devastating losses on an annual basis. The virus can be detected in healthy-looking fish tested by PCR as part of fish health surveys. The best evidence is that the virus is widespread, but only causes disease under certain conditions. Those exact conditions are unknown, but epizootics are most common during the spring and fall when water

temperatures are above 18 °C. Significant outbreaks have occurred at temperatures as high as 28 °C and as low as 15 °C.

D. Disease Signs

1. **Behavioral Signs**
   Fish may become lethargic and rest on the bottom. Increased ventilation rates and loss of equilibrium have also been described.

2. **Gross Signs**
   Gills may be generally pale or there may be pale patches on the skin and gills similar to those reported in koi infected by KHV (CyHV-3).

3. **Microscopic Signs**
   Histology of head kidneys from goldfish with acute HVHN reveals extensive necrosis of hematopoietic cells (figure 1). Nuclei of hematopoietic cells are sometime enlarged with marginated chromatin. Similar lesions may be seen in the spleen (figure 2). Many fish also have inflammation and necrosis in the sub-mucosa of the intestine (figure 3). Other lesions have been reported but are not consistently noted.

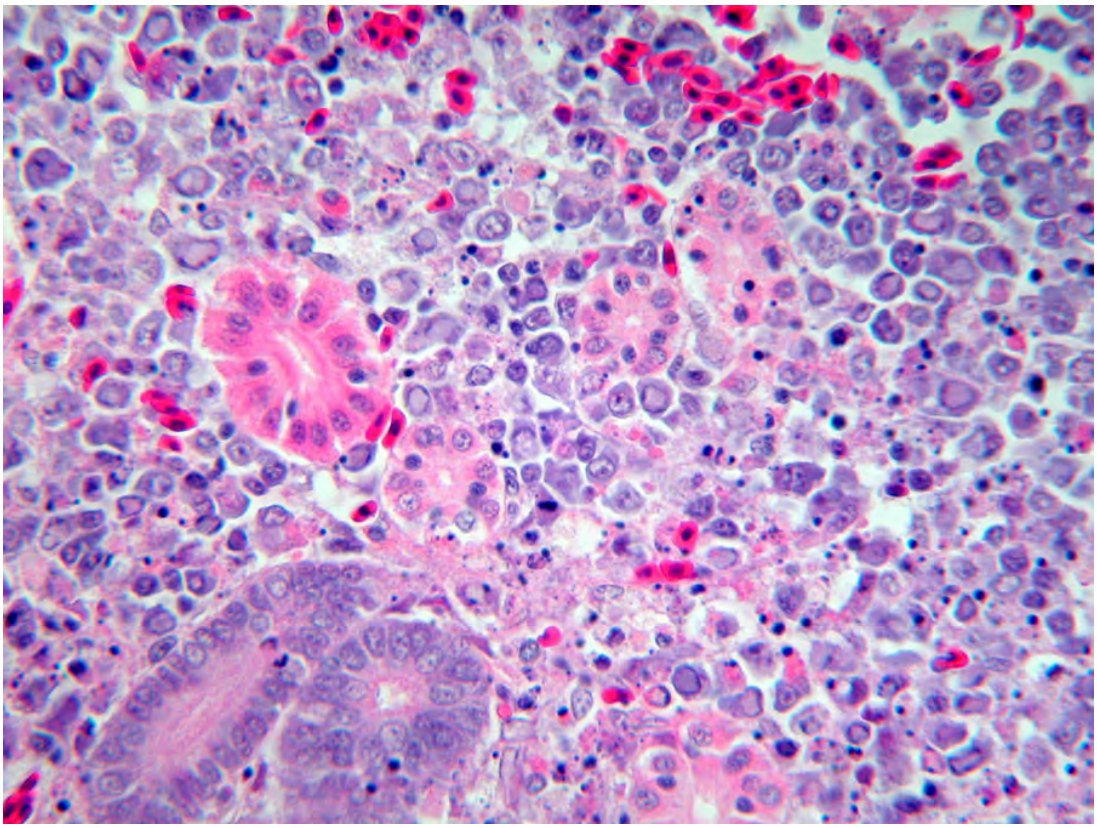


Figure 1: Necrosis of hematopoietic cells in the kidney of a goldfish with Goldfish Herpesviral Hematopoietic Necrosis Disease. Many pyknotic nuclei are evident. Other nuclei are enlarged with marginated chromatin. Picture by Dr. Lester Khoo.

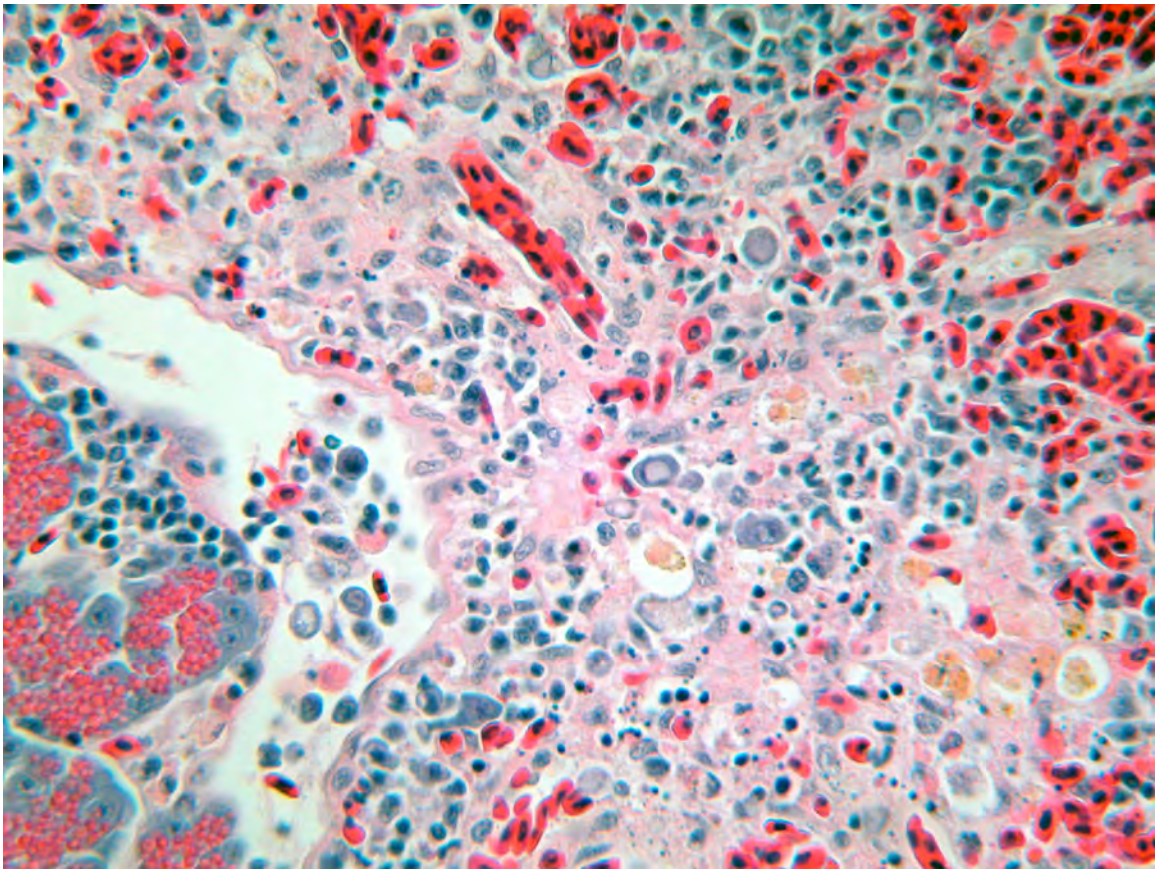


Figure 2: Necrosis of hematopoietic cells in the spleen of a goldfish with Goldfish Herpesviral Hematopoietic Necrosis Disease. Many pyknotic nuclei are evident. Other nuclei are enlarged with marginated chromatin. Picture by Dr. Lester Khoo.

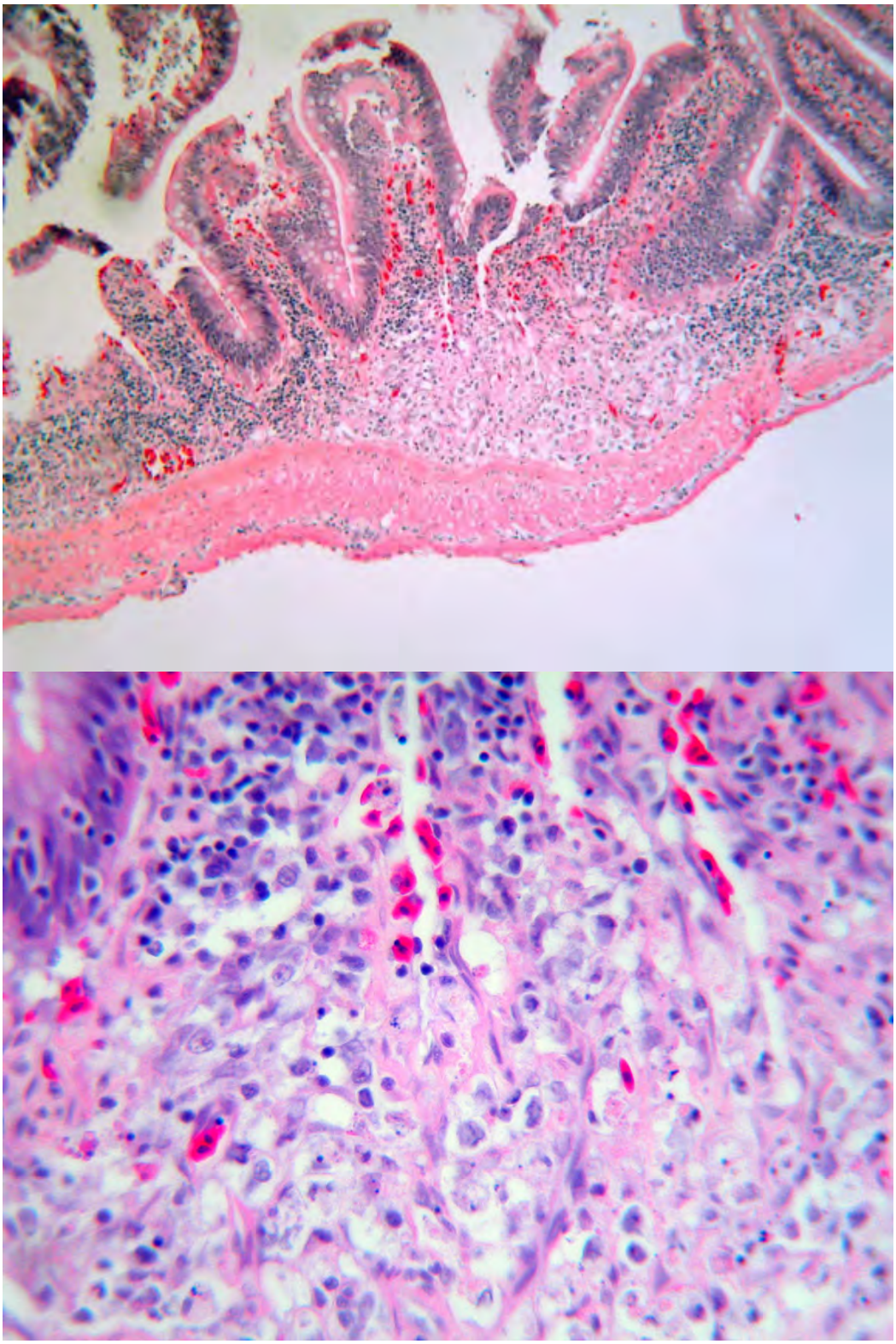


Figure 3: Necrosis of the submucosa in the intestine of a goldfish with Goldfish Herpesviral Hematopoietic Necrosis Disease. The bottom frame is a higher magnification of the focal area of necrosis evident near the center of the top frame. Picture by Dr. Lester Khoo.

E. Disease Diagnostic Procedures

The CyHV-2 virus is difficult to propagate in cell culture so successful diagnosis relies on histology or molecular methods. Most laboratories see only transient or no cytopathic effects in cell cultures. If culture is attempted, rare success is reported with the koi fin cell line (KF-1). There is a report of successful culture in a goldfish fin cell line in Japan, but that cell line is not readily available and laboratories attempting detection of CyHV-2 in that cell line often report failures. There are no antibodies available for serological testing.

1. Presumptive Diagnosis

a. Histology

Severe necrosis of the hematopoietic tissues in the head kidney and spleen is the most dependable histological lesion (figures 1 and 2). Necrosis and inflammation of the intestinal sub-mucosa is also common (figure 3).

b. Polymerase Chain Reaction

PCR may be used to detect CyHV-2 directly in the tissues of fish (Goodwin et al. 2006). However, the assay is very sensitive and will easily detect CyHV-2 in carrier fish. Care must be taken to insure that PCR-positive fish have acute HVHN and are not CyHV-2 carriers dying from another cause. Standard PCR is carried out as described below, except that the fluorescent probe is omitted and the 92 bp amplicon is resolved using a 1.5% agarose gel.

2. Confirmatory Diagnosis

a. Confirming a Histological Diagnosis

When histological lesions consistent with HVHN are evident, the presence of CyHV-2 should be confirmed by PCR or by real time quantitative PCR.

b. Confirming a diagnosis made by standard PCR

The only virus known to cross react with the CyHV-2 primer set is CyHV-1, but this pathogen has never been reported in goldfish. The primary risk involved in diagnosis by standard PCR alone is that the detection of CyHV-2 will lead to a diagnosis of HVHN when the fish was actually a CyHV-2 carrier fish moribund due to infection by another pathogen. Thus, other infectious or environmental causes should be ruled and the diagnosis confirmed either by histology or by quantitative real-time PCR. In the event that the clinical signs or PCR products are in any way atypical, further confirmation of CyHV-2 can be obtained by sequencing the PCR product and comparing it to the sequences deposited in GenBank (accession number AY939863).

1. DNA Extraction from Fish Tissues

DNA is prepared from a pool of kidney and spleen tissues using either conventional chloroform isoamyl alcohol extraction or by commercially available kits (Dneasy Tissue Kit, Qiagen Inc., CA, USA). The DNA concentration of the purified DNA is determined by spectrophotometry.

2. PCR Conditions

The conditions that provide quantitative amplification of the specific 170 bp fragment are as follows:

Cocktail: For 25 µl reactions combine 5 µl of template (concentration 50-800 µg/µl), 1.0 µl each of the forward and reverse primers (10µM each), 1.0 µl of FAM-labeled fluorescent probe (10 µM), 13 µl of 2X supermix (Bio-Rad Laboratories**,** Hercules, CA), and 4.0 µl of water. If compatible with the PCR machine, 12.5 µl reactions may also be successfully used.

Forward primer: 5'- TCGGTTGGACTCGGTTTGTG – 3'

Reverse primer: 5'- CTCGGTCTTGATGCGTTTCTTG – 3'

Fluorescent Probe: 5'- FAM-CCGCTTCCAGTCTGGGCCACTACC- BHQ1– 3'

Standards and Controls: Given that CyHV-2 has not been successfully propagated in vitro, the only suitable positive controls are DNA derived from the tissues of fish with natural CyHV-2 infections. Standards are most easily produced by cloning the product of a positive CyHV-2 reaction and then using a known concentration of purified plasmid to establish a standard curve.

Cycling conditions: Quantitative reactions with the fluorescent probe must be run on a PCR machine with the capability to perform and analyze real time PCR reactions utilizing fluorescent reporters. The cycle consists of an initial incubation of 2 min at 95 °C followed by 35 cycles of 45 s at 58 °C, 45 s at 72 °C, and 30 s at 95 °C, and a final extension of 2 min at 72 °C

3. Interpreting the Results: The copy number per reaction in fish samples is determined from the standard curve and normalized as the number of copies per µg of template DNA used. Most brood fish and carrier fish have from 0-50,000 copies of the CyHV-2 genome per µg template DNA. Fish with clinical HVHN may have millions of copies per µg template DNA. These ranges provide rough guidelines for differentiating between carriers and acutely diseased fish, however, it must be recognized that even in active infections the copy number varies as the disease progresses and there is the possibility that copy number may increase in fish in an immunocompromised state resulting from infection by another pathogen or from environmental conditions.

F. Procedures for Detecting Subclinical Infections

Subclinical infections are detected in healthy fish by quantitative real time PCR done directly on fish tissues. The calculated sensitivity of the PCR assay approaches 1 copy of the viral genome.

G. Procedures for Detecting Prior Exposure

There are no published serological methods for detecting antibodies against CyHV-2 in the blood of fish surviving HVHN. Quantitative PCR does detect the virus in healthy fish, including mature broodfish from sources where the virus is prevalent and thus may be useful in detecting populations of fish exposed to CyHV-2.

H. Procedures for Transportation and Storage of Samples

Samples for histology should be collected and fixed according to standard methods. Tissues for PCR may be stored in 95% ethanol or frozen for later DNA isolation.

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2.13 Largemouth Bass Virus Disease

John M. Grizzle

Southeastern Cooperative Fish Disease Project
Department of Fisheries and Allied Aquacultures
Auburn University
Auburn, AL 36849
grizzjm@auburn.edu

A. Name of Disease and Etiological Agent

Largemouth bass virus disease (LMBVD) is caused by an iridovirus known as largemouth bass virus (LMBV; Figure 1). In the Universal Virus Database (ICTVdB Management 2006), this virus along with guppy virus 6 (GV) and doctor fish virus (DFV) is listed in the species Santee-Cooper ranavirus. Additional names for LMBV are largemouth bass ranavirus and largemouth bass iridovirus. A *Megalocytivirus* isolated from largemouth bass in Taiwan has also been called largemouth bass iridovirus (Chao et al. 2004). For reviews, see Goldberg (2002) and Grizzle and Brunner (2003).

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Largemouth bass virus has been found only in the central and eastern United States. The closely related GV and DFV were isolated from ornamental fish being imported into the U.S. from southeast Asia (Hedrick and McDowell 1995).

2. Host Species

The most commonly reported species with LMBVD or subclinically infected with LMBV is largemouth bass *Micropterus salmoides*. Smallmouth bass *Micropterus dolomieu* are also affected by LMBVD. Subclinical infections occur in other members of the Centrarchidae family, but LMBV in fish of other families appears to be rare.

C. Epizootiology

The first known isolation of LMBV was from largemouth bass collected from Lake Weir, Florida, in 1991 (Grizzle et al. 2002). The virus is highly communicable by waterborne transmission (Grant et al. 2005), but most infected fish do not show signs of disease. Causes of subclinical infections converting to overt disease are unknown. There is no evidence that stress is a trigger for outbreaks of LMBVD in wild populations; water temperature and other variables are usually suitable for largemouth bass during LMBVD outbreaks. Increased mortality can occur in confined largemouth bass captured from populations infected with LMBV, probably because of virus transfer among fish

and increased occurrence of bacterial diseases in the LMBV-infected fish (Schramm et al. 2006). The genetics of the virus (Goldberg et al. 2003) and the fish (Goldberg et al. 2005) may be important factors affecting the severity of disease outbreaks. Fish that have been exposed to LMBV develop antibodies, but the role of antibodies in disease resistance is unknown.

This disease occurs during summer in adult fish, and subclinical infections occur during all seasons. Occasionally, LMBVD causes a noticeable fish kill in wild populations, but there can be a decreased abundance of older age classes even when a fish kill is not observed (Maceina and Grizzle 2006). Young-of-year largemouth bass can be infected experimentally, but spontaneous outbreaks of LMBVD have not been reported in age 0 fish. Most hatcheries with LMBV-infected fish have not observed an increased mortality rate.

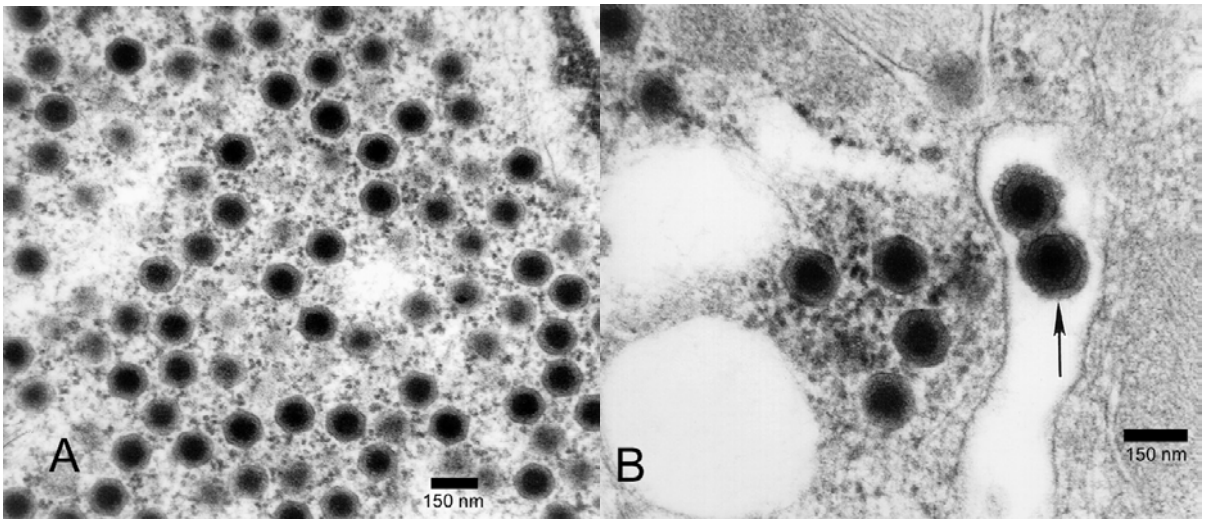


Figure 1. Largemouth bass virus. (A) Transmission electron microscope image of LMBV in cell culture. (B) Enveloped virus (arrow) after passing through the cell membrane.

D. Disease Signs

Diseased fish swim lethargically near the surface of the water and then lose equilibrium. Some moribund fish have an overinflated swim bladder (Figure 2) and less commonly have exudate in the swim bladder (Figure 3). There are no other gross lesions characteristic of LMBVD. If other gross lesions are noted, an additional disease should be suspected. Fish injected intraperitoneally with LMBV have acute peritonitis; however, histological lesions have not been adequately documented in fish with spontaneous LMBVD.

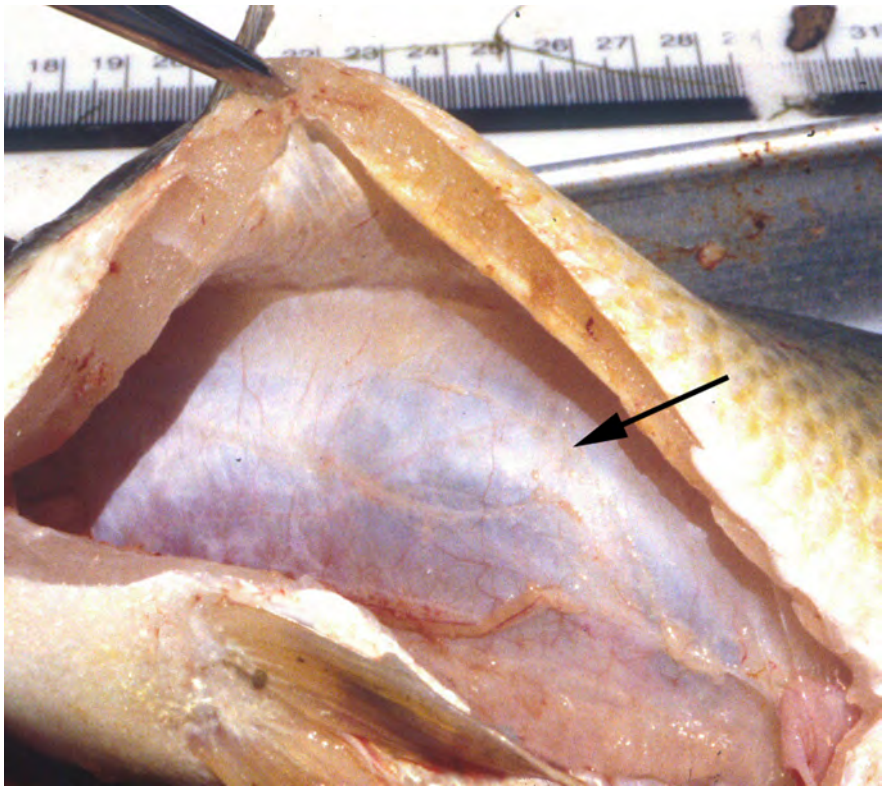


Figure 2. Largemouth bass with largemouth bass virus disease. The swim bladder is overinflated (arrow). Scale for the ruler in this photograph is centimeters.

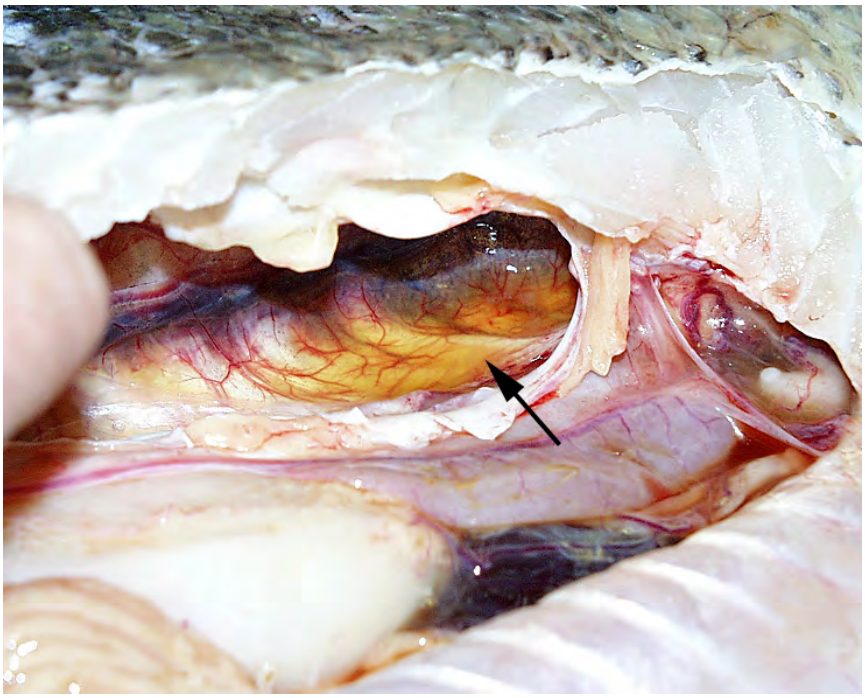


Figure 3. Yellow exudate (arrow) in the swim bladder of a largemouth bass with LMBVD. Photo by Andrew Goodwin, University of Arkansas at Pine Bluff.

E. Disease Diagnostic Procedures

Diagnosis of LMBVD requires the detection of LMBV in fish that have signs typical of LMBVD. The mere presence of LMBV does not mean that a fish is diseased, and the behavior and swim bladder lesions that have been observed in fish with LMBVD are not pathognomonic. Fish infected with LMBV can also have bacterial infections, most often *Flavobacterium columnare* or motile *Aeromonas* spp. If an LMBV-infected fish with signs of LMBVD also has additional diseases, a decision about the relative importance of LMBV as a cause of morbidity depends in part on the presence and severity of lesions not attributed LMBVD.

The organs most commonly used for LMBV testing are swim bladder, trunk kidney, and spleen. Gill may also be a suitable organ for sampling, but additional field validation is needed. No single organ always has a detectable amount of LMBV in infected fish so it is important to sample multiple organs (Beck et al. 2006).

1. Presumptive Diagnosis

a. Behavior and Gross Lesions

Lethargy and swimming near the surface, followed by a loss of equilibrium and floating in a laterally recumbent position, occur in some fish dying from LMBVD. Internally, the swim bladder may be reddened or overinflated.

b. Virus Isolation

The cell lines most commonly used are bluegill fry (BF-2) cells and fathead minnow (FHM) cells, but several other fish cell lines will support replication (Piaskoski et al. 1999; McClenahan et al. 2005a). Incubation of the inoculated cells at 25°C to 30°C provides more rapid detection of CPE than at lower temperatures, but LMBV will replicate at least as low as 15°C. Cytopathic effect (CPE) is indicated by cell rounding followed by lysis [see Section 2, 4.6.D. "Largemouth Bass Virus (LMBV)"]; cytoplasmic inclusion bodies are not visible without staining. At 30°C, CPE can be noticeable in less than one day if the sample has a high titer of LMBV, but 7 days may be necessary to detect low titers.

2. Confirmatory Diagnosis

The virus is identified by PCR [see Section 2, 4.6.D.2 "Confirmation Method for LMVD - Polymerase Chain Reaction (PCR) (Modified from Plumb et al. 1999)"]. The PCR method can be simplified by adding the culture fluid from a presumptively positive cell culture to the PCR mixture, rather than using extracted DNA for the PCR template (McClenahan et al., 2005b). Before adding the cell culture fluid to the PCR mixture, the cell culture fluid should be centrifuged for 5 min at 10,000 X gravity and then diluted 1:500 in buffer.

F. Procedures for Detecting Subclinical Infections

The virus can be isolated from subclinically infected fish at any time of the year; however, low titers of virus are often present so the use of a highly sensitive assay is important to avoid false negative results. Optimization of cell culture isolation procedures (e.g., 30°C for cell culture incubation and blind passage of initially negative samples) can increase sensitivity (McClenahan et al. 2005a) or even greater sensitivity is possible by using PCR to detect viral DNA that has been extracted from homogenized organs (Grizzle et al. 2003). Testing organs, such as gill, head kidney, and liver, in addition to swim bladder, trunk kidney, and spleen, can also increase the probability of LMBV detection (Beck et al. 2006).

G. Procedures for Determining Prior Exposure to the Etiological Agent

An enzyme-linked immunosorbant assay (ELISA) has been developed by John Hawke (Louisiana State University) for the detection of serum antibodies against LMBV, but this test has not been widely field-tested. Agar gel diffusion assay can also be used to detect antibodies against LMBV (Fraser et al. 2002).

H. Procedures for Transportation and Storage of Samples

Organs or whole fish can be frozen for shipment. Little or no loss of infectious LMBV occurs during short-term storage of frozen samples (Plumb and Zilberg 1999a). However, samples should not frozen and thawed repeatedly during storage, and long-term storage should be at -80°C.

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2.A1 Methods for Testing for the Presence of Mycoplasma in Stock Cell Cultures Appendix 1

C. N. Lannan

Hatfield Marine Science Center
Oregon State University
Newport, OR 97365
503/867-0244

The specific method used to screen stock cell cultures for mycoplasma depends on the availability of certain laboratory equipment. References describing the comparative sensitivities of the various methods of mycoplasma detection are listed. Regardless of the method used, stock cell cultures should be routinely monitored for mycoplasma.

A. General Quality Control Procedures for Cell Culture

1. Susceptible, normal appearing, and rapidly dividing cells must be used for all virus assays.

2. Mycoplasma-free cell cultures should be used whenever possible.

3. Stock cell cultures should be tested, at no more than three months intervals, for the presence of mycoplasmal contamination. Contaminated cultures should be discarded.

4. Mycoplasma-negative cultures must be handled before mycoplasma-positive cultures are handled in the laboratory. Separate media and solutions should be used for mycoplasma-negative and mycoplasma-positive cultures.

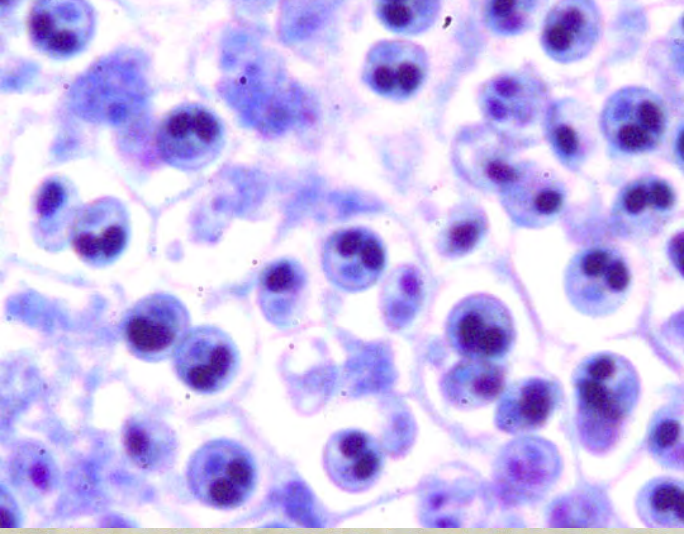
5. The antibiotics, penicillin (100 IU/mL) and streptomycin (100 µg/mL) or gentamicin (100 µg/mL), and the antifungal agents, Mycostatin R (25 µg/mL) or amphotericin B (2.5 µg/mL) are permitted for media in routine cell culture work.

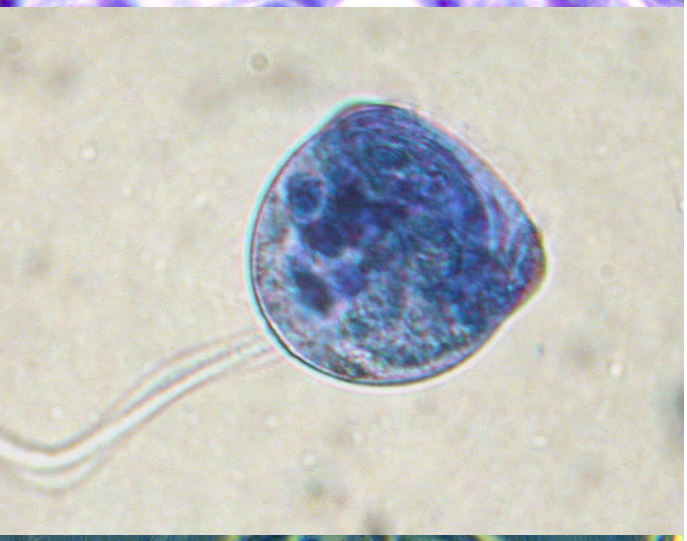
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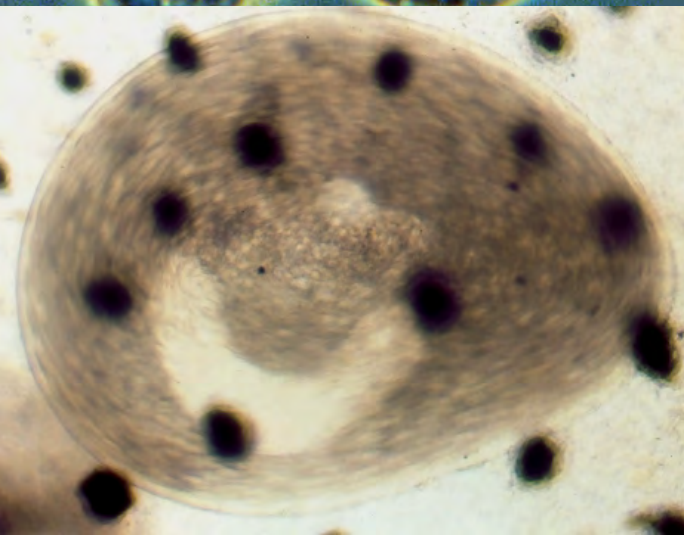
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Section 1
Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens

Chapter 3 Parasitic Diseases of Fish

3.1 General Procedures for Parasitology

3.1.A1 Key to Major Taxa of Adult Parasites of Fishes

3.2 Ichthyobodiasis

3.3 Diplomonad (Hexamitid) Flagellates: Diplomonadiasis, Hexamitosis, Spironucleosis

3.4 Ovipleistophoriasis (Pleistophora): Ovary Parasite of Golden Shiners

3.5 Salmonid Ceratomyxosis

3.5.A1 Salmonid Ceratomyxosis Methods Appendix 1

3.6 Whirling Disease of Salmonids

3.7 Proliferative Gill Disease

3.7.A1 Proliferative Gill Disease Methods Appendix 1

3.8 Proliferative Kidney Disease

3.9 Ichthyophthiriasis

3.10 External Infection by Ciliated Parasites

3.11 Monogenean Diseases

3.12 Bothriocephalosis (click here for video)

3.13 Lernaeid Parasitism

3.14 Gill Maggot Disease (Genus *Salmincola*)

3.15 Sea Lice

3.16 Branchiuran Fish Louse Disease

3.17 Centrocestiasis

3.1 General Procedures for Parasitology

Jack Frimeth

Ontario Ministry of Natural Resources
Fish Health Lab, Room 324
Department of Microbiology
College of Biological Science
University of Guelph
Guelph, Ontario N1G2W1
Canada
591/824-4120 ext. 3819

A. Introduction

The examination of fish for parasites may be required as part of a routine monitoring program for all disease agents including bacterial and virological agents, as part of a routine monitoring program for inspection of parasites only, or as a diagnostic search for a specific disease/mortality causing agent.

An external parasitological examination can be the first part of a routine monitoring program or diagnostic process. When an internal parasitological examination is required, it should follow the bacteriological examination, which requires aseptic conditions. Where multiple fish samples are required for bacteriology, virology, histology, and parasitology, the sampling methods and order of process will be dictated by the number of fish samples available. Where numbers are not restrictive, the sample size may be increased accordingly and bacteriology samples taken from fish selected for this process only. Tissues for virology, parasitology, and/or histology may be taken from remaining fish.

In this chapter, suggested methodologies are presented to facilitate the recovery, storage, and shipping of parasite specimens. Only those parasites that are currently recognized as being responsible for the majority of "disease" situations in fish culture are individually discussed. A simple key to the major taxa of adult parasites of fish is also provided in Section 1, 3.1.A1 A Key to Major Taxa of Adult Parasites of Fishes Appendix 1.

The specific parasites included in this section were determined by discussion with fish pathologists and aquaculturists throughout North America from the information provided from the Blue Book questionnaire (AFS Newsletter, Fish Health Section Volume 17, 1989). Additional parasites will be added as deemed necessary.

B. Sample Submission

The proper recovery and identification of fish parasites is dependent upon the state of freshness of the host. Thus, live fish samples are always preferred. If this is not possible, the usual order of preference for the submission of samples is: (i) freshly killed and packed on ice, (ii) preserved in an appropriate fixative, and (iii) deep frozen. The sediment in each container should be examined for external parasites that have fallen off or internal parasites that have been regurgitated or exited via the anus.

The survival of parasites following the host's death will be dependent upon the temperature at which the fish is maintained. The colder (i.e. on ice, not frozen) the host is kept, the better the survival of the parasites. Certain parasites may exhibit morphological changes following the death of the host. In addition, internal parasites may migrate from their sites of infection and external parasites may detach from their dead host. Whatever the process by which the fish host samples are submitted to a diagnostic lab, the host should be prepared as soon as is possible following its death.

A guide to methods for the packing and transport of fish samples is given by Mitchell and Hoffman (1985). The examination of whole, preserved fish is sometimes difficult due to the rigidity of the fixed muscle. Under certain conditions, where examination of fresh material is not practical or required, the viscera may be removed *in toto* by cutting at the pharyngeal/esophageal junction and at the anus. The swim bladder and kidney are also removed at this time. The viscera are then fixed and stored until examined. Once fixed, the viscera may also be shipped by wrapping them in a 5 to 10% formalin-soaked paper towel inside a sealed plastic bag. The two ends at the esophagus and anus can each be tied with a piece of thread to prevent any loss of their contents.

C. General Necropsy Procedures

The following is a general procedure for the examination of a fish for parasites. Where numbers of fish, time, or facilities are limiting, this procedure may be adapted to suit a particular situation. Specific methods for the recovery and treatment of specific parasites are given within the individual chapters.

1. Examination of Blood

If an examination for blood parasites is required, the sample must be collected from freshly killed fish before the blood clots and/or hemolysis occurs.

Blood may be collected via any of the following methods and host sites: (i) cardiac puncture with a syringe or sharpened microhematocrit tube as described by Goede (1989), (ii) entering the dorsal aorta via the oral cavity or along the lateral line, (iii) from the caudal vein by syringe or by severing the caudal peduncle, or (iv) by cutting a gill vessel.

Regardless of the route and method used, the collecting vessel should be heparinized to prevent clotting. The collecting syringe need not be heparinized if the blood is transferred immediately to the collecting vessel.

Motile living parasites (e.g. flagellates and larval nematodes) may be examined in a fresh drop of blood diluted with physiological saline (8.5 gm NaCl/L of distilled water). Flagellates are easily recovered from the white cell or "buffy" layer of the blood sample after microhematocrit

centrifugation. Fresh blood should be mixed with an equal or excess amount of saline to facilitate examination.

A blood smear may be prepared for later examination. A single drop of blood is required. This is easily achieved by touching a blood filled microhematocrit tube to one end of a glass slide. A second glass slide is then drawn to the edge of the drop, and pulled quickly but smoothly, creating a film, one cell layer thick (Figure 1). The smear is air dried, fixed for 10 minutes in methanol, and stained with a Romanovsky stain (Humason 1979).

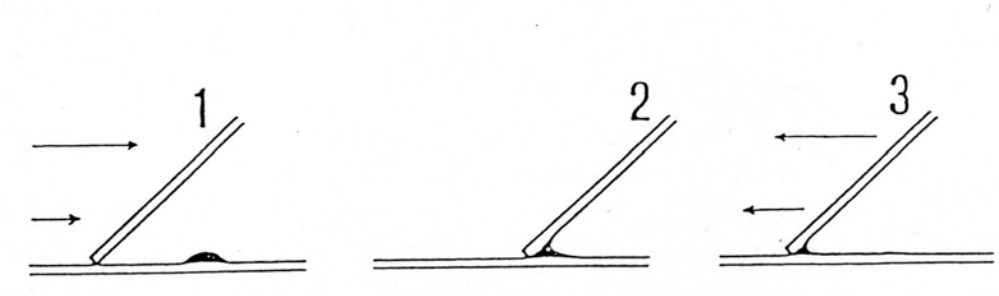


Figure 1. Preparation of a blood smear (from Moller and Anders, 1986).

2. External Examination

The entire surface of the fish should be thoroughly examined including the oral and opercular cavities, the nares, gills, and fins. If there is a time restraint or a large number of fish are to be inspected, most external parasites can be detected by examining a mucous scraping from a number of sites along the body, including the head, pectoral and caudal fins. The external surface of small fish (i.e. fingerlings) may be examined with the aid of a dissecting microscope, whereas the use of a hand-held lens can be useful in examining the surface of larger fish.

Protozoans and monogenetic trematodes may be removed from the external surfaces and gills by placing the fish or the individual tissues in a solution such as 1:4000 formalin. Small parasitic specimens are easily handled with a pasteur pipette. The nares may be examined by flushing with saline in a pasteur pipette and a wet mount or smear made of the wash fluid.

A mucous smear can be made by touching a glass or cover slip onto the side of the fish or mucus may also be collected with the non-cutting edge of a scalpel and wiped onto a slide. A fresh preparation may be mixed with saline and examined with a compound microscope, using a 10 to 40X objective. The use of phase contrast may be helpful for the recognition of protozoans. When preparing a fresh smear, care must be taken when placing the cover slip onto the material. Artifacts can result from excessive pressure. If necessary, tap water may be substituted for saline in any of the preparations given in these procedures. However, the use of saline may be critical to maintain a suitable osmotic environment for certain parasites, and is the medium of choice.

If there is enough saline on the slide and the cover slip is lowered properly, no air bubbles should be present. Excess saline can be removed by touching a paper towel to one edge of the cover slip. In the event there is insufficient saline, it may be added at the edge of the cover slip.

If protozoan cysts or encysted helminths are observed upon examination, pressure may be gently applied to the cover slip to free the organisms. Encysted worms should be freed before fixing.

a. Opercula and Gills
The opercles are cut off, placed into a petri dish, and examined under a dissecting microscope. The gill arches are removed separately and placed into a petri dish filled with saline and examined with a dissecting microscope. A small number of gill filaments can be more closely examined with a compound microscope. If the fish is small enough, an entire gill arch can be placed on the slide in slaine and covered with a cover slip. Otherwise, a section of gill arch is cut out, placed onto the slide, the filaments cut off with a scalpel, and the cartilaginous arch removed. If the fish is very large, some filament tips can be removed with scissors and placed directly onto a drop of saline on a slide. If desired, gill preparations can be minced on the slide with a scalpel to facilitate parasite detection. A drop or two of 1% methylene blue or 1% methyl green nuclear stain onto a wet mount will facilitate protozoan detection. These methodologies can also be applied to fin and mucous preparations.

b. Eyes
The eyes should first be examined while still in the orbits. Each eye can be removed using forceps and scissors and placed into a small petri dish with enough saline to cover the eye. The eye is cut open and the lens, humor, and retina examined. If the lens is removed, the remaining contents of the eye can be prepared as a wet mount and examined with a compound microscope.

3. Internal Examination

If a bacteriological examination is to be done, in addition to a parasitic examination, the external surface of the fish should be disinfected before exposing the body cavity.

The fish is laid on one side and is opened from the mouth to the anal region. This is accomplished by a ventral incision. A pair of blunt/sharp scissors should be used, with the blunt end going into the body cavity to minimize damage to the internal organs. The lateral musculature may be removed (Post 1987), but this is an individual preference.

a. Abdominal Cavity
The body cavity is examined first for encysted parasites. The heart is then removed and placed into a petri dish filled with saline. The viscera are removed *in toto*, by cutting the pharyngeal/esophageal junction and the large intestine at the anus. The viscera are placed into a saline filled petri dish. If a detailed examination is required, the liver, gall bladder, spleen, esophagus, stomach, pyloric caeca/pancreas, and intestine (which may be sub-divided as required) are removed and placed into individual petri dishes for examination. The swim bladder is then removed and similarly examined. Care should be taken not to deflate the swim bladder as the initial examination is facilitated when it is full. Removal and examination of the kidneys, ureters, and urinary bladder is now possible.

Smears of the ureter, gall bladder, and urinary bladder contents may be made by simply teasing these organs apart on a slide or utilizing a tuberculin needle and syringe to remove the contents of the latter two. Squashes may also be prepared of these organs as well as the kidney, liver, spleen, and gonads.

Cysts should be removed, placed onto a microscope slide, and the cyst wall teased apart to release the contents. If this is not possible, a cover slip may be placed on top of the cyst and sufficient pressure applied to squash the cyst. Encysted helminths should be freed before fixing.

The alimentary canal segments are stretched out in the petri dish and cut open longitudinally and the contents examined. Smears (wet and fixed) of the intestinal epithelium and contents should be made and examined for the presence of parasites. The alimentary tract of larger fish may be flushed through with saline and the gut contents examined separately from the tract wall.

A representative number of pyloric caeca can be examined by expressing their contents. This is easily done by cutting caeca off at their intestinal end and grabbing their blunt terminal end with a pair of forceps. A second pair of forceps is used to run down the individual caecum, from the blunt end to the open end and squeezing out the contents. It is often not practical to cut open large numbers of caeca, unless a quantitative study is required.

b. Brain

The brain of the fish is usually the last organ to be removed as this is the most difficult to access. It is not necessary that the brain remain totally intact for a parasitological examination. For fish less than 15 cm in length, the brain case may be cut longitudinally with the use of a pair of blunt/sharp scissors. The blunt end is placed in the mouth and the pointed end is lined up directly between the eyes. A single cut will usually expose the brain, which can be removed with forceps, squashed, and examined. In the case of larger fish, a pair of bone cutters may be required.

c. Musculature

The musculature is usually examined last by: (a) slicing the epaxial musculature at regular intervals, or (b) removing the two fillets, slicing, and/or candling them.

Candling is the use of transmitted light through a fish fillet to aid in the illumination and detection of parasite stages within the musculature (pp 338-339, Moller and Anders 1983). The efficiency of this method is dependent upon the thickness of the fillet. Squashes may also be necessary for the detection of cysts. Digestion with 0.5% pepsin powder in water and 0.5% HCl at 37°C may facilitate quantitative studies (pp 7-8, Hoffman 1967).

D. Fixation and Treatment of Parasites

There are a number of fixatives that are useful for the preservation and storage of fish parasites. These include formol-alcohol (i.e. AFA), Bouin's, and formalin (Humason 1979). Formalin is the most common fixative used, but there is confusion with regards to its use.

The formalin product purchased directly from the manufacturers is a saturated aqueous solution containing between 37 and 40% formaldehyde gas, by weight. This solution is a 100% stock solution of formalin. To make a 10% solution of formalin for preserving samples, it is necessary to dilute 1 part of the 100% stock solution with 9 parts of water. A 5% solution will require that 1 part of the 100% stock solution be diluted with 19 parts of water. These solutions may be buffered.

Certain literature may refer to the use of a 4% formaldehyde solution (Roberts 1989; Moller and Anders 1986). This is a 10% aqueous solution of the commercial stock formalin, which contains 40% by weight formaldehyde gas. In other words, it is calculated based upon the stock formaldehyde gas concentration (i.e. 10% x 40% of stock = 4%).

1. **Protozoan Smears/Small Helminths**

   If a temporary preparation is required for protozoan smears or small helminths, this may be done in one of two ways. A preparation may be stored overnight as follows: a dampened paper towel is placed in the bottom of a petri dish, the specimen slide placed on top of the paper towel, the lid placed on the dish, and then put into a 4°C refrigerator. A sample preparation may be retained for several days by adding a ring of nail polish or petroleum jelly around the perimeter of the cover slip to prevent dehydration and keeping this under refrigeration.

   If any temporary preparation is to be fixed, the fixative can be added to one side of the cover slip. A dry paper towel is touched to the opposite side of the cover slip. This will cause the saline under the cover slip to be drawn into the paper and create a capillary action under the cover slip, which will draw the fixative through the sample.

2. **Helminths, Acanthocephalans, and Leeches**

   When necessary, living trematodes, cestodes, and leeches should be fixed under light pressure of a cover slip or glass slide. The saline is replaced slowly with warm fixative.

   All helminth parasites should be cleaned with saline before fixation. This is accomplished by placing the parasite into an appropriate sized vial filled with saline, capping it and shaking it vigorously. Small parasites are best handled by sucking them up with a pasteur pipette to minimize damage, whereas larger parasites can be handled with forceps. The fluid is decanted and the procedure repeated as necessary. (Note: decant into a jar, not over a drain.)

   Monogeneans, trematodes, cestodes, acanthocephalans, and leeches can be fixed in 5-10% neutral buffered formalin for 24 hours and transferred to 70% ethanol for storage and/or shipping. Many helminth parasites may be relaxed in fresh water before fixation. Some parasitologists suggest relaxing cestodes in 80°C tap water and then slowly replacing the water with fixative. Water or fixative that is too hot may result in blisters to the tegument or cuticle of certain parasites, therefore, the use of warm (ca. 50°C) formalin to relax and fix live parasites is recommended.

   Live acanthocephala should be left in distilled water overnight in the refrigerator to induce eversion of the proboscis before fixation in warm formalin. Large nematodes and acanthocephalans may have to have holes poked in them with fine dissecting needles. Entomology pins mounted onto the end of small wooden dowels are excellent for this purpose as well as for manipulating small, delicate parasites. Live nematodes and crustaceans are fixed for 24 hours in warm solution of 5 to 10% glycerin in 70% ethanol and transferred to fresh solution for storage. These may also be fixed in 10% formalin then transferred to glycerin/ethanol for storage.

   Make sure that all containers, regardless of their contents, are properly labeled. Labels should be written in pencil and the label placed inside the container. A second label can be put on the outside, but the inside is most important.

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3.1.A1 Key to Major Taxa of Adult Parasites Of Fishes*

1. Individual organism microscopic (except *Ichthyophthirius*) and single-celled, but may be multinuclear……Protozoa

   Individual organism usually visible without a microscope and multicellular……2

2. Body worm-like……..3

   Body not worm-like……...….8

3. Body dorso-ventrally flattened, not round in cross-section……4

   Body not dorso-ventrally flattened, round in cross-section……7

4. Body with segmentation or distinct external annulations or rings……..5

   Body without segmentation or distinct external annulations or rings…….6

5. Anterior attachment organ present, which may include hooks and muscular suckers, posterior attachment organ not present; gut absent……Cestoda

   Anterior and posterior attachment organs present with well defined posterior sucker; gut present……...Hirudinea

6. Posterior attachment organ present which usually includes hooks (hamuli) and marginal hooks; external parasites……...Monogenea

   Posterior attachment organ not present, possesses circumoral and ventral suckers except for blood dwelling species; internal parasites……..……...Digenea

7. Anterior spined proboscis present……..Acanthocephala

   Anterior spined proboscis not present……..……..Nematoda

8. Body in form of two hinged shell-valves; organism encysted on surface of gills or fins…….……Glochidia

   Body not in form of two hinged shell-valves, possesses appendages for attachment to host surface; organism not encysted……..Crustacea

*Does not apply to larval Cestoda, Digenea, or Nematoda

3.2 Ichthyobodiasis

Wilmer Rogers

Alabama Agricultural Experiment Station
Auburn University, AL 36849-5419
205/844-9223

A. Name of Disease and Etiological Agent

Ichthyobodiasis is caused by *Ichthyobodo* (*Costia*) spp. This is a protozoan flagellate belonging to the Class *Zoomastigophorea*, Order *Kinetoplastida*, Family *Bodonidae*. There are two species of *Ichthyobodo* commonly reported for fish: *I. pyriformis* and *I. necator*. A synonym for *Ichthyobodo*, is *Costia*. *Ichthyobodo* is sometimes spelled *Ictyobodo*, *Icthyobodo*, or *Ichtyobodo*.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

This parasite occurs throughout the world.

2. Host Species

This parasite has been reported from many fish species. It is reported that all species of freshwater fishes are susceptible to attack. It has been a problem in virtually all cultured fish ranging from tropical fish to salmonids.

C. Epizootiology

Ichthyobodo is an obligate parasite that must have a fish host or it will die in a short time. It is attached to the host but adverse conditions may cause the parasite to encyst, either on the fish or free in the water. Electron microscopy has shown attachment to the host to be by a small disc with microtubules that extend into host cells. Young fish are reported to be more susceptible to *Ichthyobodo* than older fishes.

Temperature plays an important part in the infectivity of the parasite. Temperatures of 25^{o} to 30^{o}C are prohibitive for the infection.

Ichthyobodo is transmitted from fish to fish in the water, and the infective stage may come from host fish or cysts. The disease can be dispersed geographically by water or infected fish.

Ichthyobodo is a small parasite 5 to 20 µm in length (Figure 1). Reproduction takes place by simple transverse division, but the parasite is able to encyst after leaving the fish.

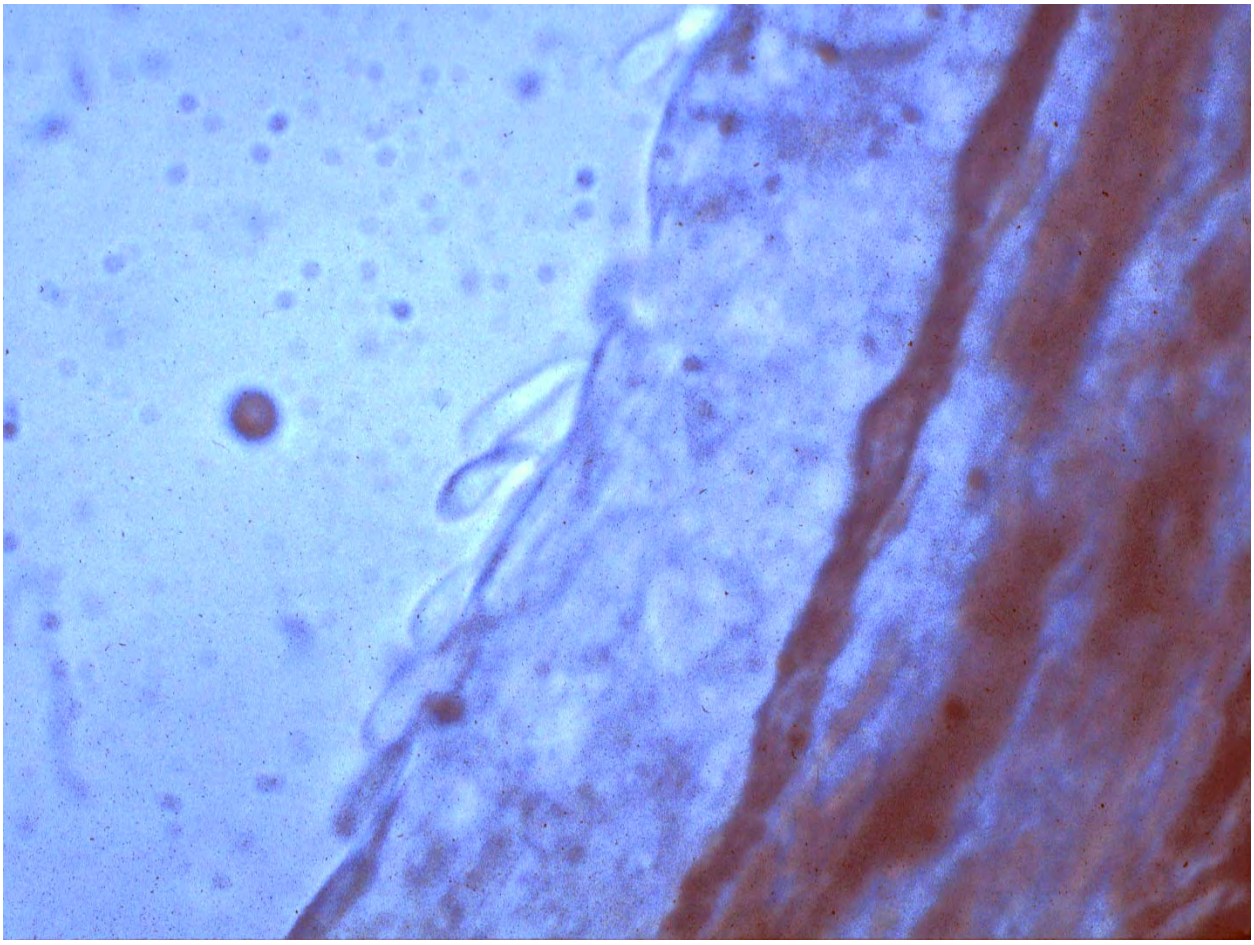


Figure 1. *Ichthyobodo* on the epithelium of a catfish gill (picture by Fred Meyer).

D. Disease Signs

1. **Behavioral Changes Associated with the Disease**
   Infected fish will commonly flash or scrape against objects. Infected fish may also stop eating and may gasp at the water surface.

2. **External Gross Signs**
   A characteristic sign of ichthyobodiasis is excess mucus production, which has been referred to as blue slime. Sometimes complete removal of epithelium may be seen and the pigment may be missing from the skin (Figure 2).

3. **Internal Gross Signs**
   Infected fish that have been off feed for a period of time may show signs of starvation.

4. **Histopathological Changes Associated with the Disease**
   Since *Ichthyobodo* spp. are found on the skin and gills of fish, these organs are affected. Histological sections will usually show a proliferation of mucous cells but inflammation at the sites of infection is not always seen (Figure 3).

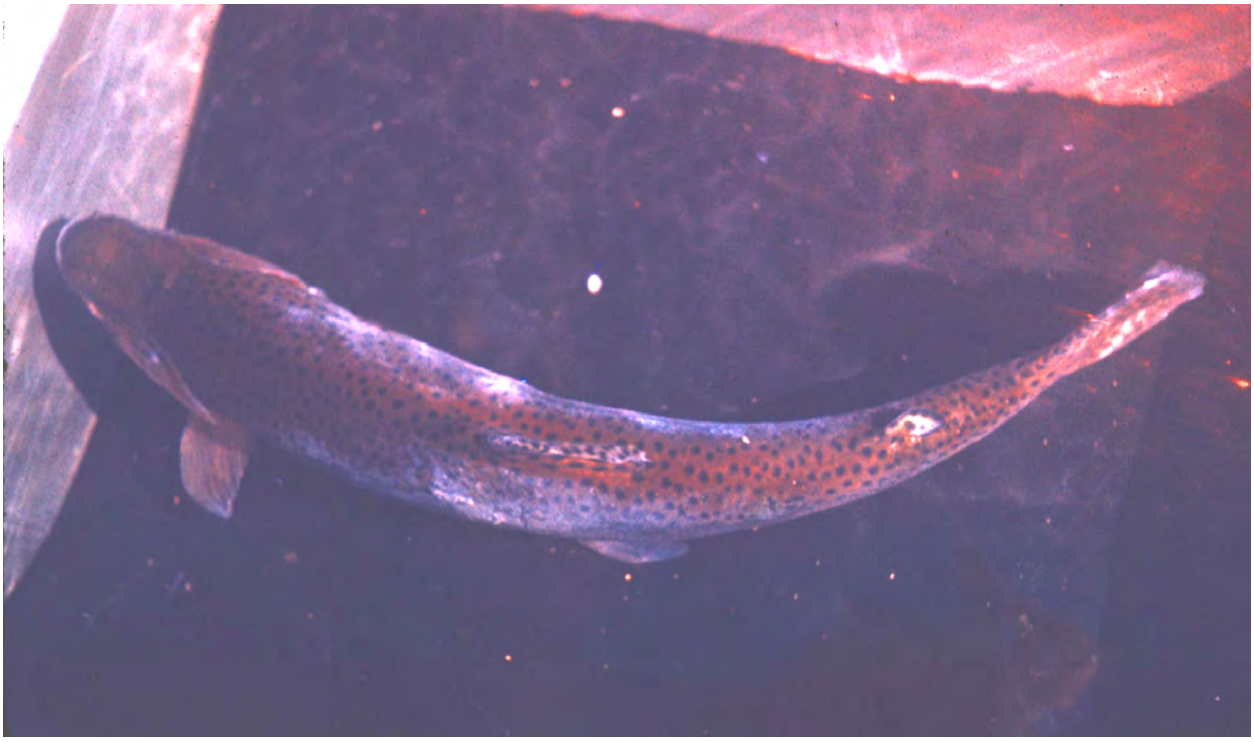


Figure 2. Blue slime signs in a trout (picture by Fred Meyer).

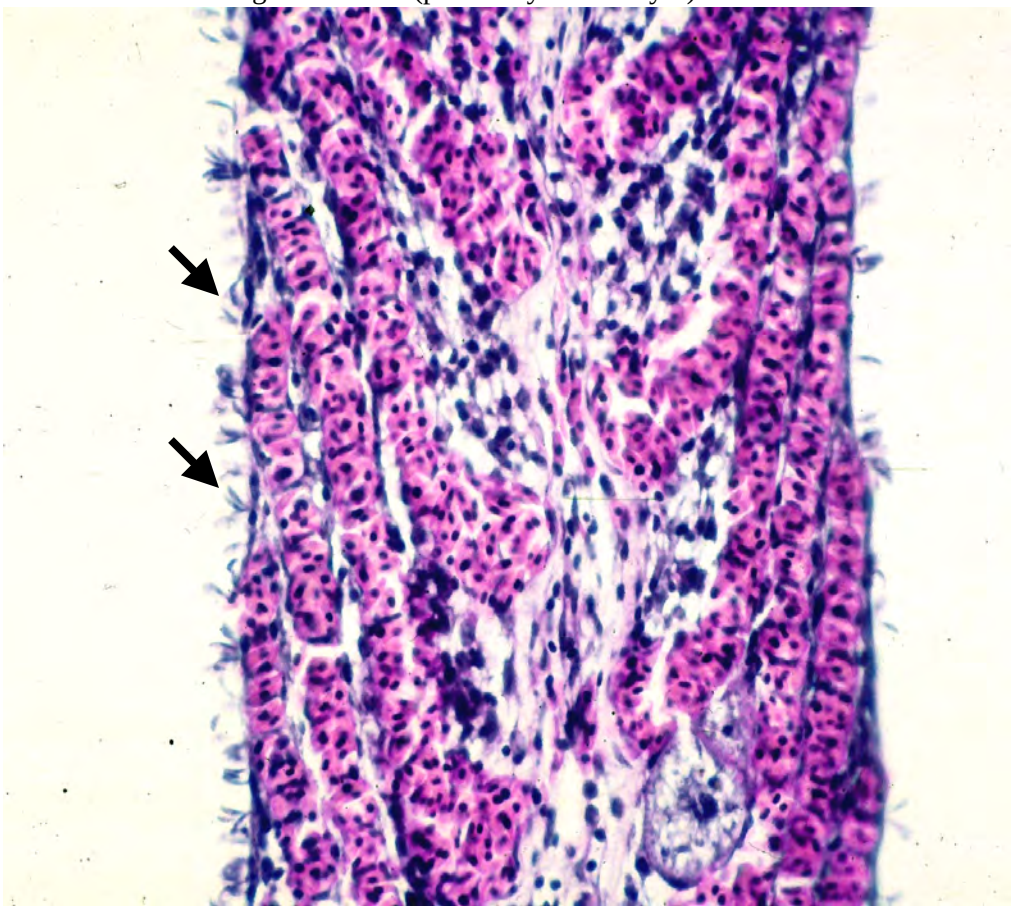


Figure 3. Numerous *Ichthyobodo* on the surface of a catfish gill. Histology with H&E stain (picture by Andy Goodwin).

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

a. Isolation/Detection of Pathogen
Diagnosis of *Ichthyobodo* is dependent upon disease signs and observation of the parasite in wet mount preparations. The disease signs will often, but not always, show a lesion with increased mucus production and discoloration, which is generally loss of pigment. Skin scrapings from the margin of the lesion will show the small oval, free swimming, almost kidney-shaped forms and the pear-shaped attached forms. Typically, one long and one short flagellum is present, however, when the parasite is ready to divide, two long and two short flagella may be seen. The attached forms may be seen with the free flagellum waving, which gives the parasite a flickering motion. This has been compared to the light of a candle flickering. Because of the characteristic movement, *Ichthyobodo* is easily diagnosed from live specimens.

b. Clinical Signs
Clinical signs associated with the disease include excess mucus production (blue slime) and discoloration, which is usually loss of pigment. Gills may become swollen. Behavioral changes may include loss of appetite, lethargy, and flashing or rubbing against objects apparently in an attempt to rid themselves of the irritation caused by the parasite. Complete removal of the epithelium may be seen.

c. Histopathological Examination
Histopathological examination will show the small, pear-shaped parasites attached to the skin and gills.

2. Confirmatory Diagnosis

Histological sections will confirm the presence of the parasite, but are not necessary for diagnosis. Observation of the parasite in wet mounts will confirm the disease.

F. Procedures for Detecting Subclinical Infections

Low levels of infection may be detected from apparently healthy fish using skin and gill scrapings (wet mount technique as described above).

G. Procedures for Determining Prior Exposure to the Etiological Agent

None available.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Ichthyobodo is an obligate parasite and is difficult or near impossible to culture under experimental conditions. Thus, survival of the parasite depends on how fish hosts are treated. If live fish hosts cannot be transported then freshly killed hosts should be stored and shipped on ice.

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3.3 Diplomonad (Hexamitid) Flagellates: Diplomonadiasis, Hexamitosis, Spironucleosis

Sarah L. Poynton

Department of Comparative Medicine
Johns Hopkins University School of Medicine
1-127 Jefferson Building, Johns Hopkins Hospital
600 North Wolfe Street
Baltimore, MD 21287
410/502-5065
fax: 443/287-2954
spoynton@jhmi.edu
spoynton@igb-berlin.de

A. Name of Disease and Etiological Agent

Diplomonadiasis or hexamitosis is infection by diplomonad flagellates (Order Diplomonadida, suborder Diplomonadina, Family Hexamitidae). If the exact genus is known, the infections may be reported as hexamitiasis (*Hexamita*), octomitosis (*Octomitus*), or spironucleosis (*Spironucleus*); of these, probably only the latter is applicable to fish (see below). Infections may be reported as localized (commonly in the intestine, and possibly also including "hole-in-the head disease" of cichlids (Paull and Matthews 2001), or disseminated or systemic (Ferguson and Moccia 1980; Kent et al. 1992; Poppe et al. 1992; Sterud et al. 1998).

Light microscopy studies have reported three genera from fish - namely *Hexamita*, *Octomitus*, and *Spironucleus*. However, transmission electron microscopy (TEM) is needed to confirm genus (Poynton and Sterud 2002), and light microscopy studies are therefore taxonomically unreliable. If TEM is not available, the organisms should be recorded as diplomonad or hexamitid flagellates. All recent comprehensive ultrastructural studies show only the genus *Spironucleus* infecting fish, and it is probable that this is the genus to which all diplomonads from fish belong (Poynton and Sterud 2002).

Some 15 to 20 species of diplomonads have been reported from fish (Poynton and Sterud 2002). However, most descriptions do not include comprehensive surface and internal ultrastructure and thus are incomplete. To date, only three species are well characterized: *S. barkhanus* (Sterud et al. 1997), *S. torosa* (Poynton and Morrison 1990), and *S. vortens* (Poynton et al. 1995) (possibly synonymous with *S. elegans*); the commonly reported "*H. salmonis*" is currently being redescribed, and will probably also be assigned to the genus *Spironucleus*.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Diplomonad flagellates commonly infect freshwater and marine fish in many parts of the world, and occur in wild, farm, and aquarium environments (Kulda and Lom 1964a; Kulda and Nohýnková 1978; Woo and Poynton 1995). Some species may have a wide geographic range, for example *S. barkhanus* and *S. torosa* occur in freshwater and marine waters, and *S. vortens* occurs in the USA, England, and Norway.

2. Host Species

Infections occur in many families of fish including Acipenseridae, Anguillidae, Catostomidae, Centrarchidae, Cichlidae, Cyprinidae, Cyprinodontidae, Gadidae, Gasterosteidae, Mugilidae, Percichthyidae, Percidae, Salmonidae, Siganidae, and Sparidae. Two species appear to be host specific: *S. barkhanus* from salmonids, and *S. torosa* from gadids (Poynton and Sterud 2002). In contrast, *S. vortens* has been reported from cichlid and cyprinid fish. Pathogenic infections are most commonly reported from farmed salmonids (*S. barkhanus* and "*H. salmonis*") and cichlids (*S. elegans, S. vortens*).

C. Epizootiology

The reporting of disease from captive but not wild fish, and the absence of disease in wild fish with heavy infections suggests that: a) the stressors of captivity (particularly poor husbandry) may predispose fish to pathogenic infections, and b) certain species and stains of diplomonads are pathogenic, while others are not. Furthermore, in some instances pathogenic infections occur when infection passes to a novel host. For example, in Norway, *S. barkhanus* is a serious pathogen in sea-farmed Atlantic salmon, *Salmo salar*, yet is a commensal in feral Arctic char, *Salvelinus alpinus*.

The life cycle is believed to be direct, with oral or rectal acquisition of infection. Although trophozoites (motile feeding stage) and cysts are known, their relative roles in the life cycle have not yet been clearly demonstrated.

D. Disease Signs

Disease signs are not the same for each well-characterized species of diplomonad, thus each is treated separately below. No disease is associated with infection by *S. torosa*.

1. Behavioral Changes Associated with the Disease

a. *S. barkhanus*
In Atlantic salmon: sluggish behavior (inconsistent finding).
In chinook salmon: none reported.

b. *S. vortens*
Variable: in light infections, none; in very heavy infections, decreased appetite, anorexia, lethargy, occasionally floating on side yet continuing to eat.

c. *"H. salmonis"*
Fish may be anorexic, weak, and excessively nervous.

2. External Gross Signs

a. *S. barkhanus*
In Atlantic salmon: diseased fish significantly smaller than other fish in same age group, morbidity, and mortality.
In chinook salmon: anemia, swollen abdomen, and mortality.

b. *S. vortens*
Variable: none to weight loss, malaise, poor growth rate and mortality in juveniles; "hole-in-the-head" lesions.

c. *"H. salmonis"*
Emaciation, dull and dark color, red vent, pale shiny feces, abdominal distension, and mortality.

3. Internal Gross Signs

a. *S. barkhanus*
In Atlantic salmon: whitish granulomatous nodules in kidney and liver; red-brown putrid boils in muscle; hemorrhage in cerebral meninges, contents of posterior gut more fluid than normal with a greenish color, fibrinous perihepatitis, severe muscular degeneration, multifocal encephalitis, and suppurative meningitis.
In chinook salmon: serosanguinous ascites, enlarged mottled and congested liver, and enlarged kidney and spleen.

b. *S. vortens*
Variable: none to fluid-filled intestine.

c. *"H. salmonis"*
Gastrointestinal infections may be accompanied by enteritis, and yellow and watery or jelly-like gut contents.

4. Histopathological Changes Associated with the Disease

a. *S. barkhanus*
In Atlantic salmon: multifocal coagulation and caseous necrosis in liver and kidney.
In chinook salmon: liver edema, congestion and inflammation; renal interstitium hyperplastic.

b. *S. vortens*
Sometimes intestinal dilation, in "hole-in-the-head disease" flagellates present in kidney, liver, spleen, head lesions, and intestine.

c. *"H. salmonis"*
Gastointestinal infections exhibit excess mucus, cytoplasmic blebbing, and apoptotic bodies in epithelial cells.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

a. Isolation/Detection of Pathogen

Live diplomonad flagellates can be readily recognized in fresh wet mounts, usually made from material from the gastrointestinal tract, and covered by a cover slip (Figure 1 and Figure 2). The trophozoites are highly motile, pyriform to elongate, 5 to 20 µm long (excluding flagella), with six anterior flagella and two posterior recurrent flagella. Detection may be improved if the preparation is diluted with tap or distilled water, or 0.9% NaCl, and the motility of the organisms can be reduced by the addition of a viscous medium such as Protoslo (Carolina Biological Supply Company). Preparations should be examined under bright field, phase contrast, or Normarski illumination at 400x magnification or higher.

Stains may be used to show general features of a diplomonad flagellate (most notably the paired nuclei) and species-specific features (see "Confirmatory Diagnosis" below and Figures 3 and 4).

If a systemic infection is suspected, blood smears stained with Giemsa may be examined (Figure 6). The characteristic paired nuclei and two recurrent flagella passing through the cell can be seen.

Preparations may also be stained by DAPI (4'-6-diamidino-2-phenylindole) and exposed to bright field and UV illumination; the characteristic paired nuclei of the diplomonad appear blue/violet (Figure 7) (Sterud 1998).

b. Clinical Signs

See "Disease Signs" above.

c. Histopathological Examination

Hematoxylin and eosin and the Feulgen reaction (nuclei magenta pink, cytoplasm turquoise) may be used to show the characteristic paired nuclei (Figure 8 and Figure 9). The diplomonads may be seen in the gastrointestinal lumen, aligned along the brush border of the intestine, and present in viscera and somatic musculature.

2. Confirmatory Diagnosis

For determination of genus, transmission electron microscopy is required (Brugerolle 1974, 1975; Brugerolle et al., 1973, 1974; Poynton and Sterud 2002). Of particular importance are: the shape of the nuclei, location of kinetosomes (flagellar bases), and presence or absence of a flagellar pocket (Figure 10), (Table 1). Keys for identification to genus are given in Poynton and Sterud (2002) (for diplomonads from fish), and in Brugerolle and Lee (2000) (all diplomonads).

For determination of species, scanning and transmission electron microscopy are required (Poynton and Sterud 2002). Important taxonomic features are: surface architecture (especially at the posterior end of the body), pattern of the bands of microtubules that accompany the recurrent flagella (when viewed in transverse section though the middle of the cell), and cytoplasmic organelles (Table 2). Special light microscopy stains such as protargol silver protein may be used to demonstrate the cytoskeleton, particularly at the posterior end of the body (Figure 3, Figure 4, and Figure 5).

Suitable electron microscopy fixatives are glutaraldehyde or a combination of glutaraldehyde/paraformaldehyde (Ferguson 1979; Poynton and Morrison 1990; Poynton et al. 1995; Sterud et al. 1997). Samples may be processed as tissue pieces or as a cell suspension (in the case of lumen infections).

The molecular taxonomy and protein code sequencing of diplomonads from fish is still in its infancy because few species are well recognized by classical methods, and few species have been isolated and maintained in *in vitro* culture.

Table 1. Ultrastructural features used to distinguish three genera of diplomonads reported from fish (from Poynton and Sterud 2002).

Character	*Spironucleus*	*Hexamita*	*Octomitus*
Flagellar pockets (cytostomal canals)	+	+	-
Central axis formed by recurrent axonemes, microtubular bands, endoplasmic reticulum	-	-	+
Two terminal spikes	-	-	+
Shape of nuclei	S- shaped	spherical	reniform
Location of kinetosomes relative to nuclei	sub-apical	external surface	between
Position of recurrent flagella relative to nuclei	medial	lateral	medial
Supra-nuclear microtubular band	+	+	reduced
Infra-nuclear microtubular band	+	+	reduced

F. Procedures for Detecting Subclinical Infections

Since the relationship between prevalence and density of infection and disease are not well established, no recommendations are given.

G. Procedures for Determining Prior Exposure to the Etiological Agent

No methods are currently available to detect previous infections with diplomonad flagellates affecting fish.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Samples should be collected within minutes from a freshly killed fish. Flagellates may survive for 15 to 30 minutes in freshly killed fish kept in the refrigerator.

For long term studies, diplomonad flagellates can be successfully maintained in *in vitro* culture. Protocols are available for "*H. salmonis*" (Buchmann and Uldal 1996), *S. barkhanus* (Sterud 1998), and *S. vortens* (Sangmaneedet and Smith 2000).

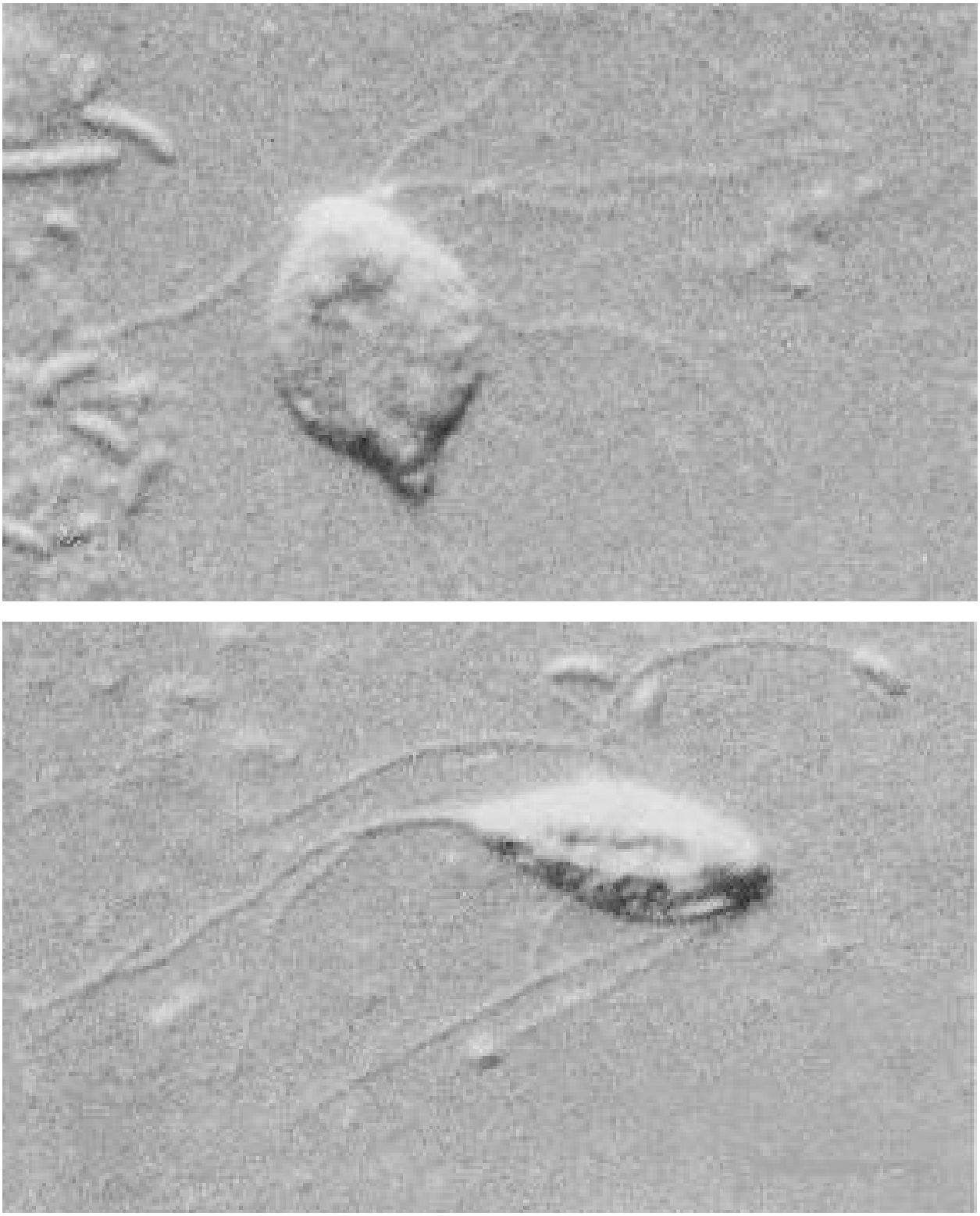
Table 2. Ultrastructure of currently recognized diplomonad species from fish (from Poynton and Sterud 2002).

	External	Internal	
Species	surface and ornamentation	pattern of microtubular bands accompanying flagellar pocket (a)	other cytoskeletal elements
S. barkhanus	unadorned body, rf emerge from barhans (b)	3 radiate	-
S. torosa	unadorned body, rf emerge from tori (c) caudal projection, trophozoites can attach to host microvilli	3 concentric	mt + lamellae support tori and flanges
S. vortens	adorned body with compound lateral ridges (d) counter-crossing at posterior end with papillae, rf emerge from counter-crossing ridges	3 staggered	mt support right peripheral ridge, striated fibrous structures supports central ridge
*S. elegans**	adorned body with compound lateral ridges (e), rf emergence not known	staggered?	mt support peripheral ridge, striated fibrous structure supports central ridge

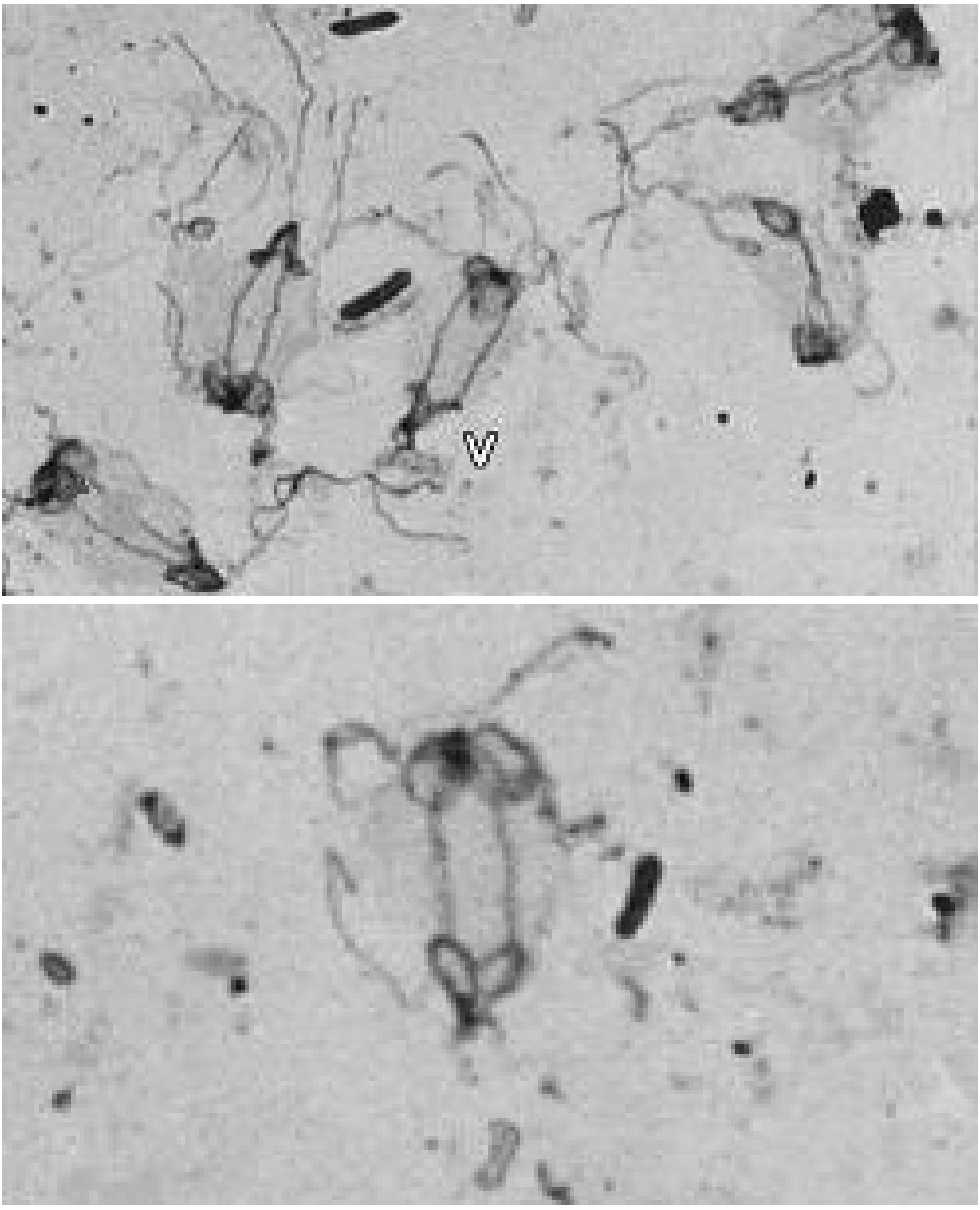
(a) viewed in transverse section through the middle of the body
(b) barkhans are crescent shaped ridges
(c) tori are raised ring-shaped areas, surrounded by flanges
(d) compound lateral ridges comprise a smooth central part, and a rope-like peripheral ridge narrower on the left than on the right, peripheral ridges bear tufts of microfibrillar material
(e) compoud lateral ridges comprise a smooth central part, and peripheral ridges bearing tufts of microfibrillar material

mt = microtubules
rf = recurrent flagellum

* Although *S. elegans* has been described from fish only by light microscopy, the internal ultrastructure has been described for diplomonads from amphibians. In the absence of SEM, the morphology of the taxonomically important posterior end is not known, so its affinity to other species in this table cannot be fully assessed. Current evidence suggests that *S. elegans* and *S. vortens* may be conspecific. Cross infection studies (Kulda and Lom 1964b) have confirmed that freshwater angelfish, *Pterophyllum scalare*, can be successfully infected with diplomonads from amphibians, *Triturus alpestris*.



Figures 1 and 2. Diplomonad flagellates prepared by different techniques and viewed by light microscopy. Fresh preparation of spherical and elongate trophozoites of *Spironucleus torosa* viewed by Normarski illumination. Originally published in the Journal of Protozoology 1990 (**37,** 369-383) and reproduced with permission of the Society of Protozoologists.



Figures 3 and 4. Smear preparations of *S. torosa* stained by Protargol silver protein, note V-shaped structure (v) at the posterior end of the body [which TEM shows to be two rings of microtubules supporting the flanges around the tori (compare with Figure 4)], note also the passage of recurrent flagella near centre of the body. Originally published in the Journal of Protozoology 1990 (**37,** 369-383) and reproduced with permission of the Society of Protozoologists.

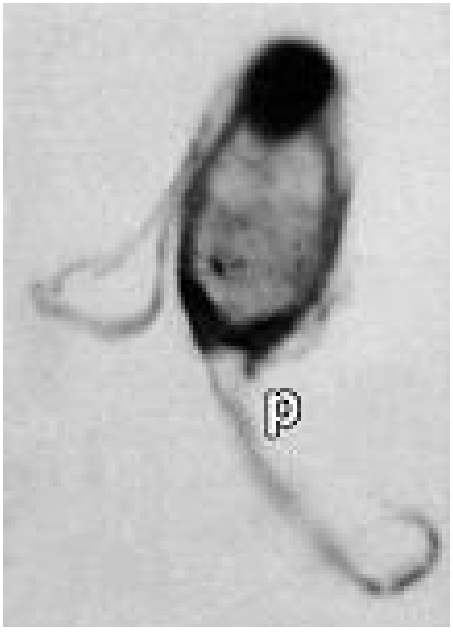


Figure 5. Preparation of *S. vortens* stained by Protargol silver protein (filter method); note dark area across posterior end of body (which TEM shows to be an extensive symmetrical system of microtubules supporting the elaborate architecture of the counter-crossing ridges at the posterior end of the body), the papillae (p), and passage of recurrent flagella just beneath the surface of the cell. Originally published in the Journal of Eukaryotic Microbiology 1995 (**42,** 731-742) and reproduced with permission of the Society of Protozoologists.

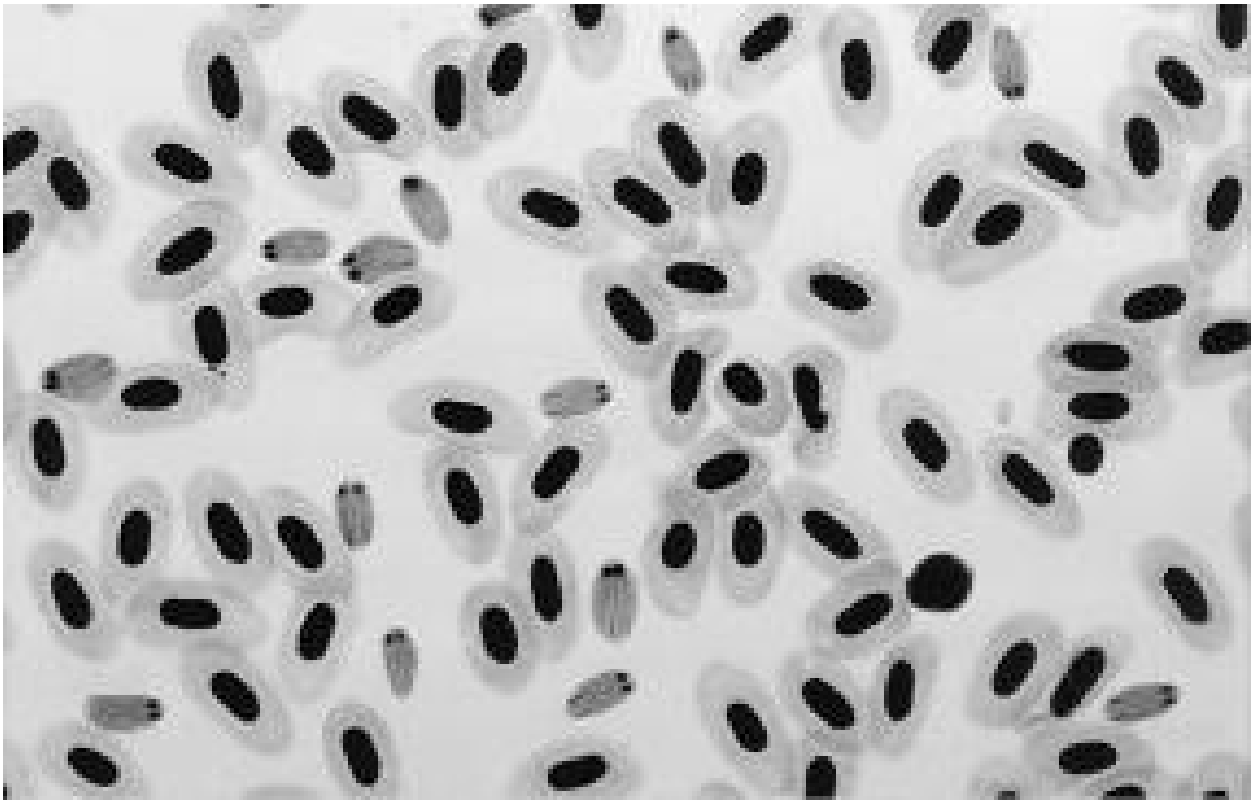


Figure 6. Unidentified diplomonads from blood smear stained with Leishman's Giemsa; note paired nuclei and passage of recurrent flagella through the cell. Originally published in Diseases of Aquatic Organisms 1992 (**14,** 81–89) and reproduced with permission of Inter-Research.

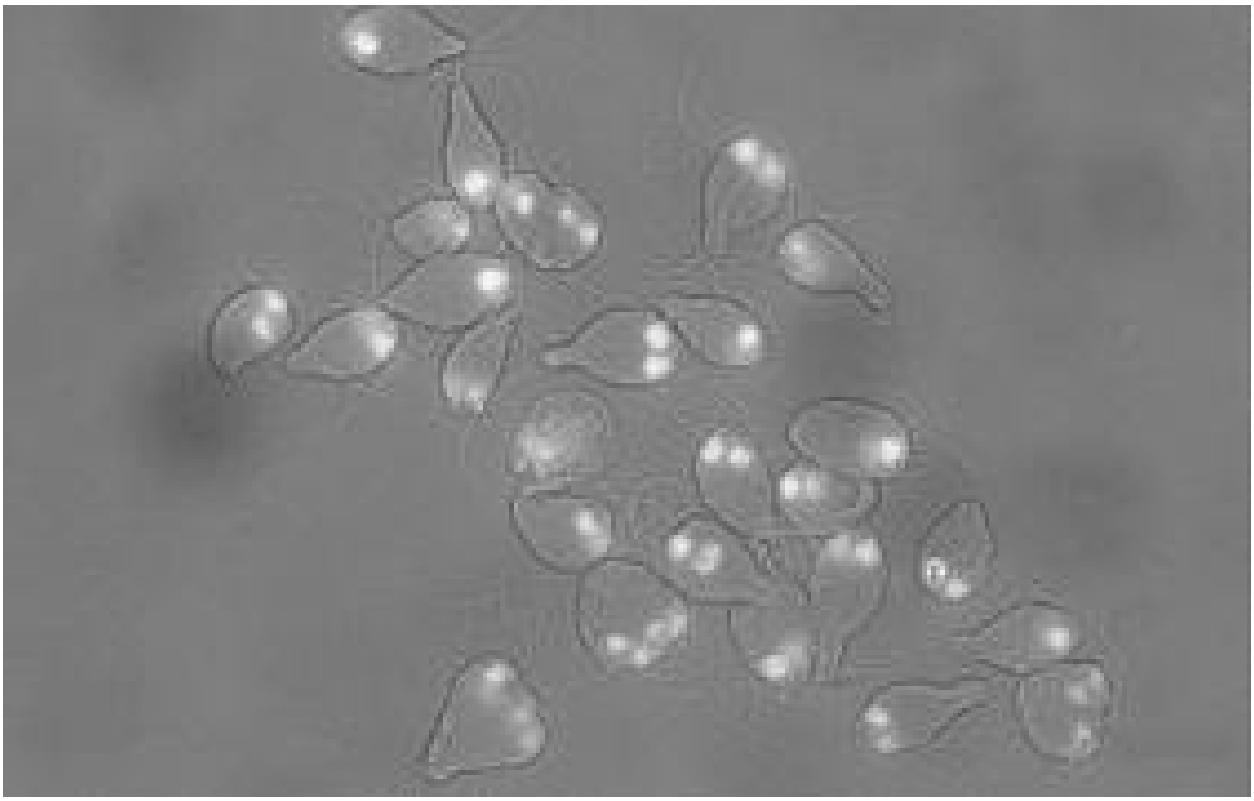


Figure 7. Culture of *S. barkhanus* stained with DAPI (4'-6-diamidino-2-phenylindole) and exposed to bright field and UV illumination, nuclei appear blue/violet (white in this photograph).

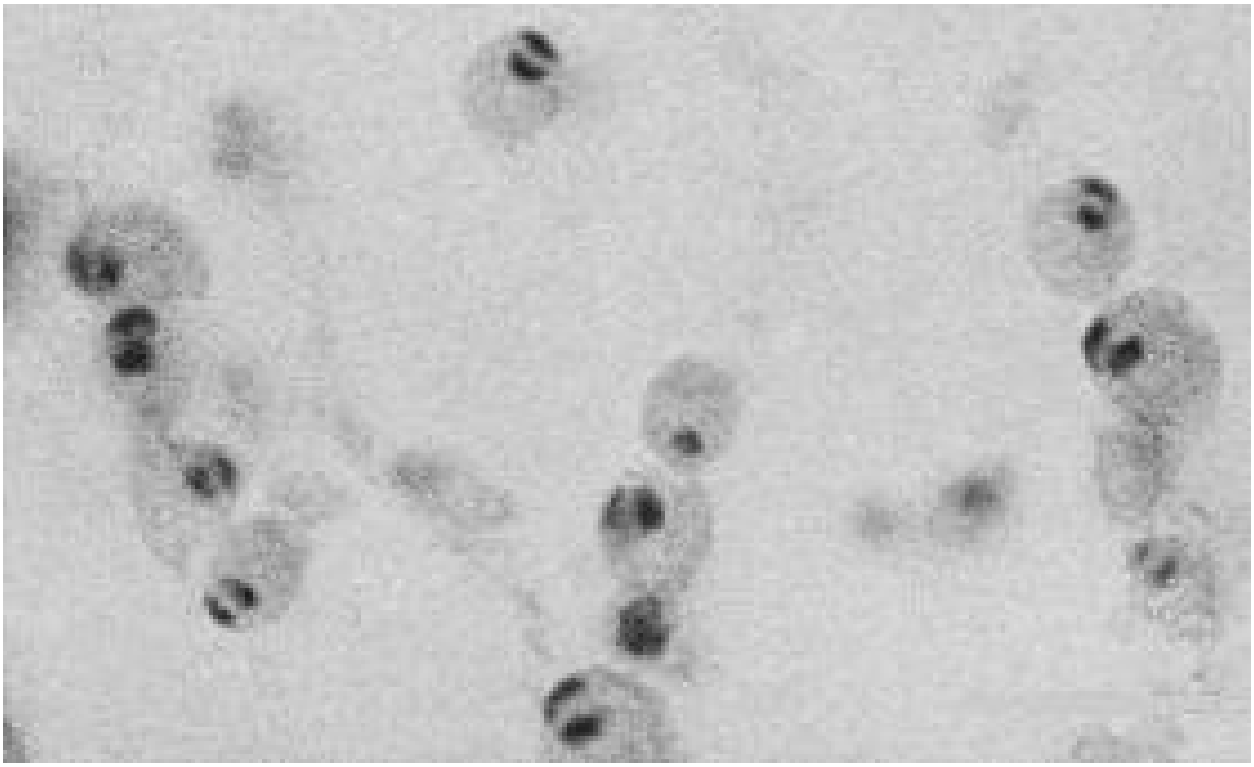


Figure 8. *S. torosa* in tissue section (lumen of rectum) stained by the Feulgen reaction. Originally published in the Journal of Protozoology 1990 (**37,** 369-383) and reproduced with permission of the Society of Protozoologists.

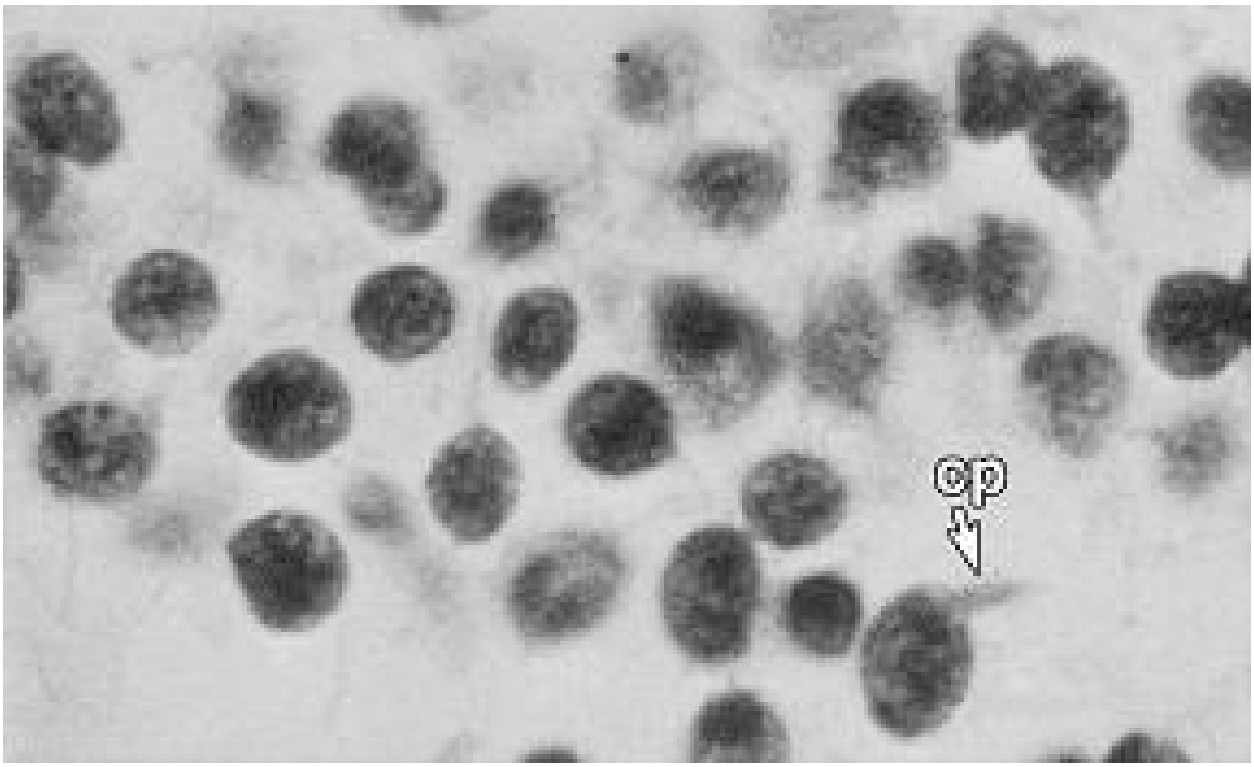


Figure 9. *S. torosa* in tissue section (lumen of rectum) stained with haematoxylin and eosin; (cp) caudal projection. Originally published in the Journal of Protozoology 1990 (**37,** 369-383) and reproduced with permission of the Society of Protozoologists.

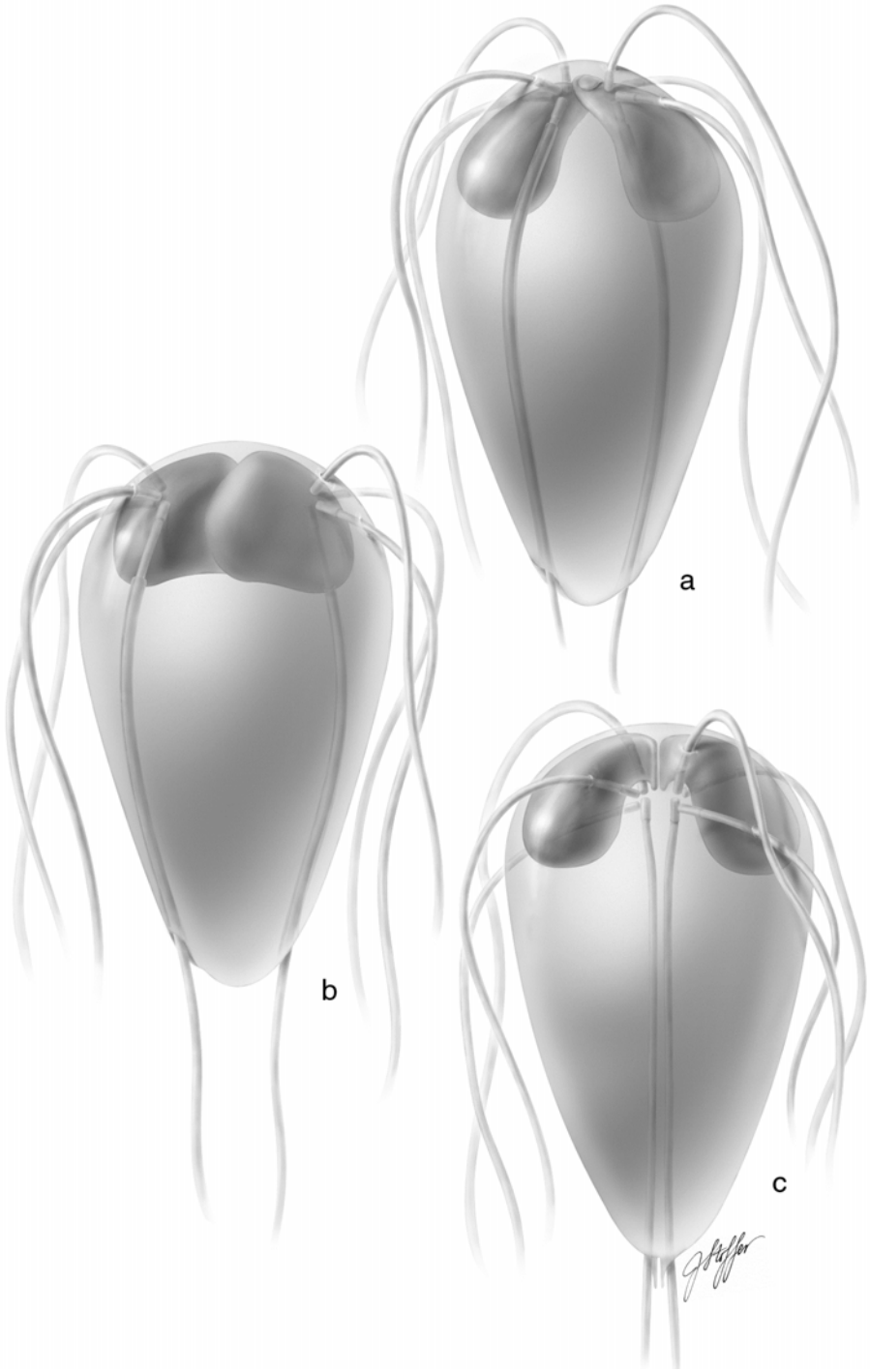


Figure 10. Principal distinguishing features of the three genera of diplomonads within the suborder Diplomonadina that have been reported from fish, (a) *Spironucleus*; (b) *Hexamita*; (c) *Octomitus*. Organisms shown in ventral view. Note especially the presence or absence of flagellar pockets (cytostomal canals) (evident as sheaths around the recurrent flagella of *Spironucleus* and *Hexamita,* and absent from *Octomitus*), shape of the nuclei, and locations of kinetosomes and tract of the recurrent flagella passing posteriorly. Surface ornamentation, microtubular bands, and endoplasmic reticulum are excluded for simplicity, and flagella are shortened in this illustration. Original by Judith A. Stoffer after Brugerolle (1974), Brugerolle *et al*. (1973b, 1974) and Kulda & Nohýnková (1978). © 2001 Judith A. Stoffer.

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3.4 Ovipleistophoriasis (Pleistophora): Ovary Parasite of Golden Shiners

Robert Summerfelt

Department of Animal Science
124 Science, Addition 2
Iowa State University
Ames, IA 50011
515/294-6107
rsummerf@iastate.edu

A. Name of Disease or Etiological Agent

The ovary parasite of golden shiner, *Notemigonus crysoleucas*, is the microsporidan *Ovipleistophoria ovariae* (Phylum *Microsporida*, commonly called the microsporidia, formerly named *Pleistophora ovariae*). This parasite causes a disease known as ovipleistophoriasis.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Areas where ovipleistophoriasis has been reported include: Alabama, Arkansas, California, Illinois, Kansas, Kentucky, Louisiana, Mississippi, Missouri, North Carolina, Oklahoma, Tennessee, and Texas. Ovipleistophoriasis should be expected in golden shiners from commercial sources.

2. Host Species

The type host for ovipleistophoriasisis is the golden shiner and it has been reported once in fathead minnows, *Pimemphales promelas*. The parasite was not detected in goldfish *Carassius auratus* reared in the same hatchery ponds used for golden shiners. Efforts to experimentally transmit the parasite by intraperitoneal or intramuscular injection of spores from infected shiners to goldfish were unsuccessful (Nagel and Summerfelt 1977a). This technique has been used to infect goldfish and other cyprinid fish with *Pleistophora hypessobryconis.* *Ovipleistophoria ovariae* was found in golden shiners from a creek and in shiners collected from a pond in the same watershed. It was not possible to ascertain whether these infected fish were feral or released from an angler's bait bucket. It is unlikely that a predatory fish would become infected by consuming an infected bait minnow. For example, *Pleistophora tahoenis*, which parasitize intramuscular connective tissue of the Piute sculpin, *Cottus beldingi* (Summerfelt and Ebert 1969), has not been reported in lake trout, *Salvelinus namaycush,* in Lake Tahoe, Nevada-California (Hoffman 1967; Canning and Lom 1986), even though the sculpin is their major prey.

C. Epizootiology

Ovipleistophoria ovariae is an obligate intracellular parasite, which carries out its entire development in the developing oocytes of its host. As a consequence of this infection, a reduced fecundity and castration may result. The life stages in the oocytes of the fish host are merogony (schizogony; the proliferative phase), and sporogony (the spore forming stage). The life cycle of *Ovipleistophoria ovariae* is synchronized with the reproductive cycle of the host so that spore formation peaks at the time of ovulation. There is no intermediate host, however, mechanical vectors such as zooplankton, which have ingested spores of *Ovipleistophoria ovariae*, may infect fish. Transmission may be either horizontal or vertical in nature. Spores may be ingested or infected females may pass spore-containing ova. *Ovipleistophoria ovariae* infections are economically important when prevalence and intensity of infection are heavy. Fish are infected for life and the intensity of infection increases with age. Producers unaware of the infection may find that older fish have poor reproduction. The parasite has yet to be associated with host mortality.

D. Disease Signs

1. Behavioral Changes Associated with the Disease

No behavioral changes associated with the disease have been described.

2. External Gross Signs

No external gross signs of infection have been described, however, infected adult female fish are often longer and heavier than uninfected females from the same population.

3. Internal Gross Signs

The parasite infects only the developing ova (oocytes). When a mature, uninfected ovary from a freshly sacrificed fish is viewed with the unaided eye, it appears as a uniformly colored translucent mass. Ovaries of prespawning fish infected with *Ovipleistophoria ovariae* have a conspicuously white marbling or translucent opaque spots. These spots are amorphous masses of atretic oocytes.

4. Histopathological Changes Associated with the Disease

Young-of-year fish (hatching to January) carry a latent form of the parasite that cannot be seen by light microscopy of the oogonia or stage one oocyte. Egg development is described on a numerical scale of 1 to 7, where seven is the mature ovum. The earliest stage of the parasite, the meront (schizont) is first seen in histological sections of stage 2 to 4 oocytes. Spores are present in stage six and seven oocytes as the fish approach first spawning (i.e. May or approximately 25^{o}C). Egg cytoplasm is consumed and completely replaced with sporophorous vesicles filled with spores. Atretic oocytes are filled with spores, cellular debris, fibrin, and phagocytic cells.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

Presence of conspicuous white marbling of the fresh ovary suggests an infected fish. Presumptive diagnosis is indicated by the presence of microsporidan spores in the ovary of golden shiners. However, presence of a microsporidian in fish ovaries should not be taken to imply the presence of *Ovipleistophoria ovariae*.

Presumptive diagnosis may be facilitated by preparing a wet mount from fresh chilled, frozen, or preserved ovaries. Wet mounts are effective for finding spores, especially in fish before and after spawning. Spores are not present or they are rare in juveniles or adults from fall through the winter months.

To make a wet mount, remove one ovary and macerate it in a mortar with pestle - or thoroughly dice one ovary sample with a razor blade. Add saline (0.85%), drain off fluid, centrifuge the mixture to remove larger cellular debris (optional), then examine a drop of supernatant fluid from the centrifuged sample on a slide as a wet mount under a coverslip with brightfield, transmitted light microscopy at 950X magnification or with dark phase microscopy. Spores in wet mounts are most refractile when examined by dark phase microscopy. The characteristic morphology is also evident when spores in wet mounts are examined with brightfield transmitted light (Figure 1A and Figure 1B). The ether separation method described by Landolt (1973), without enzyme digestion, is recommended for preparation of a relatively pure suspension of *Ovipleistophoria ovariae* spores (Leida 1978).

Spore measurement should be done by examining a monolayer of spores (Canning and Lom 1986) from fresh specimens. Preservation will cause shrinkage, and the amount will vary with the type of fixative (Parker and Warner 1970). Spore measurement is not required unless more than one host species is examined, due to the possibility of a different species of *Ovipleistophoria* or *Pleistophora* in a different host species.

In fresh material, the refractile portion of the spore is about 40% of the total spore length, followed by a less refractile or clear area occupying the rest of the spore. The coiled polar tube is visible in the clear area. The polar tube may be extruded by applying pressure to the coverslip (Figure 1D). Extrusion of the polar tube is a measure of spore viability.

Stained smears may be prepared from the suspension used for the wet mount. General procedures for the preparation and staining of protozoan smears are given by Kudo (1924). Canning and Lom (1986) describe some selective staining methods for microsporida. Leida (1978) described the staining response of spores in smears: 1) spores stained well with toluidine blue, the polar cap stained a dark blue-purple, the polar filament less intensively blue; 2) results with PAS were variable, at best the polar cap and polar filament stained faintly; 3) hematoxylin stained spores have a dark-blue or purple, girdle-like ring, sometimes with a dark nuclear spot (Figure 1C and Figure 1D) Giemsa stained the polar cap intensely blue and the nucleus less intensely purple.

2. Confirmatory Diagnosis

Histological confirmation is required to establish "no infection" when wet mounts were used for preliminary screening. Histological sections are not needed when spores are found in wet mounts. For identification of life stages (meronts and sporoblasts) other than spores from late summer through spring, fix an ovary from a fresh specimen in 10% neutral (or buffered) formalin or Bouin's fixative and prepare histological sections by standard methods.

Histological sections should be prepared when wet mounts are negative to confirm the diagnosis. Paraffin sections should be prepared following standard histological procedures (Luna 1968). Ovary may be sectioned longitudinally or transversely at 6 to 10 µm, with 3-5 sections per slide. Stain one slide with metachromatic blue stain containing 0.1% toluidine blue (Summerfelt and Warner 1970a), another with Mallory's aniline blue-collagen stain (Biological Staining Commission 1960:52-53), and a third with hematoxylin-eosin (H&E). The metachromatic blue stain was useful for a quick overview of a section to identify spores. Spores stain light to dark

blue, with a dark band in the middle and dark blue at the end. This stain is poor for distinguishing cytological details of the developing oocytes. Mallory's stain is highly differential for primary and secondary yolk, connective tissue, nucleoli, and the follicle. It is also excellent for differentiating meronts (schizonts), sporoblasts, and spores from the cytoplasm and yolk of the oocytes.

The intensity of infection can be estimated from the percentage of the cross-sectional area of the ovarian sections occupied by infected oocytes or atretic eggs.

F. Procedures for Detecting Subclinical Infections

When examining a juvenile fish in the fall or winter, when the spores are uncommon, the only evidence of infection will be the presence of schizonts in histological sections. Schizonts can be seen in the cytoplasm of the stage 2 to 4 oocytes. As the season progresses and later oocyte stages develop, sporoblasts will develop within sporophorous vesicles enclosed by the pansporoblast membrane. At the present time no serological tests have been developed to detect asymptomatic infections, *i.e.* the premeront (schizont) latent stage.

G. Procedures for Determining Prior Exposure to the Etiological Agent

Currently no tests are available to determine prior exposure to *Ovipleistophoria ovariae*.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Samples may consist of living or moribund fish which may be frozen or preserved in Bouin's solution or 10% buffered formalin.

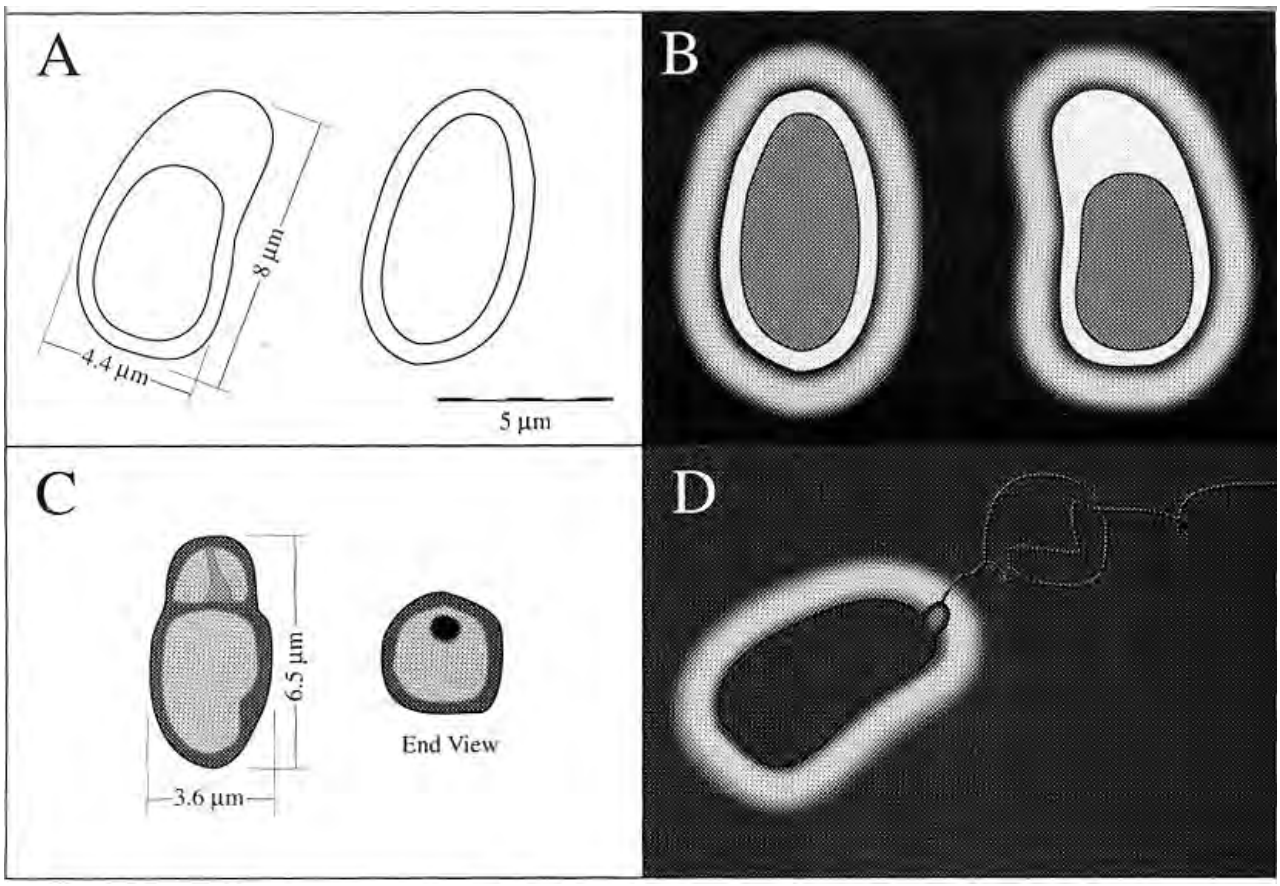


Figure 1. Spores of *Pleistophora ovariae*.

A. Fresh spores seen in brightfield microscopy typically appear slipper-shaped (left one of two spores) with a large posterior vacuole (clear area) around which the polar tube is coiled. The posterior vacuole is about equal to 60% of spore length. The vacuole is not apparent in all spores (right).

B. In darkfield microscopy, the spore wall (endospore) is highly refractile and is surrounded by a halo.

C. Fixed and stained spores (H&E) are smaller than fresh spores (Parker and Warner 1970; Summerfelt and Warner 1970a). In longitudinal view (left), the anterior end of the endospore is constricted by a dark band. The chromophilic body anterior to the constriction is the plar cap, a PAS positive, anchoring organelle at the base of the polar tube. In endview (right), the nucleus is visible as an off-centered spot.

D. Fresh spore after extrusion of polar tube (formerly called the polar filament); the polar tube may be 135 µm long. The sporoplasm (infective unit) passes through the length.

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3.5 Salmonid Ceratomyxosis

J. L. Bartholomew

Department of Microbiology and Center for Salmon Disease Research
Nash Hall 220, Oregon State University
Corvallis, OR 97331-3804
541/737-1856
bartholj@orst.edu

A. Name of Disease and Etiological Agent

Salmonid ceratomyxosis is caused by *Ceratomyxa shasta* (Myxozoa:Myxosporea).

B. Known Geographic Range and Host Species of the Disease

1. Geographical Range

The distribution of the infective stage of the parasite is restricted to the Pacific northwest of the United States and Canada. In the United States: Columbia, Cowlitz, Lewis (east fork), and Washougal rivers; LaCamas Lake; Snake River from its confluence with the Columbia River to approximately 440 miles upstream; Deschutes River including Davis, Odell, Crescent, and Suttle lakes; Willamette River from the mouth to 100 miles upstream; Nehalem, Rogue, and Klamath rivers; Klamath Lake; Sacramento, Mokelumne, Feather and Pitt River systems. In Canada: Fraser River. In Alaska: Tanana and Naknek River systems on the Alaskan Peninsula, Russel Creek (Cold Bay) in the Aleutian chain, the Togiak and Wood Rivers in Bristol Bay, and Lower Talarik Creek (Lake Iliamna). Additional isolations have been made in Alaska; however, it is unclear if these represent an established parasite presence.

Anadromous salmon may come in contact with *Ceratomyxa shasta* during migration and infected juvenile and adult fish have been reported in freshwater and marine environments outside of the parasite's established range.

2. Host Species

Natural infections of *Ceratomyxa shasta* are known to occur in the following: rainbow/steelhead trout *Oncorhynchus mykiss*, cutthroat trout *Oncorhynchus clarki*, pink salmon *Oncorhynchus gorbuscha*, chum salmon *Oncorhynchus keta*, coho salmon *Oncorhynchus kisutch*, sockeye salmon *Oncorhynchus nerka*, chinook salmon *Oncorhynchus tshawytscha*, Atlantic salmon *Salmo salar*, brown trout *Salmo trutta*, brook trout *Salvelinus fontinalis*, and Dolly Varden *Salvelinus malma*.

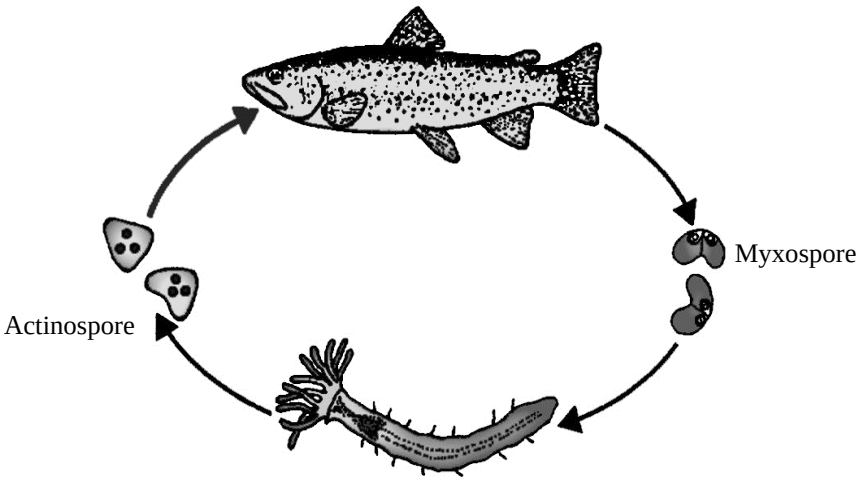
Within a species, strains of salmonids originating from enzootic waters are often more resistant compared with strains from non-enzootic waters.

C. Epizootiology

Ceratomyxosis causes losses in wild and domestic trout and salmon of all ages and sizes and has been reported as a significant contributor to prespawning mortality among infected adult fish.

Bartholomew et al. (1997) demonstrated that completion of the parasite life cycle requires development of the alternate actinosporean stages in the freshwater polychaete, *Manayunkia speciosa* (Figure 1). Natural transmission occurs when susceptible salmonids come in contact with the waterborne actinosporean stage following its release from the polychaete. The distribution of the polychaete is likely the factor that has defined the geographic distribution of the parasite.

Mortality generally occurs when water temperatures exceed 10˚C; however, fish can become subclinically infected at temperatures as low as 4˚C. Infections with *Ceratomyxa shasta* are prevented at salinities greater than 15 ppt; however, if fish are infected when they enter salt water the disease progress may continue.





Actinospore



Myxospore

Figure 1. Life cycle of *Ceratomyxa shasta* showing salmonid and polychaete host and alternating myxosporean and actinosporean life stages shown at right.

D. Disease Signs

Clinical signs of ceratomyxosis vary among fish species. In most cases, at least some of the following will be seen: anorexia, lethargy, marked darkening (especially in rainbow trout/steelhead), distended abdomen, exopthalmia, a swollen and hemorrhagic vent, and emaciation. In juvenile salmonids, the digestive tract may be grossly swollen, necrotic, and hemorrhagic with mucoid contents. The intestine and pyloric caeca may be lined with caseous material.

Additional characteristics may include ascites, kidney lesions (fluid filled blebs/pustules to firm creamy white nodules), and hemorrhaging and (or) necrosis of liver, gall bladder, spleen, gonads, kidney, heart, gills, and skeletal musculature.

In adult salmonids the walls of the intestine and pyloric caeca may be thickened and hemorrhagic. Nodular lesions may develop in the intestinal wall perforating the intestine in chinook salmon. Gross lesions (which may abscess) can occur in liver, kidney, spleen, or musculature. Abscesses of the body musculature are particularly common in coho salmon.

Depending on a number of factors (e.g. host species), a variety of organs may be affected. However, *Ceratomyxa shasta* has a predilection for the digestive tract (especially posterior intestine and pyloric caeca) and, secondarily, the kidney.

Development of *Ceratomyxa shasta* infections in the posterior intestine typically triggers acute inflammation involving polymorphonuclear leukocytes (PMNs), fibroblasts, and macrophages. The epithelial lining necrotizes, fragments, and ultimately sloughs, and is replaced by fibrous connective tissue containing host cells and trophozoites. The lumen may contain epithelial cells, epithelial cell fragments, PMNs, fibroblasts, trophozoites, pansporoblasts, and spores in later stages.

Pathological changes are less pronounced in the pyloric caeca. Trophozoites are often abundant between epithelial cells and in the muscularis externa. There may be separation of muscle layers due to the large number of trophozoites, but muscle necrosis is normally not severe.

The kidney is more severely affected in salmon than in trout. In chum salmon, kidney necrosis is severe, affecting both renal and hematopoietic elements. All normal tissue may be destroyed and replaced by developing parasites. Focal lesions are also common in the liver.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

Wet mounts can be prepared form the wall of the posterior intestine or of ascites if present. Material obtained via intestinal lavage is acceptable (Coley et al. 1983). Lesions present in any tissue should also be examined. Wet mounts can be scanned in a systematic manner under phase contrast or brightfield microscopy at 250 to 400X magnification. Presumptive diagnosis is based on identification of multicellular myxosporean trophozoites in salmonids showing signs of ceratomyxosis (Figure 2).

Histological sections of intestinal tract or other grossly infected tissues may be stained with either Giemsa or hematoxylin and eosin. In Giemsa-stained sections, multicellular trophozoites stain light blue with the nuclei contain a dark-staining karyosome surrounded by a clear halo.



Figure 2. Trophozoite stages.

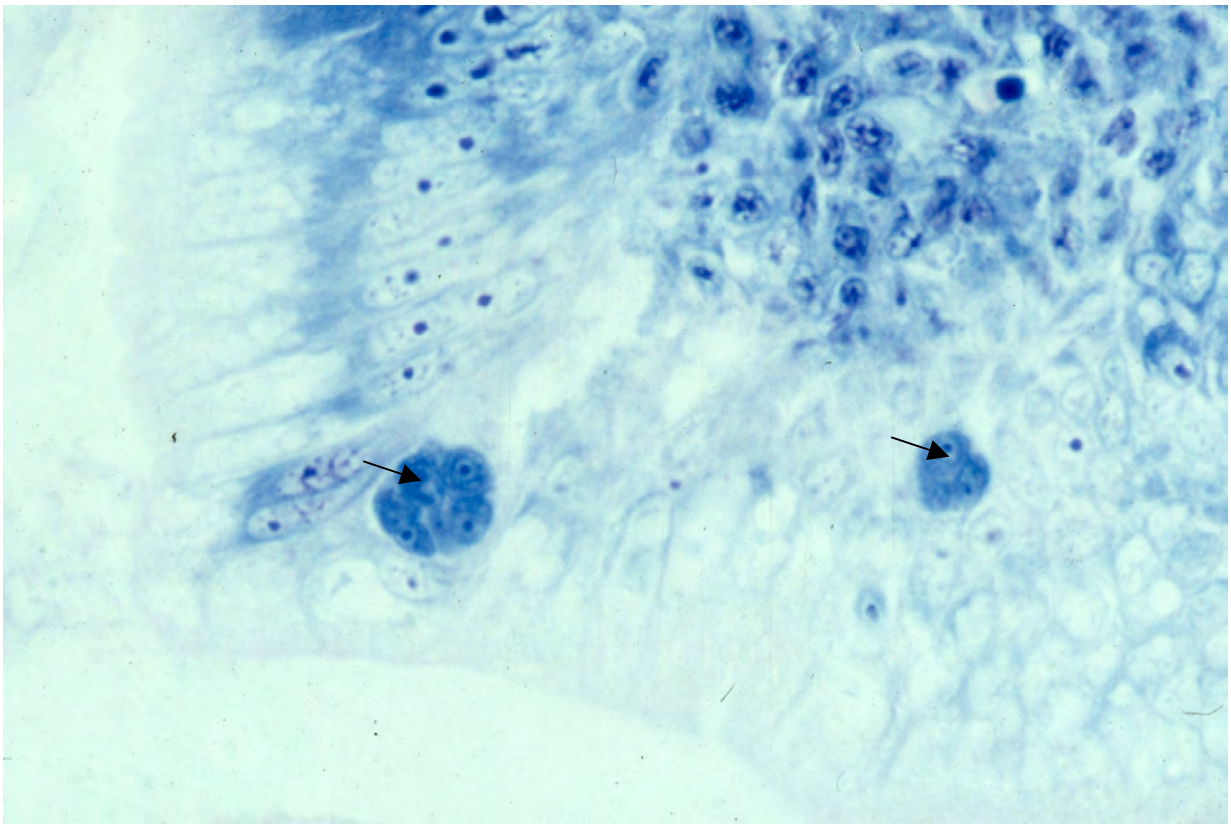


Figure 3. Giemsa stained histological section of intestine from an infected rainbow trout, arrows indicate trophozoites.

2. Confirmatory Diagnosis

Confirmatory diagnosis of ceratomyxosis can be based on the presence of the characteristic kidney bean-shaped mature spores of *Ceratomyxa shasta* in wet mounts or histological sections. Spores observed in wet mounts are about 14 to 23 µm long by 6 to 8 µm wide at the suture line (Figure 4 and Figure 5). Spores are most likely found in the posterior intestine but are often found in other tissues as well, particularly the kidney, liver, gall bladder, and pyloric caeca.

Serological identification of *Ceratomyxa shasta* trophozoites can be accomplished using monoclonal antibodies (Bartholomew et al. 1989b).

Molecular diagnosis of *Ceratomyxa shasta* can be accomplished using parasite-specific primers in a polymerase chain reaction assay [PCR; Palenzuela et al. 1999; Palenzuela and Bartholomew 2002; Section 1, 3.5.A1 Appendix 1]. A modification of the PCR assay has been made to allow for non-lethal sampling of fish (Fox et al. 2000).

F. Procedures for Detecting Subclinical Infections

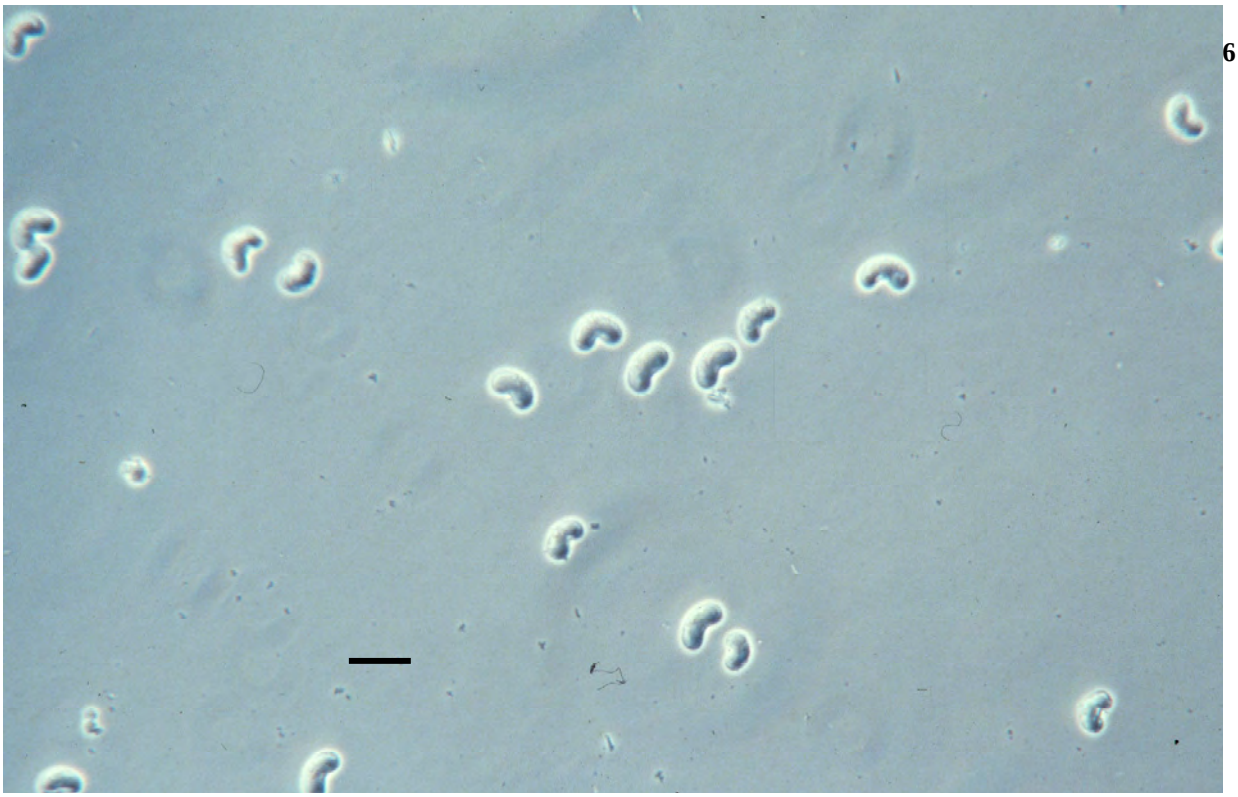
Spore formation usually does not occur until late in the infection; therefore, diagnosis of ceratomyxosis in early or subclinical infections should rely on serological or molecular detection of the parasite.

G. Procedures for Determining Prior Exposure to the Etiological Agent

No procedures have been reported.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Although spores can be detected in frozen samples, trophozoites are fragile and easily destroyed by freezing or heat. Therefore, samples for visual examination should consist of living, moribund, or dead fish (or tissues) held at low temperatures or on ice but not frozen. Samples may also be processed routinely for histology. Samples for molecular analysis should be frozen or refrigerated in lysis buffer.





Figures 4 and 5. Mature *Ceratomyxa shasta* spores. Bar = 10 µm.

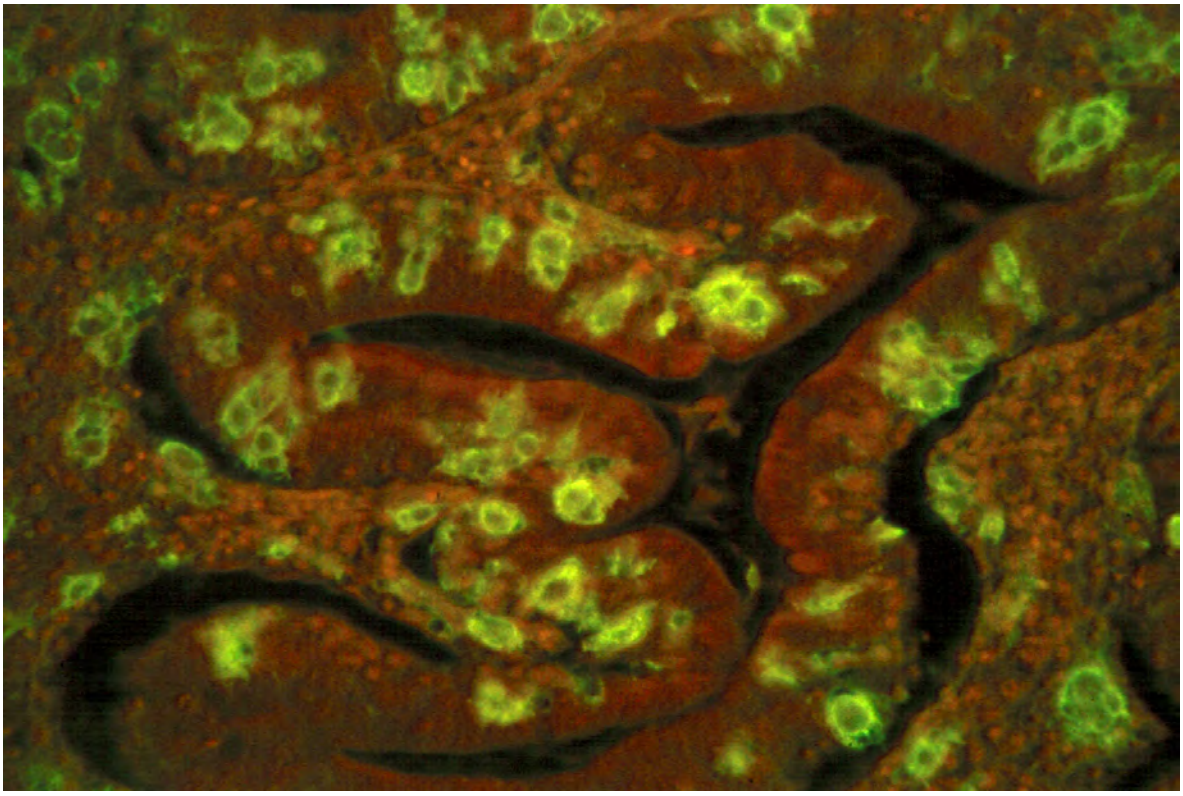


Figure 5. Trophozoite stages in histological section of intestinal tissue using a fluorescein-conjugated monoclonal antibody specific for *Ceratomyxa shasta*.

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3.5.A1 Salmonid Ceratomyxosis Methods Appendix 1

J. L. Bartholomew

Department of Microbiology and Center for Salmon Disease Research
Nash Hall 220, Oregon State University
Corvallis, OR 97331-3804
541/737-1856
bartholj@orst.edu

Molecular Detection of *Ceratomyxa shasta* Using the Polymerase Chain Reaction Assay (Palenzuela et al. 1999)

A. Sample Collection

Because of the specificity of the polymerase chain reaction assay (PCR), contamination of the samples by bacteria or other contaminants is not a problem and sterility or aseptic techniques are not strictly necessary. However, the risk of cross contamination and carry-over of DNA from other samples is high and sampling must be done with this in mind.

Samples should be thus taken as follows:

1. Use disposable material for each fish or alternatively (see notes below) destroy the DNA from the dissecting tools between fish. Process one fish at a time.

2. Remove the intestine (or a piece) from the fish and place it on a clean, disposable surface (a piece of aluminum foil works just fine).

3. Cut a small piece of the gut (25 to 100 mg, about 2 to 5 mm), preferably from the lower tract. We use disposable, nonsterile razor blades to cut this small piece, and a simple toothpick to transfer it to the screw-cap vial with 500 µL DNA extraction buffer (see next section for recipe). Conventional dissecting tools can be used but only if they are completely decontaminated between samples.

4. Dispose of the working surface, change gloves if they came in contact with the tissues, and proceed to the next fish.

Notes on decontamination of material: Conventional methods for disinfecting dissecting tools, such as spraying with ethanol and flaming, not only **DO NOT** completely destroy the DNA, but they can

actually fix it in the material. Procedures which are used to clean the material between samples include:

5. Use of the commercial products DNA AWAY or RNAse AWAY. Both destroy DNA quickly and safely. It is sufficient to wipe the material with a paper towel wetted with the product and then rinse it with fresh, distilled water from a lab bottle. These products are not recommended for use with metallic material, but we have used them liberally on forceps and scissors and have not encountered problems.

6. First wipe off any pieces of tissue, blood, or mucus from the tools, then immerse them in 10% bleach (Clorox) solution for ***at least*** one minute. Rinse before use with distilled water from a wash bottle. This concentration of bleach is corrosive for metals after long exposures, but this is not a problem if the exposure is limited to the sampling and tools are rinsed well in distilled water before storage. We normally have two containers (per person) with bleach during sampling, each containing a set of dissecting tools. After use, the tool is wiped clean with a piece of paper and put back in the bleach solution. The next time we use a tool, we get if from the other set and so on.

B. Protocol for the Diagnosis of *Ceratomyxa shasta* in Fish Intestines by PCR

1. DNA Extraction

a. Reagents:

i. DNA Extraction Buffer
The buffer is NaCl 100 mM, Tris-HCl 10 mM, EDTA 25 mM, SDS 1%.
The stock solutions are:

NaCl 5M	{50X}
Tris-HCl 1M, pH 7.8	{100X}
EDTA 0.5M, pH 8	{20X}
SDS 20%	{20X}

Stock solutions should be made using ultrapure, nuclease-free water (HPLC grade or equivalent), aliquoted and stored at -20^{o}C until needed. Pre-made stock solutions (molecular biology grade) can be purchased from a commercial supplier, aliquoted, and frozen, so the chances of contamination are reduced.

ii. Proteinase K
Obtain commercially as a stable liquid solution (store at 4^{o}C) or make at a concentration of 20 mg.mL^{-1} and freeze at -20 ^{o}C in 1 mL aliquots

iii. RNAse A
A 10 mg mL^{-1} solution can be purchased as a 50% glycerol solution that is liquid at -20^{o}C.

b. Methods:
Special care must be taken, as carry-over of DNA from other samples occurs easily. Use new blades and/or tools to take and handle the tissues. Samples can be fresh or frozen. There is no

need to clean the tissues from fat or adherent tissue.

i. Dissect intestine on a clean surface as described above. Cut a small piece (25 to 75 mg) with a new razor blade and transfer the tissue to a nuclease-free microtube (1.5 or 2 mL are O.K., but screw-cap vials are recommended because of the boiling step), containing 500 µL of DNA extracton buffer. The first few times it may be helpful to weigh the tissue, to get an idea of the size of tissue that should be cut. Samples can be stored at room temperature for up to a few weeks, but otherwise should be refrigerated or frozen.

ii. Add 5 µL of proteinase K to a final concentration of 200 $\mu g.mL^{-1}$ (from a liquid stock at 20 mg mL). Incubate at 37^{o} C, horizontally on a slow rocking platform or with frequent inversion of the tubes until the tissue is completely digested (this takes about 4-5 hours, but overnight incubation does not affect the quality of the DNA and is recommended for larger samples).

iii. Following proteinase K incubation, spike the samples with 5 µL of RNase A (at 10 $mg.mL^{-1}$) and digest one hour.

NOTE: Be very careful when opening the tubes: the samples are viscous and stick to the walls, cap and lid. Cross contamination is easy, either by aerosol when opening the tubes or just with the fingers when touching lids and caps. Spinning the tubes briefly before opening them should be done on a routine basis.

iv. Boil the tubes in a floating rack in boiling distilled water. Remove tubes and let samples cool to room temperature. Store at -20^{o} C until needed.

2. PCR Reagents

a. Primers

The sequences for the forward and reverse *Ceratomyxa shasta*-specific PCR primers are as follows. Primers can be synthesized by a variety of companies.

CS-1	GGG CCT TAA AAC CCA GTA G
CS-3	CCG TTT CAG GTT AGT TAC TTG

b. PCR Master Mix

*Taq polymerase and template (sample DNA) are not included in the master mix, but the concentrations of the reagents are calculated for 20 µL reactions with 1 µL of sample and 0.2 µL of Taq polymerase per reaction:

For **<u>10 mL</u>** of Cs 1-3 master mix:

Sterile water	7.5 mL
Taq 10x PCR Buffer (Promega)	1 mL
$MgCl_2$ 25 mM (Promega)	800 µL
dNTPs (at 100mM; Promega)	80 µL (20 µL each A,T,G,C)
Primer F (CS1 at 500 µM)	10 µL
Primer R (CS3 at 500 µM)	10 µL
(*) Taq polymerase	**[100 µL]***
(*) Sample	**[500 µL]***

Make the mix on ice and under nuclease-free conditions. Aliquot and store at -20^{o} C. Try to avoid freeze-thaw cycles.

3. PCR Protocol

Dilute the DNA samples with ultrapure, nuclease-free water. Samples need to be diluted greater than 1/10, especially at low infection levels, as the crude DNA preparation contains PCR inhibitors. Dilution of samples to 1/100 work for all the samples tested to date. Very low infection levels have tested positive when diluted 1/1000.

Assemble the reaction mixture on ice in 20 µL reactions as follows:

1 µL diluted template (sample DNA)
1 U Taq polymerase (usually 0.2 µL, depending on supplier)
18.8 µL Cs 1 to 3 master mix

Overlay with mineral oil and start the PCR machine with the cycle:

95^{o}C/3m + 35 cycles x {94^{o}C/1m + 58^{o}C/30s + 72^{o}C/1m}+72 ^{o}C/10m.

Analyze 10 µL of each reaction in a 1% agarose gel with ethidium bromide staining. Positive samples will have an amplicon of 640 bp.

3.6 Whirling Disease of Salmonids

E. MacConnell

U. S. Fish & Wildlife Service
Bozeman Fish Health Center
Bozeman, MT 59715
beth_macconnell@fws.gov

A. Name of Disease and Etiological Agent

Whirling disease is a chronic inflammatory disease in salmonid fish caused by the myxosporean parasite *Myxobolus cerebralis.*

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

The parasite, *Myxobolus cerebralis*, has been found in the United States (Arizona, California, Colorado, Connecticut, Idaho, Maryland, Massachusetts, Michigan, Montana, Nevada, New Hampshire, New Jersey, New Mexico, New York, Ohio, Oregon, Pennsylvania, Utah, Vermont, Virginia, Washington, West Virginia, and Wyoming), Europe, United Kingdom, New Zealand, Africa, and Russia.

2. Host Species

Most species of salmonids are susceptible to *M. cerebralis* infection. Prevalence of infection, appearance of clinical signs, and severity of infection vary among species. Rainbow trout *Oncorhynchus mykiss* and steelhead are highly susceptible to whirling disease. Cutthroat trout *Oncorhynchus clarki*, chinook salmon *Oncorhynchus tshawytscha*, and brook trout *Salvelinus fontinalis* are less susceptible than rainbow trout. Bull trout *Salvelinus confluentus*, brown trout *Salmo trutta*, coho salmon *Oncorhynchus kisutch*, and Atlantic salmon *Salmo salar* are partially resistant while lake trout *Salvelinus namaycush* and arctic grayling *Thymallus arcticus* are highly resistant to whirling disease. Factors such as age, size, parasite dose, and environmental factors at initial exposure determine the degree of susceptibility, onset of disease, and severity of signs.

C. Epizootiology

The life cycle of *M. cerebralis* is temperature dependent and involves two hosts: salmonid fish and aquatic oligochaete, *Tubifex tubifex* (Figure 1). The cycle in fish and worm is greatly extended in colder water temperatures. Waterborne triactinomyxons, released from *T. tubifex* attach and invade the skin of the fish. The parasite has sequential affinity for the skin, nerves, and skeletal cartilage in the salmonid host. Clinical signs of whirling disease may be seen 3 to 8 weeks after infection. Susceptibility to whirling disease varies with fish age, size, parasite dose, and environmental factors.

Mortality in young salmonids is highly variable. Survival increases with increasing age of fish at first exposure to the parasite.

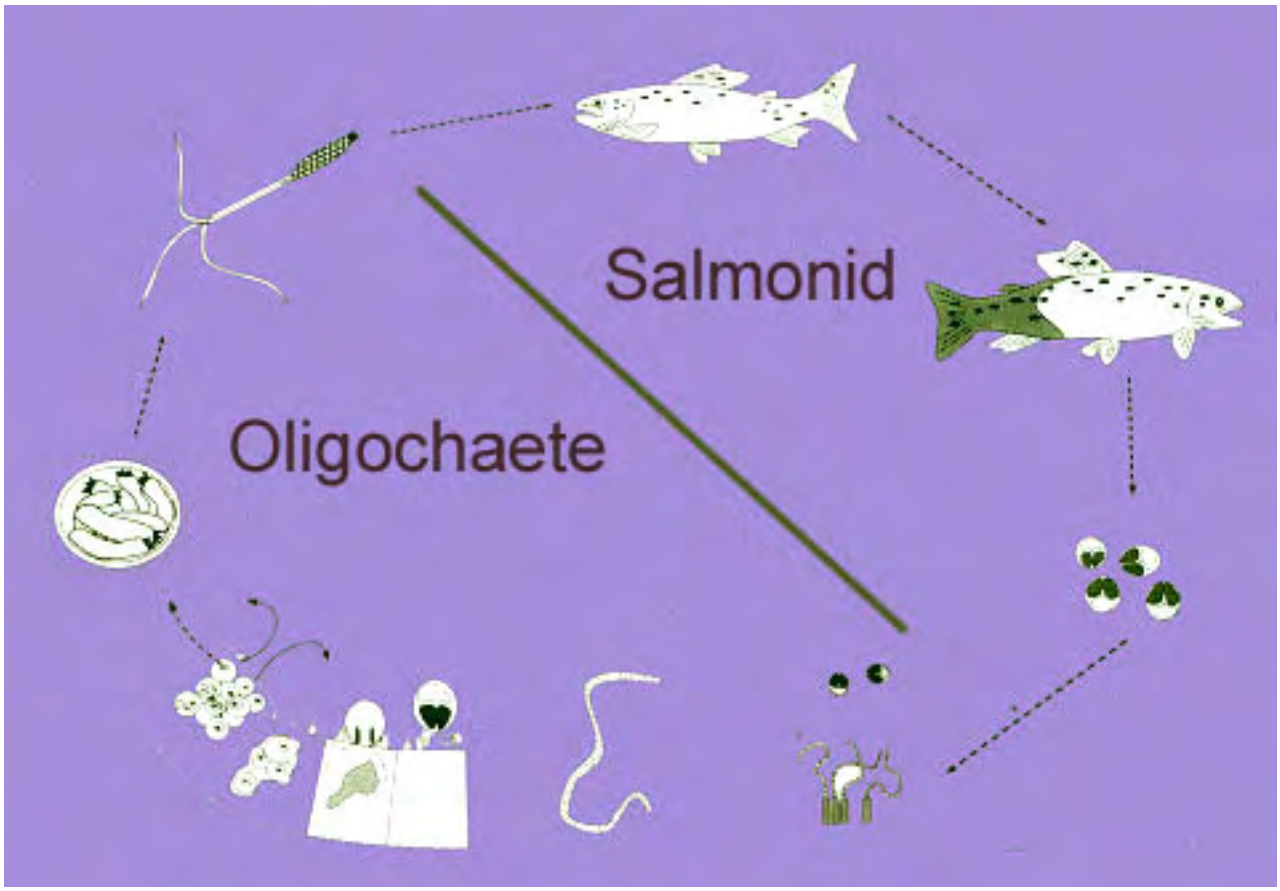


Figure 1. Life cycle of *Myxobolus cerebralis* in oligochaete and fish hosts.

D. Disease Signs

1. **Behavioral Signs**
   Whirling or radical tail-chasing behavior.

2. **Gross Signs**
   Blacktail and subtle to striking skeletal deformities (Figure 2). During the active phase of infection growth rates may be depressed.

3. **Microscopic Signs**
   Tissue sections show a range of microscopic signs from discrete foci of parasites and cartilage degeneration (Figure 3) to widespread cartilage necrosis, numerous parasites, granulomatous inflammation, and involvement of surrounding tissues (Figure 4). In adult fish, spores are found in isolated pockets in bone and are rarely associated with inflammatory lesions.

E. Disease Diagnostic Procedures

Diagnosis of *Myxobolus cerebralis* depends upon the detection and identification of prespore or sporogonic stages of the parasite. Spores of *M. cerebralis* are spherical in shape with two pyriform polar capsules (Figure 5). Trophozoites are multinucleate and much larger than spores (Figure 6).

Spore extraction procedures involve processing fish heads and may result in the observation of other *Myxobolus* spp., including unnamed species, found in cranial tissues and of similar morphology. *Myxobolus squamalis* found in scales is similar in size to *M. cerebralis* but has two distinctive ridges on either side of the suture. Several species are found in the central nervous system of salmonids: *M. kisutchi*, is similar in size to *M. cerebralis; M. neurobius* is oval in shape and larger (13.4 to 14 µm long); and *M. arcticus* is pyriform and large (14.3 x 16.5 µm). See Figure 5.2 under Section 2, 5.2 Myxobolus cerebralis (Whirling Disease). Methods for measuring myxosporean spores are described by Hoffman (1999).

Detailed procedures for screening and confirmatory tests are provided in Section 2, 5.2.A "Screening Test."

1. **Sampling Criteria**
   The greatest numbers of spores are found in young fish five months after parasite exposure (when water temperature is >10°C) spore numbers then decline throughout the life of the fish. Optimal samples for the detection of *M. cerebralis* are collected from fingerlings or yearlings of the most susceptible species. Spore development is temperature dependent. A minimum of 90 days is required at 12 to 13°C and up to 11 months at 0 to 7°C for sporogenesis to occur.

   Entire heads are removed from fingerlings and yearlings. A wedge or core sample is removed from fish too large to sample the entire head (see Section 2, 2.2.E.4 "Collection of Specimens for the Detection of *Myxobolus cerebralis* evaluation"). Processing cranial cartilage may not yield spores from all *M. cerebralis*-infected salmonids. Negative results may be obtained from bull trout and mountain whitefish cranial cartilage when cartilage from the caudal peduncle contains *M. cerebralis* spores.



Figure 2. Rainbow trout naturally infected with *Myxobolus cerebralis*. The lower four fish show blacktail and skeletal deformities.

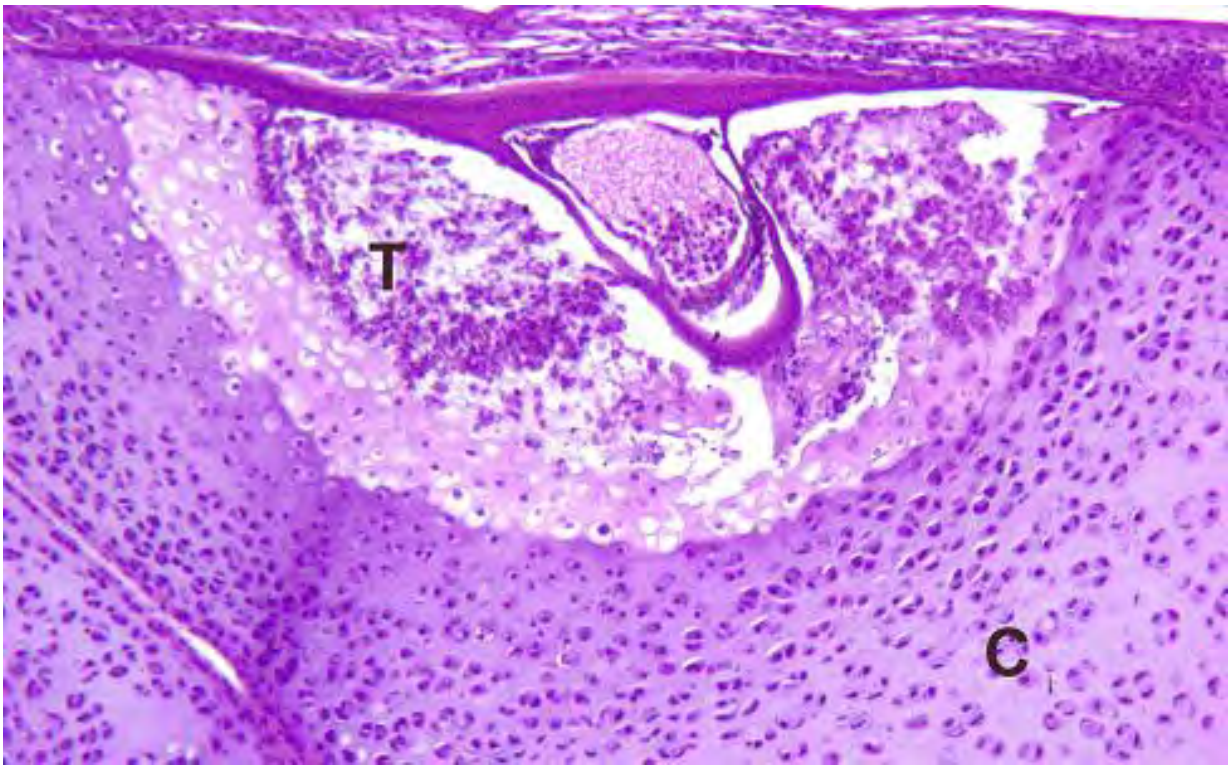


Figure 3. Focal lesion in cartilage (C) containing trophozoite stages of *M. cerebralis* (H&E).

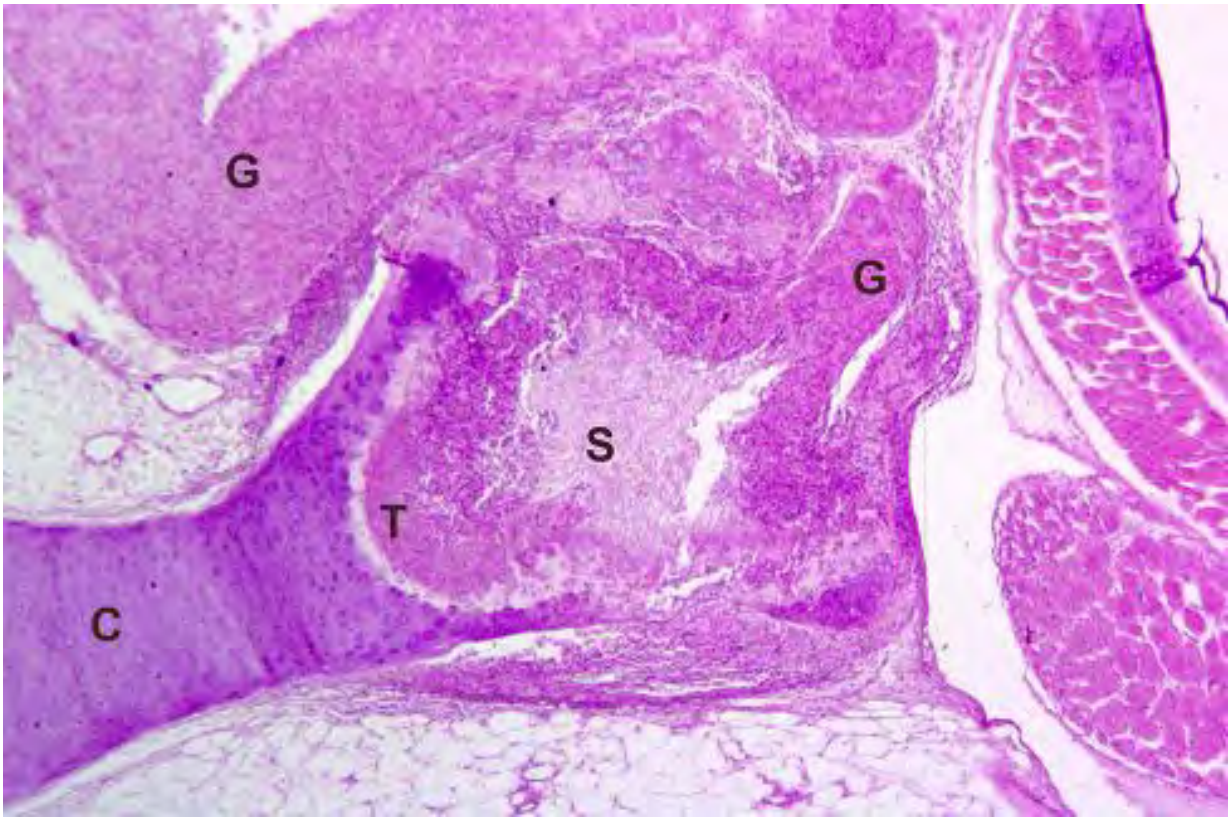


Figure 4. Replacement of cartilage with extensive granulomatous inflammation (G) containing trophozoites (T) and spores (S) in head of rainbow trout (H&E).

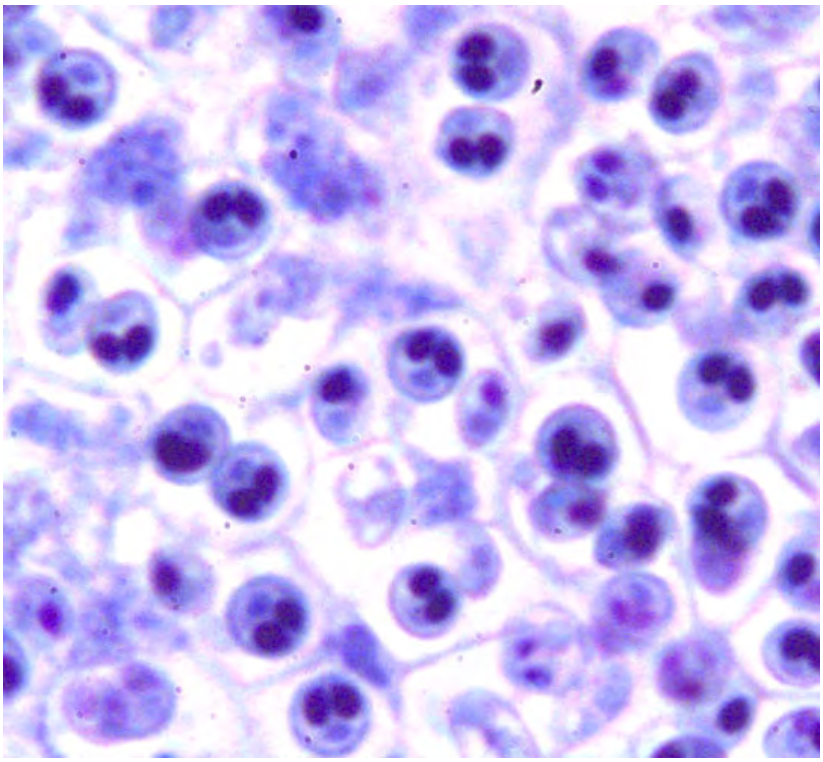


Figure 5. Spherical *Myxobolus cerebralis* spores with pyriform polar capsules.

