

Fish Health Section **BLUE BOOK**



2004 Edition



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

This edition of the American Fisheries Society Fish Health Section Blue Book is designed to incorporate revisions annually. This searchable CD contains chapters on accepted procedures for diagnosis of common bacterial, viral, parasitic, and mycotic infections of fish and shellfish. It also contains Procedures for Aquatic Animal Health Inspections, co-authored by the AFS-FHS and the USFWS.



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Preface

The new edition of the American Fisheries Society, Fish Health Section Blue Book builds upon the previous edition but in a new format designed to incorporate revisions on an annual basis. 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 you will see in this new edition.

Certain aspects of this edition will be familiar. All of the previous chapters have been included here. Many chapters were updated for this edition (thanks to the authors who were able to provide additional information) and the revision date appears at the bottom of each page. Other chapters will be updated in subsequent issues and therefore retain the original publication date. You will also note that the Blue Book no longer has a general editor. These changes require that material contained here will be cited either using the individual chapter authors, or by citing the entire document. We felt that this provided the authors recognition for their efforts and it also allows for different publication dates within a volume.

A major addition to this edition is the inclusion of the Standard Procedures for Aquatic Animal Health Inspections. This Section was 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. The detailed information contained in the Inspection Section may make some of the individual chapters in other sections of the Blue Book appear redundant, but we feel that these are complementary. The individual pathogen chapters cover topics like geographic and host range, and epizootiology, which are not included in the inspection chapter. Chapters also describe a broader range of diagnostic methods, recognizing that these methods are used for much broader purposes than inspections, and many more pathogens that are of importance to diagnosticians but that are not often included in formal fish health inspections.

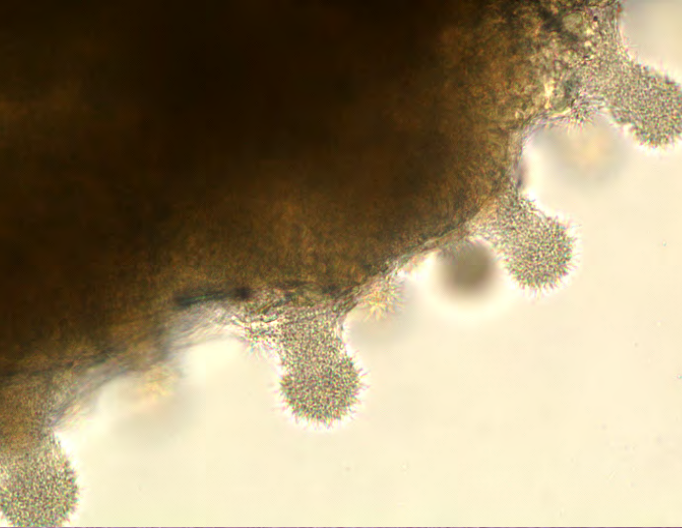
Because this is a new venture, initial subscriptions will be for five years, with the understanding that publication options are rapidly changing and we will need to re-evaluate this process during this period. We look forward to your comments and hope to have your assistance in insuring that the Blue Book continues to be a useful reference.

Time and space prevent the acknowledgment of all the many individuals who spent countless hours in the creation of this document. A special word of thanks should go to Tom Bell, Rob Bakal, Joy Evered, and Trish Barbash of the USFWS, Aaron Lerner of the American Fisheries Society, members of the FHS Technical Standards Committee that reviewed the document, all the various contributing members of pathogen committees for the inspection section, and the authors that took the time to update the chapters of the diagnostic section. A very special word of appreciation to Kari Higbee of the Utah Division of Wildlife Resources, who has spent a significant portion of her time in 2003 to compile, format, and review all the various 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.

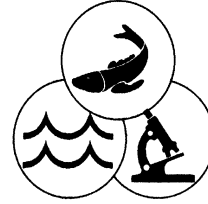
Blue Book Committee

Jerri Bartholomew
Andrew Goodwin
Christopher Wilson



Section 1

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



Chapter 1 Bacterial Diseases of Fish

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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°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 ₂ O	0.05%
CaCl ₂ ·2H ₂ O	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°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 (H₂S) 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 H₂S 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 (NO₃) as a nitrogen source,

and those that can reduce nitrate to nitrite (NO₂) 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 β-hemolytic streptococci and aerococci are LAP-negative. The test is performed using commercially available discs that are impregnated with leucine-β-naphthylamide. The LAP enzymatic activity releases β-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 (NH ₄ H ₂ PO ₄)	1.00
Dipotassium phosphate (K ₂ HPO ₄)	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](#).

Figure 1: Gram Stain

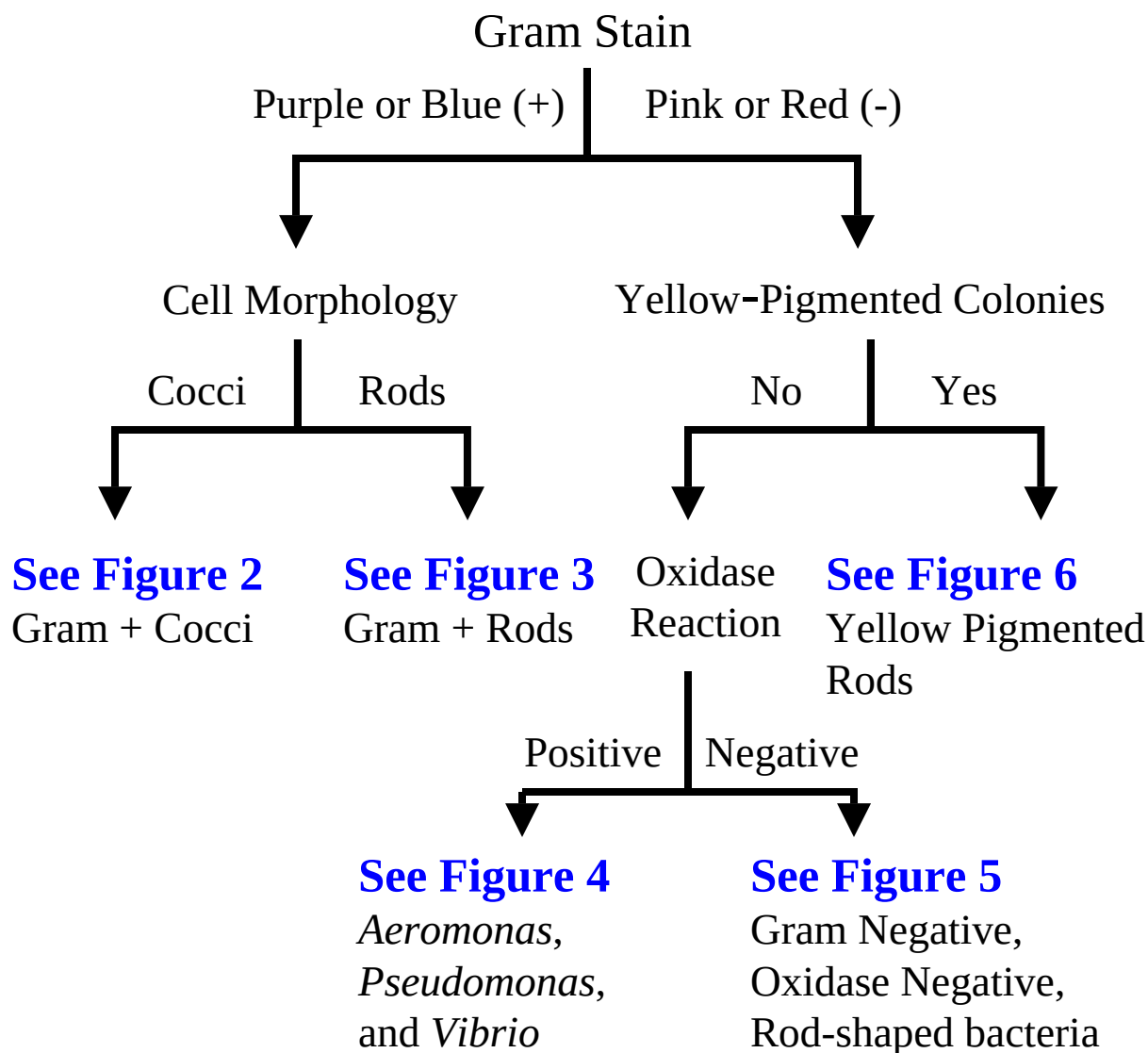
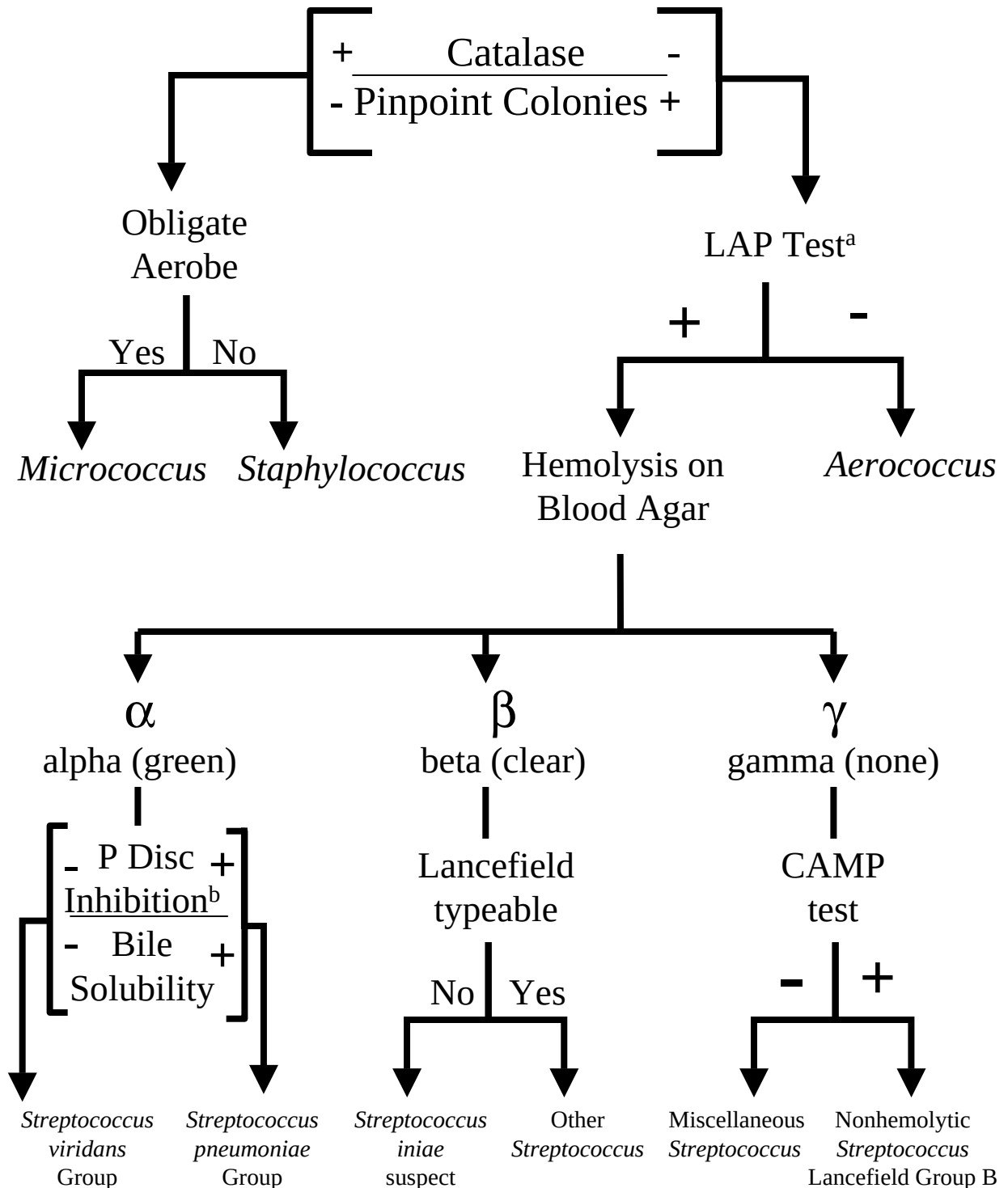


Figure 2: Gram Positive Cocci



a = leucine aminopeptidase test

b = ethyl hydrocuprein hydrochloride (5 µg) disc

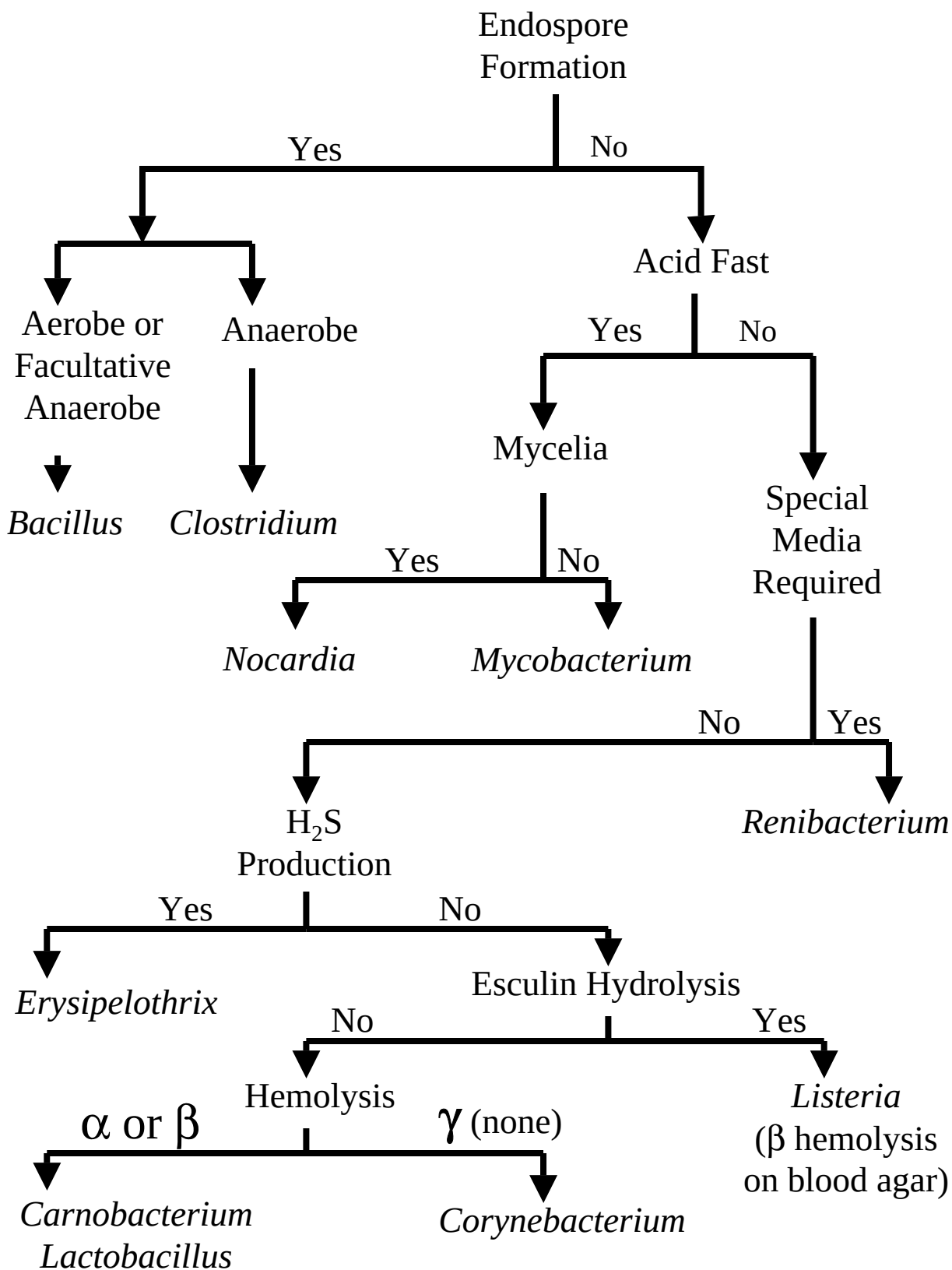
Figure 3: Gram Positive Rods

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

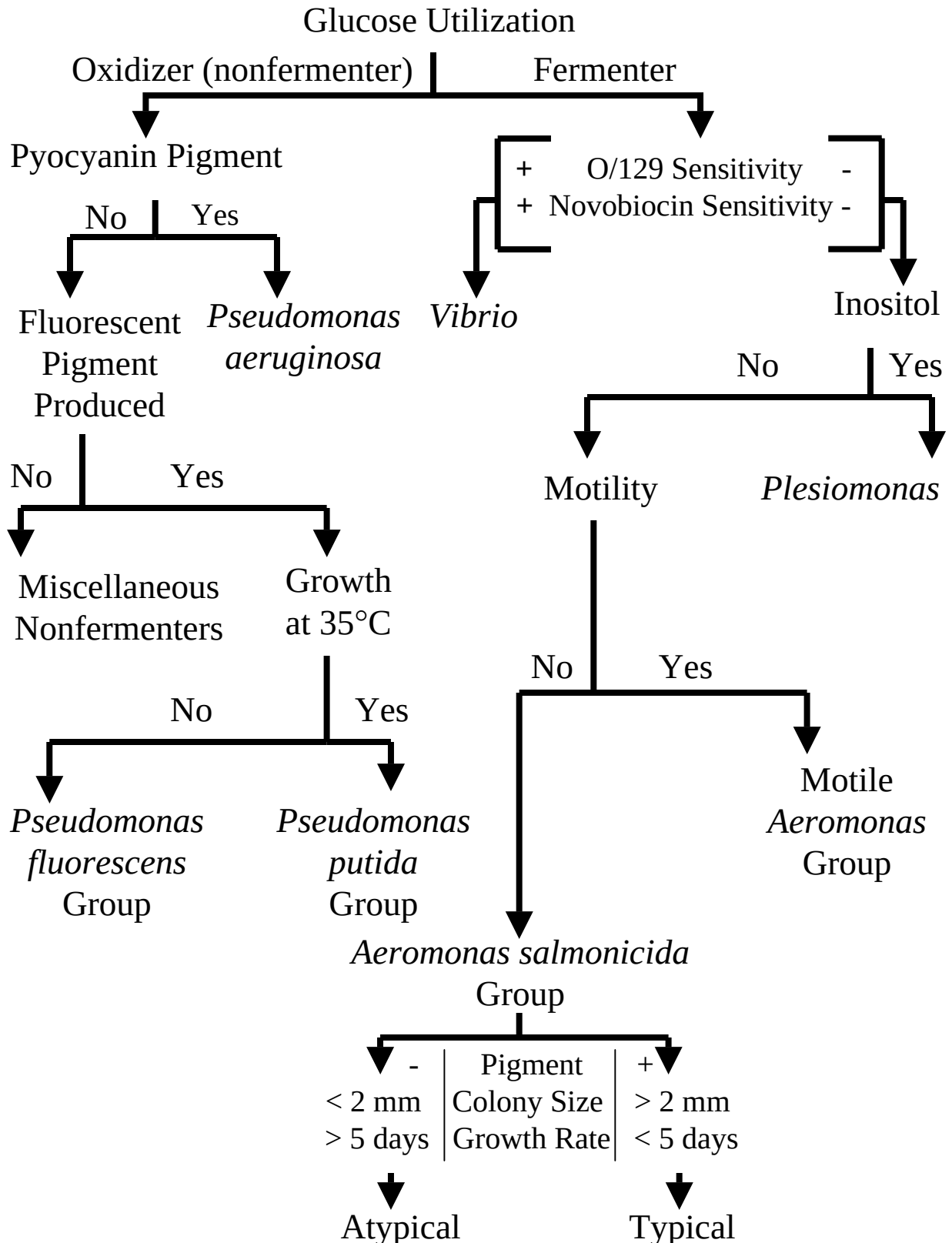


Figure 5: Gram Negative, Oxidase Negative, Rod-shaped bacteria

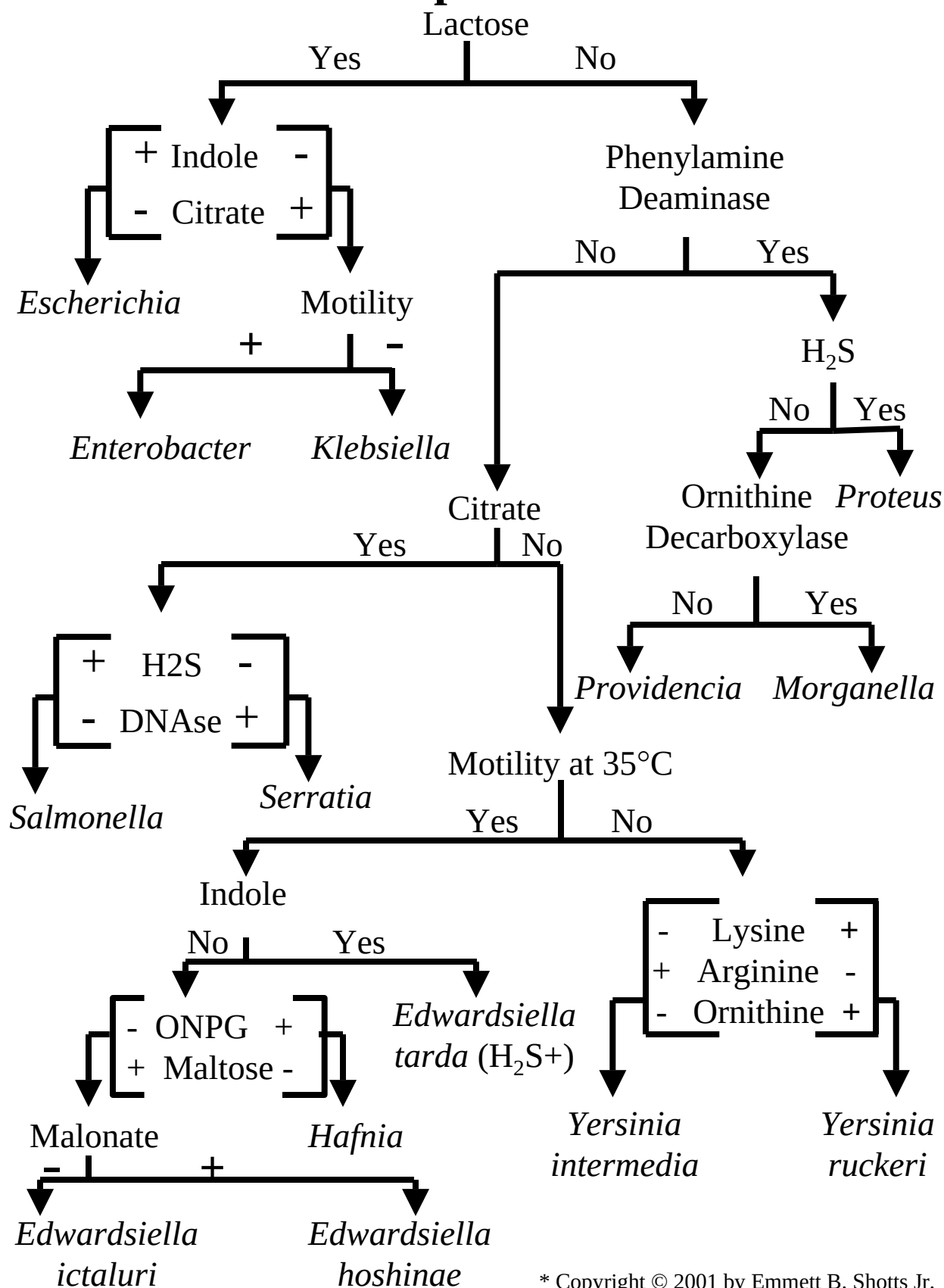
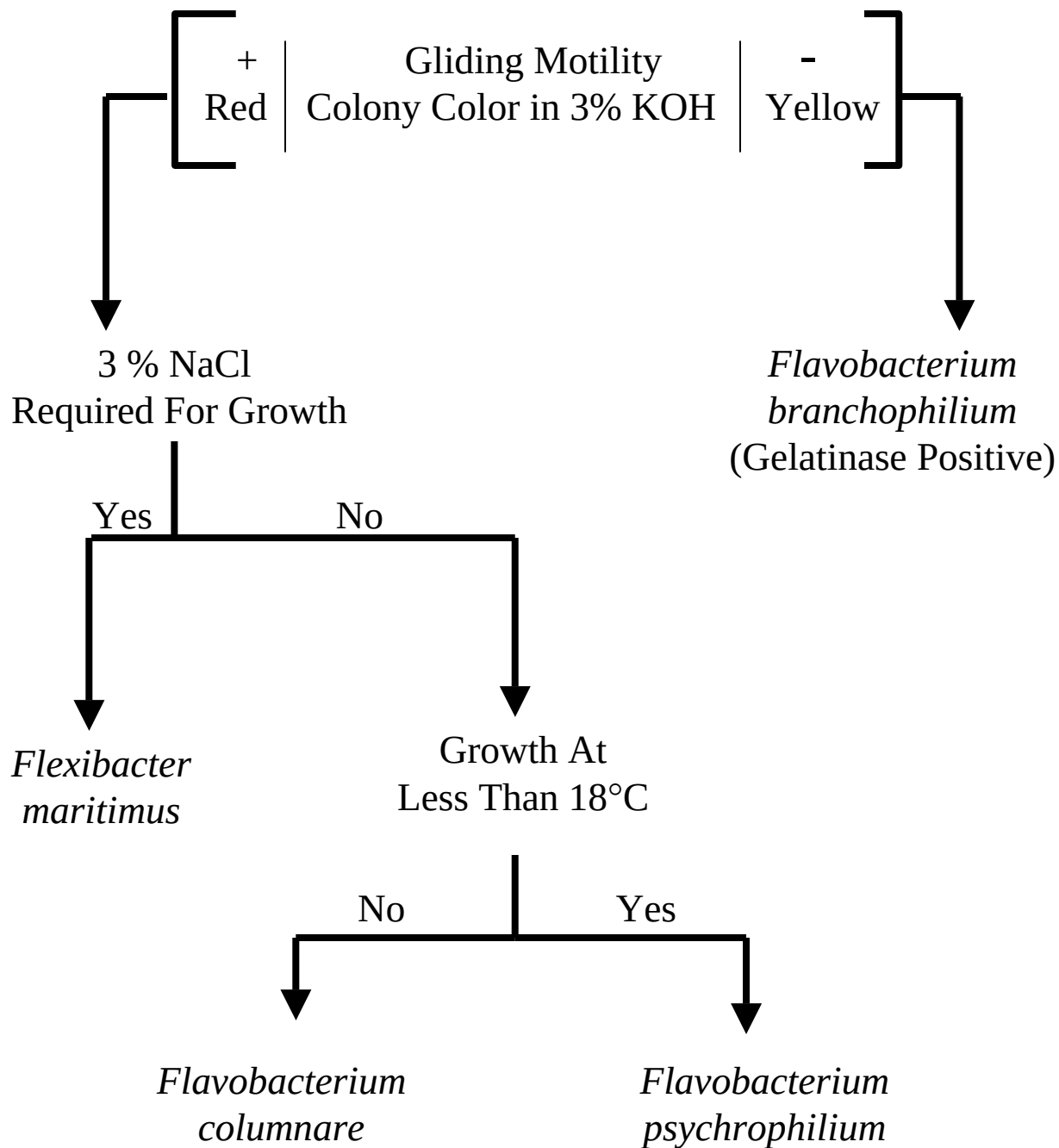


Figure 6: Yellow-Pigmented Rods



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.)

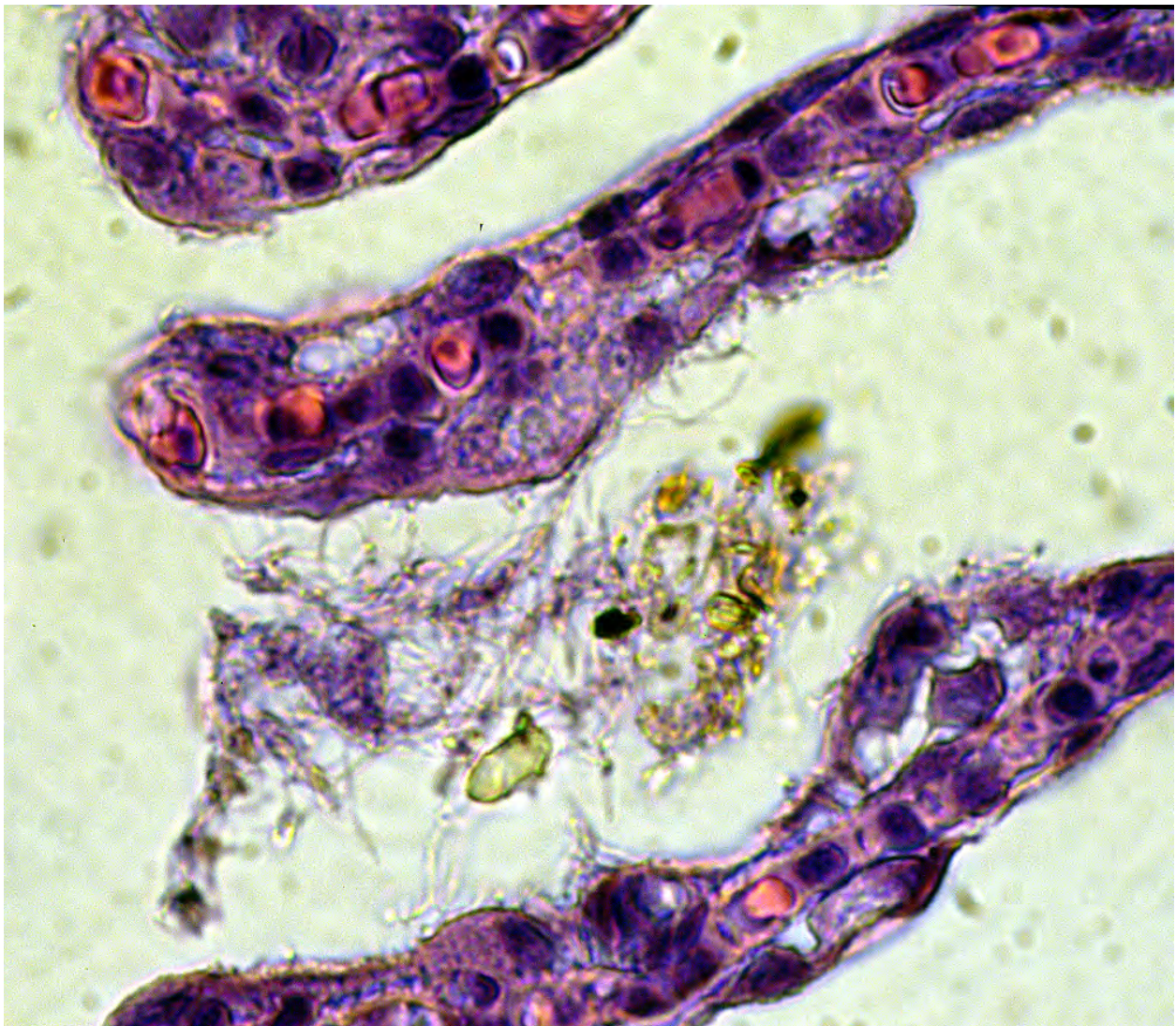


Figure 1. H&E section of gill from a rainbow trout showing large mats of flavobacterium causing bacterial gill disease. Photo courtesy of Dr. Chris Wilson.

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 branchiophilum* 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
jbortoli@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 (*Plecoglossus 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°C or below. However, some outbreaks occur or persist at temperatures up to 16°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 conditions 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 μ m). 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.

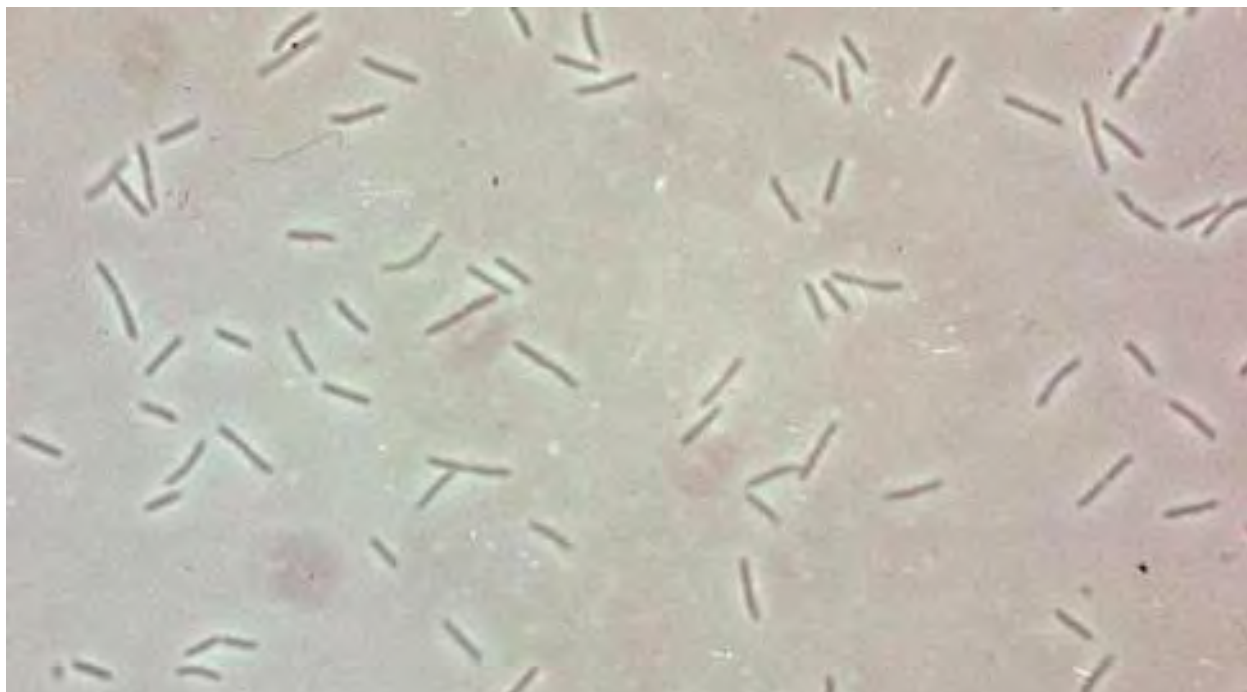


Figure 8. Cells of *F. psychrophilum* in wet mount preparation observed at 1000X.

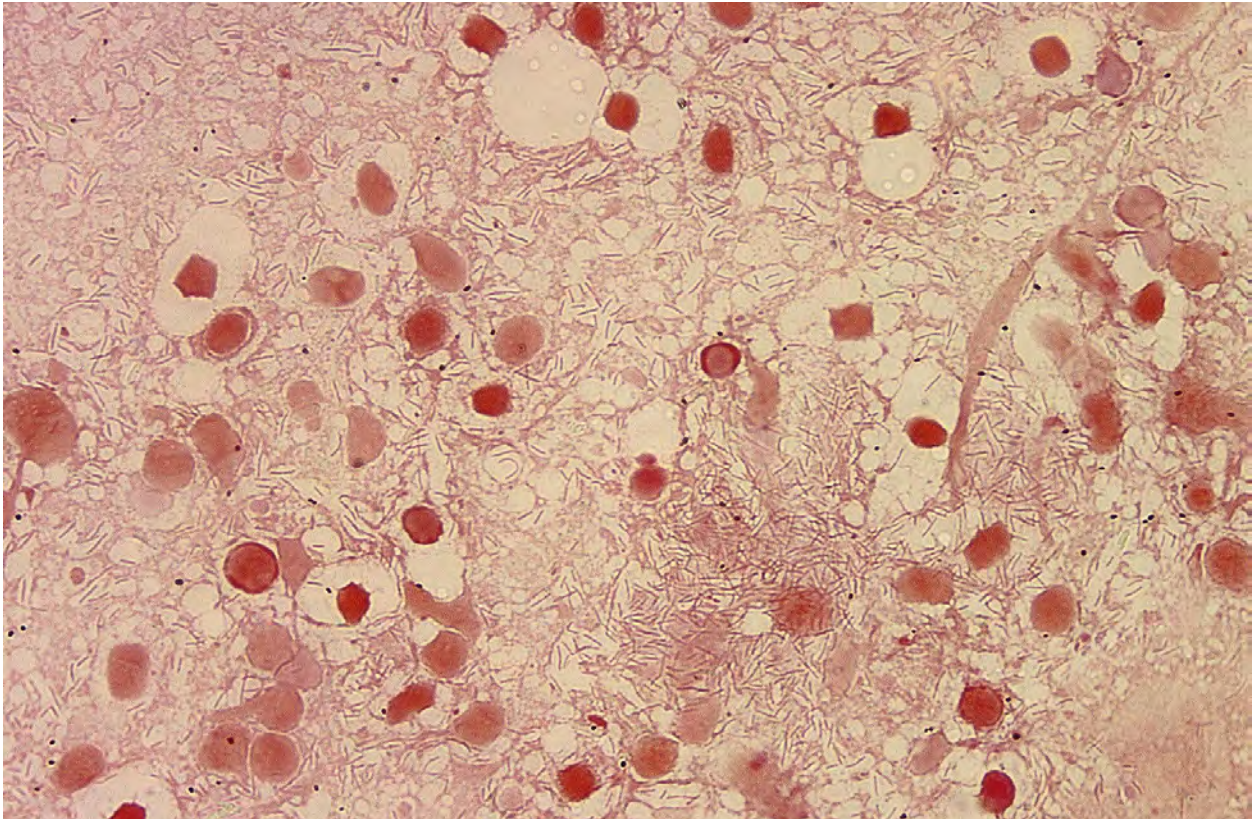


Figure 9. Gram stained *F. psychrophilum* cells. A smear prepared from the spleen of an infected fish (picture provided by C. Banner, Oregon Dept. of Fish and Wildlife).

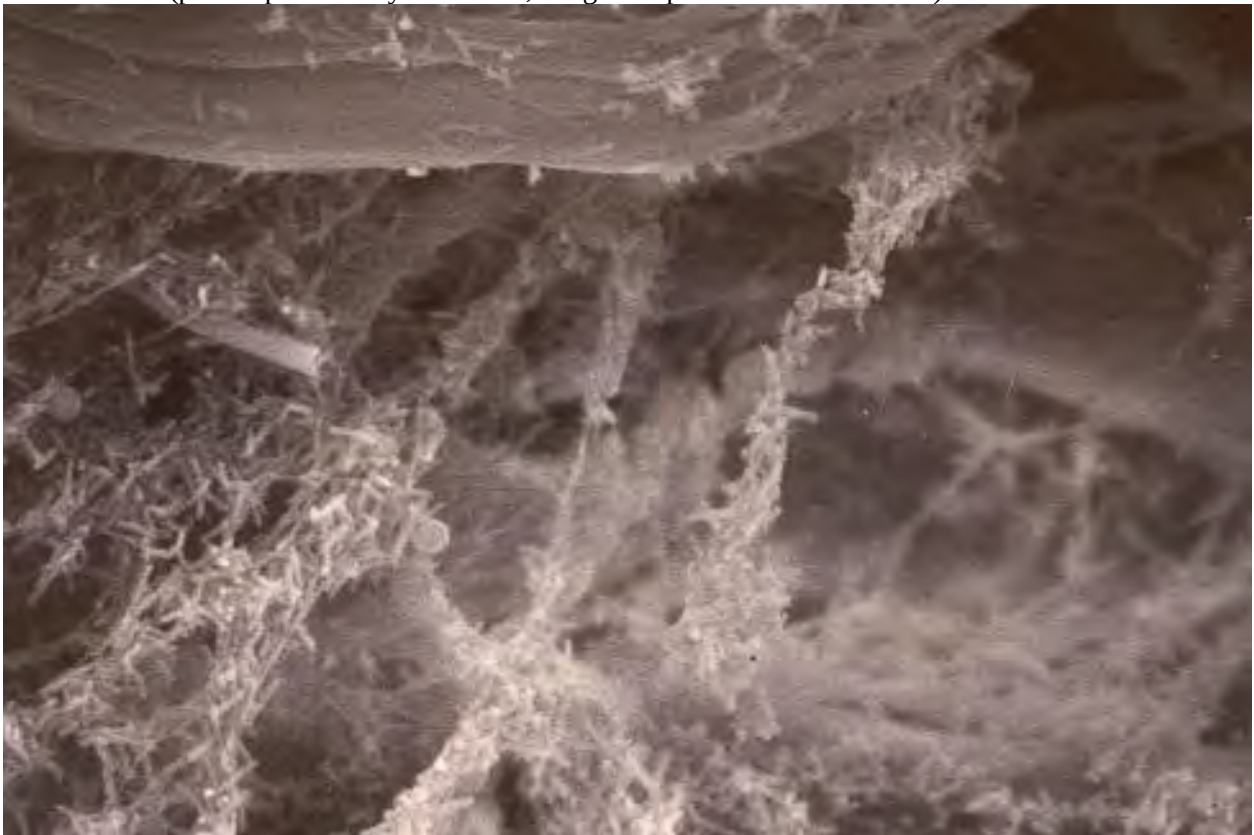


Figure 10. Scanning EM of coldwater disease cells in a muscle lesion of a juvenile coho salmon.

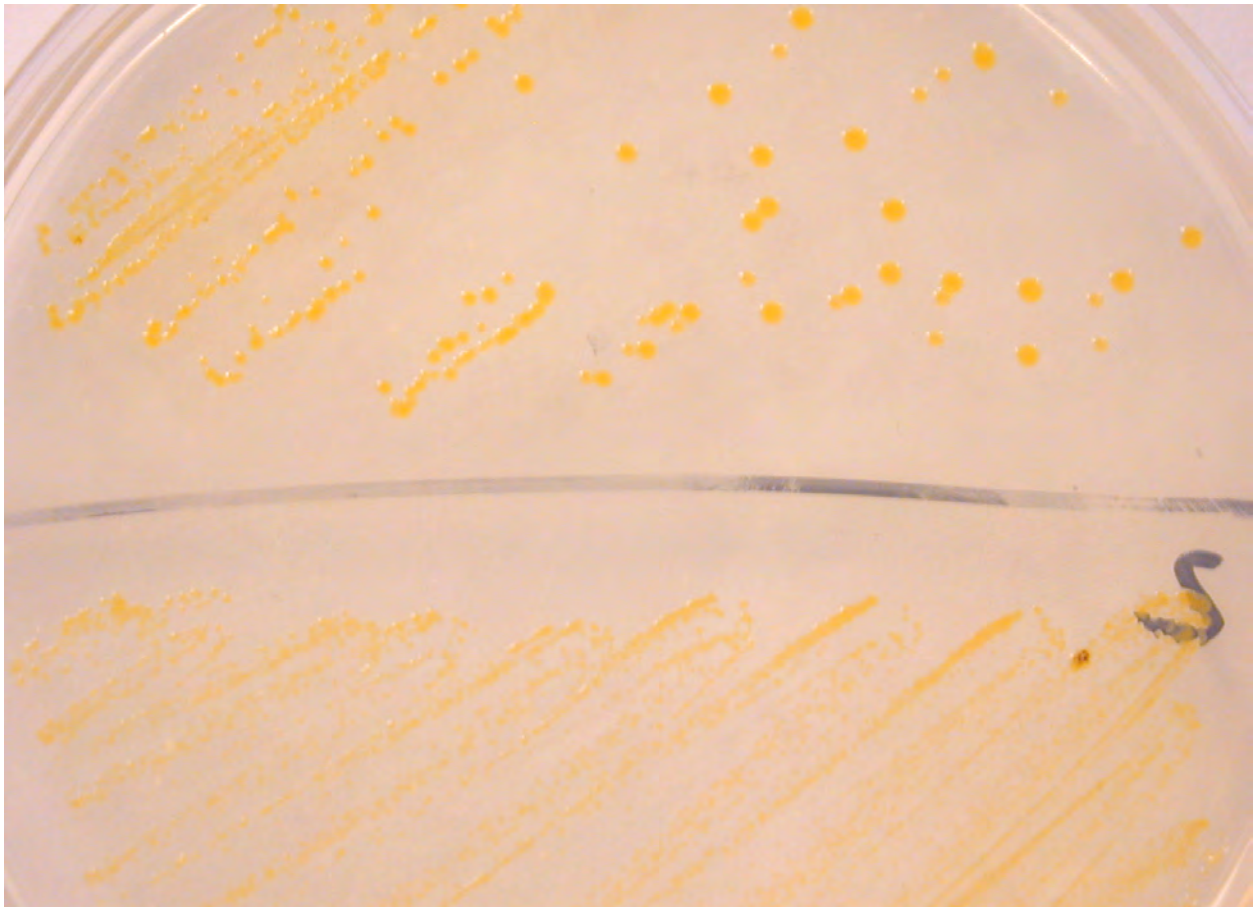


Figure 11. *Flavobacterium psychrophilum* colonies on TYES Agar.

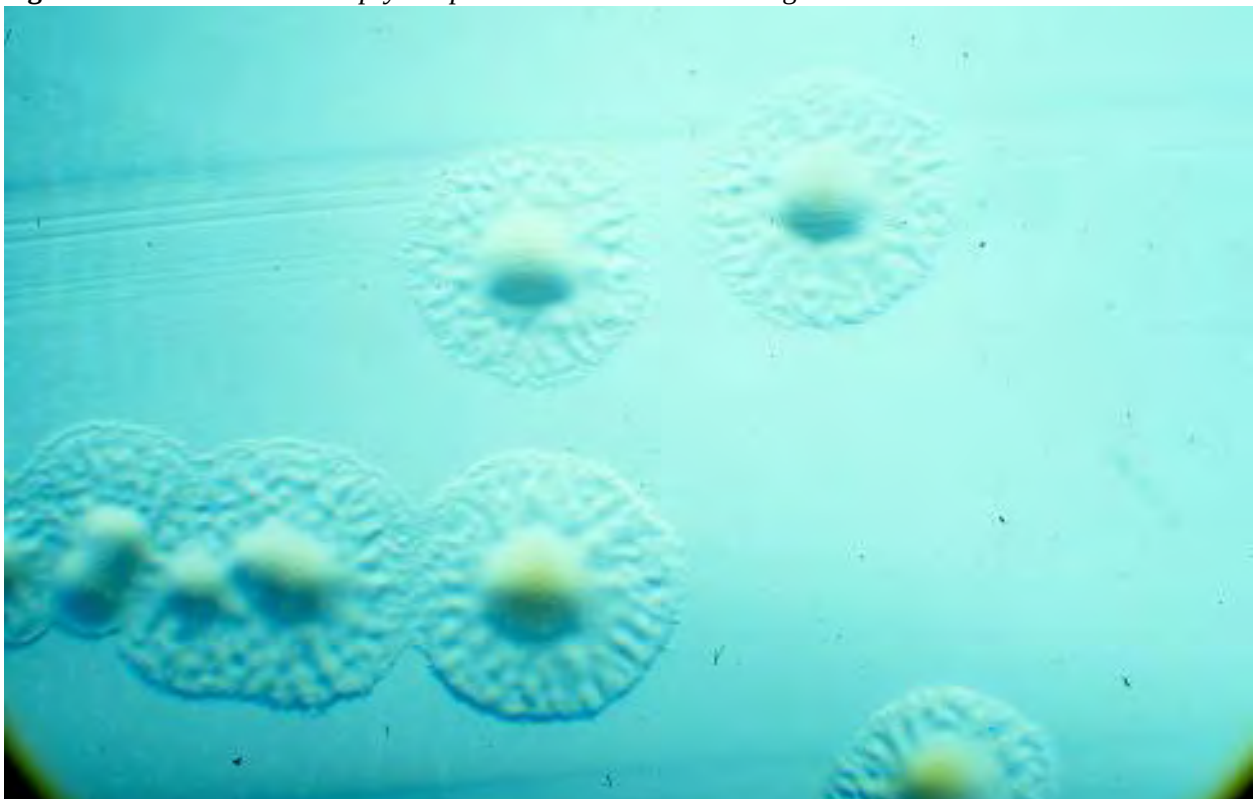


Figure 12. Typical *F. psychrophilum* "fried egg" colony morphology on cytophaga agar.

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°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).

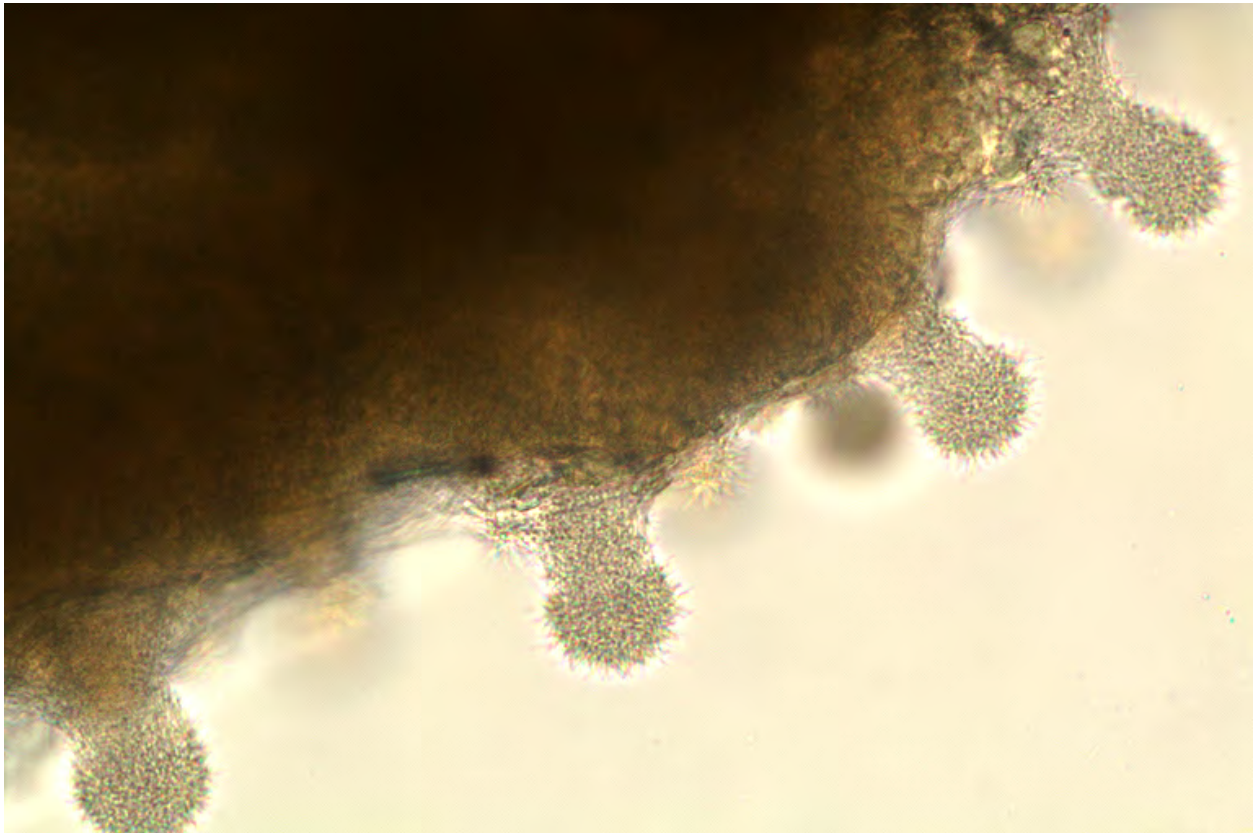


Figure 7. "Hay stacks" of *Flavobacterium columnare* on the gills of a koi (picture by Andy Goodwin).

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 μm) gram-negative rods in lesions; dry rhizoid, yellowish colonies are produced on cytophaga agar (Anacker and Ordal 1959) after three days incubation at 20°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 H₂S production.



Figure 1. *Edwardsiella 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°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°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°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” lesions.



Figure 4. Red and white mottling in the liver of a catfish with ESC (picture by Andy Goodwin).

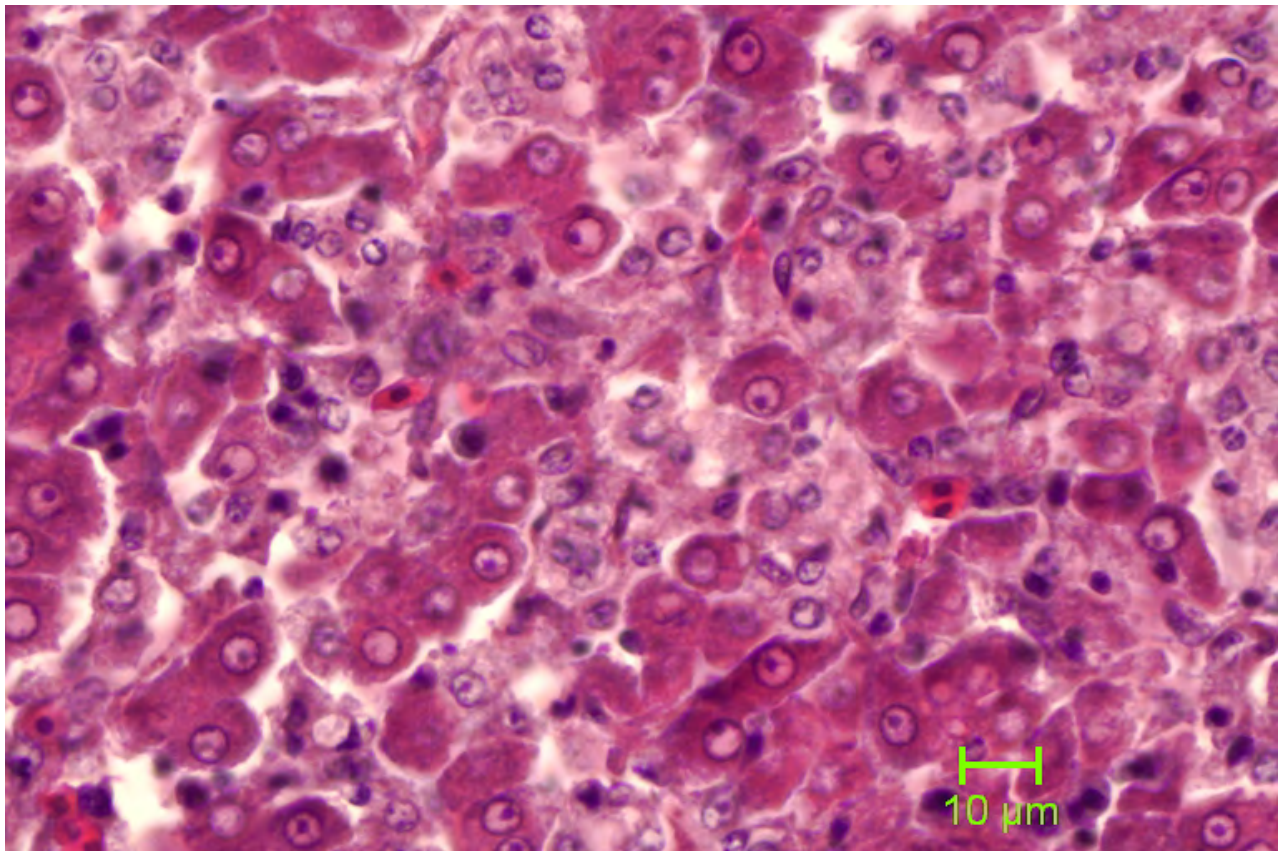


Figure 5. Granulomatous exudate (pale pink cells) in the liver of a channel catfish with ESC (picture by Andy Goodwin).



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

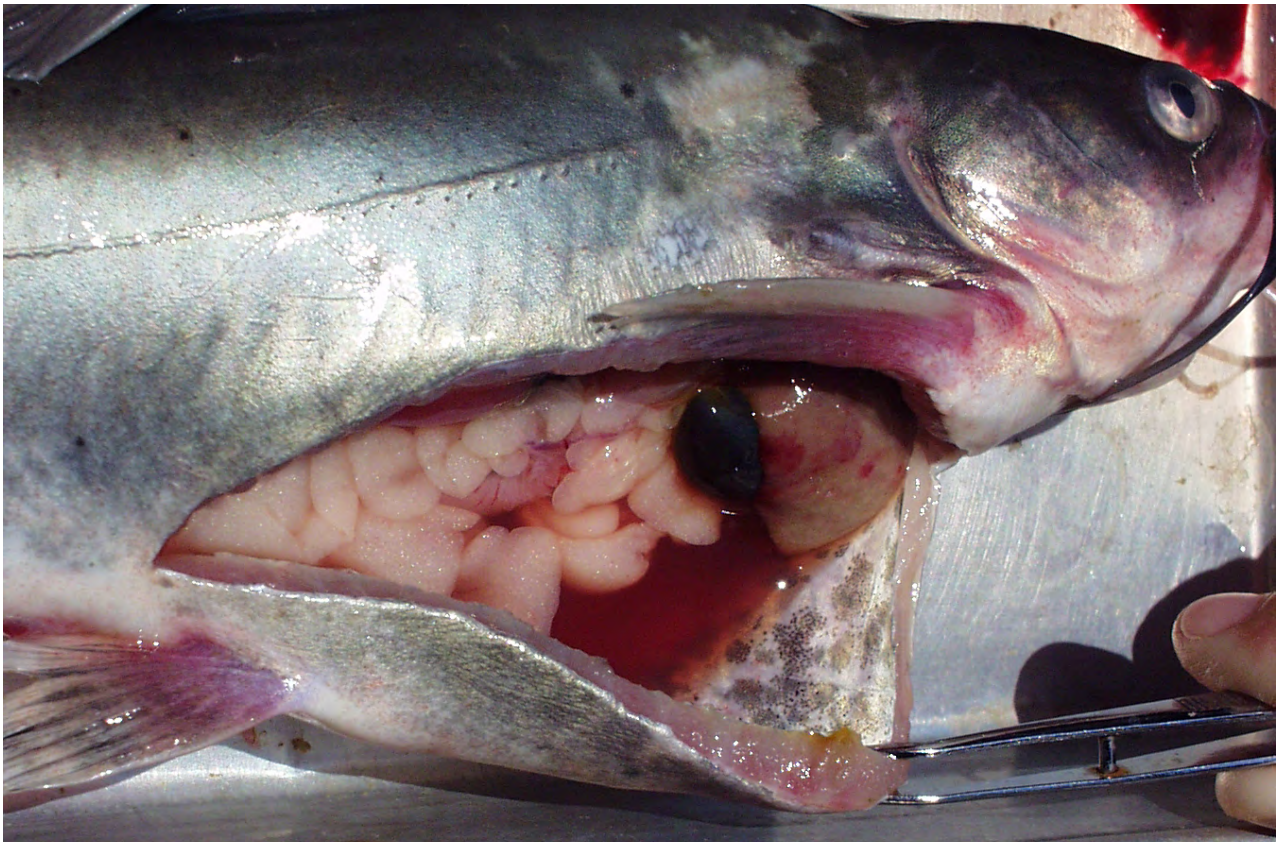


Figure 7. Hemorrhagic exudate and hepatic edema in a channel catfish with ESC (picture by Andy Goodwin).



Figure 8. Shallow white ulcers on the skin of 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 H₂S.

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°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°C, chronic infections are more likely to develop, with incubation periods up to several weeks. At temperatures below 8 to 9°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°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°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.

1.10 Other Diseases Caused by *Aeromonas salmonicida* - 2

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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1.10 Other Diseases Caused by *Aeromonas salmonicida* - 3

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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°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°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° 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@pccadmin.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 hemorrhaging 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 chloride 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 vibriostatic agent O/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.

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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 pallasii*.

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*.

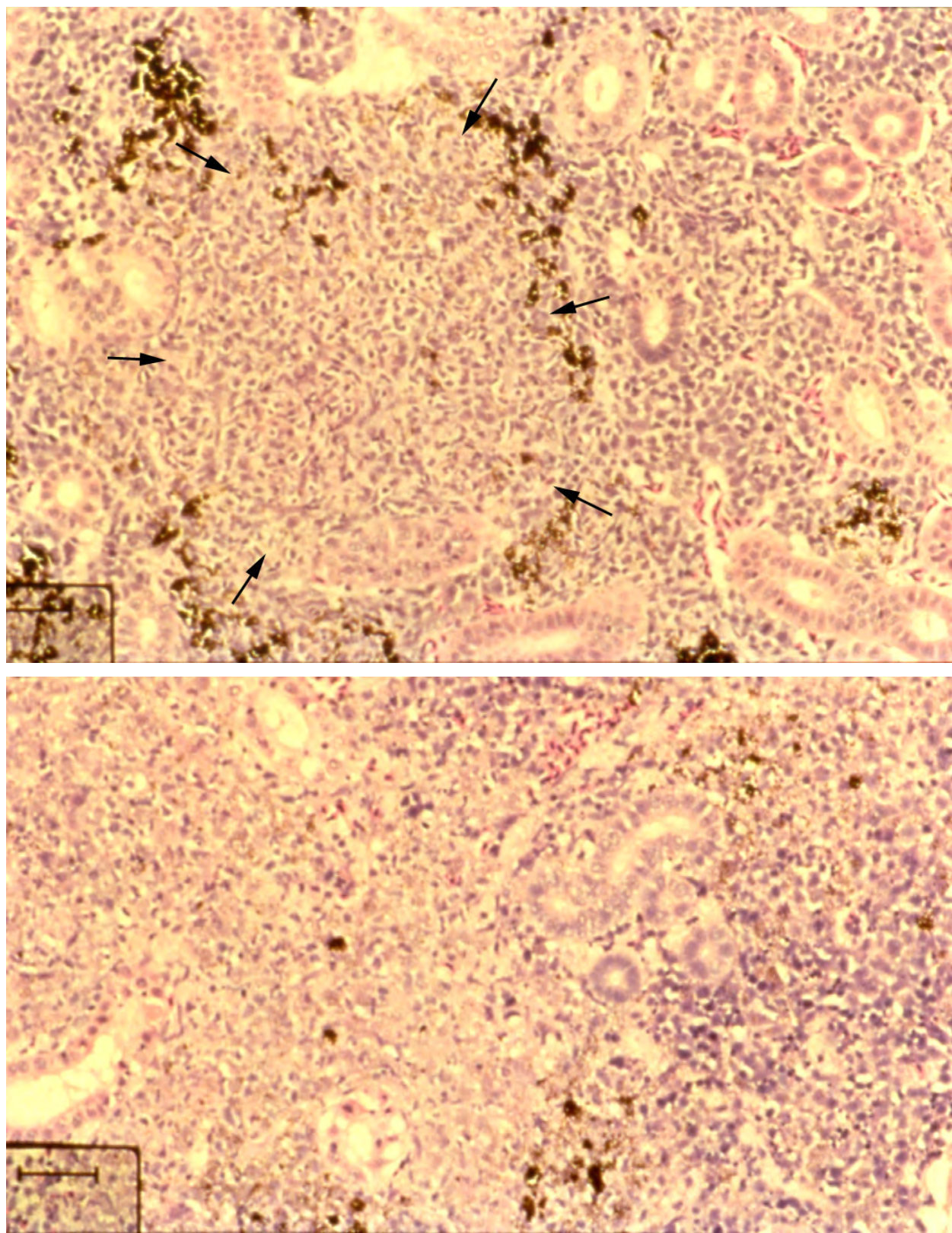


Figure 5. Focal (top photo, arrows) and diffuse (bottom photo) granulomas in the posterior kidney of juvenile chinook salmon *Oncorhynchus tshawytscha* with BKD. Hematoxylin and eosin stain; scale bars = 50 μ m. Photos courtesy of Dr. Caroline O'Farrell.

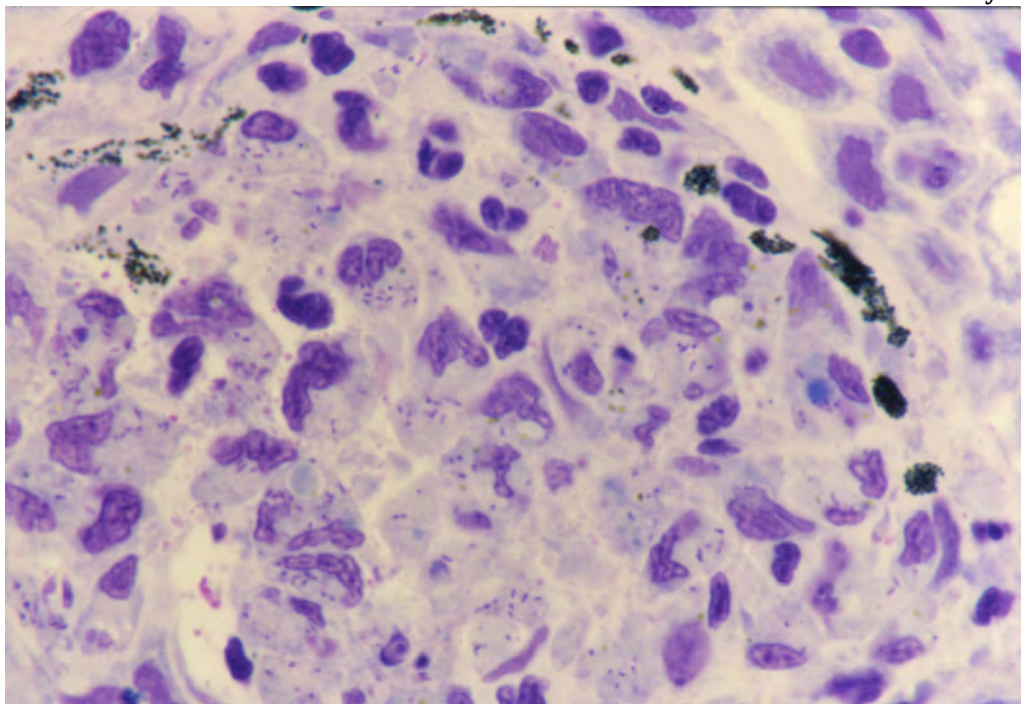


Figure 6. Histological section of a skin lesion of a juvenile chinook salmon *Oncorhynchus tshawytscha* infected with *Renibacterium salmoninarum*. Most of the small rod-shaped *R. salmoninarum* are visible within the cytoplasm of macrophages. Note that the bacteria are purple-blue in this Giemsa-stained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

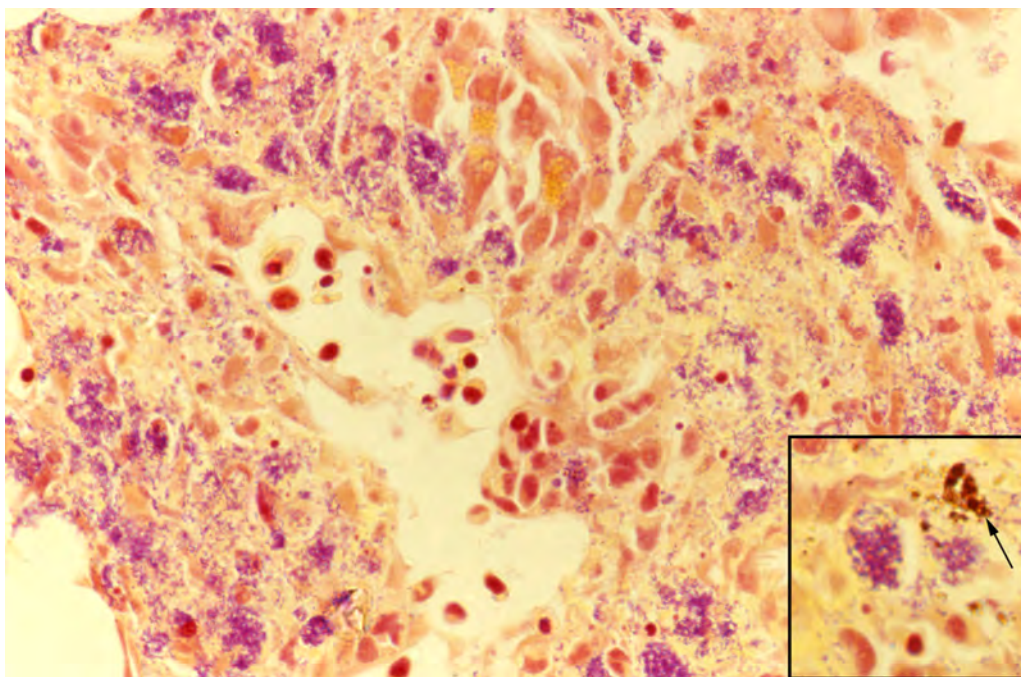


Figure 7. Gram-stained histological section of pancreatic tissue of a juvenile chinook salmon *Oncorhynchus tshawytscha* with systemic BKD. *Renibacterium salmoninarum* cells are present extracellularly and intracellularly within macrophages. Note the color difference between the gram-positive (purple-blue) 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 ($<2.4 \times 10^3$ 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 ₂ PO ₄	0.20 gram
Na ₂ HPO 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 lyophilized 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 ₂ CO 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 lyophilized 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 commercial 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 lyophilized 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 checkerboard 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

	1	2	3	4	5	6	7	8	9	10	11	12	
A													
B	N	N	B	B	CC	CC	4	4	3	3	1	1	Conjugate 1:1000
C	N	N	B	B	CC	CC	4	4	3	3	1	1	
D	N	N	B	B	CC	CC	4	4	3	3	1	1	Conjugate 1:1500
E	N	N	B	B	CC	CC	4	4	3	3	1	1	
F	N	N	B	B	CC	CC	4	4	3	3	1	1	Conjugate 1:2000
G	N	N	B	B	CC	CC	4	4	3	3	1	1	
H													

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 μ L 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.

$$(60 \text{ wells/plate}) (200 \text{ µL/well}) = 12,000 \text{ µL} = 12 \text{ mL}$$

Prepare 15 mL coating antibody at 1 µg/mL

$$X (1,000 \text{ µg/mL}) = 15,000 \text{ µL} (1 \text{ µg/mL})$$

$$X = 15 \text{ µL}$$

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.

$(192 \text{ wells}) (200 \text{ } \mu\text{L/well}) = 38,400 \text{ } \mu\text{L} = 38.4 \text{ mL}$
Prepare 45 mL.

$$\begin{aligned} 1/2000 &= X / 45 \text{ mL} \\ X &= 22.5 \text{ } \mu\text{L} \end{aligned}$$

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 .

1.14.A2 Bacterial Kidney Disease Appendix 2 - 5

- 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 preferable 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 $\times g$ 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

$$X (1000 \mu\text{g/mL}) = 105 \text{ mL} (1 \mu\text{g/mL})$$

$$X = 0.105 \text{ mL} = 105 \mu\text{L}$$

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 μ L/well) = 96,000 μ L = 96 mL
Again, make ~10 mL extra, or 105 mL.

$$\begin{aligned} 1/2000 &= X / 105 \text{ mL} \\ X &= 0.0525 \text{ mL} = 52.5 \mu\text{L} \end{aligned}$$

Combine 52.5 μ L 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 μ L 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 μ L 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 μ L 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, H₂S, 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.

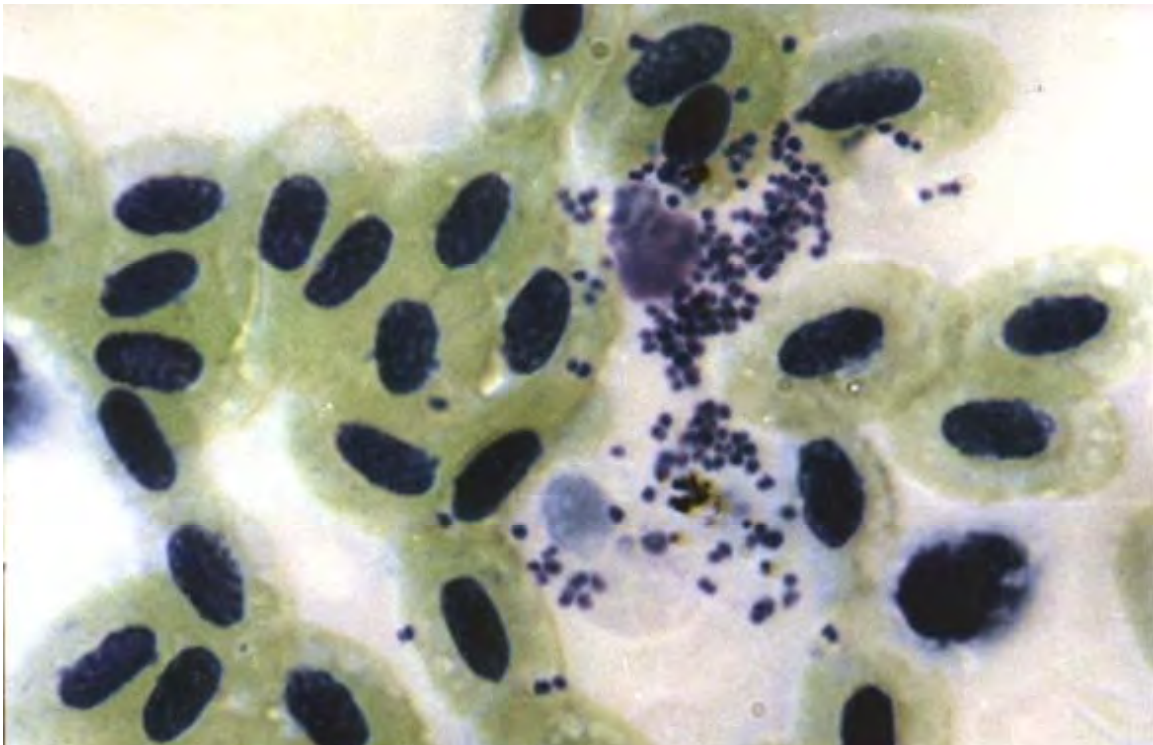


Figure 1. Blood smear from coho salmon infected with *Piscirickettsia salmonis*.