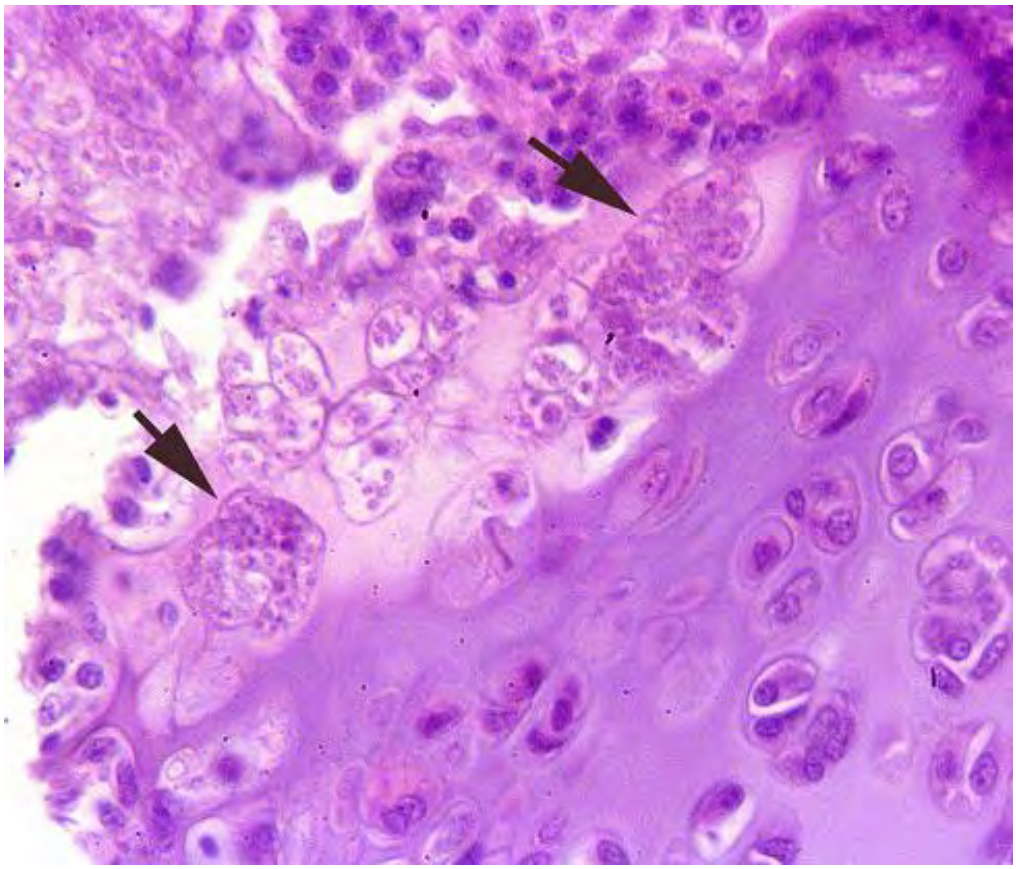


Figure 6. Trophozoite stages (arrows) of *Myxobolus cerebralis* in cartilage (H&E).

2. **Presumptive Diagnosis**
   a. Clinical Signs
   Include blacktail, whirling behavior, and skeletal deformities.

   b. Spore Extraction
   Pepsin-trypsin digest (PTD) (Markiw and Wolf 1974). Refer to Section 2, 5.2.A "Screening Test" for specific assay procedures.

   Note: Spore extraction methods will not detect presporogonic stages of the parasite. Fish (e.g. rainbow trout) exposed at young ages to *M. cerebralis* may develop clinical signs of whirling disease prior to the development of spores.

3. **Confirmatory Diagnosis**
   a. Histological Confirmation
   Confirmation of *M. cerebralis* requires the observation of presporogonic and spore parasite stages located in cartilage tissue surrounded by bone (Figure 7). Mature spores are unstained in hematoxylin and eosin (H&E) stained tissue sections. Polar capsules of the spore stain darkly and are clearly visible in Giemsa or methylene blue stained sections. Refer to Section 2, 5.2.B.1 "Histopathology" for specific procedures.

   b. Polymerase Chain Reaction (Andree et al. 1998)
   Confirmation of *M. cerebralis* is based on the amplification of parasite DNA using the nested polymerase chain reaction (PCR) assay. Refer to Section 2, 5.2.B.2 "PCR" for specific nested PCR assay procedures.

F. Procedures for Detecting Subclinical Infections

1. **Pepsin-Trypsin Digest (PTD) (Markiw and Wolf 1974)**
   Refer to Section 2, 5.2.A "Screening Test" for specific assay procedures.

2. **Polymerase Chain Reaction (PCR) (Andree et al. 1998; Schisler et al. 2001)**
   The highly sensitive PCR assays should be used to detect early or light infections.

G. Additional Procedures for Detecting *Myxobolus cerebralis* (not for Inspection Purposes)

1. **Plankton Centrifuge Method (O'Grodnick 1975)**

   a. Deflesh heads and/or whole fish. Process remaining cartilage in 20 g batches.

   b. Homogenize each batch of cartilage in 175 to 200 mL of water for 3 to 5 minutes in a high-speed blender. Blend the sample completely.

   c. Strain blended sample through a new or disinfected urinary calculi filter. Rinse any remaining sample with water and save all washings. Disinfect and discard tissues.

   d. Add the entire filtrate to a separatory funnel located directly above the plankton centrifuge (026WA106 plankton centrifuge, Kahl Scientific Instrument Corp., PO Box 1166, El Cajon,

CA 92022, or equivalent). Turn on the plankton centrifuge and adjust the separatory funnel to a slow and steady flow into the plankton centrifuge cup.

e. Run the centrifuge until the separatory funnel has been completely emptied including three rinses with water.

f. Scrape the sides of the plankton centrifuge cup with a rubber spatula to liberate spores. Rinse the spatula. Transfer the sample to labeled tubes. Samples can be stored in a solution of 10% neutral buffered formalin until examination.

g. Shake the sample well. Place a drop of the suspension: 1) under coverslip on both sides of hemocytometer and search grids for *M. cerebralis* spores; or 2) on microscope slide, add a cover glass and systematically search for spores. If the suspension is thick, a drop of water may be added.

2. *In situ* Hybridization (Antonio et al. 1998)

Digoxigenin labeled primers, specific for *M. cerebralis*, bind to the parasite and are visualized in tissue sections using standard immunohistochemical procedures. For this assay, tissues should be fixed in neutral buffered formalin without decalcification. Refer to Antonio et al. (1998) for the full procedure.

3. Polymerase Chain Reaction (PCR) Single Round Assay (Baldwin and Mykleburst 2002; Schisler et al. 2001)

One set of primers can be used in a single round of amplification to detect *M. cerebralis* DNA. The PCR assay described and tested in Colorado by Schisler et al. (2001) utilizes a primer set that has a slight sequence variation from Andree et al. (1998) in a single round format. Refer to Section 2, 5.2.B.2 "PCR" for PCR assay procedures.

a. Modified PCR Procedure
Follow procedures in Section 2, 5.2.A thru 5.2.C.

i. Amplification of *M. cerebralis* DNA (Baldwin and Mykleburst, 2002).
PCR master mix:

PCR buffer (300 mM Tris, 75 mM ammonium sulfate, pH 9.0)	
MgCl	2.5 mM
DNTPs	400 uM
Tr5-16 Primer	20 pmol
Tr3-17 Primer	20 pmol
Taq Polymerase	2 U μL^{-1}

Primers (Andree et al. 1998):

Tr5-16	(5'-GCATTGGTTTACGCTGATGTAGCGA-3')
Tr3-17	(5'-GGCACACTACTCCAACACTGAATTTG-3')

Standard reaction volume: 50 µL (45 µL master mix and 5 µL DNA template)

ii. Perform amplification in thermocycler. Denature at 95°C for five minutes.
Cycle:

95°C for 1 minute.
65°C for 2.5 minutes.
72°C for 1.5 minutes.

Repeat cycle 35 times. Conclude with 72°C for 10 minutes.

iii. Visualization of PCR Product Gel Electrophoresis
Prepare agarose gel. Dilute 10 µL PCR product with 2 µL loading dye. Load 10 µL into each well. Run for approximately one hour at 140 V.

H. Procedures for Determining Prior Exposure to the Etiological Agent

See above.

I. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Samples collected from apparently normal, moribund, or dead fish should be packed on ice for shipment. For histological examination, fresh tissue samples should be preserved in fixative containing formalin (e.g. 10% neutral buffered formalin, Davidson's).

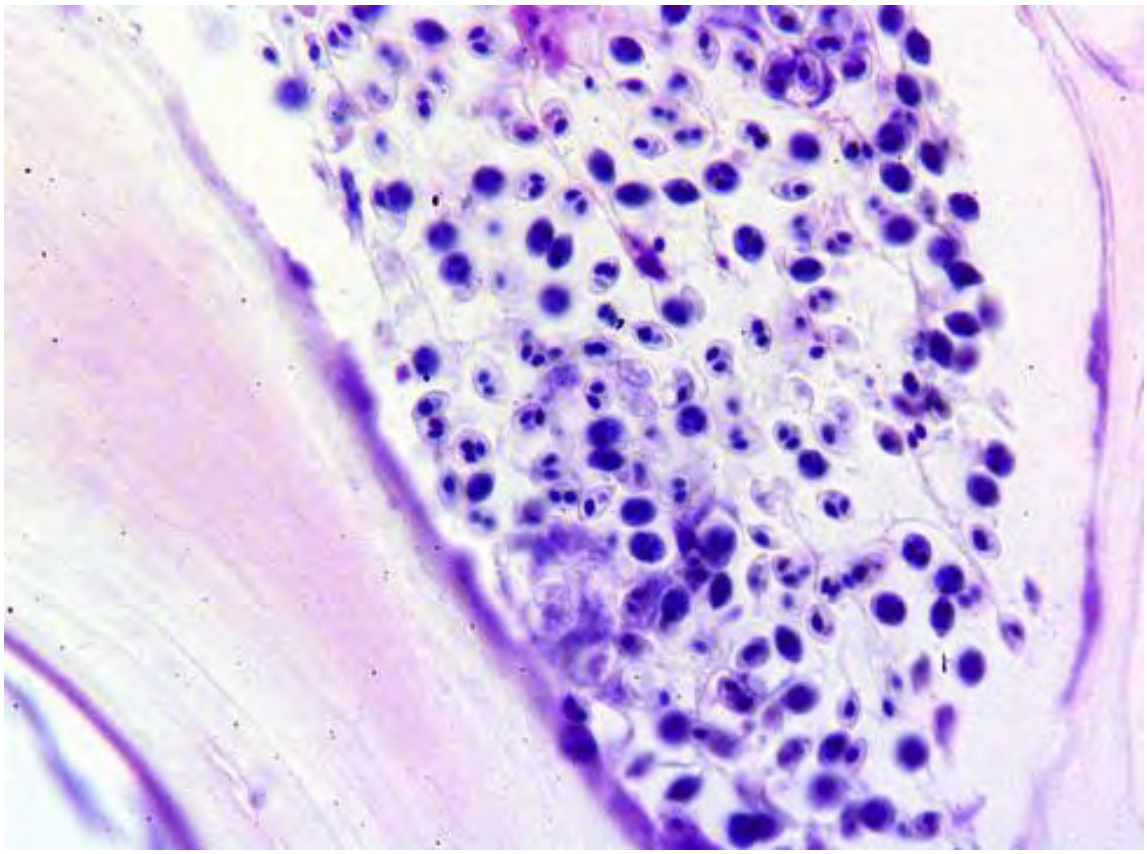


Figure 7. Giemsa-stained tissue section showing spores in cartilage surrounded by bone.

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3.7 Proliferative Gill Disease

L. M. Pote, L. A Hanson , L. Khoo*

Department of Basic Sciences College of Veterinary Medicine,
Mississippi State University, Mississippi State, MS 39762
662/325-1194
lpote@cvm.msstate.edu
662/325-1202
hanson@cvm.msstate.edu

*School of Veterinary Medicine,
University of Pennsylvania, New Bolton Center,
610/444-5800
lkhoo@vet.upenn.edu

A. Name of Disease and Etiological Agent

Proliferative gill disease (PGD) is caused by *Henneguya ictaluri* (Myxozoa: Myxosporea). Molecular data has confirmed that the actinospore stage of this parasite is *Aurantiactinomyxon* spp. and the final myxospore stage is *Henneguya ictaluri* (Pote et al. 2000).

B. Known Geographic Range and Host Species of the Disease

1. Geographical Range

This disease has been reported in commercial catfish in the Southern United States and California.

2. Host Species

Proliferative gill disease has been reported in channel catfish *Ictalurus punctatus* and Blue catfish *Ictalurus furcatus* in the United States.

C. Epizootiology

Proliferative gill disease has been attributed to major fish losses in commercial channel catfish of all ages and sizes. Major outbreaks of this parasite occur primarily in spring (April-May) and with smaller outbreaks occurring in fall (September-October) in the Southeastern United States.

Research has confirmed that this disease is associated with the actinospore *Aurantiactinomyxon* spp. (Burtle et al. 1991; Styer et al. 1991; Bellerud et al. 1995), tentatively identified as *A. ictaluri* (Bellerud et al. 1995). It has been confirmed that the oligochaete *Dero digitata*, routinely found in catfish ponds (Bellerud et al. 1995), are infected by several myxozoans, including *A. ictaluri*. In

experimental infections where catfish were exposed to worms infected with *Aurantiactinomyxon* spp. (Styer et al. 1991) or pure *A. ictaluri* (Belem 1994), PGD-like myxozoan stages were found in the gills 5 to 6 days after exposure. Based on recent molecular data, Pote et al. (2000), further demonstrated that *A. ictaluri* actinospores, the PGD myxozoan stages present in the gills, and a subsequent myxospore gill stage, *Henneguya ictaluri* n. sp. had identical rRNA gene sequences, thus confirming that the actinospore, *A. ictalur*i, is a life stage of the myxospore, H. *ictaluri* n. sp. The proposed life cycle for this parasite is: infected *D. digitata* release the actinospore stage (Figure 1) into the aquatic environment and, upon contact with the actinospore either orally or through the skin or gills (Bellem and Pote 2002), the fish becomes infected. Development to the final *H. ictaluri* myxospore occurs in the gills (Figure 2 and Figure 3), and these spores are released into water subsequently infecting *D. digitata* (Figure 4).

D. Disease Signs

Early clinical signs of affected fish include inappetence and listlessness. As a result of the respiratory insult suffered by these fish, they are often seen in schools behind supplemental aeration devices or in the shallows along the pond bank in the early morning as they are unable to breathe in spite of sufficient dissolved oxygen levels. Mortality is often severe in the early stages of the disease and this usually dissipates after two weeks. Fingerlings are usually more susceptible to PGD although larger fish are also susceptible. However, lesions (gross and microscopic) in food fish may not reflect the clinical severity the disease (i.e. there are only a few minor lesions in the gills and yet mortality is high).

Gross lesions are limited to the gills. This disease presents acutely with gills that are often mottled red and white (Figure 5). These gills are swollen and fragile and bleed easily. The disease progresses with fractures of the cartilage in the primary gill filaments often resulting in blunted and missing filaments (Figures 6,7, 8, and 9). Chronic or healed lesions may present as misshapen gill filaments.

Histologically, PGD is characterized as a granulomatous branchitis. In acute infections, there is congestion and hemorrhage, and a mixed inflammatory infiltrate composed of mononuclear inflammatory cells, together with hypertrophy and hyperplasia of the branchial epithelium. Consequently, the lamellar troughs become occluded and are obliterated. Lysis and fractures occur in the cartilage and defects in the cartilage become apparent. Parasitic trophozoites that are most often stained intensely basophilic (in hematoxylin and eosin preparations) are sometimes evident in the inflammatory milieu. Dyschondroplasia ensues with callus type formation bridging the cartilaginous defects. The gill filaments regenerate and often a misshapen focus (kink) in the cartilage is the only residual sign of infection. The parasitic trophozoites can be seen in other non-branchial tissues (spleen, liver, brain, anterior and posterior kidneys), but there is usually no associated inflammation.

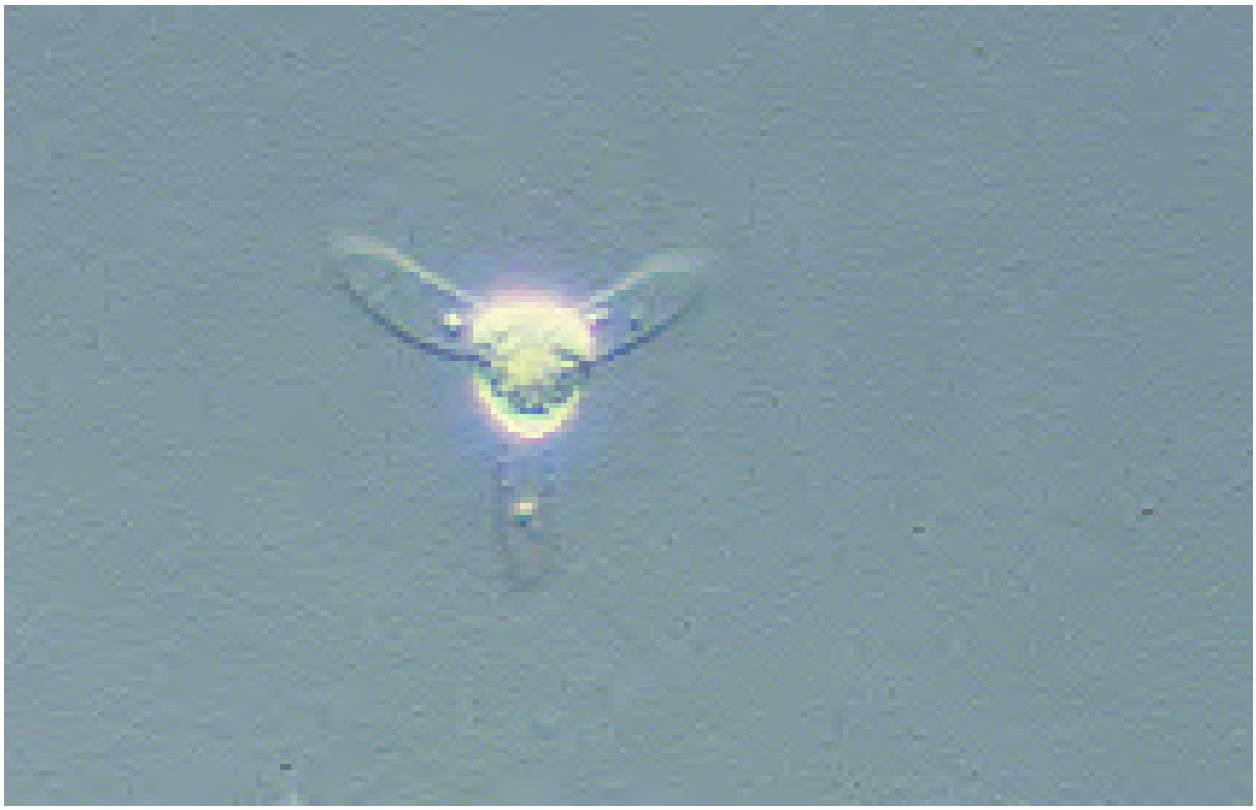


Figure 1. Actinospore stage of *H. ictaluri* in water.

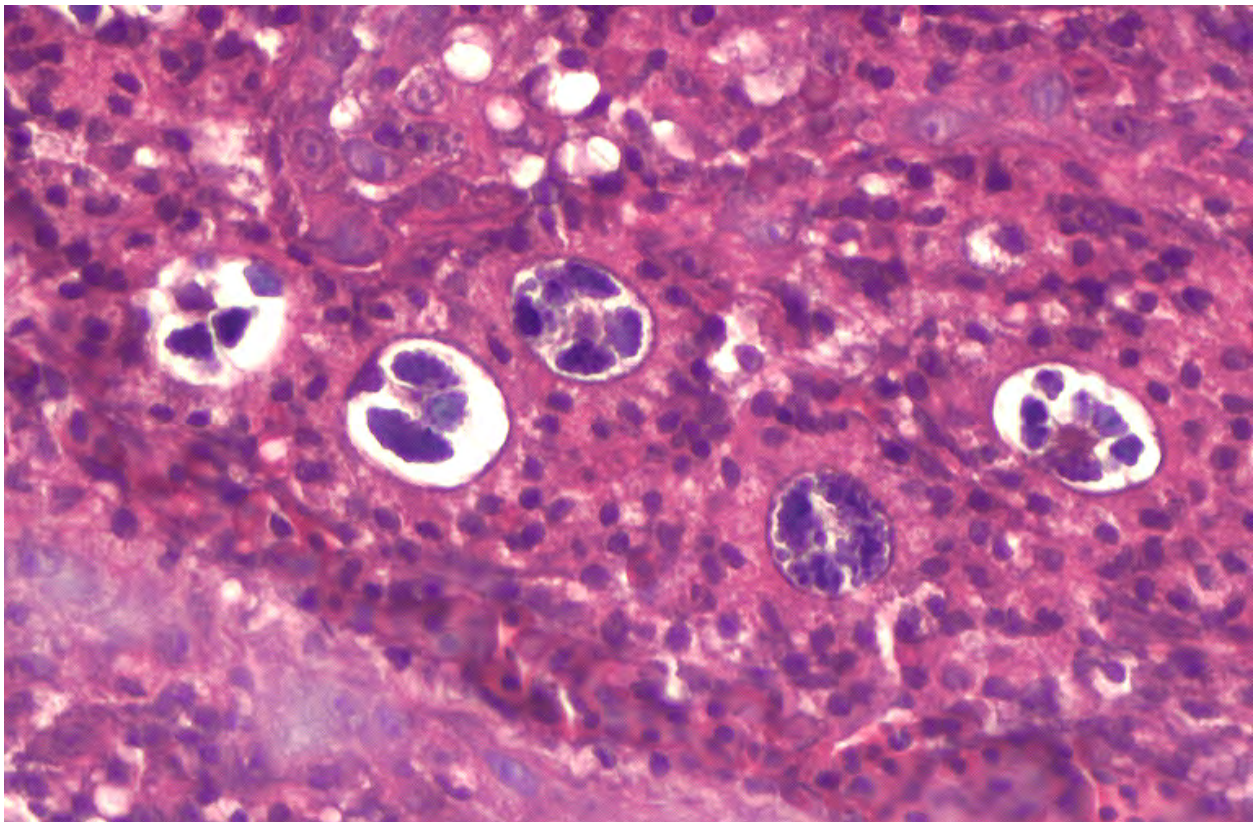


Figure 2. Histological section of early trophozoite stage of *H. ictaluri* in gills (courtesy of Andy Goodwin).

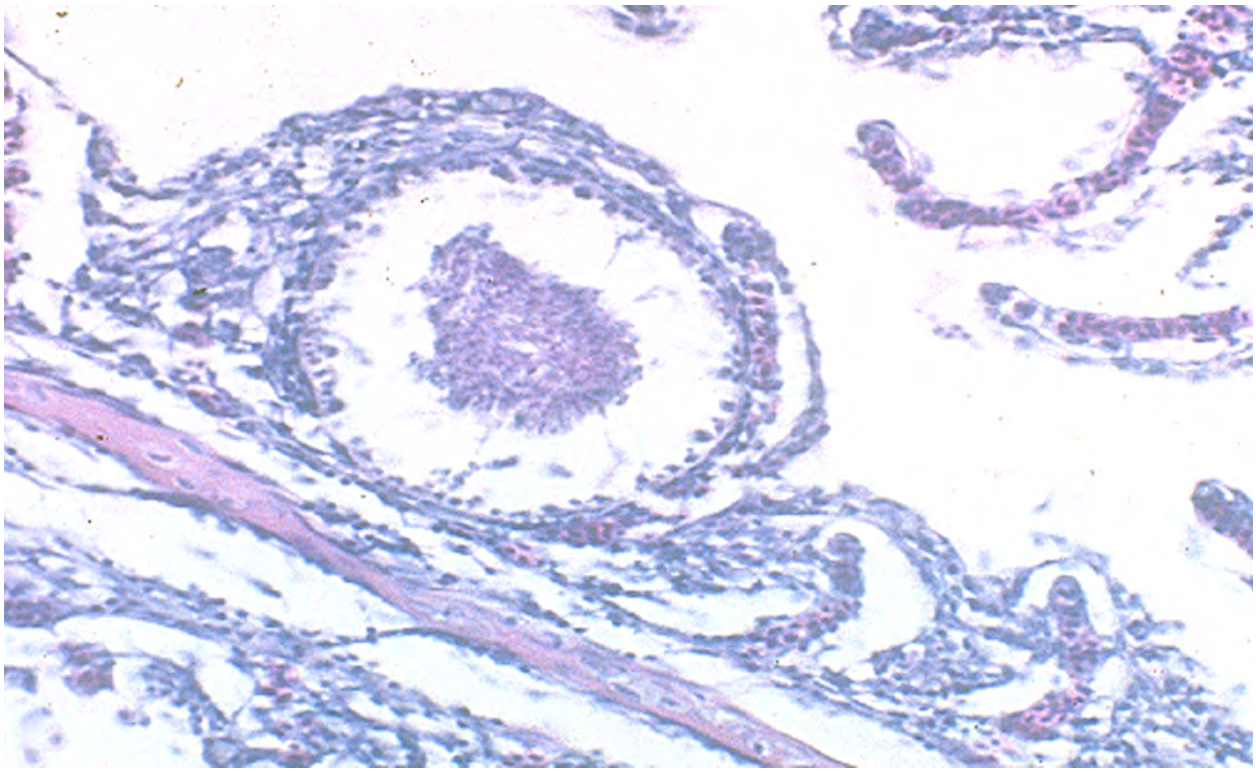


Figure 3. *Henneguya ictaluri* cyst in gills.



Figure 4. *Henneguya ictaluri* spores released from tissue cyst.

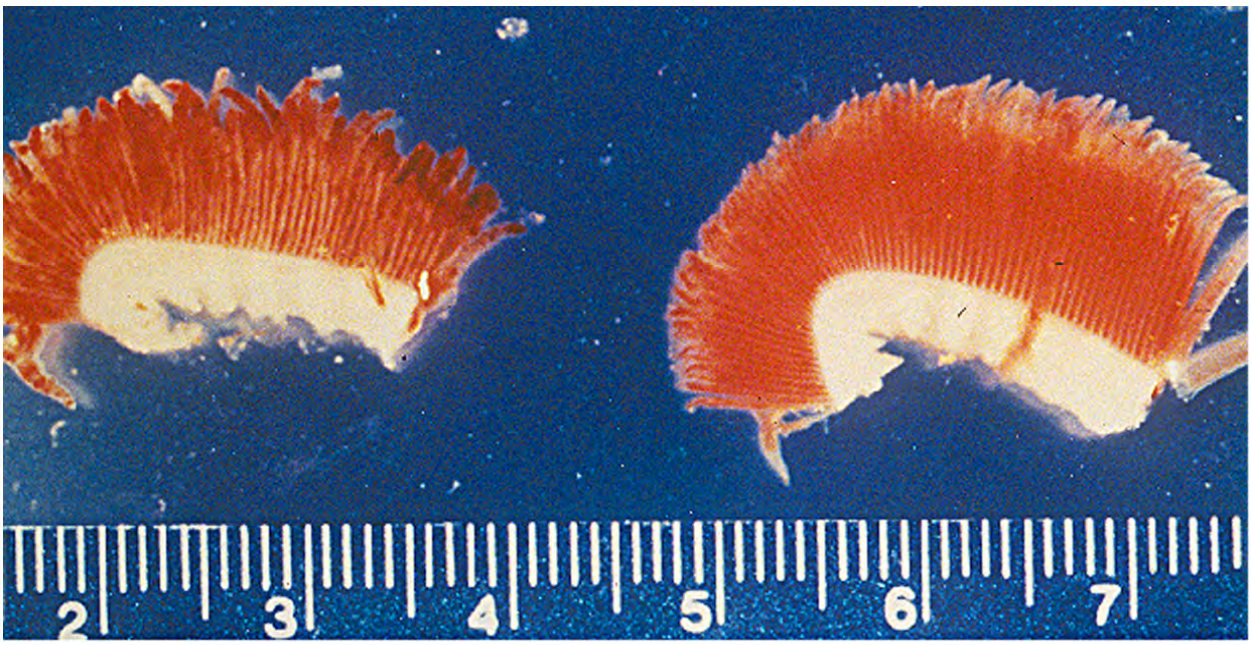


Figure 5. PGD infected gill (left) and normal uninfected catfish gill (courtesy of Larry Hanson).



Figure 6. PGD infected channel catfish demonstrating cartilage damage (courtesy of Andy Goodwin).

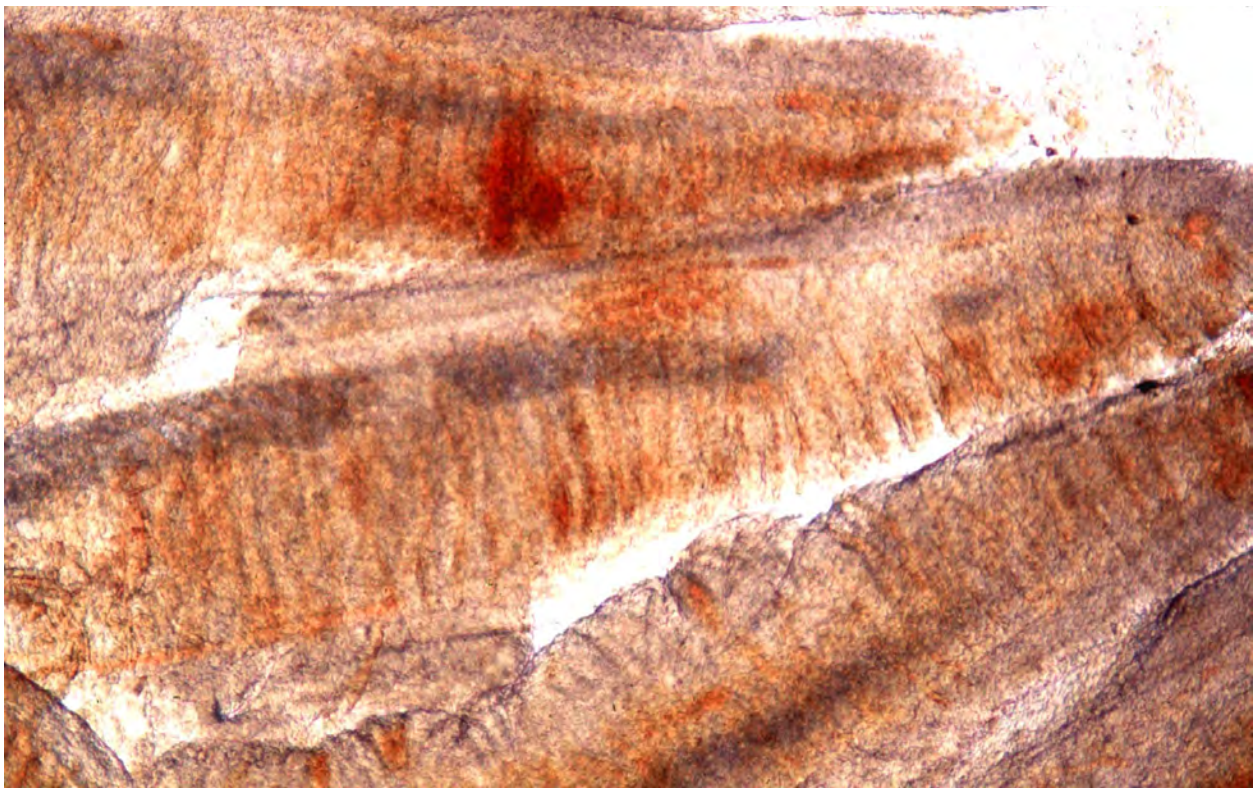


Figure 7. Wet mount of PGD infected channel catfish demonstrating cartilage damage (courtesy of Andy Goodwin).

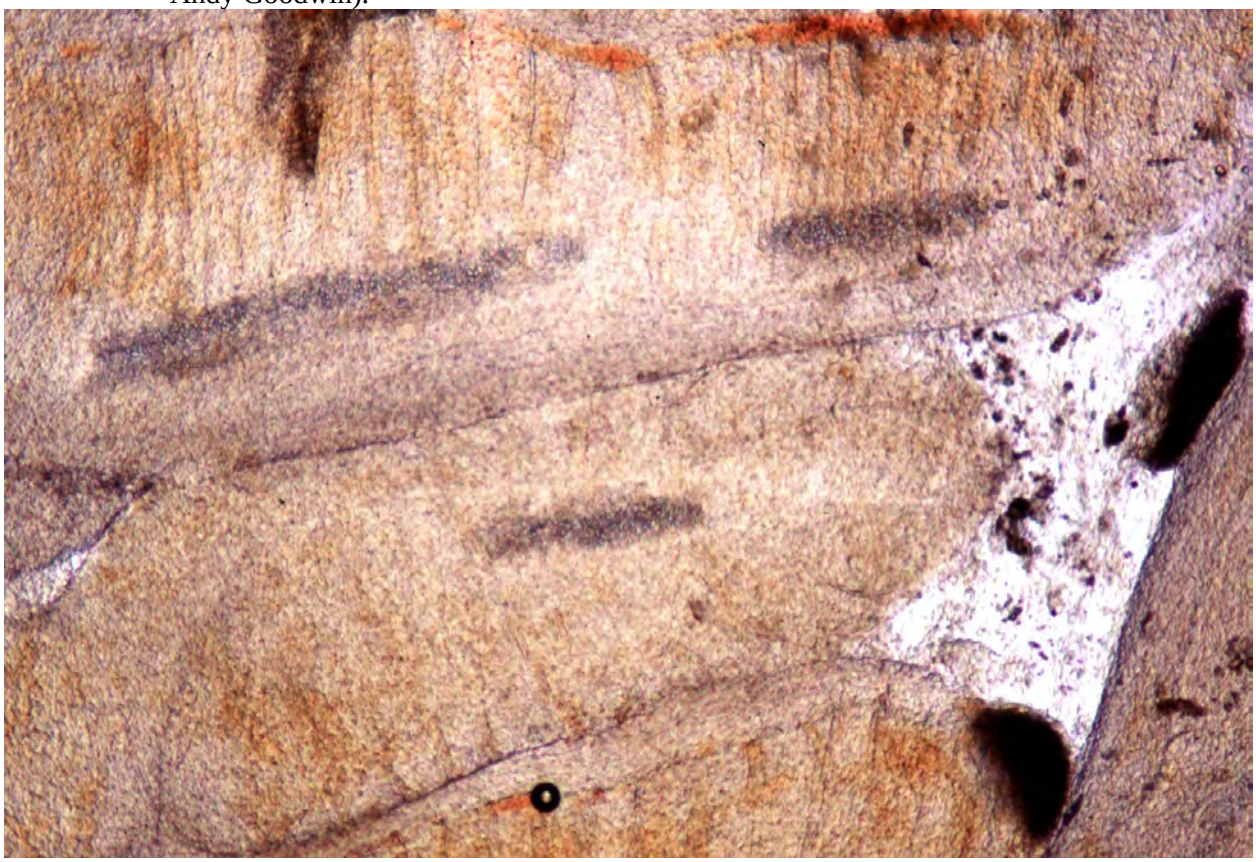


Figure 8. Wet mount of PGD infected channel catfish demonstrating cartilage damage (courtesy of Andy Goodwin).

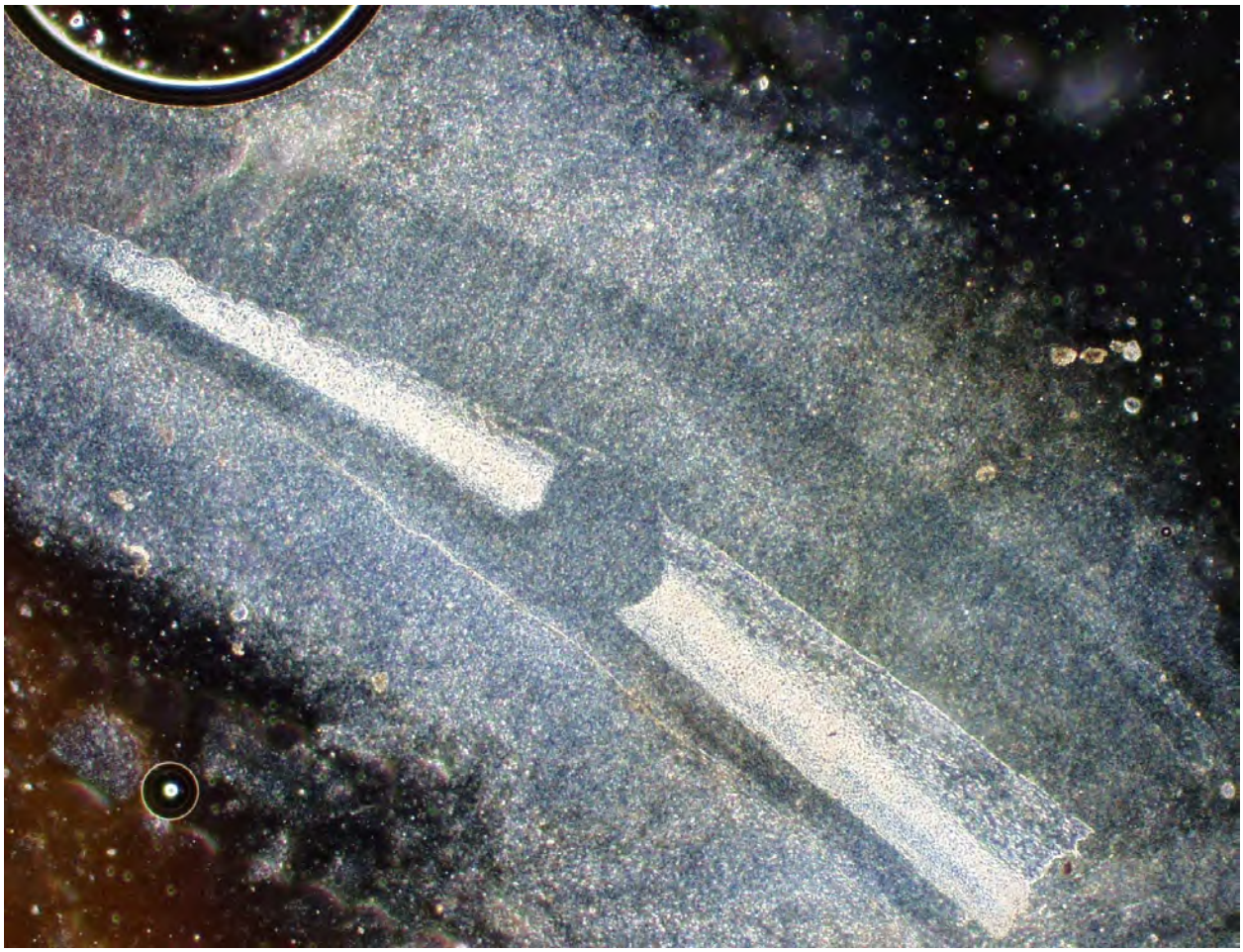


Figure 9. Phase contrast photomicrograph of a PGD infected catfish gill filament demonstrating cartilage damage and epithelial hyperplasia (courtesy of Larry Dorman).

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

Presumptive diagnosis is based on the clinical signs and the gross lesions (swollen, clubbed gill filaments together with congestion and hemorrhage). Defects in the cartilage of the branchial filaments can be detected on microscopic examination of gill wet mount preparations. These defects can also be present in subclinical cases especially during the cooler months and this may be due to a function of delayed healing.

2. Definitive Diagnosis

Presence of the parasitic trophozoites on histological preparations are required for definitive diagnosis (Figure 2). These are sometimes not readily evident and several sections may have to be examined. Only rarely are these early stages evident on gill wet mount preparations. Molecular confirmation of early gill stages of *H. ictaluri* and the final myxospore stage can be accomplished by using *H. ictaluri* specific polymerase chain reaction assay (Hanson et al. 2001; Whitaker et. al 2002; Section 1, 3.7.A1 PGD Appendix 1).

F. Procedures for Detecting Subclinical Infections

Often the only indication of a subclinical infections are the cartilaginous defects (breaks) that are evident on microscopic examination of wet mount or histological preparations of gills. The presence of *H. ictaluri* spores can not be detected in the gills until several months after initial infection. There are numerous *Henneguya* species in channel catfish with similar spore morphology, thus identification *Henneguya ictaluri* requires molecular confirmation (Section 1, 3.7.A1 PGD Appendix 1).

G. Procedures for Determining Prior Exposure to the Etiological Agent

No procedures have been reported.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

In order to detect the stages of this organism in the gills by visual examination, live or freshly dead fish should be kept at low temperatures (preferably on ice) prior to submission. Gill samples may also be placed in 10% buffered formalin for routine histology, however, early stages of this parasite (prior to day three post-infection) may not be detectable. Samples for molecular analysis can be frozen or placed in 10% buffered formalin or in 70% ethanol.

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3.7.A1 Proliferative Gill Disease Methods Appendix 1

L. M. Pote, L. A Hanson , L. Khoo*

Department of Basic Sciences College of Veterinary Medicine,
Mississippi State University, Mississippi State, MS 39762
662/325-1194
lpote@cvm.msstate.edu
662/325-1202
hanson@cvm.msstate.edu

*School of Veterinary Medicine,
University of Pennsylvania, New Bolton Center,
610/444-5800
lkhoo@vet.upenn.edu

Protocol for the Molecular Detection of *Henneguya ictaluri* in Gills Using Polymerase Chain Reaction Assay (Hanson et al. 2001; Whitaker et al. 2001)

A. Sample Collection

DNA contamination is the major problem that must be avoided in diagnostic PCR. The highest risk is from PCR products. All tissue processing, PCR product work, and PCR set-up should be performed in physically separated lab spaces. Pipette use should be similarly designated and aerosol resistant pipette tips used. Mix master mixes under a UV pretreated work space, aliquot the master mix, and add the DNA template last in a separate location from the master mix preparation site. Use gloved hands for opening tubes and change the gloves between samples of DNA template. Using new or stringently cleansed chlorox and treated or baked lab utensils, remove a sample of gill filaments.

B. DNA Extraction

1. Suspend 40 to 50 mg of fresh gill tissue in cell lysis solution (Puregene DNA Isolation Kit; Gentra Systems, Inc., Minneapolis, Minnesota) with 100 µg proteinase K/mL.

2. Incubate for two hours at 55°C.

3. Purify according to manufacturer's instructions.

4. Note: modifications of this protocol for formalin fixed tissue include incubating fixed tissue in cell lysis solution at 65°C for 15 minutes, homogenizing the tissue with a microfuge pestle, followed by the proteinase K digestion and extraction as outlined above.

C. PCR Procedure (Hanson et al. 2001; Whitaker et al. 2001)

The *A. ictaluri* specific PCR reaction mixtures contains reaction buffer (15 mM Tris, pH 8.0; 50 mM KCl; 1.5 mM MgCl 2), 20 pmol of each primer (upper 5' CAAAAGTTTCTGCTATCATTG 3' and lower 5'AGCGCACAGATTACCTCA3'), 200 µM of each of dATP, dCTP, dGTP, and dTTP, 1.25 units *Taq* Gold DNA Polymerase (Perkin-Elmer), and 2 µL of template DNA containing 1µg of DNA in a total volume of 50 µL. The PCR incubations consist of three steps.

1. Step 1 is one cycle of 94°C for 10 minutes, 50°C for one minute and 72°C for 30 seconds. This activates the TAQ Gold.

2. Step 2 consists of 20 cycles of 92°C for one minute, 50°C for 15 seconds, and 72°C for 15 seconds.

3. Step 3 is an extension cycle at 72°C for five minutes.

All diagnostic PCR should include positive (10 ng of plasmid clone containing the *H. ictaluri* rRNA gene) and negative controls (water) as template. The 104-bp PCR amplification product is expected and can be visualized by electrophoresis on a 10% polyacrylamide gel and staining using ethidium bromide or Gel Star (FMC BioProducts, Rockland, ME). We recommend the use of a 20 bp ladder such as that produced by Bio-Rad (Hercules, CA) as a size standard. For subclinical infection use 35 cycles in Step 2 instead of the 20 cycles described above. Because the subclinical infection detection method is much more sensitive than the method for confirming clinical infection, stringent measures should be observed to avoid DNA cross contamination. The use of dUTP and uracil N-glycosyalase (Applied Biosystems Foster City, CA) in the PCR reactions as well as known negative control tissue in the extraction process are quality control measures that should be considered. Confirmation of PCR product can be done by direct sequencing (Whitaker et al. 2001) the product will match nucleotides 1515 to 1619 of GenBank Accession AF195510.

ACAAAAGTTTCTGCTATCATTGTTGAAGGCATCGTATAGTTGCCAAGGGTGTATTGGTTGAG
GTGGCAACATCAAGGCTGATGCATTTGAGGTAATCTGTGCGCT

Figure 1. Nucleotide sequence of *H. ictaluri* PCR product.

3.8 Proliferative Kidney Disease

Michael Kent

Department of Microbiology
220 Nash Hall
Oregon State University
Corvallis, OR 97331
michael.kent@orst.edu

A. Name of Disease and Etiological Agent

Proliferative kidney disease (PKD) is caused by the PKX myxozoan, which was recently described as *Tetracapsuloides bryosalmonae* (syn. *Tetracapsula bryosalmonae*, *Tetracapsula renicola*) (see Canning et al. 2002).

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

The disease has been reported in major rainbow trout producing countries throughout Europe. In North America, PKD has been reported in the United States from California, Idaho, Washington, and in Canada from British Columbia and Newfoundland.

2. Host Species

Natural infections of PKX have been reported in rainbow trout and steelhead *Oncorhynchus mykiss*, cutthroat trout *Oncorhynchus clarki*, chinook salmon *Oncorhynchus tshawytscha*, coho salmon *Oncorhynchus kisutch*, Atlantic salmon *Salmo salar*, brown trout *Salmo trutta*, arctic char *Salvelinus alpinus*, grayling *Thymallus thymallus*, and pike *Esox lucius*. Infections have been observed in captive and free-living populations. Kokanee salmon *Oncorhynchus nerka* and chum salmon *Oncorhynchus keta* have been experimentally infected.

Bryozoans are required alternate host in the life cycle. Species of bryozoans that have been identified as host for PKX include *Plumatella emarginata* and *Fredericella sultana* (Canning et al. 1999; Longshaw et al. 1999).

C. Epizootiology

Proliferative kidney disease is caused by presporogonic stages of the parasite that infect the kidney interstitium and other well-vascularized organs. Incomplete sporulation occurs in kidney tubules. Clinical disease is primarily observed in young-of-the-year fish when summer water temperatures are greater than 12^{o}C. Previously uninfected yearlings are susceptible, whereas survivors show strong resistance to reinfection. Infections are detected approximately one month after exposure and clinical

disease follows about 3 to 4 weeks later. Mortality is chronic and extremely variable (5-90%). High mortalities are often associated with secondary infections of *Aeromonas salmonicida*, *Flexibacter columnaris*, or *Ichthyophthirius multifilis*. Recovery from PKD begins to occur in an infected population at about 12 weeks post-exposure and most fish that survive recover after 20 weeks.

D. Disease Signs

1. **Behavioral Changes Associated with the Disease**
   Fish with PKD are often lethargic and dark.

2. **External Gross Signs**
   Affected fish exhibit exophthalmia, lateral body swelling, and a distended abdomen. Gills are pale due to anemia.

3. **Internal Gross Signs**
   Internal gross signs are ascites and enlargement of the spleen and the posterior or entire kidney. The kidney is often grey and mottled and uniformly enlarged, and the kidney capsule is often distended ventrally in a corrugated pattern (Figure 1, Figure 2, and Figure 3).

4. **Histopathological Changes Associated with the Disease**
   Histopathological examination shows the kidney interstitium to be the primary site of infection, where the parasite evokes a chronic interstitial nephritis. The initial stage of the disease is characterized by hematopoietic hyperplasia. This is followed by diffuse, chronic inflammation. Coalescing whorls of inflammatory cells, primarily macrophages, surround the parasites (Clifton-Hadley et al. 1984, 1987; Smith et al. 1984; MacConnell et al. 1989). Parasites also infect blood vessels, where they adhere to vessel walls, occlude vessels, and evoke a necrotizing vasculitis. Well-vascularized extrarenal organs (e.g. gills, liver, spleen, and pancreas) also are infected and exhibit similar histologic changes as found in the kidney (Figure 4).



Figure 1. Macroscopic changes of PKD in rainbow trout. Note bloody ascites, enlarged spleen and pale gills. Kidney shows moderate enlargement in posterior region.



Figure 2. Chinook salmon with PKD, compared to normal (N). Note uniformly enlarged kidney in lower fish with PKD.



Figure 3. Macroscopic changes of PKD in cutthroat trout. Note enlarged spleen, kidney, and pale gills.

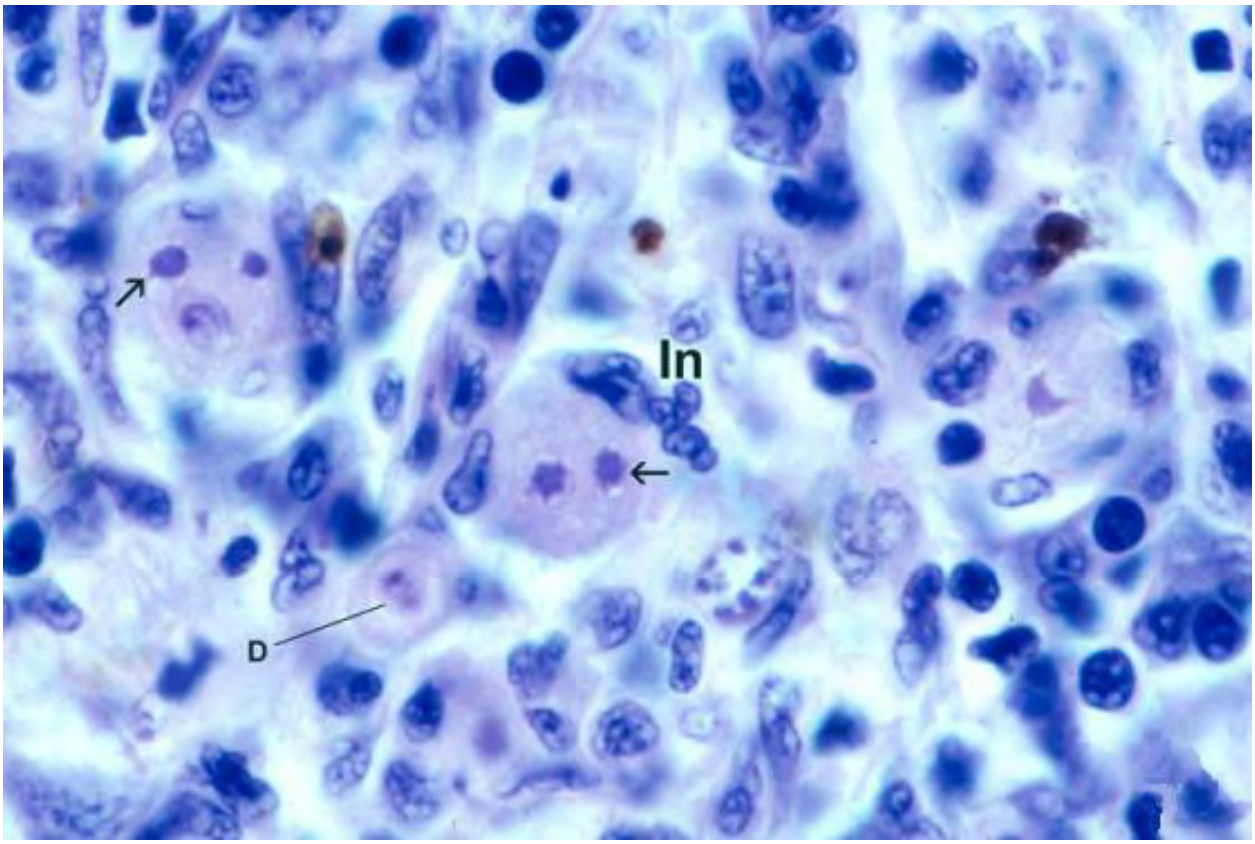


Figure 4. PKX cells in the kidney interstitium in histological section stained with H&E. Arrows = primary cell nuclei, D = daughter cell, In = inflammatory cells surrounding parasites.

E. Disease Diagnostic Procedure

1. Presumptive Diagnosis

The PKX parasite can be presumptively identified in Giemsa-stained imprints of the posterior kidney or spleen (Klontz and Chacko 1983). In these preparations, PKX parasites are round, 10 to 20 µm in diameter, and have a light staining, vacuolated cytoplasm (Figures 5 and 6). One to seven secondary (daughter) cells are found within the cytoplasm of the primary (mother) cell. Intensely stained inflammatory cells often are attached to and surround the parasite. Wet mounts may also be used for presumptive diagnosis, in which the PKX parasite occurs within the kidney interstitium. PKX organisms can be differentiated from host cells by the presence of a distinctive cell membrane and small nuclei (Figure 7).

2. Confirmatory Diagnosis

Confirmatory diagnosis is based on histological examination of tissue sections stained with hematoxylin and eosin (Figure 4) or tissue imprints stained with the GS-1 specific lectin as described by Hedrick et al. (1992). For histology, the principal diagnostic feature of PKD is the presence of the PKX parasites in the renal interstitium. The PKX parasite is amoeboid, 5 to 20µm in diameter, and has a foamy, eosinophilic cytoplasm. The primary cell contains 1 to 3 distinctive nuclei, which are characterized by the presence of a large, intensely stained, eosinophilic endosome (nucleolus). One to seven spherical, dense daughter (secondary) cells are present in most of the parasites. Some of the secondary cells contain tertiary cells. Inflammatory cells are attached to and surround many of the PKX parasites (Figure 4).

Detection of PKX organisms in tissue imprints stained with lectin (Hedrick et al. 1992) is as follows: a) Kidney or spleen pieces tissues are blotted on clean paper tissues to remove excess blood and imprints are made on clean slides. b) After air drying for 30 minutes, imprints are fixed in acetone ethanols (60:40) at -20°C for 10 minutes. c) Slides are air dried and stained immediately or stored at -70°C under desiccated conditions.

The biotinylated GS-1 lectin from *Griffonia simplicifolia* (L-37359 Sigma, St. Louis) is suspended in 0.01M phosphate buffer pH 6.8 at 5-10 µg/L. Aliquots are place on the imprints and incubated for 1 hour at 25°C. Slides are rinsed three times in PBS and fluorescine at 30 µg/L is placed over the imprints. Slides are again incubated in a moist chamber at 25°C for 30 minutes. Slides are rinsed three times, carefully blotted dry, and mounted with a coverslip using a drop of a mixture containing 1 part 0.1M N-2-hydroxy-ethylpiperazin-N'-2-ethanesulfonic acid (HEPES) pH 8.0 and 9 parts glycerol. The specimens are observed under a UV microscope.

PKX cells stain brilliant apple green with a granular cytoplasm (Figure 8). Nuclei and daughter cells appear as dark, subspherical bodies within the cytoplasm of the PKX organism. This lectin has also been used to identify PKX in tissue sections (Figure 9) (Marin de Mateo et al. 1996).

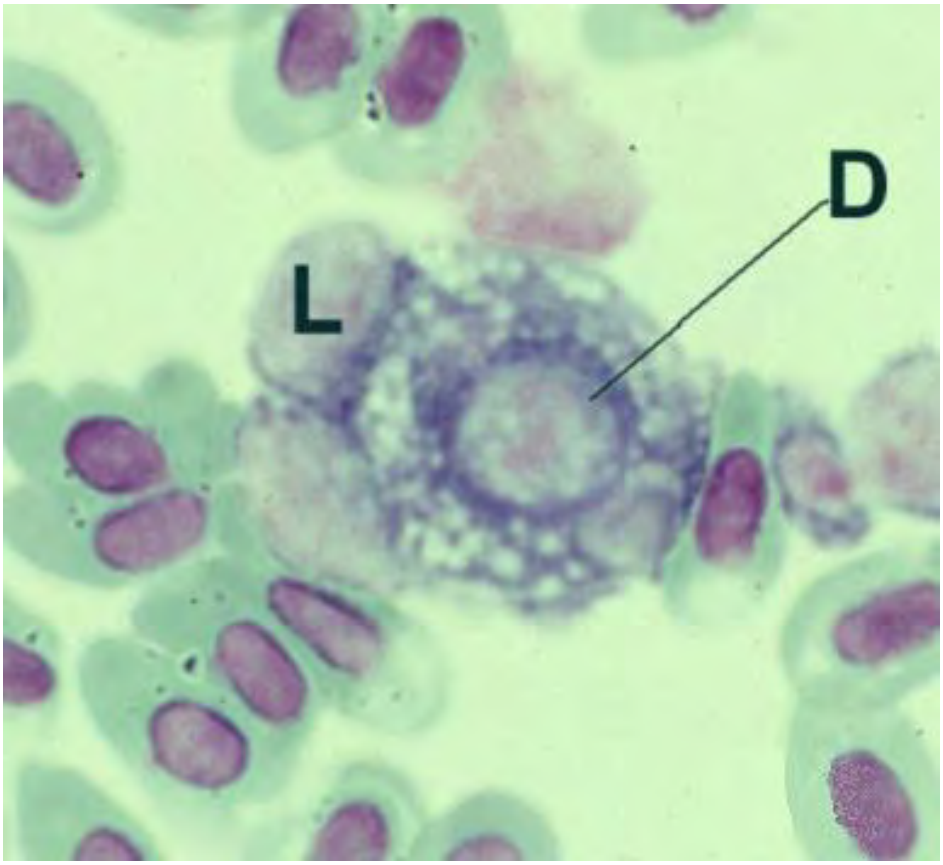
Monoclonal antibodies specific to PKX have been also used to visualize parasites in tissue sections (Adams et al. 1992; Marin de Mateo et al. 1996).

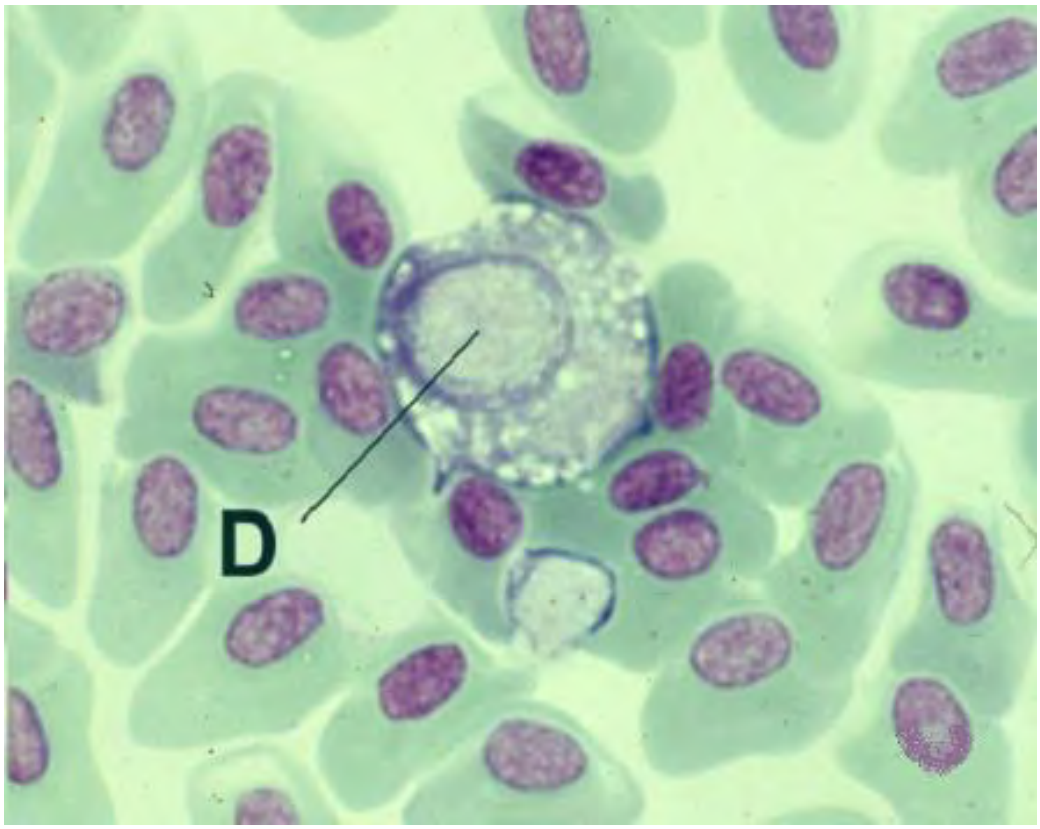
F. Procedures for Detecting Subclinical Infections

Necropsy of apparently healthy fish that are examined during the summer months from waters in which the parasite is enzootic will frequently reveal renal and splenic hypertrophy. Histological examination of the kidney will often reveal the parasite and the typical tissue reaction, although the latter is usually milder than that found in clinically affected fish.

Examination of wet mounts with phase microscopy or kidney imprints is also effective for detecting subclinical infection.

PCR are also useful for detecting light infections. PCR tests using small subunit ribosomal DNA primers were reported by Kent et al. (1998). The most sensitive and specific primer sets appear to be PKX 3f and 4r or PKX 5f and 6r (Kent et al. 1998; Morris and Adams 2002).





Figures 5 and 6. PKX cells in Giemsa imprints. Note lymphocytes (L) surronding parasite. PKX cells has foamy cytoplasm and contains daughter cells (D).

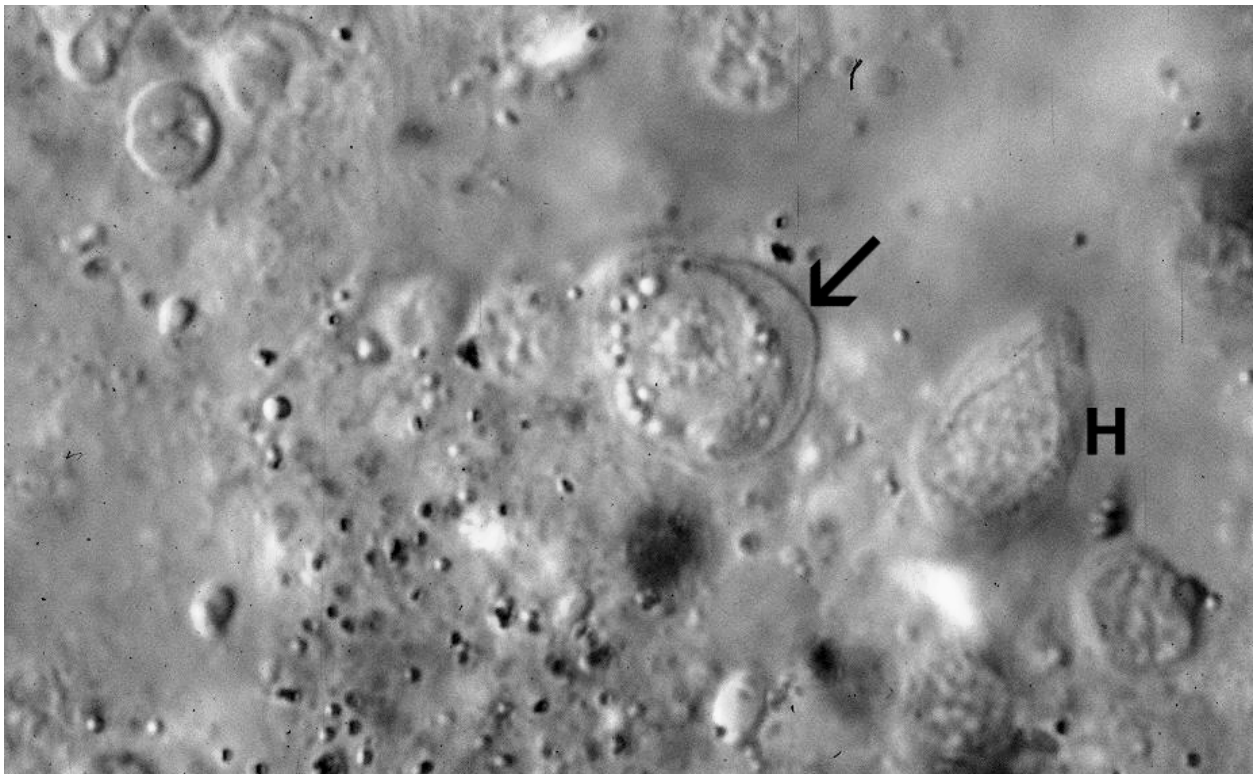


Figure 7. Wet mount preparation of salmonid kidney showing PKX. Note distinctive cell membrane (arrow) and smaller nucleus compared to host cells (H). Cytoplasm of PKX is granular. Nomarski phase contrast.

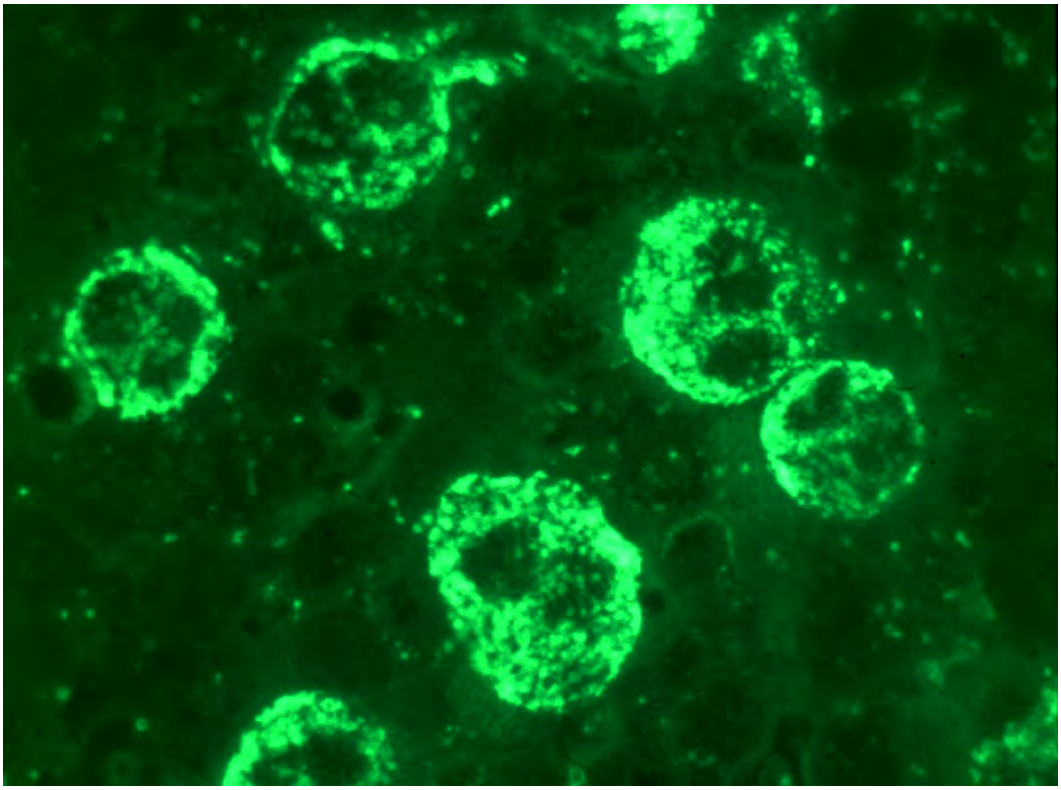


Figure 8. PKX cells in tissue imprints stained with biotinylated GS-1 lectin. Note granular cytoplasm of primary cells.

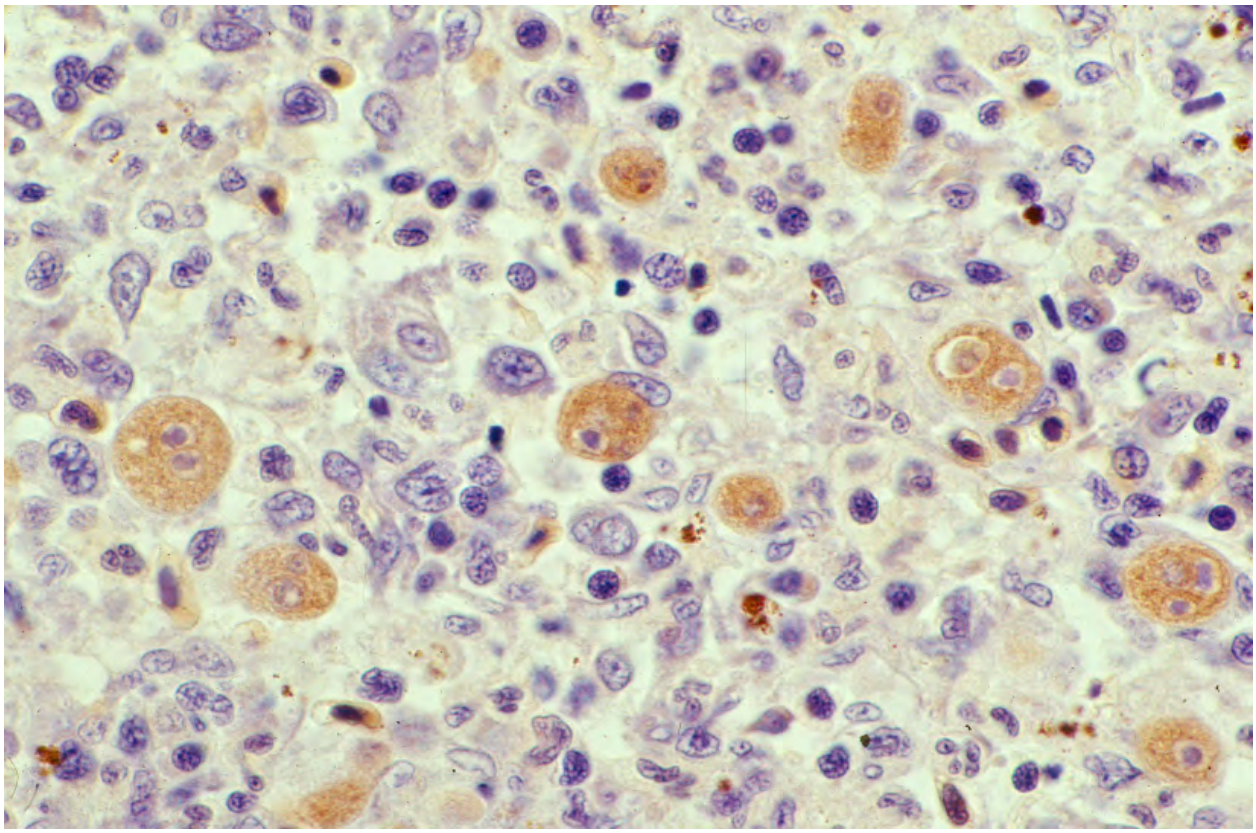


Figure 9. PKX in tissue sections stained with GS-1 lectin.

G. Procedures for Determining Prior Exposure to the Etiological Agent

Sporogonic forms (pseudoplasmodia; Figure 10) are found in the lumina of kidney tubules late in the disease and persist for several months in recovered fish. These forms are comprised of an enveloping cell with many refractile granules and contain a monosporous sporoblast. In wet mounts, the sporoblasts are oblong, 12 X 7 µm and often contain two spherical polar capsules 2 µm in diameter (Kent and Hedrick 1986; Kent et al. 2000). The sporoblast is pliable due to apparent incomplete development of the spore valves. The presence of these sporoblasts in the lumina of kidney tubules during the fall or winter may indicate prior infection by the interstitial form of PKX. The diagnosis of PKX based on the presence of these forms only, however, should be tentative because an inexperienced diagnostician may confuse these incomplete spores with those of other coelozoic (lumen dwelling) myxosporeans that infect the kidney. The PCR tests are useful for confirming the identity of sporogonic forms of PKX (Kent et al. 1998). These PCR tests have also been used to confirm the identity of PKX in bryozoans.

Morris and colleagues (see review by Morris and Adams 2002) have used these PCR primers in *in situ* hybridization tests to identify the parasite in tissue sections. Using this approach, Morris et al. (1999) further linked the intraluminal sporogonic forms to PKX

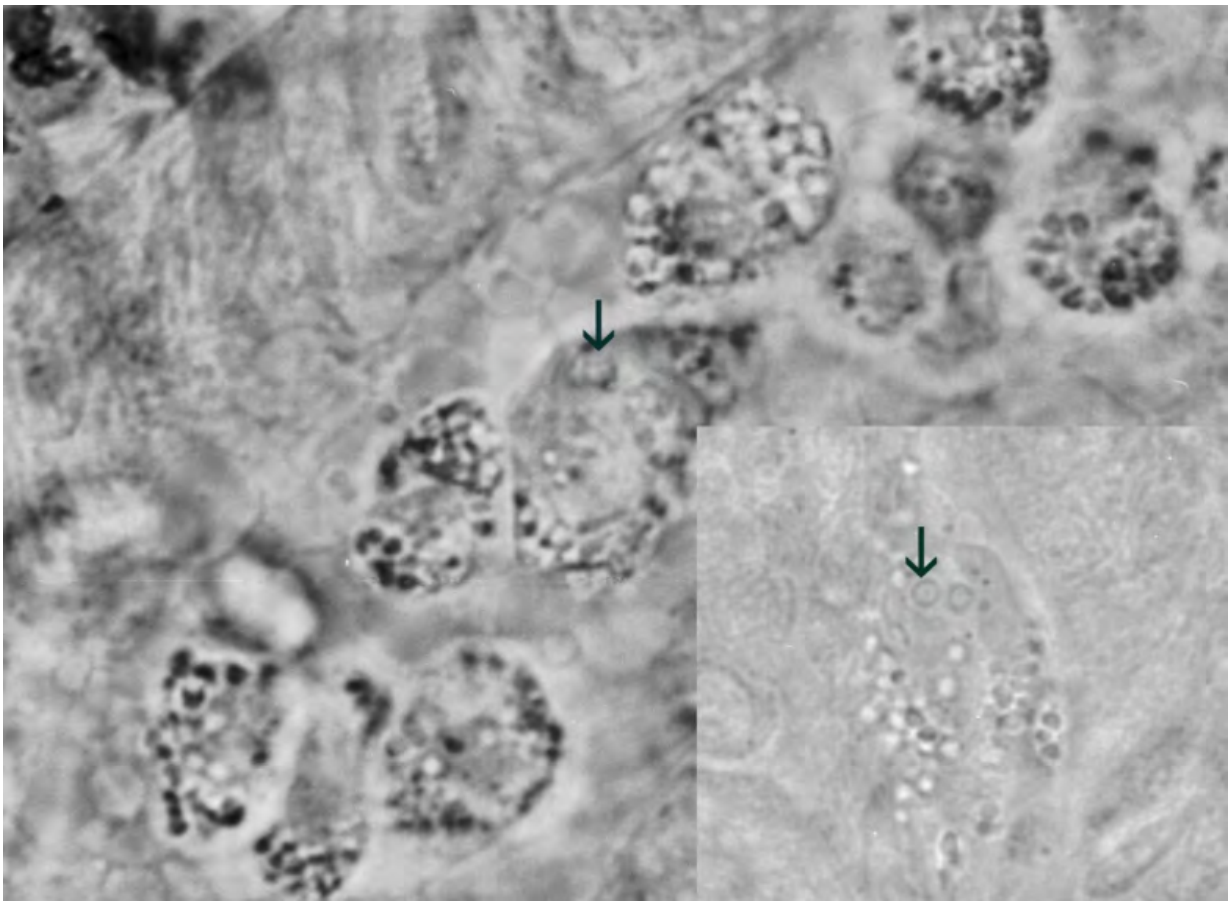


Figure 10. Wet mount of kidney showing PKX sporogonic forms in the lumen of kidney tubules. Arrow = polar capsules.

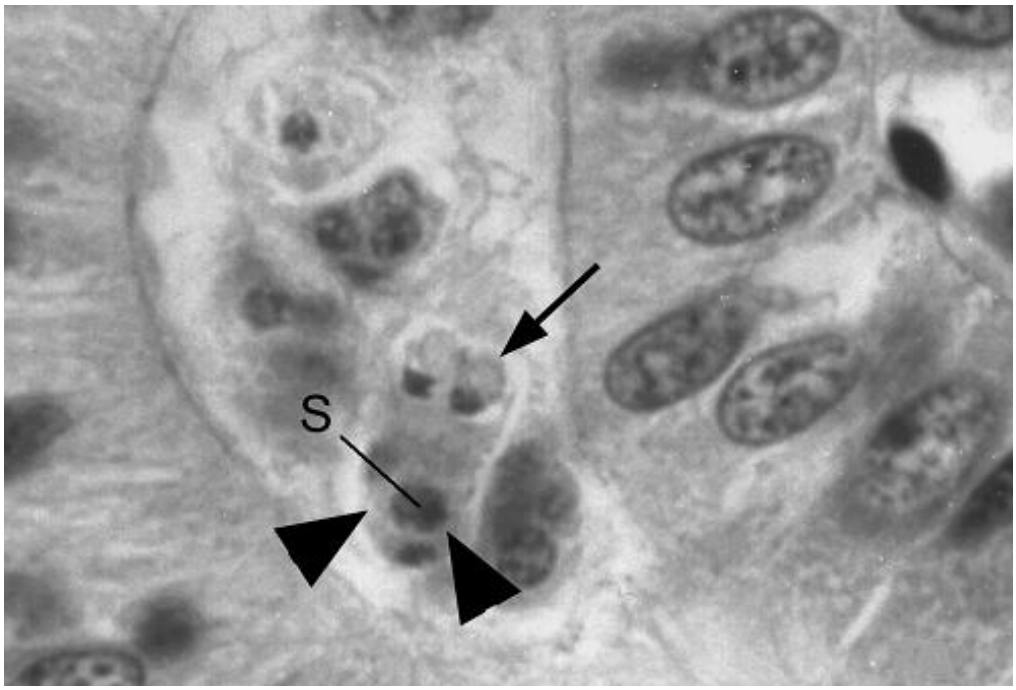


Figure 11. Histological sections of kidney tubules with spores of PKX. Hematoxylin and eosin stain showing polar capsules (arrows), sporogonic nuclei (S).

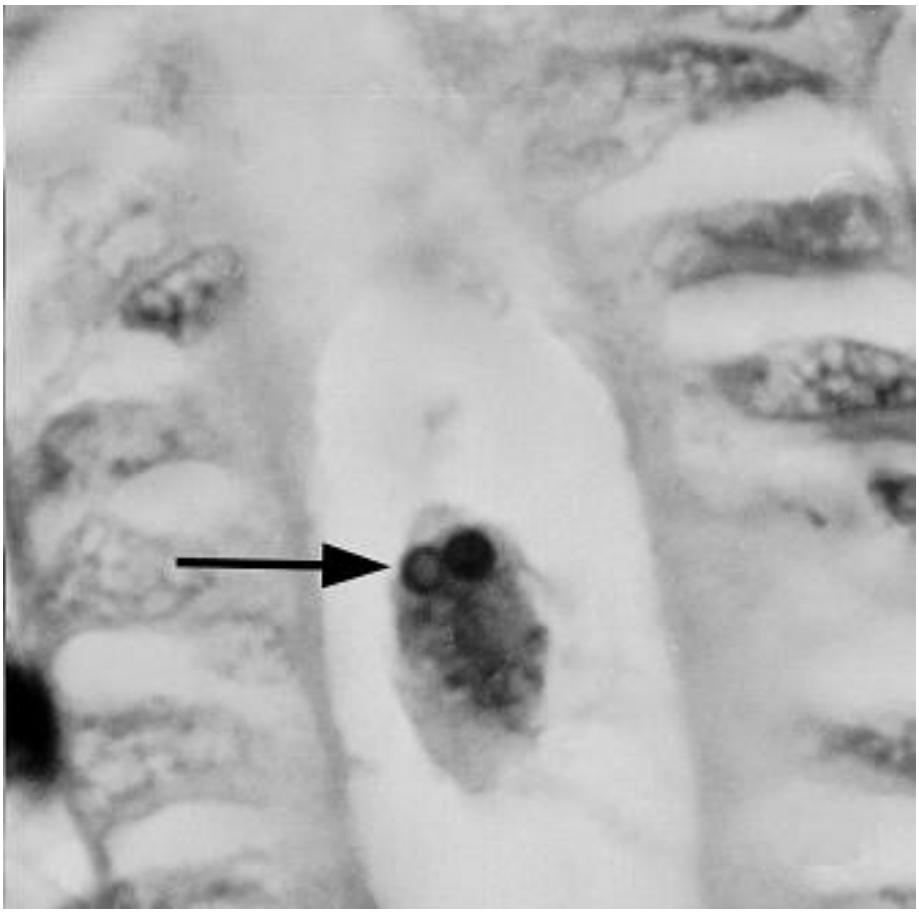


Figure 12. Histological sections of kidney tubules with spores of PKX.). Giemsa stain showing dark blue polar capsules (arrows).

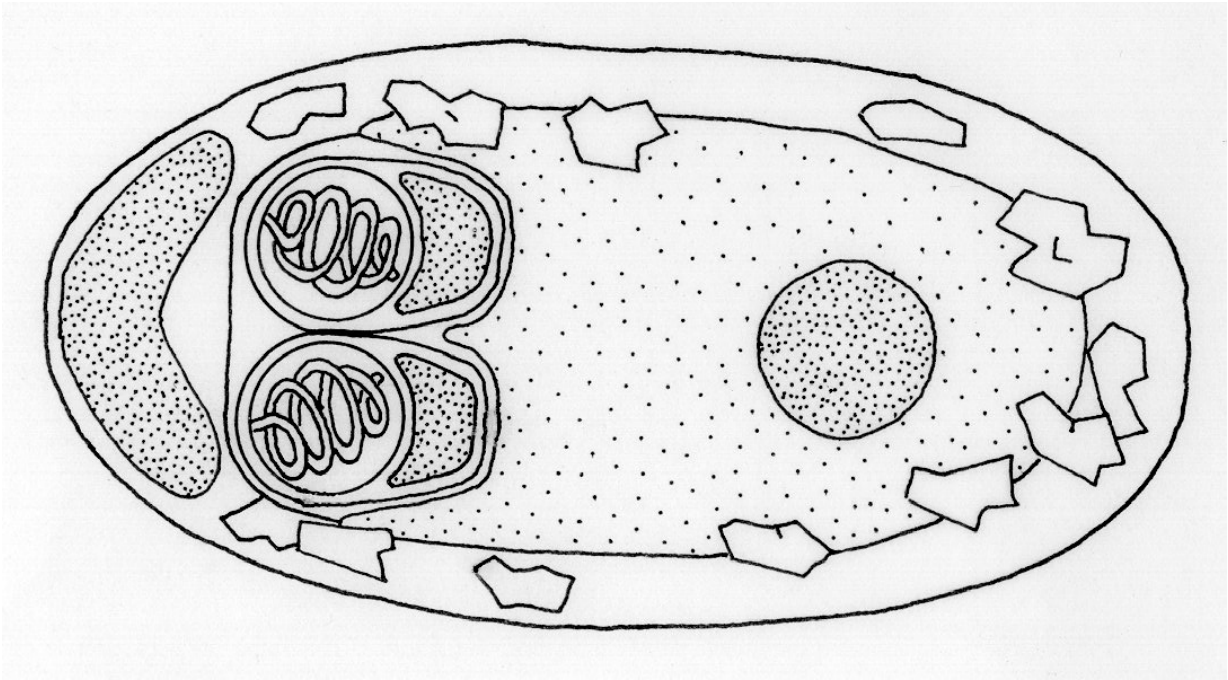


Figure 13. Line drawing of PKX sporoblast within pseudoplasmodium from kidney tubules.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Tissues for histological examination should be collected from freshly killed fish and fixed immediately on site in either Davidson's or Bouin's solution. Imprints should also be prepared from freshly killed fish. The PKX parasite may deteriorate very rapidly in iced or frozen samples.

I. Acknowledgements

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3.9 Ichthyophthiriasis

Margaret S. Ewing

Department of Zoology
Oklahoma State University
LSW 430
Stillwater, OK 74078
405/744-9689
msewing@okway.okstate.edu

A. Name of Disease and Etiological Agent

Ichthyophthiriasis is caused by the ciliate *Ichthyophthirius multifiliis*, a hymenostomatid protozoan.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Ichthyophthirius multifiliis is worldwide in distribution.

2. Host Species

This parasite infects virtually every species of freshwater fish. Mosquito fish *Gambusia affinis*, grass carp *Ctenopharyngdon idella*, and sunfishes *Lepomis* spp. appear to be somewhat less susceptible to infection than other species.

C. Epizootiology

The parasite is commonly found in small numbers on wild fish, and natural populations rarely experience heavy infections unless stressed. Disease outbreaks in cultured fish are common when naive fingerlings are brought in contact with older fish that may be carrying a few trophonts (the tissue-dwelling stage of the parasite), and the fish are crowded or otherwise stressed (Figure 1). As water temperatures increase in spring, the life cycle of the parasite proceeds more quickly and the trophonts and theronts (the free-living infective stage) become more abundant, sometimes resulting in disease outbreaks (Figure 2). Estimates of the length of the life cycle in the literature vary a good deal (Figure 3). At 21^{o}C the life cycle is completed in 5 to 7 days and at 10^{o}C in about 30 to 40 days. Water temperatures of 32^{o}C kill the theront. Outbreaks in summer and early fall are uncommon for nonsalmonids. However, the majority of reports of Ich in salmonid culture may occur during this same period, due to the differences in water temperature requirements. Outbreaks with extensive mortalities have also been reported in late winter, even at the time of ice break-up. Such epizootics appear to be associated with the late winter, poor nutritional status of fish and perhaps the existence of cold temperature strains of the parasite. About 10% of the trophonts on a fish may reproduce within the host epithelium, providing an additional mechanism for an increase in the intensity of

infection (Figure 4). Mortality may be high in fingerlings but is usually much lower in older fish that presumably have survived infection and acquired at least partial resistance to the parasite.

D. Disease Signs

1. Behavioral Changes Associated with the Disease

Flashing often results from the irritation the parasite causes in the epidermis. Heavy infections of the gill will interfere with respiratory exchange and may cause such infected fish to gasp or "pipe" for air at the surface.

2. External Gross Signs

Fairly discrete white spots up to 1 mm in diameter can be seen on the skin of the body and fins (Figure 5). In moderate to heavy infections, the skin appears "peppered" with white dots. Upon lifting the operculum, white spots may also be seen on the gills.

3. Internal Gross Signs

White spots sometimes are seen in the lining of the mouth or nares. The white spots (trophonts) have occasionally been observed in the peritoneal cavity.

4. Histopathological Changes Associated with the Disease

In the skin, the trophont may compress the overlying epidermis as it grows. Mixed inflammatory cells are commonly associated with trophonts in infections of more than five days duration (at 21^{o}C). About five days after infection, the overlying, often hyperplastic tissue lifts and tissue fluid accumulation contributes to the appearance of a fluid-filled cyst containing the trophont. Growth of trophonts as well as reproduction of the trophont and active aggregation of trophonts contribute to enlarging the cysts (Figure 4).

In the gill, the growing parasites cause distortion of the lamellae (Figure 6). In heavy infections epithelial hyperplasia is common, as are hyperplasia and hypertrophy of chloride cells. Lamellae may be fused. The exit of mature trophonts from gill epithelium leaves significant holes in the tissue, which contributes to osmoregulatory problems.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

a. Detection of the Pathogen
Presence of discrete white spots on skin suggests ichthyophthiriasis.

b. Clinical Signs
The signs described under D.1 through D.3 are characteristic. The absence of the parasite from the skin does not necessarily indicate absence of infection, as some fish may be infected only in the gills.

c. Histopathological Examination
Any or all the changes described under D.4 may be observed.

2. **Confirmatory Diagnosis**
   Squash preparation of gill or skin scrapings examined with at least 40X magnification, e.g., under a dissecting microscope, should reveal larger trophonts. These are uniformly ciliated and contain a horseshoe-shaped macronucleus (Figure 1). Staining is not needed.

F. Procedure for Detecting Subclinical Infection

Very small trophonts, such as those found within the first two days of infection at 21^{o}C, may be observed grossly on the pectoral fins. Scrapings at this stage will reveal the parasite. Examination of gill may be important in detecting subclinical infection although finding trophonts on the gill in very light infections is usually difficult.

G. Procedure for Determining Prior Exposure to the Etiological Agent

Prior exposure of a group of fish may be determined by exposing several fish in isolation to theronts to see whether they become infected. Typically, recovered fish are partially resistant to further exposure.

Alternatively, when a suspension of theronts is mixed with a serum sample from previously exposed fish, theronts are immobilized by specific immune serum.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Infected live fish may be shipped in water or a mixture of water and ice.

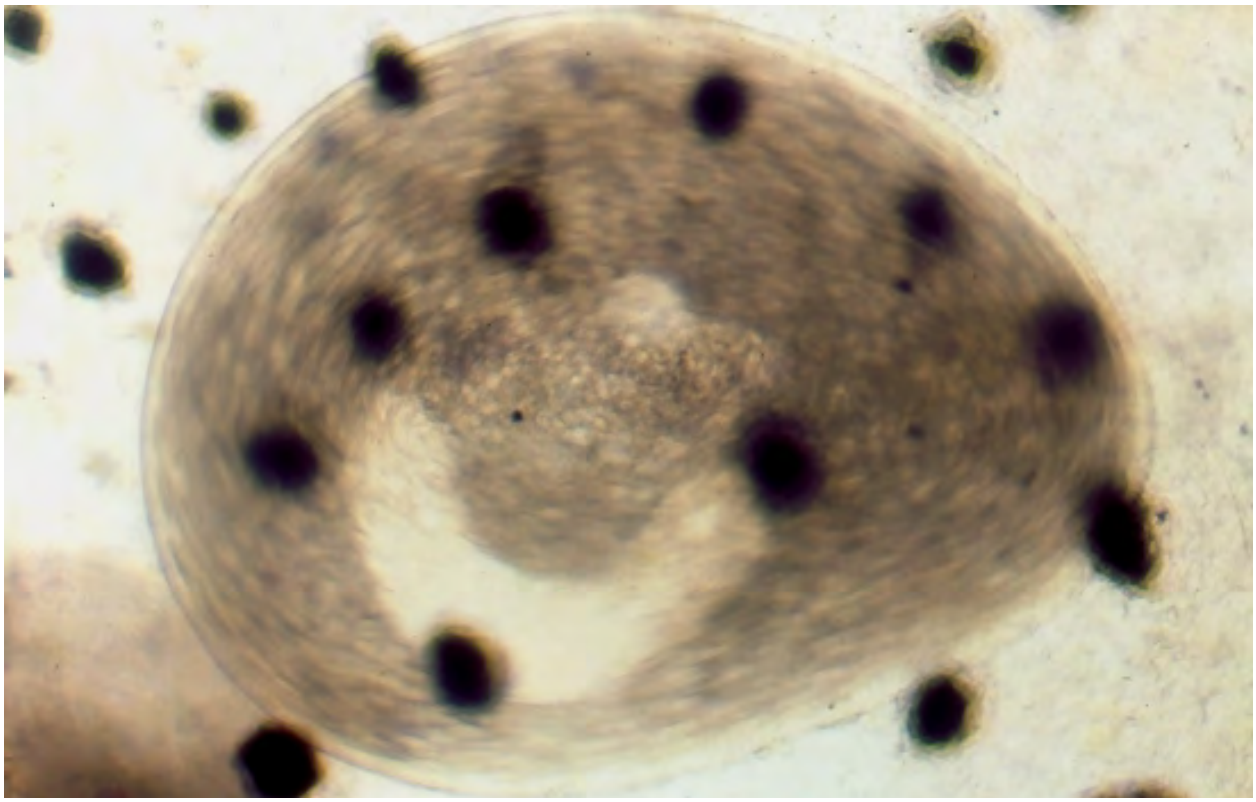


Figure 1. *Ichthyophthirius multifiliis* trophont in channel catfish epidermis. Note large horseshoe-shaped macronucleus, which is pale. Margin is blurred because trophont is moving in this fresh preparation. Black spots are in fish skin.

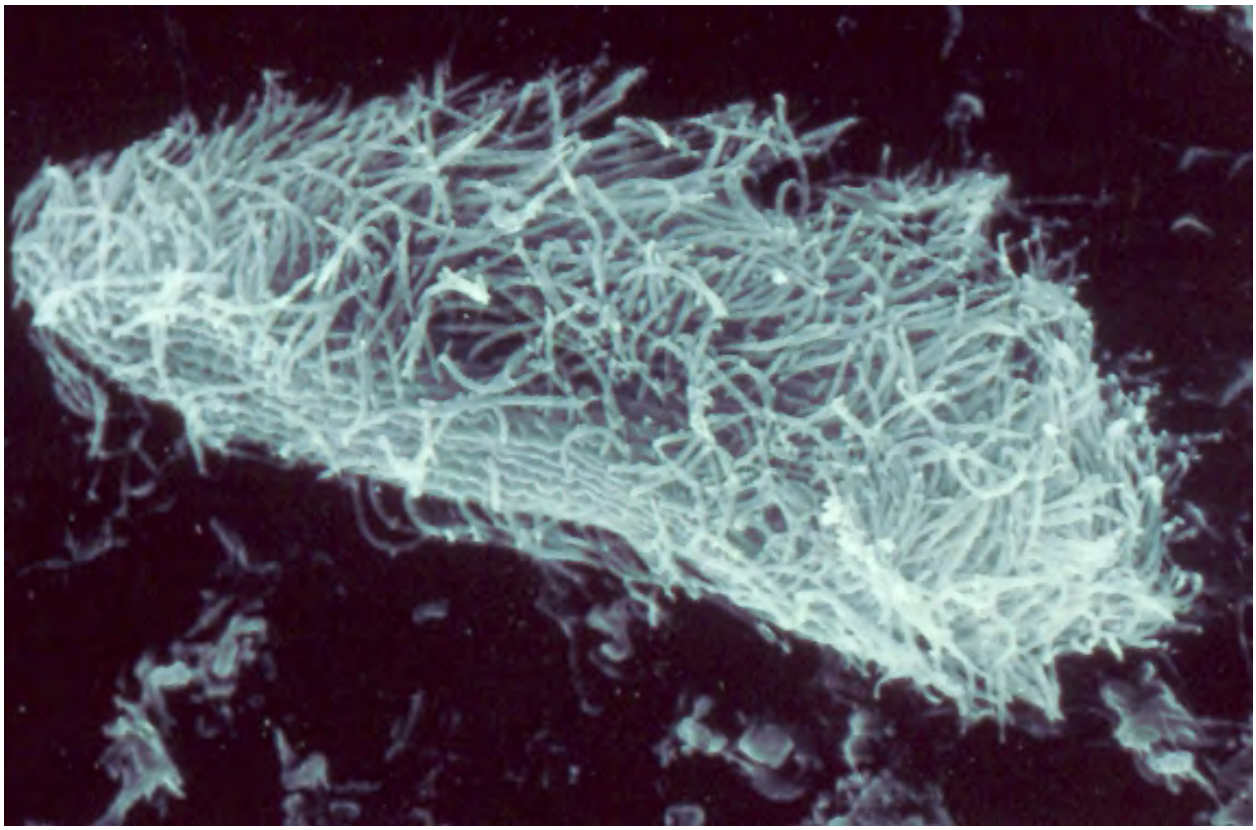


Figure 2. Theront as seen by scanning electron microscopy. Approximately 15 µm in length.

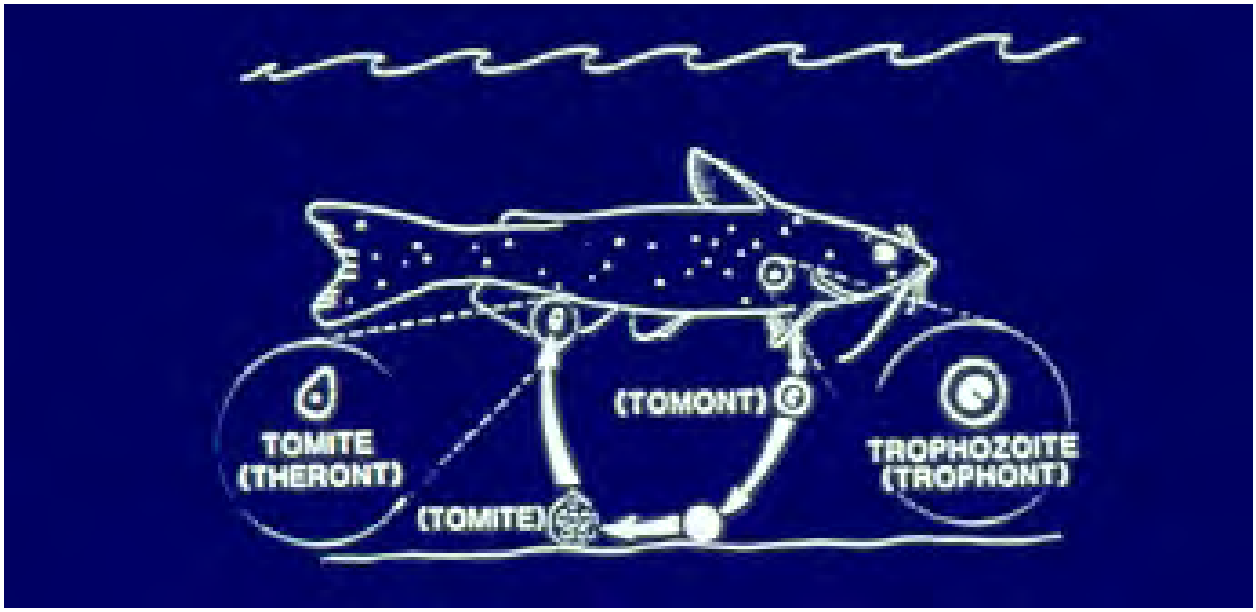


Figure 3. Life cycle of *Ichthyophthirius multifiliis.*

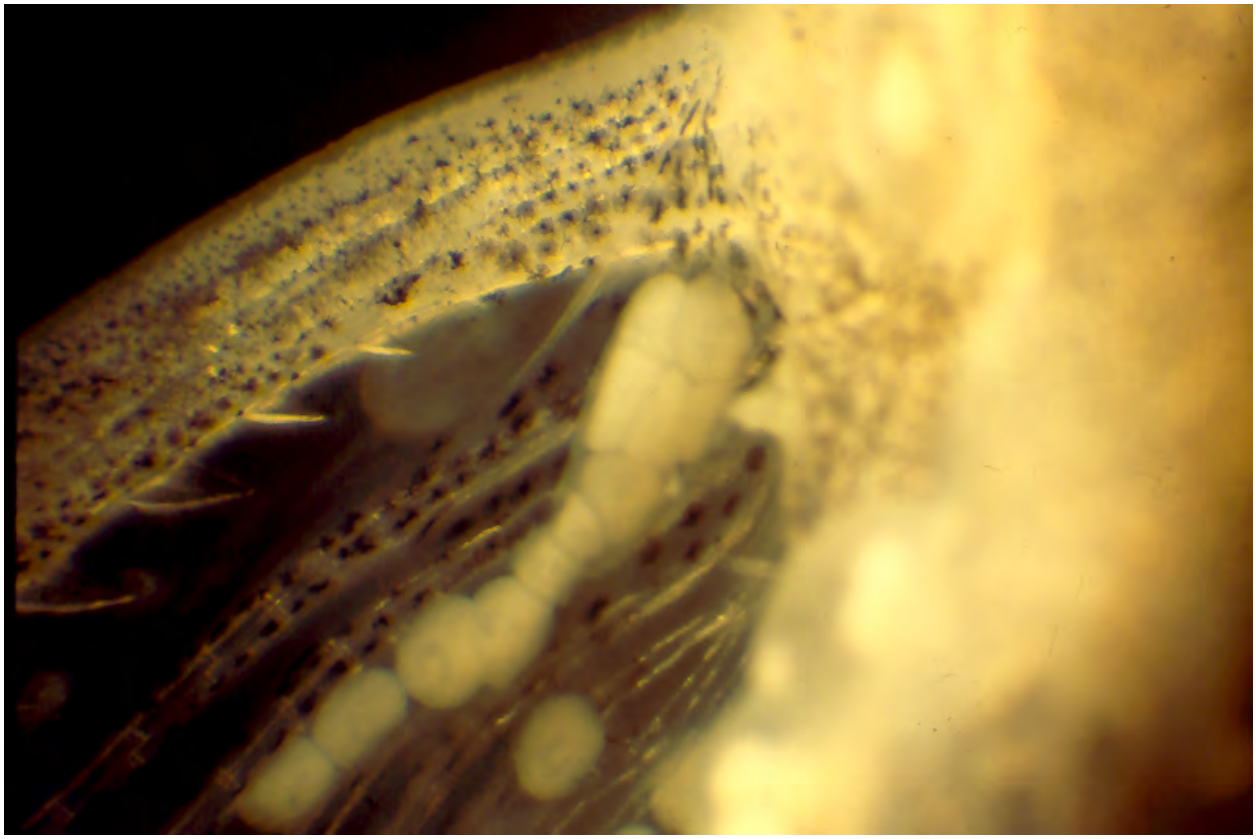


Figure 4. Group of at least 11 trophonts within a single cyst-like space within the epithelium of a channel catfish pectoral fin.



Figure 5. Channel catfish infected with *Ichthyophthirius multifiliis.* White spots are cystlike spaces containing trophonts.

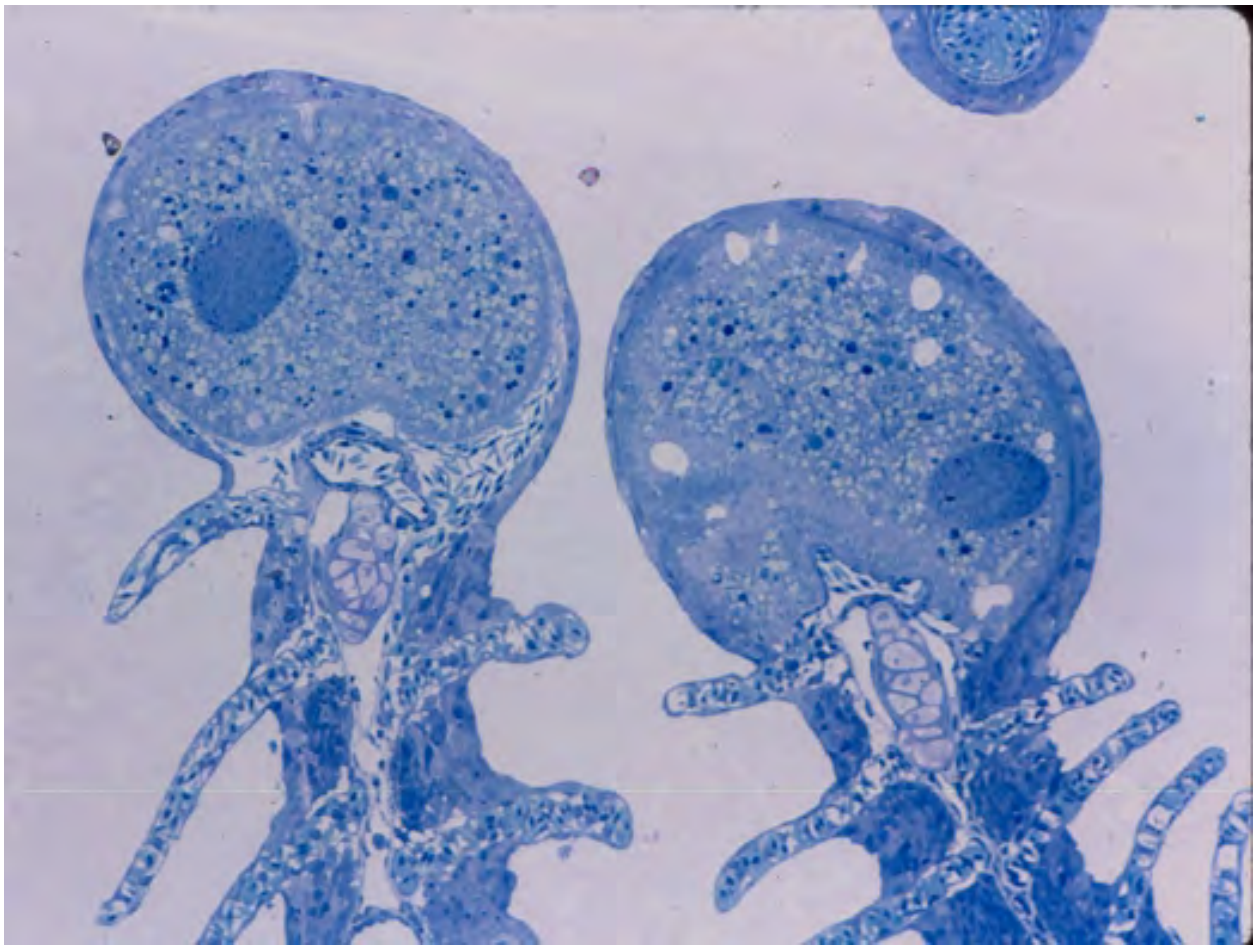


Figure 6. Gill filaments showing distortion caused by growth of the trophont. A small portion of the macronucleus appears in the trophonts in this section, stained a darker blue with Mallory's.

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3.10 External Infection by Ciliated Parasites

Peter W. Taylor and Andrew E. Goodwin*

Abernathy Fish Technology Center
1440 Abernathy Rd.
Longview, WA 98632
pete_taylor@r1.fws.gov

*University of Arkansas at Pine Bluff
Aquaculture/Fisheries Center
1200 N. University Dr.
Mail Slot 4912
Pine Bluff, AR 71601
870/543-8137
agoodwin@uaex.edu

The protozoans inhabiting the gills and body surfaces of fish are a diverse group of parasites. They occur in small numbers in almost all populations of fishes and only cause problems when predisposing factors in the environment place undue stress upon the host allowing heavy infestations to develop. In natural waters, these protozoa usually occur in very low numbers on the host species. Heavy infections in the wild are rare because water quality is usually good and fish are thinly dispersed. Heavy infections are more common in aquacultural situations where fish are crowded and water quality conditions may deteriorate. Presence of these parasites is to be expected in almost all lots of fish from almost all sources. Concern should not be over the presence of these parasites but with the degree of infection.

A. Name of the Disease and Etiological Agents

"External parasite infection" is the most common reference to this condition. It is comprised of four general groups of ciliated parasites: (1) motile Peritrichs (*Trichodina* spp., *Trichodinella* spp., *Tripartiella* spp.); (2) sessile Peritrichs (*Ambiphrya* spp., *Apiosoma* spp., *Epistylis* spp.); (3) motile Holotrichs (*Chilodonella* spp.); and (4) sessile Suctoria (*Capriniana spp*. formerly *Trichophrya* spp.). Other protozoan parasites including the holotrich *Ichthyophthirius* , the flagellated *Ichthyobodo* and *Spironucleus*, the *Myxosporea*, and the *Microsporea* are described in separate chapters in the Bluebook.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

These parasites are cosmopolitan and occur throughout the world. All are found in freshwater fish. *Trichodina, Apiosoma,* and *Chilodonella* have all been reported on fish from marine or brackish water systems.

2. Host Species
Most species of fishes are considered susceptible to these parasite groups.

C. Epizootiology

In a natural state these protozoans occur in low numbers on the fish and usually cause no harm. Under certain conditions these protozoans can increase in such numbers that the host becomes severely compromised. Crowding and poor water quality can stress the host rendering it less resistant and more susceptible to parasitization. Crowding of fish will facilitate fish to fish contact and parasitic transmission.

D. Disease Signs

1. Behavioral Changes Associated with the Disease
Moderate to heavily infected fish may go off feed. Infected fish may scrape against objects and display flashing behavior. Fish may also gasp at the water surface.

2. External Gross Signs
Skin may display changes in pigmentation and have excess mucus production. *Epistylis* spp. may produce bloody lesions on scaled fish and cottony growths associated with erosion of fins and spines in all species. Gills may appear swollen, hemorrhagic, or with heavy mucus.

3. Internal Gross Signs
None recorded.

4. Histopathological Changes Associated with the Disease
Parasites will be present in tissue sections. Gill tissue may show epithelial hyperplasia, underlying inflammatory cells, epithelial necrosis, and an increase in mucus cells. Skin tissue may show the same responses.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

a. Isolation and Detection of Pathogen
Diagnosis of external parasites is dependant upon clinical signs and observation of the organism in wet mounts of gill clips or skin scrapings using the 10X or 40X objective of a compound microscope.

i. Motile peritrichs appear saucer-shaped and vary in diameter from 30 µ to over 100 µm. They possess a sclerotized denticular ring and a ring of cilia on their outer margins.

ii. Sessile peritrichs may be barrel or urn-shaped and vary from 30 µ to 150 µ in length. Individually attached or in groups by a dichotomous stalk.

iii. Motile holotrichs are heart-shaped varying in body length from 30 µm to 150 µm. A pharyngeal basket is present and there are parallel rows of cilia on the ventral surface.

iv. Sessile suctoria have cilia present only on their larval stages whereas tentacles present on adult stages. They appear orange to brown in color and are from 50 µm to 100 µm in diameter.

b. Clinical Signs
Described above in sections D.1 and D.2.

c. Histopathological Examination
Not necessary for diagnostic purposes.

2. Confirmatory Diagnosis

Presence of organisms in wet mounts is sufficient for confirmation of an external parasite infection. However, presence of a few parasites is not enough to cause or suggest a pathological situation; the parasite load must be heavy before this group can be considered a detriment to fish health. Confirmation of this group can be considered a detriment to fish health. Confirmation of this group down to Genus can be made using a compound microscope with 10X, 40X, and 100X oil immersion objectives. If speciation of the organism is deemed necessary, it would be best to submit the sample to a qualified specialist in protozoology.

F. Procedures for Detecting Subclinical Infections

Subclinical infections are probably universal among fish populations and should be considered a natural condition.

G. Procedures for Determining Prior Exposure to the Etiological Agent

None available.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

The sample of choice would be live fish that show clinical signs of the disease. Another alternative is to place fish in plastic bags (no water) and then these pack bags in ice for shipment. The number and distribution of protozoan parasites will change quickly in iced samples so these samples should only be considered useful for 10 hours. The least desirable method is to preserve the sample. Gill tissue and skin scrapings should be placed in 5% buffered formalin for 24 hours and then transferred to 70% ethanol or AFA for shipment and/or storage.

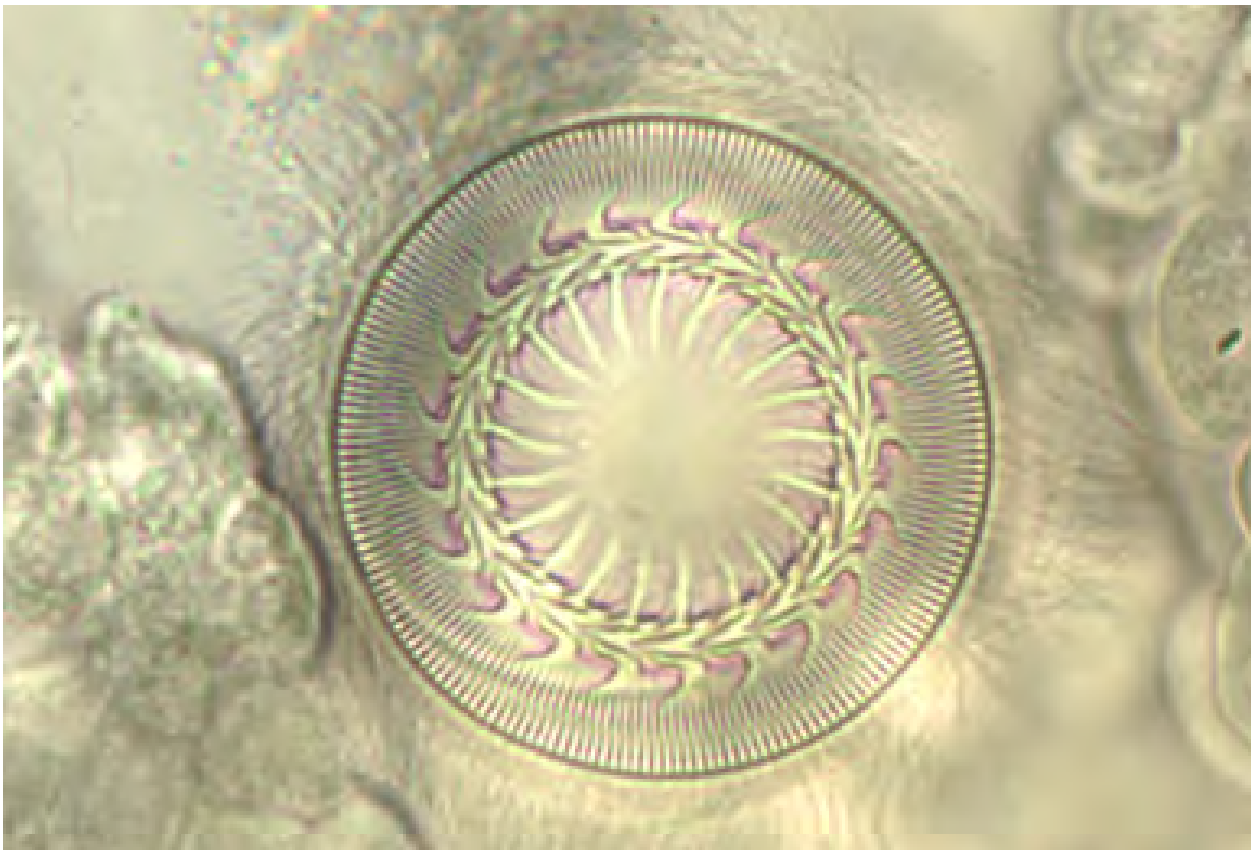


Figure 1. *Trichodina sp*. on the skin of a Channel Catfish.

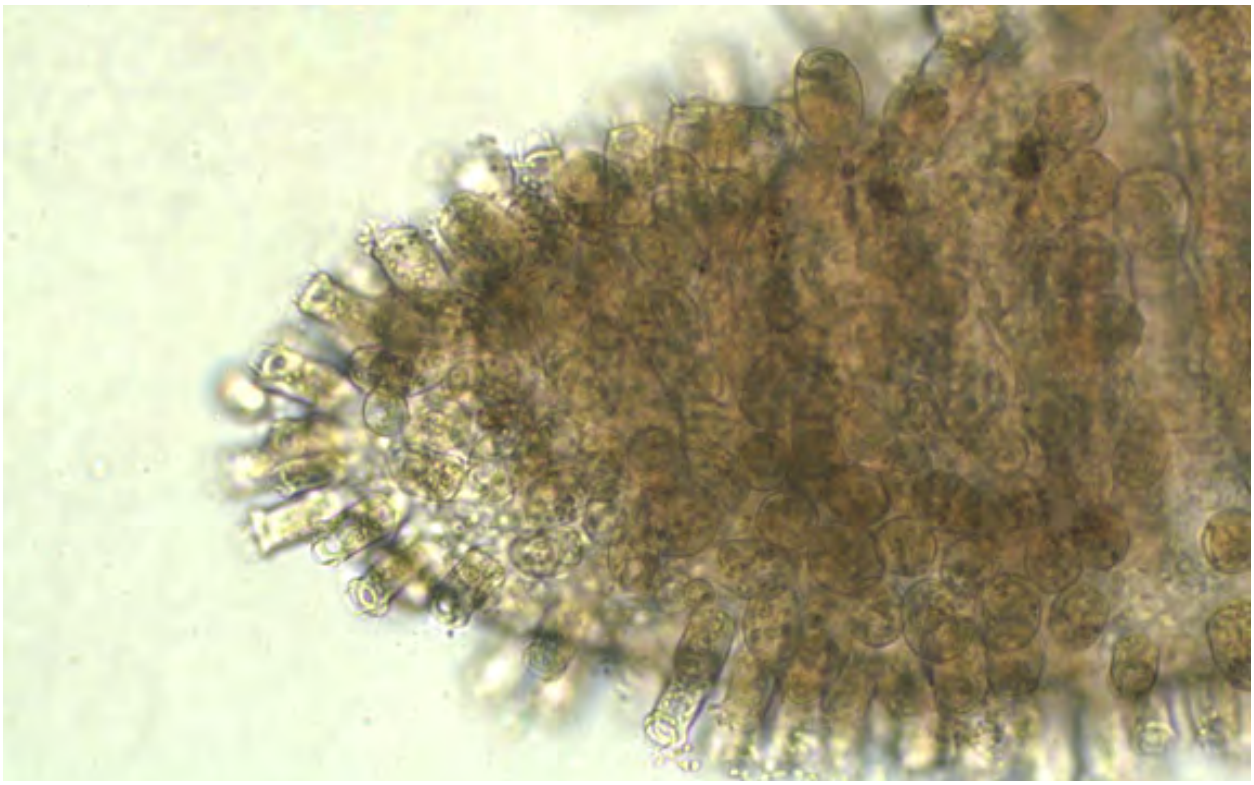


Figure 2. Large numbers of Ambiphrya on the gill of a Channel Catfish.

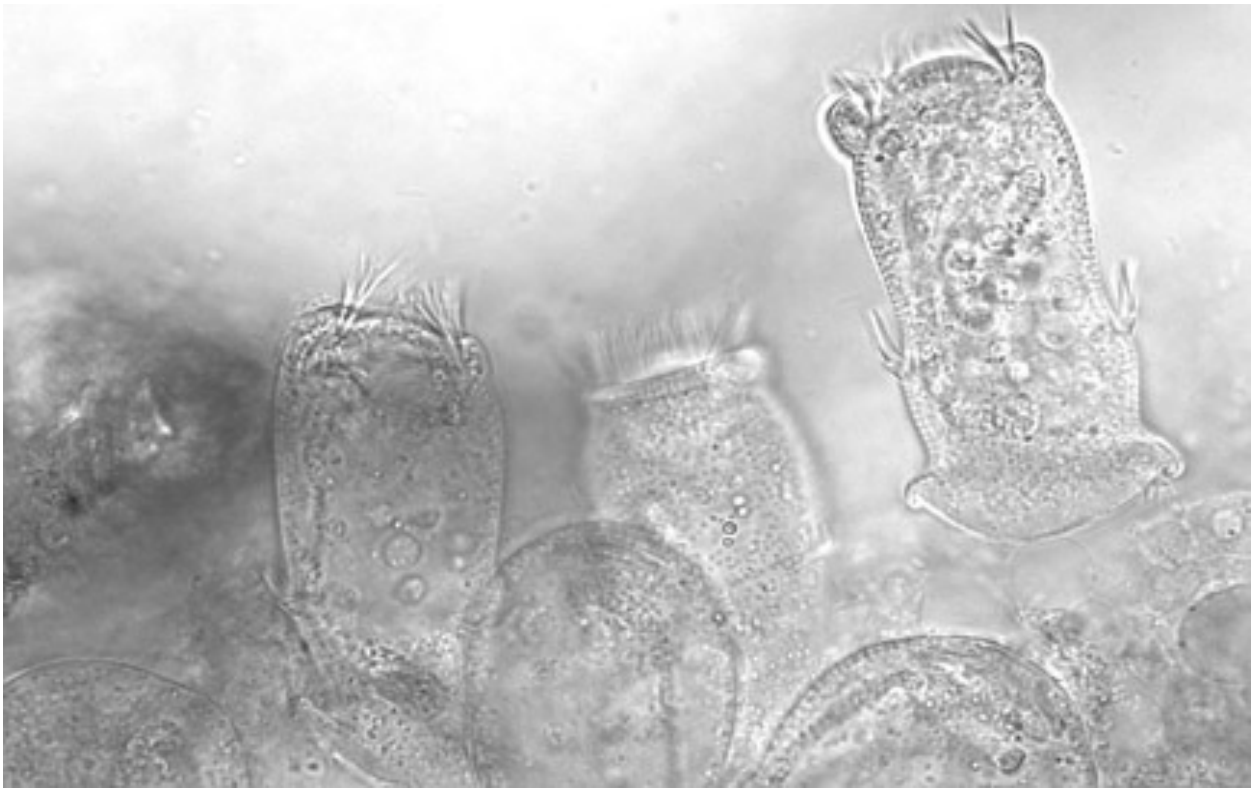


Figure 3. Ambiphrya on the gill of a Channel Catfish.



Figure 4. *Apiosoma* on the gill of a black crappie.

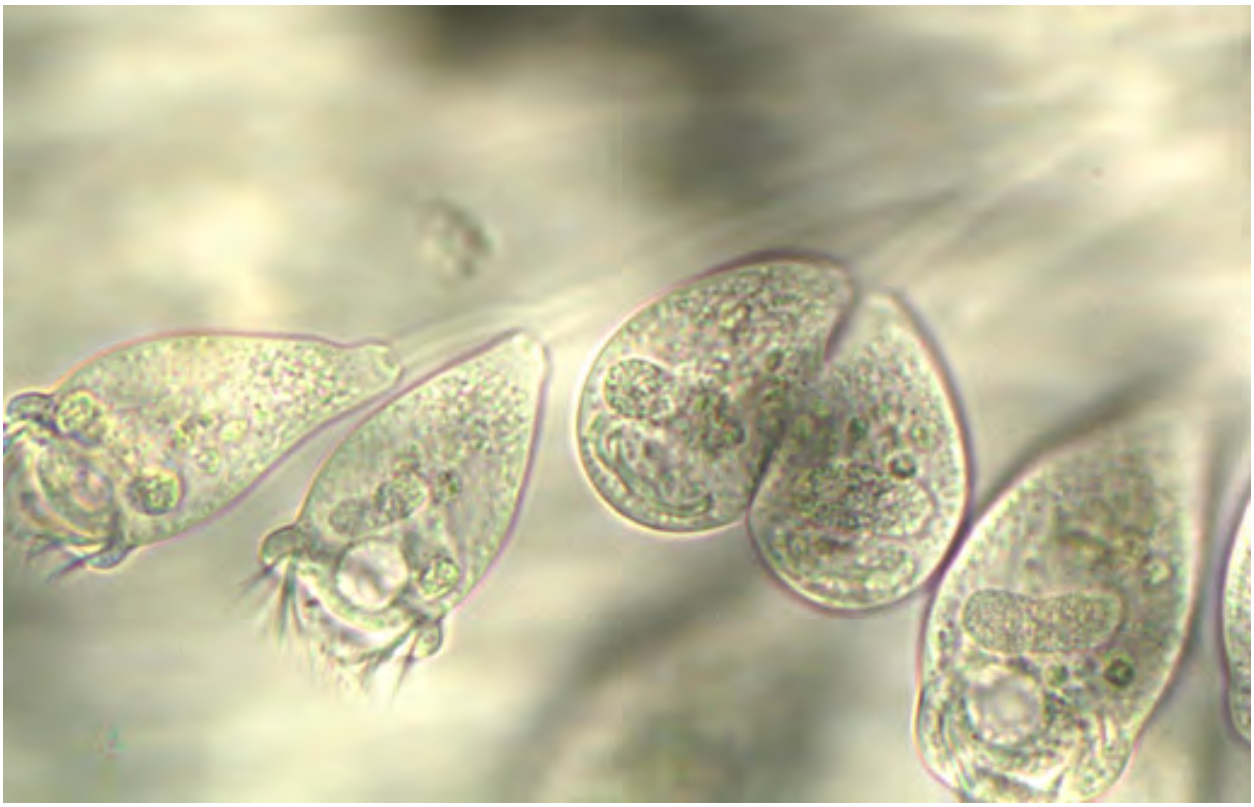


Figure 5. *Epistylus* on the fin of a Channel Catfish.

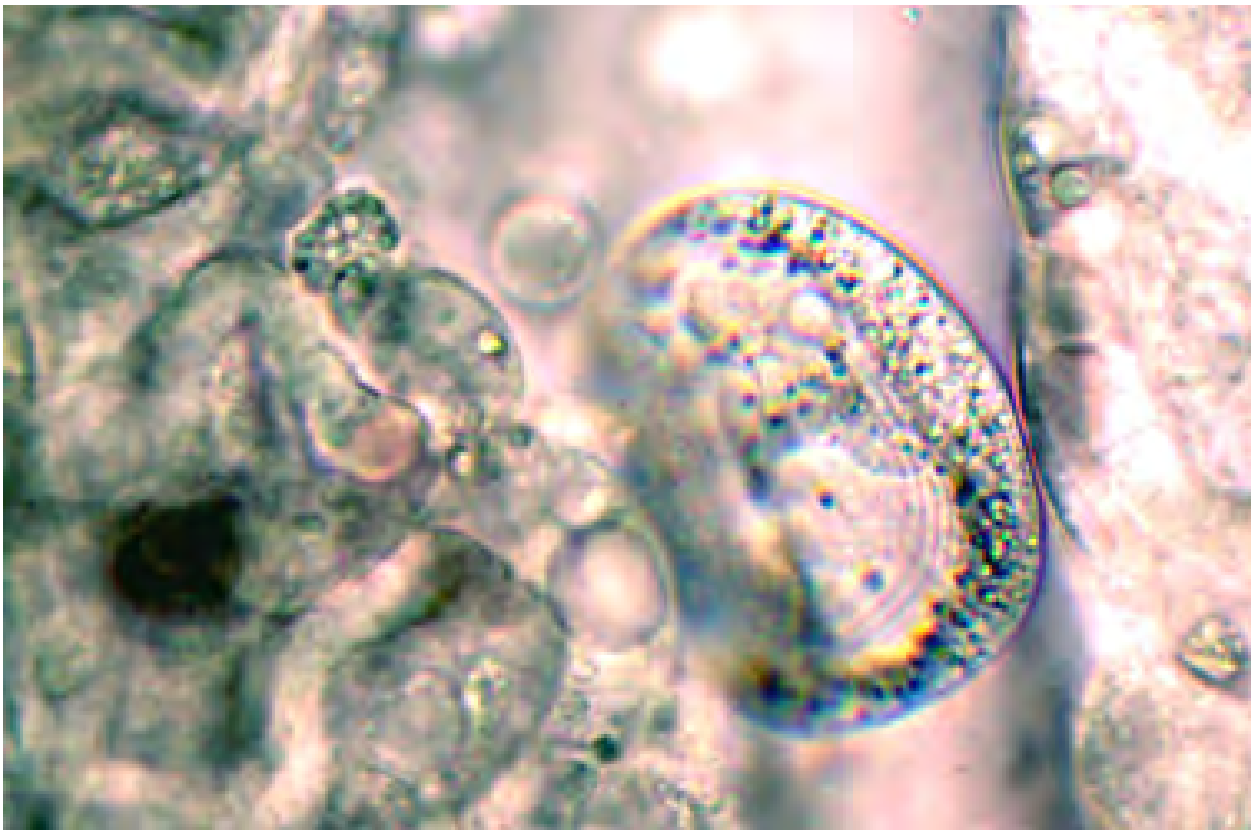


Figure 6. *Chilodonella* on a Goldfish gill.

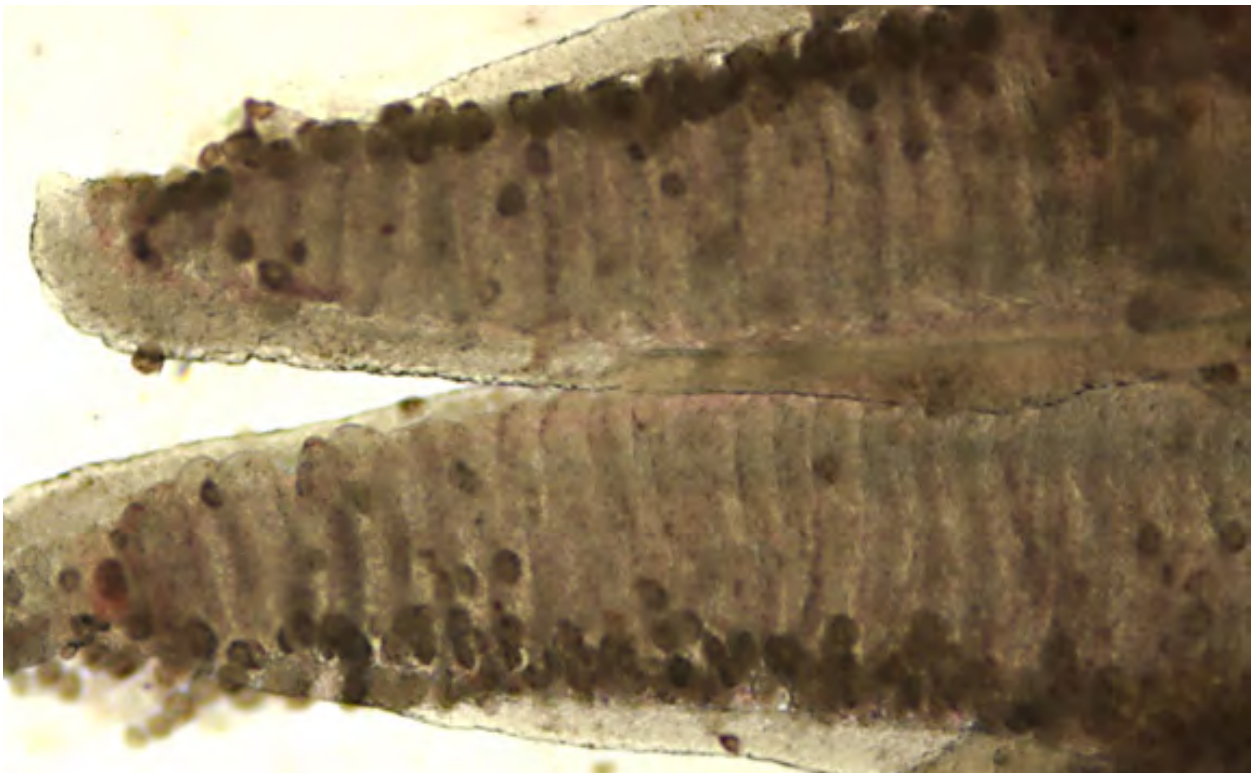


Figure 7. *Capriniana* on a Channel Catfish gill.

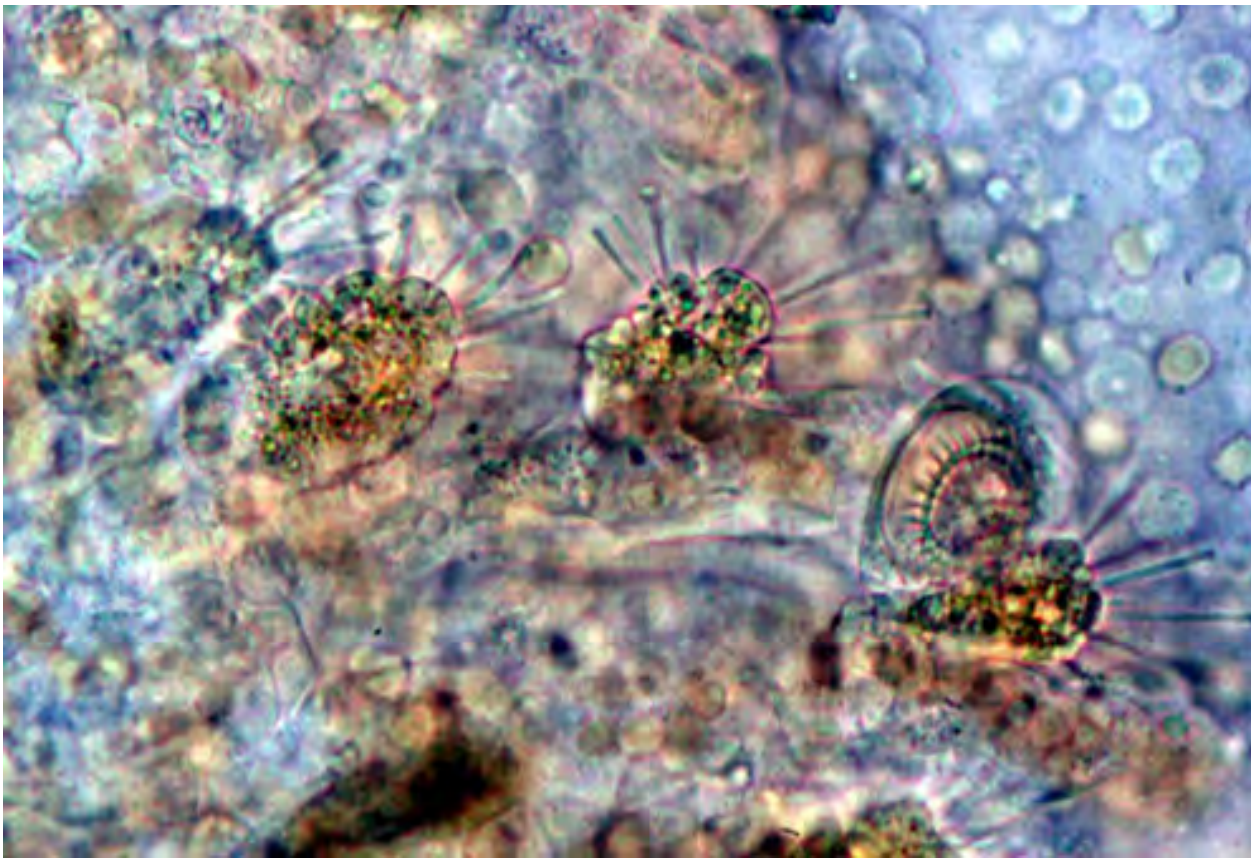


Figure 8. *Capriniana* on a Channel Catfish gill.

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3.11 Monogenean Diseases

Mary Beverley-Burton

Department of Zoology
College of Biological Science
University of Guelph
Guelph, Ontario N1G 2W1
Canada

Monogenea are ubiquitous parasites of the body surface (skin, fins, and gills) of many freshwater fishes. Three groups are known to have the potential to cause disease in fishes of economic importance in North America: (1) *Gyrodactylus* spp., (2) ancyrocephalids including *Ligictaluridus* spp. and *Cleidodiscus* spp., and (3) dactylogyrids including *Dactylogyrus* spp. All of these worms are small (generally 1 mm or less) and host specific. Each species of parasite will generally only parasitize one host species (or closely related group of hosts).

I. *Gyrodactylus* spp.

A. Name of Disease and Etiological Agent

Gyrodactyliasis is caused by *Gyrodactylus* spp. (*Platyhelminthes:Monogenea*).

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Gyrodactylus spp. occurs on freshwater fishes throughout North America. These worms have, in fact, been reported from every continent except Antarctica.

Although early reports of *Gyrodactylus* spp. in salmonid culture facilities in North America implicated these parasites as the cause of considerable mortality, and gyrodactyliasis has been listed as a "threatening" disease of the salmonids in the Pacific region. There have been almost no recent records of gyrodactyliasis, apart from brief mention of "problems" on Vancouver Island and in Washington State. This situation probably reflects the efficacy of currently used treatments rather than an absence of these worms. The closely related *Laminiscus strelkowi* (Family *Gyrodactylidae*) has been detected in large numbers on the gills of salmonids reared in Pacific netpens. However, it is still unclear as to the precise role that this parasite may play in disease.

2. Host Species

Most salmonid species are susceptible to infection by one or more species of *Gyrodactylus*. The following are of interest in the context of disease: coho salmon *Oncorhynchus kisutch*, sockeye salmon *Oncorhynchus nerka*, rainbow trout *Oncorhynchus mykiss*, Atlantic salmon *Salmo salar*, brown trout *Salmo trutta*, and salmonid hybrids e.g. splake *Salvelinus fontinalis* (brook trout) x *Salvelinus namaycush* (lake trout).

Ictalurid (catfish) species including channel catfish *Ictalurus punctatus* are also susceptible.

Cyprinids including goldfish *Carassius auratus*, common carp *Cyprinus carpio*, grass carp *Ctenopharyngodon idella*, and golden shiner *Notemigonus crysoleucas* are also susceptible.

C. Epizootiology

The only documented, overt disease in wild salmonids involves *Gyrodactylus salaris* on *Salmo salar* in Norwegian rivers. It is of interest that in brook trout fry, experimentally infected with *Gyrodactylus salmonis*, a mortality of 44% was observed over 22 days. Larger fish were less susceptible. Both channel catfish and golden shiner in the southeastern U. S. and in Illinois, respectively, have been adversely affected by *Gyrodactylus* spp.

Fry and fingerlings of the common carp can be killed by severe outbreaks of gyrodactyliasis. While most information comes from Eastern Europe and the Commonwealth of Independent States (C. I. S.), there are reports of "problems" in the southeastern U. S. A.

1. Conditions Under Which Disease Occurs

Gyrodactylus spp. is viviparous (i.e. producing live young) and hermaphroditic (i.e. have both female and male reproductive organs). Thus, in a population all adult worms may carry a fully developed embryo which in turn, while still in the parental uterus, carry young of the next generation. Thus, *Gyrodactylus* populations can multiply extremely quickly. The finding of *Gyrodactylus* on cultured fishes (especially salmonids) should always be a reminder of their disease potential. This is particularly true under conditions where fish are stressed.

Although fish of all ages may be parasitized, young fish appear to be the most affected. The number of worms infecting an individual (i.e. intensity) generally decreases with increased host age. Overcrowding, especially of young fish, often leads to increased intensities of infection, which may or may not produce signs of disease.

Secondary infections by bacteria and/or fungi have often been considered part of the cause of mortality associated with gyrodactyliasis. However, there is no experimental evidence from observations in North America to support these hypotheses, although in Norway, the fungus *Saprolegnia* seems to be an established secondary pathogen.

With the scanty evidence available, it appears that on salmonid fishes, *Gyrodactylus* spp. is most abundant at cooler times of the year (winter and early spring) when water temperatures are 8^{o}C or less. Warm-water species have higher optimum temperatures, and the parasites are usually well adapted to the temperature ranges tolerated by their hosts.

D. Disease Signs

1. **Behavioral Changes Associated with the Disease**
   Some fish (e.g. rainbow trout) with moderate or heavy infections of skin-dwelling *Gyrodactylus* spp. may flash, turning sideways as if attempting to rub away the irritation. Under experimental conditions, intensely infected brook trout fry were observed to be weakened and lethargic before becoming moribund.

2. **External Gross Signs (in heavy infections)**
   Gyrodactylus spp. are site specific as well as host specific parasites. In salmonids and cyprinids, the general body surface (skin, fins, and/or tail) is affected while in catfish the barbels, underside of the head, and fins are particularly prone to attack. In heavily infected cyprinids, the gills may become affected as well as the body surface.

   External gross signs in heavy infections include overall darkening in color in fry, erosion of the fins (particularly the dorsal fin), pale discolored flanks, and thickened cuticle, obvious secretions of mucus sometimes described as a blue/grey slime, and emaciation (especially in young fish). In catfish detachment of the barbels due to necrosis may occur.

3. **Internal Gross Signs**
   None reported.

4. **Histopathological Changes Associated with the Disease**
   Gyrodactylus spp. are tissue browsers, feeding on the epidermis (skin) of the body surface, fins, or gills. Mechanical damage may also be inflicted by the attachment apparatus (haptor) of the parasites. Sections of skin from heavily infected fish show epidermal hyperplasia with zones of degeneration and necrosis.

   Extensive kidney damage, including degeneration and necrosis of the epithelium lining of the renal tubules, was observed in experimentally infected brook trout fry. These fish were heavily infected and had become moribund. The renal pathologies were thought to have been associated with epidermal disruption, caused by the"grazing" of parasites, leading to osmotic imbalance with both inflow of surrounding water and loss of tissue fluids.

E. Disease Diagnostic Procedures

1. **Presumptive Diagnosis**

   a. Isolation and Detection of Pathogen
      With heavy infections of small fish (e.g. salmonid fry), it is possible to see *Monogenea* on the body surface of a dead or anaesthetized fish in a dish of water using the low power of a dissecting microscope or a 10X magnifying glass. A preliminary identification (to decide which group of *Monogenea* is present) can be made using a temporary preparation. Put a skin scrape or gill filament squash on a microscope slide and add a small drop of water and cover with a coverslip. The worms can then be examined under a compound microscope.

      In order to identify worms to genus (see Figure 1), it is necessary to collect and preserve specimens and make a more permanent preparation for examination under the high-dry

objective (40X) of a compound microscope. Pritchard and Kruse (1982) have described the following techniques: small *Monogenea* are easily collected by placing infected material (whole small fish or tissue from larger fish e.g. gills, fins, barbels, etc.) in a container of 1:4,000 formalin for 15 to 45 minutes; shake the container vigorously for 2 to 3 minutes and remove fish remains; pour the resultant "soup" into a tall vessel and allow worms to settle for about 30 minutes. Decant supernatant fluid and examine sediment by placing small amounts in a Petri dish in order to scan under a dissecting microscope. Remove any specimens with a pipette and either transfer them to a clean slide for immediate examination (in a drop of water under a cover slip) or into fixative (AFA-alcohol-formalin-acetic acid or 5% formalin) pending the making of more permanent preparations. The worms in the temporary wet preparation should be adequate for identification to genus with reference to the diagrams provided in this manual (see Figure 1).

b. Clinical Signs
As described above

c. Histopathological Examination
Is not necessary for diagnostic purposes.

2. Confirmatory Diagnosis
To identify *Monogenea* to the species level, it is necessary to have a compound microscope, preferably fitted with a 100X oil-immersion lens and a measuring device. It would usually be preferable to send material requiring specific identification to an appropriate diagnostic center or specialist, who would have access to the relevant literature.

F. Procedures for Detecting Subclinical Infections

Subclinical infections are probably universal in salmonid hatchery and culture facilities and may also occur where non-salmonids are reared. A sample of fish can either be killed and examined as described above, or worms can be collected from living fish as described by Cone and Cusack (1988): a sample of fish is placed in a plastic net box containing 1:4000 formalin solution (the size of the net box and volume of formalin solution would vary with the size of the sample; for 60 yearlings four liters of solution is appropriate) and the fish allowed to thrash about for two minutes. After removal of the fish, worms can be concentrated by sedimentation and processed as described above.

G. Procedures for Determining Prior Exposure to *Gyrodactylus* spp.

None available for Monogenean diseases.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Although, under ideal conditions, small *Monogenea* can be more easily studied while alive, this is usually not practical for diagnostic work. Living worms can survive on whole, small fishes or gill

material for about 6 hours if kept in a small quantity of chilled water in a cooled container. However, fixation is generally preferable.

Small fishes can be fixed whole in a 5% formalin solution for 48 hours, and then transferred for shipping and/or storage to 70% ethanol. Similarly, tissues from larger fishes (fins, barbels, gills, etc.) can be fixed and shipped separately.

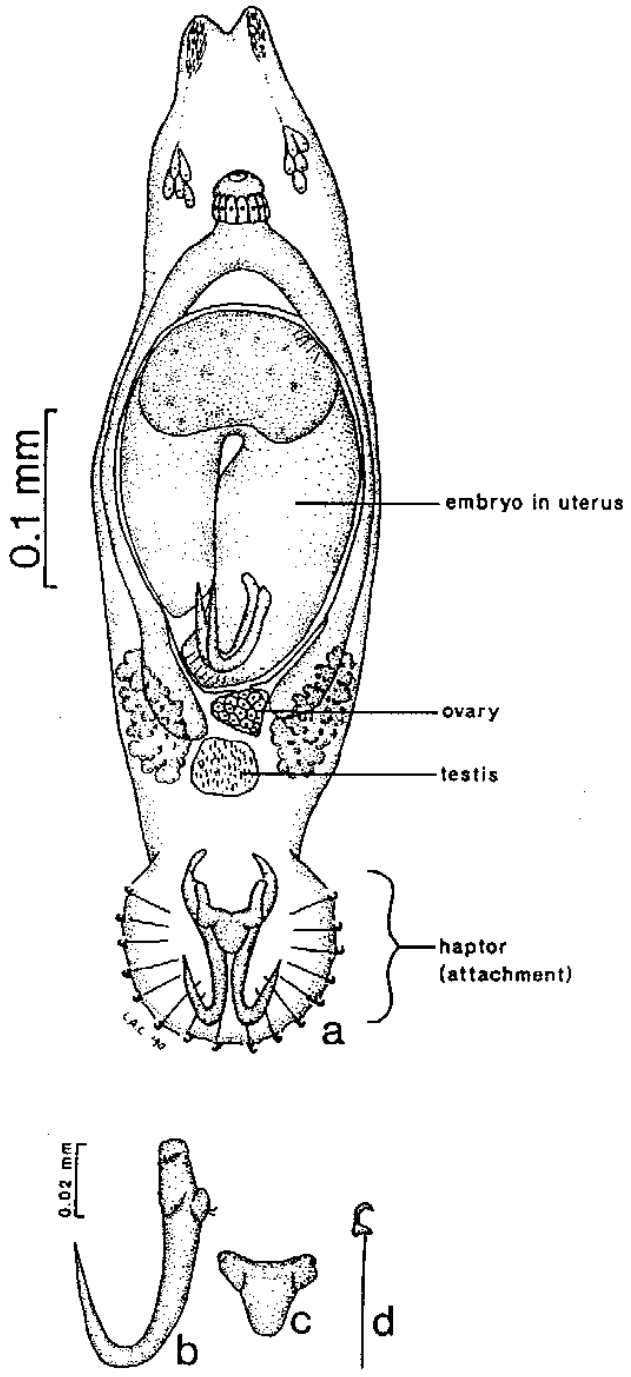


Figure 1. Generalized *Gyrodactylus* sp.

II. Ancyrocephalids - Including *Ligictaluridus* spp. and *Cleidodiscus* spp.

A. Name of Disease and Etiological Agent

"Monogenean Disease" is an acceptable, general term. *Ligictaluridus* spp.(= *Cleidodiscus*, in part, of previous texts) and *Cleidodiscus* spp. are both in the family *Ancyrocephalidae* (*Platyhelminthes : Monogenea*).

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Ligictaluridus spp. are enzootic to North America, occuring only on ictalurid fishes. Where catfish have been introduced into other countries (e.g. Poland and the C. I. S.), these parasites were introduced at the same time. *Cleidodiscus* spp. are found on fishes in North America and in Eurasia.

2. Host Species

Channel catfish *Ictalurus punctatus* are parasitized by *Ligictaluridus* spp., and bluegill *Lepomis macrochirus* by *Cleidodiscus* sp.

Parasitized wild fish have been found in many areas throughout the natural ranges of both hosts with no records of disease. However, under culture conditions, problems are known to occur.

C. Epizootiology

1. Conditions Under Which Disease Occurs

Ancyrocephalids, in contrast to *Gyrodactylus* spp., are oviparous (i.e. they do not give birth to live young). Shelled eggs are laid and development of the embryos is external to the body of the worm. The period of development is temperature-dependent, e.g. at 20^{o}C, and generation time is 14 days. As temperatures increase (to an optimum level), generation time becomes less. On hatching, the oncomiracidium, a minute ciliated larva emerges and actively swims about searching for a suitable host. Build-up of intensity is thus potentially not as "explosive" as that of *Gyrodactylus* spp. Both *Ligictaluridus* spp. and *Cleidodiscus* spp. parasitize the gills of their hosts. Overcrowding, especially of fry and/or fingerlings, often leads to increased intensity of infection, which may or may not produce signs of disease.

Ancyrocephalids are most abundant on wild freshwater fishes in mid to late summer; however, there is much variation and generalizations are not possible. Fish of all ages are found to be parasitized, but young fish appear to be the most affected.

D. Disease Signs

1. **Behavioral Changes Associated with the Disease**
   Ancyrocephalids live on the gills of their hosts and respiratory distress is not uncommon. Gasping may occur in heavy infections on young fishes, especially if water temperatures are high. Under these conditions fish are often lethargic and unable to feed.

2. **External Gross Signs**
   In heavy infections, there is an obvious production of mucus on the gills of young fish with slight flaring of the operculum.

3. **Internal Gross Signs**
   None reported.

4. **Histopathological Changes Associated with the Disease**
   Mechanical damage to the epithelium of the gill lamellae is caused by the attachment disc (haptor), which is armed with hamuli and marginal hooks, and by the tissue-grazing method of feeding used by these parasites. A mild tissue reaction, involving hyperplasia, at the site of attachment has been reported. In *Cleidodiscus* infections on adult bluegills, lesions are not uncommon and appear as off-white to yellow cysts on the gill filaments. These cysts are composed of hyperplastic, squamous epithelium covering well developed granulation tissue. Necrosis may occur in areas where vascularization is diminished.

E. Disease Diagnostic Procedures

See "Disease Diagnostic Procedures" for *Gyrodactylus* spp.

F. Procedures for Detecting Subclinical Infections

See "Procedures for Detecting Subclinical Infections" for Gyrodactylus spp.

G. Procedures for Determining Prior Exposure to Etiological Agent

None available for Monogenean diseases.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

See "Procrdures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent" for *Gyrodactylus* spp.

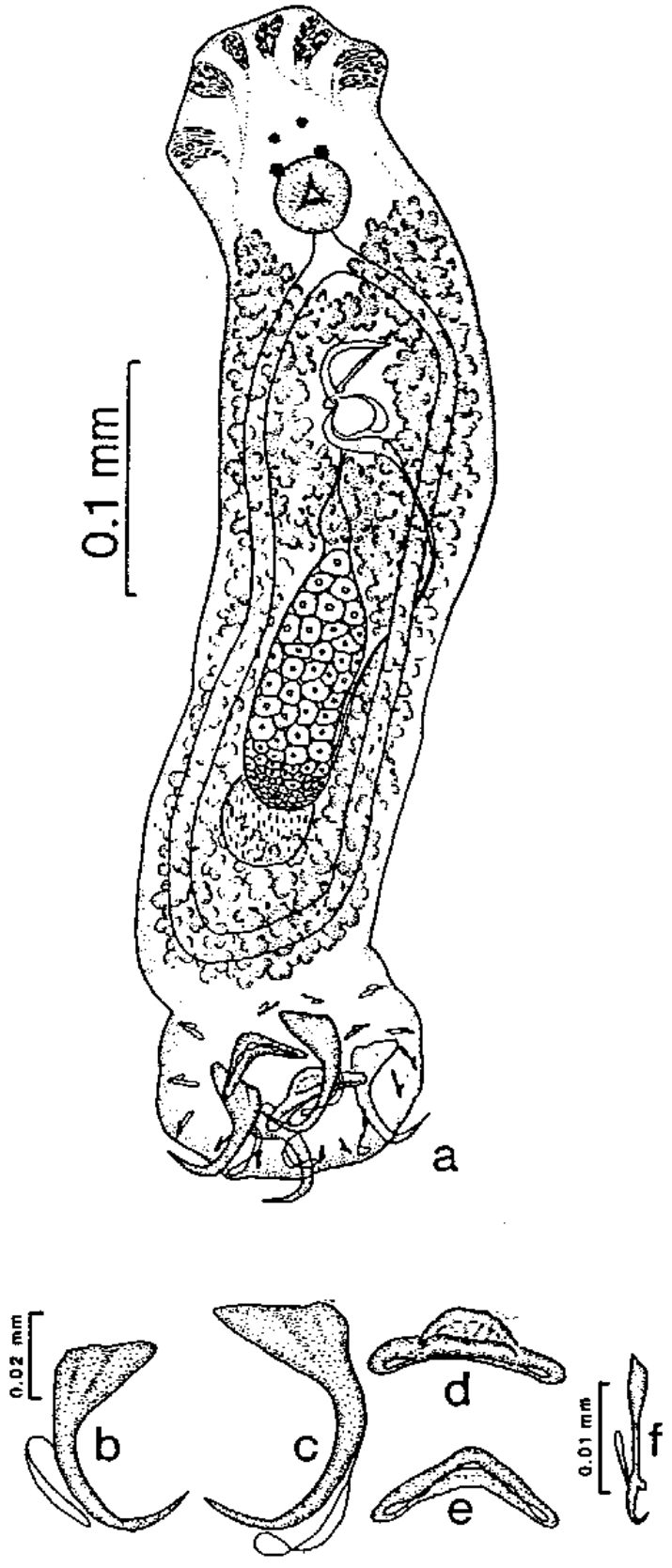


Figure 2. Generalized *Ligictaluridus* sp.

III. Dactylogyrids - Including *Dactylogyrus* spp.

A. Name of Disease and Etiological Agent

"Monogenean Disease" is an acceptable general term. *Dactylogyrus* spp. (*Platyhelminthes : Monogenea*).

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Dactylogyrus spp. occurs on a wide variety of freshwater fishes throughout North America.

2. Host Species

Cyprinids, including goldfish *Carassius auratus*, common carp *Cyprinus carpio*, grass carp *Ctenopharyngodon idella*, and golden shiner *Notemigonus crysoleucas*, are the major hosts of *Dactylogyrus* spp. Parasitized fish probably occur throughout the natural ranges of the above hosts.

C. Epizootiology

Fry and fingerlings of carp are particularly susceptible to *Dactylogyrus* infection and high mortalities occur in culture situtations in eastern Europe and the C. I. S.

1. Conditions Under Which Disease Occurs

Dactylogyrus spp., like the ancyrocephalids, produce shelled eggs and have life cycles in which generation time is temperature-dependent. *Dactylogyrus* spp. on carp kept in colder climates generally have a longer generation time (e.g. 5 to 6 months at 1 to 2^{o} C) than those in warmer areas (e.g. only a few days at 22 to 24^{o} C).

Overcrowding, especially of young fish, may produce stressful conditions in which infected fish succumb and die. Mortalities of 80-100% have been recorded in some carp fry populations. *Dactylogyrus* spp. typically occur on the gills of their hosts and, in water with a low concentration of oxygen, respiratory impairment may lead to death of fry and fingerlings. In Israel, fish over 35mm long were found to tolerate relatively high intensities of infection and some larger carp are resistant to infection when exposed to *Dactylogyrus* larvae.

D. Disease Signs

1. Behavioral Changes Associated with the Disease

These are similar to those noted for the ancyrocephalids above.

2. External Gross Signs

As above for heavy infections.

3. Internal Gross Signs

None reported.

4. Histopathological Changes Associated with the Disease

Dactylogyrus spp. are tissue browsers and cause mechanical damage by feeding with their attachment apparatus (haptor). Marked gill hyperplasia is seen in heavy infections and results in serious malfunction of the respiratory surface and deformation of the gill lamellae. Heavily infected fish may exhibit blood changes, e.g. low hematocrits and depletion of hemoglobin. Host growth rate is not normally affected unless culture conditions are unsatisfactory. If the young fish are able to feed, they grow rapidly and overcome the deleterious effects of the parasite and if culture conditions are not good, growth rate may be retarded.

E. Disease Diagnostic Procedures

See "Disease Diagnostic Procedures" for *Gyrodactylus* spp.

F. Procedures for Detecting Subclinical Infections

See "Procedures for Detecting Subclinical Infections" for Gyrodactylus spp.

G. Procedures for Determining Prior Exposure to Etiological Agent

None available for Monogenean diseases.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

See "Procrdures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent" for *Gyrodactylus* spp.

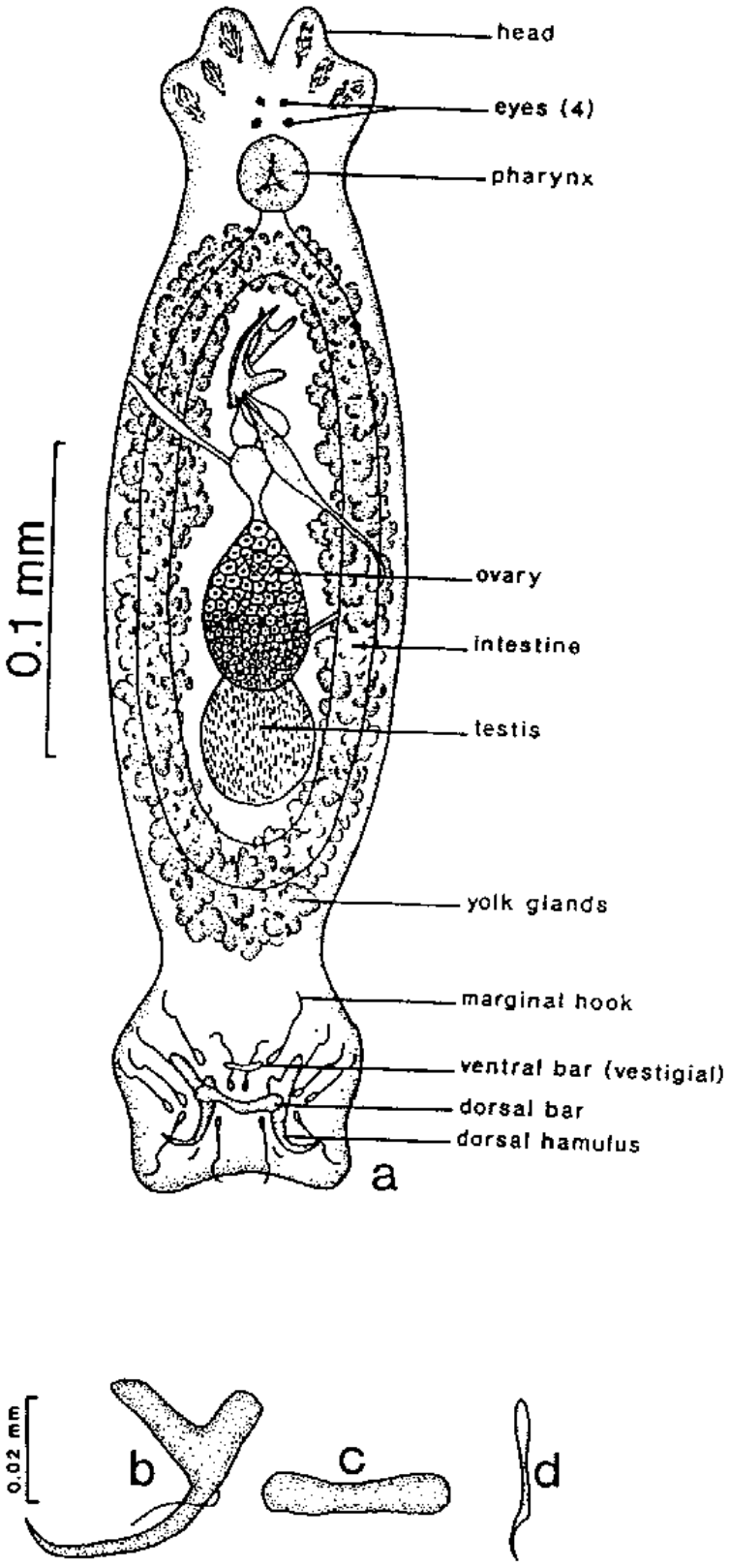


Figure 3. Generalized *Dactylogyrus* sp. (a) entire animal, ventral view, (b) dorsal hamulus, (c) dorsal transverse bar, (d) larval hook.

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3.12 Bothriocephalosis

Andrew Mitchell

U. S. Department of Agriculture
Agricultural Research Service
H K Dupree-Stuttgart National Aquaculture Research Center
PO Box 1050
Stuttgart, AR 72160-1050
870/673-4483
dmitchell@spa.ars.usda.gov

A. Name of Disease and Etiological Agent

Bothriocephalosis is the intestinal infection of certain fish by the cestode *Bothriocephalus acheilognathi* (Yamaguti 1934), a Pseudophyllidean tapeworm. The infecting organism is also known as the Asian fish tapeworm and as the Chinese tapeworm and has had several synonymous scientific names, including: *Bothriocephalus opsariichthydis = Bothriocephalus opsalichthydis*, *Bothriocephalus fluviatilis*, *Schyzocotyle fluviatilis*, *Bothriocephalus gowkongensis*, and *Bothriocephalus phoxini*.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

The Asian tapeworm has been reported in Asia, Australia, Europe, South Africa, and North America. In North America, it has been reported in Mexico, British Columbia, throughout the lower half of the United States, and in Colorado, Nebraska, New Hampshire, New York, Nevada, and Utah.

2. Host Species

Most members of the family *Cyprinidae* should be considered as potential hosts. However, goldfish *Carassius auratus*, are apparently not susceptible to *B. acheilognathi*. This worm also infects some silurids, poecilids, percids, and centrarchids. In the United States, the fish host species include: goby *Awaous guamensis*, grass carp *Ctenopharygodon idella*, common carp *Cyprinus carpio*, sleeper *Eleotris sandwicensis*, plains killifish *Fundulus zebrinus*, mosquito fish *Gambusia affinis*, humpback chub *Gila cypha*, roundtail chub *Gila robust*, green sunfish, *Lepomis cyanellus*, virgin spinedace *Lepidomeda mollispinis*, peamouth *Mylocheilus oregonensis*, golden shiner *Notemigonus crysoleucas*, emerald shiner *Notropis atherinoides*, red shiner *Notemigonus lutrensis*, spotfin shiner *Notropis spilopterus*, fathead minnow *Pimephales promelas*, woundfin *Plagopterus argentissimus*, guppy *Poecilia reticulata*, Colorado pikeminnow *Ptychocheilus lucius*, speckled dace *Rhinichthys osculus*, and green swordtail *Xiphophorus helleri*.

C. Epizootiology

Acute infection with the Asian tapeworm occurs when intensively cultured larval fish feed on copepods infested with high levels of *Bothriocephalus acheilognathi* procercoids. Up to 80% mortality among larval fish has been reported by fish producers; however, death as a direct result of tapeworm infection is not a common occurrence. In the United States, most problems associated with this worm involve reduced growth and the inability of infected fish to withstand harvesting procedures. The worm evidently shortens the life span and stunts the growth of feral fish.

Operculated eggs of the Asian tapeworm are shed with fish feces into the water. A motile coracidium emerges from these eggs and is eaten by cyclopoid copepods. Larval worm development proceeds within the copepod. Copepods are eaten by fish and the developing worm is released in the anterior portion of the alimentary canal. The worm attaches to the mucosal lining of the intestine and matures producing gravid segments which release eggs. Worms up to 60 cm have been reported; however, most mature worms measure between 5 to 8 cm.

The Asian tapeworm is a thermophile that prefers temperatures of 20 to 30^{o}C. Growth and maturation are fastest at temperatures above 25^{o}C. However, in small fish the density of the worms decreases at these temperatures probably because of limited space and nutrient availability. Egg maturation, hatching, and coracidium movement are maximized at 25 to 30^{o}C. Spring to late summer is the peak recruitment period because most worms are gravid at this time.

D. Disease Signs

1. **Behavioral Changes Associated with the Disease**
   Occasionally, fry hang listlessly around the edge of the pond.

2. **External Gross Signs**
   Heavily infected golden shiners appear emaciated with a swelling in the anterior portion of the abdomen. Weakened fish often develop bacterial problems resulting in signs typical of columnaris disease or motile aeromonas septicemia in grass carp (20 to 30 cm in length). Bloating and raised scales may also occur with massive tapeworm infections.