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.

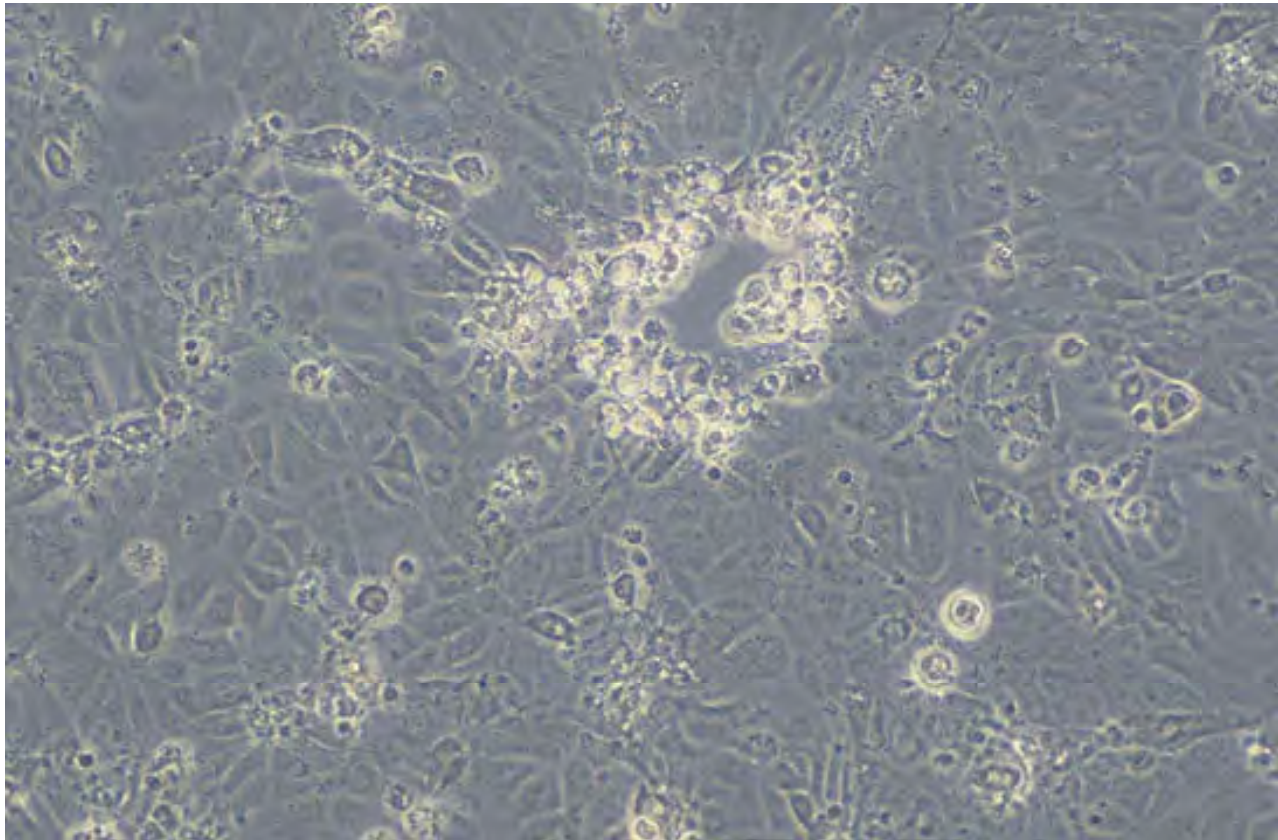


Figure 2. Cytopathic effect (CPE) of *Piscirickettsia salmonis* grown in a CHSE-214 cell culture.

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.

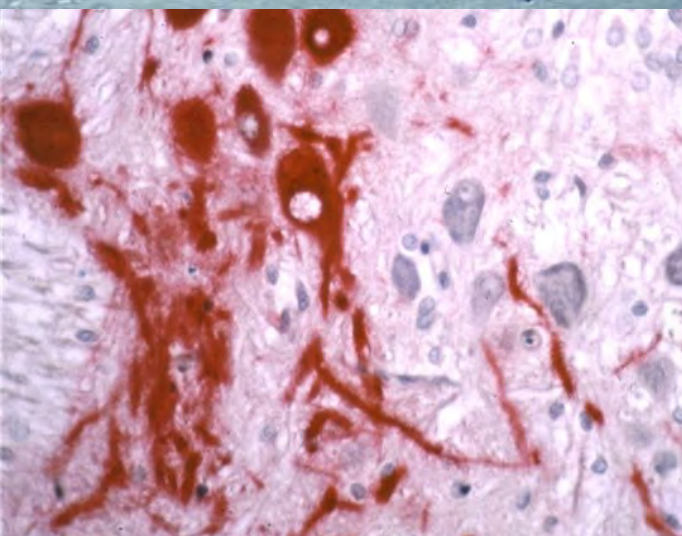
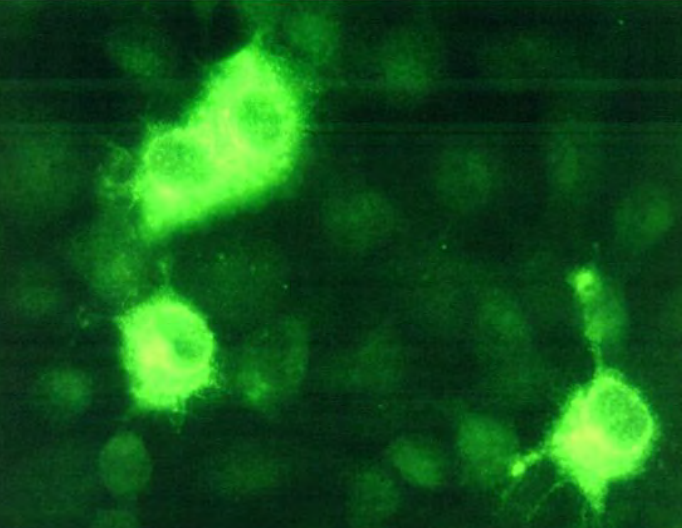
H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

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 $\leq -20^{\circ}\text{C}$.

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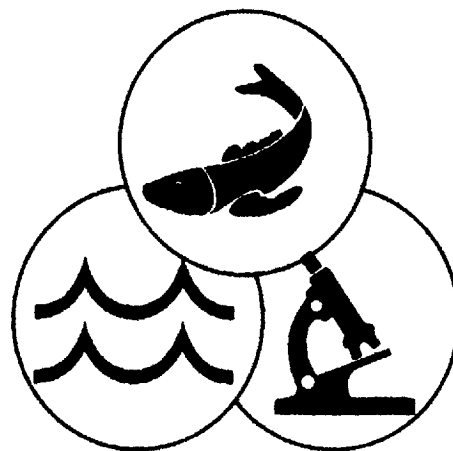
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Section 1

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.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.

<u>Size/maturity of fish</u>	<u>Tissue assayed</u>
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 1.0 cm² 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 cytopathological 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 (TCID₅₀) 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](#).

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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°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°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.

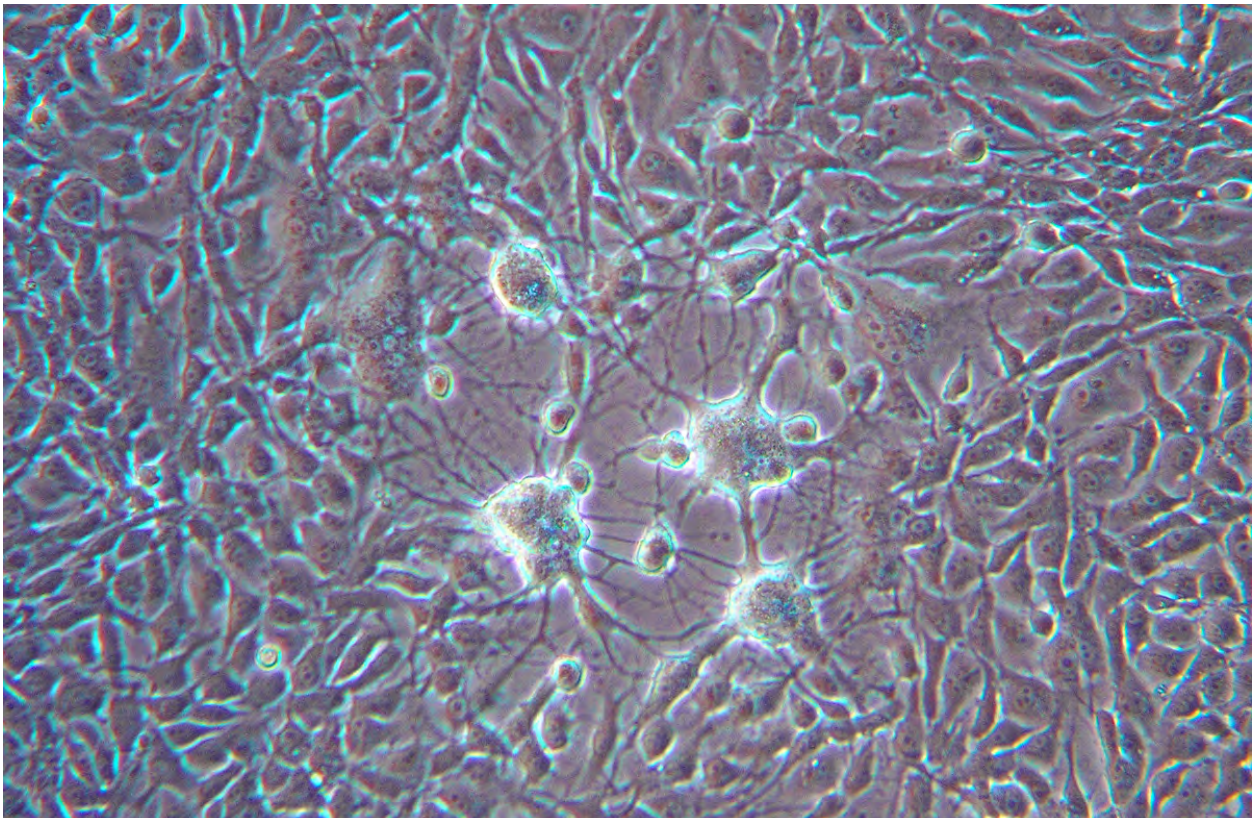
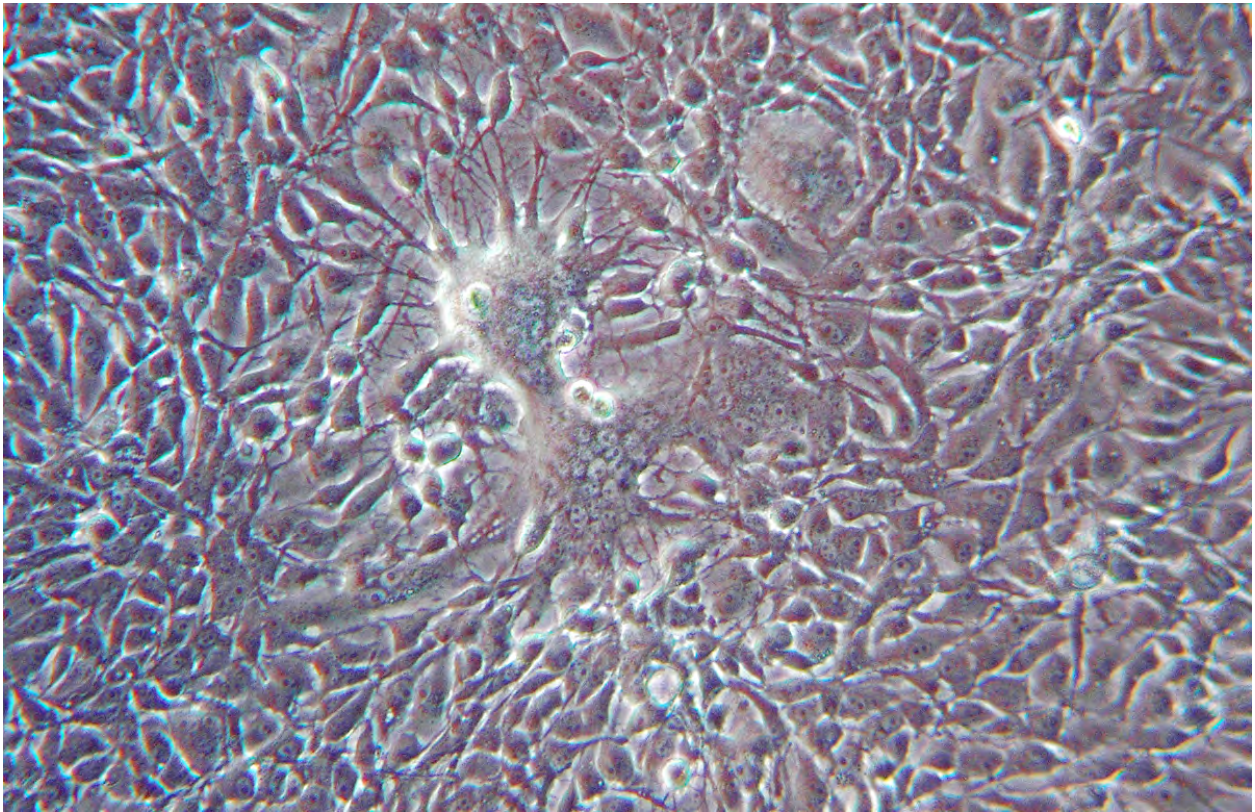
E. Disease Diagnostic Procedures

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°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°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 TCID₅₀ or PFU/0.1 mL of the serum and virus mixture. Sera demonstrating greater than 50% plaque or TCID₅₀ 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 rehydrated 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. Muroid 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). NeVTA 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 NeVTA, 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 mandibular 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 congregated 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°C rather than 10°C. OMV, YTV, and NeVTA replicate at 10 to 20°C and virus replication at 15°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.

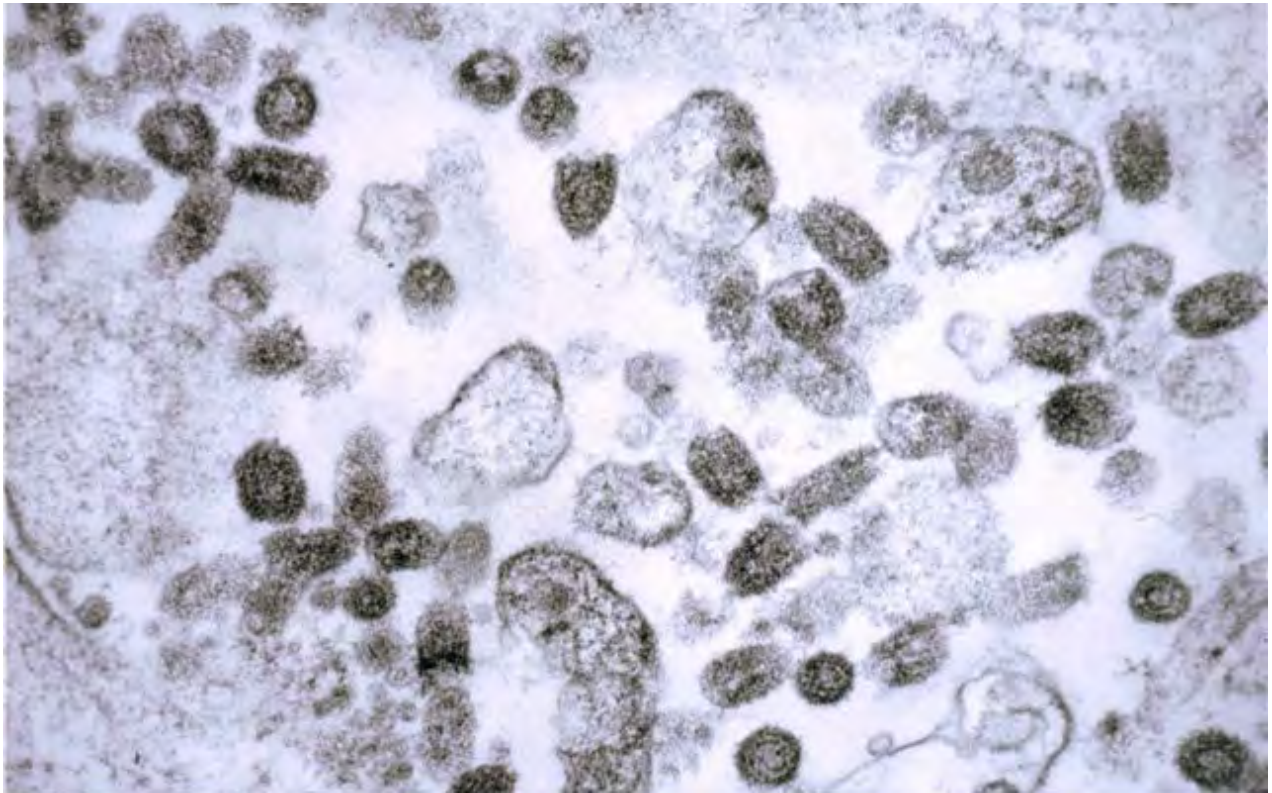


Figure 1. Electronmicrograph image of IHN virus in tissue culture.



Figure 2. Electronmicrograph image of negative stained IHN virus 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°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 than 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.b “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).

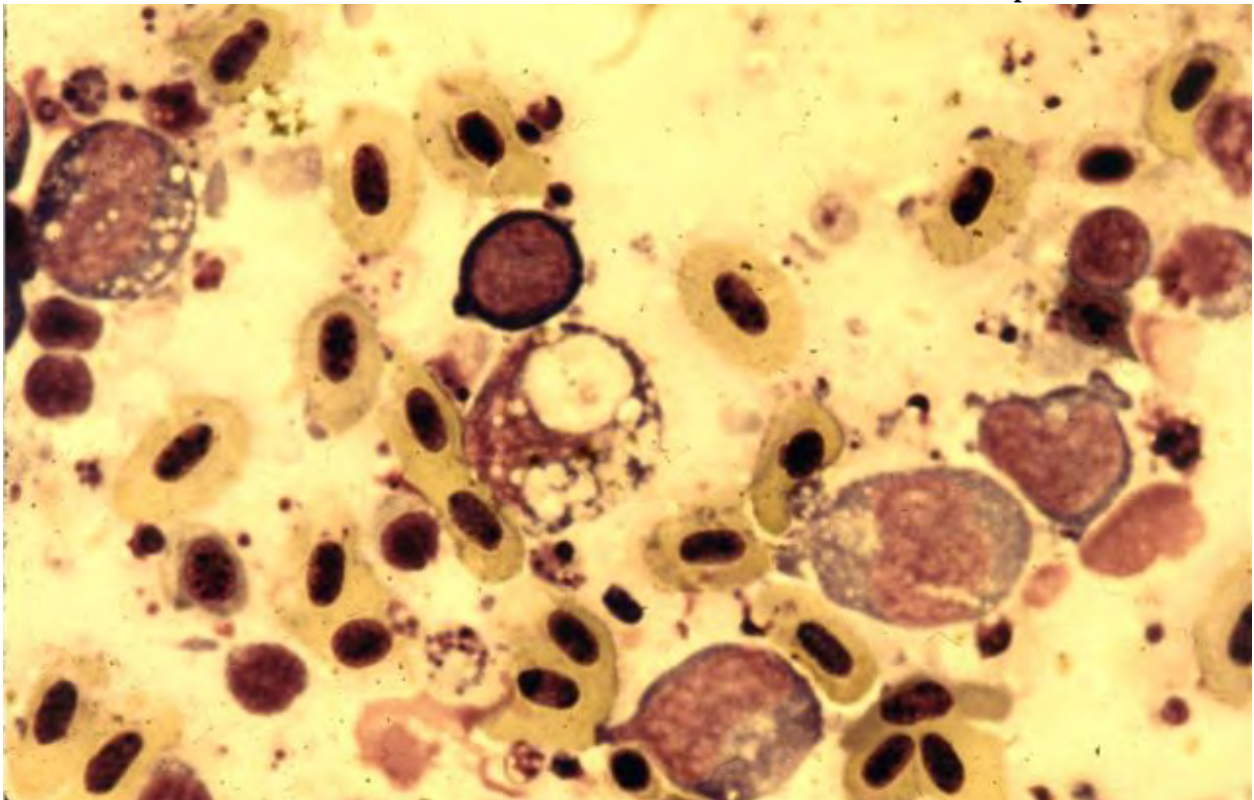


Figure 3. A kidney imprint from an IHN infected rainbow trout stained with Diff Quik®.

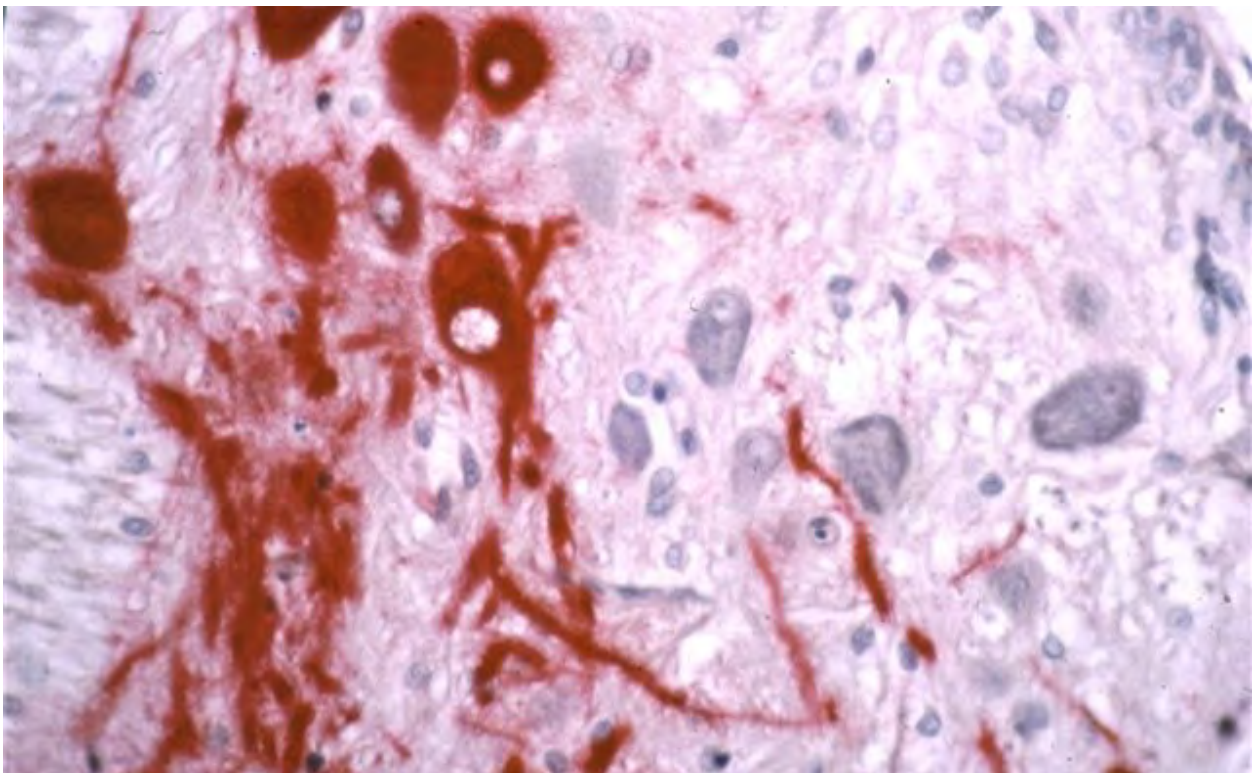


Figure 4. Alkaline phosphatase immunohistochemistry procedure (Drolet et al. 1994) on deparaffinized sections of brain tissue from an effected rainbow trout; the presence of IHNV antigen is indicated by the red staining).

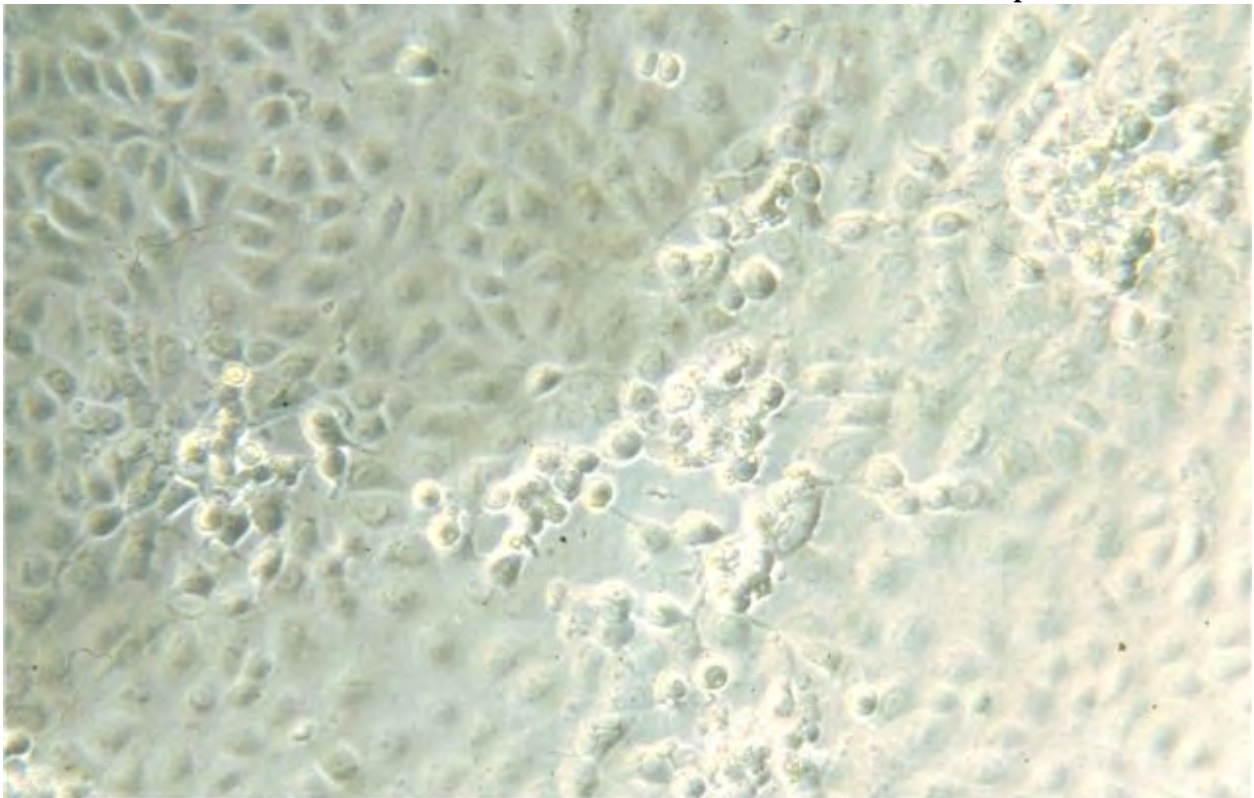


Figure 5. IHNV induced cytopathic effect on the CHSE-214 cell line 72 hours post-inoculation. The CPE also can be observed by studying plaque morphology.



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

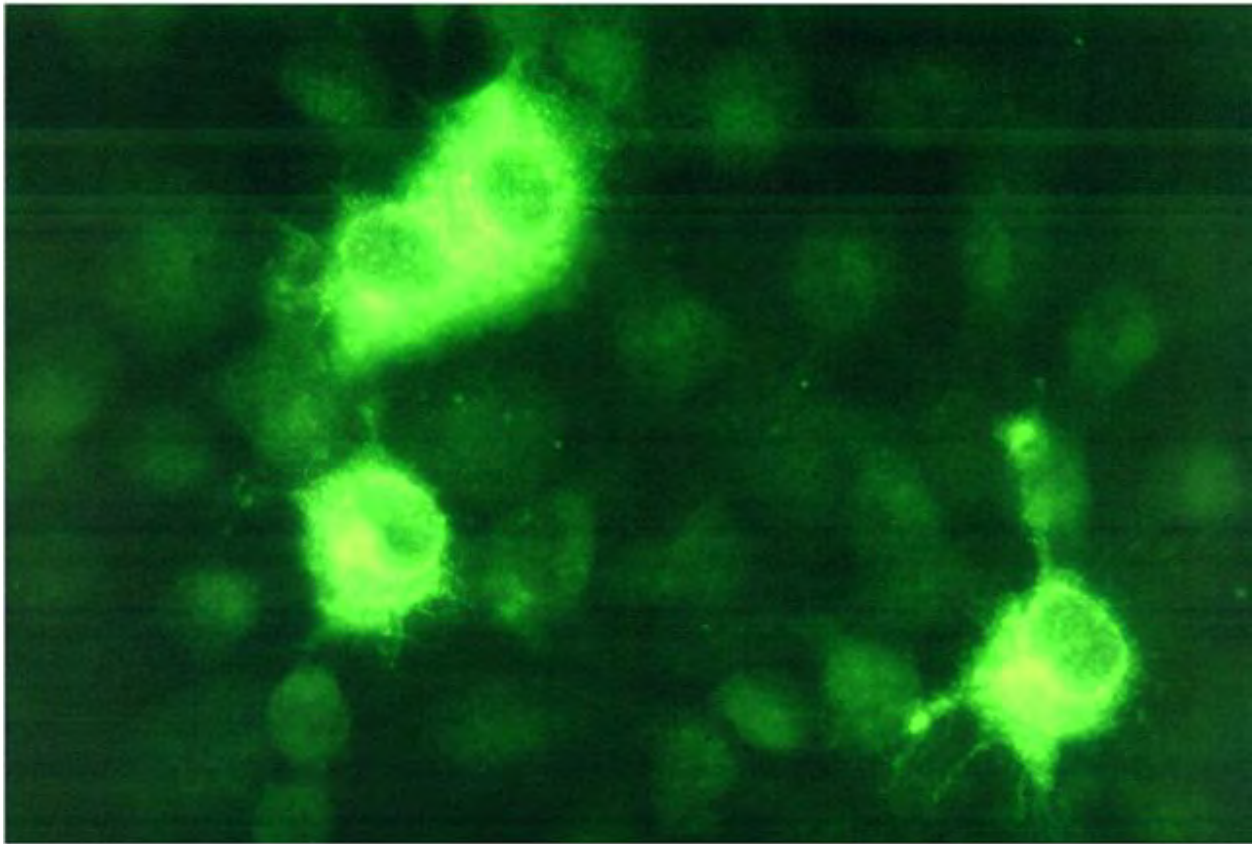


Figure 7. Confirmation of IHN by FAT on infected cell cultures; the presence of IHN antigen is indicated by the apple green fluorescence).

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 IHN, 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 IHN detection. IHN 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 IHN 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

P. E. McAllister

USFWS, National Fish Health Research Laboratory
PO Box 700
Kearneysville, WV 25430
304/725-8461
phil_mcallister@usgs.gov

A. Name of Disease and Etiological Agent

Infectious pancreatic necrosis (IPN) is a viral disease principally associated with salmonids, although IPN and IPN-like viruses (IPNV) have been isolated from various nonsalmonid fish and from invertebrates. Several IPNV serotypes are recognized including VR-299, Ab, and SP. Serogroups also proposed are: serogroup I that contains nine viruses and serogroup II that contains the IPNV-TV. The IPN and IPN-like viruses belong to a distinct taxonomic group of viruses called the birnaviruses. Some designations given to the disease caused by IPNV in nonsalmonid fish are branchionephritis in eels, spinning disease in menhaden, and viral ascites in yellowtail.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Infectious pancreatic necrosis occurs in North America (Canada and the United States), most of eastern and western Europe, Asia (China, Japan, Korea, Taiwan, and Thailand), Africa (South Africa), New Zealand, and South America (Chile).

2. Host Species

The earliest isolations of IPNV were made from brook trout *Salvelinus fontinalis*, rainbow trout *Oncorhynchus mykiss*, and brown trout *Salmo trutta*. IPNV and IPN-like viruses have been recovered from at least 67 freshwater and marine species representing salmonid fishes, nonsalmonid fishes, crustaceans, and molluscs. Some isolations have been made during natural epizootics while others were made from ostensibly healthy hosts.

C. Epizootiology

In salmonids, acute infections occur in one- to four-month-old fish and can result in cumulative mortality approaching 100%. Mortality develops rapidly at about 10 to 14°C, is protracted at lower temperatures, and can be reduced at higher temperatures. Isolates of IPNV show a broad range of pathogenicity. Older fish can undergo subclinical or inapparent infection with negligible mortality. Some survivors of infection become virus carriers, shedding virus with feces, urine, and sex products. These fish are the reservoirs of virus for contemporary and subsequent generations. The carrier state can continue for years in both

hatchery and natural environments. Disease outbreaks that occur in older fish involve virus carriers and are usually stress activated. The antibody response to infection is variable, and environmental conditions and stress factors affect the host response to infection. Inapparent infection, but rarely disease, occurs in the wild.

D. Disease Signs

A variety of clinical signs and histopathological changes can occur in infected fish, but none distinguishes IPN from the other fish viral diseases. Some hosts develop patent clinical signs of disease while others, although infected, appear normal.

External clinical signs can include dark pigmentation, exophthalmia, abdominal distention, mucoid fecal pseudocasts, and hemorrhages at the bases of and in the fins and on the body surface. Fish can swim erratically, rotating about the long axis or whirling violently. Internally, the liver and spleen are pale, and the stomach and intestine are without food but filled with mucoid material. Hemorrhages can be evident in the visceral organs.

Marked pathological changes can occur in the pancreatic, in renal and hepatic tissues, and in the intestinal mucosa. Pancreatic tissue can undergo severe necrosis characterized by pyknosis, karyorrhexis, and intracytoplasmic inclusions. The pylorus, pyloric caeca, and anterior intestine can undergo extensive necrosis, and sloughing intestinal epithelium combines with mucus forming a white catarrhal exudate. 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

Clinical signs and histopathological changes associated with IPN are variable and cannot be used for presumptive or definitive diagnosis or to distinguish IPN from other fish viral diseases. In an intensive culture setting, clinical signs for the species of fish and the history of the fish population and the culture station are used as part of a presumptive diagnosis. The absence of clinical signs does not indicate that the fish are free of IPNV. Virological examination is required for diagnosis of IPN and for inspection of fish stocks.

Virological examination consists of the assay of clinical samples (tissues and reproductive fluids) in cell cultures of blue gill fry (BF-2), chinook salmon embryo (CHSE-214), or rainbow trout gonad (RTG-2). Sonication (10 seconds at 75-100W) of the pellets obtained from ovarian fluid samples provide the same level of detection as tissue samples (McAllister et al. 1987).

2. Confirmatory Diagnosis

Confirmatory identification of IPNV can be done by the serum neutralization assay, the immunoblot (McAllister and Schill 1986), the enzyme-linked immunosorbent assay (ELISA), and FAT. Because several serotypes of IPN virus can be distinguished by infectivity neutralization, antiserum to each serotype or a polyvalent antiserum must be used in the serum neutralization assay. Similarly for ELISA, it is best to use antiserum to each serotype or a polyvalent antiserum even though significant cross-reactivity is apparent in ELISA.

F. Procedures for Detecting Subclinical Infections

Subclinical infections can be detected by cell culture assay.

G. Procedures for Determining Prior Exposure to the Etiological Agent

No definitive procedures have been reported. Monitoring virus-specific antibody is not routinely practiced because some IPN viruses react with components of normal trout serum.

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

See [Section 2, 2.2.A.2 "Receiving Jurisdiction Information."](#)

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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

P. E. McAllister and W. N. Batts*

USFWS, National Fish Health Research Laboratory
PO Box 700
Kearneysville, WV 25430
304/725-8461
phil_mcallister@usgs.gov

*USFWS National Fisheries Research Center
Naval Station-Bldg 204
Seattle, WA 98115
206/526-6282

A. Name of Disease and Etiological Agent

Viral hemorrhagic septicemia (VHS) is caused by a rhabdovirus designated viral hemorrhagic septicemia virus (VHSV). The disease has been known by various names of which Egtved disease and infectious kidney swelling and liver degeneration (INuL) disease are the best known. The virus has been known as the Egtved virus.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Viral hemorrhagic septicemia is enzootic in most countries of continental Europe and in Sweden and Finland. Viral hemorrhagic septicemia virus has been isolated in the Puget Sound area in the State of Washington and in the Gulf of Alaska near Cordova, Alaska. As of 1992, no disease outbreaks or virus isolations have been reported elsewhere in the United States.

2. Host Species

Epizootics of VHS occur primarily in rainbow trout *Oncorhynchus mykiss*, in brown trout *Salmo trutta*, and to a lesser extent northern pike *Esox lucius*. Natural infections have been diagnosed in chinook salmon *Oncorhynchus tshawytscha*, coho salmon *Oncorhynchus kisutch*, steelhead *Oncorhynchus mykiss*, Pacific cod *Gadus macrocephalus*, grayling *Thymallus thymallus*, and whitefish *Coregonus* sp. Atlantic salmon *Salmo salar*, brook trout *Salvelinus fontinalis*, lake trout *Salvelinus namaycush*, golden trout *Oncorhynchus aguabonita*, rainbow trout x coho salmon hybrids, gibel *Carassius auratus gibelio*, sea bass *Dicentrarchus labrax*, and turbot *Scophthalmus maximus* are susceptible to experimental challenge.

C. Epizootiology

VHS is readily transmissible to all ages of fish, and survivors of infection can become lifelong virus carriers. The virus ostensibly gains access to the fish through gills. Mortality can occur at temperatures from 3 to 12°C (mortality is greatest at 3 to 5°C); whereas at higher temperatures, mortality and the proportion of virus carriers decrease. At temperatures above 15°C, deaths from VHS rarely occur. 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. Inapparent infection, but rarely disease occurs in the wild. In captive fish, disease can recur seemingly as a consequence of culture and environmental stress.

D. Disease Signs

A variety of clinical signs and histopathological changes can be apparent in infected fish. 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, anemia, lethargy, hyperactivity, and hemorrhages in the eyes, skin, gills, and at the base of fins. Internally, visceral mesenteries can show diffuse hemorrhage, 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, kidneys, spleen, and skeletal muscle. In the liver, kidneys, and spleen, focal to extensive necrotic changes can occur, i.e. vacuolation, pyknosis, karyolysis, lymphocytic infiltration, and occasionally inclusion body formation. The hematopoietic areas of the kidney and spleen are the initial foci of infection. In skeletal muscle, blood cells (primarily erythrocytes) accumulate in muscle bundles and fibers but little damage occurs.

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 and for inspection of fish stocks. Virological examination consists of the assay of clinical samples in cell culture. During cell assay incubation, the pH of the medium must remain within the range of 7.4 to 7.8. Cell cultures of chinook salmon embryo (CHSE-214), epithelioma papulosum cyprini (EPC), fathead minnow (FHM), or rainbow trout gonad (RTG-2) may be used.

The EPC cell line is recommended because of pH stability and its ability to be pretreated with polyethylene glycol (PEG). Virus adsorption to EPC cells can be enhanced by pretreating the cells with a 7% solution (final concentration) of polyethylene glycol (PEG, 20,000 MW; Batts and Winton 1990) or by adding DEAE dextran (Campbell and Wolf, 1969; 50 µg/mL final concentration) or PEG to the sample diluent during adsorption.

2. Confirmatory Diagnosis

Confirmatory diagnosis of the VHSV can be done by the serum neutralization assay, the immunoblot assay (McAllister and Schill 1986; McAllister and Owens 1987), the enzyme-linked immunosorbent assay (ELISA) (Way and Dixon 1988; Olesen and Jorgensen 1991), or the FAT (Lorenzen, et al. 1988). For the serum neutralization assay, the cell cultures and the conditions of incubation and pH control must be maintained as indicated above. Antiserum to each serotype must be used in the serum neutralization assay because three serological types of VHS can be distinguished by infectivity neutralization. For the ELISA, only antibody to VHSV (serotype F1) is needed because this antibody binds all three VHSV serotypes.

F. Procedures for Detecting Subclinical Infections

Subclinical infections can be detected by cell culture assay. In some instances, VHS virus has only been detected by examination of brain tissue.

G. Procedures for Determining Prior Exposure to the Etiological Agent

The immune response of survivors of VHS epizootics and of inapparent VHS virus carriers varies with both the fish and season of the year. Nevertheless, detection of VHS virus-specific neutralizing antibody can be a useful tool for VHS surveillance (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 to 7.8) or cell culture medium (pH 7.4 to 7.8) and shipped on ice or at 4°C. Glycerol should never be used to preserve specimens because VHS virus has been shown to be inactivated.

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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 (1cm³ 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.

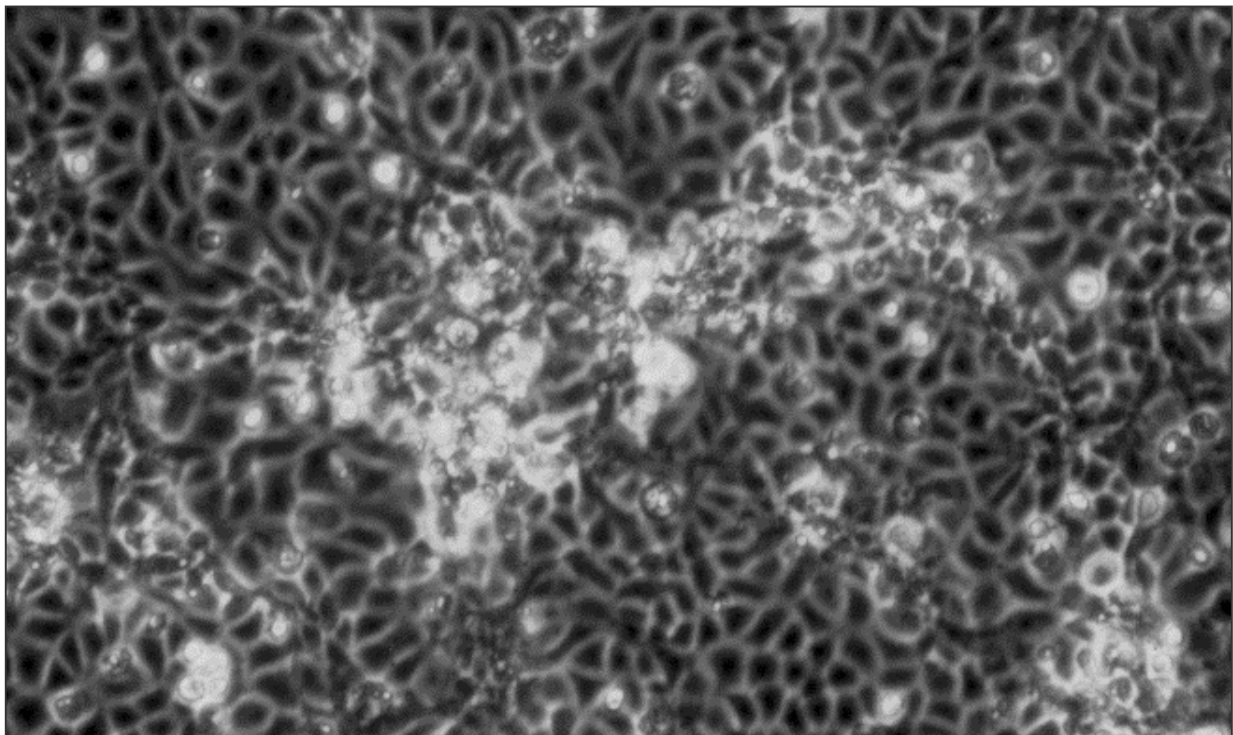


Figure 1. Cytopathic effect on CHSE-214 cells.

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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-H₂O 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 37°C, one week at 25°C, one month at 4°C, or indefinitely at -20°C without degradation. The tissue is considered compromised unless it is placed in RNA Later™ directly after sampling from the fish and stored below 37°C 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)
 Microcentrifuge (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 μ L 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 μ L of the supernatant to another microfuge tube containing 750 μ L 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 μ L 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 μ L 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 μ L 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 105°C
 1. 42°C for 15 minutes.
 2. 94°C for 5 minutes.
 3. 59°C for 45 seconds.
 4. 72°C for 105 seconds.
 5. 94°C for 45 seconds.
 6. 59°C for 45 seconds.
 7. 72°C for 105 seconds.
 Go to (5) for 36 cycles.
 72°C for 7 minutes.
 Hold 4°C.
 - 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-H₂O 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 1cm³ 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, ($>10 \text{ field}^{-1}$) 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 and, in one incomplete report, South America. The Office International des Epizooties (OIE) reference laboratory for SVCV, the Centre for Environment, and the Fisheries and Aquaculture Science (CEFAS) in Weymouth, England, have isolated SVCV from fish newly imported from Asia. SVCV has been isolated 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.

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.

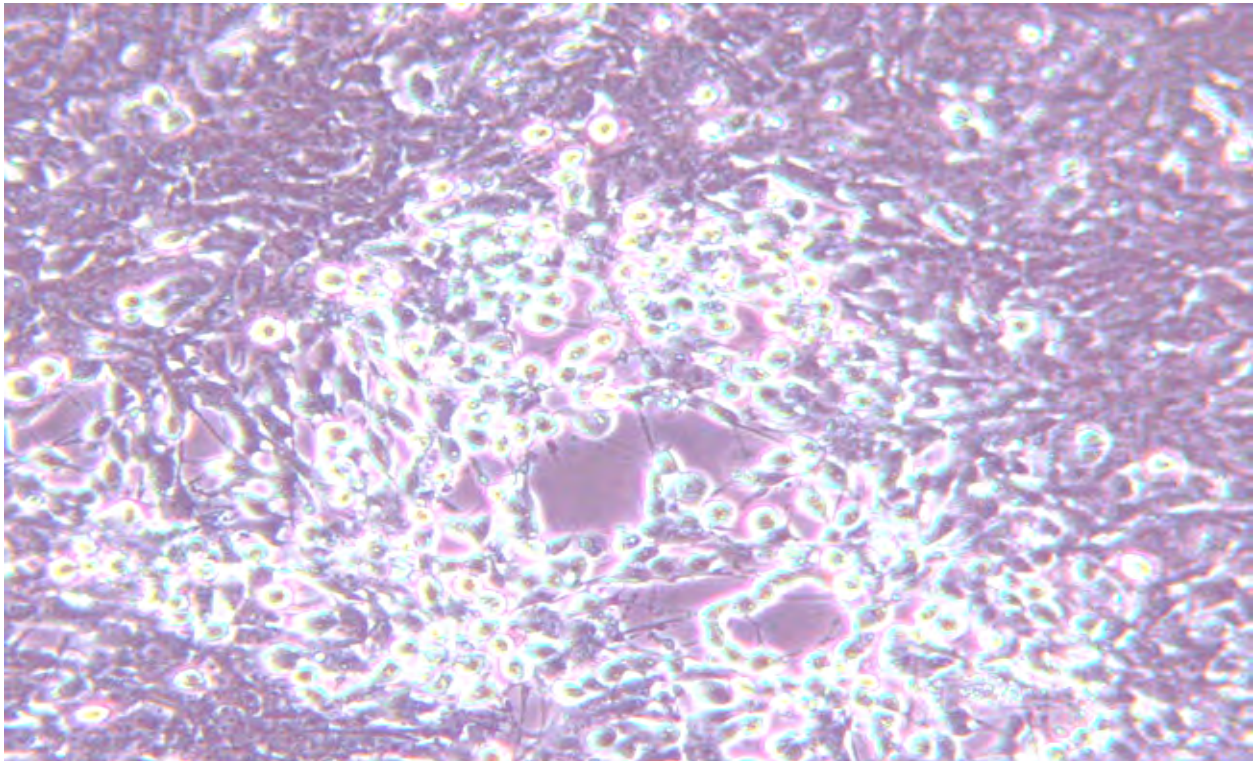


Figure 2. Cytopathic effects of SVCV in a monolayer of EPC cells. Note the foci of rounded and dead cells. Phase contrast.

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.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

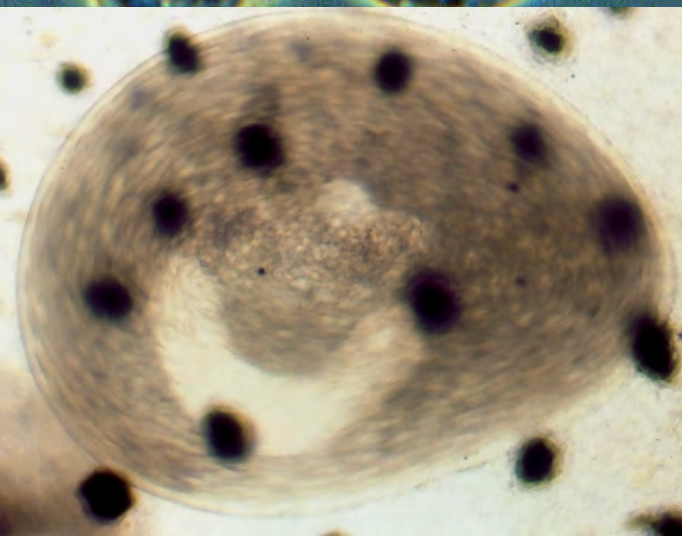
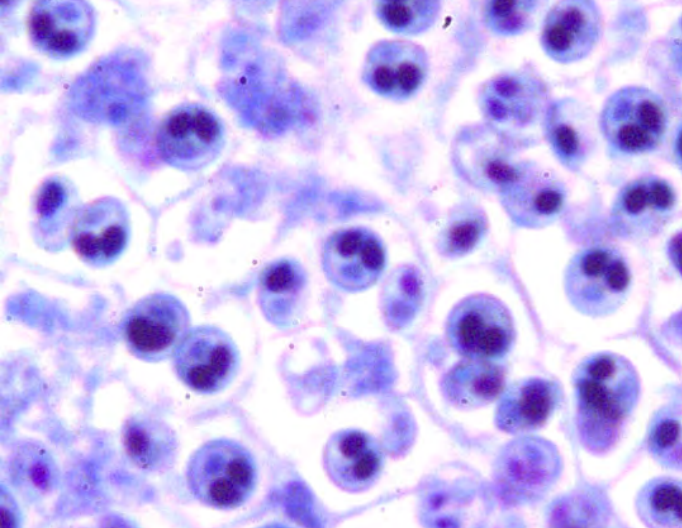
The specific method used to screen stock cell cultures for mycoplasma depends on the availability of certain laboratory equipment. References describing the comparative sensitivities of the various methods of mycoplasma detection are listed. Regardless of the method used, stock cell cultures should be 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 mycoplasma 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.

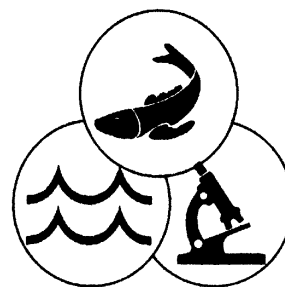
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Section 1

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

3.1 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).

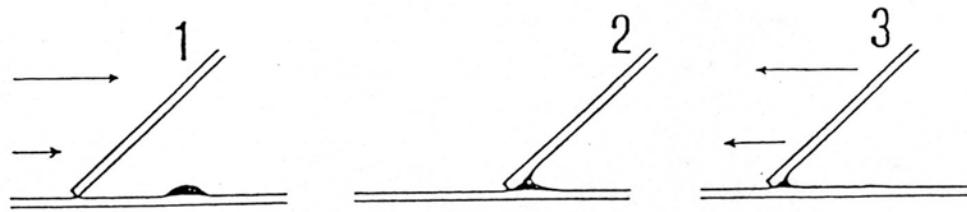


Figure 1. Preparation of a blood smear (from Moller and Anders, 1986).

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 saline 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. $10\% \times 40\% \text{ of stock} = 4\%$).

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*, *Ichtyobodo*, 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° to 30°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.

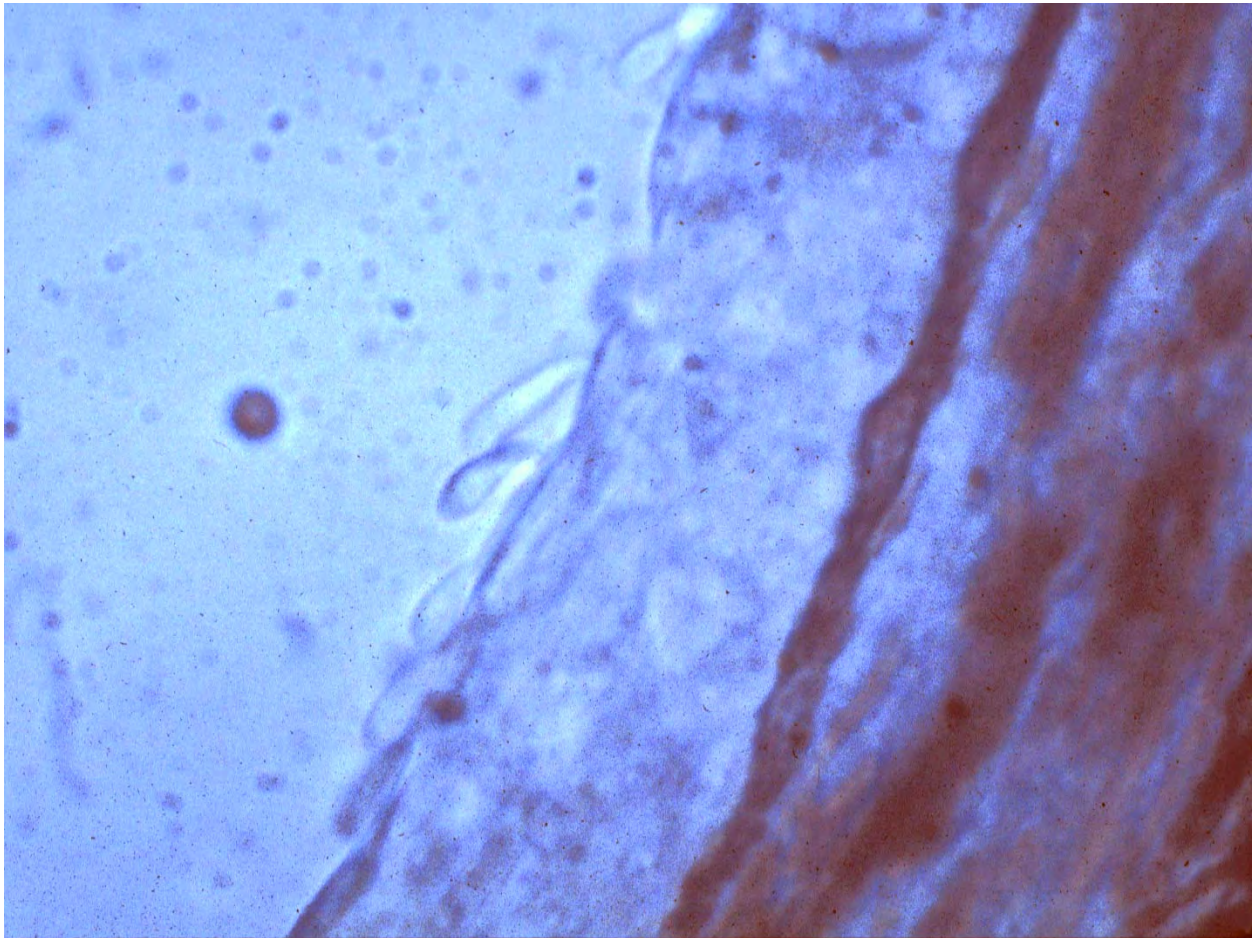


Figure 1. *Ichthyobodo* on the epithelium of a catfish gill (picture by Fred Meyer).

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).

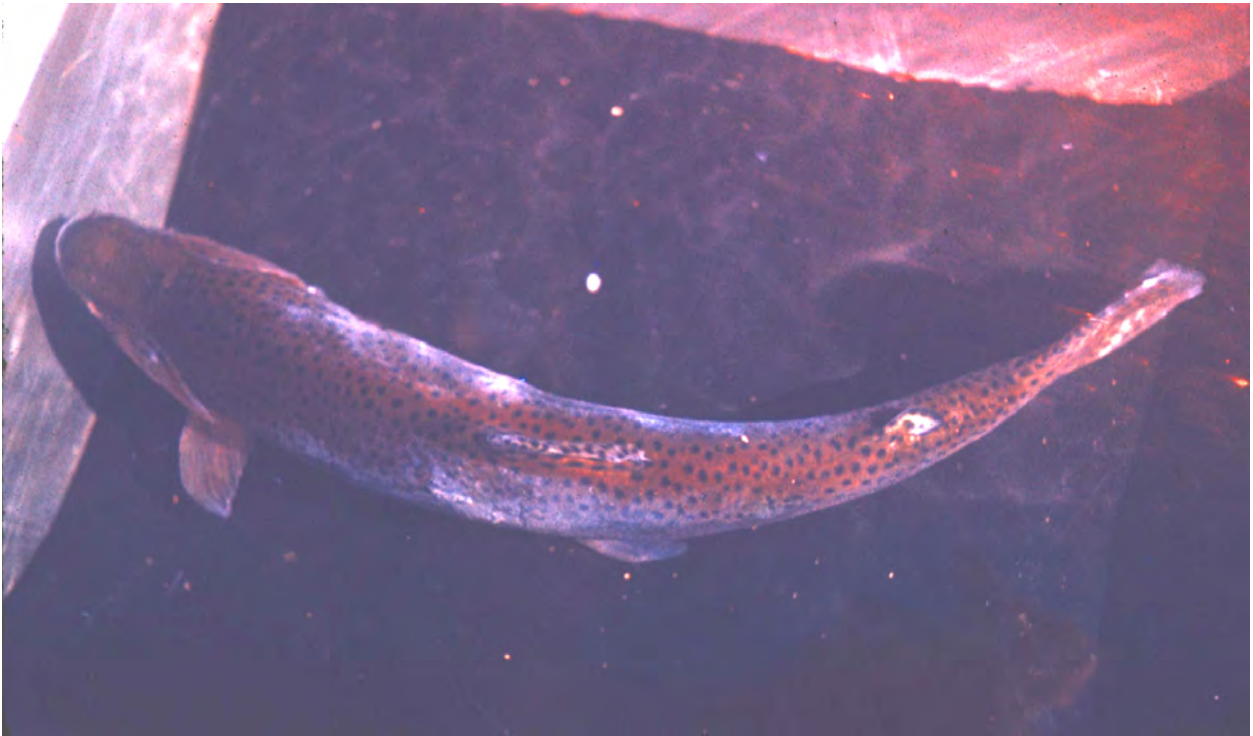


Figure 2. Blue slime signs in a trout (picture by Fred Meyer).

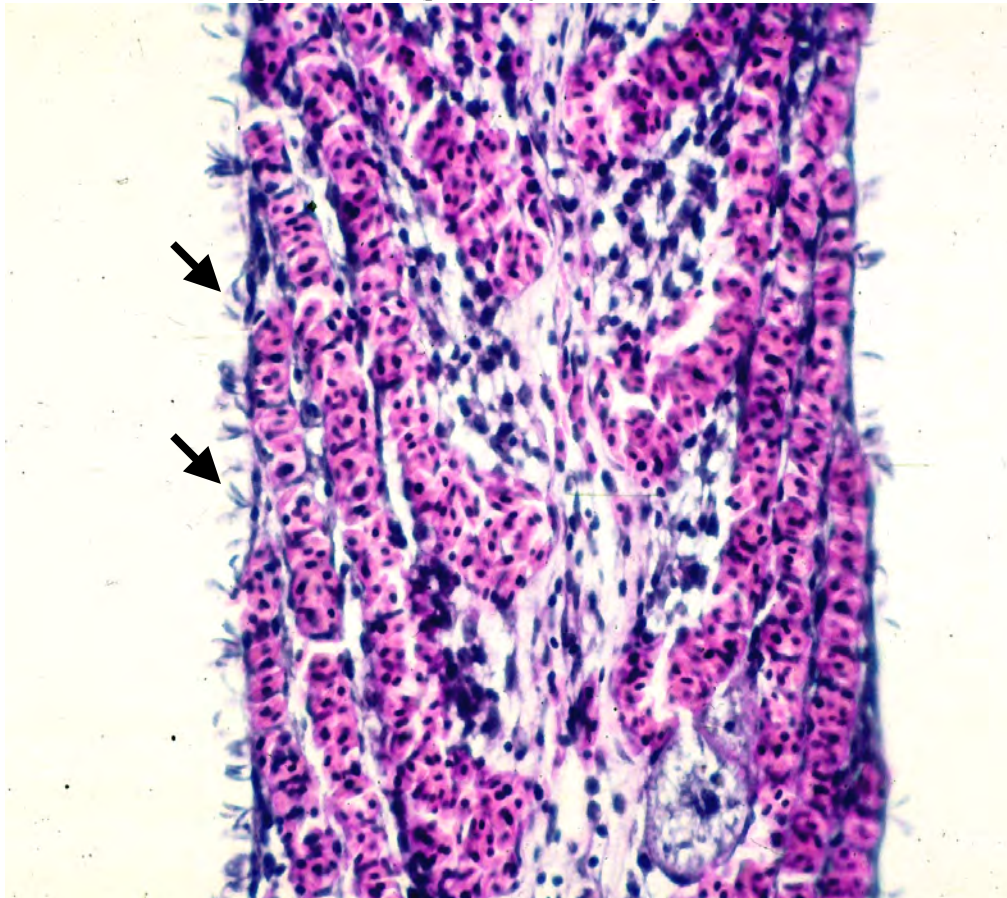


Figure 3. Numerous *Ichthyobodo* on the surface of a catfish gill. Histology with H&E stain (picture by Andy Goodwin).

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, Spirotrichuriasis

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*), octomitos (Octomitus), or spirotrichuriasis (*Spirotrichurus*); 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 *Spirotrichurus*. 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 *Spirotrichurus* 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 *Spirotrichurus*.

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 strains 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*”
Gastrointestinal 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 through 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	<i>Spironucleus</i>	<i>Hexamita</i>	<i>Octomitus</i>
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.

3.3 Diplomonad (Hexamitid) Flagellates - 6

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).

Species	External	Internal	
	surface and ornamentation	pattern of microtubular bands accompanying flagellar pocket (a)	other cytoskeletal elements
<i>S. barkhanus</i>	unadorned body, rf emerge from barhans (b)	3 radiate	-
<i>S. torosa</i>	unadorned body, rf emerge from tori (c) caudal projection, trophozoites can attach to host microvilli	3 concentric	mt + lamellae support tori and flanges
<i>S. vortens</i>	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
<i>S. elegans</i> *	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

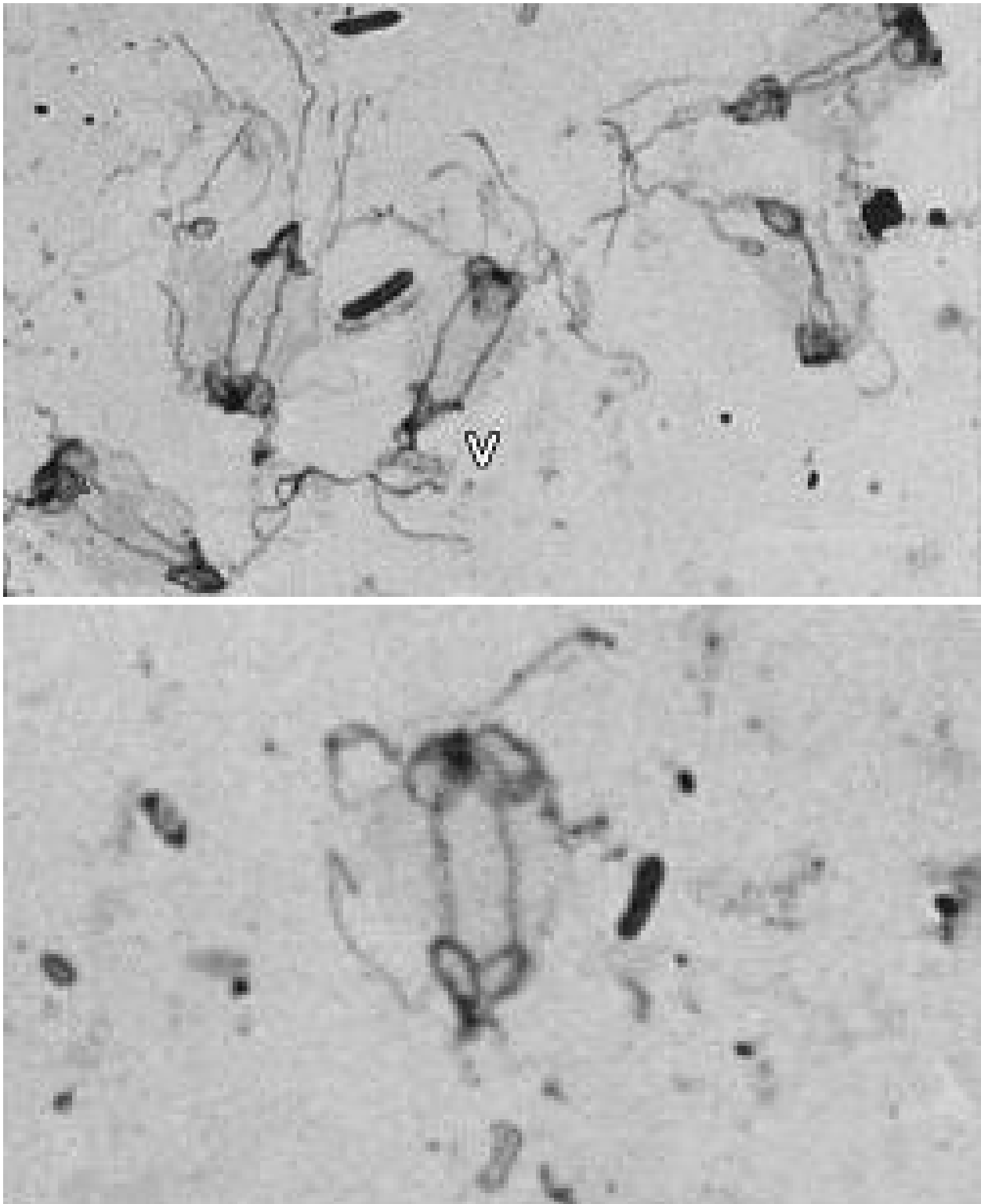
mt = microtubules

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.

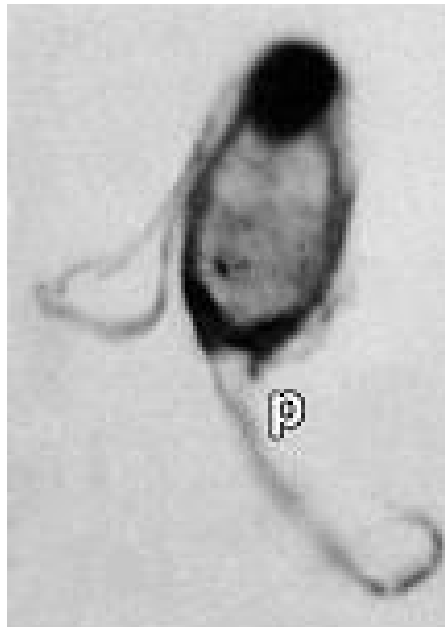


Figure 5. Preparation of *S. vortens* stained by Protargol silver protein (filter method); note dark area across posterior end of body (which TEM shows to be an extensive symmetrical system of microtubules supporting the elaborate architecture of the counter-crossing ridges at the posterior end of the 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.

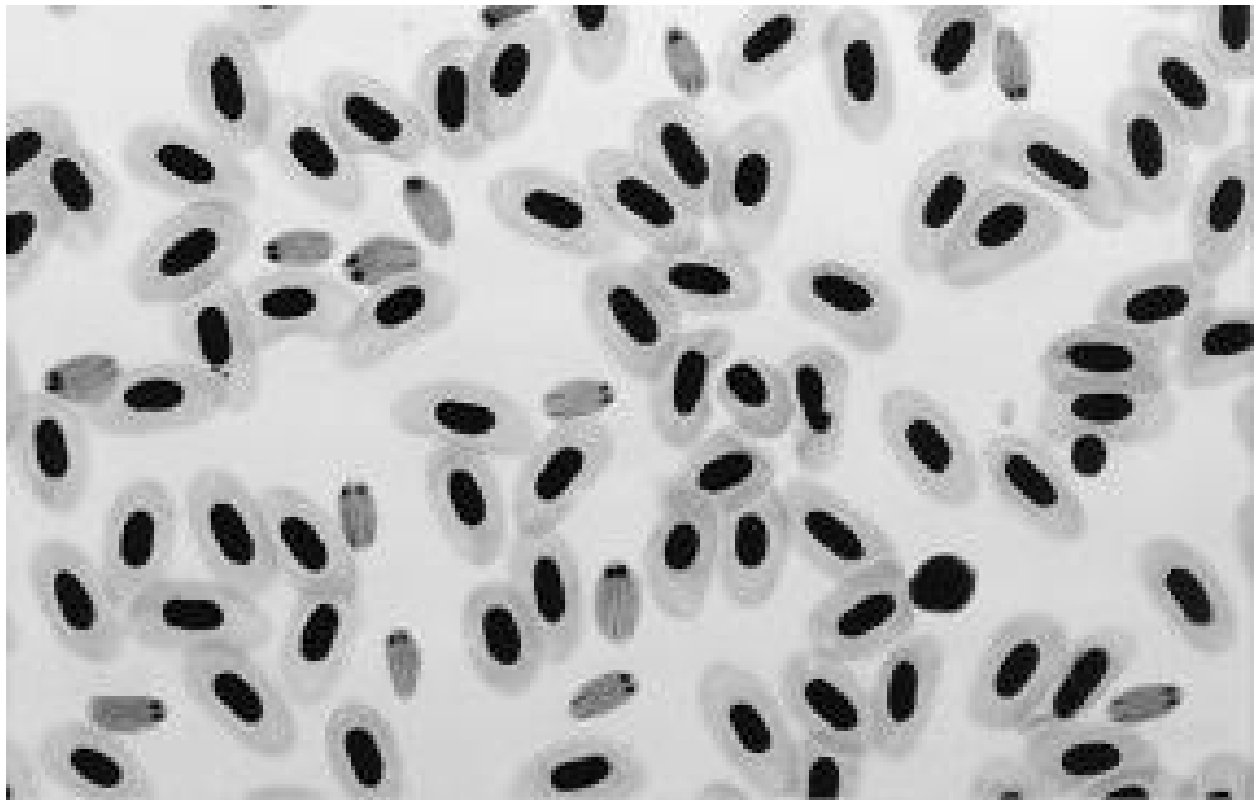


Figure 6. Unidentified diplomonads from blood smear stained with Leishman's Giemsa; note paired nuclei and passage of recurrent flagella through the cell. Originally published in Diseases of Aquatic Organisms 1992 (14, 81-89) and reproduced with permission of Inter-Research.

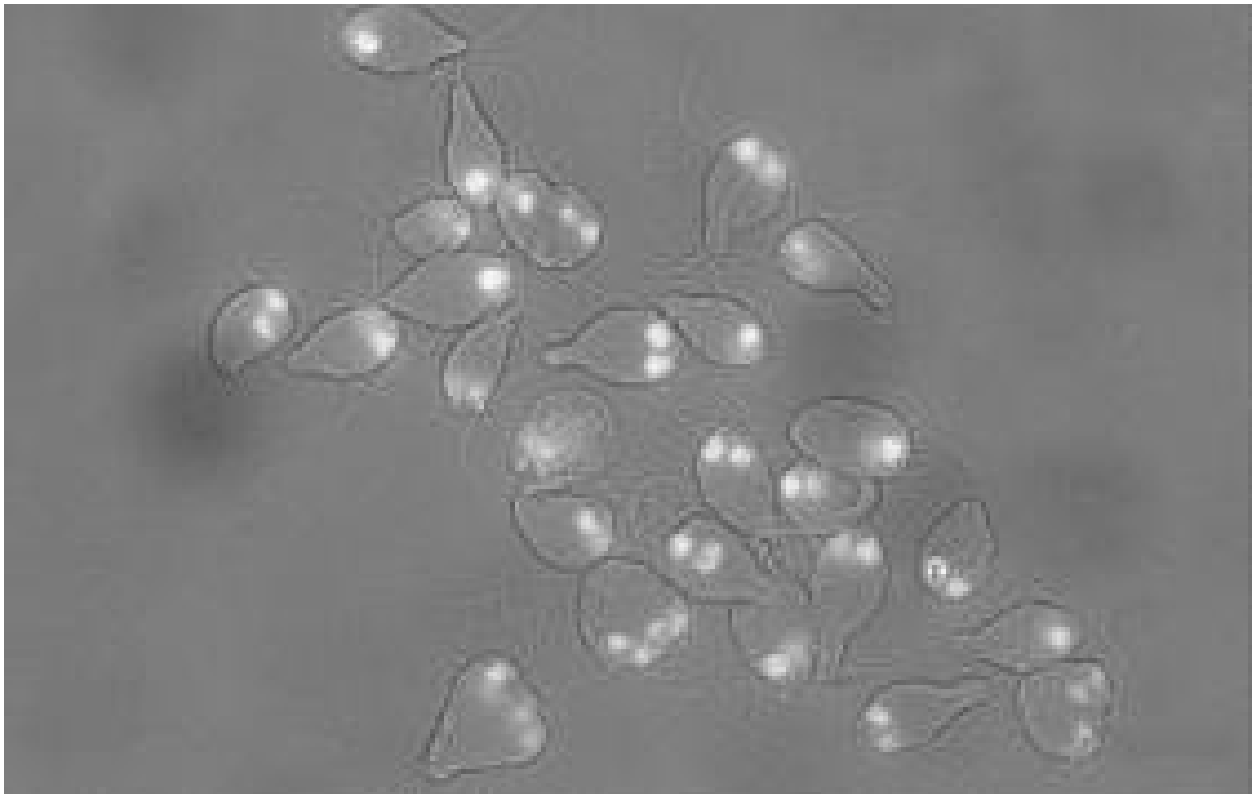


Figure 7. Culture of *S. barkhanus* stained with DAPI (4'-6-diamidino-2-phenylindole) and exposed to bright field and UV illumination, nuclei appear blue/violet (white in this photograph).

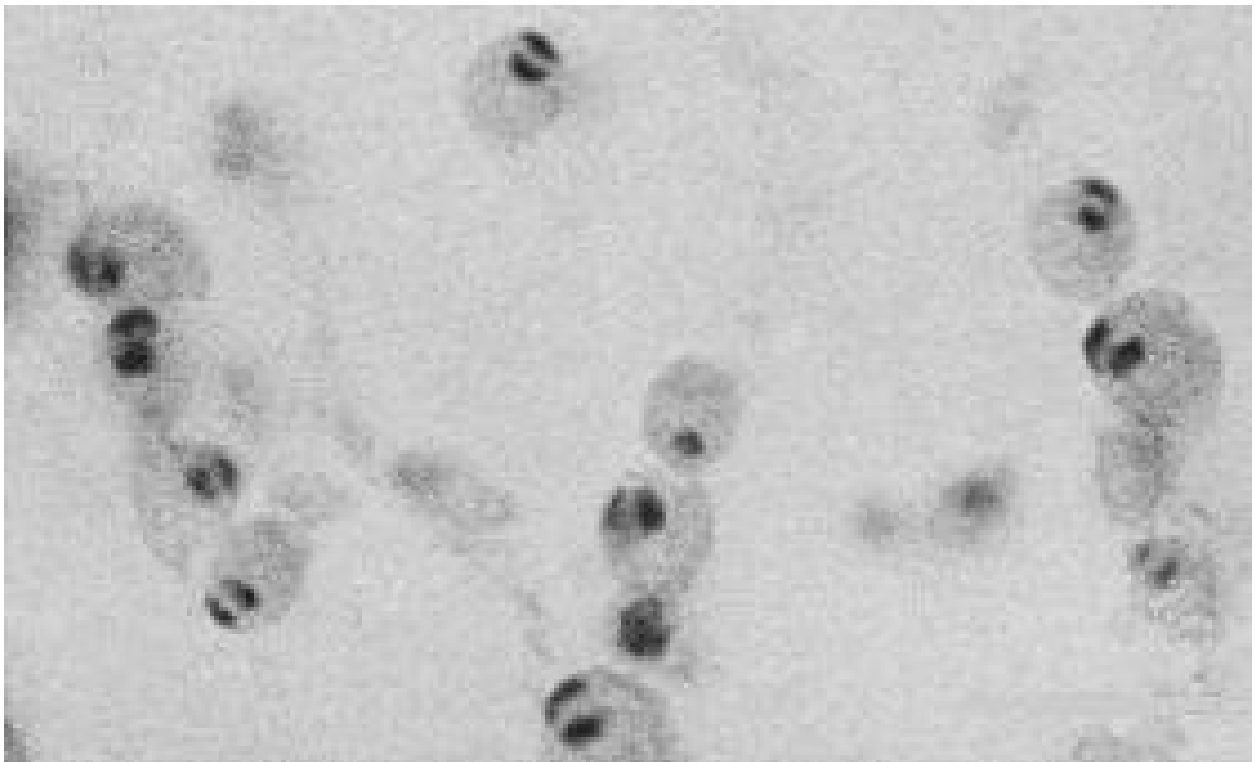


Figure 8. *S. torosa* in tissue section (lumen of rectum) stained by the Feulgen reaction. Originally published in the Journal of Protozoology 1990 (37, 369-383) and reproduced with permission of the Society of Protozoologists.

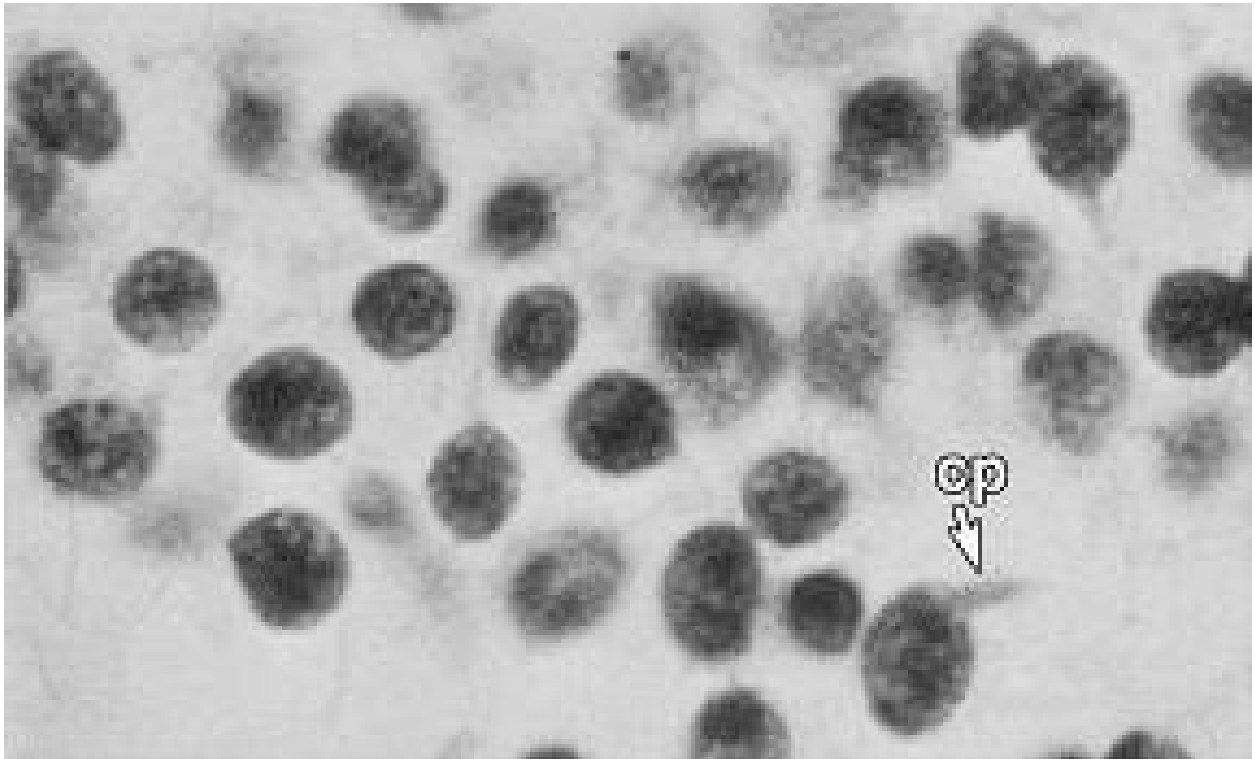


Figure 9. *S. torosa* in tissue section (lumen of rectum) stained with haematoxylin and eosin; (cp) caudal projection. Originally published in the Journal of Protozoology 1990 (37, 369-383) and reproduced with permission of the Society of Protozoologists.

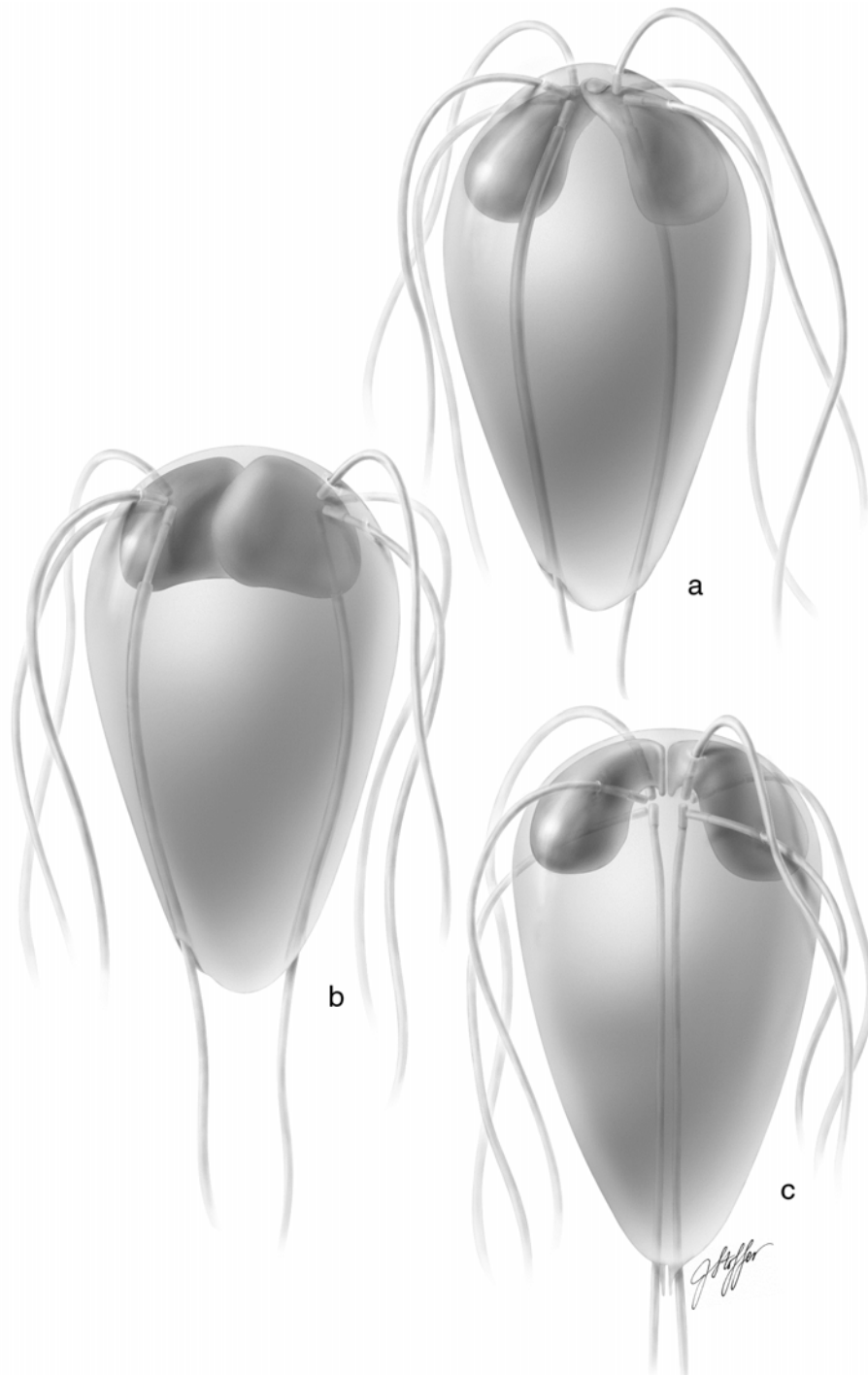


Figure 10. Principal distinguishing features of the three genera of diplomonads within the suborder Diplomonadina that have been reported from fish, (a) *Spironucleus*; (b) *Hexamita*; (c) *Octomitus*. Organisms shown in ventral view. Note especially the presence or absence of flagellar pockets (cytostomal 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 Ovipleistophoriosis (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
rsommerf@iastate.edu

A. Name of Disease or Etiological Agent

The ovary parasite of golden shiner, *Notemigonus crysoleucas*, is the microsporidan *Ovipleistophora ovariae* (Phylum *Microsporida*, commonly called the microsporidia, formerly named *Pleistophora ovariae*). This parasite causes a disease known as ovipleistophoriosis.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Areas where ovipleistophoriosis has been reported include: Alabama, Arkansas, California, Illinois, Kansas, Kentucky, Louisiana, Mississippi, Missouri, North Carolina, Oklahoma, Tennessee, and Texas. Ovipleistophoriosis should be expected in golden shiners from commercial sources.

2. Host Species

The type host for ovipleistophoriosis is the golden shiner and it has been reported once in fathead minnows, *Pimephales 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*. *Ovipleistophora 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 tahoensis*, 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°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 microsporidian 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*.

3.4 *Ovipleistophoriasis* (*Pleistophora*): Ovary Parasite of Golden Shiners - 3

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 microsporidia. 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 intensively blue and the nucleus less intensively 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

3.4 Ovipleistophoriosis (Pleistophora): Ovary Parasite of Golden Shiners - 4

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 *Ovipleistophora 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.

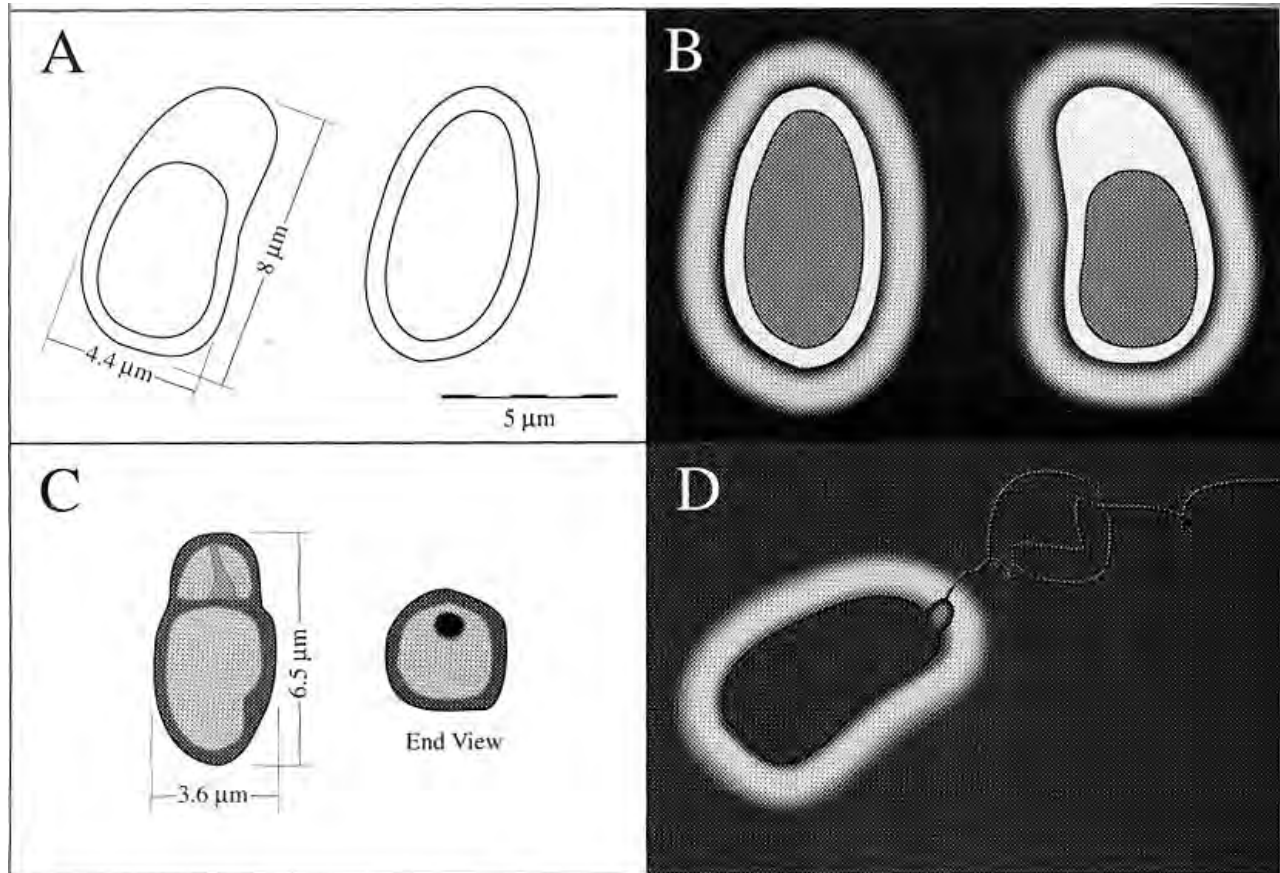


Figure 1. Spores of *Pleistophora ovariae*.

- 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:Myxosporidia).

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.

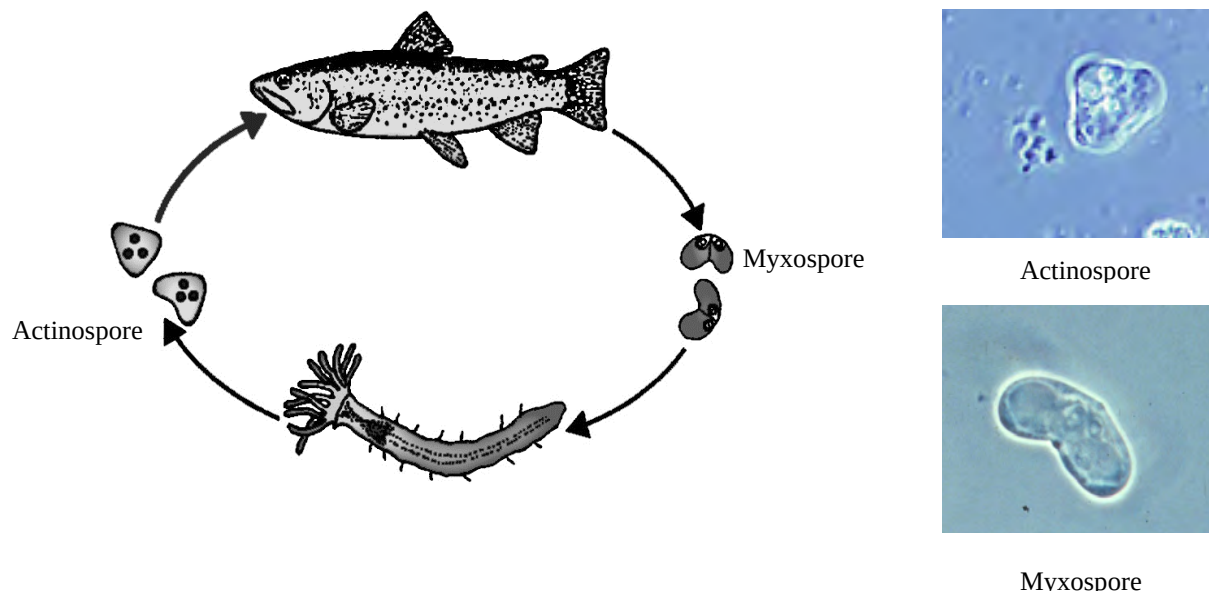


Figure 1. Life cycle of *Ceratomyxa shasta* showing salmonid and polychaete host and alternating myxosporean and actinosporean life stages shown at right.

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, exophthalmia, 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 from 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.

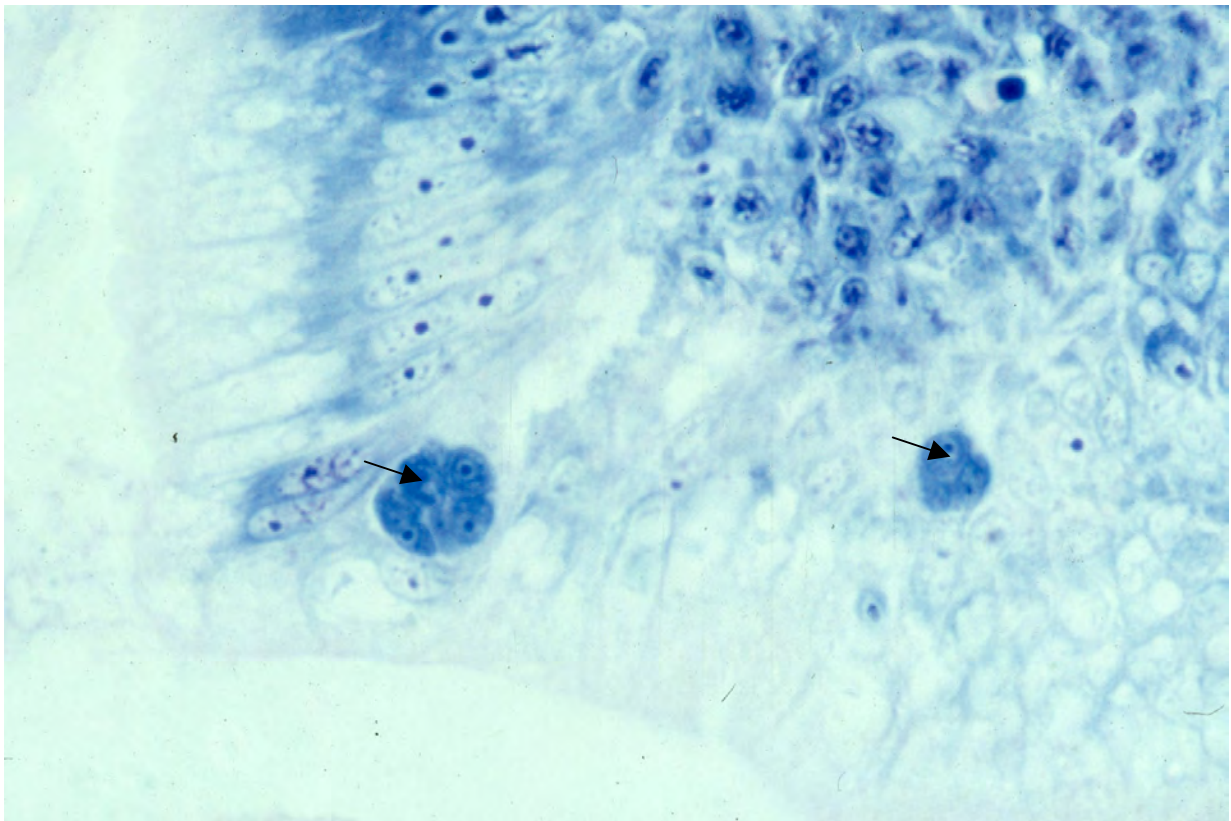


Figure 3. Giemsa stained histological section of intestine from an infected rainbow trout, arrows indicate trophozoites.

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.



6



Figures 4 and 5. Mature *Ceratomyxa shasta* spores. Bar = 10 μm .

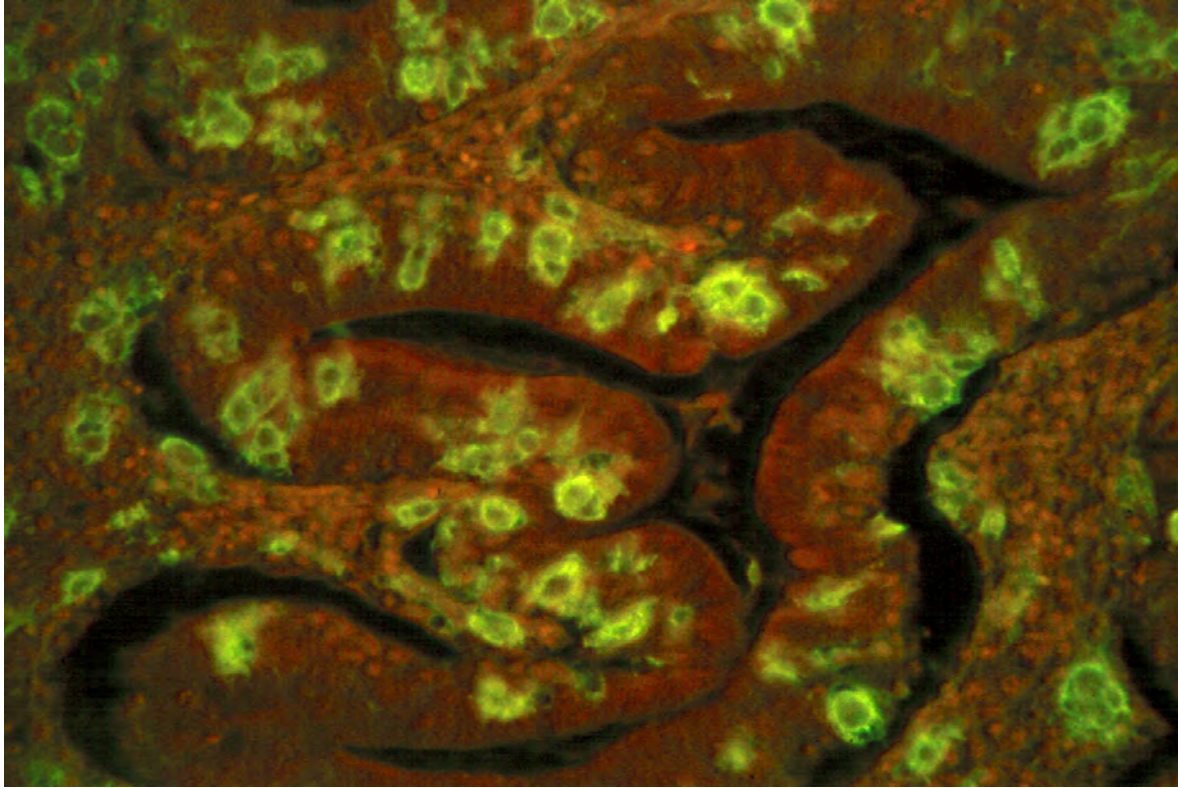


Figure 5. Trophozoite stages in histological section of intestinal tissue using a fluorescein-conjugated monoclonal antibody specific for *Ceratomyxa shasta*.

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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 it 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°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°C) or make at a concentration of 20 mg.mL⁻¹ and freeze at -20 °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°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

3.5.A1 Salmonid Ceratomyxosis Methods Appendix 1 - 3

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 $\mu\text{g.mL}^{-1}$ (from a liquid stock at 20 mg mL). Incubate at 37° 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° 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 **10 mL** 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]*

3.5.A1 Salmonid Ceratomyxosis Methods Appendix 1 - 4

Make the mix on ice and under nuclease-free conditions. Aliquot and store at -20° 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°C/3m + 35 cycles x {94°C/1m + 58°C/30s + 72°C/1m}+72 °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.

3.6 Whirling Disease of Salmonids - 2

Mortality in young salmonids is highly variable. Survival increases with increasing age of fish at first exposure to the parasite.

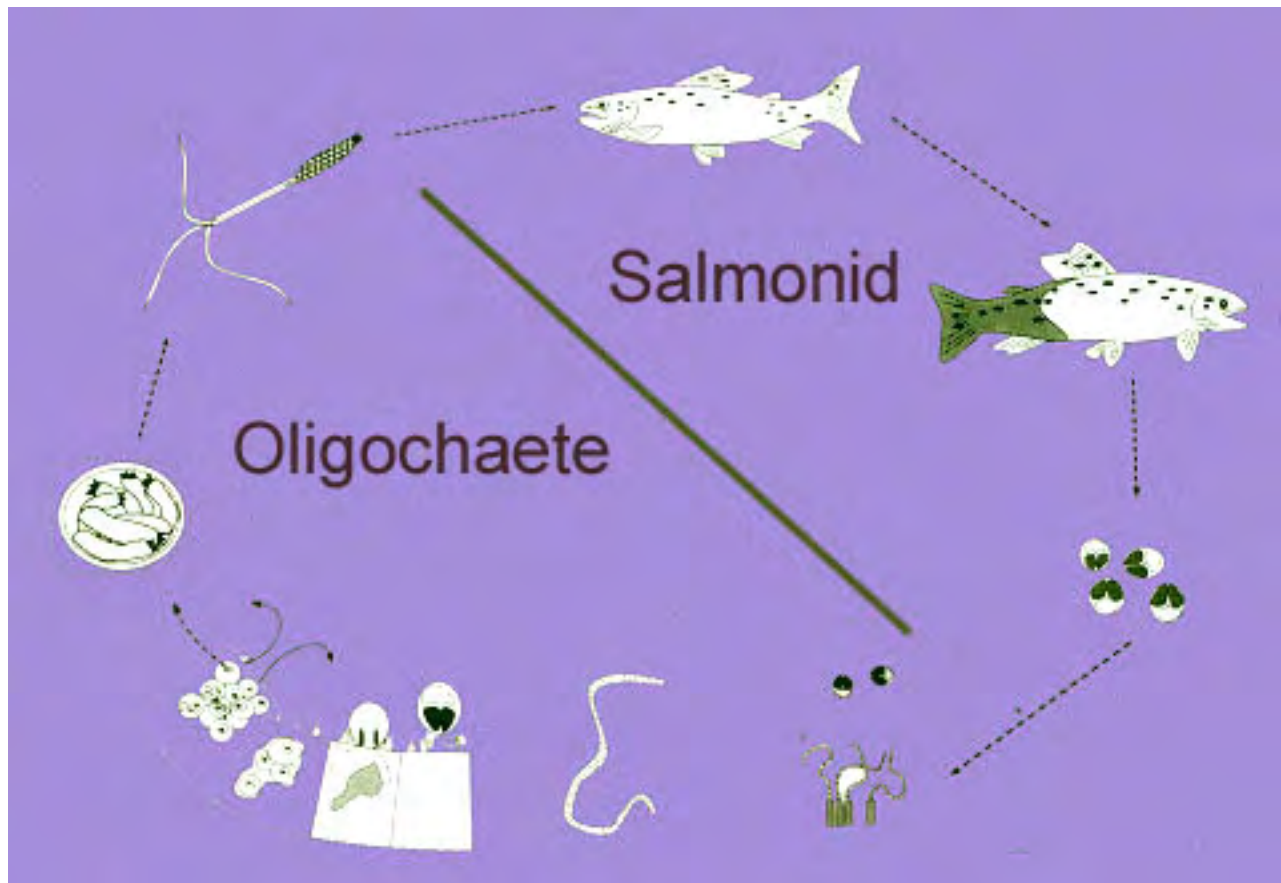


Figure 1. Life cycle of *Myxobolus cerebralis* in oligochaete and fish hosts.

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.

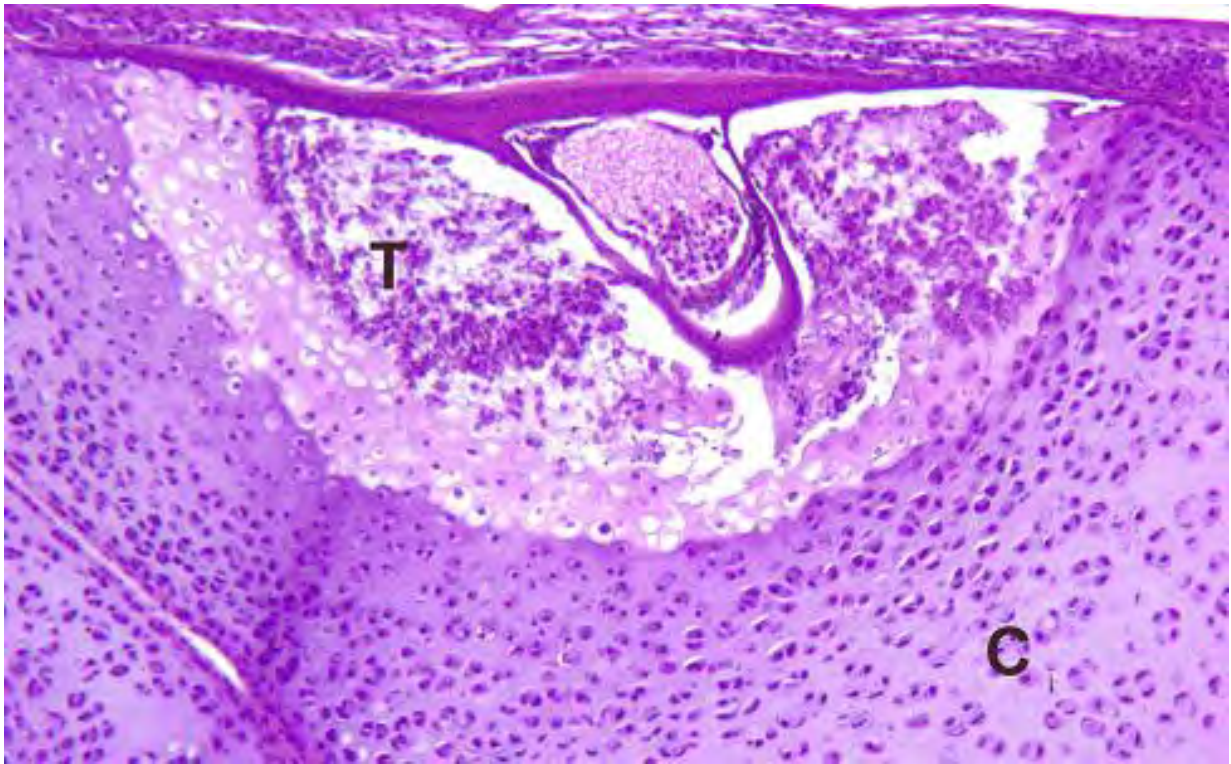


Figure 3. Focal lesion in cartilage (C) containing trophozoite stages of *M. cerebralis* (H&E).

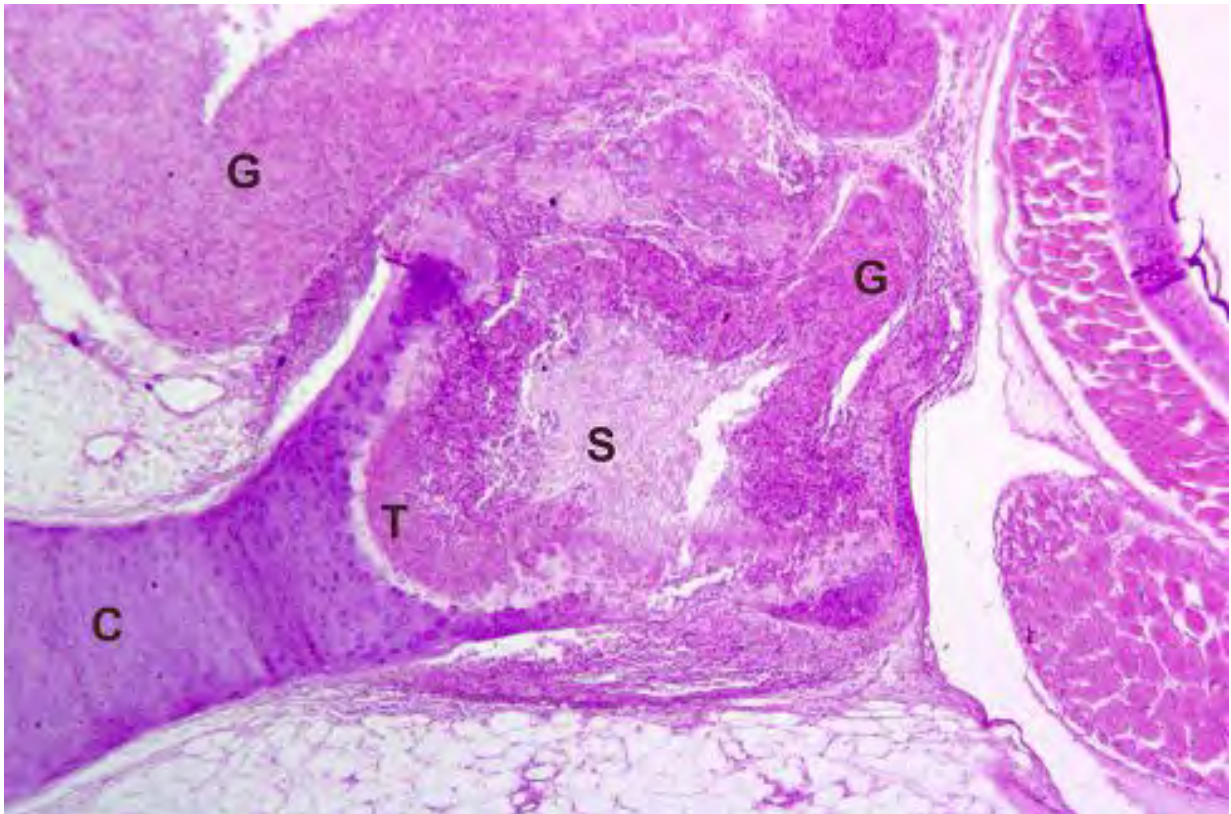


Figure 4. Replacement of cartilage with extensive granulomatous inflammation (G) containing trophozoites (T) and spores (S) in head of rainbow trout (H&E).

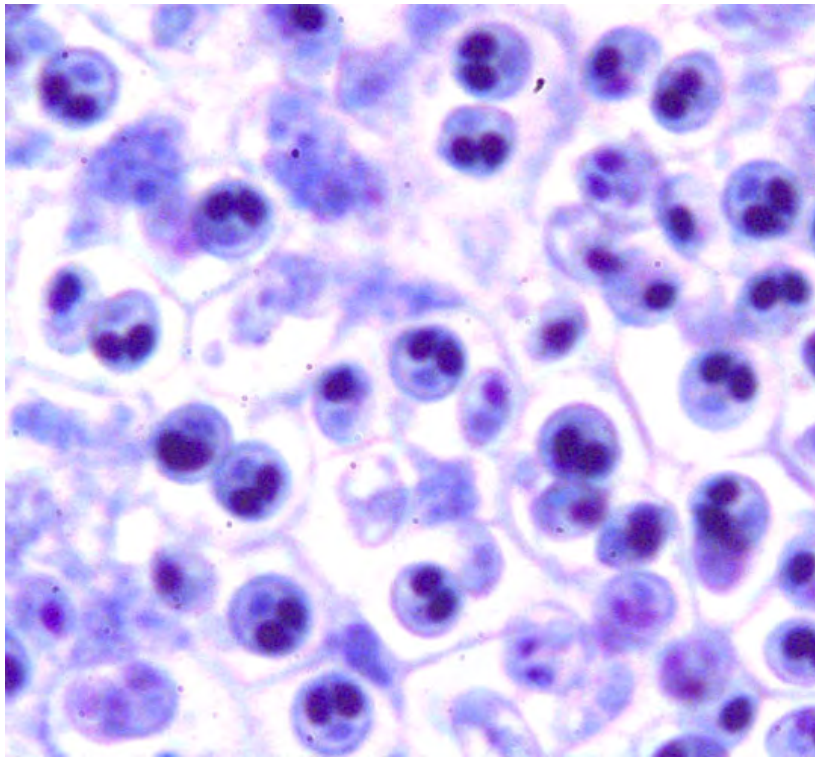


Figure 5. Spherical *Myxobolus cerebralis* spores with pyriform polar capsules.

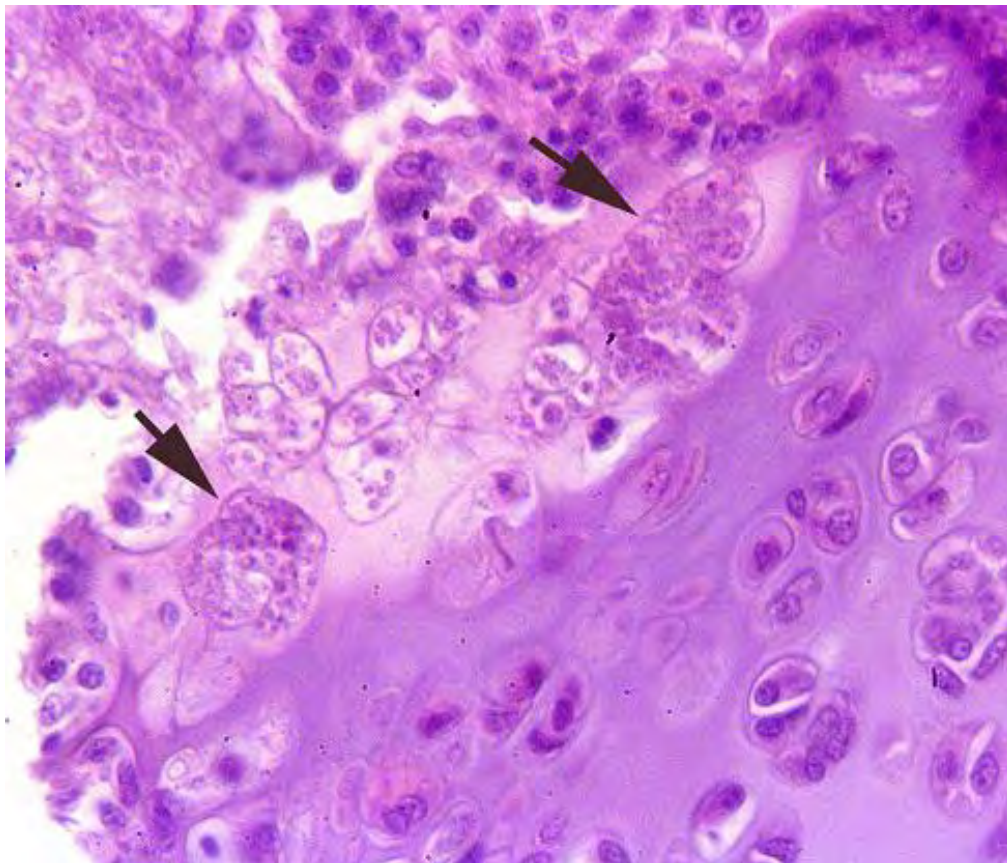


Figure 6. Trophozoite stages (arrows) of *Myxobolus cerebralis* in cartilage (H&E).

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).

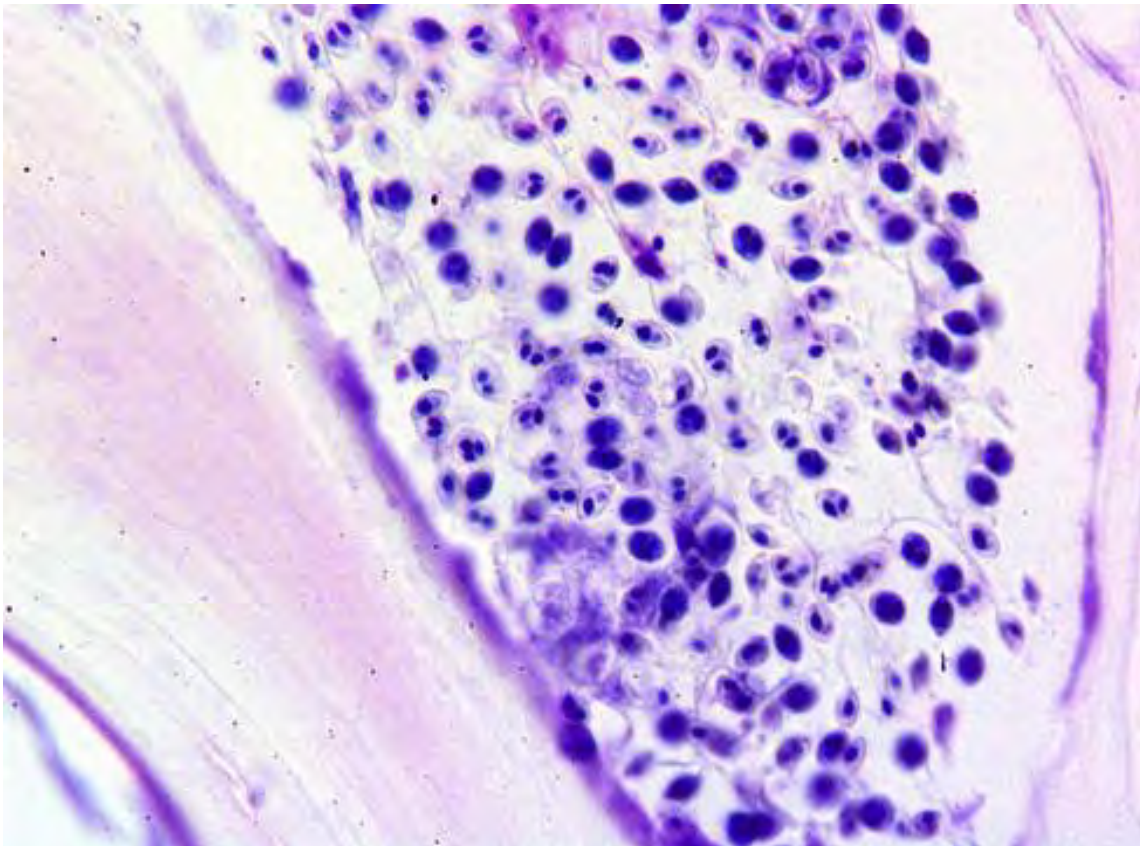


Figure 7. Giemsa-stained tissue section showing spores in cartilage surrounded by bone.

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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. ictaluri*, 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.

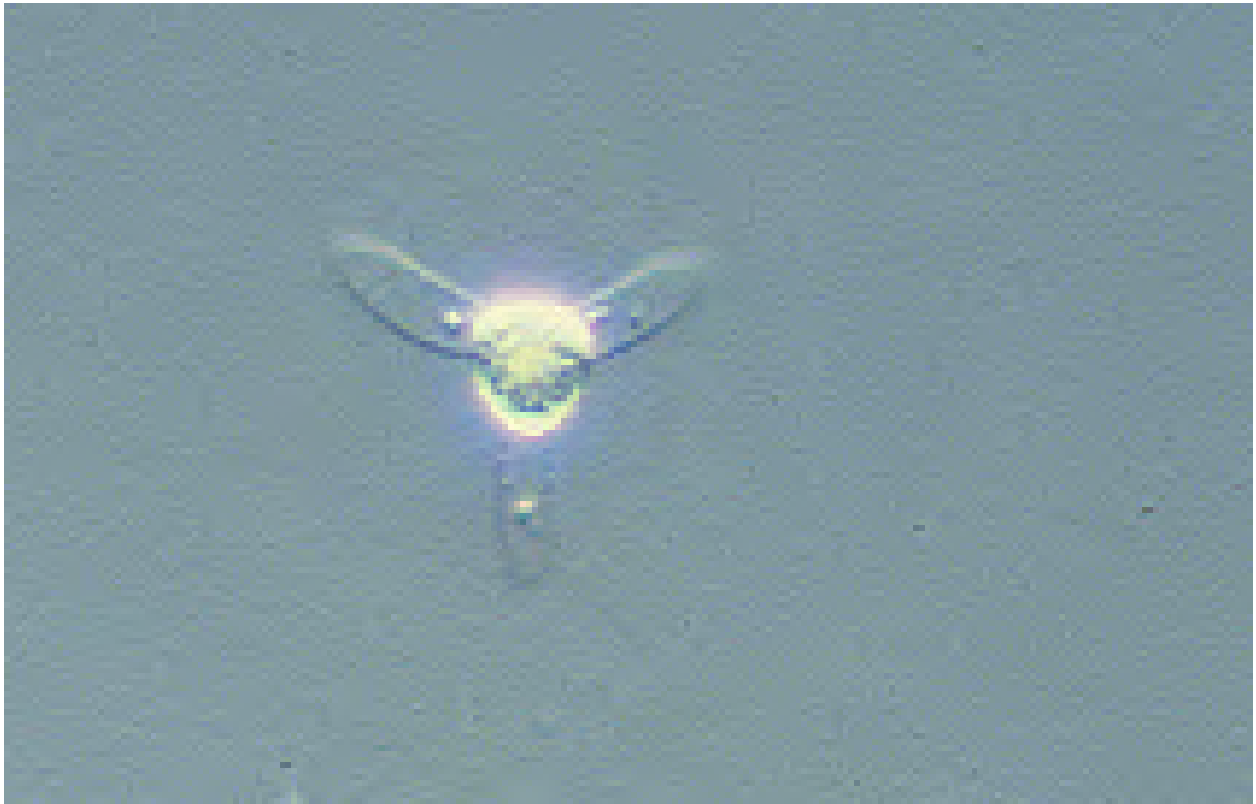


Figure 1. Actinospore stage of *H. ictaluri* in water.

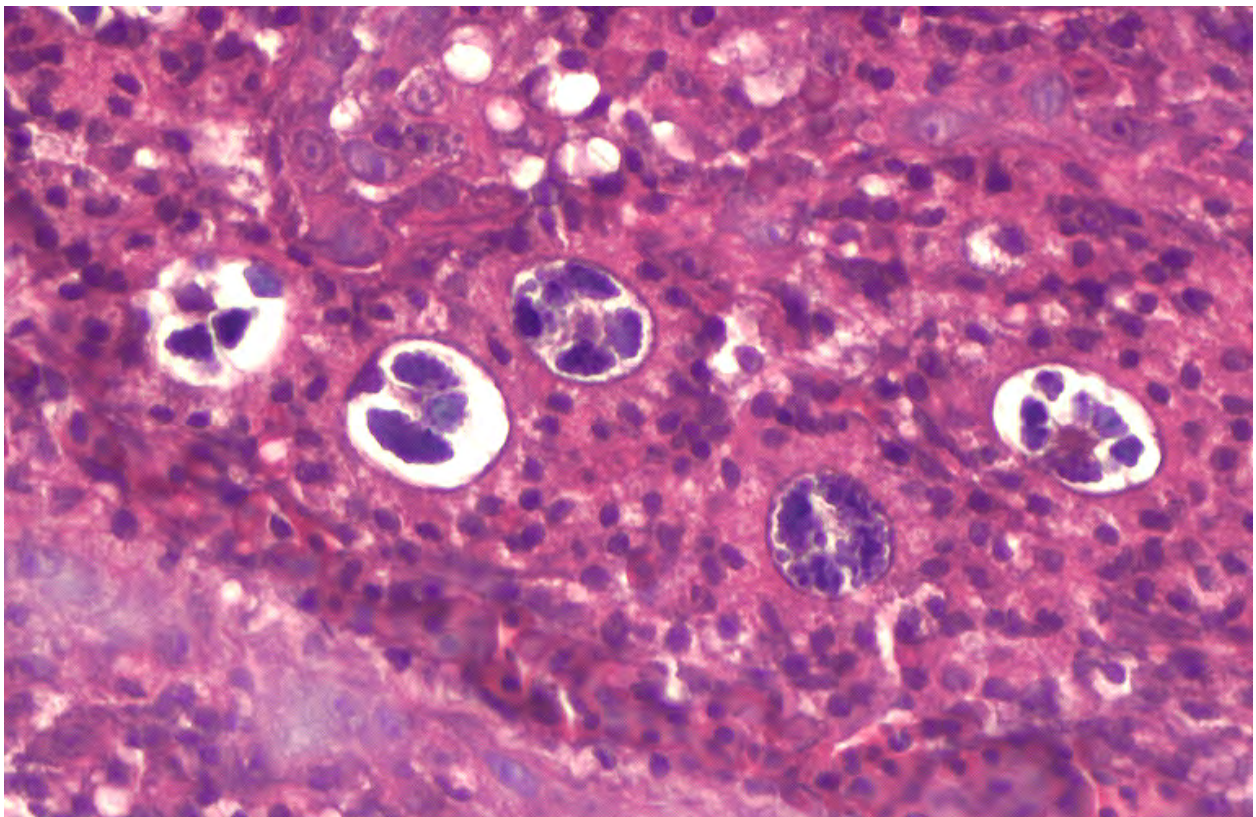


Figure 2. Histological section of early trophozoite stage of *H. ictaluri* in gills (courtesy of Andy Goodwin).

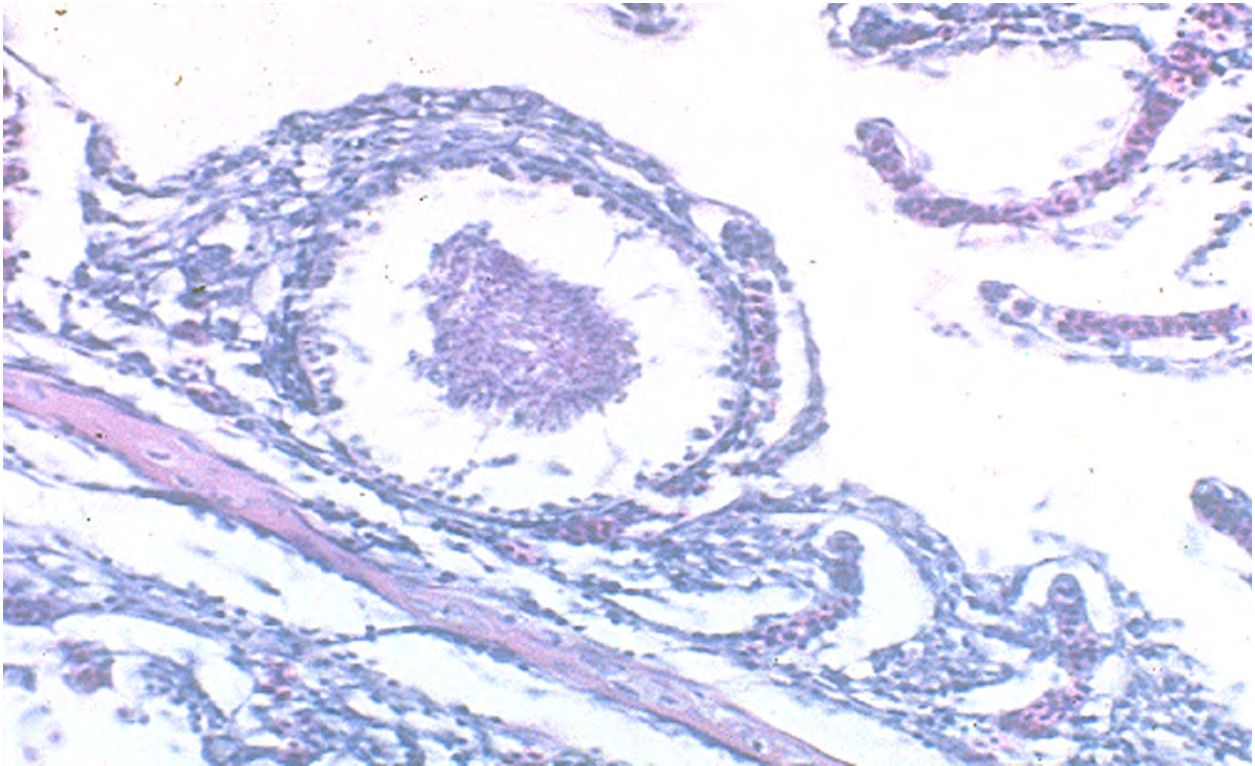


Figure 3. *Henneguya ictaluri* cyst in gills.



Figure 4. *Henneguya ictaluri* spores released from tissue cyst.

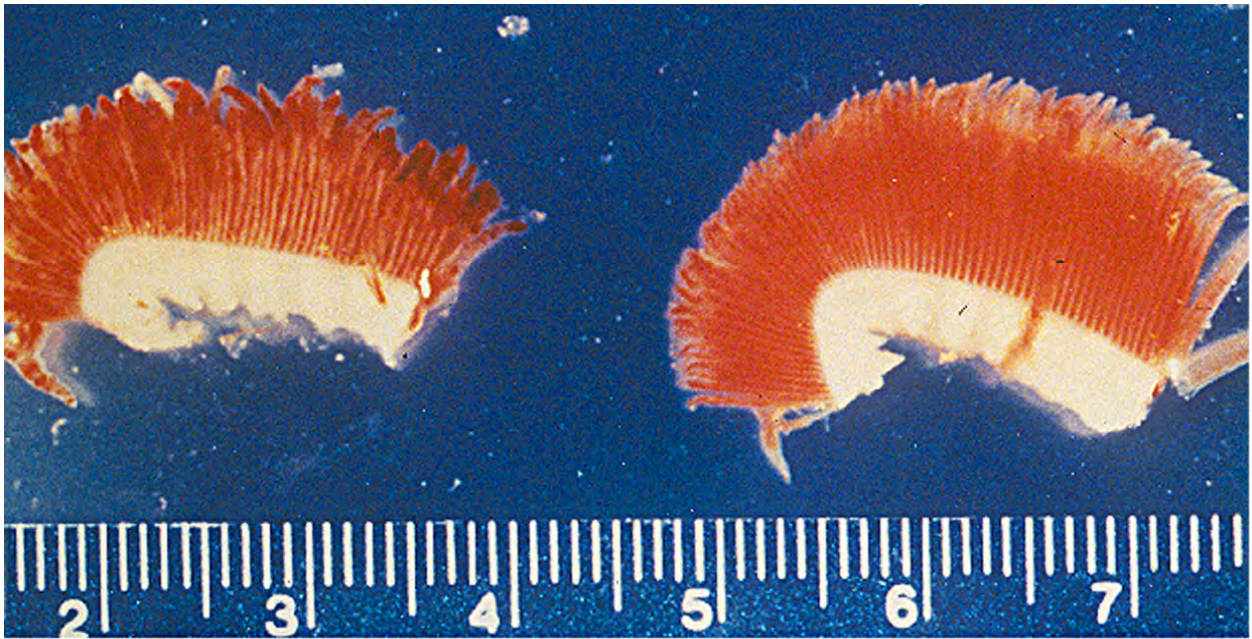


Figure 5. PGD infected gill (left) and normal uninfected catfish gill (courtesy of Larry Hanson).



Figure 6. PGD infected channel catfish demonstrating cartilage damage (courtesy of Andy Goodwin).

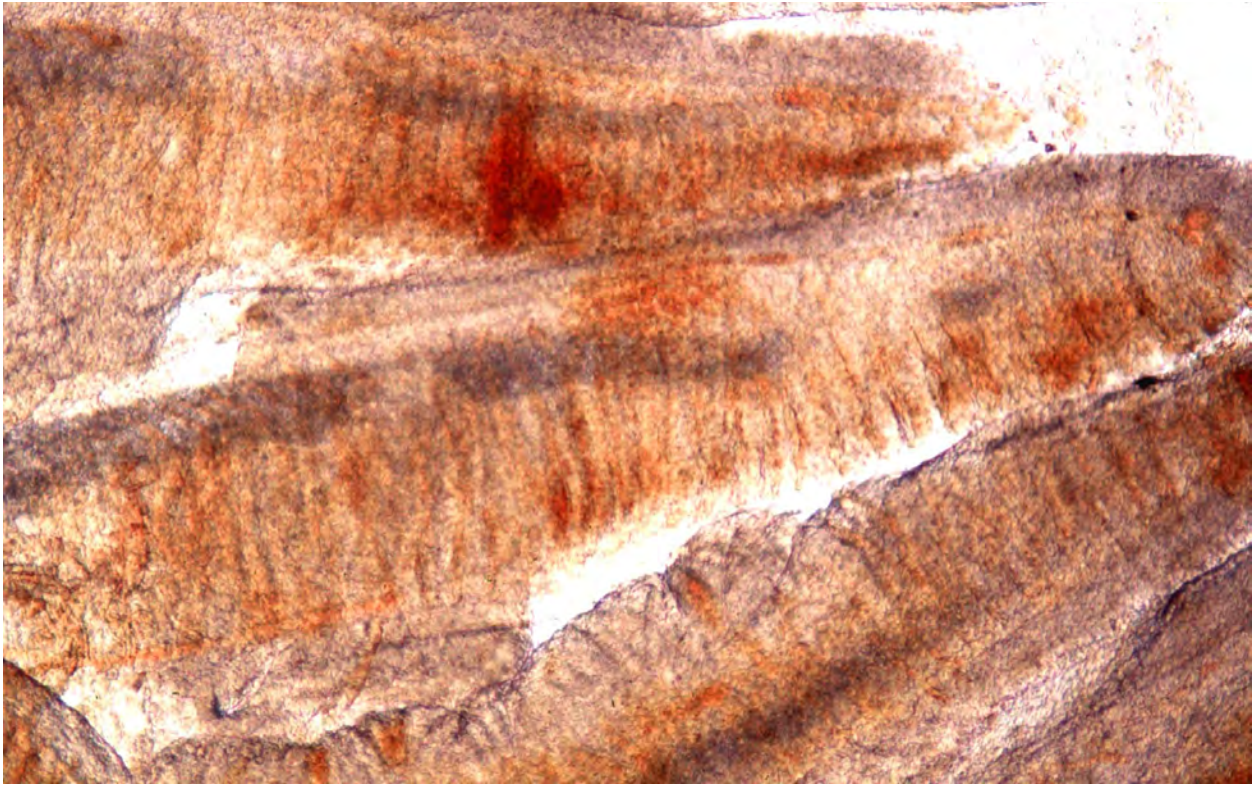


Figure 7. Wet mount of PGD infected channel catfish demonstrating cartilage damage (courtesy of Andy Goodwin).

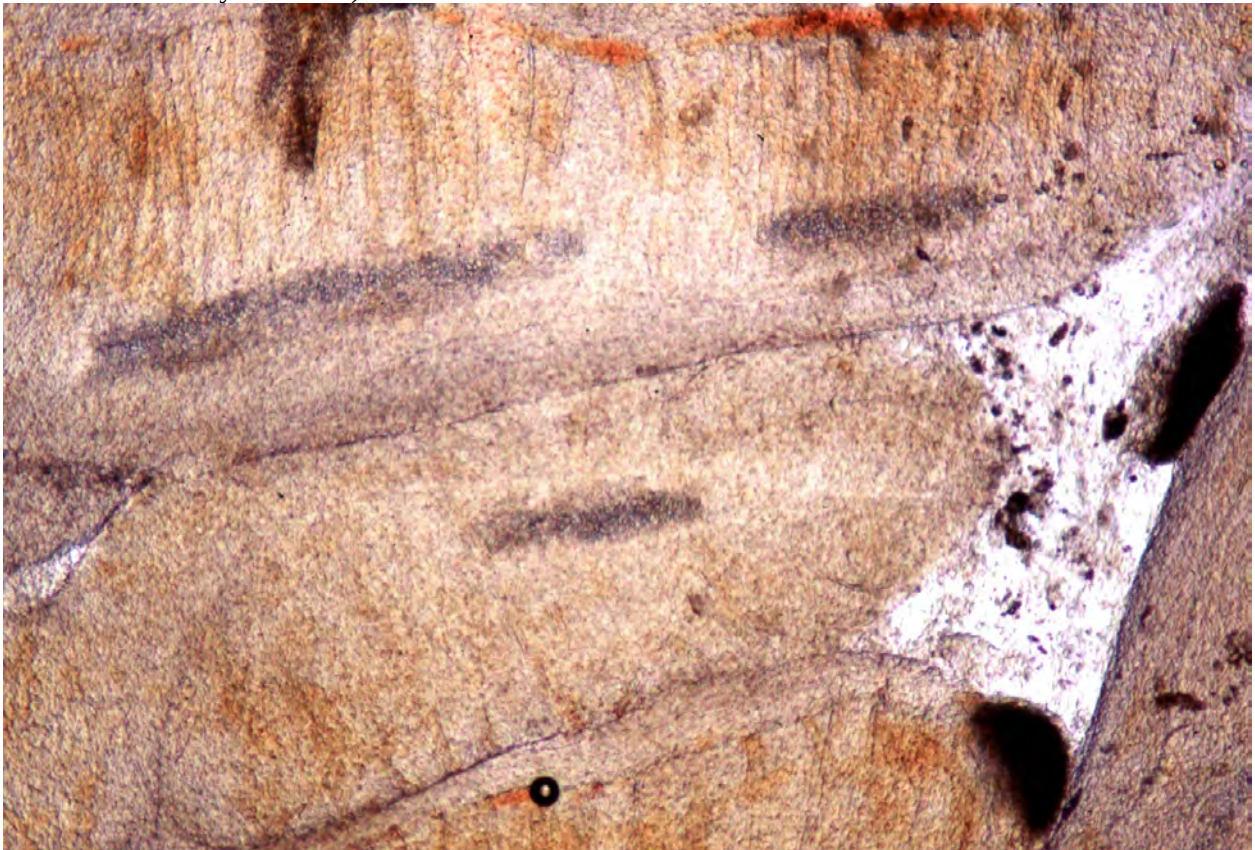


Figure 8. Wet mount of PGD infected channel catfish demonstrating cartilage damage (courtesy of Andy Goodwin).

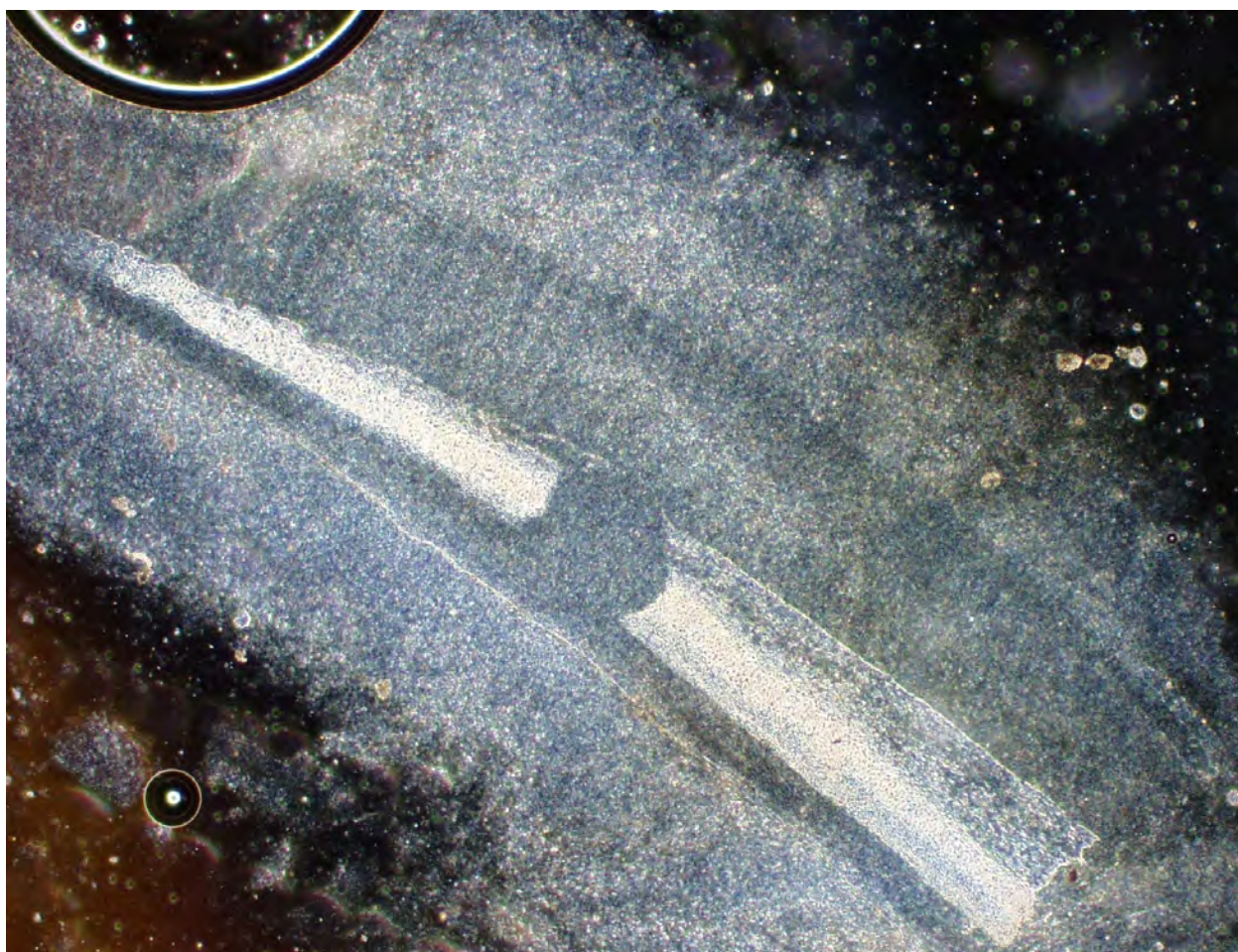


Figure 9. Phase contrast photomicrograph of a PGD infected catfish gill filament demonstrating cartilage damage and epithelial hyperplasia (courtesy of Larry Dorman).

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 MgCl₂), 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-glycosylase (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°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 multifiliis*. 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.

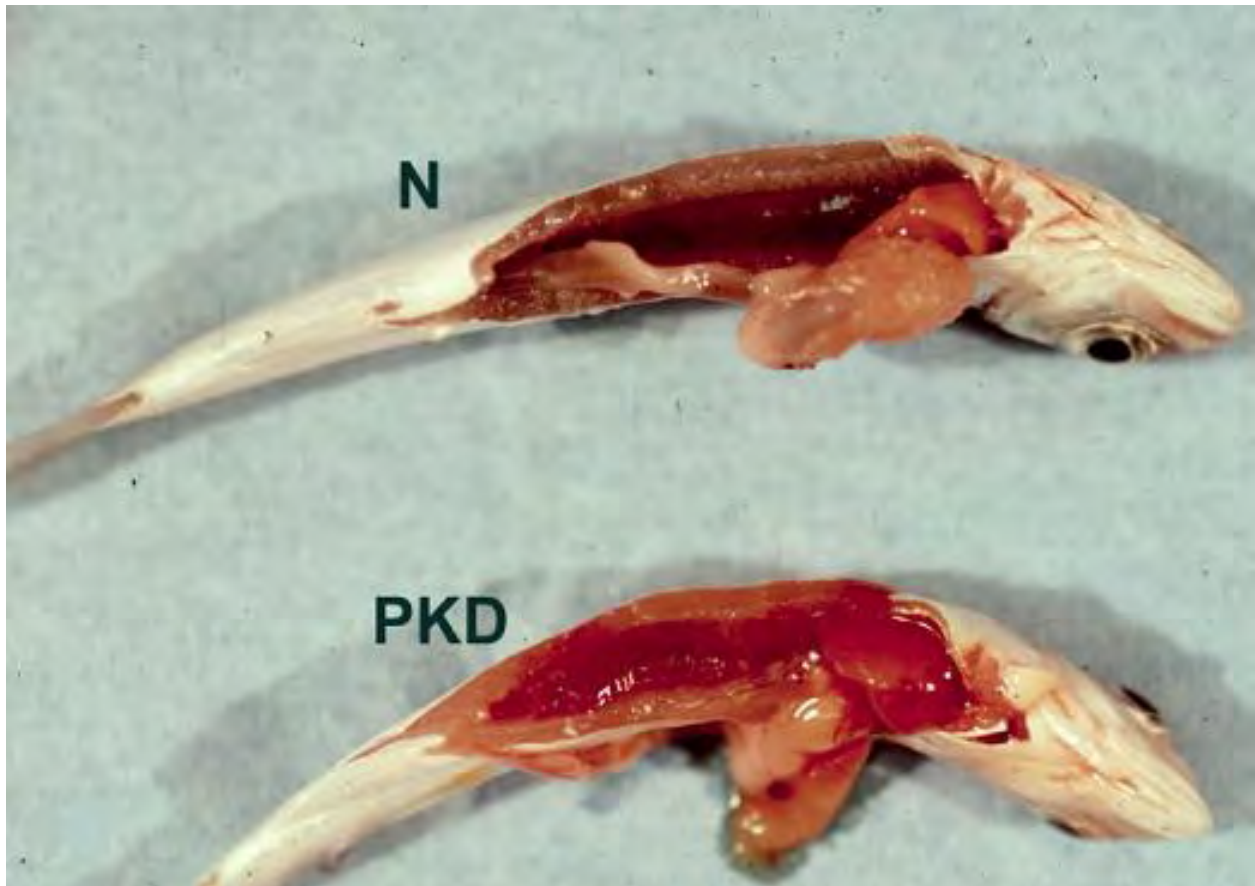


Figure 2. Chinook salmon with PKD, compared to normal (N). Note uniformly enlarged kidney in lower fish with PKD.



Figure 3. Macroscopic changes of PKD in cutthroat trout. Note enlarged spleen, kidney, and pale gills.