3. **Internal Gross Signs**
   The intestinal tract just posterior to the first bend is greatly enlarged and appears yellow to white from the massive worm infection. The intestines become stretched, thin, flaccid and may rupture. In 20 to 30 cm fish, the body cavity may be filled with a cloudy yellow fluid. Death is due to physical blockage of the intestine by the worms or to intestinal rupture.

   No signs are apparent when *Bothriocephalus acheilognathi* infections are light.

4. **Histopathological and EM Observed Changes Associated with the Disease**
   The attachment of the scolex to the intestine results in mechanical damage, localized hemorrhages, inflammation, and focal pressure necrosis of the mucosa. More severe damage including loss of microvilli and enterocytes has been reported. Lymphocytes, macrophages, and eosinophils accumulated in the wall of the infected gut and migrated into the lumen where they adhered to the parasite tegument.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

Asian tapeworms are in the anterior section of the intestinal tract, just posterior to the first bend. The scolex (head) of the tapeworm assumes a pit viper or arrowhead shape during a cycle of extension and contraction. For an example, see the movie showing Bothriocephalus under various lighting conditions. The shape of the scolex is difficult to determine if the worm is dead, frozen, or preserved. Histological or clinical signs that are useful to the diagnosis of infected fish have not been demonstrated.

2. Confirmatory Diagnosis

Once tapeworms with pyramidal scolices are found, they must be distinguished from all others with similar shaped scolices. Some worms of the genera *Anoncocephalus*, *Atractolytocestus*, *Bathybothrium*, *Echinophallus*, *Eubothrioides*, *Eubothrium*, *Fistulicola*, *Glaridacris*, *Marsipometra*, *Proteocephalus*, *Ptychobothrium*, and *Schistocephalus* species have a pit viper or pyramid-shaped scolex. To aid in the differentiation of these tapeworms, the following definition and key for *Bothriochephalosis acheilognathi* is given.

Bothriocephalus acheilognathi is a complete and distinctly segmented, thin tapeworm that can reach a length of over 50 cm, but is usually less than l0 cm. Segmentation is evident on worms l mm or more in length. *Atractolytocestus*, *Glaridacris*, and *Ptychobothrium* species are not segmented.

Bothriocephalus acheilognathi has a flattened scolex with two bothria (deep, elongated sucking grooves dorsal and ventral as seen in Figure l), no hooks, no spines, no suckers (sucking devices surrounded by a muscular fringe are usually circular as seen in Figure 1), and no proboscides (short tentacles). In the lateral view (normal viewing position of the worm), the scolex takes a strong pit viper or arrowhead appearance when extended and a balled or fist-shaped appearance when contracted. The posterior portion of the scolex is wider than the first few segments in both the extended and contracted positions. *Schistocephalus* species have scolices less than the width of the first few segments.

Bothriocephalus acheilognathi has no neck. The neck, which is present and very obvious on *Marsipometra* species and sometimes present on *Eubothrium* species, is a nonsegmented area posterior to the scolex, anterior to the first obvious segment, and two or more times the length of the anterior segments (see Figure 1).

Bothriocephalus acheilognathi has no dorsal or ventral median furrow. This furrow, present in *Eubothrium* and *Bathybothrium* spp., is not always clearly visible for the full length of the worm, but careful viewing reveals definite short sections of the furrow. The furrow may appear as a small indentation on the posterior edge of several proglottids (see Figure 1).

F. Procedures for Detecting Subclinical Infections

Sample size should be adequate to detect the presence of infected fish at a 5% level of prevalence (see Section 1, 3.1 General Procedures for Parasitology).

1. Sever the head of the fish just behind the opercular flap.

2. The tip of blunt scissors can then be inserted into the body cavity and the ventral wall cut to the anus without severing the intestine.

3. Cut the intestinal tract at the anus and at the esophagus or just posterior to the stomach and remove the intestine with the fingers or forceps.

If large worms are present, they will be apparent as a yellow to white bulge in the intestine. Large worms can be seen with the naked eye; however, a microscopic examination is required to detect small worms. If none are obvious, the intestine should be uncoiled and the attached tissues removed; this can usually be done by rubbing the intestine gently between the fingers. However, if the intestinal tract is small and thus easily damaged, it can be placed directly on a microscope slide or glass plate (9 x 9 x 0.3 cm) without removal of attached material. The uncoiled intestinal tracts from several small fish can be placed side by side on a glass plate. An uncoiled intestine can be folded if it is longer than the plate. A second slide or glass plate placed over the excised intestine spreads the intestine for easy visibility. Usually, the two slides or glass plates are pressed together with two or four small binder clips placed on opposite sides. Small specimens less than 3 cm long may not require binder clips for flattening.

Parasites can be detected in the intestine with a 15 to 30-power dissecting microscope and reflected light. The scan objective (2 to 4 X magnification) on a binocular microscope can be used if the specimen is small enough to be placed between two slides. Once an investigator has learned to recognize small intestinal worms (350 μm minimum length), the technique can be used rapidly and with confidence. Asian tapeworms sometimes take on a silvery cast and movement will be detected if the specimen is viewed for 15 seconds. Because these tapeworms occupy the anterior part of the intestinal tract, this portion should be examined thoroughly. This technique can be used for fish up to 20 cm long, but it is most effective for fish less than 13 cm. For fish longer than 20 cm, the anterior third of the intestine can be removed and slit open lengthwise, and the contents can be scraped out with a scalpel. The contents can then be spread and flattened between two glass plates (no binder clips are necessary) and examined microscopically. Because food particles may obscure worms, best results can be obtained with fish that have not eaten for at least 24 hours.

G. Procedures for Determining Prior Exposure to the Etiological Agent

None available.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival or Recognition of the Etiological Agent

Submitting live fish is best; if this is not possible, carefully remove the tapeworms, keeping the scolex intact, and place them in 80^{o}C water for about five minutes and then transfer to 10% formalin. Confirmatory diagnosis is unlikely from preserved specimens.

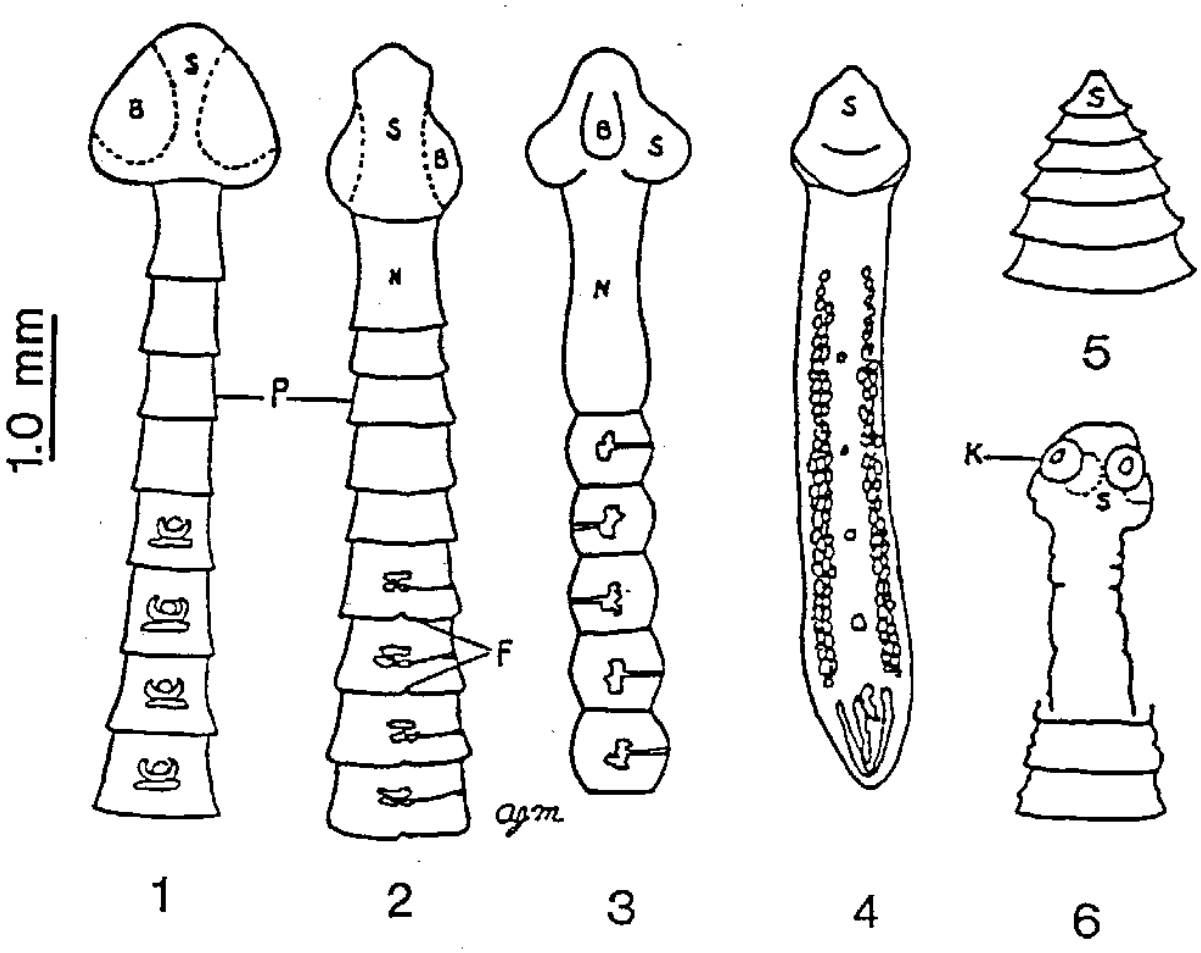


Figure 1. (1) *Bothriocephalus acheilognathi*; (2) *Eubothrium salvelini*; (3) *Marsipometra parva*; (4) *Glaridacris larvei* (unsegmented); (5) *Schistocephalus solidus*; (6) *Proteocephalus ambloplitis*; B – bothria; F – furrow; K – sucker; N – neck; P – proglottid; S – scolex.

I. Key for the Separation of Adult* Bothriocephalus Acheilognathi From Other Tapeworms with Pyramidal or Arrowhead-shaped Scolices

1a. Non-segmented or segmentation incomplete or indistinct……*Atractolytocestus*
…….. *Glaridacris*
……...…….. Ptychobot*hrium*
1b. Segmentation complete and distinct……2
2a. Scolex with 4 or 5 suckers…….……. *Proteocephalus*
2b. Scolex with 2 bothria no suckers…… 3
3a. Tapeworms of marine fish…….. *Anoncocephalus*
…….…….… *Echinophallus*
…….. *Eubothrioides*
…….…….… *Fistulicola*
3b. Tapeworms of freshwater fish…….…4
4a. Dorsal-ventral median furrow……. *Bathybothrium*
…….. *Eubothrium*
4b. No dorsal-ventral median furrow……..... 5
5a. Neck……..... *Marsipometra*
5b. No neck……..... 6
6a. Wide portion of scolex (constricted) is the same or less than the width of the anterior segments……..……..... *Schistocephalus*
6b. Solex clearly wider than anterior segments……..... *Bothriocephalus acheilognathi*

* Scolex characters are usually found in juvenile worms in fish.

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3.13 Lernaeid Parasitism

Ken Johnson

Department of Wildlife and Fisheries
Room 202, Nagle Hall, TXA&M
College Station, TX 77843

A. Name of Disease and Etiological Agent

Anchor parasite infection is caused by parasites of the genus *Lernaea*. Harding (1950) recognized 28 species worldwide. Lernaeid parasitism is most often associated with *Lernaea cyprinacea*. It is assumed here that *Lernaea cyprinacea* has two synonyms, *Lernaea elegans* (Leigh-Sharp 1925) and *Lernaea carassii* (Tidd 1933). Most work citing epizootics of *Lernaea* continue to be associated with the taxon *Lernaea cyprinacea*.

B. Known Geographical Range and Host Species of the Disease

1. **Geographical Range**
   Most lernaeid epizootics are associated with *Lernaea cyprinacea*. The parasite range is warmer temperate fresh waters of all continents and most islands where cyprinids have been introduced.

2. **Host Species**
   In North America, one would expect to find its distribution associated with that of the goldfish *Carassius auratus* and the common carp *Cyprinus carpio* and as a result of baitfish marketing, wherever golden shiners *Notemigonus crysoleucas* and fathead minnows (*Pimemphales promelas*) are used. It also infects a number of noncyprinid species but does not usually result in death of the fish. Among other North American species, *Lernaea cruciata* is occasionally seen as a problem with centrarchids.

C. Epizootiology

The infection cycle consists of the following: eggs that undergo final development within and hatch from dual egg sacs affixed posteriorly and exteriorly on bodies of parasitic adult females; larvae which develop by passing through a series of stages until terminal-stage larvae of females become parasitic and penetrate their host's skin; and adult females that after undergoing a metamorphosis into mature, anchor-shaped adults will complete the cycle by producing the egg-containing sacs close to their posterior ends that protrude from the host into the water medium. Optimum temperature for the infection cycle is 23 to 40^{o}C and dormancy occurs below 15^{o}C. Typical cycling occurs every 20 to 25 days at 20 to 30^{o}C.

Transmission between systems can occur as larval stages are passed with water but most dispersal is by infected fishes. Tadpoles are also parasitized and they and their semi-terrestrial forms are known to act in dispersal. *Lernaea cyprinacea* has a strong affinity for cyprinids and will accumulate in culture systems on these hosts to a degree that infections of companion groups such as ictalurids or centrarchids also obtain epizootic numbers. *Lernaea* spp. are not found in waters with salinity greater then 1.8 parts per thousand (Hoffman 1976).

D. Disease Signs

1. **Behavioral Signs**
   Behavioral changes are similar to infections of other external parasites (flashing, listlessness, and eventual morbidity).

2. **External Gross Signs**
   If there is opportunity to observe fishes closely or in hand, the parasites are readily apparent. They appear as bristle-like projections usually up to 6.5 mm long attached to the body surface. Usual attachment sites are fin bases and the oral cavity, but any exterior surface may be parasitized. Irritation by the parasites and local microbial infection at attachment sites will usually show some degree of inflammation and ulceration. The presence of several parasites does not equate to morbidity unless they provide opportunity for a secondary infection by microbes or unless fishes are particularly small.

3. **Internal Gross Signs**
   Internal signs are not evident unless the anchors penetrate the visceral cavity on small fish. Any organ penetrated will receive obvious physical damage.

4. **Histopathology**
   A fibrinous nodule eventually forms around the head of the parasite.

E. Disease Diagnostic Procedures

1. **Presumptive Diagnosis**
   Occurrence of a high parasite intensity or an apparent correlation between a population portion hosting large parasite numbers per fish and a diseased state is evidence for a presumptive diagnosis.

2. **Confirmatory Diagnosis**
   The entire animal should be examined, including the oral cavity. Verification of parasite identity may be conducted by examination of carefully dissected parasites (Figure 1). The characteristic anchor-shape is helpful in distinguishing lernaeids from other copepods, among them the copepod genus *Achtheres* whose members commonly occur in oral cavities. *Lernaea* spp. adults are usually less than 1.5 cm in length. The arm-like extensions of the anterior end (embedded) and the pencil-shaped posterior portion are relatively stiff but may be inadvertently torn apart during dissection. Twin egg sacs are usually seen at their connection near the posterior end. Infective stages, which are similar in appearence to free-living copepods, are sometimes observed by preparing wet-mounts of skin scrapings or gill filament clippings.

Morphology of dorsal and ventral arms of the cephalic region are primary characters used in distinguishing species (see Harding 1950 for key) but have the disadvantage of being somewhat variable. It is common to see specimens of *Lernaea cyprinacea* with dorsal arms that are less branched. *Lernaea cruciata*, a species sometimes associated with centrarchids, has dorsal and ventral arms of about equal size.

Specimens can be preserved in 5 to 10% formalin and then, if desired, mounted by placing directly into Hoyer medium.

F. Procedures for Detecting Subclinical Infections

A search for parasites in the manner described above.

G. Procedures for Determining Prior Exposure to the Etiological Agent

None available.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

When there is a need to keep the parasites alive for experimental work, infected fish carriers should be kept alive after capture. Shields (1968) describes methodology for maintaining a continuous culture of these parasites.

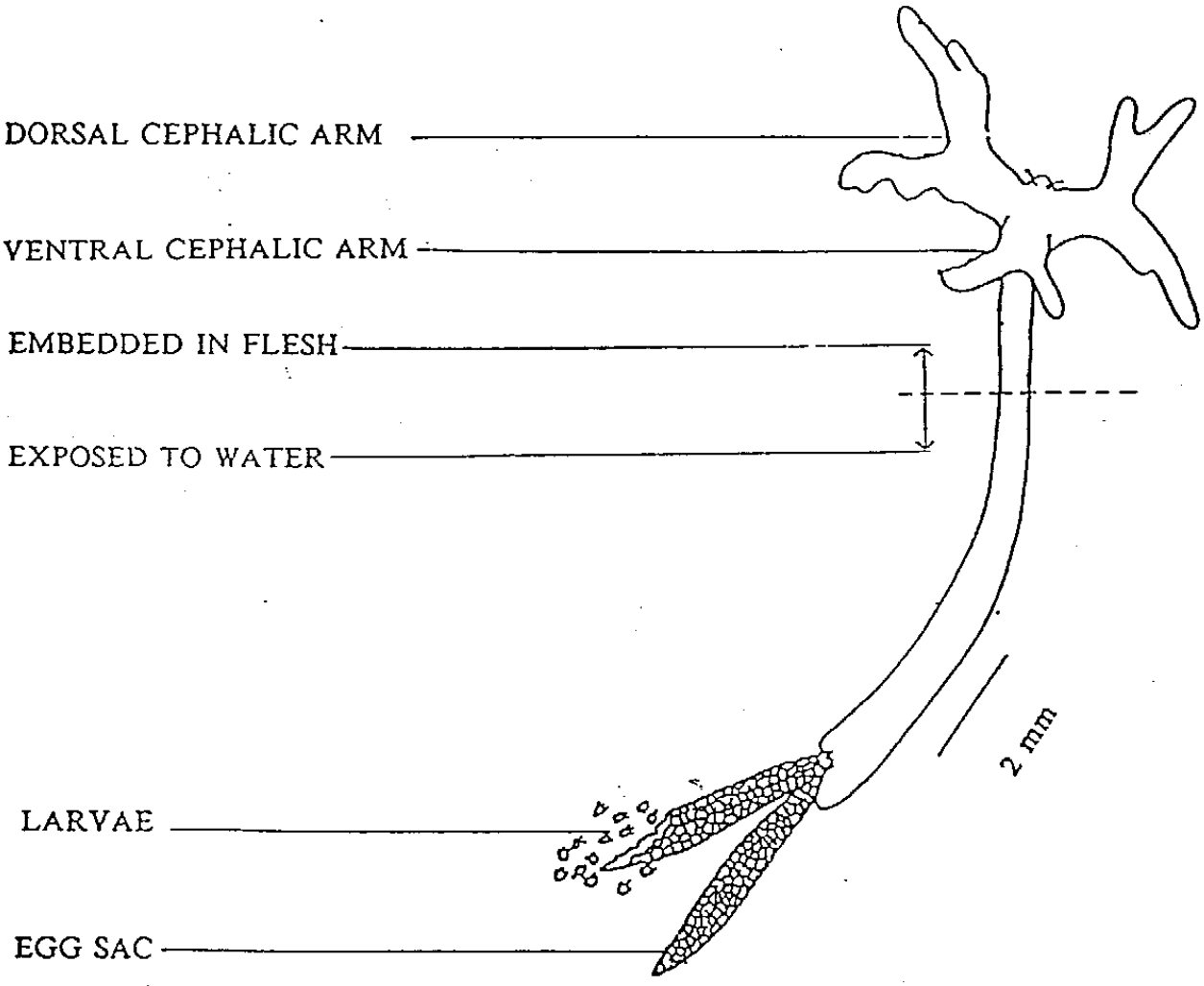


Figure 1. Diagnostic features of *Lernaea cyprinacea*, a species commonly associated with a wide variety of hosts.

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3.14 Gill Maggot Disease (Genus *Salmincola*)

David Conley

Institute of Parasitology
McGill University
MacDonald College
Ste. Anne-de-Bellevue, Quebec H9X 1C0 Canada
514/398-7722

A. Name of Disease and Etiological Agent

Gill maggot of salmon and trout is also called fish louse or gill louse.

1. *Salmincola salmoneus*

2. *Salmincola californiensis*
   Synonyms include: *Salmincola bicauliculata, Salmincola carpenteri,* and *Salmincola falculata*.

3. *Salmincola edwardsii*
   Synonyms include: *Lernaeopoda edwardsii, Lernaeopoda arcturi,* and *Salmincola exsanguinata*.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range
Circumpolar, occurring in freshwater habitats of the northern hemisphere.

a. *Salmincola salmoneus*
   Natural distribution straddles Atlantic Ocean and is found in North America and Europe.

b. *Salmincola californiensis*
   Natural distribution straddles Pacific Ocean and is found in North America and Asia.

c. *Salmincola edwardsii*
   Natural distribution includes eastern and central North America and Europe.

2. Host Species
Found in fish of the Family *Salmonidae*.

a. *Salmincola salmoneus*
   Found in Atlantic salmon (*Salmo salar*).

b. *Salmincola californiensis*
Reported on coho salmon *Oncorhynchus kisutch*, sockeye salmon *Oncorhynchus nerka*, pink salmon *Oncorhynchus gorbuscha*, chum salmon *Oncorhynchus keta*, chinook salmon *Oncorhynchus tshawytscha*, rainbow trout *Oncorhynchus mykiss*, and cutthroat trout *Oncorhynchus clarki*. Also reported on dolly varden *Salvelinus malma*, lake trout *Salvelinus namaycush*, and mountain whitefish *Prosopium williamsoni*.

c. *Salmincola edwardsii*
Reported on brook trout *Salvelinus fontinalis*, arctic char *Salvelinus alpinus*, lake trout *Salvelinus namaycush*, and dolly varden *Salvelinus malma*. Also reported on mountain whitefish *Prosopium williamsoni*. Fingerlings of Atlantic salmon *Salmo salar*, brown trout *Salmo trutta*, and rainbow trout *Oncorhynchus mykiss* have been experimentally infected but the copepods were unable to survive and develop to maturity (D. C. Conley, unpublished data).

C. Epizootiology

Infections leading to epizootics may occur when unfiltered surface waters containing infected fish are used for fish culture or when infected fish are concentrated into high densities. Temperature and intensity of infection are directly related such that higher temperatures accelerate egg development and shorten the generation time. Heavy infections usually occur in mid to late summer and result in impaired respiration and host mortality.

D. Disease Signs

1. Behavioral Changes Associated with the Disease
No particular signs in light to moderate infections (i.e. adults on mature fish). Heavily infected fish will "flash" or jump, trying to rid themselves of the parasite. Rubbing along the surfaces of solid objects, such as the sides and bottom of tanks, is common; frayed or completely eroded fins may be observed. Fish may become darker and stay near the surface or congregate near inlets, outlets, or aeration devices, especially when water temperatures rise above optimal ranges. Rate of opercular movement may increase, accompanied by flaring of the opercula, and fish may exhibit fatigue in swiftly flowing waters. Fish may also go off their feed and become listless or solitary. Culture tanks may develop a scum on the surface due to excessive production of fish mucus. Salinity tolerance is reduced, and mortality may occur following transfer of infected fish to salt water.

2. External Gross Signs
Adult females (Figure 3) are conspicuous and readily observed on the gills and fins of hosts. They are pale yellowish, normally with two egg sacs dangling from the trunk or posterior region. Egg sacs of adults attached to gill filaments may be observed streaming from behind gill covers. Adult size ranges from 2.5 to 8 mm total length, depending on species and age. The infective copepodid stage (Figure 1) and juvenile stages (chalimus I-IV; Figure 2) range between 0.5 and 1.5 mm in size and may be detected by using a dissecting microscope. Secondary bacterial and fungal infections are sometimes present.

3. Internal Gross Signs
None reported.

4. **Histopathological Changes Associated with the Disease**
   The bulla or frontal filament and feeding of the copepod elicit an epithelial hyperplasia at the site of attachment. Hypertrophy of gill epithelium tissue leads to fusion of adjacent lamellae and gill filaments. Hemorrhages and/or "blood blisters" may occur near the site of attachment, which may lead to anemia in severe infections. Infected gill filaments become pale and swollen or "clubbed" in appearance. The gills become mucous coated and ragged looking or "crypted" due to atrophy or growth inhibition of affected filaments. Respiration, excretion, and osmoregulation may be impaired.

E. Disease Diagnostic Procedures

1. **Presumptive Diagnosis**
   Presence of adult female copepods and/or juvenile stages on gills, in gill chambers, and on fins and skin of the hosts.

2. **Confirmatory Diagnosis**
   Salmincola can be identified using fish disease texts with good illustrative drawings and photos (e.g. Kabata 1988). For expert diagnosis to the species level, remove the copepods from fish and place in a glass or plastic vial with 70% alcohol or 5% formalin and forward to a fish parasitologist.

F. Procedures for Detecting Subclinical Infections

Sampling of fish populations or lots using a sample size adequate to detect a 5% level of prevalence (see Ossiander and Wedemeyer 1973). The infective stage (copepodid) and juvenile stages (chalimus I-IV) may be detected by using a dissecting microscope with objectives 6 to 12X.

G. Procedures for Determining Prior Exposure to the Etiological Agent

Removal of the operculum and examination of gill filaments using a dissecting microscope may reveal tissue damage or crypting due to previous infections. Frontal filaments (of juveniles) and bullae (of adults) may sometimes be distinguished from surrounding tissue. Gill tissues may recover after a light to moderate infection thus obscuring signs of a prior infection. There are no serological tests available at this time to determine prior exposure.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Live or fresh fish (on wet ice) are preferred. If transport of whole fish is inconvenient, use forceps to remove the adults from infected gills, fins, or skin, and place the copepods in cold water in a plastic bag or suitable container and ship on wet ice. The copepods may live up to several days when kept cold.

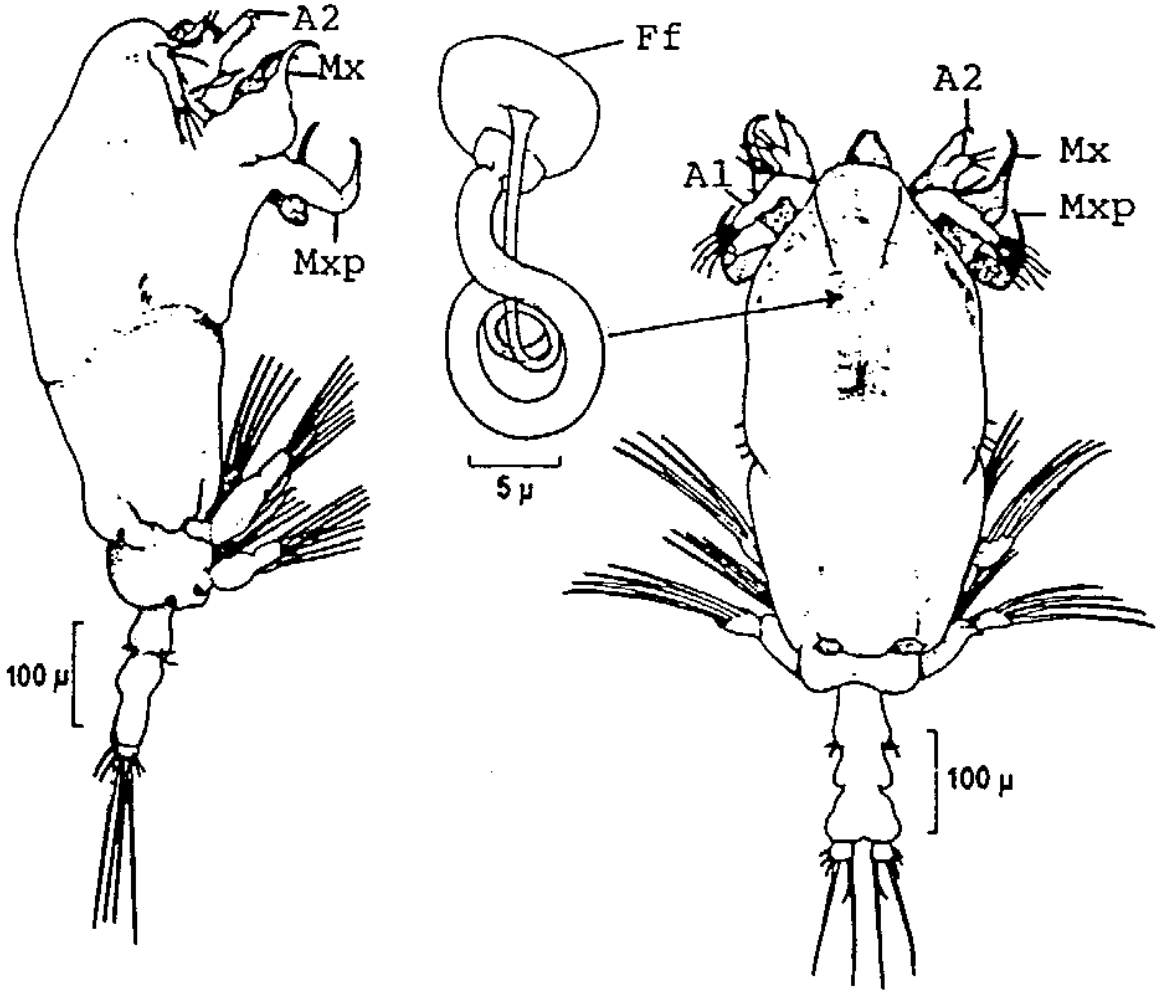


Figure 1. *Salmincola*, copepodid (infective stage). A1 - antennule. A2 - antenna. Mx – Maxilla. Mxp – maxilliped. Ff – frontal filament. (From Kabata and Cousins 1973).

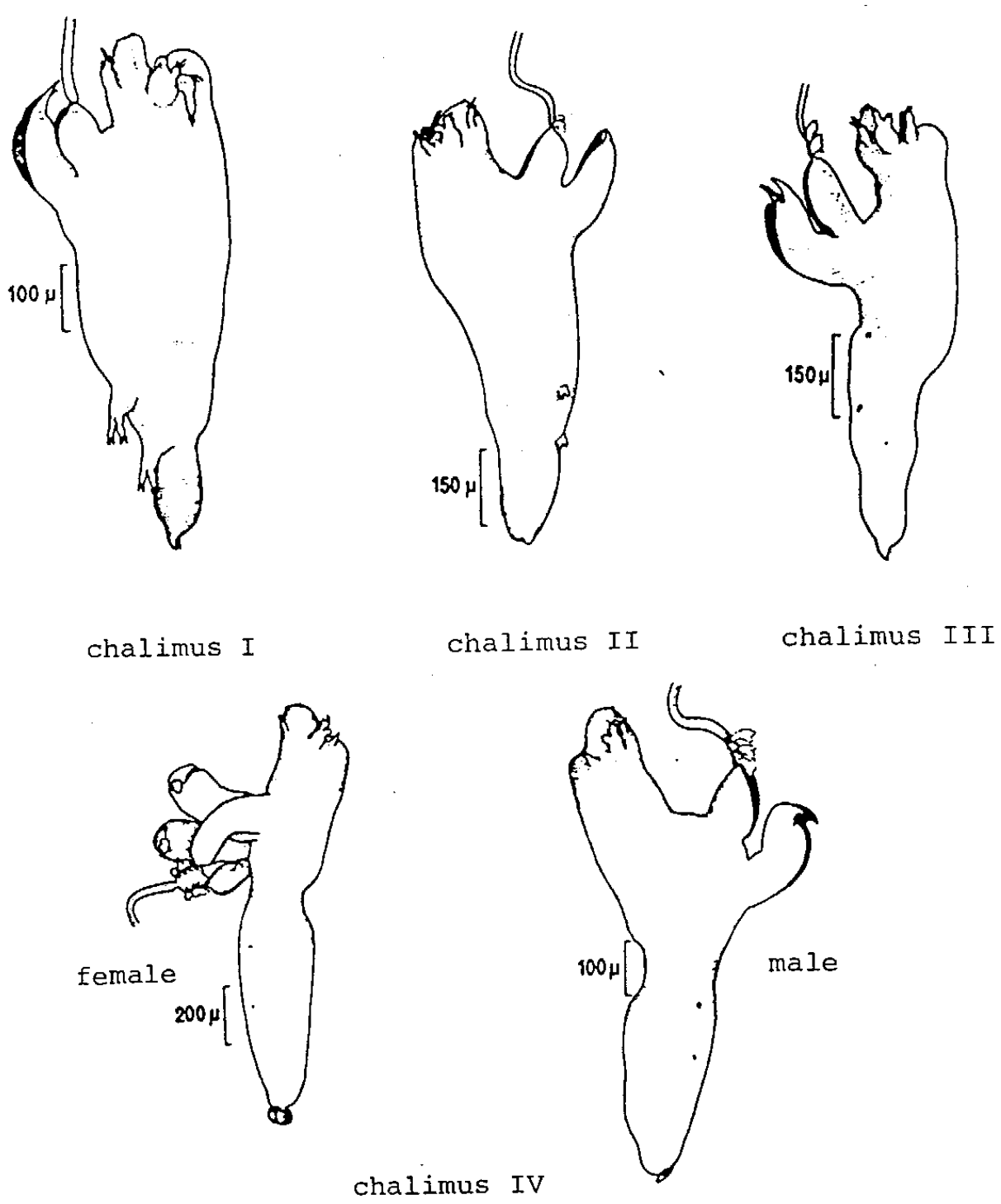


Figure 2. *Salmincola*, chalimus stages (attached by frontal filament). (From Kabata and cousins 1973).

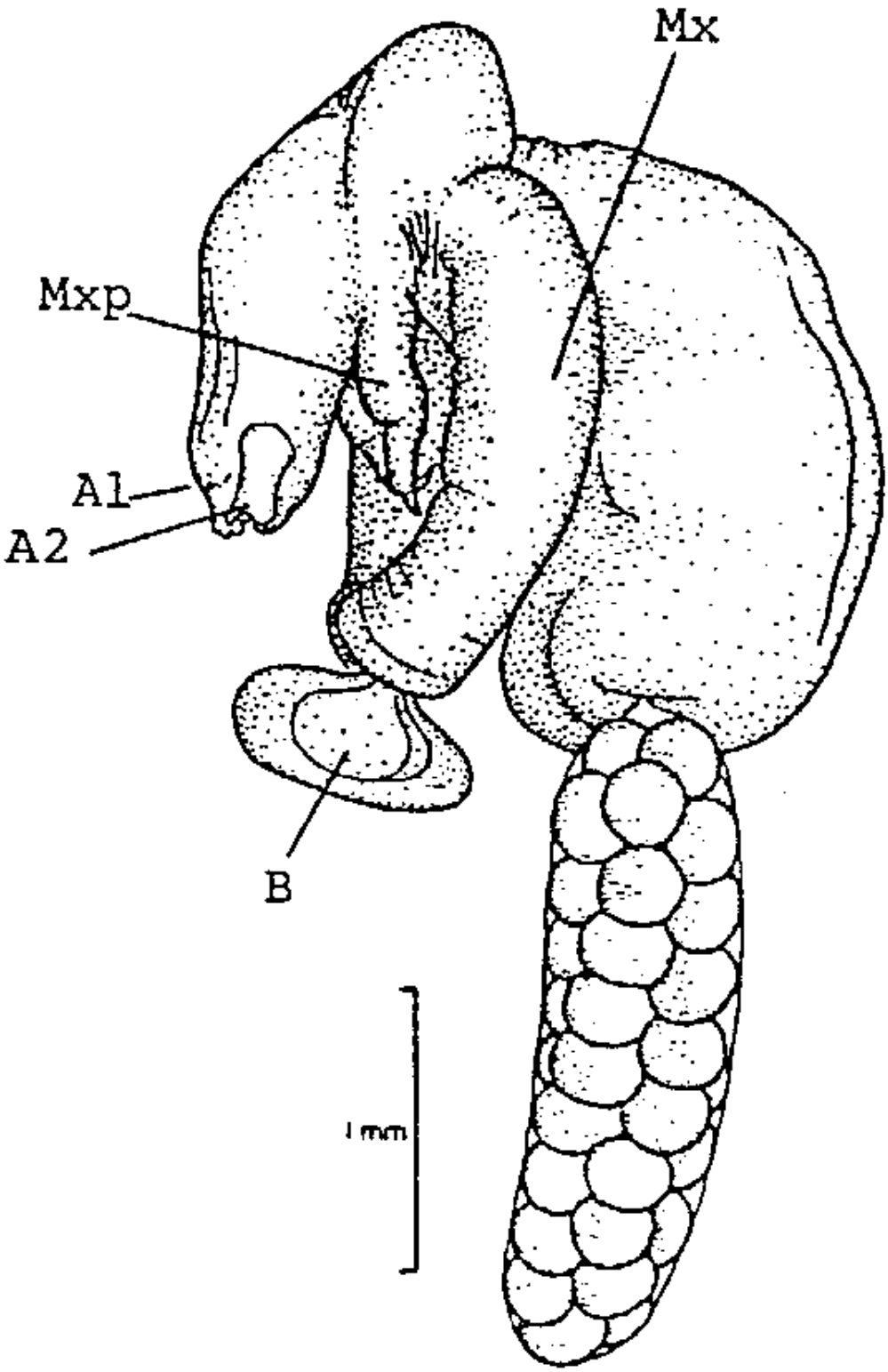


Figure 3. Salmincola, adult female. A1 – antenna. Mx – maxilla. Mxp – maxilliped. B – bulla. (From G. Fryer 1981).

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3.15 Sea Lice

Stewart Johnson and Leo Margolis

Department of Fisheries and Oceans
Biological Sciences Branch
Pacific Biological Station
Nanaimo, British Columbia V9R 5K6
Canada
604/756-7000

A. Name of Disease and Etiological Agent

The term sea lice is commonly used to refer to several species of marine ectoparasitic copepods of the family *Caligidae* (order *Copepoda*: suborder *Siphonostomatoida*) that infect salmonids. These species include *Caligus clemensi*, *Caligus curtus*, *Caligus elongatus*, and *Lepeophtheirus salmonis*. Other species in the family *Caligidae* are parasites of a wide variety of marine fishes. Emphasis here is only on those species that affect farmed salmonids.

Synonyms include: salmon louse, salmon lice, sea louse, white spot, and summer lesion syndrome. The use of the term "salmon louse" should be restricted to the common name for *L. salmonis.*

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Sea lice have been reported on wild and farmed salmonids in the North Atlantic and North Pacific oceans and adjacent seas. The sea lice species found on sea-farmed salmonids vary according to geographic region, as follows:

a. *Caligus clemensi*
Found in the Pacific coast of Canada (British Columbia) and probably the northwest coast of the United States (Washington).

b. *Caligus curtus*
Found in the Atlantic coast of Canada (Bay of Fundy).

c. *Caligus elongatus*
Found in the Atlantic coast of Canada, Ireland, and Scotland.

d. *Lepeophtheirus salmonis*
Found in the Pacific coast of Canada, Atlantic coast of Canada, Ireland, Scotland, and Norway. Although this is a marine species, it may be found on the body surfaces of salmonids that have recently entered fresh water.

2. Host Species

The species of *Caligus* occur naturally on a wide variety of hosts. *Lepeophtheirus salmonis* is essentially limited to salmonids.

a. *Caligus clemensi*

Salmonid hosts include: pink salmon *Oncorhynchus gorbuscha*, chum salmon *Oncorhynchus keta*, coho salmon *Oncorhynchus kisutch*, sockeye salmon *Oncorhynchus nerka*, and rainbow or steelhead trout *Oncorhynchus mykiss*. This species likely occurs also on chinook salmon *Oncorhynchus tshawytscha*.

Other hosts include: Pacific herring *Clupea harengus pallasi*, three spine stickleback *Gasterosteus aculeatus*, greenling *Hexagrammos* sp., Pacific ratfish *Hydrolagus colliei*, copper rockfish *Sebastes caurinus*, and walleye pollack *Theragra chalcogramma*.

b. *Caligus curtus*

Salmonid hosts include: Atlantic salmon, *Salmo salar*.

Other hosts include: generally considered a parasite of gadid fishes, but has a broad host range, including more than 35 species of teleosts and elasmobranchs.

c. *Caligus elongatus*

Salmonid hosts include: rainbow trout, brown trout *Salmo trutta*, Atlantic salmon, and brook trout *Salvelinus fontinalis*.

Other hosts include: a very broad host range, with more than 80 species of teleost and elasmobranch hosts reported.

d. *Lepeophtheirus salmonis*

Salmonid hosts include: pink, chum, coho, sockeye, chinook, Atlantic, cherry or masu salmon, *Oncorhynchus masou*, rainbow trout, coastal cutthroat trout *Oncorhynchus clarki*, and brook trout.

Other hosts include: far east rudd *Leuciscus brandti*, flag rockfish *Sebastes rubrivinctus*, sand lance *Ammodytes hexapterus*, and white sturgeon *Acipenser transmontanus*.

Current studies indicate differences in susceptibility to *Lepeophtheirus salmonis* among three salmonid species tested. The order of susceptibility is 1) Atlantic salmon, 2) chinook salmon, and 3) coho salmon.

C. Epizootiology

1. Conditions Under which Disease Occurs

All species of sea lice studied have 10 developmental stages. These stages include two free-living planktonic nauplius stages, one free-swimming infectious copepodid stage, four attached chalimus stages, two preadult stages, and one adult stage. All stages except the nauplii feed on mucus, skin, and blood. The adults usually cause the most serious disease state.

In wild salmonids, sea lice rarely cause disease, although localized tissue damage commonly occurs on wild salmonids through the grazing activities of adult *Lepeophtheirus salmonis*.

In farmed salmonids, sea lice have been reported to cause serious disease in many seawater netpen facilities and in some seawater-fed landbased facilities. Important biological and environmental factors that may determine the severity of infections include water temperature, presence of reservoir hosts, water circulation, salinity, species cultured, general fish health, and stocking density. Few of these factors have been sufficiently investigated.

In general, prevalence of sea lice increases with higher temperatures, independent of time of season. Generation times of sea lice are temperature dependent. *Caligus elongatus* is reported to have a generation time of approximately five weeks at 10 to 13^{o}C. The generation time of *Lepeophtheirus salmonis* is approximately six weeks at 9 to 12^{o}C and 7.5 weeks at 10^{o}C.

Egg-bearing female *Caligus elongatus* and *Lepeophtheirus salmonis* are present throughout the year. Hatching of the eggs and development to the infective copepodid stage in both species occurs at temperatures as low as 5^{o}C.

The presence of wild hosts may serve as a reservoir of sea lice. Many non-salmonid hosts of *Caligus elongatus* are often attracted to and present in large numbers in the vicinity of netpen sites. Wild hosts were the source of *Caligus clemensi* that infected salmonids in a seawater tank facility in British Columbia.

In British Columbia, Atlantic salmon and rainbow trout are generally more heavily infected with sea lice than chinook or coho salmon when raised at the same site.

Some evidence suggests that the severity of infections at particular sites increases with time, although the reason for this increase is unknown.

All salmonid stages reared in salt water are susceptible to infection with sea lice. The relationship of the number of sea lice to severity of the disease is dependent on 1) size and age of the fish, 2) the general state of health of the fish, and 3) the species and developmental stages of the sea lice present.

D. Disease Signs

1. Behavioral Changes Associated with the Disease

Studies on leaping behavior in salmon suggest a relationship between the presence of sea lice and leaping frequency, but the evidence is not conclusive.

2. External Gross Signs

Copepodids and chalimus larvae are small (<4 mm in length) and can occur on all exterior surfaces of the body and fins as well as in the buccal cavity and on the gills. Preadult and adult sea lice are visible to the naked eye. They occur on the body surfaces, especially on the head and back and in the perianal region.

Damage by copepodid and chalimus larvae is limited to a small area around their point of attachment. They erode the epidermis and sub-epidermis. Heavy infections of *Caligus clemensi* on the fins of pink salmon resulted in various stages of fin damage up to complete removal. Because the preadult and adult parasites are larger and capable of moving on the surface of the fish, damage is more severe and widespread. Heavily infected salmon commonly show grey

patches (extensive areas of skin erosion and hemorrhaging) on the head and back, and a distinct area of erosion, dark coloration, and sub-epidermal hemorrhages in the perianal region. The most severe damage is seen on the head of infected Atlantic salmon. Death may be caused by secondary bacterial infections (e.g. vibriosis), fungal infections when fish are re-introduced to fresh water, or, in severe cases, by osmotic stress.

3. **Internal Gross Signs**
   None reported.

4. **Histopathological Changes Associated with the Disease**
   In areas of copepod feeding, the epidermis of Atlantic salmon is eroded and the dermis is edematous with hemorrhagic areas. Fins of Atlantic and chinook salmon infected with chalimus larvae of *Lepeophtheirus salmonis* show erosion of the dermis. The skin and fins of coho salmon infected with the chalimus larvae of *Lepeophtheirus salmonis* show a pronounced epithelial hyperplasia. In many instances chalimus larvae on coho fins are surrounded by epithelial tissue and inflammatory infiltrate.

E. Disease Diagnostic Procedures

1. **Presumptive Diagnosis**
   A presumptive diagnosis is made by detecting the presence of copepods and determining their specific identity through microscopic examination. The small size of the copepodid and chalimus stages requires the use of a dissecting microscope to detect their presence. Easily recognized features used for distinguishing among the four species of sea lice found on sea-farmed salmon and trout include the presence or absence of lunules, body shape, and structure of the first and fourth legs. General body shape can be determined using a dissecting microscope. Determining the structures of the first and fourth legs requires their removal and examination under a compound microscope.

2. **Confirmatory Diagnosis**
   In general, the preadult and adult stages of *Caligus* species can be easily distinguished from those of *Lepeophtheirus salmonis* by the presence of lunules (Figure 1).

   On the Pacific coast of Canada, the preadult and adult stages of *Caligus clemensi* can be distinguished from those of *Lepeophtheirus salmonis* by the presence of lunules (Figure 1). Identification of preadult and adult *Caligus clemensi* can be confirmed by reference to Kabata (1972; 1988) and Parker and Margolis (1964). Identification of adult *Lepeophtheirus salmonis* can be confirmed by reference to Kabata (1973; 1979; 1988). Earlier developmental stages of *Caligus clemensi* can be identified by reference to Kabata (1972). Earlier developmental stages of *Lepeophtheirus salmonis* can be identified by reference to Johnson and Albright (1991).

   In Atlantic waters and adjacent seas, the preadult and adult stages of *Caligus curtus* and *Caligus elongatus* can be distinguished from *Lepeophtheirus salmonis* by the presence of lunules (Figure 1). Adult *Caligus curtus* can be distinguished from *Caligus elongatus* by differences in the shape of the genital complex and abdomen as well as differences in the number of setae on the exopod of the fourth leg (Figures 1 and 2). The fourth leg has four setae in *Caligus curtus* and five setae in *Caligus elongatus*. Species identification can be confirmed by reference to Parker et al. (1968) and Kabata (1979; 1988). The earlier developmental stages of *Caligus curtus* and *Caligus elongatus* have not been described.

F. Procedures for Detecting Subclinical Infections

Same as Disease Diagnostic Procedures above.

G. Procedures for Determining Prior Exposure to the Etiological Agent

There are no procedures available at present.

H. Procedures for Transport and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Samples of sea lice can be preserved in 10% formalin or 70% ethyl alcohol. If not available, any other histological preservative (e.g., Bouin's; Davidson's) can be used.

For long-term storage, fix for two weeks in 10% formalin and then transfer to 10% glycerine alcohol (10 ml glycerine in 90 ml of 70% ethyl alcohol).

I. Key to Adult Sea Lice of Salmon and Trout

The following key serves to distinguish the adults of the four species of sea lice reported from sea-farmed salmon and trout.

1. Lunules absent *Lepeophtheirus salmonis*……..……..(Figures 1A and 1B) (Atlantic and Pacific)
   Lunules present…..……2
2. Exopod of fourth leg with 4 setae……(Figure 2A)
   Distal margin of exopod of first leg with 4 undivided setae, seta 4 longer than others...(Figure 3A)
   Caligus curtus……(Figures 1C and 1D) (Atlantic)
   Exopod of fourth leg with 5 setae (Figures 2B and 2C)……...……3
3. Distal margin of exopod of first leg with setae 1 and 4 undivided and unarmed, setae 2 and 3 bifid……(Figure 3B)
   Caligus clemensi……...(Figures 1E and 1F) (northeast Pacific)
   Distal margin of exopod of first leg with setae 1 and 4 undivided and armed, setae 2 and 3 appearing chelate due to presence of secondary process arising near midlength...(Figure 3C)
   Caligus elongates……..….(Figures 1G and 1H) (Atlantic)

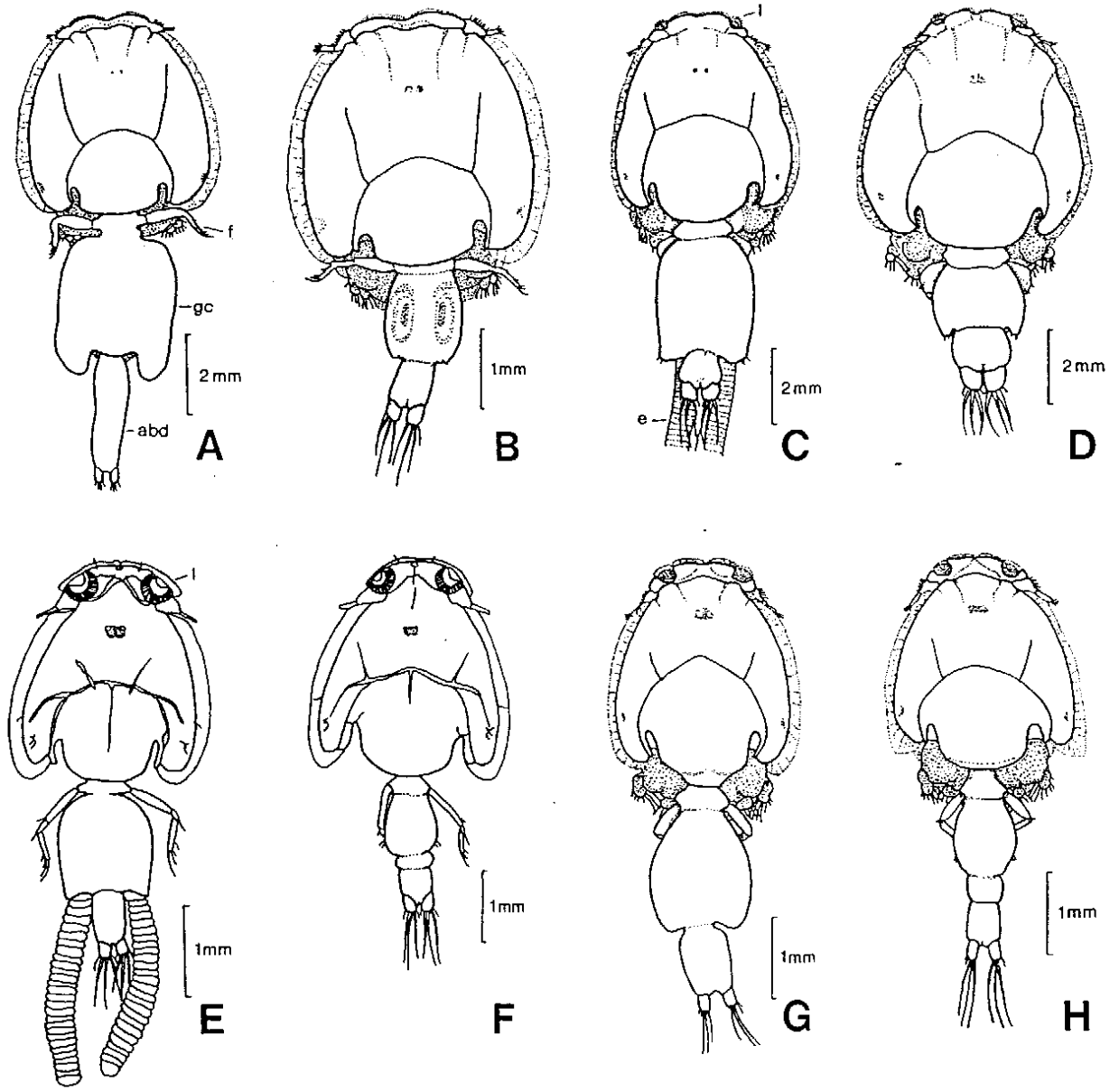


Figure 1. Adult stages of sea lice: (A) *Lepeophtheirus salmonis*, female; (B) same, male; (C) *Caligus curtus*, female; (D) same, male; (E) *Caligus clemensi*, female; (F) same, male; (G) *Caligus elongates*, female; (H) same, male.

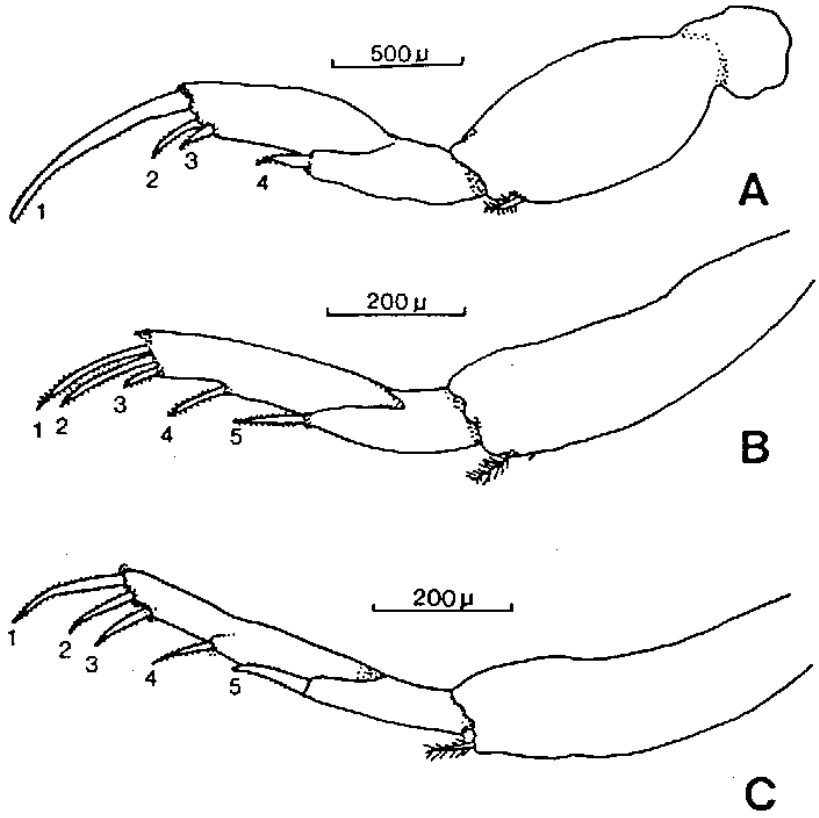


Figure 2. Structure of the fourth leg: (A) *Caligus curtus*; (B) *Caligus clemensi*; (C) *Caligus elongates* (A, redrawn from Kabata 1979; B, redrawn from Kabata 1972; C, original).

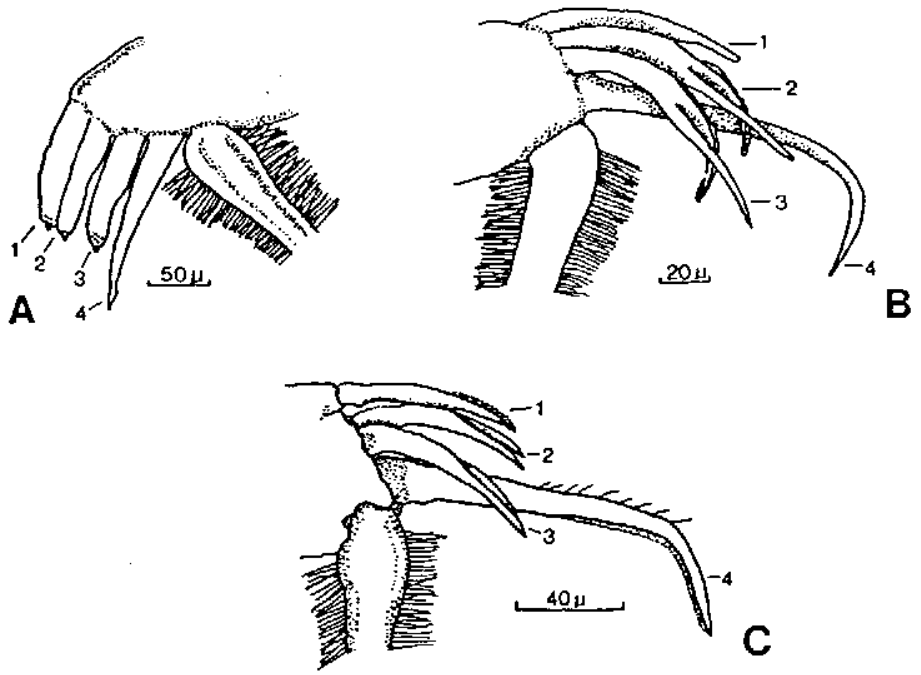


Figure 3. Distal margin of the exopod of the first leg: (A) *Caligus curtus*; (B) *Caligus clemensi*; (C) *Caligus elongates* (A, C, modified from Kabata 1979).

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3.16 Branchiuran Fish Louse Disease

Bill Hogans

Atlantic Reference Center
Huntsman Marine Science Center
St. Andrews, New Brunswick E0C 2X0
Canada
506/529-8895

A. Name of Disease and Etiological Agent

1. Name of Disease
Fish louse or fish lice.

2. Etiological Agent
Argulus. Over 120 species reported from freshwater and marine fishes. The genus is in poor taxonomic condition. Many species presently documented are based on inadequate original descriptions and are probably synonomous with the more common and established species.

3. List of Common or Economically Important Species of Argulus

a. North America
Argulus americanus, Argulus alosae, Argulus appendiculosus, Argulus borealis, Argulus catastomi, Argulus coregoni, Argulus flavescens, Argulus funduli, Argulus japonicus, Argulus laticauda, Argulus megalops, Argulus maculosus, Argulus pugattensis, Argulus stizostethii, and Argulus versicolor.

b. Eurasia
Argulus coregoni, Argulus indicus, Argulus japonicus, Argulus foliaceus, Argulus scutiformis, and Argulus viridis.

c. Africa
Argulus africanus, Argulus amblopites, Argulus brachypeltis, Argulus exiguus, Argulus jollymanni, Argulus rhipidophorus, and Argulus striatus.

d. South America
Argulus chromidis, Argulus juparensis, Argulus nattereri, Argulus pestifer, and Argulus violaceus.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

The majority of species are pan-continental in North and South America, Africa, and Eurasia; a few species are circumglobal. All species are found in freshwater or marine environments (16 species have been described and no localities given). Many species reported as being "restricted" to fresh or marine waters often occur on anadromous hosts in estuarine habitats. Some species are restricted to Atlantic or Pacific coasts of continents or restricted to freshwater lakes and rivers.

2. Host Species

Majority of *Argulus* species show extremely low host-specificity (e.g. *Argulus flavescens* has been reported from 18 species of freshwater fishes in North America). A single species may infect fishes from several different orders and families. The argulids can be considered opportunistic parasites in most reported cases, particularly on cultured fishes.

C. Epizootiology

The fertilized female leaves the host skin or gills and lays several hundred eggs on submerged objects. A free-swimming second copepodid larva hatches after 48 to 72 hours, and must find a host within 48 to 98 hours or die.

Larvae are positively phototaxic, and development occurs more rapidly in bright illumination. Several molts of copepodid and sub-adult stages occur on the host. The adult stage is reached in approximately 15 to 40 days, depending on water temperature. Both males and females are parasitic on the host.

Egg laying begins at water temperatures between 14 and 16°C, egg development ceases below 12°C. At temperatures less than 8°C, the parasites cease growth, encase themselves in mucus, and remain on the host until the advent of warmer water temperatures (Bauer et al. 1973). Optimal temperature for adults is 23 to 28°C. Adults may live free from a host for up to 15 days. *Argulus* infects all age classes of the hosts on which they occur, being particularly infective to and occasionally causing sever mortalities of juvenile fishes.

Mortalities of both cultured and wild fish have been attributed to *Argulus* infections, although those involving wild hosts are rare (or at least rarely reported). In particular, cultured fishes of several species and age classes have suffered mass mortalities due to *Argulus* infections in North and South America, Africa, and Eurasia. The pattern of mortality is generally simple. Optimal water temperatures induce a relatively low number of parasites to breed successfully and realize a high biotic potential. Mortality of individual hosts and progress of the disease is proportional to intensity of infection. Heavily infected fish die more quickly, and transmission and production of the parasites enhanced. Initial infection of cultured fishes is usually caused by introduction of parasitized hosts. Crowding of fishes, low dissolved oxygen levels, and slow current conditions enhance the spread and patholgenicity of the disease.

D. Disease Signs

1. **Behavioral Changes Associated with the Disease**
   Light to moderate infections of *Argulus* caused cultured fishes to rub against the sides of the enclosure in an attempt to rid themselves of the parasite (i.e. flashing). Heavy infections cause the fish to dart about until exhausted or alternatively, to become lethargic and seek the sides and bottoms of tanks. Equilibrium loss has been reported as a consequence of heavy infections.

2. **External Gross Signs**
   The argulids are large parasites (when adult, 5 to 20 mm), and are usually visible on the host's skin or gill surface. Older wounds are occasionally observable after becoming necrotic or ulcerated or when affected by secondary bacterial and fungal infections. The skin, gills, and fins secrete excess mucus in response to the feeding of recently attached copepodids.

3. **Internal Gross Signs**
   Internal effects of argulid infections have not been reported.

4. **Histopathological Changes Associated with the Disease**
   The parasites pierce the host tissue with the pre-oral stylet, inject a cytolytic toxin, and feed on the blood released by the resultant wound. The surface of the host at the point of stylet entry can become erythemic and hemorrhagic. A hemorrhagic factor is produced by some species. Several parasites feeding in close proximity may cause edema and localized swelling of tissues. Proliferation surrounding the stylet entry wound has been observed.

E. Disease Diagnostic Procedures

1. **Presumptive Diagnosis**

   a. Isolation and Detection of Pathogen
      Isolation of the parasite is not necessary to determine the cause of the disease. The relatively large size and characteristic external morphology of the adults allows quick detection and identification. Recently attached juveniles are small (1 to 3 mm) and are not easily seen with the unaided eye. The larval stages of most species are unknown.

   b. Clinical Signs
      Presence of the parasites occurs on the host skin, fins, or gills. Recognition of external tissue pathology is associated with feeding behavior of *Argulus* sp.

   c. Histopathological Examination
      Not necessary for diagnosis of *Argulus*.

2. **Confirmatory Diagnosis**
   All species of *Argulus* are relatively large external parasites and are usually easily seen. All exhibit an external morphology that is characteristic and not easily confused with any other parasitic crustacean. The large dorsal shield and maxillary suckers are distinguishing features (Figure 1).

   Identification to species is based on the structure and position of the respiratory area, the number and shape of the sclerites of the supporting rods of the maxillary suckers, and the armature of the

basal plate of the second maxillae (Kabata 1988). These morphological characters serve only to differentiate the adults and subadults of species and are not adequate or applicable for larvae. There are presently no methods for differentiating species using the larval (copepodid) stage.

Microscopic examination of adults and subadults is best accomplished by clearing the whole fixed specimen in 85% lactic acid. If necessary, small appendages or parts may be dissected from the parasite and examined separately, for species determination. Staining the parasite or any of its parts with lignin pink (while in lactic acid) is helpful in differentiating fine structure.

F. Procedure for Detecting Subclinical Infections

Periodic examination of fish will reveal the parasites.

G. Procedures for Determining Prior Exposure to the Etiological Agent

No methods are presently known for determining prior exposure to *Argulus*.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Specimens of *Argulus* are best fixed in 10% neutral buffered formalin for two or three days, then transferred to 70% ethanol for preservation. If formalin is unavailable, 50% isopropyl alcohol is a suitable fixative and preservative.

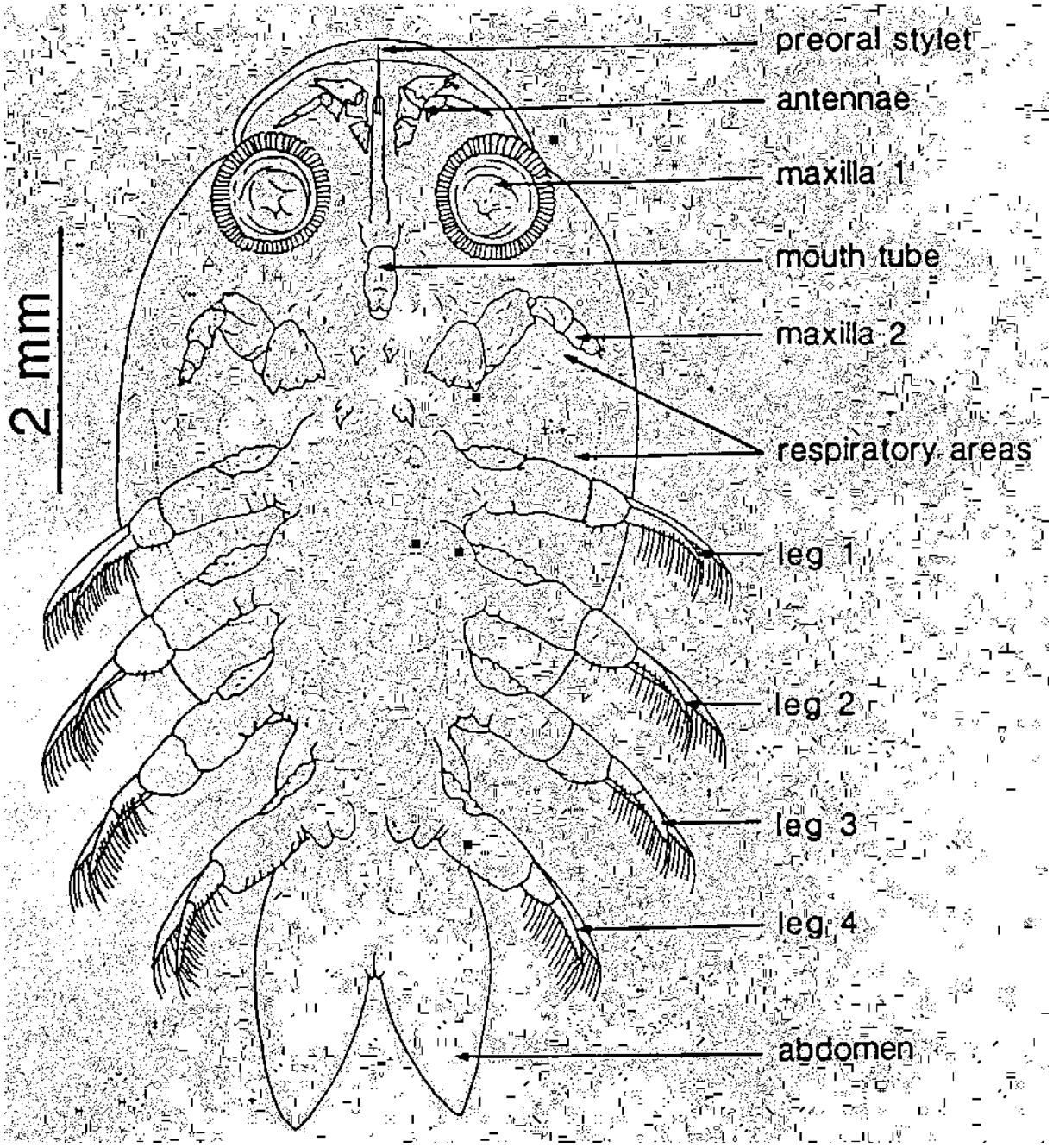


Figure 1. Ventral view of *Argulus alosae* showing pertinent diagnostic features (redrawn from Kabata 1988).

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Centrocestiasis (gill trematode disease)

Andrew Mitchell and Andrew Goodwin*

U.S. Department of Agriculture
Agricultural Research Service
H K Dupree-Stuttgart National Aquaculture Research Center
P.O. Box 1050
Stuttgart, AR 72160-1050
870/673-4483

*Aquaculture and Fisheries Center
University of Arkansas at Pine Bluff, Mail Slot 4912
1200 North University Drive, Pine Bluff, Arkansas 71601,
870/575-8137

A. Name of Disease and Etiological Agent.

Centrocestiasis is an infection of the gills of fish by a heterophyid trematode *Centrocestus formosanus* (Nishigori, 1924). Common names for the parasite include "gill trematode" and "mystery fluke."

B. Known Geographical Range and Host Species of the Disease.

1. Geographical Range

The gill trematode has been reported from fish in China, India, Israel, Japan, Malaysia, Mexico, Philippines, Taiwan, Thailand, and Vietnam. In the United States, it has been reported in Florida, Hawaii, Texas and Utah. Cases in the US have all been associated with tropical climates or warm springs.

2. Host Species

The life cycle of *C. formosanus*, includes two intermediate and one definitive or final host.

First intermediate hosts – aquatic mollusks.
Three aquatic snails from the family Thiaridae - *Melanoides tuberculata*, the red-rimmed melania, *Stenomelania newcombi* and *Thiara scabra* - are known to carry developing stages of *C. formosanus* but only *M. tuberculata*, an exotic snail, is now found in the United States in about 15 western, northwestern and southern states. *Melanoides tuberculata* is viviparous and reproduces parthenogenetically. These two factors enhance its ability to reproduce and spread. Young are born live at about 2 mm and can reach a length of 70 to 80 mm although they are more commonly 10 and 40 mm (Figure 1).



Figure 1: Red-rimmed melania (*Melanoides tuberculata*); snail vector of the gill trematode (*Centrocestus formosanus)*. Scale bar major divisions in cm.

Second intermediate hosts – fish and some frogs

The gill trematode is not host specific for freshwater fish, therefore all species should be considered as potential hosts (the parasite is found in the branchial tissue). Frogs, particularly of the genus *Rana*, should also be considered as potential hosts (the trematodes are found in the stomach wall and in the muscle).

Definitive or final hosts – birds and mammals

Aquatic birds in North America known to host the parasite include the black crown night heron *Nycticoras nycticorax* and the green backed heron *Butorides striatus*. Because several aquatic bird genera are reported as final hosts worldwide, almost any fish eating bird should be considered as a possible host. Some fish-eating mammals including rats, cats and dogs, are also reported as natural definitive hosts throughout the world.

C. Epizootiology

Trematodes are released from their cysts in the branchial tissues of fish by the digestive juices in the intestinal tract of their final hosts. The worms released into the lumen of the gut develop into egg producing adults in a few days. Eggs are shed by adult trematodes into the lower intestines of the final host and are defecated into the water. Miracidia, the first larval stages, hatch from the eggs, then enter the snail; reports on the way of entry differ. Some report that the eggs hatch in the water in about two weeks, releasing miracidia that penetrate the snail's foot. Others report that the eggs are eaten by the snails and the miracidia hatch in the snail's intestine and penetrate through the snail intestinal wall. Either way, the miracidia locate in the snail's digestive gland and begin to produce asexually giving rise to cercariae (hundreds of thousands of cercariae can be produced from one miracidium). Cercariae are the second larval stage and infective to fish. They emerge from the snails, are shed in the surrounding water, and search for suitable fish hosts.

The highest shedding rate occurs from snails 20 to 25 mm in length. The average number of cercariae released daily from one red-rimmed melania is more than 1,600 however a release rate of 63,400 cercariae in 24 h from one snail has been observed. Most shedding occurs during daylight. The cercariae of *C. formosanus* survive in waters with temperatures of 15 – 35°C (ideal range being 15 – 25°C). The survival temperature range of the host snail (17-32°C) is within that of the cercaria, therefore, anywhere the snail survives, year round infections of this trematode can occur. The cercariae can survive for 2 to 5 days in water without the host fish but are probably only infective to fish within the first 48 h. The cercaria possesses a straight slender tail, heart-shaped body, two prominent eye spots, and two suckers (an oral and a well-developed ventral sucker) (Figure 2).



Figure 2: Cercaria of *Centrocestus formosanus*. Note eye spots and straight, slender, non-forked tail. Total length of cercaria (including tail) is about 260 μm.

The cercarial body is 113 to 154 µm long and 76 to 103 µm wide and the tail length is 114 to 155 µm long and 18 to 24 µm wide. The cercariae penetrate the fish host gill tissues and migrate to positions near the gill filament cartilage (Figure 3 video).

Figure 3 Video: Cercaria of *Centrocestus formosanus* orient along the filament cartilage shortly after penetration [link].

They become metacercariae, surrounded by cysts of parasite origin that have thin inner walls about 2µm thick. As they mature the cysts are further surrounded by a cyst of host origin. Immature metacercariae still have the paired eye spots evident and their cysts are about 100 µm in length (Figure 4 video).

Figure 4 Video: Immature metacercaria of *Centrocestus formosanus* (parasite cyst about 100 µm in length) encysted in the gill filament of a fish. Eye spots are still present [link].

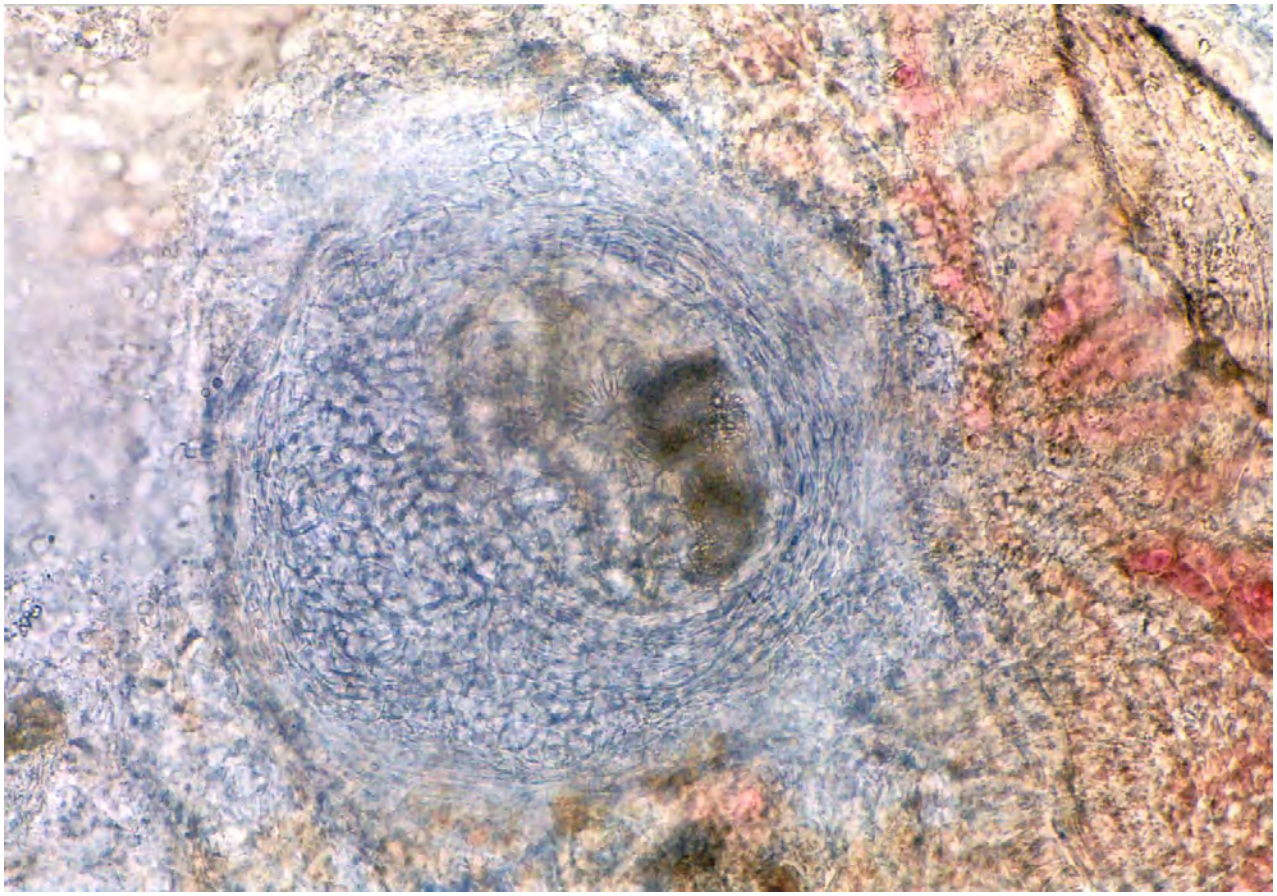


Figure 5. Maturing *Centrocestus formosanus* (parasite cyst about 150 µm in length) encysted in the gills of a fish: a) photo above and b) video showing circumoral spines [link].

They begin to mature and grow becoming ovoid metacercarial cysts of about 150 µm (107 – 225 µm) in length and of about 110 µm (78 - 159 µm) in width. Maturing metacercariae usually develop two offset rows of circumoral spines (16 spines per row around the oral sucker - Figure 5a, 5b video) and X-shaped excretory vesicles containing dark excretory granules (Figure 6a, 6b video).

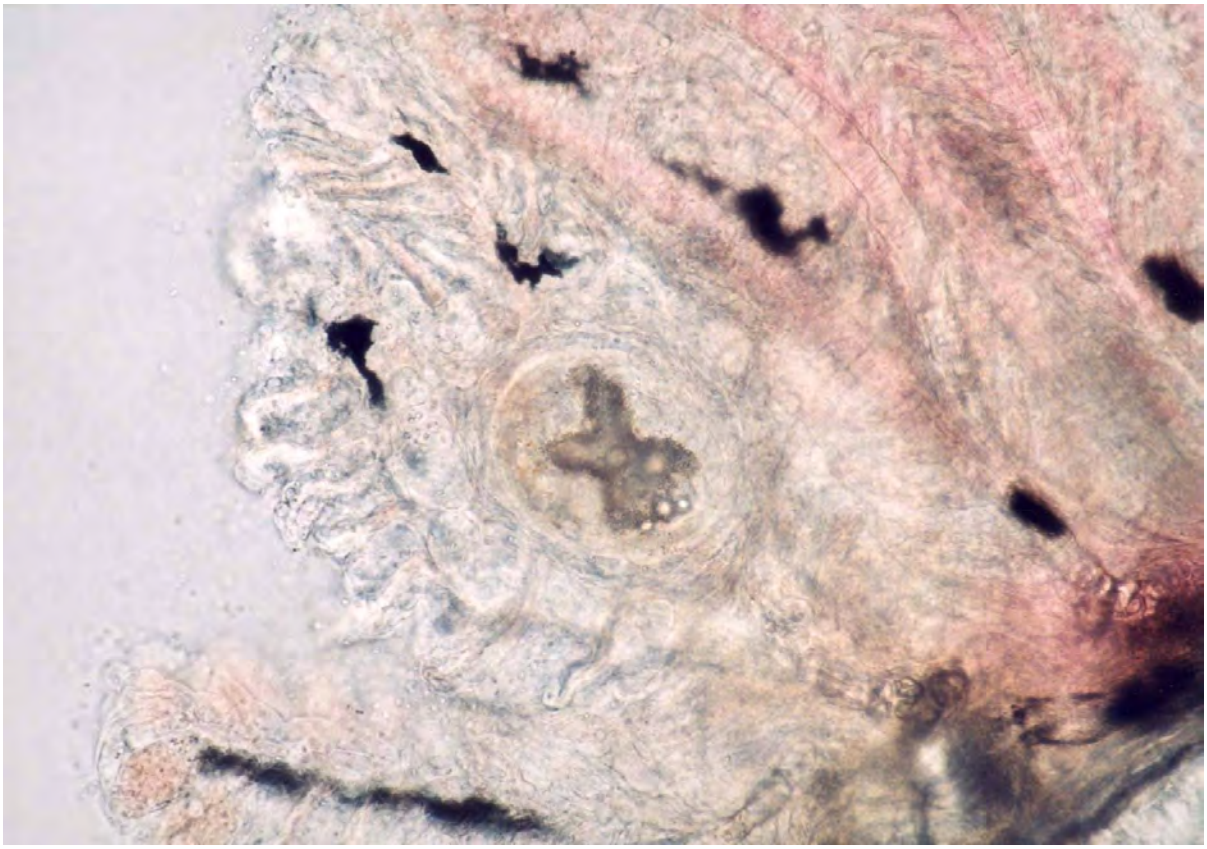


Figure 6: Maturing *Centrocestus formosanus* (parasite cyst about 150 µm in length) encysted in the gills of a fish: a) photo above and b) video showing X-shaped excretory vesicles [link].

Further development of the metacercaria is delayed until a final host eats the fish. Some metacercariae grow slower, remain smaller, retain eyespots and develop no circumoral spines. Some fish, such as greenthroat darters *Etheostoma leidum* and fountain darters *Etheostoma fonticola*, have host responses that kill the maturing metacercariae and these dead cysts (eye spots remain visible) measure about 95 µm (73 – 114 µm) by 49 µm (36 – 66 µm) (Figure 7).

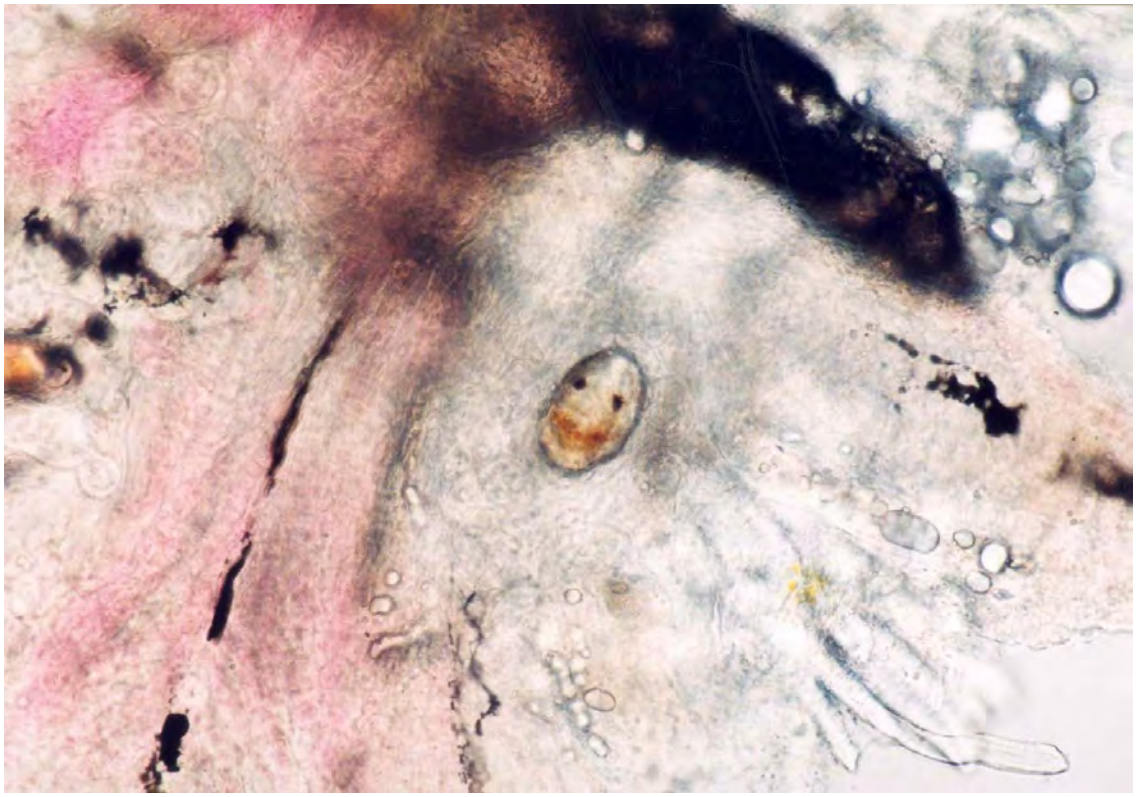


Figure 7: Dead metacercaria (parasite cyst about 95 µm in length) of *Centrocestus formosanus* encysted in the gills of a fountain darter (*Etheostoma fonticola*). Eye spots are still apparent.

The impact that this parasite has on the fish host depends on the number of metacercariae infecting the gill tissues and the size of the fish infected. Serious damage to the gills occurs in small darters (20 to 25 mm) infected with 25 cysts/gill arch while fat sleepers *Gobiomorus maculates* (up to 26 cm) survive infections of more than 350 cysts/gill arch). Also, individual fish species may have different host reactions that could affect the impact of the infection.

D. Disease Signs

1. Behavioral Changes Associated with the Disease

Increased respiration rates in experimentally infected fish have been observed. Infected fish may appear somewhat listless and lay on the bottom. Fish with a heavy gill trematode infection may die more readily following handling than fish with light infections or no infection at all.

2. External Gross Signs

No clinical signs are apparent when *C. formosanus* infections are light. In heavy infections, the opercular flaps, particularly of smaller fish, become flared by distorted branchial tissues. The gills may press against the wall of the pharynx and opercular flap producing an intense red color visible externally through the operculum (Figure 8).



Figure 8: Ventral view of a fountain darter (*Etheostoma fonticola*) with massive infection of *Centrocestus formosanus* and an uninfected fish (lower). Opercula flared outward by deformed and hyperplastic gill tissue (each division of scale = 1 mm).

3. Internal Signs

By light microscopy of gill wet mounts, cysts are evident in the gills (sizes given above) often with areas of clearing surrounding the cysts (Figure 9).



Figure 9: Gills of channel catfish (*Ictalurus puncatus*) experimentally infected with *Centrocestus formosanus*. Host cyst walls are seen as areas of clearing (cartilage proliferation) around metacercaria (parasite cyst about 100 μm in length).

In fish with numerous cysts, the filaments are shortened and thickened, there is epithelial hyperplasia, fusion of filaments, and lamellae engorged with red blood cells (Figure 10).



Figure 10: Gill of a fountain darter (*Etheostoma fonticola*) infected by metacercaria of Centrocestus formosanus. Low and higher magnification views show shortened and thickened gill filaments, epithelial hyperplasia, and damaged lamellae engorged with blood (parasite cyst about 95 μm in length).

Gill cartilage is often deformed and may have thickened areas that bend or branch (Figure 11 video).

Figure 11 Video: Gill of a channel catfish (*Ictalurus puncatus*) experimentally infected with *Centrocestus formosanus.* Gill filaments are deformed (thickened and bent) in areas adjacent to parasite cysts [link].

4. Histopathological Changes Associated with the Disease

The trematodes migrate to just outside the cartilage rod and are encysted in chondrocytes and cartilage closely associated with the filamental cartilage. These cysts produce the clear areas seen in wet mounts of gill tissue. Distortion of the gill architecture frequently leads to a loss of lamellae in areas adjacent to the encysted parasite (Figure 12).

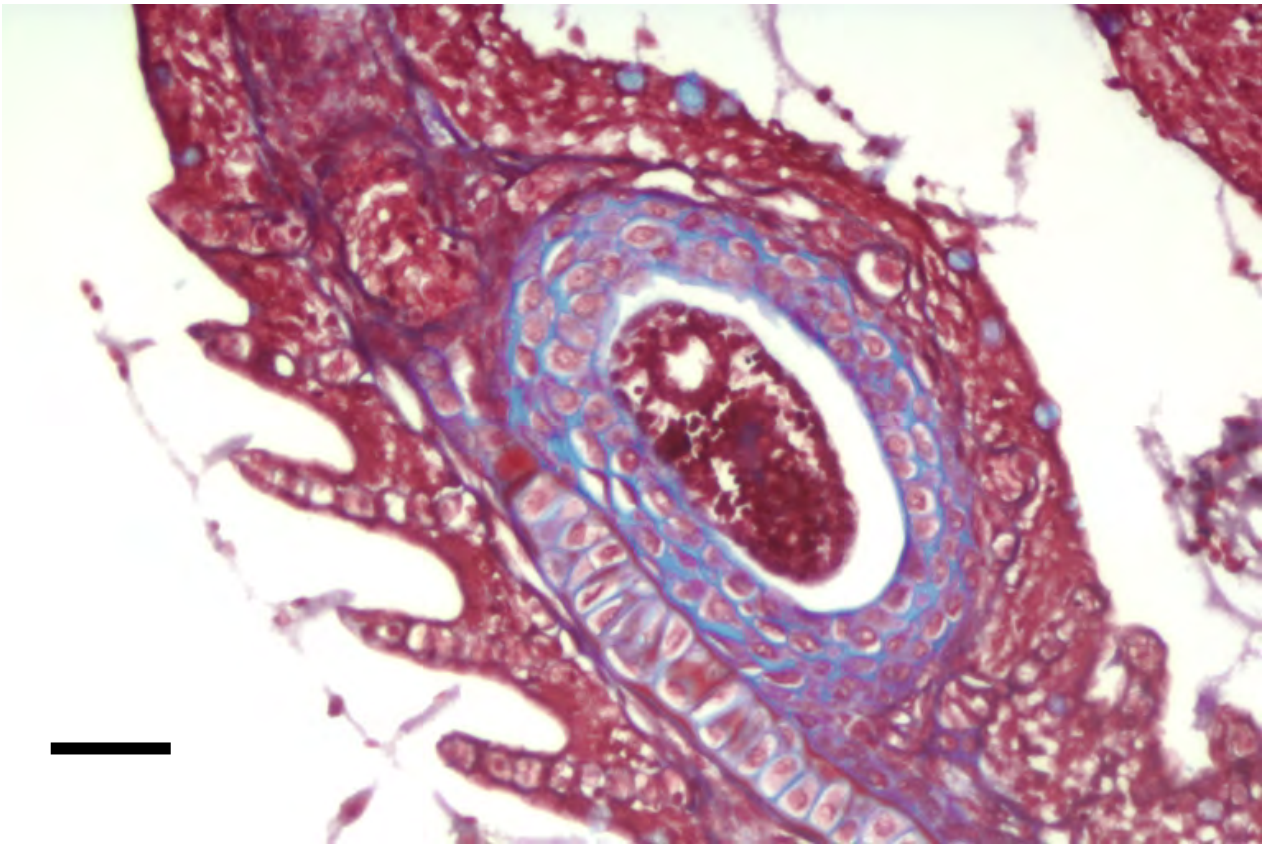


Figure 12: Histological section of a gill filament from a sunshine bass (*Morone chrysops* x *M. saxatilis*), experimentally infected with *Centrocestus formosanus* (Masson's trichrome stain). The host cyst wall stains blue, demonstrating that it is composed of cartilage. Lamellae adjacent to the cyst are lost. Scale bar = 100 μm.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

In wet mounts of gill tissue, the presence of a 75 to 225 µm ovoid cyst containing an organism with two eye spots (young metacercariae) or a dark X-shaped structure (mature metacercariae) provides presumptive evidence of the presence of *Centrocestus formosanus*.

2. Confirmatory Diagnosis

There are no definitive techniques available for the diagnosis of *C. formosanus*; specific PCR methods are under development, but not yet sufficiently validated for use. Centrocestiasis is therefore confirmed by a combination of morphometric characters and a few distinctive pathological features associated with the infection. Under a dissection scope the cysts are teased from the gill tissue with a scalpel and using two scalpels (#11 blades) the metacercariae can be freed from the cyst. Coverslip pressure will sometimes free the worms from the cysts. When liberated from the cyst the metacercariae take a pyriform or conical shape and the body is entirely covered with tegumental spines (very small spines seen on the periphery of the worm – 100 to 400X) (Figure 13 video).

Figure 13 Video: Metacercaria of *Centrocestus formosanus* removed from cysts. Tegumental spines are seen along the periphery of the parasite [link].

In mature metacercariae, in addition to having X-shaped excretory bladders, two offset rows of 16 circumoral spines (total = 32 spines) can be counted. Further characterization using morphometric and meristic characters can be made by a parasitologist. Some distinctive pathological features include the distortion of gill cartilage and the presence of broad, clear-walled cysts surrounding the metacercariae. Histological confirmation that the host tissue cyst wall is composed of chondrocytes is important. Additional evidence may be gained by collecting red-rimmed melania from suspect waters and observing them for the release of cercariae that are about 230 to 300 µm total length with straight non-bifurcated tails and two prominent eye spots. Place five to ten snails in about 100 mL of water (20 to 30°C) and leave in the dark overnight, remove and expose to bright light for one hour, and examine water at 20 to 100X for cercariae.

The only other trematode species infecting freshwater fish in the USA that could be described with an X-shaped excretory bladder and circumoral spines are *Ascocotyle spp*., but they are not found encysted in the gills. In one case, trematodes in cartilaginous cysts were found in the gill filaments of steelhead trout *Oncorhynchus mykiss* caged in the Willamette River in Oregon. These trematodes were reported to have only one row of circumoral spines and therefore thought not to be *C. formosanus*.

F. Procedures for Detecting Subclinical Infections

Fish located in waters in USDA Plant Hardiness Zones of 9a or higher (usna.usda.gov/Hardzone/ushzmap.html) have the potential to be infected with this trematode. These areas are found in the southern parts of Florida, Texas, Arizona, Nevada and California. Also, fish in rivers that originate from springs or geothermal water with year round temperatures between 17 and 32°C are also candidates for infection especially if red-rimmed melania are found in the water. There is potential for the infection to occur in fish in waters that are heated by industrial effluents year round.

To detect subclinical infections in fish, the second or third gill arch should be removed in its entirety and, if the fish is less than 40 mm, put on a glass slide covered with a coverslip and viewed under a dissection scope or binocular scope at 20X. For fish larger than 40 mm, the cartilaginous gill arch will keep the coverslip from flattening the gills and trematodes may remain hidden. Therefore, carefully remove the filaments from the arch for viewing. Any ovoid objects around 75-225 µm in diameter should be further examined at higher powers (100 to 400X) to determine if characteristic cysts and metacercariae are present. Suspect tissues may be fixed in 10% formalin for later confirmation by histology and in 70 to 95% ethanol for light microscopy.

G. Procedures for Determining Prior Exposure to the Etiological Agent

Cyst elimination may begin 50 days post infection and probably will be complete within a year. There may be residual thickening, branching or bending of the filament cartilage but other parasites, including at least one myxosporian, may leave similar lesions. There is no conclusive method for determining the presence of prior infections if the parasite has been eliminated.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival or Recognition of the Etiological Agent

Submitting live fish is best; if this is not possible, put fish on ice and ship to a diagnostic laboratory within 48 h. As a last resort put specimens in 70 to 95 % ethanol (if the fish is large, remove and fix the second or third gill arch). For histology, fix fish in 10% formalin.

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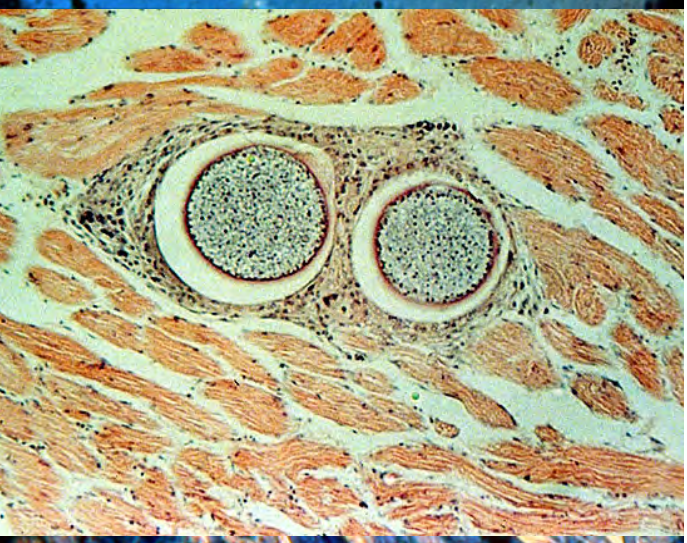
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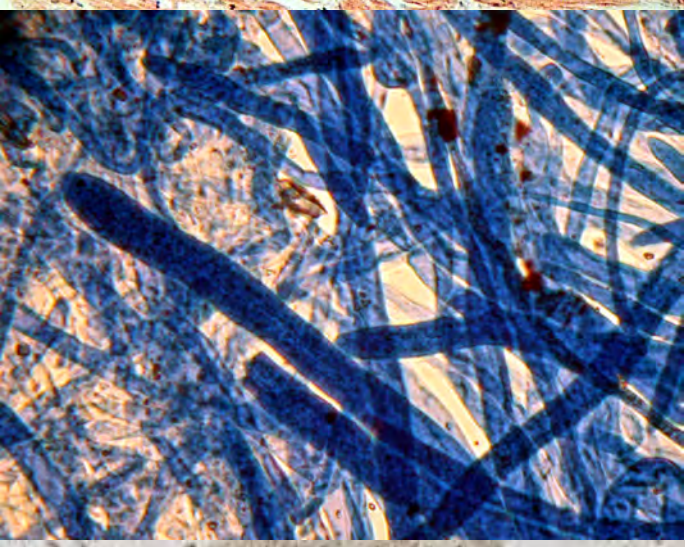
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Section 1
Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens

Chapter 4 Mycotic Diseases of Fish

4.1 General Procedures for Mycology

4.2 Branchiomyces

4.3 Ichthyophonus Disease

4.4 Saprolegnia

4.5 Miscellaneous Mycotic Diseases

4.1 General Procedures for Mycology

Tom A. Bailey

U. S. Environmental Protection Agency
401 M. Street SWH7507 C
Washington, DC 20460

A. External Infections

Many fungal infections are quite visible to the naked eye. All body surfaces and gills should be examined carefully for the presence of "tufts," nodules, or other epithelial lesions indicative of the presence of fungi.

Wet mounts should be prepared not only from lesions but also from mucus scraped from the surface of the fish. The specimen should be examined microscopically at 10X, 100X and 500X magnification for the detection and identification of mycoses.

B. Internal Infection

After the fish has been opened and bacteriological samples collected, examine all organs carefully for the presence of cysts, nodules, or unusual appearance. The gills, brain, viscera, and kidney should be examined with a hand lens or dissecting microscope for the presence of fungi and associated lesions. Wet mount preparations should be made of all the above tissues as well as the air bladder and the contents of the stomach and intestine. Microscopic examinations should be made on all the wet mounts from 10X to 500X. Histopathology may be necessary to detect mycosis in some instances, i.e. *Phoma sp.*

C. Procedures for Killing and Preserving Fungi

Killing and preservation in 10% formalin or lactophenol are adequate if further study is needed.

D. Staining, Processing, and Mounting of Specimens for Study

See Section 1, 3.1 General Procedures for Parasitology.

4.2 Branchiomyces

Tom A. Bailey

U. S. Environmental Protection Agency
401 M. Street SWH7507 C
Washington, DC 20460

A. Name of Disease and Etiological Agent

Branchiomycosis (Gill Rot) is caused by *Branchiomyces sanguinis* and *B. demingrans*.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Branchiomycosis has been reported from Eastern Europe and from the United States in Alabama, Arkansas, Florida, Georgia, Missouri, Ohio, Rhode Island, and Wisconsin (Piper et al. 1982).

2. Host Species

Branchiomyces is a major problem in Eastern European fish culture (Meyer 1973) where it infects pike *Esox lucius*, tench *Tinca tinca*, and common carp *Cyprinis carpio*. In the United States *Branchiomyces* has been found in rainbow trout *Oncorhynchus mykiss*, largemouth bass *Micropterus salmoides*, smallmouth bass *Micropterus dolomieu*, striped bass *Morone saxatilis*, northern pike *Esox lucius*, pumpkinseed *Lepomis gibbosus*, and guppies *Poecuilia reticulata*.

C. Epizootiology

Branchiomyces invades the gill blood vessels and is most prevalent in fish raised in warm water with high organic content. The fungus can be recognized in gill filament wet mounts. Non-septate, branched hyphae filled with protoplasmic mass, which divides into granular "spherical spores," can often be seen in gill lamellae (see micrograph, pp 41 Hoffman and Meyer 1974).

D. Disease Signs

External signs for branchiomycosis include pale, whitish gills with accompanying necrosis, fish gasping at the surface, and high mortality.

E. Disease Diagnostic Procedures

Examine wet gill tissue microscopically (100X or 400X magnification). Observe nonseptate hyphae and spores ("beads") of the fungus in the capillaries and tissue of gill lamellae.

F. Procedures for Detecting Subclinical Infections

No procedures for detecting subclinical infections are available.

G. Procedures for Determining Prior Exposure to the Etiological Agent

No procedures for detecting prior exposure to the etiological agent are available.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Live or freshly killed fish are preferable. Samples should be properly sealed, labeled with host species name, date of collection and other pertinent data, packed into cardboard specimen tubes, and shipped on ice to a diagnostic laboratory immediately.

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4.3 Ichthyophonus Disease

Tom A. Bailey

U. S. Environmental Protection Agency
401 M. Street SWH7507 C
Washington, DC 20460

A. Name of Disease and Etiological Agent

Ichthyophonus disease is caused by the intrusion of endospore bodies of *Ichthyphonus hoferi* into new hosts or within the body of an already infected host. The disease is sometimes referred to as "swinging disease." Swinging disease of trout is the abnormal swimming motion due to lordosis and scoliosis caused by *Ichthyophonus.*

B. Known Geographical Range and Host Species of the Disease

Cultured trout in the Western United States, Australia, Europe, Japan, and Taiwan. Also found in marine fish and aquarium fishes worldwide.

C. Epizootiology

Ichthyophonus is an internal and obligate fungal parasite with low host specificity. Similar organisms have been reported from Amphibia and Copepoda, but it is uncertain whether these organisms are the same as occur in fish. *Ichthyophonus* has occurred in carp, trout, and salmon, and could occur whenever the flesh of raw fish is fed to cultured fishes or the spores allowed to accumulate in ponds. No sexual cycle has been determined. The proposed life cycle involves the subdivision of multinucleate spores forming small, motile, endospore bodies (with or without prior formation of hyphae and terminal hyphal bodies). These enter new hosts or are distributed within the body of an already infected host and subsequently grow into large multinucleate spores. The major infection sites are the blood-rich organs (kidney, heart, spleen, and liver).

D. Disease Signs

Infected fish cease feeding and become lethargic. In acute infections, trout develop a gross lumpy appearance. Behavorial changes may also occur due to deformities. Spinal deformities may develop; nodules may develop in the kidney, liver, or muscle. Spores also may occur in the spleen and brain. In chronic infections, no visible signs may develop. Secondary effects may include severe emaciation.

E. Disease Diagnostic Procedures

Examine microscopically wet mounts of fresh kidney or other suspect tissue for fungal "resting spores" (spherical bodies of various sizes ranging from 10 μm to 200 μm encapsulated by infected host tissue) using a lower power (100X) magnification. Observe spheres closely for hyphal protrusions. The germination of hyphae after host death is diagnostically significant and is a definitive characteristic of *Ichthyophonus* (see Figure 4, pp251, Mc Vicar 1982).

F. Procedures for Detecting Subclinical Infections

Smears of kidney tissue, which are air dried and stained with methylene blue, have been successfully used in some laboratories as a screening procedure. The statistical reliability of this method is unknown.

Ichthyophonus can be grown *in vitro* on artificial medium for confirmation. Spores or spore-containing tissue can be transferred to tubes or petri dishes containing Minimum Eagle's Medium or Hagem's Fungus Medium (McVicar 1982).

G. Procedures for Determining Prior Exposure to the Etiological Agent

Experimentally, antibodies against *Ichthyophonus* have been detected using standard serological techniques and indirect fluorescent antibody tests, however, these findings have not yet been reduced to practical use.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Live or freshly killed fish are preferable. Samples should be properly sealed, labeled with host species, name, date of collection and other pertinent data, packed into cardboard specimen tubes and shipped on ice to a diagnostic laboratory immediately.

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4.4 Saprolegnia

Tom A. Bailey

U. S. Environmental Protection Agency
401 M. Street SWH7507 C
Washington, DC 20460

A. Name of Disease and Etiological Agent

Saprolegniasis is caused by the attachment of the swimming zoospores (etiological agent) of common water molds to the gills, skin, or fins.

The water molds belong to the Subdivision Diplomastigomycotina and the Class Oomycetes.

The most prevalent species associated with infections are *Saprolegnia diclina* type 1 and *Saprolegnia ferax*. Other genera of importance within this family include *Achyla, Aphanomyces, Dictyuchus, Leptolegnia, Leptomitus,* and *Phythium*. One or several representatives from the above genera can be found at the same infection site.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Water molds are ubiquitous and inhabit all freshwater. These organisms are universally distributed and can even be found in brackish water with salinities up to 2.8 percent.

2. Host Species

The water molds are parasitic to insects, amphibians, and fish and fish eggs of both warmwater and coldwater species.

C. Epizootiology

In fish propagation, handling injuries, malnutrition, temperature shock, external parasitism, and spawning increase the susceptibility of fish to infection by water molds.

Whenever fungal zoospores are present in excess of 23,000 spores/liter, there is potential for infection. If these infections are left unchecked, high mortality can result.

D. Disease Signs

The skin or other surfaces of infected fish and the surface of fish eggs become covered with white, cottony tufts of non-septate filamentous hyphae.

E. Disease Diagnostic Procedures

1. Isolation

Although there are numerous media available for isolating and culturing aquatic fungi, the two simplest media to use are corn meal agar (CMA) or Saboraud's agar (SAB) (Fuller 1978). Isolates grow well at room temperature (20 to 25^{o}C).

Isolates should be made from live or freshly killed fish to prevent isolation of saprophytic species. The fungus is isolated from the infection site (see Section 1, 4.1 General Procedures for Mycology) by removing small pieces of infected tissue or hyphae from fish or fungused eggs and placing them in petri dishes or test tubes containing sterile agar (CMA or Sabouraud's) or sterile water plus halved hemp or clover seeds. The petri dishes or test tubes containing the fungal isolates may then be shipped to a diagnostic laboratory for reisolation, purification, and identification.

2. Histological Examination

Infections by any of the water molds results in the formation of visible, cottony, wool-like lesions on the integument, gills, or fins of fish or on the surface of eggs. Willoughby (1971) has documented that *Saprolegnia* infections of salmonids form a delicate ring of hyphae around an apparently uninfected area. Fungal hyphae penetration is limited primarily to the epidermis and dermis. Although muscular intrusions are rare, muscular lesions may develop when bacterial pathogens accompany the fungus. In the case of small fish, fungal hyphae may deeply invade muscular tissue as well as penetrate vital organs and the central nervous system.

Histopathologically, water mold infections degenerate epidermal and dermal tissues. Specific sequelae include necrosis, spongiosis, acantholysis, intercellular oedema, and sloughing of epidermal cells. Lesions manifest a pale appearance possibly due to the clumping of melanin granules in dermal melanophores. There is little or no inflammatory response associated with fungal infections in fish. Death is largely due to osmoregulatory malfunctions.

F. Procedures for Detecting Subclinical Infections

The hyphae and spores of water molds are best detected from early lesions of moribund fish or from viable eggs adjacent to dead fungus laden or infected eggs. Excise a small number of hyphae from the lesion, place them into sterile distilled water (to induce sporulation), and observe microscopically (400X). Primary zoospores are pyriform and have two flagella at the apex. After swimming for a short period, they encyst. The primary cysts form secondary spores, which are reniform (kidney-shaped), have two flagella (1 anterior and 1 posterior), and swim for a prolonged period of time. The secondary spore also encysts, but later germinates to form hyphae.

G. Procedures for Determining Prior Exposure to the Etiological Agent

There is no procedure for determining prior exposure to fungal pathogens.

H. Procedures for the Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Live or freshly killed fish and eggs are preferable. Samples should be sealed, properly labeled with host species, date of collection and other pertinent data, and packed into cardboard shipping tubes. Specimen should be shipped on ice to a diagnostic laboratory immediately.

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4.5 Miscellaneous Mycotic Diseases

Tom A. Bailey

U. S. Environmental Protection Agency
401 M. Street SWH7507 C
Washington, DC 20460

A. Deuteromycetes

Deuteromycosis is caused by imperfect fungi found in the class Deuteromycetes. Representative genera and reported hosts include:

1. *Cryptococcus* is a yeast reported to be associated with exophthalmos in tench *Tinca tinca*.

2. *Candida sake* is a yeast reported from the gut of Amago trout *Oncorhynchus rhodurus*. Clinical signs are distended stomachs filled with viscous fluid and gas bubbles.

3. *Phoma herbarum* is capable of infecting salmonid fry and fingerlings. Geographic range includes the Pacific Northwest of the United States and Southwest England. Clinical signs reported are swollen and hemorrhaging vents, compressed abdominal areas, caudal petechiae, and loss of equilibrium. The pneumatic duct of the swimbladder is generally the first site of infection and elicits an inflammatory response.

4. *Fusarium* is a form genus in the class Hyphomycetes and has been reported to infect carp, lobster, and other crustaceans. *Fusarium* is associated with "Burn Spot Disease" in lobster and "Black Gill Disease" of prawn in Japan.

B. Dematiaceous Hyphomycetes

The disease condition caused by this group of fungi are commonly known as phaeohyphomycosis or chromomycosis. These fungi are systemic and have pigmented hyphae.

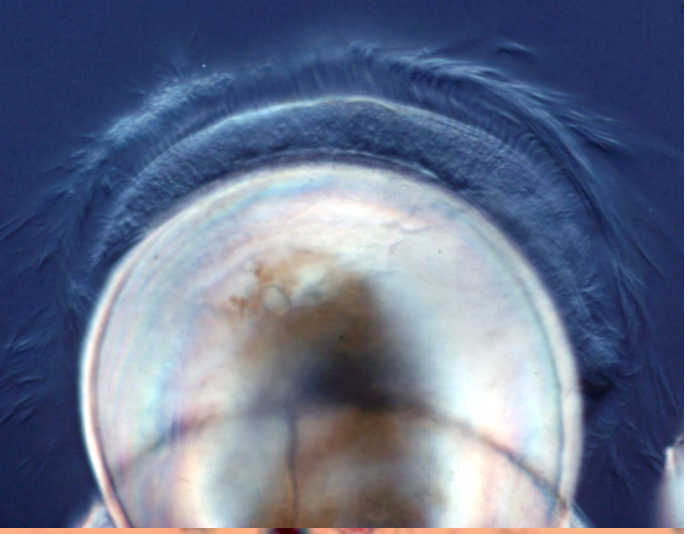
1. *Ochroconis tshawytschae* is pathogenic to young chinook salmon. The major infection site is the posterior kidney.

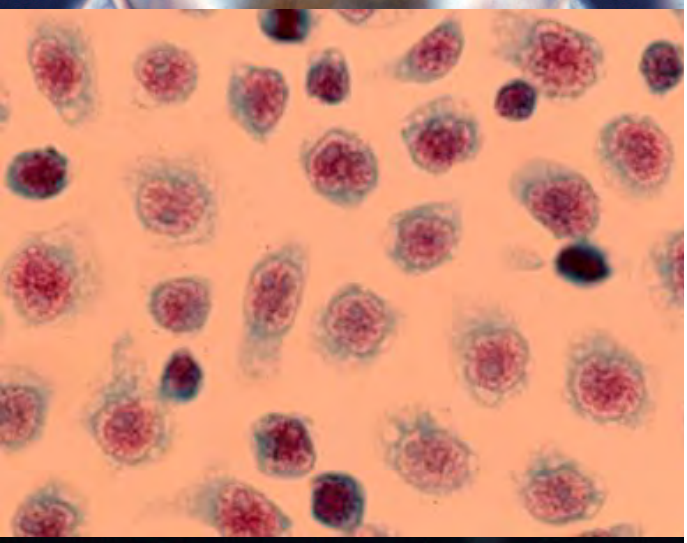
2. *Ochroconis humicola* is pathogenic to coho salmon, rainbow trout, and frogs. A major site of infection is the kidney. Disease signs may include enlarged abdomen, small ulcers, hemorrhaging, edema, and exophthalmus.

3. *Exophiala* has been reported to infect brain tissue of channel catfish (non-proliferative or proliferative granulomatous) and trout (exophthalmos and cranial ulcers) or the kidneys (inflammatory, necrotic and granulomatous) of Atlantic salmon.

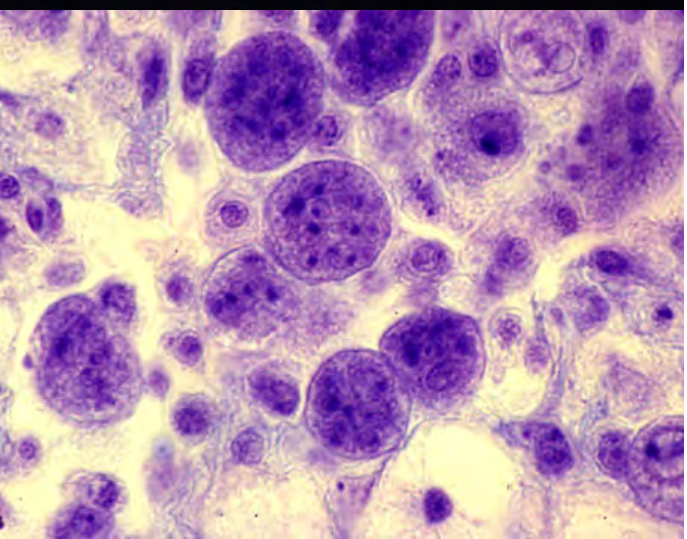
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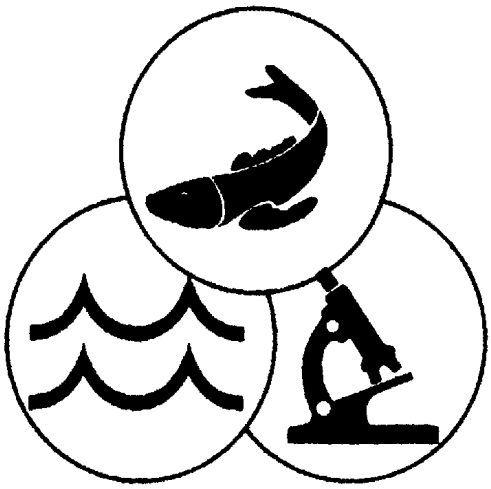








Section 1
Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens



Chapter 5 Diseases of Molluscan Shellfish

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5.1 Diseases of Shellfish Introduction

Ralph Elston

Battelle Marine Sciences Laboratory
439 W Sequim Bay Rd
Sequim, WA 98382
206/683-4151
aquatech@olypen.com

The inclusion of molluscan diseases in the Bluebook represents a departure into a relatively less studied area of animal medicine in which the biology of the host animals is substantially unlike fish. Nonetheless, the same principles of health management, including diagnosis, apply. Until recently, the tools of investigation available for application to the study of natural and experimental diseases were largely descriptive, consisting of morphological pathology. While many important advances have been made, the study of viral diseases and many basic areas of physiology have lagged as a result of little development in the areas of cell and tissue culture. Invertebrates do not produce antibodies as we know them in fish, so while vertebrate antibodies can be used to detect antigens in invertebrates, we do not find the same record of infectious agent exposure available by antibody detection as is possible in many of the diseases of higher animal.

As the commercial cultivation of molluscs has increased, there has been increasing activity in the study of basic mechanisms of diseases of these animals, using contemporary tools. Most of these activities are so new that their benefits are not yet available to apply to the practical diagnosis of molluscan diseases. Thus, many diagnoses will rely on histological evaluation of tissues. This is both a strength and a limitation. The strength is that the field of molluscan pathology is relatively well founded in an understanding of the pathogenesis of diseases by virtue of morphological studies. The limitation is the relative lack of quantitative methods for identifying and enumerating infectious microorganisms and the relative lack of ability to construct a history of prior exposure or carrier status of animals.

Clinical signs of the diseases are listed, although, these are rarely pathognomonic for the specific disease. They are included, however, since they offer some guidance to the alert diagnostician and help form a list of differential diagnoses. It is worth reminding the diagnostician that the formation of presumptive and confirmatory diagnoses is relative to the methodologies available and the knowledge base regarding a particular disease. Thus, many of the confirmatory diagnoses are based on histological observation. In some cases, where the disease is distinctive, this is a defensible approach and may remain the definitive method even when other technologies are available. However, in other cases, histological diagnosis is not definitive but must suffice until more advanced methods are available.

5.2 *Perkinsus* sp. Infections of Marine Molluscs

Kimberly Reece[1] and Christopher Dungan[2]

[1] Virginia Institute of Marine Science
College of William and Mary
P.O. Box 1346
Gloucester Point, VA 23062
kreece@vims.edu

[2] Maryland DNR
Cooperative Oxford Laboratory
904 S. Morris Street
Oxford, MD 21654
cdungan@dnr.state.md.us

A. Name of Disease and Etiological Agent

In the earliest-reported cases in the USA (Mackin et al. 1950), the name of the disease in *Crassostrea virginica* oysters was popularly abbreviated as 'dermo', in reference to the now-obsolete identification of its protistan agent, *Perkinsus marinus*, as *Dermocystidium marinum* (=*Labyrinthomyxa marina*). Recent evidence suggests that the current placement of the genus *Perkinsus* among the Apicomplexa may be incorrect, because member species also show consistent affinities with the dinoflagellates, or with a proposed new alveolate protozoan phylum Perkinsozoa. Since 1981, several new *Perkinsus* spp. parasites and diseases have been described and reported in a variety of bivalve and gastropod molluscs, worldwide (reviewed by Villalba et al. 2004). Other currently described *Perkinsus* species include *P. olseni* from Australian abalone, *P. atlanticus* from European clams, *P. chesapeaki* and *P. andrewsi* from Chesapeake Bay clams, *P. mediterraneus* from Mediterranean flat oysters, and *P. qugwadi* (*incertae sedis*) from Pacific Canada scallops. *Perkinsus olseni* is proposed to be a priority synonym of *P. atlanticus* (Murrell et al. 2002). *Perkinsus chesapeaki* and *P. andrewsi* are proposed as synonymous clam parasite species, with the former name in priority (Burreson et al. 2005). Affinities of *P. qugwadi* with the genus *Perkinsus* are tenuous (Bower et al. 2003).

B. Known Geographic and Host Ranges of the Diseases

Perkinsus marinus (Mackin et al.1950) infections (dermo disease) occur widely in *C. virginica* oysters along the Gulf of Mexico and Atlantic coasts of North America, from Tabasco, Mexico to Maine, USA. The disease was reported once in moribund, introduced *C. virginica* from Hawaii waters (Kern et al. 1973).

Perkinsus chesapeaki (McLaughlin et al. 2000) and *P. andrewsi* (Coss et al. 2001) infections are reported at high respective prevalences in Chesapeake Bay *Mya arenaria, Tagelus plebeius,* and *Macoma balthica* clams.

Perkinsus olseni (Lester and Davis 1981) infections are reported in moribund southern Australia abalone *Haliotis rubra, H. laevigata, H. cyclobates*, and *H. scalaris*. Similar parasites are reported in diverse bivalve molluscs from northern, southern, and eastern Australian coasts, as well as from northern New Zealand waters.

Perkinsus atlanticus (Azevedo 1989) infections are reported in moribund *Ruditapes decussatus, Venerupis philippinarum, V. pullastra,* and *V. aureus* clams from the Atlantic coast of the Iberian Peninsula. Similar infections are reported in *Crassostrea angulata* and *Ostrea edulis* oysters from the Iberian Atlantic and Mediterranean coasts, and in Italian *R. decussatus* and *Callista chione* clams.

Perkinsus mediterraneus (Casas et al. 2004) infections are reported in commercially cultured *Ostrea edulis* flat oysters from the western Mediterranean, Balearic Islands.

Perkinsus qugwadi (incertae sedis) (Bower et al. 1998) infections were transiently reported in Japanese scallops, *Patinopecten yessoensis*, grown in Canadian Pacific waters.

Infections by undescribed *Perkinsus* sp. with affinities to *P. olseni* and *P. atlanticus* are reported in moribund *V. philippinarum* clams from the Japan, Korea, and China coasts; as well as in *Paphia undulata* clams from the Thailand coast.

C. Epizootiology

The severity of oyster dermo disease cycles seasonally in enzootic North American waters, with highest infection prevalences, infection intensities, and disease mortality during late summer and fall. Similar seasonality characterizes *Perkinsus* spp. infections in several Chesapeake Bay clam species. *Perkinsus marinus* proliferates maximally at temperatures above 20 °C and salinities above 15 ppt. Since molluscan hosts are nominal osmoconformers, disease morbidity and mortality are reduced during low-salinity periods in affected estuaries, but infections persist in oysters over-wintering in 5 ppt waters. Mortality in affected oyster populations can reach 100%, with 30-50% mortality in the first year of exposure, and cumulative mortality of 75-100% during the second year of exposure. Direct waterborne transmission of infections occurs. During periods of high-salinity Chesapeake Bay epizootics, >90% of juvenile oysters planted both near and distant (>1 km) from infected oyster populations, harbored infections after 12 weeks of exposure. In Gulf of Mexico waters, the parasitic gastropod *Boonea impressa* serves as an active vector in transmitting parasite cells between oyster hosts.

D. Disease signs

Reduced growth rate occurs prior to the onset of mortality.

Non-specific behavioral and gross signs include valve gaping, emaciation, and pale digestive gland.

Histological presentation in oysters (Figure 1) includes 5-20 µm vacuolated, spherical parasite trophozoites and sub-dividing schizont cells, with eccentric nuclei and eosinophilic nucleoli (signet ring cells). Oyster defensive response includes hemocytic infiltration of infected areas. Parasite cells are distributed systemically, and occur phagocytized in hemocytes. Digestive system epithelia of the intestine, digestive gland ducts, and stomach are often heavily colonized and damaged (Mackin 1951). *Perkinsus* spp. infecting Chesapeake Bay clams show similar morphology, but occur mostly in the connective tissue compartment, especially of the gills, and rarely colonize digestive or other epithelia. Host responses in clams include hemocyte infiltration of infected tissues, phagocytosis of parasite cells, and hemocyte encapsulation of proliferating parasite cell groups.

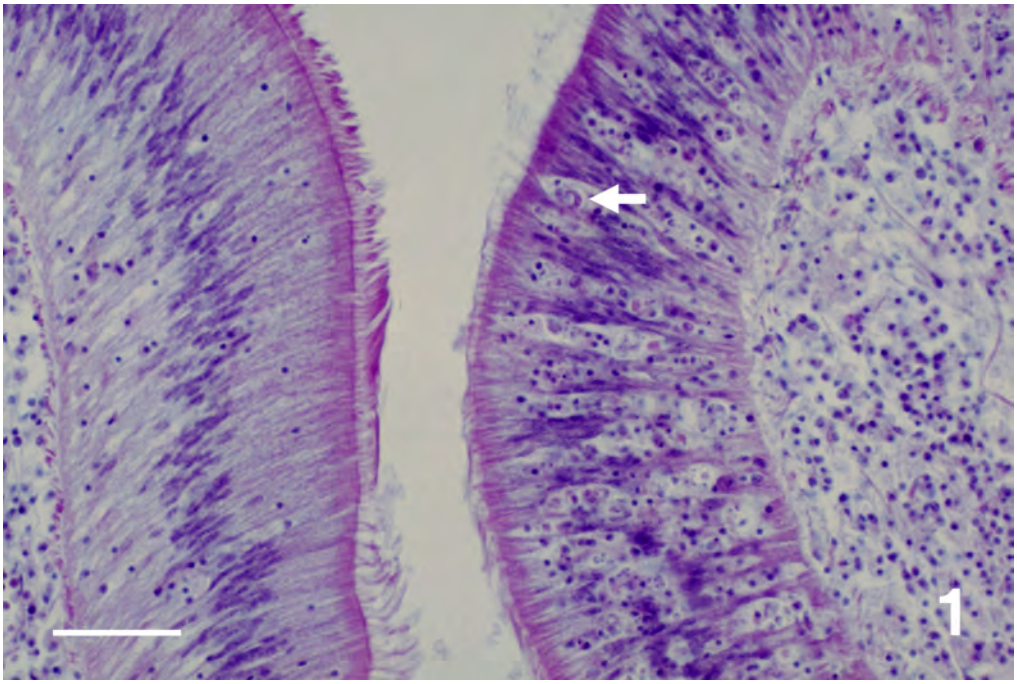


Figure 1. Apposed oyster intestinal epithelia heavily infected by *P. marinus* (right) and lightly infected (left). Hematoxylin and eosin staining (H&E) shows signet ring parasite trophozoites (arrow) with large central vacuoles and eccentric nuclei, as well as sub-dividing schizonts; but neither cell type is strongly contrasted with host tissue components. Foci of infection in digestive epithelia are characterized by epithelial disruption and necrotic abscesses where parasites proliferate among epithelial cells (right). Hemocyte infiltration occurs basal to epithelial lesions. Parasites occur systemically, and are frequently phagocytized by hemocytes. Bar = 50µm.

E. Disease Diagnostic Procedures

Table 1. Mollusc *Perkinsus* sp. infection diagnostic methods attributes.
Increasing relative assay sensitivities are categorized (1-4). Due to small (section or tissue) sample size, and inherent attendant sampling error, histological methods are excluded from the level-4 sensitivity category.

Method	Relative sensitivity [1]	Pathogen specificity	Presumptive diagnosis[2]	Confirmatory diagnosis
Gross signs	0		-	-
Histopathology	1-2	genus	++	++
Histological immunoassay	3	genus	++	++
ELISA assays	3-4	genus	++	++
RFTM assays 1. hemolymph 2. solid tissue subsample 3. whole body burden	 1-3 1-3 4	genus *	 ++ ++ +++	 ++ ++ +++
PCR assays	3-4	genus* or spe	+++	+++
ISH assays	3	genus *	+++	+++

[1] 0-4 increasing sensitivity
* except *Perkinsus qugwadi*
[2] applicability: - unsuitable, + limited utility, ++ standard method, +++ recommended method

1. Ray's high-salt fluid thioglycollate medium (RFTM) assay
Non-differentially detects all *Perkinsus* species, except *P. qugwadi* (*incertae sedis*), which enlarge but do not proliferate in RFTM. Spherical, enlarged parasite hypnospores or pre-zoosporangia measure 10-250 µm, with refractile walls that stain blue-black in 20-33% (v/v) Lugol's iodine (Figure 2).

Possible analytes include solid tissue sub-samples, hemolymph, and whole host bodies. Choice depends on desired level of sensitivity, quantitative results quality, and tolerance for lethal sampling (see Ray 1963; Bushek et al. 1994 for reviews and methods).

Optimum solid tissue sub-sample types are selected according to parasite tissue tropisms, where known: rectum or mantle for *P. marinus* infections in oysters; gill or labial palp for *Perkinsus* spp. infections in Chesapeake Bay clams.

The most sensitive and only strictly quantitative RFTM assay is the whole body burden (WBB) assay (see g.3 below). Wet weights of shucked host tissues are recorded before homogenization and incubation at 20-27 °C in 30-50 volumes of RFTM for 4-7 d. Host tissues are then hydrolyzed at 60 °C in 2M NaOH, leaving killed, unlysed, enlarged parasite cells. Parasite cell counts are normalized to initial host tissue wet weights (Bushek et al. 1994).

Since *Perkinsus* species are aerobes, anaerobic gradients in RFTM are not required.

a. Re-hydrate fluid thioglycollate medium with dextrose (Difco 0256-, BBL 01-140, Sigma A0465) in distilled water plus 20 g/L NaCl. Boil briefly to dissolve.

b. Aliquot RFTM to assay tubes or bulk containers. Autoclave to sterilize. If bulk RFTM medium is autoclaved, aliquot aseptically to sterile assay tubes or culture plate wells, before use.

c. Fortify autoclaved RFTM to 50 µg/ml chloramphenicol (mutagen), or to 100 U/ml penicillin and 100 µg/ml streptomycin.

d. Before use, fortify RFTM aliquots with 50-200 U/ml nystatin, applied as a surface overlay, where possible.

e. Aseptically inoculate RFTM aliquots with solid tissue sub-samples, hemolymph samples, or whole oyster homogenates, at ≤ 0.1 g tissue/3 ml RFTM.

f. Cap or cover RFTM assay containers. Incubate at 20-28 °C for 7-4 d, respectively.

g.1 Observe RFTM-incubated tissues, unstained, for thick-walled, refractile, vacuolated, 10-250 µm spherical, enlarged parasite hypnospore cells, using a microscope equipped for contrasting live cells. Rank and select optimal inocula for parasite *in vitro* propagation.

or

g.2 Transfer and macerate solid tissue sub-samples in pools of 20-33% Lugol's iodine on microscope slides, add additional drops of dilute Lugol's iodine to cover macerated tissue, and coverslip. Suspension samples (hemolymph) may be iodine-stained in microplate wells, or on filter membranes.

or

g.3 WBB assay. Pellet suspended solids by centrifugation at 1,500 x g for 10 min. Conservatively aspirate and discard RFTM supernate. Hydrolyze oyster tissue homogenates in 5-10 volumes of 2M NaOH, at 60 °C for 1-6 h, vortexing every 30 min., until tissue lysis is complete. Pellet parasite hypnospores by centrifugation at 1,500 x g for 10 min. Aspirate and discard NaOH supernate. Resuspend parasite cell pellet and wash 3x for 10 min. at 1,500 x g, in 0.1M phosphate-bufferd saline (PBS) + 0.5% (w/v) bovine serum albumin (BSA) (PBS/BSA). Resuspend washed pellet in 1 ml of PBS/BSA. Stain

entire cell suspension with 20% filtered Lugol's iodine for microscopic enumeration, or quantitatively dilute parasite cell suspension sub-samples in PBS/BSA, to yield countable dilutions (100-400 cells), before staining and enumerating parasite cells. Estimate total parasite cells/oyster from enumerated sample dilution factors, and normalize total parasite cells/g. host tissue wet weight. See Bushek et al. 1994; La Peyre et al. 2003 for review and details.

h. Examine stained preparations for 10-250 µm smooth, spherical cells with refractile walls that are stained blue-black. (Figure 2). Staining may vary with iodine penetration. Distinguish pollen grains and possible iodine crystals by morphology.

2. Histopathology

Their low-contrast H&E staining characteristics render *Perkinsus* species detection in histological sections a less-sensitive diagnostic method than RFTM, or other, assays. See **D. Disease Signs** and Figure 1.

3. Histological immunoassays

Fluorescent or colorimetric antibody labeling increases immuno-stained parasite contrast, and enhances detection sensitivity of histological assays. Neither polyclonal nor monoclonal antibodies developed to date to *P. marinus* immunogens are parasite species-specific (Dungan and Roberson 1993) (Figure 3). Limited quantities of rabbit anti- *P. marinus* antibodies are available by request (cdungan@dnr.state.md.us).

a. De-wax paraffin sections, and hydrate to 0.1M phosphate-buffered saline (PBS), pH 7.2.

b. Block sections with PBS + 0.05% (v/v) Tween-20 (PBST) + 2% (w/v) bovine serum albumin (BSA) + 1% normal rabbit serum.

c. Incubate sections 30-60 min. in 10-20 µg/ml primary antibody IgG diluted in PBST + 1% BSA (PBST/BSA).

d. Wash sections in PBST.

e. Incubate sections 30-60 min. in darkened, 1-4 µg/ml affinity-purified secondary antibody/FITC conjugate, diluted in PBST/BSA.

f. Wash sections in PBST.

g. Counterstain sections with 0.01-0.05% Evan's blue.

h. Dip sections in PBST to rinse.

i. Coverslip in pH 9-buffered glycerol, or equivalent mounting medium, and observe under fluorochrome-appropriate (490 nm for FITC) epifluorescence excitation.

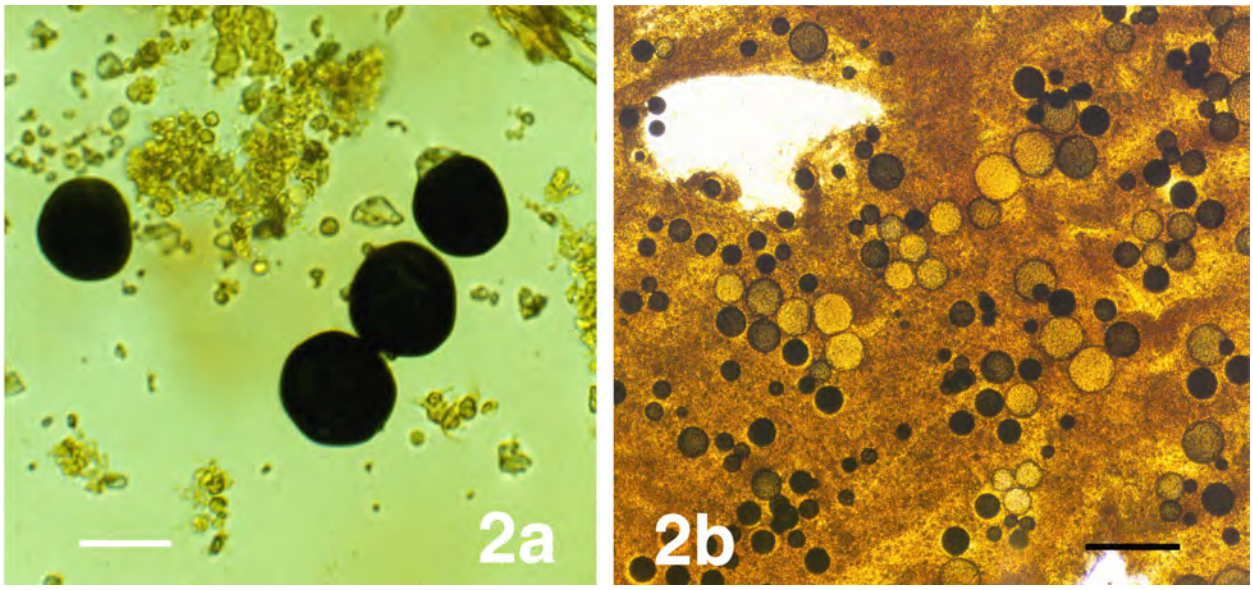


Figure 2. RFTM-enlarged *Perkinsus* sp. hypnospores stained with Lugol's iodine. 2a) Following WBB assay NaOH hydrolysis of oyster tissues. Bar = 50µm. 2b) In macerated *Mya arenaria* labial palp tissue with heavy infection. Incomplete iodine access or activity leaves many parasite hypnospores lightly stained or unstained. Bar = 250 µm.

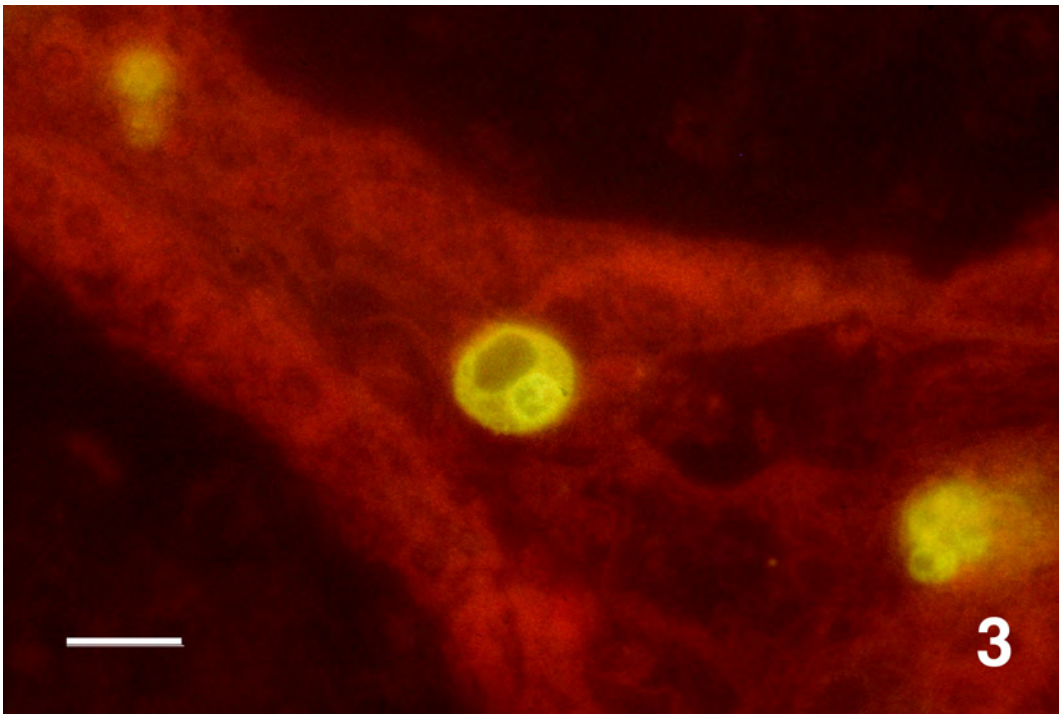


Figure 3. Fluorescence-immunostained *P. marinus* signet ring trophozoite in oyster digestive gland connective tissue. Rabbit antibodies label parasite cell and nuclear membranes, as well as cytoplasm and nucleoplasm. A poorly focused group of four small daughter trophozoites resulting from recent schizogany of a mature neighbor trophozoite, is shown at lower right. Bar = 10 µm.

4. ELISA assays

Polyclonal antibody-based analyte-capture ELISA assays are reported to detect *P. marinus* extracellular secretions (ECP) in tissue homogenates from oysters with infection intensities of one parasite cell/g oyster tissue (Ottinger et al. 2001). The specificity of polyclonal antibodies against *P. marinus* ECP was not tested against other *Perkinsus* spp. or marine mollusc protistan associates.

Monoclonal murine IgG_1 antibodies against *P. marinus* in vitro trophozoites also label *P. atlanticus* cells, but do not recognize several other protistan oyster pathogens (Romestad et al. 2001). A competitive ELISA assay using these antibodies is reported to detect 1,000 parasite cells in 50 µl of oyster tissue homogenate.

***P. marinus* ECP capture ELISA assay** (Ottinger et al. 2001)

a. Coat ELISA plate wells with 50 µl of 40 µg/ml anti-*P. marinus* extracellular proteins (ECP) IgG in coating buffer for 1 h at 21 °C.

b. Wash plate wells 3x with 0.1M TRIS-buffered saline (TBS, pH 7.2) + 0.1% (v/v) Tween-20 (TTBS).

c. Block assay wells 1 h with 240 µl of TTBS + 1% (w/v) BSA (TTBS/BSA).

d. Wash plate wells, as above.

e. Add 50 µl/well of oyster tissue homogenates or positive-control ECP samples, serially-diluted in TTBS/BSA, and incubate for 2 h at 21 °C.

f. Wash plate wells, as above.

g. Add 50 µl/well of 30 µg/ml biotinylated anti-*P. marinus* ECP IgG in TTBS/BSA, for 1 h at 21 °C.

h. Wash plate wells, as above.

i. Add 50 µl/well of streptavidin-horseradish peroxidase (HRPO) conjugate, diluted 1:1,000 in TTBS/BSA, for 1 h at 21 °C.

j. Wash plate wells, as above.

k. Add 50 µl/well of ABTS chromagenic HRPO enzyme substrate; incubate reaction for 30 min in the dark at 21 °C.

l. Read assay well absorbances of 405-nm light; using ECP standard and negative-control well absorbances to interpret diagnostic sample well absorbances.

5. PCR assays

Perkinsus genus-specific primers have been designed (Casas et al. 2002) to target the internal transcribed (ITS) region of all described *Perkinsus* species except *P. qugwadi* (*incertae sedis*) (Figure 4a). These primers amplify *Perkinsus* spp. DNA isolated from cultured cells, as well as from DNA extracts of infected host tissue, and are able to detect less than 0.01 genome equivalents in a reaction (Figure 4b). DNA sequence analysis of the amplified ITS fragment enables species identification by comparison to GenBank (HTTP://WWW.NCBI.NLM.NIH.GOV/ENTREZ/) deposited ITS region sequences for *Perkinsus* species. In addition, several species-specific PCR assays have been developed. Most of the species-specific primers also target portions of the ribosomal RNA gene complex. Many assays are available to specifically detect *P. marinus* DNA (Marsh et al. 1995; Robledo et al. 1998; Yarnall et al. 2000; Audemard et al. 2004). To quantify the amount of parasite present in the host or the environment, Marsh et al. (1995) and Yarnall et al. (2000) developed semi-quantitative PCR assays, while Audemard et al. (2004) recently developed a very sensitive real-time PCR assay for quantification of *Perkinsus* spp. DNA in environmental samples. In addition, a multiplex PCR assay was developed to allow simultaneous and differential detection of *Haplosporidium nelsoni*, *H. costale* and *P. marinus*, all parasites of the eastern oyster *Crassostrea virginica* (Penna et al. 2001). Species-specific PCR assays have also been developed for other *Perkinsus* species. Nested ITS region primers were developed to diagnose the *Perkinsus* sp. infecting Japanese short-necked clams (Hamaguchi et al. 1998), which, based on the DNA sequence of the ITS region, is likely to be *P. olseni*. Several PCR primer pairs were designed to specifically target the non-transcribed spacer (NTS) region of *P. olseni/P. atlanticus* (de la Herrán et al. 2000; Robledo et al. 2000; Murrell et al. 2002) and Coss et al. (2001) developed a specific PCR assay targeting the NTS region for *P. andrewsi* to discriminate this species from *P. marinus* and *P. atlanticus*.

***Perkinsus* genus PCR assay**

a. Extract genomic DNA from cultured cells or infected host tissue using the Qiagen (Qiagen™, Valencia, California) DNeasy tissue kit. Alternatively, DNA can be extracted using phenol/chloroform based (Reece et al. 1997) or CTAB-based DNA extraction methods (Carlini and Graves 1999).

b. Quantify DNA spectrophotometrically.

c. PCR amplification

1. Primers: Perk ITS 85 (5'CCGCTTTGTTTRGATCCC3')
   Perk ITS 750 (5'ACATCAGGCCTTCTAATGATG3')

2. Reagent concentrations for 25µl reactions.
   10 ng (cultured cell)-100 ng (infected host tissue) of genomic DNA.
   20 mM Tris-HCl (pH 8.4)
   50 mM KCl, 1.5 mM $MgCl_2$
   0.2 mM each of dATP, dGTP, dCTP, dTTP
   25 pmol of each primer (above)
   0.625 units of *Taq* DNA polymerase

0.2 mg/ml of BSA

3. Cycling parameters (modified from Casas et al. 2002)
   Initial denaturation of 4 min at 94 °C
   40 cycles of:
      1 min at 94 °C
      1 min at 55 °C
      1 min at 72 °C
   Final extension of 10 min at 72 °C

d. PCR products are electrophoresed on 2% agarose (in 1x TBE) gels, stained with ethidium bromide, and visualized using UV light.

6. SSU rRNA ISH assays

A genus-*Perkinsus* DNA probe was developed to specifically target SSU rRNA sequences of *Perkinsus* species and was shown to hybridize specifically to a variety of *Perkinsus* spp. cells in paraffin-embedded histological sections of host species (Elston et al. 2004) (Figure 5). It does not hybridize, however, to *Perkinsus qugwadi* (*incertae sedis*), or to parasitic dinoflagellates or haplosporidian species.

Perkinsus probe: Perksp700DIG (5'CGCACAGTTAAGTRCGTGRGCACG3'), probe is 5' end-labeled with digoxigenin.

In situ hybridizations are performed as previously described (Stokes and Burreson 1995, 2001), with modifications indicated below.

a. Dewax paraffin-embedded tissue sections mounted on positively charged slides in xylene, and rehydrate to 70% ethanol.

b. Wash slides in P buffer (50 mM Tris-HCl, pH 7.5; 5 mM EDTA) and treat with 125 µg/ml pronase solution in P buffer for 30 min. at 37 °C for permeablization, followed by two washes in P buffer and a 10 min incubation in 2X SSC.

c. Prehybridize in a standard prehybridization solution for 1 h at 42 °C.

d. Hybridize overnight in a humid chamber at 42 °C in a solution with a probe concentration of 7 ng/ul in prehybridization buffer.

e. Wash in a series of solutions descending from 2x SSC at 21°C to 0.5x SSC at 42 °C.

f. Immunological detection of the digoxigenin-labeled probe is done with an anti-digoxigenin antibody-alkaline phoshatase conjugate and NBT/BCIP substrate, following blocking and rinsing.

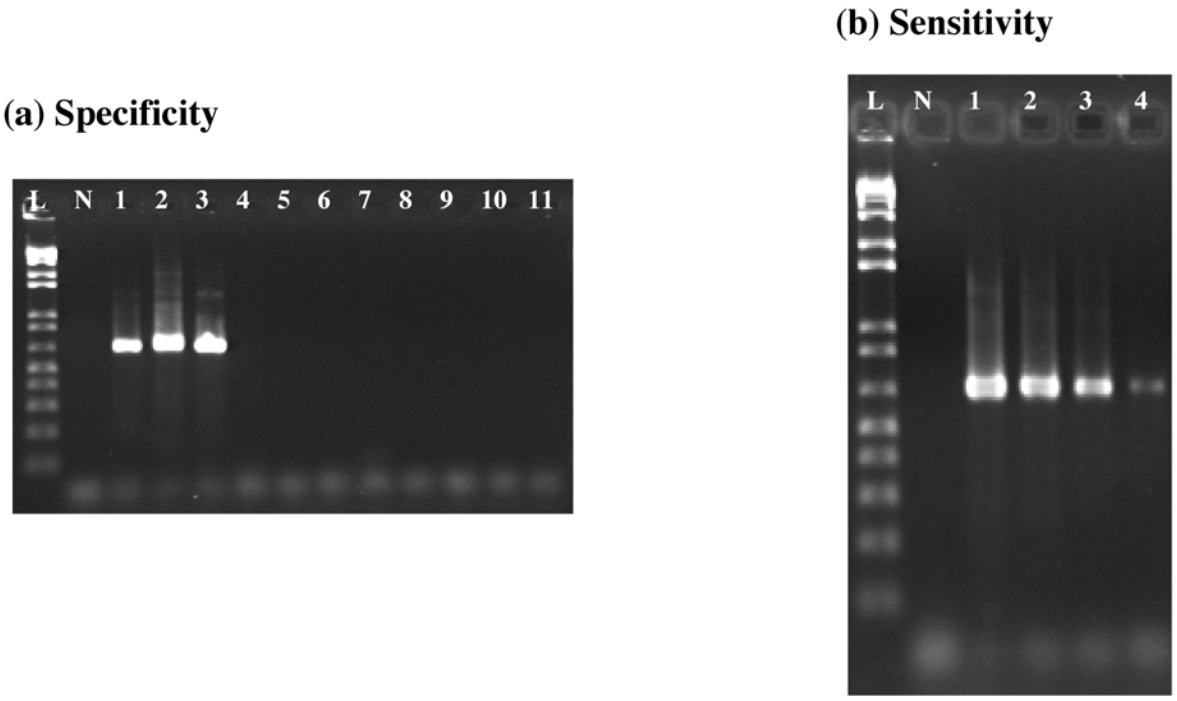


Figure 4. Ethidium bromide-stained PCR gels demonstrating the specificity and sensitivity of the *Perkinsus* genus-specific primer pair PerkITS-85/ PerkITS-750 (PerkITS) (a) Specificity of PerkITS primers tested on: no DNA (lane N), *P. marinus* (lane 1), *P. chesapeaki* (lane 2), *P. olseni* (lane 3), *Hematodinium perezi* (lane 4), *Pfeisteria piscicida* (lane 5), *Pf. schumwayae* (lane 6), *Amphidinium carterae* (lane 7), *Crypthecodinium cohnii* (lane 8), *Karlodinium micrum* (lane 9), *Peridinium foliaceum* (lane 10) and *Prorocentrum micans* (lane 11) DNAs. (b) Sensitivity of PerkITS primers tested on: no DNA (N), and serial dilutions of *P. marinus* DNA isolated from a determined (counted) number of cultured cells. DNA in each reaction was estimated to correspond with the following genome equivalents: 8 (lane 1), 0.8 (lane 2), 0.08 (lane 3), 0.008 (lane 4). Lanes L: 100-bp DNA size standards. Gel photos courtesy of C. Audemard.

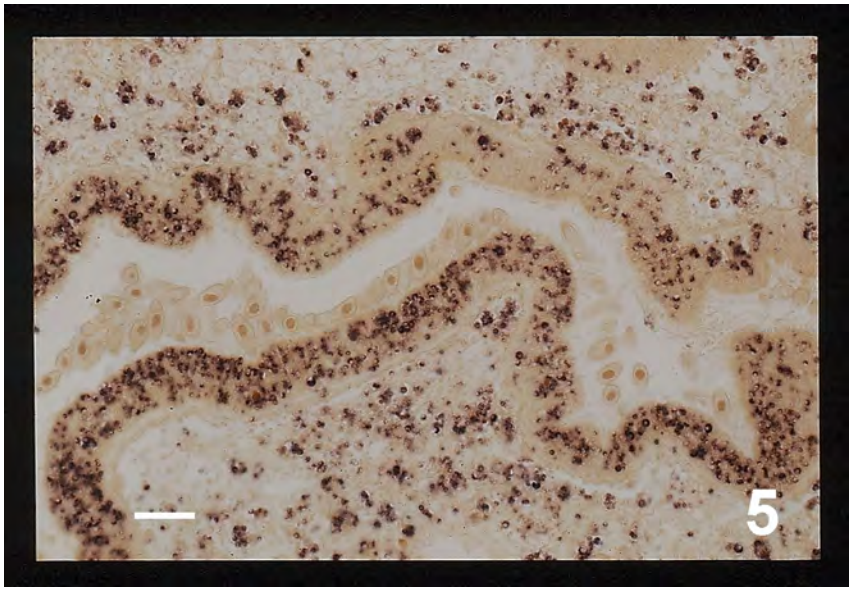


Figure 5. In situ hybridization of genus *Perkinsus* SSU rRNA gene probe (violet-blue) to a heavy *P. marinus* infection in *C. virginica* intestinal epithelium and visceral connective tissues. Both parasite nuclear rDNA and cytoplasmic rRNA share sequence homology with, and hybridize, the digoxigenin-conjugated oligonucleotide DNA probe. Large ciliates infesting the intestinal lumen and epithelium counterstain with Bismark brown. Bar = 100 µm.

F. Procedures for Detecting Subclinical Infections

1. Histological immunoassays have been successfully employed to visualize initial lesions in experimental oysters. In situ DNA/RNA probes should be similarly useful.

2. ELISA immunoassays may detect infections of one parasite cell/g oyster tissue.

3. RFTM whole body burden assays detect one parasite cell/host.

4. PCR assay amplification may detect subclinical infections when potentially rare parasite DNA target templates are present in amplified, 50-150 ng DNA sub-samples. Multiple-copy gene templates, such as rRNA gene targets, may permit detection of infections when <1 parasite genome complement is present in the amplified DNA sample.

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability or Preservation of the Etiological Agent

1. RFTM assays

a. Ship or transport live molluscs humidified and refrigerated (10-20 °C) to an analytical facility, or process aseptically where collected.

b. Aseptically dissect and inoculate antimicrobial-fortified RFTM tubes or plate wells with selected tissue biopsies, to minimize contaminant microbial over-growth and sample cross-contamination of assay tubes/wells.

c. In vitro *Perkinsus* sp. parasite cultures are successfully initiated in isohaline DME:Ham's F-12, or other culture media, from hypnospores enlarged in RFTM at 27 °C for 48-96 h (Dungan et al. 2002).

2. PCR assays

a. Aseptically, and with due precautions against sample cross-contamination, excise optimal host tissue biopsy for parasite detection in extracted DNA. Alcohol-flame instruments and dissect each mollusc on a disposable, virgin plastic weigh boat or Petri dish. Sodium hypochlorite oxidation and hydrolysis of work surface and instrument contaminants between samples is an alternative decontamination method.

b. Preserve tissue for subsequent DNA extraction by dehydration in 10 volumes of uncontaminated, 95-100% ethanol in sterile, virgin screw-capped containers. Store samples at or below 21 °C.

3. Histological assays

a. Excise desired 5 mm-thick tissue samples, place in labeled histological cassettes, and fix immediately for 48 h in Davidson's, or other, fixative solution.

b. Decant fixative; replace with 70% ethanol. Infiltrate, embed, section, and stain tissue samples.

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5.3 Haplosporidiosis of Oysters

Eugene M. Burreson and Nancy A. Stokes

Virginia Institute of Marine Science
College of William and Mary
P.O. Box 1346
Gloucester Point, VA 23062
gene@vims.edu

A. Name of Disease and Etiological Agent

Haplosporidiosis is a chronic to acute disease in oysters and perhaps other marine and freshwater bivalve molluscs caused by a variety of species in the protistan phylum Haplosporidia. There are four species of ***Haplosporidium*** and one species of ***Minchinia*** that occur in bivalve molluscs. Species in the enigmatic genus ***Bonamia*** are also now considered to be in the phylum Haplosporidia, but these important pathogens are considered in chapter VII. Although the end result of ***Haplosporidium*** spp. and ***Minchinia*** spp. infections is likely death of the host, most species are rare and are not considered important disease agents because of their very low prevalence. However, ***Haplosporidium nelsoni*** and ***Haplosporidium costale*** are important pathogens of the eastern oyster, ***Crassostrea virginica***. ***Haplosporidium nelsoni*** was first known as Multinucleate Sphere Unknown and the acronym MSX has persisted as the name of the disease. ***Haplosporidium costale*** was called Seaside Organism and the disease is still known by the acronym SSO.

B. Known Geographic Range and Host Species of the Disease

Haplosporidium nelsoni infections occur in ***C. virginca*** along the entire East Coast of the US and in the Bras d'Or Lake system in Nova Scotia, Canada; prevalence is often over 50%. Infections also occur at low prevalence (< 5%) in ***Crassostrea gigas*** in Japan, Korea, California and France (Burreson et al. 2000).

Haplosporidium costale infections occur in ***C. virginica*** from Virginia to Maine and throughout Atlantic Canada. This parasite has also been reported from ***C. gigas*** on the West Coast of the US, but prevalence is very low.

C. Epizootiology

Based on data from Delaware Bay and Chesapeake Bay, the infection cycle of ***H. nelsoni*** begins each year in May and continues through the summer with maximum oyster mortality in late July or early August. Infections acquired late in the summer usually

overwinter and then proliferate when temperature increases in the spring, resulting in a second mortality event in April or early May. MSX disease is acute and oyster mortality can reach > 90% the first summer of exposure. Parasite distribution and abundance is controlled by temperature and salinity. Maximum abundance occurs at temperatures above 20°C and salinities above 15 psu. The parasite does not occur where salinities are consistently less than 10 psu. There is some evidence that winter water temperatures below 4°C limit ***H. nelsoni*** abundance the following summer. The life cycle of ***H. nelsoni*** is unknown. Attempts at direct transmission using either plasmodia or spores have been unsuccessful and most investigators think that an as yet unidentified intermediate host is a required component of the life cycle.

Infections of ***H. costale*** are acquired in early summer but do not become patent until the following March. Plasmodia multiply rapidly with synchronous sporulation in late May/early June causing host death. Prevalence of ***H. costale*** and, thus, oyster mortality is usually less than 20%, but can reach 40% in some years along the Virginia coast. Oyster mortality from SSO disease seems to be much lower in the northeast. ***Haplosporidium costale*** is restricted to high salinity coastal bays; it does not occur where salinity is less than about 25 psu. The life cycle of ***H. costale*** is unknown.

D. Disease Signs

There are no reliable gross clinical signs of infection. Both MSX and SSO disease are acute and mortality is so rapid that infected oysters can appear healthy.

Tissue sections show 4-30 µm spherical, multinucleate plasmodia. Plasmodia of ***H. nelsoni*** (Figure 1) occur systemically in both epithelium and connective tissue; plasmodia of ***H. costale*** occur only in connective tissue. Sporulation in ***H. nelsoni*** occurs only in the epithelium of the digestive tubules (Figure 2). Spores measure about 8 µm in length. Sporulation in ***H. costale*** occurs throughout connective tissue (Figure 2). Spores measure about 4 µm in length. There is often intense hemocyte infiltration associated with infections of both species, but phagocytosis of plasmodia is rare.

E. Disease Diagnostic Procedures

Table 1. ***Haplosporidiuim*** spp. surveillance, detection and diagnostic methods.

Method	Surveillance	Presumptive *diagnosis*	Confirmatory *diagnosis*
Gross signs	-	-	-
Histopathology Plasmodia only Spores present	+++	 ++[a] +++	 - +++
PCR	+	++	++[b]
In situ hybridization	n/a	n/a	+++

a: histopathology can establish that an infection is present, but cannot reliably distinguish ***H. nelsoni*** and ***H. costale*** if only plasmodia are present.
b: useful as a confirmatory diagnosis only if plasmodia have been visualized by histopathology.
n/a: not applicable, assay used only after a presumptive diagnosis.

– = the method is presently unavailable or unsuitable
+ = the method has application in some situations, but cost, accuracy, or other factors severely limits its usefullness
++ = the method is a standard method with good diagnostic sensitivity and specificity
+++ = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity.

1. Surveillance

Fixation with Davidson's fixative for 24 hours followed by standard paraffin histology and hematoxylin and eosin (H&E) staining is used for surveillance. Tissue sections of oysters should include digestive gland, gill and mantle. Sections are cut at 5-6 µm. Histology is reasonably sensitive for routine surveillance, but can miss very light plasmodia infections. PCR, if properly validated against histology, can be used for surveillance, but no information is acquired on host response or intensity of infection.

2. Presumptive diagnosis

Histology

If only plasmodial stages are present histology cannot be used for presumptive diagnosis in coastal waters where ***H. nelsoni*** and ***H. costale*** overlap in range because the plasmodia of ***H. nelsoni*** and ***H. costale*** cannot be distinguished morphologically (Stokes and Burreson, 2001). Within Chesapeake Bay and Delaware Bay in areas

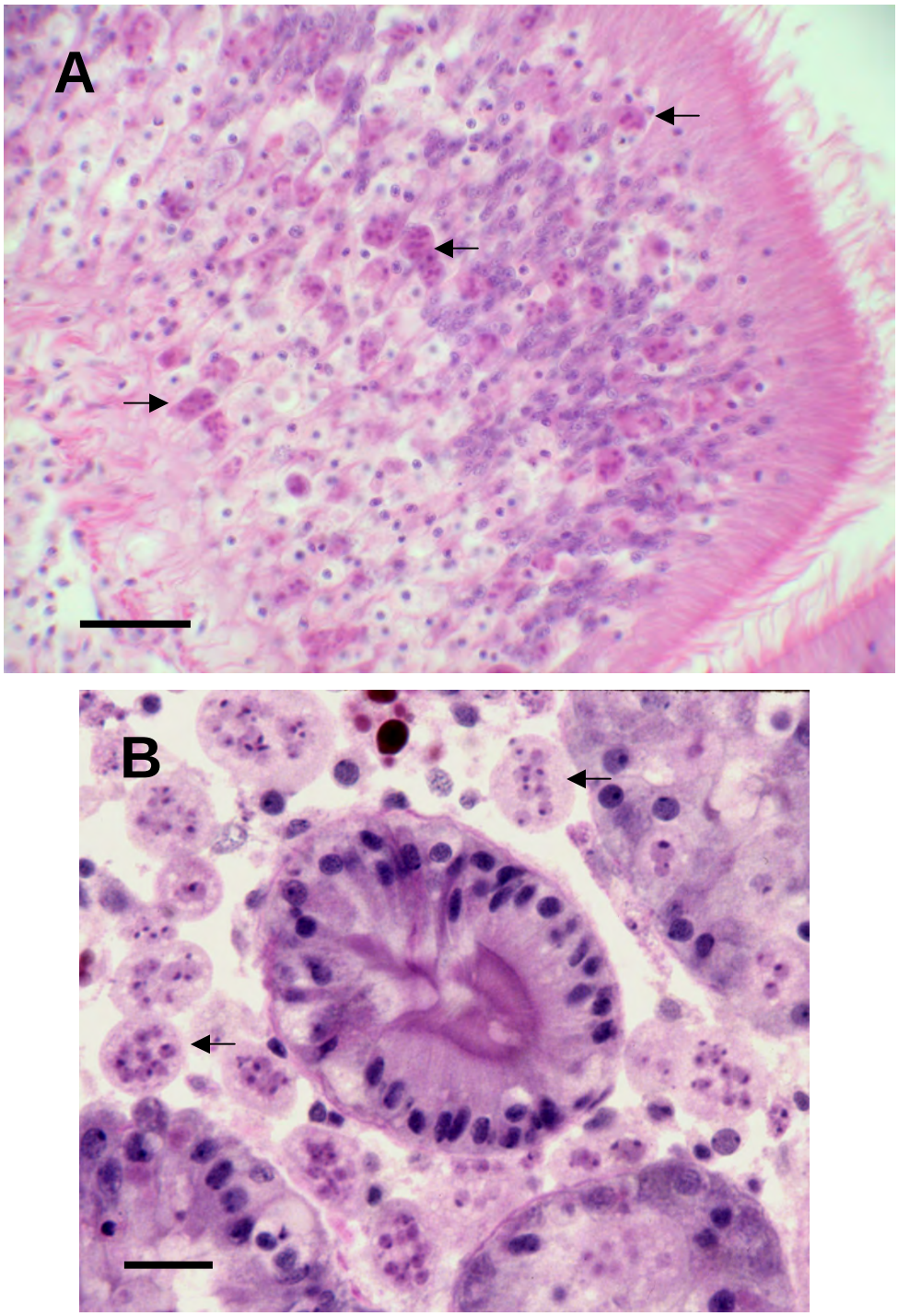


Figure 1. Multinucleate plasmodia of ***Haplosporidium nelsoni***. A. 6 µm paraffin section showing plasmodia (arrows) in the gut epithelium, H&E stain. Scale bar = 30 µm. B. 1 µm plastic section stained with toluidine blue illustrating plasmodia (arrows) in connective tissue and the eccentric nucleolus of the nuclei. Scale bar = 15 µm.