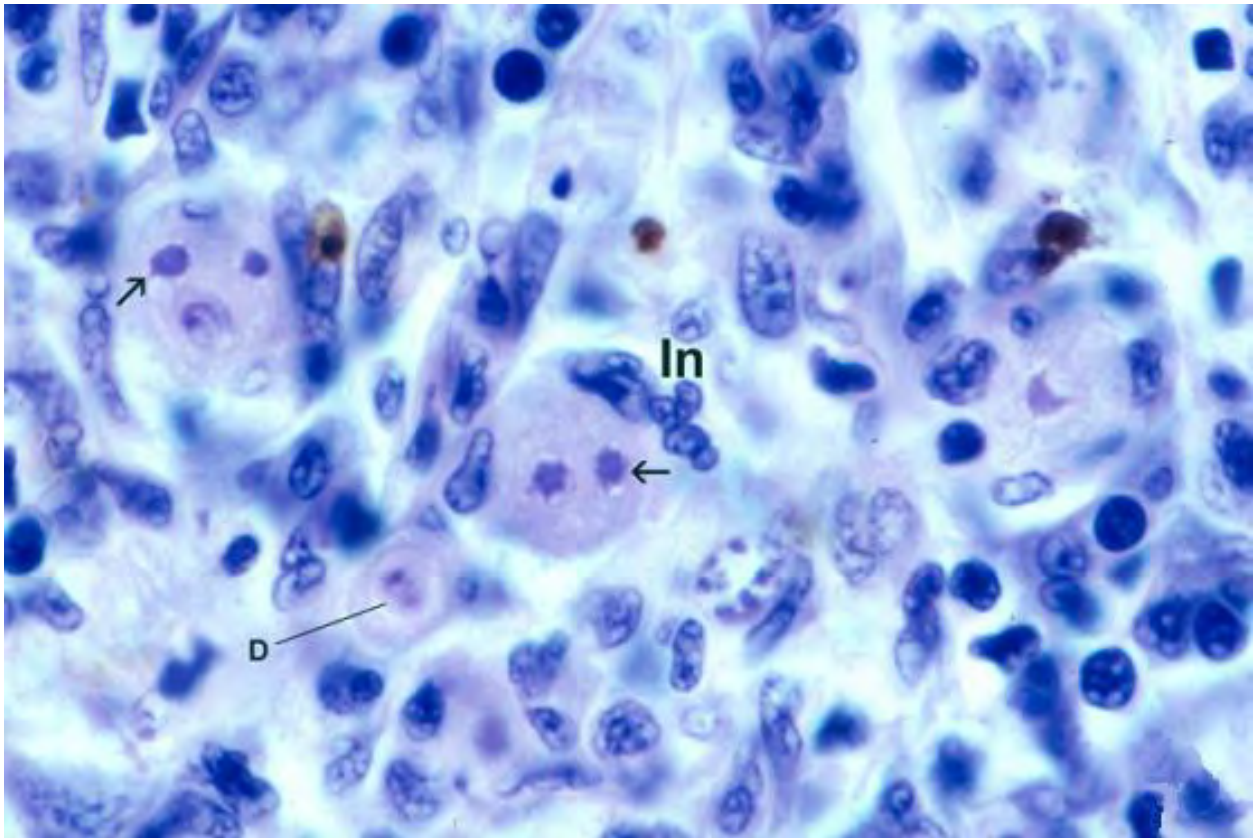


Figure 4. PKX cells in the kidney interstitium in histological section stained with H&E. Arrows = primary cell nuclei, D = daughter cell, In = inflammatory cells surrounding parasites.

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 20 μm 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 placed on the imprints and incubated for 1 hour at 25°C. Slides are rinsed three times in PBS and fluorescein 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).

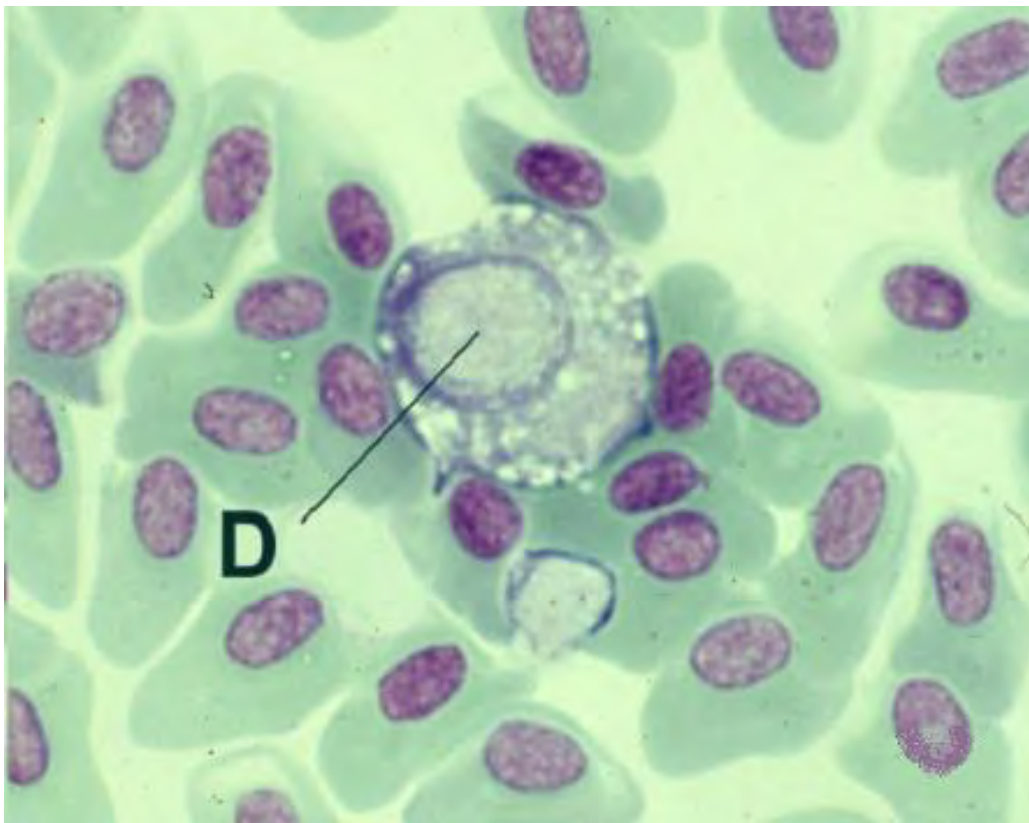
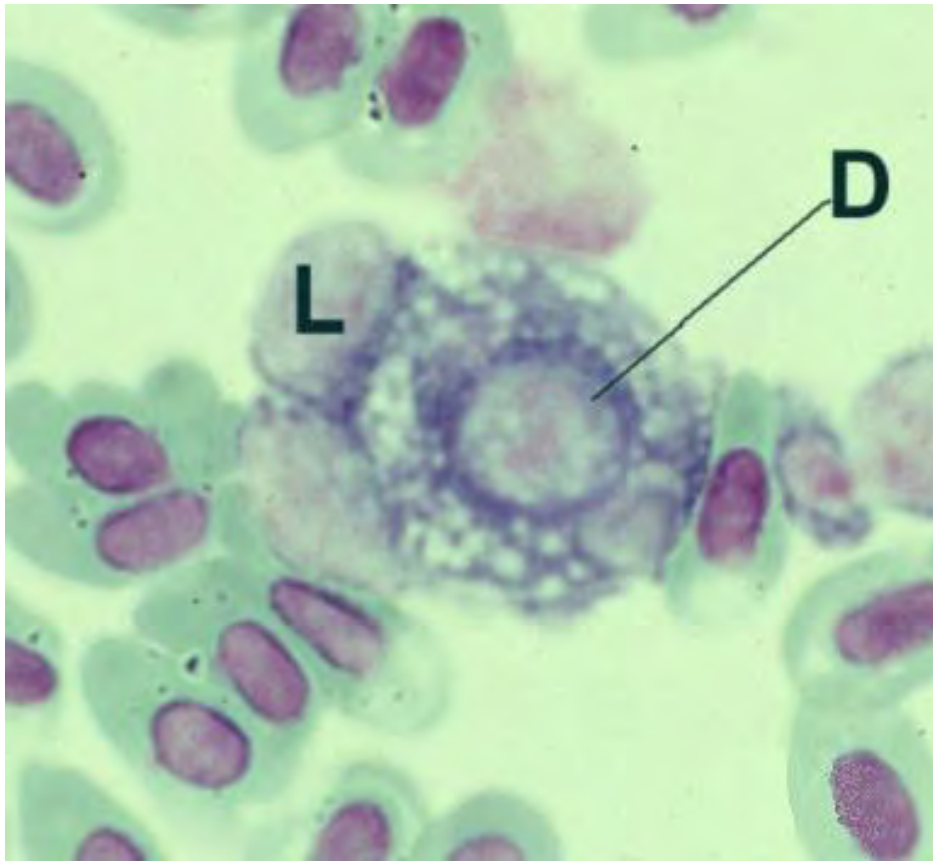
Monoclonal antibodies specific to PKX have been also used to visualize parasites in tissue sections (Adams et al. 1992; Marin de Mateo et al. 1996).

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) surrounding parasite. PKX cells has foamy cytoplasm and contains daughter cells (D).

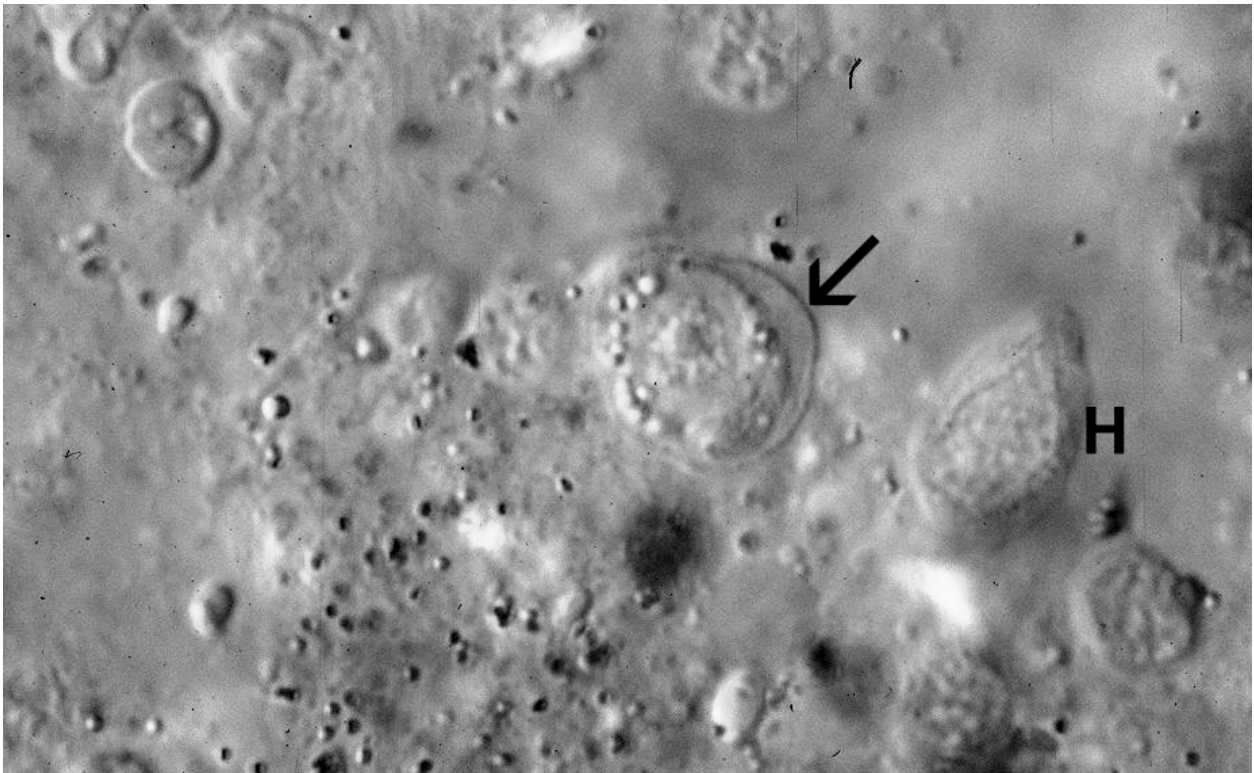


Figure 7. Wet mount preparation of salmonid kidney showing PKX. Note distinctive cell membrane (arrow) and smaller nucleus compared to host cells (H). Cytoplasm of PKX is granular. Nomarski phase contrast.

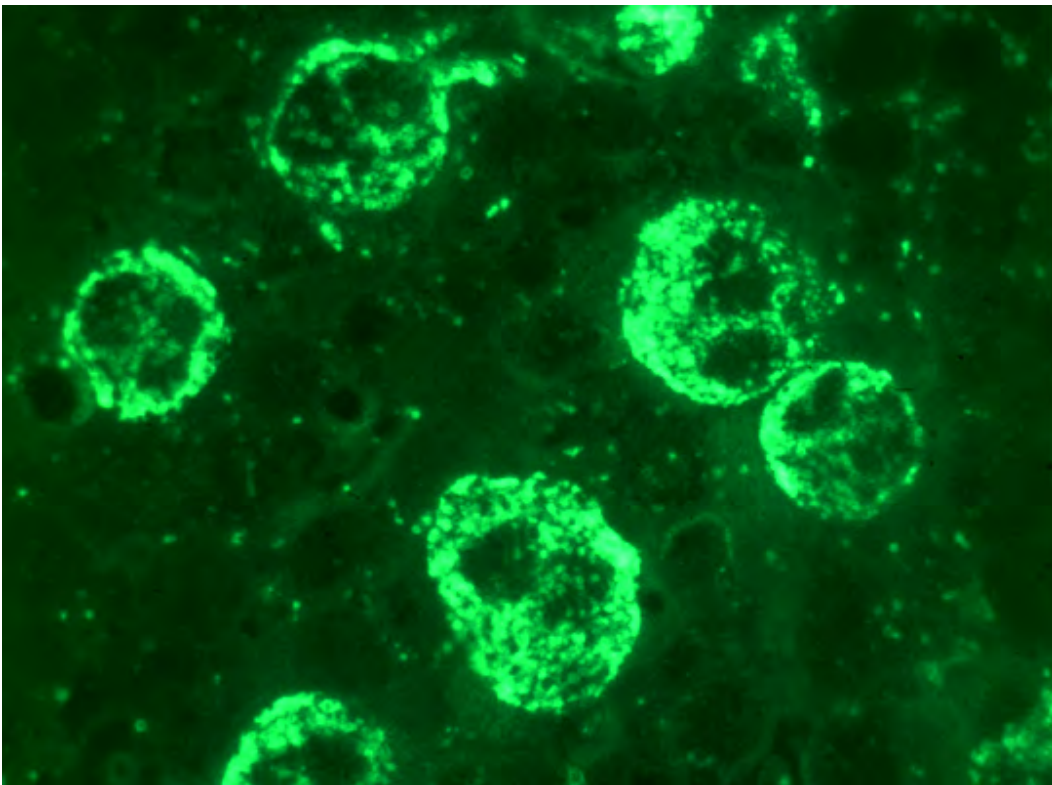


Figure 8. PKX cells in tissue imprints stained with biotinylated GS-1 lectin. Note granular cytoplasm of primary cells.

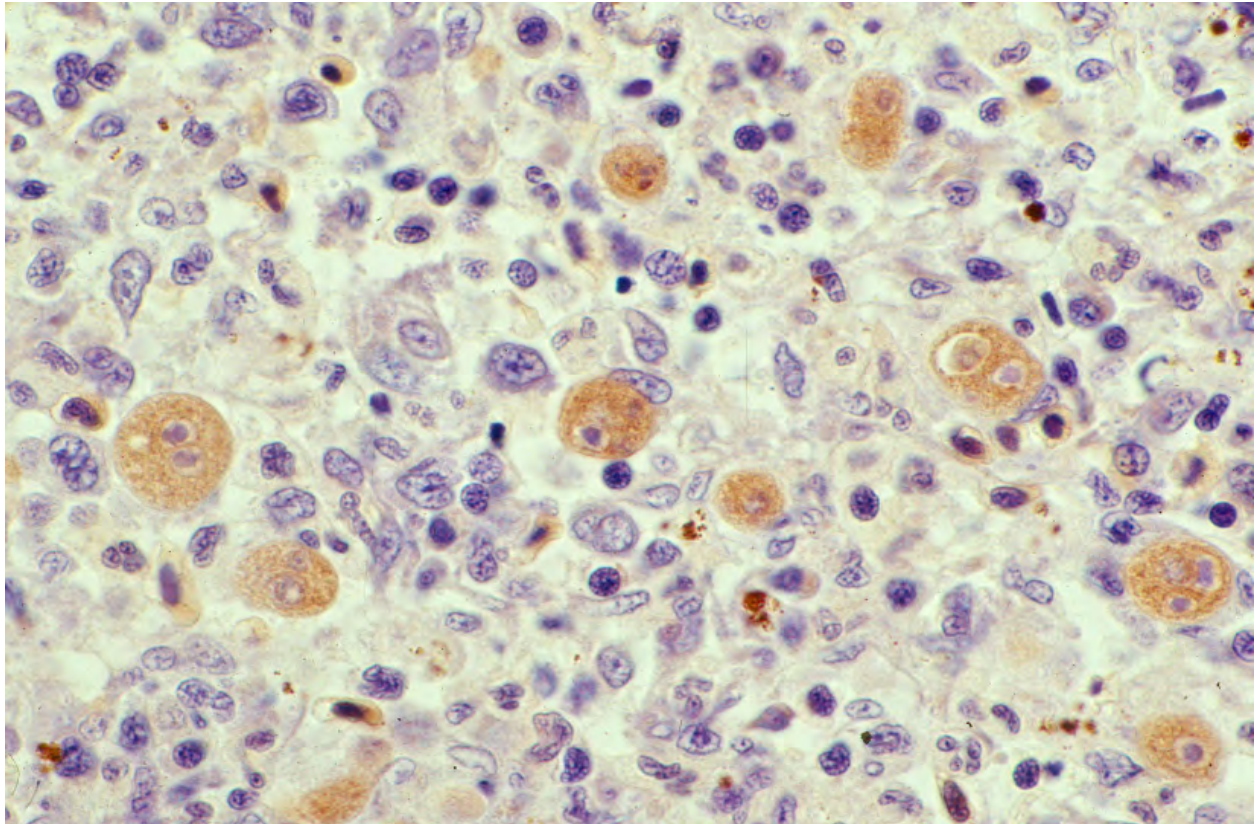


Figure 9. PKX in tissue sections stained with GS-1 lectin.

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.

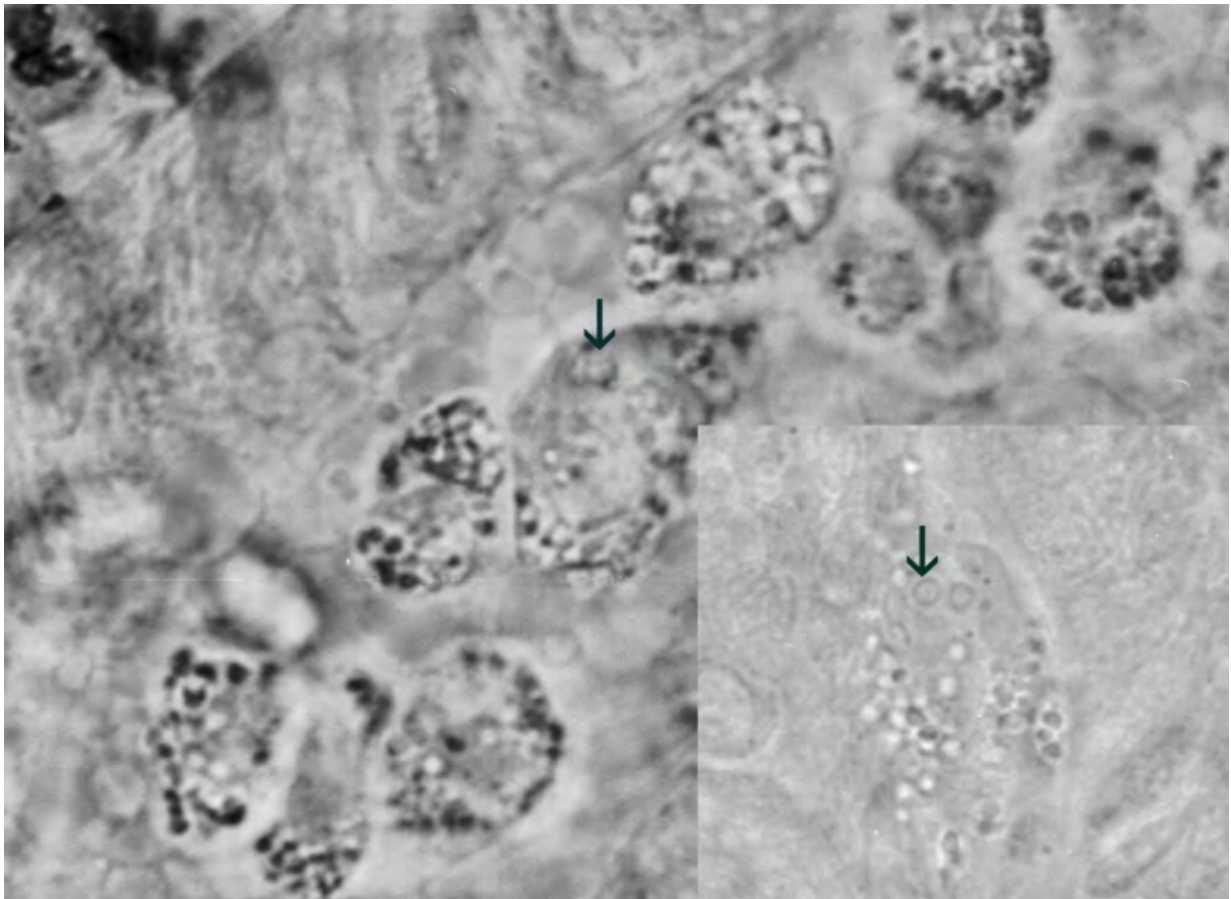


Figure 10. Wet mount of kidney showing PKX sporogonic forms in the lumen of kidney tubules. Arrow = polar capsules.

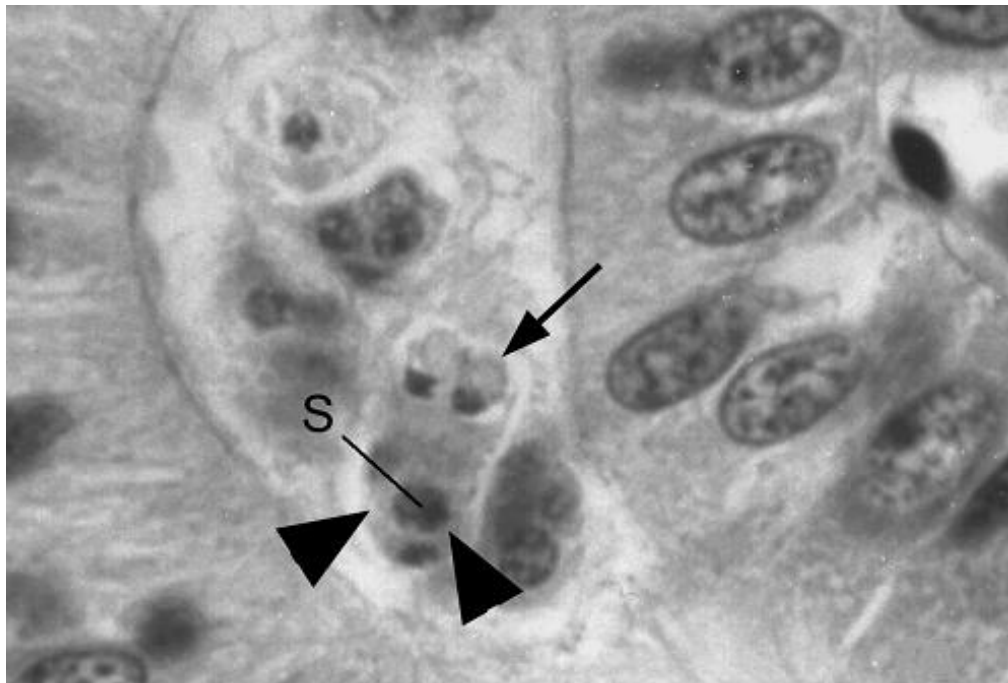


Figure 11. Histological sections of kidney tubules with spores of PKX. Hematoxylin and eosin stain showing polar capsules (arrows), sporogonic nuclei (S).

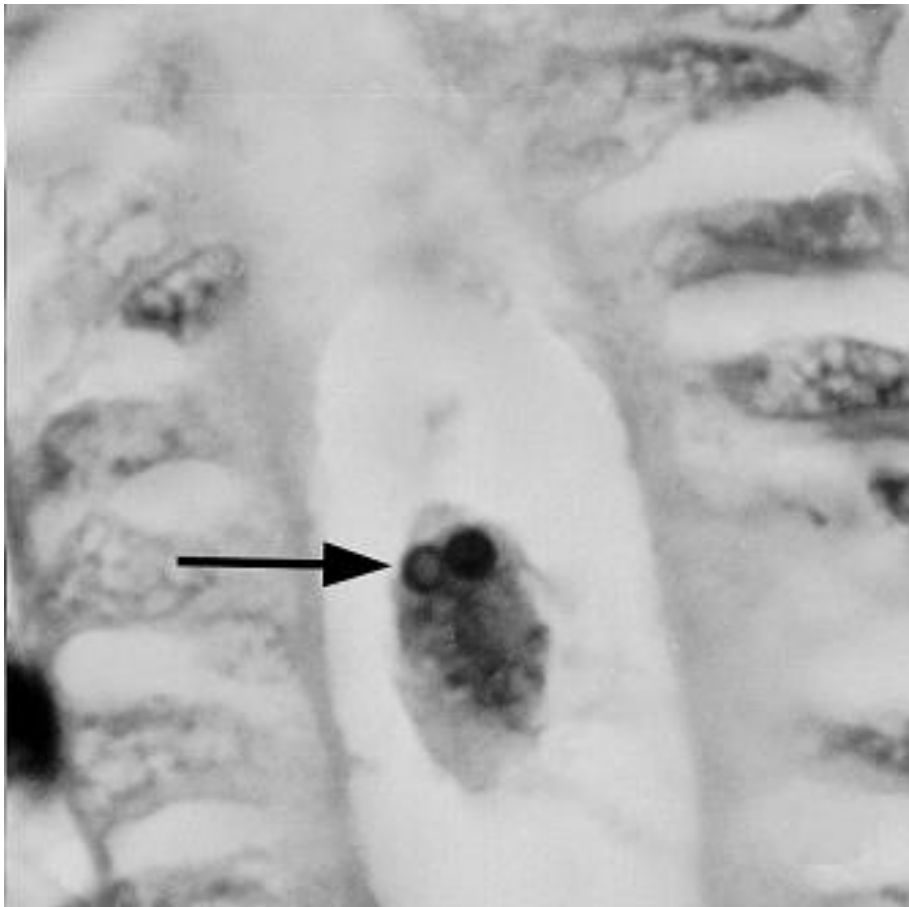


Figure 12. Histological sections of kidney tubules with spores of PKX.). Giemsa stain showing dark blue polar capsules (arrows).

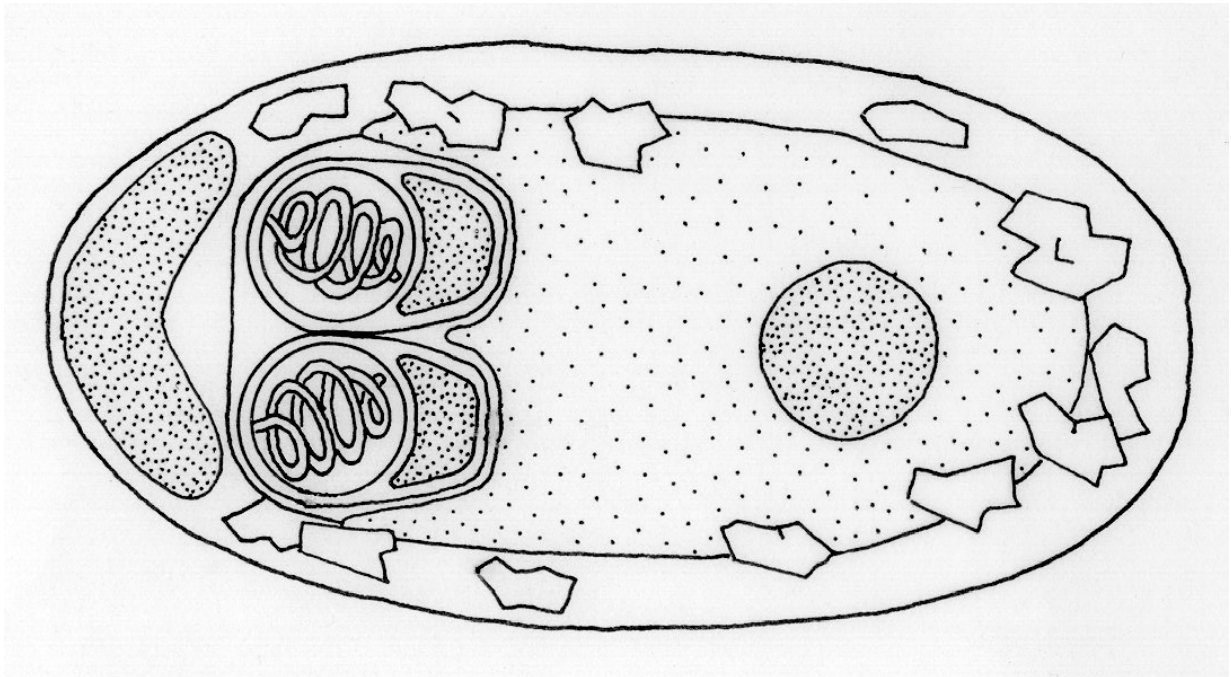


Figure 13. Line drawing of PKX sporoblast within pseudoplasmodium from kidney tubules.

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

Certain photographs used in this chapter were kindly provided by Ronald P. Hedrick (Figures 2, 4, 5, 7, and 8) and Beth MacConnell (Figure 3).

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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@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 *Ctenopharyngodon 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°C the life cycle is completed in 5 to 7 days and at 10°C in about 30 to 40 days. Water temperatures of 32°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°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°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.

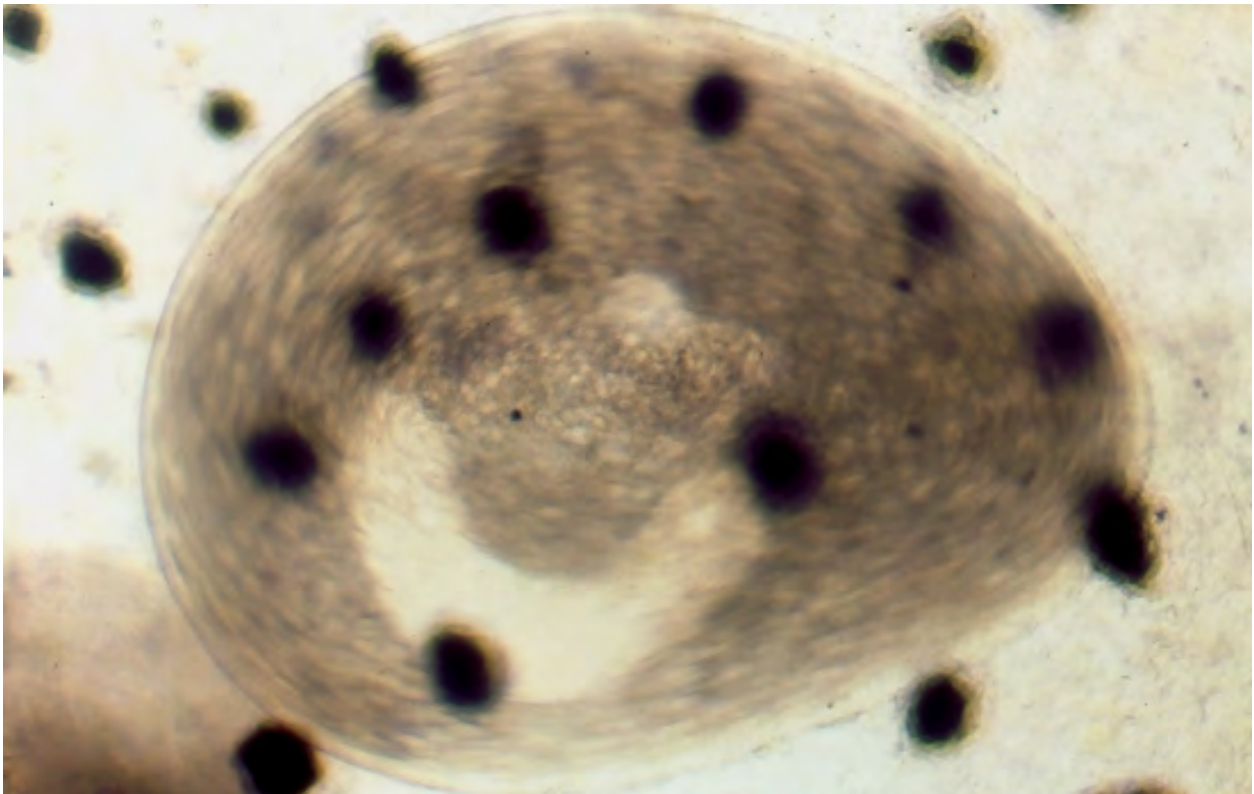


Figure 1. *Ichthyophthirius multifiliis* trophont in channel catfish epidermis. Note large horseshoe-shaped macronucleus, which is pale. Margin is blurred because trophont is moving in this fresh preparation. Black spots are in fish skin.

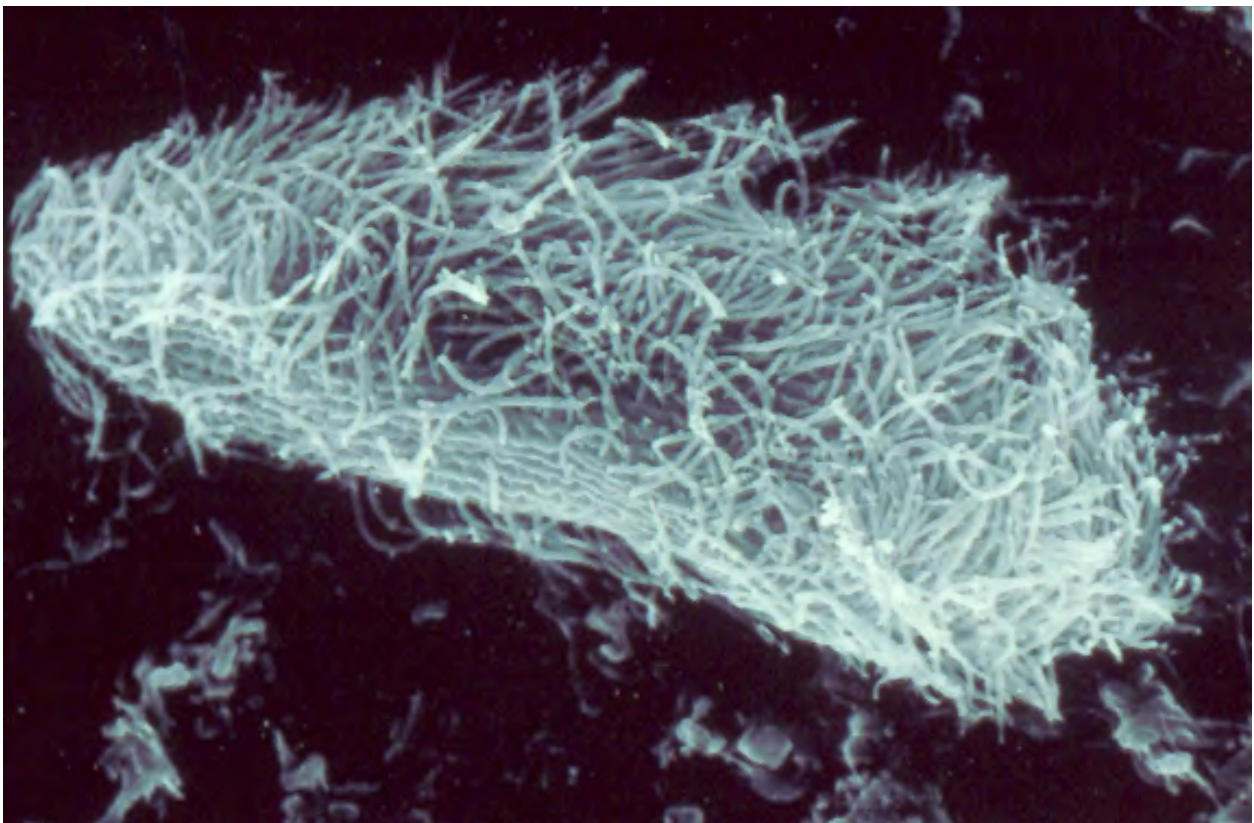


Figure 2. Theront as seen by scanning electron microscopy. Approximately 15 μm in length.

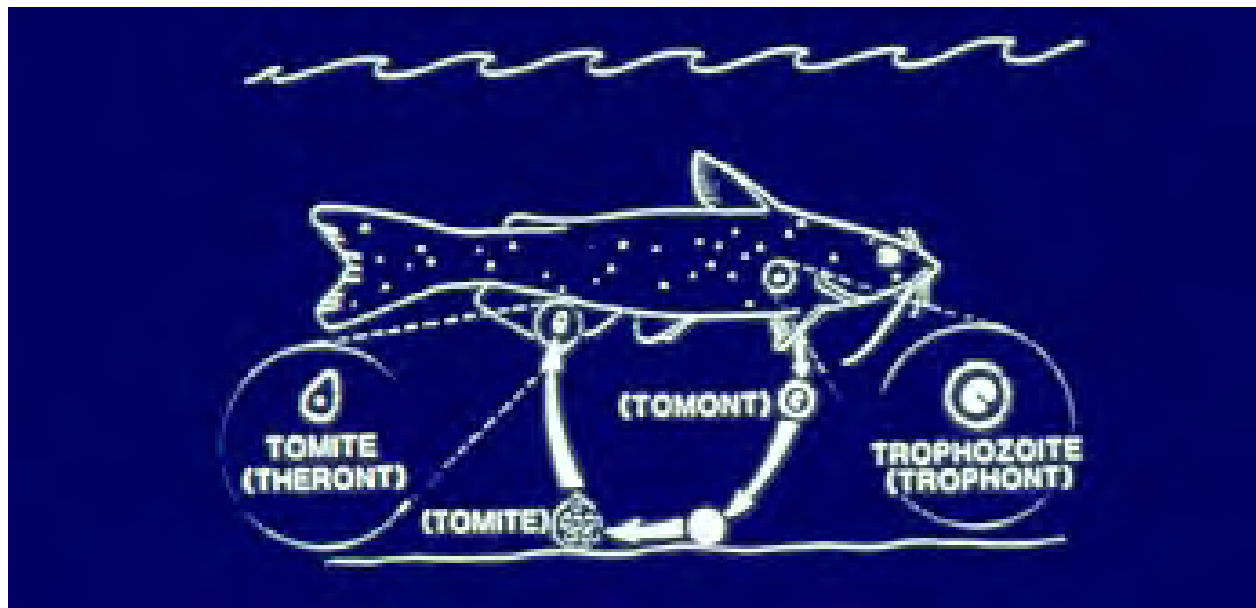


Figure 3. Life cycle of *Ichthyophthirius multifiliis*.

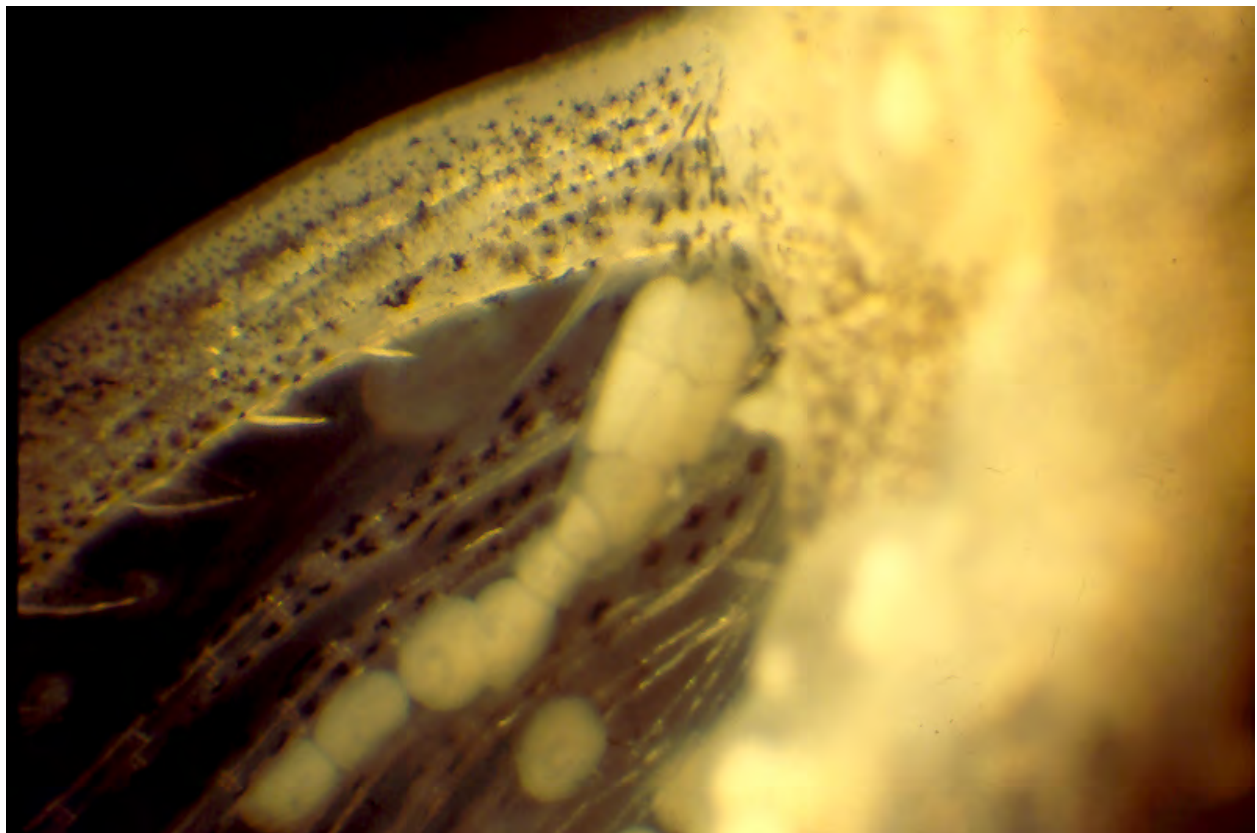


Figure 4. Group of at least 11 trophonts within a single cyst-like space within the epithelium of a channel catfish pectoral fin.



Figure 5. Channel catfish infected with *Ichthyophthirius multifiliis*. White spots are cystlike spaces containing trophonts.

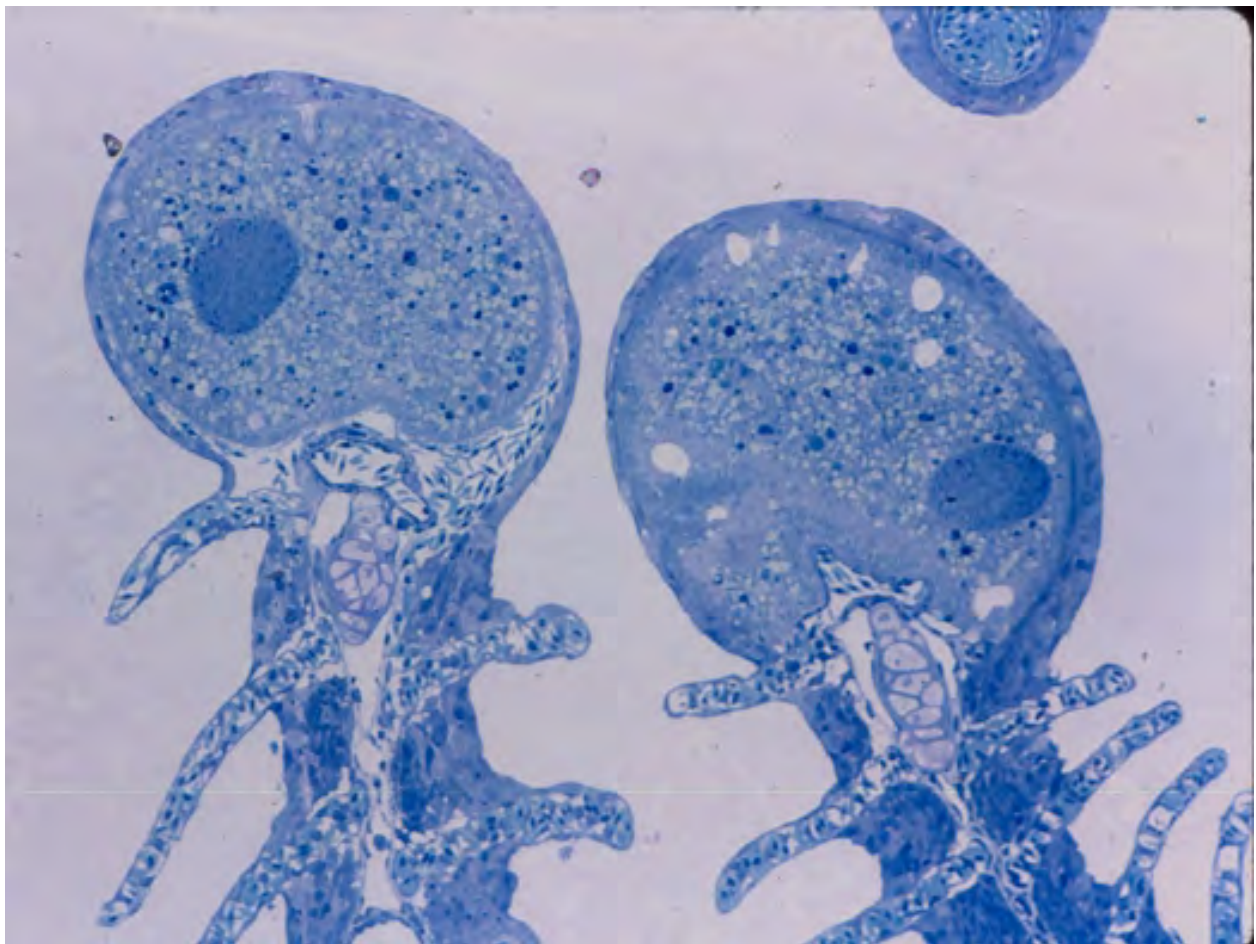


Figure 6. Gill filaments showing distortion caused by growth of the trophont. A small portion of the macronucleus appears in the trophonts in this section, stained a darker blue with Mallory's.

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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 Suctorians (*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 μ m. 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 μ m to 150 μ m. 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.

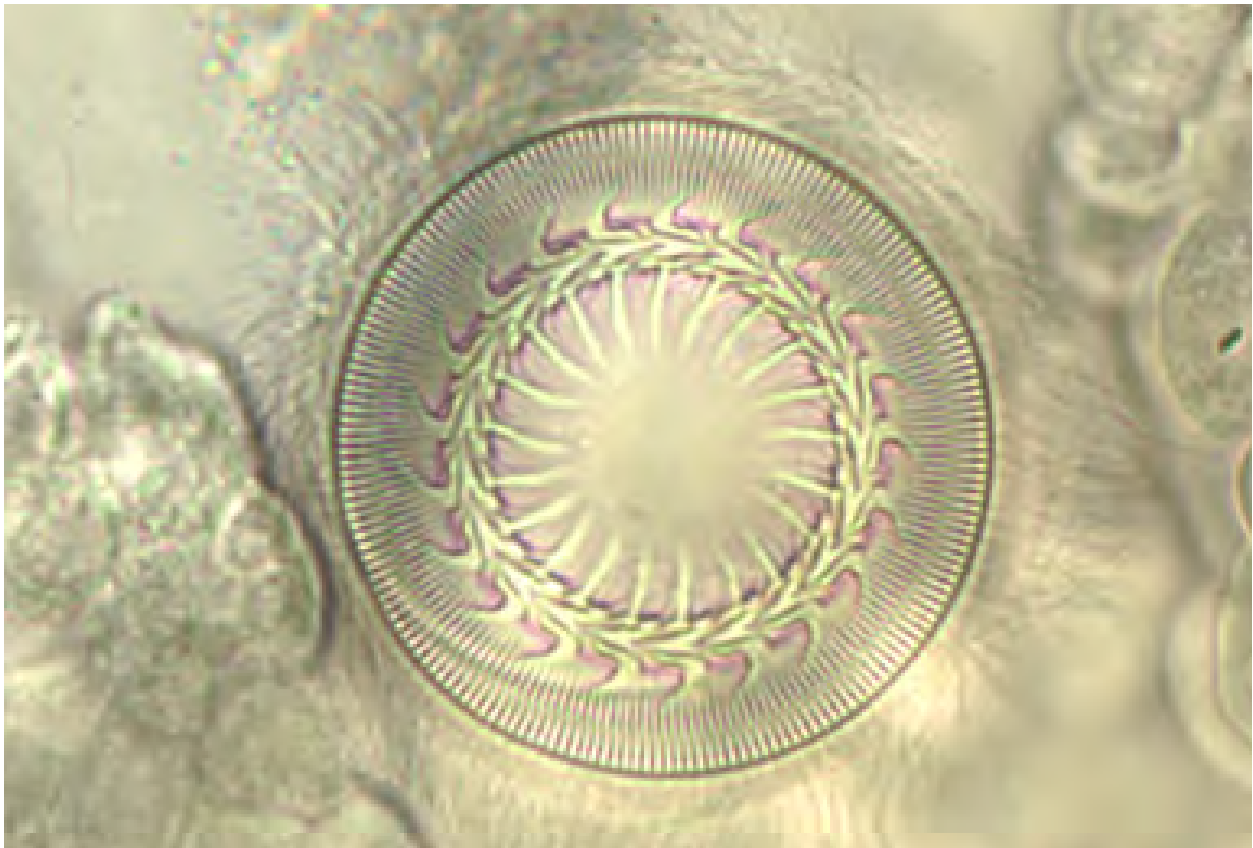


Figure 1. *Trichodina* sp. on the skin of a Channel Catfish.

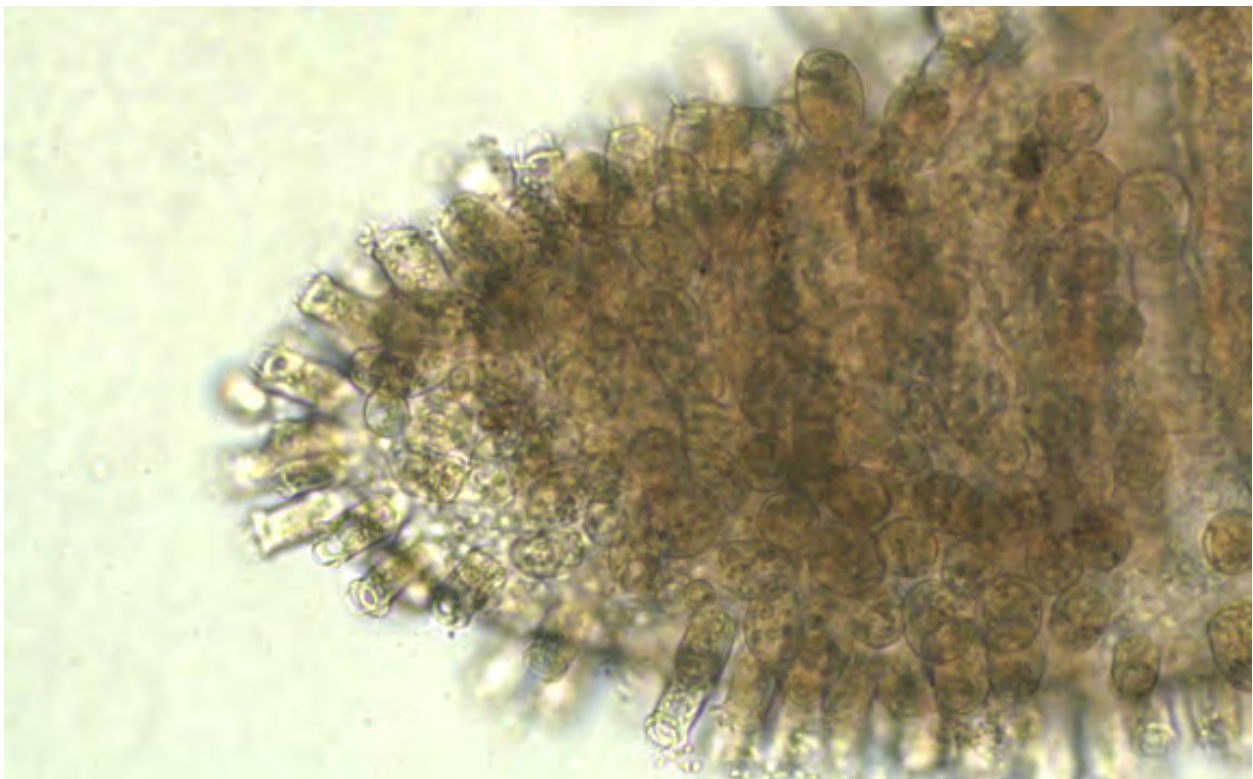


Figure 2. Large numbers of Ambiphrya on the gill of a Channel Catfish.

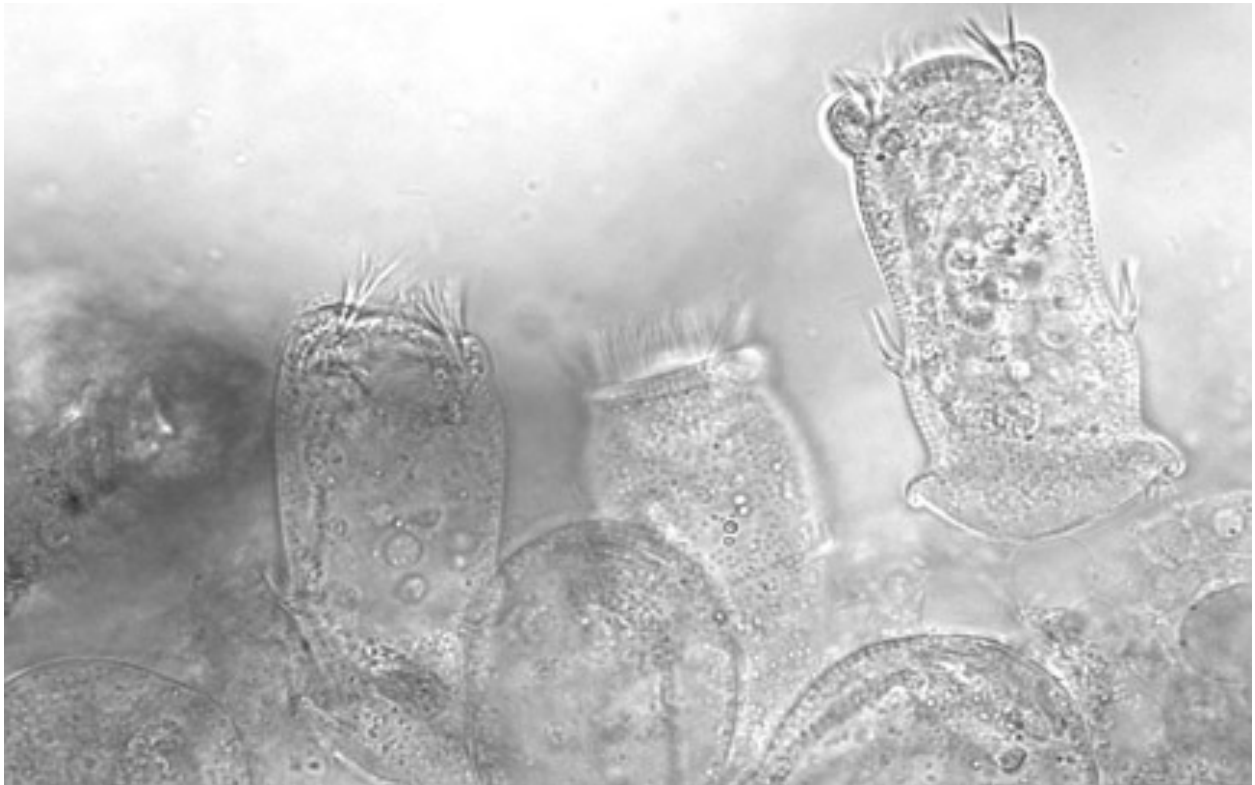


Figure 3. Ambiphrya on the gill of a Channel Catfish.



Figure 4. *Apiosoma* on the gill of a black crappie.

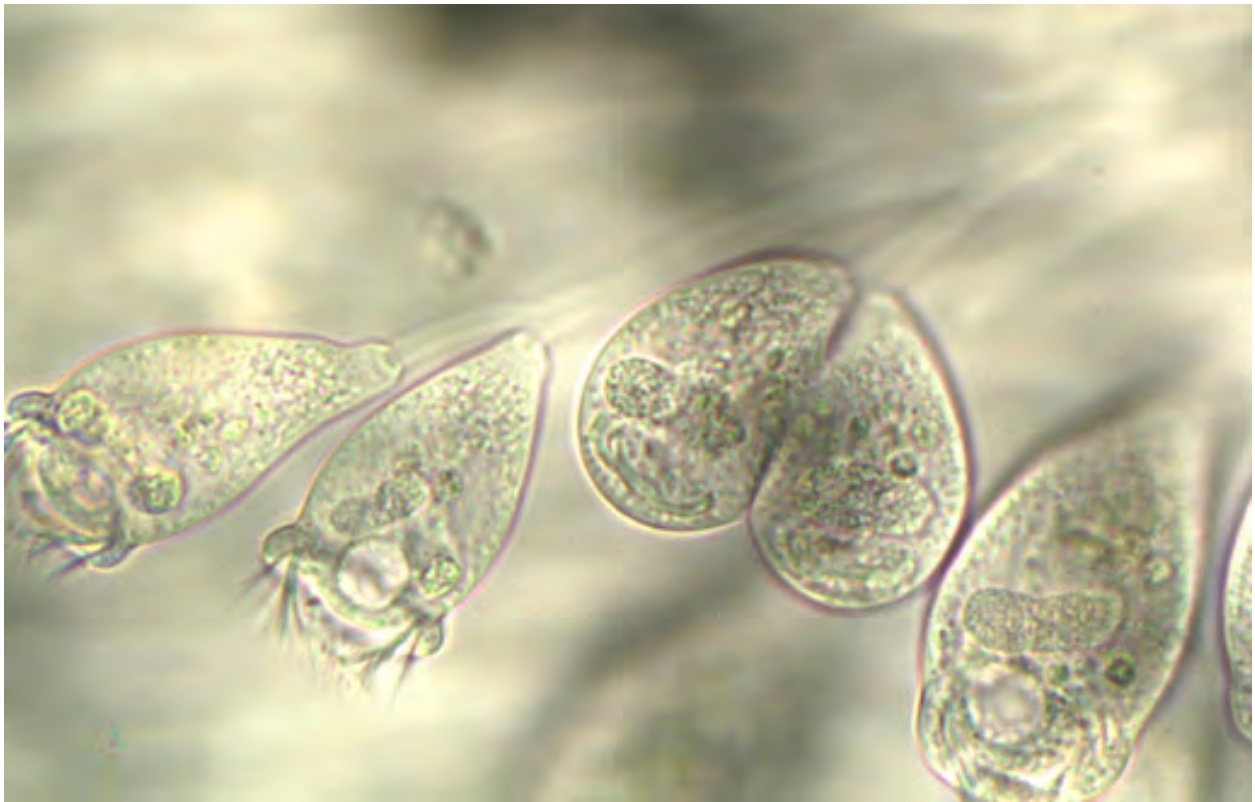


Figure 5. *Epistylus* on the fin of a Channel Catfish.

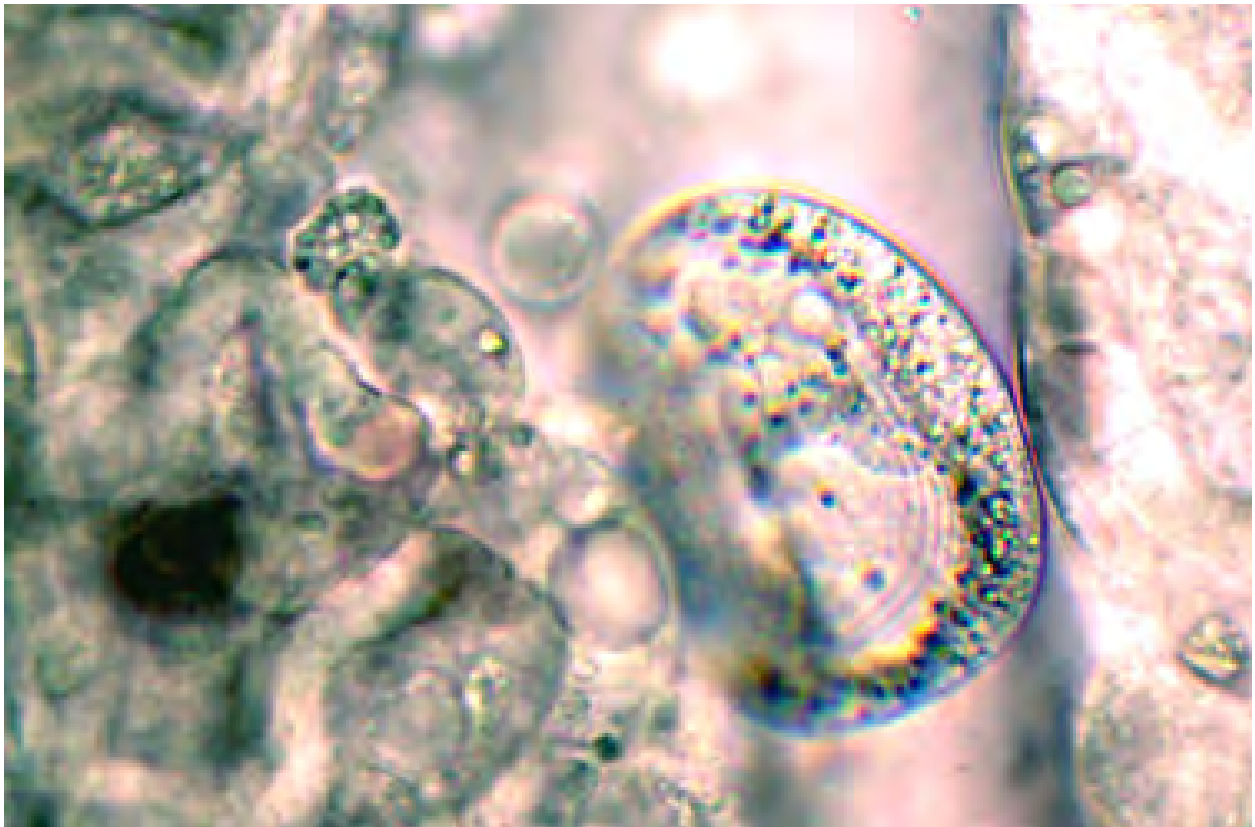


Figure 6. *Chilodonella* on a Goldfish gill.

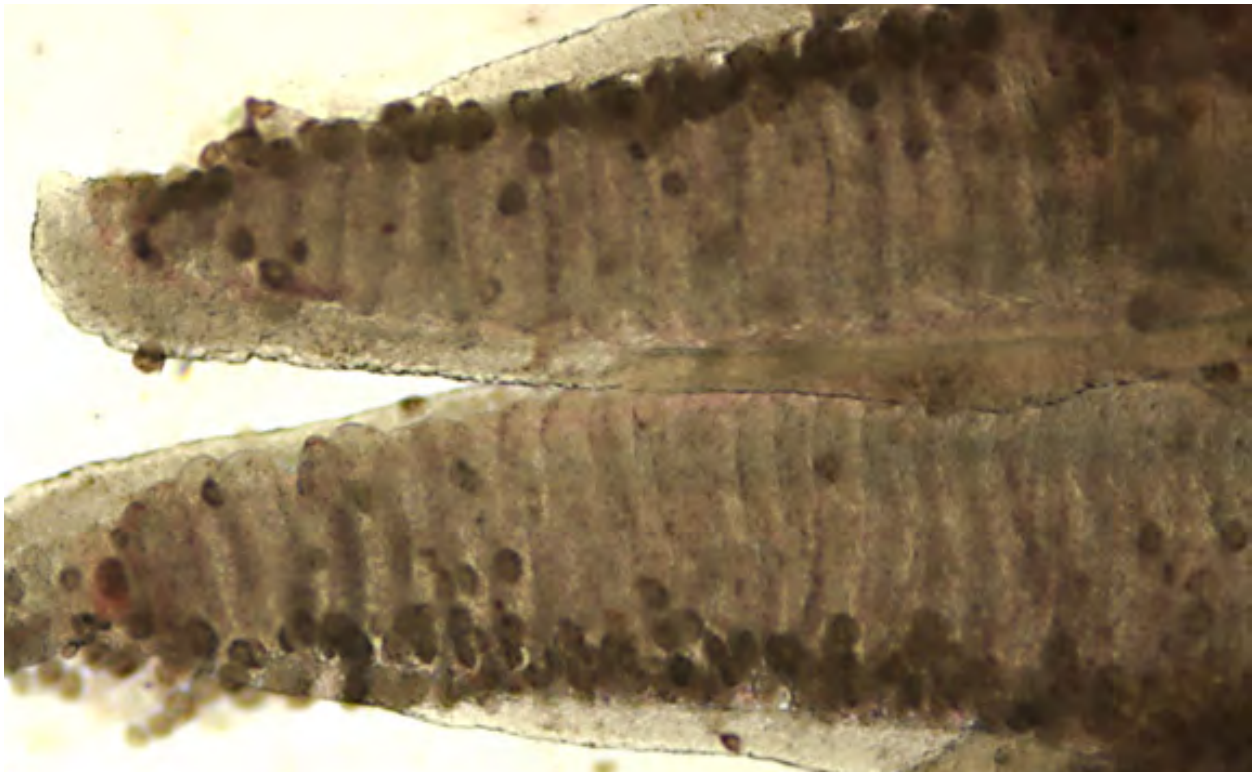


Figure 7. *Capriniana* on a Channel Catfish gill.

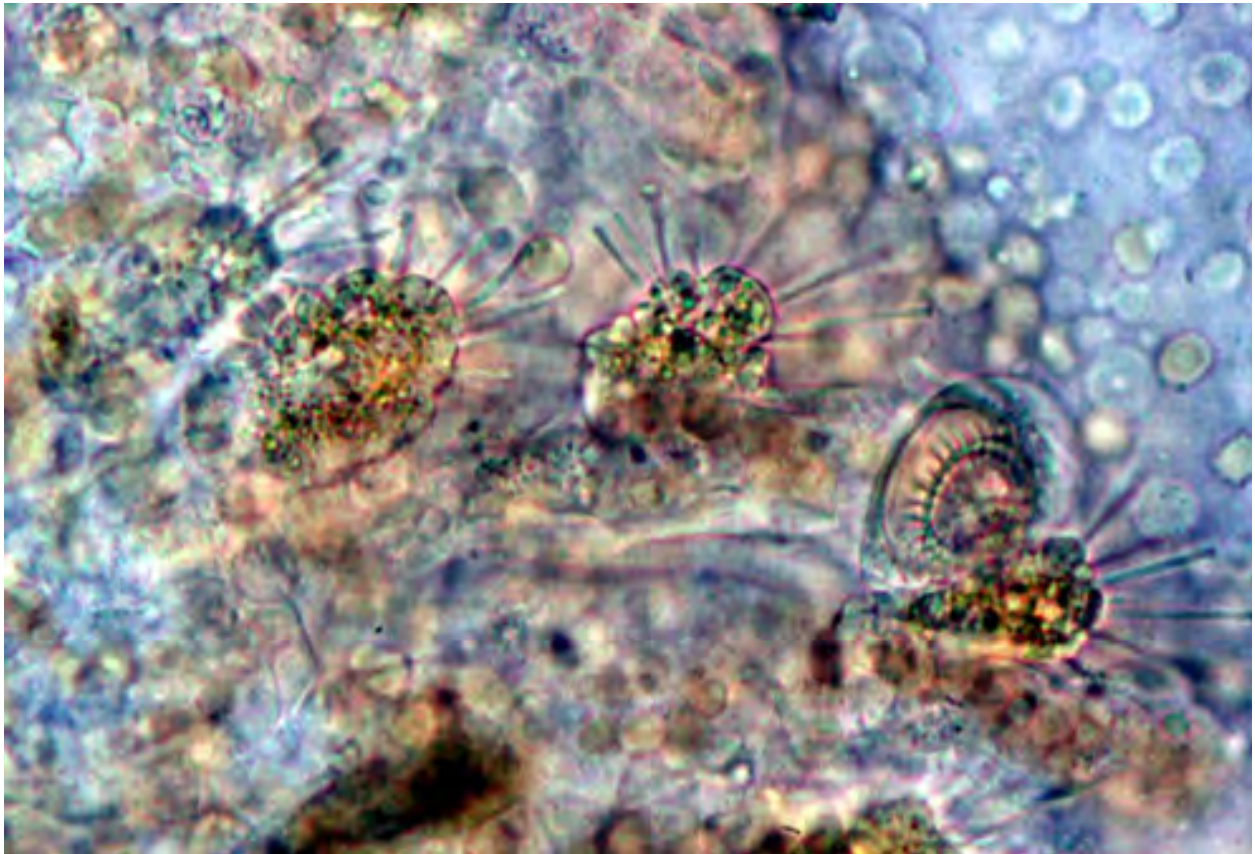


Figure 8. *Capriniana* on a Channel Catfish gill.

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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°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.

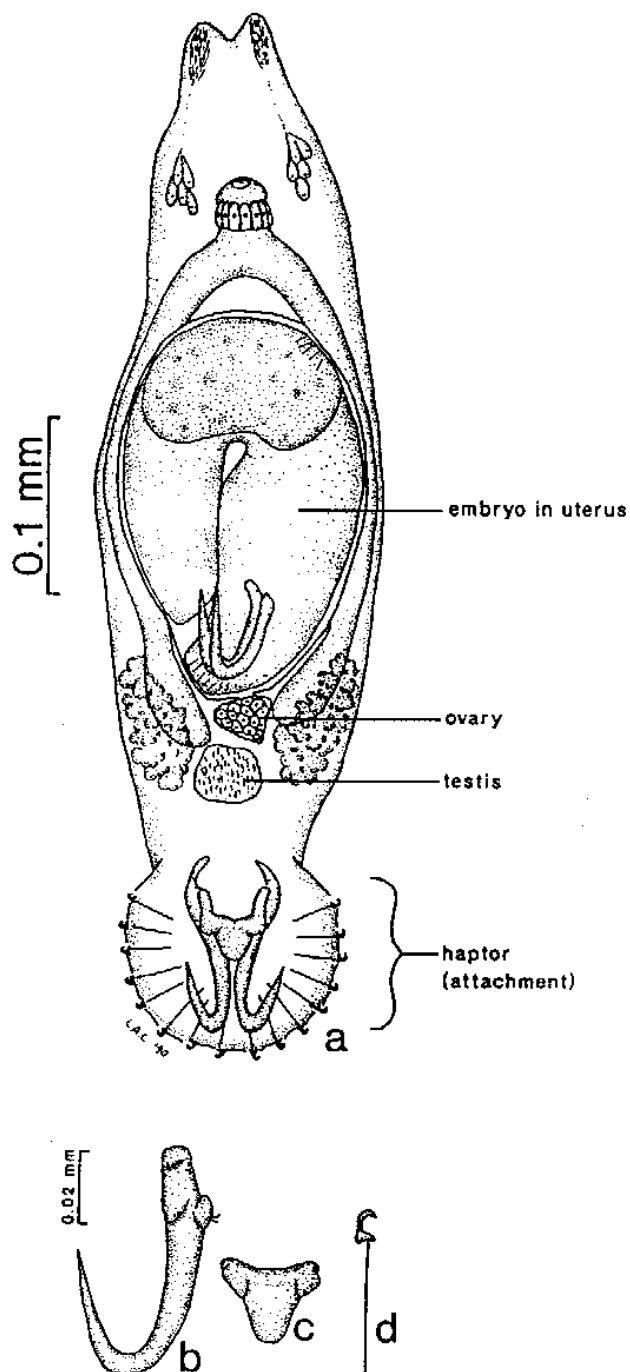


Figure 1. Generalized *Gyrodactylus* sp.

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, occurring 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°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 [“Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent” for Gyrodactylus spp.](#)

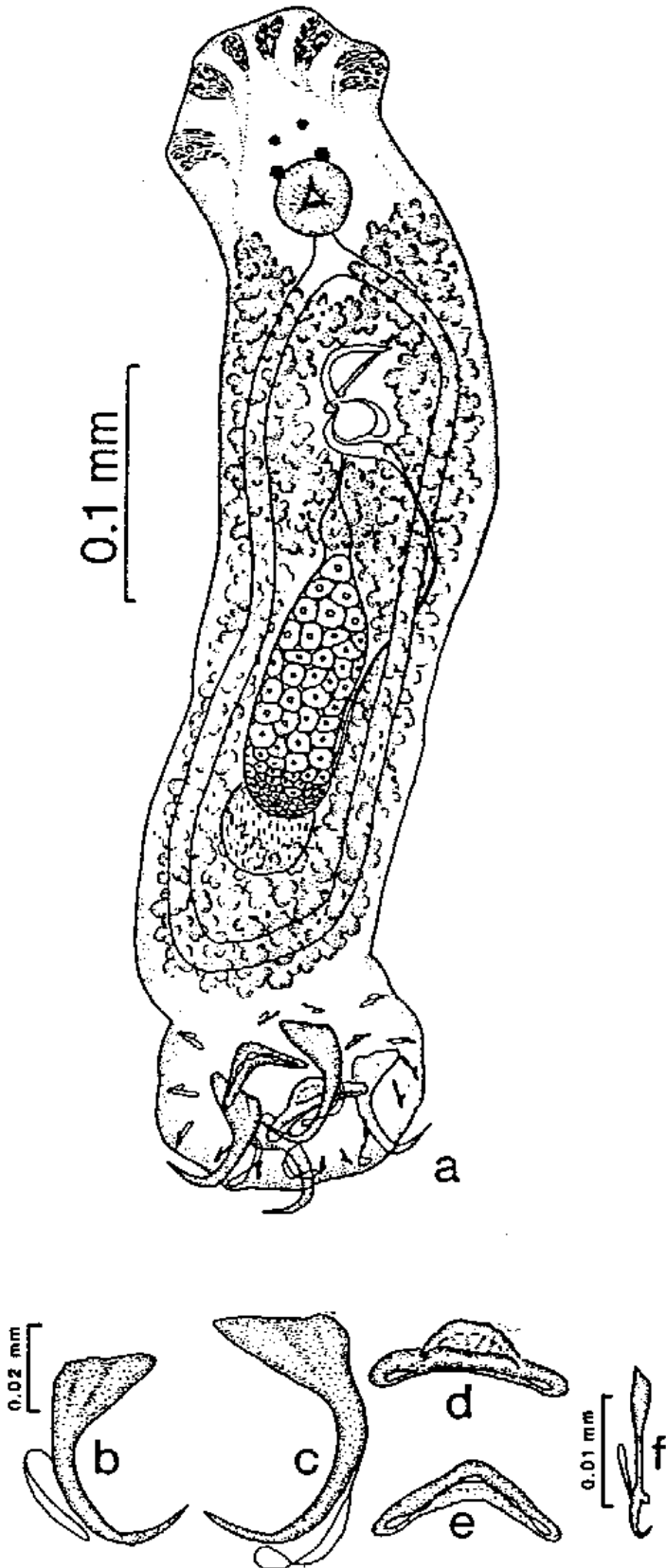


Figure 2. Generalized *Ligictaluridus* sp.

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 situations 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° C) than those in warmer areas (e.g. only a few days at 22 to 24° 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 [“Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent” for *Gyrodactylus* spp.](#)

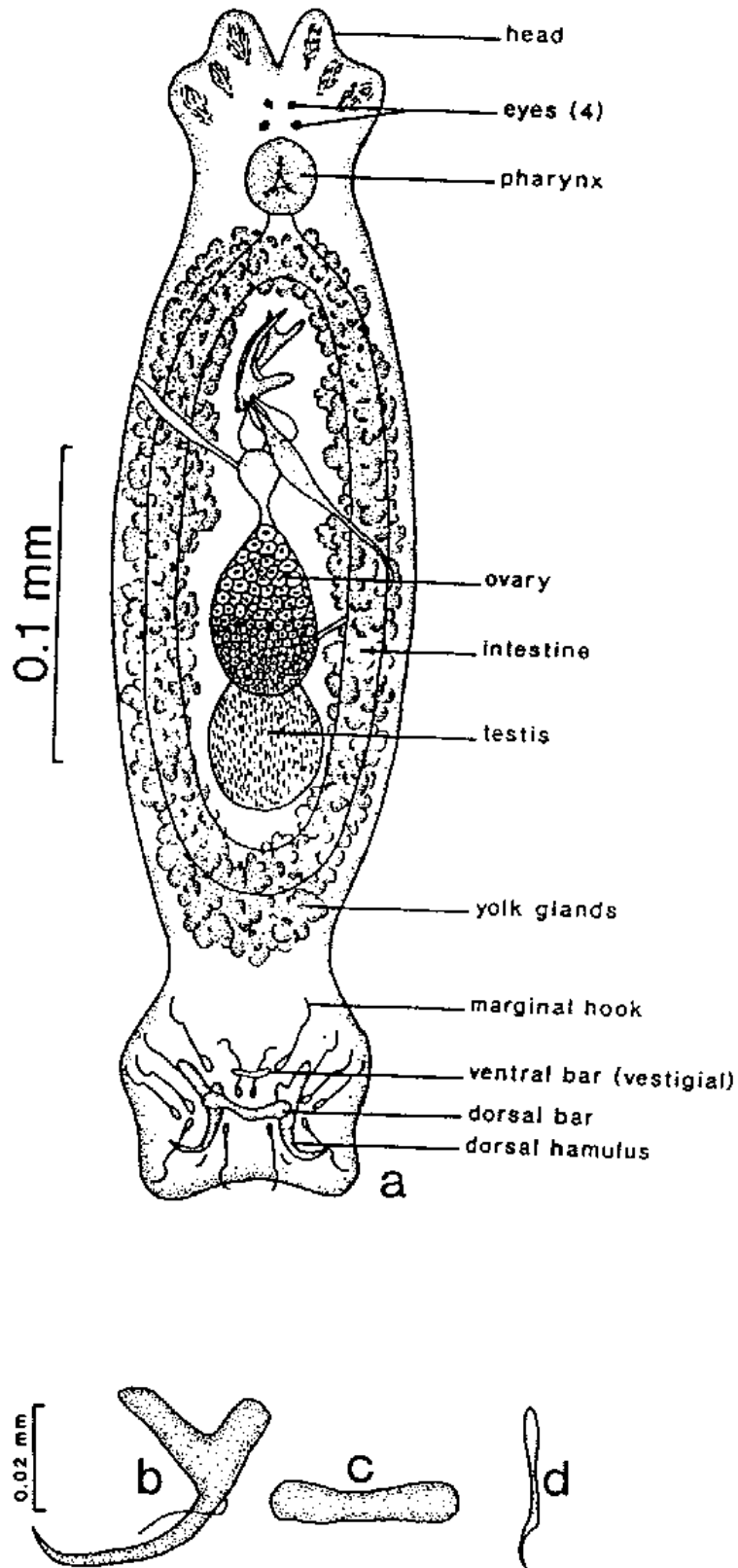


Figure 3. Generalized *Dactylogyrus* sp. (a) entire animal, ventral view, (b) dorsal hamulus, (c) dorsal transverse bar, (d) larval hook.

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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* proceroids. 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°C. Growth and maturation are fastest at temperatures above 25°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°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

Changes include thinning of the intestinal walls, loss of microvilli and the separation and shedding of enterocytes at the site of attachment. 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 *Bothriocephalosis 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 10 cm. Segmentation is evident on worms 1 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 1](#)), 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°C water for about five minutes and then transfer to 10% formalin. Confirmatory diagnosis is unlikely from preserved specimens.

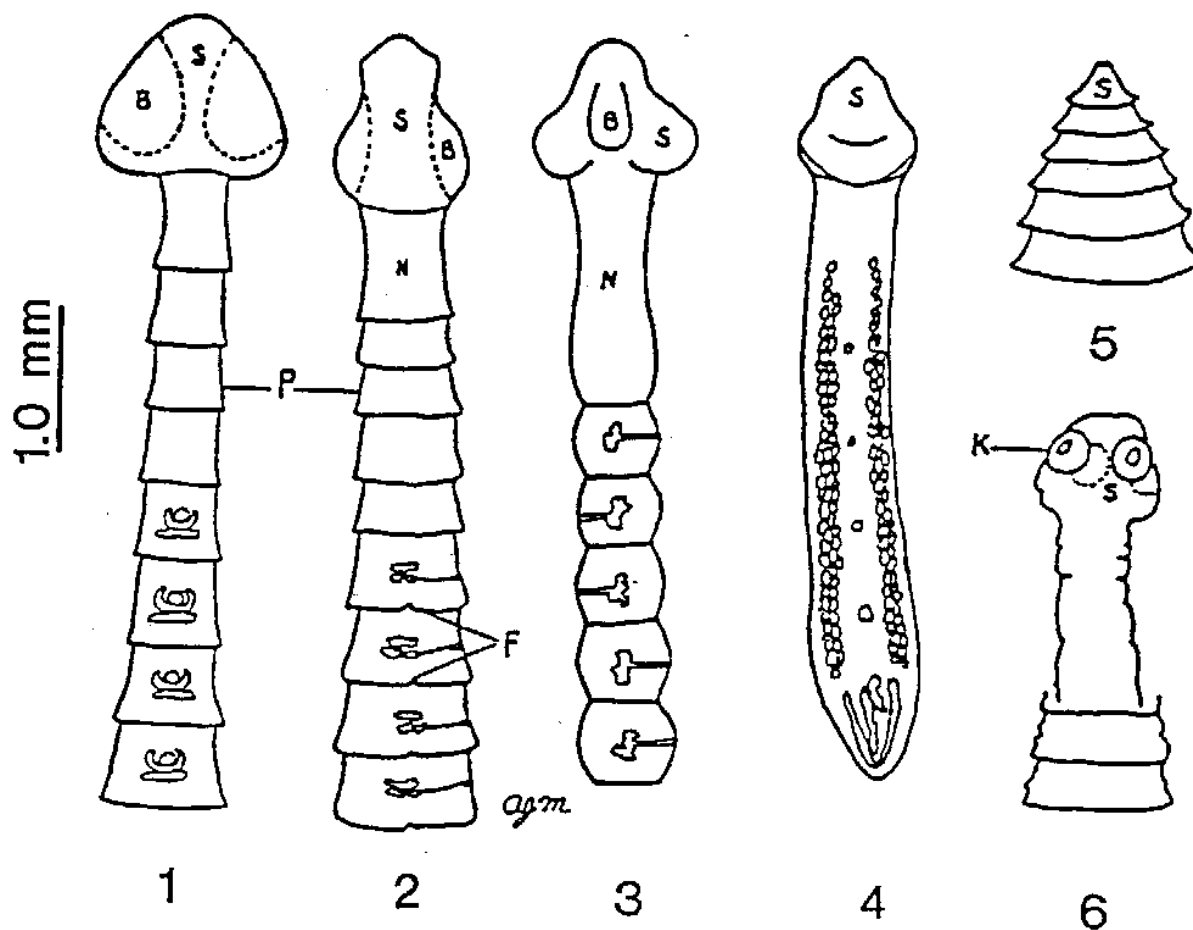


Figure 1. (1) *Bothriocephalus acheilognathi*; (2) *Eubothrium salvelini*; (3) *Marsipometra parva*; (4) *Glaridacris larvei* (unsegmented); (5) *Schistocephalus solidus*; (6) *Proteocephalus ambloplitis*; B – bothria; F – furrow; K – sucker; N – neck; P – proglottid; S – scolex.

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.....	<i>Atractolytocestus</i>
.....	<i>Glaridacris</i>
.....	<i>Ptychobothrium</i>
1b. Segmentation complete and distinct.....	2
2a. Scolex with 4 or 5 suckers.....	<i>Proteocephalus</i>
2b. Scolex with 2 bothria no suckers.....	3
3a. Tapeworms of marine fish.....	<i>Anoncocephalus</i>
.....	<i>Echinophallus</i>
.....	<i>Eubothrioides</i>
.....	<i>Fistulicola</i>
3b. Tapeworms of freshwater fish.....	4
4a. Dorsal-ventral median furrow.....	<i>Bathybothrium</i>
.....	<i>Eubothrium</i>
4b. No dorsal-ventral median furrow.....	5
5a. Neck.....	<i>Marsipometra</i>
5b. No neck.....	6
6a. Wide portion of scolex (constricted) is the same or less than the width of the anterior segments.....	<i>Schistocephalus</i>
6b. Scolex clearly wider than anterior segments.....	<i>Bothriocephalus</i> <i>acheilognathi</i>

* 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 (*Pimephales 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°C and dormancy occurs below 15°C. Typical cycling occurs every 20 to 25 days at 20 to 30°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 than 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 appearance 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.

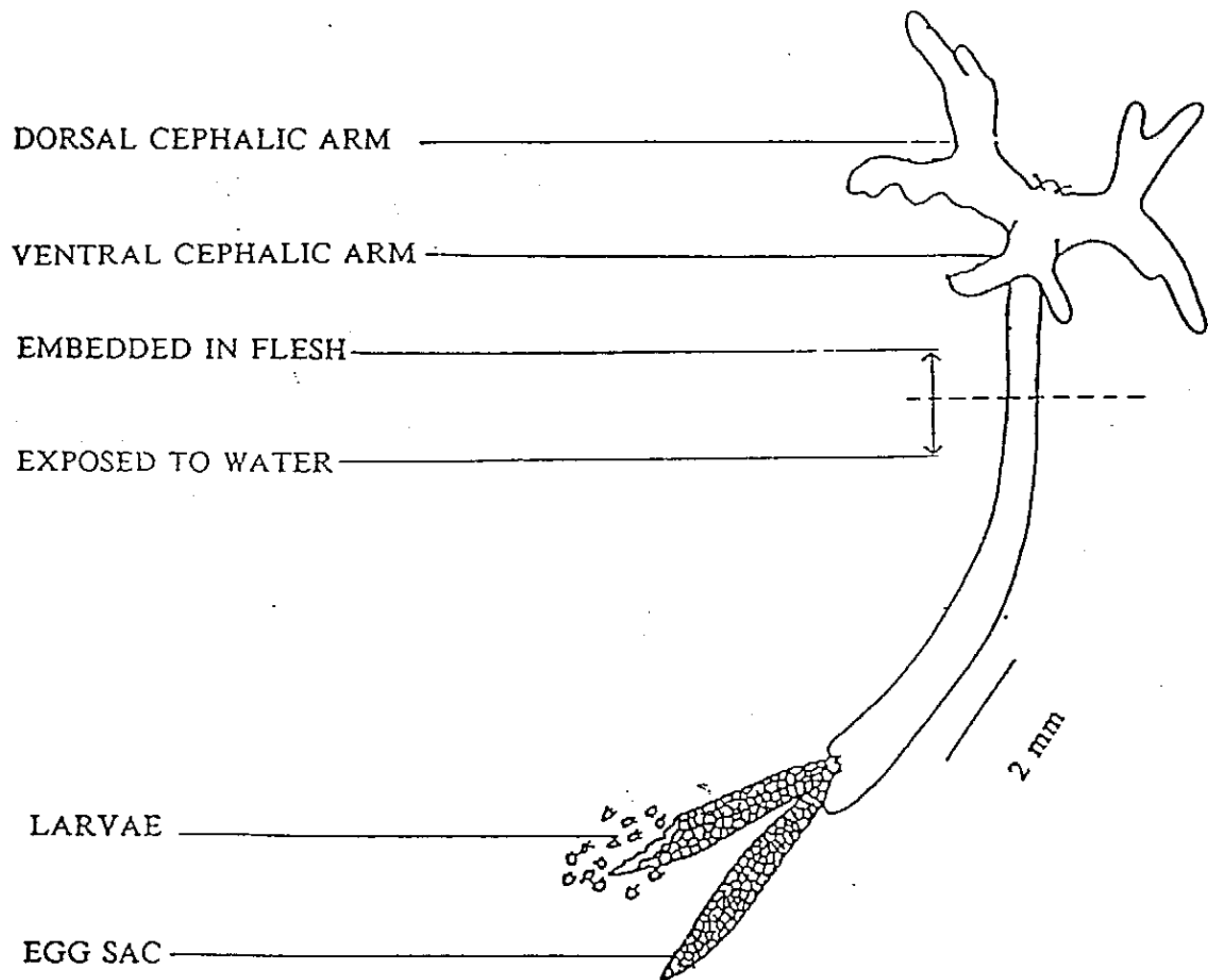


Figure 1. Diagnostic features of *Lernaean cyprinacea*, a species commonly associated with a wide variety of hosts.

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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 Ossiannder and Wedemeyer 1973). The infective stage (copepodid) and juvenile stages (chalmus 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.

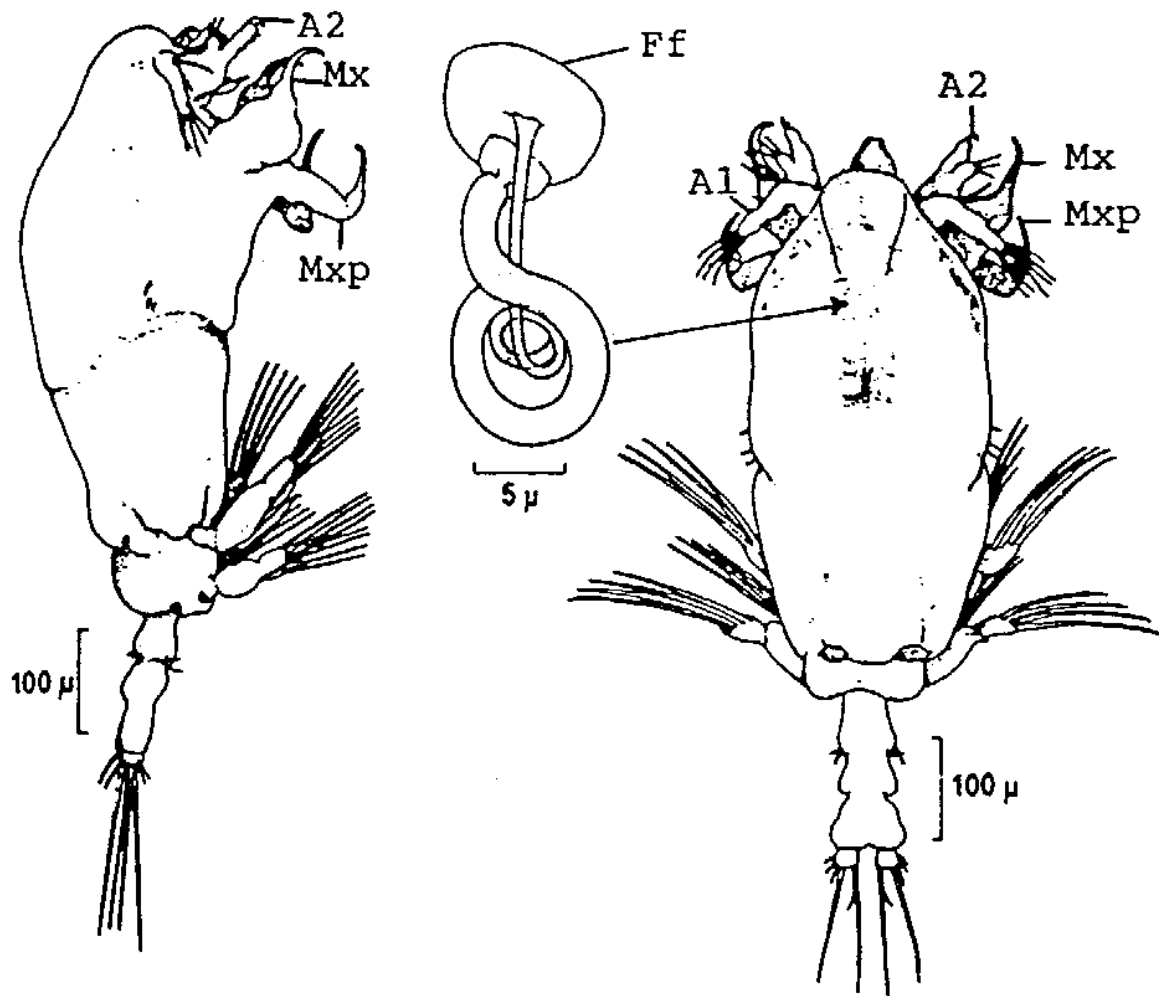


Figure 1. *Salmincola*, copepodid (infective stage). A1 - antennule. A2 - antenna. Mx – Maxilla. Mxp – maxilliped. Ff – frontal filament. (From Kabata and Cousins 1973).

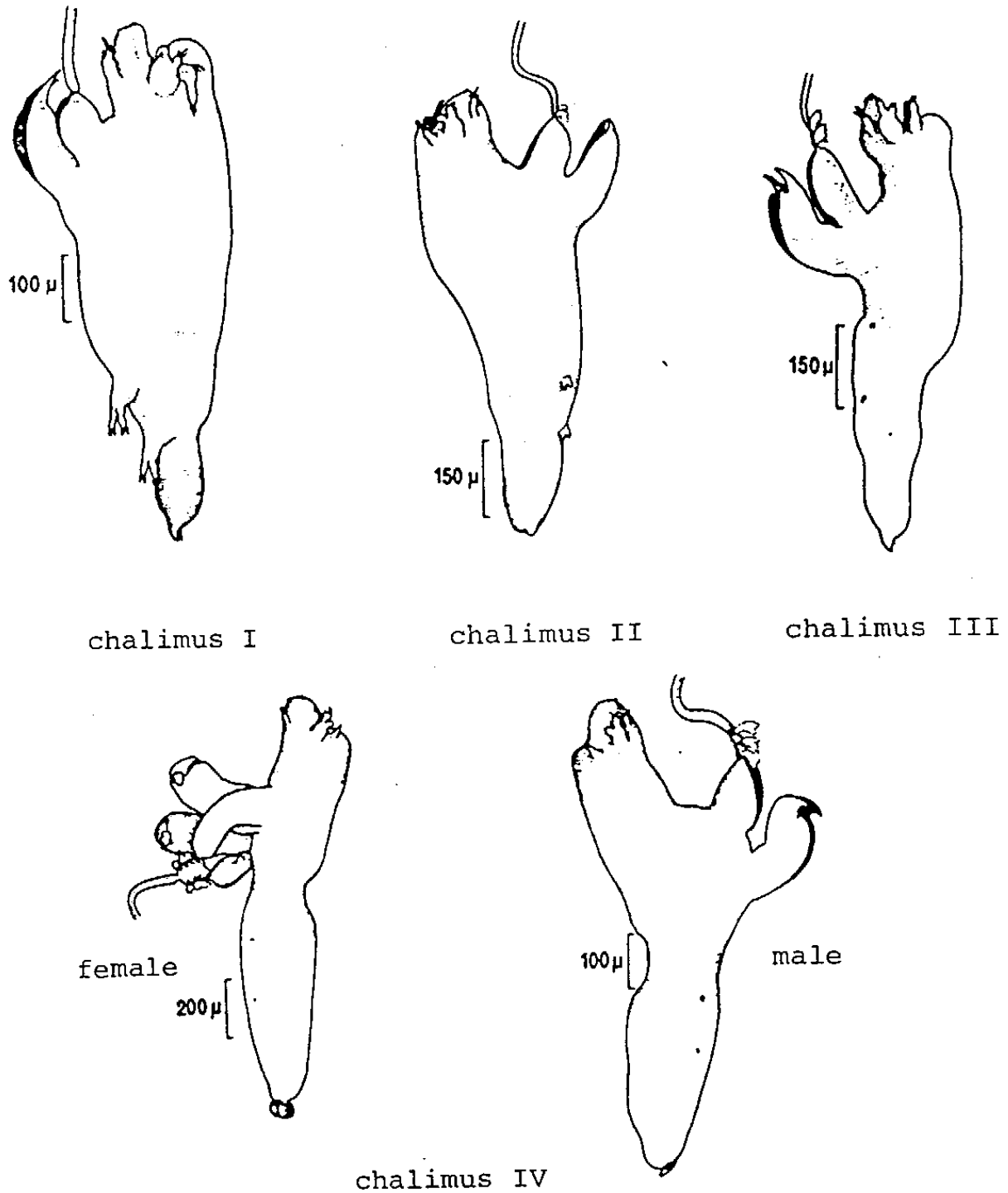


Figure 2. *Salmincola*, chalimus stages (attached by frontal filament). (From Kabata and cousins 1973).

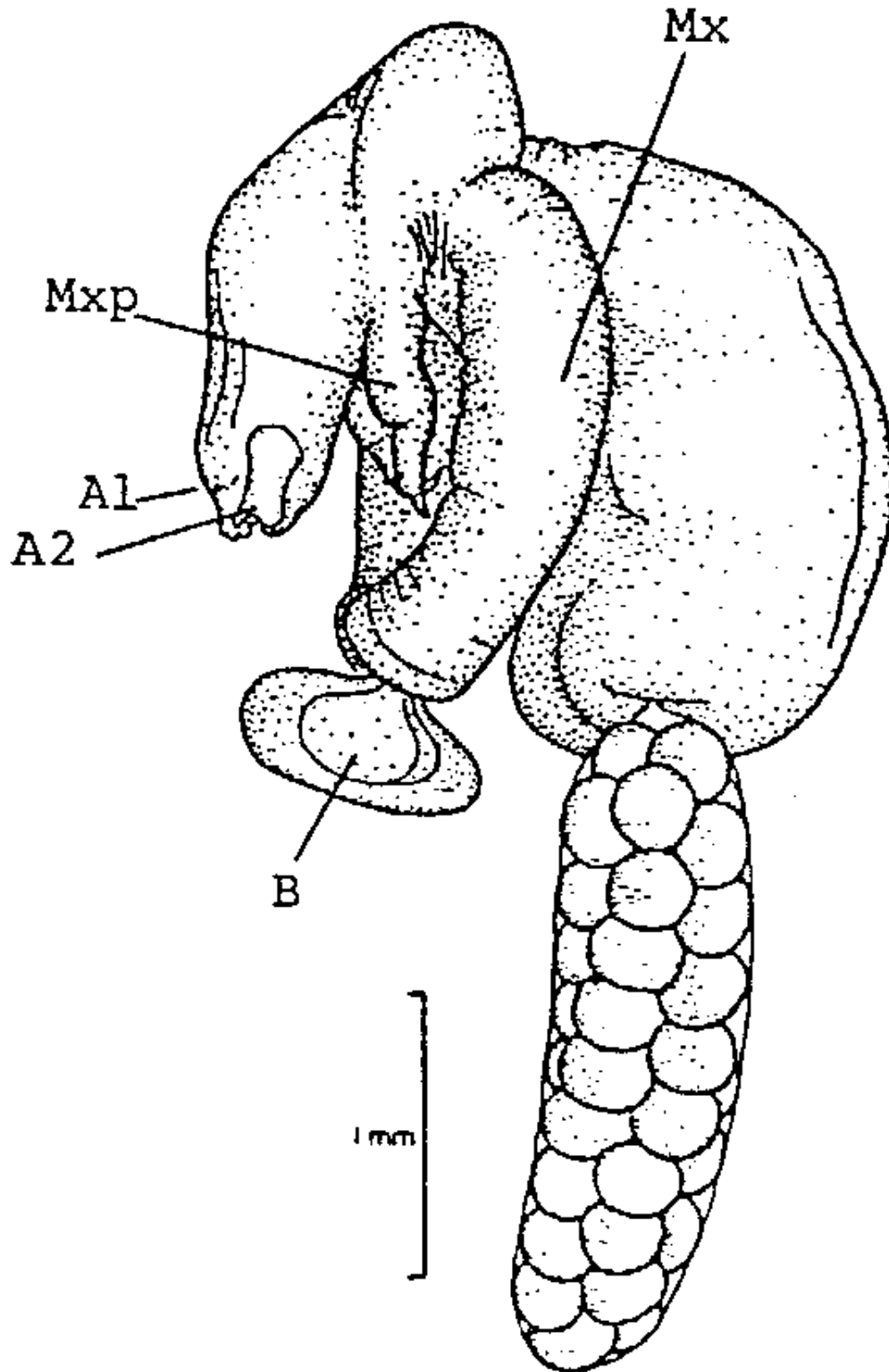


Figure 3. *Salmincola*, adult female. A1 – antenna. Mx – maxilla. Mxp – maxilliped. B – bulla. (From G. Fryer 1981).

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3.14 Gill Maggot Disease (Genus *Salmincola*) - 8

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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 pallasii*, three spine stickleback *Gasterosteus aculeatus*, greenling *Hexagrammos* sp., Pacific ratfish *Hydrolagus coliei*, 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°C. The generation time of *Lepeophtheirus salmonis* is approximately six weeks at 9 to 12°C and 7.5 weeks at 10°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°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)

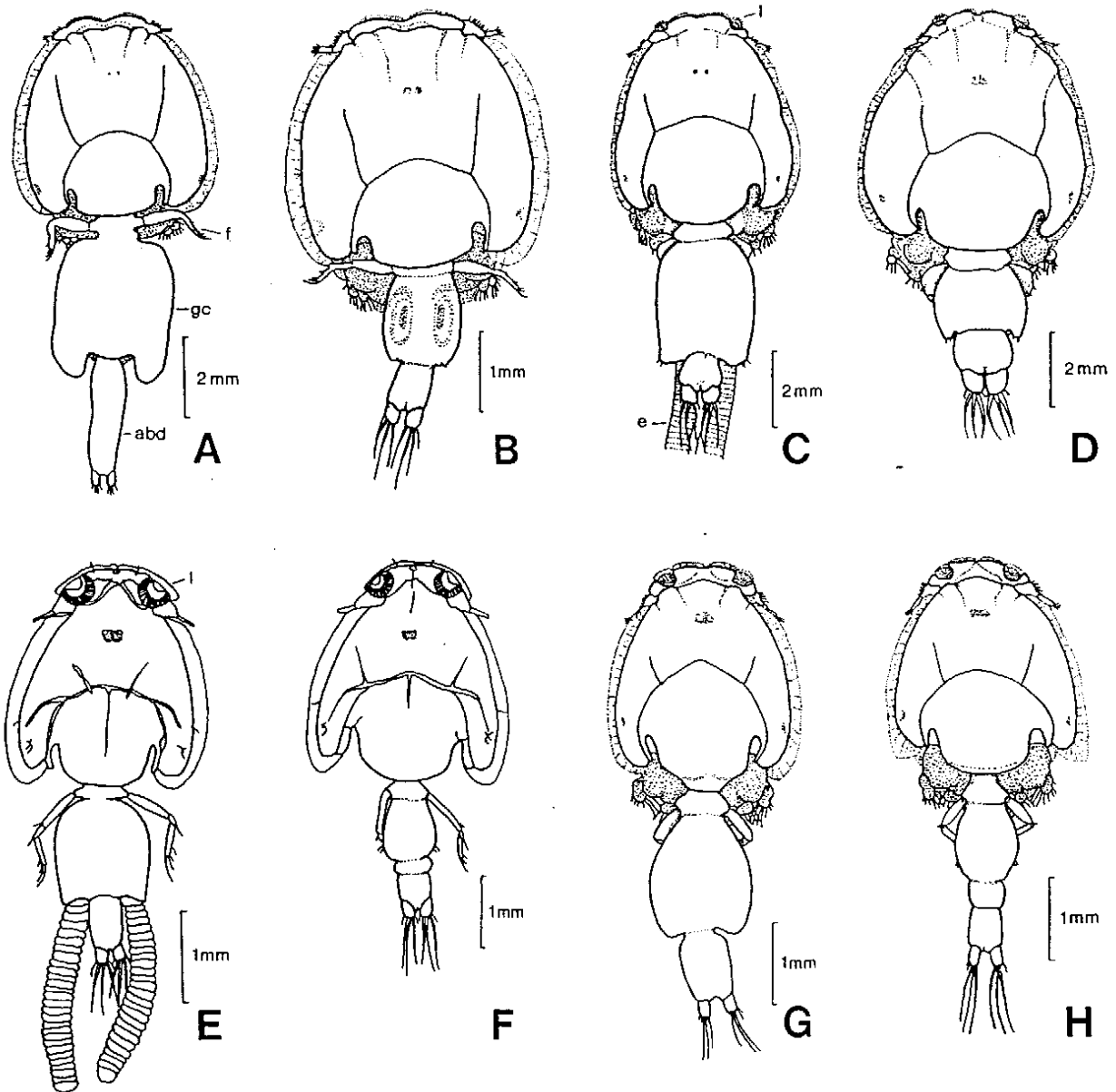


Figure 1. Adult stages of sea lice: (A) *Lepeophtheirus salmonis*, female; (B) same, male; (C) *Caligus curtus*, female; (D) same, male; (E) *Caligus clemensi*, female; (F) same, male; (G) *Caligus elongates*, female; (H) same, male.

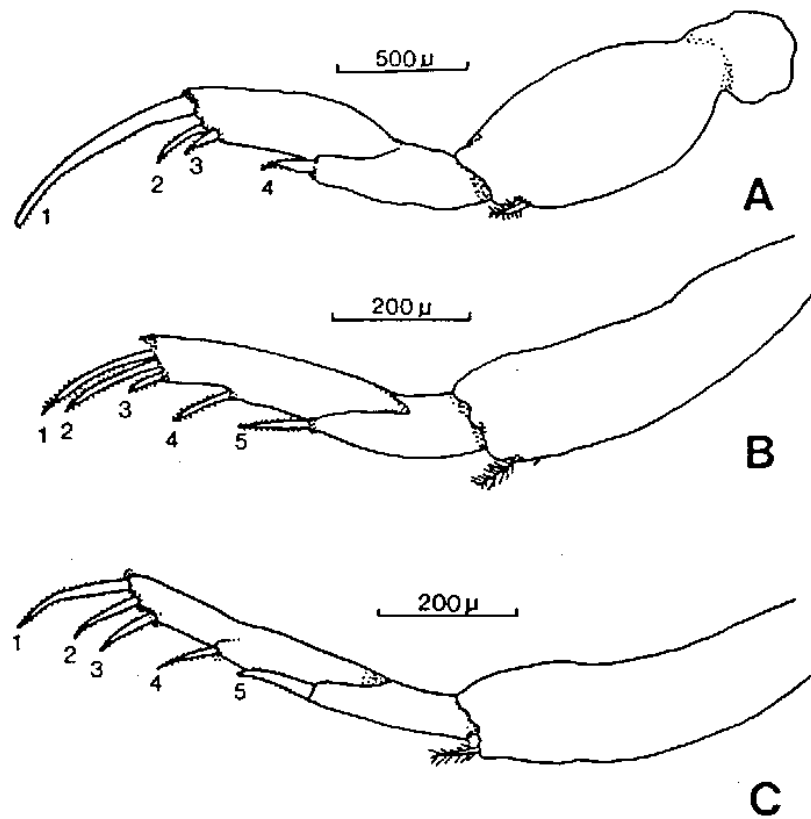


Figure 2. Structure of the fourth leg: (A) *Caligus curtus*; (B) *Caligus clemensi*; (C) *Caligus elongates* (A, redrawn from Kabata 1979; B, redrawn from Kabata 1972; C, original).

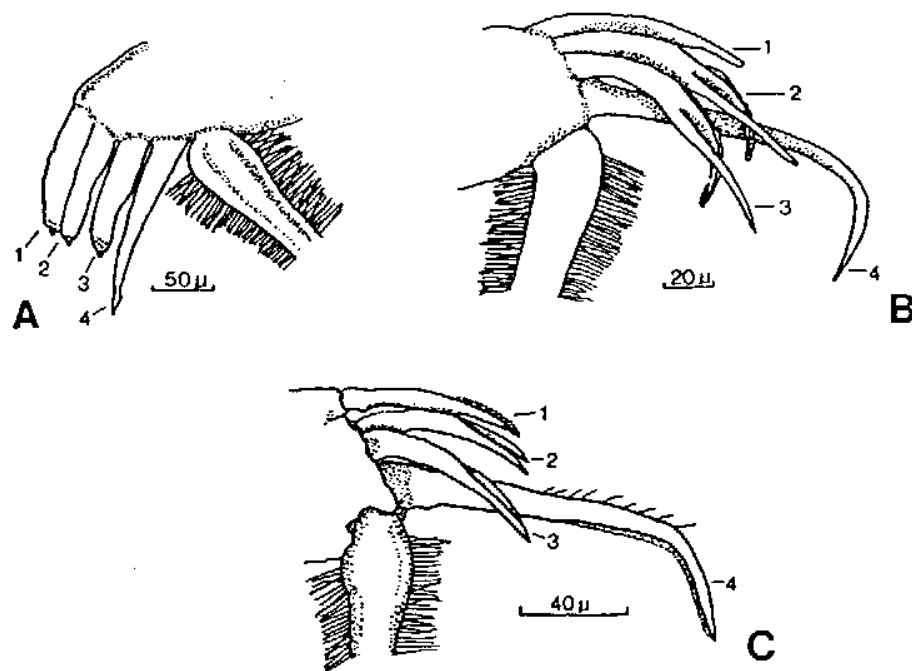


Figure 3. Distal margin of the exopod of the first leg: (A) *Caligus curtus*; (B) *Caligus clemensi*; (C) *Caligus elongates* (A, C, modified from Kabata 1979).

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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 synonymous 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 flavescentis* 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 phototactic, 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 severe 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 pathogenicity 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.

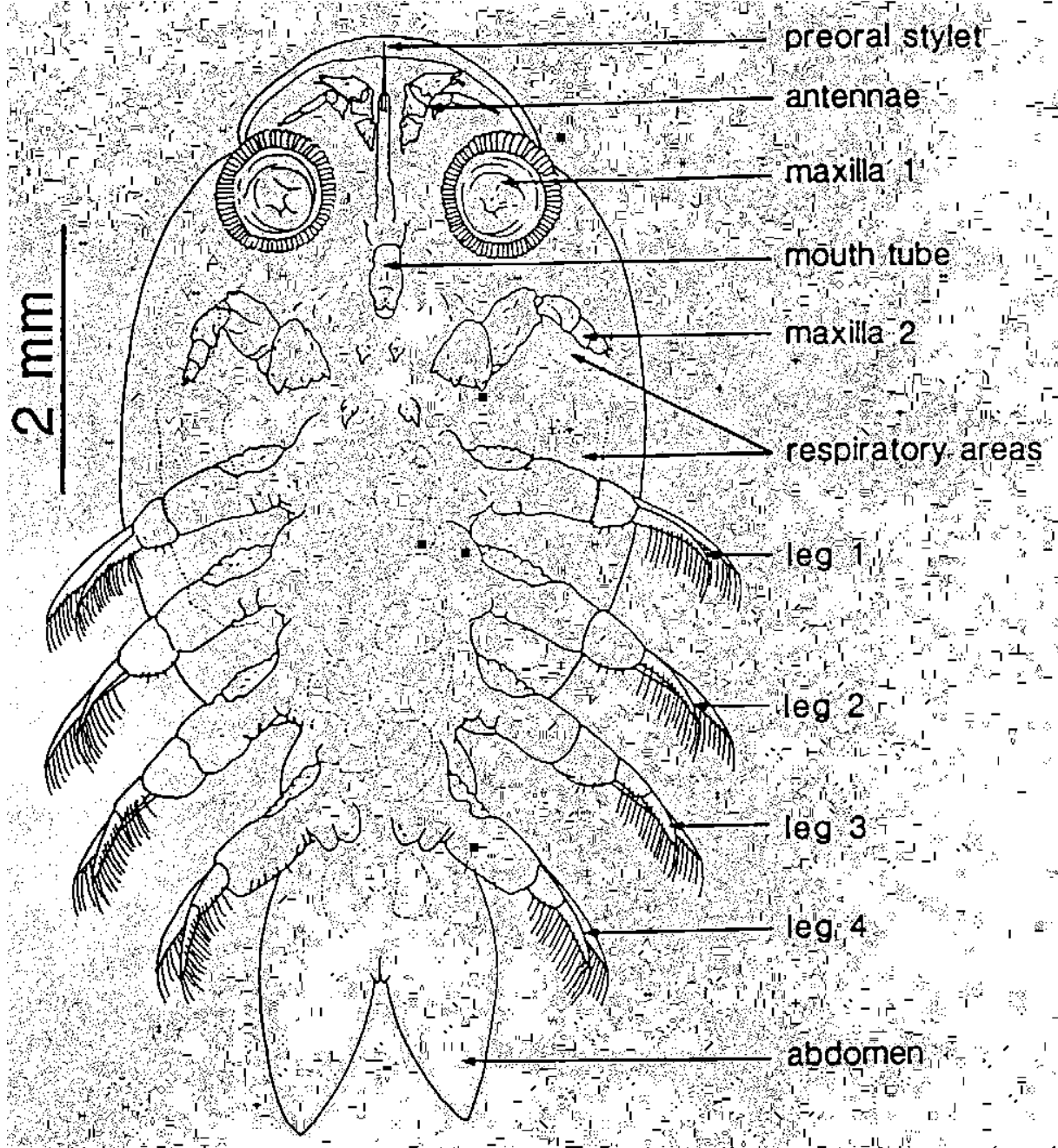
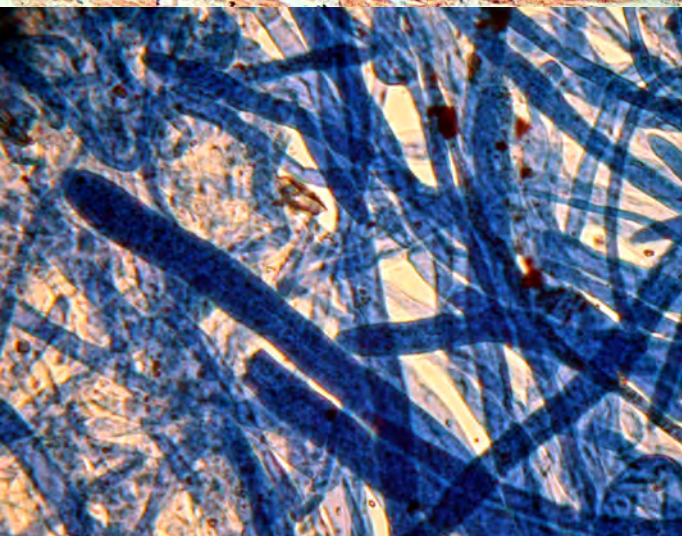
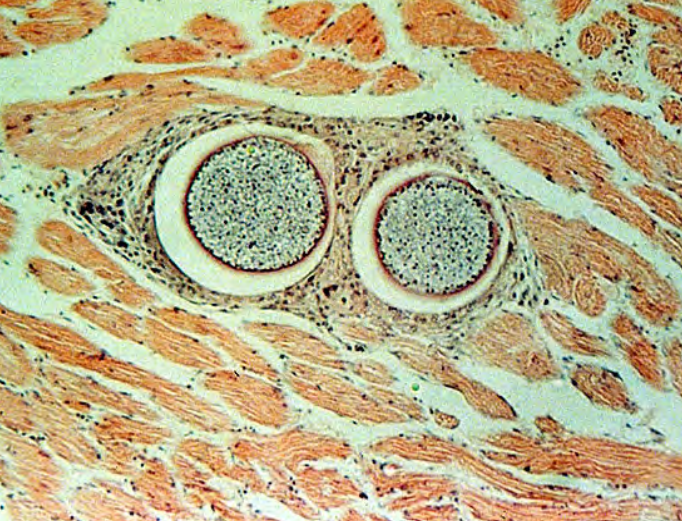


Figure 1. Ventral view of *Argulus alosae* showing pertinent diagnostic features (redrawn from Kabata 1988).

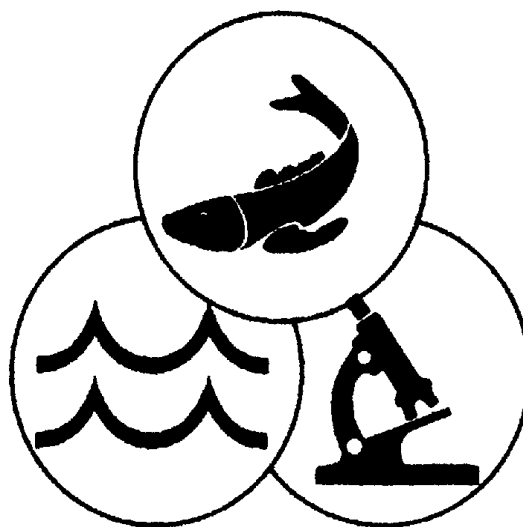
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Section 1

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. demingrands*.

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 *Cyprinus 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 *Poecilia 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 *Ichthyophonus 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. Behavioral 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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4.3 Ichthyophonus Disease - 3

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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*, *Leptomitosis*, 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 Sabouraud's agar (SAB) (Fuller 1978). Isolates grow well at room temperature (20 to 25°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.

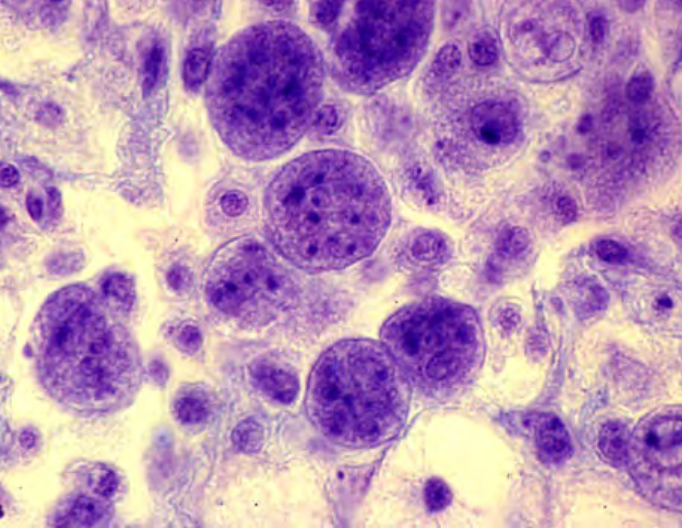
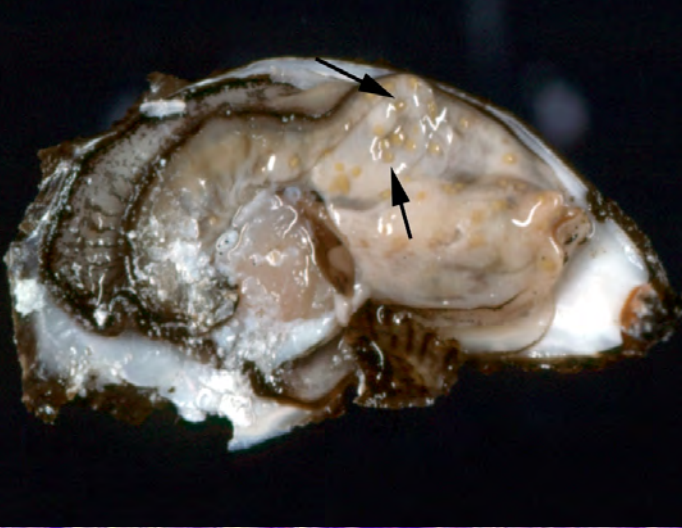
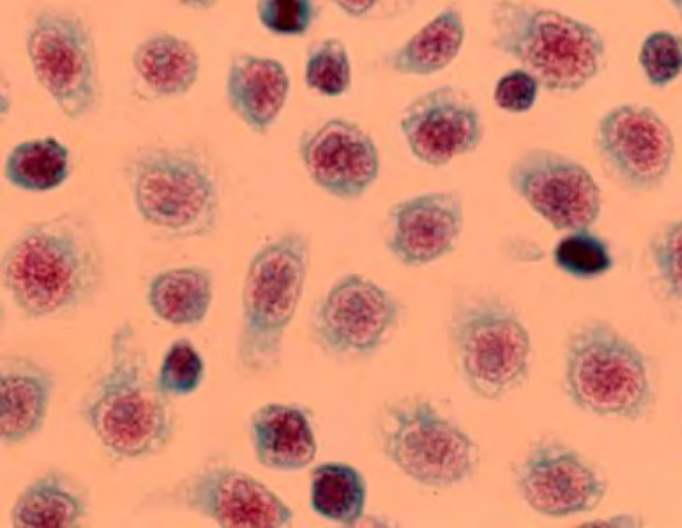
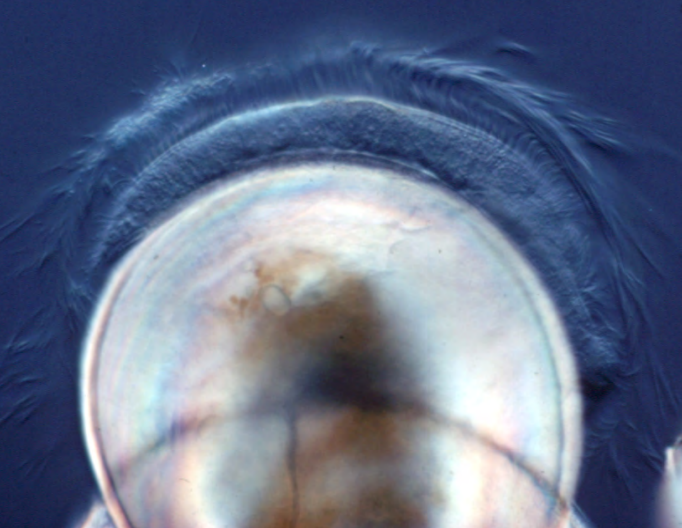
B. Dematiaceous Hyphomycetes

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.

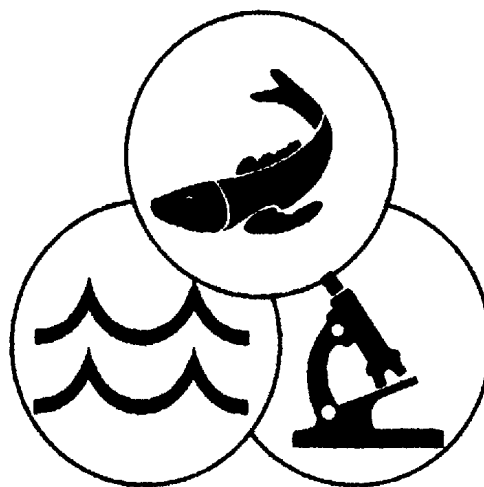
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Section 1

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



Chapter 5 Diseases of 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 marinus* Infection of American 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

The disease is popularly known as "Dermo" due to its obsolete taxonomic designation as *Dermocystidium marinum* (also obsolete: *Labyrinthomyxa marina*). Currently, the etiological agent resides in the phylum Apicomplexa as *Perkinsus marinus*. Thus, the proper name of the disease is perkinsiosis.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Perkinsus marinus occurs along the Atlantic coast of North America from Delaware Bay south and in the Gulf of Mexico. Its distribution in these waters is not continuous. It has been observed in oysters from Hawaii, but it is not known if it is now established there.

2. Host Species

Perkinsus marinus is only known to occur in *Crassostrea virginica*. Another similar parasite, *Perkinsus olseni*, has been reported in the black-lipped abalone *Haliotis ruber*. Several other species of bivalve molluscs are also reported to be infected with similar parasites, presumably *Perkinsus* spp.

C. Epizootiology

The severity of the disease increases periodically in infected areas. Mortalities can reach 100% and have been reported to be 30 to 50% in the first year with cumulative mortalities of 75% and higher in the second year in oysters introduced to an infected area. The disease does not cause serious mortalities below salinities of 12 to 15 ppt (parts per thousand) but can persist in overwintering oysters in salinities below 5 ppt. Perkinsiosis is a warm temperature disease with outbreaks and mortalities occurring in the summer months (e.g. June through October in the Chesapeake Bay region). Epizootics occur typically in warm temperatures (18 to 30°C). The disease is known to increase dramatically in infected areas that receive heavy plantings of oysters and has been shown to

be transmissible over 50 ft in the water, although it is possible that it is transmissible over much greater distances. The disease can be spread from one oyster to another by the gastropod parasite *Boonea impressa*. *Boonea* can increase the infection intensity of oysters already infected with *Perkinsus* as well as initiate new infections in oysters on which it feeds.

D. Disease Signs

Reduced growth rate prior to the onset of mortalities occurs. Non-specific signs include emaciation, gaping, and pale digestive gland.

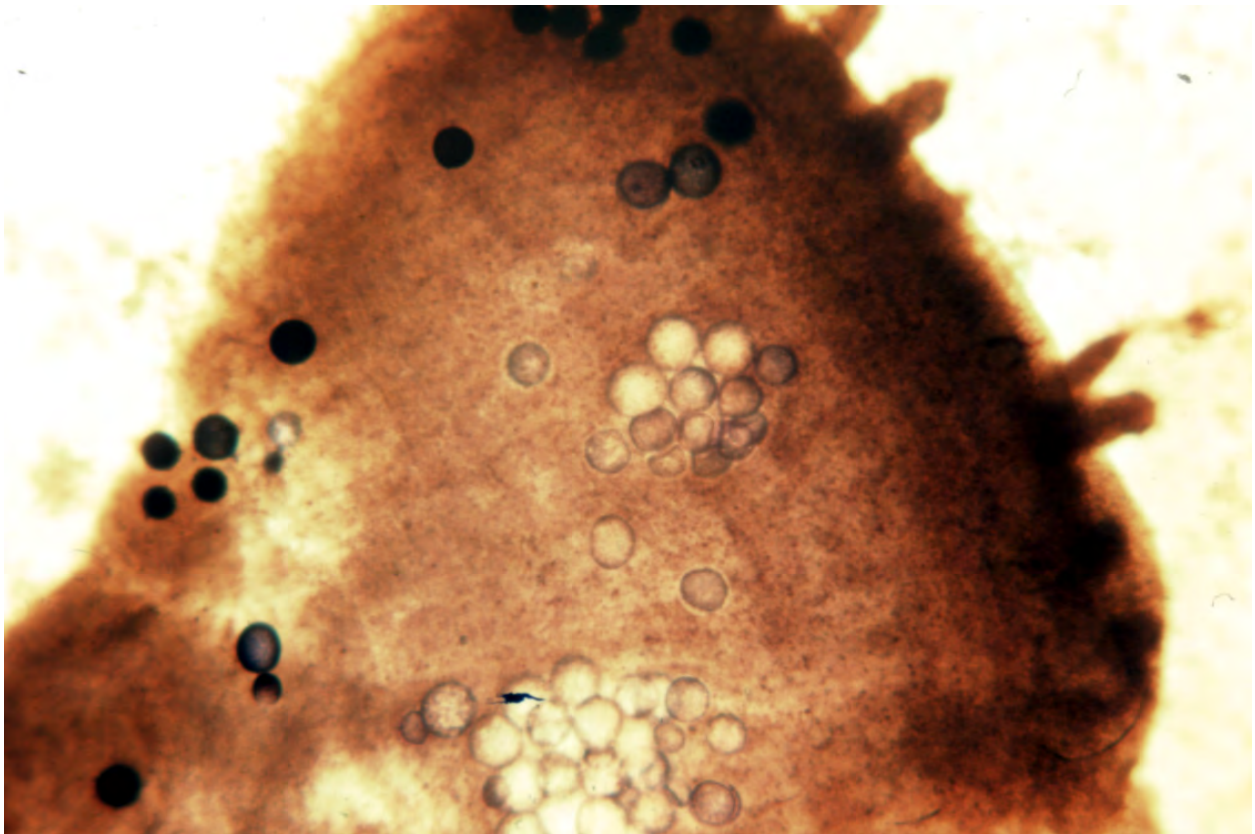


Figure 1. Mantle tissue of American oyster infected with *Perkinsus marinus* incubated in FTG medium and stained with Lugol's stain. The enlarged aplanospores near the periphery of the tissue stain blue to brown and appear dark in the photomicrograph while those deeper in the tissue are unstained and appear clear, 90X.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

Presence of spherical bodies are seen in wet mounts from heavily infected animals (see tissue sampling below). The parasite is typically in the 5 to 10 μm range and with an eccentric vacuole.

2. Confirmatory Diagnosis

a. Fluid Thioglycollate Method (FTG) (Ray 1966)

FTG is preferred because of presumed greater sensitivity and simplicity of interpretation. Examination of cultured tissues for enlarged sporangia (up to 250 μm diameter, depending on length of culture period) as follows:

- i. Rehydrate 29.3 g of FTG Medium (Difco, No. 1.0256-02 or Baltimore Biological Laboratory, No. 01-140) and 20 g of NaCl in 1 L of distilled water.
- ii. Dispense rehydrated medium in 10 mL amounts into culture tubes and autoclave. Store sterile tubes of medium in the dark at room temperature until needed, making certain that the medium remains anaerobic when used (yellow, rather than red-tinged from indicator).
- iii. Fortify each tube of medium with 200 units of mycostatin (nystatin), 500 μg of dihydrostreptomycin, and either 200 μg of chloromycetin (chloramphenicol) or 500 units of penicillin G per mL of medium just prior to use.
- iv. Plant test tissues (gill, mantle, and/or rectum) in the tubes of fortified medium and incubate in the dark at room temperature for at least one week.
- v. Blot the incubated tissue on absorbent paper toweling, flood with 2 or 3 drops of diluted Lugol's solution, and then tease tissues into fine bits.
- vi. Examine stained tissues microscopically at 25X to 100X magnification for brown, green, blue, and blue-black spheres ([Figure 1](#)).

- b. Histological examination can also provide a confirmatory diagnosis although the thioglycollate method is generally regarded as more sensitive. Examine tissues for daughter cells formed by protoplast cleavage and for the characteristic vegetative uninucleate aplanospores (typically 5 to 10 μm diameter, rarely to 20 μm or larger, [see Figure 2](#)) which develop eccentric vacuoles as they enlarge and which occur in most tissues in heavy infections ([Figure 2](#)). Aplanospores occur extracellularly or within host hemocytes. In light infections, they are found in major digestive epithelia with foci, including a marked infiltration of hemocytes, developing in various tissues as the infection progresses. All tissues, with the usual exception of non-digestive epithelium and nervous tissues, are infected in late stage infections. Infection of rectal epithelium is regarded as a sign of late stage infection.

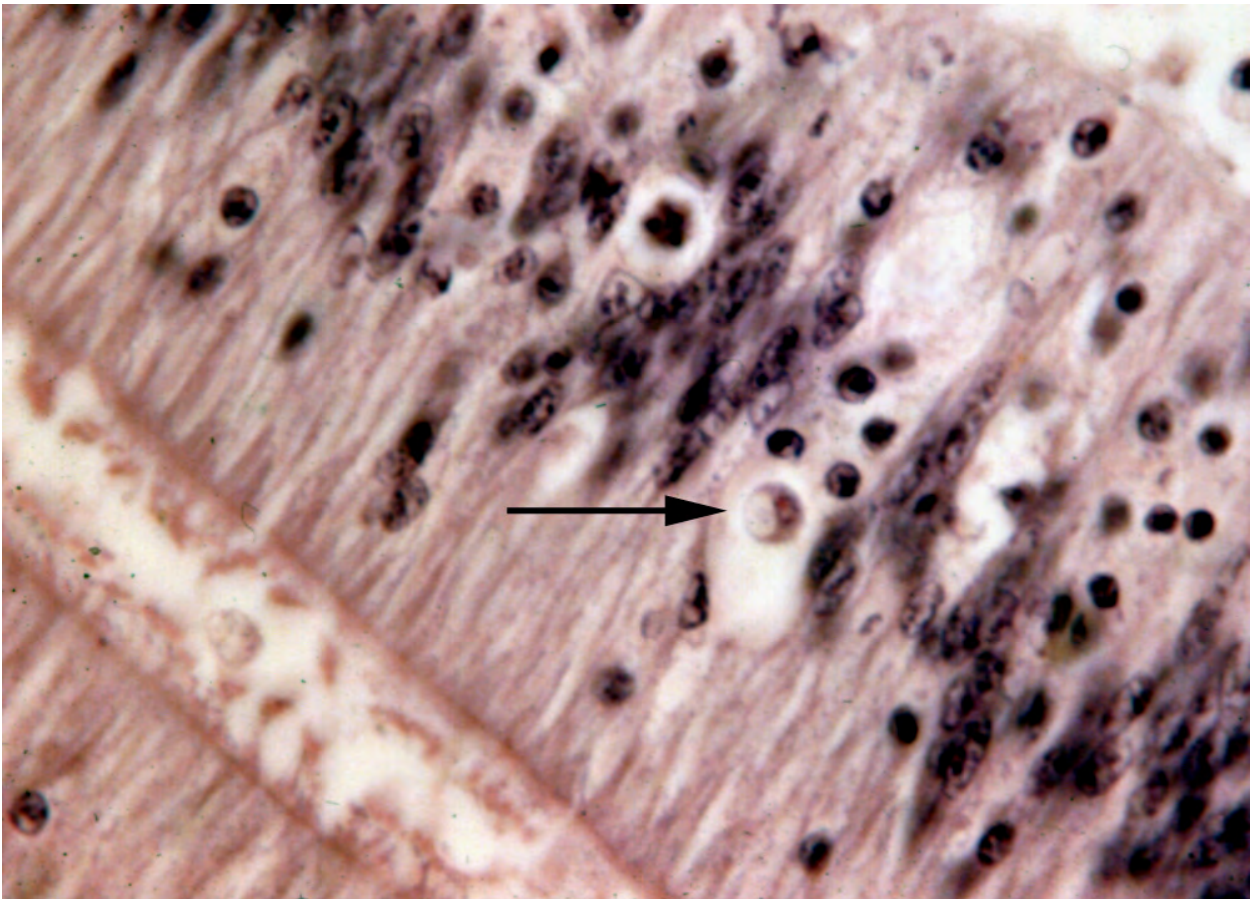


Figure 2. Arrow shows typical uninucleate aplanospore (“hypnospore”) of *Perkinsus marinus* in the intestinal epithelium. The eccentric vacuole giving the signet ring appearance is characteristic of the microorganism in the tissue, 1800X.

F. Procedures for Detecting Subclinical Infections

Use the FTG method or histological examination described above.

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

For the FTG test, oyster tissues that have not been frozen must be used. Oysters tissues for the test should be removed from the live oysters and placed directly into the FTG medium. Oysters may be held at 15 to 20°C for up to 24 hours during transport to the laboratory for examination.

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5.3 Haplosporidiosis of American 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

This disease is caused by *Haplosporidium nelsoni* (*Minchinia nelsoni*, Acetosporea). The causative agent was first known as multinucleate sphere unknown and the acronym MSX has persisted as a reference to the disease. The disease is also known as Delaware Bay disease but is differentiated from another haplosporidian disease of the same host caused by *Haplosporidium costale*.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Found in the Atlantic coast of North America discontinuously from Maine to Florida. Periodically epizootic in Delaware and Chesapeake Bays.

2. Host Species

Occurs in the American oyster *Crassostrea virginica*. Note that a variety of other morphologically similar parasites occur in other bivalve molluscs.

C. Epizootiology

Infections in Delaware Bay resulted in mortalities approaching 100% over three years when the disease first appeared in the late 1950's. On seed beds in Delaware Bay, annual mortalities of stocks subjected to the disease, and thus partially resistant, were estimated to be in the 4 to 9% range before the drought that began in 1980. About 30% of these seed were estimated to die within one year when transplanted to infected high salinity growout beds. Mortalities have exceeded 50% in recent years, even among the resident Delaware Bay stocks. Salinity and temperature are known to be important in the severity of MSX disease. In general, the disease is rarely acquired below about 10 ppt; salinities of about 15 ppt are required for the parasite to appear in substantial numbers in host tissues and serious mortalities occur only above 20 ppt. There is some indication that the disease may be limited above a salinity of 30 ppt. Oysters become infected during the warm months (late May through October) with peak mortalities in late summer and early fall, and again the following summer. The

disease reappears or increases in severity in drought years. The parasite appears to be sensitive to high temperatures, and in oysters with some resistance, the disease is reported to go into remission or disappear when temperatures exceed about 20°C.

D. Disease Signs

Disease signs include mantle recession in heavily infected oysters and heavy fouling along periphery on the inside of the left valve. Raised yellow-brown conchiolin deposits on internal valve surfaces of chronically infected oysters have been reported, but these are not consistent signs of the disease. Animals are typically thin and watery with pale-colored digestive diverticula.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

Presumptive diagnosis is based on demonstration of plasmodial forms (described below) in hemocytological preparations or in wet mounted material stained with 1% methylene blue.

2. Confirmatory Diagnosis

Histological documentation of the disease syndrome is as follows. Plasmodia occur in vascular spaces of potentially all tissues (Figure 1) and are roughly spherical and usually 4 to 30 µm in diameter but occasionally up to 50 µm in diameter. Plasmodia have one to more than 60 nuclei ranging from 1.5 to 7.5 µm in diameter, all of which, except the smallest, have a peripheral endosome and/or nuclear bar. Acid-fast staining spores (about 8 x 6 µm), believed to represent the same organism, are extremely rare, but when found are always found in digestive epithelia. Stages of the disease are hypothesized as follows:

- a. Initial Stage
Relatively small plasmodia are confined to the gill and palp epithelium.
- b. Intermediate Stage
Characterized by widespread focal infections and hemocytic infiltration.
- c. Advanced Stage
Massive parasite proliferation in most tissues.
- d. Terminal Stage
Characterized by necrosis of host and parasite tissues.
- e. Remission Stage
Hemocytic infiltration is reduced, and there is an increase in the number of pigment cells and increased phagocytosis of parasites.

F. Procedures for Detecting Subclinical Infections

Histological observation of the parasite in early stages of the disease as described above.

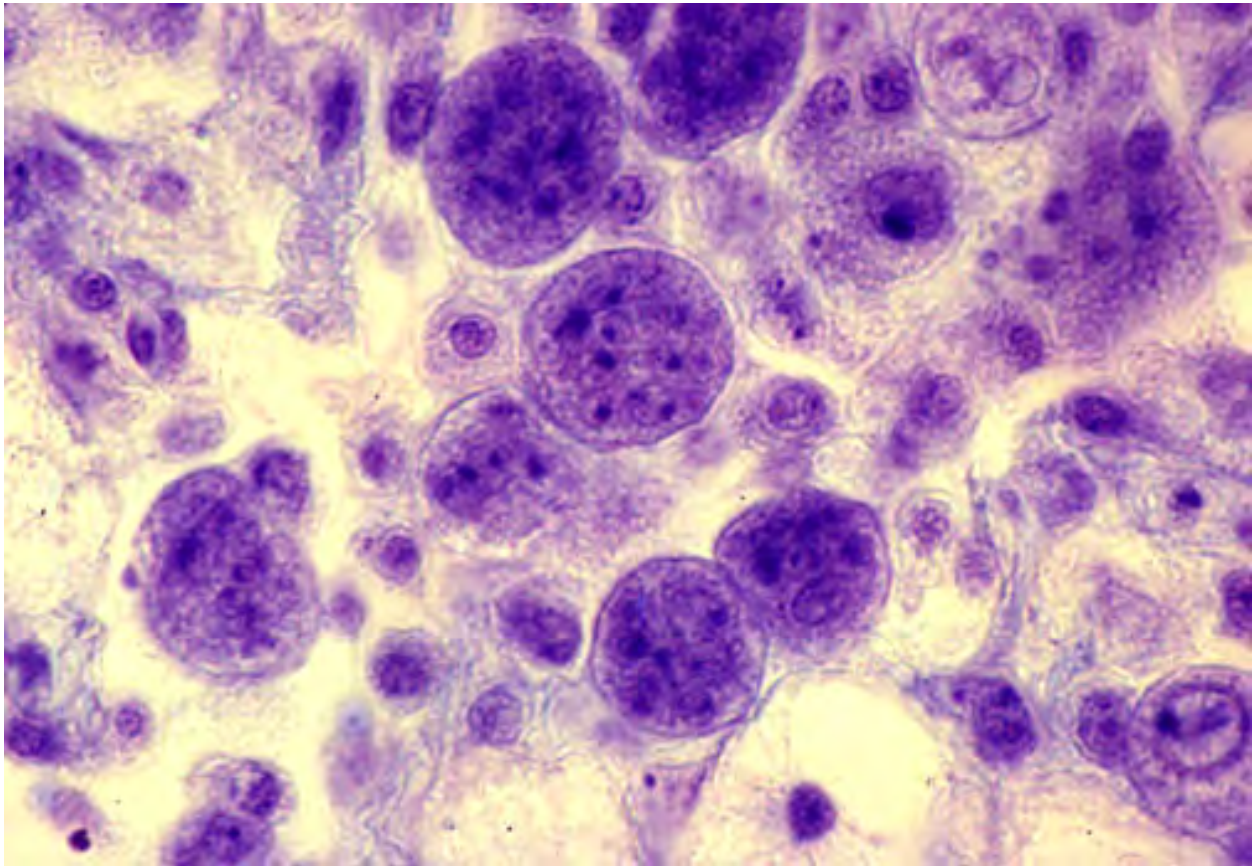


Figure 1. Plasmodia of *Haplosporidium nelsoni* in tissues of American oyster. Note the multiple nuclei with endosomes, 1125X. Photomicrograph courtesy of Susan Ford.

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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.

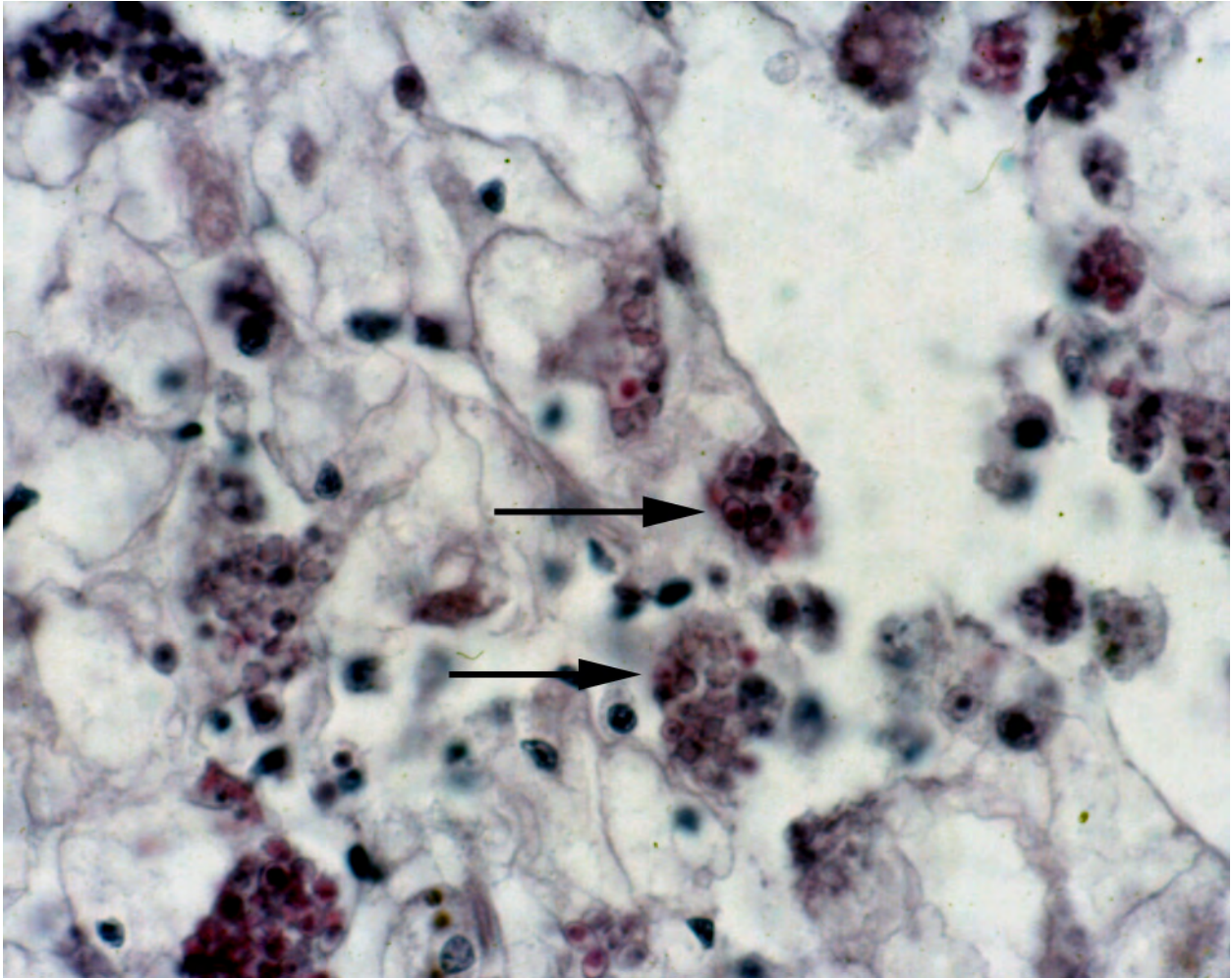


Figure 1. Sporulation of *Haplosporidium costale* in an American oyster (arrows), 1125X.

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](#)).

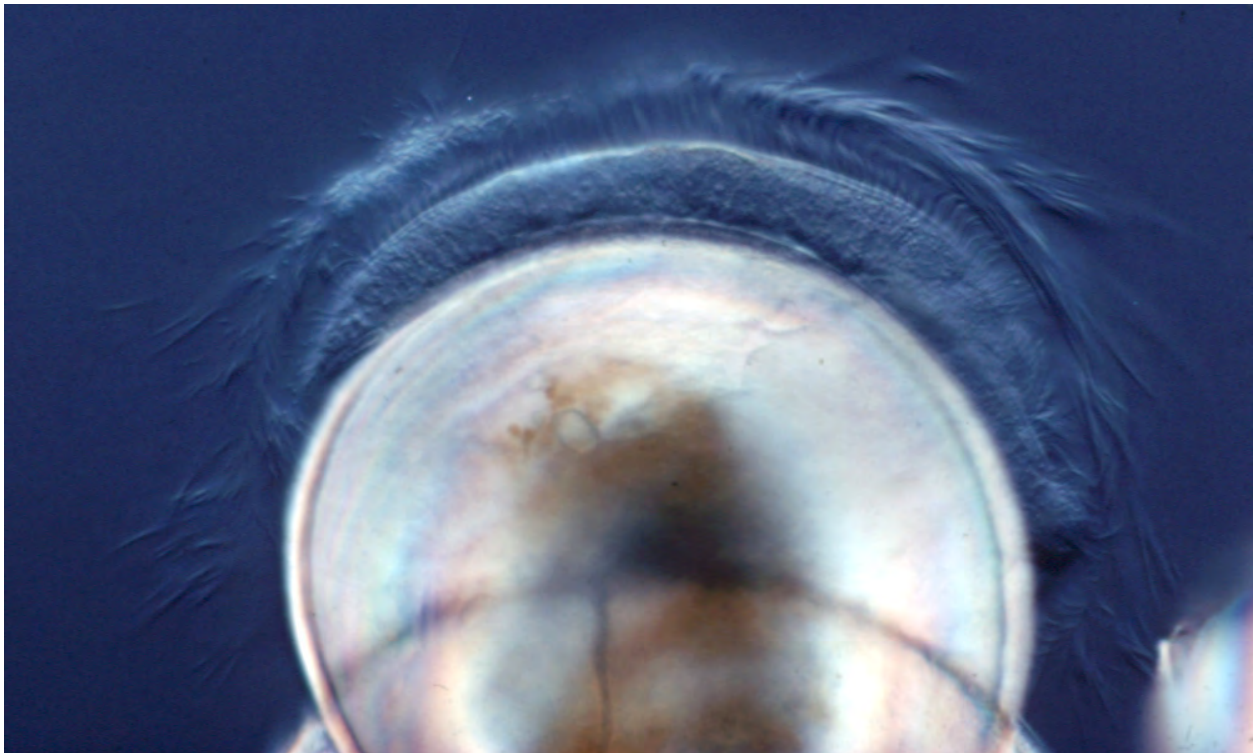


Figure 1. Wet mount of normal Pacific oyster larvae showing the extended intact ciliated velum, 383X. From Elston and Wilkinson 1985, with permission.