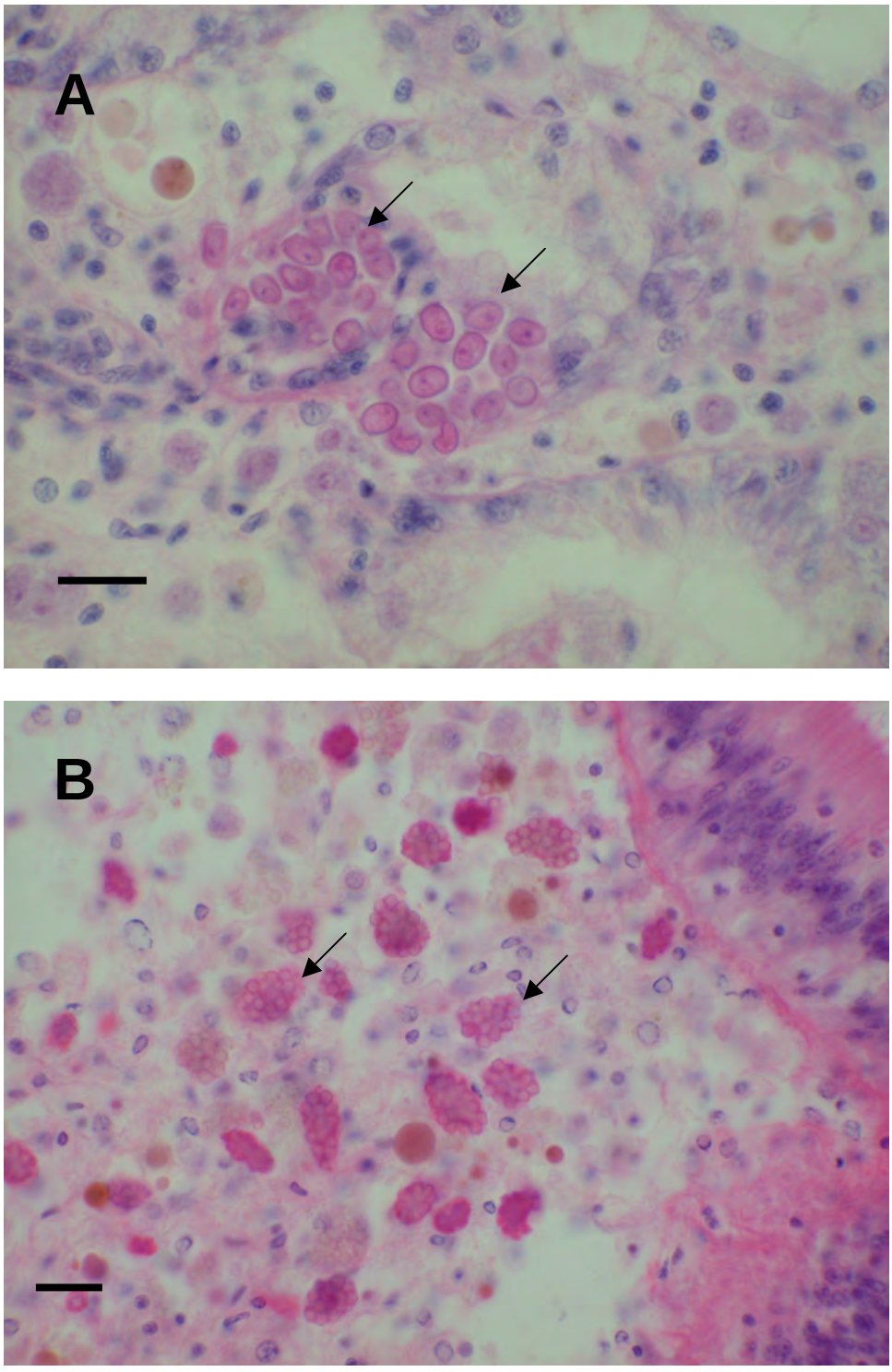


Figure 2. H&E-stained 6 µm paraffin sections of oyster tissue. A. Spores (arrows) of ***Haplosporidium nelsoni*** in epithelium of digestive tubules (scale bar = 20µm). B. Spores (arrows) of ***Haplosporidium costale*** in connective tissue (scale bar = 20µm).

where salinity is consistently below 25 psu, plasmodia can be presumptively identified as ***H. nelsoni*** because ***H. costale*** does not occur in low salinity waters. If spores are present histology can be used for both presumptive and confirmatory diagnosis because spores of ***H. nelsoni*** are 8 µm in size and occur only in the epithelium of the digestive tubules, and spores of ***H. costale*** are 4 µm in size and occur throughout the connective tissue. Unfortunately, spores of ***H. nelsoni*** are rarely present in adult oysters.

PCR assay

PCR can be used for presumptive diagnosis, or confirmatory diagnosis if plasmodia have been observed in histological sections. Specific PCR primers have been developed for both *H. nelsoni* and *H. costale* (Stokes et al. 1995, Renault et al. 2000, Stokes and Burreson 2001). Positive controls must be used.

a. Extract genomic DNA from gill/mantle and digestive gland tissue using the Qiagen DNeasy tissue kit (Qiagen™, Valencia, California).

b. Quantify DNA spectrophotometrically.

c. PCR amplification

1. Primers:
   H. nelsoni: MSX-A' (5'CGACTTTGGCATTAGGTTTCAGACC3')
   MSX-B (5'ATGTGTTGGTGACGCTAACCG3')

   H. costale: SSO-A (5'CACGACTTTGGCAGTTAGTTTTG3')
   SSO-B (5'CGAACAAGCGCTAGCAGTACAT3')

2. Reagent concentrations for 25 µl reactions.
   Reaction buffer (10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl, 10 µg/ml gelatin)
   400 µg/ml bovine serum albumin
   200 µM each of dATP, dCTP, dGTP, dTTP
   0.6 units Ampli*Taq* DNA polymerase
   25 pmoles of each primer (MSX-A' and MSX-B or SSO-A and SSO-B)
   200-400 ng template DNA

3. Cycling parameters for *H. nelsoni*
   Initial denaturation of 4 min at 94°C
   35 cycles of:
   30 sec at 94°C
   30 sec at 59°C
   1.5 min at 72°C
   Final extension of 5 min at 72°C

4. Cycling parameters for *H. costale*

Initial denaturation of 4 min at 94°C
10 cycles of touch-down PCR:
  30 sec at 94°C
  30 sec at 65°C, -1°C per cycle
  1.5 min at 72°C
30 cycles of:
  30 sec at 94°C
  30 sec at 55°C
  1.5 min at 72°C
Final extension of 5 min at 72°C

d. PCR products are electrophoresed on 2% agarose (in 1X TAE or TBE) gels, stained with ethidium bromide, and visualized using UV light. Expected PCR products are 573 bp for *H. nelsoni* and 557 bp for *H. costale*.

3. Confirmatory Diagnosis

If plasmodia infections have been observed in histology, PCR can be used as a confirmatory diagnosis. However, it is easier to cut additional sections and use an in situ hybridization assay as a confirmatory diagnosis. Mixed infections of *H. nelsoni* and *H. costale* occur in high salinity coastal waters, so it is important to assay for both pathogens. Positive and negative controls must be used in in situ hybridization assays. DNA probes that target SSU rRNA sequences have been developed for both *H. nelsoni* and *H. costale* (Figure 3) (Stokes and Burreson 1995, 2001). The use of two specific probes in a hybridization cocktail increases assay sensitivity for *H. costale*.

In situ hybridization assay

H. nelsoni DNA probe: MSX1347 (5'ATGTGTTGGTGACGCTAACCG3')
H. costale DNA probes: SSO1318 (5'CGAACAAGCGCTAGCAGTACAT3')
SSO785 (5'CAAAACTAACTGCCAAAGTCG3')
All probes are 5' end-labeled with digoxigenin.

a. Cut a transverse section through the visceral mass that includes mantle, gill and digestive gland and place it in Davidson's AFA fixative (glycerin [10%], formalin [20%], 95% ethanol [30%], dH_2O [30%], glacial acetic acid [10%]) for approximately 24 hours, then transfer to 70% ethanol until processed by histological procedures (step b). The ratio must be no more than one volume of tissue to ten volumes of fixative.

b. The samples are subsequently embedded in paraffin by conventional histological procedures. Sections are cut at 5–6 µm and placed on positively-charged slides or 3-aminopropyl-triethoxylane-coated slides. Histological sections are then dried overnight in an oven at 40°C.

c. The sections are deparaffinized by immersing them in xylene or another less toxic clearing agent for 10 minutes. The solvent is eliminated by immersion in two successive absolute ethanol baths for 10 minutes each and rehydrated by immersion in an ethanol series. The sections are then washed twice for 5 minutes in phosphate buffered saline (PBS).

d. The sections are treated with proteinase K, 50 µg/ml in PBS, at 37°C for 18 minutes. The reaction is then stopped by washing the sections in PBS with 0.2% glycine for 5 minutes. The sections are then placed in 2X SSC (20X SSC = 3M NaCl; 0.3M Na-citrate; pH 7.0) for 10 minutes.

e. The sections are prehybridized for 1 hour at 42°C in prehybridization solution (4X SSC, 50% formamide, 5X Denhardt's solution, 0.5 mg/ml yeast tRNA, and 0.5 mg/ml heat-denaturated salmon sperm DNA).

f. The prehybridization solution is then replaced with prehybridization buffer containing 2 ng/µl of the digoxigenin-labelled *H. nelsoni* probe or 3 ng/µl each of the digoxigenin-labelled *H. costale* probes. The sections are covered with *in-situ* hybridization plastic cover-slips and placed on a heating block at 90°C for 12 minutes. The slides are then cooled on ice for 1 minute before hybridization overnight at 42°C in a humid chamber.

g. The sections are washed twice for 5 minutes each in 2X SSC at room temperature, twice for 5 minutes each in 1X SSC at room temperature, and twice for 10 minutes each in 0.5X SSC at 42°C. The sections are then placed in Buffer 1 (100 mM Tris, pH 7.5, 150 mM NaCl) for 1–2 minutes.

h. The sections are placed in Buffer 1 (see step g) supplemented with 0.3% Triton X-100 and 2% sheep serum for 30 minutes. Anti-digoxigenin alkaline phosphatase antibody conjugate is diluted 1/500 (or according to the manufacturer's recommendations) in Buffer 1 supplemented with 0.3% Triton X-100 and 1% sheep serum and applied to the tissue sections. The sections are covered with *in-situ* hybridization cover-slips and incubated for 3 hours at room temperature in the humid chamber.

i. The slides are washed twice in Buffer 1 for 5 minutes each (see step g) and twice in Buffer 2 (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 5 minutes each. The slides are then placed in color development solution (337.5 µg/ml nitroblue tetrazolium, 175 µg/ml 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, 240 µg/ml levamisole in Buffer 2) for 2 hours in the dark. The color reaction is stopped by washing in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA [ethylene diamine tetra-acetic acid]).

j. The slides are then rinsed in dH_2O. The sections are counterstained with Bismarck Brown Y, rinsed in dH_2O, and cover-slips are applied using an aqueous mounting medium. The presence of *H. nelsoni* and/or *H. costale* is demonstrated by the purple-black labelling of the parasite cells.

F. Procedures for Detecting Subclinical Infections

Because of its high sensitivity, PCR amplification is the best method for detecting early infections.

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability or Preservation of the Etiological Agent

1. **Histological/in situ hybridization assays**
   a. Transport or ship live oysters humidified and cooled to the analytical facility.

   b. Shuck oysters and excise a 5 mm-thick tissue section that includes digestive gland, gill and mantle. Place section in labeled histological cassette and fix in Davidson's fixative for 24-48 h. Transfer immediately to 70% ethanol. Embed in paraffin as soon as possible.

2. **PCR assays**
   a. Aseptically excise a small piece of gill/mantle and digestive gland and place into 1.5 ml microcentrifuge tubes containing 10 volumes of 95-100% ethanol. Do not use denatured alcohol. Dip dissection equipment in ethanol and flame between oysters to prevent cross contamination.
   b. Store samples at room temperature or in a refrigerator until processed.

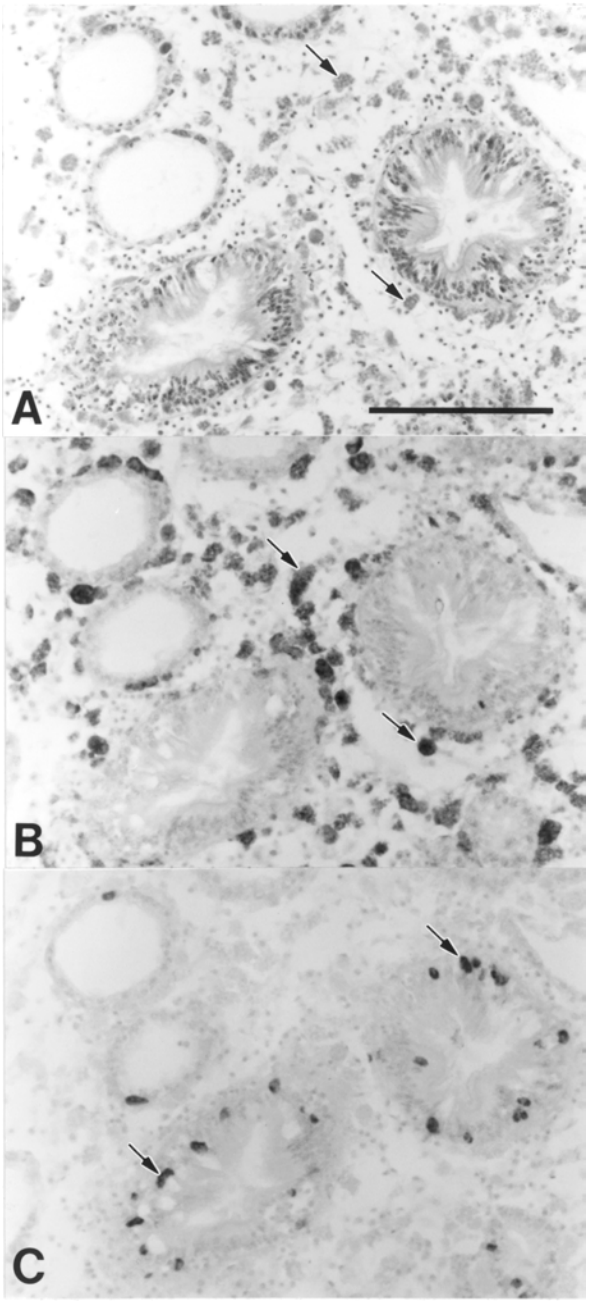


Figure 3. In situ hybridization of mixed haplosporidian infection in ***Crassostrea virginica***. A, B and C are consecutive sections from the same region of the oyster. A. H&E stained section showing many plasmodia in connective tissue (arrows). B. In situ hybridization with the ***H. costale*** DNA probe showing positive reaction with plasmodia in connective tissue (arrows). C. In situ hybridization with the ***H. nelsoni*** DNA probe showing positive reaction with plasmodia in digestive tubule epithelium (arrows), but not with plasmodia in connective tissue.

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5.4 Oyster Seaside Haplosporidiosis

Ralph Elston

Battelle Marine Sciences Laboratory
439 W Sequim Bay Rd
Sequim, WA 98382
206/683-4151
aquatech@olypen.com

A. Name of Disease and Etiological Agent

Oyster seaside haplosporidiosis is caused by *Haplosporidium costale* (*Minchinia costalis*). The agent associated with the disease was first known as seaside organism and the acronym SSO is still used in reference to the disease.

B. Known Geographical Range and Host Species of the Disease

1. **Geographical Range**
   The disease occurs from Virginia north to Massachusetts in high salinity coastal waters.

2. **Host Species**
   Fount in the American oyster *Crassostrea virginica*.

C. Epizootiology

The disease is detectable only from March through June of each year and is associated with mortalities in May and June. Infections acquired in the spring of one year may not cause death until the following spring when the mortality rate can reach 50%. Oysters may also be infected with MSX disease, which often kills the oyster before *Haplosporidium costale*. Thus, the apparent rate of mortality due to *Haplosporidium costale* is lower than if the MSX organism were not present. The fact that this disease occurs only on seaside coasts rather than in the more inland embayments apparently results from its high salinity requirement.

D. Disease Signs

1. Extremely rapid onset and course of disease to mortality (occurring in June in Chincoteague Bay, Virginia) of oysters exposed the previous year.

2. Non-specific signs of gaping, discoloration, lack of shell growth, and mantle recession may occur in oysters affected by this disease.

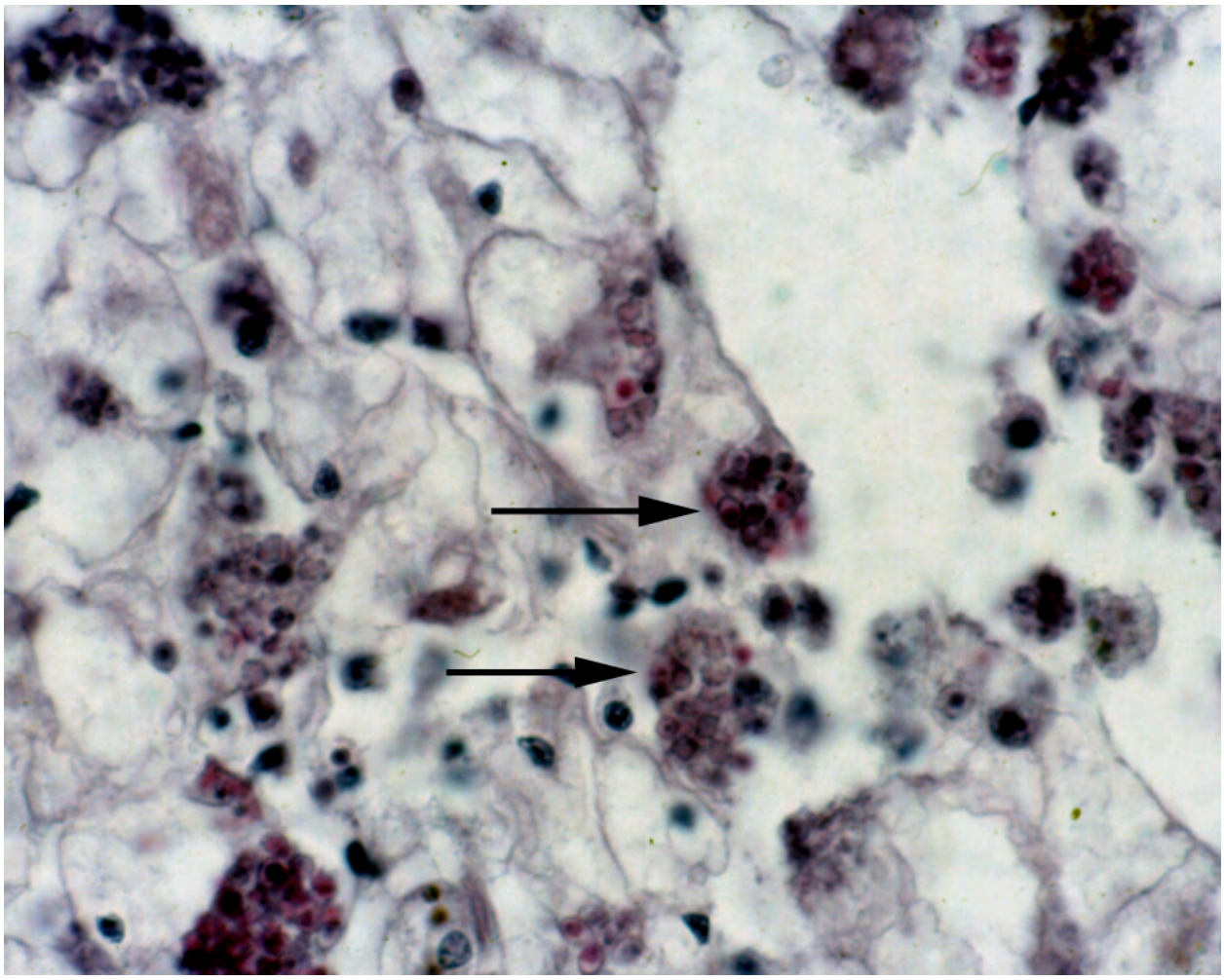


Figure 1. Sporulation of *Haplosporidium costale* in an American oyster (arrows), 1125X.

E. Disease Diagnostic Procedures

1. Confirmatory Diagnosis

Diagnosis is based on histological examination: All three stages of the organism occur only in the months of March through June. Plasmodia are not readily distinguishable from *Haplosporidium nelsoni* plasmodia, although all observed stages of *Haplosporidium costale* occur systemically in March and April, contain several nuclei, and are smaller (less than 10 μm) in diameter. Sporonts are between 10 and 20 μm in diameter. Spores (Figure 1) (4 μm length) are commonly found in connective tissue in terminally sick oysters. *Haplosporidium costale* spores are not found in digestive epithelium while *Haplosporidium nelsoni* spores, when present, are found only in digestive epithelium. Microscopically, sporonts and spores cause the typical curdled appearance in connective tissue, which is considered diagnostic for this disease.

F. Procedures for Detecting Subclinical Infections

No methods are known for detection of the disease outside of the March through June time frame when the stages described above are histologically observable.

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5.5 Velar Virus Disease of Pacific Oysters

Ralph Elston

Battelle Marine Sciences Laboratory
439 W Sequim Bay Rd
Sequim, WA 98382
206/683-4151
aquatech@olypen.com

A. Name of Disease and Etiological Agent

Oyster velar virus disease (OVVD) is also referred to as "blisters" by oyster hatchery biologists. Morphologically, the etiological agent is an iridovirus.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

The disease has been reported only from oyster hatcheries in Washington state. Given the historical commerce of this oyster, it is likely that the range is much broader along the Pacific Rim.

2. Host Species

The etiological agent is known to infect only veliger larvae of the Pacific oyster *Crassostrea gigas*. Morphologically similar viruses have been reported in adult Portuguese and Pacific oysters, but have not been definitively linked to OVVD.

C. Epizootiology

Oyster velar virus disease can cause nearly 100% mortality in affected hatchery tanks. Environmental factors that affect the disease or the oysters' susceptibility are not known. The disease typically appears in March to May, but it has also been reported throughout the summer. Mortalities occur in larvae greater than 150 μm in shell length and typically begin at about 10 days of age when cultured at 25 to 30°C.

D. Disease Signs

Larvae with advanced lesions accumulate on tank bottoms and show deciliation of the velum. Loss of ciliated velar epithelial cells, which may appear as blebs on the periphery of the velum as they are sloughed, form the characteristic "blisters" associated with the disease (see Figure 1 and Figure 2).

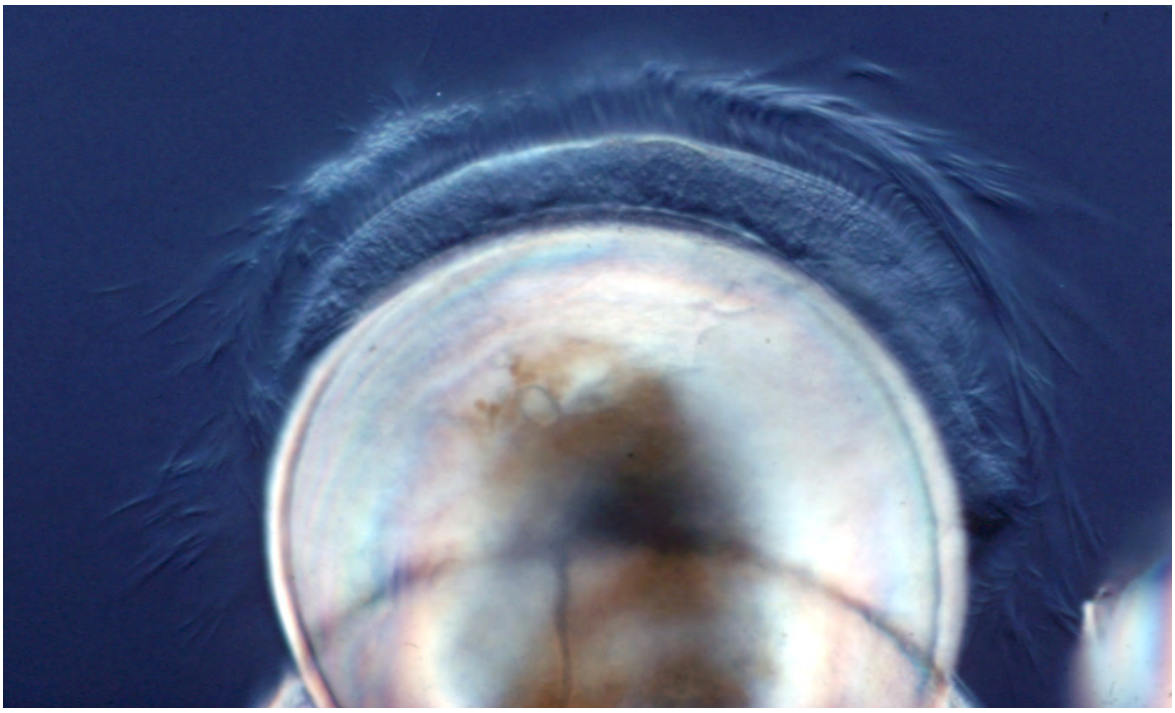


Figure 1. Wet mount of normal Pacific oyster larvae showing the extended intact ciliated velum, 383X. From Elston and Wilkinson 1985, with permission.

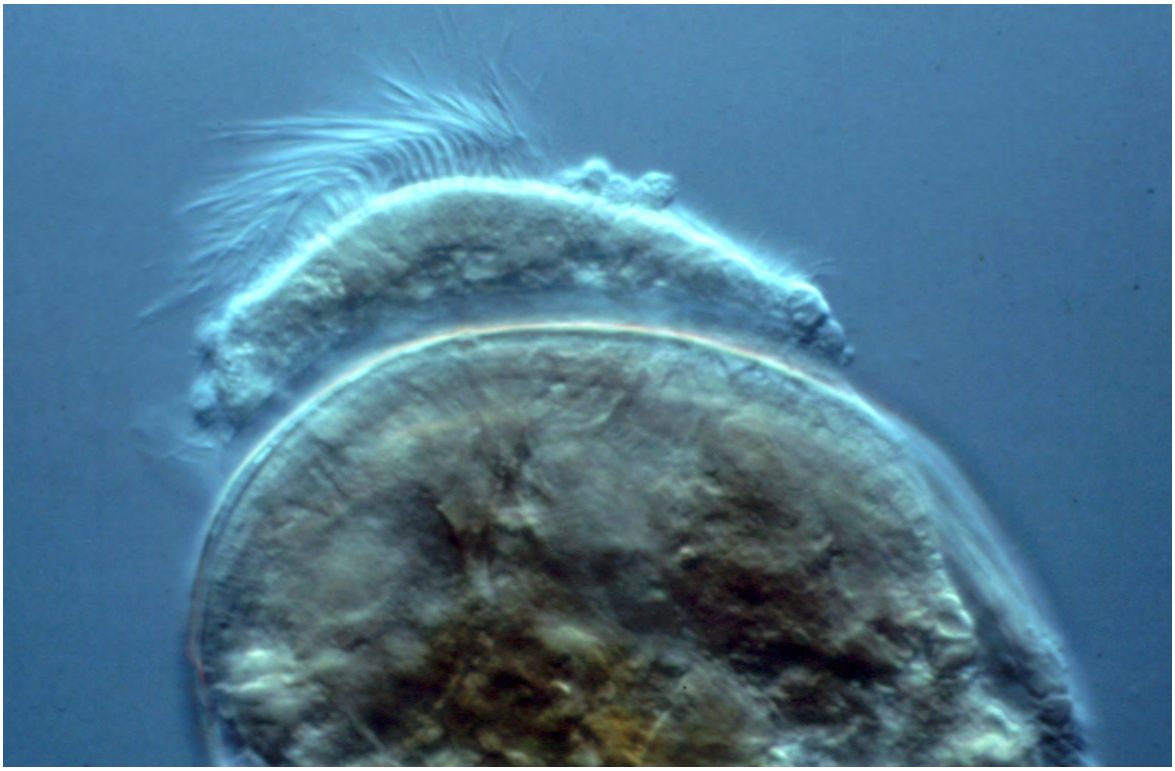


Figure 2. Wet mount of OVVD infected Pacific oyster larva showing velar degeneration including loss of cilia and loss of epithelial cells. As the cells round up and are in the process of detaching from the velum, they form the characteristic blisters.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

A strong presumptive diagnosis can be made if characteristic intracytoplasmic inclusion bodies and associated lesions (Figure 3) are observed in conjunction with the clinical signs noted above. Inclusion bodies are located most commonly in ciliated velar epithelium, but may occasionally be observed in velar supporting epithelium, esophageal, and oral epithelium. In velar ciliated epithelium, inclusion bodies are often found in sloughing cells, which may be rounded and detached within the velar cavity as the velar architecture degenerates. Inclusion bodies are spherical, dense, and basophilic in early stage infections, but become irregular and less basophilic as virions form.

2. Confirmatory Diagnosis

Electron microscopic observation of virus-like particle profiles in velar or other infected epithelium. These particles have icosahedral symmetry are about 228 nm in diameter.

F. Procedures for Detecting Asymptomatic Infections

No methods are reported.

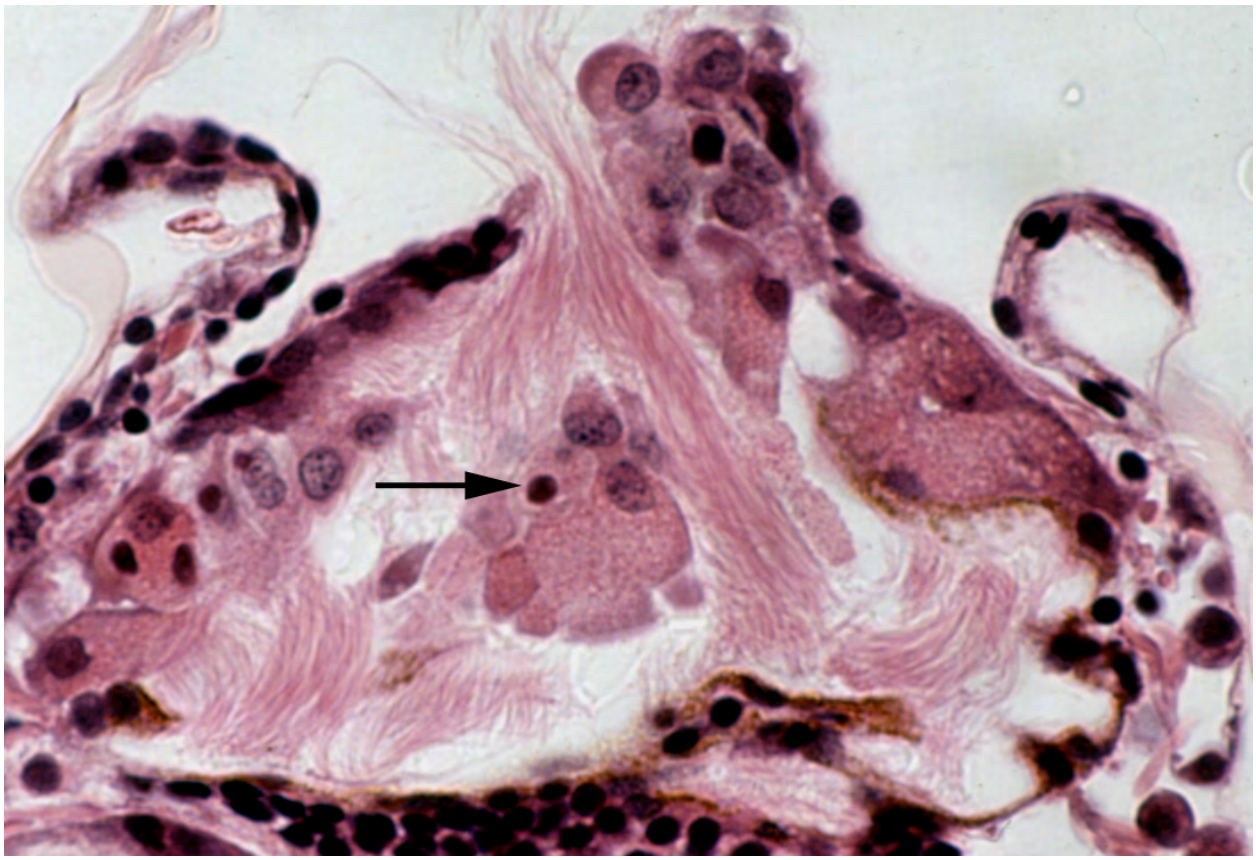


Figure 3. The intracytoplasmic inclusion body in detached velar epithelial cell (arrow) of *Crassostrea gigas* is characteristic of oyster velar viral disease, 2225X.

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5.6 Mikrocytosis (Denman Island Disease of Oysters)

S.M. Bower and G.R. Meyer

Fisheries and Oceans Canada
Pacific Biological Station
3190 Hammond Bay Road
Nanaimo, BC V9T 6N7
BowerS@dfo-mpo.gc.ca

A. Name of Disease and Etiological Agent

Mikrocytosis (also referred to as Denman Island disease) is caused by *Mikrocytos mackini*, a small unicellular organism of unknown taxonomic affiliation that is commonly referred to as a microcell. However, the use of the term "microcell" is discouraged because this term has been used to refer to *Bonamia* spp. which are distinctly different microorganisms that also parasitize oysters.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

The disease and associated mortality were first reported from Henry Bay on Denman Island in British Columbia, Canada in the early 1960s. It has subsequently been observed at other sites in the southern part of British Columbia. Although *M. mackini* has recently been detected in older beach cultured oysters in the northern parts of the state of Washington, mortalities attributable to *M. mackini* have not been reported in oysters from this area. The disease has been found only in old feral oysters at prevalences of less than 6%, using both histology and PCR detection methods.

2. Host Species

Mikrocytos mackini is infective to at least four species of oysters: Pacific oysters, *Crassostrea gigas*; eastern oysters, *Crassostrea virginica*; flat oysters, *Ostrea edulis*; and Olympia oysters, *Ostrea conchaphila* (=*lurida*).

C. Epizootiology

Although juvenile oysters are susceptible to infection, the disease caused by *M. mackini* is only apparent in older oysters in some locations in British Columbia and associated mortality only occurs in the spring (March through May). Diseased oysters that survive the spring infection period may express the disease again the following spring. Young oysters (spat or seed) can acquire the parasite when held with diseased oysters during the spring. Data suggest that most newly acquired infections do not develop into a clinical disease until the following spring.

The life cycle of *M. mackini* is temperature dependent. Exposed oysters, held at 18 °C, can retain *M. mackini* at subclinical levels for at least 6 months and *M. mackini* is only capable of causing disease in

oysters held at less than 10 °C for at least 3 months (Hervio et al. 1996). This apparent requirement for long periods at low temperatures for pathogenic expression and the prolonged prepatent period at warm temperatures may explain why the disease only occurs during the spring and is expressed in oysters from more northerly locations. Possibly, subclinical infections occur in oysters from enzootic areas throughout the year. *Mikrocytos mackini* can be directly transmitted between oysters but transmission seems to be limited to periods when the infection is active in diseased oysters. To date, no evident mechanisms of protection in *M. mackini* for existence outside the oyster host (i.e., no spore-like stage) and no alternate hosts have been detected.

D. Disease Signs

1. Gross Signs

Disease signs consist of the formation of green or yellow-green focal pustules up to 5 mm in diameter throughout the body but are most frequently observed on the surface of the body, labial palps and occasionally within the adductor muscle. Often, brown scars occur on the white nacre layer of the shell adjacent to pustules on the mantle surface. Similar lesions occur with other infectious diseases of the oyster. For example, the pustules caused by *Nocardia crassostreae*, a bacterial pathogen of oysters, are grossly indistinguishable from those caused by *M. mackini*. Thus, microscopic examination is required to identify the etiology of pustules.

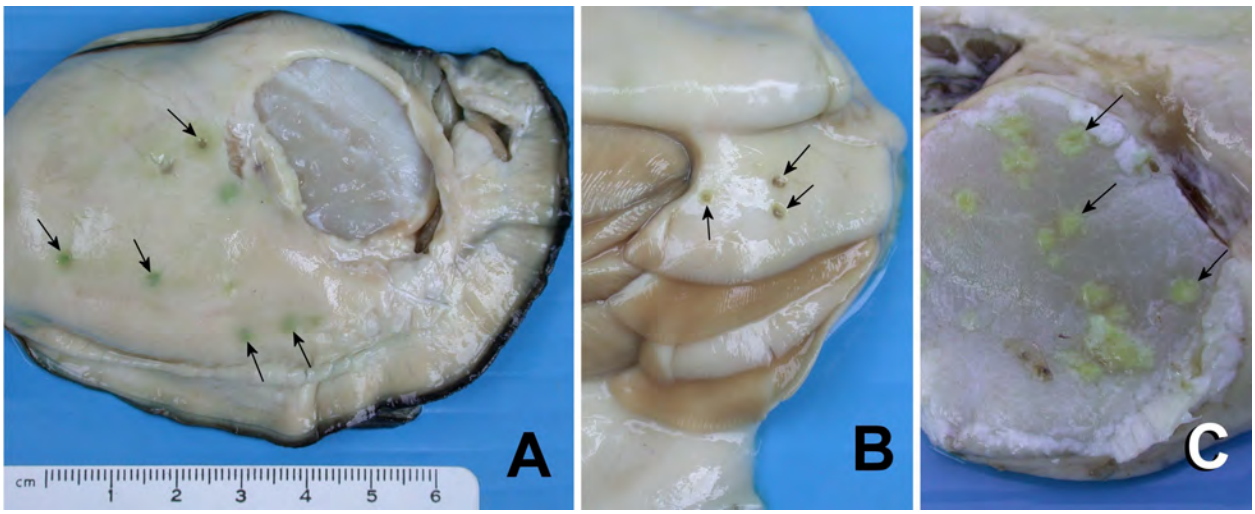


Figure 1. *Crassostrea gigas* removed from the shell and illustrating pustules (arrows) characteristic of *Mikrocytos mackini*. Figure 1A. Typical pustules on the mantle surface. Figure 1B. Pustules on the surface of the labial palps. Figure 1C. Obvious green pustules in the adductor muscle. Usually, the pustules are not as evident as shown in this adductor muscle but are often more diffuse and pale in color.

2. Microscopic Signs

Diagnosis is based on the characteristic histological signs (see below). Pustules consist of focal infiltration of hemocytes associated with *M. mackini* in the tissues (Figures 3A and 6B). Infected vesicular connective tissue cells contain the spherical unicellular parasites about 2 to 3 µm in diameter with a central nucleus (Figures 3B, 4A and 6D). *Mikrocytos mackini* also occurs in hemocytes and adductor muscle cells, and extracellularly within the pustules. Recently, the application of a digoxigenin-labelled DNA probe *in situ* hybridization technique (Meyer et al. 2004) has revealed the presence of *M. mackini* in the tissues of the gills and digestive tract (Bower et al. 2004). However, in these locations, the parasite is very difficult to discern because of its tiny size and pale staining characteristics and is thus usually masked by the denser staining of the oyster tissues. As the lesions resolve in surviving oysters late in the infection period, *M. mackini* is usually not evident in the tissues.

E. Disease Diagnostic Procedures

1. Stained Tissue Imprints

Imprints of pustules, air dried, stained with Wright, Wright-Giemsa or equivalent stain (e.g. Hemacolor, Merck; Diff-QuiK, Baxter) and examined under high power 1000× (oil immersion) for 3-5 µm spheroid or ovoid organisms with a central nucleus. Note: in tissue imprints, *M. mackini* is usually found outside of host cells and is slightly enlarged in size by this method when compared to *M. mackini* in histological preparations. Also, in imprints, *M. mackini* can not be differentiated from other microcell parasites of oysters.

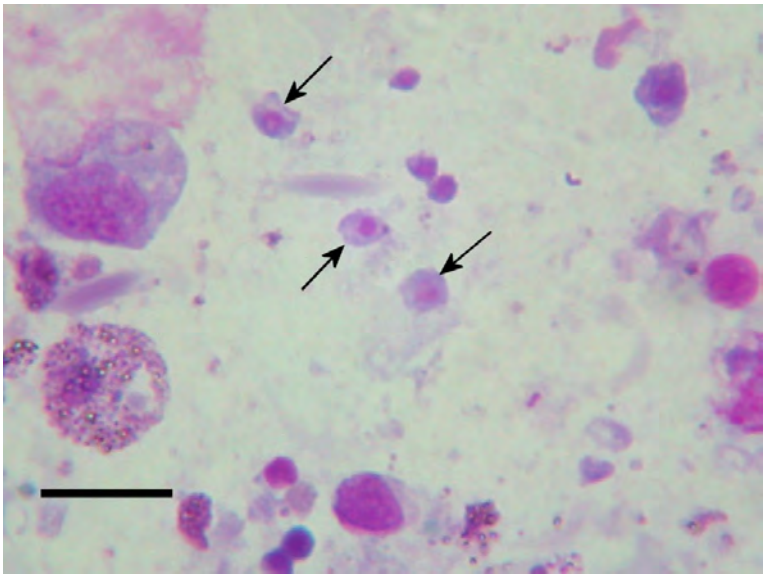


Figure 2. Imprint of a lesion in the adductor muscle of *Ostrea edulis* containing various hemocytes, tissue debris and *Mikrocytos mackini* (arrows). Hemacolor stain. Scale bar = 10 µm.

2. Histopathology

Standard histological tissue sections stained with hematoxylin and eosin stain and examined under high power 1000× (oil immersion) for the presence of intracellular protozoa 2-3 µm in diameter within vesicular connective tissue cells immediately adjacent to foci of hemocytic infiltration. This parasite also occurs in muscle cells (myocytes) and hemocytes within the lesions but is difficult to detect in these tissues. Other microcell parasites of oysters (e.g., *Bonamia* spp. which commonly occur in hemocytes) are not known to occur in the cytoplasm of vesicular connective tissue cells and myocytes. Thus, the occurrence of microcells in these host cells of oysters from enzootic areas can be used to identify *M. mackini*. Apart from host cell differences, *M. mackini* can not be reliably differentiated from other microcells based on parasite morphology at light microscopic levels of magnification (Fig. 4).

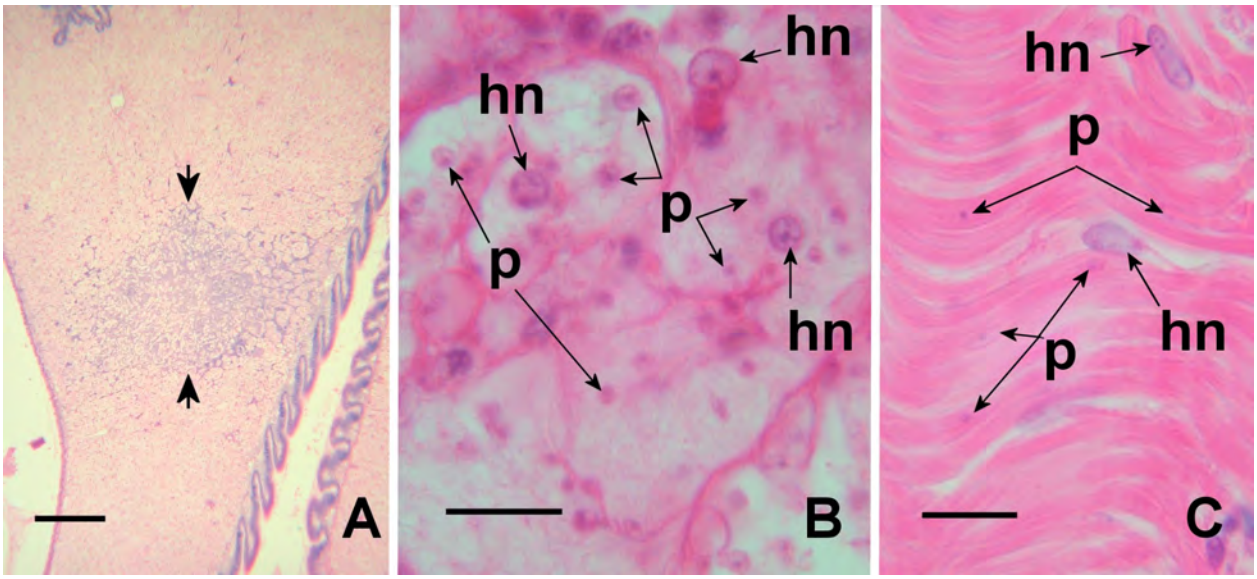


Figure 3. *Mikrocytos mackini* in the tissues of *Crassostrea gigas*. Figure3A. Foci of hemocyte infiltration (between arrows) in the labial palps. Scale bar = 0.5 mm. Figure 3B. Many *M. mackini* (p) in the cytoplasm of the vesicular connective tissue cells (hn indicates the nuclei of the host cells) on the periphery of the foci of hemocyte infiltration. Scale bar = 10 µm. Figure 3C. Cryptic *M. mackini* between the fibers of the adductor muscle (hn indicates the nuclei of the myocytes). Hematoxylin and eosin stain. Scale bar = 10 µm.

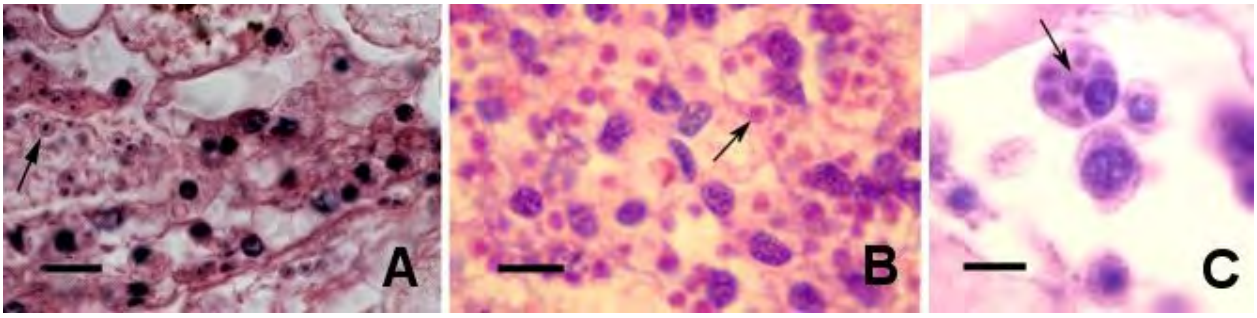


Figure 4. Images of various microcells illustrating the difficulty of differentiating between species at light microscopic levels of magnification. Figure 3A. *Microcytos mackini* (courtesy of Ralph Elston, FHS Blue Book 2004). Figure 3B. *Bonamia exitiosa* (courtesy of Ben Diggles, www.digsfish.com), Figure 3C. *Bonamia ostreae*. Hematoxylin and eosin staining variations. Scale bars = 10 µm.

3. Transmission Electron Microscopy (TEM)

Ultrastructural details of *M. mackini* from various host cells has facilitated the description of a sequence of development of this parasite in its oyster host (Hine et al. 2001). However, the salient features that distinguish *M. mackini* from all other microcells are the absence of mitochondria and haplosporosomes and its frequent close association with the mitochondria, nucleus and golgi cisternae of its host cell.

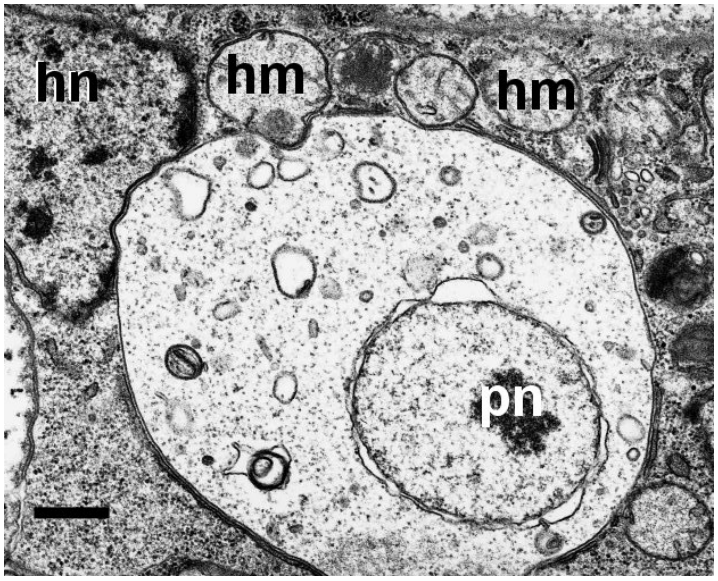


Figure 5. Transmission electron micrograph of a *Mikrocytos mackini* in close association with mitochondria (hm) and the nucleus (hn) of a hemocyte of *Crassostrea gigas*. Note the lack of mitochondria in the cytoplasm of *M. mackini* and presence of a centrally located osmophilic nucleolus (pn). Lead citrate and uranyl acetate stain. Scale bar = 0.5 µm.

4. Antibody-based Methods

Hybridomas that produce monoclonal antibodies specific for *M. mackini* have been produced (Hervio et al. 1995). However, they have not been applied to the development of diagnostic tool(s).

5. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was developed from a 1457 base pair segment of the small-subunit ribosomal DNA gene of *M. mackini* (GenBank assession number AF477623). Details on the PCR procedure are described by Carnegie et al. (2003). This *M. mackini*-specific PCR detects 3 to 4 times more *M. mackini* infections than standard histopathology (Carnegie et al. 2003).

6. *In Situ* Hybridization

Although a fluorescent *in situ* hybridization (FISH) assay was developed (Carnegie et al. 2003), this technique proved problematic because fluorescence quenched during examination, stained preparations had a short storage time, and background staining was inadequate for oyster tissue orientation. These problems were solved by labeling the 3' end of one of the oligonucleotide probes developed for FISH (MACKINI-1-OG: AGCCCACAGCCTTCAC) with digoxigenin. The digoxigenin *in situ* hybridization (DIG-ISH), specific for *M. mackini* binds to the parasites in tissue sections and are visualized using standard immunohistochemical procedures (Meyer et al. 2005). With this technique *M. mackini* stain a dark (blue/black) color and are more visible than with routine histopathology.

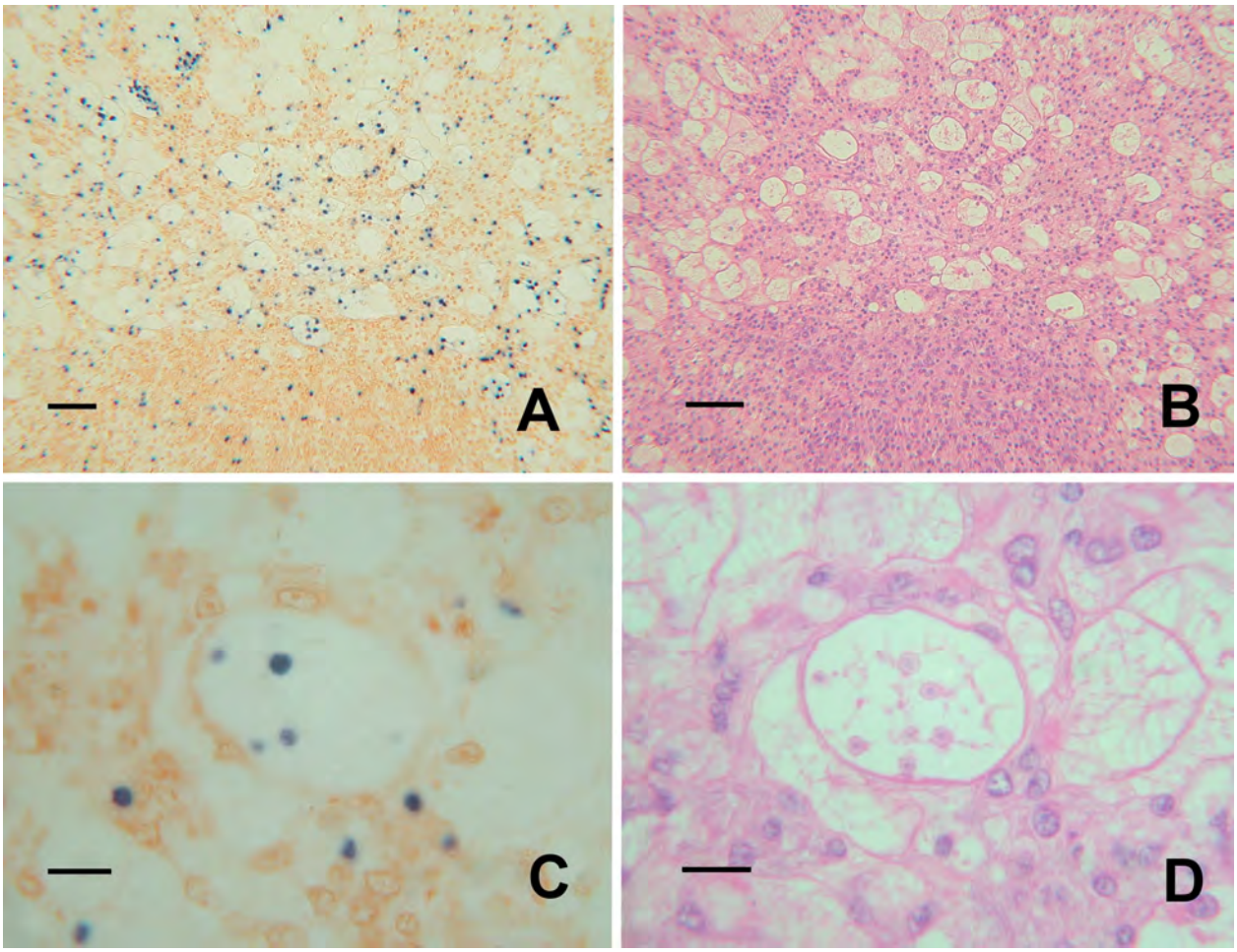


Figure 6. Comparison DIG-ISH and routine histology staining techniques for the detection of *Mikrocytos mackini* in serial sections of *Crassostrea gigas*. Figures 5A and 5B. At low magnification, staining with DIG-ISH reveals *M. mackini* as blue/black dots within the connective tissue and accumulated hemocytes at the bottom of the image (Fig. 5A). At this same magnification stained with hematoxylin and eosin stain, *M. mackini* is not visible (Fig. 5B). Scale bars = 50 µm. Figures 5C and 5D. At high magnification, DIG-ISH stained *M. mackini* are evident within a vesicular connective tissue cell and adjacent hemocytes (Fig. 5C) but are only apparent within the same vesicular cell when stained with hematoxylin and eosin stain.(Fig. 5D) Scale bar = 10 µm.

7. Summary of Diagnostic Procedures

A summary of the methods currently available for surveillance, detection, and diagnosis of mikrocytosis infections is listed in Table 1.

Table 1. Mikrocytos mackini *surveillance, detection and diagnostic methods*

Method	Screening			Presumptive	Confirmatory
	Larvae	Juveniles	Adults		
Gross signs	–	–	+	+	–
Stained tissue imprints	–	–	++	++	–
Histopathology	–	+	+++	+++	+++
TEM	–	–	–	–	+++
Antibody-based methods	–	–	–	–	+
PCR	–	++	++	++	–
DIG-ISH	–	+++	+++	+++	+++

TEM = transmission electron microscopy
PCR = polymerase chain reaction (assessment of reliability on-going)
DIG-ISH = digoxigenin *in situ* hybridization
– = the method is presently unavailable or unsuitable
+ = the method has application in some situations, but cost, accuracy, or other factors severely limits its usefullness
++ = the method is a standard method with good diagnostic sensitivity and specificity
+++ = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity.

F. Procedures for Transportation and Storage of Samples

Samples collected from apparently normal or moribund oysters should be kept cold (but not frozen) and moist, and preserved for diagnosis within 48 hours of the time of collection. For histological examination, fresh tissue samples should be preserved in fixative containing formalin (e.g. Davidson's Solution or 10% neutral buffered formalin). For PCR assays, tiny samples (about 2 mm^3) of the labial palps, mantle or pustules should be preserved in 95% ethanol.

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5.7 Nocardiosis of Pacific Oysters

Ralph Elston

Battelle Marine Sciences Laboratory
439 W Sequim Bay Rd
Sequim, WA 98382
206/683-4151
aquatech@olypen.com

A. Name of Disease and Etiological Agent

Nocardiosis is caused by an actinomycete bacterium that belongs to the genus *Nocardia*, but of uncertain species designation. The disease has previously been referred to as "focal necrosis" and "fatal inflammatory bacteremia." It is also likely synonymous with a condition described as multiple abscesses in Japan.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Found in Matsushima Bay, Japan; discontinuously on the west coast of North America from Tomales Bay, California to British Columbia, Canada, and including oyster culture areas in Willapa Bay and Puget Sound, Washington.

2. Host Species

Occurs in Pacific oyster *Crassostrea gigas*. A morphologically similar condition has been reported in European flat oysters *Ostrea edulis* cultured in areas where the disease is enzootic in the Pacific oyster.

C. Epizootiology

The mortality rate due to this disease has not been accurately measured. However, the severity of the disease in individual oysters and the high prevalence in some populations suggest that it is a significant mortality factor. In one study it was reported to occur in about 30% of oysters sampled from south Puget Sound, Washington during September and October. The widespread geographic occurrence of the disease suggests that it potentially occurs wherever the Pacific oyster is cultured and that the causative bacterium is possibly ubiquitous and acquired from the environment. In Puget Sound, areas that appear to have the most frequent and severe occurrence of the disease are shallow bays that are subject to warm summer temperatures. In British Columbia, the disease has been found in areas other than warm shallow embayments such as on firm, rocky, and sand bottoms. The disease is found in Puget Sound in oysters from both mud and gravel-bottom bays. The disease is a summer and fall phenomenon, typically observed from August through November. The disease may be present in some populations at other

times of the year but at a lesser intensity.

D. Disease Signs

Formation of yellow pustules up to 2 mm in diameter on the surface of the mantle, gill, and typically in the vicinity of the adductor muscle and heart in animals with advanced cases of the disease (Figure 1). It must be noted that similar lesions apparently result from a variety of other infections in oysters and are reported to represent a generalized host response to certain infectious agents. In nocardiosis, clumps of branching bacteria may be visible in wet mounts made from the pustules.

E. Disease Diagnostic Procedures

1. **Presumptive Diagnosis**
   Presumptive diagnosis is based on observation of gram-positive branching colonies in stained impression smears made from the pustular lesions described above.

2. **Confirmatory Diagnosis**
   Confirmatory diagnosis is based on characteristic histological signs of the disease: dense clumps of deeply staining, basophilic, gram-positive, PAS-positive, branching bacteria in vascular spaces of the connective tissues near the stomach, style sac, and intestine and in gonadal follicles and ducts (Figure 2). The bacteria elicit a marked infiltration of host hemocytes around the bacterial cells. In histological sections, it is typical to observe accumulations of hemocytes in which the core of eliciting bacteria is not visible. In advanced infections, the hemocytic infiltration may occupy most of the connective tissue of the oyster, and the connective tissue degenerates into densely staining tissue.

F. Procedures for Detecting Subclinical Infections

Observation of the characteristic lesions by histological examination.

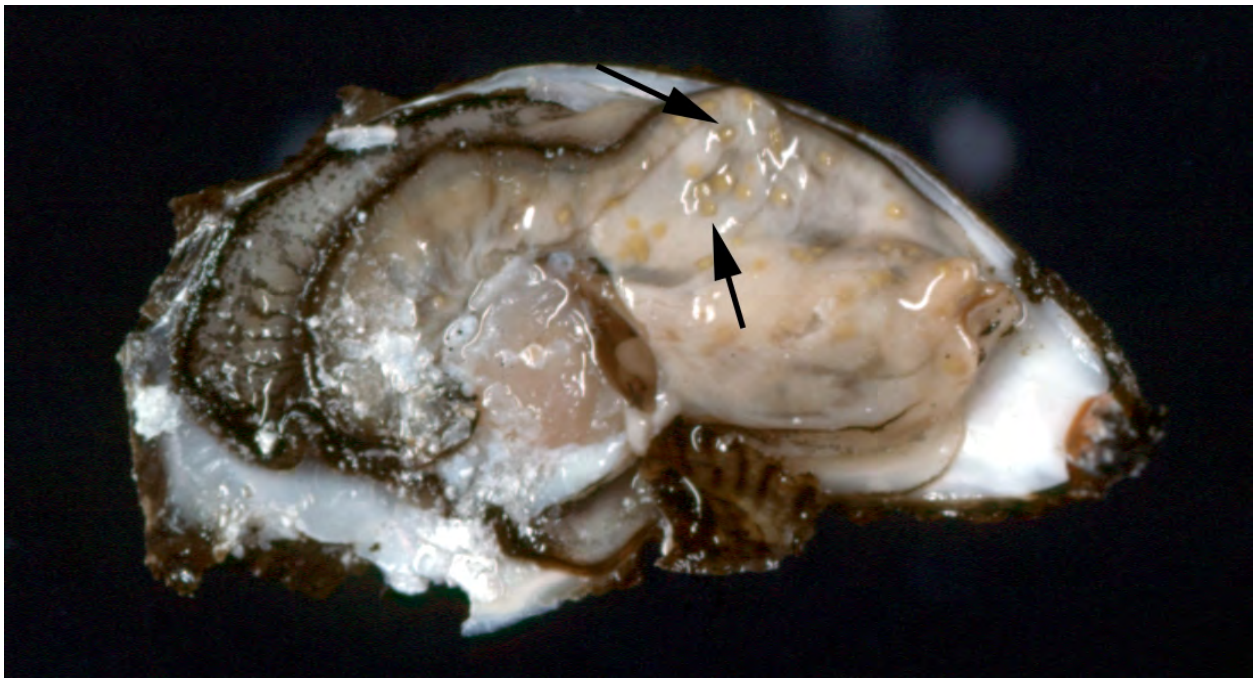


Figure 1. Pacific oyster with nocardiosis. This disease as well as others such as Denman Island disease is characterized by the formation of yellow to green pustules on the mantle and other surfaces as shown here (arrows). From Elston et al. 1987, with permission.

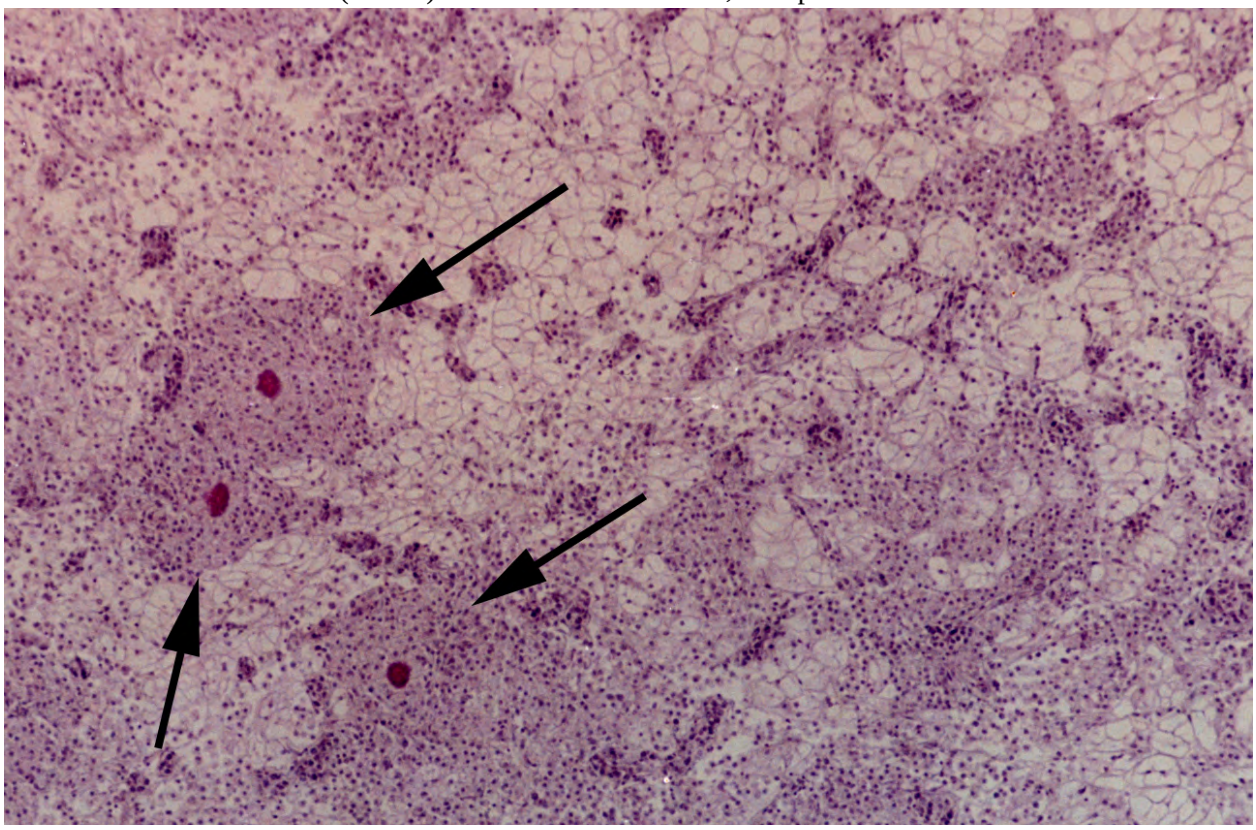


Figure 2. Low magnification of vesicular tissue of Pacific oyster with several foci of *Nocardia* sp. (arrows) surrounded by prominent zones of oyster hemocytes. The other zones of hemocyte infiltration in which the foci of bacteria are not seen in the histological section are typical of the disease, 90X.

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5.8 Bonamiasis of Oysters

James D. Moore
California Department of Fish and Game
Bodega Marine Laboratory
2099 Westside Road
Bodega Bay CA 94923
jdmoore@dfg.ca.gov

A. Name of Disease and Etiological Agent

Bonamiasis, or hemocytic parasitosis, is a disease of oysters caused by members of the genus *Bonamia* (Pichot et al. 1980). Molecular studies (Carnegie et al. 2000, Reece et al. 2004) confirmed ultrastructural evidence that these protistan parasites belong in the Haplosporidia despite the absence of a spore-forming stage in the life cycle. *Bonamia* spp., together with *Mikrocytos roughleyi and M. mackini* were historically termed 'microcell' disease agents due to their small size (2-3µm). There are two described species, *Bonamia ostreae* and *B. exitiosa* (formerly *B. exitiosus*, Berthe and Hine, 2003). Solid genetic evidence indicates that *Mikrocytos roughleyi* should also be placed in the genus *Bonamia* (Cochennec-Laureau et al. 2003b, Carnegie and Cochennec-Laureau 2004, Reece et al. 2004).

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

a. *Bonamia ostreae*
Present in Western Europe from Mediterranean and Atlantic Spain northward to Denmark, including England and Ireland. Also present in British Columbia, Canada and California, Maine and Washington, U.S.A.

b. *Bonamia exitiosa*
Present in New Zealand.

c. *Bonamia* with uncertain species designation. Parasites that clearly belong in the genus *Bonamia* but have not been assigned to either described species or as new species have been reported in Australia, Chile and North Carolina, U.S.A (Burreson et al. 2004). *Mikrocytos roughleyi* which is described from Australia also appears to belong in this genus.

2. Host Species

a. *B. ostreae*
Found in cultured and wild European flat oyster *Ostrea edulis* populations in Europe and cultured *O. edulis* in the United States and British Columbia, Canada. The Suminoe oyster *Crassostrea ariakensis* [= *rivularis*] and the Southern Hemisphere flat oysters *Ostrea chilensis*,

O. puelchana, and *O. angasi* are also susceptible to infection. Attempts to establish infections did not succeed for the Pacific oyster *Crassostrea gigas,* the Manila clam *Venerupis [= Ruditapes] philippinarum*, the carpet-shell clam *Ruditapes decussatus,* the European cockle *Cerastoderma edule* and the mussels *Mytilus edulis* and *M. galloprovincialis*.

b. *B. exitiosa*
Found in *Ostrea chilensis* in New Zealand.

C. Epizootiology

Characteristics are generally similar for *Bonamia ostreae* and *B. exitiosa*. Transmission is direct and can be achieved by injection of infected tissue, by laboratory cohabitation with infected oysters or by deployment in endemic locations. Oysters begin to die 4-6 months after exposure and mortality can reach 80% or more in naïve host oyster populations, while those with a multi-year exposure history experience 20-60% mortality each year. Mortality in cultured oysters may be reduced by practices such as off-bottom culture. Prevalence and intensity increase during warm water periods. Prevalence of *B. ostreae* is much higher in *O. edulis* two years or older compared to younger oysters. Populations of *O. edulis* with a degree of resistance to *B. ostreae* infection have developed in Washington, U.S.A. (Elston et al. 1987), Ireland (Culloty et al. 2001), and elsewhere in Europe (Culloty et al. 2004), and have been experimentally selected for in France (Naciri-Graven et al. 1998). Bonamiasis was apparently transplanted from California, U. S. A. to France (Elston et al. 1986), Spain (Cigarría and Elston, 1997), Washington, U.S. A. (Elston et al. 1986) and Maine, U. S. A. (Friedman and Perkins 1994) via the importation of *B. ostreae*-infected *O. edulis*. Infection of New Zealand *Ostrea chilensis* with *Bonamia exitiosa* is enhanced by the presence of an undescribed apicomplexan parasite (Hine, 2002).

D. Disease Signs

Most oysters infected with *B. ostreae* or *B. exitiosa* have a normal appearance. Gill ulceration may be present in advanced cases (Bucke and Feist, 1985, Dinamani et al. 1987). The key disease feature is the presence of one, several or more 2-3 µm cells in the cytoplasm of granular hemocytes wherever they occur in the open circulatory system, such as in connective tissue of the mantle, heart, viscera or gills. Focal aggregations of infected hemocytes are often associated with intense infiltration of uninfected hemocytes. *B. exitiosa* may be abundant in the gonad (Dinamani et al. 1987, Hine, 1991, Hine et al. 2001).

E. Disease Diagnostic Procedures

1. Screening and Presumptive Diagnosis

Screening and presumptive diagnosis are accomplished by examining heart imprints, histological sections or hemolymph preparations as described below (Section E.2. and Table 1). A polymerase chain reaction protocol (Section F.1.) may also be useful for screening.

A fluorescent *in situ* hybridization method (Carnegie et al. 2003) using DNA probes based on the *B. ostreae* small subunit (18S) rDNA gene sequence may support the conclusion of a histological diagnosis. The probes hybridized with *B. ostreae* and not with the related protist *Haplosporidium nelsoni* although other protists (including *B. exitiosa*) were not examined. Positive hybridization results should not be interpreted as genus- or species-level confirmation until specificity is further defined.

A separate *B. ostreae* chromogenic *in situ* hybridization method using different DNA probes (also based on the small subunit rDNA gene sequence) has been described (Cochennec et al. 2000, Diggles et al. 2003). These probes hybridized with *B. exitiosa*, *H. nelsoni* and another unrelated Haplosporidian as well as with *B. ostreae*, but did not hybridize with *Mikrocytos mackini*. This protocol is not recommended because it is not genus-specific.

Presumptive differential diagnosis of *B. ostreae* (Europe, British Columbia, Canada and California, Maine and Washington, U.S.A) and *B. exitiosa* (New Zealand) can be made based on geographic location since the two species have not been reported to co-occur at these locations. Presumptive differentiation of *B. exitiosa* from *M. roughleyi* can be made on the basis of host species (*B. exitiosa* in *Ostrea chilensis*; *M. roughleyi* in *Saccostrea glomerulata*). The PCR product from a protocol that amplifies DNA of *B. ostreae*, *B. exitiosa* and *Mikrocytos roughleyi* can be used in a restriction fragment length polymorphism analysis to distinguish the species (Section F.1.b and Table 1 below; Cochennec-Laureau et al. 2003b; Carnegie and Cochennec-Laureau 2004).

2. Confirmatory Diagnosis

Histological preparations, stained heart ventricle imprints or stained hemolymph preparations can be used (Table 1). Histology may have slightly lower sensitivity (Diggles et al. 2003, Mirella da Silva and Villalba, 2004). Definitive differential diagnosis of *B. ostreae* and *B. exitiosa* requires transmission electron microscopy (E.2.c below) preferably in conjunction with restriction fragment length polymorphism analysis (F.1.b below).

a. Heart ventricle imprint or hemolymph preparation

Excise heart ventricle and make impressions on a standard microscope slide. Air dry, fix in methanol and stain with Giemsa, Wright-Giemsa or an equivalent stain (e.g., Hemacolor, Merck). The organisms are present in the cytoplasm of granular hemocytes and may be larger (2-5µm) than in histological preparations. Hemolymph drawn from the adductor muscle or pericardial cavity may also be used, and cytocentrifugation, if available, may improve visualization and sensitivity (Mirella da Silva and Villalba, 2004). The heart imprint method can be used with tissues from previously frozen oysters (Rogan et al. 1991). A representative heart imprint is shown in Figure 1.

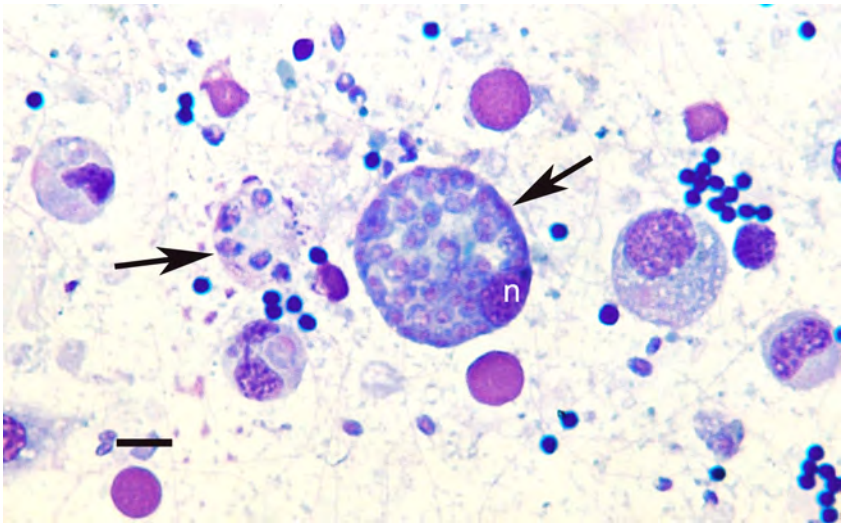


Figure 1. *Ostrea edulis* heart ventricle imprint, showing infection with *Bonamia ostreae*. The arrow on the right points to an intact hemocyte (with nucleus, n) filled with the parasites. The left arrow points to a burst hemocyte containing several of the parasites. The small, intensely blue spheres scattered throughout the preparation are heads of sperm. Scale bar = 10 µm, micrograph courtesy of Ralph Elston.

b. Histology

Routine histological procedures are used. Place 5 mm thick sections of oyster containing gill, cardiac cavity, visceral mass and mantle in a fixative such as Davidson's solution for 24 hours. Stain 5-6µm deparaffinized tissue sections with Harris' hematoxylin and eosin. With *Bonamia* spp. infection, 2-3µm cells are observed within hemocyte cytoplasm at 600-1000x magnification (Figure 2). Infected hemocytes may be present throughout the circulatory system, particularly in vascular sinuses of the stomach, intestine and digestive gland. Severe infections are often associated with massive inflammation that aids in diagnosis (Balouet et al. 1983, Bucke and Feist 1985, Cochennec-Laureau et al. 2003a). The parasites may be present free within the hemolymph or, based on one report, within gill epithelial cells (Montes et al. 1994). Hemocytes infected with *B. exitiosa* may be abundant in the gonad, particularly in males (Hine 1991).

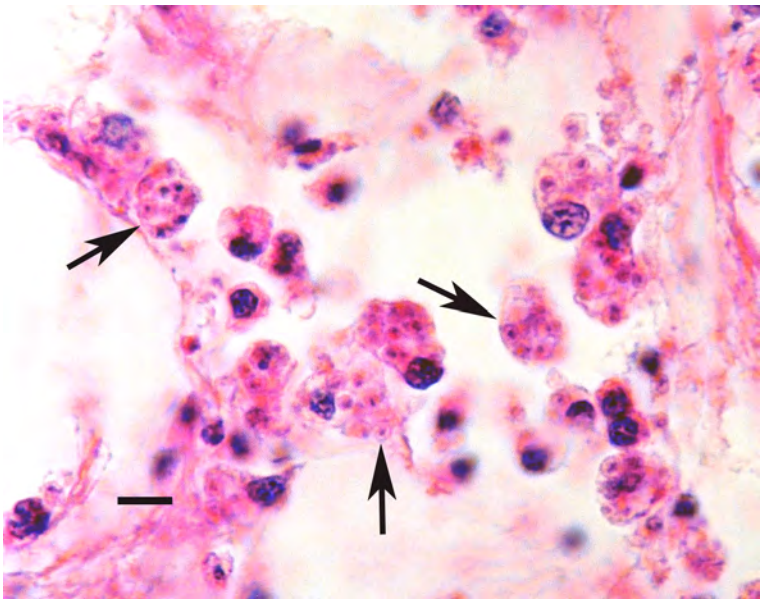


Figure 2. Histological preparation of *Ostrea edulis* infected with *Bonamia ostreae.* Arrows point to infected hemocytes. Hematoxylin and eosin. Scale bar = 10 µm, micrograph courtesy of Ralph Elston.

c. Transmission Electron Microscopy

Fix 1mm cubes of heart ventricle in 2.5% glutaraldehyde in 0.2µm filtered seawater for 2 hours, wash twice in filtered seawater, post-fix in 1% OsO_4 in filtered seawater, stain with 5 % uranyl acetate in 0.1M sodium acetate buffer for 45 min., dehydrate through an ascending series of 50-100% ethanol and embed in Embed It™ Low Viscosity Epoxy Kit (Polysciences). Stain sections in 5 % uranyl acetate for 10 min and 5 % lead citrate for 6 min (Hine et al. 2001). The parasites are present in parasitophorous vacuoles in granular hemocytes throughout the circulatory system. Key features include the presence of haplosporosomes, large mitochondria with sparse cristae, a relatively large nucleus with prominent, peripheral nucleolus and intact nuclear membrane during division. The two species differ in mean size and mean number of haplosporosomes, lipid droplets and mitochondria (Balouet et al. 1983, Dinamani et al. 1987, Hine et al. 2001).

F. Procedures for Detecting Subclinical Infections

1. Polymerase Chain Reaction

A PCR protocol based on 18S-ITS rDNA sequence has been developed for detection of *B. ostreae* (Protocol C, Carnegie et al. 2000). It provides an efficient and apparently sensitive method for screening large numbers of oysters, although specificity remains undefined. A separate PCR protocol based on the SSU rDNA sequence of *B. ostreae* was reported by Cochennec et al. 2000. This protocol also amplifies DNA of *B. exitiosa* (Diggles et al. 2003) and *Mikrocytos roughleyi* (Cochennec-Laureau et al. 2003b) although RFLP analysis provides distinction between the three species.

a. PCR to detect *Bonamia ostreae* (adapted from Carnegie et al. 2000, Protocol C).
Remove the right or upper valve and excise 0.1 gm gill tissue or draw 200 µl hemolymph from the pericardial cavity with a 22g needle on a sterile syringe. Extract and purify DNA using a QIAamp DNA Mini Kit (Qiagen, Inc.) following the manufacturer's 'Tissue Protocol' for gill tissue and 'Blood and Body Fluid Spin Protocol' for hemolymph respectively.
Forward primer C_F: CGGGGGCATAATTCAGGAAC
Reverse primer C_R: CCATCTGCTGGAGACACAG
Prepare 25 µl reaction mixtures containing 23 µl of master mix (1x PCR buffer [Invitrogen], 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.25 µM of forward and reverse primers, 0.6 U Platinum® Taq DNA polymerase [Invitrogen]) and 2 µl of template DNA at a concentration of 100 ng/µl. Follow an initial denaturation at 94 ºC for 4 minutes with 35 reaction cycles (94 °C for 1 min, 59 ºC for 1 min, 72 ºC for 1 min) and a 10 min extension at 72 ºC. Visualize the 760 base-pair amplicon using agarose gel electrophoresis (Chapter 6).

b. PCR to detect *B. ostreae* or *B. exitiosa* (adapted from Cochennec et al. 2000).
Extract DNA from gill or hemolymph using the procedure described above.
Forward primer BO: CATTTAATTGGTCGGGCCGC
Reverse primer: BOAS: CTGATCGTCTTCGATCCCCC
Prepare 50 µl reaction mixtures containing 45 µl of master mix (1x PCR buffer [Promega], 2.5 mM $MgCl_2$, 0.2mM dNTP mix, 1.0 µM of forward and reverse primers, and 1 U Taq DNA polymerase [Promega]) and 100 ng template DNA in a volume of 5 µl. Denature for 5 min at 94 ºC, then follow 30 reaction cycles (94 °C for 1 min, 55 ºC for 1 min, 72 ºC for 1 min) with a 10 min extension at 72 ºC. Visualize amplicons (300 bp for *B. ostreae*, 304 bp for *B. exitiosa*) using agarose gel electrophoresis (Chapter 6). A PCR-restriction fragment length polymorphism (RFLP) assay on the resulting product can be used to differentiate *B. ostreae*, *B. exitiosa* and *M. roughleyi* (Carnegie and Cochennec-Laureau 2004). In individual 200 µl tubes, add 16.3 µl of sterile distilled water, 2 µl of (10X) buffer provided with each enzyme, 0.2 µl of acetylated BSA (10.0 µg/µl, provided with enzyme) and 1.0 µl of BO-BOAS PCR product. Mix by pipetting, then add 0.5 µL of the restriction enzyme Hae II or Bgl I (Promega). Incubate at 37 ºC for 4 hours and visualize by electrophoresis in 2 % agarose gels. The 300 bp *B. ostreae* product is digested by both Hae II (producing 115- and 189-bp fragments) and Bgl I (producing 120- and 180-bp fragments). The 304-bp *B. exitiosa* product is digested by Hae II (producing 115- and 189-bp fragments) but not by Bgl I. The 304-bp *M. roughleyi* product is not digested by either enzyme. A 25-bp ladder (Invitrogen) will assist in size estimation.

2. *In situ* Hybridization

In situ hybridization for *B. ostreae* (adapted from Carnegie et al. 2003). Reaction with *B. exitiosa* is undefined.
Order oligonucleotide probes labeled with 5' fluorescein isothiocyanate (FITC) and purified by HPLC (Invitrogen).

Probe cocktail:
UME-BO-1: CGAGGCAGGGTTTGT
UME-BO-2: GGGTCAAACTCGTTGAAC
UME-BO-3: CGCTCTTATCCACCTAAT

Reaction positive control probe, binds oyster tissue and *Bonamia* spp:
UME-OE-385: TCATGCTCCCTCTCCGG

The following treatments should be included:

1. Diagnostic sample treated with the probe cocktail. *Bonamia* spp should be labeled if present.
2. Diagnostic sample treated with reaction positive control probe. This demonstrates probe binding and the ability to detect fluorescence signals when present.
3. Diagnostic sample with no probe (prehybridization buffer only). This is essential to determine the degree and nature of tissue autofluorescence.
4. Known *Bonamia*-positive sample treated with probe cocktail.
5. Known *Bonamia*-negative sample treated with probe cocktail.

Fix 5 mm transverse sections of oyster tissue, including gill, mantle and digestive gland in Davidson's solution for 24 hr. Place 5 µm sections on aminoalkylsilane-treated slides (Silane-Prep, Sigma). Deparaffinize in xylene or a xylene substitute (10 min, 3x) and rehydrate through 100 %, 95 %, 80 % and 70 % ethanol for 10 min each followed by PBS (Sigma) for 10 min and a separate volume of PBS for 5 min. Digest in Proteinase K (Sigma P4850 diluted to 3.0U/ml in PBS, 15 min, 37 °C) and wash in PBS with 0.2 % glycine for 5 min. Acetylate in a freshly made solution of 5 % (v/v) acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min at room temperature. Wash in PBS for 10 min and place in 5 x SET (750 mM NaCl, 6.4 mM EDTA, 100 mM tris base) for 10 min. Drain excess SET and place prehybridization buffer (5 x SET with 0.02% bovine serum albumin and 0.025 % sodium dodecyl sulfate) on each tissue section, incubate 30 min at 45 °C. Replace with prehybridization buffer containing the oligonucleotide probe or oligonucleotide cocktail. For the oligonucleotide cocktail set the highest probe concentration to 12 ng/µl. Coverslip with Parafilm and incubate in a humid chamber overnight at 45 °C. Wash in 0.2 x SET (25-fold dilution of 5x SET; 5min, 3x, 45 °C), and coverslip using Vectashield mounting medium (Vector Technologies). View under an epifluorescence microscope with a filter set appropriate for viewing FITC (absorbance = 490-496 nm, emission = 514-521 nm).

2. Immunological

ELISA (Cochennec et al. 1992) and IFAT (Boulo et al. 1989) techniques using a monoclonal antibody that binds the parasite membrane have been described for detection of *B. ostreae*. These methods do not appear to be widely used since the advent of molecular methods. Antigenic differences may exist between geographic isolates (Zabaleta and Barber, 1996).

Table 1. Methods for screening, presumptive diagnosis and confirmatory diagnosis of *Bonamia* spp. infection. Within the table, (-) indicates that the method is unsuitable due to cost or accuracy; (+) indicates the method has merit in some situations; (++) indicates a useful method with adequate sensitivity and specificity; (+++) indicates a recommended method on the bases of efficiency, cost, sensitivity and specificity. Consideration of the geographic location and host species will play a significant role in diagnostic interpretations and choice of diagnostic method.

Method	Screening	Presumptive	Confirmatory
Stained heart imprint or hemolymph preparation	+++	+++	+++
Histopathology	+++	+++	+++
Transmission Electron Microscopy	-	+	+
PCR for *B. ostreae*[1]	+++	+	-
PCR for *B. ostreae* or *B. exitiosa*[2]	++	-	-
In situ hybridization for *B. ostreae*[3]	-	++	-

1: Carnegie et al. 2000, Protocol C. Reactivity with *B. exitiosa* is undescribed.
2: Cochennec et al. 2000, Diggles et al. 2003.
3: Carnegie et al. 2003. Reactivity with *B. exitiosa* is undescribed.

G. Procedures for Transportation and Storage of Samples

Whole, live oysters should be kept cold (with gel ice packs) and moist (with towels or foam soaked in seawater) during transportation, and should be processed within 24-48 hours. Gaping, moribund oysters should be kept cold and processed as soon as possible. Air-dried heart imprints can be stored indefinitely at room temperature before staining. If only frozen samples are available, PCR screening should be used along with preparation of imprints from thawed heart tissue.

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5.9 Hexamitiasis of Oysters

Ralph Elston

Battelle Marine Sciences Laboratory
439 W Sequim Bay Rd
Sequim, WA 98382
206/683-4151
aquatech@olypen.com

A. Name of Disease and Etiological Agent

Hexamita nelsoni of the phylum Sarcomastigophora is the reputed etiological agent, frequently referred to as *Hexamita* sp. in the literature. Hexamitiasis is also referred to as pit disease.

B. Known Geographical Range and Host Species of the Disease

Some controversy exists concerning whether or not this parasite, often found systemically in dying oysters, is a pathogen. It is reported to have been responsible for mortalities of *Ostrea edulis* in recirculating seawater basins (pits) in Holland. Experimental water-borne transmission of *Hexamita* has been demonstrated for *Ostrea lurida* (*Ostreola conchaphila*). In the *Crassostrea* species, evidence shows that injected organisms will kill oysters but the pathogenicity under field conditions is equivocal. The causative agent is considered to be cosmopolitan. The following species at the locations indicated have been reported to be infected:

1. *Saccostrea commercialis* (Australian rock oyster), Australia
2. *Crassostrea gigas* (Pacific oyster), Pacific Northwestern United States
3. *Crassostrea virginica* (American oyster), Prince Edward Island, Canada
4. *Ostrea edulis* (European flat oyster), Holland; Maritime region, Eastern Canada
5. *Ostrea lurida* (*Ostreola conchaphila*), (Olympia oyster), Puget Sound, Washington

C. Epizootiology

Mortality rates have not been precisely recorded, but in certain years, oyster farmers have estimated mortalities of *Ostrea lurida* at about 75% over a two-month period. This is definitely a cold temperature disease in this species. Experiments show that infection and debilitating disease occur at 6^{o}C and lower but not at 12^{o}C or higher. Mortalities associated with this disease are usually reported in winter, but in northern zones such as Alaska, the disease has been found at other times of the year.

D. Disease Signs

Infected oysters may appear “fat” but both gaping and apparently healthy individuals may harbor large numbers of the organism systemically.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

Observation of the motile flagellates in microscopic wet preparations of blood from *Ostrea lurida* during cold water periods.

2. Confirmatory Diagnosis

Observation of the histological signs of the disease. These include hemocytosis around blood vessels and sinuses and occlusion of blood vessels by trophozoites in heavy infections, necrosis of intestinal epithelium when the flagellates are observed in the digestive lumen, and degeneration of connective tissue and branchial tissue in the presence of trophozoites. Intracellular forms of the parasite can, in some cases, be observed in hemocytes. These forms display a characteristic macronucleus.

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5.10 Marteiliasis and Marteilioidiasis of Shellfish

Ted Meyers

Alaska Department of Fish and Game
Commercial Fisheries Division
Juneau Fish Pathology Laboratory
P.O. Box 25526
Juneau, AK 99802-5526
(907) 465-3577

A. Name of Disease and Etiological Agent

The disease in shellfish known as Marteiliasis may result from infection of the upper digestive tract (rarely gills) by protozoan parasites in the phylum Paramyxea, class Marteiliidae of the genus *Marteilia*. Related parasites of the same class in the genus *Marteilioides* infect gills and oocytes. The type species is *Marteilia refringens* causing Abers Disease and "maladie de la glande digestive" in the European flat oyster *Ostrea edulis*. *Marteilia maurini* (Comps et al. 1982) was suggested to be a junior synonym of *M. refringens* based on the lack of significantly different ultrastructural morphologies (Longshaw, et al. 2001). Although this continues to make differentiation difficult, *maurini* has been validated by sequencing of the rRNA gene cluster (Berthe et al. 2000, Le Roux et al. 2001, Zrncic et al. 2001). *Marteilia sydneyi* is responsible for QX Disease in Australian Sydney rock oysters *Saccostrea commercialis (glomerata)*.

B. Known Geographic Range and Host Species of the Disease

Table 1. List of parasites, hosts and geographic ranges

Agent	Host	Range
Marteilia refringens (maurini)	*Ostrea edulis, O. angasi, Mytilus edulis, M. galloprovincialis, Cardium eduli, Tiostrea chilensis,*	W. Brittany, France to N. Mediter. Sea
Marteilia sydneyi	*Saccostrea commercialis (glomerata) Saccostrea echinata*	Australia
Marteilia lengehi	*Saccostrea cucullata*	Persian Gulf Australia
Marteilia christenseni	*Scrobicularia piperata*	France (Ronce-les-Bains)

Table 1 (contd)

Agent	Host	Range
Marteilia sp.	*Crassostrea gigas, C. virginica*	France
	Ostrea puelchana	
	Argopecten gibbus	Florida, USA
	Tridacna crocea(maxima)	Fiji
Marteilioides branchialis	*Saccostrea commercialis*	Australia
Marteilioides chungmuensis	*Crassostrea gigas*	Korea, Japan
Marteilioides-like	*Venerupis philippinarum*	S. coast Korea (Lee et al. 2001)

C. Epizootiology

1. *Marteilia refringens*: "Maladie des Abers" was first described from Bretagne (Brittany), France in the European flat oyster *Ostrea edulis* in 1967, causing losses of up to 90% annually. The causative agent was first described by Comps (1970) from oysters held in "claires" in Marennes-Oleron Bay while Grizel et al. (1974) described the organism in more detail and named it *Marteilia refringens*. Epizootics generally have begun in May, peaking in June to August and gradually diminishing by December and January, leaving an eclipse period of March to April when the parasite cannot be found. In colder waters to the north, subclinical infestations have been observed throughout the winter. Observations have shown that old mature sporangia, present from May or June onward, are eliminated by the end of January and that young plasmodia persist during the winter to re-initiate new infestations during the following May. Transplantation experiments indicated that new infestations occur from May to August. Interestingly, oysters may become parasitized without the occurrence of disease and epizootic mortality that may be regulated, in part, by environmental conditions such as water temperature and high salinities. Increasing water temperatures in spring favor development of the organism while increasing salinities and water renewal may inhibit development and transmission. Nonetheless, these parameters alone cannot explain the apparent differences in virulence of this parasite for oysters in French and Dutch waters (van Banning 1979 a, b) and the reported geographic range of the parasite from as far north as Western Brittany, France to as far south as the northern Mediterranean Sea. Control of the disease has included: restriction of oyster transfers from infested areas; delay or reduction of oyster out-planting during May to August when disease transmission is high; and grow-out of oysters in areas with high salinities (35-37 ppt) to retard parasite development.

Experiments using cohabitation and inoculation have shown no strong evidence for direct transmission of the parasite. Instead, the parasite's life cycle is likely complex, involving one or more intermediate hosts. Most recently, the calanoid copepod *Paracartia grani*

Sars was found to be a host for *M. refringens* in French claires (Audemard et al. 2004). In *Ostrea edulis*, early stages of the organism are usually observed in the palps and stomach and less frequently in the gills. Sporulation of the parasite occurs in the digestive gland epithelium, generally from May to September. The sporulation process involves internal cleavage within a stem cell producing spores consisting of several cells enclosed inside one another, typical of paramyxean parasites. Specifically, the inner cell of a 2-celled plasmodium divides to form 8 sporangia (sporonts) within a sporangiosorus. Within each sporont, spore primordia divide internally to form 3-4 spores each containing 3 concentric cells (Roubal et al. 1989). Polymorphic birefringent inclusion bodies appear in the extraspore cytoplasm and become a dominent feature of the sporangia when viewed by light optics, hence the species name. Sporangia are discharged as propagules through the gut with fecal material. More detailed morphological and life-cycle descriptions of the *Marteilia* are provided by Lauckner (1983).

2. *Marteilia sydneyi*: QX Disease was first reported from Sydney rock oysters *Saccostrea commercialis* (*glomerata)* in Moreton Bay, Queensland, Australia by Wolf (1972). The causative agent was later identified as a *Marteilia* and named *M. sydneyi* by Perkins and Wolf (1976). QX Disease has caused oyster losses of up to 80% from southern Queensland and northern New Wales, Australia. The incubation period for the parasite is less than 60 days from early infestation to oyster death and development is favored by lower salinities. The developmental cycle and target tissues are similar to *M. refringens* but the disease is not seasonal whereby mortalities can occur anytime of the year. There also are morphological differences described in the diagnostic section **E**. The disease is limited to the subtropical and tropical waters of Australia and apparently does not occur in the colder waters south of Richmond River. Control measures used to avoid infestation or reduce pathogen loads include: growout of oysters at higher levels in the intertidal zone; delayed or reduced out-planting of oysters in areas of risk during the austral summer months of January to March; grow-out of juvenile oysters in high salinity waters until after the high risk period of infestation is past and harvest of larger oysters by late December before the beginning of the transmission period.

3. *Marteilia sp*. of Calico Scallops: In December of 1988, mass mortality of nearly 100% in a calico scallop *Argopectin gibbus* population occurring over a 2,500 square mile area of the eastern coast of Florida, USA was attributed to a *Marteilia sp*. resembling *M. refringens* (Moyer et al. 1993). This mortality resulted in the loss of 11-40 million pounds of adductor muscle meats and the population had still not recovered to harvestable quantities by the spring of 1992 when last reported (Bower et al. 1994). The parasite apparently filled the tubules of the digestive gland causing dysfunction and starvation followed by rapid mortality within a one month period.

4. Other *Marteilia*: Plasmodia 8-15 µm in diameter and presporulation stages of *M. lengehi* were reported in the stomach epithelium and the digestive gland of *Saccostrea cucullata* in the Persian Gulf (Comps 1976). Similar stages were also reported from the same oyster species in Australia (Hine and Thorne 2000). However, these can only be tentative identifications as mature spores were not found. *Marteilia christenseni* from *Scrobicularia piperata* apparently can be differentiated from other *Marteilia* by the

characteristics of the cytoplasmic contents of sporangia and the morphology of the spore (Comps 1985). Nonetheless, the taxonomic validity of these *Marteilia* species will remain questionable until more investigations are completed. *Marteilia* sp. has also been described from the Argentina oyster (*Ostrea puelchana*) at French farming sites (Pascual et al. 1991).

5. *Marteilioides branchialis*: This organism, described by Anderson and Lester (1992), parasitizes the gills of Australian Sydney rock oysters *Saccostrea commercialis* that are often infested with *Marteilia sydneyi*. The resulting gill damage can cause significant losses during autumn. There are no known methods of control except for restriction of host transfer from areas where the parasite is known to occur.

6. *Marteilioides chungmuensis*: This organism, described by Comps et al. (1986, 1987), parasitizes the oocytes of Korean and Japanese Pacific oysters *Crassostrea gigas* with prevalences up to 16.3% reported in December from Gosung Bay, Korea (Ngo et al. 2003). Parasites could be found in developing and mature ova from June to January but intensity was highest in June when water temperatures were high. Parasitized ova were not observed from February to May. Because this parasite causes spawning failure and unacceptable market quality, serious economic losses to oyster farmers have resulted. There is no known control measure except to restrict transport of oysters from enzootic areas.

D. Disease Signs

1. *Marteilia refringens/maurini*:

Oysters - Parasitized oysters may appear normal but epizootic disease is characterized by glycogen depletion and tissue necrosis of the digestive gland with clinical signs of poor growth, brown to pale yellow discoloration of the digestive gland, loss of pigmentation and mucoid atrophy of the visceral mass, gaping valves followed by mortality, generally from sporulation of the parasite in the digestive tubule epithelium.

Mussels – No mortality in mussel populations has been reported that is attributable to *Marteilia* infestation. Disease signs in parasitized mussels are not described except for an overall lower condition factor and suspected disruption of the digestive gland epithelium in *M. edulis* and inhibition of gonadal development in *M. galloprovincialis*.

2. *Marteilia sydneyi*: Parasitized oysters appear similar to those infested with *M. refringens*. Glycogen depletion and tissue necrosis result in a pale brown to yellow digestive gland instead of the green color in healthy animals; an emaciated, shrunken and translucent visceral mass with complete resorption of the gonad may be visible. Mortality from starvation generally occurs in less than 60 days from initial infestation.

3. *Marteilia sp*.: Giant clam (*Tridacna crocea/maxima*) – Parasitized clams had chalky white multiple foci observed throughout a dark red-brown kidney (Norton et al. 1993).

4. *Marteilioides branchialis* – Parasitized oysters have focal lesions in the gills

5. *Marteilioides chungmuensis* – Parasitized oysters have focal nodules in the ovaries during spawning season resulting in unacceptable appearance and marketability (Fig. I-A).

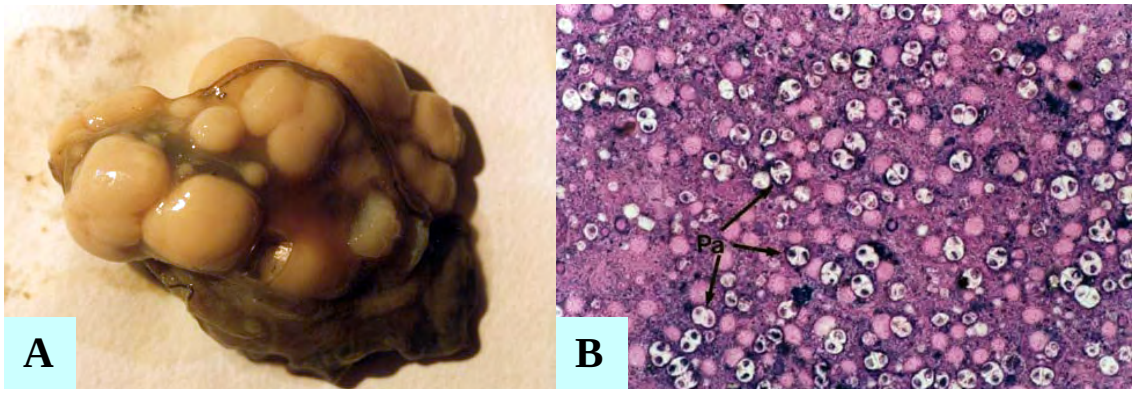


Fig. I A and B. A shows the gross appearance of a Pacific oyster infected with *Marteiliodes chungmuensi*. B shows the stained appearance of the parasite in infected ova (example parasites are indicated by Pa). B, 200x, eosin, methylene blue stained wet impression smear. From Park et al. 2003. Provided courtesy of M.S. Park for use in the BlueBook.

E. Disease Diagnostic Procedures

Table 2. Marteiliasis/Marteilioidiasis - surveillance, detection and diagnostic methods: BF = bright field; LM = light microscopy; EM = electron microscopy; IFAT= indirect fluorescent antibody test; PCR = polymerase chain reaction

Methods	**Screening**		**Presumptive**	**Confirmatory**
	Juveniles	**Adults**		
Gross signs	-	-	+	-
Direct BF/LM	++	++	+++	++
Histopathology	+++	+++	+++	++
Bioassay	-	-	-	-
Transmission EM	++	++	+++	+++
IFAT[a]	+	+	+	+
PCR[b]	+++	+++	+++	+++
DNA probe-***in situ***[c]	+++	+++	+++	+++

[a] IFAT has been used only for *Marteilia sydneyi* and reagents are limited in availability
[b] PCR reagents available for differentiation between *Marteilia refringens* and *M. maurini* and specific identification of *M. sydneyi*

[c] In situ hybridization available for *Marteilia sp., M. refringens and M. sydneyi*

– = the method is presently unavailable or unsuitable
+ = the method has application in some situations, but cost, accuracy, or other factors severely limits its usefullness
++ = the method is a standard method with good diagnostic sensitivity and specificity

+++ = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity.

1. Brightfield light microscopy: Presumptive diagnosis for *Marteilia* species can be made from smears of cut sections from fresh digestive gland tissue that has been blotted dry. The smears are dried and fixed in methanol for 2-3 minutes and stained according to instructions from any of several commercially available Giemsa or methylene blue-based staining kits (example; Dif-Quik). After staining, the rinsed and dried smears can be cover-slipped with resin and/or examined directly with oil for plasmodia of 5-8 µm in length during the early stages of the disease that can reach 40 µm in diameter during sporulation. The parasite cell cytoplasm stains variably basophilic with a bright red nucleus. The secondary cells or sporoblasts are surrounded by a bright halo.

2. Histopathology: Confirmative diagnoses for the genera *Marteilia* and *Marteilioides*, based on internal cleavage to produce cells within cells, can be made by histopathological examination of stained sections of paraffin or resin-embedded bivalve tissues using standard histological fixatives (seawater Davidson's or Carson's solutions or 10% formalin in filtered seawater), procedures and stains (ie, hematoxylin-eosin, trichrome, etc.).

1. *Marteilia refringens*- sections of palps (rarely gills), stomach and digestive gland. The younger vegetative plasmodia may be present within the epithelium of the stomach, in the connective tissues surrounding the upper intestinal tract, between the epithelial cells or within lumens of these organs. Maturing presporangia or sporangial primordia are found in the epithelium of the digestive tubules, each of which develops into a sporangiosorus of up to 30 µm in diameter containing as many as 8 sporangia. Spore formation within sporangia or sporulation results in the formation of haplosporosomes in the cytoplasm peripheral to the spores that condenses into birefringent inclusion bodies visible by light microscopy. Spores are about 2.6 to 3.5 µm in diameter. A most important overall diagnostic feature is that the phylum Paramyxea (*Marteilia* and *Marteilioides*) is distinguished from all other protozoans in shellfish by this unique morphology of internal cleavage to produce cells within cells during sporulation.

2. *Marteilia sydneyi*: - sections of digestive gland. *Marteilia sydneyi* can be distinguished from *M. refringens* by the following histological characteristics: 1) lack of striated inclusions in the plasmodia; 2) the formation of 8-16 sporangial primordia in each plasmodium instead of only 8; 3) the occurrence of 2 or rarely 3 rather than 4 spores in each sporangium and; 4) a heavy layer of concentric membranes surrounding mature spores that are lacking in spores of *M. refringens* (Bower et al. 1994).

3. Other *Marteilia sp* – little information is available to morphologically separate other *Marteilia* sp from *M. refringens* and *M. sydneyi*.

4. *Marteilioides branchialis* – sections of gills. Epithelial hyperplasia and focal inflammation surrounding parasite cells consisting of uninucleate primary cells containing 2-6, and rarely up to 12, secondary cells in the cytoplasm of epithelium, connective tissue cells, and occasionally hemocytes in the lesion.

5. *Marteilioides chungmuensis* – sections of ovary. The cytoplasm of parasitized ova and oocytes contain primary parasite cells that give rise to secondary cells that may have developing sporonts each producing one tertiary cell by endogenous budding. Each tertiary cell forms one tricellular spore by internal cleavage. The parasites can also be visualized in impression smears, which can be stained with eosin-methylene blue to differentiate the parasites (Fig. I B).

3. Transmission Electron Microscopy (TEM): Generally, histopathological characteristics are sufficient for confirmation of Paramyxea and for distinguishing *Marteilia refringens* from *M. sydneyi* and identification of *Marteilioides branchialis* and *chungmuensis*. If histological methods are unable to define morphological details described in section E-2 then standard TEM procedures for fixation (4% glutaraldehyde or 10% formalin in seawater), embedding, cutting and staining (uranyl acetate and lead citrate) are used to observe ultrastructural characteristics for confirmation of parasite species. Ultrastructural confirmation for *M. branchialis* may be necessary to determine that the spore contains two concentric cells rather than three and has 2-6, and rarely 12, sporonts per stem cell in comparison to only 2 or 3 for *M. chungmuensis*. Also, multivesicular bodies similar to those of *Marteilia sp*. occur in primary cells of *M. branchialis* but not *M. chungmuensis.*

4. IFAT: IFAT, as described by Roubal et al. (1989), has been used for *M. sydneyi* but is not yet a standard procedure.

5. PCR: PCR-restriction fragment length polymorphism is used for discrimination of *M. refringens*/*M. maurini* using methods described by Le Roux et al. (2001). Specific detection of *M. sydneyi* by PCR is described by Kleeman and Adlard (2000), and Kleeman et al. (2002a, 2002b).

1. Infected animals are frozen at –80^{o}C and ground to powder for DNA extraction.
2. 10 volumes of extraction buffer at pH 8 (100 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate) are added with 100 µg/ml proteinase K for overnight incubation at 50^{o}C.
3. DNA is extracted using a standard phenol/chloroform procedure and precipitated with ethanol.
4. A 50 µl volume of extracted DNA is denatured for 5 min at 94^{o}C followed by 30 cycles of: denaturation at 94^{o}C for 1 min, annealing at 55^{o}C for 1 min, and

elongation at 72°C for 1 min per kilo-base pair. This is followed by a final elongation step of 10 min at 72°C.

5. *M. refringens* is detected with primers (4 and 5) that target the internal transcribed spacer ITS1

   4: 5'-CCG-CAC-ACG-TTC-TTC-ACT-CC-3'
   5: 5'-CTC-GCG-AGT-TTC-GAC-AGA-CG-3'

6. *M. sydneyi* is detected with primers that target the ITS:

   LEG1: 5'-CGA-TCT-GTG-TAG-TCG-GAT- TCC-GA-3'
   PRO2: 5'-TCA-AGG-GAC-ATC-CAA-CGG-TC-3'

7. The restriction enzyme *Hha*1 tests for cleavage to identify polymorphism among the PCR products that differentiate *M. refringens* from *M. maurini*. The restriction fragments for *M. maurini* produce an electrophoretic profile on 2% agarose gel corresponding to three bands of 156, 157 and 68 bp. The profile for *M. refringens* consistst of two bands of 226 and 156 bp.

   The profile of *M. sydneyi* consists of a single fragment of 195 bp.

6. *In-situ* Hybridization (ISH): I*n-situ* hybridization methods have been described for *M. refringens* by Mialhe et al (1995) and Le Roux et al. (1999). The latter investigators describe a probe specific for the genus *Marteilia* detecting both *M. refringens* and *M. sydneyi* while a probe specific for *M. sydneyi* targeting the ITS (internal transcriber spacer) is described by Kleeman and Adlard (2000) and Kleeman et al. (2002b).

1. The Smart 2 probe is used to detect *Marteilia* at the genus level and is obtained by PCR (section E-5) using the primers:

   SS2: 5'-CCG-GTG-CCA-GGT-ATA-TCT-CG-3'
   SAS1 : 5'-TTC-GGG-TGG-TCT-TGA-AAG-GC-3'

   The PCR reaction is performed from *M. refringens* purified from *Ostrea edulis* DNA except the 1 µl of 25 mM digoxigenin-dUTP (DIG-dUTP) is added to the mix.

2. The ITS1 probe using the PRO2 and LEG1 primers (section E-5) is used to specifically detect *M. sydneyi* and is obtained by performing PCR from *M. sydneyi* purified from *Saccostrea glomerata* DNA, except the 1 µl of 25 mM DIG-dUTP is added to the mix.

3. In-situ hybridization including positive and negative controls:

a. The visceral mass of the sample mollusc, after fixation in Davidson's AFA (10% glycerine, 20% formalin, 30% of 95% ethanol, 30% distilled water and 10% glacial acetic acid) or 10% buffered formalin for 24 hr, is embedded in paraffin.
b. 5 µm sections are cut and placed on aminoalkylsilane-coated slides and baked in an oven at 60°C overnight.
c. Sections are deparaffinized by two 10-min soaks in xylene followed by immersion in two successive baths of 100% ethanol for 10 min each.
d. Sections are rehydrated in an ethanol series and treated with 100 µg/ml proteinase K in TE buffer (50 mM Tris, 10 mM EDTA) at 37°C for 30 min.
e. Sections are dehydrated in an ethanol series, air dried and incubated with 100 µl hybridization buffer (4 X standard saline citrate [SSC], 50% formamide, 1 X Denhardt's solution, 250 µg/ml yeast tRNA, 10% dextran sulfate) containing 10 ng (1 µl of the PCR reaction) of the DIG-labeled probe.
f. Sections are covered with plastic cover-slips, placed on a heating block at 95°C for 5 min, then cooled on ice for 1 min and placed in a 42°C moist chamber overnight for hybridization.
g. Sections are washed twice in 2 X SSC for 5 min each at room temperature and once for 10 min in 0.4 X SSC at 42°C.
h. The sections are then rinsed in distilled water, counterstained with Bismark Brown Y, rinsed again in distilled water and cover-slipped with an aqueous mounting medium.
i. *Marteilia* organisms are demonstrated by purple-black labeling of the parasite cells.

Note- additional procedural details for ISH or PCR can be found in the current O.I.E. (2003) Diagnostic Manual for Aquatic Animal Diseases. Most molecular procedures still await validation with the exception of the ISH and histology assays for *M. refringens* as recently published by Thebault et al. (2005).

F. Procedures for Detecting Subclinical Infections

Success for detection of subclinical infections may be more likely with the more sensitive assays such as PCR and ISH described in section **E**.

G. Procedures for Transportation and Storage of Samples

Optimum results can only be achieved by the performance of diagnostic methods on live shellfish. The procedures for transport and storage should include adequate insulated containers for shipping, refrigeration, reduced time out of seawater and consideration of animal health and condition when collected regarding survivability in transit.

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5.11 Shell Disease of Oysters

Ralph Elston

Battelle Marine Sciences Laboratory
439 W Sequim Bay Rd
Sequim, WA 98382
206/683-4151
aquatech@olypen.com

A. Name of Disease and Etiological Agent

Shell disease is caused by the fungus *Ostracoblabe implexa* and can thus be properly referred to as oyster ostracoblabiasis. The disease has also been referred to as "maladie du pied" and "maladie de la charniere" in reference to the effects of disease on the adductor muscle (incorrectly referred to as the foot) and the hinge ligament.

B. Known Geographical Range and Host Species of the Disease

The disease is well documented in the European flat oyster *Ostrea edulis* and the Portuguese oyster *Crassostrea angulata* in continental Europe (France and The Netherlands) and the United Kingdom and in *Crassostrea gryphoides and C. cucullata* in India. It has been reported in Atlantic Canada in *Ostrea edulis* and in the American oyster *Crassostrea virginica* on the Atlantic seaboard of the United States. The disease rarely progresses beyond the white spot stage in *Crassostrea.*

C. Epizootiology

Shell disease may have caused massive mortalities of *Ostrea edulis* in The Netherlands at various times and has also been claimed to be associated with severe oyster kills in France. Definitive proof that the disease is responsible for the oyster kills is lacking. Oysters are infected above 20^{o}C. Infection can be transmitted by waterborne contact or by direct growth of the fungus from one oyster to adjacent oysters. Young oysters are reported to be more susceptible than older oysters. In The Netherlands, cockle shells, used as spat collectors, were suspected of containing the disease-causing fungus, ensuring that new oyster spat would become infected at an early age.

D. Disease Signs

White spots from 0.5 to 3.0 mm diameter underlying the subnacreous layer on the inner surface of the shell characterize early stages of the disease (Figure 1). In those cases where the fungal hyphae penetrate into the mantle cavity from the shell matrix, the spots form a small slightly raised rough area with a dark central indentation. These infected spots coalesce to form the typical "cloud," also with a

characteristic rough surface, as the infected area of the shell matrix enlarges. The pallial surface of the shell may acquire a brownish tint in advanced infections.

Formation of "warts" is common. These consist of small dark protrusions attached to the inner shell surface often in the area of adductor muscle attachment and the hinge region, but also at other sites on the inner shell surface (Figure 2). Excessive and abnormal hinge deposition may occur and result in a beaked appearance of the dorsal region and inability of the oyster to effect normal shell closure.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

A presumptive diagnosis can be made on the basis of observation of the clinical signs noted above.

2. Confirmatory Diagnosis

A confirmatory diagnosis consists of the observation of the clinical signs plus the microscopic observation of the fungal mycelia in wet mounts of crushed shell matrix or warts. The mycelia are straight, non-septate hyphae, about 2 μm in diameter, that display rounded hyphal swellings (chlamydospore structures) 4 to 6 μm long. Branches occur irregularly at oblique angles to the mycelial axis.

F. Procedures for Detecting Subclinical Infections

No procedures have been documented.



Figure 1. Internal shell surface of *Ostrea edulis* showing signs of shell disease. Note the white spots indicative of the early stages of the disease adjacent to the more advanced lesion. Photography courtesy of David J. Alderman.

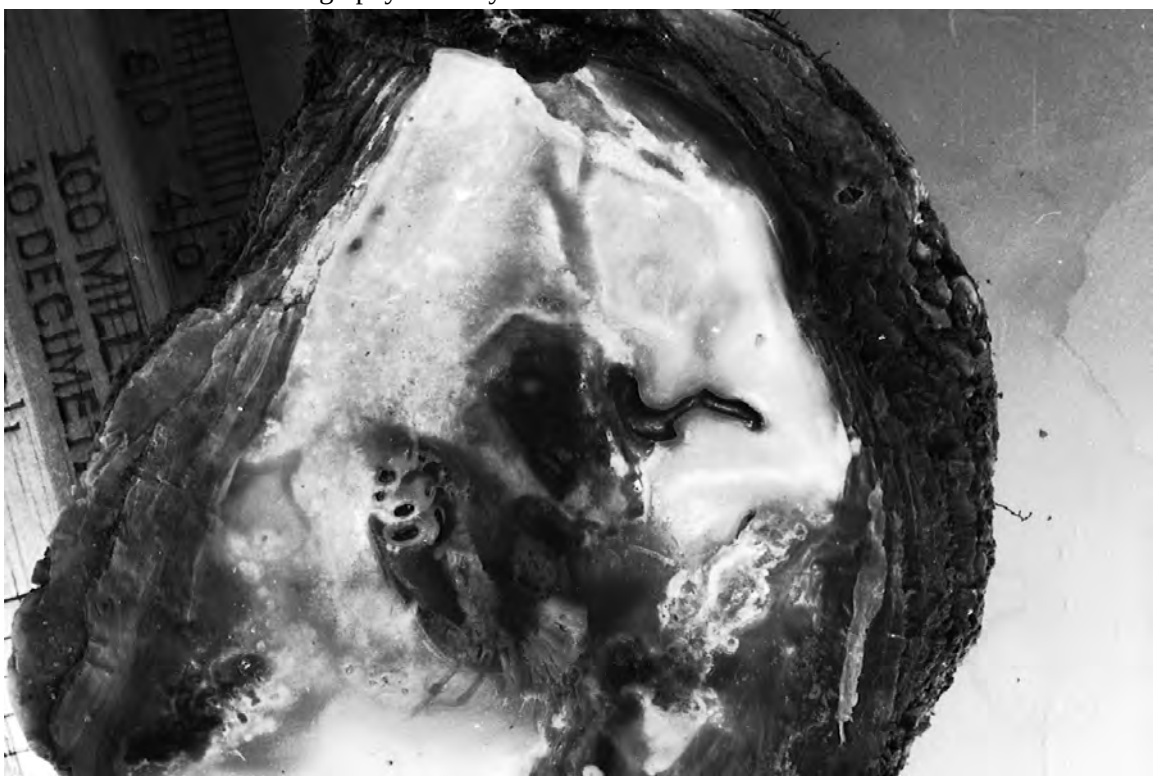


Figure 2. Shell of *Ostrea edulis* with "warts" or dark protrusions on the inner shell surface. Photograph courtesy of David J. Alderman.

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5.12 Disseminated Neoplasia of Bivalve Molluscs

Marcia House[1] and Ralph Elston[2]

[1]Northwest Indian Fisheries Commission
6730 Martin Way E.
Olympia, WA 98516
360/ 438-1181

[2]AquaTechnics
455 West Bell Street
Sequim, WA 98382
360/ 681-3122

A. Name of Disease and Etiological Agent

Disseminated neoplasia (DN) is a general grouping of proliferative disorders in the hemolymph of several bivalve species. It is generally believed that the transformed and abnormal circulating cells arise from a circulating cell (hemocyte) line but this hypothesis remains unproved and the source of hematopoietic tissue is not known with certainty in bivalve molluscs. It is clear that the cells are of host origin (Gee et al. 1994). There may actually be a complex of diseases with morphological similarities that arise from different tissues and possibly derive from infection by different etiological agents. This disease is known also known as and referred to in the literature as hemic, hematopoietic, or hemocytic neoplasia (HCN), disseminated sarcoma, hemic proliferative disease, leukocytic neoplasia, hemocytic leukemia, sarcomatous neoplasia, sarcomatoid proliferative disorder, and atypical hemocyte condition. Limited evidence suggests a retroviral etiology, but this, as well as the hemic origin of the affected tissue, has not been demonstrated in many of the species affected.

B. Known Geographical Range and Host Species of the Disease

The disease with morphologically similar or identical characteristics occurs in a variety of bivalve molluscs worldwide. Although the diseases are identical or nearly so in various species and are transmissible within a species, a common etiology has not been established. In addition, experiments attempting to demonstrate cross-species transmissibility have failed (e.g. Kent et al. 1991). The affected molluscs include:

1. *Adule californica*: west coast of North America

2. *Arctica islandica* (mahogany quahog): Rhode Island Sound, eastern North America

3. *Cerastoderma edule* (common cockle): south coast of Ireland near Cork, coast of Galacia, northwest Spain and Brittany, France.

4. *Saccostrea commercialis* (Australia rock oyster): Australia

5. *Crassostrea gigas* (Japanese or Pacific oyster): Matsushima Bay, Japan and Brittany, France

6. *Crassostra rhizophorae*: Brazil

7. *Crassostrea virginica* (Eastern or American oyster): discontinuously on the Atlantic and Gulf coasts of North America

8. *Macoma balthica* (duck clam): Chesapeake Bay, eastern North America, Finland, Gulf of Gdansk, Poland

9. *Macoma calcarea*: Baffin Island, Canada

10. *Macoma nasuta*: Yaquina Bay, Oregon

11. *Macoma irus*: Yaquina Bay, Oregon

12. *Mya arenaria* (soft shell clam): Atlantic coast of North America, discontinuously from Chesapeake Bay to Nova Scotia

13. *Mya truncata*: Baffin Island, Canada

14. *Mytilus edulis* (bay or blue mussel, also includes what may be *Mytilus trossulus* on the Pacific coast of North America): Pacific coast of North America discontinuously from Yaquina Bay, Oregon to sites in British Columbia, Canada; Atlantic coast of North America in the state of Maine; European sites in England, Denmark and Finland

15. *Mytilus galloprovincialis*: coast of Galacia, northwest Spain

16. *Ostrea chilensis* (Chilean oyster): Chiloe, Chile

17. *Ostrea lurida* (*Ostreola conchaphila*), (Olympic oyster): Yaquina Bay, Oregon

18. *Ostrea edulis* (European flat oyster): Mali-Ston area of Yugoslavia near Dubrovnic; Ria de Noya, Galacia, Spain; Mediterranean and Brittany region of France

19. *Tagelus plebeius* (razor clam): Chesapeake Bay, USA

C. Epizootiology

Some cultured populations appear to be 100% infected if individuals are monitored over several months. According to a 1992 review (Elston et al. 1992) prevalence of the disease is given in a variety of papers ranging from fractional percentages to 65% and is listed as unknown in many cases. These are generally point-in-time prevalence estimates. It is also clear that due to the transmissible nature of the disease, prevalences tend to be higher in densely cultured populations than in less dense wild populations. Annual mortality rates are reported to reach nearly 100% in some populations of affected species. Such high mortality is generally found only in cultured populations. In other cases, particularly in wild populations, the occurrence is very low and no mortality has been established. Mortality is clearly density dependant but other factors are undoubtedly important as well, but specific environmental factors that induce or enhance the disease are not well understood. Although much research

has been conducted to determine if various types of pollution contribute to the disease, no single factor has been identified.

It appears that the disease is highly infectious and dense populations of farmed shellfish maintain a high prevalence and severity of infection because close proximity facilitates the known capability for direct transmission from one animal to another. In all species in which seasonality has been investigated, the disease is reported to have highest prevalence (percentage of infected individual shellfish) during fall and winter months, typically from October through March. The prevalence drops in the spring and summer, due to the deaths of heavily diseased individuals over the winter. In farmed populations, it is clear that the disease is maintained from year to year, in spite of high mortality rates. This suggests that some individuals are either resistant to infection or do not develop advanced disease which causes death. In fact, remission has been observed in some individuals (e.g. Elston et al. 1988).

D. Disease Signs

Common clinical signs for all affected species have not been established. The following are known to apply in some cases:

1. Failure to produce mature reproductive follicles.

2. Epizootic mortalities that spread geographically in such a way as to suggest an infectious etiology.

3. Presence of high concentrations of large transformed cell types in mantle cavity fluid.

4. Swollen tissues in advanced stages of DN caused by massive systemic proliferation of transformed cells.

E. Disease Diagnostic Procedures (see Table 1)

1. Presumptive Diagnosis

Wet mount preparations of hemolymph can be prepared by bleeding the bivalve from the posterior adductor muscle with a hypodermic needle, then placing 0.1- 0.5 ml hemolymph onto a slide coated with 0.1% poly-L-lysine. The sample can be observed directly with phase contrast microscopy.

Presence of large (typically 6 to 8 µm, but often up to 10 µm diameter) hemocytes which fail to spread on a glass slide (after 15 minutes) as observed live in wet mount preparations (Figure 1). Normal hemocytes will rapidly attach and spread out on a glass substrate (Figure 2).

Also, after 15 minutes (to allow the normal cells to spread out), these samples can be fixed in 1% glutaraldehyde/ 4 % formaldehyde, and stained with Geimsa. However, a Fuelgen stain with a picro-methy blue counterstain provides very good differentiation of normal and diseased nuclei from cytoplasmic components (Moore 1993) for a permanent record.

Slide Preparation
Dip slides 10 times in a solution of 1% HCl in 70% ethanol.
Dip in 3 changes of dH_2O.
Dip in 0.1% poly-L-lysine (MW 90,000; Sigma) in dH_2O. This solution is stored frozen and re-used indefinitely.
Air dry vertically, store indefinitely at 20°C.

Schiff's Reagent
Boil 200 ml dH_2O. Remove from heat, add 1.0 g basic fuchsin. Cool to 50°C, add 20 ml 1.0 N HCl, cool to 25°C. Transfer to a clean bottle, add 1.0 g sodium metabisulfite ($Na_2S_2O_5$), shake vigorously. Store in the dark for two days until straw colored. Add 0.5 g charcoal, shake and filter; reagent should be colorless. Store at 4°C. Discard when the reagent turns purple.

Picro-methyl Blue, 0.03%
Methyl blue, 0.15 g.
Picric acid, saturated aqueous solution, 500 ml.

Staining Procedure (Adapted from Farley 1969b).
Methanol, 3 min.
10% neutral buffered formalin, 3 min.
dH_2O, 1 min x 2.
5 N HCl, 30 min.
dH_2O, 1 min x 2.
Schiff's reagent, 20min.
dH_2O, 1 min x 2.
Running tap water, 2min.
dH_2O, 1 min.
0.03% picromethyl blue, 3 min.
70% ethanol, 1 min x 2.
100% ethanol, 1 min.
Hemo-de, 2 min x 2.
Mount.

2. Confirmatory Diagnosis

Observation of transformed circulating cells (Figure 3) in hemocytological preparations with large (6 μm and larger compared with normal fixed nuclear diameters of 3 to 5 μm), often irregular nuclei with prominent single or multiple nucleoli and relatively little cytoplasm. These cells will fail to spread on glass microscope slides in advanced stages. The relative proportion of abnormal cells required to confirm a diagnosis has not been definitively determined since apparently certain normal hemocyte stem cells are morphologically similar to the transformed cells. If the disease is present, it will be progressive in many individuals and the abnormal cells will eventually comprise nearly 100% of the blood cell population in the advanced disease.

Histological examination also confirms the disease. The abnormal hemocytes are systemically distributed and are well differentiated from normal cells (Figure 4) by the above criteria even though the normal cells will not be spread out as they are on a glass substrate. In early stages of the disease, abnormal hemocytes are located in discrete foci in the connective tissue and vascular spaces (Figures 5 and 6) of connective tissue around the digestive gland. As the disease progresses, enlarged focal areas may be observed around the major digestive organs including the stomach, intestine and style sac. In advanced disease, the vascular spaces of the entire animal are filled and tightly packed with the abnormal cells. Histological preparation of a transverse section of the body, including visceral connective tissue and gills, or other organs typcially rich in circulating cells, is a good choice for observation of abnormal hemocytes. The cells are easily recognized in conventional 6 µm thick paraffin sections of tissues that have been previously fixed in either Davidson's fixative or 10% formalin in seawater.

Note: The characteristics of DN cells in *Tagelus plebeius* differ from those detected in other bivalves. The hypertrophied cells and nuclei are only about twice as large as those of normal hemocytes, marginal cytoplasm is acidophilic, and they show filopodia. Many circulating *T. plebeius* DN cells show blebbing of hypertrophied nuclei or are binucleate, but mitotic figures are rare. (Figure 7)

F. Procedures for Detecting Subclinical Infections

Use of hemocytological or histological examination as above.

G. Procedures for Transportation and Storage of Samples

Hemolymph samples and tissues for histology can be prepared and fixed as described above. If live animals can be shipped within 48 hours to the diagnostic laboratory, this is preferable because wet mounts and hemocytological preparations can be made for a preliminary diagnosis. To ship live animals, obtain required permits and ship fresh bivalves by courier service. Pack the animals in a moist insulated container with a frozen gel-pack. Insulate the animals from the gel-pack to prevent freezing of the tissues. Add sufficient packing material to protect them from breakage. Alternatively, shucked (shellfish removed from their shells) animals or sections placed in histological cassettes can be fixed in Davidson's shellfish fixative or 10% formalin in seawater. After a minimum of 24 hours fixation, drain the excess fixative and place the tissues or tissue sections in a sealable plastic bag with paper towels moistened with fixative. Seal this bag in a vacuum packed plastic bag and ship to the diagnostic laboratory by courier service.

The methods currently available for surveillance, detection, and diagnosis of DN are listed in Table 1. The designations used in the Table indicate: – = the method is presently unavailable or unsuitable; + = the method has application in some situations, but cost, accuracy, or other factors severely limits its application ; ++ = the method is a standard method with good diagnostic sensitivity and specificity; and +++ = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity.

Table 1. *DN surveillance, detection and diagnostic methods*

Method	**Screening**			**Presumptive**	**Confirmatory**
	Larvae	Juveniles	Adults		
Gross signs	–	–	–	–	–
Direct BF/LM		+++	+++	+++	+++
Histopathology		+++	+++	+++	+++
Bioassay	–	–	–	–	–
Transmission EM[1]	–	–	–	–	–
Antibody-based methods[2]	–	+	+	+	+
DNA probes – *in situ*[3]	–	–	–	–	–
RT-PCR[4]	–	–	–	–	–

BF = bright field; LM = light microscopy; EM = electron microscopy;
RT-PCR = reverse-transcription polymerase chain reaction.

[1] Electron microscopy could be used for diagnosis but histology is less time consuming and expensive and equally reliable, as well as more extensive in terms of the tissues that can be examined. Electron microscopy is therefore not an appropriate diagnostic tool.

[2] An immunocytochemical assay using a monoclonal antibody effective for detecting DN cells in *Mya arenaria* has been developed by Smolowitz and Reinisch (1986). This monoclonal antibody has not been effective for detecting DN cells in any other bivalve species, but has been a helpful tool in diagnosis of DN in *M. arenaria*.

[3] DNA probes are not available for most species and not widely available for any species.

[4] RT-PCR is more useful as a research tool and is not an appropriate application because there are less expensive definitive diagnostic tools available.

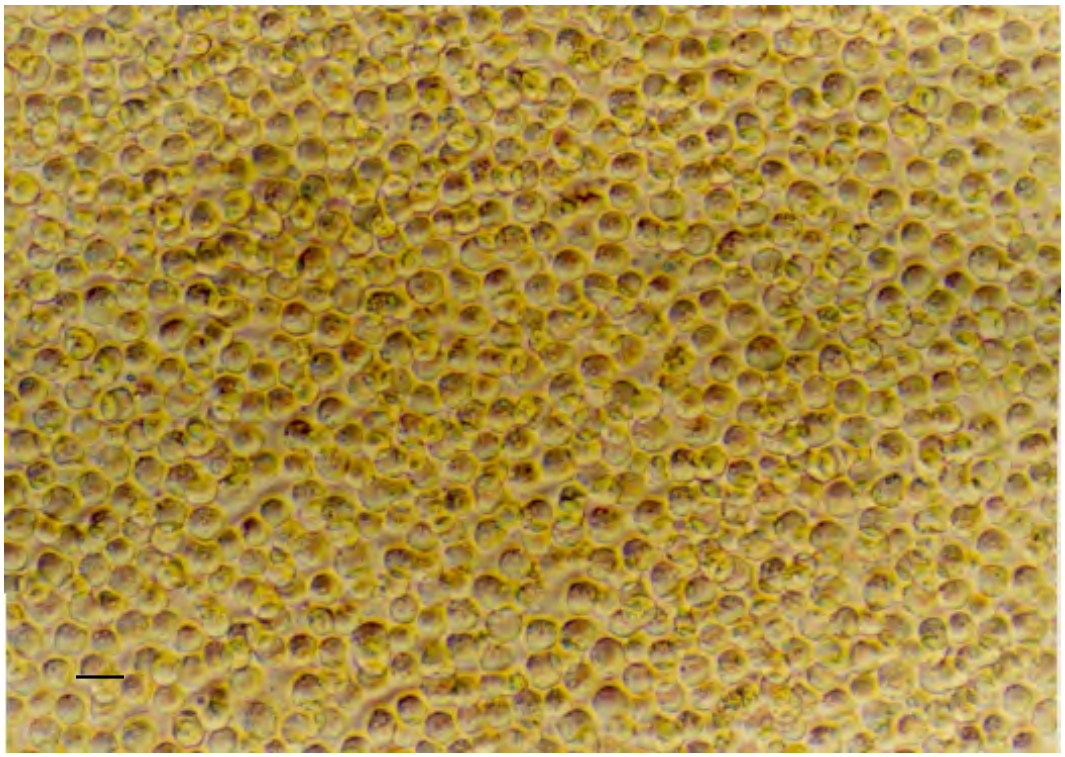


Figure 1. Neoplastic cells from *Mya arenaria*, the soft shell clam. This animal was in the final stages of the disease Scale bar = 10 um, phase contrast.

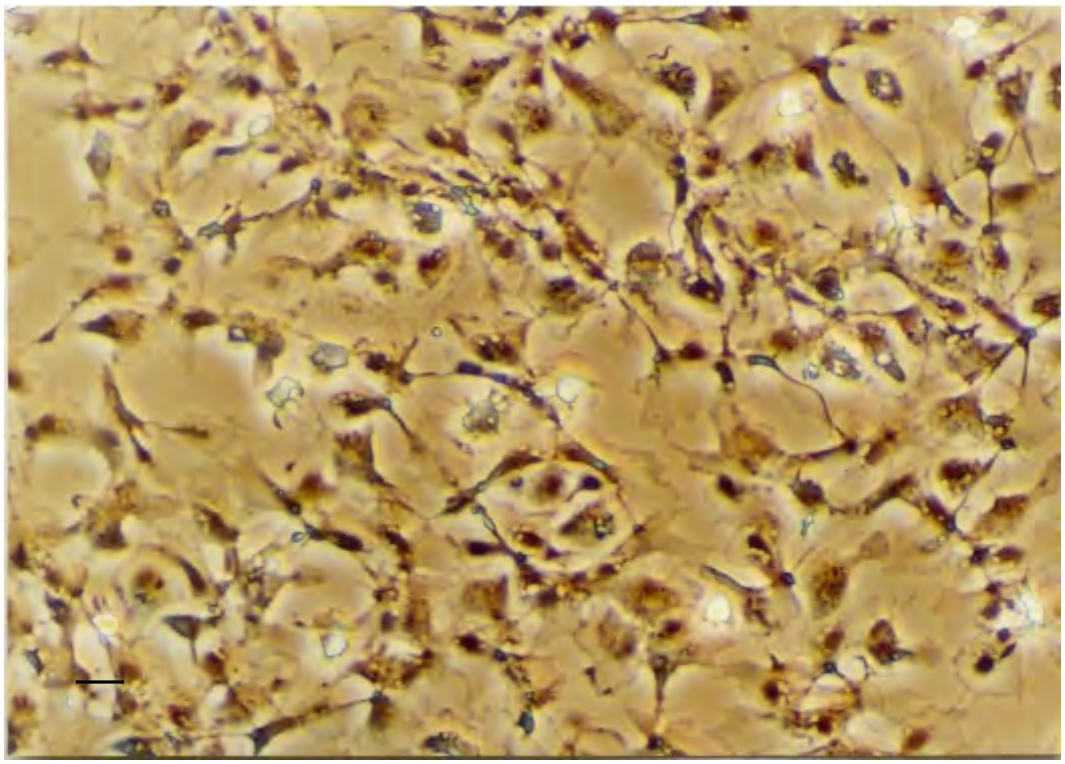


Figure 2. Normal hemocytes from *Mya arenaria* (Scale bar = 10 um, phase contrast).

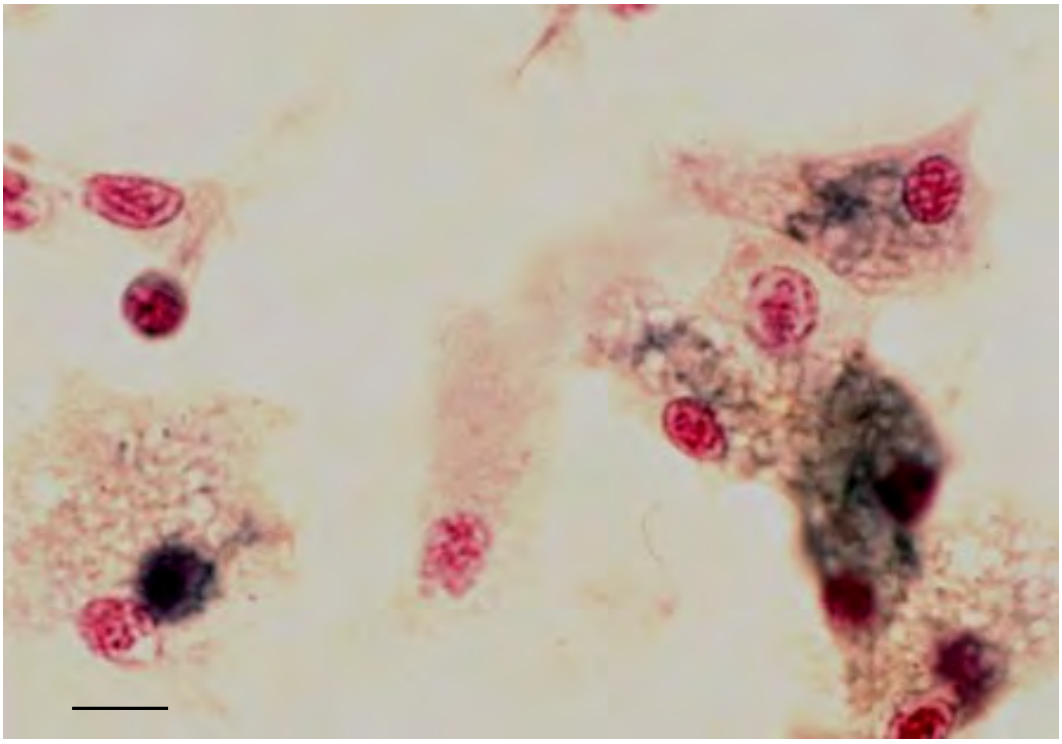


Figure 3. Hemocytological preparation of normal blood cells from *Mytilus*. Feulgen picro-methyl blue stain. Scale bar = 10 µm.

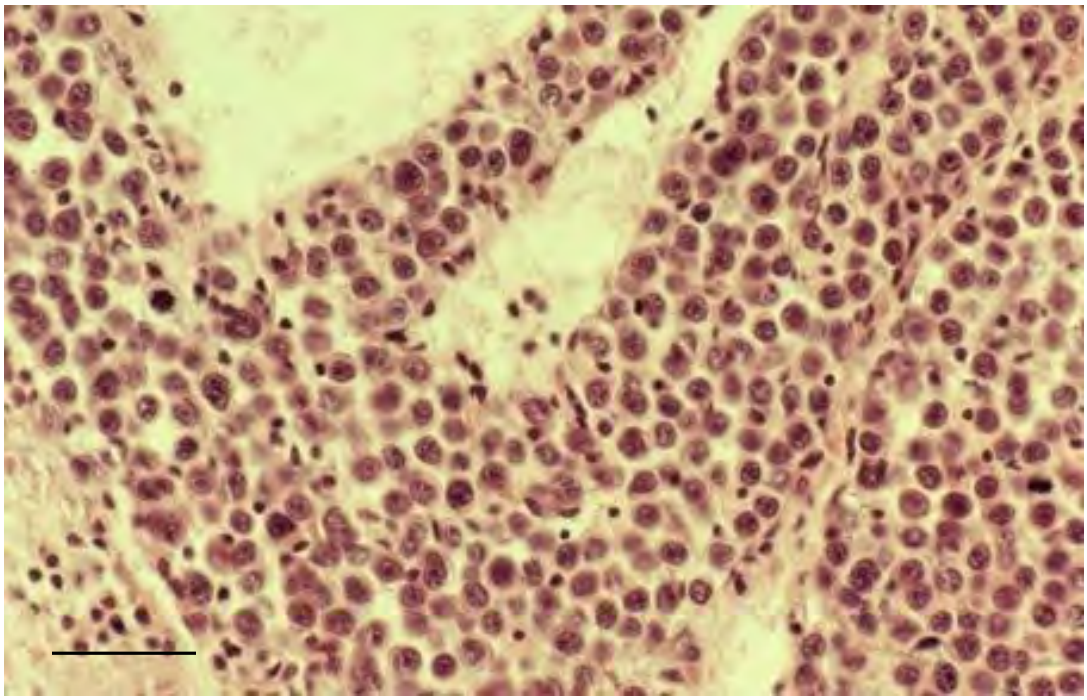


Figure 4. Hemocytological preparation of affected blood cells of *Mytilus* with hemic neoplasia in terminal stages of disease. The blood contains predominantly neoplastic blood cells that poorly attach or spread on glass substrate. Blood cells exhibit a high nucleus to cytoplasm ratio and prominent nucleoli. Scale bar = 10µm. (Feulgen-picro-methyl blue stain described in Farley, 1969b)

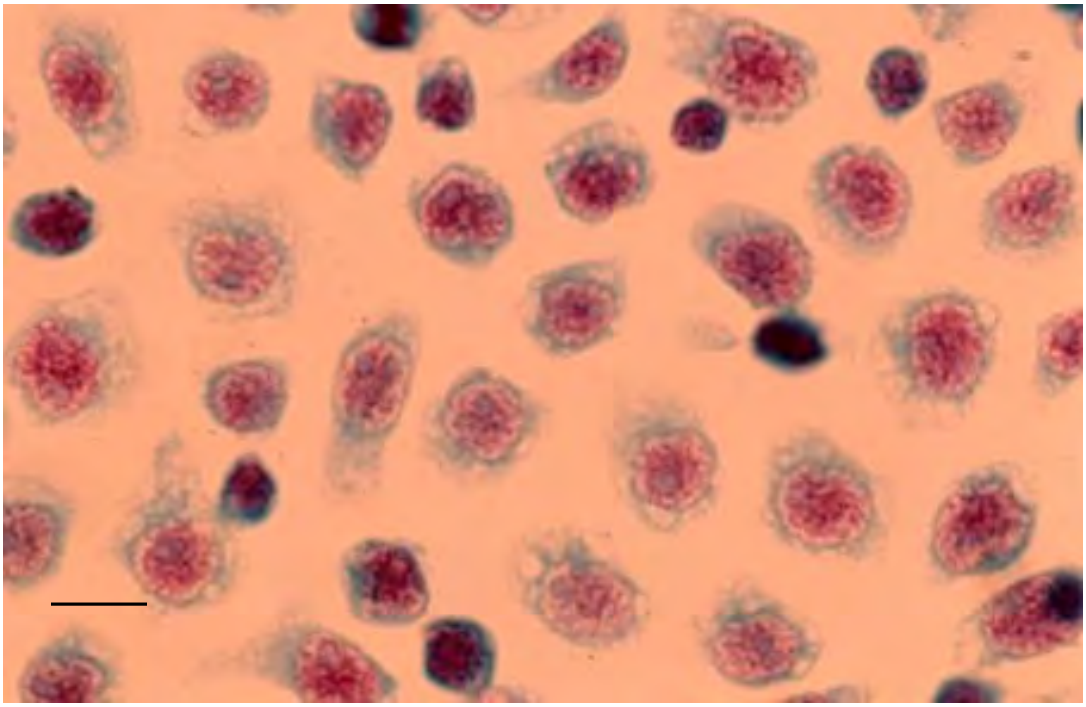


Figure 5. Histological section of *Mytilus* with DN showing the massive proliferation of neoplastic cells in the vascular spaces of the gills. Hematoxylin and eosin, Scale bar = 50 µm.

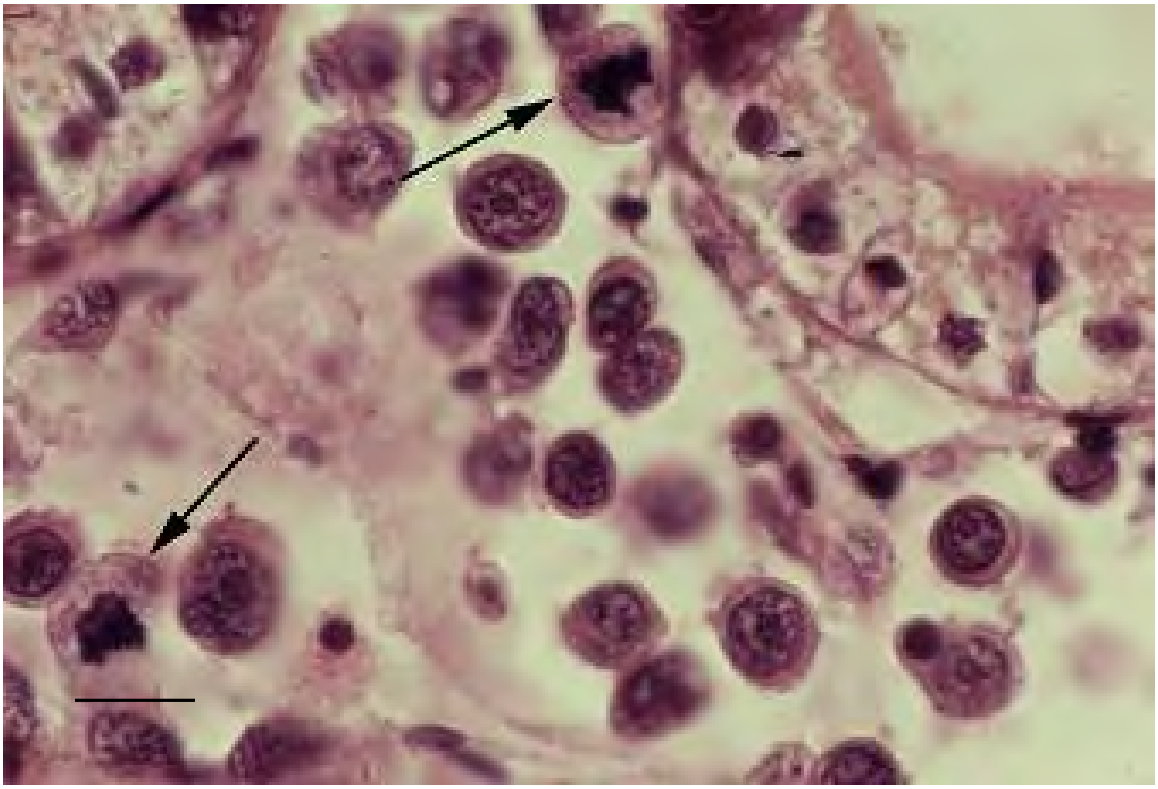


Figure 6. Histological section of the connective tissue between digestive tubules in *Mytilus* with DN showing typical enlarged hemocytes with large nuclei and scant cytoplasm. Two such cells in mitosis are shown at the arrows. Hematoxylin and eosin. Scale bar = 10 µm.

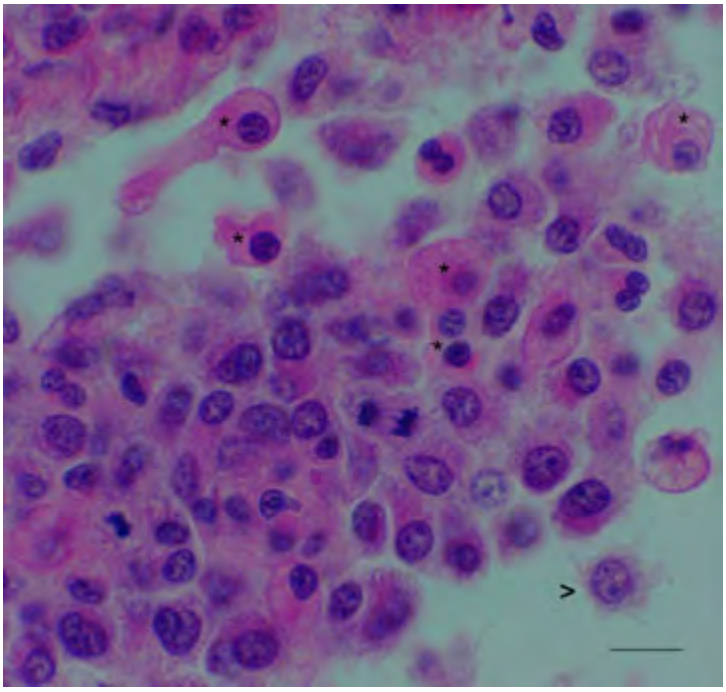


Figure 7. *Tagelus plebeius* razor clam DN disease in H&E-stained histological section of gill vasculature. Large DN cells with hypertrophied, often bi-lobed, nuclei with prominent nucleoli predominate among circulating cells. Normal hemocytes are labeled (asterisks) for comparison. Metaphase and telophase mitotic DN cells are shown, as are several bi-nucleate DN cells, and a DN cell with prominent filopodia (arrowhead). Scale bar, 10µm.

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5.13 Miscellaneous Diseases of Molluscs

Ralph Elston

Battelle Marine Sciences Laboratory
439 W Sequim Bay Rd
Sequim, WA 98382
206/683-4151
aquatech@olypen.com

This section is included to provide brief information on a variety of types of diseases that the diagnostician may hear of or be asked for information on. These diseases are not given detailed treatment for one of the following reasons: (1) they are essentially opportunistic diseases that, while important in shellfish husbandry, are not regarded to have any significance in the geographic transport of shellfish, or (2) there is insufficient information on the significance of the diseases to individuals or populations of shellfish or on their etiology and diagnosis, or (3) they are diseases of species that have limited commercial importance with the result that less is known about their geographic distribution and significance (e.g. haplosporidian infections in the gaper clam or in boring bivalves). It must also be noted that some parasites and infectious diseases are completely omitted but will likely be included in subsequent editions as their significance to aquaculture and resource management becomes more widely known. The number of reports of significant mollusc diseases is increasing rapidly. Due to the inevitable lag time in the publication of a diagnostic guide such as this one, the most recently reported diseases will not be included.

A. Rickettsia- and Chlamydia-like Diseases of Bivalve Molluscs

Rickettsia-like and chlamydia-like organisms are some of the most common histopathological observations in bivalves. There has been no definitive evidence that these are linked in terms of pathogenicity to the morphologically similar human and mammalian pathogens. They most typically are observed in epithelial cells of the digestive gland or gill (Figure 1 and Figure 2). In a few cases they are reported to be associated with mortalities of the host. However, this has not yet been definitively demonstrated and there are many examples of apparently benign infections. Chlamydia-like organisms have been reported in the bay scallop *Argopecten irradians*, the Portuguese oyster *Crassostrea angulata*, and the hard clam *Mercenaria mercenaria*. Rickettsia-like organisms have been reported in the Pacific oyster *Crassostrea gigas*, the Eastern oyster *Crassostrea virginica*, *Donax trunculus*, *Mercenaria mercenaria*, the soft shell clam *Mya arenaria*, the sea scallop *Placopecten magellanicus*, the Pacific razor clam *Siliqua patula*, the thin tellin *Tellina tenuis*, the manila clam *Tapes philippinarum*, the Japanese scallop *Patinopecten yessoensis*, the European flat oyster *Ostrea edulis*, and the Palourde clam *Ruditapes philippinarum*. The bacterial forms are usually distinguishable at the light microscope level but in some cases, as with the chlamydia-like structure in the digestive epithelium of *Mercenaria mercenaria*, the entire intracytoplasmic inclusion is referred to as an amorphous blue-body.

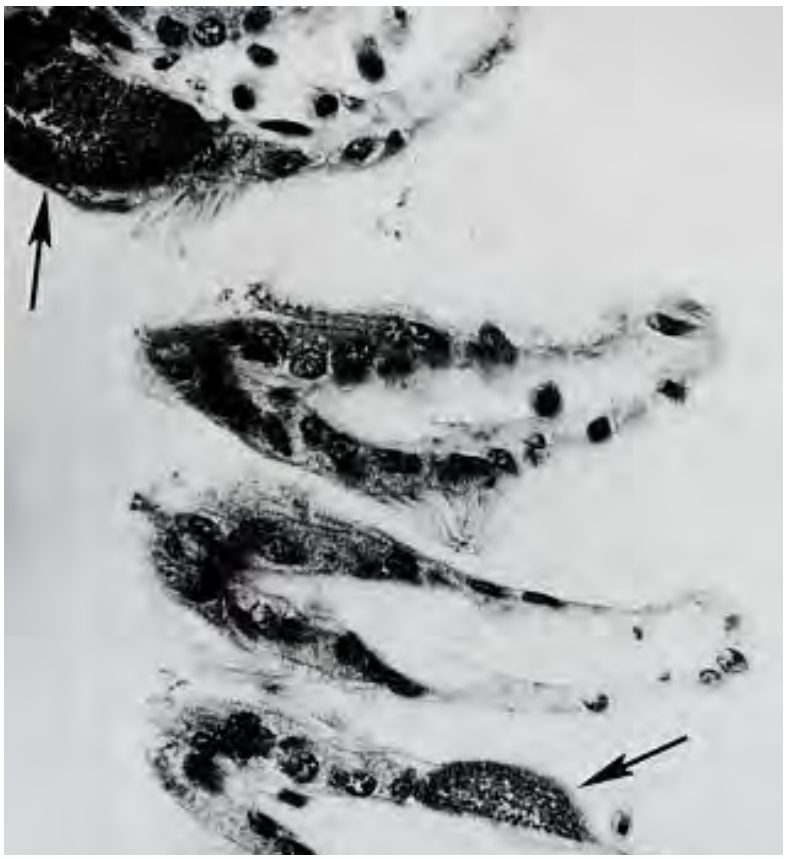


Figure 1. Rickettsia-like organisms in the branchial epithelium of the Japanese scallop (arrows) *Patinopecten yessoensis*, 580X. From Elston 1986, with permission.

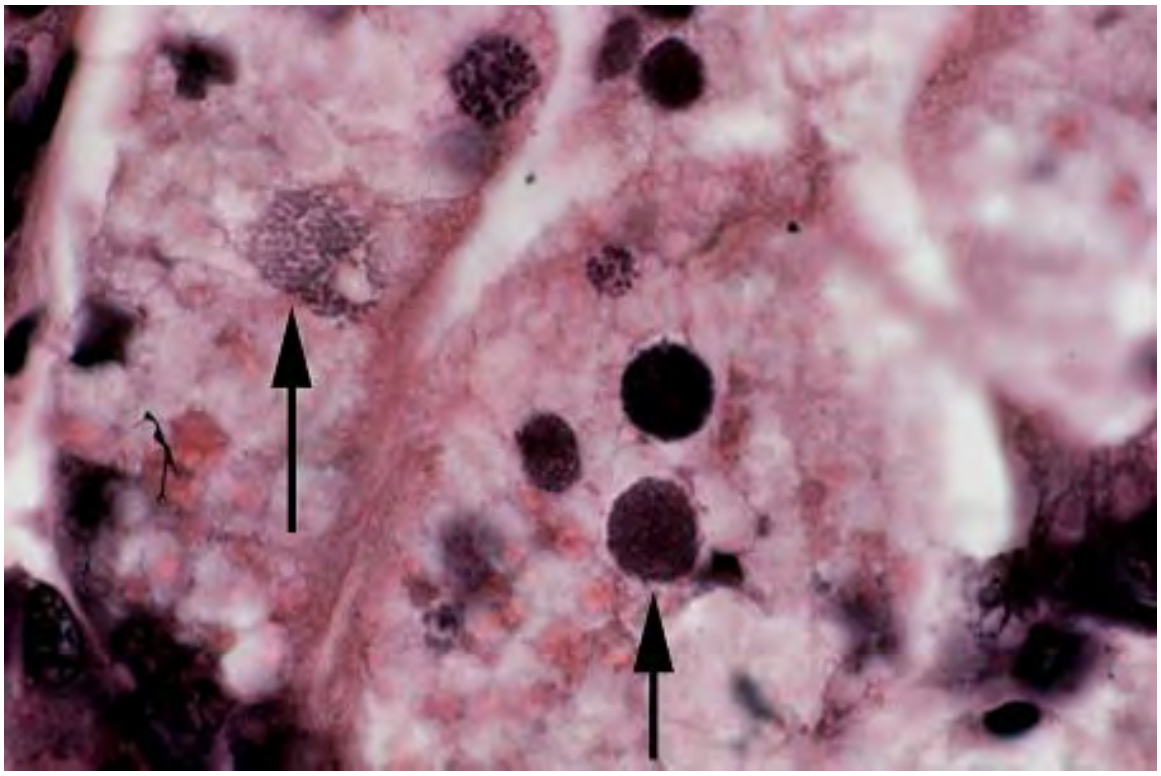


Figure 2. Rickettsia-like organisms in the digestive tubular epithelium of the Pacific razor clam *Siliqua patula* (examples at arrows), 880X.

B. Malpeque Bay Disease

This is a widely known but poorly understood disease that caused severe mortalities in American oysters *Crassostrea virginic*a in Malpeque Bay along the Canadian maritime province of Prince Edward Island starting in 1915 and continuing through the 1930s. The geographically progressive nature of the disease, which was first observed a year after substantial plantings of seed oysters imported from the United States, is considered evidence for an infectious etiology of the disease. Over 90% of original stocks are reported to have succumbed to the disease, characterized by visceral shrinkage, a translucent quality, reduced growth, and failure to spawn. The etiology has never been determined with certainty.

C. Vibriosis of Larval and Juvenile Molluscs

This is regarded as an opportunistic disease of intensively cultured molluscs. Although several species including *Vibrio tubiashi, Vibrio anguillarum*, and *Vibrio alginolyticus* are reported associated with the diseases, it is likely that other poorly characterized marine vibrios are also important in the disease in some locations and circumstances. Usually the bacteria can be reduced or eliminated in the husbandry systems by hygienic or water treatment modifications. Vibriosis is more likely to occur in the warmest months of the year. Pathogenesis based on both exotoxins and direct invasion of tissues has been reported.

D. Gill Disease of Portuguese Oysters *Crassostrea angulata*

Severe mortalities are attributed to this disease in the Ile de Oleron and Arcachon regions of France in 1967-68 and 1970-73. It has also been reported in Great Britain. The disease has effectively destroyed *Crassostrea angulata* as a cultured oyster. Subsequently, and apparently as a result of this disease, the Pacific oyster *Crassostrea gigas* has replaced the culture of the Portuguese oyster in France. In the active stages of this disease, yellow spots appear on the gill. These spots progress and their centers become brown, necrotic, and disintegrate, leaving a perforation or an indentation on the gill. In the inactive or remissive state, occurring in some individuals, the perforations or indentations occur without the necrotic tissue. Yellow or green pustules may occur on the mantle or adductor muscle. Several etiologies have been proposed but none definitively proven. Iridovirus-like particles have been observed in the gill epithelium of some oysters affected with the disease.

E. Hinge Ligament Disease of Juvenile Bivalve Molluscs

This is a disease that was discovered in a variety of cultured juvenile bivalve molluscs up to about 1 cm in shell height. It is associated with substantial mortalities. Gliding or cytophaga-like bacteria invade and erode the proteinaceous hinge ligament, often resulting in its complete liquefaction and attendant dysfunction (Figure 3 and Figure 4). A portal of entry to the soft tissues may thus be opened, but this point has not been definitively established. The frequency of observation of this phenomenon in husbanded juvenile molluscs suggests that it is a serious problem. Morphologically similar lesions have been observed in several species in many locations on both coasts of North America suggesting a common or family of similar etiological agents. The presumptive causative organism can be cultivated by careful excision of the ligament and dilution on sea-water cytophaga agar (see reference).

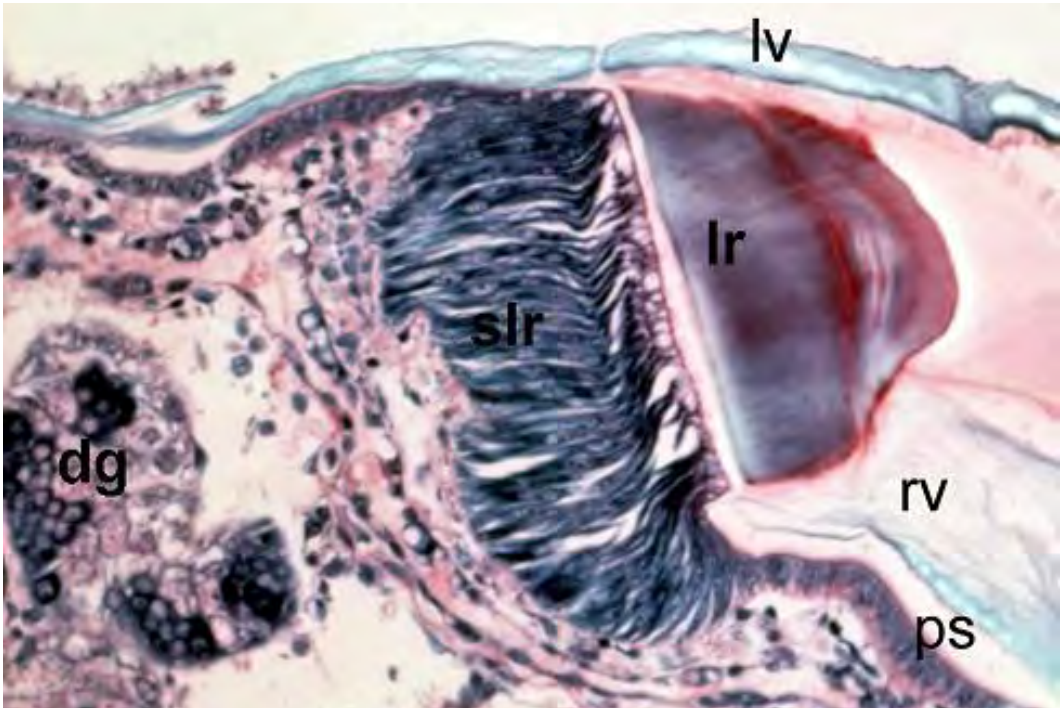


Figure 3. Normal hinge ligament of oyster showing the intact resilium (lr) and other adjacent organs (dg, digestive gland; slr, sub-ligamental ridge; lv, rv, left and right valves; ps, pallial space), 160X From Dungan and Elston 1988, with permission.

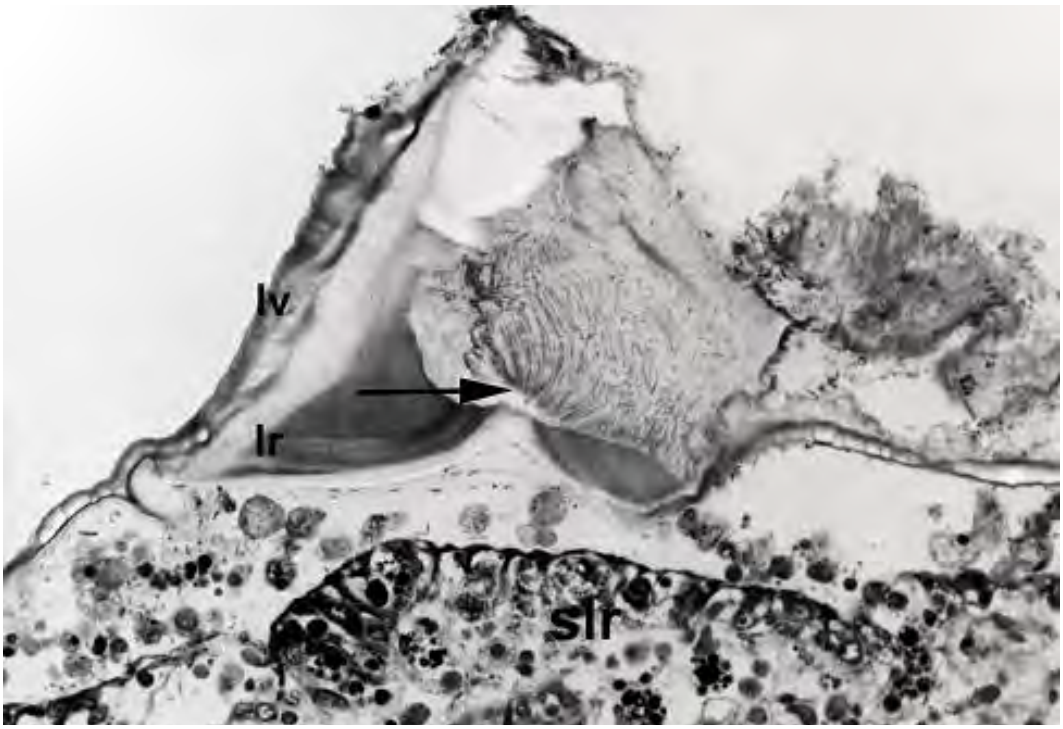


Figure 4. Hinge ligament eroded with cytophaga-like bacteria. The bacteria appear as fibers oriented at right angles to the eroding resilium margin (arrow). The sub-ligamental ridge appears necrotic and is separated from the internal resilium surface by a wide pallial space containing bacteria and rounded host cells. Organ identifiers and magnification same as Figure 3. From Dungan and Elston 1988, with permission.

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5.14 Juvenile Oyster Disease of Eastern Oysters

S. Ford

Rutgers University
Haskin Shellfish Research Laboratory
Port Norris, NJ 08345 USA
susan@hsrl.rutgers.edu

A. Name of Disease and Etiological Agent

Juvenile oyster disease (JOD) is a syndrome defined by macroscopic and microscopic signs, and high mortality, in cultured juvenile oysters. The etiology has not been confirmed, but bacterial involvement is highly likely. A newly described species of the α-proteobacteria Roseobacter group, *Roseovarius crassostreae*, has been found repeatedly in oysters with JOD symptoms at a variety of locations (Boettcher et al. 2000, 2005). Challenge experiments have so far failed to consistently induce characteristic symptoms.

B. Known Geographical Range and Host Species of the Disease

Geographical Range

JOD has been reported in aquaculture sites from New York to Maine, USA.

Host Species

JOD affects juvenile eastern oysters, *Crassostrea virginica*.

C. Epizootiology

JOD outbreaks occur at temperatures exceeding 20^{o} C and in relatively high salinity (≥25 ppt) sites (Bricelj et al., 1992; Ford and Borrero, 2001). It is a problem in nursery systems (upwellers, floats, lantern nets, etc.) and occurs during a period of rapid growth in midsummer, after the oysters have been deployed from the hatchery. There is no evidence of an infectious agent originating in the hatchery. At least some outbreaks have followed plankton blooms suggesting the possibility that plankton may provide surfaces and nutrients for subsequent bacterial proliferation (Lee et al., 1996). Mortality can reach 60 to 90% over a 4-6 week period in oysters 10 to 25 mm in shell height. Larger juveniles often show characteristic shell lesions, but experience much lower mortality. Decreased density and increased water flow through container systems can decrease mortality.

D. Disease Signs

Behavioral Signs

Not applicable

Gross Signs

JOD initially inhibits growth. Within a week or two, characteristic shell anomalies appear (Ford and Borrero, 2001). The soft, uncalcified, growing edge of the upper, flat valve often becomes eroded, resulting in the appearance of "lower valve overgrowth" or "uneven shell margins." The lower valve may become excessively cupped (Fig. 1) . The oyster's soft tissue retracts within the shell cavity and the mantle produces an anomalous deposit of light to dark brown organic material (conchiolin), which covers the retracted soft tissue and is affixed to the inner side of one or both valves (Fig. 2). The edge of the conchiolin deposit may be raised in a ring-like structure surrounding the soft tissue. Oysters that have recovered from JOD and resumed growth may show pronounced ridges (growth checks) on the external shell Lewis et al., 1996).

Microscopic Signs

Tissue sections show lesions of the mantle epithelium, including hemocyte infiltration, one to two weeks before shell deposits are observable. The lesions are highly correlated with subsequent conchiolin deposits and mortality. Histologically apparent mantle lesions become more pronounced as the shell deposits appear, and include exudates of hemocytes, debris, secondary bacteria and cilia, along with the anomalous conchiolin layers (Figs. 3, 4 and 5).

E. Disease Diagnostic Procedures

The fact that the etiological agent of JOD has not been confirmed causes some problems of diagnosis because different investigators have used one or more of the shell anomalies described above, and not always the same ones. This becomes a particular problem because all the signs are non-specific characteristics that can be caused by other factors. Growth inhibition can result from any adverse condition affecting oysters, and external growth checks are often present when growth resumes. "Lower shell overgrowth" can be seen in oysters affected by other diseases or adverse condition. Even the most distinctive symptom, the conchiolin deposit, can be occasionally induced by conditions other than JOD. Deposition of a conchiolin "barrier" on the inner surface of the shell is a protective "walling-off" reaction by the mantle epithelium to an irritant and can be observed from time to time in individual oysters, both adults and juveniles, from wild populations. Nevertheless, descriptions of the JOD syndrome indicate that the most consistent correlate with the disease is the conchiolin deposit and it is recommended that this be designated as the key diagnostic feature until an etiological agent is confirmed (Lewis, 2001).

Sampling Criteria

Cohorts suspected of suffering JOD should be sampled by collecting both live and dead (empty shells) oysters. The presence and extent of shell anomalies, specifically the conchiolin deposit on the inner shell, should be determined from a sample of 100 live and, if available, shells from 100dead oysters with valves still articulated

Presumptive Diagnosis

Rapid and high mortality of juvenile oysters during mid summer, associated with externally visible shell growth anomalies.

Confirmatory Diagnosis

Presence of conchiolin deposit on inner surface of one or both valves. Additional clinical signs include excessive shell cupping and uneven shell margins such that a several millimeter edge of the lower, cupped, valve is exposed and often fouled.

Procedures for Transportation and Storage of Samples

Samples collected from apparently normal, moribund, or dead oysters should be packed on ice for shipment and subsequent bacterial, histological, or macroscopic analysis. Also, live whole oysters can be fixed in Davidson's after severing the hinge ligament and propping open the valves so that the fixative enters the shell cavity. Maintaining the shell with the soft tissues is critical since the diagnosis is based on shell anomaly symptoms.

Table 1. The methods currently available for surveillance, detection, and diagnosis of JOD are listed in below. The designations used in the Table indicate: – = the method is presently unavailable or unsuitable; ? = the method is available but untested; + = the method has application in some situations, but cost, accuracy, or other factors severely limit its application; ++ = the method is a standard method with good diagnostic sensitivity and specificity; and +++ = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity.

Method	**Screening**		**Presumptive**	**Confirmatory**
	Juveniles	Adults		
Gross signs	+++	+	+++	+++
Histopathology	+	+	+	-
Bacteriology *	+	-	+	-
Transmission EM	-	-	-	-
Bioassay	-	-	-	-
Antibody-based methods	-	-	-	-
DNA probes – in situ	-	-	-	-
PCR**	?	?	-	-

**Roseovarius crassostreae* has been found in all outbreaks identified as JOD, although its etiological role has not been confirmed. It is a gram negative, motile rod (approximately 0.25 x 1.0 µm) that is oxidase positive and grows poorly in anaerobic media. See Boettcher et al. (2000) for details.
** Gene sequences are available for *R. crassostreae.*

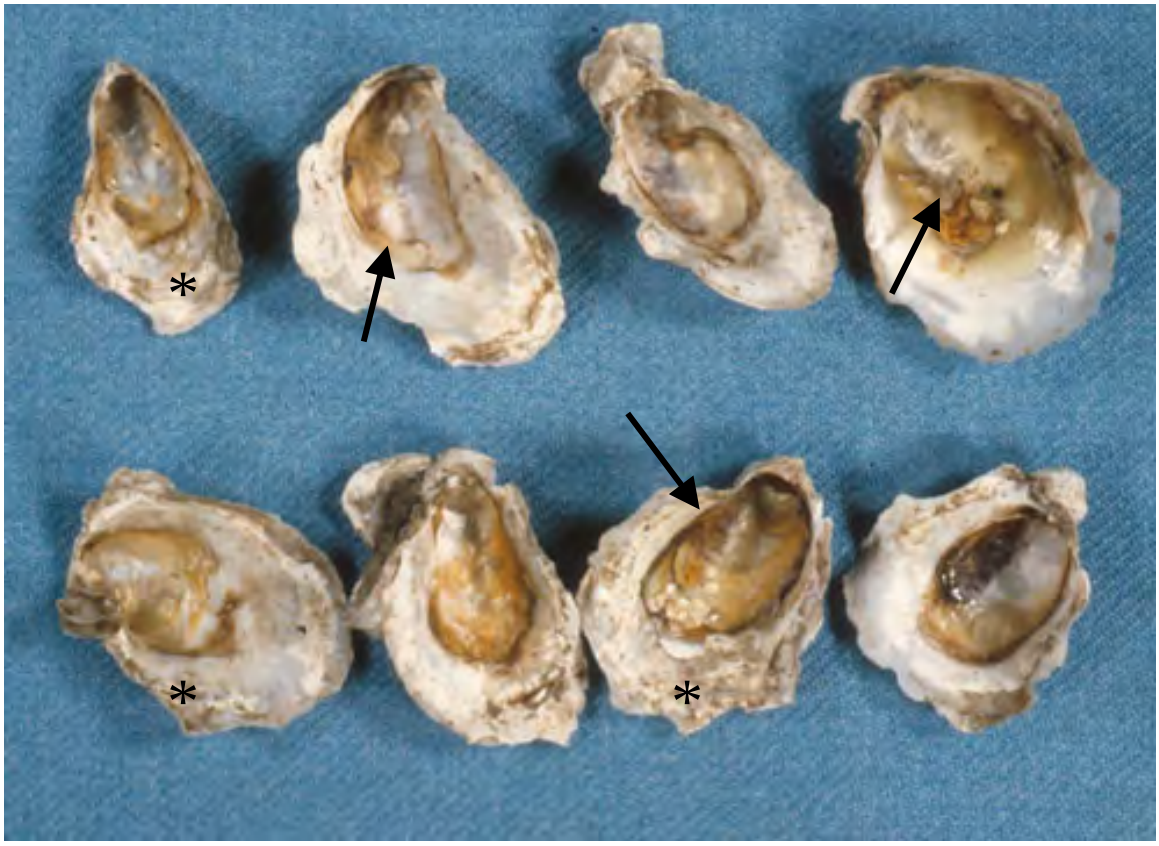


Figure 1. Inner valves of juvenile Eastern oysters affected by Juvenile Oyster Disease showing conchiolin deposits (arrows) and fouling (asterisks) on shell surface outside of deposit.



Figure 2. Top and side view of juvenile Eastern oysters affected by Juvenile Oyster Disease showing extreme cupping of lower valves and exposed inner valve edges resulting from erosion of edges of upper valves.



Figure 3. Histological section of an oyster affected by Juvenile Oyster Disease showing relationship of mantle edge, exudate of hemocytes (*), and anomalous conchiolin deposit (arrows) Hematoxylin, aniline blue, acid fuchsin. Scale bar = 100 µm.



Figure 4. Histological section of oyster affected by Juvenile Oyster Disease showing lesion on mantle surface associated with hemocyte infiltration (asterisk) and anomalous conchiolin deposit (arrow). Hematoxylin, aniline blue, acid fuchsin. Scale bar = 50 µm.

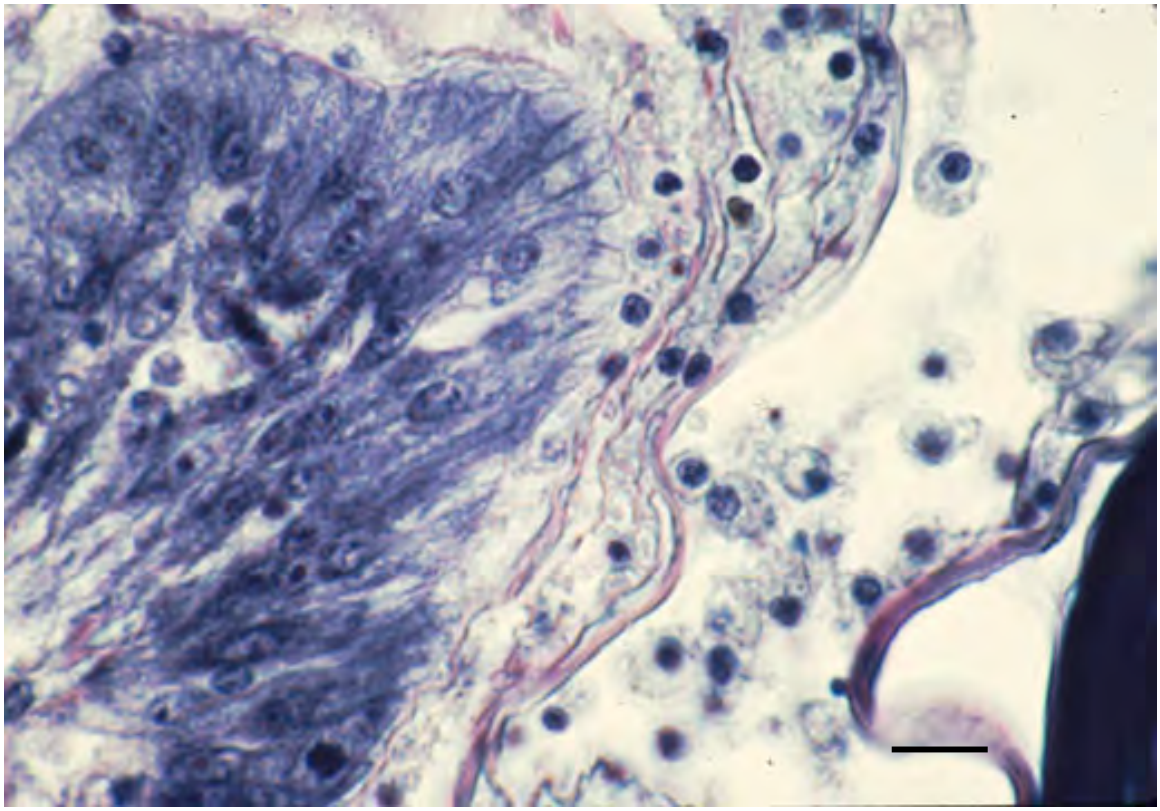


Figure 5. Higher magnification of exudate in Figure 3 showing hemocytes interspersed between layers of secreted conchiolin. Hematoxylin, aniline blue, acid fuchsin. Scale bar = 10 µm.

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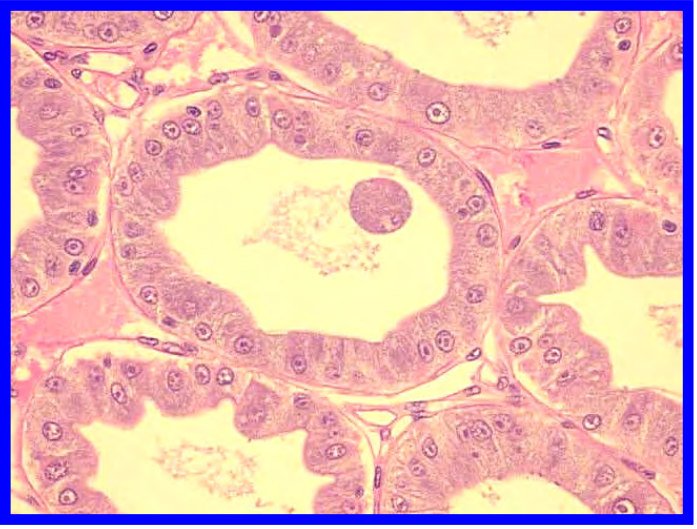
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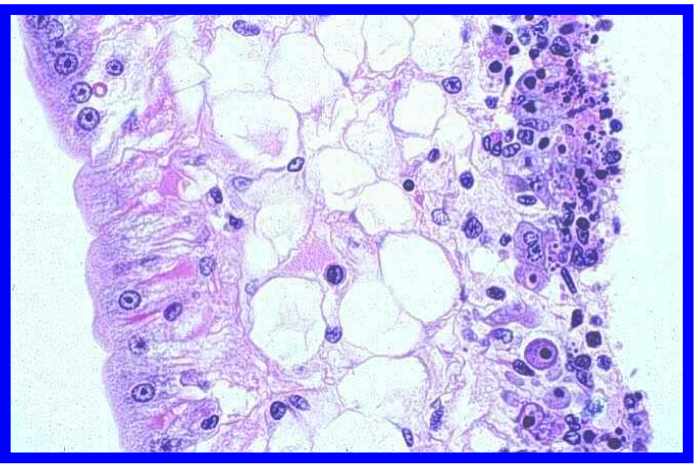
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Section 1
Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens

Chapter 6 Diseases of Crustaceans

6.1 Necrotizing Hepatopancreatitis Virus of Peneaid Shrimp

6.2 Taura Syndrome Virus of Peneaid Shrimp

6.3 Lagenidium of Decapod Crustaceans

6.1 Necrotizing Hepatopancreatitis

Patricia W. Varner

Texas Veterinary Medical Diagnostic Lab
P.O. Drawer 3040
College Station, TX 77841
PVarner@tvmdl.tamu.edu

A. Name of Disease and Etiological Agent

Necrotizing Hepatopancreatitis (NHP), initially referred to as Texas Pond Mortality Syndrome (McVey, 1993), was first reported as a seasonal disease problem in Texas pond-reared *Litopenaeus vannamei* (Pacific white shrimp) populations (Johnson, 1990; Frelier et al., 1992). The etiologic agent was identified as a dimorphic, obligate intracellular rickettsial-like organism (RLO) that targets the shrimp hepatopancreas (Frelier et al., 1992; Lightner et al., 1992) and was classified as a member of the α-Proteobacteria group (Loy et al., 1996a). Ultrastructural studies have demonstrated the presence of a predominant rod form (0.25 X 0.9 μm), as well as a helical form (0.25 X 3.5 μm) with 8 flagella at its basal apex. Since this organism is non-culturable in *in vitro* systems, its life cycle has not been fully elucidated and speculated to be vector-mediated (Frelier et al., 1993; Loy et al., 1996a).

B. Known Geographic and Host Ranges of the Diseases

1. Geographical range.

Since its initial detection in 1985, NHP has been reported in various countries in the Western Hemisphere, including the United States, Belize, Guatemala, Columbia, Ecuador, Nicaragua, Costa Rica, Brazil, Peru, Mexico, Panama and Venezuela. (Jory, 1997; Lightner, 1996; Bradley-Dunlop et al., 2004)

2. Host Species.

Natural NHP infections have been detected in *Litopenaeus vannamei, L. setiferus, L. stylirostis, Farfantepenaeus aztecus* and *F. californiensis* (Lightner, 1996).

C. Epizootiology

Since mortality rates can vary between 20-95%, economic losses can be significant to commercial shrimp producers (Loy et al., 1996b). NHP infections appear to have a seasonal occurrence in both cultured and wild shrimp populations with natural clinical outbreaks associated with increases in both water temperature and salinity (Lightner & Redman, 1994; Frelier et al., 1995). This association with these two environmental factors may reflect an increase in pathogen virulence and/or vector mediation. In a recent experimental *per os* study, salinity was demonstrated to not be a major determining factor in the transmission of the NHP organism (Vincent & Lotz, 2007).

Transmission is horizontal via ingestion. Cannibalism is believed to be a major contributing factor to the rapid spread of infection within a shrimp pond population (Vincent et al., 2004). In endemic regions, routine monitoring of ponds is recommended when existing environmental conditions are conducive for clinical disease (Bondad-Rantaso et al., 2001). Early detection has been demonstrated to be important for successful control with oxytetracycline-medicated feeds (McVey, 1993; Brock & Main, 1994; Lightner & Redman, 1994).

D. Disease signs

The clinical signs displayed by infected shrimp are nonspecific in nature and characterized by lethargy, reduced feed intake, decreased growth rate, softened shell and an atrophied hepatopancreas (Brock & Main., 1994; Lightner, 1996; Bondad-Reastasco et al., 2001). Variable pond mortality rates of up to 95% have been reported. Infected shrimp display empty midguts on pond-side exam.

The hepatopancreas is the target tissue for this organism. Infected cells initially become hypertrophied due to the intracytoplasmic proliferation of this organism. Three stages of infection were defined by Frelier et al. (1992). In a *per os* time course study, early NHP infection (stage I) was detected in the resorptive, fibrillar and/or B cells of infrequent scattered tubules by 6 days post exposure (Vincent & Lotz, 2005). Stage II of NHP infection was demonstrated to occur at 16-37 d post-exposure with moderately frequent scattered to widespread tubular epithelial cell hypertrophy/attenuation present and associated with variable numbers of intracytoplasmic rickettsial-like organisms. Stage III was characterized by frequent scattered tubular necrosis and mortality at 16 to 51 days post-exposure.

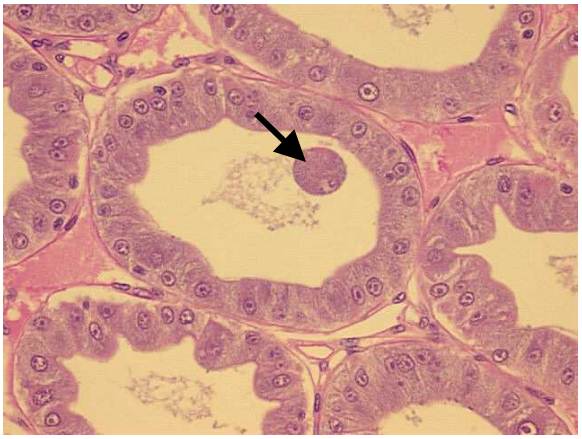


Figure 1. The generalized basophilic granularity to the cytoplasm of the tubular epithelial cells is due to the presence of numerous intracytoplasmic rickettsial-like organisms. A desquamated rickettsial-infected cell (arrow) is present within a tubule (H&E; 400X).

E. Disease Diagnostic Procedures

Presumptive

1. Gross observations

Affected shrimp may display a variety of gross and/or clinical signs that can be used to identify suspect infected shrimp to submit for diagnostic testing (Brock & Main, 1994; Lightner, 1996; Vincent et al., 2004). These may include decrease in the size of the hepatopancreas, empty midgut (Figure 2), reduced growth, soft flaccid shells and/or increased surface fouling with epicommensal/opportunistic organisms (e.g. black spot disease, etc.).

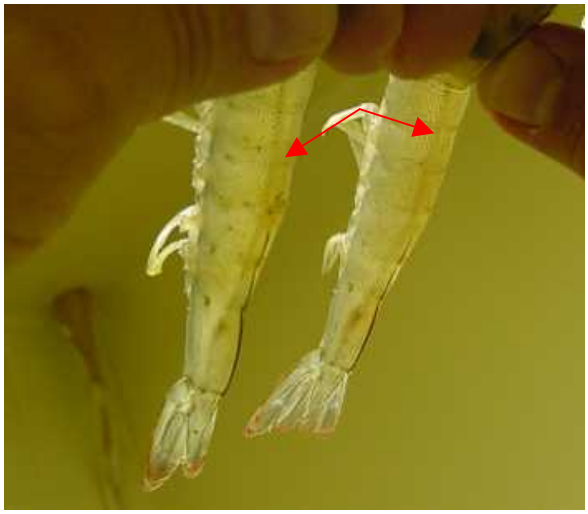


Figure 2. Gross examination for midgut fullness. Both midguts are empty in this view (arrows). The midgut will appear much darker (almost black) when ingesta is present.

2. Wet Mount Analysis

Squash preps of the hepatopancreas from suspect shrimp (Figure 3) can be evaluated microscopically for the presence of melanized necrotic tubules and/or reduced lipid content (Lightner, 1996). The presence of necrotic tubules, however, can also be due to infection by extracellular bacteria.

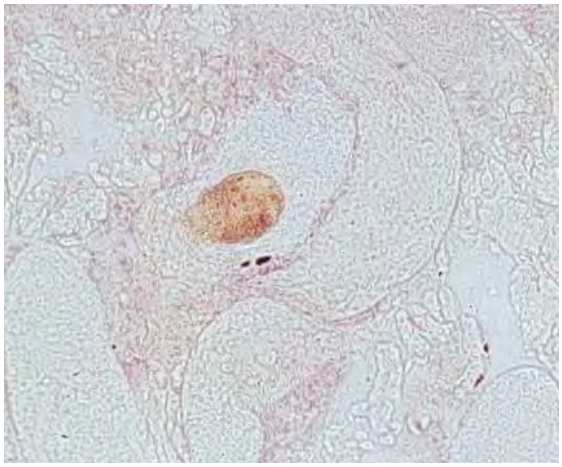


Figure 3. Necrotic tubule in squash prep made of the hepatopancreas (200X).

3. Histopathology

Davidson-fixed shrimp tissues (Humason 1972) are processed, sectioned and stained with a modified Mayer's hematoxylin and Phloxine/eosin stain (H&E) as described by Bell & Lightner (1988). In NHP-infected shrimp, varying degrees of epithelial hypertrophy/attenuation, lipid reduction and tubular necrosis may be detected in microscopic examination of H&E-stained histologic sections of the hepatopancreas (Frelier et al., 1992; Lightner & Redman, 1994; Lightner, 1996). Infected hepatopancreocytes will have a generalized basophilic granularity to the cytoplasm (Figure 1). The presence of desquamated RLO-infected cells within the tubules is a common histologic finding in Stage II NHP infection. Other histologic features include interstitial hemocytic infiltrates and tubular dilatation. Steiner's & Steiner's stain (Frelier et al., 1992; Lightner, 1996) is a good special stain to demonstrate the presence of the intracytoplasmic NHP organisms as dark brown to black organisms within infected cells (Figure 4).

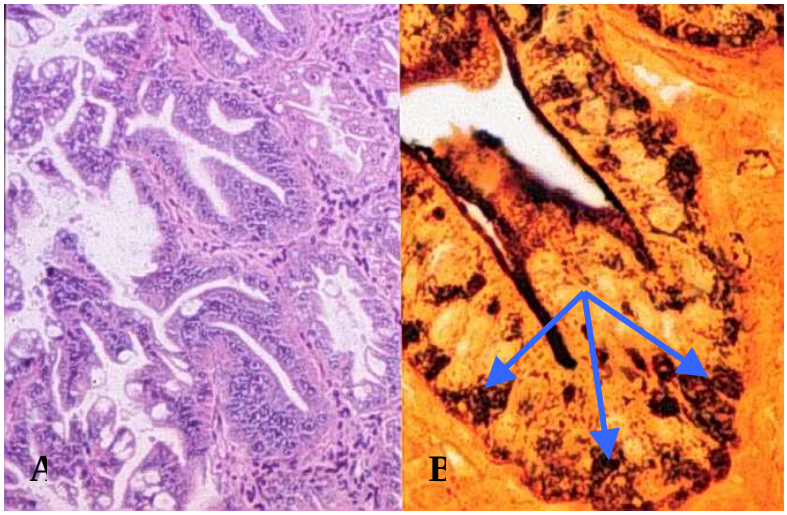


Figure 4. Stage II NHP-infected hepatopancreas with intracytoplasmic NHP organisms demonstrated with H&E (A; 200X) and Steiner's & Steiner's stain (B; 400X; arrows).

Confirmatory

1. *In situ* hybridization assay

In situ hybridization, using a PCR-generated**,** NHP-specific probe as outlined by Loy et al. (1996b), is performed to confirm suspect histologic findings. The NHP probe is generated by PCR and randomly-labeled with digoxigenin (see the following PCR reaction).

a. Slide processing

i. De-paraffinize embedded tissue sections mounted on positively-charged slides in a xylene-based solvent.
ii. Rehydrate tissue sections through a series of graded ethanols from 100% to 50% ethanol including one 10 min acid/ethanol soak.

b. Permeabilization

i. Incubate sections in Proteinase K for 8 min. at 37°C to permeabilize the tissues to allow probe penetration.
ii. Inactivate the Proteinase K by heating slides up to 95-100°C for 10 min.
iii. Then incubate the slide in 0.4% paraformaldehyde/PBS for 10 min at 4°C to enhance tissue adherence to the slide.

c. ISH hybridization & stringency washes

i. Incubate tissue section(s) in the hybridization solution at 100°C for 10 min followed by a 2 hr (or overnight) incubation at 42°C.
ii. Wash slides in decreasing concentrations of SSC (2X to 0.2X with the last soak at 37°C.

d. ISH detection step

i. Incubate slides in an Anti-digoxigenin-alkaline phosphatase conjugate for 30 min at 37°C.
ii. Then incubate in development solution (NBT/BCIP substrate) for 30-60 min. at 37°C.
iii. Counterstain with Bismarck Brown stain and dehydrate slides through graded ethanols (95%-100%) and a xylene-based solvent prior to cover-slipping for histologic evaluation (Figure 5).

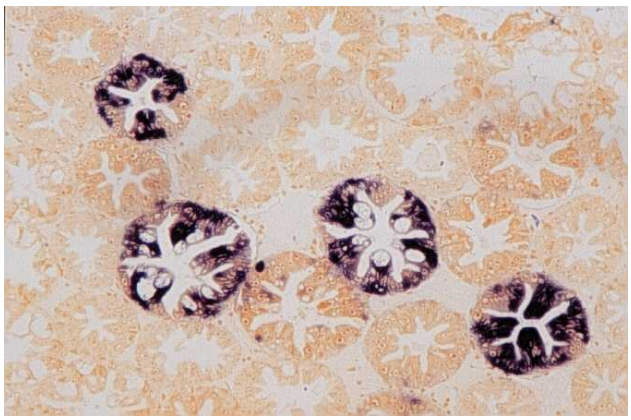


Figure 5. In situ hybridization of NHP-infected hepatopancreatic section with intracytoplasmic probe uptake (dark blue-black) by NHP-infected cells. Counterstain with Bismark Brown stain (200X).

2. Polymerase Chain Reaction (PCR)

NHP-specific PCR can be conducted on feces and/or hepatopancreata from suspect NHP-infected shrimp (Loy et al., 1996b; Brinez et al., 2003). Using NHP-specific oligonucleotide primers, a unique 209 bp target sequence, located within the 16S ribosomal RNA sequence of the NHP genome, is amplified exponentially by the polymerase chain reaction (Loy & Frelier, 1996c). The PCR-generated, NHP- specific amplicon is visualized via electrophoresis and UV-trans-illumination.

a. Sample processing

Genomic DNA is extracted and purified based on a protocol outlined by Loy et al (1996). A homogenate, made of 25-300 mg ethanol-preserved or fresh frozen hepatopancreatic tissue (or feces), is combined with lysis buffer and Proteinase K and incubated for 1-2 hr at 60-65ºC. The Proteinase K is inactivated in a ten minute 95-100ºC water bath. After centrifugation, 100 µl of the supernatant is purified on a spin column prior to use in the PCR assay.

b. DNA amplification

i. Primers[1]: NHP-F27: 5' **ACA TGC AAG TCG AAC GCA ATA GG** 3'
NHP-R235: 5' **ACA GAT CAT AGG CTT GGT AGG CTG** 3'

ii. Master mix[1] (for 25 µl volumes):

Reagent	Volume	Initial Concentration	Final Concentration
10X Buffer	2.5µl	10X (15mM Mg)	1X (1.5mM Mg)
10X dNTPs	2.5µl	2.0 mM ea.	0.2 mM ea.
NHP-F 27	0.3µl	67.32µM	0.81µM
NHP-R235	0.3µl	67.32µM	0.81µM
Taq	0.25µl	5U/µl	1.25U/25µl rxn
H_2O	16.65µl	----	-----
DNA	2.5µl	----	-----

iii. Cycling parameters[1]**:**

Initial denaturation is conducted at 95°C for 5 min., followed by 35 cycles of:
45 sec at 95°C
45 sec at 58°C
45 sec at 72°C
With a final extension step of 10 min at 72°C.

1: Alternate primer set developed at University of Arizona (Lightner, unpublished data); generate 379 bp amplicon:

Forward (F2): 5' CGT TGG AGG TTC GTC CTT CAG T 3' (0.31 µM).
Reverse (R2): 5' GCC ATG AGG ACC TGA CAT CATC 3' (0.31 µM).

Final concentration of the PCR master mix is similar to above, except for $MgCl_2$ at 2mM and AmpliTaq Gold at 2.5 units. Cycling parameters consist of 25 cycles each comprised of 30 sec intervals at 60°C, 72°C & 95°C with final extensions at 60°C for 1 min and at 72°C for 2 min.

c. Visualization of PCR products.

The PCR products are electrophoresed on a 2% agarose gel, stained with ethidium bromide and visualized with UV-transillumination. The generated banding pattern (209 bp amplicon) is compared to a reference DNA ladder and the gel photographed for documentation.

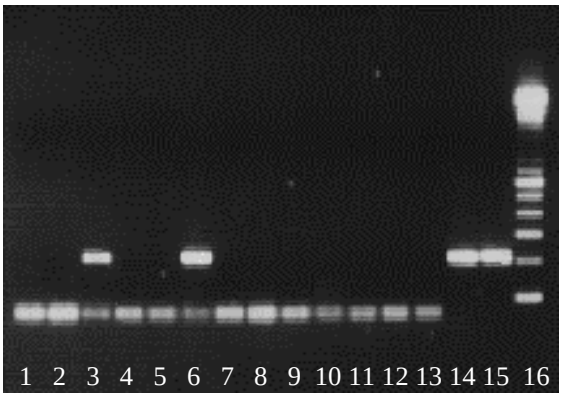


Figure 6. Ethidium bromide-stained gel demonstrating the 209 bp amplicon for NHP positive test samples (lanes 3 & 6), as well as for NHP positive extraction and molecular controls (lanes 14 & 15). Lane 1 is the negative control and lane 16 the reference DNA ladder.

3. Serology (e.g. dot-blot, immunohistochemistry)

Preliminary experimental investigations have demonstrated the feasibility for development of confirmatory serologic tests using monoclonal antibodies produced against the NHP bacterium (Bradley-Dunlop et al., 2004, Vincent & Lotz, 2005), but diagnostic reagents or kits are not commercially available at present.

Table 1. Comparative table of diagnostic methods used for screening, presumptive diagnosis and confirmatory diagnosis of NHP infection.

Method	**Relative sensitivity** [1]	**Presumptive diagnosis**[2]	**Confirmatory Diagnosis**
Gross signs	+	-	-
Histopathology	2-3	++	++
Serologic assays	3	+	+
PCR assays	3-4	+++	+++
ISH assays	4	+++	+++

[1]0-4 increasing sensitivity
[2]applicability: - unsuitable, + limited utility, ++ standard method, +++ recommended method

F. Procedures for Detecting Subclinical Infections

1. Pond-side examinations of statistically significant numbers of shrimp should be conducted during times when environmental conditions are conducive for clinical disease expression. Selected samples should be injected with the appropriate fixative (Bell & Lightner, 1988):
   a. Davidson's fixative (Histology /in situ hybridization).
   b. Ethanol (PCR)

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability or Preservation of the Etiological Agent

1. Histologic/ in situ hybridization analysis
   a. Ship or transport triple-sealed, Davidson-fixed shrimp samples (injected whole body or hepatopancreata) in either Davidson's AFA fixative or 70% ethanol to an aquatic diagnostic laboratory for further diagnostic processing.

2. PCR analysis
   a. Ship or transport triple-sealed, ethanol-fixed shrimp samples (injected whole body or hepatopancreata) in 70-95% ethanol to an aquatic diagnostic laboratory for further diagnostic processing. Samples should be properly labeled (e.g. pond number, date, species, etc), and collected in a manner to minimize sample cross contamination, as well as maintained at or below 21 °C.

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6.2 Taura Syndrome Virus of Penaeid Shrimp

Kenneth W. Hasson

Texas Veterinary Medical Diagnostic Laboratory
1 Sippel Rd
College Station TX 77841
khasson@tvmdl.tamu.edu

A. Name of Disease and Etiological Agent

Taura Syndrome (TS) (a.k.a. "colita roja" or "little red tail") is a viral disease of penaeid shrimp whose name was derived from a region of Ecuador located southwest of Guayaquil (Taura River) where the disease was first recognized in farmed Pacific White shrimp, *Litopenaeus vannamei,* during 1992 (Jimenez 1992, Wigglesworth 1994, Lightner 1996a, Lightner et al. 1994, 1995, Brock et al. 1995, 1997). The early descriptive name given to the disease,"little red tail", was based on one of the gross clinical signs observed in acutely infected *L. vannamei*. TS disease was initially attributed to waterborne toxic effects of two agricultural fungicides (Tilt™ (Propicanizole), Ciba-Geigy and Calixin™ (Tridemorph), BASF) being utilized in Ecuador to control Black Leaf Wilt disease in banana plantations within the vicinity of the stricken shrimp farms (Brock et al. 1995, 1997; Stern 1995, Wigglesworth 1994, Lightner et al. 1994, 1995, Intriago et al. 1997). During 1993 to early 1994, the spread of TS into other *L. vannamei* shrimp farming regions (Peru, Colombia) where neither of the two reported fungicides were being employed, together with the failure of repeated attempts to experimentally induce the disease through fungicide exposure bioassays, cast doubt on the toxic etiology theory (Brock et al. 1995, 1997; Hasson et al. 1995, 1999a; Lightner et al. 1994, 1995, 1996). During 1994 a series of infectivity studies were conducted culminating with fulfillment of River's postulates for a previously unknown virus, which definitively demonstrated that TS was virus-caused and the agent named Taura Syndrome virus (TSV) (Brock et al. 1995, Hasson et al. 1995, Lightner et al. 1995). Investigators in Ecuador have suggested changing the name of the virus to Infectious Cuticular Epithelial Necrosis virus (ICENV) (Jimenez et al. 2000), but TSV will be used herein. Since 1992, TSV has caused global losses among cultured *L. vannamei* stocks conservatively estimated at ~1-2 billion dollars (Lightner 2003) and is categorized as a notifiable viral pathogen by the OIE (OIE, 2003). TSV was originally classified as a member of the Picornaviridae based on limited physicochemical characteristics including intracytoplasmic replication, 32 nm diameter, nonenveloped, icosahedral morphology (Figure 1), linear positive sense ssRNA genome, a buoyant density of 1.338 g/ml and containing three major (VP1, 55kDa; VP2, 40 kDa; VP3, 24 kDa) and one minor (58 kDa) capsid proteins (Hasson et al. 1995, Bonami et al. 1997). These characteristics, together with subsequent sequence analysis of the 10,205 kb genome and finding of two distinct open reading frames (ORFs) resulted in reclassification of TSV as a member of a newly designated genus, Cripavirus within the family Dicistroviridae (Mari et al. 2002, Dhar et al. 2004, Robles-Sikisaka et al. 2001, Mayo 2002a,b). Like many other RNA viruses, TSV has been found to rapidly mutate and at least three distinct variants (1994 Hawaii reference isolate, 2000 Mexican isolate and 2002 Belize isolate) have been reported in the Americas to date based on genomic comparisons and immuohistochemical reactions of different geographic isolates (Erickson et al. 2002, Robles-Sikisaka et al. 2002, Dhar et al. 2004). Changes in virulence and host specificity among these TSV isolates have been documented (Erickson et

al. 2002, 2005; Robles-Sikisaka et al. 2002, Tang et al. 2005). A recent comprehensive phylogenetic analysis of 40 TSV geographic isolates originating from both hemispheres by Tang et al. (2005) determined that three distinct groups of TSV genetic variants exist; Americas, SE Asia and Belize.

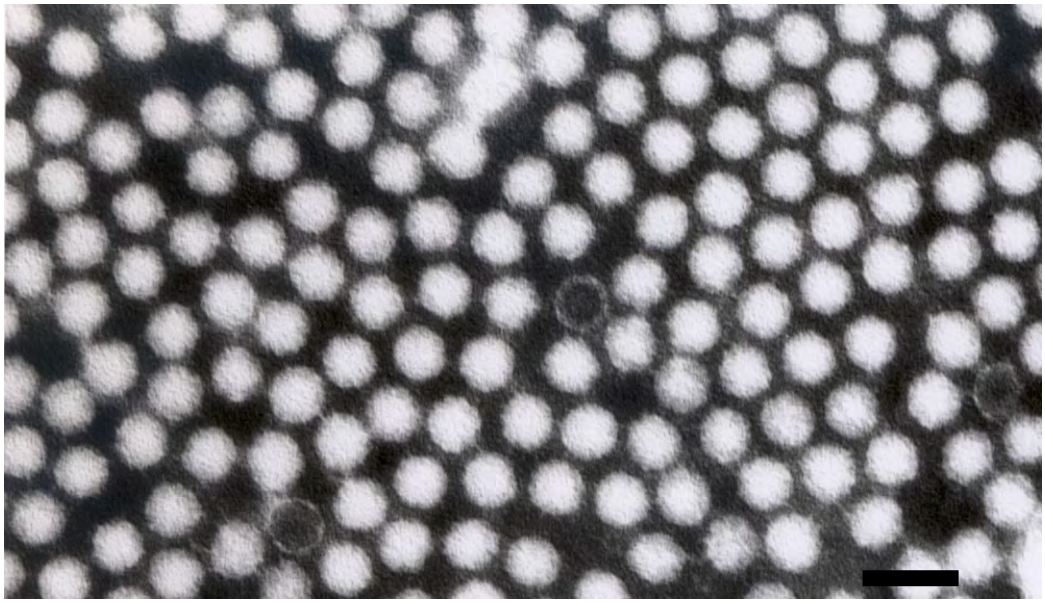


Figure 1. Transmission electron micrograph of negatively stained (2% PTA) CsCl gradient-purified TSV virions. Scale Bar= 64 nm

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Following the recognition of TS disease in Ecuador in 1992, the virus spread to 13 shrimp farming countries (Colombia, Peru, USA, Honduras, Costa Rica, Guatemala, Panama, Mexico, Nicaragua, El Salvador, Brazil, Belize, Dominican Republic) in the Western hemisphere within a four year period and is currently considered endemic along the Pacific coast from northern Peru up into southern Mexico (Lightner 1996a,b, Hasson et al. 1999a, Brock et al. 1995, 1997, Zarain-Herzberg and Ascencio-Valle, 2001). The most recent major shrimp farming country to be affected in the West was Venezuela during 2005. During 1998, TSV was detected in *L. vannamei* stocks in Taiwan (Tu et al.1999, Yu and Song 2000), marking the first incursion of the virus into the Eastern Hemisphere. International sales and movement of *L. vannamei* stocks of unknown health history within Asia is believed to be responsible for the subsequent spread of the disease into China, Thailand, Indonesia and South Korea (Nielsen et al.2005, Hanggono et al. 2005, Do et al. 2006).

2. Host Species

The Pacific White shrimp, *Litopenaeus vannamei,* is considered the principal shrimp host of TSV. All stages of this species are TSV susceptible from postlarvae (PL11) to adults (Wigglesworth 1994, Lightner et al. 1994, Lightner 1996a, Brock et al. 1995, Brock 1997, Lotz 1997). However, mortalities are highest among early juvenile stocks and outbreaks typically occur between 15-40 days post-stocking of nursery or growout ponds (Wigglesworth 1994, Lightner et al. 1994, Lightner 1996a, Brock et al. 1995). TSV has been reported in wild and cultured *L. vannamei* in the Western hemisphere (Hasson et al. 1999a, Brock

1997, Brock et al. 1997, Lightner 1995, 1996a,b) and cultured *L. vannamei* in the Eastern hemisphere (Tu et al. 1999, Yu and Song 2000, Nielsen et al.2005, Hanggono et al. 2005, Do et al. 2006). Juvenile *L. setiferus* and *Penaeus schmitti* are susceptible to TSV and can develop severe infections (Brock et a. 1997, Overstreet et al. 1997, Lightner 1996a). Laboratory exposure studies have demonstrated that *P. japonicus, P. chinensis, F. aztecus,* and *F. duorarum* are only mildly susceptible to TSV, resulting in low grade acute and chronic infections with little or no mortality (Brock et al. 1997, Overstreet et al. 1997, Hasson 1998). Recent injection-mediated exposure studies of *P. monodon* juveniles have shown this species is susceptible to TSV (Srisuvan et al. 2005). *Litopenaeus stylirostris* stocks were initially found to be refractive to TSV infection and, as a result, were widely farmed throughout Mexico from the mid to late 1990's (Brock et al 1995, 1997; Clifford 2000). However, during 1999-2000, a TSV genotypic variant emerged in Mexico that caused high mortality in farmed *L. stylirostris* stocks and resulted in a rapid industry-wide return to *L. vannamei* culture (Robles-Sikisaka et al 2002, Erickson et al 2002). A third distinct strain of TSV was found in farmed *L. vannamei* stocks in Belize where it caused significant losses (Erickson et al. 2005). In response to the threat posed by TSV to U.S. shrimp mariculture, the US Marine Shrimp Farming Program has developed various strains of TSV-resistant (SPR) specific-pathogen-free (SPF) *L. vannamei* stocks for distribution to the U.S. industry. These SPF-SPR lines are currently the principal shrimp strain cultured in the U.S. and selection programs for additional TSV-resistant lineages are ongoing (Carr et al. 1997, Argue et al. 2002, Lightner 1995).

C. Epizootiology

Cumulative mortality among TSV-susceptible *L. vannamei* stocks typically range from 60-95% (Wigglesworth 1994, Brock et al. 1995, 1997, Lightner et al. 1994, 1995, Hasson et al. 1995). Vertical transmission of TSV has not been documented and remains an open question, although rare findings of acute phase TSV lesions within the ovaries and epithelium of the vas deferens of severely infected *L. vannamei* have been reported (Lightner 1995, Dhar et al. 2004, Hasson 1998). Transmission within a pond population occurs as a result of cannibalism of moribund or dead infected shrimp by healthy, naive members of the same population (Brock et al 1995, Lightner et al. 1995, Hasson et al. 1995). Prior et al. (2000) demonstrated that the virus remains viable within dead shrimp carcasses for up to two weeks postmortem and waterborne transmission is possible if viral concentrations are high. *L. vannamei* survivors of a TSV epizootic develop a long term chronic TSV infection localized within the lymphoid organ that may last for life (Hasson et al. 1995, 1999a,b). These TSV carriers can initiate renewed epizootics if they are not removed from farms between culture cycles and are consumed by newly stocked naïve shrimp during subsequent production cycles. Fecal transmission of viable TSV and its movement between ponds, farms or neighboring countries has been linked to marine birds (Laughing Gulls, *Larus atricilla*) and a flying aquatic insect (Water Boatmen, *Trichocorixa reticulata*) following their ingestion of TSV-infected shrimp tissue (Garza et al. 1997, Lightner 1995, 1996b, Hasson et al. 1995). International movement of TSV is principally attributed to the sale of infected *L. vannamei* nauplii, postlarvae and broodstock (Brock et al. 1997, Lightner 1996a,b, Hasson et al. 1999a). The spread of the virus into the Eastern hemisphere has been linked to the importation and culture of TSV-infected *L. vannamei* (Tu et al. 1999, Yu and Song 2000*)*. As with many penaeid viruses, TSV can withstand long term freezing in shrimp tissues and still remain viable (Lightner 1995, Hasson et al. 1995). As a result, imported frozen shrimp pose a risk of virus introduction if used as fishing bait or if solid waste from shrimp processing plants or roadside markets are introduced into salt water systems (Lightner et al. 1997, Prior et al. 2001, Lightner 2002).

D. Disease Signs

TSV infection in susceptible *L. vannamei* populations has three clinically and histologically distinct overlapping phases; a ~7 day peracute to acute phase, a short term transition phase (~5 d) and a long term chronic phase (10 mo- life) (Hasson et al. 1999b,c). Acutely infected *L. vannamei* typically display lethargy, anorexia, atactic swimming behavior, flaccid bodies, soft cuticles, muscle opacity and chromatophore expansion resulting in reddening or darkening of the uropods, antennae and general body (Figure 2) (Brock et al. 1995, Lightner 1995, 1996a, Lightner et al. 1994, 1995, Hasson et al. 1995). A high percentage of TSV-infected *L. vannamei* typically die during or immediately post-molt and this accounts for the high percentage of moribund and dead shrimp with flaccid bodies and soft cuticles (Lightner 1996a, Hasson et al. 1999b). Transition phase infections are grossly identifiable by the presence of numerous, irregularly shaped, black melanized superficial cuticular lesions on the cephalothorax, abdomen and appendages (Figure 2). Shrimp with transition phase infections are typically lethargic and anorexic. Mortality continues among transition phase *L. vannamei*, but rates are greatly reduced when compared to losses among acutely infected shrimp (Hasson et al. 1999b). Chronic phase infections begin following shrimp molt and loss of the melanized exocuticle that formed during the transition phase infection. Chronically infected *L. vannamei* can easily go undetected as they feed and behave normally, displaying no outward clinical signs of disease (Hasson et al. 1999b,c). This phase of a TSV infection can persist for 10-12 months, possibly for the remainder of the shrimp's life.

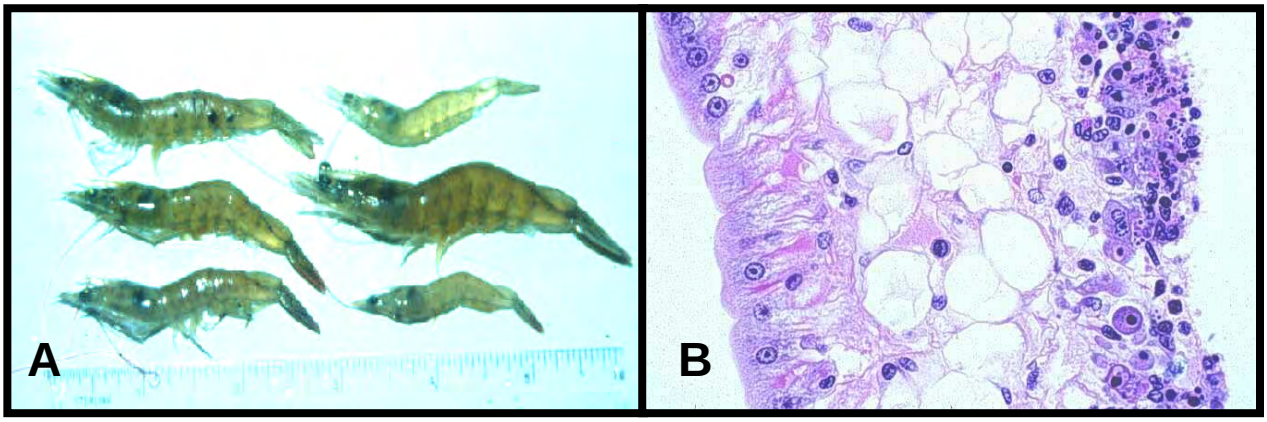


Figure 2. A) Gross clinical signs of acute phase TSV infection in *Litopenaeus vannamei*. Note chromatophore expansion producing a dark to reddish coloration of the general body, uropods and antennae. Shrimp in upper right corner is a normal, healthy specimen and appears transparent in contrast to the other five TSV-infected specimens. B) Histological section of an *L. vannamei* juvenile appendage illustrating an acute phase TSV lesion. The right side of the tissue illustrates the characteristic nuclear pyknosis, karyorrhexis and desquamation of rounded cuticular epithelial cells, which typically display cytoplasmic eosinophilia. The left side illustrates normal, uninfected cuticular epithelial cells. The exocuticle normally covering the epithelium was lost during processing. Hematoxylin and eosin stain. 600X magnification.

E. Disease Diagnostic Procedures

Numerous diagnostic methods have been developed for the detection of TSV including histological, immunological and molecular techniques (Lightner 1996a, 1999, Mari et al. 1998, Poulos et al. 1999, OIE 2003). The three principal diagnostic methods currently used for this disease and advocated by the OIE are histopathology, in situ hybridization and conventional RT-PCR (OIE 2003).

1. Histology

Shrimp are preserved with Davidson's AFA fixative (Humason 1972) through injection of the hepatopancreas, anterior cephalothorax and first, third and sixth tail segments following the methods of Bell and Lightner (1988). Tissues or whole shrimp are maintained in Davidson's for up to 24 hr prior to transfer into 70-95% ethanol for temporary storage or shipment. Dissected gill tissue, mid-sagittal sections of the cephalothorax, transverse sections of the first and third tail segments and a mid-saggital section of the sixth tail segment are dehydrated through a series of alcohol and xylene (or xylene substitutes) baths and paraffin embedded according to the methods of Bell and Lightner (1988). Larval through early juvenile stages can be processed whole and multiple shrimp embedded together. Stain 4-5 μm sections using Mayer-Bennett hematoxylin-eosin phloxine (H&E), coverslip and observe by light microscopy (400 to 600 X).

Acute TSV infections induce a distinctive histological lesion within the cuticular epithelium and subcutis of the foregut, gills, appendages, body cuticle and hindgut that is considered pathodiagnostic for this disease (Jimenez 1992, Lightner et al. 1995, Lightner 1996a, Brock et al. 1995, 1997, Hasson et al. 1995, 1999b). In susceptible moribund shrimp species, cuticular epithelial lesions are typically severe, multifocal to diffuse, and characterized by cytoplasmic eosinophilia, nuclear pyknosis and nuclear karyorhexsis in the absence of any inflammatory response. Rounding and desquamation of infected epithelial cells is commonly seen within lesions during this phase of the disease (Figure 2), which terminates with cell lysis. Numerous pyknotic and karyorrhectic nuclei impart a "peppered" or "buckshot laden" appearance to the lesion in H&E histological sections (Jimenez 1992, Lightner et al. 1995, Lightner 1996a, Brock et al. 1995, 1997, Hasson et al. 1995, 1999b). Lesions within the subhypodermal skeletal muscle, connective tissues, antennal gland, hematopoietic tissue and gonads have been observed in severely infected *L. vannamei*, but are rare to infrequent.

Marked hemocytic infiltrates and melanization within foci that previously contained cuticular epithelial cells are the primary characteristics of transition phase TSV lesions (Figure 3). Rare to infrequent scattered mild acute phase lesions of the cuticular epithelium may still persist and low numbers of spheroids may be observed within the lymphoid organ during this phase.

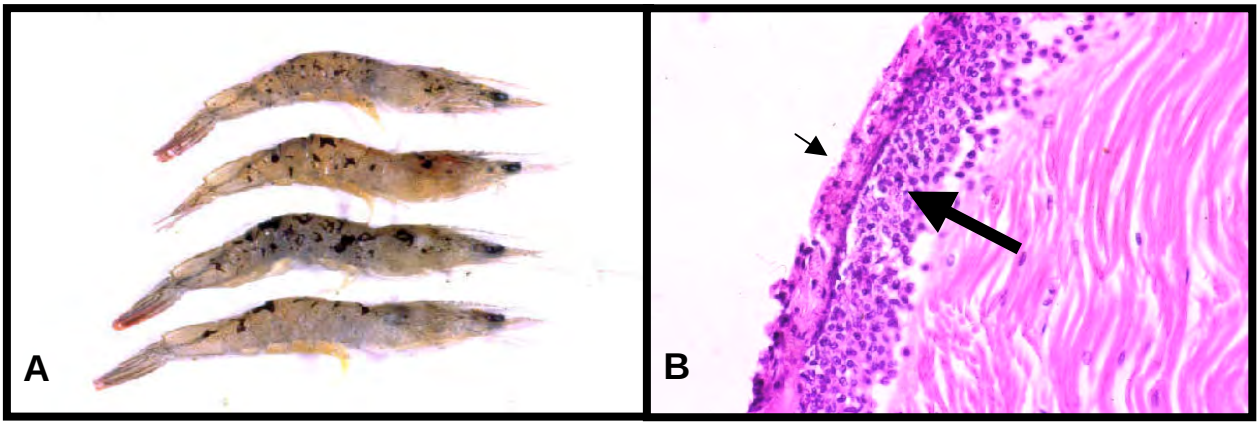


Figure 3. A) *Litopenaeus vannamei* juveniles with grossly visible TSV transition phase melanized lesions (black spots) on the abdomen and cephalothorax. B) H&E histological section of a transition phase melanized lesion illustrating numerous layered infiltrating hemocytes (large arrow), lack of epithelial cells and a melanized layer covering the surface of the lesion (small arrow).

A chronic TSV infection is characterized by numerous irregularly shaped and sized aggregates of hemocytes called spheroids (Figure 4) that are predominantly observed within the interstitium of the

lymphoid organ and along the external surface of the subgastric artery (Hasson et al. 1999b,c, Anggraeni et al. 2000). No acute or transition phase lesions are detectable histologically in chronic phase TSV infections. In severely infected shrimp, spheroids are sometimes observed in association with tegmental glands and within connective tissues of the cephalothorax and appendages (ectopic spheroids) (Hasson et al. 1999c). Lymphoid organ spheroid production is found in conjunction with at least 7 different penaeid shrimp viral diseases (TSV, LPV, IMNV, YHV, LOVV, RPV, MoV) and were recently shown to be induced by bacterial antigens (Hasson et al. 1999c, Van de Braak et al. 2002). The development of spheroids is believed to represent a cell-mediated immune response mounted by the shrimp host to sequester and eliminate foreign biotic and abiotic substances. As a result, only a presumptive diagnosis of chronic TSV infection can be made based on histologic observation of spheroids. Additional diagnostic tests (e.g. ISH or PCR) are necessary to arrive at a definitive diagnosis of chronic TSV infection (Hasson et al. 1997, 1999b, c).

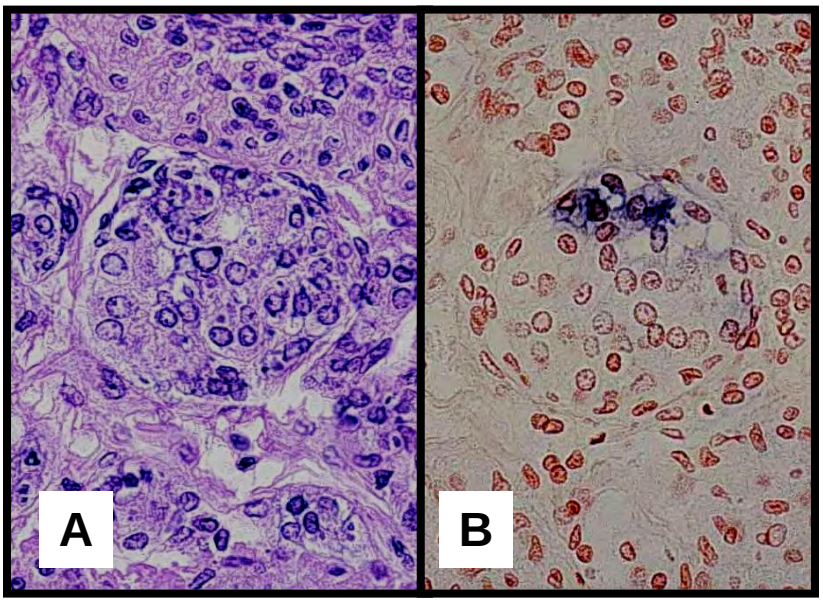


Figure 4. Consecutive histological sections illustrating a chronic phase TSV spheroid within the lymphoid organ of a *Litopenaeus vannamei* juvenile by routine H&E histology (A) and following in situ hybridization analysis with a TSV-specific DNA probe (B). Note presence of pyknotic and karyorrhectic cells within the H&E stained spheroid and a TSV probe positive signal (blue stain) within the ISH section. 400X.

2. In situ hybridization

In situ hybridization can be conducted to confirm suspect histologic findings (Figures 4 & 5). Since TSV-specific *in situ* hybridization kits (Mari et al., 1998) are no longer commercially available, randomly-labeled, digoxigenin TSV-specific probes can be generated by PCR based on the TSV PCR protocol outlined by Nunan et al. (1998).

a. Slide processing

1. De-paraffinize embedded tissue sections mounted on positively-charged slides in a xylene-based solvent.
2. Rehydrate tissue sections through a series of graded ethanols from 100% to 50% ethanol including one 10 min acid/ethanol soak.

b. Permeabilization

1. Incubate sections in Proteinase K for 8 min. at 37°C to permeabilize the tissues to allow probe penetration.
2. Inactivate the Proteinase K by heating slides up to 95-100°C for 10 min.
3. Then incubate the slide in 0.4% paraformaldehyde/PBS for 10 min at 4°C to enhance tissue adherence to the slide.

c. ISH hybridization & stringency washes

1. Incubate tissue section(s) in the hybridization solution at 100°C for 10 min followed by a 2 hr (or overnight) incubation at 42°C.
2. Wash slides in decreasing concentrations of SSC (Standard Saline Citrate Buffer, 2X to 0.2X) with the last soak at 37°C.

d. ISH detection step

1. Incubate slides in an Anti-digoxigenin-alkaline phosphatase conjugate for 30 min at 37°C.
2. Then incubate in development solution (NBT/BCIP substrate) for 30-60 min. at 37°C.
3. Counterstain with Bismarck Brown stain and dehydrate slides through graded ethanols (95%-100%) and a xylene-based solvent prior to cover-slipping for histologic evaluation (Figures 4 & 5).

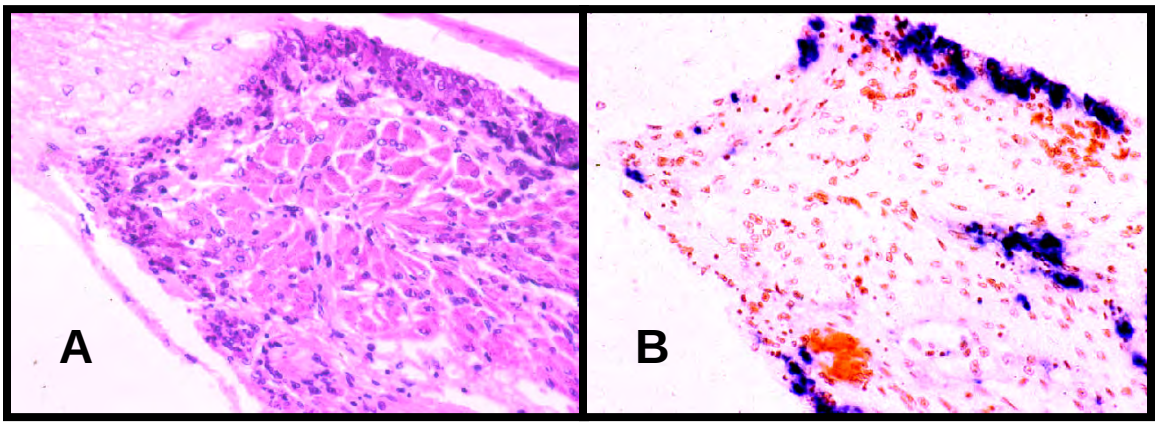


Figure 5. Consecutive histological sections of an *L. vannamei* pleopod containing acute phase TSV lesions by routine H&E histology (A) and following in situ hybridization (ISH) with a digoxigenin-labeled, TSV-specific cDNA probe (B). The probe positive signals (blue stain) observed in the ISH section identify foci containing TSV RNA within the cuticular epithelium along the outer perimeter of the appendage and skeletal muscle/connective tissue within the center of the appendage. 200X.

3. Polymerase Chain Reaction

The reverse transcription PCR protocol of Nunan et al (1998) is utilized for routine screening and diagnostic purposes and is recommended by the OIE Manual of Diagnostic Tests for Aquatic Animals (2003). Other conventional RT-PCR assays (Erickson et al. 2002, Tu et al. 1999, Do et al. 2006) and Real-time PCR assays (Dhar et al. 2002, Tang et al. 2003, Mouillesseaux et al. 2003) for TSV detection have also been developed. This technique is used to screen shrimp populations for both acute or chronic phase TSV infections, as well as to confirm suspect TSV-positive histological findings. Reverse

transcription is followed by cDNA synthesis in this one step protocol, which amplifies a portion of the VP1 gene producing a 231 bp product (Nunan et al. 1998, OIE 2003). All currently known TSV serotypes (A, B and C) are detectable by this method. Tissues used for RNA extraction can consist of pleopods, hemolymph, cephalothoracies or whole animals in the case of larval (Nauplii, Zooplankton, Mysis stages) or postlarval samples (PL5-10). Fresh, frozen (-80^{o}C) or alcohol (70-95% ethanol) preserved tissues can be utilized. Removal of eyestalks during tissue preparation of whole body postlarval samples is recommended as they may contain PCR inhibitors.

a. RNA extraction

Excise a 1-2 mm^3 piece of pleopod or cephalothorax from up to 30 fresh, frozen or ethanol preserved shrimp to obtain a pooled sample not exceeding 150 mg or pooled samples of 10% sodium citrate (anticoagulant) treated hemolymph not exceeding 100 µl can be utilized. According to Nunan et al. (1998), frozen hemolymph samples lacking anticoagulant can be assayed without prior RNA extraction. Ethanol preserved tissues are soaked in ddH_2O until saturated (15-30min) prior to extraction to remove the alcohol. Tissues are then homogenized in 1000 µl Trizol and the extracted RNA precipitated and resuspended in 100 µl of DEPC treated water for immediate use or frozen at -20 to -80^{o}C for <24 hr prior to PCR analysis. Both undiluted and diluted (1:100) samples are run for each test sample due to possible inhibition by excessive extracted RNA.

b. Amplification

1. The primers utilized are as follows:
Forward primer TSV9195: 5' TCA ATG AGA GCT TGG TCC 3'
Reverse primer TSV 9992: 5' AAG TAG ACA GCC GCG CTT 3'

2. Prepare a 25 µl reaction using the GeneAmp EZ rTth RNA PCR kit (Applied Biosystems):

Reagent	Vol/RXn	Initial Conc.	Final Conc.
DEPC Water	10.25 µl	-	-
5X EZ Buffer	5.0 µl	5X	1X
10 X DNTP's	3.75 µl	2.0 mM each	0.3 mM each (dATP, dGTP, dCTP, dTTP)
rTH polymerase	0.5 µl	2.5U/µl	1.25U
25mM Mn^{+2}	2.5µl	25mM	2.5 mM
TSV-9195	0.5 µl	50µM	1.0 µM
TSV-9992	0.5 µl	50µM	1.0 µM
RNA	2.0 µl	-	-

3. Cycling parameters
Reverse Transcription: 30 min at 60 °C, 2 min at 94°C
40 cycles of:
45 s at 94 °C (denaturation)
45 s at 60 °C (annealing and extension)
Final extension of 7 min at 60 °C
4°C hold.

PCR products (10 µl) are electrophoresed in a 2% agarose gel submerged in 1x TBE, stained with ethidium bromide for 20 min, de-stained under running tap water for 3 min, and the resulting 210 bp band visualized by UV transillumination (Figure 6).

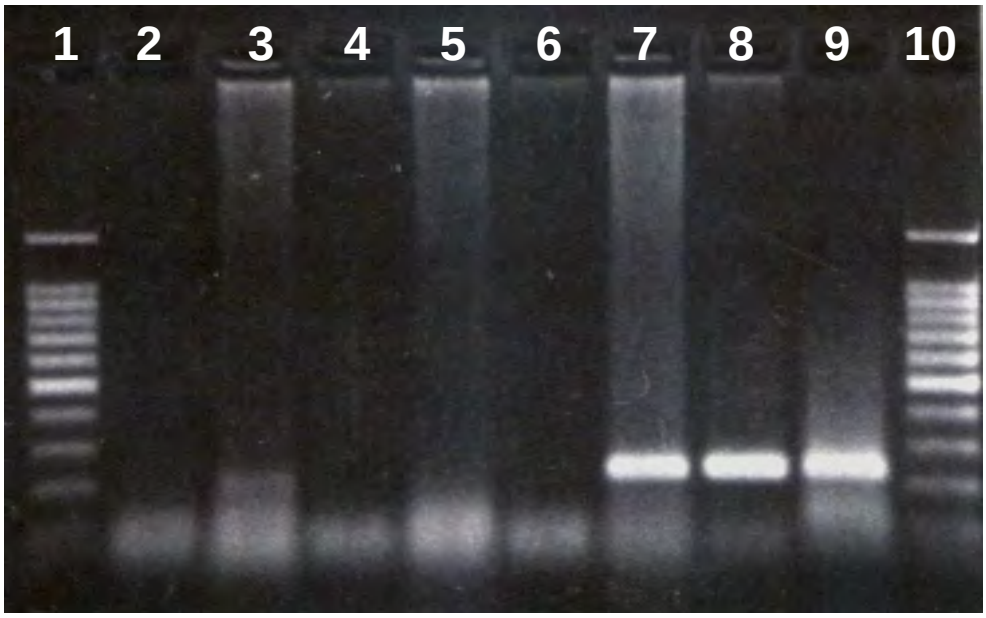


Figure 6. Electrophoretic band patterns of RT-PCR amplified TSV in an ethidium bromide- stained 2% agarose gel. A 231bp product is produced and observable in Lanes 7-9. Lanes 1 and 10: 1 kb ladder. Lane 2: Negative control (water template). Lanes 3 and 4: Undiluted and 1:100 dilution of a sample of shrimp RNA. Lanes 5 and 6: Undiluted and 1:100 dilution of a second sample of shrimp RNA. Lanes 7 and 8: Undiluted and 1:100 dilution of RNA extracted from TSV infected shrimp tissue (Positive control). Lane 9: TSV amplicons (positive control).

F. Procedures for Transportation and Storage of Samples

Davidson's preserved samples for routine histology should be transferred to either 70 or 95% ethanol within 24 hr post-fixation and prior to shipping or transport as described by Bell and Lightner (1988). Ideally, tissues for histological analysis should be processed and embedded within 24-48 hr following preservation to avoid potential hydrolysis of the viral RNA and consequent false-negative in situ hybridization results (Hasson et al. 1997). Alternatively, samples collected specifically for TSV in situ hybridization analysis can be preserved with R-F fixative (Hasson et al. 1997). Ethanol preserved pleopods, tissues or whole animals should be shipped in 70-95% ethanol. Frozen hemolymph, tissue samples or whole animals should be shipped on dry ice and maintained at -80^{o}C for long term storage.

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6.3 *Lagenidium* of Decapod Crustaceans

Tom A. Bailey

U. S. Environmental Protection Agency
401 M. Street SWH7507 C
Washington, D. C. 20460

A. Name of Disease and Etiological Agent

Lagenidiasis is caused by the motile spores of the marine fungi *Lagenidium. Lagenidium* is from the subdivision Diplomastigomycotina and class Oomycetes. Two key species are *Lagenidium callinectes* and *Lagenidium chthamalophilum*.

B. Known Geographical Range and Host Species of the Disease

1. **Geographical range**
   Found in Virginia, Texas, Florida, Oregon, California, Mexico, Tahiti, and Honduras.

2. **Host Species**
   Occurs in the ova of the blue crab *Callinectes sapidus*, cultured shrimp, Dungeness crab, and American lobster *Homerus americanus*.

C. Epizootiology

Although the complete life cycle for *Lagenidium* has not been worked out, it is most likely the motile zoospore stage which serves as the etiological agent. Mortality can reach 100%.

D. Disease Signs

Lagenidium is an internal parasite of ova and juvenile marine crustacea held under artificial or experimental rearing systems. Septate, irregular hyphae (5 to 12 μm diameter) fill the egg or body of the young crab. Zoosporangia form and penetrate the host wall serving as discharge tubes. The fungus is invasive and causes massive tissue destruction. The only tissue reaction reported is melanized areas associated with the hyphae.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

Microscopic observation of stout hyphae inside egg or discharge tubes with vesicles, which protrude the surface of eggs or the gills of juvenile crabs. In sterile sea water, observe the release of biflagellate zoospores from vesicles.

2. Confirmatory Diagnosis

Remove infected ova or gill tissue and place in Cantino's PYG agar or broth prepared in sea water (30 ppt salinity). Hyphae are branched, septate, irregular, and have a diameter of 5 to 12 µm. Vesicles form at the tip of the hyphae and differentiate into zoospores. Zoospores range in size from 9 to 12 µm and contain two laterally attached flagella. Specimen should be shipped immediately for confirmation.

F. Procedures for Detecting Subclinical Infections

No procedures for detecting sub-clinical infections are available.

G. Procedures for Determining Prior Exposure to the Etiological Agent

No procedures for detecting sub-clinical infections are available.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Live or freshly killed fish and eggs are preferable. Samples should be sealed, properly labeled with host species, date of collection, other pertinent data, and packed into cardboard shipping tubes immediately. Specimen should be shipped immediately.

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1.1 Introduction

A. Purpose

The purpose of this document is to serve as a set of standardized testing protocols to be followed when carrying out health inspections on aquatic animals. Using standardized procedures and protocols allows fisheries biologists and managers to better compare data and make more informed management decisions when evaluating the intrastate, interstate, or international movement of aquatic animals. However, the final decision to test for specific aquatic pathogens remains in the hands of those regulating bodies requesting/requiring aquatic animal health inspections. When a regulating body requests/requires testing for specific aquatic pathogens the standardized protocols included in Section 2 of the AFS Blue Book should be utilized to provide comparable test results. The aquatic pathogens listed in Section 2 of the AFS Blue Book are in no way intended to establish, dictate, or supplant the aquatic animal health policies of any regulating body. The pathogens listed and their corresponding protocols are included solely to provide standardized testing methods that produce comparable data. Inclusion of pathogens in Section 2 of the AFS Blue Book is not meant to suggest that testing for all listed pathogens is appropriate or required in all situations or in all aquatic species (see section D. "Selection of Pathogens" below).

B. Composition

This handbook reflects the combined efforts and expertise of the United States Fish and Wildlife Service Fish Health Centers and the American Fisheries Society Fish Health Section. It has been assembled by a vast array of individuals with academic and field expertise. It is a compilation of methodologies determined to be most appropriate for detecting the presence of specific pathogens during an aquatic animal health inspection. The methodologies are based on numerous sources, including the United States Fish and Wildlife Service's National Wild Fish Health Survey Manual, the American Fisheries Society Blue Book, the 5th edition of the Office International des Epizooties (OIE) Manual of Diagnostic Tests for Aquatic Animals, Alaska Department of Fish and Game, Fish Pathology Section Laboratory Manual, and the peer reviewed literature. Without the substantial contributions of these documents and the individuals that assembled and bench tested them, the development of this handbook may not have been possible.

C. Design

To be effective and to meet the needs of what is a rapidly changing and expanding field, this handbook must be extremely dynamic. Every section, including this first chapter, is open to revision. The design of this handbook will allow it to grow and change as time progresses and standardized inspection techniques are required for additional pathogens or better testing methods are made available. The guidelines for introducing changes are outlined in Section 2, A1 Appendix 1. Section 2, A3 Appendix 3 summarizes the decision making process for the selected assays and pathogens so that future changes can be made efficiently.

D. Selection of Pathogens

The pathogens considered in this handbook are those that meet the following criteria:

a. They have the potential to produce severe epizootics of clinical disease.
b. They are known to exist subclinically in apparently healthy fish.
c. There are state, federal, or international requirements that fish be inspected for the pathogen.
d. There are both screening and confirmatory tests available that are effective for the detection and identification of each pathogen in the absence of clinical signs.

Inclusion of pathogens in this document is not meant to imply that it is appropriate to test for all pathogens in all situations. Testing requirements should be based on a careful regional assessment of the risks posed by particular pathogens. The protocols in this document should then be used to test susceptible species for pathogens deemed to be of importance in that region.

E. Selection of Methodologies

The appropriateness of methodologies was determined based not only on performance characteristics (sensitivity, specificity, repeatability, and reproducibility) but also on the appropriateness in a given species or population, scientific acceptance and citable reference materials, cost, availability of reagents, availability of technology, time necessary to process samples and time required before a report can be issued, sample type and its viability, manpower requirements, number of samples to be done, the existence of reference standards, and safety.

The assays specified in this handbook are of two types. Generally, the screening method is one with a proven track record for isolation of a fish pathogen or, less commonly, for direct observation of a causative agent or detection of a component of a fish pathogen. In many cases (e.g. cell culture isolation of fish viruses), these were the first methods developed for this purpose. Although many of these assays have been used for sufficient periods of time to engender confidence in their utility, few if any of the screening methods have been subjected to the formal validation process outlined by the OIE (see “Validity of Chosen Methodologies”). Typically, this is because for many years, few competing assays were available for the purpose; however, there remains a need for the validation process to be applied to these screening tests. Nevertheless, specifying a single screening method ensures that the sensitivity of the assay used to detect a pathogen, while not know with precision, is relatively uniform wherever the assay is applied.

The second type of assay is a confirmatory, test used to verify the identity of a suspect agent. These assays are also in need of formal validation, but are used here only to accurately identify an agent isolated in culture or identified visually, with less regard as to their absolute level of sensitivity. Various assays may be appropriate as confirmatory tests if the specificity of each assay is high. Knowledge of the sensitivity of a confirmatory test becomes of increased importance when the assay is used to confirm a screening test that does not propagate the pathogen in culture. An example is the use of a DNA-based test (e.g. PCR) to confirm a serological test (e.g. FAT). In this case, both assays detect proxies for the actual agent. Better information on the sensitivity, specificity, repeatability and reproducibility of these assays would allow fish health workers to choose optimal combinations of such proxy assays.

F. Validity of Chosen Methodologies

Validation is the evaluation of a process to determine its fitness for a particular use. For diagnostic assays, this generally involves measuring the ability of a certain test to accurately predict the infection status of an animal from which a sample was obtained. Naturally, many factors affect the ability of any assay to achieve absolute predictive power. In recent years, the development of assays based upon molecular approaches and the increased attention paid to issues of quality assurance have led to concerns about the relative performance of various diagnostic assays for fish diseases.

The principles involved in validation of diagnostic assays for infectious diseases are included as a chapter in the Manual of Diagnostic Tests for Aquatic Animals published by the Office International des Epizooties (OIE) in Paris, France. This material is available on the web (www.oie.int) and in hard copy from the OIE. In addition to presenting the principles involved, the OIE lists five stages for diagnostic assay validation and discusses these in some detail with references for further reading. The chapter also describes the procedures by which the important parameters of sensitivity, specificity, repeatability, and reproducibility are determined within a known degree of statistical precision.

Finally, as new diagnostic tests for fish diseases become available, there is interest in comparing the relative performance of various assays. Formal validation methods provide a basis to accurately quantify the actual performance of assays used in fish health and provide information that can help to determine when newer assays should replace the established assays as screening or confirmatory methods. Validation methods can also resolve uncertainties as to the relative performance of materials from different commercial sources or molecular assays that are similar in type, but target different proteins or genes of a pathogen or that use different protocols or reagents. Unfortunately, few if any fish disease detection assays have ever been validated in this way. While validation testing involves much work, considerable expense, and the assembly of sets of standard samples of known infection status, the fish health community is very much in need of this information.

G. Other Important Considerations

It should be noted that as with any inspection manual or handbook, the techniques employed provide only a snapshot in time. There is no guarantee that any inspected animal or population of animals is disease free. The only statement that can be made is that at the time the animals were sampled the disease organism was not detected by the testing methods utilized. Additionally, this handbook cannot cover every scenario that may occur. For this reason, it remains the obligation and responsibility of the individual inspector to determine what the best sampling protocols are for a given inspection and that any inspection methods or protocols used meet the requirements of the requesting entity.

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U. S. Fish and Wildlife Service and/or the United States government. Any comparable instrument, laboratory supply, or reagent may be substituted in this document if operation and performance are deemed comparable to the items specified.









Section 2
USFWS/AFS-FHS Standard Procedures for Aquatic Animal Health Inspections

Chapter 2 Sampling

2.1 Sampling Introduction

2.2 Sampling

2.3 Reagents, Media, and Media Preparation

2.4 Glossary

2.5 References

2.1 Sampling Introduction

This section describes procedures for the collection of samples for both lot and facility inspections. Note that State, Tribal, inter-jurisdictional, and foreign aquatic animal health protection guidelines, policies, and /or regulations may require sample collection procedures that differ from this manual. The procedures in this manual provide an acceptable minimum standard for conducting fish health inspections in the absence of guidance from other policies or regulations as listed above.

Size and age group classifications, lot definitions, sample size, general sampling considerations, and a recommended protocol for sample collection are also included. Screening and confirmatory tests to be run on the samples collected are provided, in detail, for each pathogen in subsequent chapters of this handbook (see table of contents).

The following necropsy and sample collection protocol applies specifically to the pathogens included in this manual. A brief description of clinical signs of the diseases caused by these pathogens is provided in this manual. However, references such as the AFS/FHS Blue Book (2004 Edition) and others listed herein should be consulted for more detailed descriptions and information.

It is the responsibility of the inspector to evaluate the facility and select the appropriate fish from which to collect samples, check the policies and regulations of the jurisdictions requiring the inspection, and coordinate sample submission requirements with the laboratory that will be performing the assays. This will ensure recognition of the inspection results by the competent authorities. A guideline for inspection preparation is provided in Section 2, 2.2.A "Guidelines for Preparation of a Fish Health Inspection."

The inspection criteria outlined in this chapter is based on the testing of a statistically valid number of individuals to assess the health of the entire population and is intended only to provide information for risk management involving the movement of fish from one location to another. The lack of detection of a particular pathogen resulting from an inspection indicates only that the pathogen was not isolated by the methods used on the individual fish examined on the date of sampling. It does not indicate that every fish in that population is free of that pathogen or will be free of that pathogen at any time in the future.

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2.2 Sampling

A. Guidelines for Preparation of a Fish Health Inspection

It is the responsibility of the inspector to obtain the appropriate information from the facility manager, receiving jurisdiction, and testing laboratory in order to assess all appropriate samples to collect from each lot at the facility. The following is a checklist of information to consider when planning and preparing for an inspection.

1. Facility Information

a. Facility water source(s) – do any of the sources contain live fish?

b. Facility water temperature regime.

c. Identification of all lots of fish present at the facility at the time of inspection and during the previous 12 months (see Section 2, 2.2.C "Lot and Inspection Definitions" for definition of lot).

d. Origin and history of each lot: strain information, transfer information, and previous inspection history.

e. Location of fish in each lot at the facility (by both water source and rearing unit).

f. General health history, including any therapies administered to fish during the previous 12 months.

2. Receiving Jurisdiction Information

a. Name(s) of state contacts.

b. State regulations that apply.

c. Regional policy that may apply.

d. Pathogens listed by regulation or policy.

e. Sampling and laboratory analysis methods required by regulation or policy.

f. Does testing laboratory meet all requirements of receiving jurisdiction(s)?

Listed in Table 2.1 are the target fish species, size/age group and tissue to be selected for inspection for each pathogen. To the extent that inspection requirements allow for it, sampling efforts will be directed at the most susceptible species, age, temperature, and rearing units for that pathogen. For some species, strain susceptibility or resistance, as well as other performance factors can be obtained and reviewed from the National Fish Strain Registry (http://159.189.37.201/).

All samples shall be processed as soon as possible after collection. If the collected animals are not maintained alive before processing, samples shall be stored chilled (0 to 4°C) but not frozen and shall be processed as soon as possible after collection. All samples for virology must be inoculated onto cell cultures within 72 hours post-collection. Fish selected should be representative of the lot being inspected and shall include fish with lesions and moribund fish when present.

Samples are collected by, submitted by, and accepted from a federally accredited veterinarian, a state or federal animal health official, or an American Fisheries Society (AFS) certified inspector or pathologist. Contact the laboratory that will be performing the assays to coordinate sampling.

3. Sample information will include the following information at a minimum:

    a. Name and address of owner.

    b. Location of sample collection.

    c. Type of water source (well, spring, surface).

    d. Whether a water source is fish-free.

    e. Name and address of the submitting individual.

    f. Age, species, origin of fish (fish or eggs).

    g. Number of fish present in each lot.

    h. Number of fish sampled in each lot.

    i. For *M. cerebralis* sampling, age should be given in temperature degree-days. If continuous temperature data is not available, report age in months. It should be indicated how long the fish have resided in the water supply.

B. Size/Age Groups

Environmental and species differences can markedly affect the growth rate of fish. In addition, some pathogens are most readily detected when fish are a certain size, whereas others are most readily detected when fish are a certain age. For the purpose of fish health inspections, fish are assigned to one of four groups based on either size or age depending on the pathogen of interest. The following table provides a general reference for these classifications. These classifications may not fit all species.

Table 2.1. A matrix to assist in selecting fish, the appropriate tissue, screening and confirmation methods to use to detect the specific pathogens listed below.

ORGANISM	COMMON NAME OF DISEASE	KNOWN SUSCEPTIBLE SPECIES	TISSUE FOR SAMPLING	PRIMARY (SCREENING TECHNIQUE)	CONFIRMATORY TECHNIQUE	COMMENTS
Bacterial Pathogens						
Aeromonas salmonicida	Furunculosis	Any freshwater fish	Kidney	Bacterial culture of kidney on TSA or BHIA media	Fluorescent Antibody Test (FAT)	May be isolated from many species of fish, birds, and protozoan parasites
Yersinia ruckeri	Enteric Red Mouth (ERM)	Any freshwater fish	Kidney	Bacterial culture of kidney on TSA or BHIA media	FAT	May be isolated from many species of fish and birds
Edwardsiella ictaluri	Enteric Septicemia of Catfish (ESC)	Ictalurids	Kidney	Bacterial culture of kidney on TSA or BHIA media	FAT	
Renibacterium salmoninarum	Bacterial Kidney Disease (BKD)	Salmonids	Kidney, ovarian fluid	Direct fluorescent antibody test on kidney smear or ovarian fluids	Bacterial culture using SKDM-2 media for a total of 6 weeks *or* nested Polymerase Chain Reaction (PCR) technique	
Piscirickettsia salmonis		Salmonids, freshwater, and marine fish	Kidney/Spleen/Liver/Blood	Cell culture on CHSE for 28 days. Hold for an additional 14 days. Or tissue impression stained with Giemsa.	IFAT, Immuno-histochemistry or PCR	Use antibiotic-free media in cell cultures.
Viral Pathogens						
Infectious Hematopoietic Necrosis Virus	IHN	Salmonids	Whole fry, viscera, or kidney/spleen - depending on size, ovarian fluid	Cell culture on EPC cells for 14 days at 15°C. Followed by a 14-day blind pass.	Serum neutralization or nested PCR or IFAT	Target tissues should be kidney/spleen from larger fish and ovarian fluid from spawning broodstock.
Infectious Pancreatic Necrosis Virus	IPN	Wide variety of freshwater and saltwater fish and shellfish	Whole fry, viscera, or kidney/spleen - depending on size, ovarian fluid	Cell culture on CHSE-214 cells for 14 days at 15°C. Followed by a 14-day blind pass.	Serum neutralization or nested PCR or IFAT	Target tissues should be kidney/spleen from larger fish and ovarian fluid from spawning broodstock. May be isolated from many species of aquatic organisms
Infectious Salmon Anemia Virus	ISA	Salmonids and Atlantic herring	Whole fry, viscera, or kidney/spleen - depending on size; ovarian fluids	Cell culture on SHK-1 cells for 14 days at 15° C. Followed by a 14-day blind pass.	PCR or IFAT	In addition to sampling kidney spleen, when available sample ovarian fluid from spawning broodstock. Most mortality occurs in saltwater with fluctuating temperatures
Oncorhynchus masou Virus	OMV	Salmonids	Viscera, ovarian fluids	Cell culture on CHSE-214 cells for 14 days at 15° C. Followed by a 14-day blind pass.	PCR technique or send to reference lab for confirmation	Target tissues should be kidney/spleen from larger fish and ovarian fluid from spawning broodstock. Only known to occur in Japan

Table 2.1. (Continued) Target fish species, size/age group, and tissue to be selected for inspection for each pathogen.

ORGANISM	COMMON NAME OF DISEASE	KNOWN SUSCEPTIBLE SPECIES	TISSUE FOR SAMPLING	PRIMARY (SCREENING TECHNIQUE)	CONFIRMATORY TECHNIQUE	COMMENTS
Viral Hemorrhagic Septicemia Virus	VHS	Salmonids, pike, turbot, herring, pilchard	Kidney/spleen	Cell culture on EPC cells for 14 days at 15° C. Followed by a 14-day blind pass.	PCR	In addition to sampling kidney spleen, when available sample ovarian fluid from spawning broodstock.
White Sturgeon Herpesvirus	WSHV	White sturgeon, possibly shortnose sturgeon	Kidney/spleen, ovarian fluids	Cell culture on WSS-2 cells for 14 days at 20° C. Followed by a 14-day blind pass.	Send to reference lab for confirmation	
Largemouth Bass Virus	LMBV	Centrarchids and ecocids	Kidney/spleen/swim bladder	Cell culture on FHM or BF-2 cells for 7 days at 25 to 30° C. Followed by a 7-day blind pass.	PCR	
Spring Viremia of Carp Virus	Infectious carp dropsy	Cyprinids, also brown trout, pike, shrimp and copepods	Kidney/spleen	Cell culture on EPC cells for 14 days at 20 to 25° C. Followed by a 14-day blind pass.	Serum neutralization or PCR	Most easily isolated in the spring during and for several weeks after epizootics.
Parasite Pathogens						
Myxobolus cerebralis	Whirling Disease	Salmonids	Cranial cartilage (entire head or wedge/core sample from larger fish	Pepsin-trypsin digest	Histological observation of spores/lesions consistent with infection in cranial cartilage or nested PCR	For a facility inspection only one lot of the most susceptible species on each water source need be inspected. When possible select fish that have been on that water supply, while at a susceptible age, for a minimum of 1800 degree-days C or for six (6) months.
Ceratomyxa shasta	Ceratomyxosis	Salmonids	Intestine (posterior)	Wet mounts of intestinal scraping	Detection of spores or PCR	When possible select fish 1) in earth ponds or ponds receiving untreated surface water, 2) that have been on that water supply for a minimum of six (6) months and 3) that are moribund or lethargic.
Tetracapsula bryosalmonae	Proliferative Kidney Disease (PKD)	Salmonids	Kidney	Smears of kidney stained with Leishman-Giemsa or Lectin	Histology	When possible: 1) select fish from earth ponds or raceways receiving untreated surface water, 2) sample moribund fish and 3) conduct sampling during summer or early fall months.
Bothriocephalus acheilognathi	Asian Tapeworm	Cyprinids, silurids, poeciliids, percids, centrarchids, gobiids, cyprinodontids	Intestine (anterior one third)	Visualization of cestode with pyramidal scolex in the semi-contracted state	Positive identification by use of a key	Late summer and fall sampling optimal for detection.

Table 2.2. Suggested categories for grouping fish for sample collection.

Designation	Total length	or	Age
Fry	< 4 cm		0–3 months of age
Fingerlings	4 – 6 cm		4-12 months age
Yearlings/Adults	> 6 cm		Non-brood fishes greater than 12 months of age
Broodstock	> 6 cm		Sexually mature fish greater than 12 months of age and used as broodstock

C. Lot and Inspection Definitions

Refer to Table 2.1 and to the pathogen specific sections of this handbook for detailed information on what species of fish are susceptible to each pathogen and the conditions under which it is most readily detected.

1. **Lot of Non-Broodstock Fish**
   A group of non-brood fish of the same species and age group (see definitions of age group in Table 2.2) that have continuously shared a common water source throughout their life history. A representative sample of all strains and rearing units containing this lot shall be included.

2. **Lot of Broodstock Fish**
   A group of sexually mature fish of the same species that share a common water source. The sample must be representative of all age groups (e.g. three-, four-, and five-year-old brood fish) and strains present at the facility.

3. **Lot Inspection**
   The collection and examination of a statistically valid number of the appropriate samples from a specific lot of a susceptible species for any pathogen listed in this handbook. Moribund fish will be included when present. Unless otherwise stated in the policies and/or regulations of the jurisdictions involved, sampling for the required pathogens will be performed at the 5% APPL with a 95% confidence level. See Table 2.3 for further explanation of the number of samples required.

   a. Exception
      In broodstock lots where there is access to ovarian fluid, sampling for the required viral pathogens will be performed at the 5% APPL with a 95% confidence level in both kidney/spleen tissues and ovarian (coelomic) fluid. Kidney/spleen and ovarian fluid samples must come from different individuals.

      ***Example*:** In a broodstock population consisting of 2500 individuals, kidney/spleen samples will be collected from 60 fish (males and/or females) and ovarian fluid will be collected from an additional 60 females for a lot inspection requiring IHNV testing.

b. Exception
A lot of anadromous salmon regularly monitored for *Renibacterium salmoninarum* through ELISA or quantitative PCR techniques may be considered positive for this pathogen without additional testing. Results of the monitoring must be provided to the jurisdictions involved when requested.

4. Facility Inspection
Lot inspection of each and every susceptible lot of fish held on the facility for any of the bacterial, viral, and parasitic pathogens listed in this handbook.

a. Exception
For *Myxobolus cerebralis*, only one lot of the most susceptible species on each water source at the facility needs to be inspected. It is essential that the lot chosen has had sufficient exposure to create a detectable infection

b. Inspection Frequency
Most regulating jurisdictions require that a history of annual inspections be submitted with the inspection report prior to permitting the importation, stocking, and/or transfer of aquatic animals. It is, therefore, recommended that a program of annual facility inspections be encouraged for any facility participating in intrastate, interstate, and/ or international commerce of their animals.

D. Sample Number

Unless otherwise dictated by the receiving jurisdiction, the number of fish to be collected from each lot must be in accordance with a plan that provides 95% confidence that at least one infected fish will be collected if the minimum assumed pathogen prevalence level (APPL) of infection equals or exceeds 5%. Examples of the number of fish to sample for various population sizes are listed in Table 2.3. Table 2.3 also includes examples of the number of fish to sample if a 2% or 10% APPL is required by the requesting authority. If the population size is estimated to be between two grouping levels, the sample is taken from the next higher population class (Amos 1985; OIE 2000; Ossiander and Wedemeyer 1973; Thoesen 1994).

Table 2.3. Sample number based on an assumed pathogen prevalence level (APPL) in the population of 10%, 5 %, or 2%.

Lot Size	**Number of Fish Required for Sample**		
(number of fish)	**10% APPL**	**5% APPL**	**2% APPL**
50	**20**	**35**	**50**
100	**23**	**45**	**75**
250	**25**	**50**	**110**
500	**26**	**55**	**130**
2000	**27**	**60**	**145**
>100,000	**30**	**60**	**150**

E. Sample Collection

The order in which tissues are collected will vary depending on the tests to be run. What tissues and fluids are collected, will vary depending on the size of the fish, age of the fish, purpose for which the inspection is being performed, and the requirements of the assays used by the receiving laboratory.

1. Necropsy

A detailed necropsy procedure can be found in "Fish Disease: Diagnosis and Treatment" (Noga 1996).

a. Examine and note presence of gross external lesions. If lesions are collected for histological examination, it must be done in a manner that will not compromise the aseptic collection of samples for bacteriology and virology.

b. Collected fish are humanely euthanized immediately prior to sample collection.

Note: If confirmation of *M. cerebralis* infection is to be done histologically, fish should not be killed by a blow to the head as this may compromise the integrity of skeletal elements.

c. Fry are generally only examined for viruses. Fingerling, yearling, and adult sized fish may be examined for bacteria, virus, and/or parasites.

d. The instruments used during sample collection are at a minimum cleaned between sample pools and disinfected between lots.

e. The body cavity is opened being careful not to compromise the target sample tissues with contents from the intestinal tract.

f. If it blocks access to the kidney, the swim bladder is moved.

2. Collection of Kidney Cultures for the Detection of *Aeromonas salmonicida, Edwardsiella ictaluri, Yersinia ruckeri, and/or Renibacterium salmoninarum*

Samples for bacteriology should always be taken first with proper aseptic technique to minimize contamination. These cultures are not usually collected from fry.

a. A sterile swab or inoculating loop is inserted into the posterior kidney and streaked on a plate or slant (Section 2, 2.3.B "Media Preparation") of the appropriate media. Streaks from up to four fish in the same lot may be made on a single culture plate. When multiple cultures will be made from one fish, reinsert the sterile inoculation loop or swab into the kidney before each plate or slant is streaked. If fish are large enough, a piece of kidney tissue may be excised and used to streak the media.

i. *Aeromonas salmonicida, Edwardsiella ictaluri, and Yersinia ruckeri:* Brain Heart Infusion Agar (BHIA) or Trypticase Soy Agar (TSA) (Section 2, 2.3.A.1 "Growth Media")

ii. *Renibacterium salmoninarum*: SKDM-2 (Section 2, 2.3.A.2 "Growth Media") if the receiving laboratory will be using bacterial culture for the confirmation of R. salmoninarum.

b. Sample any organs with visible lesions.

c. Incubate media and identify pathogens by the methods described in Section 2, Chapter 3 Bacteriology.

d. A smear for the *R. salmoninarum* FAT (Section 2, 3.5.A "Summary of Screening Test") is made from the posterior kidney on a microscope slide. The slides will be screened by FAT as described in Section 2, 3.5.A "Summary of Screening Test."

e. If the polymerase chain reaction technique (PCR) (Section 2, 3.5.B.2 "Nested Polymerase Chain Reaction (PCR) for Confirmation of *R. salmoninarum* DNA") will be used to confirm a positive *R. salmoninarum* FAT slide, a kidney sample is collected after sampling of the kidney is completed for bacteriology and virology.

Note: Approximately 25 mg of kidney is collected into a sterile vial and frozen. Tissues collected for PCR archiving should be labeled so that those tissues can be identified individually if corresponding FAT slides are found positive for *R. salmoninarum*.

f. Ovarian fluid, when available, may be collected from spawning female broodstock for detection of *R. salmoninarum*. The ovarian fluid sample may be obtained from an aliquot of the sample collected for viral analysis (Section 2, 2.2.E.3.e) and processed at the laboratory as described in Section 2, 3.5.A.1.b "Ovarian Fluid Pellet Smear."

3. Collection of Tissues for the Detection of Viral Agents

During collection, transport, and storage prior to processing, samples should be kept chilled (0 to 4°C). **Do not freeze.** During processing, samples should be kept on ice and at no time exceed 15°C or virus viability may be compromised. Tissues for viral testing may be collected and stored in a viral transport media such as Hank's Buffered Salt Solution (HBSS) with or without antibiotics (Section 2, 2.3.C.2 "Hanks Balanced Salt Solution (HBSS)"). The pH should be maintained within the 7.2 to 7.6 range. The samples must be processed and inoculated onto cell cultures within 72 hours of collection and 48 hours or less is recommended.

a. From fry, the entire fish is collected and placed into sterile containers; when present, yolk sacs should be removed to reduce toxicity in cell culture and muscle tissue may be trimmed off as needed to maintain a reasonable sample volume.

b. From fingerling-sized fish, the visceral mass including the kidney is collected and placed into sterile containers. If the stomach is filled with feed, it may be removed.

c. From yearling/adult fish, approximately equal amounts of the spleen and kidney are obtained using aseptic technique and placed into sterile containers. Tissues from up to five fish may be pooled in the same container with approximately an equal amount of tissue from each fish. Total sample volume should not exceed 1.5 grams of tissue.

d. From broodstock fish, approximately equal amounts of the spleen and kidney are obtained using aseptic technique and placed into sterile containers. Tissues from up to five fish may be pooled in the same container with approximately an equal amount of tissue from each fish. Total sample volume should not exceed 1.5 grams of tissue.

e. From female broodstock at spawning, ovarian fluid is collected into an appropriately sized sterile container. Approximately equal volumes (1 mL per fish) of ovarian fluid from up to five fish may be pooled in the same container.

4. Collection of Specimens for the Detection of *Myxobolus cerebralis* Evaluation

Selection of appropriate species and age should be made using Table 2.2 and information in Section 2, 5.2 *Myxobolus cerebralis* (Whirling Disease) (Lorz and Amandi 1994; Meyers 1997).

a. Fish from the same lot may be processed in pools of up to five fish by pepsin-trypsin digest (PTD). For confirmation by PCR or histology, head/core samples are processed individually. Therefore, in order to track positive pools, all corresponding samples must be labeled appropriately.

b. From fingerling and yearling fish (less than 15 cm), the entire head, including opercles, is severed from the body.

c. For larger fish where size makes collecting the entire head impractical, a wedge or core samples may be taken. Include gill arches for more resistant species (Section 2, 5.2 *Myxobolus cerebralis* (Whirling Disease)).

i. A triangle-shaped wedge is cut posterior to the orbit at the dorsal surface almost to the ventral edge of the opercula. The top (dorsal) portion of the wedge should measure 1.5 cm (Figure 2.1).

ii. A core sample is taken by inserting a biopsy or boring tool (at least 19mm diameter; boring drill bit or sharpened pipe fitted to a drill work well) into the dorsal surface of the head just posterior to the eyes and forcing it ventrally until it penetrates into the mouth (Figure 2.2).

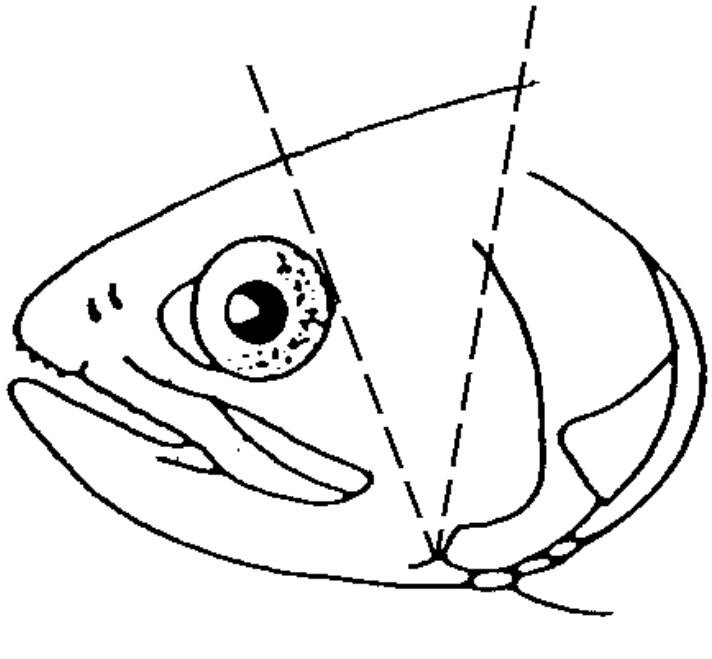


Figure 2.1. Diagram of location of wedge sample from adult fish.

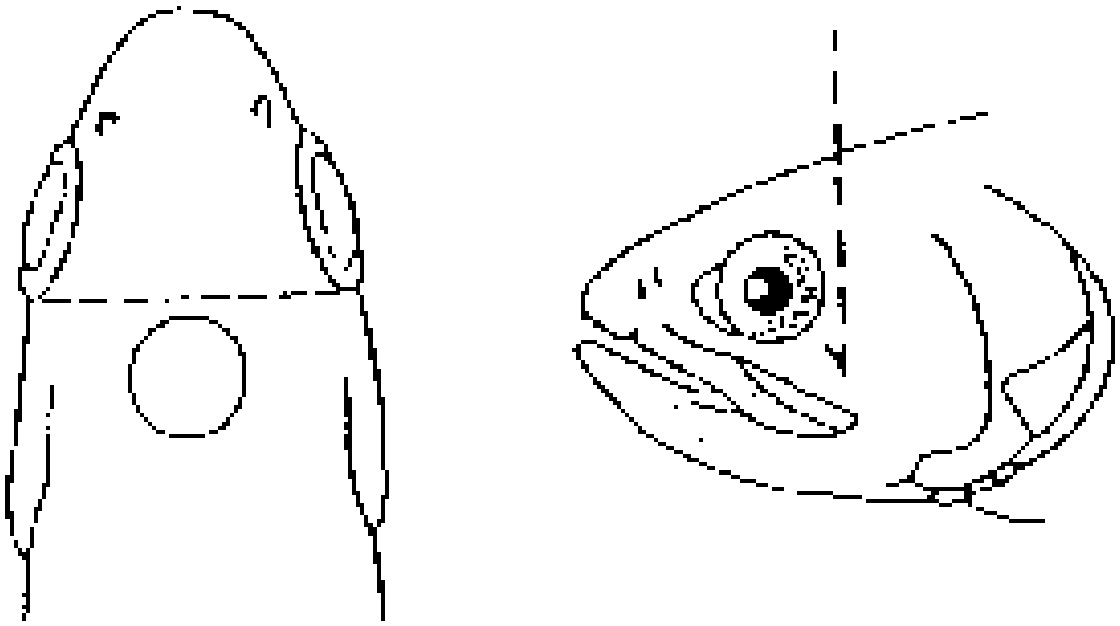


Figure 2.2. Diagram of location of core sample from adult fish.

d. Each head, wedge, or core sample should be split such that each piece contains all the tissue layers. The tissue that is to be processed by PTD for screening should be placed in a plastic bag and transported to the laboratory on ice. Tissues representing up to five fish may be combined for PTD assay.

e. The other half-head, wedge, or core is preserved in a manner suitable for confirmatory testing as follows:

   i. For PCR confirmation, at the time of collection, refrigerate, place on ice or freeze. Upon receipt by the laboratory, samples may be frozen at -20°C.

      Note: extreme care should be taken in the collection of samples in which PCR confirmation may be used. Refer to Chapter 6 for appropriate precautions.

   ii. For histological confirmation, at the time of collection place tissue in a fixative suitable for histology, such as 10% neutral buffered formalin (Section 2, 2.3.C.3 "10% Neutral Buffered Formalin (10% NBF)") or Davidson's (Section 2, 2.3.C.4 "Davidson's Fixative") fixative. Use a 10:1 (volume/volume) volume of fixative to sample.

f. Number individual samples to correspond to the tissue pool to be analyzed by PTD.

5. Collection of Tissues for the Detection of *Ceratomyxa shasta* (Bartholomew 2001)

a. For detection of *C. shasta*, fish are sampled individually and tissues are not pooled for examination.

b. Wet mounts are prepared from intestinal scrapings from the posterior intestine and from any lesions. When possible, fish should be examined immediately after death, but whole fish or intestines can be shipped on ice and examined within 24 hours.

c. For PCR confirmation of *C. shasta*, excise a small portion of the lower intestine (about 2 to 5 mm) and transfer to a vial with 500 µL DNA extraction buffer (Section 2, 5.6.G "DNA Extraction Buffer"). Alternatively, the sample may be frozen or fixed in 100% ethanol (EtOH). Samples in ethanol may be stored at room temperature; those in extraction buffer should be refrigerated or frozen for long-term storage.

Note: extreme care should be taken in the collection of samples in which PCR confirmation may be used. Refer to Section 2, Chapter 6 Polymerase Chain Reaction (PCR) for appropriate precautions.

6. Collection of Tissues for the Detection of *Bothriocephalus acheilognathi*

a. Collected fish are best processed shortly after euthanasia, but may be transported on ice for up to 24 hours. Fixed specimens are not acceptable because of recovery and identification problems. This is especially true for small tapeworms.

b. If fish larger than 20 cm are to be examined, the anterior third of intestinal tracts can be removed and placed in bags on ice to avoid transporting whole large fish.

c. To remove the intestine, cut at the anus and just posterior to the stomach. Unravel intestines gently with fingers and cut off the anterior third and place in a bag on ice. Discard the lower two-thirds of the intestines.

d. Fish smaller than 20 cm are best transported to the laboratory alive but may be shipped whole on ice in plastic bags.

7. Collection of Samples for the Detection of *Tetracapsula bryosalmonae* (Klontz and Chacko 1983; Hedrick et al. 1986; Kent 1994)

a. For *T. bryosamonae* fish are sampled individually and tissues are not pooled for examination. However, impressions of more that one fish can be made on a single microscope slide.

b. Impressions of kidney tissue of each fish are made by excising a small ($5mm^2$) piece of tissue, blotting the tissue on paper to remove the excess blood, and making serial impressions on an alcohol-cleaned slide. Tissue is fixed in either absolute (100%) methanol (MeOH) for five minutes for Leishman-Giemsa staining or in acetone-ethanol (60:40) at -20°C for 10 minutes for lectin staining. The slide is labeled appropriately (location, reference) and stored in a slide box. For prolonged storage of slides for lectin staining, they should be stored dessicated at -70°C.

c. A small piece of kidney from each fish is saved in 10% neutral buffered formalin (Section 2, 2.3.C.3 "10% Neutral Buffered Formalin (10% NBF)") or other suitable tissue fixative (Section 2, 2.3.C.4 "Davidson's Fixative") for confirmation of the presence of *T. bryosalmonae* by histopathology.

d. For PCR confirmation of *T. bryosalmonae*, excise a small portion of the kidney (about 2 to 5 mm^2) and transfer to a vial with 500 µL DNA extraction buffer (Section 2, 5.6.G "DNA

Extraction Buffer"). Alternatively, the sample may be frozen or fixed in 100% ethanol (EtOH). Samples in ethanol may be stored at room temperature; those in extraction buffer should be refrigerated or frozen for long-term storage.

Note: extreme care should be taken in the collection of samples in which PCR confirmation may be used. Refer to Section 2, Chapter 6 Polymerase Chain Reaction (PCR) for appropriate precautions.

2.3 Reagents, Media, and Media Preparation

A. Growth Media

Most of these media are commercially available as pre-made formulas or as bases, which can be easily made in the laboratory. These commercial products are entirely acceptable and should be made and stored according to the manufacturer's recommendations.

1. **Brain Heart Infusion Agar (BHIA) and Tryptic Soy Agar (TSA)**
   These two basic agars are interchangeable for bacterial cultures obtained during an inspection. They are both commercially available.

2. **Selective Kidney Disease Medium-2 (SKDM-2) (Austin, et.al.1983)**
   Used for the selective isolation of *Renibacterium salmoninarum.*

Peptone	10 g
Yeast extract	0.5 g
L-Cysteine HCL	1 g
Agar	15 g
Distilled water	to 1000 mL

Adjust pH to 6.5 before adding agar. Autoclave for 15 minutes at 121°C. Cool to ~ 50°C and add:

Fetal Bovine Serum	200.0 mL

The following antibiotics can also be added to the SKDM-2 to reduce overgrowth from other bacterial organisms (Austin et. al. 1983).

4.0 mL Cyclohexamide (1.2 g Cyclohexamide in 96 mL dH_2O)
1.0 mL D-Cycloserine (0.3 g D-Cycloserine in 24 mL of dH_2O)
2.0 mL Polymyxin B-sulfate (0.3 g Polymyxin B-sulfate in 24 mL of distilled H_2O)
1.0 mL Oxolinic Acid (0.06 g Oxolinic Acid in 24 mL of 5% NaOH)

B. Media Preparation

1. **Plate Media**

   a. Prepare media in stainless steel beakers or clean glassware according to manufacturer instructions. Check pH and adjust if necessary. Media must be boiled for one minute to completely suspend agar. Use of a stir bar will facilitate mixing of agar.

b. Cover beaker with foil or pour into clean bottles being sure to leave lids loose. Sterilize according to manufacturer's instructions when given or at 121°C for 15 minutes at 15 pounds pressure.

c. Cool media to 50°C.

d. Alternatively, media can be autoclaved and cooled to room temperature and refrigerated for later use. Store bottles labeled with media type, date, and initials. When media is needed, boil, microwave, or use a water bath to completely melt the agar. Cool to 50°C. Avoid reheating media multiple times before use.

e. Before pouring media, disinfect hood or counter thoroughly and place sterile petri dishes on the disinfected surface. Aseptically mix any antibiotic solutions, sheep's blood, or Fetal Bovine Serum into the media at this temperature.

f. Label the bottom of each plate with medium type and date prepared.

g. Remove bottle cap and pour plates or dispense with a sterile Cornwall pipette, lifting each petri dish lid as you go. Pour approximately 15 to 20 mL per petri dish. Replace lids as soon as the plate is poured.

h. Immediately wash medium bottle, cap, and pipette in hot water to remove agar and clean up any spilled agar.

i. Invert plates when the media has cooled completely (~ 30 to 60 minutes) to prevent excessive moisture and subsequent condensation on the plate lid.

j. Allow plates to sit overnight at room temperature. Store plates upside down in the refrigerator in a tightly sealed plastic bag or plate storage tin.

k. Follow manufacturer's recommendation for storage period of prepared media. Each batch should be labeled with date of preparation and/or an expiration date.

2. Tube Media

a. Prepare media in stainless steel beakers or clean glassware according to manufacturer's instructions. If the medium contains agar, boil for one minute to completely suspend the agar. Use of a stir bar will facilitate mixing of agar.

b. Media with indicators must be pH adjusted. This can be done when medium is at room temperature; otherwise, compensation for temperature needs to be made.

c. Arrange test tubes in racks. Disposable screw cap tubes can be used for all tube media.

d. Use an automatic pipetter or Pipette-aid™ to dispense the medium. If using the Brewer or Cornwall pipette, prime with deionized water and then pump the water out of the syringe prior to pipetting. Discard the first few tubes of media that are dispensed. Dispense approximately 5 to 10 mL media in 16 x 125mm or 20 x 125mm tubes. Close caps loosely.

e. Immediately after use, rinse the automatic pipetter in hot tap water followed by distilled water to remove all media and prevent clogging of the instrument.

f. Follow manufacturer's recommendation for autoclave time and temperature.

g. If making slants, put tubes in slant racks after autoclaving. Adjust the slant angle to achieve the desired slant angle and butt length (i.e. long butt and short slant for TSI or a standard slant over ¾ of the tube length for TSA or BHIA).

h. Cool completely to room temperature in the slanted position. Tighten caps.

i. Label the tubes or the tube rack with type of medium and date made.

j. Store at 2 to 8°C, following manufacturer's recommendation for long-term storage.

C. Reagents

1. 70% Ethanol (EtOH)

Ethanol (95%)	737 mL
Distilled water	to 1000 mL

2. Hanks Balanced Salt Solution (HBSS)

10X HBSS	100.0 mL
Cell culture grade water	895.0 mL
$NaHCO_3$ (7.5%)	5.0 mL

If antibiotics are used, subtract 320 mL of water and add in its place:

Penicillin/Streptomycin (16%)	160.0 mL
Penicillin G (10,000 units/mL)	
Streptomycin sulfate (10,000 ug/mL)	
Fungizone	160.0 mL
250 ug/mL Amphotericin B	
205 ug/mL Desoxycholate	

Mix. Filter with 0.22 um filter. Store at 4°C.

3. 10% Neutral Buffered Formalin (10% NBF)

Formalin (37% formaldehyde)	100 mL
Distilled water	900 mL
Sodium phosphate (monobasic)	4 g
Sodium phosphate (dibasic)	6.5 g

Store at room temperature.

4. Davidson's Fixative

95% Ethanol	600 mL
Formalin (37% formaldehyde)	400 mL
Acetic acid (glacial)	200 mL
Distilled water	600 mL

Store at room temperature.

2.4 Glossary

Anterior kidney - portion of the kidney containing hematopoietic tissues with little or no urinary function. Generally it is the portion of the kidney closest to the head.

Aseptic technique - prevention of contact with microorganisms not contained in the target tissue.

Disinfect - to free from pathogenic organisms or render those organisms non-infectious.

ELISA - Enzyme-Linked Immunosorbent Assay.

Inspector - a federally accredited veterinarian, a state or federal animal health official, or an American Fisheries Society (AFS) certified inspector or pathologist.

Health history - past events pertaining to infectious or noninfectious agents found during routine monitoring or diagnostic testing.

Life history -all life stages including eggs.

PCR - Polymerase Chain Reaction.

Posterior kidney - portion of the kidney containing excretory elements and responsible for performing urinary function.

Statistically valid number - the number of tissue or fluid samples sufficient to assess the risk of a particular pathogen being present in the population of interest. Selection of this sample number involves specifying both a minimum assumed prevalence level of the pathogen in the population and the minimum acceptable confidence interval for the detection of that pathogen.

Sterile - free from living microorganisms.

Strain - a fish population that exhibits reproducible physiological, morphological, or cultural performance characteristics that are significantly different from other fish populations or a broodstock derived from such a population and maintained therafter as a pure breeding population.

Strain resistance - the development of disease resistance by a particular strain of fish either through natural or artificial selection.

Susceptible species - any species capable of becoming infected with a particular pathogenic organism.

Therapies - the use of any drugs and/or chemicals for the treatment of disease.

Visceral mass - contents of the abdomen of the fish that includes the liver, spleen, stomach, pyloric caeca, intestine, and kidney.

Water source - a spring, lake, river, stream, creek, or aquifer. Multiple wells from the same aquifer or multiple inlets from the same surface water supply constitute a single water source for a facility. Wells in different aquifers or with demonstrably different water chemistry must be considered different water supplies even if present on a single facility. Unless they are known to come from the same aquifer, every well should be considered a separate source.

2.5 References

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Section 2
USFWS/AFS-FHS Standard Procedures for Aquatic Animal Health Inspections

Chapter 3 Bacteriology

3.1 Bacteriology Introduction

3.2 *Aeromonas salmonicida* (Furunculosis)

3.3 *Yersinia ruckeri* (Enteric Redmouth Disease, ERM)

3.4 *Edwardsiella ictaluri* (Enteric Septicemia of Catfish, ESC)

3.5 *Renibacterium salmoninarum* (Bacterial Kidney Disease, BKD)

3.6 *Piscirickettsia salmonis*

3.7 Reagents, Media, and Media Preparation

3.8 Bacterial Identification Techniques

3.9 Glossary

3.10 References

3.A1 Laboratory Reference Flow Chart Appendix 1

3.A2 Profiles Obtained with API-20E for Known Fish Pathogens

3.A3.A Worksheet A – PCR Sample Data/Log Sheet

3.A3.B Worksheet B – Initial Amplification of Nucleic Acid by PCR for the Confirmation of *R. salmoninarum*

3.A3.C Worksheet C – Nested Amplification of Nucleic Acid by PCR for the Confirmation of *R. salmoninarum*

3.A3.D Worksheet D – Initial Amplification of Nucleic Acid by PCR for the Confirmation of *P. salmonis*

3.A3.E Worksheet E – Nested Amplification of Nucleic Acid by PCR for the Confirmation of *P. salmonis*

3.A3.F Worksheet F – Direct Amplification of Nucleic Acid by PCR for the Confirmation of *P. salmonis*

3.A3.G Worksheet G – Photodocumentation of the PCR Product Gel

3.1 Bacteriology Introduction

The following chapter describes inspection procedures for bacterial pathogens of fish that may be required for a fish health inspection. The target bacterial species include *Aeromonas salmonicida, Yersinia ruckeri, Edwardsiella ictaluri, Renibacterium salmoninarum,* and *Piscirickettsia salmonis*. Section 2, Chapter 2 Sampling describes procedures for proper sampling of fish tissues to ensure detection of any of these pathogens during a fish health inspection.

Presumptive identifications of *A. salmonicida (*subspecies *salmonicida and achromogenes)* (Section 2, 3.2 *Aeromonas salmonicida* (Furunculosis)), *E. ictaluri* (Section 2, 3.4 *Edwardsiella ictaluri* (Enteric Septicemia of Catfish, ESC)), and *Y. ruckeri* (Section 2, 3.3 *Yersinia ruckeri* (Enteric Redmouth Disease, ERM)) are based on Gram staining properties, and characteristic biochemical reactions. Confirmatory identification consists of fluorescent antibody testing using fluorescein-conjugated, species-specific antibody (Section 2, 3.8.E "Fluorescent Antibody Test (FAT)"). Known isolates of *A. salmonicida, E. ictaluri,* and *Y. ruckeri* are purchased from ATCC and are used as positive controls. Single, unknown isolates may be used to test for all three of these organisms.

The presumptive identification of the Gram-positive bacterium *R. salmoninarum* (Section 2, 3.5 *Reibacterium salmoninarum* (Bacterial Kidney Disease, BKD)) is based upon serological methods. For purposes of initial screening and detection of the pathogen, the direct fluorescent antibody technique (FAT) on kidney smears and ovarian fluid samples is employed (Section 2, 3.8.E "Fluorescent Antibody Test (FAT)"). Documentation exists which indicates the possibility for false positive results caused by bacterial organisms which cross react with antibodies prepared against *R. salmoninarum* (Austin et al., 1985; Bullock et al., 1980; Brown et al. 1995). For this reason, it is important to follow steps described below to confirm that a positive FAT result is due to the presence of this pathogen. **Exception:** Anadromous salmonids regularly monitored for *R. salmoninarum* with ELISA or quantitative PCR techniques may be considered positive without additional testing by FAT.

Any FAT results which appear positive for *R. salmoninarum* should be confirmed by either culture of kidney tissue on selective kidney disease medium (SKDM-2) (Section 2, 3.5.B.1 "Bacterial Culture") or by testing the positive tissues with the polymerase chain reaction (PCR) technique (Section 2, 3.5.B.2 "Nested Polymerase Chain Reaction (PCR) for Confirmation of *R. salmoninarum* DNA").

The presumptive identification of the gram-negative, intracellular bacterium *P. salmonis* (Section 2, 3.6 *Piscirickettsia salmonis*) is based on isolation in tissue cell culture without antibiotics and/or detection in stained tissue impressions (Section 2, 3.6.A "Summary of Screening Tests"). Confirmatory testing is by serological methods or PCR (Section 2, 3.6.B "Confirmatory Tests").

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U. S. Fish and Wildlife Service, the United States government, and/or the American Fisheries Society. Any comparable instrument, laboratory supply, or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.

3.2 *Aeromonas salmonicida* (Furunculosis)

Aeromonas salmonicida may be difficult to detect based on a variety of environmental, physiological, and host factors. At water temperatures between 14 to 24°C, fish can develop clinical disease within 4 to 12 days after bacterial exposure in the water supply. At temperatures below 13°C, chronic and latent infections are more likely to develop. This pathogen has been associated with disease in a variety of salmonid and non-salmonid species. It is generally accepted that any freshwater fish can carry the bacteria with or without showing signs of disease (Bullock et al. 1983; Thoesen 1994). The typical form of the bacterium (subspecies *salmonicida*) produces a soluble brown pigment on tryptic soy agar after two to three days of growth at 20°C. The less common and atypical strain of this pathogen (subspecies *achromogenes*) does not produce pigmentation under these incubation conditions. Both strains, however, have been associated with disease in a variety of fish species (Paterson et al. 1980).

A. Summary of Screening Test

1. Bacterial Culture and Biochemical Analysis

a. Aseptically inoculate samples into TSA tubes or onto plates as described in Section 2, 2.2 Sampling.

b. Incubate for 24 to 48 hours at 20 to 24°C (room temperature). If no growth occurs at either 24 or 48 hours, record this information on the data sheet. **If no growth occurs after 96 hours, samples are reported as negative for *A. salmonicida*.**

c. When primary culture occurs on tubes or plates use a sterile loop or needle to select a single, isolated colony to subculture onto fresh TSA plates. If colonies are not well isolated on the original media, a new plate will have to be streaked over the entire plate surface to achieve isolation of bacteria.

d. Incubate at 20 to 24°C for 24 hours to allow bacterial growth; all tests should be performed on 24 to 48 hour cultures.

e. Using a sterile needle or small loop, pick individual distinct bacterial colonies representing each colony type. Use of a dissecting microscope can aid in distinguishing between differing colony types. Assign an isolate number to each isolated colony and record all colony characteristics on the data sheet.

f. Begin initial identification of pure strain bacterial cultures (Section 2, 3.A1 Laboratory Reference Flow Chart Appendix 1).

i. Colony Pigmentation
Typical strains of *A. salmonicida* have brown diffusible pigment after 2 to 3 days of incubation. Some strains may not be pigmented (subsp. *achromogenes*).

ii. Gram Determination (Section 2, 3.8.A "Gram Reaction")
A. salmonicida is Gram-negative. Gram-positive isolates may be reported as negative for *A. salmonicida*.

iii. Presence of Cytochrome Oxidase (CO) (Section 2, 3.8.B "Cytochrome oxidase")
Most are CO positive. Rarely, CO negative strains of *A. salmonicida* subsp. *salmonicida* have been encountered (Chapman et al. 1991).

iv. Motility (Section 2, 3.8.C "Motility")
A. salmonicida is non-motile. Motile isolates may be reported as negative for *A. salmonicida*.

g. Perform biochemical testing on each isolate (Section 2, 3.A1 Laboratory Reference Flow Chart Appendix 1).

i. Tube Method (Section 2, 3.8.D.1 "Tube Method")

1. Glucose fermentation (Section 2, 3.8.D.1.a "Glucose Fermentation") using OF basal medium containing glucose will produce an oxidative/fermentative (O/F) or a fermentative (F) result with gas (most strains produce gas, but some may be weak or variable in this production). **Any isolate with a result other than this may be reported as negative for *A. salmonicida*.**

2. Gelatinase (Section 2, 3.8.D.1.c "Gelatinase") and Indole (Section 2, 3.8.D.1.d "Indole Test") are considered together with the pigmentation of the isolate for interpretation of the results.

a. Brown diffusible pigmented isolates that are Gelatinase positive and Indole negative are **PRESUMPTIVELY positive** *A. salmonicida* sub-species *salmonicida*.

b. Non-pigmented isolates that are Gelatinase negative and Indole positive are **PRESUMPTIVELY positive** *A. salmonicida* sub-species *achromogens.*

3. **Isolates yielding any other combination of these results may be reported as negative for *A. salmonicida*.**

4. Additional biochemical tests will differentiate *A. salmonicida salmonicida* from *A. salmonicida achromogens*.

a. Maltose fermentation (Section 2, 3.8.D.1.e "Carbohydrate Utilization") is determined using OF basal medium containing maltose, producing a fermentative F result. These tests may be done in a closed (i.e., over-layed) tube as is described for glucose (Section 2, 3.8.D.1.a "Glucose Fermentation") or by interpreting the reaction at the bottom of an open tube that contains sufficient medium to distinguish fermentation from oxidation (e.g., minimum of 5 mL in a 16 x 25 mm tube). *A salmonicida* sub-species *salmonicida* ferments maltose. *A. salmonicida* subspecies *achromogens* typically does not ferment maltose; however, maltose utilization has been documented for a few isolates.

b. Esculin hydrolysis (Section 2, 3.8D.1.h "Esculin Test") is determined on Bile Esculin Agar slants with a resulting positive hydrolysis by *A. salmonicida* sub-species *salmonicida*, whereas strains of *A. salmonicida* sub-species *achromogens* are negative.

ii. Commercial Identification Systems (Section 2, 3.8.D.2 "Commercial Identification Systems")

1. Biolog (Section 2, 3.8.D.2.b "Biolog")

2. API
If isolates are tested with the commercial system API described in Section 2, 3.8.D.2.a "API-20E," it is recommended that the reference profiles be consulted in Section 2, 3.A2 Profiles Obtained with API-20E for Known Fish Pathogens.

h. When testing is complete, either cryopreserve isolates of interest or discard bacterial plates and biochemical tubes in a biohazard bag and autoclave before proper disposal.

i. Positive control isolates of *Aeromonas salmonicida* can be obtained from the American Type Culture Collection (ATCC). The Internet location for ATCC is http://www.atcc.org. Below are suggested isolates to use for positive control cultures:

i. *A. salmonicida* subspecies s*almonicida* – ATCC # 14174.

ii. *A. salmonicida* subspecies *achromogenes* – ATCC # 10801.

iii. A **cytochrome oxidase negative** isolate is also available – ATCC # 49385.

B. Confirmatory Test

1. Fluorescent Antibody Test (FAT) (Section 2, 3.8.E "Fluorescent Antibody Test (FAT)")
FAT is performed on at least one representative isolate from each lot inspected and found positive during screening. Positive bacterial isolates will fluoresce strongly and have the same morphology as the positive control. A list of sources from which antibodies may be obtained is provided in Section 2, 3.8.E.6 "Commercial Sources for Antibodies."

3.3 *Yersinia ruckeri* (Enteric Redmouth Disease, ERM)

Yersinia ruckeri affects fingerlings of potentially all salmonids. In salmonids, mortality increases dramatically following periods of stress due to environmental conditions and handling. Survivors of enteric redmouth disease outbreaks become carriers, after which the bacteria are shed from the intestinal tract in large numbers during a regular 36 to 40 day cycle. Severity of infection decreases at water temperatures below 10°C. *Yersinia ruckeri* has been isolated from the kidney of some non-salmonids as well. It is generally accepted that, although not ubiquitous in the environment, any freshwater fish can carry the bacteria with or without exhibiting signs of the disease (Bullock 1984; Thoesen 1994).

A. Summary of Screening Test

1. Bacterial Culture and Biochemical Analysis

a. Aseptically inoculate samples onto tubes or plates as described in Section 2, 2.2 Sampling.

b. Incubate for 24 to 48 hours at 20 to 24°C, (room temperature). If no growth occurs at either 24 or 48 hours, record this information on the data sheet. **If no growth occurs after 96 hours, samples are discarded and reported as negative for *Y. ruckeri*.**

c. When primary culture occurs on tubes or plates use a sterile loop or needle to select a single isolated colony to subculture onto fresh TSA or BHIA plates. If colonies are not well isolated on the original media, a new plate will have to be streaked over the entire plate surface to achieve isolation of bacteria.

d. Incubate at 20 to 24°C for 24 hours to allow bacterial growth; all tests should be performed on 24 to 48 hour cultures.

e. Using a sterile needle or small loop, pick individual distinct bacterial colonies to represent each colony type. Use of a dissecting microscope can aid in distinguishing between differing colony types. Assign an isolate number to each pure colony and record all colony characteristics on the data sheet.

f. Begin initial identification of pure strain bacterial cultures (Section 2, 3.A1 Laboratory Reference Flow Chart Appendix 1).

i. Gram Determination (Section 2, 3.8.A "Gram Reaction")
Yersinia ruckeri is Gram-negative. **Gram-positive isolates may be reported as negative for *Y. ruckeri*.**

ii. Presence of Cytochrome Oxidase (CO) (Section 2, 3.8.B "Cytochrome Oxidase")

Yersinia ruckeri is CO negative. **CO positive isolates may be reported negative for *Y. ruckeri*.**

iii. Motility (Section 2, 3.8.C "Motility")
Yersinia ruckeri is motile. **Non-motile isolates may be reported as negative for *Y. ruckeri*.**

g. Perform biochemical testing on each isolate (Section 2, 3.A1 Laboratory Reference Flow Chart Appendix 1)

i. Tube Method (Section 2, 3.8.D.1 "Tube Method")

1. Triple Sugar Iron (TSI) (Section 2, 3.8.D.1.b "Triple Sugar Iron (TSI)")
Yersinia ruckeri will yield an alkaline over acid (K/A) or alkaline over acid with gas (K/AG) result. **Any isolate with a result other than this may be reported as negative for *Y. ruckeri*.**

2. Carbohydrate Utilization (Section 2, 3.8.D.1.e "Carbohydrate Utilization")

a. Arabinose cannot be utilized (fermented) by *Y. ruckeri*.

b. Rhamnose cannot be utilized (fermented) by *Y. ruckeri*.

c. Sucrose cannot be utilized (fermented) by *Y. ruckeri*.

i. Salicin cannot be utilized (fermented) by *Y. ruckeri*.

ii. **Isolates yielding positive results for any of these tests may reported as negative for *Y. ruckeri*.**

3. Malonate Test (Section 2, 3.8.D.1.g "Malonate Test")
Isolates yielding positive results for this test may be reported as negative for *Y. ruckeri*.

4. Indole Test (Section 2, 3.8.D.1.d "Indole Test")
Isolates yielding positive results for this test may be reported as negative for *Y. ruckeri*.

5. Esculin Test (Section 2, 3.8.D.1.h "Esculin Test")
Isolates yielding positive results for this test may be reported as negative for *Y. ruckeri*.

6. Decarboxylase Test (Lysine) (Section 2, 3.8.D.1.f "Decarboxylase Test (Lysine)")
Isolates yielding negative results for this test may be reported as negative for *Y. ruckeri*.

7. Sorbitol (Section 2, 3.8.D.1.e "Carbohydrate Utilization")

a. Isolates that satisfy all previous conditions in this section are **PRESUMPTIVELY positive** for *Y. ruckeri*.

b. Sorbitol is used to differentiate between Type I and Type II *Y. ruckeri*.

i. Isolates yielding negative results for this test are **PRESUMPTIVELY positive** for *Y. ruckeri* Type I.

ii. Isolates yielding positive results for this test are **PRESUMPTIVELY positive** for *Y. ruckeri* Type II.

ii. Commercial Identification Systems (Section 2, 3.8.D.2 "Commercial Identification Systems")

1. Biolog (Section 2, 3.8.D.2.b "Biolog")

2. API
If isolates are tested with the commercial system API described in Section 2, 3.8.D.2.a "API-20E," it is recommended that the reference profiles be consulted in Section 2, 3.A2 Profiles Obtained with API-20E for Known Fish Pathogens."

3. When testing is complete, either cryopreserve isolates of interest or discard bacterial plates and biochemical tubes in a biohazard bag and autoclave before proper disposal.

4. Positive control isolates of *Yersinia ruckeri* can be obtained from the American Type Culture collection (ATCC). The Internet location for ATCC is http://www.atcc.org. Below are suggested isolates to use for positive control cultures:

a. *Yersinia ruckeri* serovar Type I – ATCC # 29473.

b. *Yersinia ruckeri* Type II – ATCC # 29908.

B. Confirmatory Test

1. **Fluorescent Antibody Test (FAT)** (Section 2, 3.8.E "Fluorescent Antibody Test (FAT)")
FAT is performed on at least one representative isolate from each lot inspected and found positive during screening.

a. A separate antibody must be utilized for confirmation of either Type I or II strains.

b. Positive bacterial isolates will fluoresce strongly and have the same morphology as the positive control.

c. A list of sources from which antibodies may be obtained is provided in Section 2, 3.8.E.6 "Commercial Sources for Antibodies."

3.4 *Edwardsiella ictaluri* (Enteric Septicemia of Catfish, ESC)

The primary host species of this pathogen include all species of catfish and tilapia, as well as other warm water species. Both fingerlings and adults can be affected by the disease. In catfish, the bacterium is transmitted through the olfactory system to the brain, where the typical "hole-in-the-head" lesions can be observed during an *E. ictaluri* epizootic. Disease typically occurs at water temperatures between 20°C and 30°C. Experimental infections have been established in salmonids. It is generally accepted, that any warmwater species of fish can carry this pathogen with or without exhibiting signs of disease (Hawke et al 1981; Bullock and Herman 1985; Austin and Austin 1987; Thoesen 1994).

A. Summary of Screening Test

1. Bacterial Culture and Biochemical Analysis

a. Aseptically inoculate samples onto tubes or plates as described in Section 2, 2.2 Sampling.

b. Incubate for 24 to 48 hours at 28 to 30°C. Alternatively, this organism can be grown at 20 to 24°C (Plumb et al. 1989). If no growth occurs at 24 and 48 hours, record this information on the data sheet. **If no growth occurs after 96 hours, samples are discarded and reported as negative for *E. ictaluri*.**

c. When primary culture occurs on tubes or plates use a sterile loop or needle to select a single colony to subculture onto fresh TSA or BHIA plates. If colonies are not well isolated, a new plate will have to be streaked over the entire plate surface to achieve isolation of bacteria.

d. Incubate at the temperature used above for 24 hours to allow bacterial growth; all tests should be performed on 24 to 48 hour cultures.

e. Using a sterile needle or small loop, pick individual distinct bacterial colonies to represent each colony type. Use of a dissecting microscope can aid in distinguishing between differing colony types. Assign an isolate number to each pure colony and record all colony characteristics on the data sheet.

f. Begin initial identification of pure strain bacterial cultures (Section 2, 3.A1 Laboratory Reference Flow Chart Appendix 1).

i. Gram Determination (Section 2, 3.8.A "Gram Reaction")
Edwardsiella ictaluri is Gram-negative. **Gram-positive isolates may be reported as negative for *E. ictaluri*.**

ii. Presence of Cytochrome Oxidase (CO) (Section 2, 3.8.B "Cytochrome Oxidase")
E. ictaluri is CO negative. **CO positive isolates may be reported negative for *E. ictaluri*.**

g. Perform biochemical testing on each isolate (Section 2, 3.A1 Laboratory Reference Flow Chart Appendix 1).

i. Tube Method (Section 2, 3.8.D.1 "Tube Method")

1. Triple Sugar Iron (TSI) (Section 2, 3.8.D.1.b "Triple Sugar Iron (TSI)")
E. ictaluri will yield an alkaline over acid (K/A) or alkaline over acid with gas (K/AG) result. **Any isolate with a result other than this may be reported as negative for *E. ictaluri*.**

2. Carbohydrate Utilization (Section 2, 3.8.D.1.e "Carbohydrate Utilization")
The following carbohydrates cannot be utilized (fermented) by *E. ictaluri*:

Arabinose
Rhamnose
Sucrose
Salicin

Isolates yielding positive results for any of these tests may be reported as negative for *E. ictaluri*.

3. Malonate Test (Section 2, 3.8.D.1.g "Malonate Test")
Isolates yielding positive results for this test may be reported as negative for *E. ictaluri*.

4. Indole Test (Section 2, 3.8.D.1.d "Indole Test")
Isolates yielding positive results for this test may be reported as negative for *E. ictaluri*.

5. Esculin Test (Section 2, 3.8.D.1.h "Esculin Test")
Isolates yielding positive results for this test may be reported as negative for *E. ictaluri*.

6. Decarboxylase Test (Lysine) (Section 2, 3.8.D.1.f "Decarboxylase Test (Lysine)")
Isolates yielding negative results for this test may be reported as negative for *E. ictaluri*.

7. Manitol Utilization Test (Section 2, 3.8.D.1.e "Carbohydrate Utilization")

a. **Isolates yielding positive results for this test may be reported as negative for *E. ictaluri*.**

b. Isolates yielding negative results for this test and that satisfy all previous conditions in this section are **PRESUMPTIVELY positive** for *E. ictaluri.*

ii. Commercial Identification Systems (Section 2, 3.8.D.2 "Commercial Identification Systems")

1. Biolog (Section 2, 3.8.D.2.b "Biolog")

2. API
If isolates are tested with the commercial system API described in Section 2, 3.8.D.2.a "API-20E," it is recommended that the reference profiles be consulted in Section 2, 3.A2 Profiles Obtained with API-20E for Known Fish Pathogens Appendix 2."

3. When testing is complete, either cryopreserve isolates of interest, or discard bacterial plates and biochemical tubes in a biohazard bag and autoclave before proper disposal.

4. Positive control isolates of *Edwardsiella ictaluri* can be obtained from the American Type Culture collection (ATCC). The Internet location for ATCC is http://www.atcc.org.

B. Summary of Confirmatory Test

1. **Fluorescent Antibody Test (FAT)** (Section 2, 3.8.E "Fluorescent Antibody Test (FAT)")
FAT is performed on at least one representative isolate from each inspection. Positive bacterial isolates will fluoresce strongly and have the same morphology as the positive control. A list of sources from which antibodies may be obtained is provided in Section 2, 3.8.E.6 "Commercial Sources for Antibodies."

3.5 *Renibacterium salmoninarum* (Bacterial Kidney Disease, BKD)

Renibacterium salmoninarum infections can occur at any life stage in salmonid populations. Clinical signs of disease are uncommon in fish less than six months of age. Mortality has been reported at water temperatures between 4°C and 20.5°C, with the disease progressing rapidly between 15°C to 20.5°C. Acute and sub-acute forms of disease are less common than the more typical chronic form of disease, characterized internally by a large edematous kidney that appears gray and corrugated (Austin and Austin1987; Bullock and Herman 1988; Thoesen 1994).

A. Summary of Screening Test

1. **Fluorescent Antibody Test (FAT)** (Section 2, 3.8.E "Fluorescent Antibody Test (FAT)")

   a. Collect tissues as described in Section 2, 2.2 Sampling and prepare FAT slides (**Exception:** Anadromous salmonids regularly monitored for *R. salmoninarum* with ELISA, quantitative PCR, or MFAT techniques may be considered positive without additional testing by FAT).

      i. Kidney
      Prepare kidney smear on a non-coated or acetone-cleaned glass slide.

         1. Place a piece of posterior kidney or homogenized preparation on the slide.

         2. Create a thin smear on the surface of the glass slide.

      ii. Ovarian Fluid Pellet Smear

         1. After pooled ovarian fluid samples are processed and the appropriate amount of supernatant removed for virology assays (see Section 2, 4.4.C "Processing of Coelomic (Ovarian) Fluid Samples"), the pellet is re-suspended in the remaining ovarian fluid by thorough vortexing or repeat pipetting.

         2. Transfer two 1.5 mL aliquots from each original pool of ovarian fluid (or 0.5 mL per fish) to sterile, labeled 1.5 mL microcentrifuge tubes (see **Note**). Freeze the remainder of the sample at -20°C for PCR confirmation.

         3. Centrifuge the 1.5 mL aliquots at 10,000 X g for 15 minutes (see **Note**).

         4. The pellet is carefully removed with a small amount of supernatant using a sterile pipette and a thin smear is prepared on a glass slide. Pellets originating from the same ovarian fluid sample tube may be combined on one slide.

Note: Elliott and McKibben (1997) document the importance of using a minimum of 10,000 X g for sufficient sedimentation of *R. salmoninarum* from 0.5 mL of ovarian fluid collected from individual fish. Enough ovarian fluid must be transferred from each pool to represent 0.5 mL for each fish represented in the pool (i.e. 2.5 mL from a five-fish-pool). Most 15 mL centrifuge tubes used for collection and processing of ovarian fluid samples cannot be centrifuged at a relative centrifugal force (rcf) as high as 10,000 X g. It is, therefore, necessary to aliquot the appropriate amount of fluid into microcentrifuge tubes suitable for the required rcf.

b. After the tissue smear is heat fixed or air dried, slides are fixed in acetone for five minutes.

c. Stain slides with FITC-conjugated *R. salmoninarum* antisera as described in Section 2, 3.8.E.2 "Direct FAT (DFAT) Staining."

d. Examine at least 50 fields using oil immersion at 1000X magnification to detect the 1.0 X 0.5 µm bacterial cells, which should appear stained as an "apple green" fluorescence.

   i. **Smears which do not show any fluorescent bacterial cells may be discarded and reported as negative for *R. salmoninarum*.**

   ii. Any smears, which have "apple green" fluorescent, diplobacilli bacterial cells present measuring approximately 1.0 X 0.5 µm, shall be considered **PRESUMPTIVELY positive** for *R. salmoninarum.*

e. It is preferable to use tissue infected with *R.salmoninarum* for a positive FAT control. Positive control culture isolates of *Renibacterium salmoninarum*, however, can be obtained from the American Type Culture collection (ATCC). The Internet location for ATCC is http://www.atcc.org. Suppliers of commercially prepared antibodies for FAT may also provide positive control materials for use in this assay (see Section 2, 3.8.E.6 "Commercial Sources for Antibodies").

B. Confirmatory Tests

1. Bacterial Culture (Austin et al. 1983)

a. At the time samples are collected during the inspection (Section 2, 2.2.E.2 "Collection of Kidney Cultures"), aseptically inoculate samples of tissues onto plates containing selective kidney disease media (SKDM-2) (Section 2, 3.7.A.3 "Selective Kidney Disease Medium-2 (SKDM-2)").

b. Incubate for 2 to 3 weeks at 15^{o}C in a humid chamber to prevent dehydration of media.

c. At 2 to 3 weeks, observe plates for growth of pinpoint bacterial colonies.

   i. If *R. salmoninarum* is presumptively identified in FAT, corresponding samples inoculated onto SKDM-2 should be examined weekly.

   ii. If no growth, continue to incubate plates for up to six weeks, and examine them several times per week for growth.

iii. **If no growth after six weeks, samples may be discarded and reported as negative for *R. salmoninarum*.**

iv. If growth of small (2 mm diam.), smooth, white round colonies is observed, obtain inoculum from colony and confirm identification using FAT or PCR.

1. **If FAT or PCR results on culture are positive, sample is reported as positive for *R. salmoninarum*.**

2. **If FAT or PCR results on culture are negative, sample is reported as negative for *R. salmoninarum***

Note: The slow growth of this organism makes phenotypic characterization of suspect isolates difficult and time consuming. The inspector may consider pursuing phenotypic characterization if the detection of *R. salmoninarum* by these techniques continues to be questionable (consult Austin and Austin 1993).

2. Nested Polymerase Chain Reaction (PCR) for Confirmation of *R. salmoninarum* DNA

The polymerase chain reaction technique employs oligonucleotide primers to amplify segments of the gene that codes for the 57 kDa protein of *R. salmoninarum* (Chase and Pascho 1998). DNA is extracted from fish tissues and amplified initially with forward and reverse primers. The amplified DNA product is then re-amplified using a "nested PCR" technique. The DNA products from both amplifications are then visualized by agarose gel electrophoresis. The following procedures have been adapted from those of Chase and Pascho (1998), and have been reviewed and approved by the authors.

a. Extraction of DNA from Kidney and Ovarian Tissues
(**NOTE**: The following procedure employs an extraction kit available from Qiagen, Inc. (http://www.qiagen.com). DNA Extraction kits of similar efficacy are available from many other sources, and can be utilized as alternatives for extraction of DNA in this protocol. These kits utilize "spin columns" for binding and elution of DNA from tissue lysates. Most do not require the use of highly toxic reagents and reduce the chance of contamination during extraction).

i. Procedures

1. Transfer 25 to 50 mg of kidney tissue, or 50 µL ovarian fluid, into a 1.5 mL microcentrifuge tube. Tissue can be fresh or previously frozen.

2. Add 180 µL of lysozyme lysis buffer (Section 2, 3.7.G.1 "Lysozyme Lysis Buffer"). Incubate at 37°C for one hour, vortexing occasionally.

3. Add 25 µL of Proteinase K stock solution and 200 µL of buffer AL lysis buffer (provided by extraction kit manufacturer), then mix by vortexing and incubate at 70°C for 30 minutes. Vortex occasionally. Tissues should be well lysed by the end of 30 minutes.

4. Incubate at 95°C for another 10 minutes. Vortex occasionally.

5. Add 210 µL of ethanol, mix thoroughly on vortex.

6. Place a spin column in a 2 mL collection tube. Place sample mixture over the filter in the spin column, being careful not to moisten the rim of the column. Close the cap and centrifuge at 6000 x g for 1minute at room temperature.

7. Place the spin column in a clean 2 mL collection tube and discard the tube containing the filtrate.

8. Carefully open spin column and add 500 µL buffer AW1 (wash buffer provided by kit manufacturer). Centrifuge again as described above.

9. Repeat steps 7 and 8, using 500 µL buffer AW2. Centrifuge at full speed for three minutes to dry the membrane.

10. Place spin column in clean 1.5 mL micro centrifuge tube and add 200 µL of buffer AE (elution buffer provided by kit manufacturer) for elution of DNA (TE buffer pH 8.0 or water can also be used). Incubate for five minutes at room temperature. Centrifuge at 6000 x g for one minute.

11. Repeat step 10 so that the total volume of DNA is 400 µL. Discard spin column and store DNA solution at -20-70^{o}C until use.

12. Quantify the amount of DNA extracted with a spectrophotometer (see Section 2, Chapter 6 Polymerase Chain Reaction (PCR)).

a. 25 to 50 mg of fish kidney tissue should produce between 100 and 300 ng DNA per µL using this procedure. A greater concentration of DNA should be diluted with elution buffer before performing PCR.

b. 50 µL ovarian fluid produces a much lower amount of DNA per µL using this procedure. Dilute the template if DNA exceeds 300 ng/µL.

b. Initial Amplification of *R. salmoninarum* DNA

i. General QA/QC considerations must be considered before performing PCR (see Section 2, Chapter 6 Polymerase Chain Reaction (PCR)).

ii. Procedures for initial round:

1. Using Section 2, 3.A3.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.

2. Using Section 2, 3.A3.B Worksheet B – Amplification of Nucleic Acid by PCR for the Confirmation of *Renibacterium salmoninarum*, record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according to the number of samples to be processed and the amount of MM needed per reaction (40 µL). Add 4 to the number of samples so that there is enough to run controls.

3. Under UV cabinet, add PCR reagents **except for sample DNA** to the MM tube in the order listed on Section 2, 3.A3.B Worksheet B – Amplification of Nucleic Acid by PCR for the Confirmation of *Renibacterium salmoninarum*, adding water first and Taq last.

Keep all reagents cold during mixing, and return them to freezer immediately after use. Do not expose enzymes, Primers, or dNTP's to UV light.

a. Water to make a 40 µL total volume per reaction.

b. PCR Buffer mix (1X).

c. $MgCl_2$ (1.5 mM per reaction).

d. dNTP mix (0.2 mM per reaction).

e. Primers (20 pmole each per reaction).

i. Forward 5' – A GCT TCG CAA GGT GAA GGG – 3'

ii. Reverse 5' – GC AAC AGG TTT ATT TGC CGG G – 3'

f. TAQ polymerase (2 units per reaction).

4. Place 40 µL of MM into each 0.5 mL PCR tube and close caps tightly. Move PCR tubes to sample loading area.

5. In sample loading area, load 10 µL of each sample DNA to the appropriately labeled PCR tubes. Close caps tightly. Remove sample tubes from UV cabinet to thermocycler.

6. Load the sample tubes into the thermocycler wells.

7. Thermocycler should be programmed for 30 to 40 cycles of the following temperature regime and recorded on Section 2, 3.A3.B Worksheet B – Amplification of Nucleic Acid by PCR for the Confirmation of *Renibacterium salmoninarum*:

a. Preheat sample to 94°C for two minutes.

b. Denaturing at 93°C for 30 seconds.

c. Annealing at 60°C for 30 seconds.

d. Extending at 72°C for one minute.

e. Post dwell at 4 to 16°C for holding samples after cycling is complete.

c. "Nested" PCR-secondary amplification of *R. salmoninarum* DNA
Materials, methods, and general QA/QC considerations of this section and Section 2, Chapter 6 Polymerase Chain Reaction (PCR) also apply to the nested PCR process:

i. Using Section 2, 3.A3.C Worksheet C – Nested Amplification of Nucleic Acid by PCR for the Confirmation of *Renibacterium salmoninarum*, record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples and controls to be processed and the amount of MM needed for each reaction (49 µL).

ii. Add PCR reagents except the first round amplified DNA into the Master Mix (MM) tube. Return reagents to freezer.

1. Primers

a. Forward 5' – AT TCT TCC ACT TCA ACA GTA CAA GG – 3'

b. Reverse 5' – C ATT ATC GTT ACA CCC GAA ACC – 3'

iii. In PCR tubes (0.5 mL), pipette 49 µL of MM. Close caps tightly. Remove tubes from UV cabinet to amplified DNA area.

iv. Load 1 µL of amplified sample DNA into the appropriate PCR tubes.

v. Load PCR tubes into thermocycler wells.

vi. Program thermocycler for 10 to 20 cycles of the following regime:

1. Preheat sample to 94°C for two minutes.

2. Denaturing at 93°C for 30 seconds.

3. Annealing at 60°C for 30 seconds.

4. Extending at 72°C for one minute.

5. Post dwell at 4 to 16°C for holding samples after cycling is complete.

Note: PCR Products can be refrigerated for one month or frozen at -70°C for long-term storage.

d. Visualization of PCR Product by Electrophoresis
See Section 2, Chapter 6 Polymerase Chain Reaction (PCR) for general procedures.

i. Visualization of amplified products resulting from PCR for detection of *R. salmoninarum* DNA is best accomplished after electrophoresis on a 1.5 or 2% agarose gel (Section 2, 6.3.C "Detection of Product").

ii. Using Section 2, 3.A3.C Worksheet C – Nested Amplification of Nucleic Acid by PCR for the Confirmation of *Renibacterium salmoninarum*, record location of each sample on the agarose gel at the time samples are loaded.

iii. After electrophoresis, stain gel with ethidium bromide and visualize on an UV transilluminator.

iv. Carefully record locations of bands on positive control samples in relation to DNA ladder bands.

1. Band locations of positive controls should be at anticipated locations according to primers used in both the first and second (nested) round PCR assays (first round primer M21=383bp; nested primer M38=320 bp). **Bands occurring at these locations are confirmatory for *R. salmoninarum* and are reported as POSITIVE.**

2. Note any unusual band occurrences. Negative controls should not have any bands. Suspicion of contamination indicates PCR should be re-run on samples from template DNA tube.

e. Document the electrophoresis results (Section 2, 6.3.G "Visualize the DNA"). Photograph all gels and attach the photo to Section 2, 3.A3.G Worksheet G – Photodocumentation of the PCR Product Gel. Attach to case history information**.**

3.6 *Piscirickettsia salmonis*

Piscirickettsia salmonis has primarily been reported as an emerging marine pathogen of salmonids (Bravo and Campos 1989; Fryer et al. 1990; Cvitanich et al. 1991; Brocklebank et al. 1992; Rodger and Drinan 1993; Grant et al. 1996; Cusack et al. 1997; Olsen et al. 1997), although there are reports of isolations in other species (Chen et al. 2000b) and in juvenile salmonids in freshwater (Bravo 1994; Gaggero et al. 1995). Additionally, there are an increasing number of reports on isolations of *P. salmonis*-like organisms from other fish species (Chern and Chao 1994; Chen et al. 1994; Chen et al. 2000a; Mauel et al. 2003). Clinical signs of disease and mortality have been reported in fish in marine net pens (Cvitanich et al. 1991; Branson and Diaz- Munoz 1991; Brocklebank et al. 1992; Olsen et al. 1997; Kent and Poppe 1998). The first evidence of disease may be the appearance of small white lesions or shallow hemorrhagic ulcers on the skin. Affected fish appear dark and lethargic. The major gross pathological changes are gill pallor, peritonitis, ascites, enlarged spleen, swollen gray kidney, and in some cases, a liver with large pale necrotic lesions.

A. Summary of Screening Tests

These techniques represent the best methods available for isolating and identifying *Piscirickettsia salmonis*, but they have not been demonstrated to be sensitive enough to detect a covert infection or subclinical infections. These methods are most effective in fish with clinical cases of infection.

1. **Culture of Obligate Intracellular Bacteria in Cell Culture** (Lannan and Fryer 1991)
   Piscirickettsia salmonis is a Gram-negative, non-motile, highly fastidious intracellular bacterial pathogen. *Piscirickettsia salmonis* does not replicate on bacteriological media, but instead it must be grown in cell culture, and therefore escapes detection by routine techniques used for bacterial isolation. Additionally, *in vitro*, this organism is sensitive to many antibiotics used in routine virus isolations, and will not grow even if inoculated onto suitable host cells if such compounds are included in the culture medium.

   a. Samples of kidney, spleen, liver and blood suitable for virology testing are aseptically collected from diseased fish during either overt or covert infections as described in Section 2, 2.2.E.3 "Collection of Tissues for the Detection of Viral Agents." DUE TO SENSITIVITY OF *P. SALMONIS* TO ANTIBIOTICS *IN VITRO*, NONE SHOULD BE USED IN MEDIA DURING COLLECTION OF TISSUE OR THE CULTURE OF CELLS and because no antibiotics will be used, stringent aseptic techniques should be used to collect tissues. Musculature underlying skin lesions may also be a suitable tissue if collected aseptically by removing the contaminated outer skin layer.

   b. Tissue should be homogenized at 1/20 (w/v) in sterile antibiotic-free HBSS. Do not centrifuge. *Piscirickettsia salmonis* cells are bound in membranes and centrifugation will remove the bacteria from the supernatant. The 1:20 homogenate should then be further diluted 1/5 and 1/50 in antibiotic-free HBSS. These final two dilutions of the homogenate are the inocula for cell cultures, and are 1:100 and 1:1,000 dilutions of the original tissue.

c. The diluted homogenate can be inoculated directly (0.1 mL/culture per well of a 24-well plate) into the antibiotic-free culture medium overlaying the CHSE-214 cell monolayer (refer to Section 2, 4.3 Cell Culture).

d. The cell cultures must be incubated at 15 to 18°C for 28 days and observed for the appearance of cytopathic effect (CPE). The piscirickettsial CPE consists of plaque-like clusters or rounded cells (Figure 3.1). With time, the CPE progresses until the entire cell sheet is destroyed. A drop of the suspect cell culture supernatant can be dried on a glass slide, fixed with methanol and stained with Giemsa stain (described below). Examine for presence of the bacteria.

e. If CPE does not occur (except in positive control) cultures should be incubated at 15 to 18°C for an additional 14 days.

f. If CPE is absent after the 42 day combined incubation period, samples are reported as negative and may be discarded using the proper decontamination procedures. If CPE associated with the bacteria occurs at any time during this assay, it is considered a PRESUMPTIVE positive and the identification of *P. salmonis* should be confirmed by the appropriate method.

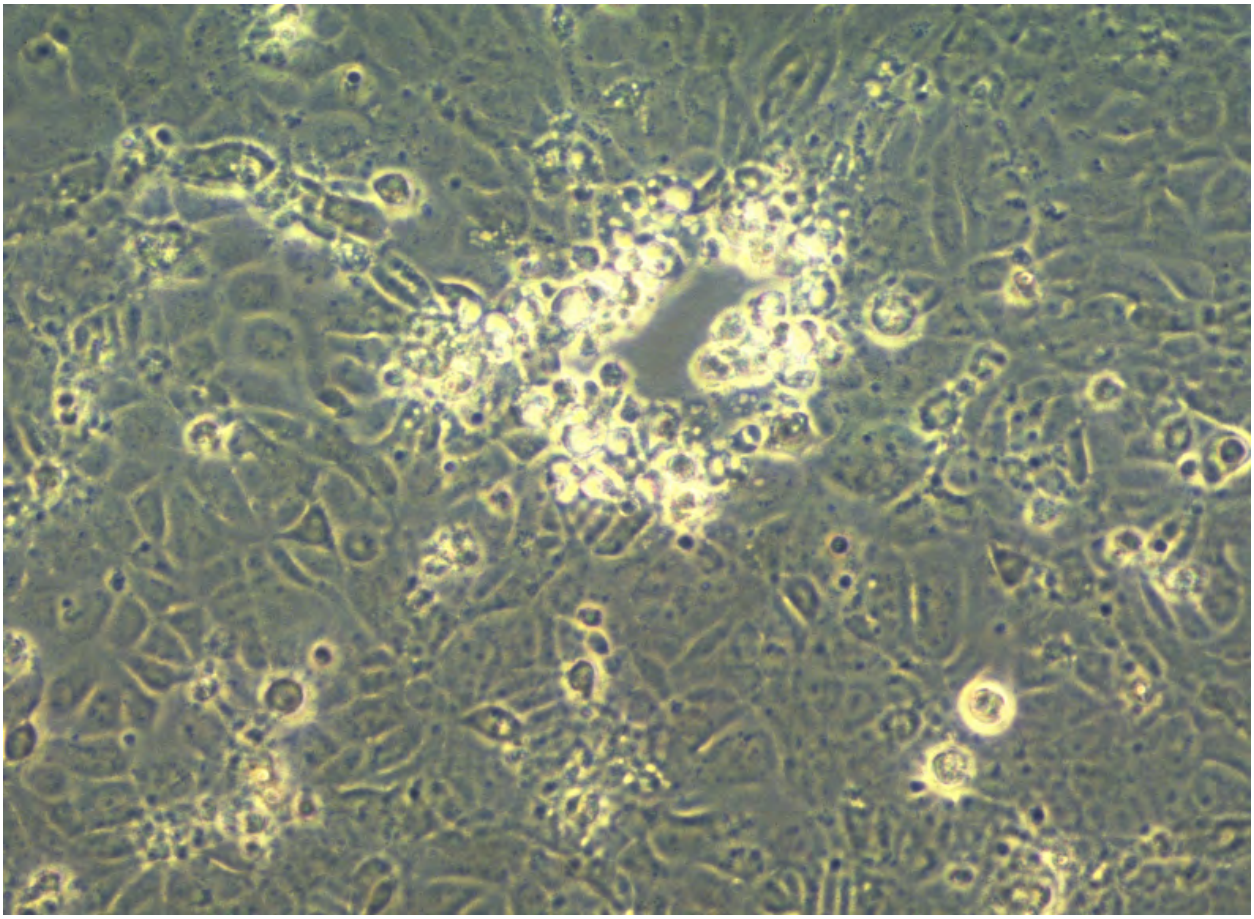


Figure 3.1. Cytopathic effect typical of *Piscirickettsia salmonis* on a monolayer of CHSE-214 cells. (Photo courtesy of Marcia House, NWIFC.)

2. Tissue Impressions of Kidney Stained with Giemsa Stain

Piscirickettsia salmonis can also be diagnosed based on clinical signs and detection in Giemsa stained tissue impressions, or sections (refer to **Note** below). It is pleomorphic, ranging from 0.5 to 1.5 µm in diameter and frequently is seen in pairs (Figure 3.2). Positive identification is confirmed by IFAT using *P. salmonis*-specific antibodies (Section 2, 3.8.E.3 "Indirect FAT (IFAT) Staining").

a. Preparations of tissue culture supernatant, smears or impressions of the kidney, liver, and spleen should be prepared, air-dried, and fixed for five minutes in absolute methanol.

b. Immerse slides in working solution of Giemsa stain for 30 minutes. Stock solution: 0.4 w/v in buffered methanol solution, pH 6.9 (commercially available). Working solution: diluted 1:10 in phosphate buffer pH 6.0 (0.074M NaH_2PO_4, 0.009M Na_2HPO_4).

c. Destain with tap water.

d. Observe slides under oil immersion. Examine a minimum of 50 fields. Tissue smears from infected organs show darkly stained pleomorphic organisms occurring in coccoid or ring forms, frequently in pairs, with a diameter of 0.5-1.5 µm (Figure 3.2). Visualization of organisms in smears is considered a PRESUMPTIVE positive result and the identification of *P. salmonis* should be confirmed by the appropriate confirmatory method.

Note: Every effort should be made to culture *P. salmonis* because it is the most sensitive screening method.