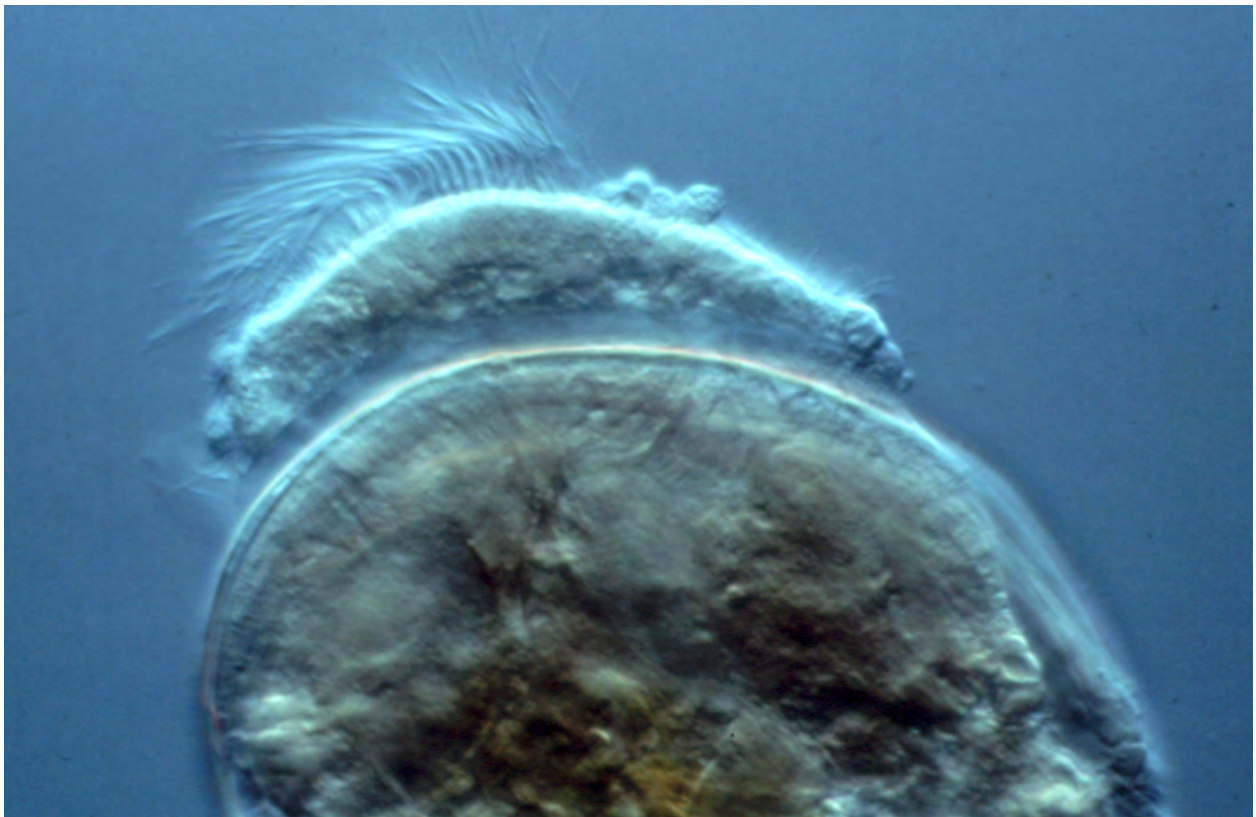


Figure 2. Wet mount of OVVD infected Pacific oyster larva showing velar degeneration including loss of cilia and loss of epithelial cells. As the cells round up and are in the process of detaching from the velum, they form the characteristic blisters.

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 and are about 228 nm in diameter.

F. Procedures for Detecting Asymptomatic Infections

No methods are reported.

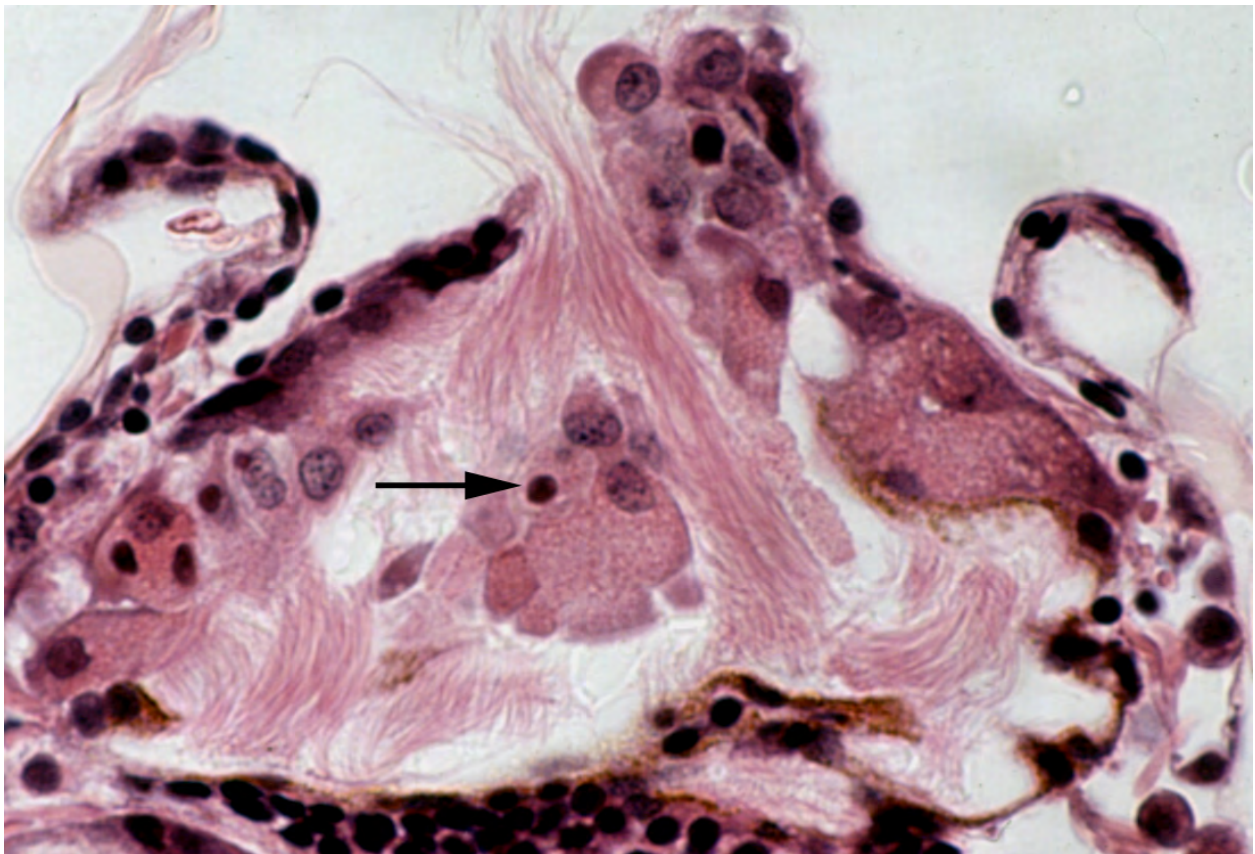


Figure 3. The intracytoplasmic inclusion body in detached velar epithelial cell (arrow) of *Crassostrea gigas* is characteristic of oyster velar viral disease, 2225X.

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5.6 Denman Island Disease

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

Denman Island disease is associated with infection by small unicellular organisms, recently named *Mikrocytos mackini*, but commonly referred to as "microcells." Although the disease has been referred to as "microcell disease," the term "microcell" in reference to the microorganism or the disease is discouraged because the term has been used to refer to other distinctly different microorganisms such as *Bonamia ostreae*.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

The disease was first reported at Henry Bay on Denman Island in British Columbia, Canada. It has subsequently been observed at other sites on Denman Island and in the Strait of Georgia in British Columbia.

2. Host Species

This disease is reported only in the Pacific oyster *Crassostrea gigas*.

C. Epizootiology

Mortality rates up to 53% in a single season have been reported, but the severity fluctuates from year to year. Infection and mortalities increased at lower tide levels (monitored at 4.0, 2.5, and 1.0 ft). In the original report of the disease, only five- to seven-years-old oysters were affected while two-year-old oysters appeared healthy. Signs of the disease can appear in April and develop through July. Mortality occurs by July and the disease is considered to have run its course by August. Some of the infected oysters will recover.

D. Disease Signs

Signs of the disease occur in five- to seven-year-old oysters (as reported in the original report) in otherwise healthy condition. Disease signs consist of the formation of yellow or yellow green pustules

on the surface of the body (as also true with other infectious diseases of the oyster). The digestive gland may be light brown in diseased oysters compared to dark green or dark brown in healthy oysters. Brown scars may occur on the white nacre layer of the shell adjacent to pustules, although pustules may occur deep within the tissues.

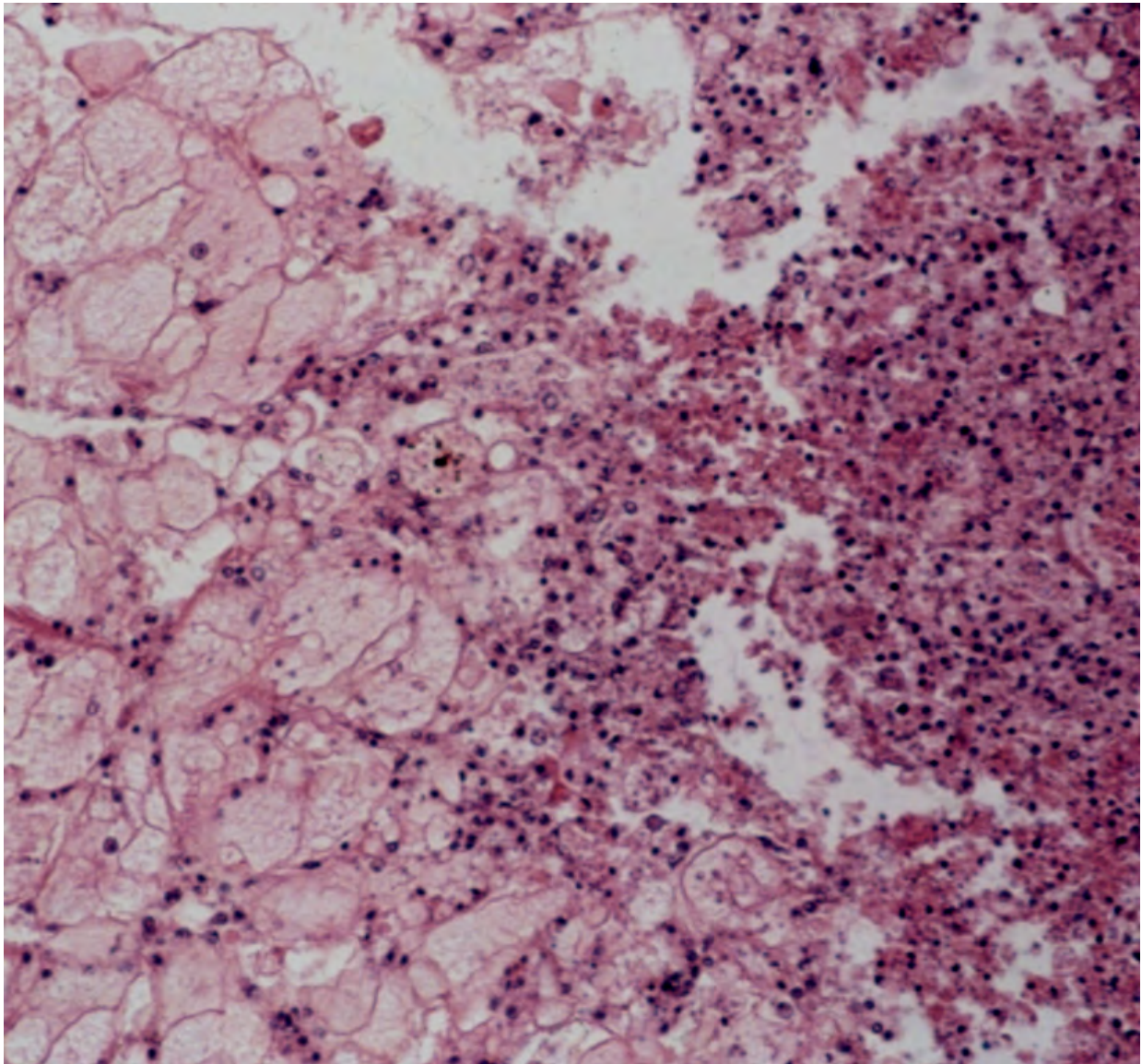


Figure 1. Histological section showing normal vesicular tissue of the Pacific oyster on the left and vesicular tissue infected with *Mikrocytos mackini* on the right, 310X.

E. Disease Diagnostic Procedures

Diagnosis is based on the characteristic histological signs. Pustules are focal infections of the vesicular connective tissue with marked hemocytic infiltration (Figure 1). Infected connective tissue cells contain spherical unicellular parasites about 2 to 3 μm in diameter with a central nucleus (Figure 2). The parasites may occasionally be observed extracellularly within the lesions. On rare occasions, the infection may occur in adductor muscle fibers. As the lesions resolve in some individuals late in the infection period, the microorganisms may be difficult to differentiate.

F. Procedures for Detecting Subclinical Infections

No methods are available.

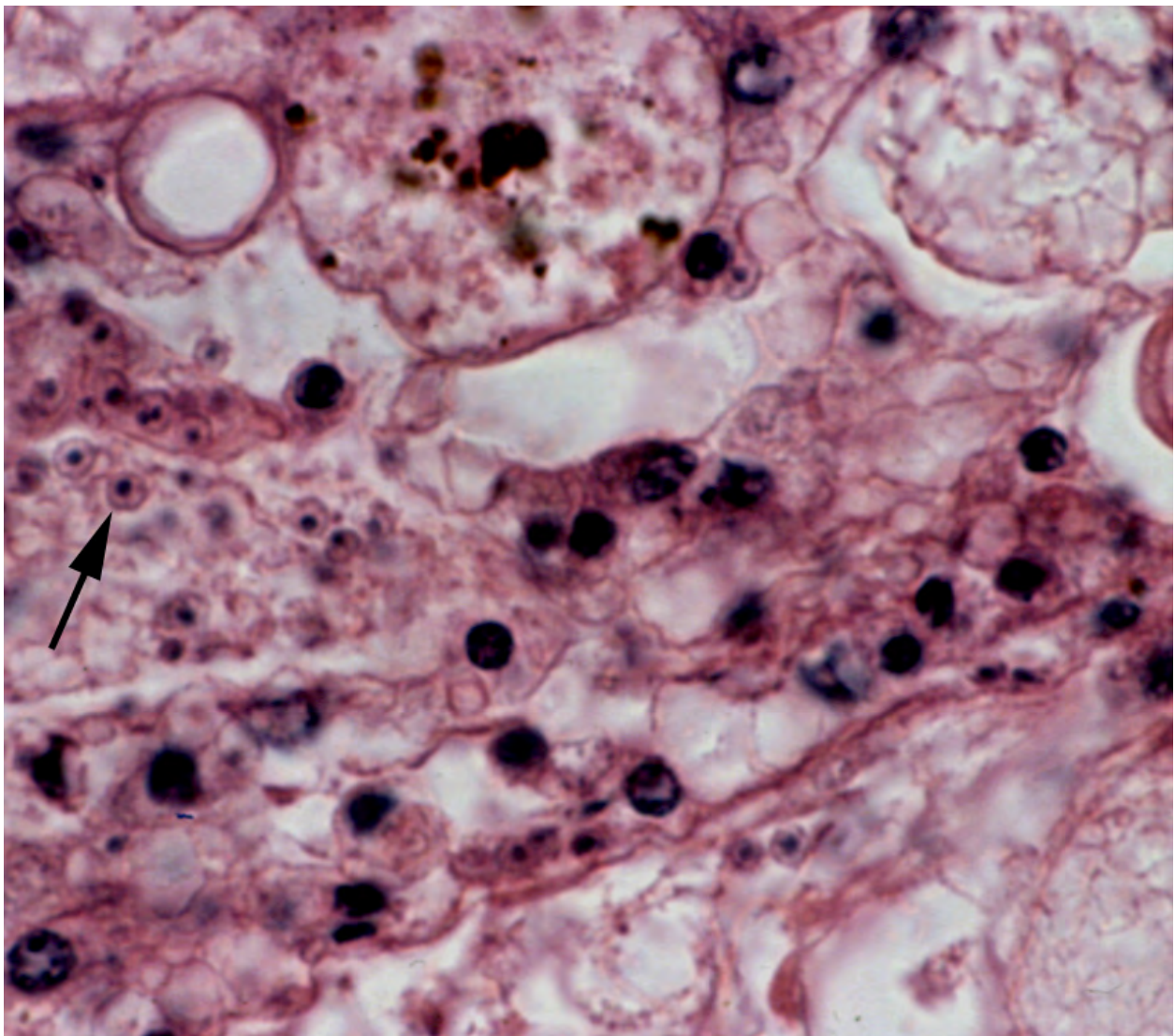


Figure 2. *Microcytos mackini* infection in the Pacific oyster showing the characteristic 2 to 3 μm organisms with central nuclei, example at arrow, 1560X.

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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.

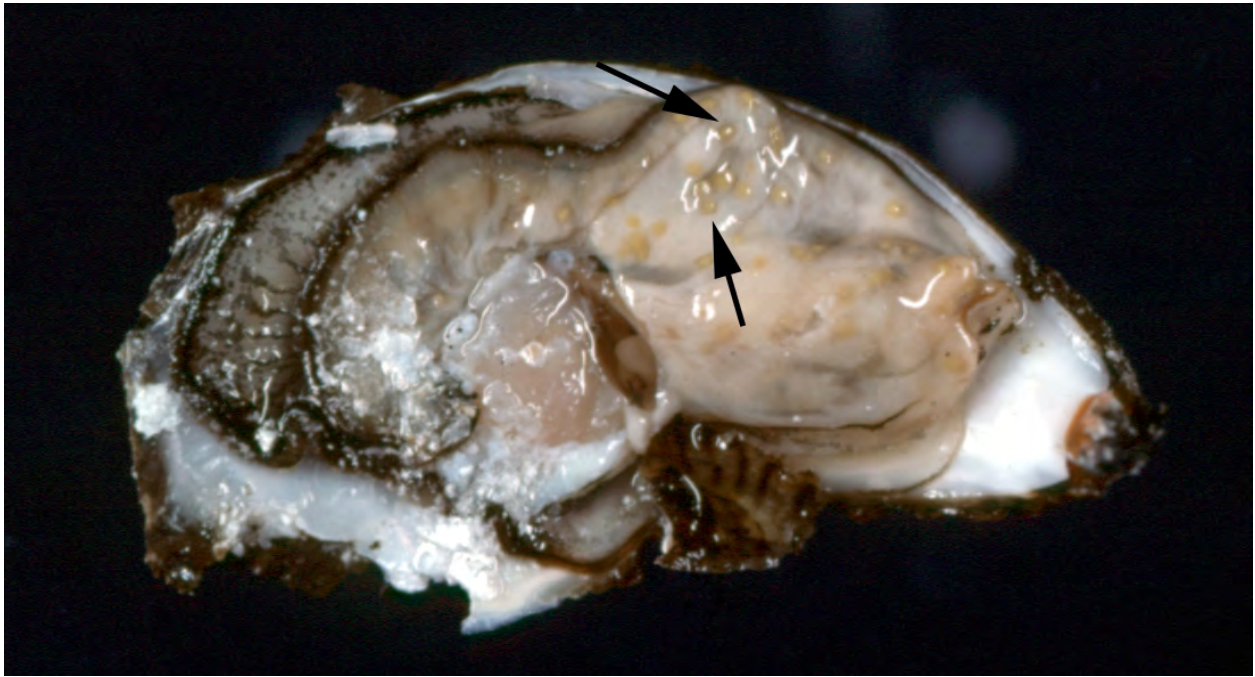


Figure 1. Pacific oyster with nocardiosis. This disease as well as others such as Denman Island disease is characterized by the formation of yellow to green pustules on the mantle and other surfaces as shown here (arrows). From Elston et al. 1987, with permission.

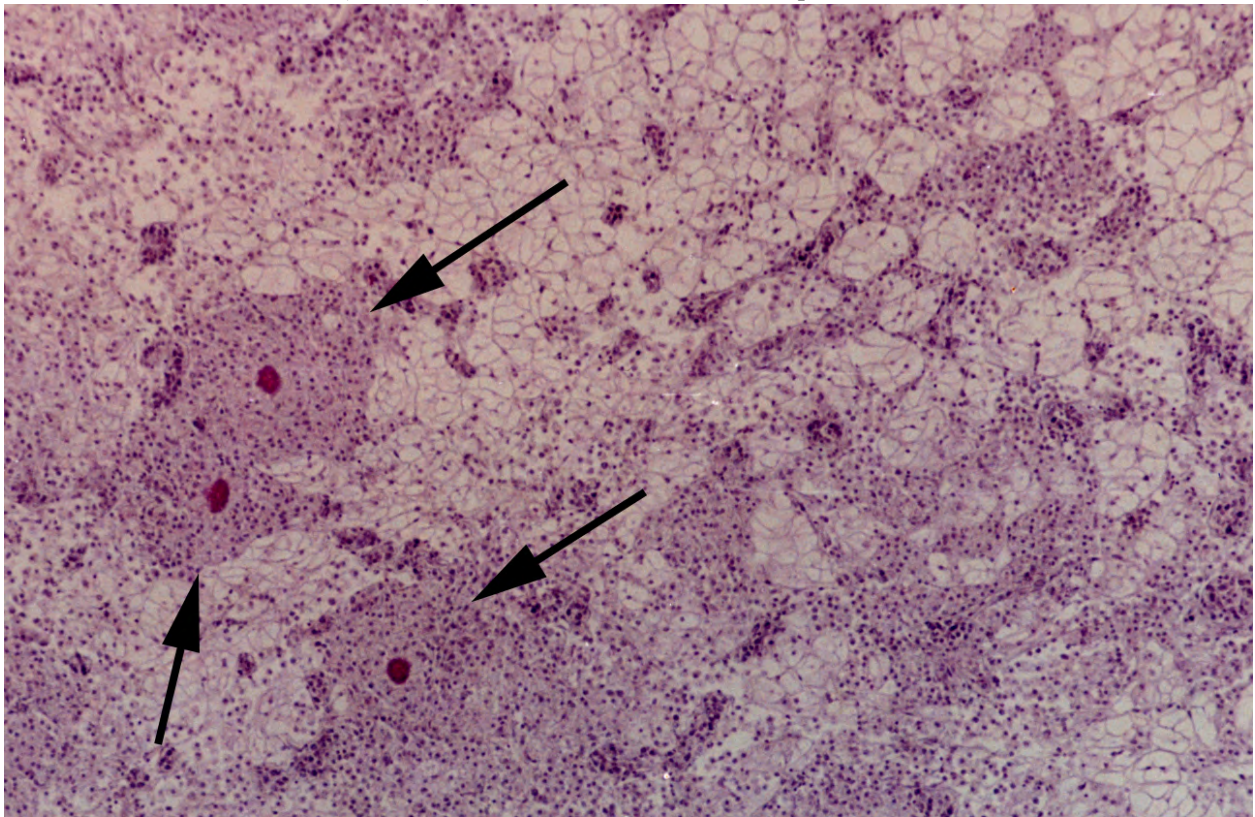


Figure 2. Low magnification of vesicular tissue of Pacific oyster with several foci of *Nocardia* sp. (arrows) surrounded by prominent zones of oyster hemocytes. The other zones of hemocyte infiltration in which the foci of bacteria are not seen in the histological section are typical of the disease, 90X.

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5.8 Bonamiasis of Ostreid 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

Bonamiasis is caused by *Bonamia ostreae* and other similar *Bonamia* sp. provisionally assigned to the Ascetospora. Microcell disease has been used synonymously but this non-specific term is discouraged since it has also been used to refer to other diseases.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

a. *Bonamia ostreae*

Found in Europe: Atlantic and Mediterranean France and Spain, The Netherlands, England, Ireland, and Denmark. Also found in North America: California and Washington.

b. *Bonamia* sp.

Found in New Zealand.

2. Host Species

Bonamia ostreae can cause epizootic disease in the European flat oyster *Ostrea edulis* and has been shown to experimentally infect the Chilean oyster *Ostrea chilensis* and the New Zealand dredge oyster *Tiostrea lutaria*. A similar but distinctive *Bonamia* sp. also infects *Tiostrea lutaria*.

C. Epizootiology

Mortality rates approaching 100% within one year were reported in newly infected populations in Europe. Mortality rates tend to decline the longer a population has been infected and are typically reported in the 20 to 60% range after the disease has been in a population for two or more years. In populations with a long history of the disease, mortality tends to be highest in younger oysters with the annual mortality rate declining as the oysters age. For example, studies in Washington state showed a 20%, 7%, and 4% mortality rate, respectively, for two-, three-, and four-year-old infected oysters. The disease can appear throughout the year but is generally associated with warm spring and summer temperatures. Bonamiasis can cause significant mortalities between 12 and 20°C but not at higher temperatures.

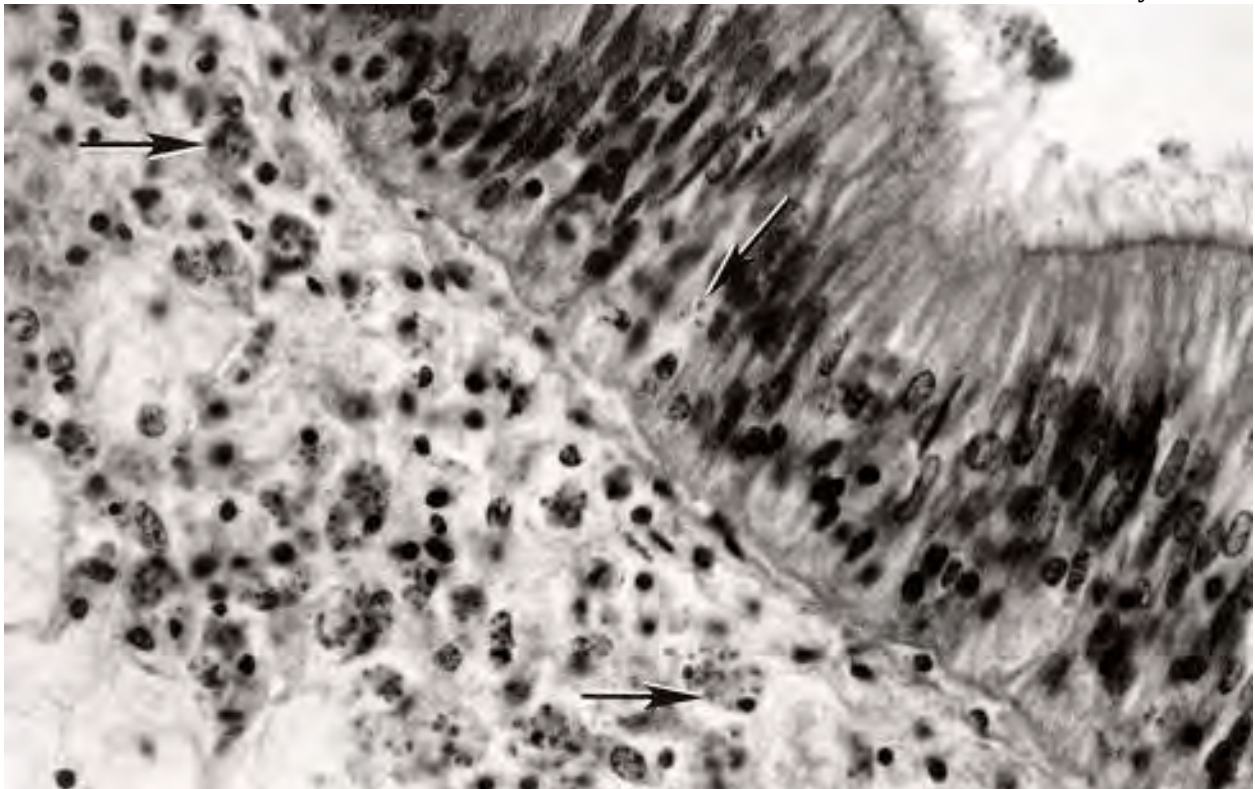


Figure 1. Histological section of *Ostrea edulis* heavily infected with *Bonamia ostreae*. Hemocytes infected with multiple organisms are shown at the arrows in the vascular sinus underlying the stomach epithelium. Organisms in hemocytes between epithelial cells are also evident (arrow), 480X.

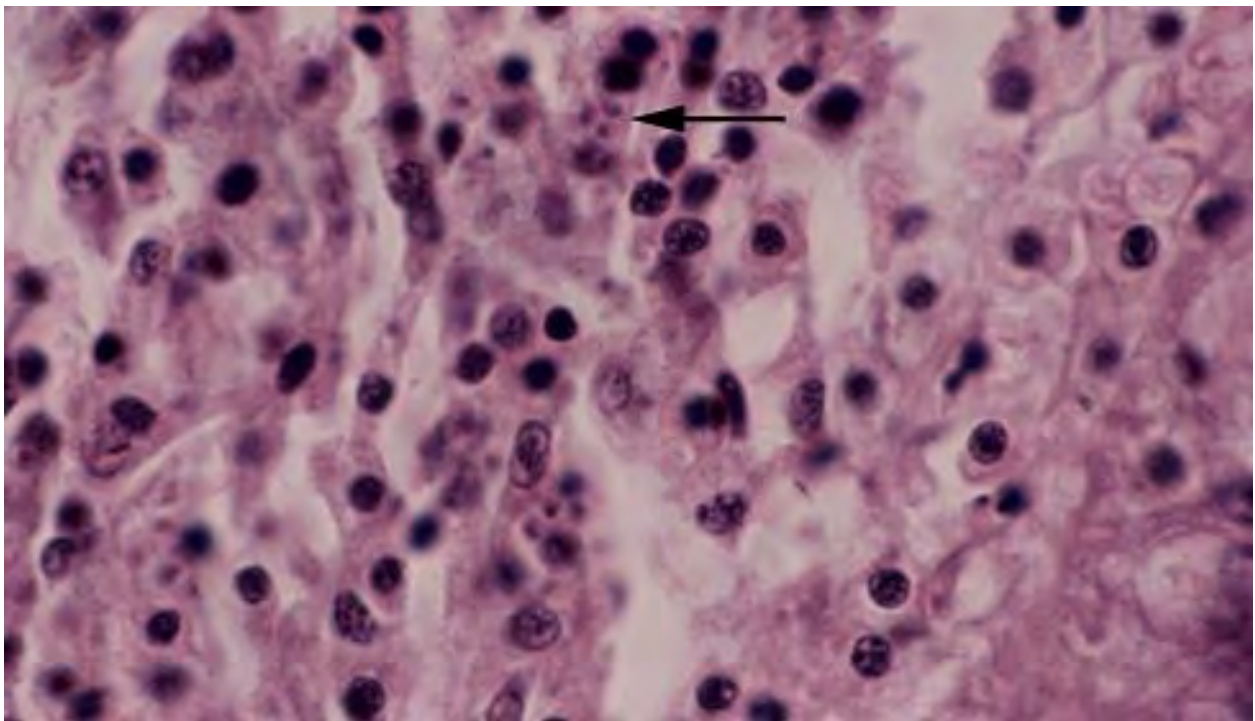


Figure 2. A relatively light infection of *Bonamia ostreae* in the European flat oyster. Arrow indicates a hemocyte containing the 2 to 3 μm organisms with eccentric nuclei, 1200X.

D. Disease Signs

1. Weak shell closure and gaping are non-specific signs.
2. Oysters are frequently secondarily infected with opportunistic bacteria. Some oysters with bonamiasis may die with relatively light infections while others are at an advanced stage of infection at death.

E. Disease Diagnostic Procedures

1. Confirmatory Diagnosis

- a. Make acetone- (or methanol-) fixed impression smears from heart tissue (use of the ventricle is recommended since the auricles contain an abundance of serous cells that make identification of the parasite difficult) and stain with Wright, Wright-Giemsa, or equivalent stain (e.g. Hemacolor, Merck). Examine for 2 to 5 μm ovoid to spherical organisms with eccentric (*Bonamia ostreae*) or central (*Bonamia* sp.) nucleoplasm within or outside of hemocytes. *Bonamia ostreae* is characterized by a prominent eccentric vacuole. The organisms are larger in fixed smears than they are in fresh or histological preparations.

Less sensitive diagnosis can be made histologically by identifying the 2 to 3 μm *B.ostreae* within hemocytes in tissue sections (Figure 1). Organisms are distributed systemically in advanced infections but accumulations are often found in branchial tissue in early infections. Infected blood cells accumulate in the vascular sinuses around the stomach and intestine (Figure 2).

- b. Reactivity with monoclonal antibodies specific for epitopes found on *Bonamia ostreae* or *Bonamia* sp. Antibodies can be used in the ELISA microtiter system evaluating whole blood preparations, in indirect FA on impression smears, or frozen tissue sections. A kit for performing the ELISA technique is manufactured by SANOFI pharmaceutical company in France. Contact the company's U. S. representative in New York, NY for information on its availability in North America. At the time of publication, the ELISA kit had not been tested in North America and testing was not complete in Europe.

F. Procedures for Detecting Subclinical Infections

ELISA with monoclonal antibodies on whole blood.

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5.8 Bonamiasis of Ostreid Oysters - 4

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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 Sarcodistophora 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°C and lower but not at 12°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 of *Ostrea edulis*

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

Marteiliasis of the European flat oyster is caused by *Marteilia refringens* (Ascetospora). The condition is also known as Aber disease and "maladie de la glande digestive."

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

The disease has been reported from virtually all beds of susceptible oysters in Spain and along the Atlantic coast of France beginning with the 1967 epizootic in Brittany, France. The parasite has been reported in French oysters moved to The Netherlands but not in Dutch oyster stocks.

2. Host Species

The disease occurs only in the European flat oyster *Ostrea edulis*. One report describes early stages in the digestive epithelium of the Pacific oyster *Crassostrea gigas*, but these oysters do not develop any evidence of the disease even when cultured in disease enzootic areas.

C. Epizootiology

Mortality rates of 90% annually were reported in the first epizootics of marteiliasis in France. When disease-free spat or two- and three-year-old oysters were planted in infected areas in March, they became infected between the first of May and the end of August. Severe mortalities occurred before the end of the first winter, but the parasite could not be found in the surviving oysters. The fact that the parasite occurs in oysters in some areas without causing disease suggests that environmental factors or oyster stock differences are important in determining whether or not the disease becomes a significant problem. In addition, mortality seems to be related to the formation of the spore stages (known as sporulation) of the parasite within the oyster's tissues. The sporulation process may result in the release of toxic substances that affect the oyster.

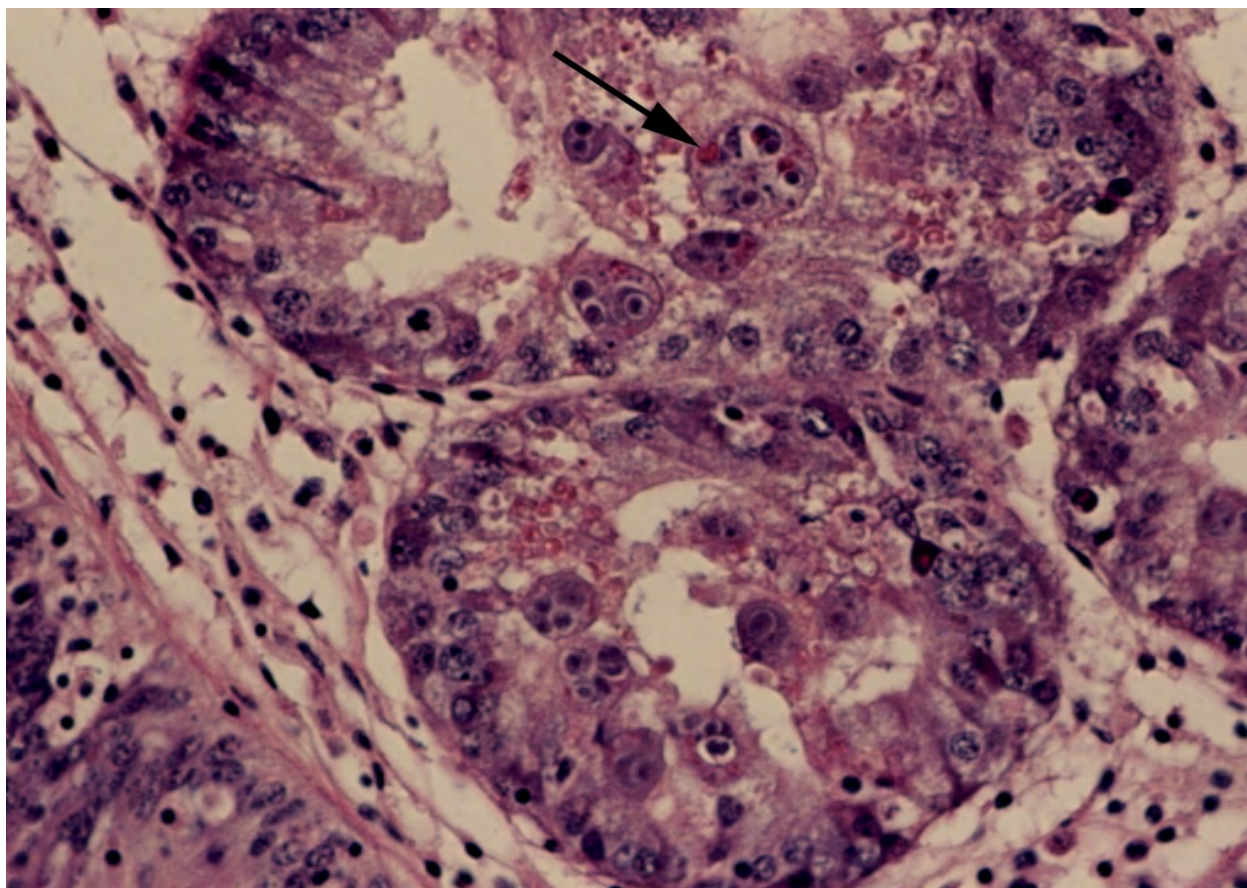


Figure 1. Digestive tubules of the European flat oyster infected with *Marteilia refringens*. Several sporangiosori are evident within the tubular epithelial cells. The characteristic birefringent material resulting from sporulation is shown at the arrow, 600X.

D. Disease Signs

Heavily infected oysters appear normal, but death may result when the parasite undergoes sporulation.

E. Disease Diagnostic Procedures

Diagnosis is based entirely on histological and ultrastructural examination of tissues. Early stages of the parasite appear in the stomach epithelium. Vegetative stages are also found in connective tissue near digestive organs, between epithelial cells of these organs, and in the lumina of the organs. Sporulation occurs in the epithelial cells of the digestive tubules. Endogenous budding forms a presporangium and is followed by the formation of a sporangiosorus (up to 30 μm in diameter) that contains up to eight sporangia (Figure 1). Haplosporosomes are formed in the peripheral cytoplasm and, as spores are formed, the cytoplasm outside of the spore wall condenses into birefringent inclusion bodies visible by light microscopy. The spores are about 2.6 to 3.5 μm in diameter.

F. Procedures for Detecting Subclinical Infections

Observation of parasite stages by histology or electron microscopy as described above.

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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 ostracoblabiliasis. 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°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.

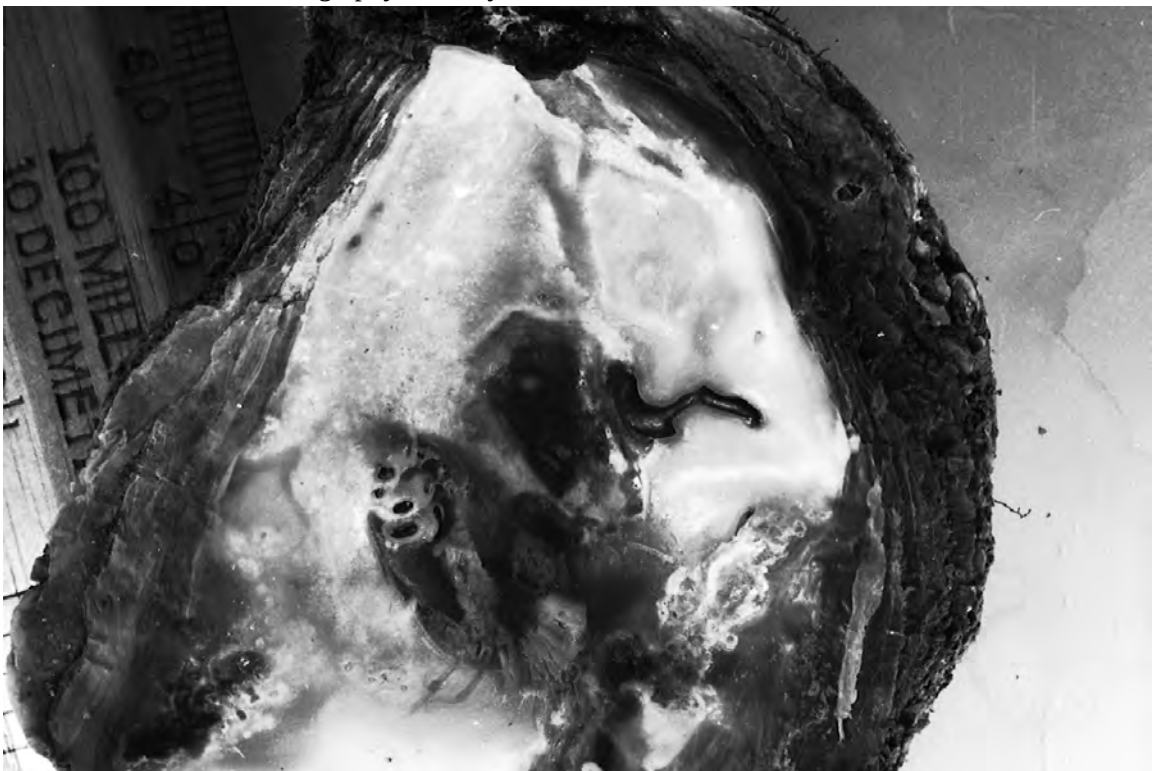


Figure 2. Shell of *Ostrea edulis* with "warts" or dark protrusions on the inner shell surface. Photograph courtesy of David J. Alderman.

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5.12 Hematopoietic Neoplasm of Bivalve Molluscs

Ralph Elston

Battelle Marine Sciences Laboratory
439 W Sequim Bay Rd
Sequim, WA 98382
206/683-4151
aquatech@olympen.com

A. Name of Disease and Etiological Agent

This disease is known as hemic, hematopoietic, or hemocytic neoplasia (HCN). Also referred to as disseminated sarcoma, hemic proliferative disease, leukocytic neoplasia, 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. Thus, there may actually be a complex of diseases with morphological similarities that arise from different tissues and possibly arise from different etiological agents.

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. Common etiology or cross-species transmissibility is not established. 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 and the Brittany region of France
4. *Saccostrea commercialis* (Australia rock oyster): Australia
5. *Crassostrea gigas* (Japanese or Pacific oyster): Matsushima Bay, Japan and Breton, France
6. *Crassostrea rhizophorae*: Brazil
7. *Crassostrea virginica* (Eastern or American oyster): discontinuously on the Atlantic and Gulf coasts of North America

5.12 Hematopoietic Neoplasm of Bivalve Molluscs - 2

8. *Macoma balthica* (duck clam): Chesapeake Bay, eastern North America and Finland
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. *Ostrea chilensis* (Chilean oyster): Chiloe, Chile
16. *Ostrea lurida* (*Ostreola conchaphila*), (Olympia oyster): Yaquina Bay, Oregon
17. *Ostrea edulis* (European flat oyster): Mali-Ston area of Yugoslavia near Dubrovnic; Ria de Noya, Galicia, Spain; Mediterranean and Brittany region of France

C. Epizootiology

Some cultured populations appear to be 100% infected if individual animals are monitored over several months. Mortality rates are reported to approach 100% over an annual period in some species. In other cases, in cultured populations, annual mortality rates of 30 to 50% are typical. Specific environmental factors that induce or enhance the disease are not known. 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 high disease levels because of facility of transmission from one animal to another. In all species in which seasonality has been investigated, the disease is reported to have highest prevalences (percentage of infected individual shellfish) during fall and winter months, typically from October through March. The prevalence drops in the spring and summer period, possibly because heavily infected individuals die in the winter and the disease does not start another cycle of increasing infection until autumn.

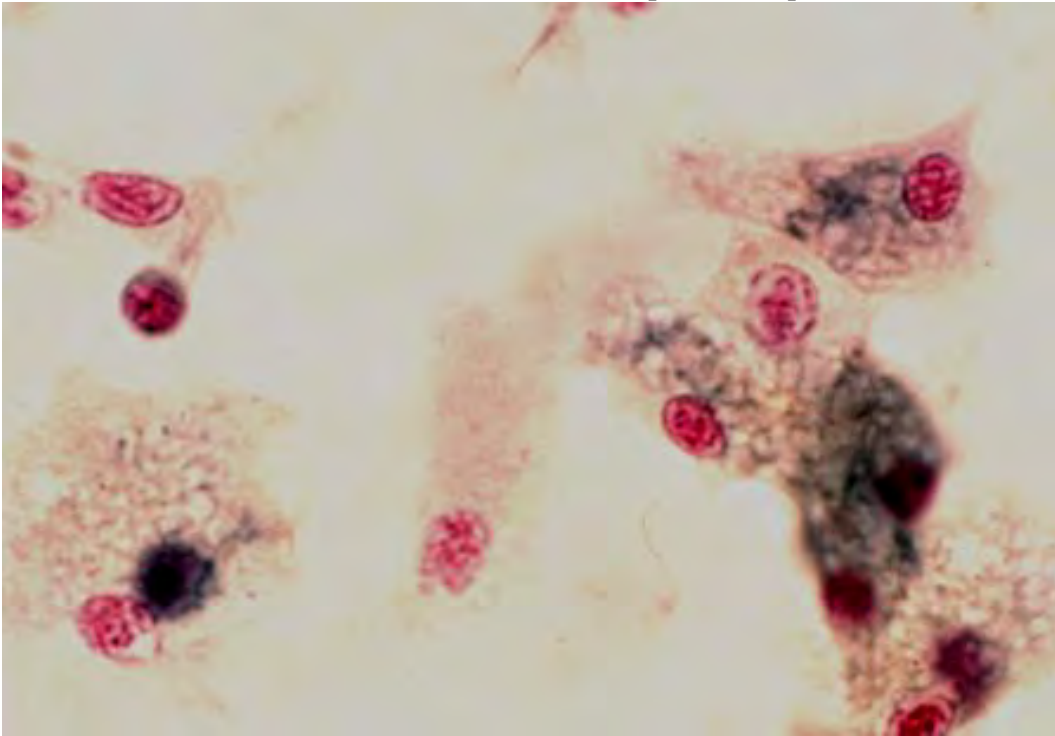


Figure 1. Hemocytological preparation of normal blood cells from *Mytilus*.

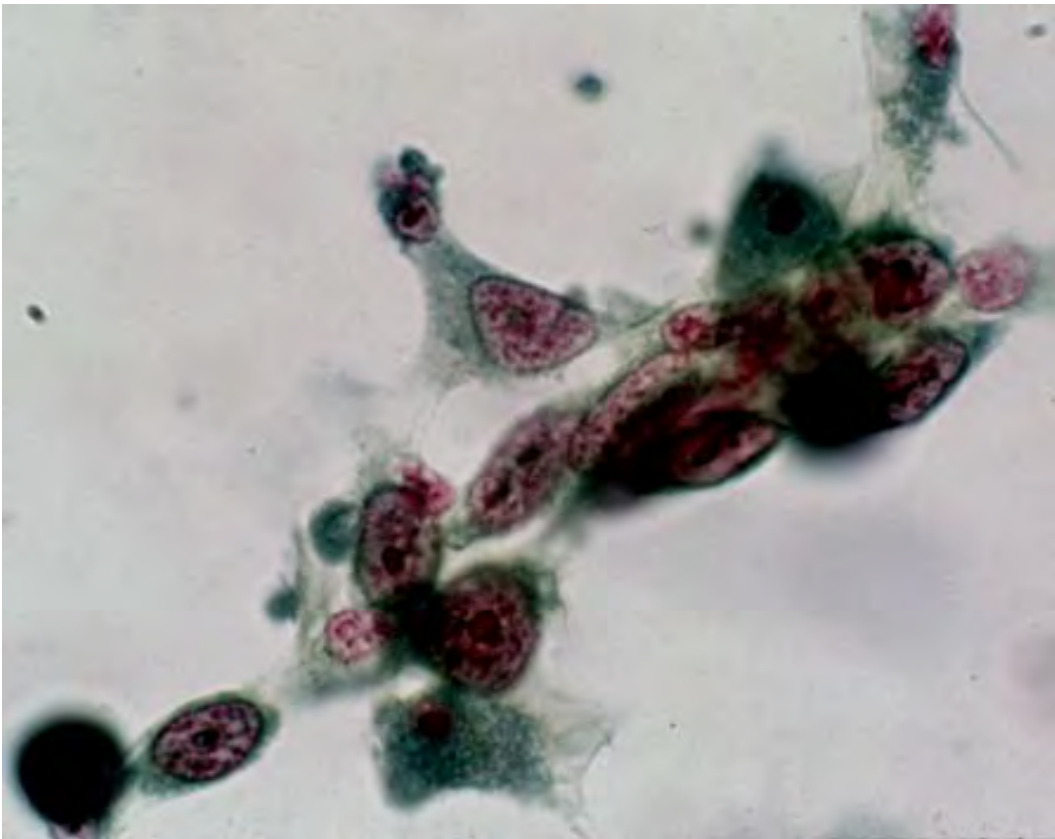


Figure 2. Hemocytological preparation of affected blood cells of *Mytilus* with hemic neoplasia. These cells are characterized by enlarged pleomorphic nuclei with prominent nucleoli, but the cells are still attached and well spread out on the glass.

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 stage individuals resulting from the massive systemic proliferation of transformed cells.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

Presence of large (typically 6 to 8 μm , but often up to 10 μm diameter) blood cells which fail to spread on a glass slide as observed live in wet mount preparations. Normal hemocytes will rapidly attach and spread out on a glass substrate (Figure 1).

2. Confirmatory Diagnosis

Observation of transformed hemocytes (Figure 2 and 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 (Figure 3) will fail to spread on glass microscope slides in advanced stages. The relative proportion of abnormal to normal 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 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 (Figure 4 and Figure 5) 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 the advanced disease, the vascular spaces of the entire animal are filled and tightly packed with the abnormal cells.

F. Procedures for Detecting Subclinical Infections

Use of hemocytological or histological examination as above.

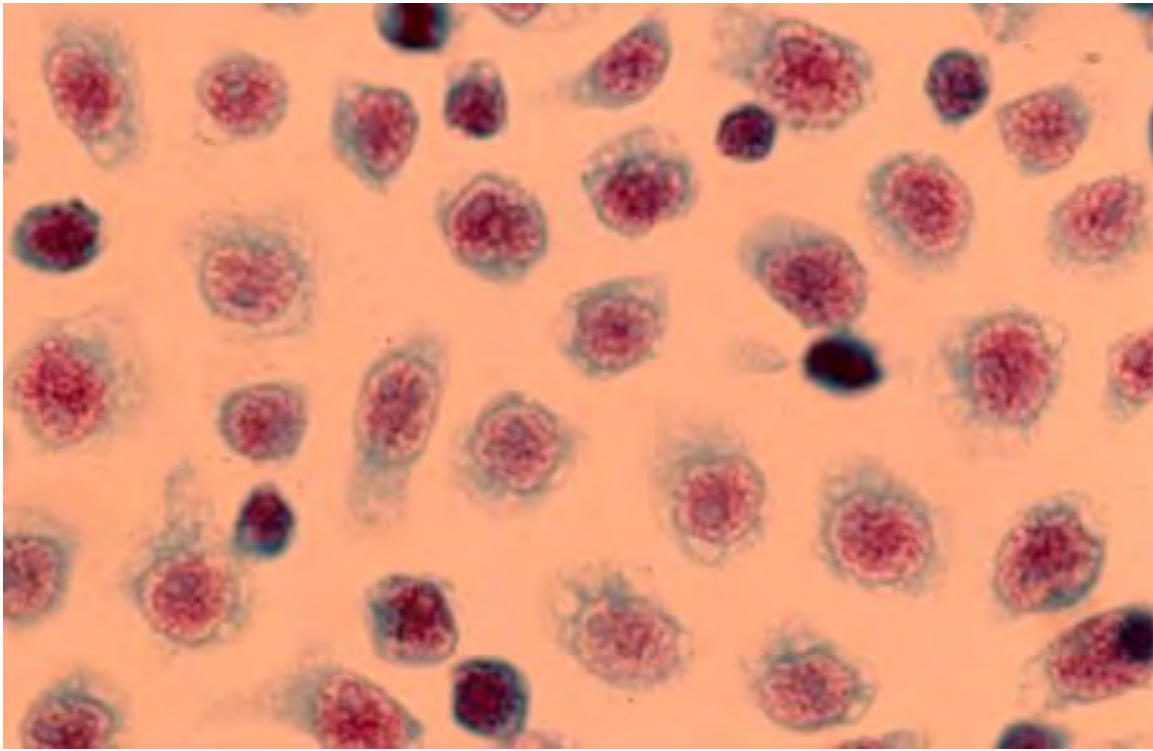


Figure 3. Hemocytological preparation of affected blood cells of *Mytilus* with hemic neoplasia. This mussel was in the terminal stages of the disease. The blood contains predominantly neoplastic blood cells that poorly attach or spread on the glass substrate. Blood cells exhibit a high nucleus to cytoplasm ratio and prominent nucleoli, 1860X.

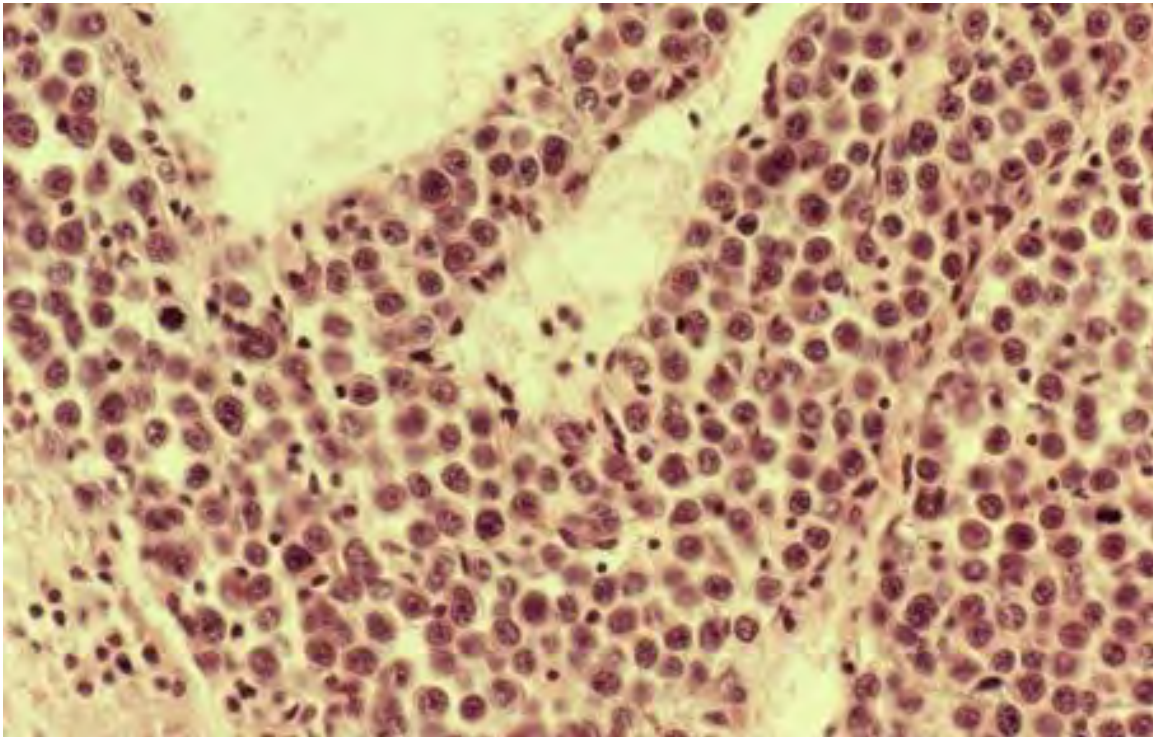


Figure 4. Histological section of *Mytilus* with hemic neoplasia showing the massive proliferation of neoplastic cells in the vascular spaces, 400X.

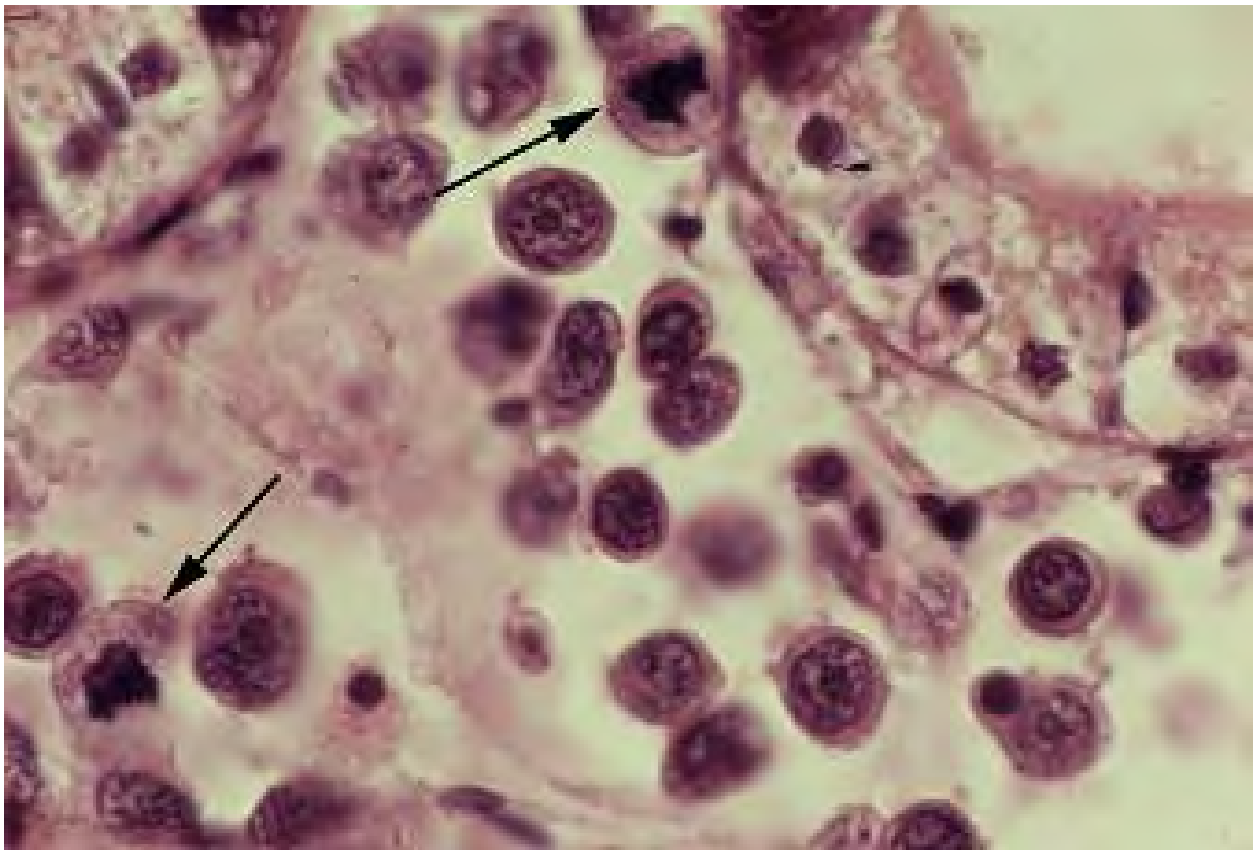


Figure 5. Histological section of *Mytilus* with hemic neoplasia showing typical enlarged hemocytes with large nuclei and scant cytoplasm. Two such cells in mitosis are shown at the arrows, 1200X.

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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.

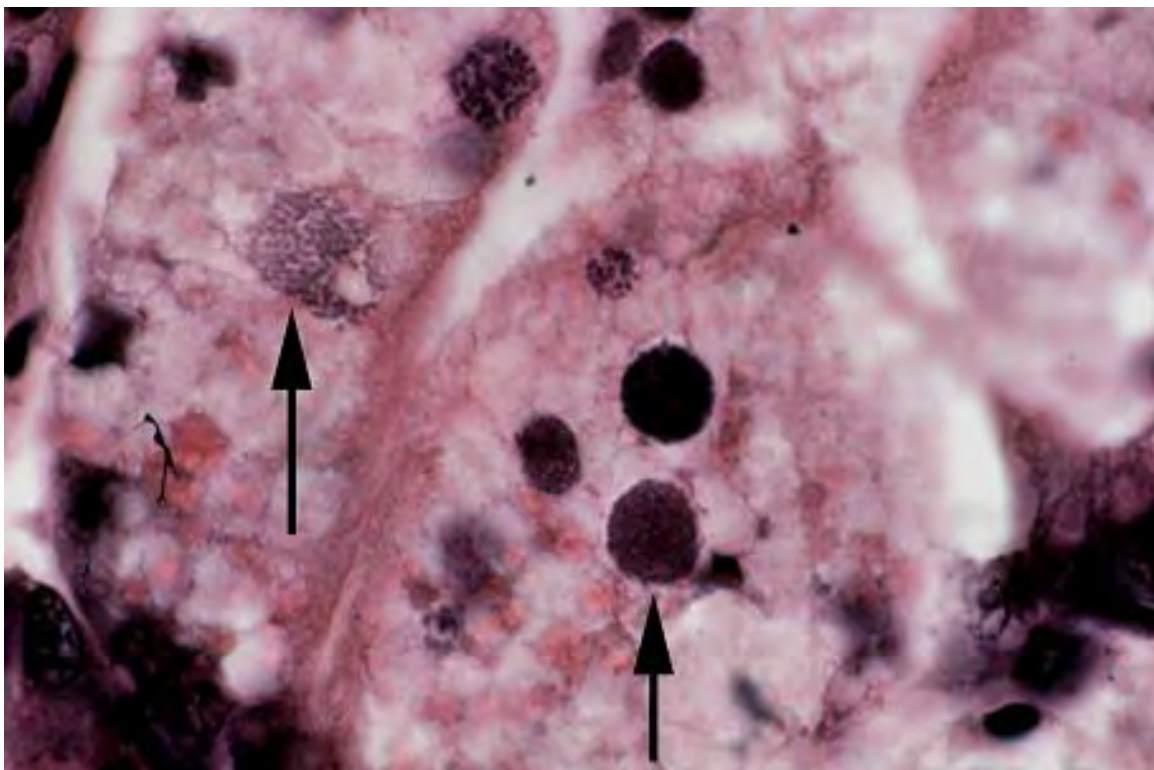


Figure 2. Rickettsia-like organisms in the digestive tubular epithelium of the Pacific razor clam *Siliqua patula* (examples at arrows), 880X.

B. Malpeque Bay Disease

This is a widely known but poorly understood disease that caused severe mortalities in American oysters *Crassostrea virginica* 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](#)).

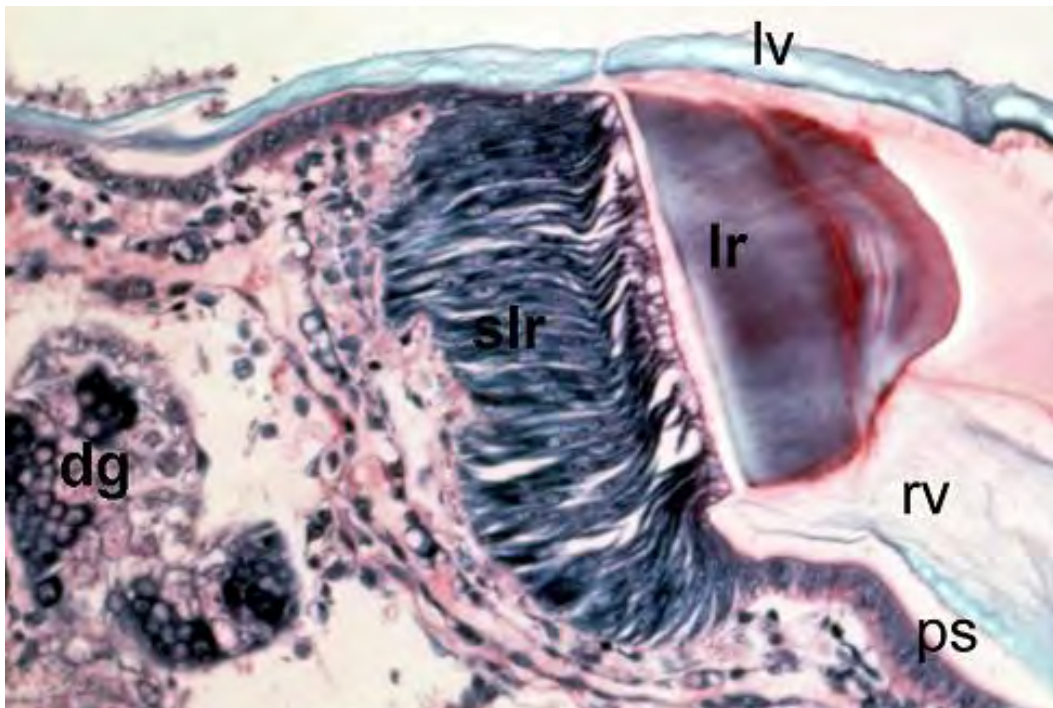


Figure 3. Normal hinge ligament of oyster showing the intact resilium (lr) and other adjacent organs (dg, digestive gland; slr, sub-ligamental ridge; lv, rv, left and right valves; ps, pallial space), 160X From Dungan and Elston 1988, with permission.

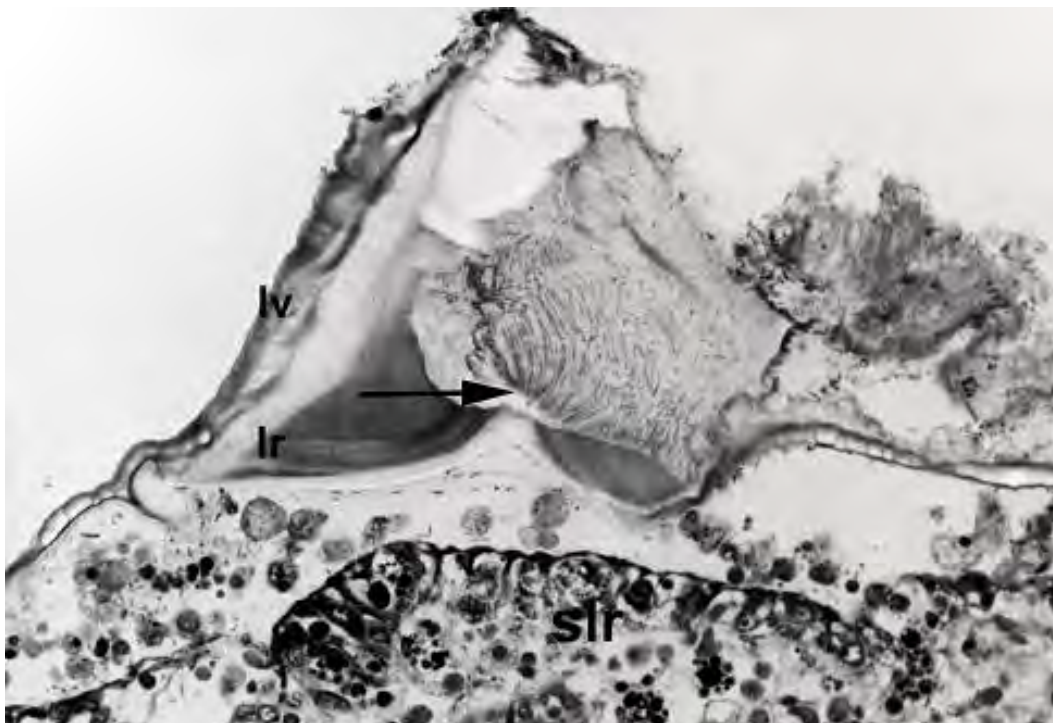


Figure 4. Hinge ligament eroded with cytophaga-like bacteria. The bacteria appear as fibers oriented at right angles to the eroding resilium margin (arrow). The sub-ligamental ridge appears necrotic and is separated from the internal resilium surface by a wide pallial space containing bacteria and rounded host cells. Organ identifiers and magnification same as Figure 3. From Dungan and Elston 1988, with permission.

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5.13 Miscellaneous Diseases of Molluscs - 6

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5.14 *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 *Homarus 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 μ m 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 minimum standard protocols to be followed when carrying out health inspections on aquatic animals. These inspections may be used for intrastate, interstate, or international movement of animals. Using a standard set of procedures and protocols allows fisheries managers to better compare data and make better management decisions. However, the final decision to require the use of these protocols remains in the hands of those regulating bodies requesting/requiring health inspections. This handbook is in no way meant to provide, dictate, or supplant aquatic animal health policies of any entity and is solely intended to be a procedural handbook.

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 have been taken from numerous sources, including the United States Fish and Wildlife Service's National Wild Fish Health Survey Manual, the 4th edition of the American Fisheries Society Blue Book, the 3rd edition of the Office International des Epizooties (OIE) Diagnostic Manual for Aquatic Animal Diseases, 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 have the potential to produce severe epizootics of clinical disease but are also known to exist in a carrier state. They are pathogens of regulatory concern, for which there are both screening and confirmatory tests available. The

methodologies described herein are effective for the detection and identification of each pathogen in the absence of clinical signs.

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 known 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 Diagnostic Manual for Aquatic Animal Diseases 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

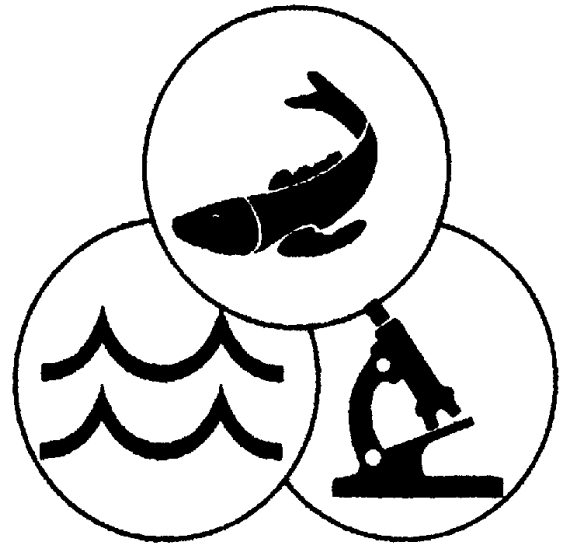
It should be noted that as with any inspection manual or handbook, the techniques employed provide only a snapshot in time. There is no guarantee that any inspected animal or population of animals is disease free. The only statement that can be made is that at the time the animals were sampled the disease 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. These guidelines represent the **minimum acceptable standard** and may be superseded by applicable State, Tribal, interjurisdictional, and foreign aquatic animal health protection guidelines, policies, and /or regulations.

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 is for four bacteria (*Aeromonas salmonicida*, *Yersinia ruckeri*, *Edwardsiella ictaluri*, and *Renibacterium salmonarium*), eight viruses (Infectious Hematopoietic Necrosis Virus, Infectious Pancreatic Necrosis Virus, Infectious Salmon Anemia Virus, Largemouth Bass Virus, *Oncorhynchus masou* Virus, Spring Viremia of Carp Virus, Viral Hemorrhagic Septicemia Virus, and White Sturgeon Herpesvirus), and four parasites (*Myxobolus cerebralis*, *Ceratomyxa shasta*, *Tetracapsula bryosalmonae*, and *Bothriocephalus acheilognathi*). A brief description of clinical signs of the diseases caused by each of these pathogens is included in this handbook. However, references such as the AFS/FHS Blue Book (Thoesen 1994) 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.

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 protocol if operation and performance are deemed comparable to the items specified.