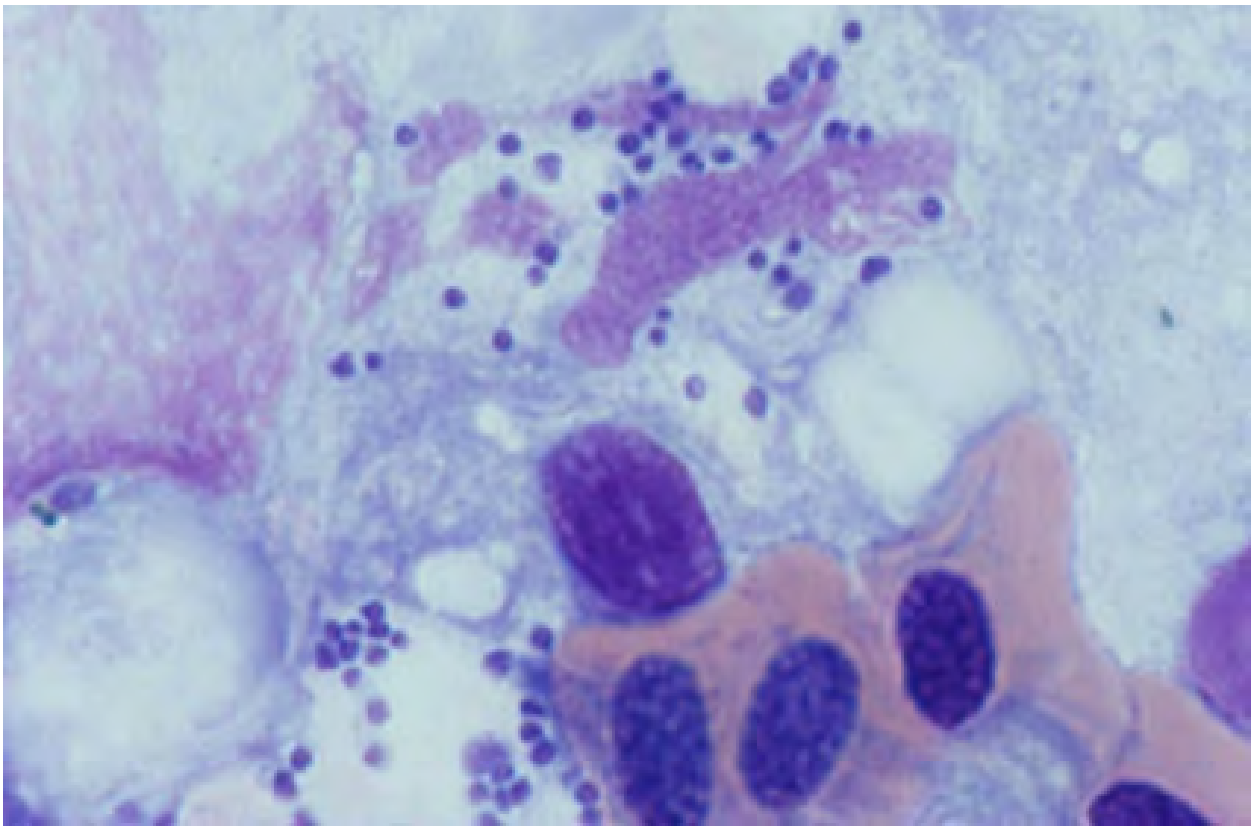


Figure 3.2. Giemsa stained liver impression from *Piscirickettsia salmonis* infected coho salmon (1000X magnification). Photo courtesy of Marcia House, NWIFC.

B. Confirmatory Tests

1. Serological Methods

The positive identity of *P. salmonis* isolated in cell culture or observed in Giemsa stained smears may be determined by serological methods, including IFAT or immunocytochemistry of histological sections.

a. Indirect Fluorescent Antibody Test (IFAT) (refer to Section 2, 3.8.E.3 "Indirect FAT (IFAT) Staining") (Lannan et al. 1991)

i. Smears or impressions of the kidney, liver, and spleen should be prepared, air-dried, and fixed for five minutes in absolute methanol.

ii. Tissues smears to be examined by IFAT must be freshly prepared or stored at -20°C.

iii. The sample is first incubated with anti-*P. salmonis* polyclonal or monoclonal antibody, then washed and incubated with a secondary antibody conjugated with fluorescein isothiocyanate (FITC).

iv. Following washing, apply glycerol-based mounting media and coverslip, then examine with a microscope equipped with epi-fluorescence.

b. Immunohistochemistry of Tissue Sections (Alday-Sanz et al. 1994)\

i. Sections (5 µm) of formalin fixed, paraffin-embedded tissues are deparaffinized and treated to eliminate endogenous peroxidase activity.

ii. The tissue is first incubated with anti-*P. salmonis* polyclonal or monoclonal antibody, then washed and incubated with a secondary antibody conjugated with horse-raddish peroxidase.

iii. Following washing, the tissue is exposed to a chromagen (hydrogen peroxide and 3,3-diaminobenzidine), counterstained with hematoxylin, dehydrated and prepared for examination under a light microscope.

2. Polymerase Chain Reaction (PCR)

A nested polymerase chain reaction (PCR) has been developed to detect genomic DNA of *P. salmonis* using general bacterial 16S rDNA primers in the first amplification and *P. salmonis*-specific primers in a second reaction. A direct (single amplification) PCR can be run using the *P. salmonis* specific primers.

a. Preparation of Infected Cell Culture Supernatant or Tissue
Use of a commercially available spin column to purify DNA from cell culture supernatant or tissue is recommended for PCR sample preparation. In addition to following the manufacturer's instructions on use of the columns, initial digestion of the sample in lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, 4 mg/mL lysozyme) at 37°C for 30 minutes is suggested (refer to Section 2, 3.5.B.2 "Nested Polymerase Chain Reaction (PCR) for Confirmation of *R. salmoninarum* DNA").

i. The cell culture supernatant preparation:

1. Triturate the overlying cell culture media to disrupt the suspect cell culture, as if you were preparing a re-inoculation in a virology assay.

2. Transfer 0.2 mL of the suspension to a clean sterile 1.5 mL microcentrifuge tube and centrifuge at 4°C.

3. Discard the supernatant and treat the pellet as a tissue sample in the extraction procedure.

b. Nested Polymerase Chain Reaction (Mauel et al. 1996)

i. Initial amplification using eubacterial primers:

1. General QA/QC considerations must be reviewed before performing PCR (see Section 2, Chapter 6 Polymerase Chain Reaction (PCR) for more specific QA/QC considerations for PCR).

2. Procedures for the initial round:

a. Using Section 2, 3.A3.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.

b. Using Section 2, 3.A3.D Worksheet D – Initial Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis*, record the date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according to the number of samples to be processed and the amount of MM needed per reaction (45 µL). Add four to the number of samples so that there is enough to run controls.

c. Under a UV cabinet, add PCR reagents except for sample DNA to the MM tube in the order listed in Section 2, 3.A3.D Worksheet D – Initial Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis*, adding water first and Taq last. Keep all reagents cold during mixing, and return them to the freezer immediately after use. Do not expose enzymes, primers, or dNTP's to UV light.

i. Water to make a 45 µL total volume per reaction.

ii. 1X PCR buffer (10 mM Tris HCl, pH 9, 50 mM KCl, and 0.1% Trition X100).

iii. 1.5 mM $MgCl_2$.

iv. dNTP mix (0.2 mM).

v. Primers (1 mM each)

1) EubA 5'-AAG-GAG-GTG-ATC-CAN-CCR-CA-3'

2) EubB 5'-AGA-GTT-TGA-TCM-TGG-CTC-AG-3'

vi. Taq polymerase (2.5 units per reaction).

d. Place 45 µL of MM into each 0.2 mL PCR tube and close the caps tightly. Move PCR tubes to sample loading area.

e. In sample loading area, load 5 µL of each sample DNA to the appropriately labeled PCR tubes. Close caps tightly. Remove sample tubes from UV cabinet to thermocycler.

f. Load the sample tubes into the thermocycler wells.

g. The thermocycler should be programmed for the following regime:

i. Denature the mixture at 94°C for two minutes.

ii. Amplify by 35 cycles of:

1) Denaturing at 94°C for one minute.

2) Annealing at 50°C for two minutes.

3) Extending at 72°C for three minutes.

4) Post-dwell at 4 to 15°C after cycling is complete.

ii. "Nested" PCR-secondary amplification using *P. salmonis* specific primers.

1. Materials, methods, and general QA/QC considerations of this subchapter and Section 2, Chapter 6 Polymerase Chain Reaction (PCR) also apply to the nested PCR process.

2. Procedures for the second round:

a. Using Section 2, 3.A3.E Worksheet E – Nested Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis*, record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according to the number of samples and controls to be processed and the amount of MM needed for each reaction (48 µL).

b. Add PCR reagents except the first round amplified DNA to the Master Mix (MM) tube. Return reagents to the freezer. The reaction mixture is the same as the initial with the exception of the primers, which are:

i. PS2S (223F) 5'-CTA-GGA-GAT-GAG- CCC-GCG-TTG-3'

ii. PS2AS (690R) 5'-GCT-ACA-CCT-GAA-ATT-CCA-CTT-3'

c. Place 48 µL of the MM into each PCR tube (0.2 mL) and close caps tightly. Remove the tubes from UV cabinet to amplified DNA area.

d. Load 2 µL of amplified sample DNA into the appropriate PCR tubes.

e. Load the tubes into the thermocycler wells.

f. Thermocycler should be programmed for the following regime:

i. Denature the mixture at 94°C for two minutes.

ii. Amplify by 35 cycles of:

1) Denaturing at 94°C for one minute.

2) Annealing at 65°C for two minutes.

3) Extending at 72°C for three minutes.

4) Post-dwell at 4 to 15°C after cycling is complete.

c. Direct (single round) polymerase chain reaction.

i. Materials, methods and general QA/QC considerations of this subchapter and Section 2, Chapter 6 Polymerase Chain Reaction (PCR) also apply to the direct PCR process.

1. Using Section 2, 3.A3.E Worksheet E – Nested Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis*, record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according to the number of samples and controls to be processed and the amount of MM needed for each reaction (45 µL). Add four to the number of samples so that there is enough to run controls.

a. Add PCR reagents except the first round amplified DNA into the Master Mix (MM) tube. Return reagents to the freezer. The reaction mixture is the same as the nested with the exceptions of the volume of water and primers. The primers are:

i. PS2S (223F) 5'-CTA-GGA-GAT-GAG- CCC-GCG-TTG-3'

ii. PS2AS (690R) 5'-GCT-ACA-CCT-GAA-ATT-CCA-CTT-3'

b. Place 45 µL of the MM into each PCR tube (0.2 mL) and close caps tightly. Remove the tubes from UV cabinet to amplified DNA area.

c. Load 5 µL of sample DNA into the appropriate PCR tubes.

d. Load the tubes into the thermocycler wells.

e. The therrmocycler should be programmed for the following regime:

i. Denature the mixture at 94°C for two minutes.

ii. Amplify by 35 cycles of:

1) Denaturing at 94°C for one minute.

2) Annealing at 65°C for two minutes.

3) Extending at 72°C for three minutes.

4) Post-dwell at 4 to 15 °C after cycling is complete.

d. Visualization of PCR product by electrophoresis (see Section 2, Chapter 6 Polymerase Chain Reaction (PCR) for general procedures).

i. Visualization of amplified products resulting from PCR for detection of *P. salmonis* DNA is best accomplished after electrophoresis on a 2% agarose gel (Section 2, 6.3C "Detection of Product").

ii. Using Section 2, 3.A3.G Worksheet G – Photodocumentation of the PCR Product Gel, record location of each sample on the agarose gel at the time samples are loaded.

iii. After electrophoresis, stain gel with ethidium bromide and visualize on an UV transilluminator.

iv. Carefully record locations of bands on positive control samples in relation to DNA ladder bands.

1. Band locations of positive controls should be at anticipated locations according to primers used in both the first (EubA/ EubB) and second (PS2S/ PS2AS) round PCR assays (first round (EubA/ EubB) band = 1540 bp, nested and direct PCR (PS2S/ PS2AS) = 476 bp). **Bands occurring at the PS2S/ PS2AS product locations are confirmatory of *P. salmonis* and are reported as POSITIVE.**

2. Note any unusual band occurrences. Negative controls should not have any bands. Suspicion of contamination indicates that PCR should be re-run on samples from the extracted DNA tube.

v. Document the electrophoresis results (Section 2, 6.3.G "Visualize the DNA"). Photograph all gels and attach the photo to Section 2, 3.A3.G Photodocumentation of the PCR Product Gel. Attach to case history information.

Note: Other PCR assays have been developed to detect *P. salmonis* (Marshall et al. 1998). These primer sequences and reaction conditions would also be suitable for confirmation of the presence of *P. salmonis*. Furthermore, additional diagnostic tests, such as transmission electron microscopy (TEM) and enzyme-linked immunosorbent assay (ELISA) (Aguayo 2001) may also aid in the detection of *P. salmonis*.

3.7 Reagents, Media, and Media Preparation

A. Growth Media

Most bacteriological culture medias are commercially available as pre-made or as bases, which can be easily prepared in the laboratory. These commercial products are entirely acceptable and should be made and stored according to the manufacturer's recommendations. Recipes for those media which are not available in a commercial preparation are included below.

1. **Brain Heart Infusion Agar (BHIA)** (Difco 1998)
   A basic agar for most bacterial cultures.

2. **Tryptic Soy Agar (TSA) or Tryptic Soy Broth (TSB)** (Difco 1998)
   A basic media for most bacterial cultures.

3. **Selective Kidney Disease Medium-2 (SKDM-2)** (Austin et al. 1983)
   A media used for selective isolation of *Renibacterium salmoninarum.*

Peptone	10g
Yeast extract	0.5g
L-Cysteine HCL	1g
Agar	15g
Distilled water	874mL

Adjust pH to 6.5 before adding agar. Autoclave for 15 minutes at 121^{o}C. Cool to ~ 50^{o}C and add:

Fetal Bovine Serum	100.0 mL

The following antibiotics are added to reduce overgrowth from other bacterial organisms):

4.0 mL Cycloheximide (1.2 g Cyclohexamide in 96 mL dH_2O)
1.0 mL D-Cycloserine (0.3 g D-Cycloserine in 24 mL of dH_2O)
2.0 mL Polymyxin B-sulfate (0.3 g Polymyxin B-sulfate in 24 mL of dH_2O)
1.0 mL Oxolinic acid (0.06 g Oxolinic acid in 24 mL of 5% NaOH)

B. Media to Identify Growth and Biochemical Characteristics

Most of these media are commercially available as pre-made or as bases, which can be easily prepared in the laboratory. These commercial products are entirely acceptable and should be made and stored according to the manufacturer's recommendations. Unless otherwise specified, these media can also be prepared from basic laboratory ingredients according to recipes found in the following references: MacFaddin 1980, Difco 1998, and MacFaddin 2000.

1. Motility Test Medium

A semi-solid media used as a tube test to detect the ability of a microorganism to exhibit motility. Several types are commercially available, including MIO (motility, indole, ornithine) which allows for the detection of motility, and the reaction of two biochemical tests in the same tube.

2. Trytic Soy Broth (TSB)

A nutrient broth media used to determine motility of a microorganism with the hanging drop method.

3. Triple Sugar Iron Agar (TSI)

A commercially prepared dehydrated media used to evaluate the utilization of glucose and two additional carbohydrates, as well as the production of hydrogen sulfide.

4. Oxidation/Fermentation (OF) Medium

A basal media for carbohydrate utilization tests, available in a commercially prepared dehydrated powder. The OF basal is prepared according to manufacturer's recommendations prior to the addition of individual carbohydrates as described below:

a. To prepare final medium aseptically add 10 mL of a filter-sterilized (0.45 µm) 10% carbohydrate solution to autoclaved and cooled (50°C) media resulting in a 1% final concentration, with the exception of salicin, which should be made as a 5% solution resulting in a 0.5% final concentration (see below). Only one carbohydrate is added to the basal medium for each test to be run.

10% Arabinose (1 g Arabinose to 10 mL in dH_2O)
10% Rhamnose (1 g Rhamnose to 10 mL in dH_2O)
10% Sucrose (1 g Sucrose to 10 mL in dH_2O)
10% Sorbitol (1 g Sorbitol to 10 mL in dH_2O)*
10% Maltose (1 g Maltose to 10 mL in dH_2O)
10% Glucose (1 g Glucose to 10 mL in dH_2O)
5% Salicin (0.5 g Salicin to 10 mL in dH_2O)

b. Mix flask thoroughly and aseptically dispense into sterile tubes. Store at 2 to 8°C. Final pH = 6.8 $\pm$ 0.2 at 25°C.

*A sorbitol utilization slant media can also be prepared and utilized as described in Cipriano and Pyle (1985).

5. Nutrient Gelatin

A dehydrated medium for determining the presence of Gelatinase.

6. Tryptone Broth

For use with the indole test.

Tryptone	10 g
Distilled water	1000 mL

Heat gently to dissolve. Dispense into tubes. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool. Store at 2 to 8°C.

7. Decarboxylase Medium Base

A basal media for use in Lysine test. The basal media, without addition of lysine, serves as the control.

To make L-Lysine media add 5 g L-Lysine to 1 liter of prepare basal decarboxylase media. Dispense into tubes. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool. Final pH = 6.8 $\pm$ 0.2 at 25°C. Store at 2 to 8°C.

8. Malonate Broth
A media used for the malonate test.

9. Bile Esculin Agar
A commercially prepared, dehydrated medium used to determine a bacterium's ability to hydrolyze esculin into glucose and esculetin.

C. Media Preparation

1. Plate Media

a. Prepare media in stainless steel beakers or clean glassware according to manufacturer instructions. Check pH and adjust if necessary. Media must be boiled for one minute to completely suspend agar. Use of a stir bar will facilitate mixing of agar.

b. Cover beaker with foil or pour into clean bottles being sure to leave lids loose. Sterilize according to manufacturer's instructions when given, or at 121^{o}C for 15 minutes at 15 pounds pressure (consult with the manual provided by the autoclave manufacturer for adjustment of time when large volumes of media are being sterilized).

c. Cool media to 50°C.

d. Alternatively, media can be autoclaved and cooled to room temperature and refrigerated for later use. Store bottles labeled with media type, date, and initials. When media is needed, boil, microwave, or use a water bath to completely melt the agar. Cool to 50°C.

e. Before pouring media, disinfect hood or counter thoroughly and place sterile petri dishes on the disinfected surface. Mix any added ingredients into the media at this temperature.

f. Label the bottom of each plate with medium type and date prepared.

g. Remove bottle cap and pour plates or dispense with a sterile Cornwall pipette, lifting each petri dish lid as you go. Pour approximately 15 to 20 mL per 100 X 15mm petri dish. Replace lids as soon as the plate is poured.

h. Invert plates when the media has cooled completely (~ 30 to 60 minutes) to prevent excessive moisture and subsequent condensation on the plate lid.

i. Allow plates to sit overnight at room temperature. Store plates upside down in the refrigerator in a tightly sealed plastic bag or plate storage tin.

j. Follow manufacturer's recommendation for storage period of prepared media. Each batch should be labeled with the date of preparation.

2. Tube Media

a. Prepare media in stainless steel beakers or clean glassware according to manufacturer's instructions. If the medium contains agar, boil for one minute to completely suspend the agar. Use of a stir bar will facilitate mixing of agar.

b. Media with indicators must be pH adjusted. This can be done when medium is at room temperature; otherwise, compensation for temperature needs to be made.

c. Arrange test tubes in racks. Disposable, autoclavable, screw cap tubes can be used for all tube media.

d. Use an automatic pipetter or Pipette-aid™ to dispense the medium. If using the Brewer or Cornwall pipette, prime with deionized water, then pump the water out of the syringe prior to pipetting and discard the first few tubes of media that are dispensed. Dispense approximately 5 to 10 mL media in 16 X 125 mm or 20 X 125 mm tubes. Close caps loosely.

e. Immediately after use, rinse the automatic pipetter in hot tap water followed by distilled water to remove all media and prevent clogging of the instrument.

f. Loosely place screw caps on tubes. Do not tighten caps. It is necessary to allow pressure to release from tubes while heating in the autoclave.

g. Follow manufacturer's recommendation for autoclave time and temperature.

h. If making slants, put tubes in slant racks after autoclaving. Adjust the slant angle to achieve the desired slant angle and butt length (i.e. long butt and short slant for TSI or a standard slant over ¾ of the tube length for TSA or BHIA). Then tighten caps.

i. Cool completely to room temperature in the slanted position.

j. Label the tubes or the tube rack with type of medium and date made.

k. Store at 2 to 8°C, following manufacturer's recommendation for period of long-term storage.

D. Reagents

Most of these reagents are commercially available pre-made. These commercial products are entirely acceptable and should be stored according to the manufacturer's recommendations. The formulations provided below were obtained from the references cited.

1. Gram Stain Reagents

These stains can be ordered as a complete kit or can be prepared as follows:

a. Crystal Violet

Crystal violet (90% dye content)	20.0 g
Ethanol (95%)	200 mL
Ammonium oxalate	8.0 g
dH_20	800 mL

Mix first two ingredients and let sit overnight or until dye goes into solution. Add remaining

ingredients and filter before use.

b. Gram's Iodine

Iodine crystals	1.0 g
Potassium iodide	2.0 g
dH_20	300 mL

c. De-Colorizer

Acetone	40 mL
Ethanol (95%)	60 mL

d. Safranin

Safranin O	2.5 g
95% ethanol	100 mL
dH_20	900 mL

Filter safranin solution before use.

2. FAT Mounting Fluid (pH 9.0)

Glycerol	90.0 mL
DABCO*	2.5 g
PBS	10.0 mL

Suspend the DABCO in glycerol over low heat. Then add 1X PBS (see below). Adjust pH to 8.6 to 9.0 with 1N hydrochloric acid or 0.1N sodium hydroxide. The pH of the mounting media is important, as an acid pH will quench fluorescence. Check the pH frequently. Store at room temperature.

* Optional ingredient - DABCO is 1,4-diazabicyclo-(2,2,2)-octane. Its addition to mounting fluid can reduce quenching of fluorescence.

3. Phosphate-Buffered Saline for FAT (PBS), pH 7.1

a. 1x concentration (0.15 M NaCl, 0.01 M phosphate; makes 1 L)

NaCl	8.50 g
Na_2HPO_4 (anhydrous)	1.07 g
$NaH_2PO_4 \cdot H_2O$ (monohydrate)	0.34 g
DH_2O	to 1 L

Adjust pH to 7.1 with 1N hydrochloric acid or 0.1N sodium hydroxide.

b. 5x concentration (makes 10 L of 1x PBS)

NaCl	85.00 g
Na_2HPO_4 (anhydrous)	10.70 g
$NaH_2PO_4 \cdot H_2O$ (monohydrate)	3.45 g
DH_2O	to 2 L

Adjust pH to 7.1 with 1N hydrochloric acid or 0.1N sodium hydroxide.

4. Kovac's Indole Reagent

Isoamyl alcohol	30 mL

p-Dimethyl aminobenzaldehyde	2 g
Hydrochloric acid (HCl)	10mL

Dissolve the aldehyde in the alcohol. Slowly add the acid to the mixture. Store solution at 2 to 8°C in amber dropper bottle.

5. Counter Stains

a. Rhodamine
Rehydrate the rhodamine to 1 mg/mL in distilled water. If the rhodamine does not completely dissolve, add a small drop of 0.1 M sodium hydroxide. Store at 2 to 8°C.

b. Evans Blue
Prepare 0.1% stock solution with distilled water and decontaminate with 0.45 µm membrane filtration. Store at room temperature. Prepare a 0.01% working dilution with sterile PBS (Section 2, 3.7.D.3 "Phosphate-Buffered Saline for FAT (PBS), pH 7.1") (Cvitanich 1994).

c. Eriochrome Black T
Prepare a solution at 1:60 (w/v) in PBS (Section 2, 3.7.D.3 "Phosphate-Buffered Saline for FAT (PBS), pH 7.1"), and filter through Whatman #1 and Whatman # 42 filter papers before initial use (Elliott & McKibben 1997).

6. FITC Conjugated Rabbit Anti-X /Rhodamine Counter Stain

Rhodamine stock	10.0 µL
FITC conjugated antibodies	10.0 µL
Phosphate buffered saline	480.0 µL

Store at 4°C.

Note: Evans Blue or Eriochrome Black T can also be used as counterstain in FAT in a separate step during the staining process, but neither can be added directly to the conjugate solution.

E. Cytochrome Oxidase Spot Test

Individual test strips can be purchased from several suppliers (Catalogue # 38-191, Remel, Tel. 800-255-6730).

F. Determination of Antiserum and Conjugate Working Dilutions for FAT

Commercially prepared anti-sera and conjugates should be reconstituted according to the manufacturer's instructions. Aliquots of 0.5 mL can be frozen for later dilution into a working solution of the reagent. Reagents are more stable if frozen as a stock solution. The proper working dilution is the highest dilution that still maintains maximum brightness of the fluorochrome on positive control FAT preparations. Generally, the manufacturer will recommend between 1:20 to 1:50 using a suitable buffer (PBS) as the diluent. However, in all cases, each laboratory must establish the proper working dilution by starting with the manufacturer's recommendation and bracketing, or testing dilutions on either side of the recommended concentration. The following example shows how to determine the correct working dilution of FAT conjugate where the manufacturer recommends a working dilution of 1:40.

1. Using the stock solution, dilute the antiserum at 1:20, 1:30, 1:40, 1:50, 1:60 using PBS or another buffer as recommended by antibody manufacturer.

2. Rhodamine counter stain is added directly to the optimum antiserum working dilution at a 1:50 (alternatively, Evans Blue may be used to counterstain FAT stained slides – do not add Evans Blue to the conjugate directly).

3. The FAT is performed on replicates of a known positive control, each replicate using a different dilution of the conjugated antiserum. In this way, the working dilution can be determined as the endpoint of optimum fluorescence (the highest dilution that still provides a bright specific fluorescence with little or no background staining).

4. Prepared conjugate solutions should be filtered through 0.2 to 0.4 micron filter prior to use and storage. Store frozen in small aliquots. Do not thaw and re-freeze antibodies repeatedly.

G. PCR Reagents

The following are formulations for extraction reagents used specifically in the protocols described in Section 2, 3.5.B.2 "Nested Polymerase Chain Reaction (PCR) for Confirmation of *R. salmoninarum* DNA."

1. Lysozyme Lysis Buffer

100 mL (this formulation is used for lysis of gram positive bacteria with Qiagen extraction kits).

Lysozyme	2 g
Tris HCl Stock	2 mL
EDTA stock	2 mL
Triton	1.2 mL

Bring these components to 100 mL with sterile distilled water (molecular grade).

2. Tris HCl Stock Solutions

100 mL at 1M pH 8.0 (for use in lysozyme buffer).

Trizma base	5.7 g
Tris HCl	8.9 g
dH2O	85.4 mL

3. EDTA Stock Solution

100 mL at 0.1 M (for use in lysozyme buffer).

EDTA	3.72 g

Bring to 100 mL with sterile distilled water (molecular grade).

3.8 Bacterial Identification Techniques

Unless otherwise specified, all of the materials and techniques described in 3.8 Bacterial Identification Techniques are described in detail in MacFaddin's (2000) and or the 11th Edition Difco Manual (1998). Each of the tests listed are provided with a set of control bacterial species available from ATCC, which will provide quality control for each biochemical test. It is not necessary, however, to set up control isolates for every test run in these protocols. It is strongly suggested that newly prepared batches of media and reagents be tested using the control bacterial isolates listed for each.

A. Gram Reaction

Gram staining detects a fundamental difference in the cell wall composition of bacteria.

1. Gram Stain
(Kits are available commercially, or formulas for reagents are listed in Section 2, 3.7.D "Reagents.")

a. Prepare a bacterial smear from a pure culture.

i. Put a drop of saline, distilled water, PBS (Section 2, 3.7.D.3 "Phosphate-Buffered Saline for FAT (PBS), pH 7.1"), or formalin saline (0.4% formalin, 0.85% NaCl) on a clean glass slide.

ii. Using a sterile loop or needle, touch an isolated colony and mix in the water drop.

iii. Mix until just slightly turbid (light inoculum is best, excess bacteria will not stain properly).

iv. Let air dry and heat fix. Do not overheat; slide should not be too hot to touch.

v. Allow to cool.

b. Flood the slide with crystal violet (Section 2, 3.7.D.1.a "Crystal Violet"), and allow to remain on the slide for 60 seconds.

c. Wash off the crystal violet with running tap water.

d. Flood the slide with Gram's iodine (Section 2, 3.7.D.1.b "Gram's Iodine"), and allow to remain on the slide for 60 seconds.

e. Wash off with running tap water.

f. Decolorize with decolorizer solution (Section 2, 3.7.D.1.c "De-Colorizer") until the solvent flows colorless from the slide (approximate 5 to 10 seconds). Excessive decolorization

should be avoided since it may result in a false gram-negative reading. Too little decolorization can result in a false positive result.

g. Rinse immediately with running tap water.

h. Counter stain with safranin (Section 2, 3.7.D.1.d "Safranin") for 60 seconds.

i. Rinse with tap water and allow to air dry.

j. Results

i. Gram-Negative
Cells are decolorized by the alcohol-acetone solution and take on a pink to red color when counter stained with safranin.

ii. Gram-Positive
Cells retain the crystal violet and remain purple to dark blue.

k. Quality Control
Use a known bacterial culture as a control for each case history to assess correct staining and interpretation (cultures can be obtained from ATCC and maintained at 2 to 8°C for long term use).

i. Positive
Staphylococcus sp. (ATCC – any isolate)

ii. Negative
Yersinia ruckeri

iii. Commercially prepared Gram stain control slides are available (Fisher Scientific, #08-801).

2. 3% Potassium Hydroxide
Alternative test for Gram reaction.

a. Add a heavy inoculum of a pure culture of bacteria grown on a solid medium to a drop of 3% potassium hydroxide (KOH) solution (3 grams KOH per 100 mL distilled water) on a clean glass slide.

b. Stir for about one minute, occasionally lifting the loop to look for thickening and "stringing" of the slurry.

c. Results

i. Gram-Positive
Bacteria will not appear to change the viscosity of the KOH solution.

ii. Gram-Negative
Bacteria will cause the KOH solution to become stringy or mucoid in appearance and consistency.

d. Quality Control
Use a known bacterial culture as a control for each case history to assess correct staining and interpretation (cultures can be obtained from ATCC and maintained at 2 to 8°C for long term use).

i. Gram-Positive
Staphylococcus sp.

ii. Gram-Negative
Yersinia ruckeri

B. Cytochrome Oxidase

See Section 2, 3.7.E "Cytochrome Oxidase Spot Test." This test determines the presence of cytochrome oxidase enzymes. The use of an iron-containing metal inoculation loop can lead to a false-positive reaction. Use only plastic or platinum loops for this test.

1. Add an inoculum of a pure 18 to 24 hour old bacterial culture to the surface of the test strip impregnated with reagent.

2. Results

a. Positive
Purple color within 5 to 10 seconds (reactions that occur after 10 seconds are negative).

b. Negative
No purple color.

3. Quality Control

a. Positive
Pseudomonas aeruginosa (ATCC 10145)

b. Negative
Yersinia ruckeri

c. Observe expiration dates of reagent strips.

C. Motility

This test determines if a bacterial isolate is motile by means of flagella.

1. Hanging Drop Method

a. Inoculate a tryptic soy agar (TSA) slant or tryptic soy broth (TSB (Section 2, 3.7.A.2 "Tryptic Soy Agar (TSA) or Tryptic Soy Broth (TSB) (Difco 1998)")) with the isolate. Note: Use

suitable medium for those organisms that do not grow on TSA or in TSB (e.g., yellow-pigmented organisms).

b. Incubate at room temperature until growth is obtained, usually 24 hours.

c. For isolates grown on agar, place a drop of sterile distilled water or PBS onto the center of a clean cover slip. Inoculate the center drop with pure strain culture using a sterile loop. For isolates grown in broth, use a sterile loop or sterile dropper and place a drop in the center of a clean clover slip.

d. Carefully invert the cover slip and place over the concave portion of a hanging drop slide.

e. Observe for motility at 400X magnification on a compound microscope. Care should be taken to not interpret "drift" or "Brownian motion" as motility.

f. Record results as motile or non-motile. Note: If the hanging drop slide was prepared from an isolate grown on agar and the bacterium appears to be non-motile, an additional hanging drop test using TSB (or other suitable broth medium) or semi-solid medium method must be done to confirm true non-motility.

2. Semi-Solid Medium Method
Refer to Section 2, 3.7.B.1 "Motility Test Medium."

a. Stab the semi-solid medium with a small amount of inoculum.

b. Incubate overnight at room temperature.

c. If the bacterial species is motile, the medium will become turbid with growth that radiates from the line of inoculum. If the bacterial species is non-motile, only the stab line will have visible bacterial growth.

d. Confirmation of results using the hanging drop method is recommended.

3. Quality Control

a. Positive
Escherichia coli (ATCC 25922)

b. Negative
Aeromonas salmonicida

D. Biochemical Testing

1. Tube Method

a. Glucose Fermentation
An OF basal medium (Section 2, 3.7.B.4 "Oxidation/Fermentation (OF) Medium") is used to test the fermentation of glucose by bacterial isolates.

i. With a sterile needle, inoculate two tubes of OF-glucose by stabbing to the bottom of the tube and then streaking the surface of the slant as the needle is drawn out of the tube. Screw the cap on loosely.

1. Fermentation Test
One tube is over-layed with sterile mineral oil or paraffin. Sterile petroleum jelly (heated to melting) should be used for more accurate observation of gas production.

2. Oxidation Test
The second tube is not overlayed.

	Fermentation Test Tube	**Oxidation Test Tube**
Fermentative	A or AG	A or AG
Oxidative	N	A or AG
Non-reactive	N	N

ii. Incubate at 20 to 24°C and read after 18 to 24 hours.

iii. Results
A = Acid (yellow)
AG = Acid + Gas
N = No change or Alkaline (green or blue-green).

iv. Quality Control

1. Fermentative
Aeromonas species

2. Oxidative
Pseudomonas fluorescens

b. Triple Sugar Iron (TSI)
TSI agar (Section 2, 3.7.B.3 "Triple Sugar Iron Agar (TSI)") contains the three sugars in varying concentrations: glucose (1X), which is a simple monosaccharide, and lactose and sucrose (10X each), both of which are disaccharides. It also contains the pH indicator phenol red. All organisms that utilize glucose will yield an initial acidic reaction throughout the tube (yellow - see below) regardless of whether they utilize sucrose or lactose. Reversion will not occur until all the glucose in the slant portion is completely utilized. At this point, the bacterium will utilize either one or both of the disaccharides, continuing the production of acids in the media (the slant remains yellow). If, after all the glucose in the slant is used and the bacterium cannot utilize either lactose or sucrose, the bacterium is forced to revert to protein (peptone) present in the agar. In this case, nitrogenous bi-products are produced and the pH in the media rises until the pH indicator shows a reversion from yellow (acid) to red (alkaline). Blackened medium is caused by hydrogen sulfide production, which changes ferrous sulfate to ferrous sulfide. In addition, splitting of the medium or presence of bubbles in the butt of the tube can determine gas production.

i. With a sterile needle, inoculate the TSI slant by stabbing to the bottom of the tube and then streaking the surface of the slant as the needle is drawn out of the tube. Screw the cap on loosely.

ii. Incubate at 20 to 24°C. Read after 18 to 24 hours.

iii. Results

A = Acid
K = Alkaline
H_2S= Hydrogen sulfide produced

Slant / Butt	Color	Interpretation
K / N or K / A	Red / Orange	Only Peptone Utilized
	Red/ Yellow	Only Glucose Fermented
A /A	Yellow /Yellow	Glucose plus Lactose and/or Sucrose Fermented
Gas	Splitting or Bubbles	Gas Production
H_2S	Black Butt	Hydrogen Sulfide Produced

N = No change

iv. Quality Control

1. K/A
Shigella flexineri (ATCC 12022)

2. A/AG
Escherichia coli (ATCC 25922)

c. Gelatinase
A test to determine bacterial production of gelatinase enzymes that liquefy gelatin.

i. Inoculate by stabbing ½ to 1 inch deep into the nutrient gelatin media (Section 2, 3.7.B.5 "Nutrient Gelatin") with a heavy inoculum from an 18 to 24 hour pure culture.

ii. Incubate 18 to 24 hours at 20°C.

iii. Results

1. Positive
Media is liquefied. Weak results can be visualized by rapping the tube against the palm of the hand to dislodge droplets of liquid from the media. Any drops seen are considered positive.

2. Negative
No liquefaction occurs in media.

iv. Quality Control

1. Positive
Proteus vulgaris (ATCC 8427)

2. Negative
Escherichia coli (ATCC 25922)

v. Precautions

1. The liquid will generally appear turbid due to bacterial growth.

2. Nutrient gelatin softens at temperatures above 20°C. Keep refrigerated until ready to inoculate, and do not let tubes reach room temperature or warmer. This will make interpretation of results difficult. Tests, which are incubated at 35°C, should be refrigerated prior to recording results.

d. Indole Test
A test to determine bacterial ability to split indole from the tryptophan molecule. Certain bacteria are able to oxidize the amino acid, tryptophan, with tryptophanase enzymes to form three indolic metabolites - indole, skatole (methyl indole), and indoleacetate. Indole, pyruvic acid, ammonia, and energy are principle degradation products of tryptophan. Indole, when split from the tryptophan molecule, can be detected with the addition of Kovac's reagent. The reagent is not a dye or stain, but reacts with indole to produce an AZO dye.

i. Inoculate tryptone broth (Section 2, 3.7.B.6 "Tryptone Broth") with a light inoculum from an 18 to 24 hour pure culture.

ii. Incubate 24 to 48 hours at 20°C

iii. At the end of 24 hours incubation do the following:

1. Aseptically remove 2 mL of media and place in an empty sterile test tube. Save extra tube for 48-hour incubation, if necessary.

2. Add about 5 drops of Kovac's reagent (Section 2, 3.7.D.4 "Kovac's Indole Reagent") to one of the tubes and agitate tube.

3. If a positive reaction is observed, the test is complete.

4. If the 24 hour incubated sample is negative, incubate the remaining tube for an additional 24 hours, and test again for the presence of indole with Kovac's reagent.

iv. Results

1. Positive
Within 1 to 2 minutes, a cherry red ring will form at the surface of the media.

2. Negative
No color formation is observed at the surface; the color remains that of the reagent – yellow.

3. Variable
An orange color may develop. This indicates the presence of skatole, which may be a precursor of indole formation.

v. Quality Control

1. Positive
Escherichia coli (ATCC 25922)

2. Negative
Pseudomonas aerugenosa (ATCC 27853)

vi. Precautions

1. Avoid inhaling fumes of Kovac's. Wear gloves to avoid skin contact.

2. Tests for indole should be conducted after both 24 and 48 hours of incubation before a test can be declared negative. Split the broth culture prior to performing the 24-hour test. If negative, incubate the untested tube (without Kovac's) for another day and try again.

3. Do not eliminate the 24-hour test, because some organisms may have produced indole by 24 hours, but have broken it down by 48 hours. DO BOTH!

4. Kovac's reagent should be fresh. A color change from yellow to brown indicates aging and results in reduced sensitivity of the test.

5. The procedure described here produces more reliable results than those obtained from MIO (motility-indole-ornithine) medium.

e. Carbohydrate Utilization (MacFaddin 1980)
The following carbohydrates are utilized to aid in bacterial species identification: Arabinose, Rhamnose, Mannitol, Salicin, Sorbitol, and Sucrose (saccharose). The procedures to be followed for each of these media are identical.

i. Inoculate carbohydrate tube (Section 2, 3.7.B.4 "Oxidation/Fermentation (OF) Medium") with growth from an 18 to 24 hour pure culture.

ii. Incubate with loosened cap 18 to 24 hours at 20^{o}C. A prolonged incubation of up to four days may be necessary for some negative results.

iii. Results

1. Positive
Acid is produced from fermentation, which turns media yellow.

2. Negative
No fermentation of carbohydrate, media remains green.

3. Aerogenic
Gas bubbles are present within the media.

iv. Quality Control

Carbohydrate	**Positive Control Isolate**	**Negative Control Isolate**
Arabinose	*Escherichia coli* (ATCC 25922)	*Yersinia ruckeri*
Sorbitol	*Escherichia coli* (ATCC 25922)	*Y. ruckeri* Type I
Rhamnose	*Enterobacter aerogenes* (ATCC)	*Yersinia ruckeri*
Salicin	*Enterobacter aerogenes* (ATCC)	*Yersinia ruckeri*
Maltose	*Yersinia ruckeri*	*Pseudomonas fluorescens*

v. Precautions

1. Difficulty in interpreting test results may occur with slow growing bacteria. Prolonged incubation may be required.

2. Heavy bacterial growth throughout the media can offset the color of a negative (green) reaction, giving the appearance of a weakly positive (yellow) reaction. This is especially true with yellow-pigmented bacteria. These tubes should be retested if a true yellow color is not noted within several days.

f. Decarboxylase Test (Lysine)
A determination of bacterial enzymatic capability to decarboxylate an amino acid to form an amine with resultant alkalinity.

i. For each isolate to be tested, it is necessary to inoculate a decarboxylase control tube and lysine test tube (Section 2, 3.7.B.7 "Decarboxylase Medium Base"). Use light inoculum from 18 to 24 hour pure culture.

ii. Add 1 to 2 mL oil overlay to each tube.

iii. Incubate 24 hours at 20°C. A prolonged incubation of up to four days may be necessary.

iv. Results

Test Result	**Lysine Tube**	**Control Tube**
Positive	Turbid to faded purple (glucose fermented, decarboxylase produced)	Yellow (glucose fermented)
Negative	Yellow (glucose fermented)	Yellow
Negative	Purple (glucose not fermented, decarboxylase not produced	Purple (glucose not fermented)

v. Quality Control

1. Positive
Yersinia ruckeri

2. Negative
Enterobacter cloacae (ATCC 13047)

vi. Precautions

1. At the end of incubation, the lysine tube might show a layer of purple over yellow. Gently shake the tube before interpreting the result.

2. An indistinct yellow-purple color may be difficult to interpret. Use the control tube for comparison. Any trace of purple color after a 24-hour incubation in the amino acid tube denotes a positive result.

3. Do not interpret tests prior to 18 to 24 hours. During the first 12 hours, only glucose is fermented which produces a yellow color. Decarboxylase enzymes do not form until the acidic environment is established by the fermentation of glucose.

g. Malonate Test
A method to establish if a bacterial isolate is able to utilize sodium malonate as its only source of carbon.

i. Inoculate malonate media (Section 2, 3.7.B.8 "Malonate Broth") with a light inoculum from an 18 to 24 hour pure culture.

ii. Incubate 24 to 48 hours at 20°C.

iii. Results

1. Positive
Light blue to deep blue color throughout the media.

2. Negative
Color remains the same as un-inoculated tube - green.

iv. Quality Control

1. Positive
Enterobacter aerogenes (ATCC 13048)

2. Negative
Yersinia ruckeri

v. Precautions
The test tube must be incubated for at least 48 hours before it may be called negative. Since some bacteria produce only slight alkalinity, it is useful to compare the test to an un-inoculated tube. Any trace of blue color denotes a positive reaction.

h. Esculin Test
To determine the ability of an organism to hydrolyze the glycoside esculin (aesculin) to esculetin (aesculetin) and glucose in the presence of bile (10 to 40%).

i. Inoculate the surface of the bile esculin slant (Section 2, 3.7.B.9 "Bile Esculin Agar") with inoculum from an 18 to 24 hour old pure culture.

ii. Incubate 20°C for 24 to 48 hours.

iii. Results. Note: Bile Esculin Agar was originally designed for isolation and presumptive characterization of Group D Streptococcus spp. As the bile salts inhibit nearly all other Gram-positive bacteria. The presence of bile in the medium does not affect differentiation of esculin by A. salmonicida; this medium is used because it is commercially available and easily prepared.

1. Positive

Dark brown to black color diffuses into the medium beginning at the site of inoculation. Often, the entire slant and butt become colored.

2. Negative

Bacterial growth is present on the slant, but the color of the medium remains similar to un-inoculated.

iv. Quality Control

1. Positive
Enterobacter aerogenes (ATCC 13048)

2. Negative
Yersinia ruckeri

v. Precautions
False positives may occur with hydrogen sulfide producing organisms, such as S*hewanella putrefaciens.* Neither of the target organisms for these protocols will, however, produce hydrogen sulfide.

2. Commercial Identification Systems

Several commercial test strips or kits are available for biochemical testing of bacteria. Bear in mind that these kits are designed for human and/or animal testing and the manufacturer's recommended incubation temperature is 37°C. The decreased incubation temperature (22°C room temperature) required for most fish pathogens results in slightly different reactions and longer incubation periods. Therefore, test results may not follow the manufacturer's identification profiles exactly. Therefore, it may be necessary to refer to the charts in Section 2, 3.A2 Profiles Obtained with API-20E for Known Fish Pathogens Appendix 2 for identification when API strips are employed.

a. API 20E™
The API 20E™ system is a standardized, miniaturized version of conventional procedures for the identification of *Enterobacteriaceae* and other Gram-negative bacteria. It is a microtube system designed for the performance of 23 standard biochemical tests from isolated colonies on plating medium. Refer to the instructions enclosed with each kit for more detailed information. The API system™ is available from bioMérieux (1-800-638-4835, catalog #20-109/20-179). Reference charts of API profiles for *A. salmonicida* and *Y. ruckeri* are listed in Section 2, 3.A2 Profiles Obtained with API-20E for Known Fish Pathogens Appendix 2.

b. Biolog
MicroLog™ is a microbial identification system able to identify and characterize a wide variety of organisms based on carbon source utilization. The system has identification databases that contain over 1400 different species/genera of aerobic and anaerobic bacteria and yeasts. The identification databases include a wide variety of organism including animal, plant, and water pathogens. The system also allows the user the capability to build customized organism databases. Products are available directly from Biolog, 3938 Trust Way, Hayward, CA. 94545 (1-510-785-2564 or website www.biolog.com).

E. Fluorescent Antibody Test (FAT)

The Fluorescent Antibody Test (FAT) is one serological method for corroboration testing of bacterial isolates. FAT can be performed either with a direct antibody staining (DFAT) or indirect (IFAT) technique, depending upon the availability of pathogen-specific FITC-conjugated or unconjugated antibody preparations from the manufacturers listed below (Section 2, 3.8.E.6 "Commercial Sources for Antibodies"). There are three basic steps for FAT: preparing and fixing bacterial samples; staining the slides with antibody reagents; reading and interpreting the slides.

1. Preparing the Slides

a. Pure Bacterial Cultures
(Confirmatory testing of pure isolates of *A. salmonicida*, *Y. ruckeri*, or *E. ictaluri*.)

i. Pure isolates of bacteria are diluted in sterile PBS (Section 2, 3.7.D.3 "Phosphate-Buffered Saline for FAT (PBS), pH 7.1") and applied to two replicate wells of an FAT slide.

ii. Allow air-drying completely or heat fix.

b. Kidney
(For presumptive detection of *R. salmoninarum*) – prepare kidney smear on a non-coated or acetone-cleaned glass slide.

i. Homogenize a piece of posterior kidney tissue and create a thin smear on the surface of a slide.

ii. Allow to air dry completely or heat fix.

c. Ovarian Fluid Pellet Smear
(DFAT-for presumptive detection of *R. salmoninarum*) – ovarian fluid is collected (Section 2, 2.2.E.2.f).

i. After pooled ovarian fluid samples are processed and appropriate amount of supernatant removed for virology assays (Section 2, 4.4.C "Processing of Coelomic (Ovarian) Fluid Samples"), the pellet is re-suspended in the remaining supernatant by thorough vortexing or repeat pipetting.

ii. Transfer two 1.5 mL aliquots from each pool of ovarian fluid (or 0.5 mL per fish) to sterile, labeled 1.5 mL micro-centrifuge tubes (see **Note**). Freeze the remainder of the sample at -20^{o}C for PCR confirmation.

iii. Centrifuge the 1.5 mL aliquots at 10,000 X g for 15 minutes.

Note: Elliot and McKibben (1997) document the importance of using a minimum of 10,000 X g for sufficient sedimentation of *R. salmoninarum* from 0.5 mL ovarian fluid collected from individual fish. Enough ovarian fluid must be transferred from each pool to represent 0.5 mL for each fish represented in the pool (i.e. 2.5 mL from a five-fish-pool). Most 15 mL centrifuge tubes used for collection and processing of ovarian fluid samples cannot be centrifuged at a relative centrifugal force (rcf) as high as 10,000 X g. It is, therefore, necessary to aliquot the appropriate amount of fluid into microcentrifuge tubes suitable for the required rcf.

iv. The pellet is carefully re-suspended and a thin smear prepared on a glass slide. Pellets originating from the same ovarian fluid sample tube may be combined on one slide.

v. Allow to air dry completely or heat fix.

d. After the tissue is completely air dried or heat fixed, slides are fixed in acetone for five minutes. Other fixing solutions are suitable as long as a component of the solution contains a

lipid-dissolving reagent such as acetone or xylene, which helps remove lipids and improve the overall fluorescence quality and intensity.

2. **Direct FAT (DFAT) Staining** (Thoesen 1994)
Once slides are prepared, the staining procedure is the same regardless of the sample type (bacterial isolate or tissue).

   a. Positive and Negative Controls
   Slides containing both a non-cross-reacting bacterial species and a known positive control can be prepared in quantity and stored refrigerated for use as controls for FAT staining. Positive controls are always used in confirmation testing to correctly identify morphology and fluorescence of the bacteria in question. The negative control is important in determining overall staining technique, background debris, and non-specific fluorescence.

   b. Place slides in dark, humidified chamber, and place one drop of specific FITC conjugate (Section 2, 3.7.D.6 "FITC Conjugated Rabbit Anti-X/Rhodamine Couter Stain") on each sample slide and control slides.

   c. Incubate for 30 to 60 minutes at room temperature, according to manufacturer's recommendation.

   d. Using a squirt bottle or transfer pipette, GENTLY rinse the slides with PBS (Section 2, 3.7.D.3 "Phosphate-Buffered Saline for FAT (PBS), pH 7.1").

   e. If rhodamine counterstain has not been incorporated in the FAT stain, apply the counterstain of choice (Section 2, 3.7.D.5 "Counter Stains") at this point for 1-2 minutes.

   f. Final rinse/soak in PBS for 5 to 10 minutes. Air-dry completely.

   g. Add a small drop of FA mounting fluid, pH 9 (Section 2, 3.7.D.2 "FAT Mounting Fluid (pH 9.0)") to each slide, being careful not to touch the dropper to the slide to prevent the possibility of cross contamination.

   h. Place a 24 x 50 mm cover slip over the slide using care not to trap air bubbles.

   i. Spread the mounting fluid by gently pressing the cover slip with the blunt end of a pen or lab marker.

   j. Add one drop of immersion oil to the cover slip and examine at 1000X using the epifluorescent filter.

3. **Indirect FAT (IFAT) Staining** (Thoesen, 1994)
IFAT is a double layered antibody technique, where the first layer consists of unconjugated, purified immunoglobulin (IgG) or antibody prepared in one animal species (e.g. rabbit) against the target antigen. The second antibody applied is a FITC-conjugated antibody prepared in a second animal species (e.g. goat), and specific for IgG of the first animal species (e.g. goat anti-rabbit IgG). Once slides are prepared, the staining procedure is the same regardless of the sample type (bacterial isolate or tissue).

a. Positive and Negative Controls
Slides containing both a non-cross-reacting bacterial species and a known positive control can be prepared in quantity and stored refrigerated for use as controls for FAT staining. Positive controls are always used in corroboration testing to correctly identify morphology and fluorescence of the bacteria in question. The negative control is important in determining overall staining technique, background debris, and non-specific fluorescence.

b. Place slides in dark, humidified chamber, and place one drop of unconjugated, pathogen specific antibody on each sample slide and control slides.

c. Incubate at room temperature for 30 to 60 minutes, according to manufacturer's recommendations.

d. Gently rinse slides with PBS (Section 2, 3.7.D.3 "Phosphate-Buffered Saline for FAT (PBS), pH 7.1").

e. Place a drop of FITC-conjugated second antibody on slides and incubate at room temperature in dark chamber according to manufacturer's recommendation.

f. Rinse briefly with PBS. If rhodamine counterstain has not been incorporated into the FAT stain, apply the counterstain of choice (Section 2, 3.7.D.5 "Counter Stains") at this point for 1 to 2 minutes.

g. Rinse and wash in PBS for 5 to 10 minutes.

h. Air dry completely and apply a small drop of FA mounting fluid, pH 9 (Section 2, 3.7.D.2 "FAT Mounting Fluid (pH 9.0)"). Apply a cover slip to mounting fluid.

i. Add one drop of immersion oil to the coverslip and examine at 1000X using the epifluorescent filter.

4. Reading Results

a. Slides are read at 1000X on a compound fluorescence microscope (refer to the microscope manufacturer for correct wavelength and filters required for FITC epifluoresence microscopy). The positive and negative control slides are read first. This has two purposes: (1) quality assurance for the staining process (positive control should have myriad numbers of fluorescing bacteria, the negative control should have no fluorescence); and (2) to familiarize the reader with the correct bacterial size, shape, and magnitude of the fluorescent halo of bacteria in the positive control. The reader can refer back to the positive control as a reference, if needed, to confirm suspect bacteria in the sample wells. Positive fluorescence appears "apple green" in color.

i. Pure Culture Bacterial Confirmatory Testing
Positive bacterial isolates will fluoresce strongly and have the same morphology and size as the positive control. Negative isolates will not fluoresce.

ii. *R. salmoninarum* Screening
Examine at least 50 fields. Positive bacterial cells are 1.0 X 0.5 µm, which fluoresce. Negative smears will not fluoresce.

iii. *P. rickettsia* Screening

Examine at least 50 fields. Positive bacterial cells are 0.5 to 1.5 µm in diameter, pleomorphic, occur in coccoid or ring forms which fluoresce. Observation of fluorescing cells within host tissue cells provides strong evidence of *P. salmonis* infection. Negative smears will not fluoresce.

5. Hints for Good Results

a. Use FITC conjugates at optimum working dilution. Follow manufacturer's recommendation to test for optimum working concentration (Section 2, 3.7.F "Determination of Antiserum and Conjugate Working Diutions for FAT").

b. Filter all conjugated antibody reagents (.45 µm filter) prior to use to reduce background debris that fluoresce nonspecifically and cause difficulty in reading and interpreting the slides.

c. Prepare thin smears; thick smears will not fix properly and are more easily washed off during the staining process, and thick slides require frequent focusing to observe multiple focal planes.

d. Evenly distribute the kidney material in PBS (Section 2, 3.7.D.3 "Phosphate-Buffered Saline for FAT (PBS), pH 7.1") or use a very light inoculum of pure bacterial culture for each well (excess bacteria will stain poorly).

e. Heat-fix slides prior to fixing in acetone. If there is not possible to heat-fix the slides they can be air-dried and sent to the lab without fixation. Fixed slides should be stored refrigerated until stained.

f. Control slides should be rinsed in separate Coplin jars to avoid any potential for cross contamination during the staining process. Fixing solutions should be completely changed, at a minimum, between separate inspection cases and/or when positive results occur.

g. Use anhydrous acetone to fix slides; the acetone reduces the lipid content of the preparation (de-fatting) increasing the overall fluorescence quality and intensity.

6. Commercial Sources for Antibodies

a. Kirkegaard and Perry Laboratories, Inc.
2 Cessna Court, Gaithersburg, MD 20879-4145 USA
Phone: 800/638-3167, 301/948-7755
Web Site: http://www.kpl.com

Antibodies available: Polyclonal antibodies available in FITC-conjugated and other preparations for *Renibacterium salmoninarum* only. KPL also provides positive control material for FAT.

b. Microtek International, LTD (Bayotek)
6761 Kirkpatrick Crescent, Saanichton, B.C., CA
Phone: 250-652-4482
Web Site: http://www.microtek-intl.com

Antibodies available: Polyclonal antibodies available for Indirect FAT for he following bacterial pathogens: *Renibacterium salmoninarum, Piscirickettsia salmonis, Aeromonas salmonicida,* and *Yersinia ruckeri* serotypes 1 and 2.

3.9 Glossary

Acute - a disease having rapid onset, severe symptoms and a relatively short course – not chronic.

Anneal - the attachment of oligonucleotide primers to a specific site on a single stranded DNA segment.

Aseptic - the nature of preventing contamination of foreign microorganisms.

Basal media - a media formulation to which additional components may be added for a particular test.

Carbohydrate - a particular sugar used in a test.

Chronic - describes the course of a disease which is long and drawn out – not acute.

Commercially prepared - a component or test, which is available from a commercial source for purchase.

Confirmatory identification - identification of a pathogen through the completion of both initial screening techniques and another confirmatory test as described in this document, the results of which concur with each other for positive identification of a particular organism.

Counterstain - a stain used in FAT to achieve a dark background color of tissues and materials stained, aiding in the observation of fluorescing bacterial cells.

Decolorize - the application of a solution to stained material to remove excessive stain.

Denature - the enzymatic/temperature dependent activity which converts double stranded DNA to single stranded DNA.

DNA extraction - the process of obtaining pure, double stranded DNA from sample tissues and materials.

Elution - the washing of DNA from a spin column filter membrane.

Extension - the synthesis of a new, copied segment of DNA following denaturing and annealing processes involved with PCR.

Fermentation - bacterial utilization of a compound in the absence of oxygen.

Filtrate - the resultant liquid obtained from filtration.

FITC - fluorescein isothiocyanate, a reagent, which is used as an antibody label for the fluorescent antibody test.

FITC-conjugated (antibody) - describes the existence of a fluorescent label on an antibody used for the fluorescent antibody test.

Lysate - the product solution of tissue lysis.

Nested-PCR - a second PCR is performed, targeting a sub-segment of DNA produced in the first round PCR specific for a particular organism.

Oxidation - bacterial utilization of a compound in the presence of oxygen.

Pellet - the product of sedimentation of solid materials from a liquid resulting from centrifugation.

Phenotypic characterization - the detectable expression of a bacterial isolate to environmental conditions, biochemical testing and morphological observations recorded for the purpose of differentiation and determination of the species of bacteria.

Polymerase chain reaction (PCR) - a rapid procedure for in vitro enzymatic amplification of a specific segment of DNA.

Positive control isolates - bacterial isolates may be obtained from the American Type Culture Collection (ATCC: http://www.atcc.org).

Post-dwell - the period of time after cycles are completed in PCR.

Presumptive identifications - identification of a pathogen through the completion of initial screening tests as described in this document, where no other test has been performed to confirm the positive identification of the organism.

Primary culture - bacterial cultures achieved from media inoculated directly from fish tissues.

Primer - oligonucleotides that, in the presence of DNA and excess dNTP's, hybridize specifically to a target sequence and "prime" new DNA synthesis.

Pure bacterial culture - a culture of bacteria originating from an isolated colony.

Quality control - taking steps to assure that testing and results are accurate and reliable.

Selective media - a medium containing ingredients, which may either exclude growth of some microorganisms, select for growth of a particular species of microorganism, or both.

Serological methods - detection methods, which employ the use of an antibody against the target organism.

Spin column - a small filter unit provided by many commercial DNA extraction kits used to bind, wash, and elute DNA from tissue lysates.

Stock suspension - suspension of a compound, which must be further diluted before direct application in a particular test.

Sub-acute - a course of disease which exhibits some acute and some chronic symptoms.

Subculture - the transfer of an established bacterial culture to an uninoculated medium.

Supernatant - the surface fluid resulting from centrifugation of a liquid.

Working suspension (dilution) - suspension of a compound at the correct dilution for direct application in a particular test.

Yellow-pigmented bacteria - bacterial isolates which exhibit a yellow pigmentation on colony formations (e.g. *Flavobacterium* species).

3.10 References

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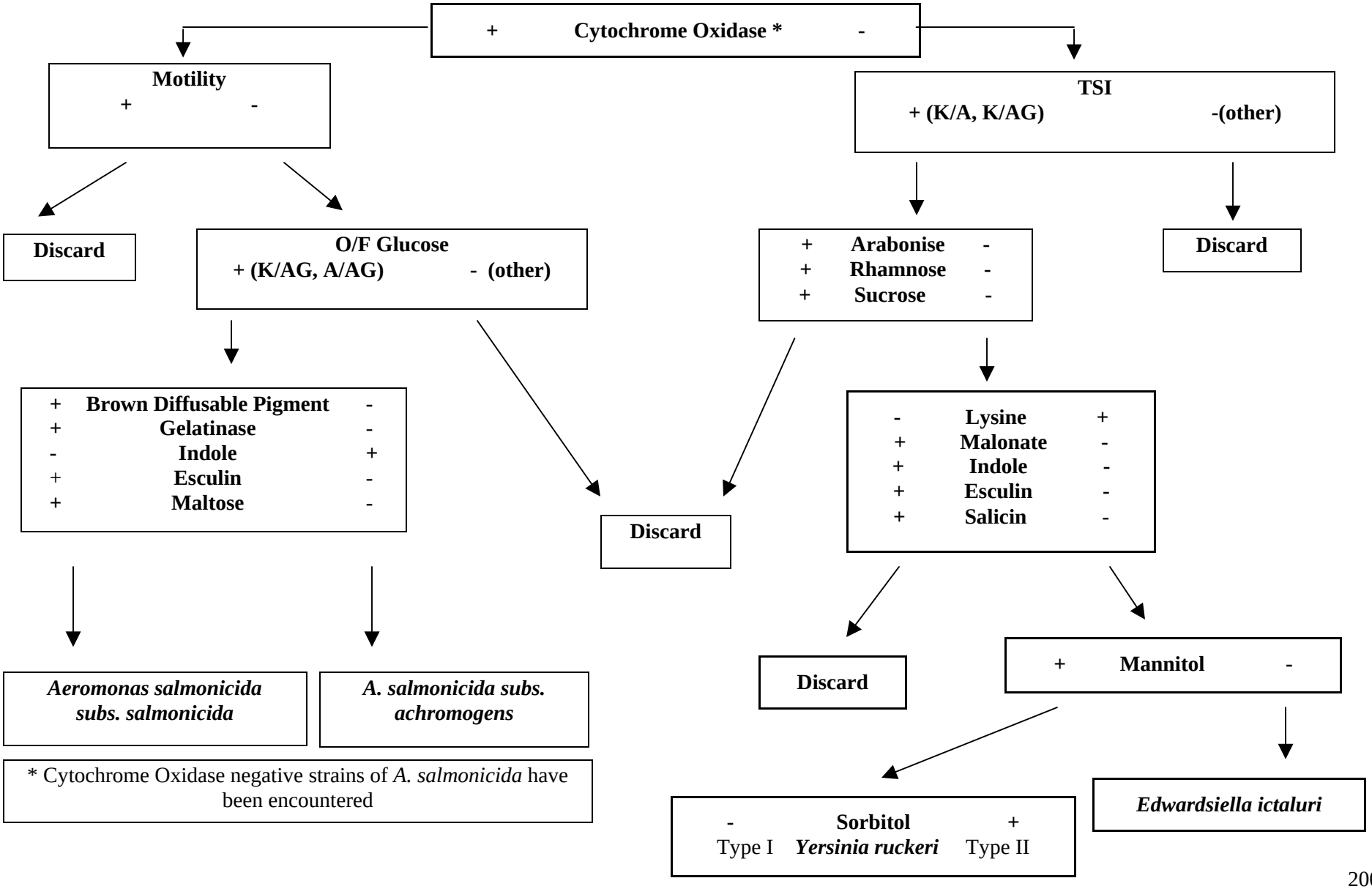
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3.A1 Laboratory Reference Flow Chart for Identification of Gram-Negative Bacterial Pathogens Which Grow on TSA or BHIA and are Targeted for Detection to Complete Fish Health Inspection Requirements



* Cytochrome Oxidase negative strains of *A. salmonicida* have been encountered

2004

3.A2 Profiles Obtained with API-20E for Known Fish Pathogens

The following table represents API20E profiles for ***Yersinia ruckeri*** when cultures were tested at 22°C rather than the manufacturer's recommended incubation temperature of 35 to 37°C. All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT. Based on the profile submitted to API, bacterial identification is given in order of probability, then remarks as to the likelihood of the profile are provided when profiles are poorly matched to the manufacturer's database (National Wild Fish Health Survey Lab Procedures Manual, USFWS).

Bacterial Isolate ID	API PROFILE*	API Manual or Computer Identification
1. Eastern Fishery Disease Laboratory (EFDL) Positive Control #1- Type II (11.29) 2. Nisqually Fall chinook (3/88)	**5307500**	1. *Serratia marcescens* 2. *Serratia liquefaciens* 3. *Hafnia alvei*
1. Eastern Fishery Disease Laboratory (EFDL) Positive Control #2- Type II (11.29) 2. Fall Chinook, Suquamish R, WA (3/88) 3. Spring Chinook, Skookum Creek, WA (2/88)	**5107500**	(Same ID as 5307500 above) 1. *Serratia marcescens* 2. *Serratia liquefaciens* 3. *Hafnia alvei*
Unknown source – Isolate confirmed by biochemical and serological testing.	**5144100**	1. *Escherichia coli* 2. *Yersinia ruckeri*
Eastern Fishery Disease Laboratory (EFDL) Positive Control - Type I (11.4)	**5107100**	"Unacceptable profile"
Coho, Quinault River, WA	**5106100**	"Questionable ID"
Late Fall Chinook, Battle Creek, CA (11/94)	**5105100**	"Acceptable ID" 1. *Hafnia alvei*
Notes from ERM archived files – previous testing	**5104500**	"Questionable ID"

Bacterial Isolate ID	API PROFILE*	API Manual or Computer Identification
1. Hagerman – Type I (11.4)	**5104100**	"Very good ID" 1. *Yersinia ruckeri*
Coho, Quilcene R., WA (11/88)	**5104000**	"Very good ID" 1. *Yersinia ruckeri*
Unknown source	**5100100**	"Excellent ID" 1. *Yersinia ruckeri*
Unknown source	**4105100**	1. *Hafnia alvei*
Unknown source	**4104100**	1. *Yersinia ruckeri* 2. *Salmonella gallinarum*
Unknown source	**4104000**	"Acceptable ID" 1. *Yersinia ruckeri*
Unknown source	**0104100**	"Acceptable ID" 1. *Yersinia ruckeri*

* *Yersinia ruckeri* generally fails to produce a positive citrate reaction when incubated at room temperature (22 to 25°C). Refer to the API Manual for specific biochemical tests and interpretation of API20E™ profiles. Also see references listed on page 5-27.

The following represents API20E profiles for ***Aeromonas salmonicida*** isolates following manufacturer's instructions but incubating test strips at room temperature (22°C). All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT.

Bacterial Isolate ID (collection date)	**API PROFILE***	**Computer/Manual ID**
spp not identified, Makah NFH, WA (8/88)	**0006104**	"Acceptable ID" 1. *Pseudomonas pseudomaleae*
Winter Steelhead, Makah NFH, WA (1/89) Chum, Makah NFH , WA (12/89)	0006104	Same as above
Winter Steelhead, Quinault NFH, WA (1/89)	0006104	Same as above
Spring Chinook, Entiat NFH, WA (8/89)	0006104	Same as above
Spring Chinook, Quilcene NFH, WA (3/91)	**2006104**	1. *Aeromonas salmonicida*
Profiles given in **API MANUAL** for *Aeromonas salmonicida*	**6006104** **6006504** **4006104** **2006104**	"Good to Excellent ID" 1. *Aeromonas salmonicida*

**Aeromonas salmonicida* generally fails to produce positive relations for ONPG, ADH, and LDC when incubated at room temperature (22 to 25° C).

Profiles provided in the API Manual are based on positive reactions for some or all of these first three biochemical tests, therefore the first digit of the "acceptable" profiles for *A.salmonicida* include the values 2,4, or 6. More often, a zero value is obtained after 24 to 48 hours incubation at room temperature. Longer incubation periods are required for these tests.

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3.A3.A Worksheet A – PCR Sample Data/Log Sheet

Case Number_____ **Sample Site**_____ **Date**_____
Species_____
Tissue Type_____

Tissue Sample ID	Extraction Method	PCR Result	Notes
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			
9.			
10.			
11.			
12.			
13.			
14.			
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16.			
17.			
18.			
19.			
20.			

3.A3.B Worksheet B – Initial Amplification of Nucleic Acid by PCR for the Confirmation of *Renibacterium salmoninarum*

Case Number_____ **Date**_____

PCR Reagent	Lot#	Final Concentration	Stock Concentration**	Volume per Reaction (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		Add to total 40 µL		30.1	
10XBuffer		1X	10X	5.0	
$MgCL_2$		1.5 mM	50 mM	1.5	
dNTP's		0.2 mM	10 mM	1	
(+)Primer		20 pMole	20 pMole/µL	1	
(-)Primer		20 pMole	20 pMole/µL	1	
TAQ		2 units/Rx	5U/µL	0.4	
DNA$^{\pm}$		-	-	10 µL	-

*Add water to Master Mix first, TAQ last.

**Change "Stock Concentration" parameters as necessary. Different reagent sources supply varying stock concentrations.

$^{\pm}$Do not add DNA template until Master Mix reaction tubes have been removed from the reagent mixing (MM) area.

Primer Sets for *R. salmoninarum* 1st Round Amplification

P3 (round 1 forward)	5'-A GCT TCG CAA GGT GAA GGG-3'
M21 (round 1 reverse)	5'-GC AAC AGG TTT ATT TGC CGG G-3'

3.A3.C Worksheet C – Nested Amplification of Nucleic Acid by PCR for the Confirmation of *Renibacterium salmoninarum*

Case Number_____ **Date**_____

PCR Reagent	Lot#	Final Concentration	Stock Concentration**	Volume per Reaction (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		Add to total 49 µL		39.1	
10XBuffer		1X	10X	5.0	
$MgCL_2$		1.5 mM	50 mM	1.5	
dNTP's		0.4 mM	10 mM	1	
(+)Primer		20 pMole	20 pMole/µL	1	
(-)Primer		20 pMole	20 pMole/µL	1	
TAQ		2 units/Rx	5U/µL	0.4	
DNA$^{\pm}$ (round 1)		-	-	1 µL	-

*Add water to Master Mix first, TAQ last. **Change "Stock Concentration" parameters as necessary. Different reagent sources supply varying stock concentrations. $^{\pm}$Do not add DNA template until Master Mix reaction tubes have been removed from the reagent mixing (MM) area.

Primer Sets for *R. salmoninarum* 2nd (Nested) Round Amplification

P4 (round 2 forward)	5'-AT TCT TCC ACT TCA ACA GTA CAA GG-3'
M38 (round 2 reverse)	5'-C ATT ATC GTT ACA CCC GAA ACC-3'

Gel Concentration		**Weight of agarose (grams)**	**Volume of Buffer (mL)**

Gel Template (Sample Placement Map)
Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

3.A3.D Worksheet D – Initial Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis*

Case Number_____ **Date**_____

PCR Reagent	Lot#	Final Concentration	Stock Concentration**	Volume per Reaction (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		Add to total 45 µL		35.0	
10XBuffer		1X	10X	5.0	
$MgCL_2$		1.5 mM	50 mM	1.5	
dNTP's		0.2 mM	10 mM	1	
(+)Primer		1 mM	1 mM	1	
(-)Primer		1 mM	1 mM	1	
TAQ		2.5 units/Rx	5U/µL	0.5	
DNA$^{\pm}$		-	-	5 µL	-

*Add water to Master Mix first, TAQ last.

**Change "Stock Concentration" parameters as necessary. Different reagent sources supply varying stock concentrations.

$^{\pm}$Do not add DNA template until Master Mix reaction tubes have been removed from the reagent mixing (MM) area.

Primer Sets for *P. salmonis* 1st Round Amplification

EubA (round 1 forward)	5'- AAG-GAG-GTG-ATC-CAN-CCR-CA -3'
EubB (round 1 reverse)	5'- AGA-GTT-TGA-TCM-TGG-CTC-AG -3'

3.A3.E Worksheet E – Nested Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis*

Case Number_____ **Date**_____

PCR Reagent	Lot#	Final Concentration	Stock Concentration**	Volume per Reaction (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		Add to total 48 µL		38.0	
10XBuffer		1X	10X	5.0	
$MgCL_2$		1.5 mM	50 mM	1.5	
dNTP's		0.2 mM	10 mM	1	
(+)Primer		1 mM	1 mM	1	
(-)Primer		1 mM	1 mM	1	
TAQ		2.5 units/Rx	5U/µL	0.5	
DNA$^{\pm}$ (round 1)		-	-	2 µL	-

*Add water to Master Mix first, TAQ last. **Change "Stock Concentration" parameters as necessary. Different reagent sources supply varying stock concentrations. $^{\pm}$Do not add DNA template until Master Mix reaction tubes have been removed from the reagent mixing (MM) area.

Primer Sets for *P. salmonis* 2nd (Nested) Round Amplification

PS2S (223F) (round 2 forward)	5'- CTA-GGA-GAT-GAG- CCC-GCG-TTG -3'
PS2AS (690R) (round 2 reverse)	5'- GCT-ACA-CCT-GAA-ATT-CCA-CTT -3'

Gel Concentration		**Weight of agarose (grams)**	**Volume of Buffer (mL)**

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

3.A3.F Worksheet F – Direct Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis*

Case Number_____ **Date**_____

PCR Reagent	Lot#	Final Concentration	Stock Concentration**	Volume per Reaction (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		Add to total 45 µL		35.0	
10XBuffer		1X	10X	5.0	
$MgCL_2$		1.5 mM	50 mM	1.5	
dNTP's		0.2 mM	10 mM	1	
(+)Primer		1 mM	1 mM	1	
(-)Primer		1 mM	1 mM	1	
TAQ		2.5 units/Rx	5U/µL	0.5	
$DNA^{\pm}$		-	-	5 µL	-

*Add water to Master Mix first, TAQ last. **Change "Stock Concentration" parameters as necessary. Different reagent sources supply varying stock concentrations. $^{\pm}$Do not add DNA template until Master Mix reaction tubes have been removed from the reagent mixing (MM) area.

Primer Sets for *P. salmonis* 2nd Direct Amplification

PS2S (223F) (round 2 forward)	5'- CTA-GGA-GAT-GAG- CCC-GCG-TTG -3'
PS2AS (690R) (round 2 reverse)	5'- GCT-ACA-CCT-GAA-ATT-CCA-CTT -3'

Gel Concentration		**Weight of agarose (grams)**	**Volume of Buffer (mL)**

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

3.A3.G Worksheet G – Photodocumentation of the PCR Product Gel

Case Number _____ Date _____

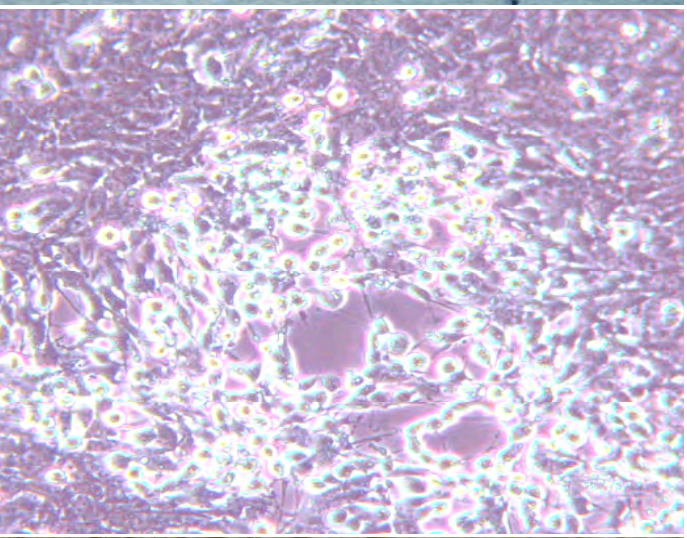
Samples _____

Affix gel photo documentation to this sheet and make notes on results:

AFFIX PHOTODOCUMENTATION

Notes:









Section 2
USFWS/AFS-FHS Standard Procedures for Aquatic Animal Health Inspections

Chapter 4 Virology

4.1	Virology Introduction
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4.A1.C	Worksheet C – Photodocumentation of the PCR Product Gel
4.A2	Cell Enumeration

4.1 Virology Introduction

The procedures described in this chapter are specifically for the detection of Infectious Hematopoietic Necrosis Virus (IHNV), Infectious Pancreatic Necrosis Virus (IPNV), Infectious Salmon Anemia Virus (ISAV), Largemouth Bass Virus (LMBV), *Oncorhynchus masou* Virus (OMV), Spring Viremia of Carp Virus (SVCV), Viral Hemorrhagic Septicemia Virus (VHSV), and White Sturgeon Herpesvirus (WSHV).

The initial detection method for all of these viruses is by observing cytopathic effect (CPE) in cell culture using virus isolation procedures. The presence of IHNV may be confirmed using serum neutralization, indirect fluorescent antibody test (IFAT), Alkaline Phosphatase Immunocytochemistry stain, or polymerase chain reaction (PCR) techniques**. In this edition, the primer sequences for the IHNV RT-PCR assay have been modified from those in previous editions of this manual. The new primers target the central portion of the G gene, instead of the previously published primers which targeted the N gene. The G gene primer set has been tested extensively (Emmenegger et al., 2000, Kurath et al., 2003) and avoids using a series of six consecutive 'G's' that were less than optimal for PCR in the N gene forward primer.** The presence of IPNV and VHSV may be confirmed using serum neutralization, indirect fluorescent antibody test (IFAT), or polymerase chain reaction (PCR) techniques. **Also new in this edition, the primer sequences for VHSV RT-PCR have been modified compared to those provided in previous editions of this manual. The new sequences now match the OIE (2006) VHSV primers.** The presence of ISAV may be confirmed using IFAT or PCR techniques. The presence of LMBV and OMV are confirmed using PCR techniques. The presence of SVCV may be confirmed using serum neutralization or PCR techniques. WSHV suspect cultures will be sent to an appropriate laboratory for confirmation.

These procedures may also detect other replicating agents not listed here. When this occurs, every attempt will be made to complete the identification of the organism. Some of these viruses may occur in combination and the finding of one agent will not preclude following procedures that may identify other agents.

If one of these viruses or an unknown replicating agent is found, the proper parties and authorities will be notified in a timely manner and at least one representative sample of each isolate should be archived at -70°C to be used for future reference.

Blind passage of samples not exhibiting CPE after 14 days (7 days in the case of LMBV) of primary incubation is included in these procedures to determine if it provides a significant increase in detection of viral agents. It is requested that laboratories using these procedures summarize their findings of primary and blind passage detections by virus and provide it to the Handbook Revision and Oversight Committee annually. If the data shows that blind passage of these samples is not providing a sufficient increase in viral detection, it will be removed from the procedures.

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U. S. Fish and Wildlife Service, the United States government, and/or the American Fisheries Society. Any comparable instrument, laboratory supply, or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.

4.2 Selection of Appropriate Cell Lines

Selection is based on the ability of the cell lines to detect the viruses of interest and, whenever possible, utilizing cell lines capable of detecting different viral agents to increase the efficiency of the laboratory procedures. At the minimum, one cell line specified for the virus of interest will be used. At least two cell lines should be used for all samples to maximize the detection of viral agents present in the samples (Bouchard et al., 1999; OIE 2000; Plumb 1999; Thoesen 1994; Wolf 1988).

A. General Considerations

1. All viral testing will utilize cell lines traceable to cell lines from the American Type Culture Collection (ATCC) when available.

2. At the minimum, all cell lines should be tested annually for viral sensitivity and mycoplasma infection.

3. Work with only one cell line at a time.

4. Aseptic technique is required for cell culture work.

B. Cell Line Sensitivities

1. The EPC cell line provides high sensitivity for IHNV and SVCV.

2. The EPC, FHM and BF-2 cell lines provide high sensitivity for VHSV.

3. The SHK-1 and ASK cell lines provide high sensitivity for ISAV.

4. The FHM and BF-2 cell lines provide high sensitivity for LMBV.

5. CHSE-214 cell line provides high sensitivity for IPNV and OMV.

6. The WSS-2 cell line provides high sensitivity for WSHV.

Table 4.1. Recommended cell lines to detect target viruses.

Virus[a]	Cell Line	Common Name	ATCC[b] Designation
IHNV	Epithelioma Papulosum Cyprini	EPC	CRL-2872
IPNV	Chinook Salmon Embryo	CHSE-214	CRL-1681
ISAV	Salmon Head Kidney	SHK-1	
	Atlantic Salmon Kidney	ASK	CRL-2747
LMBV	Fat Head Minnow	FHM	CCL-42
	Bluegill Fry	BF-2	CCL-91
OMV	Chinook Salmon Embryo	CHSE-214	CRL-1681
SVCV	Epithelioma Papulosum Cyprini	EPC	CRL-2872
VHSV	Epithelioma Papulosum Cyprini	EPC	CRL-2872
	Fat Head Minnow	FHM	CCL-42
	Bluegill Fry	BF-2	CCL-91
WSHV	White Sturgeon Spleen	WSS-2	

[a] Viruses: IHNV- Infectious Hematopoietic Necrosis Virus; IPNV- Infectious Pancreatic Necrosis Virus and other related birnaviruses; ISAV – Infectious salmon anemia virus; LMBV – Largemouth Bass Virus and other related iridoviruses; OMV - *Oncorhynchus masou* Virus; SVCV – Spring viremia of carp; VHSV - Viral Hemorrhagic Septicemia Virus; WSHV - White Sturgeon Herpesvirus.

[b] American Type Culture Collection, Rockville, MD

4.3 Cell Culture

Standard animal cell culture techniques are used with adaptations for fish cell lines when necessary i.e., incubation temperature. Normal appearing cultures composed of rapidly dividing cells will be used for all assay work. Cells will be routinely subcultured to maintain healthy cells, approximately once every two weeks for most cell lines, or split weekly for seeding plates. Aseptic technique is required when working with any cell line. Only one cell line is worked with at a time (Freshney 1983; Jakoby and Pastan 1979; Lannan 1994; Merchant et al., 1964; Rovozzo and Burke 1973; True 2000).

A. Subculture Procedures for Flasks

1. Suggested split ratios and seeding rates are given in Table 4.2.

2. Remove tissue culture medium by decanting fluid.

3. Slowly add trypsin-versene (EDTA) (Section 2, 4.9.E "Trypsin-Versene (EDTA)") solution and rock the flask gently for one minute. In a 75 cm^2 flask a volume of 3 to 4 mL is sufficient to coat cells.

4. Decant again.

5. Carefully observe the cell layer and repeat steps 3 and 4 as necessary until cells start detaching from flask. Dislodging of the cells may be completed by sharply striking the edge of the flask against the heel of a hand.

6. Add tissue culture medium to neutralize the trypsin. In a 75 cm^2 flask, 10 mL is sufficient.

7. Triturate to break up cell clumps and add an appropriate volume of fresh tissue culture medium for transferring to other flasks. Enumeration of cells in the suspension may be done at this time to determine the necessary volume to transfer (Section 2, 4.A2 Cell Enumeration Appendix 2)

8. Aspirate and dispense into new flasks. The sub-cultivation ratio is generally 1:4 to 1:6. Following manufacturers recommendations, bring total volume in each new flask up to the acceptable level with the appropriate tissue culture medium. For a 75 cm^2 flask, this will usually be about 20 mL. MEM-10/Hepes (Section 2, 4.9.G "MEM-10/Hepes (Tissue Culture Medium for all Cell Lines Except SHK-1 and ASK)") works well in an open system for all cell lines listed in Table 4.2 except SHK-1 and ASK, which do best with L-15 (Section 2, 4.9.H "Leibovitz's L-15 (Tissue Culture Medium for SHK-1 and ASK Cell Lines)").

9. Incubate flasks at room temperature (20 to 25°C) until they reach confluence and then held at the appropriate temperature for that cell line.

    a. SHK-1, ASK and CHSE-214 cells should be incubated at 15°C.

    b. WSS-2 cells should be incubated at 20°C.

c. FHM and BF-2 cells should be incubated at 25°C (25 to 30°C for LMBV).

d. EPC cells may be incubated at 15 to 25°C.

Table 4.2. Seeding guidelines for the subculture of fish cell lines.

CELL LINE		Suggested Seeding Rate (per cm^2)	INCUBATION TEMP (°C)	
Common Name	Nominal split Ratio		Suggested	Range
BF-2	1:2 - 3	100,000	25 - 30	20 - 30
CHSE-214	1:3 - 6	100,000	15 - 20	4 - 27
EPC	1:3 - 6	250,000	15 - 25	15 - 30
FHM	1:4 - 6	250,000	25 - 30	10 - 36
WSS-2	1:4 - 8	150,000	20 - 25	20 - 30
SHK-1	1:2 - 3	250,000	15 - 20	15 - 20
ASK	1:2 - 3	250,000	15 - 20	15 - 20

B. Seeding Procedures for Plates

1. Monolayers on plates are prepared approximately 24 to 48 hours prior to inoculation with the sample.

2. A flask of visually healthy cells approximately 7 to 10 days old is selected and trypsinized as described previously. After neutralizing the trypsin with tissue culture medium, the total volume in the flask is adjusted to provide a cell concentration appropriate for the seeding rate listed in Table 4.2 and the area of the wells to be seeded. The appropriate volume of the cell suspension is then pipetted into each well of the plate.

   Example: When seeding EPC cells in a 24-well plate, 0.5 mL of a 1 X 10^6 cells per mL suspension is dispensed per well.

3. At a minimum, control wells are included in each plate set and should be included on each plate whenever possible. Control wells are made by dispensing tissue culture medium into plate wells that contain normal looking cell monolayers that have not been inoculated with a sample. These wells are observed during the incubation along with the sample wells for abnormalities that may arise due to media or cell problems. A plate set refers to the group of plates seeded from a single flask at the same time.

4. Incubate plates overnight at room temperature (approximately 20 to 25°C).

4.4 Sample Processing Procedures

Tissue processing for viral culture is described below. Ingredients and preparation procedures for buffers and other solutions/media are listed in Section 2, 4.9 Reagents and Media (Amos 1985; OIE 2000; Rovozzo and Burke 1973; True 2000; Wolf 1988).

A. General Considerations

1. As during sampling and transport, care is taken to protect tissues and fluids from exposure to UV light, freezing, or high temperatures lethal to the viruses of interest.

2. During sample processing, dilution levels are selected from the acceptable range to maintain maximal virus titers and minimize cell toxicity.

3. Following inoculation of test media, remaining tissue/fluid products are kept at 4°C until all assays are completed. Subsequent to the completion of the assays, all material is decontaminated and discarded.

B. Processing of Kidney and Spleen Samples

1. If transport medium (Section 2.3.C.2 "Hanks Balanced Salt Solution (HBSS)") is used, it is poured off and disinfected before discarding. Tissue samples are weighed to the nearest 0.1 g and sterile sample dilution medium (Section 2, 4.9.A "Sample Dilution Medium - Hanks Balanced Salt Solution (HBSS)") is added to make a dilution of 1:10 to 1:100 (w/v). Unless there is a high potential for cell toxicity from the sample, the 1:10 dilution will be used.

2. Tissues are homogenized and a measured amount of homogenate is pipetted into a sterile tube for centrifugation.

3. Tubes are centrifuged at 4°C for 15 minutes at 2000-3000 times gravity relative centrifugal force (X g).

4. An aliquot of supernatant (for virologic evaluation) is transferred to a tube containing an equal amount of antibiotic incubation medium (anti-inc) (Section 2, 4.9.B "Antibiotic Incubation Medium (Anti-Inc) Made with HBSS for Sample Disinfection" or Section 2, 4.9.C "Antibiotic Incubation Medium (Anti-Inc) Made with Minimum Essential Medium (MEM-0) for Sample Disinfection"). Sample dilution is now 1:20 volume/volume.

   Tubes are vortexed and incubated for two hours at 15°C or 12 to 24 hours at 4°C. Samples are then re-centrifuged at 2000-3000 X g and the supernatant is inoculated onto tissue culture plates as described in the inoculation procedures (Section 2, 4.5.A "Plate Inoculation Procedures for Primary Culture").

C. Processing of Coelomic (Ovarian) Fluid Samples

1. An equal volume from each ovarian fluid sample is pipetted into a sterile tube for centrifugation.

2. Centrifuge the tubes at 2000-3000 X g for 15 minutes at 4°C.

3. Undiluted ovarian fluid may be used to inoculate cell cultures as described in the inoculation procedures or up to a 1:5 dilution (1 part ovarian fluid to 4 parts anti-inc) (Section 2, 4.9.B "Antibiotic Incubation Medium (Anti-Inc) Made with HBSS for Sample Disinfection" or Section 2, 4.9.C "Antibiotic Incubation Medium (Anti-Inc) Made with Minimum Essential Medium (MEM-0) for Sample Disinfection") may be made.

4. If a dilution is made, tubes are vortexed and incubated for two hours at 15°C or 12 to 24 hours at 4°C. Samples are then re-centrifuged at 2000-3000 X g and the supernatant is inoculated onto tissue culture plates as described in the inoculation procedures.

4.5 Screening Method for Viral Isolation

The initial detection or screening method used for all listed viral agents is the observation of CPE in cell culture. Standard viral propagation techniques include plate inoculation and initial incubation for approximately 14 days (7 days for LMBV) during which time samples exhibiting CPE are re-inoculated. At approximately 14 days (7 days for LMBV), blind passage of samples not exhibiting CPE is performed and monitored for a total combined incubation period (initial plus blind pass) of 28 days (14 days for LMBV). Blind passage is included to optimize detection of low titer and/or slow growing viruses. To further maximize detection of viral agents, samples should be inoculated on at least two different cell lines (Amos 1985; OIE 2006; Bouchard et al., 1999; Plumb et al., 1999; Rovozzo and Burke 1973; True 2000; Wolf 1988).

A. Plate Inoculation Procedures for Primary Culture

1. General Considerations

a. All cell monolayers to be inoculated are to be at least 80% confluent, approximately 24 hours old, and visually healthy.

b. Tissue culture plates are identified by labeling with the cell line, date of inoculation, and sample information.

c. Aseptic technique is required.

d. It should not be assumed that one set of conditions is optimal for all viruses. Please refer to information on specific pathogens for detailed information on culture conditions.

2. Tissue culture medium is decanted from plates.

3. Inoculate with replication at least 2 cm^2 of cell monolayer with a minimum of 100 µL from each sample.

   Example: If using 24-well plates (2 cm^2/well), 100µL of each sample would be inoculated onto each of two wells of the plate.

4. To allow for viral adsorption, incubate plates for 30 to 60 minutes with gentle rocking at least every 15 minutes or continuously on a laboratory rocker. Do not allow cell monolayer to dry out during incubation.

   a. Incubation temperature for IHNV, IPNV, ISAV, OMV and VHSV is 15°C.

   b. Incubation temperature for WSHV is 20°C.

c. Incubation temperature for SVCV is 20 to 25°C.

d. Incubation temperature for LMBV is 25 to 30°C.

5. Dispense an adequate volume of the appropriate tissue culture medium into each well of the plate. MEM-5/Hepes (Section 2, 4.9.F "MEM-5/Hepes (Tissue Culture Medium for all Cell Lines Except SHK-1 and ASK)") works well in an open system for all cell lines listed in Table 4.2 except SHK-1 which does best with Leibovitz's L-15 (Section 2, 4.9.H "Leibovitz's L-15 (Tissue Culture Medium for SHK-1 and ASK Cell Lines)"). If using 24-well plates, 0.5 mL of medium is adequate.

6. Seal each plate.

7. Incubate plates at the appropriate temperature for a minimum of 14 days.

   a. Incubate for IHNV, IPNV, ISAV, OMV, and VHSV at 15°C.

   b. Incubate for WSHV at 20°C.

   c. Incubate for SVCV at 20 to 25°C.

   d. Incubate for LMBV at 25 to 30°C.

8. Monitor cells at least twice per week for cytopathic effect (CPE). CPE is defined as any morphological change that cells may demonstrate in response to viral or toxic agents. It may range from foaming of the cytoplasm to focal clumping or local destruction of cells. Examples of the appearance of normal cell line monolayers and the CPE typical of these viruses are shown in Figures 4.1 to 4.12.

9. Re-inoculations are made from representative wells exhibiting CPE on these primary inoculations and from at least one well of all samples not exhibiting CPE (blind passage) according to the procedure in "Re-Inoculation Procedure" below.

B. Re-Inoculation Procedure

1. General Considerations

a. Re-inoculation of wells showing toxicity and to confirm the presence of virus with typical CPE may be performed on individual wells at any time during the primary incubation. These will be maintained as individual samples and plated with replication during the re-inoculation procedure.

b. Blind passage from at least one well of all samples not exhibiting CPE will be performed after 10 to 14 days of incubation of the primary culture. These samples may be combined in up to a five-pool sample (representing up to 25 fish) and plated with replication during the re-inoculation procedure. It is suggested that the wells remaining on the initial plate be left intact and observed for at least another 7 to 10 days for a total initial incubation period of 21 days.

Example: On a lot inspection using five-fish pools and 24-well plates, two of the 12 samples exhibit CPE at day five and re-inoculation is performed as described below. The remaining 10 samples (20 wells representing 50 fish) show no evidence of CPE after 14 days of primary incubation and re-inoculation is performed by combining one well from each of five samples and inoculating this pooled sample onto two wells of a 24-well plate. This is repeated with the other five samples using a total of four wells on the re-inoculation plate.

c. As in the initial tissue processing, sample dilution levels for re-inoculation are selected from the acceptable range to maintain maximal virus titers and minimize cell toxicity.

d. Aseptic technique is required.

2. Using a pipette, stir and scrape the bottom of the well to be subcultured to dislodge the cell layer.

3. Aspirate the fluid and cell debris from the well and place in a sterile tube for centrifugation.

4. Tubes are centrifuged at 4°C for 15 minutes at 2000-3000 X g.

5. Remove a measured amount of supernatant and place in a separate sterile tube for dilution.

6. Dilute samples.

   a. For wells exhibiting CPE, use the lowest dilution possible for re-inoculation not to exceed 1:100 using sample dilution medium (Section 2, 4.9.A “Sample Dilution Medium - Hanks Balanced Salt Solution (HBSS)”).

   b. For blind passage samples, up to a 1:5 dilution may be made.

7. If bacterial or fungal contamination is present, the sample should be filtered through a 0.45 µ filter before inoculation onto the plate.

8. Use the appropriate amount of each of these solutions to inoculate a new plate as described above in Section 2, 4.5.A “Plate Inoculation Procedures for Primary Culture.”

9. Monitor these re-inoculation plates at least twice per week for CPE. Total incubation time for both the primary and re-inoculation or blind pass samples is 28 consecutive days.

   Example: If the blind pass is performed on day 14 of the primary incubation, the re-inoculation plate is observed for at least an additional 14 days. If the blind pass is performed on day 10, the re-inoculation plate is observed for at least 18 days.

10. Results

    a. **If no CPE is noted after the 28 day combined incubation period (14 days for LMBV) with no apparent problems in the assay, samples are reported as negative and may be discarded using the proper decontamination procedures.**

    b. If CPE occurs at any time during this assay, it is considered a **PRESUMPTIVE positive** result and the identification of the virus should be confirmed by the appropriate method.

4.6 Identification of Viruses

Methods used for confirmation must have high specificity for the agents they are used to identify but high sensitivity is not required since the cell culture screening method amplifies the virus. The serum neutralization and indirect fluorescent antibody test (IFAT) have long been used for viral identification, Alkaline Phosphatase immunocytochemical staining has been useful in the identification of IHNV in archived samples and historical studies, and polymerase chain reaction (PCR) procedures have now been developed for many of the listed viruses. With the exception of WSHV, procedures for one or more of these three confirmation methods have been included for each virus. The identification of IHNV, IPNV, SVCV, and VHSV may be confirmed using serum neutralization. The identification of IHNV, IPNV, ISAV, and VHSV may be confirmed using IFAT. The identification of IHNV, IPNV, ISAV, LMBV, OMV, SVCV, and VHSV may be confirmed using PCR. The identification of IHNV may be confirmed using APIC stain. WSHV suspect cultures and all replicating agents not identified with these procedures will be sent to an appropriate reference laboratory for identification.

A. Infectious Hematopoietic Necrosis Virus (IHNV)

Infectious Hematopoietic Necrosis Virus (IHNV) is an enveloped bullet shaped single stranded RNA virus belonging to the *Novirhabdovirus* genus of the *Rhabdoviridae*. IHNV has a wide geographic range that includes North America, Europe, and the Far East. The virus is primarily found in salmonids with rainbow trout fry being highly susceptible to disease. Older fish are more resistant to infection but may become carriers. Transmission is primarily horizontal but cases of egg associated transmission have been recorded as well as transmission by fomites. The virus may be shed in ovarian fluid and excretory products such as feces and may also be isolated from the kidney, spleen, encephalon, and digestive tract of clinically ill fish. Under natural conditions, most clinical disease from IHNV is seen in fry when water temperature is between 8 to 15°C with fish exhibiting darkening of the skin, ascites, exophthalmia, and petechial hemorrhages internally and externally. Degeneration and necrosis of the hematopoietic tissue in the kidney is thought to be the actual cause of mortality (Egusa 1991; OIE 2006; Wolf 1988).

1. Screening Method

a. Cell culture on EPC cell line incubated at 15°C.

b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for IHNV.**

d. If CPE typical of a rhabdovirus is produced at any time during the incubation, that sample is considered **PRESUMPTIVELY positive** for IHNV, SVCV, or VHSV and confirmatory tests must be completed to distinguish among these viruses.

i. The appearance of CPE typical of a rhabdovirus is described as rounded and granular cells in grape-like clusters that may exhibit margination of nuclear chromatin (optical density of nucleoli increases and nuclear membranes appear thickened). Plaques in the confluent cell monolayers are ragged in outline and contain coarsely granular debris and rounded cells. Rounded, infected cells also accumulate at plaque margins and can be present within the plaque. Polyethylene glycol (PEG) has been used to enhance plaque formation but is not necessary to detect IHNV in open systems without an overlay (Batts and Winton 1989) see Figures 4.1 to 4.3.

ii. The Polymerase Chain Reaction (PCR), serum neutralization, or Indirect Fluorescent Antibody Test (IFAT) methods may be used to confirm the cause of the CPE is due to the presence of IHNV.

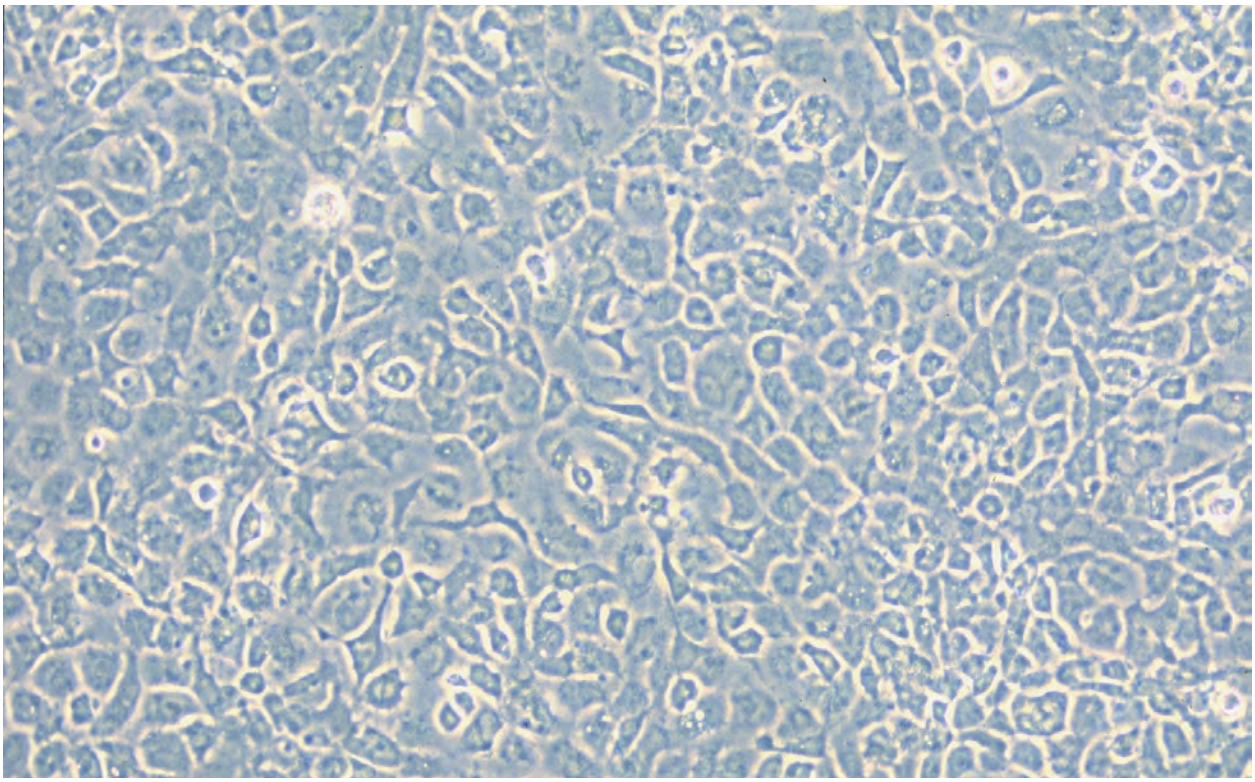


Figure 4.1. Normal EPC monolayer. Photo courtesy of Jim Winton, USGS.

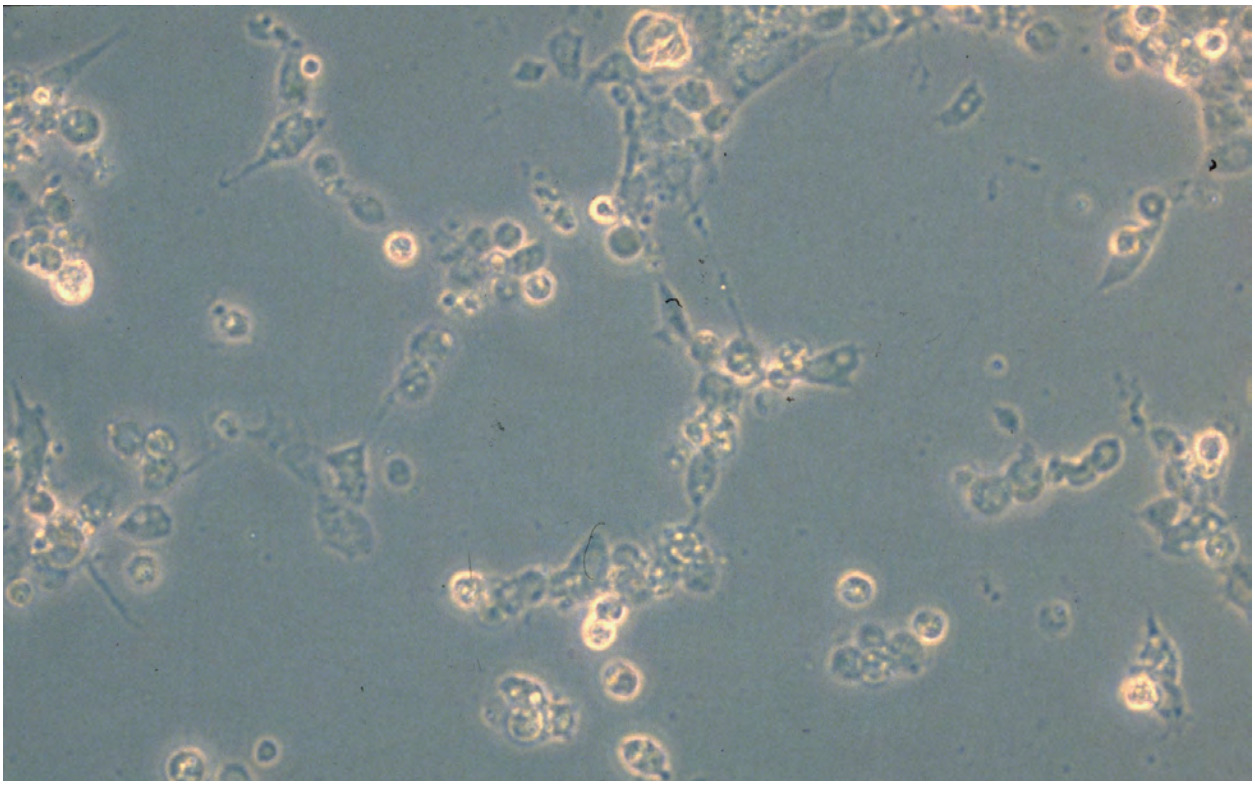


Figure 4.2. CPE typical of a rhabdovirus on EPC monolayer. Photo courtesy of Jim Winton, USGS.

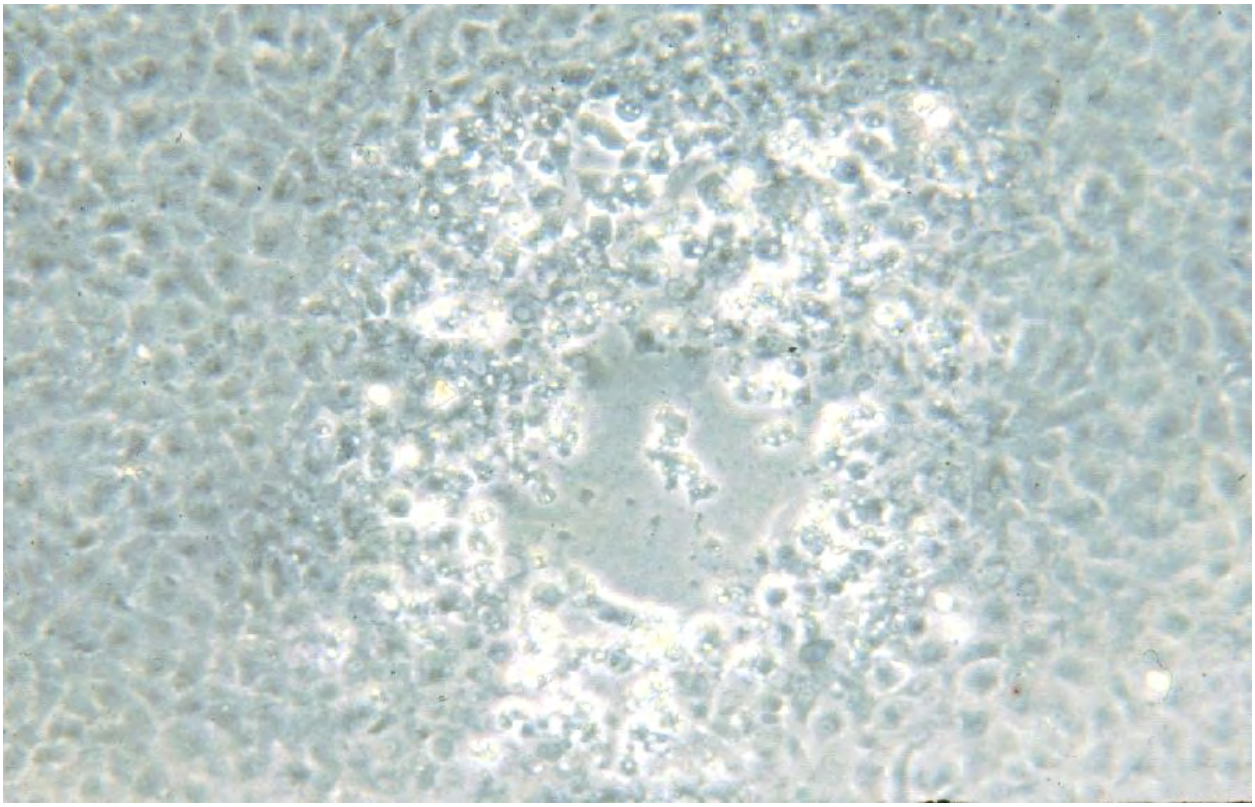


Figure 4.3. CPE plaque of IHNV on EPC monolayer. Photo courtesy of Scott LaPatra, Clear Springs. Foods, Inc.

2. Confirmation Methods for IHNV

a. Polymerase Chain Reaction (PCR) Method for Confirmation of IHNV
The Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) technique employs oligonucleotide primers to amplify base pair segments of the complementary DNA (cDNA) produced in RT-PCR reactions containing the target virus. Total RNA is extracted from cell culture supernatant, subject to reverse transcription for production of appropriate cDNA, which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.

i. Extraction of RNA from Cell Cultures
Total RNA from infected cells is extracted using a phase-separation method (eg. phenol-chloroform or TRIZOL, Invitrogen) or by RNA affinity spin columns (eg. RNeasy Total RNA kit, Qiagen) according to the manufacturer's instructions. While all of these methods work well for drained cell monolayers or cell pellets, RNA binding to affinity columns can be affected by salts present in tissue culture media and phase-separations methods should be used for extraction of RNA from cell culture fluids.

ii. Production of DNA by Reverse Transcription and Amplification by PCR

1. QA/QC (See Section 2, 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)

2. Using Section 2, 4.A1.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.

3. Using Section 2, 4.A1.B.1 Worksheet B.1 – Infectious Hematopoietic Necrosis Virus (IHNV), record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add four to the number of samples so that there is enough to run controls.)

4. Primers for IHNV*

a. Forward: 5'-AGA-GAT-CCC-TAC-ACC-AGA-GAC-3'

b. Reverse: 5'-GGT-GGT-GTT-GTT-TCC-GTG-CAA-3'

*2007 edition note: The primer sequences for the IHNV RT-PCR assay have been modified from those in previous editions of this manual. The new primers target the central portion of the G gene, instead of the previously published primers which targeted the N gene. The G gene primer set has been tested extensively (Emmenegger et al., 2000, Kurath et al., 2003) and avoids using a series of six consecutive 'G's' that were less than optimal for PCR in the N gene forward primer.

5. Thermocycler Program for IHNV

a. Incubate at 50°C for 30 minutes for Reverse Transcriptase reaction.

b. Denature sample at 95°C for two minutes.

c. 30 cycles as follows:

i. Denaturing at 95°C for 30 seconds.

ii. Annealing at 50°C for 30 seconds.

iii. Extending at 72°C for 60 seconds.

d. Final extension at 72°C for seven minutes.

Hold samples at 4°C after cycling is complete. PCR Products can be refrigerated for one month or frozen at -20°C for long-term storage.

iii Visualization of PCR Product by Electrophoresis (Section 2, 6.3.C "Detection of Product")

1. Visualize the DNA
Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in PCR assays.

a. **Bands occurring at the 693 bp location are confirmatory for IHNV and are reported as POSITIVE.**

b. **If there is an absence of appropriate bands with no indication of problems with the assay, consider testing for other viruses or consult an appropriate reference laboratory.**

2. Photograph the Gel (Section 2, 6.3.G "Visualize the DNA")
Photo document all gels and attach the photo to the case history information. (Section 2, 4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)

b. Serum Neutralization Method
See Section 2, 4.7 Serum Neutralization for the general procedure.

i. Use the cell line on which the initial CPE was produced.

ii. Incubate plates at 15°C.

c. Indirect Fluorescent Antibody Test (IFAT) Method for the Confirmation of IHNV
See Section 2, 4.8 Indirect Fluorescent Antibody Test (IFAT) Procedures for the general procedure.

i. Use an EPC cell line.

ii. Incubate virus inoculated monolayers at 15 to 18°C.

d. Direct Alkaline Phosphatase Immunocytochemistry (APIC) Staining Method for the Confirmation of IHNV (modified from Drolet et. al, 1993) – The Alkaline Phosphatase Immunocytochemical (APIC) procedure is a method for confirming the identity of isolates

suspected to be Infectious Hematopoietic Necrosis Virus. This is a direct assay that can detect antigen in formalin fixed and stained tissue culture cells for up to one year. There are four basic steps for the APIC assay: preparing and fixing cell monolayers; infecting the cell monolayers with the virus; staining the plate; reading and interpreting the stained plate. The procedure outlined below uses reagent kits obtained from Vector Laboratories, Burlingame, CA and DiagXotics Inc., Wilton, CT but suitable stain kits and antibodies are also be available from other sources.

i. Preparation of Materials

1. Prepare the reagents according to the instructions provided by the supplier.

2. Appropriate QA/QC testing should be performed periodically on the antibodies, antisera, stains, and cell lines to ensure accurate results are obtained during this assay.

ii. Preparation of Cell Monolayers in 96-well plates

1. At least four sets of plates are made for each assay to provide sufficient room for 6-8 replicates of each sample, positive control (reference) viruses, and negative control (uninoculated) cell lines. One set of plates is then fixed, stained and examined at approximately 8, 12, 24, and 48 hours as necessary for confirmation. Observing the cultures at multiple time points allows for visualization of the virus at the most appropriate stage of infection of the cell monolayer.

2. Working in a clean hood, seed sufficient wells of the 96-well plate with the appropriate volume of that cell suspension as in Section 2, 4.3.B "Seeding Procedures for Plates."

3. Incubate at 20 to 25^{o}C for 18 to 24 hours or until the cells are 80 to 100% confluent as this minimizes excessive cell loss during fixation and staining.

iii. Virus Sample Inoculation and Incubation

1. Preparation of the Suspect and Known Virus Sample

a. Using a pipette, stir and scrape the bottom of the suspect virus well or reference virus-infected flask (Section 2, 4.7.C.2 "Procedure for Producing Reference Viruses") to be subcultured to dislodge the cell layer.

b. Aspirate the fluid and cell debris from the well or flask and place in a sterile tube for centrifugation. Samples may be diluted up to 1:100 in HBSS.

c. Tubes are centrifuged at 4°C for 15 minutes at 2000-3000 X g.

d. Use supernatant from this tube for inoculation of the cell cultures in the plates.

2. Inoculation of the Cell Cultures with the Suspect and Known Virus Samples

a. Aspirate media from each well of the wells to be inoculated, leaving a small

amount to inhibit cell dehydration.

b. Inoculate 50 to 100 µL of supernatant prepared in "Preparation of the Suspect and Known Virus Sample" (above) from each sample onto four replicate wells in each of four sets of plates.

c. Inoculate 50 to 100 µL of reference virus onto replicate wells in each of four sets of plates, one set to be used at each time period.

d. At least one negative (uninoculated) control well is made on each plate set for each cell line used so that one will be stained and examined at each time period.

e. Absorb 30-60 minutes without rocking at 15 to 18°C, sample removal is not necessary.

f. Add 100 µL MEM-5 (Section 2, 4.9.F "MEM-5/Hepes (Tissue Culture Medium for all Cell Lines Except SHK-1)") per well.

3. Incubate at 15 to 18°C.

iv. Plate Fixation

1. Working under the hood at room temperature, add 50 µL 10 % Neutral Buffered Formalin (NBF) (Section 2, 2.3.C.3 "10% Neutral Buffered Formalin (10% NBF)") to each well without dumping the media.

2. Fix for five minutes.

3. Remove media and fixative and disinfect prior to disposal.

4. Add 200 µL/well tap water and let stand for 1 minute.

5. Remove water and disinfect prior to disposal.

6. Allow plate to air dry.

7. Store plate at 5-25 °C until stained.

v. Staining (Modified from Vectastain kit instructions)

1. Pre-heat incubator to 37-45 °C. Buffer may be heated in the incubator for more rapid staining.

2. Add 200 µL of wash buffer to each well and let soak for 1 minute. Remove buffer.

3. Repeat step 4.12.E.2 above to remove any remaining fixative.

4. Add 50 µL of blocking reagent to each well.

5. Incubate at 37-45 °C for 30 minutes.

6. Remove blocking reagent by turning the plate upside down and shaking. Do not wash.

7. Dilute DiaXotics 14D primary antibody 1:10 in buffer and add 50 µL to each well.

8. Incubate at 37-45 ^{o}C for 15-30 minutes.

9. Wash by repeating step 4.12.E.2 three times.

10. Add 50 µL of Vectastain Biotinylated Universal Antibody to each well.

11. Incubate at 37-45 ^{o}C for 15-30 minutes.

12. Wash by repeating step 4.12.E.2 three times.

13. Add 50 µL of Vectastain ABC-AP Reagent to each well.

14. Incubate at 37-45 ^{o}C for 15-30 minutes.

15. Wash by repeating step 4.12.E.2 five times.

16. Add 50 µL of Vector Red Alkaline Phosphatase Substrate to each well (should be used within 15 minutes of preparation).

17. Incubate in the dark at room temperature for 15-30 minutes.

18. Wash by repeating step 4.12.E.2 three times.

19. Dry and Store at 25 ^{o}C.

20. Examine at 400-1000x magnification.

vi. Results

1. Positive controls should contain individual cells that appear red and early plaques will appear as clumps of several infected cells. No red should be seen on negative controls. If red "clouds" are seen, there may have been insufficient washing or a fold in the cell sheet.

2. With no problems identified in the assay and with red cells in the suspect sample which appear similar to the positive control at one of the time points, the suspect sample is considered **POSITIVE** for IHNV.

3. With no problems identified in the assay but without appropriate red color at any of the four time periods, the suspect sample is considered **NEGATIVE** for IHNV.

B. Infectious Pancreatic Necrosis Virus (IPNV)

Infectious Pancreatic Necrosis Virus (IPNV) is a nonenveloped, icosahedral shaped bi-segmented double-stranded RNA virus belonging to the *Aquabirnavirus* genus of the *Birnaviridae*. There are many viruses in the *Aquabirnavirus* group, most of which have not been shown to cause disease in fish. If warranted, additional tests can be used to further identify the virus as IPNV. IPNV has a wide geographic range that includes North and South America, Asia, and Europe. It is very stable under a wide range of environmental conditions and is capable of surviving for several days in both fresh and saltwater. It is resistant to a wide range of chemical disinfectants including ether, chloroform, and quaternary ammonium compounds but is deactivated by 70% ethanol. Isolates display wide antigenic diversity and virulence. There are two sero-groups that do not cross-react in serum neutralization tests with the majority of strains belonging to sero-group A. IPNV has been isolated from several species of marine and freshwater fish and shellfish. Acute catarrhal enteritis has primarily been seen in salmonid fry and fingerlings with initial mortality occurring in the more robust individuals. Fish that survive the disease may become asymptomatic carriers and shed the virus through the feces and sex products. IPNV may be transmitted vertically as well as horizontally (Bruno and Poppe 1996; Egusa 1991; OIE 2000; Roberts 1982; Wolf 1988).

1. **Screening Method**

   a. Cell culture on CHSE-214 cell line incubated at 15°C.

   b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

   c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for IPNV.**

   d. If CPE typical of IPNV is produced at any time during the incubation, the sample is considered **PRESUMPTIVELY positive**.

      i. The appearance of CPE typical of IPNV is described as stellate shaped plaques with spindle-shaped cells. Some of the cells within the plaque will exhibit nuclear pyknosis (nuclei shrink in size and chromatin condenses) with other cells appearing normal. See Figures 4.4 and 4.5. Typically, positive cultures result in rapidly lytic CPE but some cells may survive and reform a normal looking monolayer.

      ii. The serum neutralization, PCR, or IFAT methods may be used to confirm the cause of the CPE is due to the presence of IPNV.

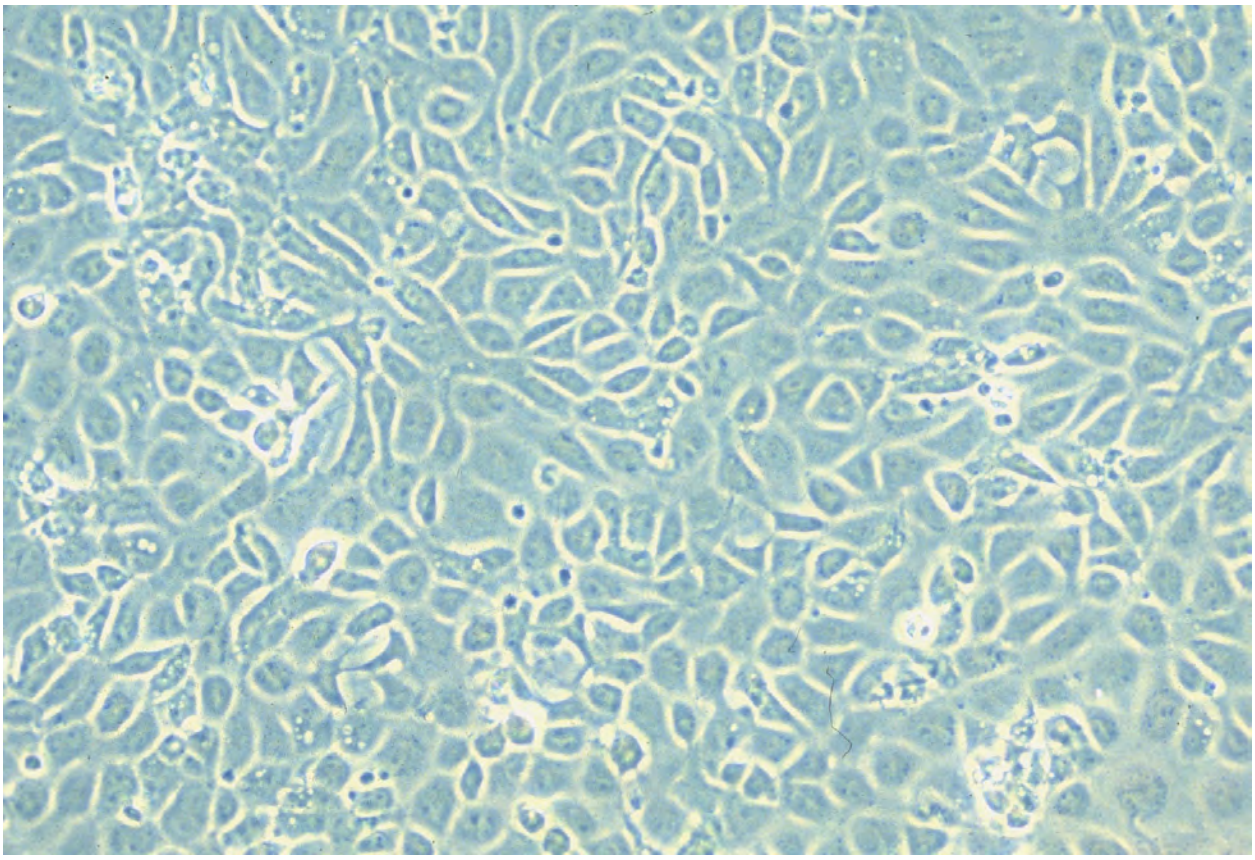


Figure 4.4. Normal CHSE-214 monolayer. Photo courtesy of Jim Winton, USGS.

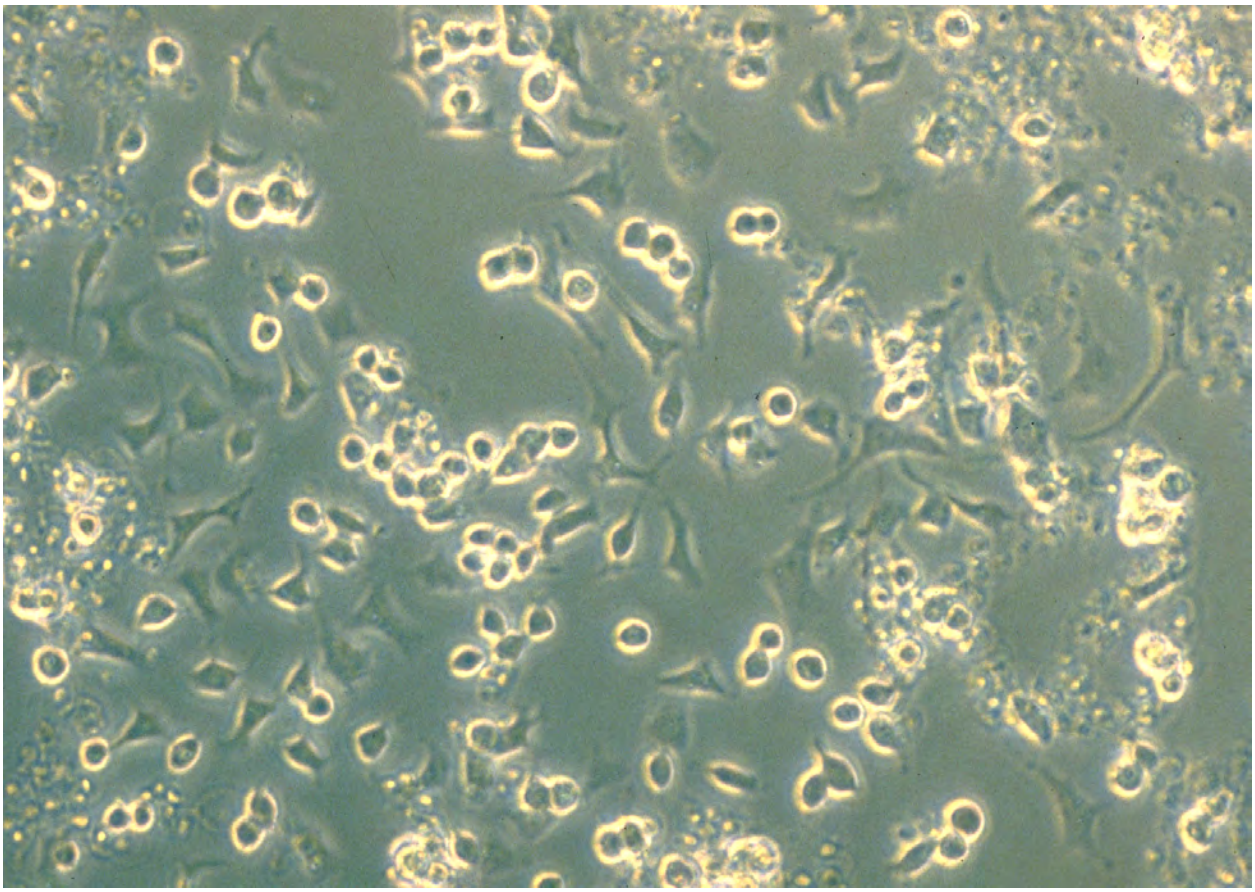


Figure 4.5. CPE typical of IPNV on CHSE-214 cells. Photo courtesy of Jim Winton, USGS.

2. **Confirmation Methods for IPNV**

   a. Serum Neutralization Method
   See Section 2, 4.7 Serum Neutralization for the general procedure.

      i. Use the cell line on which the initial CPE was produced.

      ii. Incubate plates at 15°C.

   b. Polymerase Chain Reaction (PCR) Method for Confirmation of IPNV (Modified from Blake et al., 1995): The Reverse Transcriptase
   Polymerase chain reaction (RT-PCR) technique employs oligonucleotide primers to amplify base pair segments of the complementary DNA (cDNA) produced in RT-PCR reactions containing the target virus. Total RNA is extracted from cell culture supernatant, subject to reverse transcription for production of appropriate cDNA, which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.

      i. Extraction of RNA from Cell Culture Fluid (heat RNA Release Method)

         1. Dilute cell culture fluid 1:50 in molecular grade RNase free water by adding 2 µL fluid to 98 µL water in microcentrifuge tubes.

2. Place tubes in heat block at 100°C for 10 minutes.

3. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from re-annealing on itself).

4. Quantify RNA template in the spectrophotometer. The optimum amount of RNA template should be around 100 µg/mL (or 100 ng/µL). Generally, 1 µL of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/µL or use up to 5 µL/reaction if reading falls below 50 ng/µL.

ii. Formation of DNA by Reverse Transcription and Amplification by PCR

1. QA/QC (See Section 2, 6.2 PCR Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)

2. Using Section 2, 4.A1.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.

3. Using Section 2, 4.A1.B.2 Worksheet B.2 – Infectious Pancreatic Necrosis Virus (IPNV), record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add four to the number of samples so that there is enough to run controls in the assay.)

4. Primers for IPNV

   a. Forward: 5'- AAA GCC ATA GCC GCC CAT GAA C -3'

   b. Reverse: 5'- TCT CAT CAG CTG GCC CAG GTA C -3'

5. Thermocycler Program for IPNV

   a. Pre-dwell at 50°C for 15 minutes.

   b. Preheat or "Jumpstart" sample to 95°C for two minutes.

   c. 35 cycles as follows:

      i. Denaturing at 95°C for 30 seconds.

      ii. Annealing at 50°C for 30 seconds.

      iii. Extending at 72°C for 60 seconds.

   d. Post dwell at 72°C for seven minutes.

   e. Hold samples at 4°C after cycling is complete.
   PCR Products can be refrigerated for one month or frozen at -70°C for long-term storage.

iii. Visualization of PCR Product by Electrophoresis (Section 2, 6.3.C "Detection of Product")

1. Visualize the DNA
Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in both the first and second (nested) round PCR assays.

a. **Bands occurring at the 174 bp location are confirmatory for IPNV and are reported as POSITIVE.**

b. **The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for IPNV.**

2. Photograph the Gel (Section 2, 6.3.G "Visualize the DNA")
Photo document all gels and attach the photo to the case history information. (Section 2, 4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)

c. Indirect Fluorescent Antibody Test (IFAT) Method for the Confirmation of IPNV
See Section 2, 4.8 Indirect Fluorescent Antibody Test (IFAT) Procedures for the general procedure.

i. Use a CHSE-214 cell line.

ii. Incubate virus inoculated monolayers at 15 to 18°C.

C. Infectious Salmon Anemia Virus (ISAV)

Infectious Salmon Anemia Virus (ISAV) is a spherical enveloped single-stranded RNA virus belonging to the newly proposed *Isavirus* genus of the *Orthomyxoviridae*. The disease is mostly seen in Atlantic salmon in salt water in the spring and fall associated with rapidly changing water temperature. Characteristics of the disease include anemia, ascites, petechial hemorrhages on the peritoneal surface and perivisceral fat, and congestion of the liver, spleen, kidney, and upper digestive tract. While Atlantic salmon are the only species known to suffer disease, the virus has been isolated from free ranging Atlantic salmon, Rainbow trout and Brown or sea trout; however, isolation of ISAV from carrier fish may be difficult. (Bruno and Poppe 1996; OIE 2000).

1. **Screening Method**

a. Cell culture on SHK-1 cell line incubated at 15°C (Bouchard et al., 1999).

b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for ISAV.**

d. If CPE typical of ISAV is produced at any time during the incubation, the sample is considered **PRESUMPTIVELY positive.**

   i. The appearance of CPE typical of ISAV is described as plaques of vacuolated cells that round up and loosen from the growth surface. It may progress to involve the entire cell sheet with only small, rounded, refractile, and necrotic cells observable. See Figures 4.6 and 4.7.

   ii. Polymerase chain reaction (PCR) or IFAT methods may be used to confirm the cause of the CPE is due to the presence of ISAV.

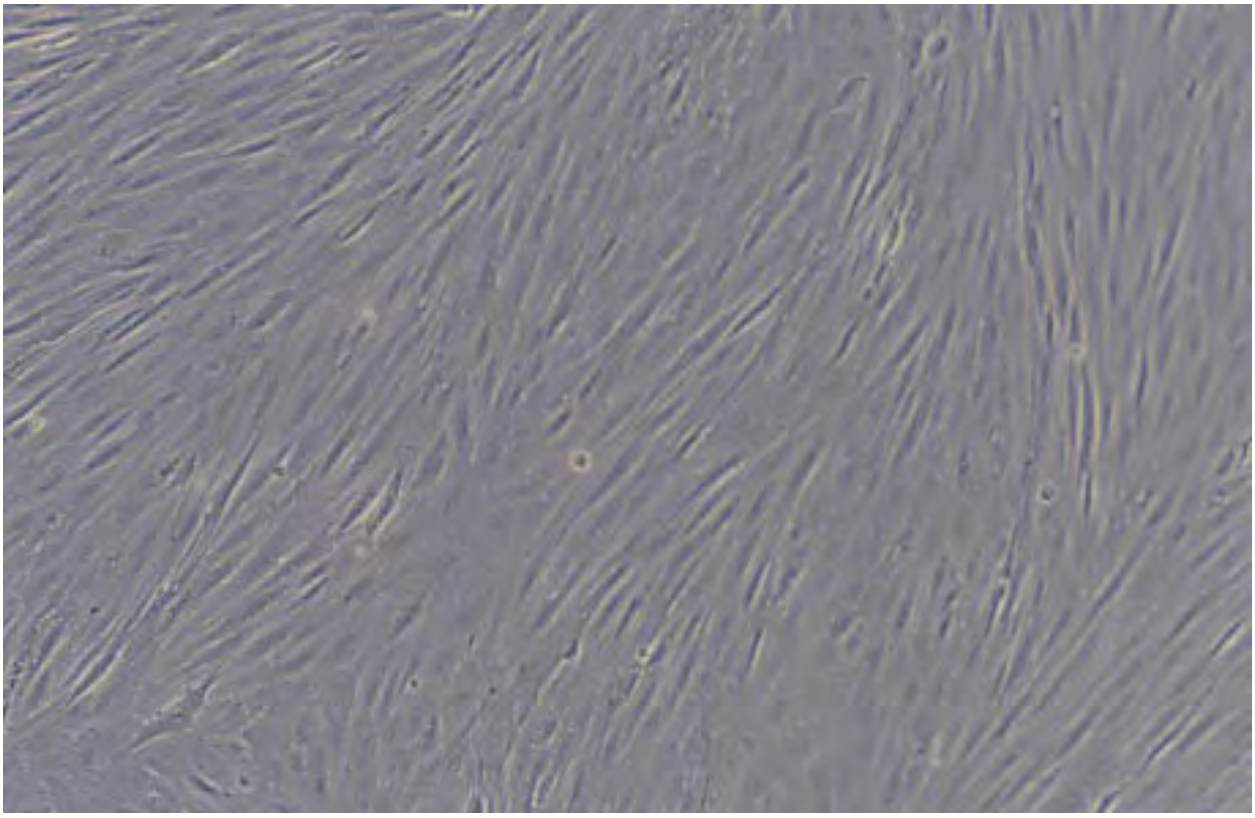


Figure 4.6. Normal SHK-1 monolayer. Photo courtesy of Jim Winton, USGS.

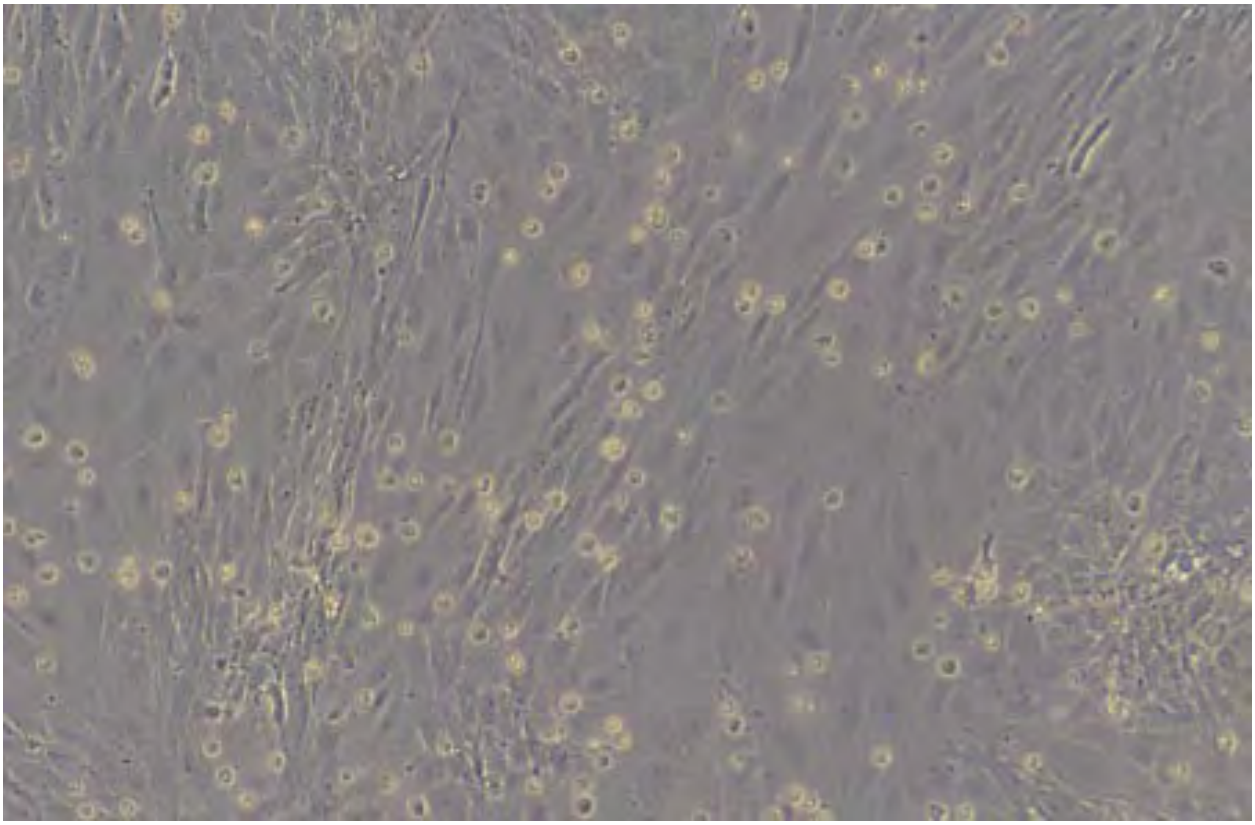


Figure 4.7. CPE typical of ISA on SHK-1 cells. Photo courtesy of Jim Winton, USGS.

2. Confirmation Methods for ISAV

a. Polymerase Chain Reaction (PCR) (Modified from Bouchard et al., 1999): The Reverse Transcriptase
Polymerase chain reaction (RT-PCR) technique employs oligonucleotide primers to amplify base pair segments of the complementary DNA (cDNA) produced in RT-PCR reactions containing the target virus. Total RNA is extracted from cell culture supernatant, subject to reverse transcription for production of appropriate cDNA, which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.

i. Extraction of RNA from Cell Culture Fluid (heat RNA Release Method)

1. Dilute cell culture fluid (with some cell scrapings) 1:50 in molecular grade RNAase free water by adding 2 µL fluid to 98 µL water in microcentrifuge tubes.

2. Heat to 95°C for two minutes in a heat block, water bath, or thermocycler.

3. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from reannealing on itself).

4. Quantify RNA template in the spectrophotometer. The optimum amount of RNA template should be around 100 µg/mL (or 100 ng/µL). Generally, 1 µL of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/µL or use up to 5 µL/reaction if reading falls below 50 ng/µL.

ii. Formation of DNA by Reverse Transcription and Amplification by PCR

1. QA/QC (See Section 2, 6.2 PCR Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)

2. Using Section 2, 4.A1.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.

3. Using Section 2, 4.A1.B.3 Worksheet B.3 – Infectious Salmon Anemia Virus (ISAV), record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add four to the number of samples so that there is enough to run controls and empty slots in the assay.)

a. Primers for ISAV

i. Forward: 5'-GGC TAT CTA CCA TGA ACG AAT C-3'

ii. Reverse: 5'-TAG GGG CAT ACA TCT GCA TC-3'

b. Thermocycler Program for ISAV

i. Pre-dwell at 42°C for 15 minutes.

ii. Preheat or "Jumpstart" sample to 94°C for five minutes.

iii. 40 cycles as follows:

1) Denaturing at 94°C for 45 seconds.

2) Annealing at 59°C for 45 seconds.

3) Extending at 72°C for 105 seconds.

iv. Post dwell at 72°C for seven minutes.

v. Hold samples at 4°C after cycling is complete.
PCR Products can be refrigerated for one month or frozen at -70°C for long-term storage.

iii. Visualization of PCR Product by Electrophoresis (See Section 2, 6.3.C "Detection of Product.")

1. Visualize the DNA
Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in the PCR assays.

a. **A band occurring at the 493 bp location is confirmatory for ISAV and the sample is reported as POSITIVE.**

b. **The lack of the appropriate band with no indication of problems with the assay are reported as NEGATIVE for ISAV.**

2. Photograph the Gel (Section 2, 6.3.G "Visualize the DNA")
Photo document all gels and attach the photo to the case history information. (Section 2, 4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)

b. Indirect Fluorescent Antibody Test (IFAT) Method for the Confirmation of ISAV
See Section 2, 4.8 Indirect Fluorescent Antibody Test (IFAT) Procedures for the general procedure.

i. Use a SHK-1 cell line.

ii. Incubate virus inoculated monolayers at 15 to 18°C.

D. Largemouth Bass Virus (LMBV)

Largemouth Bass Virus (LMBV) is an icosahedral enveloped double-stranded DNA virus in the ranavirus genus of the Iridoviridae family. LMBV infection has been found in centrarchid and esocid populations in the Mid-West and Southeastern United States and has been found experimentally to be associated with mortality in juvenile largemouth bass (Plumb 1999). During an active infection, the virus may be isolated from several tissues including the kidney, spleen, and swim bladder.

1. Screening Method

a. Cell culture on FHM or BF-2 cell lines incubated at 25 to 30^0C (Piaskoski et al. 1999; Grant et al. 2003; McClenahan et al. 2005)

b. Monitor for CPE at least twice per week for an initial incubation period of 7 days. If CPE is not observed, make a blind pass and incubate for an additional 7 days.

c. Re-inoculations are made from representative wells exhibiting CPE on the initial inoculations and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 7 days at 25-30 C. If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for LMBV.

d. If CPE typical of LMBV is produced at any time during the incubation, the sample is considered **PRESUMPTIVELY positive.**

i. The appearance of CPE typical of LMBV is described as circular cell free areas with rounded cells at the margins. See Figures 4.8 through 4.11.

ii. The polymerase chain reaction (PCR) method is used to confirm that the cause of the CPE is due to the presence of LMBV.

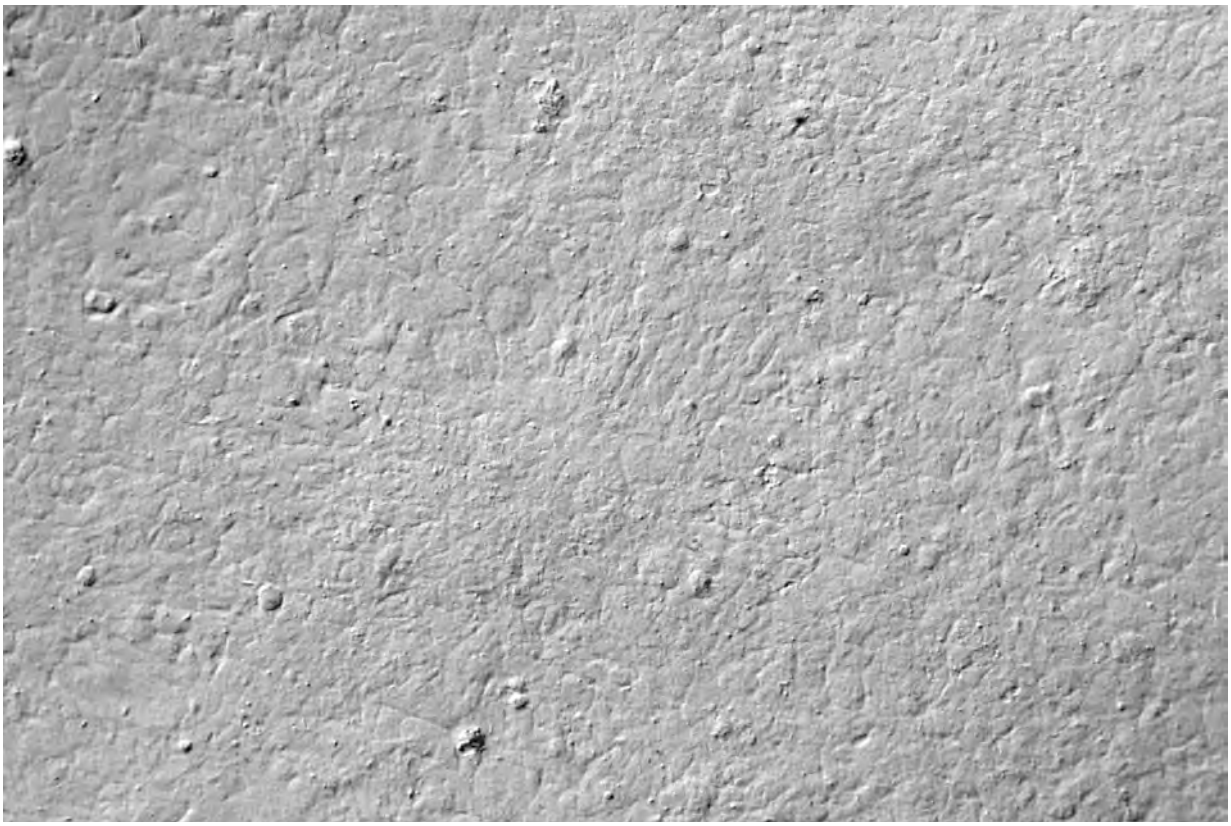


Figure 4.8. Normal FHM monolayer. Photo courtesy of John Grizzle, Auburn University.

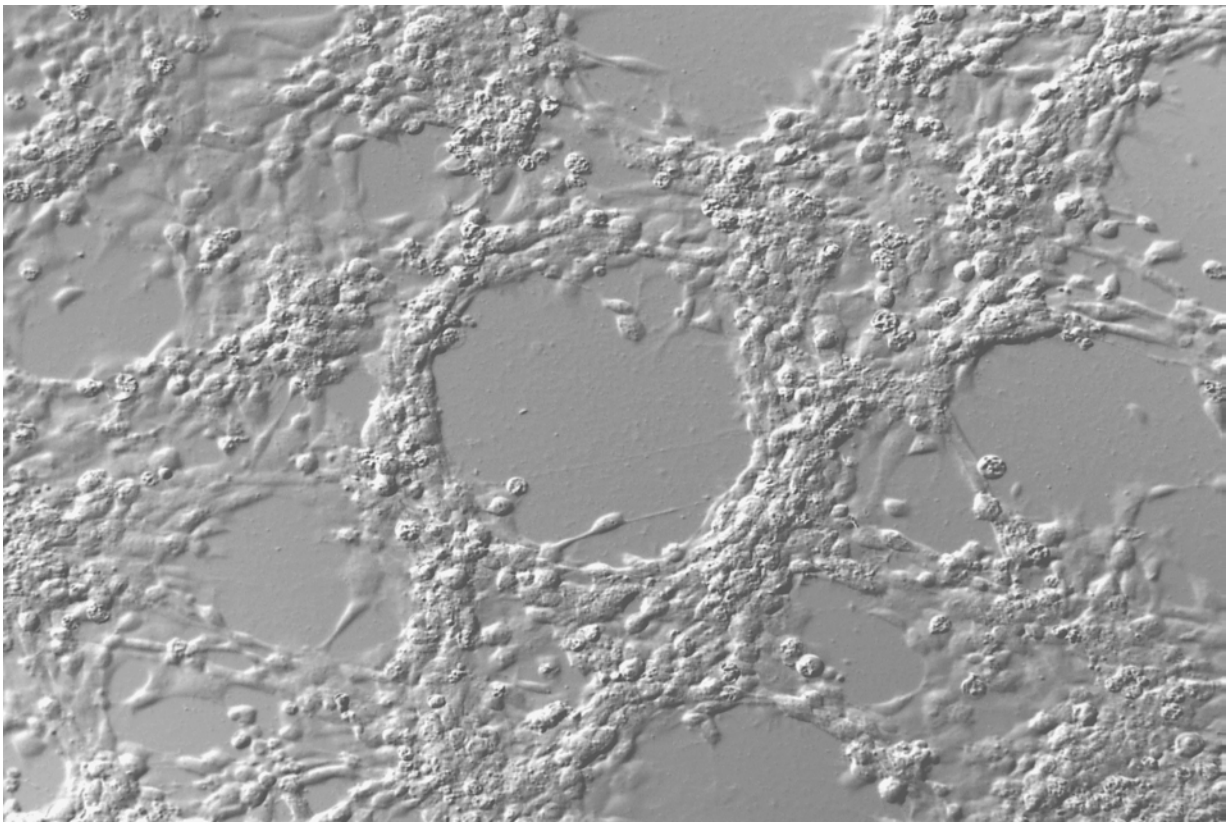


Figure 4.9. CPE typical of LMBV on FHM cells. Photo courtesy of John Grizzle, Auburn University.

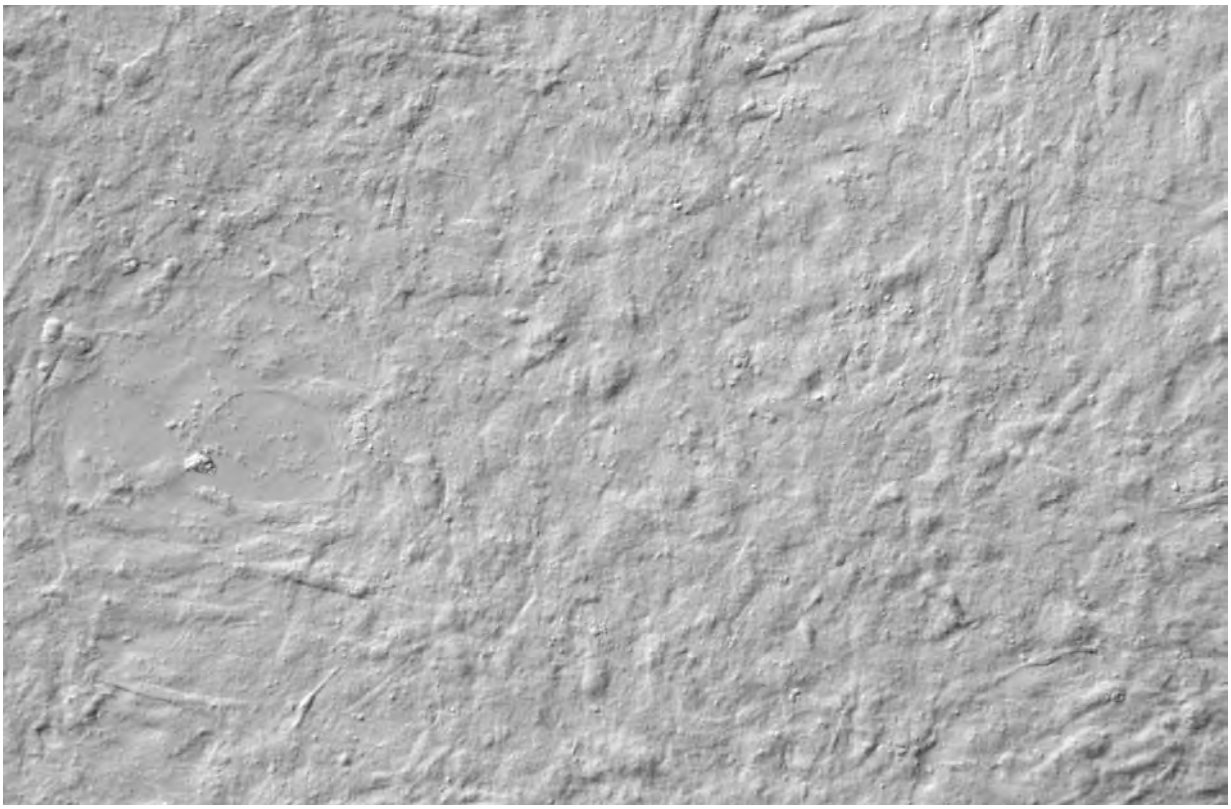


Figure 4.10. Normal BF-2 monolayer. Photo courtesy of John Grizzle, Auburn University.

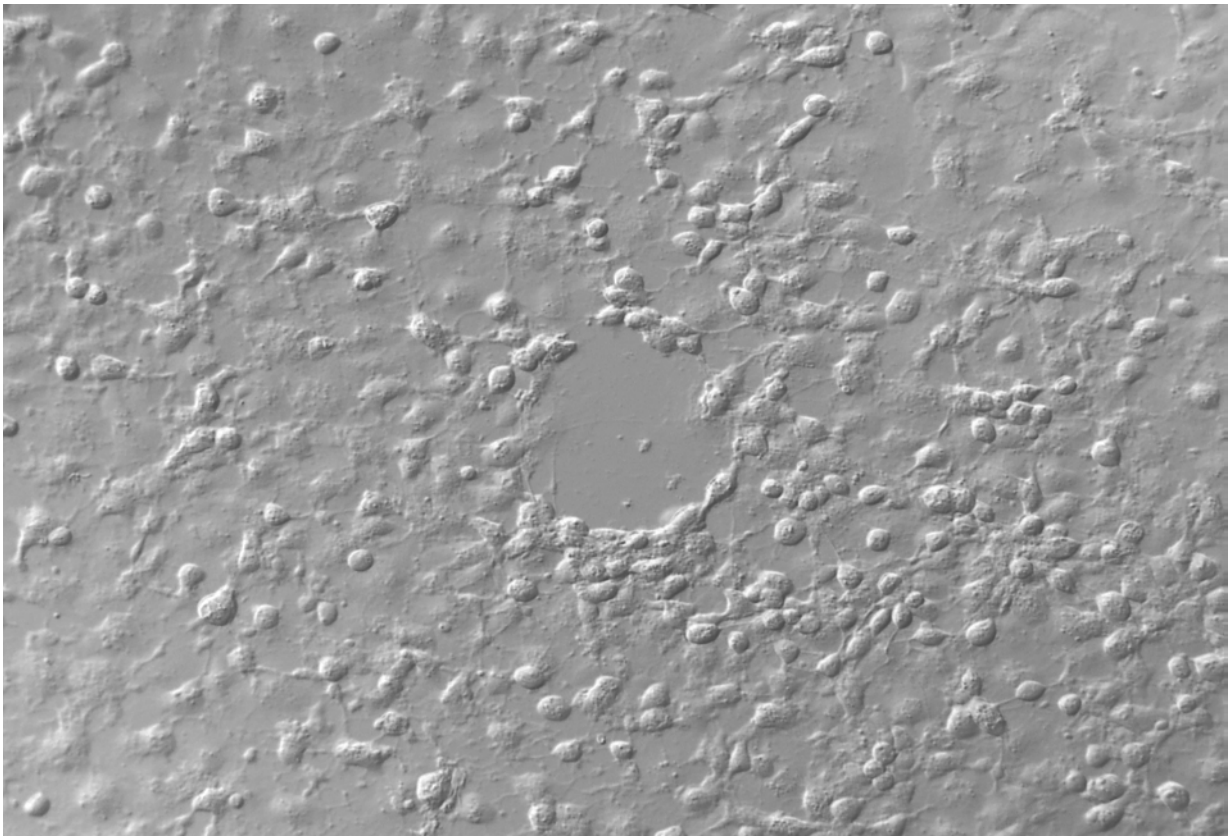


Figure 4.11. CPE typical of LMBV on BF-2 cells. Photo courtesy of John Grizzle, Auburn University.

2. **Confirmation Method for LMBV - Polymerase Chain Reaction (PCR) (Modified from Plumb et al., 1999)**
   This is a DNA containing virus so DNA is extracted from cell culture fluid and amplified with forward and reverse primers. The DNA products are then visualized by agarose gel electrophoresis.

   a. Extraction of DNA from Cell Culture-Use one of the following methods.
      BuccalAmp DNA Extraction (modified by J. Woodland, USFWS)
      Extract DNA follwing the procedures outlined in the Buccal Amp DNA extraction kit (Epicentre®). Collect sample by rotating the swab onto infected cells still attached to the well of the cell culture plate. To obtain sufficient DNA, swab 1-2 wells of a 24-well plate or 3-4 wells of a 48-well plate. Follow remaining steps provided with the kit.

      Qiagen DNA Extraction
      The following procedure employs an extraction kit available from Qiagen, Inc. http://www.qiagen.com.) DNA Extraction kits of similar efficacy are available from many other sources and can be utilized as alternatives for extraction of DNA by following their protocol. These kits utilize "spin columns" for binding and elution of DNA from cell culture lysates. Most do not require the use of highly toxic reagents and reduce the chance of contamination during extraction.

      i. Supernatant and cells from suspect sample wells are removed and centrifuged for five minutes at 300 X g.

      ii. Resuspend pellet in 200 µL PBS.

      iii. Add 20 µL proteinase K solution (Section 2, 5.6.E "Proteinase K") and 200 µL buffer AL to the sample, mix thoroughly by vortexing.

      iv. Incubate for 10 minutes at 70°C.

      v. Add 200 µL of 100% ethanol to the sample, mix thoroughly by vortexing.

      vi. Pipet the mixture, including any precipitate into the DNeasy spin column sitting in the 2 mL collection tube provided.

      vii. Centrifuge for one minute at 6,000 Xg. Discard flow-through and collection tube.

      viii. Place the DNeasy spin column in a new 2 mL collection tube, add 500 µL buffer AW1, and centrifuge for one minute at 6,000 Xg. Discard the flow-through and collection tube.

      ix. Place the DNeasy spin column in a new 2 mL collection tube, add 500 µL buffer AW2, and centrifuge for three minutes at full speed to dry the membrane.

      x. Place the DNeasy spin column in a clean 1.5 or 2 mL microcentrifuge tube and pipet 100 µL buffer AE directly onto the DNeasy membrane.

xi. Incubate at room temperature for one minute, then centrifuge for one minute at 6,000 Xg to elute.

xii. Repeat steps x and xi.

xiii. Discard spin column and store DNA solution at -20 to -70°C until used for amplification.

xiv. Quantify the amount of DNA extracted with a spectrophotometer (Section 2, Chapter 6 Polymerase Chain Reaction (PCR)). The optimum amount of DNA template should be around 100 µg/mL (or 100 ng/µL). Generally, 1 µL of DNA template is sufficient per reaction. Dilute template if more than 300 ng/µL or use up to 5 µL/reaction if reading falls below 50 ng/µL.

b. Amplification of LMBV DNA

i. General QA/QC Considerations (See Section 2, 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)

ii. Using Section 2, 4.A1.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.

iii. Using Section 2, 4.A1.B.4 Worksheet B.4 - Largemouth Bass Virus (LMBV), record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add four to the number of samples so that there is enough to run controls in the assay.)

iv. Primers for LMBV (Grizzle et al., 2003)

1. Forward: 5'-GCG GCC AAC CAG TTT AAC GCA A -3'

2. Reverse: 5'- AGG ACC CTA GCT CCT GCT TGA T -3'

v. Thermocycler Program for LMBV

1. Pre-dwell sample at 95°C for 3 min 15 sec.

2. 35 cycles of the following regime:

a. Denaturing at 95°C for 45 sec.

b. Annealing at 60°C for 45 sec.

c. Extending at 72°C for 60 sec.

3. Post-dwell at 72°C for 7 min.

4. Hold samples at 4°C after cycling is complete.

PCR products can be refrigerated for one month or frozen at -70°C for long-term storage.

c. Visualization of PCR Product by Electrophoresis (Section 2, 6.3.C "Detection of Product")
Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated location according to primers used.

i. **A band occurring at the 248 bp location is confirmatory for LMBV and the sample is reported as POSITIVE.**

ii. **The lack of the appropriate band with no indication of problems with the assay are reported as a NEGATIVE sample for LMBV.**

d. Photograph the Gel (Section 2, 6.3.G "Visualize the DNA")
Photo document all gels and attach the photo to the case history information. (Section 2, 4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)

E. Oncorhynchus Masou Virus (OMV)

Oncorynchus Masou Virus (OMV) is an enveloped double-stranded DNA virus belonging to the *Herpesvirus* genus of the *Herpesviridae*. Salmonids are the only fish known to be susceptible to infection with OMV with kokanee being the most susceptible. The geographic range has so far been limited to Japan and Eastern Asia. The initial disease is a septicemia that may cause edema and hemorrhage in fry during which time, the virus will be shed in the feces and urine and isolated from the liver, kidney, and spleen. Several months later, survivors may develop epithelial tumors around the mouth and fins with virus able to be isolated from these lesions. Most disease is seen in water temperatures below 14°C. Although OMV may be isolated from ovarian fluid at spawning, transmission is primarily by the horizontal route (OIE 2000; Wolf 1988).

1. **Screening Method**

a. Cell culture on CHSE-214 cell line incubated at 15°C.

b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for OMV.**

d. If CPE typical of OMV is produced at any time during the incubation, the sample is considered **PRESUMPTIVELY positive**.

i. The appearance of CPE typical of OMV is described as the formation of rounded cells which progress to marked syncytia and eventual lysis of the entire cell sheet. See Figures 4.12 and 4.13.

ii. The polymerase chain reaction (PCR) method may be used to confirm that the cause of the CPE is due to the presence of OMV or suspect samples may be sent to an appropriate laboratory for confirmation.

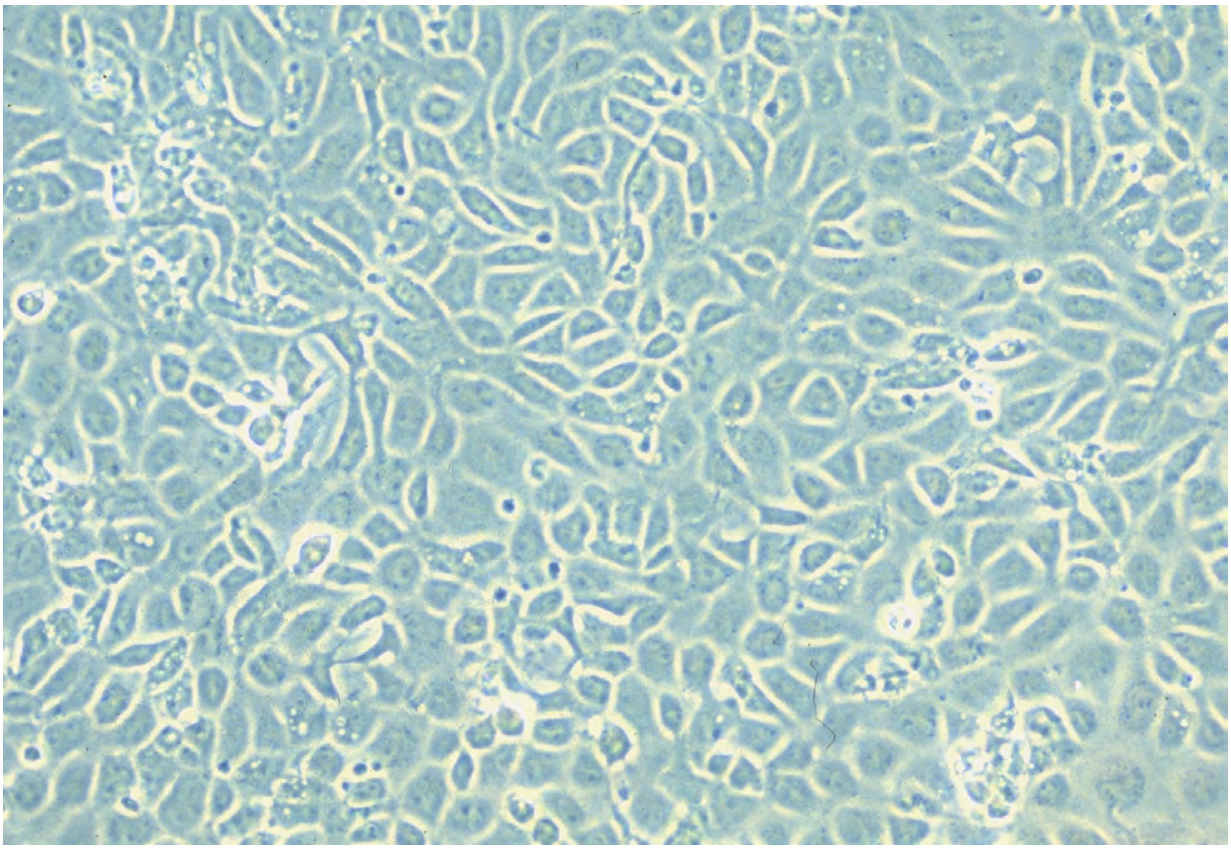


Figure 4.12. Normal CHSE-214 monolayer. Photo courtesy of Jim Winton, USGS.