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. 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
Bacterial Pathogens						
<i>Aeromonas salmonicida</i>	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
<i>Yersinia ruckeri</i>	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
<i>Edwardsiella ictaluri</i>	Enteric Septicemia of Catfish (ESC)	Ictalurids	Kidney	Bacterial culture of kidney on TSA or BHIA media	FAT	
<i>Renibacterium salmoninarum</i>	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	
<i>Piscirickettsia salmonis</i>		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
<i>Oncorhynchus masou</i> 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.**2.2 Sampling - 4**

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 14 days at 20 to 25° C. Followed by a 14-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						
<i>Myxobolus cerebralis</i>	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.
<i>Ceratomyxa shasta</i>	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.
<i>Tetracapsula bryosalmonae</i>	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.
<i>Bothriocephalus acheilognathi</i>	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%.

<u>Lot Size</u> (number of fish)	<u>Number of Fish Required for Sample</u>		
	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](#)).

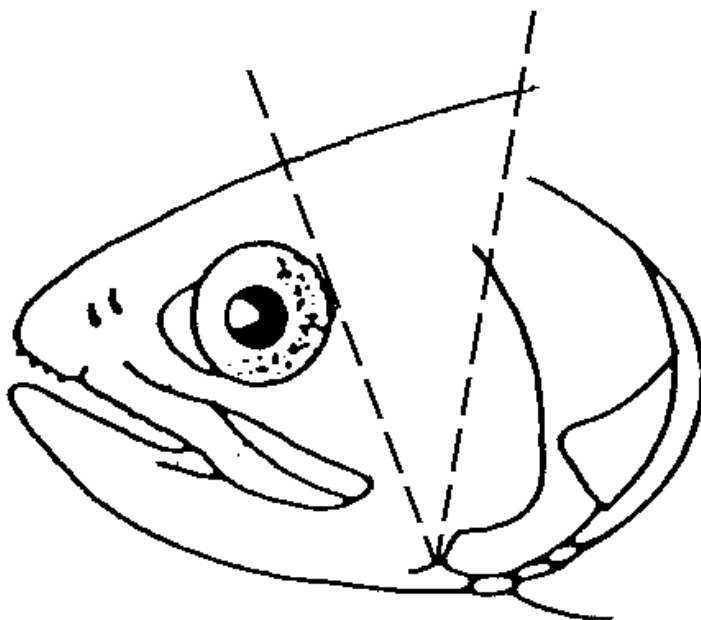


Figure 2.1. Diagram of location of wedge sample from adult fish.

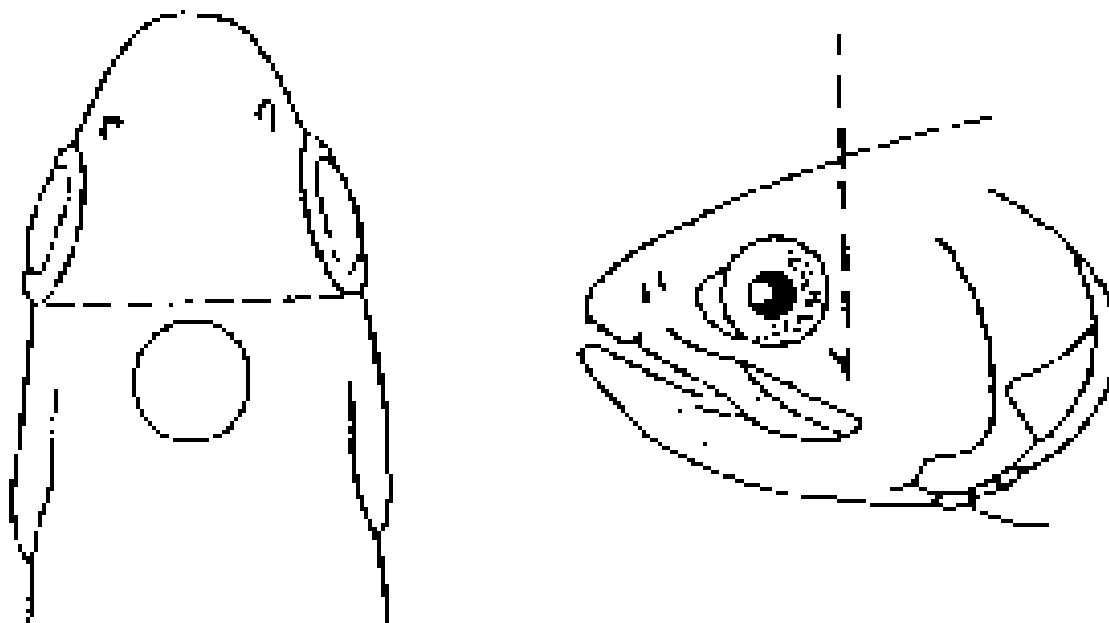


Figure 2.2. Diagram of location of core sample from adult fish.

- 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. bryosalmonae* fish are sampled individually and tissues are not pooled for examination. However, impressions of more than one fish can be made on a single microscope slide.
- b. Impressions of kidney tissue of each fish are made by excising a small (5mm²) 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 mm²) 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 ₂ O)
1.0 mL D-Cycloserine (0.3 g D-Cycloserine in 24 mL of dH ₂ O)
2.0 mL Polymyxin B-sulfate (0.3 g Polymyxin B-sulfate in 24 mL of distilled H ₂ O)
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.

2.3 Reagents, Media, and Media Preparation - 2

- 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 $\frac{3}{4}$ 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 thereafter 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.

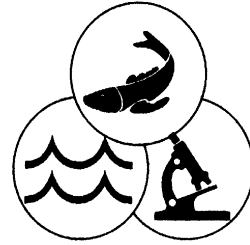
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Section 2

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)
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- 3.8 Bacterial Identification Techniques
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- 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 1985; Bullock 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*.**
 - 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 *salmonicida* – 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”](#))

3.3 *Yersinia ruckeri* (Enteric Redmouth Disease, ERM) - 2

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*.

3.3 *Yersinia ruckeri* (Enteric Redmouth Disease, ERM) - 3

- 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*.**

3.4 *Edwardsiella ictaluri* (Enteric Septicemia of Catfish, ESC) - 2

- 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. Mannitol 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”](#))

3.4 *Edwardsiella ictaluri* (Enteric Septicemia of Catfish, ESC) - 3

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 1987; 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.

3.5 *Renibacterium salmoninarum* (Bacterial Kidney Disease, BKD) - 2

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 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°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.

3.5 *Renibacterium salmoninarum* (Bacterial Kidney Disease, BKD) - 4

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 1 minute 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°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.

3.5 *Renibacterium salmoninarum* (Bacterial Kidney Disease, BKD) - 5

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 μ L 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 μ L 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 μ L 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 μ L).

3.5 *Renibacterium salmoninarum* (Bacterial Kidney Disease, BKD) - 6

- 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.**

3.5 *Renibacterium salmoninarum* (Bacterial Kidney Disease, BKD) - 7

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 a carrier state of infection. 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.

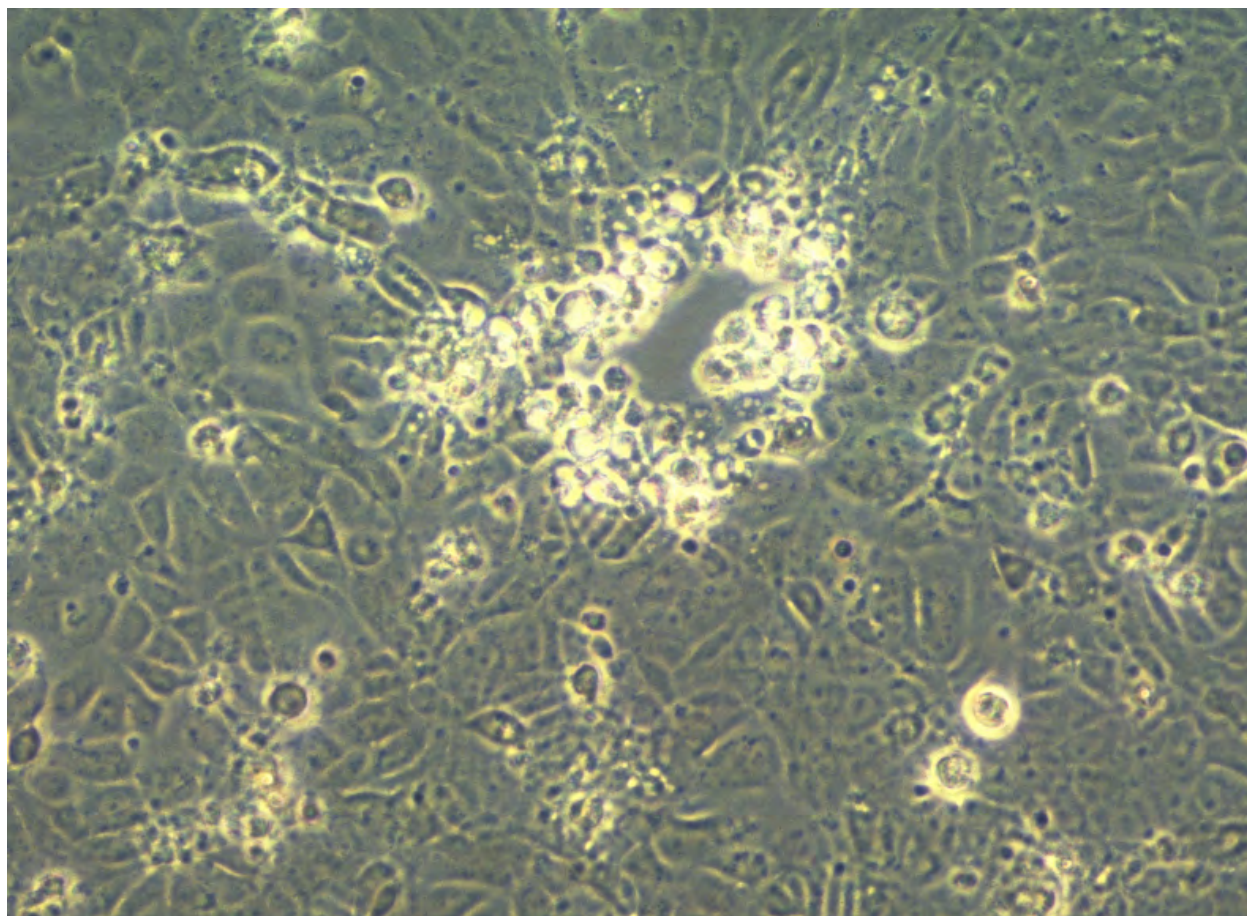


Figure 3.1. Cytopathic effect typical of *Piscirickettsia salmonis* on a monolayer of CHSE-214 cells. (Photo courtesy of Marcia House, NWIFC.)

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.

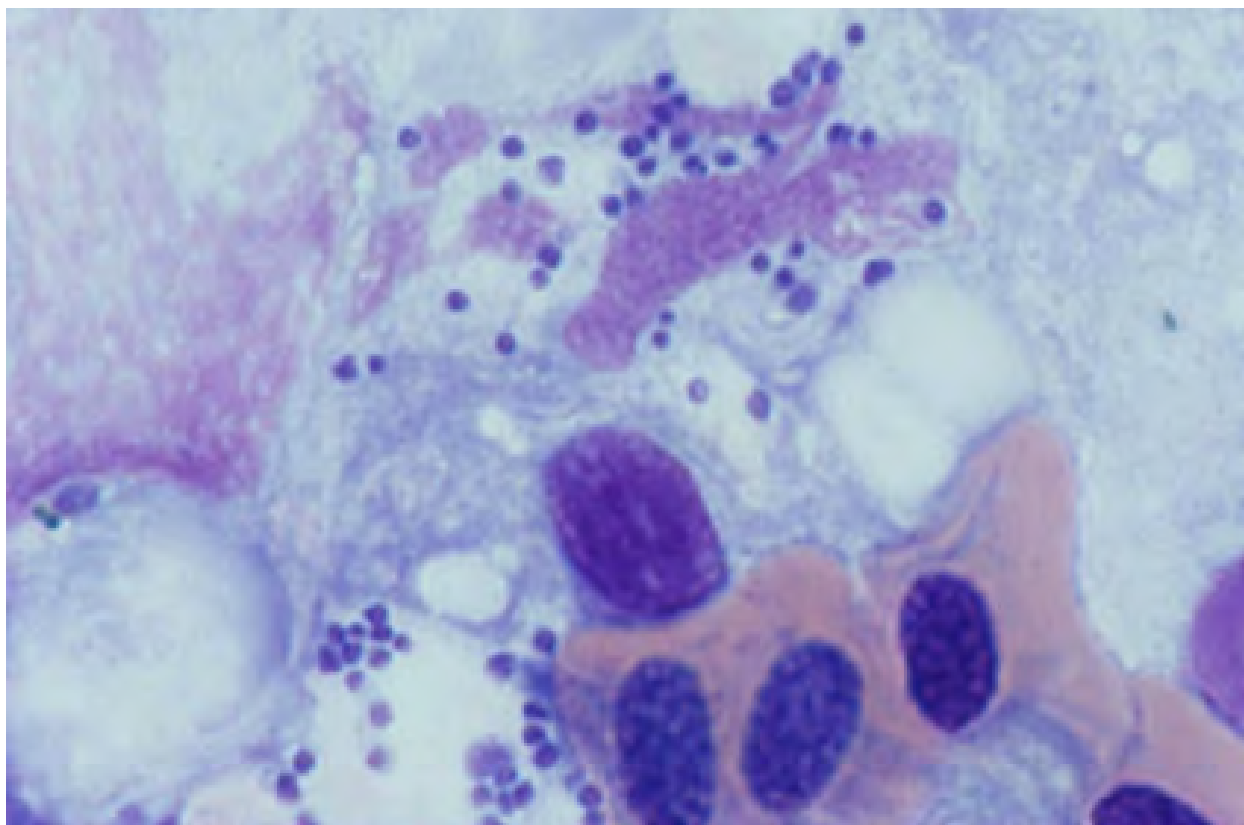


Figure 3.2. Giemsa stained liver impression from *Piscirickettsia salmonis* infected coho salmon (1000X magnification). Photo courtesy of Marcia House, NWIFC.

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% Triton X100).
 - iii. 1.5 mM MgCl₂.
 - 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 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.
- 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°C. Cool to ~ 50°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 dH₂O)
- 1.0 mL D-Cycloserine (0.3 g D-Cycloserine in 24 mL of dH₂O)
- 2.0 mL Polymyxin B-sulfate (0.3 g Polymyxin B-sulfate in 24 mL of dH₂O)
- 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. **Tryptic 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 μ m) 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 dH₂O)

10% Rhamnose (1 g Rhamnose to 10 mL in dH₂O)

10% Sucrose (1 g Sucrose to 10 mL in dH₂O)

10% Sorbitol (1 g Sorbitol to 10 mL in dH₂O)*

5% Salicin (0.5 g Salicin to 10 mL in dH₂O)

- b. Mix flask thoroughly and aseptically dispense into sterile tubes. Store at 2 to 8°C. Final pH = 6.8 ± 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 ± 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 media used for the esculin test

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°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 ₂ O	800 mL

3.7 Reagents, Media, and Media Preparation - 5

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
 - dH₂O 300 mL
- c. De-Colorizer
 - Acetone 40 mL
 - Ethanol (95%) 60 mL
- d. Safranin
 - Safranin O 2.5 g
 - 95% ethanol 100 mL
 - dH₂O 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
 - Na₂HPO₄ (anhydrous) 1.07 g
 - NaH₂PO₄·H₂O (monohydrate) 0.34 g
 - DH₂O 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
 - Na₂HPO₄ (anhydrous) 10.70 g
 - NaH₂PO₄·H₂O (monohydrate) 3.45 g
 - DH₂O 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
dH ₂ O	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

3.8 Bacterial Identification Techniques - 2

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 broth (TSB ([Section 2, 3.7.A.2 “Tryptic Soy Agar \(TSA\) or Tryptic Soy Broth \(TSB\) \(Difco 1998\)”](#))) with the isolate.

- b. Incubate at room temperature until growth is obtained, usually 24 hours.
- c. After incubation use a sterile loop or sterile dropper and place a drop on a clean cover slip.
- d. Place a tiny drop of distilled water in one corner of the same cover slip.
- e. Carefully invert the cover slip and place over the concave portion of a hanging drop slide.
- f. Observe for motility at 400X magnification on a compound microscope. Care should be taken to not interpret “drift” or “Brownian motion” as motility.
- g. Record results as motile or non-motile.

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-layered 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 overlaid.

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).

	Fermentation Test Tube	Oxidation Test Tube
Fermentative	A or AG	A or AG
Oxidative	N	A or AG
Non-reactive	N	N

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

H₂S = Hydrogen sulfide produced

N = No change

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 ₂ S	Black Butt	Hydrogen Sulfide Produced

iv. Quality Control

1. K/A
Shigella flexneri (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 aeruginosa (ATCC 27853)

vi. Precautions

1. Avoid inhaling fumes of Kovac's. Wear gloves to avoid skin contact.

3.8 Bacterial Identification Techniques - 8

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°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	<i>Escherichia coli</i> (ATCC 25922)	<i>Yersinia ruckeri</i>
Sorbitol	<i>Escherichia coli</i> (ATCC 25922)	<i>Y. ruckeri</i> Type I
Rhamnose	<i>Enterobacter aerogenes</i> (ATCC)	<i>Yersinia ruckeri</i>
Salicin	<i>Enterobacter aerogenes</i> (ATCC)	<i>Yersinia ruckeri</i>

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

1. Positive
Presence of a black to dark brown color on the slant.
2. Negative
No blackening of the medium.

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 *Shewanella 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°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 epifluorescence 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>

3.8 Bacterial Identification Techniques - 16

Antibodies available: Polyclonal antibodies available for Indirect FAT for the 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

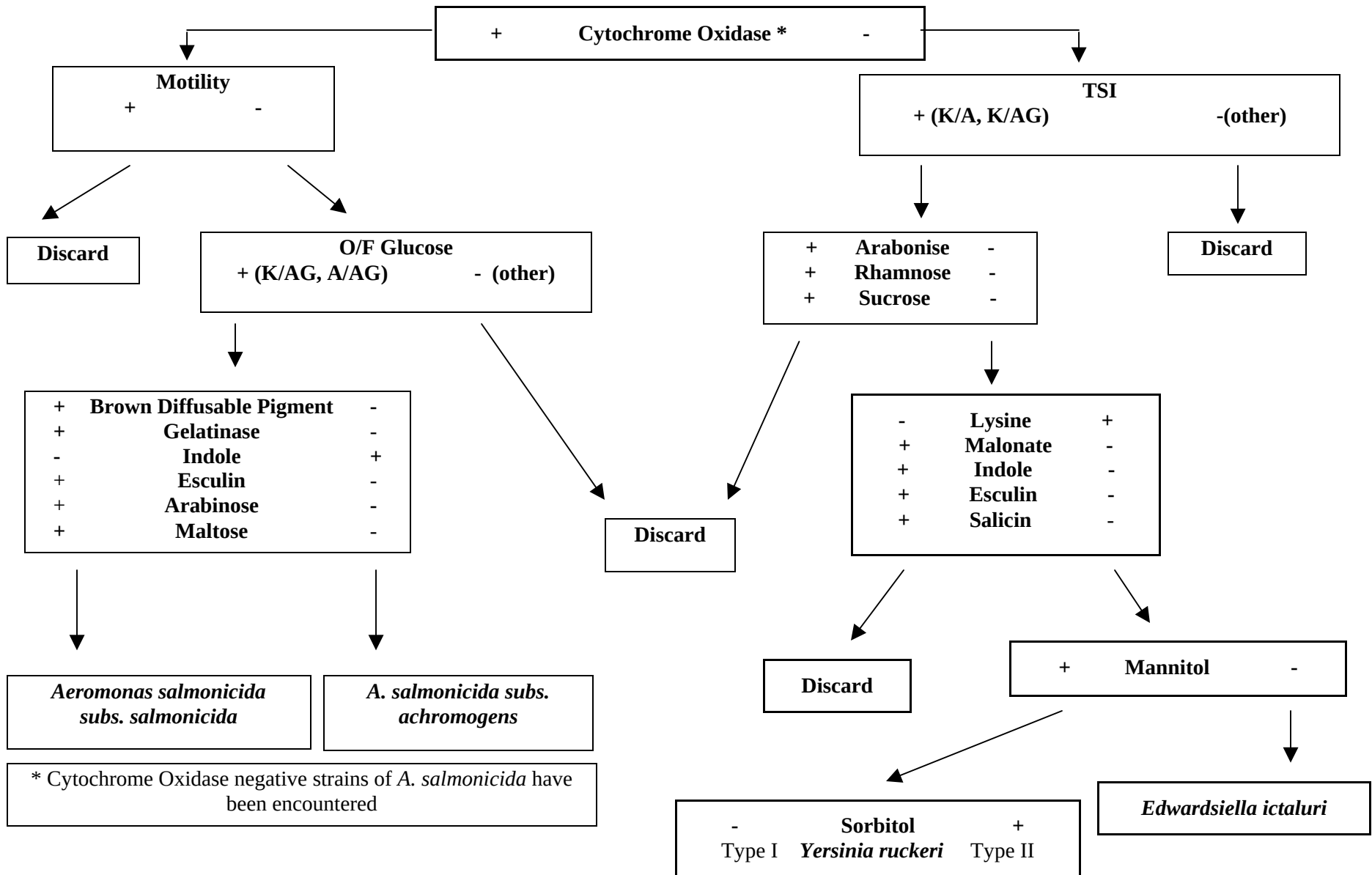
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3.A1 Laboratory Reference Flow Chart for Identification of Gram-Negative Bacterial Pathogens Which Grow on TSA or BHIA and are Targeted for Detection to Complete Fish Health Inspection Requirements



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. <i>Serratia marcescens</i> 2. <i>Serratia liquefaciens</i> 3. <i>Hafnia alvei</i>
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. <i>Serratia marcescens</i> 2. <i>Serratia liquefaciens</i> 3. <i>Hafnia alvei</i>
Unknown source – Isolate confirmed by biochemical and serological testing.	5144100	1. <i>Escherichia coli</i> 2. <i>Yersinia ruckeri</i>
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. <i>Hafnia alvei</i>
Notes from ERM archived files – previous testing	5104500	“Questionable ID”

3.A2 Profiles Obtained with API-20E for Known Fish Pathogens - 2

Bacterial Isolate ID	API PROFILE*	API Manual or Computer Identification
1. Hagerman – Type I (11.4)	5104100	“Very good ID” 1. <i>Yersinia ruckeri</i>
Coho, Quilcene R., WA (11/88)	5104000	“Very good ID” 1. <i>Yersinia ruckeri</i>
Unknown source	5100100	“Excellent ID” 1. <i>Yersinia ruckeri</i>
Unknown source	4105100	1. <i>Hafnia alvei</i>
Unknown source	4104100	1. <i>Yersinia ruckeri</i> 2. <i>Salmonella gallinarum</i>
Unknown source	4104000	“Acceptable ID” 1. <i>Yersinia ruckeri</i>
Unknown source	0104100	“Acceptable ID” 1. <i>Yersinia ruckeri</i>

* *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.

3.A2 Profiles Obtained with API-20E for Known Fish Pathogens - 3

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. <i>Pseudomonas pseudomaleae</i>
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. <i>Aeromonas salmonicida</i>
Profiles given in API MANUAL for <i>Aeromonas salmonicida</i>	6006104 6006504 4006104 2006104	“Good to Excellent ID” 1. <i>Aeromonas salmonicida</i>

**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.

References

- Romalde, J. L., and A. E. Toranzo. 1991. Evaluation of the API-20E system for the routine identification of the enteric redmouth disease. *Bulletin of European Association of Fish Pathologists* 11(4), 147.
- Kent, M. L. 1982. Characteristics and identification of *Pasteurella* and *Vibrio* species pathogenic to fishes using API-20E (Analytab Products) multitube test strips. *Canadian Journal of Fisheries and Aquatic Sciences* 39:1725-1729.
- Toranzo, A. E., Y.Santos, T. P. Nieto, and J. L. Barja. 1986. Evaluation of different assay systems for identification of environmental *Aeromonas* strains. *Applied Environmental Microbiology* 51:652-656.

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.			
15.			
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 ₂ O*		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 [±]		-	-	10 µL	-

*Add water to Master Mix first, TAQ last.

**Change "Stock Concentration" parameters as necessary. Different reagent sources supply varying stock concentrations.

[±]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 ₂ O*		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 [±] (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. [±]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)														
<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>	<u>9</u>	<u>10</u>	<u>11</u>	<u>12</u>	<u>13</u>	<u>14</u>	<u>15</u>
<u>16</u>	<u>17</u>	<u>18</u>	<u>19</u>	<u>20</u>	<u>21</u>	<u>22</u>	<u>23</u>	<u>24</u>	<u>25</u>	<u>26</u>	<u>27</u>	<u>28</u>	<u>29</u>	<u>30</u>

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 ₂ O*		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 [±]		-	-	5 µL	-

*Add water to Master Mix first, TAQ last.

**Change "Stock Concentration" parameters as necessary. Different reagent sources supply varying stock concentrations.

[±]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 ₂ O*		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 ⁺ (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. ⁺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 ₂ O*		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 [‡]		-	-	5 µL	-

*Add water to Master Mix first, TAQ last. **Change "Stock Concentration" parameters as necessary. Different reagent sources supply varying stock concentrations. [‡]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 _____

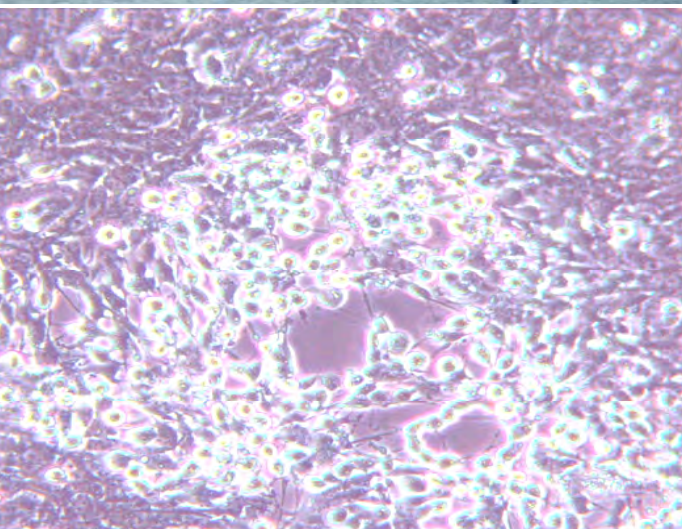
Date _____

Samples _____

Affix gel photo documentation to this sheet and make notes on results:

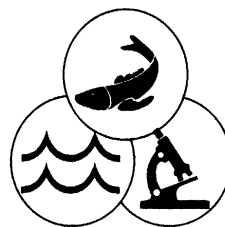
AFFIX PHOTODOCUMENTATION

Notes:



Section 2

USFWS/AFS-FHS Standard Procedures for Aquatic Animal Health Inspections



Chapter 4 Virology

- 4.1 Virology Introduction
- 4.2 Selection of Appropriate Cell Lines
- 4.3 Cell Culture
- 4.4 Sample Processing Procedures
- 4.5 Screening Method for Viral Isolation
- 4.6 Identification of Viruses
- 4.7 Serum Neutralization
- 4.8 Indirect Fluorescent Antibody (IFAT) Procedure
- 4.9 Reagents and Media
- 4.10 Glossary
- 4.11 References
 - 4.A1.A Worksheet A – PCR Sample Data/Log Sheet
 - 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.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, IPNV, and VHSV may be confirmed using serum neutralization, indirect fluorescent antibody test (IFAT), or polymerase chain reaction (PCR) techniques. 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 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 and at least two cell lines should be used for all samples to maximize the detection of viral agents present in the samples (Bouchard 1999; OIE 2000; Plumb 1999b; 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 IHN, SVCV, and VHSV.
2. The SHK-1 cell line provides high sensitivity for ISAV.
3. The FHM and BF-2 cell lines both provide high sensitivity for LMBV.
4. CHSE-214 cell line provides high sensitivity for IPNV and OMV.
5. The WSS-2 cell line provides high sensitivity for WSHV.

4.2 Selection of Appropriate Cell Lines - 2

Table 4.1. Recommended cell lines to detect target viruses.

Virus ^a	Cell Line	Common Name	ATCC ^b Designation
IHNV	Epithelioma Papulosum Cyprini	EPC	
IPNV	Chinook Salmon Embryo	CHSE-214	CRL-1681
ISAV	Salmon Head Kidney	SHK-1	
LMBV	Fat Head Minnow or Bluegill Fry	FHM BF-2	CCL-42 CCL-91
OMV	Chinook Salmon Embryo	CHSE-214	CRL-1681
SVCV	Epithelioma Papulosum Cyprini	EPC	
VHSV	Epithelioma Papulosum Cyprini	EPC	
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 1979; Lannan 1994; Merchant, 1964; Rovozzo 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 cm² 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 cm² 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 cm² flask, this will usually be about 20 mL. MEM-10/Hepes ([Section 2, 4.9.G “MEM-10/Hepes \(Tissue Culture Medium for all Cell Lines Except SHK-1\)”](#)) works well in an open system for all cell lines listed in [Table 4.2](#) except SHK-1, which does best with L-15 ([Section 2, 4.9.H “Leibovitz's L-15 \(Tissue Culture Medium for SHK-1 Cell Line\)”](#)).
9. Incubate flasks at room temperature (20 to 25°C) until they reach confluence and then incubate at the appropriate temperature for that cell line.
 - a. SHK-1 and CHSE-214 cells should be held at 15°C.
 - b. WSS-2 cells should be held at 20°C.

- c. FHM and BF-2 cells should be held at 25°C.
- d. EPC cells may be held at 15 to 25°C.

Table 4.2. Seeding guidelines for the subculture of fish cell lines.

<u>CELL LINE</u>		Suggested Seeding Rate (per cm ²)	<u>INCUBATION TEMP (°C)</u>	
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	0 - 36
WSS-2	1:4 - 8	150,000	20 - 25	20 - 30
SHK-1	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 1×10^6 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 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 during which time samples exhibiting CPE are re-inoculated. At approximately 14 days, blind passage of samples not exhibiting CPE is performed and monitored for a total combined incubation period (initial plus blind pass) of 28 days. 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 2000; Bouchard 1999; Plumb 1999b; Rovozzo 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.

2. Tissue culture medium is decanted from plates.

3. Inoculate with replication at least 2 cm² of cell monolayer with a minimum of 100 µL from each sample.

Example: If using 24-well plates (2 cm²/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.
 - a. Incubation temperature for IPNV, IHN, VHSV, ISAV, and OMV is 15°C.
 - b. Incubation temperature for WSHV, SVCV, and LMBV is 20 to 25°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 \(Tissue Culture Medium for all Cell Lines Except SHK-1\)”](#)) 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 \(Tissue Culture Medium for SHK-1 Cell Line\)”](#)). 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 LMBV and SVCV at 20 to 25°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.

4.5 Screening Method for Viral Isolation - 3

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 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 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. 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 2000; 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 1989) see Figures 4.1 to 4.3.

- ii. The serum neutralization, PCR, or IFAT methods may be used to confirm the cause of the CPE is due to the presence of IHNV

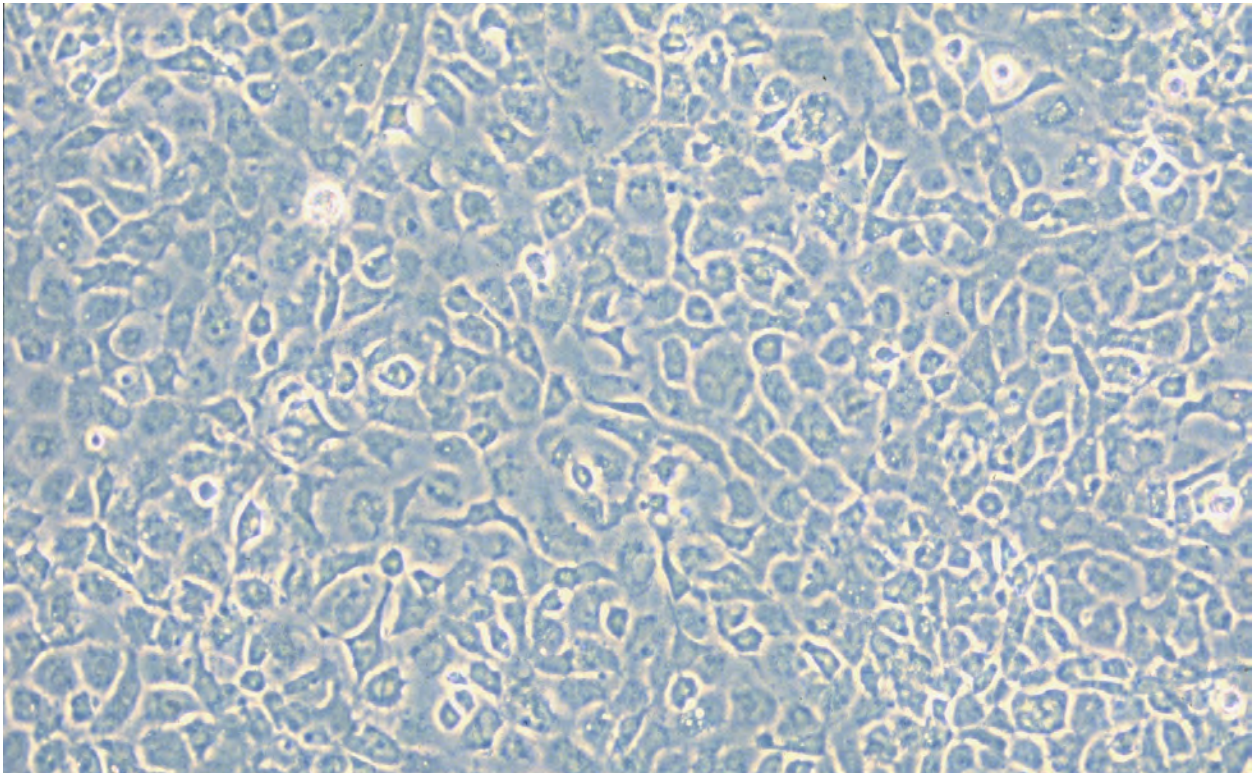


Figure 4.1. Normal EPC monolayer. Photo courtesy of Jim Winton, USGS.

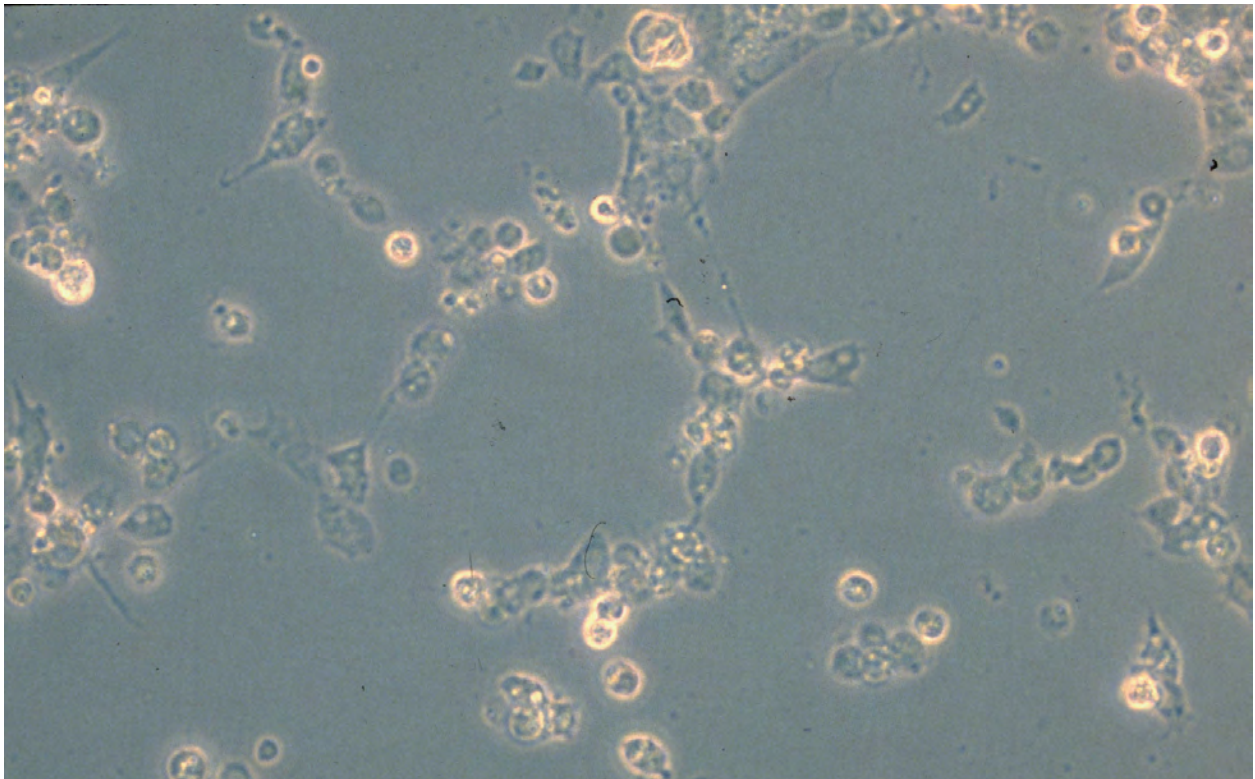


Figure 4.2. CPE typical of a rhabdovirus on EPC monolayer. Photo courtesy of Jim Winton, USGS.

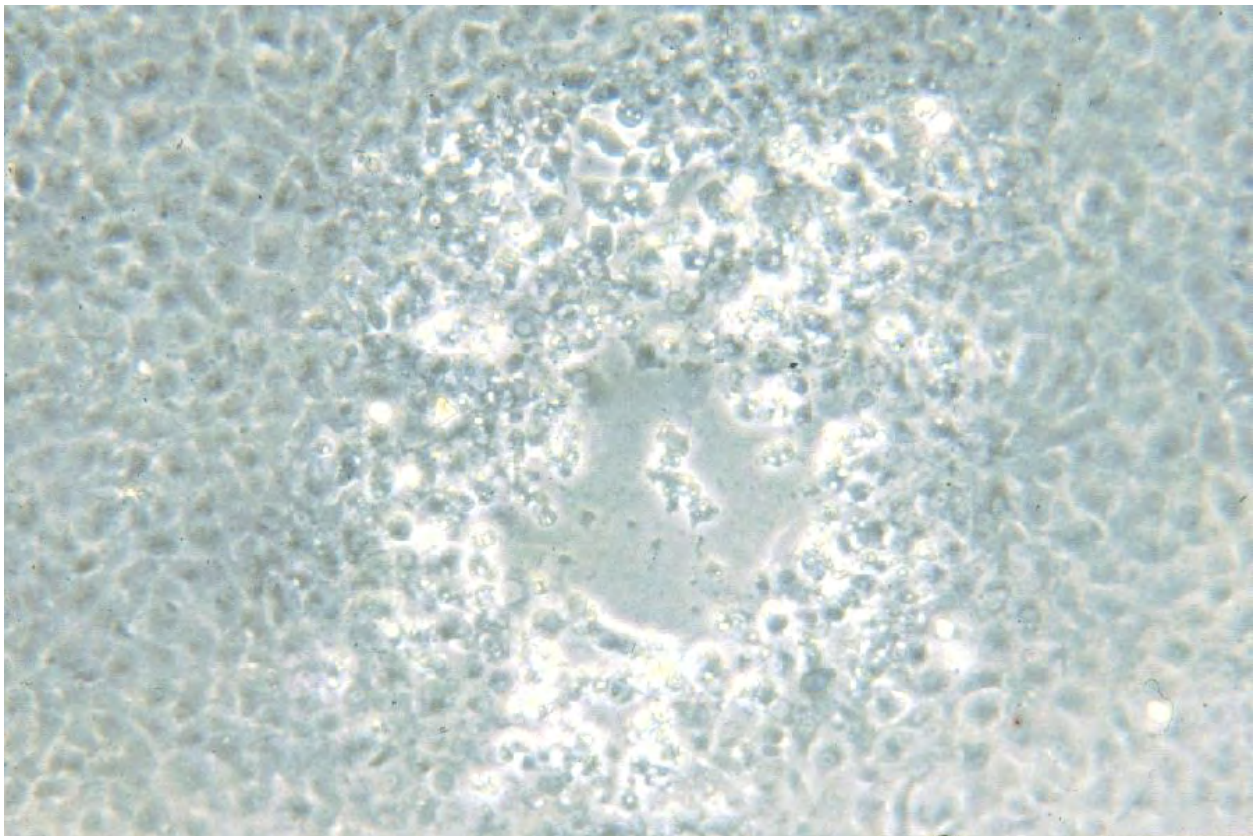


Figure 4.3. CPE plaque of IHNV on EPC monolayer. Photo courtesy of Scott LaPatra, Clear Springs. Foods, Inc.

2. Confirmation Methods for IHN

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 IHN (Modified from Arakawa 1990): 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. Heat tubes to 95°C for two minutes in a water bath, heat block, 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. Production of DNA by Reverse Transcription and Amplification by First Round 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 \(IHN\)](#), 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. First Round Primers for IHN
 - a. Forward: 5'-TCA AGG GGG GAG TCC TCG A-3'
 - b. Reverse: 5'-CAC CGT ACT TTG CTG CTA C-3'

5. First Round Thermocycler Program for IHNV
 - a. Pre-dwell at 50°C for 15 minutes.
 - b. Preheat or “Jumpstart” sample to 95°C for two minutes.
 - c. 25 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.
- iii. “Nested” Second Round PCR for IHNV
 1. QA/QC (See [Section 2, 6.2 PCR – Quality Assurance/Quality Control](#) for specific QA/QC considerations for PCR.)
 2. Again use [Section 2, Section 2, 4.A1.B.1 Worksheet B.1 – Infectious Hematopoietic Necrosis Virus \(IHNV\)](#) for the second round to 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.
 3. Second Round Primers for IHNV
 - a. Forward: 5'-TTC GCA GAT CCC AAC AAC AA-3'
 - b. Reverse: 5'-GCG CAC AGT GCC TTG GCT-3'
 4. Second Round Thermocycler Program for IHNV
 - a. Preheat or “Jumpstart” sample to 95°C for two minutes.
 - b. 25 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.
 - c. Post dwell at 72°C for seven minutes.
 - d. 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 (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 both the first and second (nested) round PCR assays.

 - a. **Bands occurring at the 786 bp location in the First Round Assay and the 323 bp location in the Second Round Assay are confirmatory for IHNV 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 IHNV.**
 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 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.

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 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.

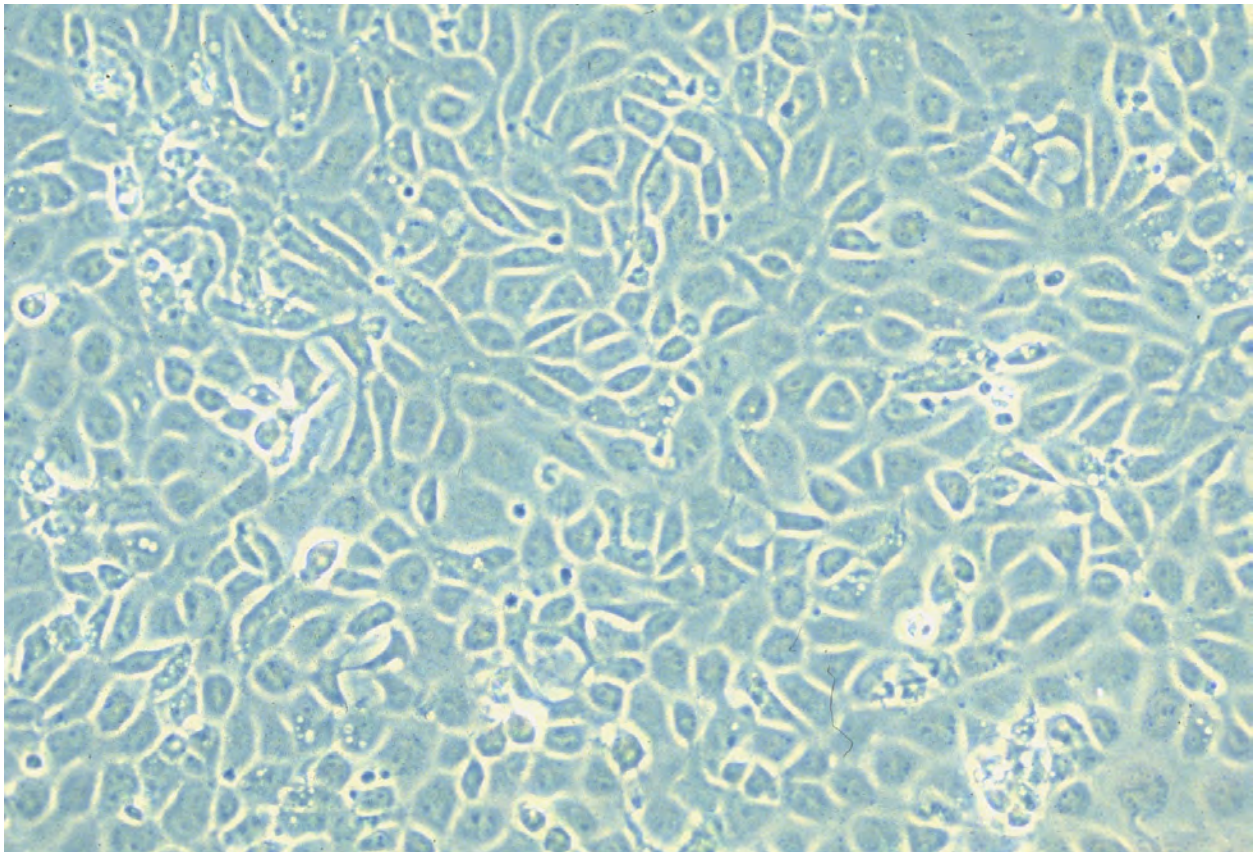


Figure 4.4. Normal CHSE-214 monolayer. Photo courtesy of Jim Winton, USGS.

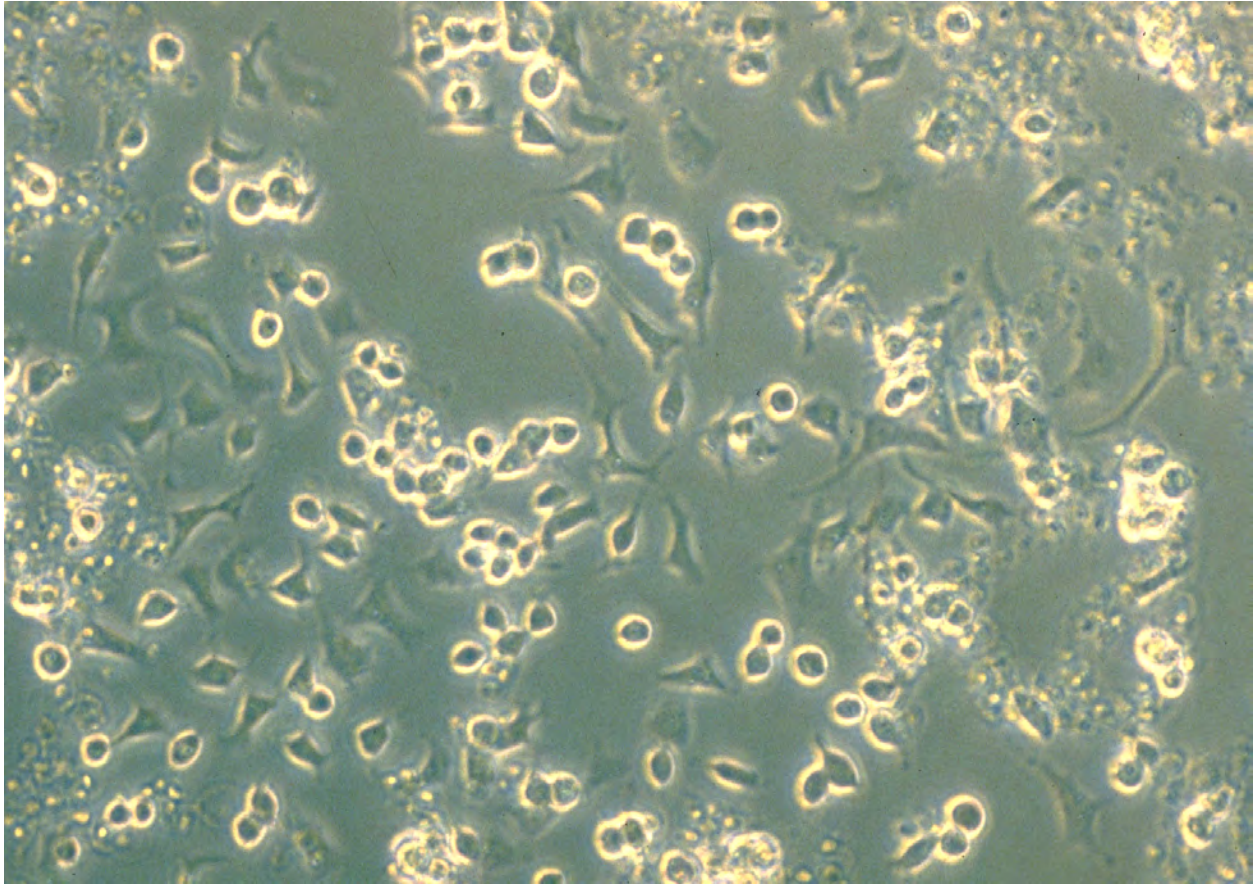


Figure 4.5. CPE typical of IPNV on CHSE-214 cells. Photo courtesy of Jim Winton, USGS.

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 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 1996; OIE 2000).

1. Screening Method

- a. Cell culture on SHK-1 cell line incubated at 15°C (Bouchard 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.

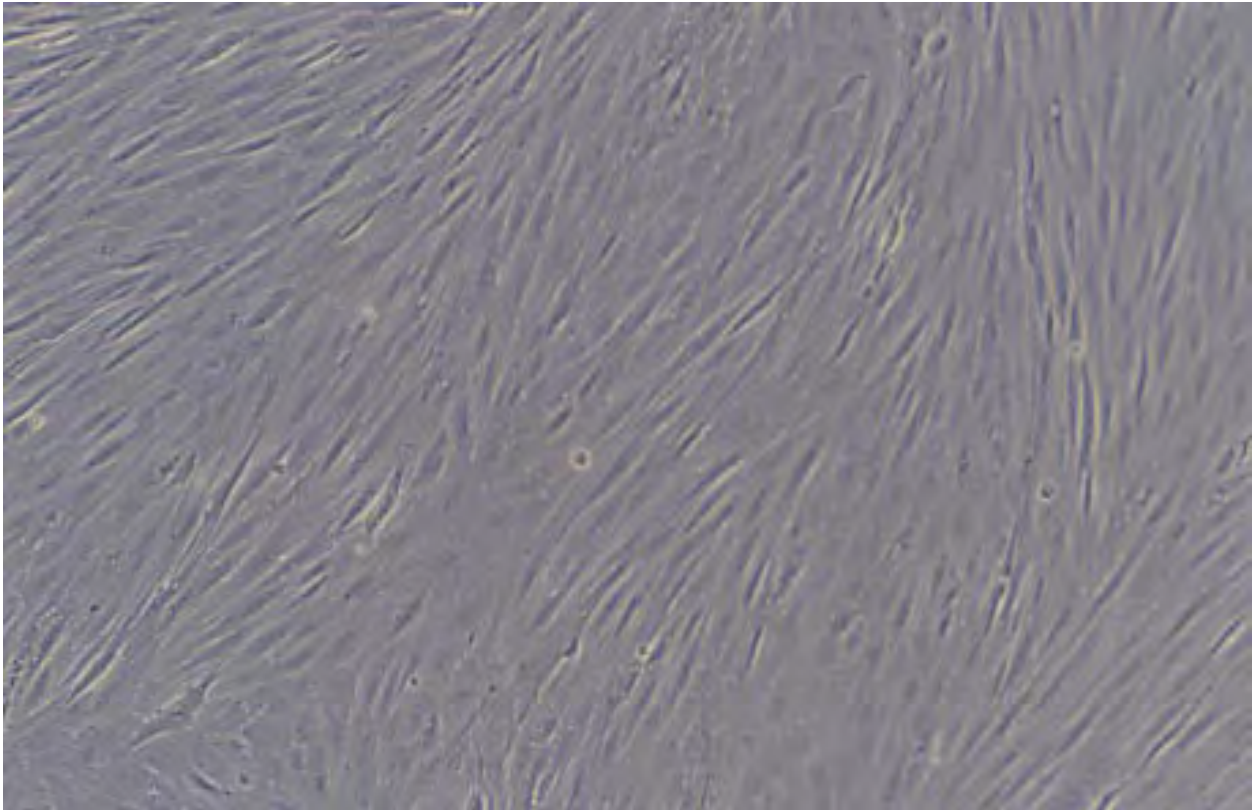


Figure 4.6. Normal SHK-1 monolayer. Photo courtesy of Jim Winton, USGS.

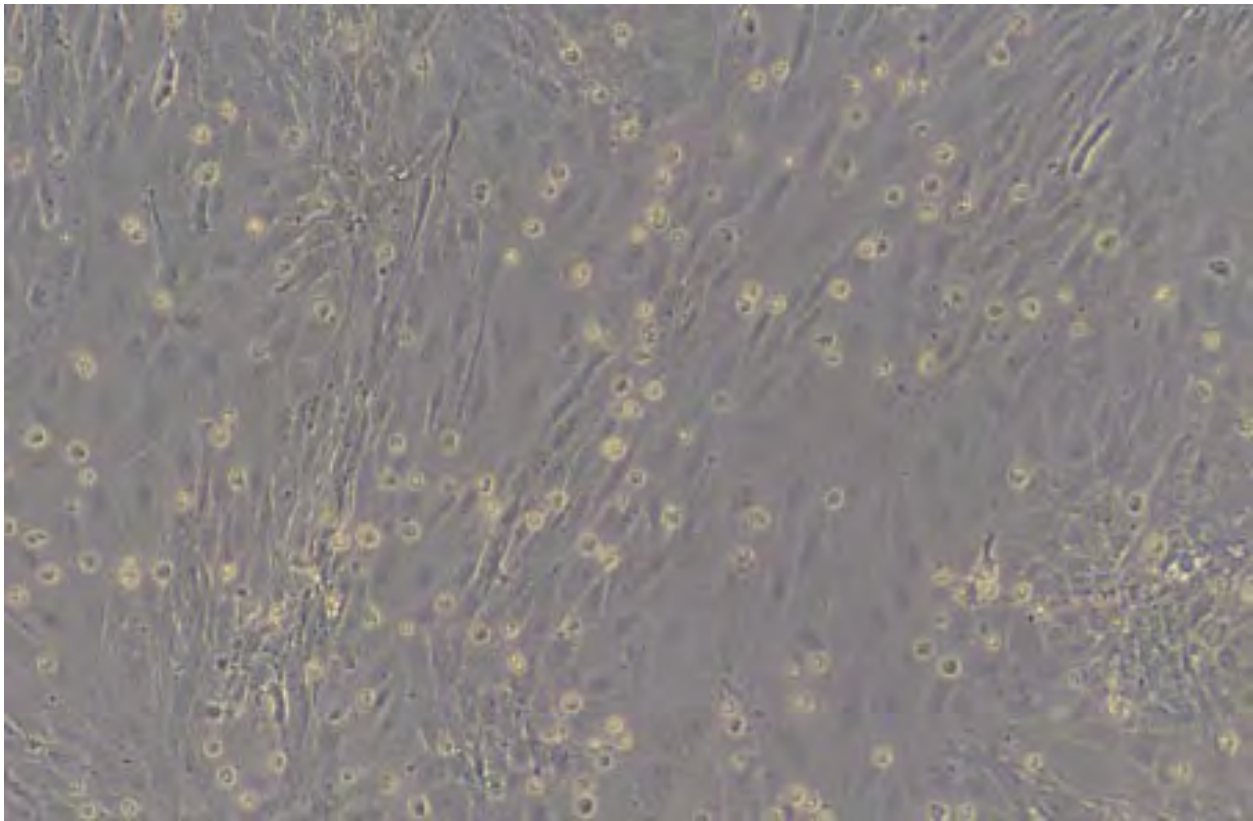


Figure 4.7. CPE typical of ISA on SHK-1 cells. Photo courtesy of Jim Winton, USGS.

2. Confirmation Methods for ISAV

- a. Polymerase Chain Reaction (PCR) (Modified from Bouchard 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 μ L fluid to 98 μ L 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 μ g/mL (or 100 ng/ μ L). Generally, 1 μ L of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/ μ L or use up to 5 μ L/reaction if reading falls below 50 ng/ μ L.

- 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 1999a). 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 20 to 25°C (Plumb 1999b).
- 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 inoculations 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 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.

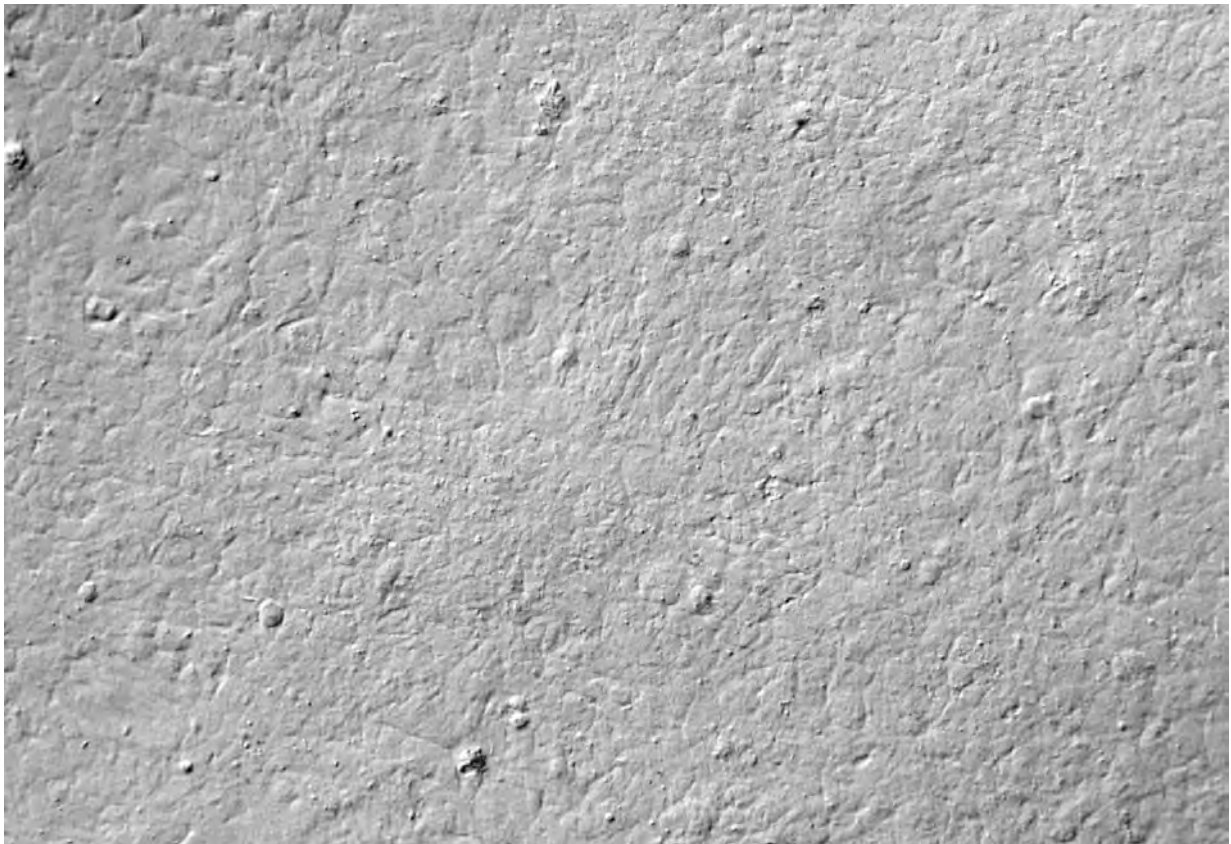


Figure 4.8. Normal FHM monolayer. Photo courtesy of John Grizzle, Auburn University.

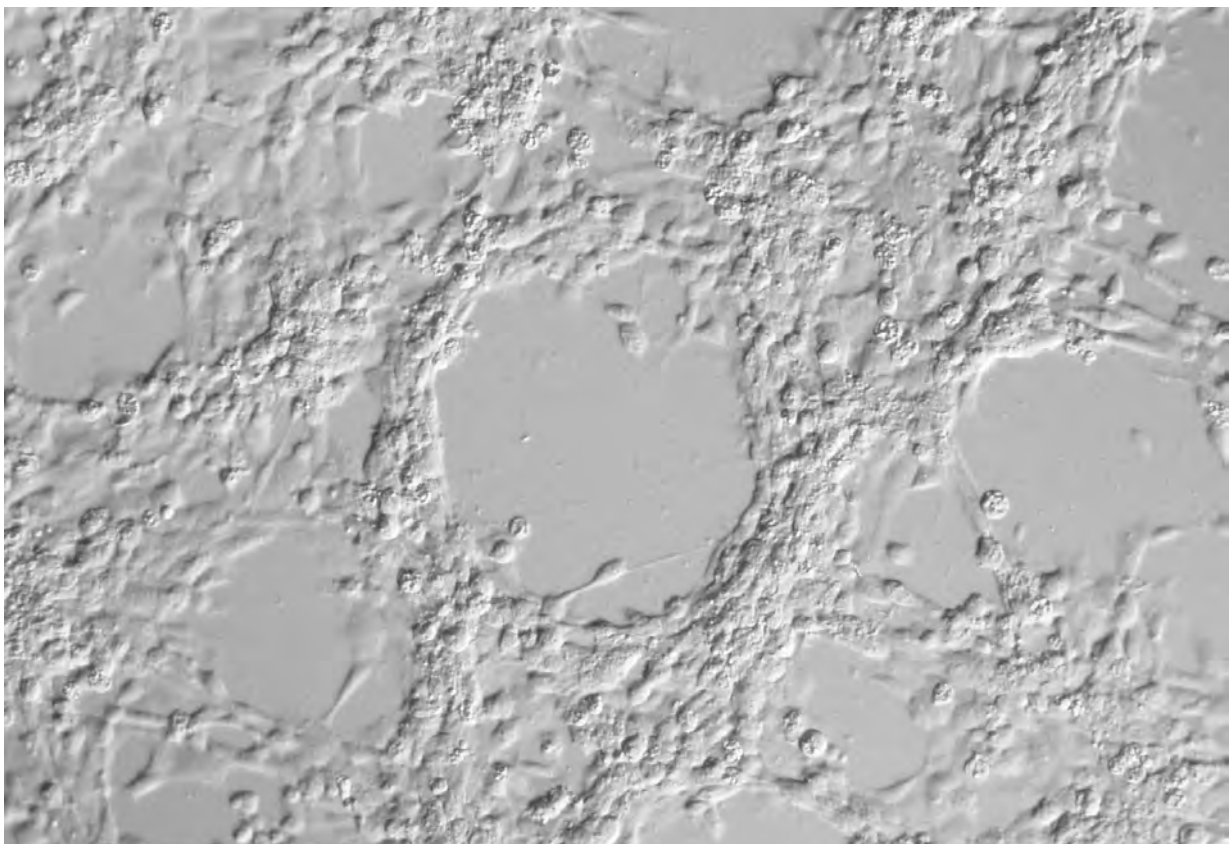


Figure 4.9. CPE typical of LMBV on FHM cells. Photo courtesy of John Grizzle, Auburn University.

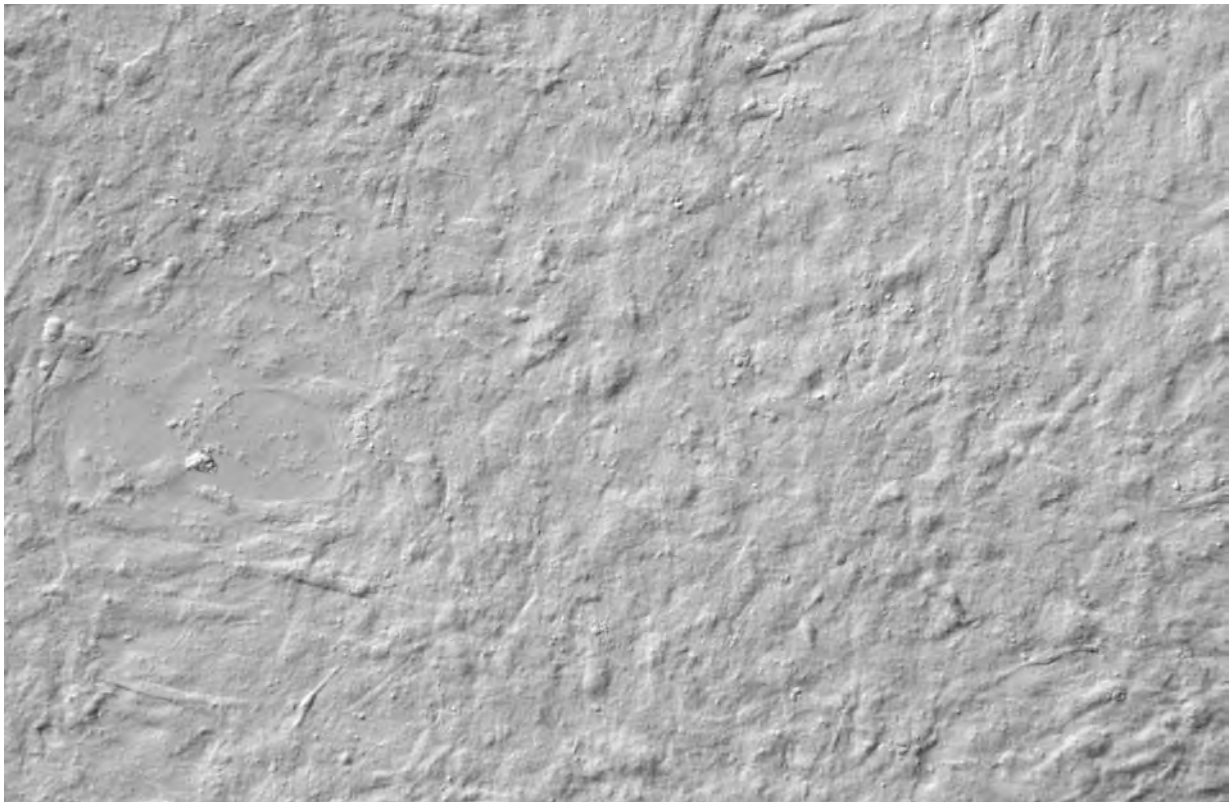


Figure 4.10. Normal BF-2 monolayer. Photo courtesy of John Grizzle, Auburn University.

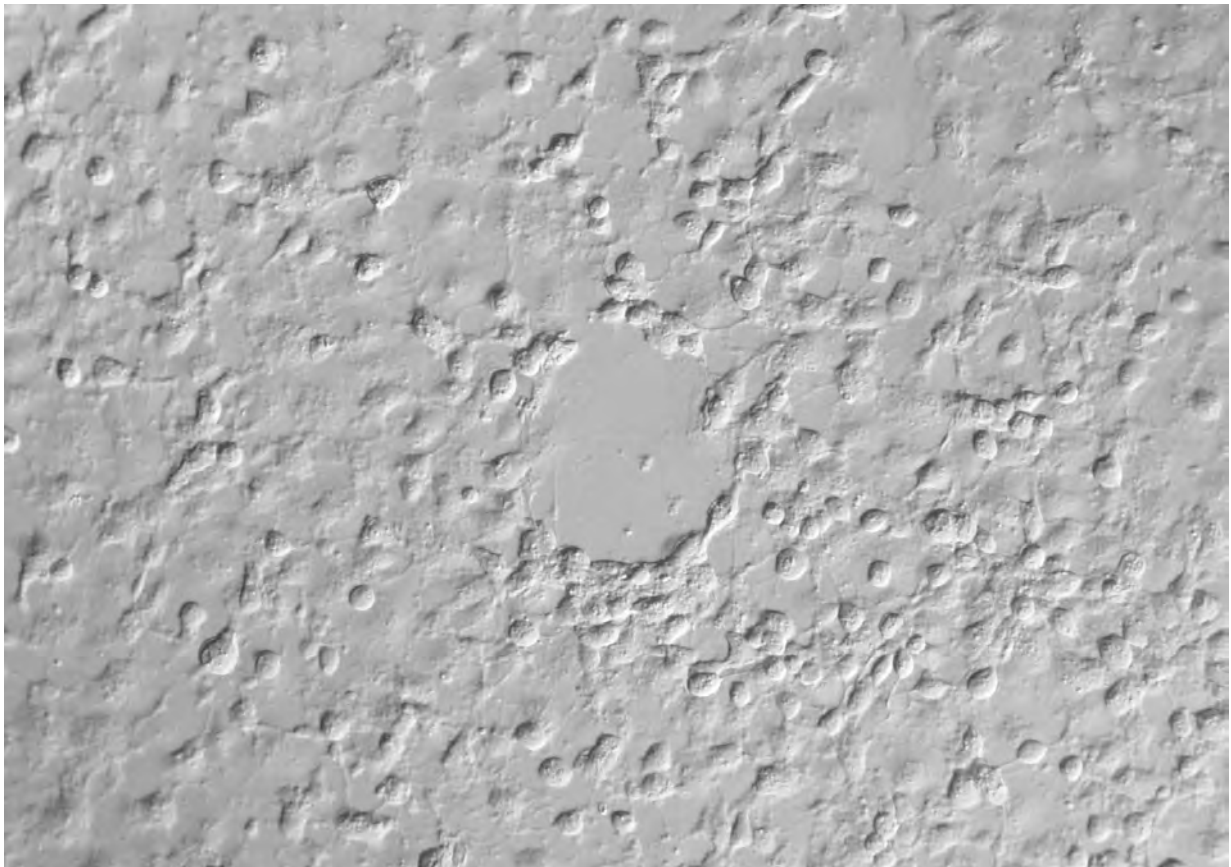


Figure 4.11. CPE typical of LMBV on BF-2 cells. Photo courtesy of John Grizzle, Auburn University.

2. Confirmation Method for LMBV - Polymerase Chain Reaction (PCR) (Modified from Plumb 1999b)

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

(**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 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 Xg.
- ii. Resuspend pellet in 200 μ L PBS.
- iii. Add 20 μ L proteinase K solution (Section 2, 5.6.E “Proteinase K”) and 200 μ L buffer AL to the sample, mix thoroughly by vortexing.
- iv. Incubate for 10 minutes at 70°C.
- v. Add 200 μ L 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 μ L 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 μ L 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 200 μ L 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 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 to 94°C for five minutes.
2. 30 cycles of the following regime:
 - a. Denaturing at 94°C for 60 seconds.
 - b. Annealing at 45°C for 60 seconds.
 - c. Extending at 60°C for 120 seconds.
3. Post dwell at 72°C for two minutes.
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)

Oncorhynchus 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.

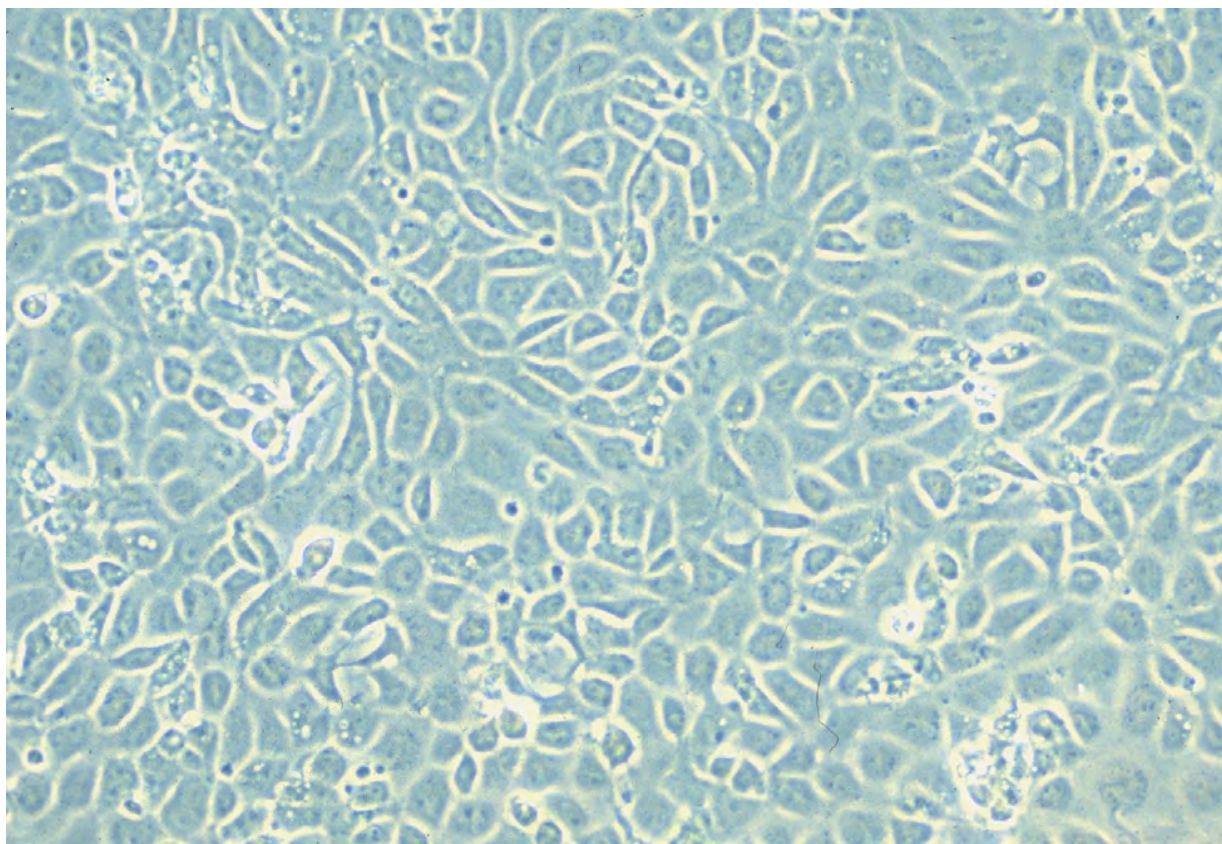


Figure 4.12. Normal CHSE-214 monolayer. Photo courtesy of Jim Winton, USGS.