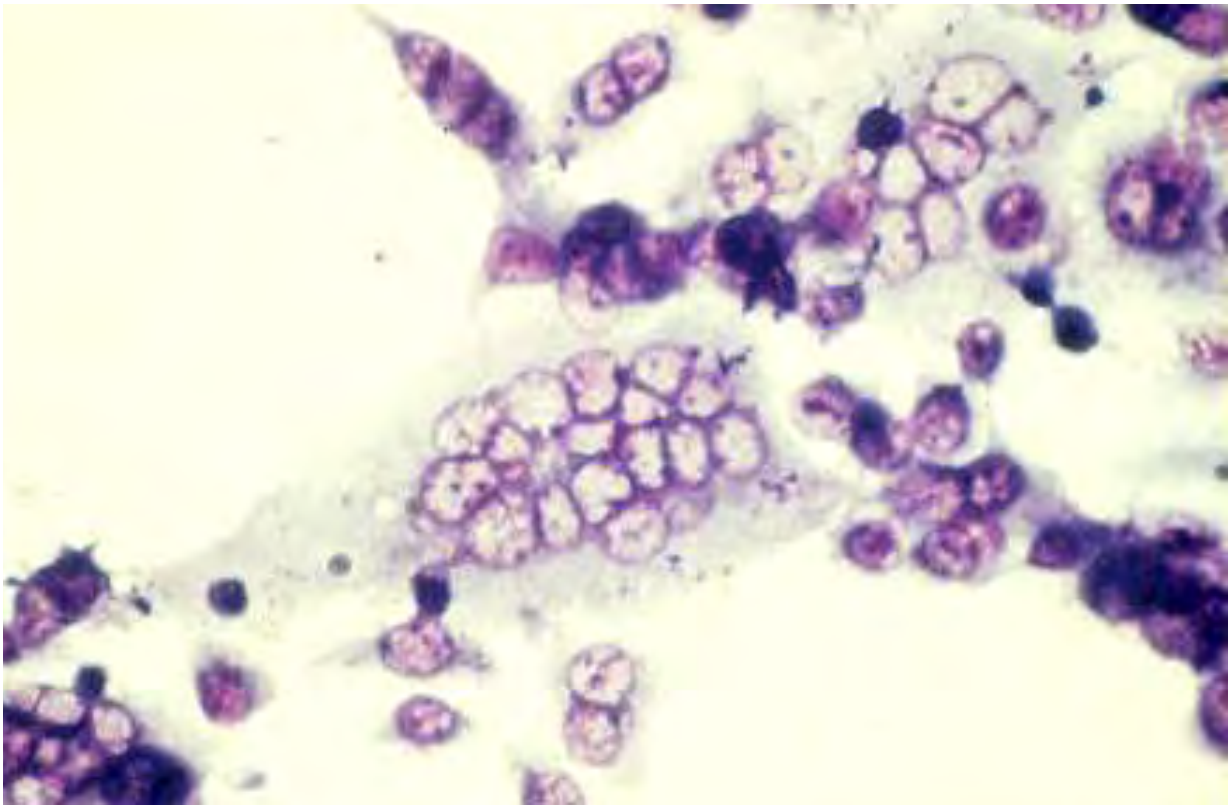


Figure 4.13. CPE typical of OMV on CHSE-214 cells. Photo courtesy of Mamoru Yoshimizu, Hokkaido University.

2. Confirmation Method for OMV

OMV is considered an exotic pathogen in the United States and the maintenance of live virus for positive controls by serological methods may not be prudent in many laboratories. A polymerase chain reaction (PCR) procedure that does not require live positive control material has been developed for this virus (Modified from Aso et al., 2001). This is a DNA containing virus so DNA is extracted from cell culture material and amplified with forward and reverse primers. The DNA products are then visualized by agarose gel electrophoresis.

a. Extraction of DNA from Cell Culture Fluid

i. Supernatant and cells from suspect sample wells are removed and a pellet is formed by centrifugation of this material at 19,000 Xg (14,800 rpm) for 15 minutes.

ii. Wash the pellets twice with 1 mL PBS and mix with 200 µL of chelating resin (Sigma).

iii. Incubate the mixture at 56°C for 20 minutes in a water bath, vortex, and then place in a boiling water bath for eight minutes.

iv. Vortex the samples and centrifuge at 8200 Xg (10,000 rpm) for 90 seconds.

b. Amplification of OMV DNA

i. General QA/QC Considerations (See Section 2, 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)

ii. Using Section 2, 4.A1.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.

iii. Using Section 2, 4.A1.B.5 Worksheet B.5 – *Oncorhynchus masou* Virus (OMV), record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add four to the number of samples so that there is enough to run controls in the assay.)

iv. Primers for OMV

1. Forward: 5'-GTA-CCG-AAA-CTC-CCG-AGT-C-3'

2. Reverse: 5'- AAC-TTG-AAC-TAC-TCC-GGG-G-3'

v. Thermocycler program for OMV

vi. 30 cycles of the following regime:

1. Denaturing at 94°C for 30 seconds.

2. Annealing at 56°C for 30 seconds.

3. Extending at 72°C for 30 seconds.

vii. Hold samples at 4°C after cycling is complete.

Note: **PCR Products can be refrigerated for one month or frozen at -70°C for long-term storage.**

c. Visualization of PCR Product by Electrophoresis (Section 2, 6.3.C "Detection of Product")

i. Visualize the DNA
Carefully record location of bands on all positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated location according to primers used.

1. **A band occurring at the 439 bp location is confirmatory for OMV and the sample is reported as POSITIVE.**

2. **The lack of the appropriate band with no indication of problems with the assay are reported as a NEGATIVE sample for OMV.**

ii. Photograph the Gel (Section 2, 6.3.G "Visualize the DNA")
Photo document all gels and attach the photo to the case history information. (Section 2, 4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)

d. A laboratory capable of confirming the identity of OMV is the Laboratory of Microbiology, Hokkaido University, 3-1-1 Minato-cho, Hokodate, Hokkaido 041-0821, Japan. Phone/fax: (81.138) 40.88.10.

F. Spring Viremia of Carp Virus (SVCV)

Spring Viremia of Carp Virus (SVCV) is an enveloped bullet shaped single stranded RNA virus belonging to the *Vesiculovirus* genus of the *Rhabdoviridae*. The reported geographic range includes European countries that experience low water temperatures during the winter and cooler regions of the USA. The host range of SVCV includes Esocids and Cyprinids with the common carp being the principle host. Characteristic lesions include hemorrhages of the skin, gills, and viscera. Mortality is usually seasonal, often most severe in 1 or 2 year old fish during spring or early summer when water temperatures rise through the permissive range. The SVCV may rarely be found in ovarian fluid at spawning but transmission is primarily horizontal and may involve passive transfer by parasites such as the louse and leech (OIE 2000; Wolf 1988).

1. **Screening Method**

a. Cell culture on EPC cell line incubated at 20 to 25°C.

b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for SVCV.**

d. If CPE typical of a rhabdovirus is produced at any time during the incubation, that sample is considered **PRESUMPTIVELY positive** for IHNV, SVCV, or VHSV and confirmatory tests must be completed to distinguish among these viruses.

i. The appearance of CPE typical of a rhabdovirus is described as rounded and granular cells in grape-like clusters that may exhibit margination of nuclear chromatin (optical density of nucleoli increases and nuclear membranes appear thickened). Plaques in the confluent cell monolayers are ragged in outline and contain coarsely granular debris and rounded cells. Rounded, infected cells also accumulate at plaque margins and can be present within the plaque. See Figures 4.1 and 4.2 above and figure 4.14 below.

ii. The serum neutralization or polymerase chain reaction methods may be used to confirm that the CPE is due to SVCV.

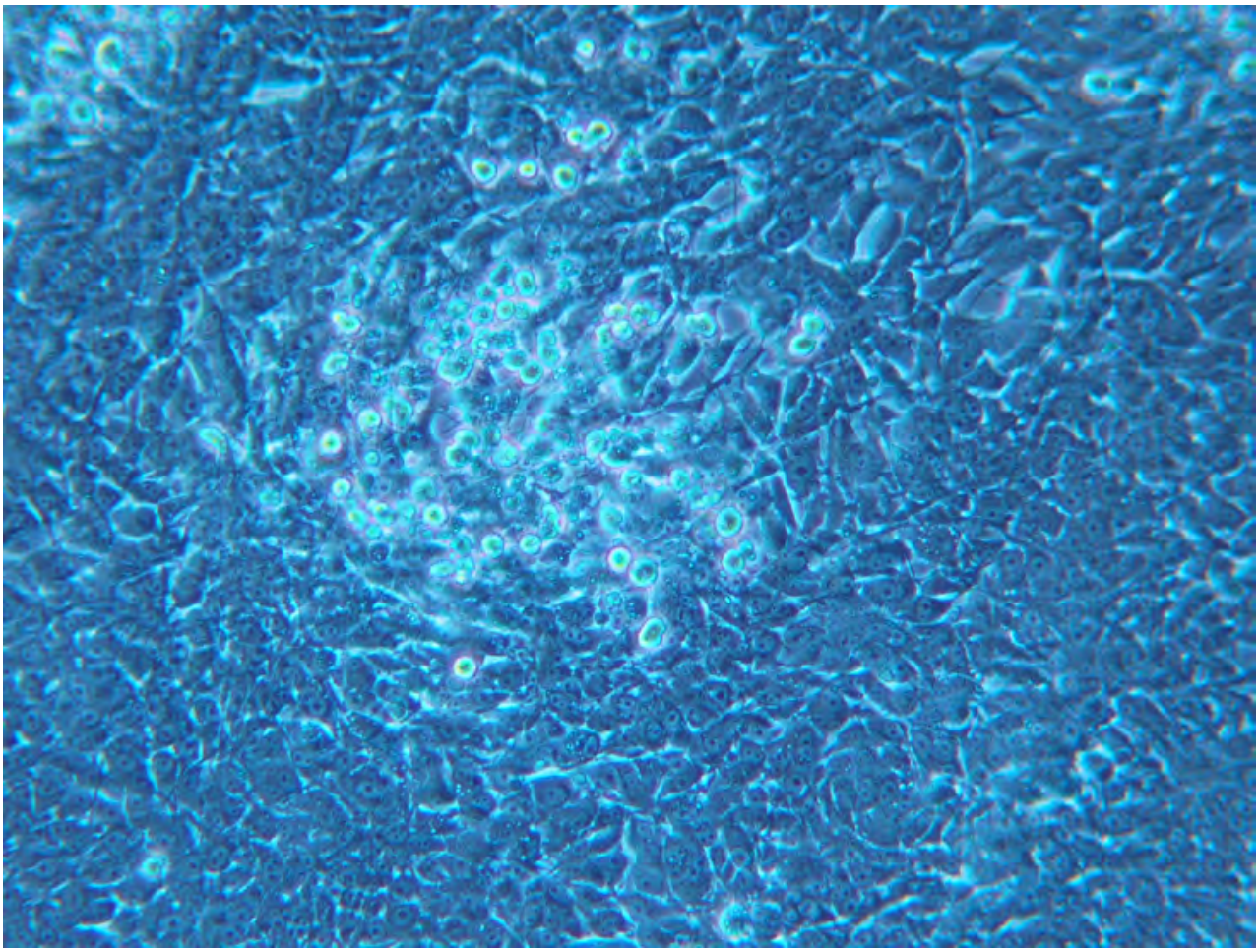


Figure 4.14. CPE plaque of SVCV on EPC monolayer. Photo courtesy of Andrew Goodwin, University of Arkansas.

2. **Confirmation Methods for SVCV**

Spring Viremia of Carp Virus is one member of a larger group of closely related rhabdovirus-like fish pathogens. Due to the similarity between members of this group, confirmatory serum neutralization tests or PCR must be carefully controlled to prevent false positive results that identify a closely-related isolate as the highly regulated SVCV. As with all serum neutralization assays, neutralizing antibodies must be carefully tested for cross reactivity with non-SVCV isolates. When PCR is used for the confirmatory test, the OIE recommends sequencing the PCR

product to confirm that the sequence represents a virus considered to be SVCV. The final identification of the virus rests on its position in a cladogram derived from a library of SVCV and related DNA sequences. Because of the taxonomic difficulties inherent in the proper classification of SVCV-like isolates, laboratories may wish to submit the virus to a reference laboratory for final confirmation. The Centre for Environment, Fisheries and Aquaculture Science (CEFAS) in Weymouth, England is the OIE reference laboratory for SVCV. In the US, the National Veterinary Services Laboratory in Ames, Iowa may also be able to provide this service. It is important to first contact the reference laboratory to confirm the availability of services and to discuss proper shipping methods.

a. Serum Neutralization Method
See Section 2, 4.7 Serum Neutralization for the general procedure

i. Use the cell line on which the initial CPE was produced.

ii. Incubate plates at 20 to 25°C.

b. Polymerase Chain Reaction (PCR) method for confirmation of SVCV (OIE proposed 2003):. Total RNA is extracted from cell culture supernatant and subject to reverse transcription for production of appropriate cDNA which is then amplified with forward and reverse primers in a PCR reaction. The DNA product from the amplification is then visualized by agarose gel electrophoresis.

i. Extraction of RNA from Cell Culture Fluid (method of Strømmen & Stone, 1998)

1. Total RNA is extracted from 100 µl of suspect viral tissue culture supernatant. The resulting RNA is dissolved in 40 µl molecular biology grade DNase- and RNase-free water. Extraction may be done using commercially available total RNA extraction kits that produce high quality RNA suitable for RT-PCR. Examples are Trizol Reagent(tm) (BRL, Life Technologies), SV Total RNA isolation system (Promega) and Nucleospin® RNA (BD Biosciences)..

2. Quantify RNA template in the spectrophotometer. The optimum amount of RNA template should be around 100µg/ml (or 100ng/µl).

ii. Formation of cDNA by Reverse Transcription

1. QA/QC (See Section 2, 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)

2. Using Section 2, 4.A1.A Worksheet A – PCR Sample Data Log Sheet, record appropriate data for each sample to be tested by PCR.

3. Using Section 2, 4.A1.B.6 Worksheet B.6 – Spring Viremia of Carp Virus (SVCV), record date of assay and then calculate the amount of each reagent to go into the "RT Master Mix" (RTMM) according to the number of samples to be processed. (Add four to the number of samples so that there is enough to run controls in the assay.). About 19 µl of RTMM will be required per sample.

4. For each sample to be tested, set up a tube with 19 µl of RTMM and 1µl of purified RNA template from above (Dilute template if more than 300ng/µl or use up to 5µl/reaction if reading falls below 50ng/µl. If

more than 1 ul of template is used, reduce the water volume accordingly). Incubate the tubes at 37 C for 1 hour.

iii. First Round "Semi-Nested" PCR for SVCV:

1. Again use worksheet B for the Second Round (Appendix 4.A1) to record date of assay and then calculate the amount of each reagent to go into the PCR "Master Mix" (PCRMM) according to the number of samples and controls to be processed.

2. Prepare PCR tubes with 45.5 µl of PCRMM and add 2.5 µl of RT product for each RNA sample. Add 1 µl of each of the primers SVCV F1 & SVCV R2 (primer concentration 50 pM/µl).

First Round Primers for SVCV:

a. Forward:

5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR-RTC-3' (SVCV F1)

b. Reverse:

5'- AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH-ACN-CAY -3' (SVCV R2)

3. Place the tubes in a thermocycler and amplify using the following program.

a. 35 cycles as follows:
i. Denaturing at 95°C for 60 seconds
ii. Annealing at 55°C for 60 seconds
iii. Extending at 72°C for 60 seconds

b. Post-dwell at 72°C for 10 minutes.

c. Hold samples at 4°C after cycling is complete.

PCR Products can be refrigerated for one month or frozen at -70°C for long-term storage.

iv. Visualization of PCR Product by Electrophoresis (6.3.C)

1. Visualize the DNA – Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at the anticipated location.

a. **Bands occurring at the 714 bp location in the First Round are confirmatory for SVCV (see note on confirmation and SVCV taxonomy in F.2 above).**

b. Photograph the gel (6.3.G) – **Photo document all gels** and attach the photo to the case history information. (Worksheet C

"Photodocumentation of the PCR Product Gel" Appendix 4.A1)

c. **If no appropriate band is seen,** proceed with Second Round "Semi-Nested" PCR for SVCV**.**

v. Second Round "Semi-Nested" PCR for SVCV

1. Prepare additional PCRMM and set up tubes for the second round of PCR exactly as for the first round, but, instead of the cDNA template (RT product), use 2.5 µl of the product from the first round of semi-nested PCR as the template and the primer set below.

2. Semi-nested Second Round Primers for SVCV:

a. Forward:

5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR-RTC-3' (SVCV F1)

b. Reverse:

5'- CTG-GGG-TTT-CCN-CCT-CAA-AGY-TGY-3' (SVCV R4)

3. Place the tubes in a thermocycler and amplify using the same program as for the first round (iii.4 above)

vi. Visualization of PCR Product by Electrophoresis (6.3.C)

1. Visualize the DNA – Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at the anticipated location.

a. **Bands occurring at the 606 bp location in the Second Round are are confirmatory for SVCV (see note on confirmation and SVCV taxonomy in F.2 above).**

b. Photograph the gel (6.3.G) – **Photo document all gels** and attach the photo to the case history information. (Worksheet C "Photodocumentation of the PCR Product Gel" Appendix 4.A1)

c. **The lack of the appropriate bands with no indication of problems with the first or second round assay are reported as NEGATIVE for SVCV**

IUPAC code	Base Combination
A	Deoxyadenine
C	Deoxycytosine
G	Deoxyguanidine
T	DeoxyThymidine
U	Deoxyuracil
I	Deoxyinosine
F	Phosphorothioate-A
O	Phosphorothioate-C
E	Phosphorothioate-G
Z	Phosphorothioate-T
V	A + C + G
D	A + T + G
B	T + C + G
H	A + T + C
W	A + T
S	C + G
K	T + G
M	A + C
Y	C + T
R	A + G
N	A + G + C + T

Table 4.3 - International Union of Pure and Applied Chemistry (IUPAC) Nucleotide Sequence Codes

G. Viral Hemorrhagic Septicemia Virus (VHSV)

Viral Hemorrhagic Septicemia Virus (VHSV) is an enveloped bullet shaped single stranded RNA virus belonging to the *Novirhabdovirus* genus of the *Rhabdoviridae*. Disease is characterized by edema and hemorrhage due to impairment of osmotic balance. In North America susceptible species include Pacific herring and pilchard on the West Coast, and significant mortality has been reported in muskellunge, freshwater drum, yellow perch, round goby, emerald shiners and gizzard shad in the Great Lakes. The virus has been isolated from several species of marine fish in the Pacific and Atlantic Oceans around North America and from returning West Coast adult coho and Chinook salmon. For a more extensive list of susceptible species, please refer to Skall et al. (2005) or to Table 2.8.1 in Section 1 of the AFS/ FHS Blue Book (2007). Fry are most susceptible to disease, which usually occurs at water temperatures between 4 to 14°C, however naïve fish of all ages have been shown to be susceptible. Subclinical infections may develop, with fish shedding virus in the feces, urine, and reproductive fluids as well as being present in the internal organs. Although present in ovarian fluid, intraovum transmission has not been demonstrated with VHSV. The European and North American strains of VHSV are indistinguishable by serologic methods but can be separated by molecular methods (OIE 2006).

1. **Screening Method**
   See section 4.5 Screening Method for viral isolation

   a. Cell culture on EPC, FHM or BF-2 cell lines incubated at 15°C (Batts et al. 1991, Gagné et al. 2007). During incubation, it is critically important that the pH of the medium remain within the range of 7.4 - 7.8 because cytopathic effects (CPE) of VHSV will not develop in acidic cultures

(Batts et al., 1991), probably due to pH-dependent conformational changes in the glycoprotein (Gaudin et al., 1999). This is especially problematic for cell lines derived from coolwater species that continue to metabolize efficiently at the incubation temperatures of the assay.

b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for VHSV**.

d. If CPE typical of a rhabdovirus is produced at any time during the incubation, that sample is considered **PRESUMPTIVELY positive** for IHNV, SVCV, or VHSV and confirmatory tests must be completed to distinguish among these viruses.

   i. The appearance of CPE typical of a rhabdovirus is described as rounded and granular cells in grape-like clusters that may exhibit margination of nuclear chromatin (optical density of nucleoli increases and nuclear membranes appear thickened). Plaques in the confluent cell monolayers are ragged in outline and contain coarsely granular debris and rounded cells. Rounded, infected cells also accumulate at plaque margins and can be present within the plaque. See Figures 4.1 and 4.2 above.

   ii. The Polymerase Chain Reaction (PCR), serum neutralization, or IFAT methods may be used to confirm the cause of the CPE is due to the presence of VHSV.

2. **Confirmation Methods for VHSV**

a. Polymerase Chain Reaction (PCR) Method for Confirmation of VHSV (Modified from Einer-Jensen 1995): This Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) technique employs oligonucleotide primers specific to the N gene of VHSV to amplify base pair segments of the complementary DNA (cDNA) produced in RT-PCR reactions containing the target virus. Total RNA is extracted from cell culture supernatant, subject to reverse transcription for production of appropriate cDNA, which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.

   i Extraction of RNA from Cell Cultures
   Total RNA from infected cells is extracted using a phase-separation method (eg. phenol-chloroform or TRIZOL, Invitrogen) or by RNA affinity spin columns (eg. RNeasy Total RNA kit, Qiagen) according to the manufacturer's instructions. While all of these methods work well for drained cell monolayers or cell pellets, RNA binding to affinity columns can be affected by salts present in tissue culture media and phase-separations methods should be used for extraction of RNA from cell culture fluids.

   ii. Formation of DNA by Reverse Transcription and Amplification by PCR

      1. QA/QC (See Section 2, 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)

      2. Using Section 2, 4.A1.A Worksheet A – PCR Sample Data/Log Sheet, record

appropriate data for each sample to be tested by PCR.

3. Using Section 2, 4.A1.B.7 Worksheet B.7 – Viral Hemorrhagic Septicemia Virus (VHSV), record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add four to the number of samples so that there is enough to run controls in the assay.)

4. Primers for VHSV*

a. Forward: 5'-GGG-GAC-CCC-AGA-CTG-T-3'

b. Reverse: 5'-TCT-CTG-TCA-CCT-TGA-TCC-3'

*2007 edition note: The primer sequences for VHSV have been modified compared to those provided in previous editions of this manual. The new sequences now match the OIE (2006) VHSV primers.

5. Thermocycler Program for VHSV

a. Incubate at 50°C for 30 minutes for Reverse Transcriptase reaction.

b. Denature sample at 95°C for two minutes.

c. 30 cycles as follows:

i. Denaturing at 95°C for 30 seconds.

ii. Annealing at 50°C for 30 seconds.

iii. Extending at 72°C for 60 seconds.

d. Final extension at 72°C for seven minutes.

Hold samples at 4°C after cycling is complete.
PCR Products can be refrigerated for one month or frozen at -20°C for long-term storage.

iii Visualization of PCR Product by Electrophoresis (Section 2, 6.3.C "Detection of Product")

1. Visualize the DNA
Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in PCR assays.

a. **Bands occurring at the 811 bp location are confirmatory for VHSV and are reported as POSITIVE.**

b. **If there is an absence of appropriate bands with no indication of problems with the assay, consider testing for other viruses or consult an appropriate reference laboratory.**

2. Photograph the Gel (Section 2, 6.3.G "Visualize the DNA")
Photo document all gels and attach the photo to the case history information. (Section 2, 4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)

b. Serum Neutralization Method
See Section 2, 4.7 Serum Neutralization for the general procedure*.

i. Use the cell line on which the initial CPE was produced.

ii. Incubate plates at 15°C.

c. Indirect Fluorescent Antibody Test (IFAT) Method for the Confirmation of VHSV
See Section 2, 4.8 Indirect Fluorescent Antibody Test (IFAT) Procedures for the general procedure*.

i. Use the cell line on which the initial CPE was produced.

ii. Incubate virus inoculated monolayers at 15-18°C.

*The Danish Institute for Food and Veterinary Research, Fish Disease Section is both the European and OIE reference lab for VHSV and should have anti-serum available. The contact is Dr Ellen Ariel. e-mail: ear@dfvf.dk.

H. White Sturgeon Herpesvirus (WSHV)

White Sturgeon Herpesvirus (WSHV) is an enveloped icosahedral shaped double-stranded DNA virus belonging to the Herpesvirus genus of the *Herpesviridae*. WSHV has been found in both feral and captive populations of sturgeon in California and Oregon. WSHV-1 has been found in juvenile cultured white sturgeon less than 10 cm. The susceptibility of other sturgeon species to WSHV-1 is not known at this time. WSHV-2 has been isolated from wild and cultured subadult and adult white sturgeon. A herpesvirus has also been isolated from shortnose sturgeon, although the relationship of this isolate to WSHV-1 or WSHV-2 has not been determined. Infected fish may present with lethargy, emaciation, excessive mucus production, fluid in the gastrointestinal tract, and focal skin lesions. Horizontal transmission has been demonstrated with both WSHV-1 and WSHV-2. WSHV-2 has been isolated from ovarian fluid but vertical transmission has not been demonstrated (LaPatra, personal communication 2002; Plumb 1999).

1. **Screening Method**

a. Cell culture on WSS-2 cell line incubated at 20°C.

b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored

for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for WSHV**.

d. If CPE typical of a herpesvirus is produced at any time during the incubation, that sample is considered **PRESUMPTIVELY positive** for WSHV.

   i. The appearance of CPE typical of WSHV and other herpesviruses includes the formation of syncytia. See Figures 4.15 and 4.16.

   ii. Suspect samples are sent to an appropriate laboratory for confirmation that the CPE is due to the presence of WSHV.

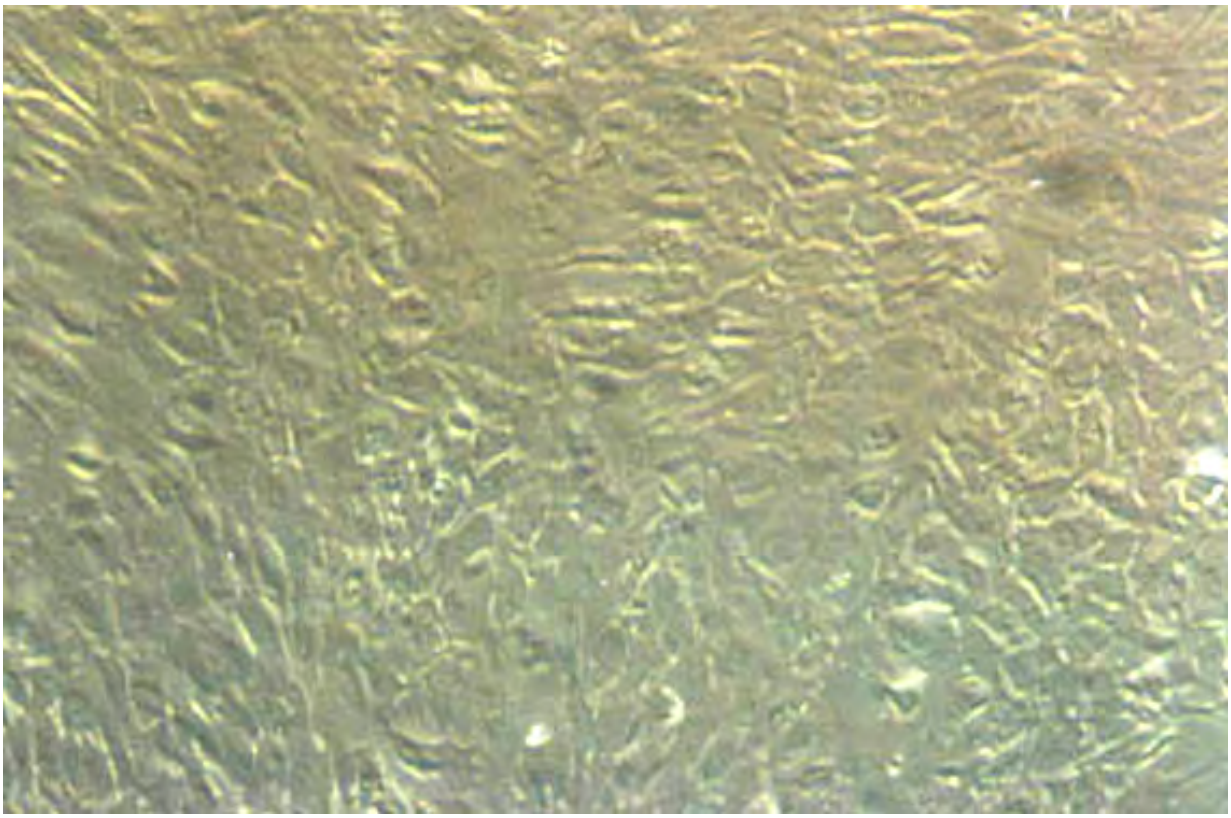


Figure 4.15. Normal WSS-2 monolayer. Photo courtesy of Scott LaPatra, Clear Springs Foods, Inc.

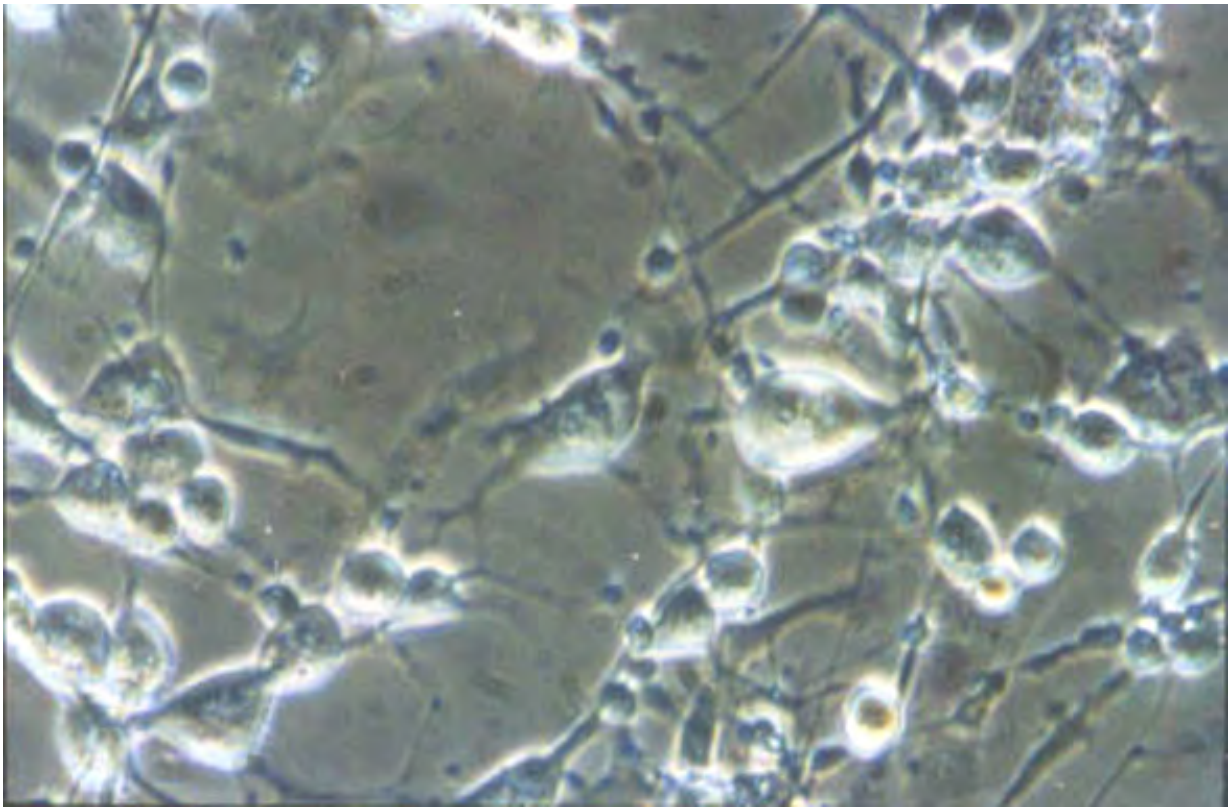


Figure 4.16. CPE typical of WSHV on WSS-2 monolayer. Photo courtesy of Scott LaPatra, Clear Springs Foods, Inc.

2. **Confirmation Method for WSHV**

   a. A PCR method has been developed for this virus, however, the necessary sequences are not available at this time. Therefore, suspect samples must be sent to a reference laboratory for confirmation.

   b. A laboratory capable of confirming the identity of WSHV is the Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA 95616. Phone: 530-752-3411.

4.7 Serum Neutralization

Serum neutralization or plaque reduction assays are serological methods used to confirm the identity of an unknown viral isolate. The procedures involve the use of a known dilution of specific neutralizing antiserum mixed with multiple dilutions of the homologous and suspect virus and subsequent observation of the ability of those viruses to produce CPE when inoculated onto a sensitive cell line. Normal serum from the species of animal used to produce the antiserum (usually rabbit or goat) is used as the negative control to account for nonspecific inhibitors of the virus.

A. Plate Preparation

1. Seed plates (Section 2, 4.3.B "Seeding Procedures for Plates") with the appropriate cell line (Section 2, 4.2 Selection of Appropriate Cell Lines) 24 to 48 hours before inoculation with virus.

2. Monolayers should be visually healthy and at least 80% confluent at the time of inoculation.

B. Virus Sample Preparation

1. A dilution of neutralizing antiserum (polyclonal or monoclonal) should be used that allows neutralization of 10^3 to 10^6 plaque-forming units (PFU) or 50% tissue culture infective dose ($TCID_{50}$) per mL of the homologous virus.

2. Dilute the suspect sample and positive control virus to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} in sterile HBSS (Section 2, 4.9.A "Sample Dilution Medium - Hanks Balanced Salt Solution (HBSS)").

3. Combine equal volumes of each dilution of the suspect sample with the diluted antiserum. Repeat the procedure for a positive control virus. Include negative controls for both the suspect and homologous virus. Incubate for one hour with agitation at the appropriate temperature.

    a. For IHNV, VHSV, and IPNV, incubate at 15°C.

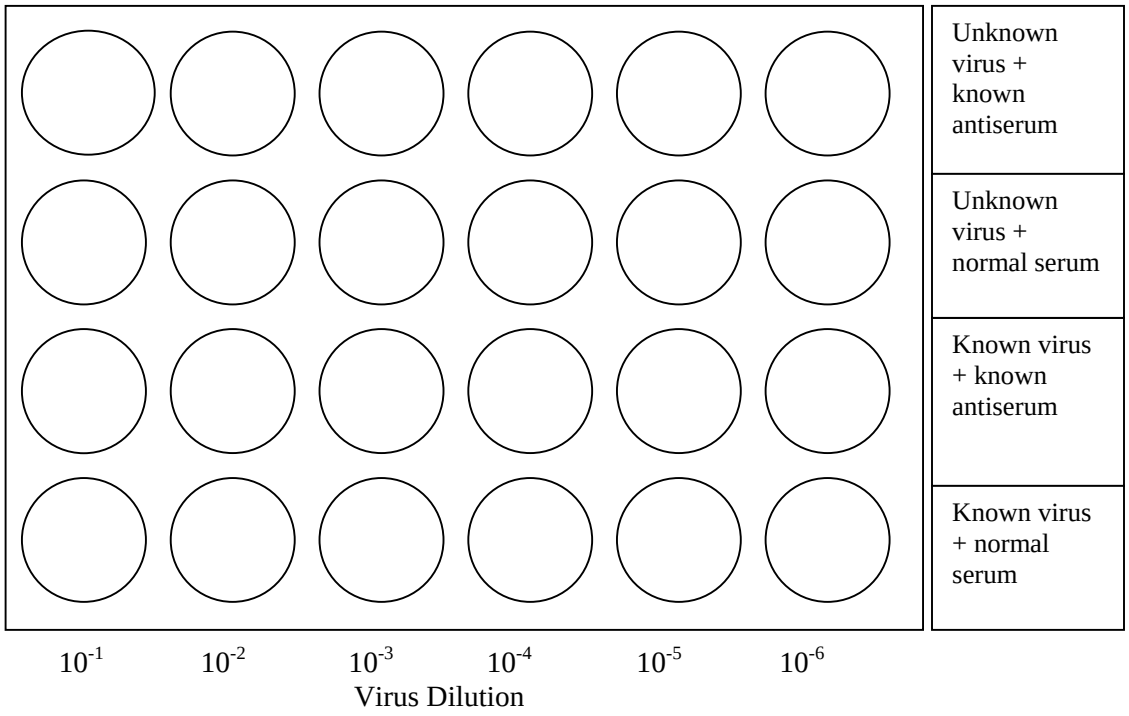
    b. For SVCV, incubate at 20 to 25°C.

4. Inoculate each of these mixtures onto the cell line in which the suspect virus was isolated as indicated in Diagram 1 below. Incubate at the above temperature for 14 days and observe plates for cytopathic effect (CPE) (see Figures 4.1 to 4.16).

    a. **Equivalent inhibition of CPE by a specific antiserum for both the suspect and homologous virus, but not for negative controls, provides confirmatory identification of the suspect virus.**

    b. Alternatively, in a sample mixed with antibody, a titer decrease of less than 2 $\log_{10}$ indicates no neutralization and the suspect sample is considered **negative** for that virus if no problems are identified with the assay.

Diagram 1. Diagram for serum neutralization assay using a 24-well plate.



C. Preparation of Reference Viruses

Where appropriate, positive controls are produced and frozen for use as needed in the serum neutralization assay.

1. Thawing of Frozen Viral Isolates

Thaw virus isolates rapidly in lukewarm water, removing the vial just before the last of the ice in the vial has melted.

2. Procedure for Producing Reference Viruses

a. Inoculate viral suspensions onto cell culture flasks containing visibly healthy monolayers of the appropriate cell line. This is done in a manner similar to the tissue inoculation described in Section 2, 4.5.A "Plate Inoculation Procedures for Primary Culture" using an appropriate volume of inoculum for the flask size. If using a 25cm^2 flask, 0.1 mL of viral inoculum is usually sufficient.

i. Use EPC cell line for IHNV and SVCV isolates.

ii. Use EPC, FHM or BF-2 cell lines for VHSV isolates. During incubation, it is critically important that the pH of the medium remain within the range of 7.4 - 7.8 because cytopathic effects (CPE) of VHSV will not develop in acidic cultures (Batts et al., 1991), probably due to pH-dependent conformational changes in the glycoprotein (Gaudin et al., 1999).

iii. Use CHSE-214 cell line for IPNV isolates.

b. To allow for viral adsorption, incubate flasks at the appropriate temperature for one hour with gentle rocking at least every 15 minutes or continuously on a laboratory rocker.

   i. For IHNV, IPNV, and VHSV, incubate at 15°C.

   ii. For SVCV, incubate at 20 to 25°C.

c. Aseptically dispense an appropriate amount of tissue culture media into the flask. For a 25 cm^2 flask this will be approximately 5 mL.

d. Incubate control sample flasks to allow replication of the viruses.

   i. For IHNV, IPNV, and VHSV, incubate at 15°C until CPE occurs or for 14 days.

   ii. For SVCV, incubate at 20 to 25°C until CPE occurs or for 14 days.

3. Procedure for Harvesting the Virus

a. Using aseptic technique, scrape the cell layer from the flask and triturate to break up.

b. Pour fluid and suspended cells into sterile tubes for centrifugation.

c. Centrifuge tubes at 4°C for 15 minutes at 2000-3000 times gravity relative centrifugal force (X g).

d. Use supernatant as positive control virus. Any fluid not needed for the assay may be aliquoted into vials and frozen at -70 C.

e. Any supernatant that is not frozen or used for the assay must be decontaminated before it is discarded.

4.8 Indirect Fluorescent Antibody (IFAT) Procedure

(Modified from LaPatra 1989)

The fluorescent antibody test (FAT) is a serological method for confirming the identity of viral isolates. The indirect FAT (IFAT) technique uses pathogen specific monoclonal anti-virus antibody (MAb) as the primary antibody (usually mouse IgM), and fluorescein isothiocyanate (FITC) conjugated antisera (usually goat or rabbit anti-mouse IgG) to the immunoglobulin used for the primary antibody. There are four basic steps for IFAT: preparing and fixing cell monolayers; infecting the cell monolayers with the virus; staining the slides with antibody reagents; reading and interpreting the slides.

A. Preparation of Materials

1. **Sterilization**

   a. Place 12 to 18 mm diameter cover glasses in a heat resistant container and sterilize at 120 ^{o}C for 30 minutes.

   b. Include dissecting forceps.

2. Dry completely in drying oven.

3. Prepare the antibody reagents according to the instructions provided by the supplier.

4. Appropriate QA/QC testing should be performed periodically on the antibodies, antisera, and cell lines to ensure accurate results are obtained during this assay.

B. Preparation of Cell Monolayers on Cover Glasses

1. Working in a clean hood, place one cover glass per well in a 24-well plate. At least four cover glasses are made for each of the unknown samples, positive control (reference) viruses, and negative control (uninoculated) cell lines. One cover glass from each of these groups is then stained and examined at approximately 8, 12, 24, and 48 hours as necessary for confirmation. Observing the cultures at multiple time points allows for visualization of the virus at the most appropriate stage of infection of the cell monolayer.

2. Using the cell line specified for the suspect virus, seed each well of the plate with the appropriate volume of that cell suspension as in Section 2, 4.3.B “Seeding Procedures for Plates.”

3. Incubate at 20 to 25^{o}C for 18 to 24 hours or until the cells are 80 to 100% confluent as this

minimizes excessive cell loss during fixation and staining.

C. Virus Sample Inoculation and Incubation

1. **Preparation of the Suspect and Known Virus Sample**

   a. Using a pipette, stir and scrape the bottom of the suspect virus well or reference virus-infected flask (Section 2, 4.7.C.2 "Procedure for Producing Reference Viruses") to be subcultured to dislodge the cell layer.

   b. Aspirate the fluid and cell debris from the well or flask and place in a sterile tube for centrifugation.

   c. Tubes are centrifuged at 4°C for 15 minutes at 2000-3000 X g.

   d. Use supernatant from this tube for inoculation of the cover glass cell cultures.

2. **Inoculation of the Cover Glass Cell Cultures with the Suspect and Known Virus Samples**

   a. Aspirate media from each well of the 24-well plate containing a cover glass, leaving a small amount to inhibit cell dehydration.

   b. Inoculate 50 to 100 µL of supernatant prepared in "Preparation of the Suspect and Known Virus Sample" (above) from each sample onto four monolayered cover glasses in each of four labeled wells.

   c. Inoculate 50 to 100 µL of reference virus onto each of four monolayered cover glasses, one of which will be used at each time period.

   d. One negative (uninoculated) control monolayered cover glass is made for each cell line used, one of which will be stained and examined at each time period.

   e. Absorb 1 hour without rocking at 15 to 18^{o}C, sample removal is not necessary.

   f. Add 1 mL MEM-5 (Section 2, 4.9.F "MEM-5/Hepes (Tissue Culture Medium for all Cell Lines Except SHK-1 and ASK)") or Leibovitz's L-15 (Section 2, 4.9.H "Leibovitz's L-15 (Tissue Culture Medium for SHK-1 and ASK Cell Lines") per well as appropriate for the cell lines being used.

3. Incubate at 15 to 18^{o}C.

D. Cover Glass Fixation and Mounting

1. Working under the hood at room temperature, aspirate media from each well to be examined at that time period (sample plus positive and negative control cover glasses). Disinfect the aspirated fluid prior to disposal.

2. Add 1 mL PBS (Section 2, 3.7.D.3 "Phosphate-Buffered Saline for FAT (PBS), pH 7.1") to each

well and let stand for five minutes.

3. Aspirate PBS and disinfect prior to disposal.

4. Repeat steps 2 and 3.

5. Add 1 mL cold (4°C) methanol and let stand for five minutes.

6. Leave some or all of the methanol in the well. Tip the plate, without spilling fluid, and use sterile forceps to remove the cover glass from the well. Leaving some fluid in the well helps decrease the adherence of the cover glass to the bottom of the plate.

7. Allow cover glasses to air dry on a labeled absorbent surface such as lab paper.

8. If not stained immediately, store at 4°C for short term (days to weeks) or at -20°C for long term (weeks to months).

E. Staining and Viewing the Cover Glasses

1. Cover fixed cell cultures with 0.1 mL of mouse monoclonal anti-virus antiserum (MAb) specific for the suspect virus and incubate at room temperature for 30 minutes in a dark humidified chamber.

2. Flood the cells twice for five minutes each time with PBS.

3. Filter the goat anti-mouse IgG fluorescein isothiocyanate (FITC) conjugated antibodies through a 0.45 µm membrane before use.

4. Cover fixed cell cultures with 0.1 mL of the second antibody (FITC conjugate) and incubate at room temperature for 30 minutes in a dark humidified chamber.

5. Flood the cells twice for five minutes each time with PBS.

6. Counter stain with a 0.01% solution of Evans blue (Section 2, 3.7.D.5.b “Evans Blue”) for one minute, rinse with PBS and air dry in the dark.

7. Place a drop of pH 9 fluorescent antibody (FA) mounting fluid (Section 2, 3.7.D.2 “FAT Mounting Fluid (pH 9.0)”) on the cells and place the cover glass cells down on a clean microscope slide.

8. Examine at 400 to 1000x magnification on a microscope equipped with epifluorescent illumination.

F. Results

1. Positive controls should contain focal clusters of cells exhibiting apple green cytoplasmic fluorescence (see Figure 4.17); no fluorescence should be observed in the negative controls.

2. With no problems identified in the assay and with fluorescence of the suspect sample which appears similar to the positive control at one of the time points, the suspect sample is considered **POSITIVE** for the virus specific to those antibodies.

3. With no problems identified in the assay but without appropriate fluorescence at any of the four time periods, the suspect sample is considered **NEGATIVE** for the virus specific to those antibodies.

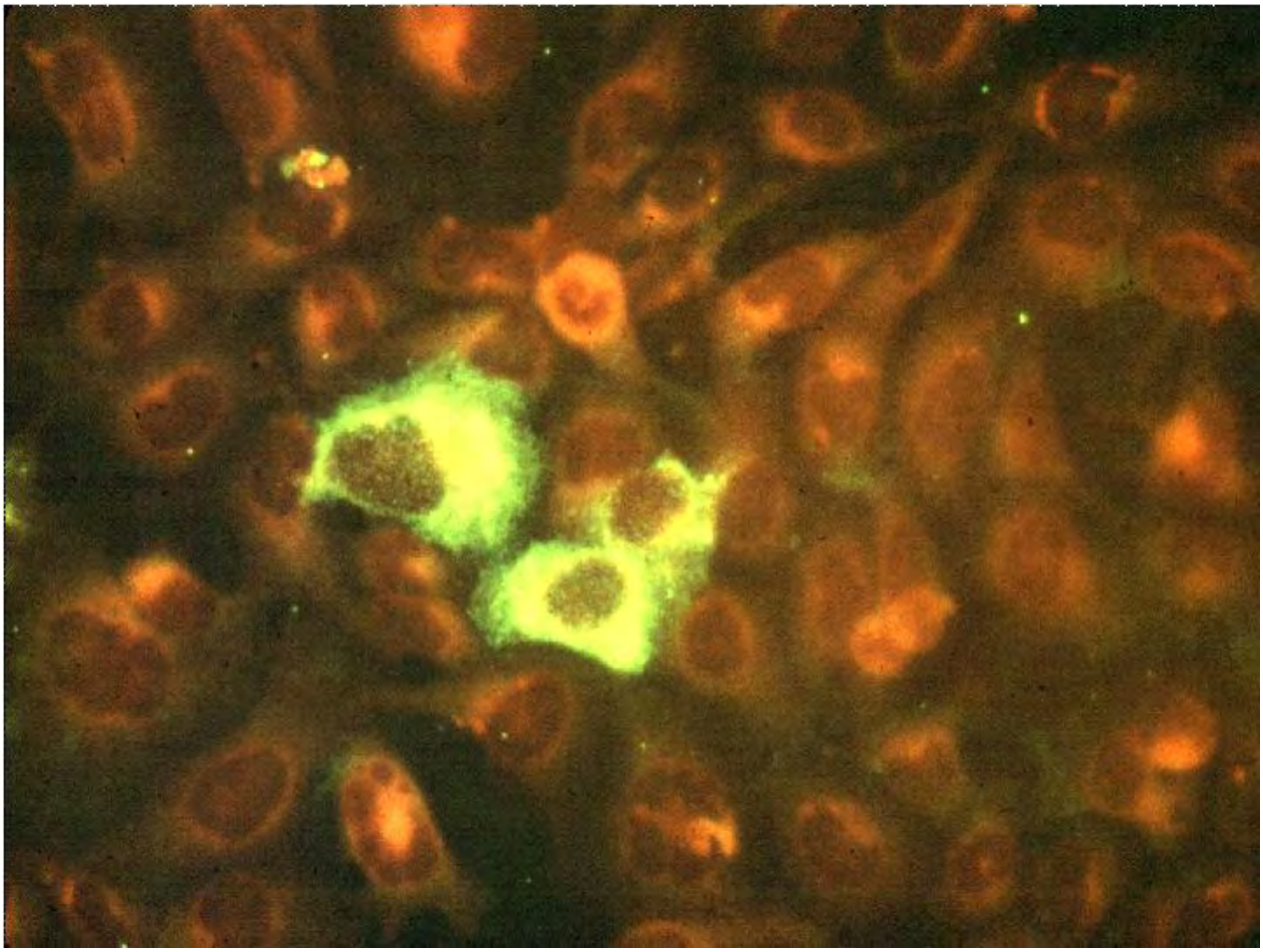


Figure 4.17. Typical fluorescence of positive IFAT for IHNV on EPC cell monolayer. Photo courtesy of Scott LaPatra, Clear Springs Foods, Inc.

4.9 Reagents and Media

Media used in tissue culture must be sterile. This may be accomplished by mixing all the ingredients and filtering with a 0.2 µm filter, or, by mixing of the stable ingredients, autoclaving, and then aseptically adding the labile ingredients such as glutamine, serum, and antibiotics.

A. Sample Dilution Medium - Hanks Balanced Salt Solution (HBSS)

10X HBSS	100.0 mL
Tissue culture grade water	895.3 mL
$NaHCO_3$ (7.5%)	4.7 mL

Mix and ensure sterility.

B. Antibiotic Incubation Medium (Anti-Inc) Made with HBSS for Sample Disinfection

10X HBSS	100.0 mL
Tissue culture grade water	575.0 mL
$NaHCO_3$ (7.5%)	5.0 mL
Penicillin/streptomycin	160.0 mL
Penicillin G (10,000 units/mL)	
Streptomycin sulfate (10,000 µg/mL)	
Fungizone	160.0 mL
250 µg/mL amphotericin B	
205 µg/mL desoxycholate	

Mix and ensure sterility. Store at 4° C. Equal volumes of sample and anti-inc are mixed together for sample disinfection.

C. Antibiotic Incubation Medium (Anti-Inc) Made with Minimum Essential Medium (MEM-0) for Sample Disinfection

10X MEM (Eagles Modified Medium)	100.0 mL
Tissue culture grade water	540.0 mL
L-Glutamine (200 mM)	10.0 mL
$NaHCO_3$ (7.5%)	30.0 mL
Tryptose phosphate broth	100.0 mL
Penicillin/streptomycin	160.0 mL
Penicillin G (10,000 units/mL)	
Streptomycin sulfate (10,000 µg/mL)	
Fungizone	160.0 mL

250 µg/mL amphotericin B
205 µg/mL desoxycholate

Mix and ensure sterility. This may be stored frozen for approximately three months. Avoid freeze-thaw cycles; thaw tubes immediately prior to use. Equal volumes of sample and anti-inc are mixed together for sample disinfection.

D. Versene (EDTA) (1:5000)

NaCl	8.0 g
$KHPO_4$	0.2 g
KCl	0.2 g
Na_2HPO_4	1.15 g
Disodium versenate (EDTA)	0.2 g
Phenol red (0.5% solution)	2.0 mL
Tissue culture grade water	to 1000 mL

Autoclave and store at room temperature.

E. Trypsin-Versene (EDTA)

Trypsin (2.5% solution)	20 mL
Versene (EDTA) (1:5000)	480 mL

Store at -20° C.

F. MEM-5/Hepes (Tissue Culture Medium for all Cell Lines Except SHK-1 and ASK)

Tissue culture grade water	810.0 mL
10X MEM	100.0 ml
Fetal bovine serum	50.0 mL
Sodium bicarbonate (7.5% solution)	10.0 mL
L-Glutamine (200 mM)	10.0 mL
Hepes buffer (1M)	15.0 mL
NaOH (1M)	5.0 mL
NaOH or HCL - as needed to adjust pH to 7.2-7.6	

If antimicrobials are included, use 796.0 mL of water above instead of 810.0 and add:

Gentamicin (50 mg/mL)	4.0 mL
Fungizone	10.0 mL
250 µg/mL amphotericin B	
205 µg/mL desoxycholate	

Mix and ensure sterility. Store at 4° C.

G. MEM-10/Hepes (Tissue Culture Medium for all Cell Lines Except SHK-1 and ASK)

10X MEM	100.0 mL
Tissue culture grade water	760.0 mL
Fetal bovine serum	100.0 mL
Sodium bicarbonate (7.5% solution)	10.0 mL
L-Glutamine (200 mM)	10.0 mL
Hepes buffer (1M)	15.0 mL
NaOH (1M)	5.0 mL
NaOH or HCL - as needed to adjust pH to 7.2-7.6	

Mix and ensure sterility. Store at 4° C.

H. Leibovitz's L-15 (Tissue Culture Medium for SHK-1 and ASK Cell Lines)

1X L-15 with 0.3g/L L-glutamine	1000.0 mL
Fetal bovine serum (5%)	50.0 mL
Gentamicin (50 mg/mL)	1.0 mL
2-mercaptoethanol (0.055 M)	0.7 mL
NaOH or HCL - as needed to adjust pH to 7.2-7.6	

Mix and ensure sterility. Store at 4° C.

4.10 Glossary

Blind passage - transfer of supernatant and inoculated tissue culture cells which are not demonstrating CPE to another plate containing fresh cells in order to dilute out possible inhibitors of viral expression and/or allow possible early viral replication due to low concentrations of virus particles to progress to detectable CPE.

Closed system - a system of incubating cells that is sealed against the transfer of air, i.e. a sealed flask.

Confluent monolayer (100%) - a single layer of tissue culture cells in which the cells have filled in all the spaces between them.

Controls

1. **Monolayer control -** tissue culture cells are grown in presence of tissue culture medium. If CPE appears in monolayer control wells, test is invalidated and must be repeated.

2. **Sham control -** diluent (MEM-0) used for suspension of samples or dilution blanks is added to cells. After adsorption, tissue culture medium is added. If CPE appears in sham control wells, test is invalidated and must be repeated.

Cytopathic effects (CPE) - changes in the morphology and metabolism of tissue culture cells. It may be due to viral or toxic agents and the appearance may range from simple foaming of the cytoplasm or focal clumping of cells to complete destruction of the cell monolayer.

FBS - fetal bovine serum taken from unborn calves in utero.

FITC - fluorescein isothiocyanate, a reagent which is used as an antibody label for the fluorescent antibody test.

FITC-conjugated (antibody) - describes the existence of a fluorescent label on an antibody used for the fluorescent antibody test.

Fomite - an inanimate object such as a net, brush, or clothing, on which a pathogenic microorganism may be transmitted from one animal to another.

Homologous virus - as used in the viral serum neutralization procedure, it is the positive control virus of the same identity used to make the neutralizing antibody.

Monoclonal antibody (MAb) - a single type of antibody produced by tissue culture cell lines derived from the spleen lymphocytes of immunized mice that have been fused (hybridoma) with mouse myeloma tumor cells. Hybridoma cells are cloned to select specific populations of cells, each producing a single antibody against one epitope or antigenic determinant on one antigen molecule among those used to immunize the mice.

Normal serum - as used in the viral serum neutralization procedure, it is serum from the same species of animal in which the neutralizing antibody is produced. It is used as a control for any nonspecific viral inhibition that may occur even with a non-homologous virus.

Open system - a system of incubating tissue culture cells that is open to the transfer of air, i.e., a plate. Requires a medium that is buffered against rising pH due to CO_2 loss. Common buffering systems are TRIS and HEPES.

Plaque - a hole or focus of degenerate or dead tissue culture cells in the cell monolayer caused by viral replication. One discrete plaque is assumed to be caused by infection with one infectious particle or aggregate (called one plaque-forming unit = pfu).

Plate set - a group of plates seeded from a single flask at the same time.

Polyclonal antisera - the entire population of antibodies produced in the sera of immunized animals that are directed against many epitopes on many of the antigenic molecules used for immunization. Most immunogens injected are whole cells or viruses that are composed of many different antigen molecules. Each antigen molecule may have more than one epitope. See "Monoclonal antibody."

Re-inoculation - transfer of inoculated tissue culture cells and supernatant from one plate to another that contains fresh cells. Used for suspected positive cultures to confirm presence of viral CPE as opposed to toxicity or contamination. Also used to replicate more viruses for storage, etc.

Serum neutralization - antibody molecules in the antiserum to neutralize or block the antigenic receptor sites or otherwise degrade the protein coat (capsid) on the corresponding virus (antigen). This prevents virus attachment to and subsequent penetration of host tissue culture cells or virus replication once inside the cell. Neutralization of viruses by antibodies is specific and used to confirm viral identity. Neutralization may be reversible.

Subculture - transfer of tissue culture cells from one container to another for the purpose of forming a new monolayer.

$TCID_{50}$ - denotes 50 percent tissue culture infective dose. This is the reciprocal of the highest dilution of virus that causes CPE in 50 percent of the wells inoculated with that dilution of infectious materials. This is determined by the Reed and Muench (1938) method.

Tissue culture-grade water - high quality water (low in ions, minerals, and contaminants) that must be used in preparation of all tissue culture media and reagents and in rinsing glassware to avoid toxicity to the cells. De-ionization at greater than 18 mOhms is sufficient to achieve this quality.

Titer - the number of infectious units or plaque-forming units per unit of sample, i.e. per gram or mL.

Toxicity - changes in cell morphology or metabolism caused by toxic substances in the medium or inoculum. This can either cause cell death or interfere with cell metabolism, thereby reducing or preventing replication of the virus. These effects may have arisen through sample toxicity, bacterial or fungal contamination, improper glassware cleaning, or improper media preparation. Usually toxicity can be distinguished from viral CPE by how rapidly it occurs (one day), abnormal cell appearance without cell death, absence of the typical pattern of CPE for the test virus, and, in the case of contamination, turbidity of the medium or visible contaminant colonies.

NOTE: Inoculation of very high-titer suspensions of certain viruses can cause an apparent toxic effect within 24 hours. If there is any doubt to whether disruption of the cell layer was caused by toxicity or CPE, a subculture should be made. This is especially true for some inocula that can produce toxic effects that may take 5 to 7 days for development.

Triturating - the act of dispersing tissue culture cells for transfer by repeatedly drawing the cell suspension into a pipet and expelling it back into the flask. This should be done until the cells are in clumps of no more than three when examined with an inverted light microscope.

Trypsin - a proteolytic enzyme used to disperse cells and causes their release from the culture surface. Serum proteins neutralize it and its action is slowed by low temperature. Trypsin will cause release of the cells more readily than versene.

Versene (EDTA) - ethylene di-amine tetra-acetic acid is a chelating agent involved in causing cells to release from the culture surface. Versene and trypsin are frequently used together.

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4.A1.A Worksheet A – PCR Sample Data/Log Sheet

Case Number_____ **Sample Site**_____ **Date**_____
Species_____
Tissue type_____

Sample ID	Extraction Method	PCR Result	Notes
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			
9.			
10.			
11.			
12.			
13.			
14.			
15.			
16.			
17.			
18.			
19.			
20.			

4.A1.B Worksheet B – Amplification of Nucleic Acid by PCR for the Confirmation of Viral Fish Pathogens

4.A1.B.1 Worksheet B.1 – Infectious Hematopoietic Necrosis Virus (IHNV)

4.A1.B.2 Worksheet B.2 – Infectious Pancreatic Necrosis Virus (IPNV)

4.A1.B.3 Worksheet B.3 – Infectious Salmon Anemia Virus (ISAV)

4.A1.B.4 Worksheet B.4 – Largemouth Bass Virus (LMBV)

4.A1.B.5 Worksheet B.5 – *Oncorhynchus masou* Virus (OMV)

4.A1.B.6 Worksheet B.6 – Spring Viremia of Carp Virus (SVCV)

4.A1.B.7 Worksheet B.7 – Viral Hemorrhagic Septicemia Virus (VHSV)

4.A1.B.1 Worksheet B.1 – Infectious Hematopoietic Necrosis Virus (IHNV)

Case Number_____ **Date**_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		-	-	23.75 µL	
PCR Buffer (no $MgCl_2$)		1X	10X	5 µL	
$MgCl_2$		2.5 mM	25 mM	5 µL	
dNTP's		0.2 mM	2 mM	5 µL	
AMV reverse transcriptase		4.5 U/Rx†	9 U/µL	0.5 µL	
(+)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
(-)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
RNasin		9.75 Units/Rx	39 Units/µL	0.25 µL	
RNA template		-	-	5 µL	-

*Add nuclease free water to Master Mix first, TAQ last.
† Rx = Reaction

Primer Sets for IHNV

Forward	Reverse
5'-AGA-GAT-CCC-TAC-ACC-AGA-GAC-3'	5'-GGT-GGT-GTT-GTT-TCC-GTG-CAA-3'

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR

Amplification (Thermocycle Process)

Date & Time	Program #	NOTES

Gel Preparation

Gel Concentration	**Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)**	**Weight of Agarose (grams)**	**Volume of Buffer (mL)**

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

4.A1.B.2 Worksheet B.2 – Infectious Pancreatic Necrosis Virus (IPNV)

Case Number_____ **Date**_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		-	-	23.75 µL	
PCR buffer (no $MgCl_2$)		1X	10X	5 µL	
$MgCl_2$		2.5 mM	25 mM	5 µL	
dNTP's		0.2 mM	2 mM	5 µL	
AMV reverse transcriptase		4.5 Units/Rx	9 Units/µL	0.5 µL	
(+)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
(-)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
RNasin		9.75 Units/Rx	39 Units/µL	0.25 µL	
RNA template		-	-	5 µL	-

*Add nuclease free water to Master Mix first, TAQ last.

Primer Sets for IPNV

	Forward	**Reverse**
1st round	5'-AAAGCCATAGCCGCCCATGAAC-3'	5'- TCTCATCAGCTGGCCCAGGTAC-3'

Control Information

	POSITIVE CONTROLS		**NEGATIVE CONTROLS**	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & Time)	Program #	NOTES

Gel Preparation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of Agarose (grams)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

4.A1.B.3 Worksheet B.3 – Infectious Salmon Anemia Virus (ISAV)

Case Number_____ **Date**_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		-	-	26.75 µL	
PCR buffer (no $MgCl_2$)		1X	10X	5 µL	
$MgCl_2$		2.5 mM	25 mM	5 µL	
dNTP's		0.2 mM	2 mM	5 µL	
AMV reverse transcriptase		4.5 Units/Rx	9 Units/µL	0.5 µL	
(+)Primer		50 pmoles/Rx	50 pmoles/µL	1 µL	
(-)Primer		50 pmoles/Rx	50 pmoles/µL	1 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
RNasin		9.75 Units/Rx	39 Units/µL	0.25 µL	
RNA template		-	-	5 µL	-

*Add nuclease free water to Master Mix first, TAQ last.

Primer Sets for ISAV

	Forward	**Reverse**
1st round	5'-GGC TAT CTA CCA TGA ACG AAT C-3'	5'-TAG GGG CAT ACA TCT GCA TC-3'

Control Information

	POSITIVE CONTROLS		**NEGATIVE CONTROLS**	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & Time)	Program #	NOTES

Gel Preparation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of Agarose (grams)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

4.A1.B.4 Worksheet B.4 – Largemouth Bass Virus (LMBV)

Case Number_____ **Date**_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		-	-	15.5 µL	
PCR buffer (no $MgCl_2$)		1X	10X	5 µL	
$MgCl_2$		1.5 mM	25 mM	3 µL	
dNTP's		0.8 mM	10 mM	4 µL	
TMAC		40 µM	100 µM	20 µL	
(+)Primer		1 µM	100 µM	0.5 µL	
(-)Primer		1 µM	100 µM	0.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
DNA template		-	-	1 µL	-

*Add nuclease free water to Master Mix first, TAQ last.

Primer Sets for LMBV

	Forward	**Reverse**
1st round	5'- GCG GCC AAC CAG TTT AAC GCA A -3'	5'- AGG ACC CTA GCT CCT GCT TGA T -3'

Control Information

	POSITIVE CONTROLS		**NEGATIVE CONTROLS**	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & Time)	Program #	NOTES

Gel Preparation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of Agarose (grams)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

4.A1.B.5 Worksheet B.5 – *Oncorhynchus masou* Virus (OMV)

Case Number_____ **Date**_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		-	-	15.5 µL	
PCR buffer (no $MgCl_2$)		1X	10X	5 µL	
$MgCl_2$		1.5 mM	25 mM	3 µL	
dNTP's		0.8 mM	10 mM	4 µL	
TMAC		40 µM	100 µM	20 µL	
(+)Primer		50 pmoles/Rx	100 pmole/µL	0.5 µL	
(-)Primer		50 pmoles/Rx	100 pmole/µL	0.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
DNA template		-	-	1 µL	-

*Add nuclease free water to Master Mix first, TAQ last.

Primer Sets for OMV

	Forward	**Reverse**
1st round	5'-GTA CCG AAA CTC CCG AGT C-3'	5'-AAC TTG AAC TAC TCC GGG G-3'

Control Information

	POSITIVE CONTROLS		**NEGATIVE CONTROLS**	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & Time)	Program #	NOTES

Gel Preparation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of Agarose (grams)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

4.A1.B.6 Worksheet B.6 – Spring Viremia of Carp Virus (SVCV)

Case Number_____ **Date**_____

Reverse Transcription Master Mix (RTMM)

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 20 µL)	Volume for ____samples
d-H_2O[1]		-	-	10 µL	
RT buffer[2]		1X	5X	4 µL	
dNTP's		1 mM	10 mM	2µL	
M-MLV reverse transcriptase		20 Units/Rx	10 Units/µL	2 µL	
Primer SVCV R2 (below)		100 pmoles/Rx	100 pmoles/µL	1 µL	

1. Add nuclease free water to Master Mix first, M-MLV reverse transcriptase last.
2. 5X RT Buffer is 250 mM tris pH 8.3, 375 mM KCl, 50 mM DTT, 15 mM $MgCL_2$

PCR Master Mix (PCRMM)

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µL)	Volume for ____samples
d-H_2O[1]		-	-	36.75 µL	
PCR buffer (no $MgCl_2$)[2]		1X	10X	5 µL	
$MgCl_2$		2.5 mM	25 mM	5 µL	
dNTP's		0.2 mM	10 mM	1 µL	
TAQ		1.25 Units/Rx	5 Units/µL	0.25 µL	

1. Add nuclease free water to Master Mix first, TAQ last.
2. 10X PCR Buffer is 100 mM Tris HCl pH 9.0, 500 mM KCl, 1 % Triton X-100

Primer Sets for SVCV

	Forward	**Reverse**
RT Reaction	None	5'- AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH-CAN-CAY -3' (SVCV R2)
1st round	5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR-RTC-3' (SVCV F1)	5'- AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH-ACN-CAY -3' (SVCV R2)

2nd round	5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR-RTC-3' (SVCV F1)	5'- CTG-GGG-TTT-CCN-CCT-CAA-AGY-TGY -3' (SVCV R4)

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1st round PCR				
2nd round PCR				

Amplification (Thermocycle Process)

Round Number (Date & Time)	Program #	NOTES

Gel Preparation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of Agarose (grams)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

4.A1.B.7 Worksheet B.7 – Viral Hemorrhagic Septicemia Virus (VHSV)

Case Number_____ **Date**_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		-	-	23.75 µL	
PCR buffer (no $MgCl_2$)		1X	10X	5 µL	
$MgCl_2$		2.5 mM	25 mM	5 µL	
dNTP's		0.2 mM	2 mM	5 µL	
AMV reverse transcriptase		4.5 Units/Rx	9 Units/µL	0.5 µL	
(+)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
(-)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
RNasin		9.75 Units/Rx	39 Units/µL	0.25 µL	
RNA template		-	-	5 µL	-

*Add nuclease free water to Master Mix first, TAQ last.

Primer Sets for VHSV

Forward	**Reverse**
5'-GGG-GAC-CCC-AGA-CTG-T-3'	5'-TCT-CTG-TCA-CCT-TGA-TCC-3'

Control Information

	POSITIVE CONTROLS		**NEGATIVE CONTROLS**	
	Extraction	PCR	Extraction	PCR

Amplification (Thermocycle Process)

Date & Time	**Program #**	**NOTES**

Gel Preparation

Gel Concentration	**Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)**	**Weight of Agarose (grams)**	**Volume of Buffer (mL)**

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel

Case Number _____ Date _____

Samples _____

Affix gel photo documentation to this sheet and make notes on results:

AFFIX PHOTODOCUMENTATION

Notes:

4.A2 Cell Enumeration

(True 2000)

Rarely are cells counted during routine propagation of cell lines; however, the use of a hemocytometer is a practical method for determining cell numbers in cell suspensions. The Improved Neubauer Hemocytometer consists of two chambers, each of which is divided into nine 1.0-mm^2 squares. A matching cover glass that is supplied with the chamber is supported 0.1 mm over the squares so that the total volume over each square is 1.0 mm^2 x 0.1 mm or 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is approximately equal to 1 mL, the cell concentration/mL is the average count per square x 10^4. Routinely, cells are counted in a total of ten 1 mm squares (fill both sides of the chamber and count the four corner and the middle squares on each side).

To reduce counting errors, count cells that touch the outer line to the left and top of the square, but do not count cells touching the outer line to the right and bottom of the square. Hemocytometer counts do not distinguish between living and dead cells unless a vital stain is used such as Trypan Blue.

Trypan Blue stain is not absorbed by living cells and can be used to distinguish between viable and nonviable cells in cell counts. Use a 1:1 dilution of cell suspension with 0.1% Trypan Blue stain and count only unstained cells. Do not count debris or dead cells that stain blue.

Trypan Blue has a greater affinity for serum proteins than for cellular protein. If the background is too dark, cells should be pelleted (10 minutes at 500 rpm) and re-suspended in protein-free medium or Hanks salt solution prior to counting.

A. Materials

Hemocytometer chamber
75 cm^2 flask of cells
Trypan Blue (0.1% in PBS)
Microscope
Dilution tubes (12 x 75 mm)
Pasteur pipet
Hanks balanced salt solution, or MEM-0 (MEM w/o serum)
Trypsin - EDTA
Pipets 1-mL, sterile, cotton plugged
22 x 22 mm cover-slips

B. Procedure

1. Select a healthy (log phase) 75 cm^2 flask of cells and remove cells from flask surface as described in Section 2, 4.3.A "Subculture Procedures for Flasks."

2. Re-suspend cells in tissue culture medium (MEM-0). For ease and accuracy in counting, the hemocytometer should be filled with cell suspensions containing approximately 20 to 50 cells/mm^2 (1 x 10^5 to 2 x 10^5 cells/mL). Dilutions vary depending on age of the cells, cell density, and cell aggregation.

3. Aseptically transfer 0.5 mL of the cell suspension into a dilution tube.

   a. Add 0.5 mL Trypan Blue stain (0.1%).

      Note: If cells are exposed to Trypan Blue for extended periods of time, viable cells, as well as non-viable cells, may begin to take up dye.

4. Gently mix to suspend the cells evenly. With a 22 x 22 mm cover-slip in place on top of the hemocytometer, use a Pasteur pipet to transfer a small drop of Trypan Blue-cell suspension mixture to both chambers. Carefully touch the edge of the cover-slip with the pipet tip and allow each chamber to fill by capillary action. Do not overfill or underfill the chambers.

5. Using a microscope with a 10x ocular and a 10x objective, count 10 squares (five from each chamber) as outlined above.

6. Calculate the number of cells/mL and the total number of cells as follows:

   a. Cells/mL = x (mean) count per square x 10^4 x Trypan Blue dilution factor.

   b. Total cells in flask = cells/mL x total volume of cell suspension.

      Example: Total number cells counted in 10 squares = 300 cells
      x count/square = 300 cells/10 squares = 30 cells

      cells/mL = 30 x 10^4 x 2 (dilution factor)
      cells/mL = 60 x 10^4 cells/mL
      cells/mL = 6.0 x 10^5 cells/mL

      Total cells = 6.0 10^5 cells/mL x 8 mL (original volume cell suspension)
      Total cells = 48.0 x 10^5 cells
      Total cells = 4.80 x 10^6 cells

   If the cells/mL calculated is not within the recommended range of cell density, use the following formula to adjust the dilution in your flask before splitting.

   a. mL medium needed = (actual cells/mL) (vol. of cell suspension)/ desired cells/mL

      Example: actual count = 6 x 10^6 cells/mL
      desired count = 1 x 10^6 cells/mL
      volume of cell suspension = 8 mL

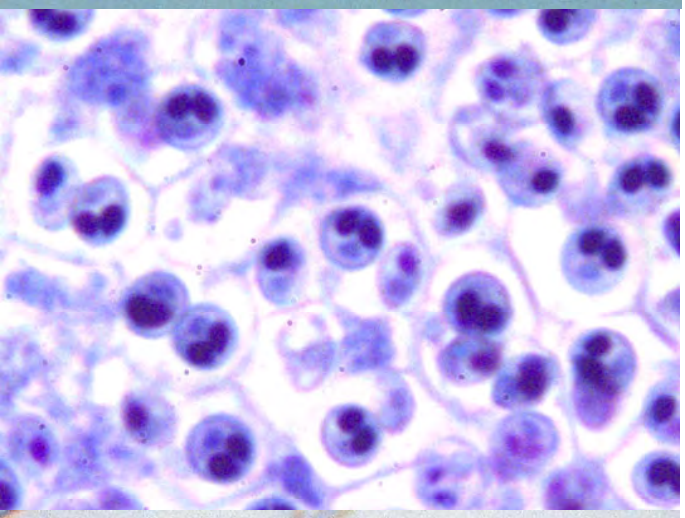
mL medium needed = x

x = mL medium needed = 6×10^6 cells/mL x 8 mL / 1×10^6 cells/mL
mL medium needed = 48×10^6 mL / 1×10^6
= 48 mL

Since you have 8 mL already in the flask, you would need to add 40 mL of medium to the flask before splitting to get the recommended seeding cell density for each new culture.









Section 2
USFWS/AFS-FHS Standard Procedures for Aquatic Animal Health Inspections

Chapter 5 Parasitology

5.1 Parasitology Introduction

5.2 *Myxobolus cerebralis* (Whirling Disease)

5.3 *Ceratomyxa shasta* (Ceratomyxosis)

5.4 *Tetracapsula bryosalmonae* (Proliferative Kidney Disease)

5.5 *Bothriocephalus acheilognathi* (Bothriocephalosis, Asian Tapeworm)

5.6 Media and Reagents

5.7 Glossary

5.8 References

5.1 Parasitology Introduction

The following chapter describes identification procedures for four parasitic infections of fish that are commonly included in a fish health inspection. The target parasite species include three myxozoan parasites of salmonid fishes: *Myxobolus cerebralis, Ceratomyxa Shasta, and Tetracapsula bryosalmonae*; and the cestode: *Bothriocephalus acheilognathi,* which infects members of the Family Cyprinidae. Section 2, Chapter 2 Sampling describes procedures for proper sampling of fish tissues to ensure detection of any of these pathogens during a fish health inspection.

For *Myxobolus cerebralis,* presumptive identification is based on identification of the myxozoan spore stage from pepsin-trypsin digested (PTD) cartilage. Tissues from up to five fish may be pooled for screening by PTD. Identification of the spores is based on morphology. Confirmatory identification is based on identification of the spores in histological sections or on amplification of *M. cerebralis* DNA by the polymerase chain reaction.

For *Ceratomyxa shasta,* presumptive identification is based on identification of myxozoan spore or trophozoite/presporogonic stages from intestinal tissue. Identification of the myxospore stage is sufficient for confirmation of infection. Identification of the earlier stages must be confirmed by amplification of *C. shasta* DNA by the polymerase chain reaction.

For *Tetracapsula bryosalmonae,* presumptive identification is based on identification of the presporogonic stages of the parasite in Leishman-Giemsa or lectin stained imprints of kidney or spleen tissue. Infection is confirmed by identification of these stages in histological sections of kidney tissue or on amplification of *T. bryosalmonae* DNA by the polymerase chain reaction.

For the cestode, *Bothriocephalus acheilognathi,* visualization of any cestode with a pyramidal scolex results in a presumptive positive classification. Confirmation requires verification of morphological characteristics of the scolex.

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U. S. Fish and Wildlife Service, the United States government, and /or the American Fisheries Society. Any comparable instrument, laboratory supply, or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.

5.2 *Myxobolus cerebralis* (Whirling Disease)

Myxobolus cerebralis may be difficult to detect because the life cycle of the parasite includes two alternate hosts: salmonids and the aquatic oligochaete worm, *Tubifex tubifex* (Wolf and Markiw 1984); and the extended time required for sporogenesis in the salmonid host. For an *M. cerebralis* inspection, sampling should target the most appropriate species, age, and rearing units that are most likely to reflect the presence of the parasite. The species most susceptible are rainbow trout, sockeye salmon, and steelhead. Brook trout, cutthroat trout, Atlantic salmon, and chinook salmon are moderately susceptible; brown and bull trout, coho salmon, and splake are partially resistant. Conflicting data are present for Arctic grayling and lake trout but in general, these species may be considered resistant or partially resistant to *M. cerebralis.* Fish are most susceptible if exposed when young; however, older fish may become infected and act as carriers of spores. Fish that have a high degree of resistance should not be selected for sampling unless they are the only species present (O'Grodnick 1979; Hedrick et al. 1999a, b; Hedrick et al. 2001; MacConnell and Vincent 2002).

Development of myxospores is temperature dependent, requiring a minimum of 90 days at 16 to 17˚C, and 120 days at 12 to 13˚C (El-Matbouli et al. 1992). When temperature data is available, sample fish that have been on the water supply for a minimum of 1800 degree-days. If continuous temperature monitoring data is not available, select fish that have been on the water supply for a minimum of six months. Select fish that reside in locations on an aquaculture facility most likely to result in exposure to the parasite such as earthen rearing containers and from locations receiving untreated surface water.

Screening for *M. cerebralis* is by examination for spores in cranial cartilage processed by pepsin-trypsin digest. Up to five fish may be pooled for screening; confirmation is on individual fish. Subsampling from large fish by using a halved head or a core sample is accepted; however, detection sensitivity may be decreased if the number of cartilaginous areas containing the parasite are few or if parasite numbers are low. Another difficulty is parasite affinity for different tissue sites may vary between salmonid species (in rainbow trout the parasite prefers the ventral calvarium; and brown trout the parasite is detected in gill arches). Confirmation of *M. cerebralis* is by identification of parasite stages in histological sections of cartilage tissue, or by amplification of parasite DNA by the polymerase chain reaction (PCR).

A. Screening Test

1. **Pepsin-Trypsin Digestion (PTD)** (Markiw and Wolf 1974; Lorz and Amandi 1994)
   This method is recommended for fresh samples; if the sample has been frozen, follow the procedure below with modifications noted in A.1.c.1 and A.1.d.

   a. Defleshing

      i. Samples should be placed in an appropriate container and heated at 45°C in a water bath until flesh is soft and eyes are opaque.

      ii. Deflesh the samples and retain all cartilage/bone including that from the gill arches and opercula.

   b. Pepsin Digestion

i. Weigh cartilage and add 0.5% pepsin solution (Section 2, 5.6.A "0.5% Pepsin Solution") at a ratio of 20 mL/g cartilage.

ii. Large heads (greater than 20 g after defleshing) may be homogenized in the pepsin solution with an electric blender.

iii. Stir at 37°C and monitor pH of pepsin solution. If pH increases above 4.0, centrifuge samples at 1200 x g for 10 minutes, decant pepsin, and add fresh pepsin to the sample.

iv. Process samples until all cartilage/bone is reduced to a consistently small, granular size (e.g. the size of beach sand; one hour is generally sufficient for small fish; two hours to overnight may be required for larger fish).

v. When digestion is complete, centrifuge at 1200 x g and decant pepsin into a solution of 1:4 bleach/water (5,000 ppm chlorine) for disposal.

c. Trypsin Digestion

i. Add 0.5% trypsin (Section 2, 5.6.B "0.5% Trypsin Solution or 0.5% Trypsin with Phenol Red") at a ratio of 20:1 (volume/g) to the pellet.

Note: If samples have been frozen prior to processing, reduce the concentration of trypsin to 0.05%

ii. Adjust to pH 8.5 with 1 N sodium hydroxide (Section 2, 5.6.C "1N Sodium Hydroxide (NaOH)").

iii. Stir at room temperature for 30 minutes.

iv. If samples are to be refrigerated at this stage, inactivate trypsin by adding serum to a final concentration of 20%, or powdered bovine serum albumin to a final concentration of 1%.

v. Pour digested sample through a non-absorbent disposable filter [e.g. paint filters (any paint store), urinary calculi filters, or synthetic material such as nylon screen] into a new tube. Mesh sizes should approximate 200 µm. Reusable filters are not recommended because of difficulties in decontamination. Autoclave filters before disposal.

vi. Centrifuge at 1200 x g for 10 minutes.

vii. Discard supernatant into a solution of 1:4 bleach/water (5,000 ppm chlorine) while retaining pelleted material.

d. Dextrose Centrifugation (Markiw and Wolf 1974)
This concentration step is recommended if spores are not found on initial examination or if a large amount of tissue debris makes microscopic examination difficult; it is not recommended if samples have been frozen because it causes distortion and decreased recovery of the spores.

i. Add 1 mL of phosphate buffered saline (PBS) (Section 2, 5.6.D "Phosphate Buffered Saline (PBS)") to the sample and vortex (this volume may need to be adjusted depending on pellet size, but should be sufficient to suspended all material).

ii. A 55% solution of dextrose is used to provide a 5 cm deep gradient in a 15 mL centrifuge tube. For larger samples, a 50 mL centrifuge tube may be required.

iii. Carefully layer the suspended sample onto the dextrose solution.

iv. Centrifuge at 1200 x g for 30 minutes.

v. Carefully decant the supernatant into a solution of 1:4 bleach/water (5,000 ppm chlorine) while retaining all pelleted material.

vi. If PTD sample will be used for PCR confirmation, the pellet should either be suspended in 0.5 to 1.0 mL of PBS and examined immediately (a 10 fold dilution of the pellet should be sufficient to disperse material enough to see clearly under the microscope). The sample may then frozen until assay or preserved using 70% ETOH.

e. Analyzing Samples

i. Vortex samples prior to preparing slide.

ii. Samples may be examined stained or unstained by wet mount or using a hemocytometer. A number of simple staining methods are suitable for staining spores; e.g. add 60 µL of a saturated solution of crystal violet biological stain to 1 mL subsample, mix and examine.

iii. A minimum of 150 fields should be examined at 200x magnification (20x objective with 10x ocular eyepiece). Measure ten representative myxospores and record the average size and size range. Identification of myxospores with the appropriate size and morphology (8 to 10 µm, rounded, two polar capsules; Figure 5.1) results in a sample designation of **PRESUMPTIVE positive.** If the size is appropriate but internal morphology is not clear, the sample should also be identified as **PRESUMPTIVE positive.** A single myxospore of appropriate size is sufficient to declare a **PRESUMPTIVE positive**. **All other samples are reported as negative.**

Note: Other myxobolid species of salmon have similar morphologies that may be confused with *M. cerebralis*. Photographs and measurements of some of these species are provided in Figure 5.2 for comparison.

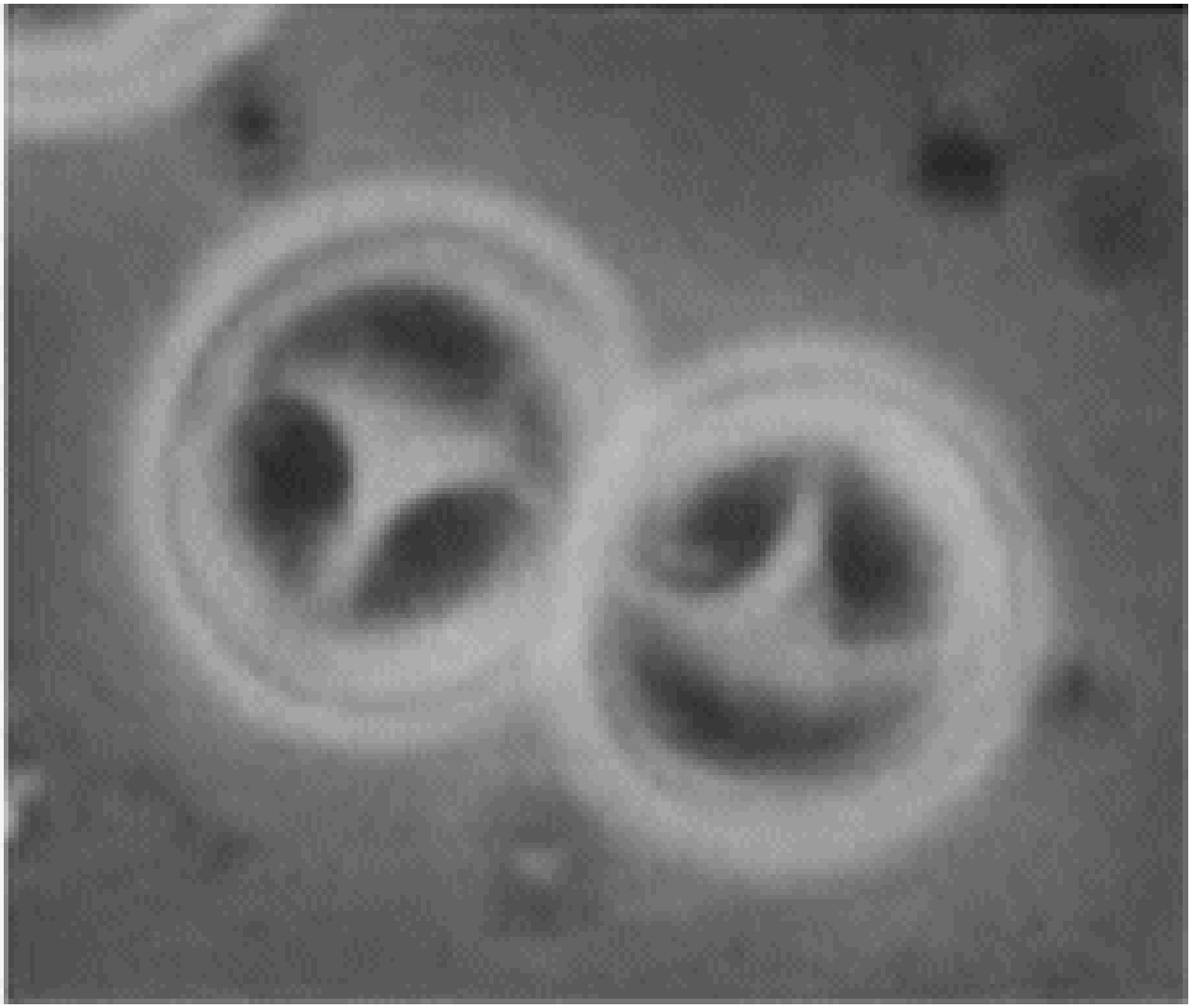


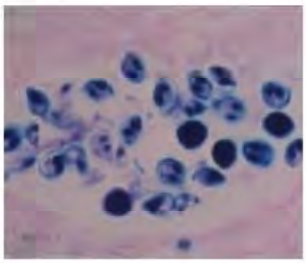
Figure 5.1. Spores of *Myxobolus cerebralis.* Photograph courtesy of R. P. Hedrick.

Figure 5.2: Myxozoans Common To Salmonid Fish



Myxobolus cerebralis

L 7.4 - 9.7 x W 7 - 10 x T 6.2 x 7.4 µm



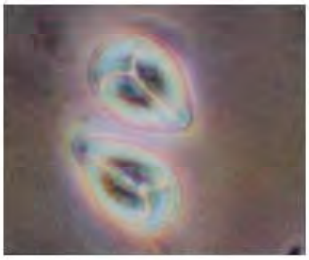
Myxobolus kisutchi

L 8.5 x W 7 µm



Myxobolus insidiosus

L 12.8 - 17.1 x W 8 - 11.9 µm



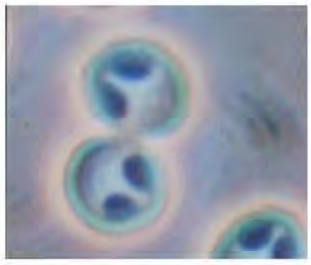
Myxobolus arcticus

L 14.3 - 16.5 µm



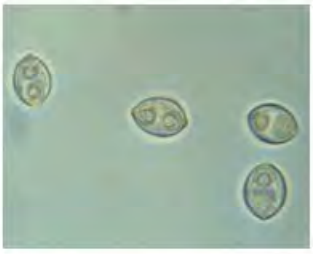
Myxobolus neurobius

L 10 - 14 x W 8 - 9.2 µm



Myxobolus squamalis

L 8.4 - 9.9 x W 7.7 - 9.9 µm



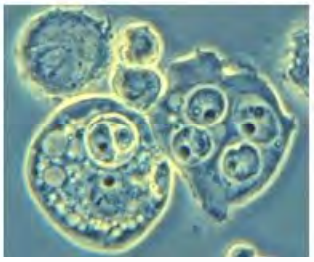
Myxidium minteri

L 9.3 - 12.6 x W 4.5 - 5.3 µm

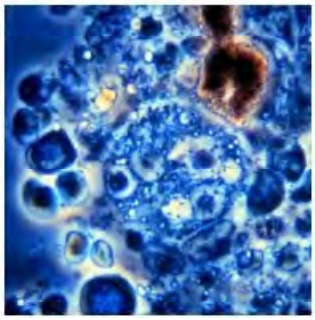


Ceratomyxa shasta

L 14 - 23 x W 6 - 8 µm



Ceratomyxa shasta trophozo



PKX organism

B. Confirmatory Tests

Confirmation of *M. cerebralis* should rely on either histopathology or the nested version of the polymerase chain reaction (PCR) (Kelly et al. 2004).

1. Histopathology

a. Confirmation of *M. cerebralis* may be done using standard histological techniques. Detection of spores with the morphological characteristics of those identified in the digest preparation in tissue other than the cartilage (e.g. brain, nervous tissue, skin, or muscle) is sufficient to report the sample as negative for *M. cerebralis*. However, failure to detect spores of the correct morphology in any tissue is not sufficient to report the sample as negative. Half heads, wedges, or core samples, which were placed in 10% neutral buffered formalin or Davidson's fixative at the time of collection (Section 2, 2.2.E.4 "Collection of Specimens for the Detection of *Myxobolus cerebralis* Evaluation") will be used for corroboration. Process tissues corresponding to a sample number in which a presumptive positive was found.

b. Small samples (from fish <15 cm) fixed in Davidson's can be transferred to 70% ethanol after 24 to 48 hours. Larger samples should remain in Davidson's for 48 hours and may require an additional decalcification step (as specified below).

c. Samples fixed in 10% formalin should be transferred after 24 to 48 hours to a commercially available chemical decalcification solution (e.g. Lerner-D-Calcifier, Decal-Stat Solution, Cal-Ex, and others) or acid decalcifying solution (e.g. 10% Nitric Acid, 5% Formic Acid).

d. Samples requiring decalcification are placed into embedding cassettes in a beaker with a magnetic stir bar and sufficient decalcifying solution (20:1 solution to gram of head tissue). Covered and place on a stirring plate for 4 to 16 hours at room temperature. After three hours, and periodically thereafter, use physical and/or chemical tests to determine if the process is complete (Hauck and Landin 1997).

   i. Physical
   Probe using a dissecting needle to determine softness of bone and/or cartilage (avoid puncturing the tissues). Soft tissues are adequately decalcified.

   ii. Chemical
   Remove 5 mL of decalcifying solution from beaker and neutralize (pH 7.0) with 0.1 N sodium hydroxide (Section 2, 5.6.C "1N Sodium Hydroxide (NaOH)" stock solution). Add 1 mL of saturated ammonium oxalate solution and mix. Allow to stand for 15 minutes and determine if a precipitate (slight cloudiness) of calcium oxalate forms. If so, the decalcification is incomplete. If incomplete, replace decalcifying solution and continue decalcification process until no calcium oxalate precipitate can be detected.

   iii. After decalcification, rinse specimens in distilled water and transfer via graded ethanol series to 70% ethanol.

e. Embed and section tissues at approximately 4 to 5 µm using standard methods. Two sections, taken at 100 µm apart, should be mounted from each sample (or each tissue block if more than one block per fish) and stained with Giemsa (May-Grunwald Giemsa works well, Luna 1968) or Hematoxylin and Eosin (H&E). The sections should target gill arches

(especially from brown trout) and the ventral calverium (floor of the brain case). Examine slide at 200X for cartilage lesions, spores and developing stages of *M. cerebralis* (Figures 5.3 and 5.4).

f. At least two additional sections (100 µm apart) should be taken, mounted and stained if histological lesions and/or spores are not observed.

Observation of any presporogonic or sporogonic stage of *M. cerebralis* in cartilage tissue is reported as positive. Observation of spores with the same morphology as those identified in the digest preparation in tissues other than cartilage are reported as negative. Confirmation cannot be reported if no spores are detected.

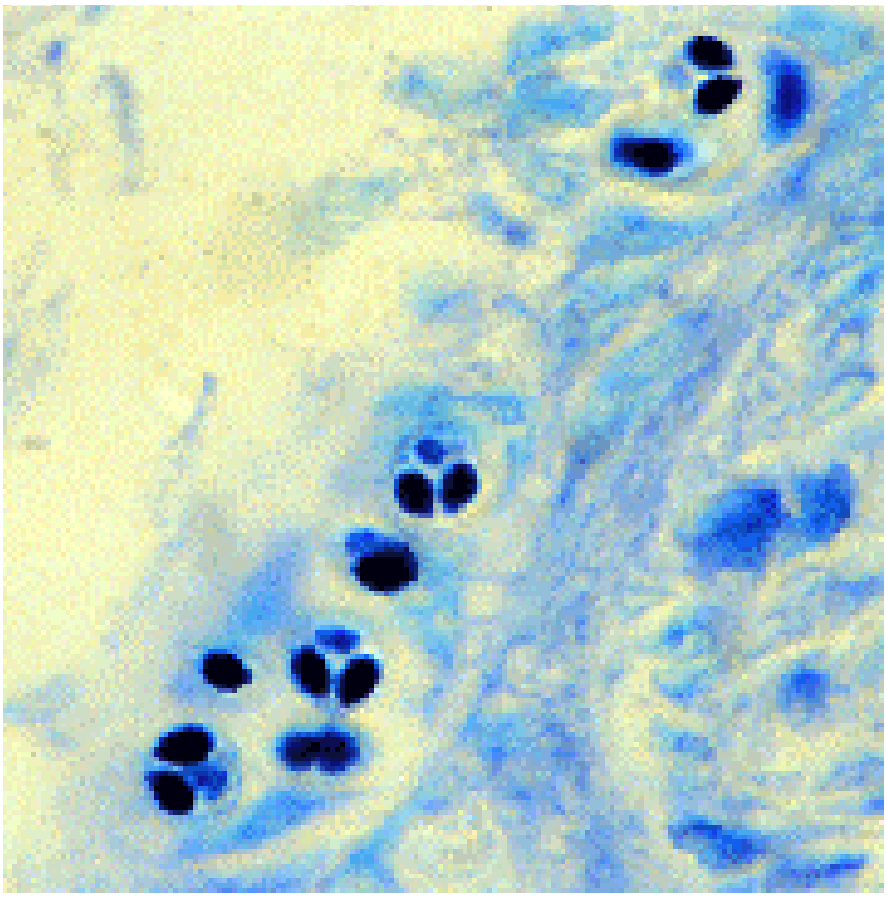


Figure 5.3. Histological sections from a fish infected with *Myxobolus cerebralis*. High magnification showing stained spores (note darkly stained polar capsules). Photograph courtesy of H. V. Lorz.

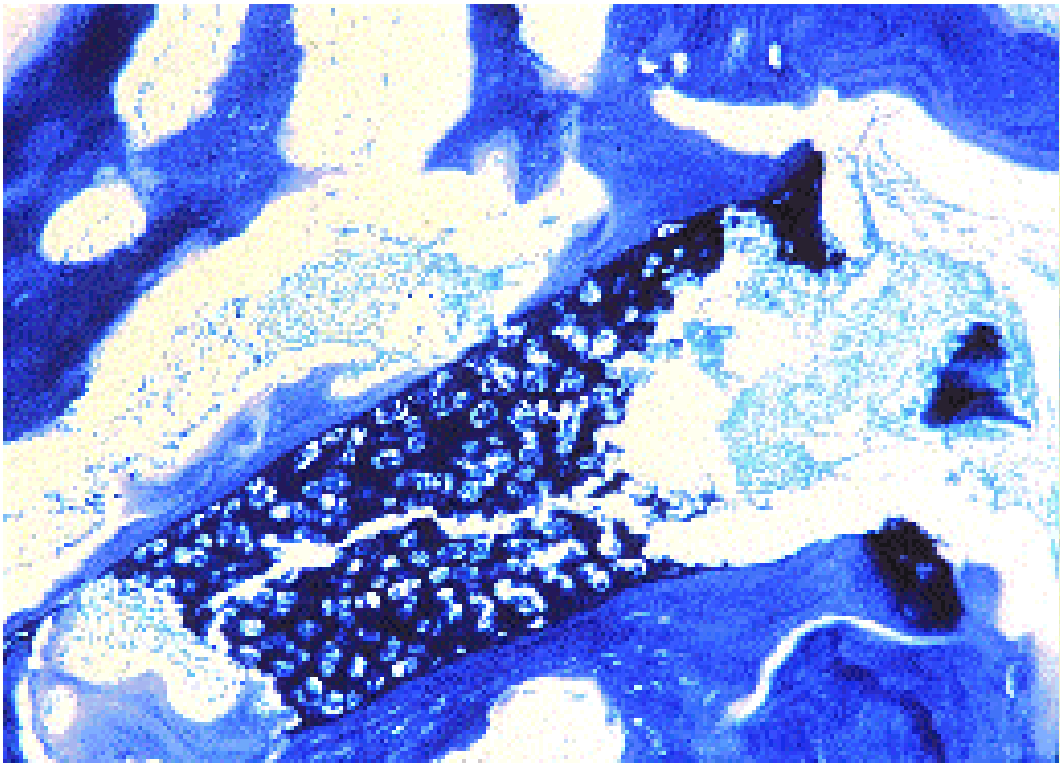


Figure 5.4. Histological sections from a fish infected with *Myxobolus cerebralis*. Low magnification showing degradation of cartilage (Leishman-Giemsa stain).

2. **Polymerase Chain Reaction (PCR)** (Andree et al. 1998; Schisler et. al. 2001)
PCR should be utilized as a confirmatory test *only* on tissue collected as described under specimen collection for PCR at the time of inspection. The assay may be performed directly on the remaining half head or core sample, allowing detection of target DNA from immature parasites as well as from spores (Section 2, 5.2.B.2.a "Extraction of DNA from a Half Head or Core for PCR Confirmation of *Myxobolus cerebralis*"). An alternative method (Baldwin and Myklebust,2002) identifies *M. cerebralis* DNA from spores concentrated by PTD (Section 2, 5.2.B.2.b "Extraction of DNA of the PTD Product for PCR Confirmation of *Myxobolus cerebralis*"). Use of this method allows the other half head to be preserved for histology. However, by applying PCR methods to a digested preparation DNA, presporogonic life stages of the parasite are destroyed, with a potential loss of detection sensitivity. The University of California has awarded an exclusive license to Biogenetic Services, Inc., for the use of the DNA-based diagnostic test for detecting the *Myxobolus cerebralis* organism described by Andree et al. (1998). Contact Biogenetic Services, Inc. for additional information on the availability and limitations to the use of this PCR-based technology. (Phone 605/697-8500; Fax 605/697-8507; Email: biogene@brookings.net; Website: www.biogeneticservices.com)

   a. Extraction of DNA from a Half Head or Core for PCR Confirmation of *Myxobolus cerebralis*

      Note: The following protocol recommends use of the Qiagen DNeasy Tissue Kit (Qiagen Inc, Valencia, CA; Qiagen #69506). Although other kits may work similarly, they have not been tested for this application. Use the kit as per the handbook, with the following modifications:

i. See Section 2, 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR.

ii. Place half-head or core sample in screw capped tubes appropriate for the sample size. Add tap water to cover the tissue and heat in a water bath at 95°C for 15 minutes. For heads ≥ 5 centimeters (2 inches), use 8 ounce sample cup or small beaker and heat for 20 minutes.

iii. Pour water into a solution of 1:4 bleach/water (5,000 ppm chlorine) for 30 minutes to disinfect and place fish sample on polypropylene cutting mat or other surface that can be disinfected or disposed of (autoclave).

iv. Deflesh head using a clean scalpel and forceps; place bone and cartilage in a 1.7 mL micro centrifuge tube or a 50 mL polypropylene screw cap centrifuge tube for larger samples.

v. Add a sufficient volume of ATL buffer, such that it is mixed with the bone material approximately 1:1 (v:w) and proteinase K solution equivalent to 1/10 volume of the buffer. If glass beads are used, extra beads can be added for the larger heads.

Tissue lysis buffer	ATL buffer (Qiagen #69504)
Proteinase K solution (See Section 2, 5.6.E "Proteinase K.")	(17.86 mg/mL)
150 mg small glass beads (optional)	710 – 1180 µm, acid washed
3 large glass beads (optional)	3 mm, acid washed

vi. Vortex until the sample is broken up.

vii. Incubate at 55°C on a rotating platform (or with occasional vortexing) for at least 3 to 4 hours to overnight.

viii. Centrifuge at 16,000 x g for five minutes.

ix. Add 200 µL *aqueous* supernatant to clean micro centrifuge tube.

Note: Occasional clear layer above the white lipid layer is oil; aqueous layer is between lipid layer and debris/glass bead pellet and may be turbid. Dark particulates do not affect DNA yield but can increase column spin times required.

x. Add 20 µL RNase A (Section 2, 5.6.F "RNAse A") (20 mg/mL; Qiagen #19101), vortex until mixed thoroughly, and incubate at room temperature for two minutes.

xi. Follow kit instructions for elution of DNA using buffers and spin column supplied in the kit (DNeasy Tissue Kit; Qiagen #69506), with the following precautions:

Note: "Dirty" preps may require longer spins. DNA preps from positive samples give consistent results when using spin columns. When expecting very small amounts of DNA, the volume of elution buffer can be reduced to 50 µL.

b. Extraction of DNA of the PTD Product for PCR Confirmation of *Myxobolus cerebralis*

i. Pellet the myxospores of the PTD product by micro centrifugation at maximum speed (at least 10,000 rpm) for one minute.

ii. Decant the supernatant and air dry for five minutes

iii. Microwave for one minute in an 800-watt microwave oven at full power.

iv. Re-suspend the pellet in 500 µL of Proteinase K lysis buffer (10 mM Tris-Cl, pH 8.0, 2 mM EDTA, 0.1 % sodium dodecylsulfate and 0.5 mg mL^{-1} Proteinase K) and incubate at 55°C for four hours frequently vortexing gently.

v. Remove sample to a clean laboratory hood and add 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1) to the lysed sample.

Note: Procedures are being developed to substitute the phenol:chloroform extraction (steps v–x) with extraction using Qiagen Kits (Qiagen Inc, Valencia, CA, Qiagen # 69506). Currently this work remains unpublished, but the procedure is as above, with the modification that after microwave treatment, 180 µL of Qiagen Buffer ATL and 20 µL of Proteinase K are added to the sample. The sample is vortexed and incubated at 55°C for 6 to 8 hours for complete cellular lysis. The DNA then can be selectively bonded to the Dneasy membrane columns, washed to reduce contaminates and inhibitors, then eluted using the Dneasy protocol for rodent tails. As will any procedure modifications, this should be thoroughly tested and documented in the laboratory.

vi. Gently invert sample several times to form an emulsion, then centrifuge at 1700 x g at room temperature for 10 minutes to separate the aqueous and organic phases.

vii. Transfer the upper aqueous phase (with the nucleic acid) to a clean micro centrifuge tube and add 500 µL of chloroform.

viii. Mix by inverting the sample several times and centrifuge at 1700 x g.

ix. Add 800 µL of ice cold ethanol and incubate at -20°C for one hour to precipitate the DNA.

x. Centrifuge at 12,000 x g for 10 minutes at 4°C, decant the supernate, and resuspend the pellet in 50 µL of TE buffer (10mM Tris, 0.1 mM EDTA, pH 8.0).

xi. Incubate sample overnight at room temperature.

c. Quantification of DNA

It is advisable that extracted products be measured using a spectrophotometer to ensure that enough DNA was successfully extracted. Quantification guidelines are in Section 2, 6.2.C.4 "Quantification of DNA."

Note: Quantify DNA of a representative sample (5% or 6%) from each group of a particular size range and assume all those within that size range have a similar concentration. If they do not range too widely, average the values and determine the DNA concentration. Then add an

appropriate volume to each PCR assay such that the amount per reaction is between 100 and 300 ng.

d. Amplification of *M. cerebralis* DNA

i. Following general PCR protocols (Section 2, 6.2 PCR Quality Assurance/Quality Control). The reagents and primers for this reaction are:

1^{o} Master Mix: 50 µL total reaction volume

H_2O – sterile, molecular biology grade	32.1 µL
Mg Cl_2 (50 mM)(5.6.K)	1.5 µL (final concentration 1.5 mM)
10X Taq Polymerase Buffer	5 µL
dNTPs (5 mM stock)	4 µL (final concentration 0.4mM)
Tr5-16 Primer 1 (20 µM)	2 µL (final concentration 0.8 µM)
Tr3-16 Primer 2 (20 µM)	2 µL (final concentration 0.8 µM)
Taq Polymerase (5 units/µL)	0.4 µL (2 units per reaction)

Primers (Andree et al. 1998)
Tr5-16 = 5'-GCA TTG GTT TAC GCT GAT GTA GCG A-3'
Tr3-16 = 5' -GAA TCG CCG AAA CAA TCA TCG AGC TA-3'

ii. Add PCR reagents **except for sample DNA** to the MM tube, adding water first and Taq last. Keep all reagents cold in frozen cryo-rack during mixing, and return them to freezer immediately after use.

iii. Place 48 µL of MM into each 0.5 mL PCR tube. Close caps tightly. Move PCR tubes to sample loading area.

iv. Load 2 µL of each sample DNA to the appropriately labeled PCR tubes.

v. Thermocycler should be programmed for 35 cycles of the following regime:

1. Cycle Parameters:

a. Denature at 95 °C for five minutes.

b. Then, 35 cycles at:
95 °C for one minute.
65 °C for 2.5 minutes.
72 °C for 1.5 minutes.

Note: For maximum sensitivity with weak positive samples these conditions are critical.

vi. For the nested amplification prepare the 2^{nd} MM as before, but substitute primers Tr5-17 and Tr3-17. Transfer 1 µL of the amplification product from the first amplification to the appropriately labeled reaction tube containing the 2^{nd} MM and load the thermal cycler using the same program as for the first amplification.

Primers (Andree et al. 1998)

Tr5-17 = 5' -GCC CTA TTA ACT AGT TGG TAG TAT AGA AGC-3'

Tr3-17 = 5'-GGC ACA CTA CTC CAA CAC TGA ATT TG-3'

e. Visualization of PCR Product by Electrophoresis

i. Prepare agarose gel as indicated in Section 2, 6.3.C "Detection of Product" and load 6.0 µL of amplified product + 1.5 µL loading buffer into sample wells.

ii. After electrophoresis of products (Section 2, 6.3.D "Electrophoresis"), stain and photograph gel as described in Section 2, 6.3.E "Staining the Gel" through 6.3.G "Visualize the DNA."

iii. Carefully record locations of bands on positive control samples in relation to DNA ladder bands. **Detection of the expected 415 bp (base pair) amplicon in a sample amplified using the nested PCR protocol will result in corroboration of the presence of *M. cerebralis* in the sample. Any samples not yielding this band with no apparent assay problems is reported as negative.**

iv. If any unusual bands are present in the samples or if bands are present in negative controls, re-run PCR reaction from template DNA tube.

v. **Photo document all gels** and attach the photo to the case history information.

5.3 *Ceratomyxa shasta* (Ceratomyxosis)

Ceratomyxa shasta is difficult to detect because the life cycle of the parasite includes two alternate hosts: salmonids and the freshwater polychaete worm, *Manayunkia speciosa* (Bartholomew et al. 1997). Salmonids are the only known fish hosts for *C. shasta.* Although susceptibility may vary between species and strain, all salmonids should be considered susceptible. The parasite is enzootic in a number of waters in California, Oregon, Washington, Idaho, Alaska, and B.C., Canada (Bartholomew et al. 1989a), but has not been reported outside of that region. Diagnosis is complicated by the long period required for development of mature myxospores in the fish and by the pleomorphic appearance of the presporogonic stages. Parasite development is temperature dependent, but in general, at 12ºC, a minimum of 40 days is required for spore development in rainbow trout in the laboratory. Confirmation of *C. shasta* is based on identification of myxospores with the appropriate morphology or by PCR confirmation of presumptive presporogonic life stages.

A. Screening Test

1. **Examination of Wet Mounts** (Bartholomew et al. 1989a, Bartholomew 2001)

   a. To prepare a wet mount, the intestine is removed, placed on a disposable surface and opened longitudinally with a clean scalpel or scissors. Scrape the posterior 1/3 of the intestinal mucosa and mix in a drop of water on a microscope slide. Prepare wet mounts from any areas of hemorrhage as well.

   b. Scan wet mount in a systematic manner under phase contrast or bright field microscopy at 200 to 440X magnification. Examine the entire smear or an area equal to that under a 22 mm^2 coverslip.

   c. Presumptive diagnosis is based on identification of multicellular myxosporidian pre-sporogonic stages (trophozoites) (Figure 5.4A). Visualization of prespore stages is not sufficient for definitive diagnosis. Any samples in which the organisms are not seen may be discarded and reported as negative.

B. Confirmatory Test

Confirmation of *C. shasta* should rely on either detection of mature spores or on amplification of parasite DNA using the polymerase chain reaction (PCR).

1. **Morphology** (Bartholomew 2001)
   Confirmatory diagnosis of *C. shasta* is based on identification of the characteristic myxospore (Figure 5.2). Myxospores of *C. shasta* measure 14 to 23 µm long and 6 to 8 µm wide at the suture line. The ends of the spores are rounded and reflected posteriorly and the suture line is distinct.

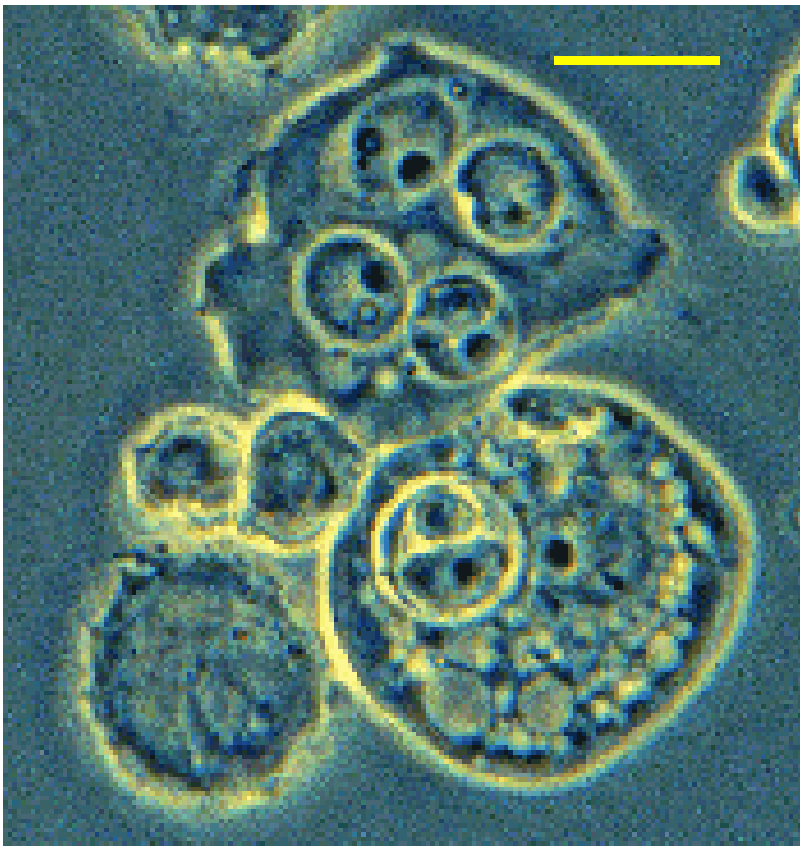


Figure 5.1. Wet mount of presporogonic stages of *Ceratomyxa shasta*. Bar equals 10 µm. Photograph courtesy of J. L. Bartholomew.



Figure 5.2. Wet mount of myxospore stage of *C. shasta***.** Bar equals 10 µm. Photograph courtesy of J. L. Bartholomew.

2. Polymerase Chain Reaction (Palenzuela et. al. 1999; Bartholomew 2001; Palenzuela and Bartholomew 2001)
PCR should be utilized as a confirmatory test *only* on tissue collected at the time of inspection, using methods described under specimen collection.

a. General Protocols and QA/QC Considerations (See Section 2, 6.2 PCR Quality Assurance/Quality Control for specific QA/QC considerations and general protocols for PCR.)

b. Extraction of DNA

i. If the sample was fixed in ethanol, transfer to micro centrifuge tube with 500 µL DNA extraction buffer (Section 2, 5.6.G "DNA Extraction Buffer"); if frozen, add 500 µL DNA extraction buffer.

ii. Add Proteinase K solution (Section 2, 5.6.E "Proteinase K") to a final concentration of 200 µg/mL (example: if stock solution is 20 mg/mL, add 5 µL).

iii. Incubate micro centrifuge tubes at 37°C in a horizontal position on a slow platform rocker or with frequent inversion by hand. Digestion of sample will require about 4 to 5 hours, but overnight incubation does not affect the quality of the DNA and is recommended.

iv. After samples are completely digested, add 5 µL of RNAse A (10 mg/mL stock) (Section 2, 5.6.F "RNAse A") to each micro centrifuge tube and incubate one hour at 37°C with rocking.

v. Heat samples at 100°C for five minutes (in a water bath, heat block, or thermocycler).

vi. Remove rack and cool at room temperature. Upon cooling, samples are ready for dilution and PCR. Samples at this stage may be stored at -20°C.

c. Amplification of *C. shasta* DNA

i. Dilute the DNA template (sample) 1:10 with ultra pure (molecular grade) sterile water.

Note: Because this assay utilizes crude DNA, quantitation by UV spectrophometer does not provide useful information. A 1:10 dilution is generally sufficient unless the tissue size is larger than recommended (if this is suspected, include a 1:100 dilution in addition)

ii. Following general PCR protocols (Section 2, 6.3 PCR Protocols), record appropriate data for PCR and calculate reagent volumes that go into the Master Mix (MM). This assay was optimized using reagents from Promega (Madison, WI, USA) and if other buffer systems are substituted, these should be tested. The primers and reagents for this reaction are:

Master Mix: 20 µL total reaction volume

H_2O – sterile, molecular biology grade	14.6 µL
Mg CL_2 (25 mM; Promega #A351B)	1.6 µL
10X PCR Poly Buffer (Promega #M190A)	2.0 µL

dNTPs (at 10mM each)	0.4 µL (final concentration 0.2 mM)
Forward Primer (100 µM)	0.1 µL (final concentration 0.5 µM)
Reverse Primer (100 µM)	0.1 µL (final concentration 0.5 µM)
Taq Polymerase (5 units/µL)	0.2 µL (1U per reaction)

Primers (Palenzuela and Bartholomew 2001):
Forward: 5' GGGCCTTAAAACCCAGTAG 3'
Reverse: 5' CCGTTTCAGGTTAGTTACTTG 3'

iii. Place 19 µL of MM into each 0.2 mL PCR tube. Close caps tightly. Move PCR tubes to sample loading area.

iv. Add 1 µL of each sample DNA to the appropriately labeled PCR tubes.

v. Thermocycler should be programmed for 35 cycles of the following regime:

1. Cycle Parameters:

a. Denature at 95°C for three minutes.

b. 35 Cycles of:
94°C for one minute.
58°C for 30 seconds.
72°C for one minute.
72°C for 10 minutes.
Hold to 4°C chill at end of program.

d. Detection

i. Prepare agarose gel as indicated in Section 2, 6.3.C "Detection of Product" and load 10.0 µL of each PCR reaction + 1.5 µL loading buffer (Section 2, 5.6.H "Loading Buffer") into sample wells.

ii. After electrophoresis of products (Section 2, 6.3.D "Electrophoresis"), stain and photograph gel as described in Section 2, 6.3.E "Staining the Gel" through 6.3.G "Visualize the DNA."

iii. Carefully record locations of bands on positive control samples in relation to DNA ladder bands. ***C. shasta* positive reactions will have an amplicon of 640 bp. Any samples not yielding this band with no apparent assay problems is reported as negative and the samples are discarded.**

iv. If any unusual bands are present in the samples or if bands are present in negative controls, re-run PCR reaction from template DNA tube.

v. **Photo document all gels** (Section 2, 6.3.G "Visualize the DNA") and attach the photo to the case history information.

5.4 *Tetracapsula bryosalmonae* (Proliferative Kidney Disease)

The myxozoan (PKX), known to cause proliferative kidney disease in salmonids, is difficult to detect because its life cycle is complex and requires a bryozoan to complete its development (Canning et al. 1999). Diagnosis of infections in fish is also complicated because the parasite does not develop completely in the fish, and resulting myxospores, if present, lack fully developed spore valves (Kent 1994). Two developmental stages occur in salmonid hosts: presporogonic stages found in blood and interstitial kidney tissue and sporogonic stages found within the lumen of kidney tubules. Although PKX-like cells have been identified from species other than in the family Salmonidae, their identity as *T. bryosalmonae* is unconfirmed.

A. Screening Tests

1. **Leishman-Giemsa Staining** (Klontz and Chacko 1983)

   a. Stain slides with tissue imprints using Leishman-Giemsa stain.

      i The fixed imprint is incubated with approximately 1 mL Leishman stain (Section 2, 5.6.L "Leishman Stain") for one minute.

      ii Giemsa stain (2 to 3 mL) (Section 2, 5.6.M "Giemsa Stain") is added to the Leishman stain and allowed to stand for an additional 10 to 15 minutes.

      iii Rinse the slide and examine.

   b. Whenever possible, a positive control slide should be examined prior to evaluating samples.

   c. Examine a minimum of 100 fields of the stained imprints at 400x using bright field microscopy for the presporogonic stages of the parasite.

   d. Presporogonic stages (Figure 5.1) are large (approximately 20 µm) with a prominent cell membrane. The primary cell (outer, surrounding cell) contains prominent granules, a nucleus with a large nucleolus, and one or more secondary or daughter cells. Macrophages are frequently adhered to the surface of the parasite.

   e. **The finding of presporogonic stages (Figure 5.1) is presumptive and warrants confirmation by histology**.

2. **Lectin-Based Staining** (Hedrick et al. 1992)

   a. Tissue imprints are stained with 50 µL biotinylated GS-1 lectin (L-3759, Sigma, St. Louis, Missouri) suspended in 0.01M phosphate buffer pH 6.8 (Section 2, 5.6.I "0.01 M Phosphate Buffer pH 6.8," pH adjusted).

b. To determine the appropriate concentration of lectin, dilutions of 25 to 0.5 µg mL^{-1} are tested on control tissue.

c. Slides are incubated in a moist chamber for 1 to 2 hours at 25˚C.

d. Rinse three times in PBS (Section 2, 5.6.D "Phosphate Buffered Saline (PBS)").

e. Apply 50 µL fluorescein avidin D (A-2001, Vector Laboratories, Inc., Burlingame, California) diluted to provide 10-30 µg mL^{-1} suspensions in PBS.

f. Incubate slides in moist chamber at 25˚C for 30 minutes.

g. Rinse three times, then carefully blot to near dry and mount with a coverslip using a drop of a mixture containing 1 part of 0.1M N-2-hydroxy-ethylpiperzine-N'-2-ethanesulfonic acid (HEPES) pH 8.0 and 9 parts glycerol.

h. Observe under a microscope with a UV light source equipped for fluorescein.

i. **The identification of fluorescing presporogonic stages (Figure 5.2) is presumptive and warrants corroboration by histology.**

B. Confirmatory Test

1. **Histopathology** (Hedrick et al. 1986)

a. Slides should be prepared from the kidney tissue that was placed in fixative at the time of collection using standard histological techniques.

b. Sections should be stained with hematoxylin and eosin (H&E) or Giemsa and examined for stages of *T. bryosalmonae*.

c. **The presence of presporogonic stages of *T. bryosalmonae* within the interstitial tissue of the kidney is corroboration of the infection (Figure 5.3).** A chronic inflammatory response is typically associated with the presence of the parasite (Figure 5.4) and a "whorled" appearance may be visualized corresponding to locations of the parasite. The presporogonic stage is approximately 20 µm in diameter and the primary cell has a lightly staining cytoplasm with a large, eosinophilic staining nucleolus (Figure 5.5). The primary cell may contain one to several spherical, dense, secondary (daughter) cells and macrophages are frequently seen adhered to the parasite. The inflammatory cell infiltrate is primarily composed of macrophages but numerous lymphocytes are also typically present. Sporogonic stages of *T. bryosalmonae* may be observed in the lumina of kidney tubules. Morphology of spores is poorly defined in fish and it appears that hardened valves typical of other myxosporidians do not form. Valveless myxospores are approximately 12 um X 7 um and have two spherical polar capsules 2 um in diameter (Figure 5.6).

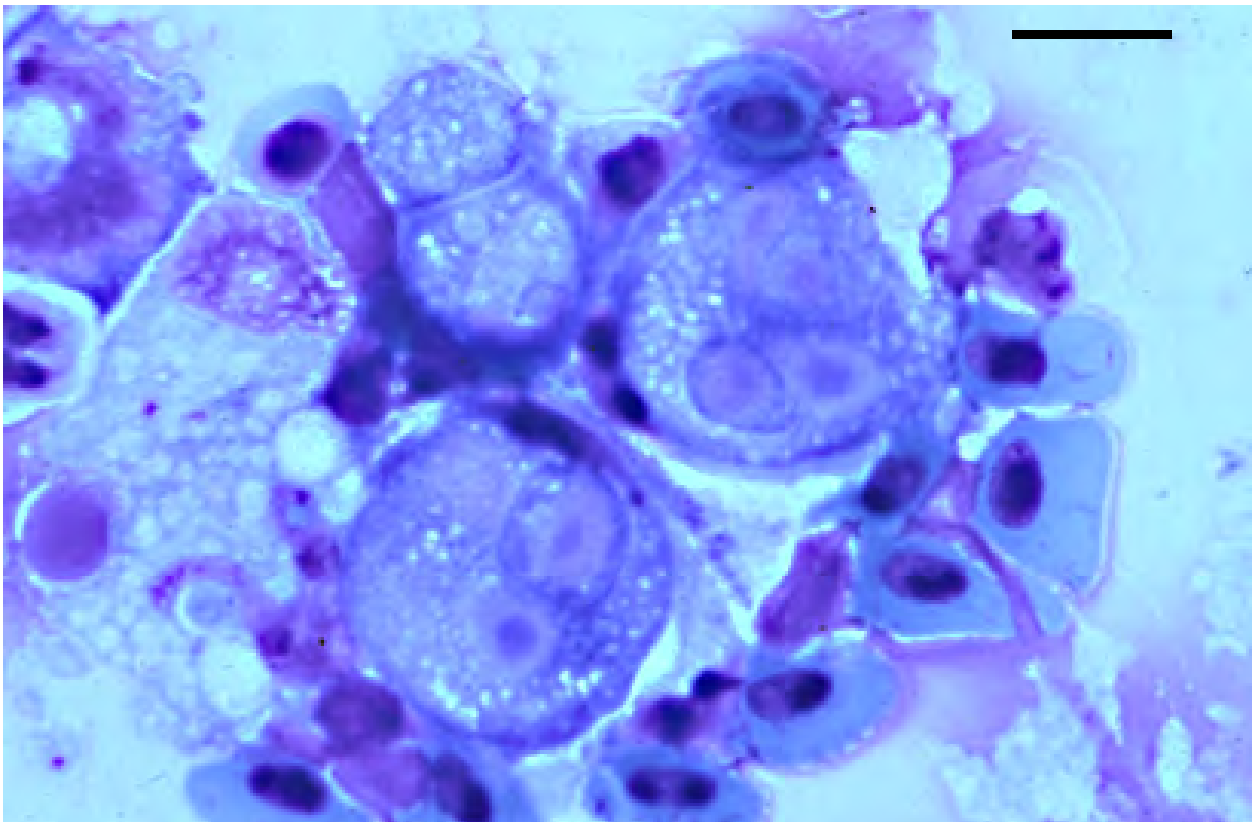


Figure 5.1. Multicellular *Tetracapsula bryosalmonae* in a Leishman-Giemsa stained imprint. Bar is 10 µm. Photograph courtesy of R. P. Hedrick.

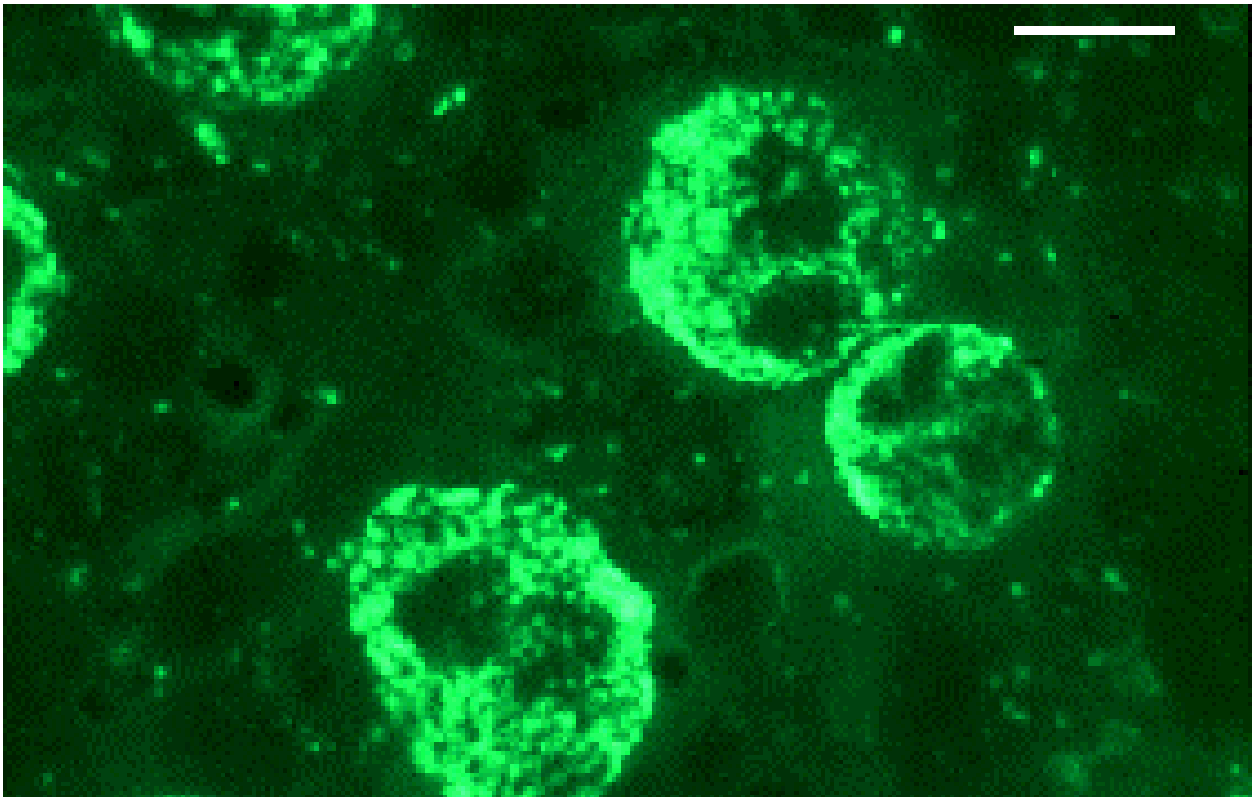


Figure 5.2. Multicellular *Tetracapsula bryosalmonae* in a lectin-stained imprint. Bar is 10 µm. Photograph courtesy of R. P. Hedrick.

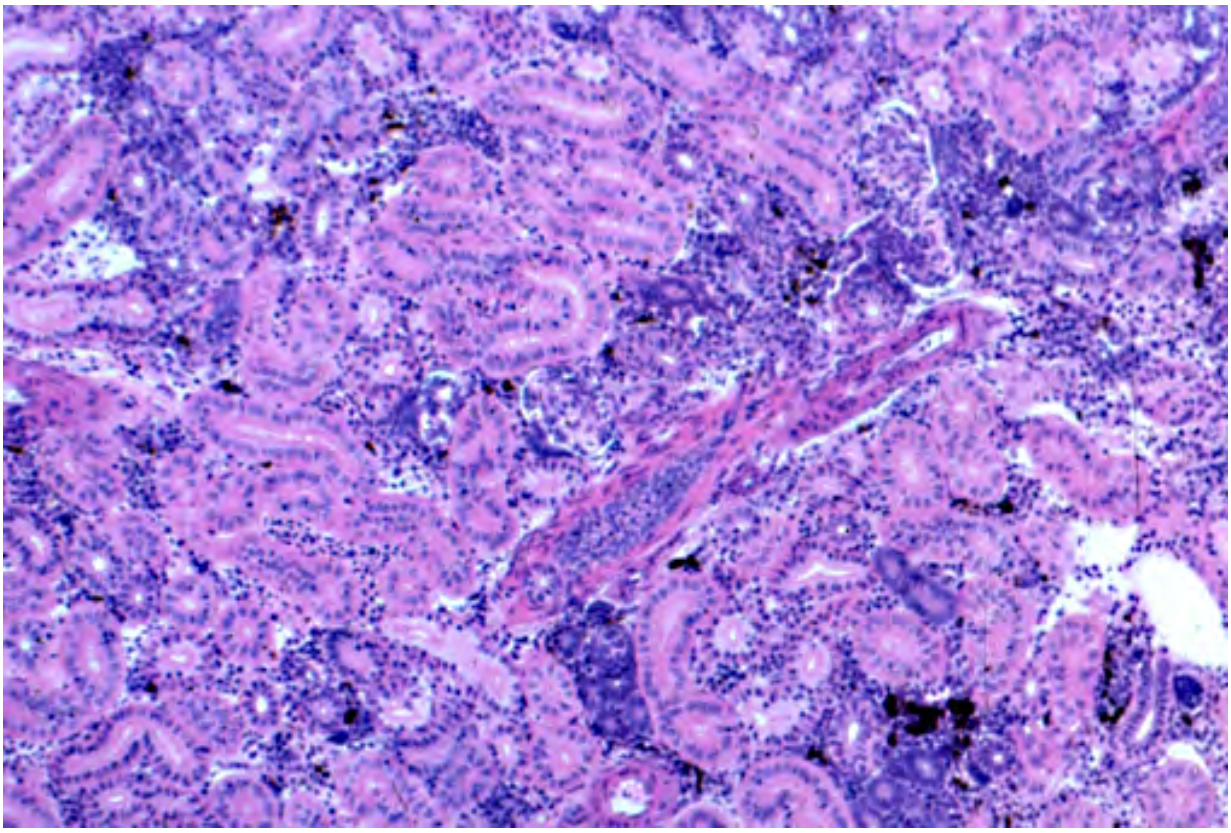


Figure 5.3. Histological sections of kidney tissue infected with *T. bryosalmonae* at low magnification of normal kidney. Photographs courtesy of R. P. Hedrick.

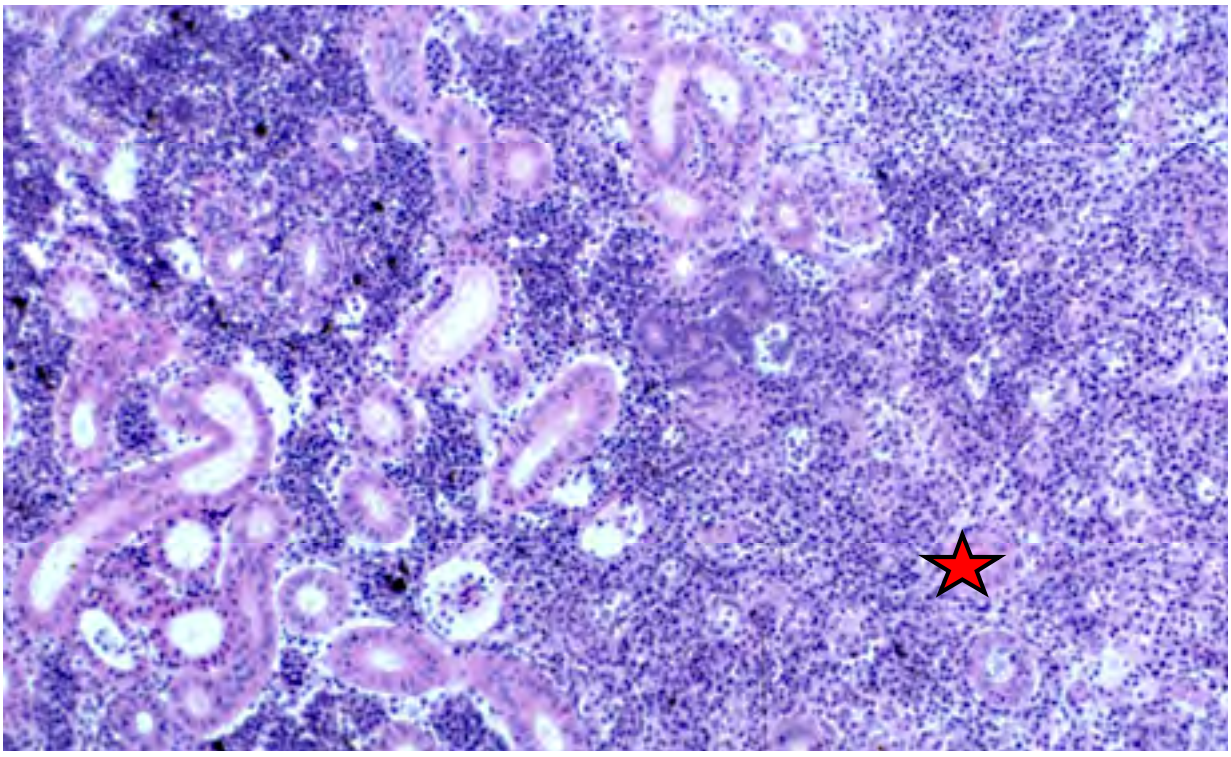


Figure 5.4. Histological sections of kidney tissue infected with *T. bryosalmonae*. at low magnification showing area of inflammatory cell infiltrate indicated by the star. Photographs courtesy of R. P. Hedrick.

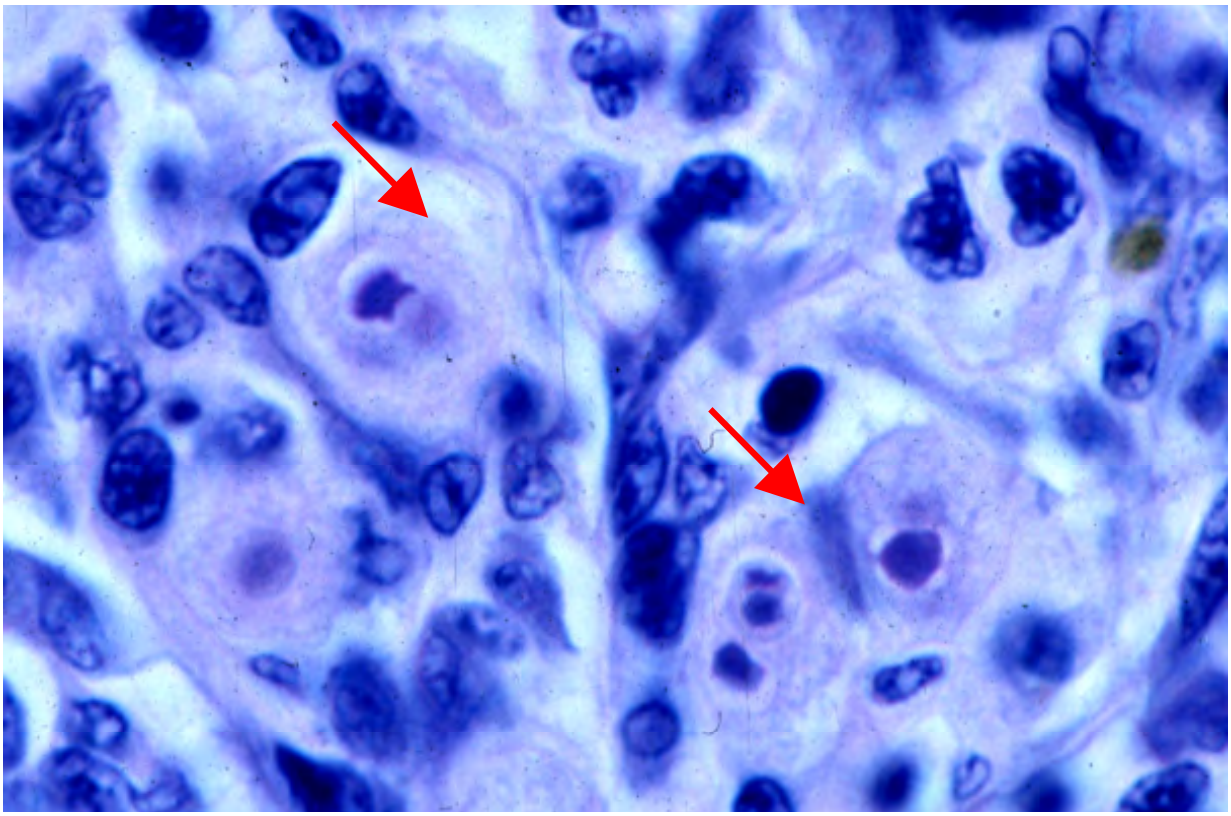


Figure 5.5. Histological sections of kidney tissue infected with *T. bryosalmonae*. at high (400X) magnification of parasites in tissue. Photographs courtesy of R. P. Hedrick.

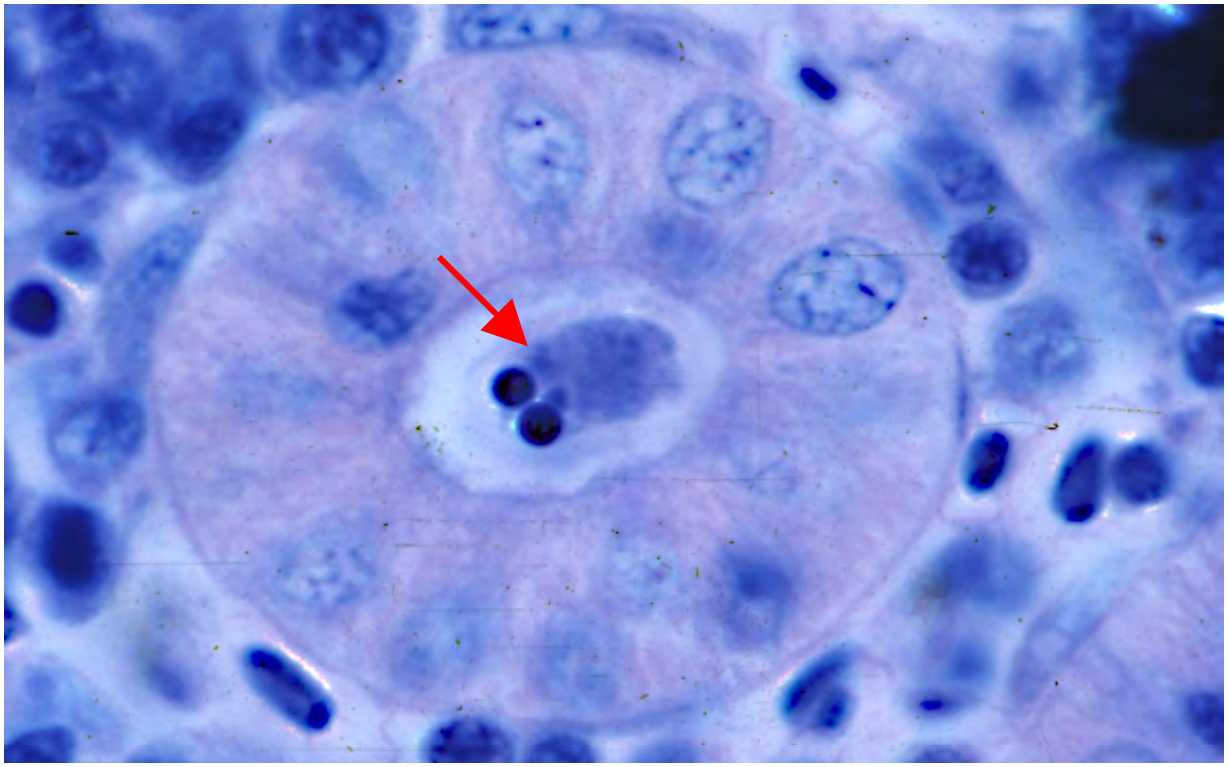


Figure 5.6. Histological sections of kidney tissue infected with *T. bryosalmonae*. sporogonic stage in kidney tubule (1000X). Photographs courtesy of R. P. Hedrick.

2. Polymerase Chain Reaction (Kent et al. 1998; Feist et al. 2001)
PCR should be utilized as a confirmatory test *only* on tissue collected at the time of inspection, using methods described under specimen collection.

a. General Protocols and QA/QC Considerations (See Section 2, 6.2 PCR Quality Assurance/Quality Control for specific QA/QC considerations and general protocols for PCR.)

b. Extraction of DNA
Note: This assay was optimized using phenol:chloroform extraction and if other extraction methods are substituted, these should be tested.

i. If the sample was fixed in ethanol, wash twice in TE buffer (Section 2, 6.4 Reagents), then soak in TE buffer for one hour. Lyse sample in 500 µL DNA extraction buffer (Section 2, 5.6.G "DNA Extraction Buffer") with Proteinase K solution (Section 2, 5.6.E "Proteinase K") to a final concentration of 200 µg/mL (example: if stock solution is 20 mg/mL, add 5 µL) with agitation for at least one hour at 60˚C, or overnight at 37˚C.

ii. Extract twice with 400 µL phenol/chloroform/isoamy alcohol (50:50:1).

iii. Precipitate DNA in 800 µL absolute ethanol and 40 µL 3 µ sodium acetate, centrifuge at 13000 x g for 15 minutes and remove ethanol. Rinse with 70% ethanol, centrifuge at 13000 x g for 10 minutes and remove ethanol. Air dry pellet and resuspend in 20 µL molecular grade water. Sample can be stored at -20˚C.

c. Quantitation of DNA
It is advisable that extracted products be measured using a spectrophotometer to ensure that enough DNA was successfully extracted. Quantification guidelines are in Section 2, 6.2.C "Extraction of DNA or RNA from Samples."

Note: Quantify DNA of a representative sample (5% or 6%) from each group of a particular size range and assume all those within that size range have a similar concentration. If they do not range too widely, average the values and determine the DNA concentration. Then add an appropriate volume to each PCR assay such that the amount per reaction is between 100 and 300 ng.

d. Amplification of *T. bryosalmonae* DNA

i. Following general PCR protocols (Section 2, 6.3 PCR Protocols), record appropriate data for PCR and calculate reagent volumes that go into the Master Mix (MM). This assay was optimized using reagents from GIBCO (Gaithersburg, MD, USA) and if other buffer systems are substituted, these should be tested. The primers and reagents for this reaction are:

Master Mix: 50 µL total reaction volume

H_2O – sterile, molecular biology grade	33.4 µL
Mg CL_2 (25 mM)	5.0 µL (final concentration 2.5 mM)
10X PCR buffer	5.0 µL
dNTPs (25mM)	0.4 µL (final concentration 0.2 mM)
5-Forward primer (20 µM)	2.0 µL (final concentration 0.8 µM)

6-Reverse primer (20 µM)	2.0 µL (final concentration 0.8 µM)
Taq polymerase (5 units/µL)	0.2 µL (1U per reaction)

Primers (Kent et al. 1998):
Forward (5F): 5' CCTATTCATTGAGTAGAGA 3'
Reverse (6R): 5' GGACCTTACTCGTTTCCGACC 3'

ii. Place 48 µL of MM into each 0.5 mL PCR tube. Close caps tightly. Move PCR tubes to sample loading area.

iii. Add 2 µL of each sample DNA to the appropriately labeled PCR tubes.

iv. Thermocycler should be programmed for 35 cycles of the following regime:

1. Cycle Parameters:
Denature at 94°C for three minutes.

2. 35 Cycles of:

94°C for one minute.
55°C for one minute.
72°C for one minute.
72°C for five minutes.
Hold to 4°C chill at end of program.

e. Detection

i. Prepare agarose gel as indicated in Section 2, 6.3.C "Detection of Product" and load 10.0 µL of each PCR reaction + 1.5 µL loading buffer (Section 2, 5.6.H "Loading Buffer") into sample wells.

ii. After electrophoresis of products (Section 2, 6.3.D "Electrophoresis"), stain and photograph gel as described in Section 2, 6.3.E "Staining the Gel" through 6.3.G "Visualize the DNA."

iii. Carefully record locations of bands on positive control samples in relation to DNA ladder bands. *T. bryosalmonae* positive reactions will have an amplicon of 501 bp. Any samples not yielding this band with no apparent assay problems is reported as negative and the samples are discarded.

iv. If any unusual bands are present in the samples or if bands are present in negative controls, re-run PCR reaction from template DNA tube.

v. **Photo document all gels** (Section 2, 6.3.G "Visualize the DNA") and attach the photo to the case history information.

5.5 *Bothriocephalus acheilognathi* (Bothriocephalosis, Asian Tapeworm)

Bothriocephalosis is an intestinal infection of certain fish by the cestode *Bothriocephalus acheilognathi* (Mitchell 1994; Scholtz 1997), a Pseudophyllidian tapeworm. The infecting organism is also known as the Asian or Asian fish tapeworm and as the Chinese tapeworm. The Asian tapeworm has been reported in Asia, Europe, Australia, South Africa, and North America. In North America, it has been reported in Mexico, British Columbia, throughout the southern half of the United States, and in New Hampshire, New York, and Hawaii. Fish become infected after ingesting infected copepods and development of the worm occurs in the anterior intestinal tract. *Bothriocephalus acheilognathi* is a thermophile that has an optimum temperature for growth and maturation above 25ºC.

Most members of the Family Cyprinidae are considered potential hosts, with the exception of goldfish, *Carassius auratus*. Infections have also been reported in species from the following families: Siluridae, Poeciliidae, Percidae, Centrarchidae, Gobiidae, and Cyprinodontidae

A. Screening Test

1. **For Fish Less than 20 cm in Length**
   The uncoiled intestines from several fish may be placed side by side on a microscope slide or glass plate (9 x 9 x 0.3 cm). When intestines are too small to uncoil they can be placed as they come onto a slide. There is no need to slit the intestines. A second slide or glass plate placed over the excised intestine spreads the intestine for easy visibility. For small intestines the weight of the glass plates is usually sufficient to flatten the contents for easy viewing. Binder clips can be used if further flattening is desired (Mitchell 1989, 1994).

   a. Examination requires the use of a 15 to 30-power dissecting microscope. Reflected light gives the best results.

   b. *Bothriocephalus acheilognathi* sometimes takes on a silvery cast and movement will be detected if the specimen is viewed for 15 seconds.

   c. Thoroughly examine the anterior intestinal tract. Small worms may measure only 350 µm in length.

2. **For Fish Longer than 20 cm**
   Slit the anterior third of the gut open lengthwise and carefully remove any cestodes while keeping the scolex intact. Scraping the inner wall of the intestine with a scalpel ensures the collection of the scolices from visible worms and from small worms not seen. Place contents on a glass plate (see above) or microscope slide (depending on volume of contents) and cover with another plate or slide. Using binder clips, press the two plates together. Examine entire sample for *B. acheilognathi*.

3. **Visualization of a cestode found in the anterior third of the intestine that forms a pyramidal scolex in the semi contracted state (Figure 5.1) is a presumptive positive classification**. In the semi contracted state the scolex is usually three times the width of the segmented portion adjacent to the scolex. If large worms or many small worms are present, they may be apparent as a yellow to white bulge in the intestine.

B. Confirmatory Test

Other cestodes have similar pyramid shaped scolices. Therefore, a key must be used to definitively identify *B. acheilognathi*. A complete key for the identification of *B. acheilognathi* and accompanying figures are found in the 4th Edition of the Blue Book (Thoesen 1994). Specific characteristics needed for definitive identification are noted below.

1. ***Bothriocephalus acheilognathi***
   Is a complete and distinctly segmented, thin tapeworm that can reach a length of over 50 cm, but is usually less than 10 cm. Segmentation is evident on worms 1 mm or more in length (Figure 5.1).

2. ***Bothriocephalus acheilognathi***
   Has a flattened scolex with two bothria (deep, elongated sucking grooves dorsal and ventral as seen in Figure 5.1, no hooks, no spines, no true suckers, and no proboscides (short tentacles). In the lateral view, the scolex takes a strong arrowhead appearance when semi contracted and a balled or squared appearance when fully contracted. The posterior portion of the scolex is wider than the first few segments in both the extended and contracted positions.

3. ***Bothriocephalus acheilognathi***
   Has no neck and no dorsal or ventral median furrow.

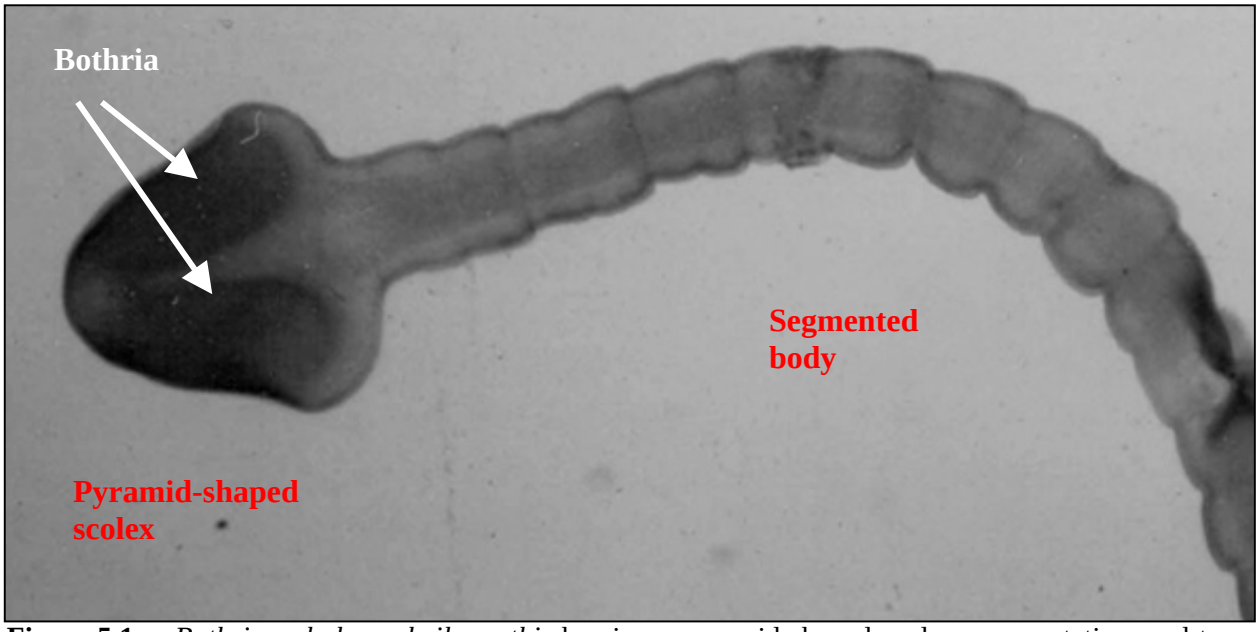


Figure 5.1. *Bothriocephalus acheilognathi* showing a pyramid shaped scolex, segmentation, and two bothria. Photograph courtesy of A. Mitchell.

5.6 Media and Reagents

A. 0.5% Pepsin Solution

Pepsin	5.0 g
HCl	5.0 mL
Sterile distilled water	to 1000 mL
Store at 4°C.	

B. 0.5% Trypsin Solutions

0.5% Trypsin Solution	
EDTA	0.2 g
NaCl	8.0 g
KCl	0.2 g
KH_2PO4	0.2 g
NaHPO4	1.15 g
Trypsin	5.0 g
Sterile distilled water	to 1000 mL
Store at 4°C.	

0.5% Trypsin with Phenol Red	
NaCl (sodium chloride)	8.0 g
KCl (potassium chloride)	0.2 g
$C_6H_5Ha_30_7 \cdot 2H_20$ (sodium citrate, dihydrate)	1.0 g
$NaH_2PO_4 \cdot H_20$ (sodium phosphate, monohydrate)	0.05 g
$NaHCO_3$ (sodium bicarbonate)	1.0 g
Glucose	1.0 g
Phenol Red (0.5%)	1.0 mL
Distilled Water	1.0 L
Trypsin	5.0 g

C. 1N Sodium Hydroxide (NaOH)

NaOH	40.0 g
Distilled water	to 1000 mL

D. Phosphate Buffered Saline (PBS)

Sodium Chloride (NaCl)	8.0 g
Monopotassium phosphate (KH_2PO_4)	0.34 g
Dipotassium phosphate (K_2HPO_4)	1.22 g

Distilled water	to 1000 mL

Filter with 0.22 um filter. Store at room temperature.

E. Proteinase K

Can be obtained as a stable liquid solution (14 to 22 $mg.mL^{-1}$) from commercial sources. If kept at 4°C, it is stable for > one year.

F. RNAse A

It is a 10 $mg.mL^{-1}$ solution and can be obtained from commercial sources [e.g. 5'-3' (Cat. # is 5-888777)] as a 50% glycerol solution that is liquid at -20°C.

G. DNA Extraction Buffer

The buffer is NaCl 100 mM, Tris-HCl 10 mM, EDTA 25 mM, SDS 1%. The stock solutions are:

NaCl 5M	{50X}
Tris-HCl 1M, pH 7.8	{100X}
EDTA 0.5M, pH 8	{20X}
SDS 20%	{20X}

Stock solutions should be made using ultrapure, nuclease-free water (HPLC grade or equivalent), aliquoted and stored at -20°C until needed. Pre-made stock solutions (molecular biology grade) can be purchased from a commercial supplier, aliquoted and frozen, so the chances of contamination are reduced.

H. Loading Buffer

Sigma P-7206 Pre-made 6X concentrate, ready to use (store -20°C)

OR

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Glycerol	30.0%

I. 0.01 M Phosphate Buffer pH 6.8

Adjust pH of PBS recipe above to 6.8.

J. 10% Neutral Buffered Formalin

Formalin, concentrated	100 mL
Distilled water	900 mL
Sodium phosphate (monobasic)	4.0 g

Sodium phosphate (dibasic)	6.5 g

K. 2.5 mM Magnesium Chloride ($MgCl_2$)

It can be obtained from commercial sources and often is supplied with Taq enzyme. Purchase as molecular biology grade.

L. Leishman Stain

Purchase from commercial source.

M. Giemsa Stain

Stock solution:

Giemsa powder	1 g
Glycerin	66 mL

Mix and place in oven at 56 to 66˚C for 0.5 to 1 hour. Add 66 mL absolute methanol after solution has cooled.

Phosphate buffer, pH 6.0

Sodium phosphate (monobasic)	35 g
Sodium phosphate (dibasic)	4.84 g
Distilled water	to 4 L

Working Giemsa

Stock Giemsa	14 mL
Phosphate buffer	200 mL

Must be made fresh.

5.7 Glossary

A. Myxozoan Life Stages

Trophozoite - vegetative, uninucleate stage with a single diploid nucleus.

Presporogonic stages - multicellular parasite stages that will eventually give rise to spores. For purposes here, this encompasses stages referred to as plasmodia, sporoblasts and sporocysts.

Spore - myxospore is the mature spore that develops in the fish. It contains polar capsules, polar filaments and sporoplasm cell(s).

Sporogenesis - the process of spore formation.

B. Cestode Morphology

Scolex - portion of a cestode that bears the organs of attachment.

Bothria - organ of attachment; characteristically they are long narrow grooves of weak muscularity; may become flattened to form an efficient sucking organ.

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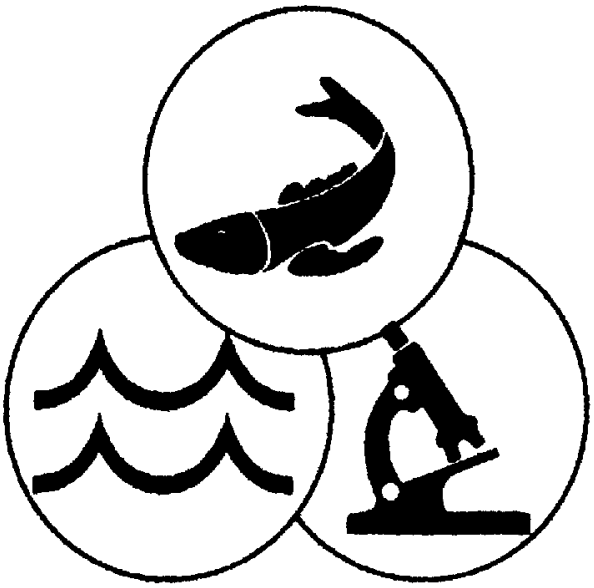
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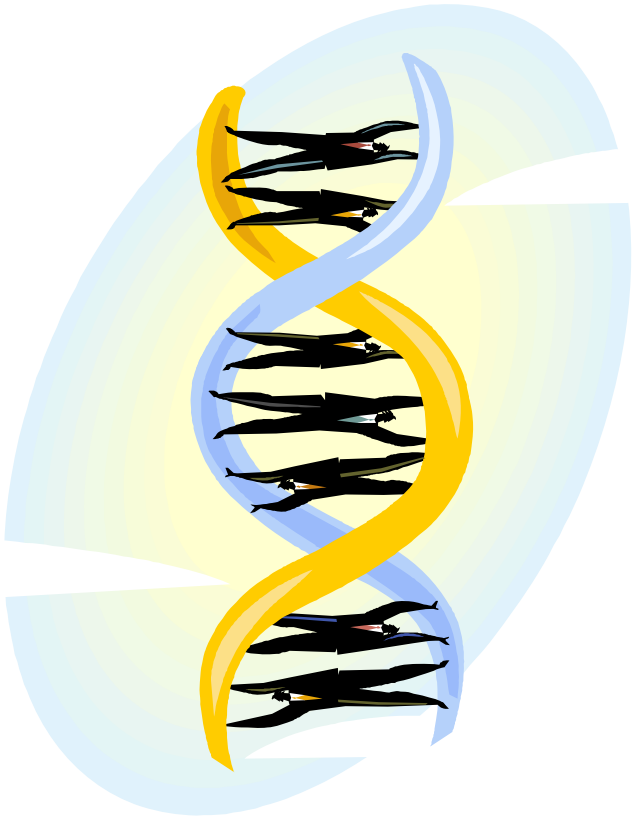
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Wolf, K., and M. E. Markiw. 1984. Biology contravenes taxonomy in the Myxozoa: new discoveries show alternation of invertebrate and vertebrate hosts. Science 225:1449-1452.



Section 2
USFWS/AFS-FHS Standard Procedures for Aquatic Animal Health Inspections





Chapter 6 Polymerase Chain Reaction (PCR)

6.1 Polymerase Chain Reaction (PCR) Introduction

6.2 PCR – Quality Assurance/Quality Control

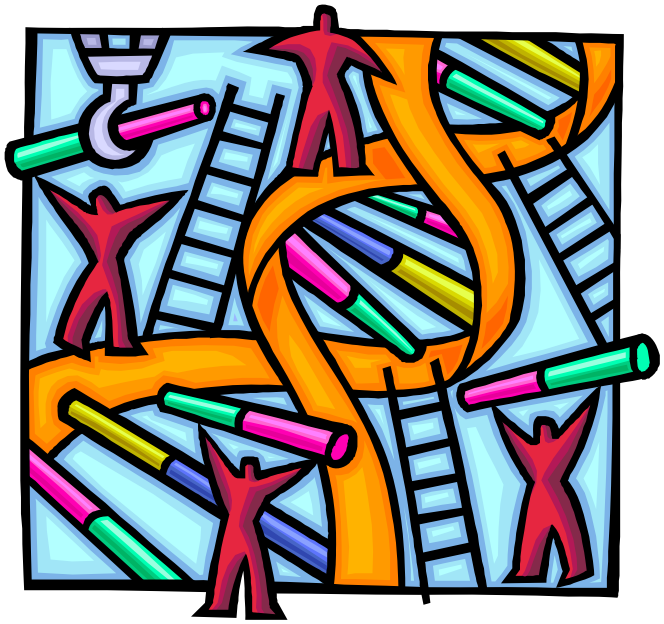
6.3 PCR Protocols

6.4 Reagents

6.5 Analysis of Extracted DNA Using an UV Spectrophotometer

6.6 Table of PCR Protocols

6.7 References



6.1 Polymerase Chain Reaction (PCR) Introduction

The polymerase chain reaction technique employs oligonucleotide primers to amplify segments of genes specific for the target pathogen. Reverse transcriptase-PCR (RT-PCR) employs an initial reverse transcription step so that complimentary DNA can be amplified from viral RNA. DNA or RNA is extracted from various fish tissues and laboratory assay products, such as cell culture supernatant containing viral agents, and amplified using forward and reverse primer sets. In some instances for either method, the initial amplified product may be re-amplified using an additional "nested PCR" technique. The DNA products are then visualized by gel electrophoresis.

Specific details for sample preparation, DNA/RNA extraction, primers and cycle conditions appear in each chapter under the specific pathogens. However, certain PCR protocols and precautions are pertinent to all assays and these are described in this chapter. The PCR quality assurance and control procedures outlined below are extremely important when performing all PCR assays (Ennis et al. 1990).

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U. S. Fish and Wildlife Service, the United States government, and/or the American Fisheries Society. Any comparable instrument, laboratory supply, or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.

6.2 PCR – Quality Assurance/Quality Control

A. General Considerations

1. Quality control is critical to all steps of the PCR process, beginning with collection of samples in the field. It is important that the person performing sample collection use the precautions outlined in Section 2, Chapter 2 Sampling to avoid cross-contamination.

2. Work surfaces should be decontaminated by washing with 10% chlorine (or commercial reagents like "DNA Away") to hydrolyze possible DNA contaminants. All sample racks and reusable equipment should be washed in DNA-away and autoclaved after use. Spray and/or wipe pipettors and working areas with DNA or RNAase -Away and turn UV on for at least 30 minutes after use (UV light damages DNA).

3. **Wear and change gloves often**. This helps prevent spread of amplified DNA or contamination of sample DNA with nucleases naturally occurring on the skin that will degrade the sample DNA. Always change to a fresh pair when leaving and entering PCR reagent mixing areas. Change gloves whenever contamination between samples is possible.

4. Employ aerosol resistant pipette tips and/or positive displacement pipettors during all extraction and amplification procedures. Separate pipettors should be dedicated for use with reagents only and another set for use with amplified products only.

5. Mix and aliquot pre-amplification ingredients under bench top UV cabinet and NEVER contaminate this area with sample material or amplified DNA product.

6. **One aerosol drop of PCR product may contain thousands of strands of DNA, which can easily contaminate reagents!** Therefore, three separate areas of lab space are necessary to reduce the risk of contamination.

    a. Master Mix (MM) Area with UV Hood
    For mixing and aliquotting master mix reagents. Supply area with dedicated pipetters, ideally positive displacement pipettor/tips. **No samples or amplified DNA is to be handled in or near this area!**

    b. Sample Loading Area with UV Hood and Dedicated Pipettor
    For loading of extracted (template) DNA from samples**.**

    c. Amplified DNA Area
    Supplied with pipettor dedicated for **amplified PCR product ONLY.** Handle any amplified PCR products in this area only, and clean area and equipment thoroughly with "DNA Away" type solutions after working with amplified DNA.

7. Provide separate storage areas for RNA and DNA samples, amplified DNA, and PCR reagents.

8. Controls

    a. Extraction controls
    A known positive tissue sample (or tissue spiked with target pathogen DNA) and a known negative tissue should be processed with the test samples to ensure that the DNA extraction was successful and contamination did not occur.

    b. PCR Controls
    Sterile water (negative) and the known positive DNA and negative controls from previous extraction (positive) will ensure that the PCR process was successful and that contamination did not occur.

    c. Internal Controls
    An internal control DNA, such as ß-actin (commercially available), should be used to verify PCR performance. If the internal control band is not produced, there may be inhibition of the PCR reaction. Therefore, the Taq DNA polymerase, storage conditions of reagents, and thermal cycler performance are potential variables to investigate.

9. Enhancing PCR specificity
    a. Uracil N-glycosylase (UNG) and dUTP can be used in PCR amplifications to prevent false positives due to contamination with carry-over PCR products generated in previous reactions. (Commercial kits are available, such as GeneAmp® PCR Carry-over Prevention Kit from Applied Biosystems.) In PCR reactions, dUTP is substituted for dTTP so the resultant PCR products contain uracil instead of thymidine. These substituted products are susceptible to degredation by UNG, thus, the products from previous PCR amplifications can eliminated from subsequent reactions by incubation with UNG which excises the uracil residues. The resulting abasic polynucleotide is destroyed by a 95ºC heat treatment at the start of the thermocycler program.

10. Newly received primers should be tested on known positive/negative controls prior to use.

11. Dispose of trash containing amplified DNA products frequently.

B. Sample Processing

1. Tissue samples should be collected on a clean bench top, which has been disinfected using a 10% chlorine (or "DNA Away") solution if possible. If collected in the field, use a disposable work surface between each lot of tissue collected (paper towel, foil, etc.).

2. Use sterile collection utensils between each lot of fish tissue collected. If data from individuals is of concern, use separate utensils for each individual. **Alcohol will not effectively decontaminate DNA from utensils.** If individual utensils are not available, flaming metal utensils between samples will effectively remove contaminants from previous samples.

3. Keep samples cold and freeze as soon as possible at or below -20^{o}C until processing can be accomplished.

4. RNA is extremely sensitive to enzymes present in sample tissues. Samples collected for RT-PCR should be frozen immediately and transported on dry ice. An RNA stabilizing buffer can also be used and does not require that samples be frozen immediately.

C. Extraction of DNA or RNA from Samples

Individual protocols will vary in specific steps for extraction of genetic material; however, the following general considerations should be employed:

1. Use micro centrifuge tubes with locking or screw-cap lids. Heating of extraction solutions causes unlocked caps to pop open, releasing aerosols that can cause cross-contamination between samples and controls. Pulse spin in the microcentrifuge before opening DNA sample tubes so that the lids are dry before opening them. This will help in preventing cross-contamination.

2. Use the accurate amount of tissue suggested by the extraction kit manufacturers. If this is exceeded, proper lysis of tissues will not be accomplished.

3. Always run positive control samples as well as negative (water and negative tissue samples) from the start of the extraction process, through amplification to electrophoresis. These controls will allow for detection of contamination as well as assure that the extraction was successful. This is the only means of assuring validity of the assay and its results.

4. **Quantification of DNA**
   If the protocol being used advises that extracted products be measured using a spectrophotometer to ensure that enough DNA or RNA was successfully extracted, refer to quantification guidelines in Section 2, 6.5 Analysis of Extracted DNA using an UV Spectrophotometer.

D. Interpretation of PCR Results

Use of the appropriate controls should allow you to assess the integrity of your PCR result.

1. False-negative reactions may result from insufficient DNA extraction, excessive amounts of DNA, PCR inhibition, improper optimization of the PCR, or human error (e.g. loading errors).

2. False-positive reactions may result from contamination either directly from the sample lot being tested or from previously amplified target DNA.

3. For further help in troubleshooting, see PCR Protocols: A Guide to Methods and Applications (Ennis et al. 1990).

6.3 PCR Protocols

A. Preparation of Amplification Reaction Mixture

Specific amplification protocols may require one or two amplification reactions.

Note: samples and reagents should be kept cold either on ice or in a frozen cryo-rack during all assembly procedures.

1. Using Section 2, 3.A3.A Worksheet A – PCR Sample Data/Log Sheet or Section 2, 4.A1.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.

2. Under a UV cabinet, prepare "Master Mix" (MM) using pathogen-specific protocols in the worksheets in each chapter. Calculate the amount of each reagent to go into the MM according to the number of samples to be processed. Add PCR reagents, **except for sample DNA,** in the order listed on the worksheet, adding water first and Taq polymerase last. Keep all reagents cold in frozen cryo-rack or on ice during mixing and return them to freezer immediately after use.

   Note: Prepare enough MM for three more samples than actually being tested to compensate for retention of solution in pipette tips and tube.

3. Place specified volume of MM into each PCR tube. Close caps tightly. Move PCR tubes to sample loading area.

4. In the sample preparation area, load specified volume of each sample DNA to the appropriately labeled PCR tubes. To avoid cross contamination, always change tips between samples and avoid touching the sides of the tube. Close caps tightly.

B. Running the PCR

All general considerations should be employed including the following:

1. Load the sample tubes into the wells (follow manufacturer's recommendations).

2. Program thermocycler for appropriate cycle conditions and run reaction.

3. Before loading into thermocycler, give tubes a "quick-spin" to ensure that all reagents and sample are drawn down from sides of tube.

4. Thermocycler should be programmed for the specific PCR condition used for each pathogen (details under pathogen).

5. After cycling, tubes may have a ring of condensation near rim of cap. Before opening tubes,

perform a "quick-spin" to draw this fluid down into the reaction area of the tube and reduce the possibility of aerosol contamination upon opening tubes.

6. PCR products can be refrigerated for up to a month following amplification (or for longer storage they may be frozen at -20˚C).

C. Detection of Product

Procedures for preparing the gel (refer to specific manufacturer's guidelines for preparation of gels and electrophoresis chambers):

1. Assemble the gel tray and position well comb in the tray according to manufacturer's recommendations.

2. Prepare 1 X TAE (Section 2, 6.4 Reagents) buffer with distilled water to volume adequate for gel and running buffer.

3. Prepare 1.5 to 2% agarose gel according to the volume recommended for specific gel forms used.

   a. Weigh appropriate amount of agarose and add to proper volume 1X TAE buffer.

   b. Heat solution to near boiling until agarose is completely dissolved.

   c. Allow solution to cool to about 65^{o}C, then pour agarose solution into gel tray. Avoid the formation of bubbles.

   d. Allow gel to cool completely for about 30 minutes and then carefully remove the comb.

   e. Position the gel into the chamber with the sample end (wells) positioned closest to the negative (black) electrode.

   f. Slowly fill the chamber with the remaining 1 X TAE buffer solution until the top of the gel surface is submerged.

4. Load samples into wells as indicated for each assay

   a. For each tube of PCR product to be visualized, mix 2 µL of gel loading dye (Section 2, 6.4 Reagents) to every 10 µL of PCR product needed to fill each well formed in the gel. Mix the sample and the dye by repeated expulsion prior to loading.

   b. When the sample and the dye are adequately mixed, carefully place the pipette tip containing the mixture over an individual well of the agarose gel, and load the well with the sample. Repeat this procedure for all the wells, being sure to include the DNA molecular weight standard (one with bands at 100 bp increments in the 100 to 1,000 bp range) for base pair (bp) reference and positive and negative controls

   c. Document gel lane assignments for each sample and control on Section 2, 3.A3.B Worksheet B – Amplification of Nucleic Acid by PCR or Section 2, 4.A1.B Worksheet B –

Amplification of Nucleic Acid by PCR and allow for at least one lane for a DNA ladder reference.

D. Electrophoresis

Approximately 80 volts for 35 minutes or until tracking dye front approaches the edge of the gel (this is dependent on gel width, so refer to manufacturer's recommendations).

E. Staining the Gel

Remove gel and tray and place in ethidium bromide (EtBr) solution (Section 2, 6.4 Reagents) for 15 to 20 minutes.

Note: EtBr solution can be reused and stored in a dark plastic tray container with a secure lid. **EtBr is very toxic** and binds with all DNA (including yours); follow appropriate manufacturer warnings! For safe proper disposal of expired EthBr solutions see Sambrook et al. or check with your local biotech supply retailer for specific products designed to remove EthBr from solution for disposal.

F. De-Staining the Gel

In water for 5 to 60 minutes. De-stain water should be handled and disposed of appropriately (see Section 2, 6.3.E "Staining the Gel").

G. Visualize the DNA

1. Place gel on a UV light source and carefully record locations of bands on positive control samples in relation to the DNA molecular weight standard. Band locations of positive controls should be at anticipated locations according to primers used.

   Note: use UV protective goggles or face shield.

2. Note any unusual band occurrences. Negative controls should not have any bands. Contamination suspicions indicate the samples should be re-run from template DNA tube.

3. Photo document all PCR gels and attach to Section 2, 3.A3.G Worksheet G – Photodocumentation of PCR Product Gel or Section 2, 4.A1.C Worksheet C – Photodocumentation of PCR Product Gel (or provide reference for finding the photo documentation).

6.4 Reagents

REAGENT:	REFERENCE:	DIRECTIONS TO PREPARE:
TAE BUFFER 10X	Sigma T-4035	Comes in prepared packets, add DI water and qs to 1L . Label as 10X- STOCK (store at RT). Also can prepare a 50X stock (see Sambrook et al.).
TAE BUFFER 1X WORKING SOLUTION	TAE-1X	Diluted 1:10 from 10X stock. Label as TAE - 1X (store at RT).
PCR LOADING BUFFER -OR- LOADING DYE	Sigma P-7206 LOADING DYE 6X	Pre-made 6X concentrate, ready to use (store -20˚C). Prepare in-house (per Sambrook et al. (store at 4˚C): Bromophenol blue 0.25%, Xylene cyanol 0.25%, Glycerol 30.0 %

ETHIDIUM BROMIDE - Recommend buying EtBr already in solution to minimize working with this hazardous compound, or it can be prepared as follows:

Note: USE CAUTION IN PREPARING EtBr SOLUTIONS: Follow all MSDS precautions. Wear gloves, avoid all contact with skin, eyes, and respiratory system. LABEL ALL BOTTLES WITH "CHEMICAL CARGINOGEN."

EtBr STOCK SOLUTION - 10mg/mL	Ethidium Bromide DI water	100 mg 10 mL Label as EtBr STOCK (10mg/mL). Protect from light (store at RT).
EtBr-WORKING SOLTION - 4.0ug/mL* WORKING STAIN SOLUTION		Add 200 µL STOCK SOLUTION to 500mL water Label EtBr - Working Solution (store at RT).

*References may suggest weaker working solutions (0.5 µg/mL) and staining periods of 45 to 60 minutes.

6.5 Analysis of Extracted DNA Using an UV Spectrophotometer

DNA quantification is achieved routinely with the use of spectrophotometry. The 230, 260, and 280 wavelengths provide the readings for quantification and purity and the 320 wavelength provides a reading for background compensation.

Standard quantitative conversion factors for nucleic acids are as follows:

1 absorbency unit at 260 nm of ds DNA = 50 ng/µL
1 absorbency unit at 260 nm of ss DNA = 33 ng/µL
1 absorbency unit at 260 nm of ss RNA = 40 ng/µL

Absorbance values for the 260 nm readings need to be in the linear range (between 0.1 and 1) for quantitation to be valid. If the absorbance at 260 nm (A260) is above 1.0 the sample needs to be diluted. If the sample is below 0.1 there is negligible DNA in the sample.

Relative purity of the DNA sample can be determined by the 260/A280 ratio. If the sample is pure nucleic acid, the ratio should be approximately 1.9. Excess protein in the sample will raise the absorbance at 280 nm thereby reducing the A260/A280 ratio.

One inexpensive instrument for this purpose is the GeneQuant II (Pharmacia Biotech) although other makes and models are available. The GeneQuant II is a spectrophotometer specific for obtaining concentrations of either double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), or RNA in units of weight, molar fraction, moles of phosphate, and total molecules. The instrument is capable of measuring the RNA or DNA using UV wavelengths at 230nm, 260 nm, 280 nm, and 320 nm simultaneously. If a standard spectrophotometer is all that is available, then conversion to pmoles /µL (i.e. uM) from ng/µL can be accomplished with the following table.

Amount of primer (ng) needed to equal 10 pmol:

Primer Length	ng of Primer Equal to 10 pmol
15	50
16	53
17	56
18	59
19	63
20	66
23	78
24	80

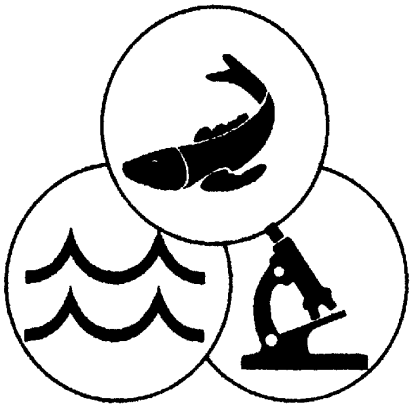
Pathogen	round	product size (amplicon)	predwell	jumpstart	cycles	denature	anneal	elongate	postdwell	hold
C. shasta		640 bp	---	95°C/3min	35	94°C/60sec	58°C/30sec	72°C/60sec	72°C/10min	4°C
IHNV	1	786 bp	50°C/15min	95°C/2min	25	95°C/30sec	50°C/30sec	72°C/60sec	72°C/7min	4°C
	2	323 bp	---	95°C/2min	25	95°C/30sec	50°C/30sec	72°C/60sec	72°C/7min	4°C
IPNV		174 bp	50°C/15min	95°C/2min	35	95°C/30sec	50°C/30sec	72°C/60sec	72°C/7min	4°C
ISAV		493 bp	42°C/15min	94°C/5min	40	94°C/45sec	59°C/45sec	72°C/105sec	72°C/7min	4°C
LMBV		248 bp	20°C/5min	94°C/3min	35	95°C/45sec	60°C/45sec	72°C/60sec	72°C/7min	4°C
M. cer		508 bp	---	94°C/2min	35	95°C/60sec	65°C/150sec	72°C/90sec	---	---
OMV		439 bp	---	---	30	94°C/30sec	56°C/30sec	72°C/30sec	---	4°C
P. sal	1	1540 bp	---	94°C/2min	35	94°C/60sec	50°C/120sec	72°C/180sec	---	4-16°C
	2	476 bp	---	94°C/2min	35	94°C/60sec	65°C/120sec	72°C/180sec	---	4-16°C
R. sal	1	383 bp	---	94°C/2min	10-20	93°C/30sec	60°C/30sec	72°C/60sec	---	4°C
	2	320 bp	---	95°C/5min	10-20	93°C/30sec	60°C/30sec	72°C/60sec	---	4°C
SVCV	1	714 bp	---	---	35	95°C/60sec	55°C/60sec	72°C/60sec	72°C/10min	4-16°C
	2	606 bp	---	---	35	95°C/60sec	55°C/60sec	72°C/60sec	72°C/10min	4-16°C
VHSV	1	950 bp	50°C/15min	95°C/2min	25	95°C/30sec	50°C/30sec	72°C/60sec	72°C/7min	4°C
	2	558 bp	---	95°C/2min	25	95°C/30sec	50°C/30sec	72°C/60sec	72°C/7min	4°C

6.7 References

Ennis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White, editors. 1990. PCR protocols: a guide to methods and applications. Academic Press, Inc.

Sambrook J., E. F. Fritsch, and T. Maniatis. 1987. Molecular cloning: a laboratory manual. 2nd Edition. Cold Springs Harbor Laboratory Press. Plainview, New York.

Section 2
USFWS/AFS-FHS Standard Procedures for Aquatic Animal Health Inspections



Appendix 1 The Handbook and Oversight Committee

A1.1 Format of the Handbook
A1.2 Handbook Revision and Oversight Committee
A1.3 Process for Amending the Handbook

Appendix 2 Handbook Committee Members

A2.1 2000 – 2002 Handbook Development Committee
A2.2 2002 – 2003 Handbook Development Committee
A2.3 2003 – 2004 Handbook Development Committee
A2.4 2004 – 2005 Handbook Development Committee
A2.5 2006 – 2007 Handbook Development Committee

Appendix 3 Position Statements

A3.1 Position Statements Introduction
A3.2 Sampling Position Statements
A3.3 Bacteriology Position Statements
A3.4 Virology Position Statements
A3.5 Parasitology Position Statements
A3.6 Polymerase Chain Reaction (PCR) Position Statements
A3.7 Appendix 1 The Handbook and Oversight Committee Position Statements

A1.1 Format of the Handbook

A. Title

1. The document itself shall be titled "Standard Procedures for Aquatic Animal Health Inspections."

B. Chapters

There shall be six chapters plus any necessary appendices.

1. **Chapter 1 Introduction**
   This chapter will contain introductory information and explanation about this handbook.

2. **Chapter 2 Sampling**
   This chapter will contain information about sampling animals for a health inspection.

3. **Chapter 3 Bacteriology**
   This chapter will contain information on sampling, testing, and corroborating samples for bacterial pathogen inspection.

4. **Chapter 4 Virology**
   This chapter will contain information on sampling, testing, and corroborating samples for viral pathogen inspection.

5. **Chapter 5 Parasitology**
   This chapter will contain information on sampling, testing, and corroborating samples for parasitic pathogen inspection.

6. **Chapter 6 Polymerase Chain Reaction**
   This chapter shall contain general protocols and quality assurance and quality control information for utilizing polymerase chain reaction methodologies.

7. **Appendices**

   a. Appendix 1 The Handbook and Oversight Committee
      This appendix will contain the format information for the handbook and procedural information for making changes to this handbook.

   b. Appendix 2 Handbook Committee and Subcommittee Members

      i. This appendix will keep a record of those individuals serving on any committee or subcommittee, their terms of appointment, and their position on the committee.

ii. The construction of this appendix shall be as follows:

1. Each subchapter of this appendix will represent one year.

2. Each committee member will be listed as a section within the subchapter of the appendix.

3. Their position on the committee will then be listed as an item under their section.

4. Subcommittee members will be listed as items under the committee member that chairs the subcommittee on which they serve.

5. Entries will include the name, title, and affilitiaion of the individual.

c. Appendix 3 Position Statements
This appendix will keep a record of all discussions by those individuals serving on any committee or subcommittee, their terms of appointment and their position on the committee. The construction of this appendix will be as follows:

i. Each subchapter of this appendix will represent one chapter in the handbook.

1. Introduction will be Appendix 3.1.

2. Sampling will be Appendix 3.2.

3. Bacteriology will be Appendix 3.3.

4. Virology will be Appendix 3.4.

5. Parasitology will be Appendix 3.5.

6. Polymerase Chain Reaction (PCR) will be Appendix 3.6.

7. Appendix 1 changes will be documented in Appendix 3.7.

ii. Each submission will be a section within the appropriate subchapter of the appendix.

iii. Each position statement and the resultant changes will then be listed as items under their section.

C. Layout

The layout of the document will be as follows:

1. The format will be in outline form.

a. The alphanumeric system will be: 1.2, **A**, 1, a, i, *1*, *a, i*

b. Appendices will be also be in outline format with an “A” being placed before the appendix number.

i. Appendix 3 of the entire document would be titled and referenced as (A3).

ii. Appendix 2 of chapter 3 would be titled and referenced as (3.A2).

2. Chapter Structure

a. Each chapter will have its own references.

b. Each chapter will have its own appendices.

c. Chapters 1 (Introduction), 2 (Sampling), 6 (Polymerase Chain Reaction (PCR)) and the appendices are to serve as references for the other chapters.

3. References to other sections within the handbook will be in the same alphanumeric form as the outline.

a. **Example:** Reference (1.2.C.3.a) will be found in chapter 1, subchapter 2, section C, number 3, item a.

D. Stringency

1. The appropriateness of methodologies shall be determined based on several factors to make these protocols realistic to accomplish.

a. The sensitivity of the assay.

b. The specificity of the assay.

c. The cost of the assay.

d. Availability of reagents.

e. Availability of technology.

f. Manpower requirements.

2. All methods and protocols provided in this handbook shall be referenced and scientifically defensible.

3. The incorporation of additional pathogens into this handbook shall be contingent on the availability of appropriate screening and COROBERATIVE tests, and because such tests are commonly required by fish health regulators.

E. Maintenance of the Handbook

1. **Handbook Revision and Oversight Committee**

    a. Annual review as requested by open invitation (see process below).

    b. Physical possession of the electronic editable version of the handbook in Microsoft Word 2000 format and Adobe PDF format.

        i. This shall be held by four individuals as follows:

            1. Handbook Revision and Oversight Committee Co-chair, United States Fish and Wildlife Service Representative.

            2. Handbook Revision and Oversight Committee Co-chair, American Fisheries Society – Fish Health Section Representative.

            3. United States Fish and Wildlife Service National Fish Health Coordinator or their duly appointed representative.

            4. Chairman of the American Fisheries Society – Fish Health Section or their duly appointed representative.

    c. Maintain an appendix of position statements to keep track of discussions so as not to revisit issues that have been previously decided when no new information has come available.

        i. Changes to the handbook shall be recorded as well, so the specifics of any given change can be examined in the future.

        ii. Dissenting opinions shall be included in the position statements as well.

F. Distribution of the Handbook

1. New versions of the handbook shall be made available by June of each year.

2. Electronic Distribution (Non-Editable) – PDF Format

    a. CD Rom distribution of current version.

    b. Web site

        i. Initial distribution.

        ii. Yearly updates.

A1.2 Handbook Revision and Oversight Committee

A. Committee Construction

1. The handbook revision and oversight committee shall consist of ten people.

    a. Three (3) from the U. S. Fish and Wildlife Service.

    b. Three (3) from the American Fisheries Society – Fish Health Section.

    c. United States Fish and Wildlife Service National Aquatic Animal Health Coordinator or their duly appointed representative (Ex-Officio).

    d. Chairman of the American Fisheries Society – Fish Health Section or their duly appointed representative (Ex-Officio).

    e. National Oceanic and Atmospheric Administration - Fisheries Service National Aquatic Animal Health Coordinator or their duly appointed representative (Ex-Officio).

    f. United States Department of Agriculture - Animal and Plant Health Inspection Service Aquaculture Program Manager or their duly appointed representative (Ex-Officio).

2. The committee will be co-chaired by one non ex-officio individual from USFWS and one non ex-officio individual AFS-FHS.

3. The terms of the non ex-officio committee members shall be three consecutive years.

    a. Terms shall begin two months prior to the individual's first annual meeting (July).

    b. Terms shall conclude 10 months after the individual's third consecutive annual meeting (July).

    c. An individual may not serve on the committee again once their three-year term has expired, unless a period of two years has elapsed.

4. Each non ex-officio committee member will rotate through the committee as follows.

    a. Year one – in-coming member.

    b. Year two – co-chair.

c. Year three – outgoing member.

d. This rotation will allow for continuity from year to year.

5. New committee members shall be invited to join the committee by the incoming committee co-chairs ninety (90) days prior to the annual meeting (June).

B. Committee Function

1. The Handbook Revision and Oversight Committee for each year shall hold its initial meeting in September.

2. At least 2/3 of the committee must be present to make official decisions.

3. The Handbook Revision and Oversight Committee shall address all requests for revision of the handbook.

4. The Handbook Revision and Oversight Committee shall keep the handbook current and shall update all distributables (CD-ROM, web page, and print version) at least once a year, no later than 30 days after final have been considered and adopted or declined (June).

5. The Handbook Revision and Oversight Committee shall maintain possession of the editable copies of the handbook.

6. The Handbook Revision and Oversight Committee shall oversee the distribution of the handbook.

A1.3 Process for Amending the Handbook

A. There may be occasions when new discoveries or editorial errors require immediate changes in the handbook. Suggestions for such changes are submitted to the co-chairs of the Revision and Oversight Committee, the President of the AFS, or the USFWS National Fish Health Coordinator. If the Committee co-chairs find that the suggestion clearly indicates that a current Handbook protocol may not produce an accurate result, the co-chairs will convene a meeting of the full committee at the soonest possible date and determine if an immediate correction is warranted. If a change is warranted, the co-chairs will coordinate the revision of the text and have it approved by the full committee. The corrected text will be provided to the USFWS; notification of the change will be included in the FHS newsletter; FHS members holding a current Blue Book subscription will be notified of the change by mail; and the changed text will be provided on the FHS web site until release of the next edition of the Blue Book. If initial review of a suggested change is not deemed urgent by the co-chairs or the Revision and Oversight Committee, it will be deferred and included in the normal revision process detailed below.

B. At least sixty (60) days prior to their annual meeting (July), the Revision and Oversight Committee shall, through the new committee co-chairs, send out a notice to all USFWS Fish Health Center Project leaders and shall publish a notice in the AFS-FHS newsletter with the date of the annual review of the inspection handbook. Additionally, comments and concerns should be solicited at this time for discussion at the meeting. Individuals should be encouraged to include any new information that may need to be considered when evaluating their comments and concerns.

C. Written requests for changes in the document or for discussion of concerns shall be submitted no later than thirty (30) days prior to the annual meeting (August) of the Revision and Oversight Committee. The requests and comments shall be submitted to the committee co-chairs who will then distribute them to the other committee members for their consideration prior to the meeting.

D. Committee members shall search the appendix for related issues or position statements that have addressed these concerns or similar concerns. This shall be done to aid in determining the need to address a particular query due to changes in available information.

E. At the annual meeting, the committee will hold discussions on each query to determine whether or not the issue merits further review.

1. If the committee calls for no further review, the committee shall write a position statement to be incorporated with the query and added to Appendix 3. Additionally, the person submitting the query shall also receive a copy of the position statement.

2. Should the committee decide the matter warrants further discussion a subcommittee shall be formed.

a. Subcommittees shall be formed with regard to the chapter under review (i.e. Bacteriology, Virology, and Parasitology), this will ensure consistency within any chapter. Subcommittees formed to deal with issues within Chapter 1, Chapter 2, Chapter 6, or Appendix 1 should focus on how changes will affect all sections of the handbook as these relate to all the other chapters.

b. A member of the Revision and Oversight Committee shall chair each subcommittee.

c. Each subcommittee will address all the queries regarding their chapter moved forward by the Revision and Oversight Committee.

d. The subcommittees shall be composed of 6 to 12 members.

   i. Members are drawn from both the USFWS and the AFS-FHS.

   ii. Equal representation is not required on subcommittees; however, there must be at least two representatives of each organization on each subcommittee.

   iii. The subcommittee chair will invite individuals to participate on the subcommittee.

   iv. The invitees shall be experts or have extensive experience in the discipline being discussed.

   v. The invitees shall represent a diverse area of the country and have a broad species interest, to ensure equal consideration of all potential culture situations.

F. The subcommittee chair shall assemble the subcommittee, provide them with the queries to be addressed and any other material needed and schedule a meeting within 30 days of the annual meeting (October).

1. At least 2/3 of the subcommittee must be present to make official decisions.

2. The subcommittees shall determine again if the query needs to be addressed.

   a. If it is determined by the subcommittee that the issue does not warrant action:

      i. The subcommittee shall write a position statement explaining why this decision was made and it will be submitted to the Revision and Oversight Committee for inclusion in Appendix 3.

      ii. The submitter of the query will also be provided a copy of the position from the subcommittee.

   b. If the subcommittee finds the query needs to be addressed they shall:

      i. Determine the most appropriate changes to be made to the document.

ii. Submit line-by-line recommendations in a position statement, which is delivered to the Revision and Oversight Committee within ninety (90) days of the subcommittee's first meeting (January).

F. At this time, the Revision and Oversight Committee shall have thirty (30) days (Febuary) to review these changes and determine if they fit with the other parts of the handbook and are in-line with the purpose and intent of the handbook.

1. If the committee accepts them:

   a. The Revision and Oversight Committee co-chairs make the changes to the handbook.

   b. The changes as well as the subcommittee position statement are added to Appendix 3.

   c. The individual submitting the query shall receive a copy of the position statement and notice of the subsequent changes to the handbook.

2. If the Revision and Oversight Committee rejects the suggested changes, they shall supply a written position statement for these actions to the subcommittee chair, who in turn shall provide it to the subcommittee members.

G. The subcommittee shall have an additional sixty (60) days (April) to:

1. Resubmit their changes by submitting a new position statement to the Revision and Oversight Committee through the subcommittee chair.

2. Drop the changes and issue a new position statement for inclusion in Appendix 3.

H. The revision and oversight committee shall have thirty (30) days (May) to re-review these changes.

1. If the new submittal is accepted by the Revision and Oversight Committee, they shall:

   a. Write a position statement.

   b. Make the changes to the handbook.

   c. Add the changes well as all position statements to Appendix 3.

   d. Supply the query submitter a copy of both the position statement and the subsequent changes to the handbook.

2. If the submission is once again rejected, the Revision and Oversight Committee shall:

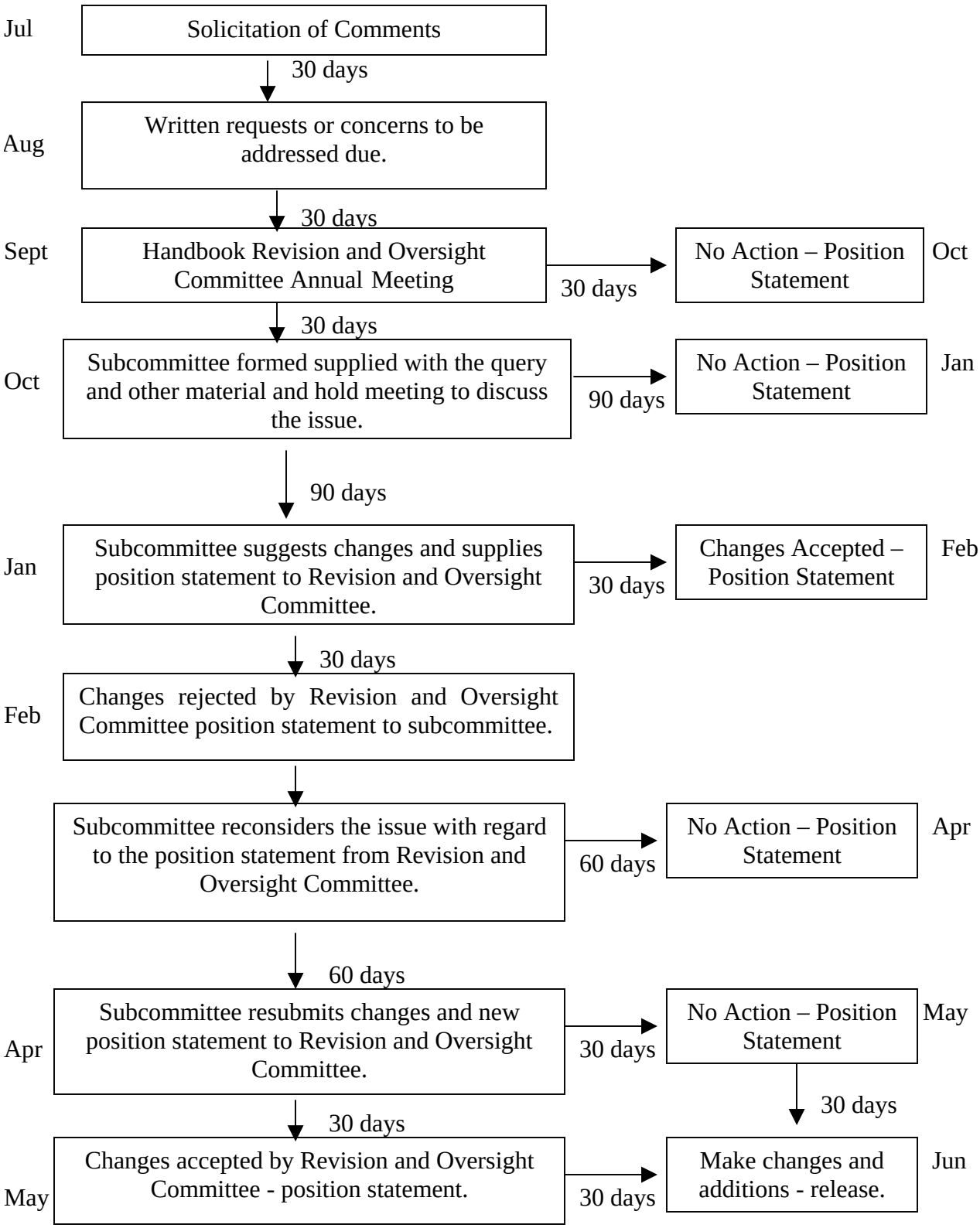
   a. Provide a position statement, which shall be included in Appendix 3 and a copy provided to the individual that made the request.

b. The request will then be considered closed and will only be reconsidered if re-submitted in the future.

I. Position statements may be written by any member of the subcommittee or Revision and Oversight Committee and signed off on by all committee members before being added to Appendix 3. It is essential that all opinions, including those dissenting from the decision, be included in the position statement.

J. The Revision and Oversight Committee shall have thirty (30) days (June) to incorporate all accepted changes, add appropriate information to Appendix 2, add the position statements to Appendix 3, and release the new version of the document.

Figure 1. Flow chart of procedures to handle requests to change the handbook.



A2.1 2000 – 2002 Handbook Development Committee

A. Dr. Robert S. Bakal

USFWS, Warm Springs Fish Health Center, Co-Chair, General Editor

B. Dr. Jerri Bartholomew

Oregon State University, Co-Chair, Fish Health Section President (Ex-Officio) Parasitology Subcommittee Chair

1. **Dr. Karl Andree**
   University of California-Davis, Dept. Veterinary Medicine and Epidemiology

2. **Dr. Andrew Goodwin**
   Aquaculture/Fisheries Center, University of Arkansas at Pine Bluff

3. **Ms. Kimberly True**
   USFWS, California – Nevada Fish Health Center

4. **Ms. Linda Vannest**
   USFWS, Bozeman Fish Health Center

5. **Dr. Becky Lasee**
   USFWS, La Crosse Fish Health Center

6. **Dr. Linda Chittum**
   Utah Division of Wildlife Resources (Served as subcommittee chair until needing to step down.)

7. **Ms. Elizabeth MacConnell**
   USFWS, Bozeman Fish Health Center

C. Ms. Patricia Barbash

USFWS, Lamar Fish Health Center, Bacteriology Subcommittee Chair

1. **Dr. Marilyn Blair**
   USFWS, Idaho Fish Health Center

2. **Dr. Larry Hanson**
   College of Veterinary Medicine, Mississippi State University

3. **Mr. Phillip Hines**
   USFWS, Pinetop Fish Health Center

4. **Mr. John Hnath**
   Michigan Department of Natural Resources

5. **Ms. Susan Marcquenski**
   Wisconsin Department of Natural Resources

6. **Mr. David Money**
   Wyoming Game and Fish, University of Wyoming

7. **Mr. Ronald Pascho**
   USGS, Western Fisheries Research Center

8. **Mr. Eric Pelton**
   USFWS, Lower Columbia Fish Health Center

9. **Dr. Clifford Starliper**
   USGS, National Fish Health Research Laboratory

10. **Dr. Chris Wilson**
    Utah Division of Wildlife Resources

11. **Dr. Doug Eib**
    New Mexico Department of Natural Resources

D. Dr. Joy Evered

USFWS – Olympia Fish Health Center, Virology Subcommittee Chair

1. **Dr. Kevin Amos**
   NMFS, National Seafood Inspection Laboratory and Washington State Department of Fish and Wildlife

2. **Mr. William Batts**
   USGS, Western Fisheries Research Center

3. **Dr. Scott LaPatra**
   Clear Springs Foods, Inc.

4. **Mr. Ken Peters**
   USFWS, Bozeman Fish Health Center

5. **Dr. John Plumb**
   Department of Fisheries and Allied Aquaculture, Auburn University

6. **Mr. Bruce Stewart**
   Northwest Indian Fisheries Commission

7. **Dr. Jim Winton**
   USGS, Western Fisheries Research Center

E. Dr. Andrew Goodwin

Aquaculture/Fisheries Center, University of Arkansas at Pine Bluff

F. Dr. Chris Wilson

Utah Division of Wildlife Resources

G. Dr. Tom Bell

USFWS - National Fish Health Coordinator (Ex-Officio)

A2.2 2002 – 2003 Handbook Development Committee

A. Dr. Joy Evered

USFWS – Olympia Fish Health Center, Co-Chair, Virology Subcommittee Chair

1. **Dr. Kevin Amos**
   NMFS, National Seafood Inspection Laboratory and Washington State Department of Fish and Wildlife

2. **Dr. Robert S. Bakal**
   USFWS, Warm Springs Fish Health Center

3. **Mr. William Batts**
   USGS, Western Fisheries Research Center

4. **Dr. Andrew Goodwin**
   Aquaculture/Fisheries Center, University of Arkansas at Pine Bluff

5. **Dr. Scott LaPatra**
   Clear Springs Foods, Inc.

6. **Mr. Ken Peters**
   USFWS, Bozeman Fish Health Center

7. **Dr. Jim Winton**
   USGS, Western Fisheries Research Center

B. Dr. Chris Wilson

Utah Division of Wildlife Resources, Co-Chair, General Editor

C. Dr. Andrew Goodwin

Aquaculture/Fisheries Center, University of Arkansas at Pine Bluff, Sampling Chapter Subcommittee Chair

1. **Dr. Kevin Amos**
   NMFS, National Seafood Inspection Laboratory and Washington State Department of Fish and Wildlife

2. **Dr. Robert S. Bakal**
   USFWS, Warm Springs Fish Health Center

3. **Ms. Patricia Barbas**
   USFWS, Lamar Fish Health Center

4. **Dr. John Grizzle**
   Professor, Dept. of Fisheries and Allied Aquacultures, Auburn University

5. **Ms. Crystal Hudson**
   USFWS, Bozeman Fish Health Center

6. **Mr. Joe Marcino**
   Minnesota Department of Natural Resources

7. **Dr. Chris Wilson**
   Utah Division of Wildlife Resources

D. Dr. Jerri Bartholomew

Center for Fish Disease Research, Oregon State University, Parasitology Subcommittee Chair

1. **Ms. Elizabeth MacConnell**
   USFWS, Bozeman Fish Health Center

2. **Dr. Danielle R. Stanek**
   Washington Animal Disease Diagnostic Laboratory, Washington State University

3. **Ms. Linda Vannest**
   USFWS, Bozeman Fish Health Center

4. **Dr. Chris Wilson**
   Utah Division of Wildlife Resources

5. **Dr. Scott Foott**
   USFWS, California / Nevada Fish Health Center

6. **Dr. Michael Kent**
   Center for Fish Disease Research, Oregon State University

E. Dr. Robert S. Bakal

USFWS - Warm Springs Fish Health Center, Appendix Subcommittee Chair

F. Ms. Becky Lasse

USFWS - La Crosse Fish Health Center, Bacteriology Subcommittee Chair

1. **Dr. Mohamed Faisal**
   Michigan State University

2. **Mr. Phil Hines**
   USFWS, Pinetop Fish Health Center

3. **Dr. Marcia House**
   Northwest Indian Fisheries Commission

4. **Ms. Crystal Hudson**
   USFWS, Bozeman Fish Health Center

5. **Dr. Hui Min She**
   Wisconsin Veterinary Diagnostic Laboratory

6. **Dr. Clifford Starliper**
   USGS, National Fish Health Research Laboratory

G. Dr. Tom Bell

USFWS - National Fish Health Coordinator (Ex-Officio)

H. Dr. Paul Bowser

Cornell University

A2.3 2003 – 2004 Handbook Development Committee

A. Becky Lasee (USFWS)

USFWS - La Crosse Fish Health Center, Co-Chair

B. Andy Goodwin (AFS-FHS)

Aquaculture/Fisheries Center, University of Arkansas at Pine Bluff**,** Co-Chair

C. Chris Wilson (AFS-FHS)

Utah Division of Wildlife Resources

D. Ray Brunson (USFWS)

USFWS - Olympia Fish Health Center, Virology Sub-Committee Chair

1. **Joy Evered**
   USFWS - Olympia Fish Health Center
2. **Jason Woodland**
   USFWS - Pinetop Fish Health Center
3. **Scott LaPatra**
   Clear Springs Foods Research
4. **Joe Marcino**
   Minnesota Department of Natural Resources
5. **Patricia Varner**
   Texas Veterinary Medicine Diagnostic Lab

E. Joy Evered (USFWS)

USFWS - Olympia Fish Health Center

F. Susan Marcquenski (AFS-FHS)

Wisconsin Department of Natural Resources, Bacteriology Subcommittee Chair

1. **Hui-Min Hsu**
   Wisconsin Veterinary Diagnostic Lab

2. **Clifford Starliper**
   USGS, National Fish Health Research Laboratory

3. **Bruce Stewart**
   Northwest Indian Fisheries Commission

4. **Danielle Stanek**
   Washington Animal Disease Diagnostic Lab

4. **Ken Cain**
   University of Idaho

5. **John Hawke**
   Louisiana State University

G. Phil Hines (USFWS)

USFWS, Pinetop Fish Health Center

H. Patricia Varner (AFS-FHS)

Texas Veterinary Medicine Diagnostic Lab

A2.4 2004 – 2005 Handbook Development Committee

A. Phil Hines (USFWS)

USFWS – Pine Top AZ Fish Health Center, Co-Chair

B. Susan Marcquenski (AFS-FHS)

Wisconsin Department of Natural Resources**,** Co-Chair

C. John Grizzle (AFS-FHS)

Auburn University

D. Ray Brunson (USFWS)

USFWS - Olympia Fish Health Center

E. Patricia Varner (AFS-FHS)

Texas Veterinary Medicine Diagnostic Lab

F. Brian Hickson (USFWS)

USFWS - Warm Springs GA Fish Health Center

A2.5

2006 – 2007 Handbook Revision and Oversight Committee

A. Brian Hickson (USFWS)
Warm Springs GA Fish Health Center, Co-Chair

B. Marcia House (AFS-FHS)
Northwest Indian Fisheries Commission**,** Co-Chair

C. Phil Hines (USFWS)
Pine Top AZ Fish Health Center, Past Co-Chair

D. Diane Elliott (AFS-FHS)
USGS Western Fisheries Research Center, Past Co-Chair

E. John Coll (USFWS)
Lamar PA Fish Health Center, Future Co-Chair

F. Jim Thompson (AFS- FHS)
Washington State University, Future Co-Chair

G. Robert Bakal (USFWS)
United States Fish and Wildlife Service, Ex-Officio

H. Andrew Goodwin (AFS-FHS)
University of Arkansas at Pine Bluff, Ex-Officio

I. Kevin Amos (NOAA)
NOAA representative

J. Janet Warg, (APHIS)
National Veterinary Services Laboratories, APHIS representative

2006-2007 Bacteriology Subcommittee

A. Diane Elliott (AFS-FHS)
USGS Western Fisheries Research Center, Chair

B. Sonia Mumford (USFWS)
Olympia WA Fish Health Center

C. Ray Brunson (USFWS)
Olympia WA Fish Health Center

D. Cliff Starliper (AFS-FHS)
USGS National Fish Health Research Laboratory

E. Patricia Barbash (AFS-FHS)
Lamar PA Fish Health Center

F. Gavin Glenney (AFS-FHS)
Lamar PA Fish Health Center

G. Andrew Goodwin (AFS-FHS)
University of Arkansas at Pine Bluff

2006-2007 Virology Subcommittee

A. Marcia House (AFS-FHS)
Northwest Indian Fisheries Commission, chair

B. Jim Winton (AFS-FHS)
USGS Western Fisheries Research Center

C. William Batts (AFS-FHS)
USGS Western Fisheries Research Center

D. Kyle Garver (AFS-FHS)
Pacific Biological Station, Department of Fisheries and Oceans, Canada

E. Susan Gutenberger (USFWS)
Lower Columbia River Fish Health Center

F. Ray Brunson (USFWS)
Olympia WA Fish Health Center

A3.1 Position Statements Introduction

A. 2000 – 2002 Position Statements

1. **Initial Position Statement**
   This chapter sums up why and how we have assembled this document. We have tried to explain the purpose and make-up of the document so there is as little confusion as possible. We had to recognize there was no way to please everyone, when so many people with differing needs and wants will use the document. We have done our best to provide what we believe is the best possible document at this time. We have adhered very closely to OIE guidelines to make this document as useful as possible for international trade inspections. We are hopeful this document will continue to grow and evolve. Individual jurisdictions are likely to require different criteria for an aquatic animal health inspection and those criteria shall supercede the recommendations set forth in this chapter.

B. 2002 – 2003 Position Statements

No changes or reviews requested.

C. 2003 – 2004 Position Statements

No changes or reviews requested.

D. 2004-2005 Position Statements

1. **Clarify the intended use of the Inspection Manual.**
   It appears that some states are referring to the inspection manual as a policy document rather than as a collection of standardized procedures. This has resulted in requirements for screening for certain pathogen in geographic areas where the pathogen's life cycle could not even be completed. The Oversight Committee agreed to amend Section 1.1 in the Introduction; Section 2.1 in the Sampling chapter; Table 2.1 in Section 2.2 to clarify the purpose of the inspection manual.

2. Does the term "carrier state" exist for all pathogens included in the inspection manual?
The Oversight Committee agreed to review all cases where "carrier state" is used in the inspection manual and replace the phrase with "subclinical" where appropriate

E. 2006 – 2007 Position Statements

No changes or reviews requested. Updated editions and titles of manuals referred to.

A3.2 Sampling Position Statements

A. 2000 – 2002 Position Statements

1. **Initial Position Statement**
   This chapter was by far the most difficult to develop, due to the vast nature of situations and scenarios an inspector might come across. It is impossible to cover all scenarios and situations; therefore, while we have done our best to cover as much as we can, it will remain incumbent on the inspector to determine how best to sample in any given situation. Individual jurisdictions are likely to require different criteria and those criteria shall supercede the recommendations set forth in this chapter.

B. 2002 – 2003 Position Statements

1. **Should a sampling exemption be recommended for facilities on SPF water, no fish introductions, and a history of negative testing?**

   a. All members of the committee recognized the importance of limiting the destruction of valuable brood stock but felt strongly that annual surveillance of all lots must be mandatory. The committee compared the lot definitions and sampling requirements of this handbook with the OIE and Title 50. The OIE does allow reduced sample sizes after two years of negative results but no exemptions are given. In conclusion, the committee does not agree that any exemption would be appropriate, but did agree to discuss, over the next year, whether sampling requirements could be modified to spare valuable broodfish while still maintaining sufficiently stringent disease surveillance.

2. **Changes made as a result of the inclusion of *Piscirickettsia salmonis.***

   a. *Piscirickettsia salmonis* added to Table 2.1.

   b. Tissue collection procedures added to Section 2, 2.2.E.3.

C. 2003 – 2004 Position Statements

No changes or reviews requested.

D. 2004-2005 Position Statements

1. **Under certain conditions, is it acceptable to use non-lethal sampling methods in lieu of killing 60 fish for a health inspection?**
   a. In some cases, captive broodstocks in hatcheries with protected water supplies have been tested at the 5% level for years without detecting pathogens covered by the inspection manual. Could samples be obtained in a non-lethal manner and used to satisfy sampling requirements for inspections?

b. In other cases, remnant populations may exist (which may be threatened or endangered species) and there may be a desire to use adult fish from these populations as a source of gametes for restoration purposes. Rather than killing 60 fish, could samples be obtained non-lethally and still meet Blue Book inspection criteria?

c. This was the first time this question was brought before the Oversight Committee. There was some discussion among the Committee members, recognizing that non-lethal sampling techniques may exist for some pathogens, however trials to show equivalency with lethal methods have not been done for all pathogens.

d. At this time, the Oversight Committee agreed that no changes be made in the inspection manual regarding the use of non-lethal methods for fish health inspections. However, the Oversight Committee encourages researchers and fish health biologists to address this question by beginning to collect comparative data to show equivalency of methods, and to consider developing new diagnostic tools that are geared toward the type of samples that can be obtained non-lethally (ovarian fluids, mucous, intestinal swabs, feces, blood, gill tissue, etc.) and in the quantities likely to be non-lethal to the fish. This topic is of interest in Canada as well as the United States; the Great Lakes Fish Health Committee has made non-lethal sample collection for inspections a top research priority.

E. 2006 – 2007 Position Statements

No changes or reviews requested.

A3.3 Bacteriology Position Statements

A. 2000 – 2002 Position Statements

1. Initial Position Statement

The pathogens selected were those the committee felt were of the greatest regulatory importance at the time the handbook was being developed. The four bacterial fish pathogens considered in this chapter represent etiological agents which are known to exist in carrier states, but which have the potential for generating severe epizootics of clinical disease under the appropriate conditions. The methods are described for detection and identification of each pathogen in the absence of clinical signs. While other bacterial pathogens exist which can cause serious disease in fish, they are often widely distributed and cannot be controlled through transfer restrictions due to their ubiquitous nature (such as the pathogenic *Flavobacterium* species), and therefore, are not the focus of these inspection procedures.

The accurate identification of a bacterial species is based upon patterns of characteristics observed when live, pure bacterial isolates are cultured under a variety of environmental and biochemical conditions. All four bacterial fish pathogens considered for identification during a fish health inspection are culturable. All have been exhaustively characterized in a variety of widely recognized bacteriological manuals (Bergey's 1984; MacFaddin's 1980 and 2000; Austin and Austin 1987). The extensive characterization of these species has lead to the establishment of simple testing schemes for presumptive identification of bacteria isolated from fish tissues as described in these protocols. *Renibacterium salmoninarum* , however, is relatively fastidious and difficult to culture and characterize phenotypically in the period of time desired to accomplish the completion of a fish health inspection. Serological techniques are also considered to be rapid, highly specific means for achieving presumptive identification of bacteria. Because of its fastidious nature, the fluorescent antibody technique has been long developed as a presumptive screening tool for the detection of *R. salmoninarum* in fish tissues.

It is generally agreed that identification of a bacterial isolate based on phenotypic or serological characteristics alone poses the possibility that a population of fish be inaccurately labeled as diseased on a fish health inspection report. Although either method of identification is acceptable as a screen for pathogens in fish, neither technique alone is precise enough to distinguish between some similar organisms. For these reasons, it is always necessary to apply a second testing regime, referred to here as “confirmatory,” to establish the accuracy of the screening test. The protocols described in this document are presented in such a manner. In past decades, studies with nucleic acids and genetic methods have furthered the accuracy in the classification and identification of bacterial species. These tools, however, were limited to research because of the difficulty in applying them accurately under clinical situations. The more recent developments in polymerase chain technology, however, have revolutionized the use of molecular biology in pathogen detection in clinical laboratories. PCR is a practical, sensitive, and accurate means to confirm the presumptive identification of a bacterial pathogen by the isolation and amplification of segments of DNA existing within fish tissues. It is presented in these protocols as an alternative to time consuming selective culture for confirmation of positive *R. salmoninarum* FAT results.

B. 2002 – 2003 Position Statements

1. Should *Piscirickettsia salmonis* be added to the handbook?

a. The bacteriology subcommittee agreed that *Piscirickettsia salmonis* inspection procedures should be included in the bacteriology chapter at this time because the pathogen has the potential for causing severe epizootics and it is of regulatory concern.

b. Two screening methods were selected: (1) isolation in tissue cell culture without antibiotics and/or (2) identification of characteristic cells in stained tissue impressions. Inoculation of susceptible cells is the most sensitive method for detecting *P. salmonis*. However, because *P. salmonis* is sensitive to low levels of antibiotics typically used in cell culture, all cultures must be free of antibiotics. Samples collected aseptically in the field may easily become contaminated by other bacteria. For this reason, an additional screening method (Giemsa stained tissue impressions) was included.

c. For confirmation of *P. salmonis*, serological (immunofluoresence or immunohistochemistry) and PCR procedures were included. Screening and confirmatory methods are consistent with those in the OIE Diagnostic Manual for Aquatic Animal Diseases.

d. It was also recognized that the screening methods may not detect covert infections; however, these methods represent the best available at this time. A statement addressing limitations of the screening assays was included.

e. The incubation times selected for tissue culture were selected based on published peer-reviewed journal articles and the OIE Manual. The committee expressed concern the combined 42-day incubation was to long, but at this time there is no available information to make a change in the incubation time.

C. 2003 – 2004 Position Statements

1. Should culture and confirmation procedures for *Flavobacterium psychrophilum*, the causative agent of coldwater disease be added to the inspection manual?

a. The bacteriology subcommittee agreed that culture and confirmation procedures for *Flavobacterium psychrophilum* should not be added to the inspection manual at this time. At present, there are no validated methods to screen fish for *F. psychrophilum*. An IFAT method is being developed that could be used on ovarian fluids and kidney smears for broodstock screening; a PCR method is also being developed; selective media are being developed to reduce contamination by other bacteria and fungi; and methods are being developed to detect the bacterium in water. Also, the distribution of *F. psychrophilum* is global, and it is naturally present in water and sediments. At present, no agency regulates this organism, however, antibiotic resistant strains of the bacterium are considered reportable in the Pacific Northwest. Antibiotic sensitivity protocols have not been standardized for F.p. When these tests, including sensitivity to antibiotics, have been validated, this question should be reconsidered.

2. Should methods for Antimicrobial Sensitivity testing of bacteria be added to the inspection manual?

a. The bacteriology subcommittee agreed to add a chapter on antimicrobial sensitivity testing to the inspection manual.

b. It is important to collect data on antibiotic sensitivity for fish pathogens because there is potential for agencies to regulate the movement of fish infected with resistant strains of bacteria. Since the disc diffusion protocols have already been peer reviewed and published, it would be very easy to incorporate them as a separate chapter in the inspection manual.

c. The subcommittee felt this was the best place for the protocols (rather than the diagnostic section of the Blue Book) due to the potential for agencies to regulate pathogens based on their resistance to antibiotics (a standardized method would be needed for this).

b. Should the requirement of growing bacteria in TSB prior to preparing the hanging drop motility test be omitted?

a. The bacteriology subcommittee agreed that bacteria do not need to be cultured in TSB before preparing a hanging drop suspension to test for motility, unless the bacteria appear non-motile.

b. Colonies may either be grown in TSB (or other suitable broth) or taken from an agar surface and suspended in sterile saline to evaluate motility.

c. If bacterial suspensions prepared from solid agar, or a stab of a semi solid agar appear non-motile, the bacteria should be cultured in TSB and the hanging drop method used to confirm motility.

4. Should the procedures to distinguish *A. salmonicida salmonicida* from *A. salmonicida achromogenes* be revised?

a. The bacteriology subcommittee agreed to remove arabinose from the list of differential tests pertaining to *A. salmonicida salmonicida* and *A. salmonicida achromogenes*. Arabinose reactions were consistent among all references, however the test itself requires longer incubation and is difficult to read/interpret for A. sal. when commercially available agar is used.

b. Indole, esculin and maltose tests were already added to the flow chart in the 2003 revision of the inspection manual, and the +/- for these tests are consistent with Bergey's manual. However, in the subcommittee's discussion, we discovered there are published references (Wiklund and Dalsgaard 1998; Wiklund and Dalsgaard 1995; Chapman et al. 1991; Austin and Austin 1987) indicating typical and atypical A. sal. do not always utilize indole and maltose according to Bergey's scheme. There is a need to compare the variability of *A. salmonicida salmonicida* and *A. salmonicida achromogenes* in utilizing

indole, maltose, and esculin to determine whether these tests should be listed as secondary tests in the flowchart.

c. If these tests are retained in the flowchart, companion text needs to be developed in the furunculosis chapter.

d. Other biochemical tests could be evaluated such as mannose, degradation of blood, or others to differentiate *A. salmonicida salmonicida* from *A. salmonicida achromogenes.*

e. The O/F box in the flow chart has K/AG, and A/AG reactions which seem to refer to TSI rather than O/F reactions. Perhaps using the words "fermenter" and "non-fermenter" or "oxidizer"should be used instead.

5. Additional Comment

The subcommittee recognized that specific research is needed to address several aspects of the diagnosis of fish bacterial pathogens. We list them here, so they are of record, but also suggest the inspection manual review team work with the technical standards committee with the goal of obtaining funding for labs to work on topics directly related to enhancing the inspection manual and Blue Book.

a. Develop screening techniques to detect *F. psychrophilum* in carrier fish. Some work has been started on IFAT and PCR methods, and there is a need to modify existing agars to enhance growth of F.p. and reduce fungal overgrowth on agar plates. There may also be selective or differential media that could be developed for F.p.
b. Although disc diffusion protocols have been developed for many fish pathogens, methods for *F. columnare* and *F. psychrophilum* have been problematic. Work continues at Louisiana State University for F. c., but to our knowledge, no one has worked to standardize a protocol for F.p. Once methods are developed, the NCCLS can coordinate field testing of the methods at fish health labs similar to what was done for other pathogens and disc diffusion protocols. If antibiotic resistant strains of F.p. are regulated in the future, there would be a need for a standardized antibiotic sensitivity method in the inspection manual.
c. Compare the variability of typical and atypical A. sal. isolates in their ability to utilize indole, maltose and esculin. In addition, evaluate other biochemical tests (such as those for mannose, lecithin or Tween 60 for example) or blood degradation to differentiate typical from atypical A. sal.

These needs arose from our discussion related to suggested changes for the inspection manual, but there may be research needs for other pathogens (protocol development, validations, etc.), which could be added to this list. The committees could brainstorm funding options and solicit specific researchers to do the work.

D. 2004 – 2005 Position Statements

No changes were suggested for Chapter 3, Bacteriology for the 2004-2005 revision year.

E. 2006 – 2007 Position Statements

1. Should the procedures be amended to allow the use of pools of kidney and spleen tissues from up to 5 fish for detection of bacteria that are screened by plate culture?

The amended procedure would enable the use of samples collected for virological analyses to be used for certain bacteriological analyses as well, provided that no antibiotics were added to the samples. The request was based on a paper (S. Mumford, C. Patterson, J. Evered, R. Brunson, J. Levine and J. Winton. 2005. Comparison of individual and pooled sampling methods for detecting bacterial pathogens of fish. J. Vet. Diagn. Invest. 17:305-310) that described the equivalency of using pooled kidney-spleen samples (from 5 fish) to individual kidney sampling techniques for detection of *Yersinia ruckeri* in culture.

Despite the potential advantages of sample pooling, the conclusion reached by the committee was that more data was needed from multiple species to validate the technique for all of the pathogens that would affected by the change. These pathogens include *Aeromonas salmonicida* and *Edwardsiella ictaluri* in addition to *Yersinia ruckeri*.

No changes were made in this edition.

A3.4 Virology Position Statements

A. 2000 – 2002 Position Statements

1. Initial Position Statement

The eight viral pathogens considered in this chapter represent agents that may exist in a carrier state, have the potential for causing severe epizootics, and/or are currently of regulatory concern. This list will likely change as these concerns vary and new control measures are developed. Techniques provided for screening and confirmation are considered to be sensitive, practical, and efficient, and applicable to the large numbers of samples necessary to detect viral pathogens in carrier states. The potential variety of techniques is limited to cell culture for screening and serum neutralization and/or PCR for confirmation to simplify the writing of this initial handbook. Other serological methods such as immunoblot and fluorescent antibody tests are available for some of these viruses and applications may be made to add these to later versions.

Cell culture is the screening method used and broad spectrum cell lines have been chosen whenever possible to aid the testing laboratory in getting the most information from the samples.

Blind passage of samples has been included to determine if it will significantly increase the ability of the laboratories to detect carrier stages of these viruses using these methods.

Since cell culture amplifies the virus, it allows for the use of a highly specific but not necessarily sensitive confirmation method (see Chapter 1). The utility of serum neutralization tests for the confirmation of IHNV, IPNV, SVCV, and VHSV has been shown with years of use and for that purpose it is included here, however, the reagents are not available for all of the viruses in this handbook. PCR is a newer technique that is also highly specific but much more rapid than serum neutralization and the detailed methods for using it to confirm IHNV, ISAV, LMBV, and VHSV are also included. PCR techniques are being developed for IPNV, OMV, and WSHV and applications may be made to include them in future version as the methods and reagents become available.

B. 2002 – 2003 Position Statements

1. Include WSIV and CCV.

a. The committee has determined that the current cell culture technique for WSIV does not have adequate sensitivity to use as a screening method and, although the CCV cell culture technique will detect overt infections, it does not detect covert infections. These limitations may lead to a false negative status for the population being inspected by these procedures. Therefore, the committee members agreed that at this time the inspection criteria for these two viruses are better handled by regional policy or on a case-by-case basis.

2. Remove the requirement for blind passage.

a. The current literature and procedures in the OIE Diagnostic Manual suggest that blind passage will increase detection of some viral agents in fish populations. With the cooperation of laboratories using the procedures in this manual, information will be gathered to determine how frequently a change in the classification of the inspected populations occurs due to the use of blind passage. The committee members agreed that the data collected from laboratories using this procedure should be reviewed each year until enough information has been gathered to support or refute the benefit of blind passage.

3. In some situations involving the movement of eggs, 28 days is too long to wait for the results of a broodstock inspection since eggs may hatch within that time period.

a. After much discussion over this concern, the committee members generally agreed to maintain the 28-day total incubation period for the virology samples at this time. This manual is intended to provide laboratories with procedures which will detect several viruses in the same assay and the longer incubation period is necessary for some of the listed viruses, specifically OMV and ISAV. Some of the alternatives discussed were that the laws, policies, and/or regulations of the jurisdictions involved may allow for a statement on the inspection certificate of the procedures used to obtain the results, and methods exist for the handling of the eggs and fry until testing is completed such as chilling the water during egg incubation to slow development and maintaining the eggs or fry in isolation.

4. Review the information available for the optimal cell line to use in screening for ISAV and the choice of primers for the PCR confirmation.

a. Although at least two cell lines other than SHK-1 have been used to detect ISAV, the current scientific literature does not support a change to either ASK or CHSE-214 cell lines in screening for ISAV. The PCR method included in the manual is capable of detecting both the North American and European strains of ISAV so no change in primers is necessary. The committee members agreed that this section should be reviewed again at a later time when more information is available.

5. Include more serological tests for confirmation of the viruses.

a. The committee members had many reservations regarding the use of serological confirmation methods that included concerns about both the availability and quality of the necessary antibodies and antisera. However, with the stipulation that appropriate QA/QC procedures are used, the committee agreed to leave serum neutralization as a confirmation method for IHNV, IPNV, SVCV, and VHSV and to add the indirect fluorescent antibody test as a confirmation method for IHNV, IPNV, ISAV, and VHSV.

6. PCR procedures for IPNV, OMV, and SVCV are now available and should be included as confirmatory methods for these viruses.

a. They have been added to the 2003 edition of the manual.

7. **Review the PCR procedure for LMBV with respect to the extraction method and primer sequences.**

    a. Some laboratories that currently work with LMBV were contacted in regard to this comment. At least three of them had used one or more of the commercially available DNA extraction kits and had found them to be adequate for identifying the virus from cell culture material. The committee members agreed to remove the extraction method that required the use of chloroform and phenol and replace it with the more worker and environmentally friendly extraction kits. The newer primer sequence was considered to be an improvement in the method, so a change has also been made to that part of the procedure.

C. 2003 – 2004 Position Statements

1. **Revise LMBV PCR protocol needs to be altered to match the new primer sets. Should the protocol exactly match that of John Grizzle's 2003 paper?**

a. The published procedure incorporated the use of uracil to substitute for thymine in the master mix due to a potential contamination issue in that lab.

b. The Oversight Committee noted that this substitution would unnecessarily complicate lab supplies/tracking/labeling in most PCR capable laboratories. A modified procedure that uses thymine (as all other PCR procedures in the Manual) has also been tested by Jason Woodland Becky Lasee, Patricia Varner, and others and found to work. This also was discussed with John Grizzle, Andy Goodwin and others who have the opinion that the modification shouldn't cause any problems with the LMBV-PCR procedure as published when doing confirmations from viruses isolated on tissue culture.

c. Although the intent of the editors and contributors to the Manual is to stick with procedures that are published and therefore "scientifically defensible", it was quickly realized by the Oversight Committee that the modified procedure would probably be the best one to adopt. However, there is some hesitation due to the lack of a definitive "bench mark test" that has been published.

d. The virology sub-committee recommends leaving the uracil technique out of the LMBV PCR and including instead as a general option in the PCR section. This justified by the following.

I. The results of current users show the procedure using thymine is robust and accurate for detecting LMBV.
II. This will improve the LMBV section for practicing labs that run inspections by standardizing the components of the master mixes used for all PCR confirmations
III. Standardizing the components will reduce QA/QC concerns and increase efficiency in the PCR lab.
IV. We are not ignoring the value or the inclusion of the uracil procedure in the Manual, but it is best put into the general PCR section to help labs deal with contamination issues as they arise in all PCR procedures.
V. The writers and editors of the Manual are considered the "experts" and should know the best course of action on any modification.

2. Should the SVCV PCR protocol be updated to utilize the new OIEprimers?

a. The subcommittee discussed conversations with Jim Winton regarding the current protocols not working on certain Asian strains of this virus. OIE has apparently accepted primer sets identified by Stone et al. 2002 that will work on all known strains of SVCV.

b. Although there is some controversy regarding the taxonomic placement of some "cross reacting" Pike Fry Rhabdovirus isolates, the subcommittee discussed the desirability of using the OIE (Stone) protocols at least until the International Convention on Taxonomy of Viruses sort out the taxonomy.

c. The subcommittee voted to recommend that the Oversight Committee adopt the OIE protocols for SVCV PCR confirmation.

3. Can we remove the blind pass from virology procedures?

a. The subcommittee discussed and reviewed the data submitted by Susan Gutenberger, USFWS. We also discussed the OIE requirements and the lack of data to support the proposition that population certifications would likely stay the same if blind passes were dropped.

b. The subcommittee members were sympathetic about the expense, time and labor expended in doing blind passes, but scientifically we could not support dropping blind passes at this time. The biggest questions are reported cases of significant changes in detection of European VHSV and LMBV through the use of blind passes. We suggested that the USFWS or the AFS-FHS attempt to query other agencies regarding data on blind passes.

c. The subcommittee voted to recommend to the Oversight Committee that the blind pass requirement be kept in the Inspection Manual at this time.

4. Add IHN-IHC (immuno-histochemical) confirmation protocol for IHNV

a. The subcommittee reviewed and discussed the protocol as submitted by Ken Nichols and Scott Foot, USFWS. We noted that although several confirmations already exist, the protocols as submitted are rigorous, referenced, and utilized defined reagents as required by the procedures of the Manual.

b. The subcommittee voted to recommend to the Oversight Committee that this protocol be added to the IHNV confirmation procedures in the Manual.

5. Change ISAV cell line for screening from SHK-1 to ASKII cell line

a. The subcommittee considered the OIE manual which allowed the use of cell lines other than SHK-1, and also considered the reference in the Vol. 23(2), 2003 Bulletin of the European Association of Fish Pathologists, pp 80-85. "Comparative isolation of infectious salmon anaemia virus (ISAV) from Scotland on TO, SHK-1 and CHSE-214 cells" by R. Grant and D.A. Smail.

b. Both Ray and Scott offered their experiences working with both cell lines in the past and their agreement that ASK cells were far easier to work with than SHK-1 cells. ASKII cells are also readily available from the ATCC collection and well referenced.

c. Based on theses discussions, the subcommittee voted to recommend to the Oversight Committee that these cells be included as acceptable for the screening of ISAV.

D. 2004 – 2005 Position Statements

1. Change the incubation temperature range from 20-25 C to 25-30 C for LMBV, and shorten the incubation period from 14 days to 7 days before doing a blind pass.

Three citations were provided showing 25-30 C is the optimal temperature range for LMBV replication (Piaskoski et al. 1999, Grant et al. 2003 and Mc Clenahan et al. 2005). At this higher temperature, CPE will occur faster and there is no need to incubate for 14 days. Seven days is sufficient.

Based on these citations, the Oversight Committee was unanimous in its vote to accept these changes.

2. Eliminate the need for blind passage for general viral isolation.

Supportive documentation was not provided with this suggested change. The same suggestion was made in 2003-2004 and was addressed above. Since no new information was provided in support of this change, the Oversight Committee voted to make no changes regarding the need for blind passage.

E. 2006 – 2007 Position Statements

1. Review and update the Viral Hemorrhagic Septicemia Virus (VHSV) protocols to reflect current knowledge in light of its recent emergence in the Great Lakes.

a. The list of susceptible species now includes those from the Great Lakes.
b. BF-2 and FHM cell lines were added to the EPC cell line as appropriate cell lines for testing.
c. RNA extraction method changed to either phase-separation method or RNA affinity spin column instead of the Heat RNA Release Method to increase efficiency.
d. PCR primers were changed to match OIE primers, and second round PCR was eliminated as it was viewed as unnecessary for a confirmatory assay.
e. Information on obtaining antisera for serum neutralization test was added.

2. Update the Infectious Hematopoeitic Necrosis Virus Primers.

a. RNA extraction method changed to either phase- separation method or RNA affinity spin column instead of the Heat RNA Release Method to increase efficiency.

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b. PCR primers were changed to target the central portion of the G gene, instead of the previously published primers which targeted the N gene. The G gene primer set has been tested extensively (Emmenegger et al., 2000, Kurath et al., 2003) and avoids using a series of six consecutive 'G's' that were less than optimal for PCR in the N gene forward primer. Additionally, second round PCR was eliminated as it was viewed as unnecessary for a confirmatory assay.

A3.5 Parasitology Position Statements

A. 2000 – 2002 Position Statements

1. Initial Position Statement

The pathogens selected were those the committee felt were of the greatest regulatory importance at the time the handbook was being developed. Rationale for selection of the screening and confirmatory assays for each of the fish parasites considered in Section 2, Chapter 5 Parasitology are detailed below. Confirmatory procedures will only be used if the sample is presumptively positive using the approved screening method. Please refer to Section 1, Chapter 1 Introduction for explanation of the acceptance of non-validated procedures for confirmation.

a. ***Myxobolus cerebralis***

i. Screening

The pepsin-trypsin digest procedure was selected as the assay of choice for isolation and concentration of spore stages from fish cartilage. Although it was acknowledged that the plankton centrifuge method offers some advantages in the ease of assay performance, review of the literature and of laboratories performing *M. cerebralis* diagnostics supported selection of the digest assay for reasons of increased sensitivity. The procedure does allow pooling of up to five fish, which is likely to decrease detection sensitivity. However, it was considered that processing of individual fish would constitute a workload beyond the capability of many laboratories, and that in some regions of the country this would be considered unacceptable. The decision was to allow pooling with the realization that in areas most affected by the parasite, there would be requirement by the states to process single fish.

ii. Confirmation

Confirmation is either by identification of spores in histological sections or detection of parasite DNA by polymerase chain reaction (PCR) assay. Detection in histological sections is the current standard. Although the committee felt that it is of lower sensitivity than the PCR assay, it will remain an acceptable confirmatory tool at this time. For DNA detection, the nested PCR assay was selected because it is scientifically acceptable and citable and it is used successfully in a number of laboratories. Because the sampling and preparation procedures described in the original publication were primarily for research purposes, the protocol described here references methods more in line with those required during field collections of fishes of different sizes. These collection and preparation methods are compatible with performing the nested assay.

b. ***Ceratomyxa shasta***

i. Screening

Presumptive identification is based on identification of any parasite stages in wet mount scrapings, the procedure currently recommended.

ii. Confirmation
Because of the distinctive morphology of the *C. shasta* spore, its identification is sufficient for confirmation. If spores are not identified, a presumptive positive can be confirmed by detection of the parasite DNA by PCR. The protocol described is published and has been developed for diagnosis in field situations. Other confirmatory procedures requiring monoclonal antibodies were not considered because these reagents are not commercially available.

c. ***Tetracapsula bryosalmonae***

i. Screening
Presumptive identification is made by identifying any parasite in stained imprints or using lectins. These two methods were proposed because identification of the parasite is difficult without practice, and the lectin has been shown to increase detection.

ii. Confirmation
At this time, confirmation is by identification of any parasite stages in histological sections. Although this method is not highly sensitive and requires a trained eye, it was agreed that scientific review of other methods made them unfeasible at this time. The lectin stain has been demonstrated to cross-react with other myxozoans and there is also question about the specificity of published PCR assays. The committee felt that this protocol would probably be updated in the near future as a demonstrated specific PCR assay becomes available.

d. ***Bothriocephalus acheilognathi***

i. Screening
Presumptive identification is by identification of basic characteristics of the cestode.

ii. Confirmation
Presumptive cestodes are confirmed by identification of key morphological characteristics. These visual identification methods are accepted in the scientific literature and are the current Blue Book standard.

B. 2002 – 2003 Position Statements

1. Review use of digest material for PCR confirmation of *Myxobolus cerebralis*.

a. Adoption of the nested PCR technique on digest material for confirmation of the presence of *Myxobolus cerebralis* can be scientifically defended at this time. Baldwin and Myklebust's work statistically determined sensitivity of single round PCR from pooled digest material from infected and non-infected reference animals, and added additional information regarding specificity. Though statistically significant, the number of samples examined was quite low, and although not determined, the confidence limits for sensitivity and specificity would likely be quite large. Qureshi et al. examined a large number of clinical samples (580 fish) using nested PCR on the digest product and compared results with the current gold standard, histologic examination, as well as with the tissue digest. Testing of additional animals should be done and levels of sensitivity need to be determined for the nested procedure applied to

digest material, but there is already more information available on this assay than for almost any other test.

2. Review histological confirmation of *Myxobolus cerebralis.*

a. The committee recommended that the criteria for determining a sample negative by histology be made more stringent. Wording will be changed to include serial step sections in samples where the initial sections examined were negative, and inability to detect any spores in tissue will no longer be considered sufficient to certify a lot of fish as negative.

3. Review protocols allowing freezing of samples for *Myxobolus cerebralis* spore recovery.

a. The committee recommended that no change be made to the current procedure, which allows PTD processing of frozen samples with modifications of enzyme concentrations. There is insufficient peer-reviewed scientific data to prove and quantify the effects of freezing on spore recovery and requiring processing of fresh heads would present a problem for many laboratories.

4. Review of PCR diagnosis of *Tetracapsula bryosalmonae*.

a. The committee agreed that recent publications on this assay demonstrate that it is a valid confirmation test and this will be added as an alternative to histology. Concerns about this and other PCR assays continue to be QA/QC issues like availability of positive control tissues.

C. 2003 – 2004 Position Statements

The full committee voted to include a modification of the whirling disease enzymatic digestion mixture to include a pH indicator. This mixture has been widely used for many years and will function at least as well as the current mixture.

D. 2004-2005 Position Statements

a. Is it possible to sample for *Tetracapsula bryosalmonae* and *Ceratomyxa shasta* in a manner similar to methods used for *M. cerebralis*?

b. Rather than sampling every lot at a hatchery for these pathogens, could only the most susceptible lot be sampled; and could samples be pooled (a 5-fish pool was suggested)?

c. No comparisons have been made regarding the detection of *T. bryosalmonae* or *C. shasta* using the most susceptible lot vs. sampling all the lots at a hatchery.

d. Nor have experiments been done to show that processing samples for screening *T. bryosalmonae* and *C. shasta* as 5- fish pools will demonstrate the true health status (positive or negative) of a lot of fish compared to sampling individual fish.

e. Based on the absence of data, the Oversight Committee agreed no changes to sampling methods for *T. bryosalmonae* and *C. shasta* will be made at this time.

E. 2006 – 2007 Position Statements

No changes or reviews requested.

A3.6 Polymerase Chain Reaction (PCR) Position Statements

A. 2000 – 2002 Position Statements

1. Initial Position Statement

This chapter was designed to supplement references to molecular techniques referenced in earlier chapters. Included are general considerations for insuring that contamination does not occur and to insure the integrity of the assay. These general protocols that can be found in many general primers for PCR and are intended to provide background information for laboratories that are just setting up PCR diagnostics.

B. 2002 – 2003 Position Statements

No changes or reviews requested.

C. 2003 – 2004 Position Statements

The committee agreed to a suggestion to provide a comprehensive table outlining all of the PCR protocols.

The virology sub-committee contributed a new section to the PCR chapter. The paper used as the source of the LMBV PCR method also included an option to use Uracil N-glycosylase (UNG) and dUTP to reduce carry-over contamination. The virology committee elected to include this method in the PCR chapter as an alternative that could be applied to PCR reactions in any laboratory where contamination is a problem, but felt that it was not reasonable to require UNG in all labs doing LMBV PCR.

A3.7 Appendix 1 The Handbook and Oversight Committee Position Statements

A. 2000 – 2002 Position Statements

1. Initial Position Statement

This appendix is truly the heart of this document. It lays out the structure of the handbook and the revision and oversight committee. It explains how the handbook will be maintained and by who. Most importantly, however, this appendix details the manner in which this handbook shall be reviewed and revised. This detailed procedure is what gives this document its advantage over previous documents of its kind. Additionally, these reviews are mandatory on an annual basis, which means the document can be kept current from a technique and pathogen standpoint, such that in the future there should be no need to create any new handbooks or manuals.

B. 2002 – 2003 Position Statements

1. Inserted months into locations that make the timeline for review easier to comprehend.

2. Removed printed document as a means of dissemination.

June 2005

Model Quality Assurance/Quality Control Program For Fish Health Laboratories

American Fisheries Society Fish Health Section

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I. **Introduction**

The purpose of the QA/QC Program is to ensure the quality, reproducibility and accuracy of the information and results generated by a fish health laboratory (FHL) for fish health inspection and diagnostic assays. Diagnostic protocols provided in updated versions of *Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens* (AFS Fish Health Section Blue Book) provide excellent standards by which the diagnostic laboratory can abide when conducting laboratory assays. Following a carefully written protocol, however, does not in itself assure that the consequence is a quality product. In order for a laboratory to assure quality services it must first establish a system of quality control by which every aspect of laboratory management, structure and upkeep are implemented. The purpose of this Model Quality Assurance/Quality Control Program for Fish Health Laboratories is to provide a structure by which laboratories providing fish disease diagnostic services can develop individual, lab-specific programs. The document is geared for adaptation at small laboratories as well as large establishments, and will serve to address many of the critical elements provided in the ISO/IEC (International Organization for Standardization/International Electrochemical Commission) International Standards 17025, as well as those standards published by OIE in (Office International de Epizooties). The present document has been adapted from the QA/QC Program established by the U.S. Fish and Wildlife Service within the *Handbook of Aquatic Animal Health Procedures and Protocols* (2004).

II. **Policy and Implementation**

A. Management Responsibilities - The FHL shall have a Director whose tasks include the following:

1. The FHL Director shall have overall responsibility for the technical integrity of the tests as well as for interpreting, analyzing, documenting and reporting result. The Director will ensure that:

   a. Employees clearly understand the functions which they are to perform, and are properly trained to perform their duties, and that training is documented;

   b. Any deviations from this QA/QC Program or unforeseen circumstances that may affected the integrity of the tests are corrected and documented, and;

   c. All test data are accurately and precisely recorded and reported.

2. Depending on the management authority overseeing the funding, policy and direction of the FHL, the FHL Director shall be accountable for QA/QC concerns of the FHL to the management authority involved.

B. Personnel/ Personnel Responsibilities

1. Each FHL maintains an adequate number of employees for all functions. All employees

possess the necessary education, training, technical knowledge, skills and experience for the tests conducted. In addition, all employees are free from pressure or inducements which might adversely influence their judgment or the results of their work.

2. Each employee shall be responsible for monitoring each test he/she is conducting to ensure that facilities, equipment, practices, and record keeping conform to this QA/QC Program.

3. Each employee shall take the necessary precautions to avoid contamination of the test, control, and reference substances.

4. Each employee shall follow established national safety and health regulations in the operation of each laboratory unit, handling of hazardous materials, and procedures for storage and disposal of hazardous wastes.

C. Quality Assurance Coordinator

1. The FHL shall have a Quality Assurance Coordinator whose responsibilities include the following:

   a. Implementing and monitoring of the QA/QC Program

   b. Implementing all necessary quality controls to ensure the accuracy and precision of reported data.

   c. Monitoring laboratory practices to verify continuing compliance with policies and procedures.

   d. Evaluating instrument calibration and maintenance records.

   e. Ensuring the validation of new technical procedures.

   f. With the Director, investigating technical problems, proposing remedial actions, and verifying their implementation.

   g. Providing recommendations for training to improve the quality of laboratory staff.

   h. Proposing corrections and improvements in the quality system.

   i. With the Director, cooperate with and accompany teams conducting external and/or internal QA/QC audits of the facility (See Appendix A).

2. Technical Qualifications Files: A technical qualifications file is maintained for each laboratory staff member. Technical qualifications files include the following items:

   a. A resume of qualifications, skills, experience, and certifications.

   b. References to all training classes, seminars, short courses, and conferences attended.

D. Laboratory Facilities

1. Sample Collection/Necropsy: All samples will be collected by qualified staff members of the fish health laboratory, under supervision of an AFS – Fish Health Section certified Fish Health Inspector or other qualified official. Necropsy and sample collection is performed in accordance with accepted protocols (Appendix D).

2. Sample Processing: Virological, parasitological, bacteriological, histopathological,

immunological (ELISA) and molecular biological (PCR) assays shall be conducted according to accepted protocols (Appendix D). Labs which do not have particular capabilities ship necessary samples for analysis to a private, academic or government laboratory with appropriate capabilities. Condition and integrity of sample submissions upon arrival should be noted (Appendix C).

3. Laboratory Space and Design

   a. Fish Heath Labs should comply with national and local standards of health and safety at or equivalent to Level II Containment (for biocontainment standards see http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm).

   b. Labs should provide separated areas for administrative activities, fish handling, and laboratory testing.

   c. Each laboratory room is equipped with adequate space and environmental conditions including cleanliness to perform assigned tasks.

   d. Storage spaces are adequate to maintain equipment, supplies, samples, and chemicals without danger of cross contamination.

E. Laboratory Equipment and Supplies

1. Equipment – The assigned Quality Assurance Coordinator (QAC) is responsible for maintenance of all laboratory equipment, and recording all maintenance records in a common log:

   a. The QAC ensures that all microscopes, balances, pipettes, thermometers, meters, incubators, refrigerators, freezers, hoods, spectrophotometers, and other instruments in use are calibrated and maintained on a routine basis by laboratory staff using the equipment (Appendix E).

   b. Each item of equipment possesses an inventory number for identification. Records of calibration and maintenance documentation are kept for each instrument and microscope.

   c. Where appropriate, equipment maintenance and temperature information is posted on its surface (hoods, balances, refrigerator/freezers, incubators).

   d. Defective or suspect equipment is taken out of service until repaired, tested and recalibrated.

   e. Equipment used for generating measurements is calibrated and/or standardized according to recommendations provided in Appendix E.

2. Reference stocks and reagents - The QAC is responsible for assuring that all reagents and reference stocks are maintained under proper storage conditions, labeled and handled appropriately by all laboratory staff:

   a. All reference stocks shall be retained with original labels from the supplier or are labeled by name, date of receipt, chemical abstracts number (CAS) or code number, batch number, expiration date if perishable, and include National Fire Protection Association (NFPA) labels indicating safe use and storage requirements.

b. Reagents are labeled to indicate identity, contents, titer or concentration, expiration date and safe use and storage requirements (NFPA labels).

c. Reference stocks and reagents are handled in a manner that precludes the possibility of contamination, deterioration, or damage to the substance.

d. All reagents, serums, cell lines, and laboratory supplies are of high quality. To ensure quality performance during all laboratory assays, specific product and manufacturer sources are provided where indicated in accepted protocols (Appendix D).

e. An inventory of all reagents is maintained for the monitoring of expiration dates. Deteriorated or outdated reference stocks and reagents are disposed of properly.

f. Current Material Safety Data Sheets (MSDS) are available to employees for all chemicals used, as required by the "Right to Know Law".

g. Necessary personal protective equipment and training in equipment use is provided to all laboratory personnel.

h. Fish Health Labs should have a "Safety and Chemical Hygiene Plan". All laboratory personnel are to utilize equipment and reagents in compliance with this plan.

i. Mixture of substances - When test, control, or reference substances are mixed, the date of preparation, initials of the preparer, and the exact contents of the mixture shall be labeled on the bottle along with storage requirements and expiration date and proper NFPA labeling.

F. Chain of Custody/Case Tracking

1. All samples are given a case history number as they are received at the laboratory.

a. The case history number uniquely identifies the test samples on receipt and tracks the case throughout the laboratory. Upon receipt the case number is assigned and labeled on all sample containers and racks following external decontamination.

b. The case history number, along with information pertaining to the specifics of the samples received, is recorded on either a Case History Record (CHR) cover sheet and/or in a Case Report book. The following information is to be included:

1) Case History Number

2) Date of Receipt

3) Date Sample Taken

4) Sample Site (including, where possible, GIS information)

5) Name of Sampler

6) Recorder Initials

7) Species and Age -class of fish

8) Condition of Samples at receipt

c. This Case History Record (CHR) cover sheet also contains specific numbers and tissue

materials sampled for the following lab assays: Bacteriology, virology, parasitology, serology, histology, molecular (PCR) and "other". In addition, any descriptive information received with the samples is attached to the CHR.

d. All sample material is assigned a number which corresponds directly with the description recorded in the CHR.

e. All tubes, bags, or other sample containers are labeled with chemical resistant laboratory markers with pertinent information to allow for accurate tracking of samples through each laboratory area.

f. All CHR's are transcribed in ink, and recorded in a manner to ensure the integrity of all samples from collection site to final analysis

2. Sample tracking in individual labs:

a. If sample items are sent to an outside laboratory for expert analysis, the transfer of that item is properly entered on the CHR. Results from the outside laboratory are obtained in writing and attached to the CHR.

b. Within each laboratory area (bacteriology, virology, etc.), a separate record system is maintained to record samples received into the area, assays requested and performed and results obtained. At completion of all assays, the results are recorded onto the original CHR, and any supporting paperwork attached.

c. When all assays are completed and results are obtained, the CHR with all necessary attachments is provided to the designated staff member for a case report write-up. All reports refer to the appropriate CHR number, and copies are maintained in laboratory files.

3. Record Retention

a. Hard copies of records are retained in office files for at least 7 years. This record retention standard also applies to computer records retained on disk.

b. Equipment logs are maintained for a minimum of two years.

G. Standard Operating Protocols and Conduct of tests

1. Each fish health laboratory shall follow specific protocols depending upon the type of case being conducted (Appendix D).

a. Fish Health Inspection samples are assayed according to the current edition of *Procedures for Aquatic Animal Health Inspections* (AFS-Fish Health Section "Blue Book"), and/or other state regulation, regional fish health compact guidelines, and/or international requirements (OIE) that may apply.

b. Directors are responsible for approving other protocols prior to their use. Critical protocol deviations must be documented on the CHR with a description of the procedures used, and/or citation from the literature.

2. Each employee conducts testing in strict accordance with established protocols as described in Section II.G.1 of these guidelines.

a. Data generated during all tests shall be documented, in ink, and attached to the CHR. Result summaries are entered directly onto the CHR cover sheet.

b. Pertinent entries are dated and initialed by the employee performing the work.

c. Any changes to the original entry should not obscure the original entry and the reason for the change should be indicated, dated, and initialed by the employee performing the change.

H. Routine Quality Control Procedures

1. Reagent Quality Control

a. Controls: Known controls and standards are tested and compared along with the samples in question with every test conducted. Standards and controls are kept separate from samples when stored. Detailed information on controls, reagents and media used for each test is described in established protocols (Appendix D).

b. Contamination Checks: On a routine basis and/or when a problem is suspected, materials and supplies used in sample testing are tested for contamination, using known controls.

2. Routine Quality Control in the Sample Processing/Necropsy Laboratory

a. Sample Tracking

1) All samples received in the sample Processing/Necropsy Lab are immediately assigned a CHR number as described in II.F.

2) The container holding the samples is externally decontaminated (Appendix F) prior to opening. Upon opening of container, the inside of the container is inspected for sample spillage which would need further disinfection.

3) Tubes, bags or other items containing tissue samples are removed, externally disinfected (Appendix F), and placed in clean laboratory racks, bags or containers bearing the appropriate CHR number.

4) Data on each tissue sample is recorded and entered onto the CHR data sheet as described in section II.F. Any written material and sample descriptions received with the samples are attached to the CHR.

5) Samples are distributed to respective laboratories for incubation, processing and/or archiving.

b. Contaminant Control - The following are basic measures to be taken to insure contaminant control in any laboratory.

1) Wear clean lab coats within laboratory.

2) Wash hands with antibacterial soap before and after lab work.

3) Clean countertops with an appropriate disinfectant (Appendix F) preceding and following any lab work.

4) Decontaminate container surfaces with 70% alcohol spray (Appendix F) before

and after use.

5) Clean countertops, floors and waste cans routinely or when needed with an appropriate solution of disinfectant.

6) Clean inside surfaces of refrigerators with an appropriate disinfectant when spills occur.

7) Soak waste materials from sample processing/ necropsies in an appropriate concentration of sodium hypochlorite solution (Appendix F) prior to disposal, or autoclave prior to proper disposal.

8) Thoroughly decontaminate tube racks, slide boxes and other items with possible contamination prior to reuse.

9) Do not eat, drink or smoke in the lab.

10) Immediately clean and disinfect any biological agent or media spills.

11) In general, avoid clutter and maintain lab equipment in an orderly and functional manner.

c. Necropsy - Live or freshly dead animals are often received for necropsy at fish health laboratories. The following are general considerations for maintaining QA/QC of samples obtained during a lab necropsy:

1) Keep all specimens cold throughout necropsy procedure.

2) Disinfect working surface prior to necropsy.

3) Set up all equipment necessary prior to commencing dissection, including separate containers for decontaminating discard items and utensils.

4) Proceed with necropsy according to established protocols.

5) Following necropsy, disinfect all work areas, equipment, microscopes, etc. involved in examination of tissues.

3. Routine Quality Control in the Parasitology Laboratory

a. Sample Tracking

1) All samples received in the Parasitology Lab are to be labeled with the proper CHR number attached to the sample container (i.e. bag of heads, whirl paks containing tissue samples, etc.)

2) Laboratory Data Log – All incoming parasitology samples must be logged into a Parasitology Lab data logging system designed to record the number and type of samples, and field sampling date. The log must contain designated spaces for logging each case out as it is completed, to include the date and initials of the employee completing the lab work.

3) Data Entry Forms – a form must be completed for all parasite assays and must include the CHR number, type of procedure used for analysis, and results.

4) All testing records must be initialed and dated by the employee performing the lab

work.

b. Contaminant Control – In addition to those listed in Section II.H.2.b., the following are measures to be taken to insure contaminant control in the parasitology lab:

1) Gloves and lab coats must be worn when handling and processing any parasitology samples.

2) Hands must be washed after removing gloves.

3) All parasitology lab waste must be autoclaved for a minimum of 20 minutes at 121^{o}C when disposing of waste materials exposed to all parasites. Heat disinfection is also effective for controlling myxosporean parasites as spore viability can be reduced to 0% with temperatures of 95^{o}C for 15 minutes (Turner, et al 1999). (When autoclaving large volumes of lab waste, time may need to be increased to insure that the core temperature of waste reaches 121^{o}C and is maintained for a minimum of 15 minutes).

4) Whenever possible, use disposable laboratory materials for assays to prevent contamination of lab ware (such as disposable paint filters for filtering pepsin-trypsin digest preparations).

5) Glassware, processing lab ware and other non-disposable or reusable lab materials must be soaked in a minimum concentration of 5000 ppm sodium hypochlorite solution for at least 10 minutes (Appendix F)

6) Store all clean glassware, equipment and reagents in designated areas free from exposure to parasite samples

c. Cross Contamination Control

1) Assays must be performed at designated parasitology work stations.

2) Work stations must be decontaminated prior to and after each lot of each case history with 5000 ppm chlorine solution (Appendix F).

3) Cover work areas with bench paper and change with each lot of each case history.

4) Work on one case history at a time, separating individual lots to prevent cross contamination between lots within a case history. Work areas, glassware and equipment used for assays must be disinfected between each lot of each case history.

d. Reagents

1) A chemical inventory must be maintained of all reagents used in the Parasitology Lab to meet the standards outlined in section II.E.2. (Reference stocks and reagents).

4. Routine Quality Control in the Bacteriology Laboratory

a. Sample Tracking

1) All samples received in the Bacteriology Lab are to be labeled with proper CHR

number attached to all racks containing culture tubes, and written on all Petri plates containing media.

2) Laboratory Data Log - All samples must be logged into the Bacteriology Lab data logging system designed to record the number and type(s) of samples, and the date each sample was taken in the field. The log must contain designated spaces for logging each case out as it is completed, to include the date and initials of the employee completing the lab work.

3) Data entry forms - for all assays conducted on each case, a form must be completed which contains at a minimum the CHR number, each sample identification number as well as description and results of all biochemical, morphological, and serological tests conducted and test results.

4) Daily testing records for each case must be initialed and dated by the employee performing the lab work.

b. Contaminant Control - In addition to those listed in Section II.H.2.b., the following are measures to be taken to insure contaminant control in the bacteriology laboratory:

1) Wash hands with antibacterial soap before and after lab work or spray with alcohol solution (Appendix F).

2) Dispose of used media (Petri plates and tubes) by autoclaving at 121^{o}C for 20 minutes. Media can then be safely discarded with the trash.

3) Glassware tops should be covered with foil prior to proper sterilization. Foil sheets to be used should be checked for pin-holes and tears, and proper fit must be ensured.

4) Soak depression-glass slides used for hanging drop motility assays in chlorine solution (Appendix F) prior to cleaning with laboratory grade detergent.

5) Soak used disposable loops, pipettes and other expendable items which become contaminated in chlorine solution before discarding in the trash.

c. General Aseptic Technique - Aseptic technique is necessary to avoid both infection and cross-contamination of microorganisms between the body and cultures of bacteria which are handled daily in the Bacteriology Lab. The primary goal of aseptic technique is to prevent the introduction of undesired microorganisms into the media and samples to be tested. The following includes materials and guidelines to ensure this goal is met:

1) Loops: Use sterile disposable loops whenever possible. Remove loops from packaging just prior to use and place in chlorine solution or hazard bag to be autoclaved immediately following use. Transfer of bacteria with loops or sterile swabs must be completed rapidly. At no time should the loop come in contact with any object other than the bacteria and the medium it is being transferred onto or into. If this should happen, dispose of the loop and get a new one to complete the transfer. Metal loops should be properly sterilized by heating to red heat between transfers. For sterilization of a used metal loop, it should be lowered

carefully into the flame to prevent splattering live organisms in the lab.

2) Avoid talking, coughing and sneezing when bacterial cultures and media are exposed to the air.

3) Petri Plates: Prior to use, the medium in each plate should be examined for possible airborne contamination which may have occurred during media preparation and/or storage. When working with plates, minimize the amount of time the lid is opened and the degree the lid is lifted.

4) Test Tubes: When working with tubes, open only one tube at a time, using the little finger of the loop hand to unthread and hold the cap (threads facing downward). Keep the time the cap is removed from the tube to a minimum.

5) Pipettes: Utilize sterile pipettes and pipette aids when transferring media, media components, serological supplies or bacterial cultures. Never pipette by mouth.

d. Cross-Contamination Control - In addition to the measures listed above, the following guidelines are followed to avoid cross contamination of microorganisms between case histories:

1) Each work station should contain only one case history at a time.

2) Work stations should be decontaminated thoroughly between cases (Appendix F).

3) Stocks of sterile media and sterile supplies are kept separately from areas of the laboratory where live organisms exist (incubators and workstations).

4) All tubes and assay containers from one case are to be racked separately from other cases, and labeled clearly with the CHR number.

e. Media and Reagent Preparation:

1) When working in a laminar flow hood allow the blower fan to operate at least 10 minutes prior to working under the hood. Spray the entire work surface and any materials to be used with 70% isopropyl alcohol or ethanol (Appendix F). Exposure of surfaces to UV light for 10 minutes before and after use is recommended for additional protection.

2) Media which require exposure while drying, such as agar plates, should be allowed to cool and harden within the aseptic environment of the laminar flow hood. Media should not be exposed to excessive amounts of ultra-violet light, as this can degrade some of the ingredients. **Warning:** Exposure to the UV light can cause skin and eye damage! Turn the UV light off whenever placing objects or working in the hood!

3) All reagents are transferred and prepared using aseptic technique.

4) All filter decontamination and dispensing of media and reagents is performed under a decontaminated laminar flow hood. A medium which is distributed to containers prior to autoclaving does not need to be dispensed under aseptic conditions.

5) All new lots of media and batches of reagents for biochemical testing of live bacterial cultures are tested using live bacterial control cultures maintained within the laboratory. Control cultures are obtained from American Type Culture Collection (ATCC) or from one of the reference labs listed in Appendix C.

6) All new batches of serological reagents are tested using both positive and negative controls.

7) All serological testing on sample material is performed side by side with positive and negative control material (cultures or tissues).

8) Identification and Confirmation of bacterial pathogens - it is important that identification of a bacterial agent be confirmed using specific techniques recommended in the appropriate Blue Book chapter, or by accepted protocols listed in Appendix D.

9) Note: laboratories utilizing live control organisms must follow appropriate containment levels according to the status of any agents considered to be exotic to the region the laboratory is located (for biocontainment standards see http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm)..

5. Routine Quality Control in the Virology Laboratory

a. Sample Tracking

1) All samples received in the Virology Laboratory are labeled with the CHR number attached to all relevant equipment (e.g. racks) containing sample material. CHR number is transcribed to all additional racks and micro titer plates used for processing that case through the lab.

2) Laboratory Data Log - All samples are logged into the Virology Lab log upon receipt in virology lab. The log is designed to record the number and type of samples, and the date each sample was taken in the field. The log must contain designated spaces for logging each case out as it is completed, to include the date and initials of the employee completing the lab work.

3) Data entry forms - for all assays conducted on each case, a form must be completed which contains at a minimum the CHR number, each sample identification number as well as description and results of all assays conducted.

4) Read micro titer plates every at least three alternating days per week, and on days when case histories are to close. Record and initial observations in the virology log and on the CHR. Indicate cell condition, presence or absence of contamination, toxicity or any apparent viral cytopathic effects (CPE), and condition of controls, including progression of CPE in virus positive controls.

5) Confirmation and identification of viral CPE – it is important that identification of a viral agent be confirmed using techniques recommended in the appropriate Blue Book chapter, or by accepted protocols listed in Appendix D.

6) Upon completion of the viral assay for a particular case history, results and dates are recorded on the data entry form and the CHR form. Also, append the data entry form to the CHR form.

b. Aseptic Technique - The goal of aseptic technique is to prevent the introduction of undesired microorganisms into the viral assay system. The goal of contaminant control is to prevent cross contamination of samples and to destroy any microorganisms present in samples, containers, or supplies prior to their disposal. The following steps will be taken to insure both aseptic technique and contaminant control:

1) When working in a laminar flow hood allow the blower fan to operate at least 10 minutes prior to working under the hood. Spray the entire work surface and any materials to be used with 70% isopropyl alcohol or ethanol (Appendix F). Exposure of surfaces to UV light for 10 minutes before and after use is recommended for additional protection.

2) Glassware stored in the virology lab first must have been rinsed thoroughly with tap water, rinsed three times with tissue culture grade water, covered and autoclaved (minute amounts of residual detergents and reagents can adversely effect tissue culture). Glassware used for harsh chemicals (chlorine, acetone, HCl, etc.) should be labeled as such and kept separate from glassware used for cell culture components. Use laboratory glassware detergent when necessary and rinse thoroughly.

3) Sterility checks must be done on media prior to addition of antibiotics. When contamination is detected, all suspect media should be discarded and each media component should be checked separately to determine the source of contamination.

4) Sterility checks involve inoculation of tryptic soy or brain heart infusion broth under the sterile hood and checking for bacterial growth for 48 hours. Sterility checks should be completed before use of media components and before adding antibiotics to complete media. Documentation of sterility checks for each batch of medium prepared should be kept on file.

5) Anything that comes in contact with samples or known virus must be disinfected or autoclaved before reuse or disposal.

6) Chlorine solution used for disinfection should be changed weekly (see Appendix F for procedures and alternative disinfection).

c. Media and Reagents

1) All chemicals, reagents and media components must be dated upon receipt in the virology lab. Whenever media, reagents or antisera are prepared, label with the date of preparation, content, and other information as described in II.E.2 (Reference stocks and reagents).

2) Each bottle, tube, flask or plate in a virology refrigerator or incubator must be properly labeled or will be discarded.

3) An inventory log will be maintained and updated on a bimonthly basis. The inventory log is primarily for much-used disposable supplies, but other supplies such as media components, balanced salt solutions, and versene salts must also be monitored regularly.

d. Cell Culture Stocks - Maintenance of healthy tissue cultures is imperative for all virology assays. The following are procedures routinely employed to ensure that healthy, sensitive cultures are used for each assay:

1) Prior to use, check each culture for possible contamination, the appearance of the cell sheet, and the pH of the medium. If the medium is turbid, bacteria or yeast are probably present. Cotton-like tufts indicate fungal contamination. Do not use contaminated cultures. Discard all suspect cultures and check into the source of contamination.

2) Examine cells microscopically to make certain that cells are healthy and the sheet is confluent.

3) Allow recently thawed versene/trypsin solution (V/T) to warm to room temperature (15-20°C). If too warm, trypsin, as an enzyme, may work too quickly and damage cells or cause clumping.

4) Label new flasks with cell line, new passage number (old number plus one), medium lot number, and date.

5) Cell lines for virology are sent to a reference laboratory for sensitivity testing and mycoplasma contamination checks according to Appendix B-4. Cell lines can be obtained from sources listed in Appendix C. Use of the most sensitive cell lines for the viruses to be detected is encouraged.

6. Routine Quality Control for the Immunoassay Laboratory (ELISA assay used to detect *Renibacterium salmoninarum* or other pathogens that may apply)– The ELISA technique discussed in the following section was developed and standardized by researchers at the USGS Western Fisheries Research Center in Seattle. Refer to Blue Book Bacterial Kidney Disease Methods Appendix 4 (1.14.A4) for controls and Appendix 5 (1.14.A5) for the detailed assay procedure. This ELISA uses reagents and antibodies presently produced by Kirkegaard and Perry Laboratories (http://www.kpl.com). It is a highly sensitive and complex protocol which requires careful preparation of assay reagents and strict adherence to a detailed protocol. Several steps are included in the overall methodology of ELISA to ensure quality control of reagents, consistent test results, and optimum performance of the assay, and are included in appendices to the protocol. **There are other ELISA systems which are commercially available and approved by USDA for certain fish pathogens. These kits must be used according to specific manufacturer's guidelines.**

a. Tissue collection and processing – fish tissues are collected and processed with care to avoid contamination of kidney tissue with foreign material or gastrointestinal contents (fish feed) which could cause false-positive reactions in the ELISA. Sterile, individual instruments are used for each tissue collected to prevent cross-contamination between positive and negative kidney tissues.

b. Sample Tracking - it is important that all individual samples or pools of samples processed for ELISA be recorded and tracked.

1) The CHR number, individual sample and pool numbers, controls and blanks are assigned to a particular well on the 96 well ELISA plates prior to setting up the assay. These are recorded on an ELISA template data sheet, and should be referenced during loading of samples and control materials onto each plate to ensure accuracy of results.

c. Checkerboard titration (optimization of antibody dilution)- the checkerboard titration assay is performed first to test antibody reagent quality and ensure optimum assay performance. Antibody testing, using a dilution matrix, allows the optimum antibody dilution to be determined for a specific lot of antibody reagents. This step ensures consistent ELISA results over time regardless of potential changes in antibodies supplied by the manufacturer. Follow the checkerboard titration assay as described in established protocols (Appendix D).

d. Standardization of antibody reagents: optimum dilutions are determined for the coating antibody (CAb) and horseradish peroxidase-labeled secondary antibody (HRP-Ab) by checkerboard titration assay. Once optimum working dilutions are established, antibody reagents are pooled and aliquoted into small working volumes. Aliquots are frozen for future use.

e. Performing the assay: the assay is performed following an established protocol (Appendix D). Special care is taken to ensure accurate dilution of antibody reagents, placement and isolation of control wells on each plate, and adherence to precise incubation periods. Dedicated equipment such as pipetters, reagent dispensing cassettes, and glassware are additional precautions that are taken to ensure accurate and consistent test results. Controls: established guidelines for the control plate, control wells on each subsequent plate, and the application of the standardized Negative Control (NC) are included in the protocol to ensure optimum assay performance and consistent data analysis. One full control plate is included for every five (5) plates in an ELISA run. The control Plate has at least two replicates of the following reagents:

1) Positive Control – Positive antigen preparation.

2) Negative Control - Negative Kidney tissue, tested by ELISA and PCR

3) Blank - Wells receive PBS-T20 diluent only and serve as a Blank negative control.

4) Conjugate Control - Wells receive Coating Solution <u>without</u> Antibody and serve as control for non-specific binding of the HRP-conjugated Ab to the well surfaces or the Coating Antibody.

5) Substrate Control - Wells receive Milk Diluent <u>without</u> HRP-Ab and serve as the control for non-enzymatic production of the ABTS color reaction.

6) Each subsequent plate also contains one column of control wells: 4 wells of Negative Control tissue and 4 Blank wells. The first Control Plate and control wells on subsequent plates allow close monitoring of assay performance between

plates during a single assay, and between assays performed on various dates.

f. Positive threshold: for data interpretation, an appropriate threshold is determined according to the desired sensitivity and particular group of samples being tested. For example, two standard deviations above the mean OD value of the Negative Control are used to establish the positive threshold used for samples collected by fish health labs of the U.S. Fish and Wildlife Service. The Negative Control tissue consists of salmonid kidney tissue tested by ELISA and PCR and found to be negative for *Renibacterium salmoninarum* antigen or DNA. The mean OD value of replicate sample wells is compared to the threshold value to determine the positive or negative status of a test tissue..

g. Annual testing program: a Quality Control program for ELISA among fish health laboratories should include an annual testing program. Sample sets with known OD values are received from an appropriate reference laboratory and tested. Results are summarized and any variation from acceptable ranges will be investigated for the source of variation and corrective steps will be taken. An annual testing program will ensure consistent and comparable test results between various fish health laboratories in North America.

7. Routine Quality Control for the Molecular Biology (PCR) Laboratory - The Polymerase Chain Reaction[1] technique (PCR) is currently used in many FHLs to corroborate results of screening procedures. PCR employs oligonucleotide primers to amplify base pair segments of genes specific for the target pathogen. Reverse Transcriptase-PCR (RT-PCR) employs an initial reverse transcriptase step so that complimentary DNA can be amplified from viral RNA. DNA or RNA is extracted from various fish tissues and laboratory assay products, such as cell culture supernatant containing viral agents, and amplified using forward and reverse primer sets. In some instances, the amplified product is re-amplified using an additional "nested PCR" technique. The DNA products are then visualized by gel electrophoresis. Detailed PCR procedures for each target pathogen are followed as written in accepted protocols (Appendix D). PCR Quality assurance and control procedures outlined below are extremely important when performing all PCR assays:

a. General Considerations:

1) Work surfaces should be decontaminated by washing with 10% chlorine bleach to hydrolyze possible DNA contaminants. Likewise, tube racks should be soaked in 10% chlorine for 30 minutes prior to use. Ultra violet decontamination is also recommended where possible.

2) Store RNA and DNA samples and templates separately from PCR reagents.

3) Employ aerosol resistant pipette tips and/or positive displacement pipetters during all extraction and amplification procedures. Separate pipetters should be dedicated for use with reagents only and amplified products only.

4) One aerosol drop of amplified DNA contains thousands of strands of DNA which can easily contaminate reagents. Therefore, three separate areas of lab space are

[1] PCR is Patented by Hoffman-LaRoche, Inc.

optimal to reduce the risk of contamination:

i. Master Mix (MM) area with UV hood - for mixing and aliquotting master mix reagents. Supply area with dedicated pipetters, ideally positive displacement Pipetter/tips. No samples or amplified DNA is to be handled in or near this area!

ii. Sample Preparation Area - for loading of extracted (template) DNA from samples (also with a UV hood).

iii. Amplified DNA Area - supplied with pipetter dedicated for **amplified PCR product ONLY.** Handle any amplified PCR products in this area only, and clean area and equipment thoroughly with "DNA-away" type solutions after working with amplified DNA Use four separate work areas for RNA and DNA extraction, reagent preparation, sample loading, and PCR amplification. Separate reagent and sample loading work surfaces must be equipped with UV decontamination.

5) Change gloves frequently when handling samples during all procedures and dispose of used gloves immediately.

6) Dispose of trash containing amplified DNA products frequently.

b. Sample Collection - When tissues are collected directly from fish for PCR assays, samples should be collected on a clean bench-top which has been disinfected using a 10% chlorine solution if possible.

1) Use sterile collection utensils between each lot of fish tissue collected. If data from individuals is of concern, use separate utensils for each individual. **Alcohol will not effectively decontaminate DNA from utensils.** If individual utensils are not available, flaming metal utensils between sampled will effectively remove contaminants from previous samples.

2) Keep samples cold and freeze as soon as possible at -70^{o}C until processing can be accomplished.

3) RNA is extremely sensitive to enzymes existent in most sample tissues. Samples collected for RT-PCR should be frozen immediately, and transported on dry ice. An RNA stabilizing reagent can also be used and does not require that samples be frozen immediately if manufacturer's recommendations are followed.

c. Extraction of DNA or RNA from samples - All general considerations should be employed including the following:

1) Use micro centrifuge tubes with sealed or locking lids so that contaminates do not escape during heating cycles that might cause breaching of lit seal.

2) Use the accurate amount of tissue suggested by the extraction kit manufacturers. If this is exceeded, proper lysis of tissues will not be accomplished.

3) Controls: all extractions should employ a negative control (sterile RNase/DNase free water, or negative tissue), and a positive control (known positive tissue) to be

run along-side of unknown samples. These controls will allow for detection of contamination as well as assure that the extraction was successful.

4) Quantification of extraction product - it is advisable that extracted products be measured using a spectrophotometer to ensure that enough DNA or RNA was successfully extracted. Refer to individual protocols for guidance on the amount of DNA or RNA recommended for each PCR reaction and dilute as needed.

d. Running the PCR - All general considerations should be employed including the following:

1) Decontaminate and UV reagent and sample loading workspace both before and after use for at least 15-30 minutes (be aware that over-use of UV lamps can break down plastics).

2) Master Mix (MM): dispense water first and TAQ polymerase last (the TAQ is the most unstable of the ingredients in the MM). Mix ingredients well before dispersing aliquots, as the TAQ is stored in a glycerol-based buffer which sinks to the bottom of the MM tube.

3) All reagent batches should be marked and recorded for each test run so they can be checked if problems occur with the assay.

4) Primers: newly received primer batches should first be tested on known positive/negative controls.

5) Controls: prepare enough MM for extraction positive/negative controls AND PCR positive/negative controls (4 extra samples). The **extraction controls** will ensure that the extraction was successful and contamination did not occur. The **PCR controls**, using sterile water (negative) and known positive DNA from previous extraction (positive) will ensure that the PCR process was successful and that contamination did not occur.

6) Before loading into thermal cycler, give tubes a “quick-spin” to ensure that all reagents and sample are drawn down from sides of tube.

7) It is recommended that all cycles begin with a 2 minute pre-dwell at 94^{o}C. This allows for all DNA to denature into single stranded form at the beginning of cycling, and reduces primer dimers and mis-priming.

8) After cycling, tubes may have a ring of condensation near rim of cap. Before opening tubes, perform a “quick-spin” to draw this fluid down into the reaction area of the tube and reduce the possibility of aerosol contamination upon opening tubes.

9) Document gel lane assignments for each sample and controls, and allow for at least one lane for a DNA ladder reference.

10) Photodocument all PCR gels and keep with the cases run (or provide reference for finding the photodocumentation.

III. **Laboratory Safety**

Copies of MSDS's (Material Safety Data Sheets) for all chemicals and reagents in the laboratory are kept on file and within easy access and viewing for all personnel. In addition, each laboratory maintains a Safety Manual and Chemical Hygiene Plan the safety manual specific for that laboratory. All personnel are to follow safety precautions published within MSDS's for each reagent used at the laboratory.

Zoonotic agents may be in exudates, tissues, and environmental samples (soil and water). Direct contact of skin or mucous membranes with infectious materials, ingestion, and accidental inoculation are the primary laboratory hazards associated with sampled material and cultures. Aerosols created during the manipulation of tissue homogenates also pose a potential infectious hazard to laboratory personnel.

Biosafety level 2 practices are recommended when working with clinical materials (tissues and bodily fluids). Laboratory personnel need not confine established cultures with low aerosol potential to an approved safety cabinet. Laboratory biosafety level 2 criteria are outlined in *Biosafety in Microbiological and Biomedical Laboratories*, 1999 U.S. Department of Health and Human Services, Public Health Services, Center for Disease Control, and National Institutes of Health, 4th edition, U.S. Government Printing Office, Washington D.C. (http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm)

IV. **Review of Guidelines**

These guidelines are to be reviewed and amended on a regular basis by the AFS/Fish Health Section QA/QC Committee. Comments are to be submitted to the Committee Chairperson. Changes, and justification for no change, will be compiled and changes will be implemented during regular Blue Book revisions.

APPENDIX A
INTERNAL AND EXTERNAL AUDIT PROGRAM

I. Introduction

The goal of a Quality Control program is to assure that all of the data generated by a scientific laboratory be scientifically valid, defensible and of known precision and accuracy. To assure that the obtained data meets these criteria, both internal and external audits should be periodically conducted under the direction of the laboratory's Quality Assurance Coordinator and Director. The information acquired from the audits will be used to estimate the quality of the analytical data, to identify deficiencies, and determine and implement corrective action.

II. Purpose

Internal and External auditing will provide an evaluation and establish the effectiveness of QA/QC programs. The audits will ensure that the requirements outlined in the QA/QC program are followed to provide quality of analytical data in terms of objectives for precision, bias, representation, comparability, and completeness.

A. The Internal Auditing Plan requires a periodic laboratory review by the Quality Assurance Coordinator (QAC) to include:

1. field and sampling activities review
2. laboratory activities review
3. laboratory performance review

Results of the internal audit will be reported to the FHL Director for assessment and correction of the observed deficiencies.

B. An External Auditing Plan should require a periodic laboratory review by a qualified team of individuals who are not employed by the FHL to be reviewed. The purpose of this assessment is to provide insight to QA/QC deficiencies not detected by the internal audit plan. The results of the external assessment should be submitted to the FHL Director for assessment and necessary corrective action.

C. Check lists are provided for guidance in performing these reviews

III. Objectives (Internal Audit Plan)

A. Field and Sampling Activities Review: the review of field and sampling activities shall be conducted by the QAC or by one or more persons knowledgeable in the activities being reviewed including:

1. Completeness of field sampling reports: this review determines whether all requirements for field sample collections have been fulfilled as outlined in accepted protocols (Appendix D). Completed records shall be maintained for each collection including sample receipt and laboratory tracking.
2. Identification of Sample Integrity: this review evaluates field sample collections to assure

samples were collected in accordance with accepted protocols (Appendix D). This will include:

a. reviewing whether samples were collected under the direction of a qualified individual.

b. identifying whether lethal and non lethal sampling were obtaining using humane methods by appropriate protocols (i.e. use of MS222 for euthanasia).

c. evaluating whether appropriate tissue was collected for each sample assay.

d. identifying whether appropriate preservation was used for collected tissues for transport to laboratory centers for processing.

e. reviewing proper and timely shipment or transport of samples to laboratory centers for processing.

3. Validation of Field Analysis: this review will evaluate whether field data obtained meets QA/QC criteria including reviews documenting instrument calibration (such as pH meters, O_2 Saturometers for water sampling), or use of commercial laboratory field test kits with the manufacturer's recommended QA/QC for onsite data collection.

B. Laboratory Activities Review: the review of laboratory activities shall be conducted by the QAC or by one or more persons knowledgeable in the activities being reviewed including:

1. Completeness of laboratory records: this review determines that incoming samples have been processed and documented according to accepted protocols (Appendix D). The review shall include an audit of completed records of all testing analysis and associated QC samples.

2. Evaluation of Sample Management: this review identifies that proper procedures were used in receipt, handling and storage of incoming samples.

3. Evaluation of General Laboratory Techniques: this review identifies that laboratory operations not addressed by accepted protocols are conducted using acceptable methods. This includes but is not limited to glassware cleaning procedures, use of analytical balances, pipetting techniques, use of sonicators, water baths, and general laboratory operations. . Other objectives of this review are to identify proper use and maintenance of laboratory apparatus. This shall include but not be limited to:

a. determining that the appropriate apparatus is used for the targeted analytical assay and that instrument location is suitable.

b. determining that equipment is adequate and meets the needs for the capacity of average number of samples analyzed.

c. determining that analytical equipment and other apparatus used is periodically cleaned, inspected, calibrated and serviced as deemed necessary (Appendix E).

4. Evaluation of Reagent and Standards Preparation: this review addresses whether all reagents and standards meet the specifications of accepted protocols (Appendix D). The review also addresses whether preparation and dilutions of these reagents/standards are properly recorded, appropriately labeled and stored in suitable containers under

recommended storage conditions.

5. Evaluation of analytical data with respect to method detection limits (MDL).

   a. ELISA – when the established protocols are employed, all data points derived from positive control titration replicates, as well as substrate negative controls are analyzed against established parameters to determine that the assay equipment is performing appropriately during every ELISA run.

A. Laboratory Performance Review: An internal laboratory performance review can be conducted as deemed appropriate or necessary. This review will evaluate the "performance" of the standard protocol of an individual assay through analysis of unknown samples or reference samples (samples containing a known target pathogen) submitted by the internal laboratory auditor (i.e. QAC). These samples can be submitted as blind samples from an independent outside source. The result of this audit provides documentation of bias of the analytical process for that individual assay for the target pathogen.

IV. **Objectives: (External Audit Plan)**

The external audit team should consist of at least three members external to the FHL to be reviewed, with knowledge of the activities to be reviewed. Since the purpose of external audits is to identify problems overlooked or undetected by the internal reviews, objectives of the external auditing committee shall include but not be limited to all objectives listed under the Internal Auditing Plan.

V. **Proficiency and Ring Testing Programs**

A. Proficiency and ring testing programs can be implemented by individual FHLs with the desire to evaluate their proficiency in performing standard protocol of a particular assay. It involves the analyses of sets of blind samples (infection status of tissues known only to the supplier) submitted to the FHL by an outside source. Participation in a ring testing program would allow the FHL to measure their level of proficiency against other FHLs performing testing on identical blind sample sets. Individual FHLs can arrange with commercial or research facilities to provide blind samples for proficiency testing, or can coordinate with other FHL's to accomplish multi-laboratory ring testing as well. Proficiency testing can also be achieved through contract of commercial labs which provide such services (see http://www.phppo.cdc.gov/mlp/EQA_qlist.asp).

B. Samples for a proficiency testing program would be processed according to the appropriate protocols by the FHL within a given time frame, and results reported to a reporting individual for analysis.

1 Evaluation of assay performance problems should involve, at minimum, the determination of the following:

   a. reagent quality (storage, preparation, shelf-life)
   b. quality, source and performance of standards and controls
   c. equipment calibration and maintenance

d. equipment performance
e. personnel training and performance
f. sample integrity (storage, processing, etc.)

APPENDIX B-1
FORMS AND DOCUMENTATION

Suggested Documentation

Documentation Files

- ✓ Technical Qualifications File – contains resume of qualifications, skills, experience and references to training received and conferences attended for each current staff member.
- ✓ Equipment Manuals
- ✓ Equipment maintenance and calibration/certification (2 year retention)
- ✓ Reagent and materials source information
- ✓ Material Safety Data Sheets
- ✓ Safety Audits
- ✓ QA/QC Audits
- ✓ Proficiency testing results
- ✓ QA/QC Quarterly meeting minutes
- ✓ Case Histories (7 year retention)
- ✓ Case History Reports (7 year retention)
- ✓ General communications relating to Case Histories

Documents

- ✓ AFS-FHS (American Fisheries Society-Fish Health Section). 2004. FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2004 edition. AFS-FHS, Bethesda, Maryland.
- ✓ OIE Manual of Diagnostic Tests for Aquatic Animal Diseases, 4th Edition. 2003. Office International Des Epizooties. Paris, France. (http://www.oie.int)
- ✓ Regional Fish Health Policies
- ✓ State/Provincial Fish Health Laws and policies
- ✓ Station Safety Plan
- ✓ Hazard Communication Plan
- ✓ Chemical Hygiene Plan

Logs to Maintain

- ✓ Equipment Maintenance
- ✓ Separate activity logs for virology, bacteriology and other laboratories (this will include documentation of protocol deviation and justification, as approved by FHL Director)

Computer files to Maintain

- ✓ Case History Database
- ✓ Reagent and media inventory
- ✓ Supply inventory

APPENDIX B-2
FORMS AND DOCUMENTATION

Audit - Field and Sampling Checklist

Quality Control Item or Procedure Noted	Suitable √	Not Suitable (Explain)
Samples are collected by, or under supervision of AFS/FHC Fish Health Inspector or veterinarian.		
Sterile utensils, loops, containers, tubes, vials used where appropriate.		
Samples labeled appropriately.		
Proper use of disinfectants.		
Proper means of disposal of contaminated waste.		
Proper means of specimen collection including humane methods for animal handling		
Sample vessels are appropriately labeled.		
Sample information recorded for case information.		
Specimens, tissues, and media are kept cool where appropriate throughout necropsy and transfer to laboratory.		
Tissue samples are obtained in a manner which avoids contamination.		
Cross contamination between samples prevented.		
Cross contamination between lots prevented.		
Sample transfer from field to lab – samples packaged to avoid leakage and cross-contamination during transfer.		

Attach notes and documentation of audit.

APPENDIX B-3
FORMS AND DOCUMENTATION

Audit - Laboratory Documentation/Sample Tracking Checklist

Documentation	Suitable √	Not Suitable (Explain)
Approved Laboratory and Diagnostic protocols available to all staff.		
Case History information entered onto CHR appropriately.		
Case History Record form printout available with appropriate information and attachments for each case.		
All racks and/or vessels containing processed samples are labeled with CHR number – all tubes with sample/lot I.D.		
Sample logs maintained in individual laboratories (bact., vir., etc) for each case.		
All data generated from all sample assays documented, in ink, on sample data forms.		
Results properly reported and dated on CHR, with appropriate sample data forms attached.		
Technical Qualifications File on all personnel.		
Appropriate equipment labeled with date of last calibration/certification.		
Equipment maintenance log with minimum of 2 years record of certification/calibration:		
Laminar flow hoods		
Fume hoods		
Balances		
Micro-pipetters		
Spectrophotometers		
Thermocyclers		
Thermometers		
Meters (pH, D.O., etc)		
CHR maintained in FHL files for minimum of 7 years.		
Material Safety Data sheets readily available for all materials.		
All reagent containers properly labeled.		

APPENDIX B-4
FORMS AND DOCUMENTATION

Audit - Laboratory and Assay Performance Checklist
BACTERIOLOGY

Quality Control Item or Procedure	Suitable √	Not Suitable (Explain)
Media Preparation		
Gram staining procedures		
Biochemical procedures and systems (API/other)		
Use and maintenance of control isolates		
Antibiotic sensitivity procedures		
Confirmatory Procedures		
Fluorescent Antibody Procedures:		
Sample preparation, fixation and storage		
Conjugate preparation and filter sterilization		
Staining procedures		
Positive/negative controls		
Slide examination and quality of preparation		
Proper disposal of wastes		
Overall aseptic technique, disinfection and sterilization procedures/equipment		
Level II Lab containment standards and building hygiene		

APPENDIX B -5
FORMS AND DOCUMENTATION

Laboratory and Assay Performance Checklist

VIROLOGY

Quality Control Item or Procedure	Suitable √	Not Suitable (Explain)
Maintenance of stock cell lines-passage if confluent cell monolayers		
Semi-annual mycoplasma screening and virus susceptibility checks of fish cell lines		
Media preparation and contamination checks		
Glassware cleaning procedures to ensure cell health		
Optimum cell lines, incubation temperatures and times used		
Centrifugation techniques		
Viral plate reinoculation procedures		
CPE examination frequency/virus identification		
Virus confirmation procedures		
Proper disposal of wastes		
Overall aseptic technique, disinfection and sterilization procedures/equipment		
Level II Lab containment standards and building hygiene		

PARASITOLOGY – Refer to Parasitology Protocols

Quality Control Item or Procedure	Suitable √	Not Suitable (Explain)
Sample storage		
Pepsin/trypsin digest procedure		
Positive controls		
Disinfection and cleaning between samples		
Confirmation procedures (PCR or histology)		
Proper sample archiving		
Slide preparation, fixation and storage		
Staining procedures		
Microscopic procedures		
Proper disposal of wastes		
Overall aseptic technique, disinfection and sterilization procedures/equipment		
Level II Lab containment standards and building hygiene		

APPENDIX B-6
FORMS AND DOCUMENTATION

Laboratory and Assay Performance Checklist
ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Quality Control Item or Procedure	Suitable √	Not Suitable (Explain)
Reagent preparation and storage		
Quality of glassware (acid washed, dedicated)		
Sample preparation		
Utilization of positive/negative controls		
ELISA plate – use of appropriate standard controls and replicates		
Adherence to protocol		
Interpretation of results		
Confirmation		
Proper disposal of wastes		
Overall aseptic technique, disinfection and sterilization procedures/equipment		

Protocol – Proper procedure can be referenced in appropriate USFWS Operational Handbook protocols.

APPENDIX B-7
FORMS AND DOCUMENTATION

Laboratory and Assay Performance Checklist
POLYMERASE CHAIN REACTION (PCR)

Quality Control Item or Procedure	Suitable √	Not Suitable (Explain)
Reagent preparation and storage		
Dedicated pipetters		
Aerosol barrier and/or positive displacement pipette tips utilized.		
Latex or nitrile gloves utilized and changed appropriately		
Appropriate laboratory set-up		
Sample handling/preparation		
DNA/RNA Extraction procedures		
Proper controls		
Quantification of template		
Master mix preparation: documentation of reagent lots and concentration		
Sample loading and controls		
Proper handling of tubes between first and second rounds in nested PCR		
Proper centrifugation		
Thermocycler properly programmed		
Gel lane documentation and loading		
Gel photodocumentation		
Interpretation of results		
Proper storage and documentation of samples, templates and PCR products		
Proper disposal of wastes		
Overall aseptic technique, disinfection and sterilization procedures/equipment		

APPENDIX C
North American Reference Laboratories

The following chart contains facilities that can be contacted for information, expertise and services listed:

Facility Information	Contact Information	Services/Expertise
American Type Culture Collection (ATCC) P.O. Box 1549 Manassas, VA 20110-2209	[To be named] Phone 703-365-2718 Fax 703-365-2730 http://www.atcc.org	Source for pathogen control isolates, cell culture stocks, reagent controls
Atlantic Veterinary College Aquatic Diagnostic Services 550 University Ave. Charlottetown, Prince Edward Island, Canada. C1A 4P3	Dr. David Groman Phone 902-566-0864 FAX 902-566-0723 aquaticdx@upei.ca	Salmonid and other finfish diseases
Auburn - Dept. of Fisheries and Allied Aquacultures Auburn University Auburn, AL 36849	John Grizzle, Ph.D. Phone 334-844-3474 FAX 334-844-9208 jgrizzle@acesag.auburn.edu	Largemouth Bass Virus
Cornell University -Aquatic Animal Health Program Dept. Microbiology and Immunology College of Veterinary Medicine Cornell University Ithaca, NY 14853	Paul R. Bowser, Ph.D Phone 607-253-3365 FAX 607-253-3384 prb4@cornell.edu Internet: http://web.vet.cornell.edu/Public/FishDisease/AquaticProg/	Finfish diseases
Department of Fisheries and Oceans Canada Fish Health and Parasitology Section 3190 Hammond Bay Road Nanaimo, British Columbia Canada V9T 6N7	Dr. Susan Bower (Shellfish Health Unit) Phone 250-756-7077 bowers@dfo-mpo.gc.ca FAX 250-756-7053 Dorothee Kieser (Fish Pathology Program) Phone 250-756-7069 Fax 250-756-7069 kieserd@dfo-mpo.gc.ca	Shellfish Diseases OIE Designated Reference Lab Salmonid and other finfish diseases; Approved lab under the Canadian Fish Health Protection Regulations
Department of Fisheries and Oceans Canada Fish Health unit 501 University Crescent Winnipeg, Manitoba Canada R3T 2N6	Brian Souter Phone 204-983-5125 FAX 204-984-2402 souterb@dfo-mpo.gc.ca	Freshwater finfish viral & parasite diseases

APPENDIX C -2
North American Reference Laboratories

Facility Information	Contact Information	Services/Expertise
Department of Fisheries and Oceans Canada Gulf Fisheries Center, Fish Health Unit P.O. Box 5030 (343 University Ave) Moncton, New Brunswick Canada E1C 9B6	Anne-Margaret MacKinnon Fish Health Section Phone 506-851-6081 mackinnona@mar.dfo-mpo.gc.ca Mary Stephenson (Shellfish Health Unit) Phone 506-851-6983 stephensonm@dfo-mpo.gc.ca FAX 506-851-2079	Salmonid and other finfish diseases; Molecular diagnostics Shellfish Diseases
Hawaii Institute of Marine Biology, School of Ocean and Earth Sciences Technology University of Hawaii, P.O. Box 1346, Kaneohe, Hawaii 96744,	Dr J.-A. Leong Phone: 808-236-7401 Fax: 808-236-7443 joannleo@hawaii.edu	OIE Reference Laboratory for IHN
Micro Technologies, Inc. 41 Main St. Richmond, ME 04357	Deborah Bouchard Phone 207-737-2637 FAX 207-737-4504 dbouchard@microtechnologies.biz	USDA Approved; Diagnostic and PCR Services; Shellfish and Finfish Inspection Services
Mississippi State Fish Diagnostic Laboratory College of Veterinary Medicine, Mississippi State University Box 9825 Spring Street, Mississippi 39762	Dr L.A. Hanson Phone: 662-325-1202 Fax: 662-325-1031 hanson@cvm.msstate.edu	OIE Reference Laboratory for Channel catfish virus disease and Enteric septicemia of catfish (*Edwardsiella ictaluri*)
Oregon State Center for Fish Diseases Research, Department of Microbiology 220 Nash Hall Oregon State University Corvallis, Oregon 97331-3804	Dr M. Kent Phone: 541-737-4441 Fax: 541-737-0496 michael.kent@orst.edu	OIE Reference laboratory for Piscirickettsiosis (*Piscirickettsia salmonis*)
Pisces Molecular, LLC 2200 Central Avenue, Suite F Boulder, CO 80301	John Wood, PhD Phone 303-546-9300 Fax 303-546-9400 jwood@pisces-molecular.com	Molecular diagnostic services, PCR assay research & development, strain genetic identification & analysis.
USDA Agriculture Research Service HKD Stuttgart National Aquaculture Research Center P.O. Box 1050 Stuttgart, AR 72160	Andrew J. Mitchell, Ph.D. Phone 870-673-4483 FAX 870-672-7710 dmitchell@spa.ars.usda.gov	Fish Disease Research
U.S. Fish and Wildlife Service Fish Health Centers Nine centers located throughout the USA	USFWS Fish Health Center locations can be obtained on the internet at: http://fisheries.fws.gov/FHC/FHCcu.htm	General and regional fish disease information

APPENDIX C-3
North American Reference Laboratories

Facility Information	Contact Information	Services/Expertise
U.S. Geological Survey National Fish Health Research Laboratory 11700 Leetown Rd. Kearneysville, WV 25430	Frank Panek Phone 304-724-4430 FAX 304-724-4435 frank_panek@usgs.gov	Fish Disease Research
U.S. Geological Survey Western Fisheries Research Center 6505 N.E. 65th St. Seattle, WA 98115	Dr. James Winton Phone 206-526-6282 Fax 206-526-5564 jim_winton@usgs.gov	Fish Disease Research; OIE Reference Laboratory for IHN and BKD
UC-Davis, Department of Medicine School of Veterinary Science University of California Davis, CA 95616	Dr. Ron Hedrick Phone 916-752-3411 rphedrick@ucdavis.edu	Fish Disease Research;
University of Maryland VA-MD Regional College of Veterinary Medicine Aquatic Animal Health Center 8075 Greenmead Drive College Park, MD 20742-3711	Ana M. Baya Phone 301-314 6837 FAX 301-935-6072 ambaya@wam.umd.edu	USDA Approved; Diagnostic Services
VA Tech - Dept. Biomedical Sciences and Pathobiology VA-MD Regional College of Veterinary Medicine Virginia Polytechnic Institute and State University Phase III, Duck Pond Dr. Blacksburg, VA 24061	Stephen A. Smith, D.V.M., Ph.D. Phone 540-231-5131 FAX 540-231-6033	Fish Disease Research; Diagnostic Services
Washington Animal Disease Diagnostic Laboratory College of Veterinary Medicine Washington State University PO Box 647034 Pullman, WA 99164-7034	Dr. Kevin Snekvik Phone 509-335-9696 FAX 509-335-7424 ksnek@vetmed.wsu.edu waddl@vetmed.wsu.edu Internet: http://www.vetmed.wsu.edu/depts_waddl/aquatic.asp	USDA Approved; Inspection and Diagnostic Services; Molecular diagnostics
Office International des Epizooties (OIE) Aquatic Animals Commission **Note: OIE Reference Labs must be reported to upon initial confirmation of an exotic pathogen in any species of fish in the US or diseases in commercially reared fish involved in international trade.**	OIE Reference Laboratories and designated Experts for aquatic animal diseases on the Internet: http://www.oie.int/fdc/eng/Diseases/en_reflabslist.htm Email: rr.americas@oie.int or fdc@oie.int	Lists all OIE reference labs and expertise in aquatic animal diseases located throughout the world.

APPENDIX D
SOURCES FOR FISH HEALTH ASSAY PROTOCOLS

American Fisheries Society, Fish Health Section. 2002. Suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 5th edition. American Fisheries Society, Fish Health Section, Bethesda, Maryland.

OIE Manual of Diagnostic Tests for Aquatic Animal Diseases, 4th Edition. 2003. Office International Des Epizooties. Paris, France. (http://www.oie.int)

Handbook of the U.S. Fish & Wildlife Service Aquatic Animal Health Procedures and Protocols. 2002. USFWS, National Fish Hatchery System, Arlington, VA (http://policy.fws.gov/fh_handbook/toc.htm)

50 CFR Chapter 1, Part 16, Code of Federal Regulations, U.S. Government Printing Office, Washington D.C. 2003. (http://www.access.gpo.gov/nara/cfr/cfr-table-search.html)

Fish Health Protection Regulations Manual of Compliance. Department of Fisheries and Oceans.1984.Fish Health Protection Regulations: manual of compliance.Fish. Mar. Serv. Misc. Spec. Publ. 31 (Revised): 32 p. (http://www.dfo-mpo.gc.ca/science/aquaculture/aquaculture_e.htm)

APPENDIX E
EQUIPMENT CALIBRATION/MAINTENANCE

The following chart provides a guide for calibration of standard equipment used in the Fish Health Laboratory. All Intervals suggested are subject to individual manufacturer and/or equipment manual.

EQUIPMENT	EXTERNAL CALIBRATION INTERVAL	INTERNAL CHECK INTERVAL	PARAMETERS TO CHECK	INSTRUMENTS REQUIRED
Autoclaves		Daily	Temperature & pressure sustained during operation	Pressure gauge, safety valve, temperature gauge, indicator strips
Balances	3 years	Weekly	Linearity, zero point, accuracy, level	Calibration reference weights
Biosafety Cabinets/hood	1 year	Weekly	Air flow, UV bulbs	Anemometer, vacuum meter
Centrifuges		During operation	Balance, speed, temperature, timer	Manufacturer provided
Electrophoresis Units		Daily / as used	Structural integrity, voltage	Volt meter (power unit)
ELISA readers	1 year	Monthly	Lamp stability, optics, filters	Calibration plate
Freezers		Daily	Visual, thermal stability	Calibrated thermometer or pyroprobe
Incubators		Daily	Temperature	Calibrated thermometer
Micro-pipettes	3 years	Daily / as used	Dirt and damage, volume delivery accurate	70% ethanol. If damaged, send for repair, graduated tips
		Every three months	Volume accuracy	Gravimetric method
Micro-pipettor (automatic)		Every three months	Volume, accuracy	Manufacturer's procedure
Microscopes	3 years (cleaned)	Daily / as used	Alignment, bulbs	Manufacturer procedure
pH meters		Daily / as used	Electrode drift or reduced response	Check against two buffer solutions
Plate washer (automatic)		Daily / as used	Nozzles, hoses, vacuum, pH of wash buffer	Distilled water for flushing, instrument gauges, pH meter
Thermal-cyclers	If needed	Annually	Block Temperature	Probe or check with sample replicate matrix
Thermometers (digital)	1 year	6 months	Check at point in working range against reference thermometer	Certified reference thermometer
Thermometers (liquid in glass)	10 Years	6 months	Check at point in working range against reference thermometer	Certified reference thermometer
Thermometers (reference)	10 years	Before use	Check at ice point	
Timers		2 years	Accuracy	
Water baths		Daily / as used	Temperature and correlation with controls	Calibrated thermometer
Water Purification (deionizer)		Daily / as used	Conductivity and meter battery	Conductivity meter, voltmeter
Water purification (glass distillation)		Weekly / as used	Conductivity, pH, hardness	Conductivity and pH meter, hardness test kit

Appendix F
Disinfectant Solution Guidelines

1) Alcohols – 70% isopropanol or ethanol; general skin and surface disinfectant
   NOTE: Ineffective against bacterial spores; evaporates rapidly; flammable; can damage rubber, plastic and flooring surfaces.

2) Iodophors - A broad spectrum disinfectant for hands (available in soap form), lab surfaces, and instrument disinfection according to the following:
   100 ppm free iodine solution – exposure for 10 minutes
   200 ppm free iodine solution – exposure for 1 minute
   25 ppm free iodine solution – exposure for 3 hours
   NOTE: "Proper dilution to 1% iodine is necessary for maximum killing effect and minimal toxicity. More concentrated solutions are actually less efficacious, presumably due to stronger complexation preventing free iodine release. It takes approximately 2 minutes of contact time for release of free iodine (Lavelle et al. 1975). Literature reports indicate that iodophors are quickly bactericidal, virucidal, and mycobactericidal but may require prolonged contact times to kill certain fungi and bacterial spores…Iodophors formulated as antiseptics are not suitable as hard-surface disinfectants, due to insufficient concentrations of iodine…Iodophor solutions retain their activity in the presence of organic matter at pH <4…" (Veterinary Pharmacology and Therapeutics 7th ed., ed by H. Richard Adams.

3) Quaternary ammonium compounds – includes Roccal, Hyamin, and other brand name agents. Use for general laboratory surface and equipment disinfection and cleaning. Dilute according to manufacturer's instructions.
   NOTE: Ineffective against bacterial spores, some viruses, and mycobacteria

4) Sodium Hypochlorite – Broad spectrum disinfectant for use on waste liquids, instrument disinfection, surface decontamination and emergency spill decontamination. Use as follows:
   a. General disinfecting of bacterial and viral contamination
      i. 200 ppm active sodium hypochlorite solution – exposure for 1 hour minimum
      ii. 500 ppm active sodium hypochlorite solution – exposure for 10 minutes to 1 hour
   b. Disinfection of parasitology lab waste containing myxosporean contamination
      i. 5000 ppm active sodium hypochlorite solution - exposure for 10 minutes minimum.
   c. Solution preparation
      i. Concentrations of active sodium hypochlorite contained within household bleach vary with brand used. It is recommended that solutions be prepared according to the following calculation:

$$\frac{(\text{ppm active sodium hypochlorite desired})\ (\text{Gal of Water})\ (128)}{(\text{Percent of active sodium hypochlorite})\ (10{,}000)} = \text{ounces of household bleach product for solution}$$

d. **NOTE:** Toxic to skin; corrosive to metals; inactivated by organic matter; deteriorated over time – shelf life of solutions exposed to air and light is less than one week.

5) Neutralization – chlorine and iodine are toxic to living organisms, so neutralization may be necessary prior to disposal. Using a 1% solution of sodium thiosulfate, the following volume can be used to neutralize solutions as follows:

Sodium hypochlorite: 28.5 mL (number of liters of disinfecting solution X concentration ppm)/100

Iodophor: 7.8 mL (number of liters of disinfecting solution X concentration ppm)/100

(Info derived from Meyer, et al, 1983; Piper, ed. 1983; and on the web – http://www.mcgill.ca/eso/newweb/lab/biosafe.htm)

See also : OIE *Manual of Diagnostic Tests for Aquatic Animal Diseases*, 4th Edition. 2003. Office International Des Epizooties. Paris, France. (http://www.oie.int) Section 1.1.5

APPENDIX G

REFERENCES

American Fisheries Society, Fish Health Section. 2004. Suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2004 edition. American Fisheries Society, Fish Health Section, Bethesda, Maryland.

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Biosafety in Microbiological and Biomedical Laboratories, 1999, U.S. Department of Health and Human Services, Public Health Services, Center for Disease Control, and National Institutes of Health, 4th edition, U.S. Government Printing Office, Washington D.C. (on the web at http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm)

Department of Fisheries and Oceans.1984.Fish Health Protection Regulations: manual of compliance.Fish. Mar. Serv. Misc. Spec. Publ. 31 (Revised): 32 p. (http://www.dfo-mpo.gc.ca/science/aquaculture/aquaculture_e.htm)

Haenen, Olga, EM Bernoth, and David Groman. 1999. Quality Assurance in Fish Disease Diagnosis. Workshop held during the 9th International Conference of European Association of Fish Pathologists (EAFP), Rhodes, Greece, Sept 20, 1999 (on the web at http://www.ifremer.fr/eafp/)

Hnath, J. 1983. Hatchery disinfection and disposal of infected stocks.p. 121 *In* F.P. Meyer, J.W. Warren & T.G. Carey (ed.) 1983. *A Guide to Integrated Fish Health management in the Great Lakes Basin.* Great Lakes Fishery Commission, Ann Arbor, MI. Spec. Pub. 83-2

International Laboratory Accreditation Cooperation (ILAC). 2000. *Guidelines for the Requirements for the Competence of Providers of Proficiency Test Schemes.* ILAC-G13:2000 NATA, Rhodes, NSW2138, Australia http://www.ilac.org

ISO/IEC 17025. 12/15/99. General requirements for the competence of testing and calibration laboratories. International Organization for Standardization Reference Number ISO/IEC 17025:1999(E). (http://www.iso.ch)

NFPA (National Fire Protection Asscoiation). *Fire Protection Guide to Hazardous Materials*. 2001. ISBN: 087765400X

OIE *Manual of Diagnostic Tests for Aquatic Animal Diseases*, 4th Edition. 2003. Office International Des Epizooties. Paris, France. (http://www.oie.int)

OIE *Quality Standard and Guidelines for Veterinary Laboratories: infectious diseases.* Office International Epizooties. ISBN: 92-9044-575-0

Piper, R.G., I.B. McElwain, L.E. Orme, J.P. McLaren, L.G. Fowler, and J.R. Leonard. 1983. Fish Hatchery Management. U.S. Department of the Interior, Fish and Wildlife Service, Washington, D.C.

Ratliff, Thomas A., Jr. 1990. *The Laboratory Quality Assurance System, A Manual of Quality Procedures with Related Forms.* Van Nostrand Reinhold, 115 Fifth Ave, NY, NY, 10003.

U.S. Fish & Wildlife Service Handbook for Aquatic Animal Health Procedures and Protocols. 2002. USFWS, National Fish Hatchery System, Arlington, VA (http://policy.fws.gov/fh_handbook/toc.htm

50 CFR Chapter 1, Part 16, Code of Federal Regulations, U.S. Government Printing Office, Washington D.C. 2003. (http://www.access.gpo.gov/nara/cfr/cfr-table-search.html)

A4.1 Citation Guidelines

In 2003, the 4th Edition of the Blue Book (1994) underwent a major revision to include the joint FHS/USFWS Fish Inspection Handbook and many new or updated diagnostic chapters. In addition, the entire document was converted to a digital format for distribution on CDs. These revisions made it necessary to change the way that the Blue Book is structured and cited.

Within the Blue Book, all of the chapters in both sections now have a footer that tells when the chapter was last revised. For purposes of citation, chapters in this document are cited as chapters in a multi-author/multi-chapter book. The editions will no longer be numbered (as in "4th edition") but will now be described only by the publication date ("2007 edition"). Chapters in the Diagnostic Section are credited to the individual authors that appear on the title page of each chapter. The year cited after the author's name should be the year in which the chapter was last revised. For the Inspection Section, the section should be cited as a single chapter with the USFWS and AFS-FHS as authors and the same date as the Blue Book edition. Specific examples for citing chapters from this new edition of the Blue Book are below.

Section 1 (the Diagnostic Section) chapters in this document may be cited as follows:

Mitchell, A., and A. Goodwin. 2004. Centrocestiasis (gill trematode disease). *In* AFS-FHS (American Fisheries Society-Fish Health Section). FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2007 edition. AFS-FHS, Bethesda, Maryland.

Johnson, K. 1994. Lernaeid parasitism. *In* AFS-FHS (American Fisheries Society-Fish Health Section). FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2007 edition. AFS-FHS, Bethesda, Maryland.

Section 2 (the Inspection Section) of this document may be cited as:

USFWS and AFS-FHS (U.S. Fish and Wildlife Service and American Fisheries Society-Fish Health Section). 2007. Standard procedures for aquatic animal health inspections. *In* AFS-FHS. FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2007 edition. AFS-FHS, Bethesda, Maryland.

Section 3 (the Quality Assurance/Quality Control Section) of this document may be cited as:

AFS-FHS (American Fisheries Society-Fish Health Section). 2007. Model Quality Assurance/Quality Control Program For Fish Health Laboratories, 2007 edition. AFS-FHS, Bethesda, Maryland.

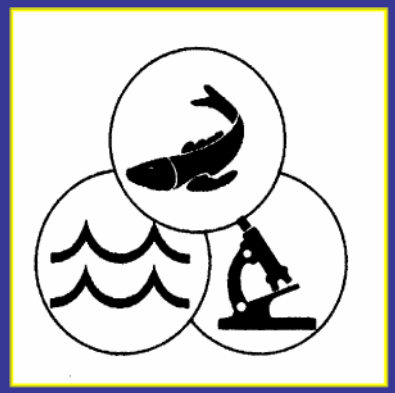
The Blue Book in its entirety may be cited as:

AFS-FHS (American Fisheries Society-Fish Health Section). 2007. FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2007 edition. AFS-FHS, Bethesda, Maryland.

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Important Information and Set-Up Instructions

Loading Adobe Acrobat®

This CD-ROM is set up for Adobe Acrobat Reader® version 7.0 or later. If you must use an earlier version (e.g. because Adobe does not have a 7.0 or later version available for your operating system) then all of the CD-ROM's features may not be active.

For WINDOWS NT, 2000, and XP Acrobat Reader® Version 7.0 can be installed from this CD-ROM.

For MACINTOSH OS X v10.2.8 or higher, Acrobat Reader® Version 7.0 can be installed from this CD-ROM.

For LINUX users, Acrobat Reader® Version 7.0 install program is provided on this CD-ROM.

Instructions for Adobe Acrobat® 7.0

File Management

It is important to adjust your Acrobat Reader® software so that it closes all files you open as you navigate this CD-ROM. Many windows opened simultaneously may slow your computer down. To get the proper setting, follow these steps:

1. **Go to *Edit* on the Menu Bar**
2. **Pull down to *Preferences***
3. **Go to *General***
4. **Check the *Open Cross-Document Links in Same Window* box**

Exiting the CD-ROM

To exit the CD-ROM, click the Exit bookmark or click Exit on the Welcome Screen.

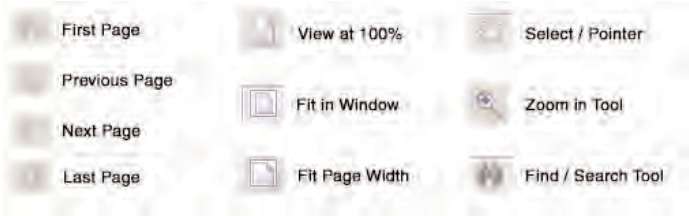
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Navigation Buttons & Descriptions

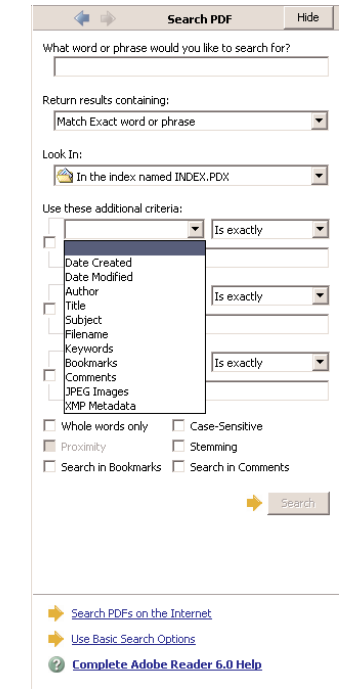
The following are buttons found in Adobe Acrobat®, followed by a short description to help you navigate this CD-ROM.



Searching this CD-ROM

To search this CD-ROM click on the binoculars button. This will open the Search PDF window to the right side of the screen. In this window, select “Use Advanced Search Option” located at the lower right. Once “Use Advanced Search Option” has been selected, a different menu page will open that will allow you to search by different criteria, including Title, Subject, Author, and Keywords. An example of this Search window is pictured to the right.

Search Window Layout →



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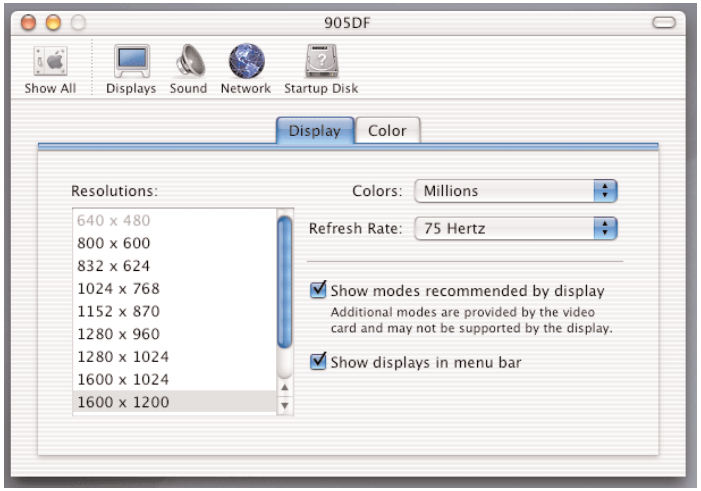
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Operating System Display Settings

So that the settings to the Adobe Acrobat Reader® program, outlined below under FILE MANAGEMENT and SETTING UP SEARCH FIELDS, can be made successfully, your computer's display settings should be set to 800x600 pixels or higher. The Adobe Acrobat Reader® program cannot be modified if your display settings are lower than 800x600 pixels. For example, a 640x400 pixels setting causes the Adobe Acrobat Reader® program's settings window to appear so large that it is not possible to click on the OK button to confirm the change made to the settings.

Mac OS X Display Settings



On an Apple computer running Mac OS X, the display settings are reached by first clicking on the apple in the upper left hand corner of the screen. From that menu, choose “System Preferences.” From the main System Preferences screen, locate and click on the icon named Displays. The dialog to the left will appear. If the check box labelled “Show modes recommended by display” is checked, only supported resolutions will be given as options. Select the highest resolution above 800x600 which is most comfortable for viewing. After making your selection, you can simply close the System Preferences by pressing command-Q or by clicking on “System Preferences” on the menu bar and choosing “Quit System Preferences.”

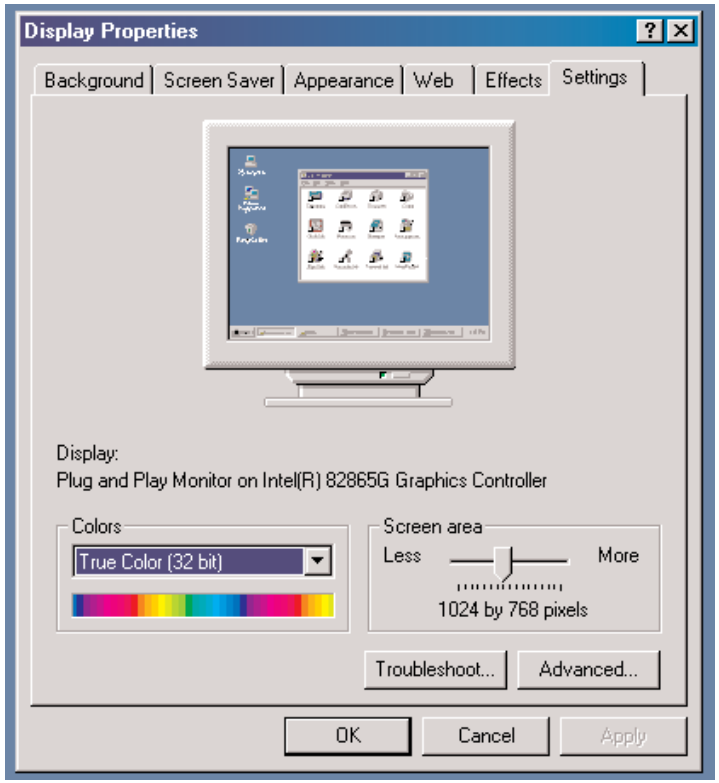
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Operating System Display Settings Continued...

Windows Display Settings



On an IBM compatible computer running Windows 98, NT, ME, 2000, or XP, the display settings are reached by first right clicking on the desktop (with no applications running; this is where your “background” or “wallpaper” is displayed). From that menu, choose “Properties.” Now displayed is the Display Properties window. Locate and click on the tab marked Settings. The dialog to the left will appear. Select the highest resolution above 800x600 which is most comfortable for viewing by moving the slider named “Screen Area.” After making your selection, you can simply press apply. A brief check of settings will appear, giving you time to preview the new resolution. If the screen seems to distort or stretch, the resolution is not supported by your monitor and a different setting must be chosen. Once an acceptable resolution is found, click yes on the window asking you to keep or discard the settings. Once you have chosen to keep the settings, click OK to leave the Display Properties panel.

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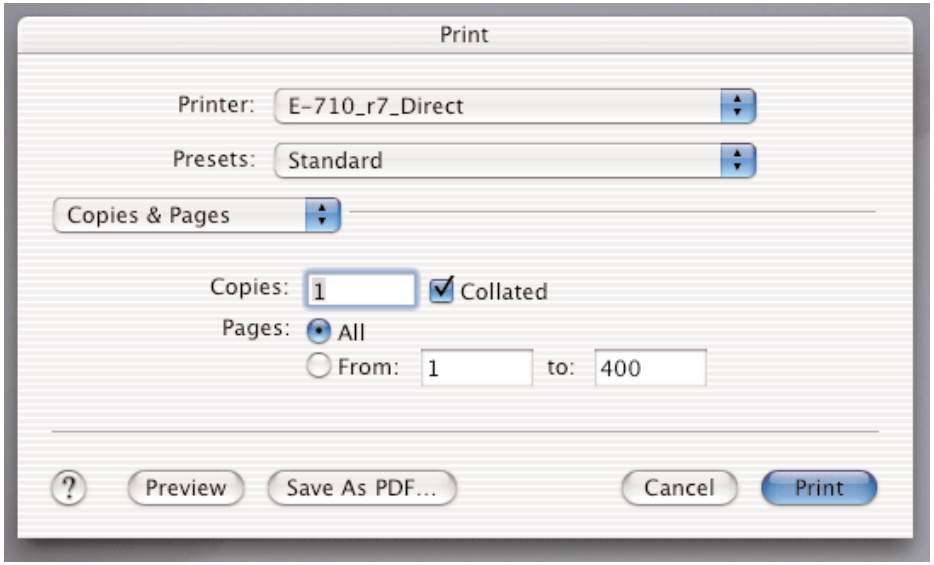
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Printing Articles and Documents

To Print all or a portion of a document on an Apple computer running Mac OS X, follow these steps:

1. With the document open in Acrobat, click File on the menu bar and then locate and click on Print. The dialog box to the left will appear.
2. Choose the desired printer from the first drop down menu entitled "Printer."
3. Select how many copies of the document you desire by placing the number into the field marked "Copies:"
4. Under the "Pages" option, choose either "All" or select a page range by choosing "From" and then entering the page range into the provided fields.
5. Click on Print.



Mac OS X Print Dialog

• Additional Printing features dependent on your system's capabilities are located under the drop down menu that says "Copies and Pages." Changing this heading will reveal system specific settings which can be changed to allow for double sided printing, page size, orientation, etc.

Fish Health Section

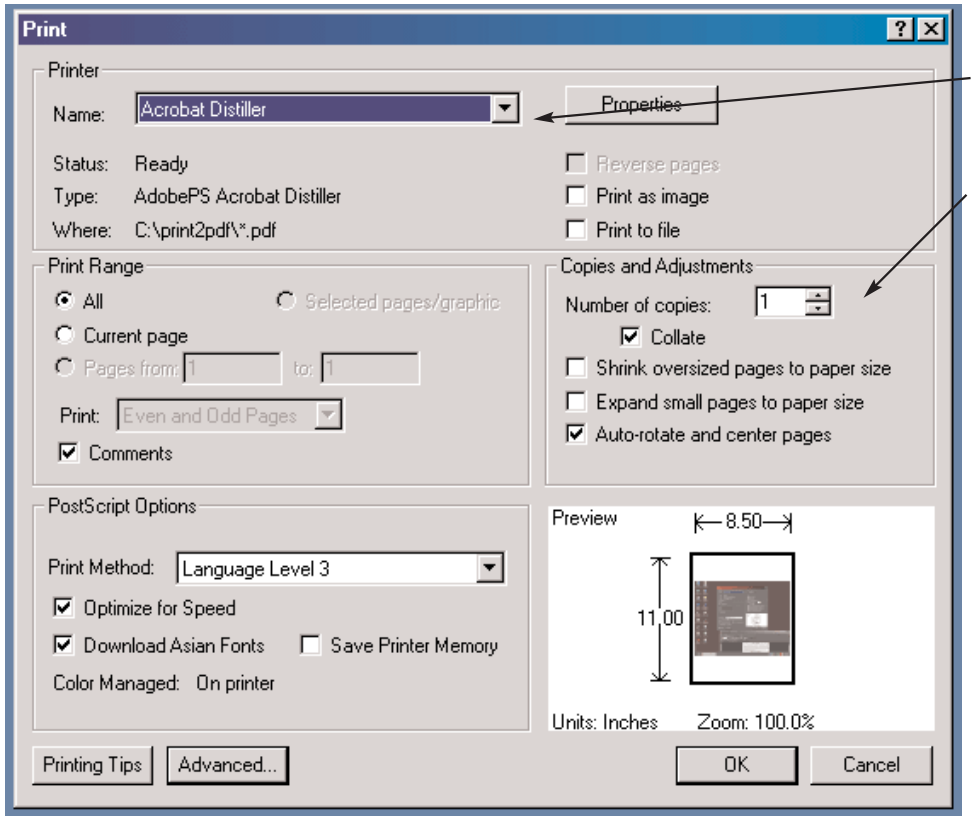
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Printing Articles and Documents

To Print all or a portion of a document on an IBM compatible computer running Windows 98, NT, ME, 2000, or XP, follow these steps:

1. With the document open in Acrobat, click File on the menu bar and then locate and click on Print. The dialog box to the left will appear.
2. Choose the desired printer from the first drop down menu entitled “Printer.”
3. Select how many copies of the document you desire by placing the number into the field marked “Copies:”
4. Under the “Page Range” option, choose either “All,” “Current page” (to print only the currently viewed page),or select a page range by choosing “Pages From” and then entering the page range into the provided fields.
5. Click on Print.



Windows Print Dialog

• Additional Printing features dependent on your system’s capabilities are accessed by clicking on the button marked “Properties.” Clicking on this option will reveal system specific settings which can be changed to allow for double sided printing, page size, orientation, etc. Several other printer specific options are listed on this main printing dialog, such as the option to print to file and to center or rotate the pages being printed.