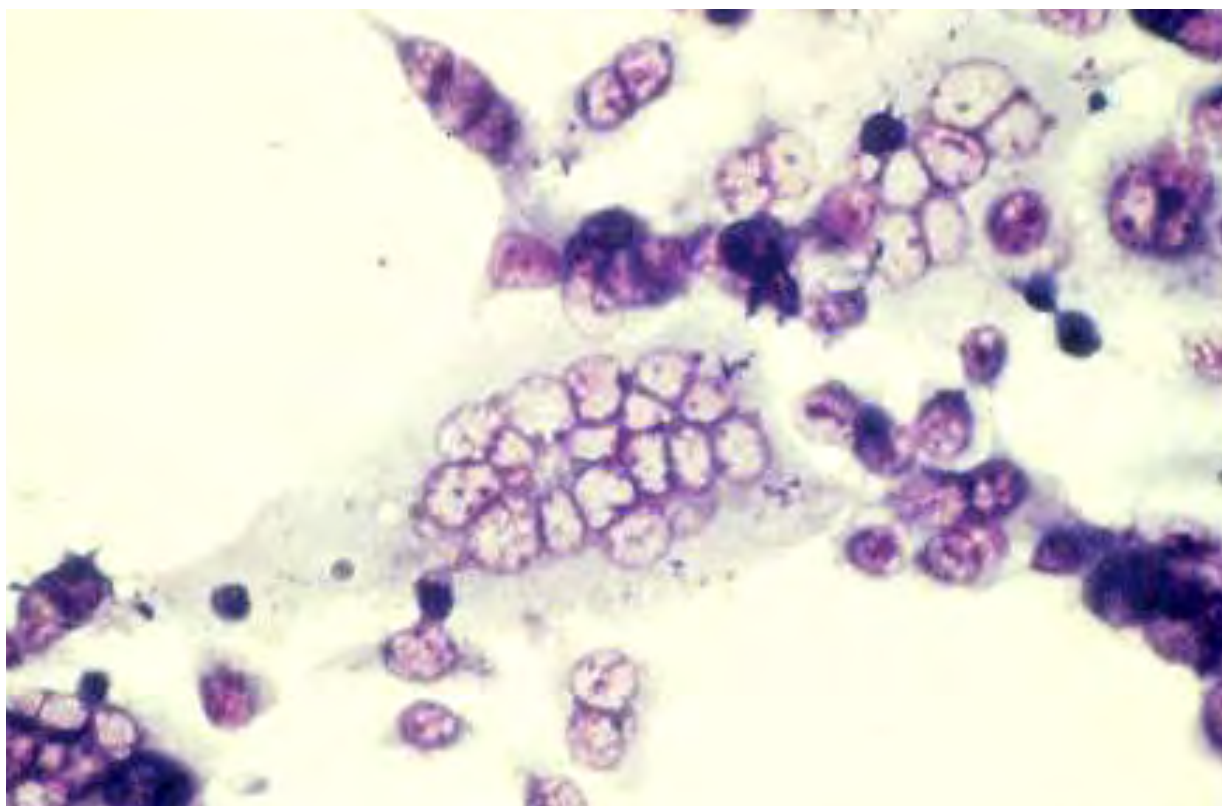


Figure 4.13. CPE typical of OMV on CHSE-214 cells. Photo courtesy of Mamoru Yoshimizu, Hokkaido University.

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 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 geographic range includes European countries that experience low water temperatures during the winter and SVCV has recently been isolated in the USA. The host range of SVCV includes Esocids and Cyprinids with the common carp being the principle host. It causes disease in Cyprinids of all ages characterized by hemorrhages on the skin, gills, and viscera. Mortality is usually seasonal, often most severe in the spring or early summer during rising water temperatures. SVCV may 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 IHN, 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 cause of the CPE is due to the presence of SVCV.

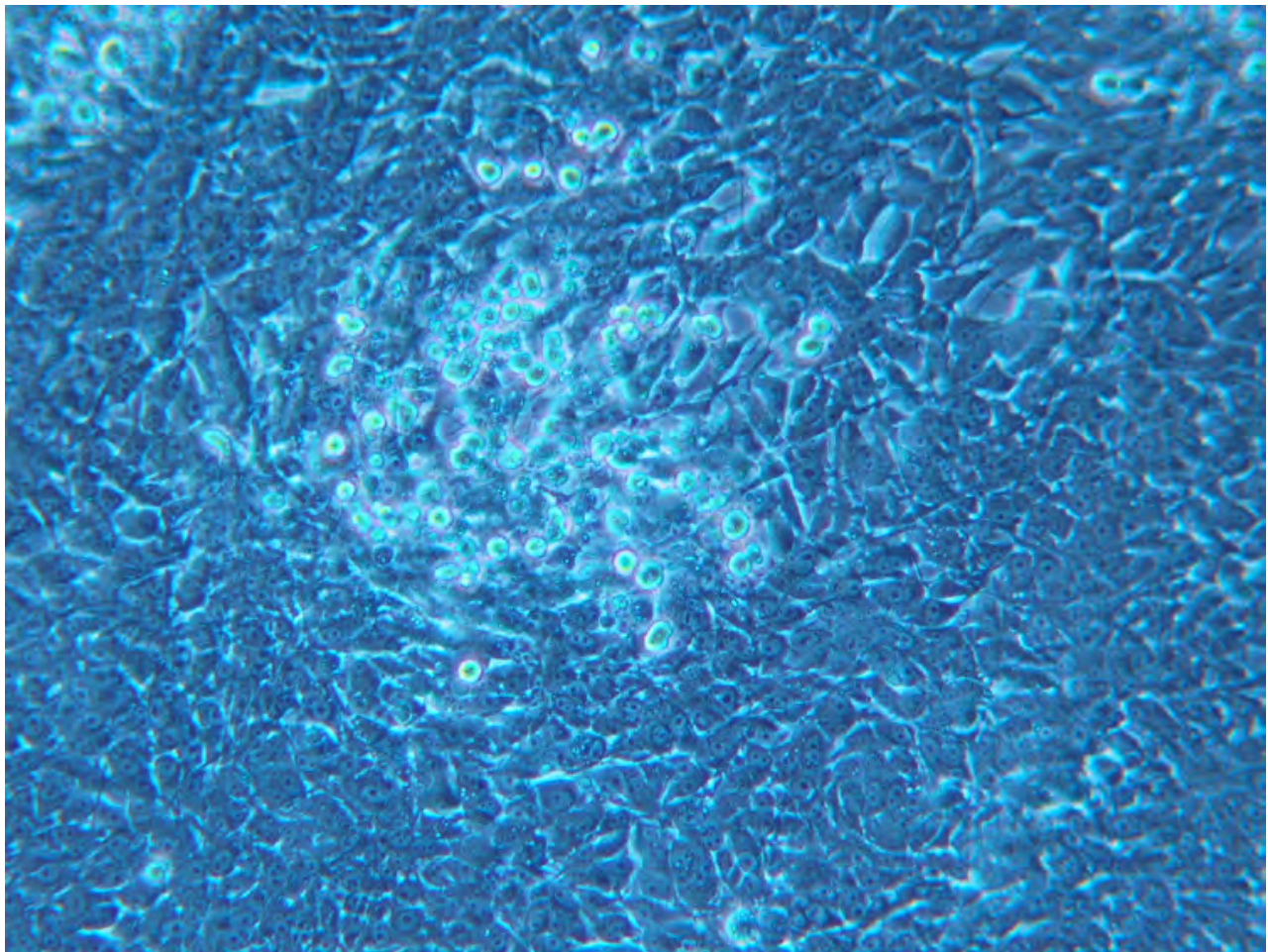


Figure 4.14. CPE plaque of SVCV on EPC monolayer. Photo courtesy of Andrew Goodwin, University of Arkansas.

2. Confirmation Methods for SVCV

- 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 (Modified from Bjorklund 1996): 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 RNase free water by adding 2 µL fluid to 98 µL water in microcentrifuge tubes.
 2. Place tubes in heat block at 95°C for two 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.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 “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 SVCV
 - a. Forward: 5'- TCA TTT AGA GCC ATA CG -3'

- b. Reverse: 5'- GAA AGC ATG TTG AAG CT -3'
- 5. Thermocycler Program for SVCV
 - a. Pre-dwell at 50°C for 15 minutes.
 - b. Preheat or “Jumpstart” sample to 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. 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 803 bp location are confirmatory for SVCV 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 SVCV.**
 - 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)

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 in Continental Europe is mostly seen in trout, grayling, white fish, pike, and turbot and is characterized by edema and hemorrhage due to impairment of osmotic balance. Disease in North America is primarily seen in Pacific herring and pilchard but the virus has been isolated from several species of marine fish in the Pacific and Atlantic Oceans around North America and from returning adult Coho and Chinook salmon. Fry are most susceptible to disease, which usually occurs at water temperatures between 4 to

14°C. A carrier state may develop with fish shedding virus in the feces, urine, and sexual fluids as well as being present in the internal organs. Although present in ovarian fluid, vertical transmission has not been demonstrated with VHSV. The European and North American strains of VHSV are indistinguishable by serologic methods but may be separated by PCR methods (OIE 2000; 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 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 serum neutralization, PCR, 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. 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 VHSV (Modified from Einer-Jensen 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 (with some cell scrapings) 1:50 in molecular grade RNase free water by adding 2 μ L fluid to 98 μ L water in microcentrifuge tubes.
 2. Place tubes in heat block at 95°C for two 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 μ g/mL (or 100 ng/ μ L). Generally, 1 μ L of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/ μ L or use up to 5 μ L/reaction if reading falls below 50 ng/ μ L.
- ii. Formation of DNA by Reverse Transcription and Amplification by First Round 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. First Round Primers for VHSV
 - a. Forward: 5'-TCT CTC CTA TGT ACT CCA AG-3'
 - b. Reverse: 5'-TTC CGG TGG AGC TCC TGA AG-3'
 5. Thermocycler Program for First Round VHSV
 - a. Pre-dwell at 50°C for 15 minutes.
 - b. Preheat or “Jumpstart” sample to 95°C for two minutes.
 - c. 25 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.
- iii. “Nested” Second Round PCR for VHSV

1. QA/QC (See Section 2, 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)
 2. Again use Section 2, 4.A1.B.7 Worksheet B.7 – Viral Hemorrhagic Septicemia Virus (VHSV) for the second round to 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.
 3. Second Round Primers for VHSV
 - a. Forward: 5'-ATG GGC TTC AAG GTG ACA C-3'
 - b. Reverse: 5'-GTA TCG CTC TTG GAT GGA C-3'
 4. Thermocycler Program for Second Round VHSV
 - a. Preheat or “Jumpstart” sample to 95°C for two minutes.
 - b. 25 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.
 - c. Post dwell at 72°C for seven minutes.
 - d. 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 (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 950 bp location in the first round assay and the 558 bp location in the second round assay are confirmatory for VHSV 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 VHSV.**
 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 VHSV
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 15-18°C.

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 1999a).

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.

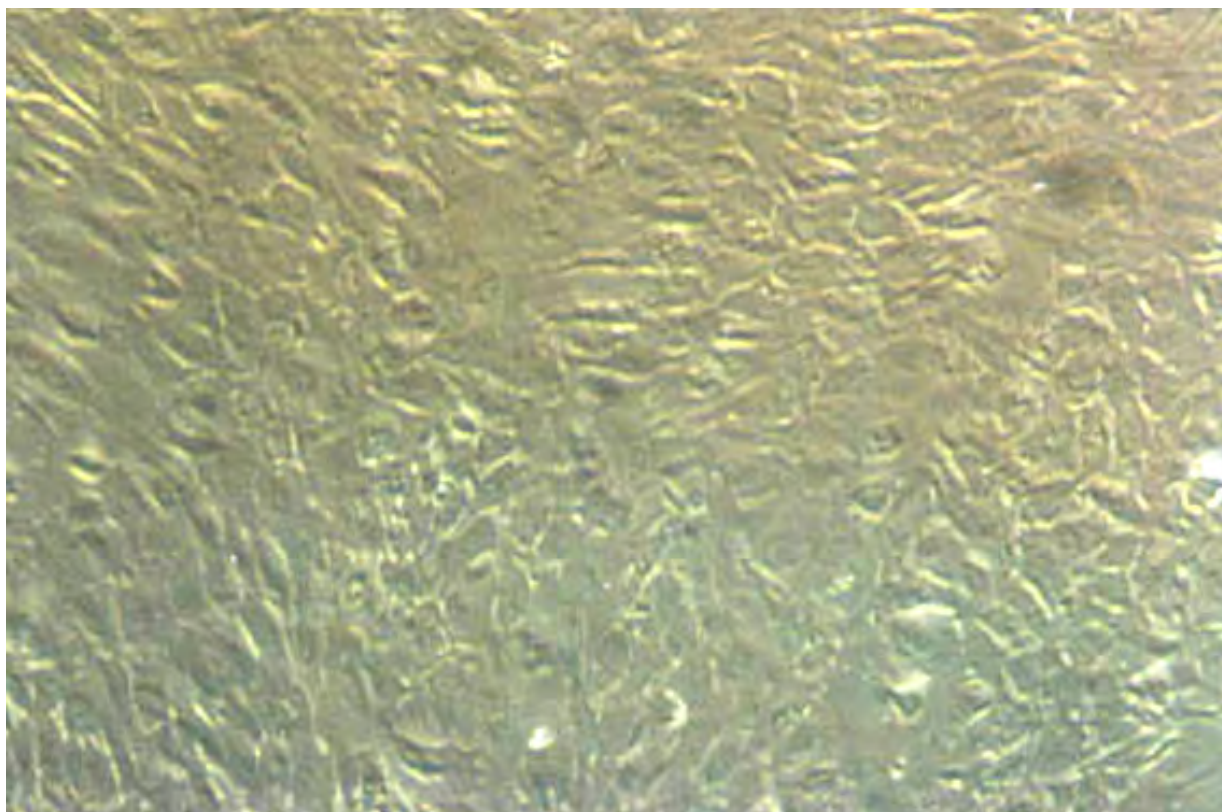


Figure 4.15. Normal WSS-2 monolayer. Photo courtesy of Scott LaPatra, Clear Springs Foods, Inc.

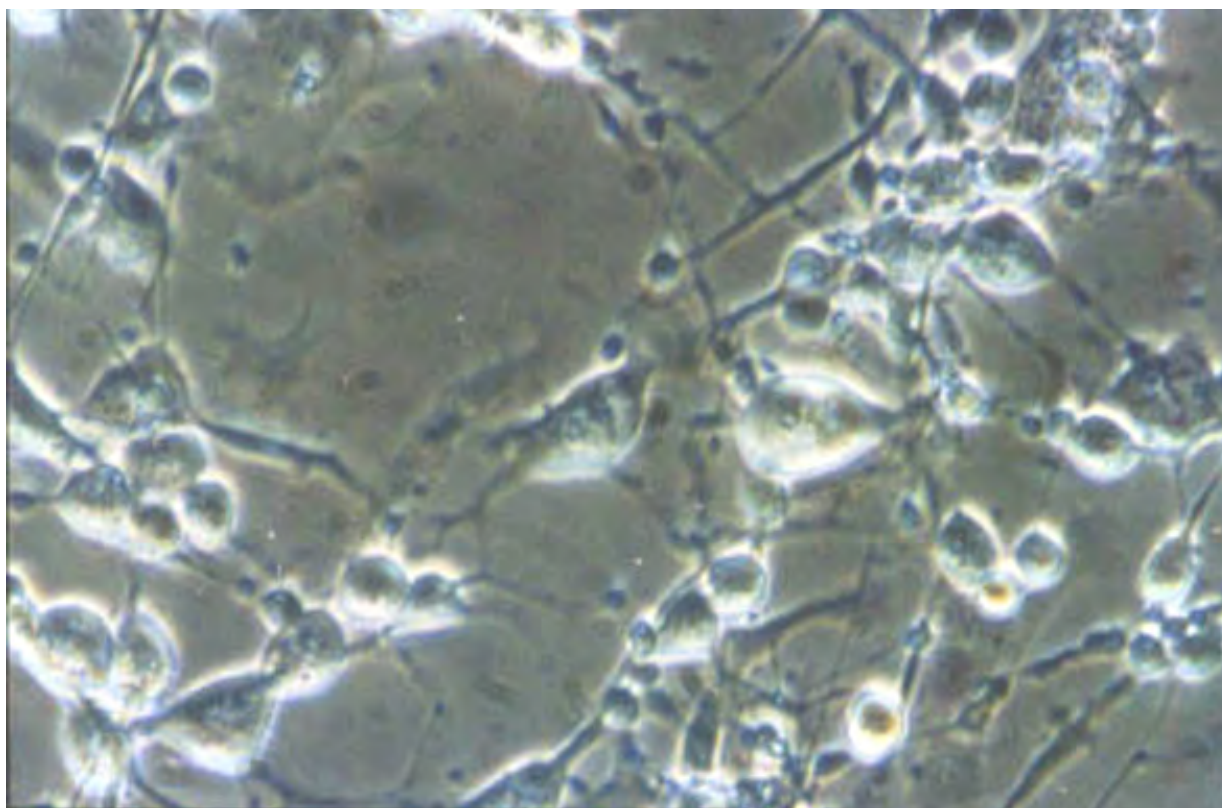


Figure 4.16. CPE typical of WSHV on WSS-2 monolayer. Photo courtesy of Scott LaPatra, Clear Springs Foods, Inc.

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

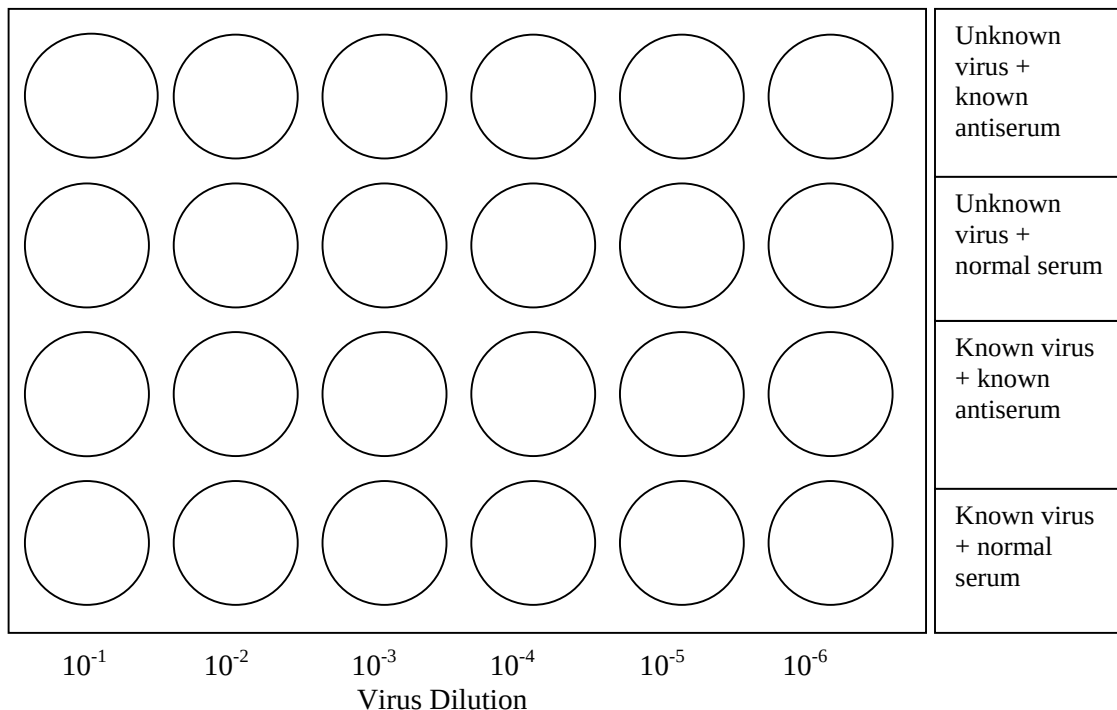
Serum neutralization or plaque reduction assays are serological methods used to confirm the identity of an unknown viral isolate. The procedures involve the use of a known dilution of specific neutralizing antiserum mixed with multiple dilutions of the homologous and suspect virus and subsequent observation 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 (TCID₅₀) 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 IHN, 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 25cm² flask, 0.1 mL of viral inoculum is usually sufficient.
 - i. Use EPC cell line for IHN, SVCV, and VHSV isolates.
 - ii. 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 IHN, 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 cm² flask this will be approximately 5 mL.
- d. Incubate control sample flasks to allow replication of the viruses.
 - i. For IHN, 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 °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°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°C, sample removal is not necessary.
 - f. Add 1 mL MEM-5 ([Section 2, 4.9.F “MEM-5/Hepes \(Tissue Culture Medium for all Ce3ll Lines Except SHK-1\)”](#)) per well.
3. Incubate at 15 to 18°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.

4.8 Indirect Fluorescent Antibody (IFAT) Procedure - 3

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

4.8 Indirect Fluorescent Antibody (IFAT) Procedure - 4

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.

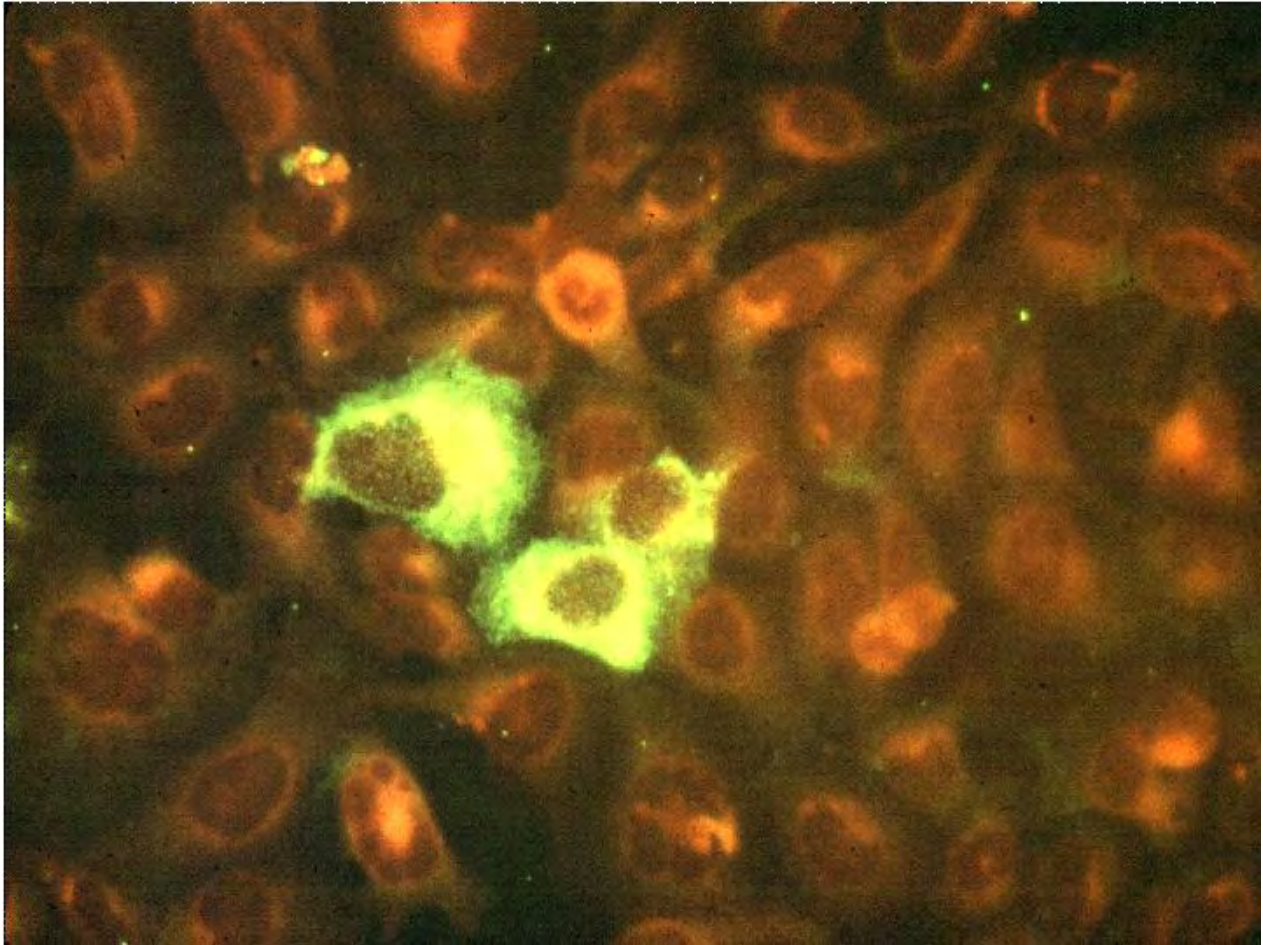


Figure 4.17. Typical fluorescence of positive IFAT for IHN virus on EPC cell monolayer. Photo courtesy of Scott LaPatra, Clear Springs Foods, Inc.

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 $\mu\text{g/mL}$)	
Fungizone	160.0 mL
250 $\mu\text{g/mL}$ amphotericin B	
205 $\mu\text{g/mL}$ desoxycholate	

Mix and ensure sterility. Store at 4° C.

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 $\mu\text{g/mL}$)	
Fungizone	160.0 mL
250 $\mu\text{g/mL}$ 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.

D. Versene (EDTA) (1:5000)

NaCl	8.0 g
KHPO ₄	0.2 g
KCl	0.2 g
Na ₂ HPO 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)

Tissue culture grade water	810.0 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)

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 Cell Line)

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 CO₂ 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.

TCID₅₀ - 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.

4.11 References

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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 ₂ O*		-	-	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

Master Mix for Nested or Second Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µL)	Volume for _____ samples
d-H ₂ O*		-	-	27.5 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	
(+)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	
Round 1 product		-	-	2 µL	-

*Add nuclease free water to Master Mix first, TAQ last.

4.A1.B.1 Worksheet B.1 - Infectious Hematopoietic Necrosis Virus (IHNV) - 2

Primer Sets for IHNV

	Forward	Reverse
1 st round	5'-TCA AGG GGG GAG TCC TCG A-3'	5'-CAC CGT ACT TTG CTG CTA C-3'
2 nd round	5'-TTC GCA GAT CCC AAC AAC AA-3'	5'-GCG CAC AGT GCC TTG GCT-3'

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1 st round				
2 nd 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)														
<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>	<u>9</u>	<u>10</u>	<u>11</u>	<u>12</u>	<u>13</u>	<u>14</u>	<u>15</u>
<u>16</u>	<u>17</u>	<u>18</u>	<u>19</u>	<u>20</u>	<u>21</u>	<u>22</u>	<u>23</u>	<u>24</u>	<u>25</u>	<u>26</u>	<u>27</u>	<u>28</u>	<u>29</u>	<u>30</u>

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 ₂ O*		-	-	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
1 st round	5'-AAAGCCATAGCCGCCCATGAAC-3'	5'- TCTCATCAGCTGGCCCAGGTAC-3'

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1 st round				

4.A1.B.2 Worksheet B.2 – Infectious Pancreatic Necrosis Virus (IPNV) - 2

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)														
<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>	<u>9</u>	<u>10</u>	<u>11</u>	<u>12</u>	<u>13</u>	<u>14</u>	<u>15</u>
<u>16</u>	<u>17</u>	<u>18</u>	<u>19</u>	<u>20</u>	<u>21</u>	<u>22</u>	<u>23</u>	<u>24</u>	<u>25</u>	<u>26</u>	<u>27</u>	<u>28</u>	<u>29</u>	<u>30</u>

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 ₂ O*		-	-	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
1 st 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
1 st round				

4.A1.B.3 Worksheet B.3 – Infectious Salmon Anemia Virus (ISAV) - 2

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)														
<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>	<u>9</u>	<u>10</u>	<u>11</u>	<u>12</u>	<u>13</u>	<u>14</u>	<u>15</u>
<u>16</u>	<u>17</u>	<u>18</u>	<u>19</u>	<u>20</u>	<u>21</u>	<u>22</u>	<u>23</u>	<u>24</u>	<u>25</u>	<u>26</u>	<u>27</u>	<u>28</u>	<u>29</u>	<u>30</u>

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 ₂ O*		-	-	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 LMBV

	Forward	Reverse
1 st 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
1 st round				

4.A1.B.4 Worksheet B.4 – Largemouth Bass Virus (LMBV) - 2

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)														
<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>	<u>9</u>	<u>10</u>	<u>11</u>	<u>12</u>	<u>13</u>	<u>14</u>	<u>15</u>
<u>16</u>	<u>17</u>	<u>18</u>	<u>19</u>	<u>20</u>	<u>21</u>	<u>22</u>	<u>23</u>	<u>24</u>	<u>25</u>	<u>26</u>	<u>27</u>	<u>28</u>	<u>29</u>	<u>30</u>

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 ₂ O*		-	-	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
1 st 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
1 st 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)														
<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>	<u>9</u>	<u>10</u>	<u>11</u>	<u>12</u>	<u>13</u>	<u>14</u>	<u>15</u>
<u>16</u>	<u>17</u>	<u>18</u>	<u>19</u>	<u>20</u>	<u>21</u>	<u>22</u>	<u>23</u>	<u>24</u>	<u>25</u>	<u>26</u>	<u>27</u>	<u>28</u>	<u>29</u>	<u>30</u>

4.A1.B.6 Worksheet B.6 – Spring Viremia of Carp Virus (SVCV)

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 ₂ O*		-	-	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 SVCV

	Forward	Reverse
1 st round	5'-TCA TTT AGA GCC ATA CG -3'	5'-GAA AGC ATG TTG AAG CT -3'

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1 st round				

4.A1.B.6 Worksheet B.6 – Spring Viremia of Carp Virus (SVCV) - 2

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)														
<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>	<u>9</u>	<u>10</u>	<u>11</u>	<u>12</u>	<u>13</u>	<u>14</u>	<u>15</u>
<u>16</u>	<u>17</u>	<u>18</u>	<u>19</u>	<u>20</u>	<u>21</u>	<u>22</u>	<u>23</u>	<u>24</u>	<u>25</u>	<u>26</u>	<u>27</u>	<u>28</u>	<u>29</u>	<u>30</u>

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 ₂ O*		-	-	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.

Master Mix for Nested or Second Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µL)	Volume for ____ samples
d-H ₂ O*		-	-	27.5 µL	
PCR bffer (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	
(+)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	
Round 1 poduct		-	-	2 µL	-

*Add nuclease free water to Master Mix first, TAQ last.

4.A1.B.7 Worksheet B.7 – Viral Hemorrhagic Septicemia Virus (VHSV) - 2

Primer Sets for VHSV

	Forward	Reverse
1 st round	5'-TCT CTC CTA TGT ACT CCA AG-3'	5'-TTC CGG TGG AGC TCC TGA AG-3'
2 nd round	5'-ATG GGC TTC AAG GTG ACA C-3'	5'-GTA TCG CTC TTG GAT GGA C-3'

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1 st round				
2 nd 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)														
<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>	<u>9</u>	<u>10</u>	<u>11</u>	<u>12</u>	<u>13</u>	<u>14</u>	<u>15</u>
<u>16</u>	<u>17</u>	<u>18</u>	<u>19</u>	<u>20</u>	<u>21</u>	<u>22</u>	<u>23</u>	<u>24</u>	<u>25</u>	<u>26</u>	<u>27</u>	<u>28</u>	<u>29</u>	<u>30</u>

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-mm² 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 mm² x 0.1 mm or 0.1 mm³ or 10⁻⁴ cm³. Since 1 cm³ is approximately equal to 1 mL, the cell concentration/mL is the average count per square x 10⁴. 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 cm² 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 cm² 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/mm² (1×10^5 to 2×10^5 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 $\times 10^4 \times$ Trypan Blue dilution factor.
 - b. Total cells in flask = cells/mL $\times$ total volume of cell suspension.

Example: Total number cells counted in 10 squares = 300 cells
 x count/square = 300 cells/10 squares = 30 cells

$$\begin{aligned}\text{cells/mL} &= 30 \times 10^4 \times 2 \text{ (dilution factor)} \\ \text{cells/mL} &= 60 \times 10^4 \text{ cells/mL} \\ \text{cells/mL} &= 6.0 \times 10^5 \text{ cells/mL}\end{aligned}$$

$$\begin{aligned}\text{Total cells} &= 6.0 \times 10^5 \text{ cells/mL} \times 8 \text{ mL (original volume cell suspension)} \\ \text{Total cells} &= 48.0 \times 10^5 \text{ cells} \\ \text{Total cells} &= 4.80 \times 10^6 \text{ cells}\end{aligned}$$

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.

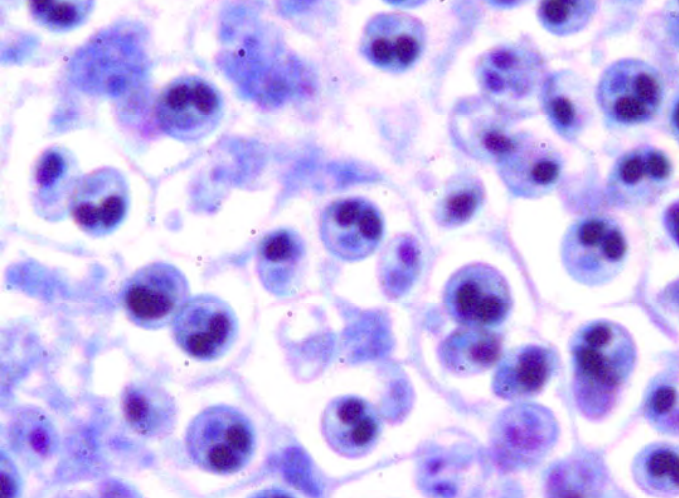
- a. mL medium needed = (actual cells/mL) (vol. of cell suspension)/ desired cells/mL

Example: actual count = 6×10^6 cells/mL
 desired count = 1×10^6 cells/mL
 volume of cell suspension = 8 mL

mL medium needed = x

$$\begin{aligned}x &= \text{mL medium needed} = 6 \times 10^6 \text{ cells/mL} \times 8 \text{ mL} / 1 \times 10^6 \text{ cells/mL} \\ \text{mL medium needed} &= 48 \times 10^6 \text{ mL} / 1 \times 10^6 \\ &= 48 \text{ mL}\end{aligned}$$

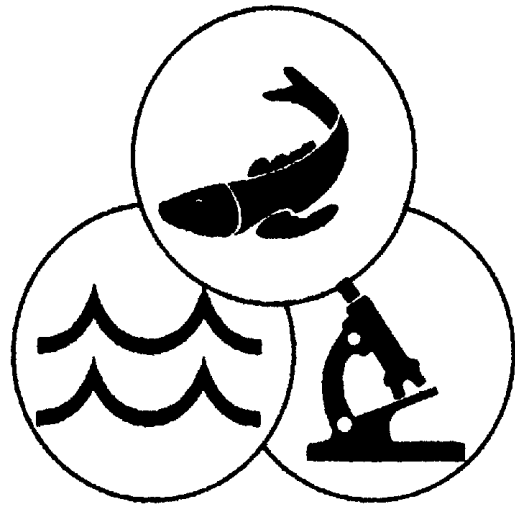
Since you have 8 mL already in the flask, you would need to add 40 mL of medium to the flask before splitting to get the recommended seeding cell density for each new culture.



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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

5.2 *Myxobolus cerebralis* (Whirling Disease) - 2

- 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”](#)) 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.

5.2 *Myxobolus cerebralis* (Whirling Disease) - 3

- 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.

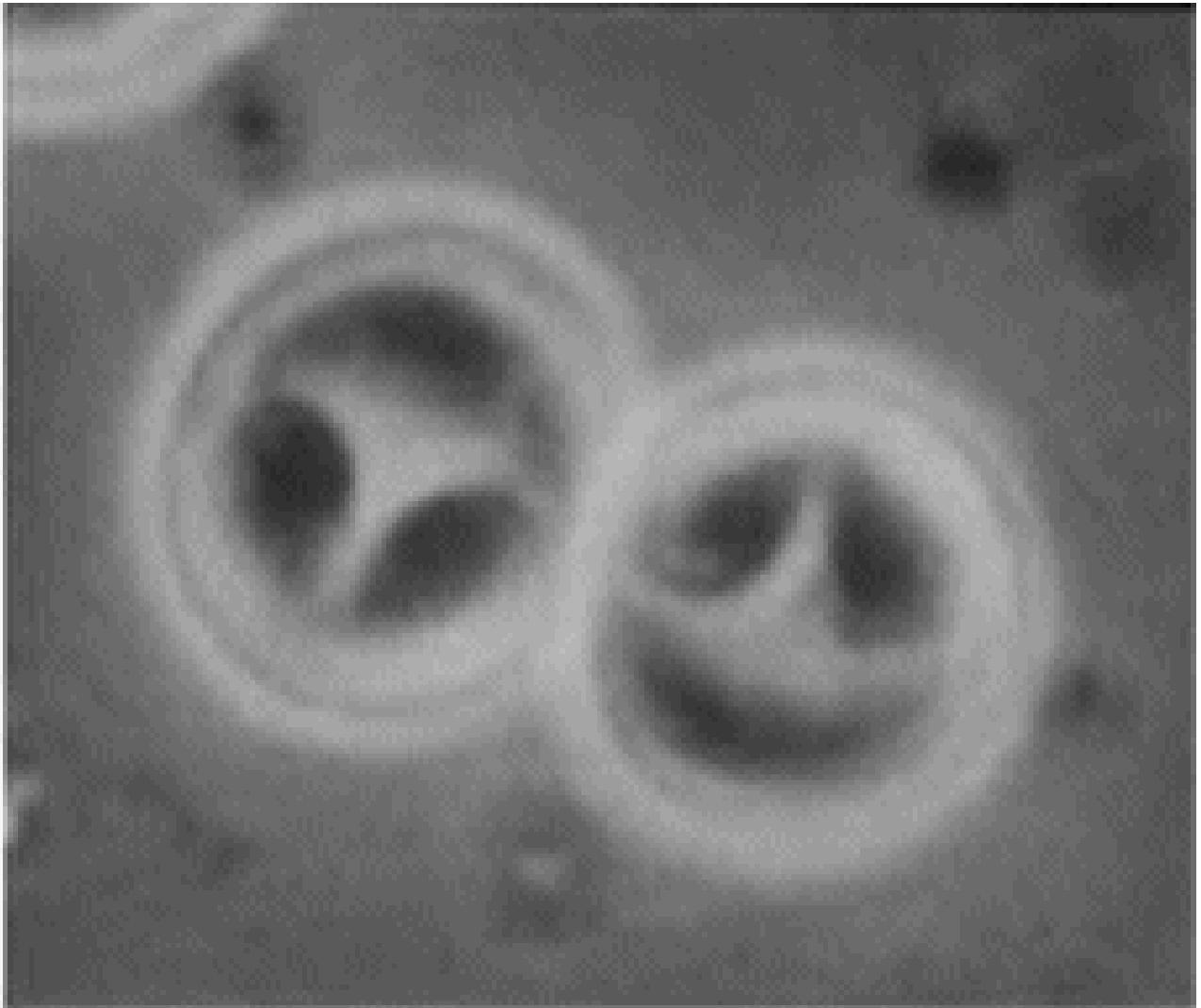


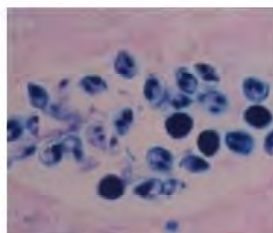
Figure 5.1. Spores of *Myxobolus cerebralis*. Photograph courtesy of R. P. Hedrick.

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



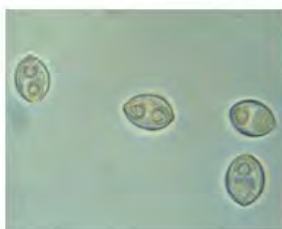
Myxobolus neurobius

L 10 - 14 x W 8 - 9.2 μm



Myxobolus squamalis

L 8.4 - 9.9 x W 7.7 - 9.9 μm



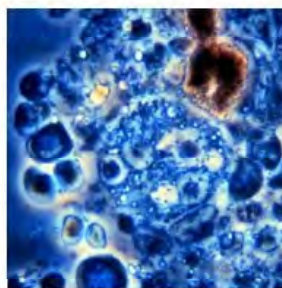
Myxidium minteri

L 9.3 - 12.6 x W 4.5 - 5.3 μm



Ceratomyxa shasta

L 14 - 23 x W 6 - 8 μm



PKX organism



Ceratomyxa shasta trophozoites

B. Confirmatory Tests

Confirmation of *M. cerebralis* should rely on either histopathology or the nested version of the polymerase chain reaction (PCR).

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

5.2 *Myxobolus cerebralis* (Whirling Disease) - 7

(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.

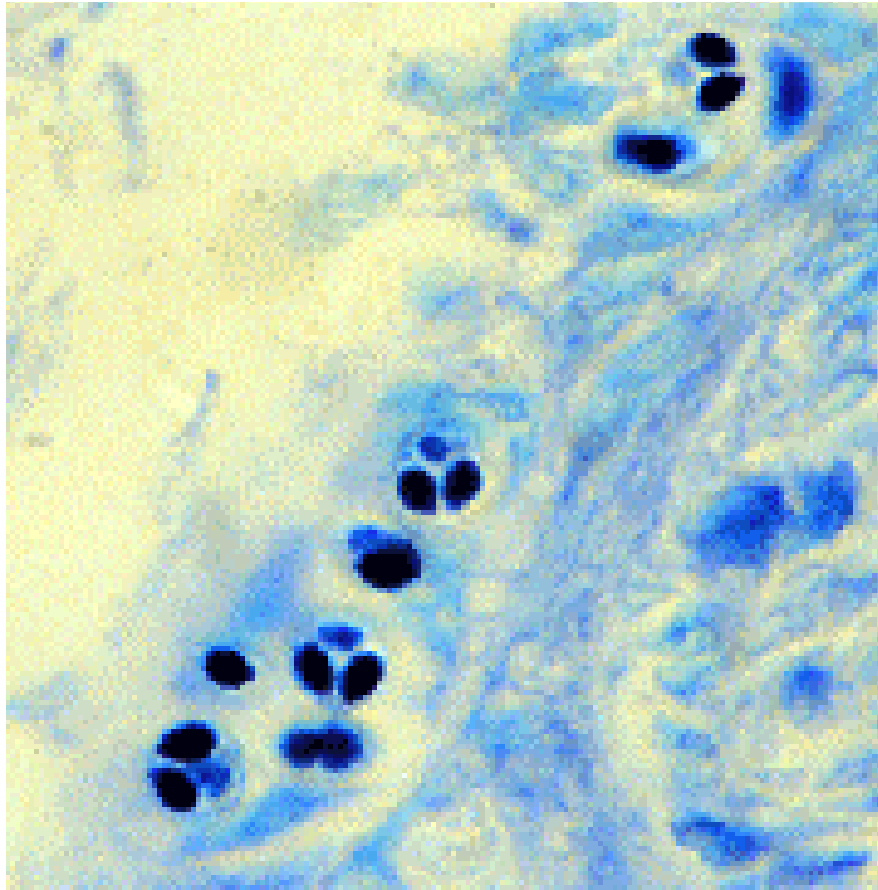


Figure 5.3. Histological sections from a fish infected with *Myxobolus cerebralis*. High magnification showing stained spores (note darkly stained polar capsules). Photograph courtesy of H. V. Lorz.

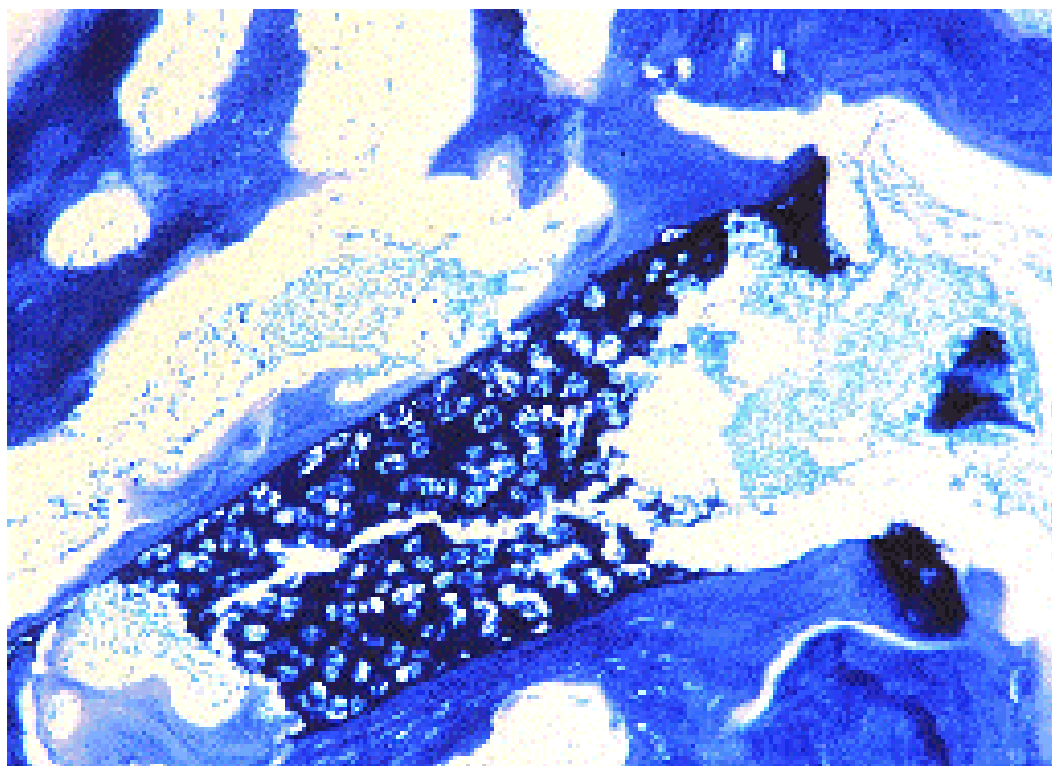


Figure 5.4. Histological sections from a fish infected with *Myxobolus cerebralis*. Low magnification showing degradation of cartilage (Leishman-Giemsa stain).

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:

5.2 *Myxobolus cerebralis* (Whirling Disease) - 9

- 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	(17.86 mg/mL)
(See Section 2, 5.6.E “Proteinase K.”)	
150 mg small glass beads (optional)	710 – 1180 μ m, 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 μ L 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 μ L 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 μ L.

- 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 μ L 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 μ L 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 μ L of Qiagen Buffer ATL and 20 μ L 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 contaminants 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 μ L of chloroform.
 - viii. Mix by inverting the sample several times and centrifuge at 1700 x g.
 - ix. Add 800 μ L 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 μ L 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° Master Mix: 50 µL total reaction volume

H ₂ O – 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 2nd 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 2nd MM and load the thermal cyclor 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 12°C, 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 mm² 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 μ m. Photograph courtesy of J. L. Bartholomew.



Figure 5.2. Wet mount of myxospore stage of *C. shasta*. Bar equals 10 μ m. 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 spectrophotometer 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 ₂ O – 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

5.3 *Ceratomyxa Shasta* (Ceratomyxosis) - 4

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)
<i>Taq</i> 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 “Detection of Product”](#)), 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).

5.4 *Tetracapsula bryosalmonae* (Proliferative Kidney Disease) - 2

- b. To determine the appropriate concentration of lectin, dilutions of 25 to 0.5 $\mu\text{g mL}^{-1}$ 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 $\mu\text{g mL}^{-1}$ 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 μm X 7 μm and have two spherical polar capsules 2 μm in diameter ([Figure 5.6](#)).

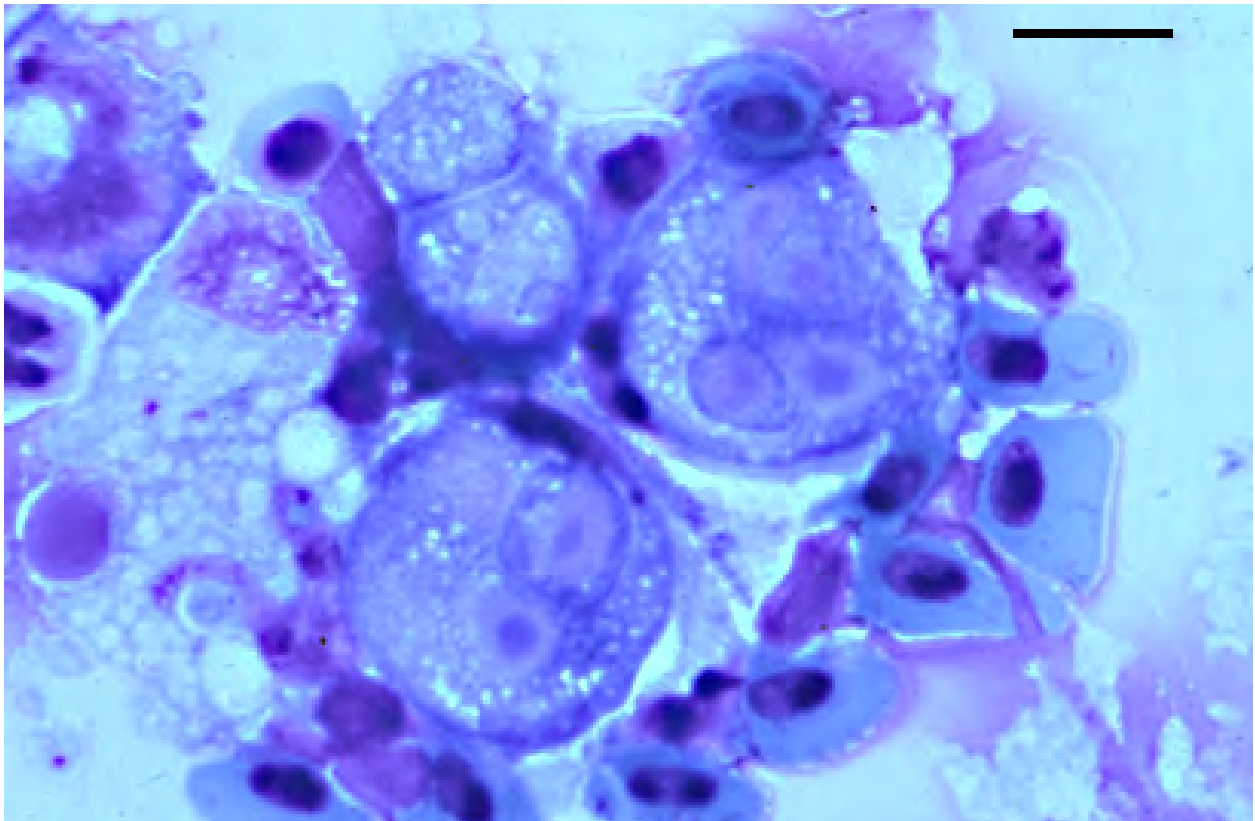


Figure 5.1. Multicellular *Tetracapsula bryosalmonae* in a Leishman-Giemsa stained imprint. Bar is 10 μ m. Photograph courtesy of R. P. Hedrick.

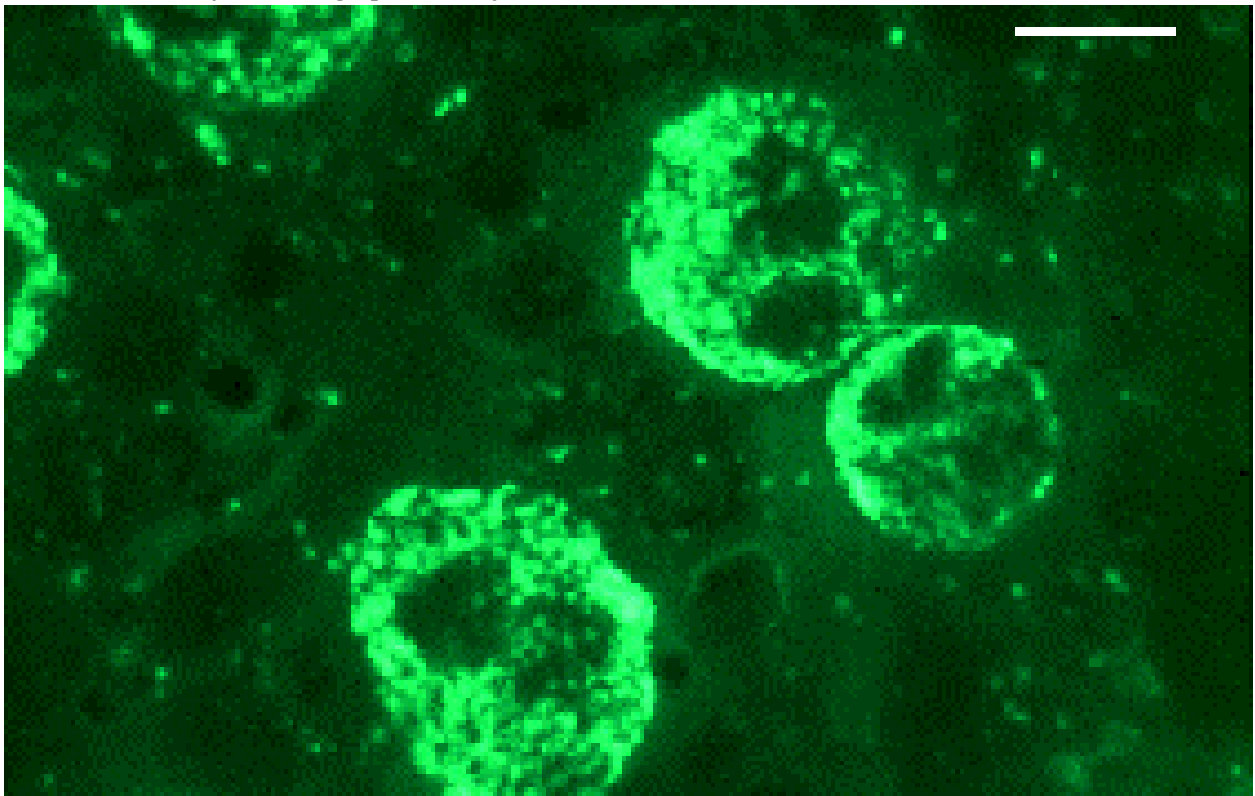


Figure 5.2. Multicellular *Tetracapsula bryosalmonae* in a lectin-stained imprint. Bar is 10 μ m. Photograph courtesy of R. P. Hedrick.

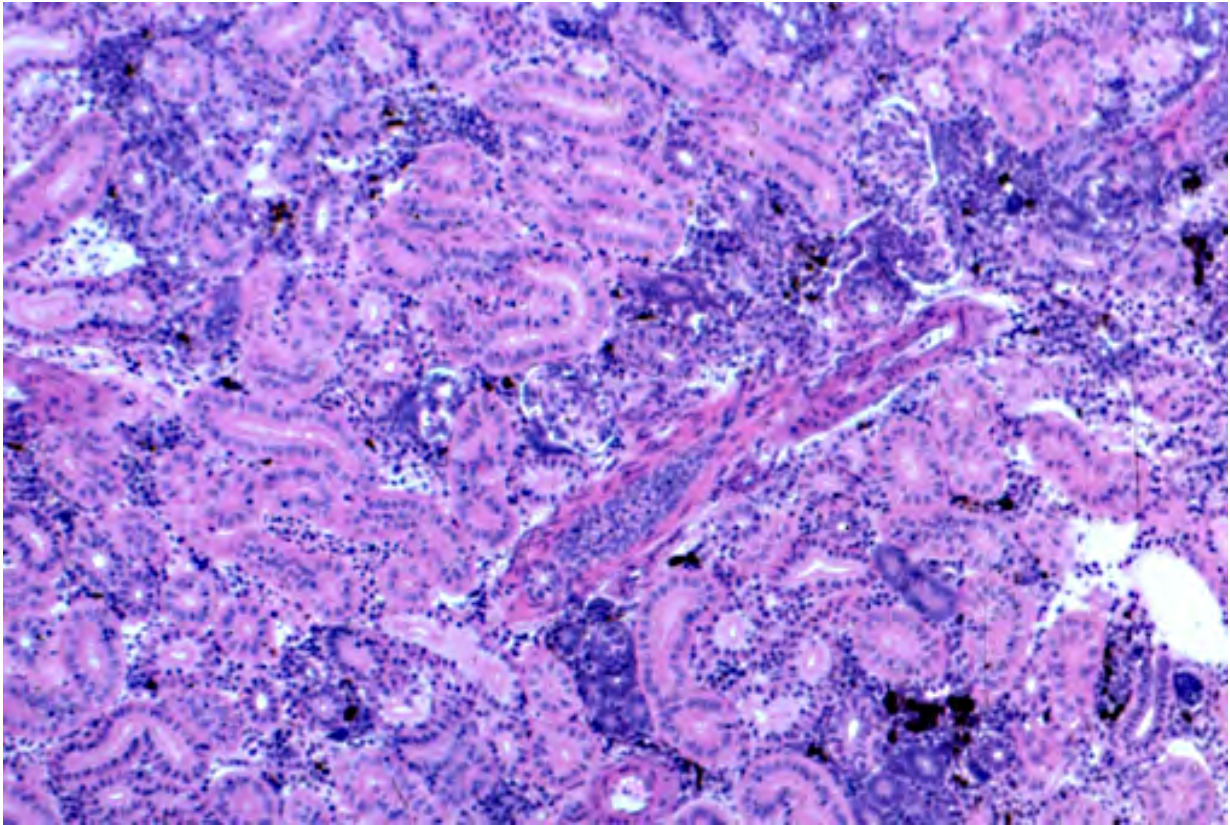


Figure 5.3. Histological sections of kidney tissue infected with *T. bryosalmonae* at low magnification of normal kidney. Photographs courtesy of R. P. Hedrick.

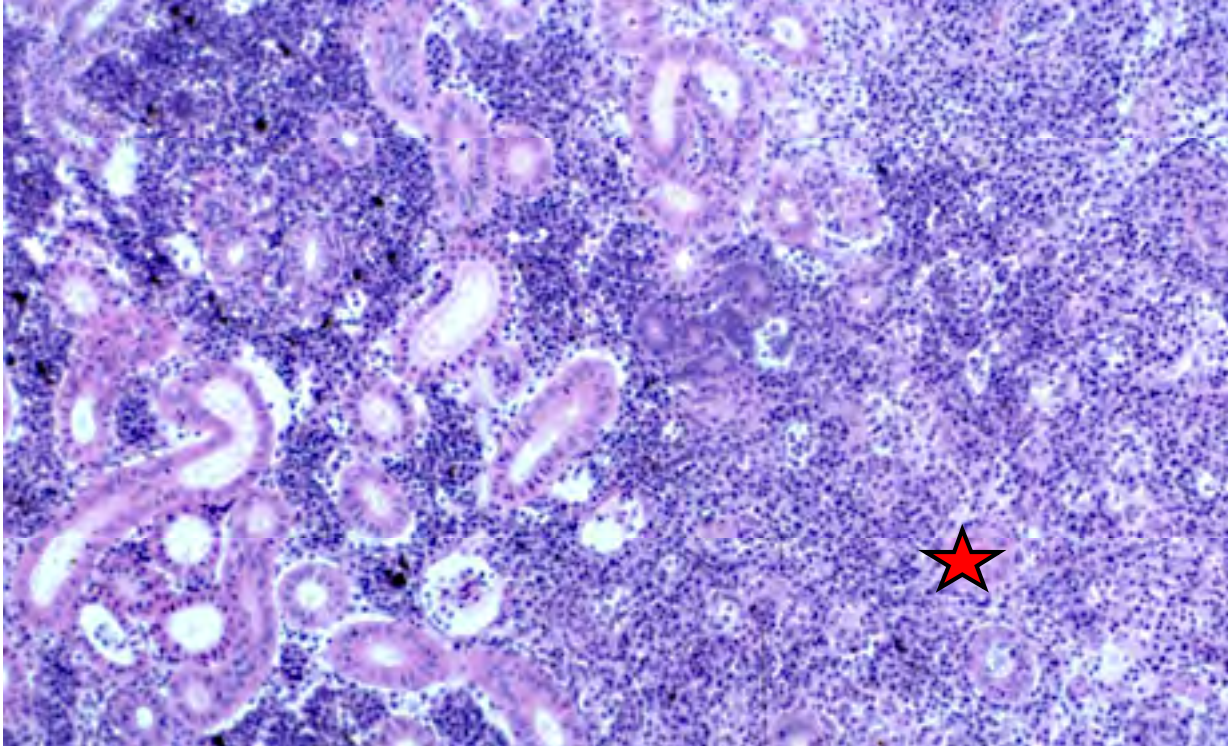


Figure 5.4. Histological sections of kidney tissue infected with *T. bryosalmonae*. at low magnification showing area of inflammatory cell infiltrate indicated by the star. Photographs courtesy of R. P. Hedrick.

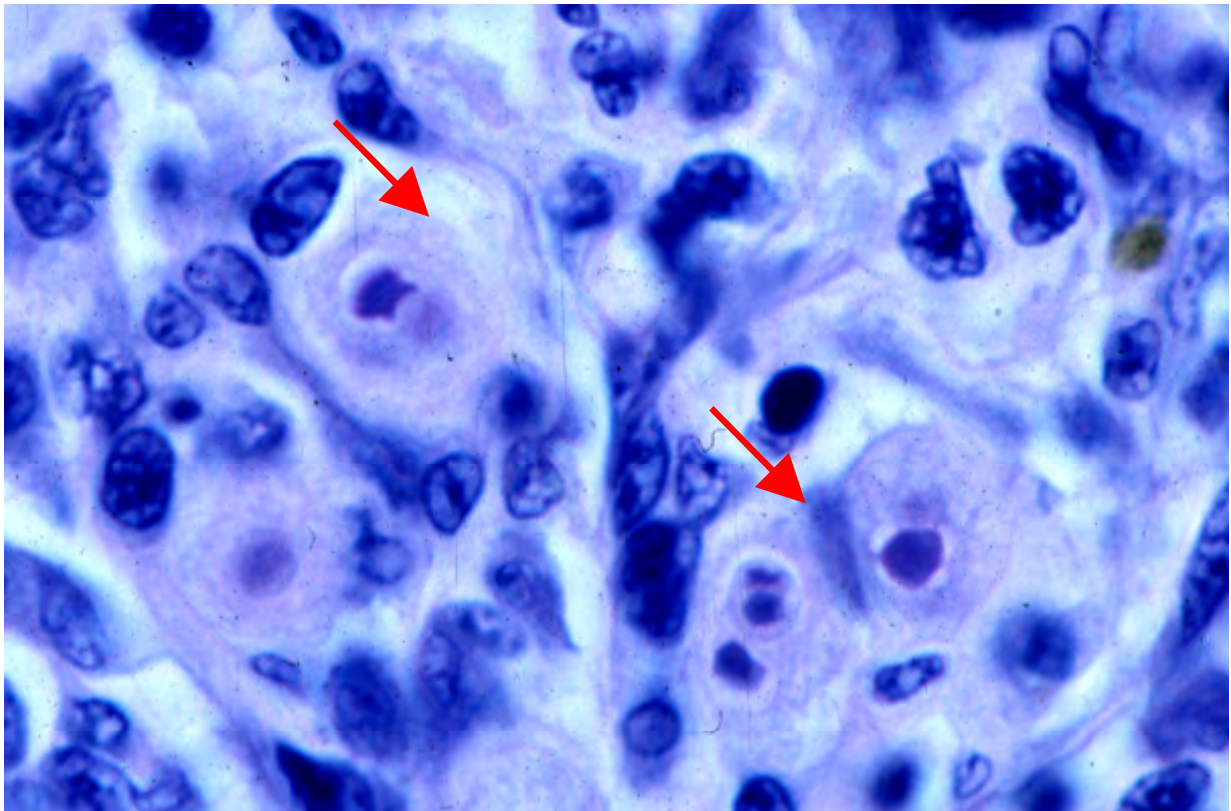


Figure 5.5. Histological sections of kidney tissue infected with *T. bryosalmonae*. at high (400X) magnification of parasites in tissue. Photographs courtesy of R. P. Hedrick.

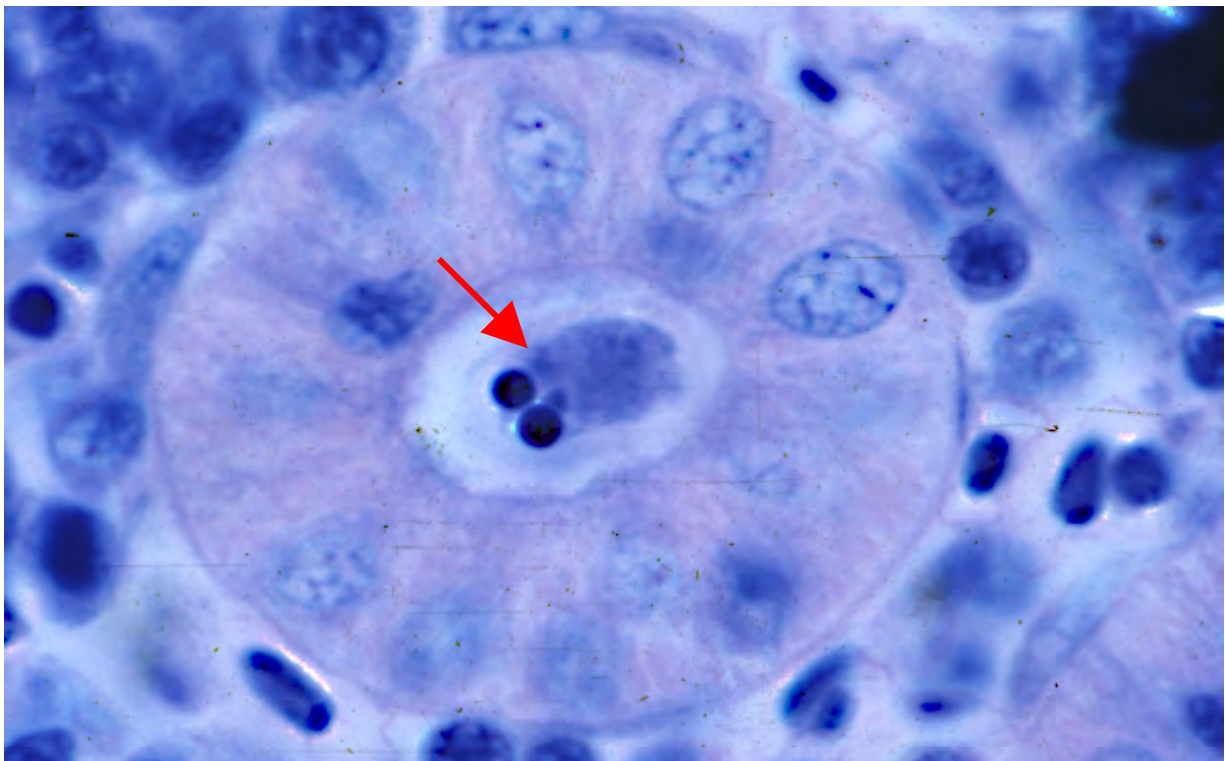


Figure 5.6. Histological sections of kidney tissue infected with *T. bryosalmonae*. sporogonic stage in kidney tubule (1000X). Photographs courtesy of R. P. Hedrick.

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 M 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 ₂ O – 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)

5.4 *Tetracapsula bryosalmonae* (Proliferative Kidney Disease) - 7

6-Reverse primer (20 µM)	2.0 µL (final concentration 0.8 µM)
<i>Taq</i> 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 25°C.

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.

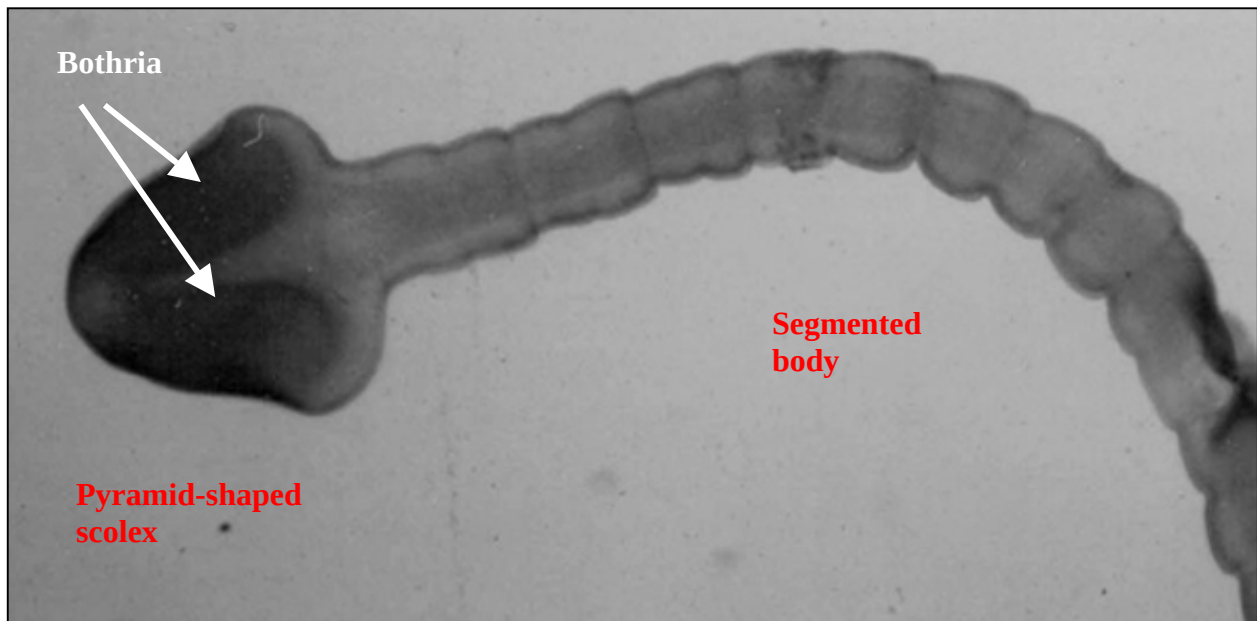


Figure 5.1. *Bothriocephalus acheilognathi* showing a pyramid shaped scolex, segmentation, and two bothria. Photograph courtesy of A. Mitchell.

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 Solution

EDTA	0.2 g
NaCl	8.0 g
KCl	0.2 g
KH ₂ PO ₄	0.2 g
NaHPO ₄	1.15 g
Trypsin	5.0 g
Sterile distilled water	to 1000 mL
Store at 4°C.	

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 ₂ PO ₄)	0.34 g
Dipotassium phosphate (K ₂ HPO 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 (MgCl₂)

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.

5.8 References

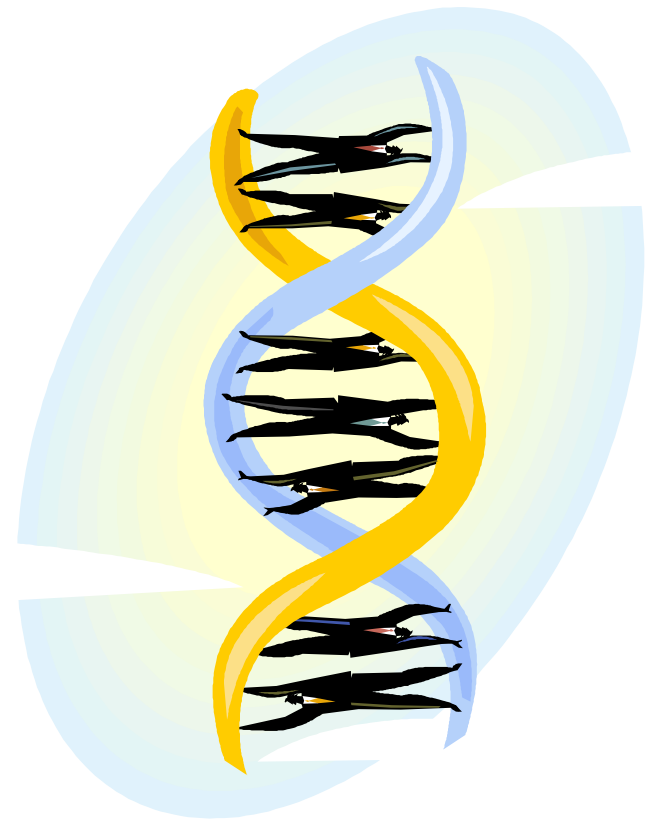
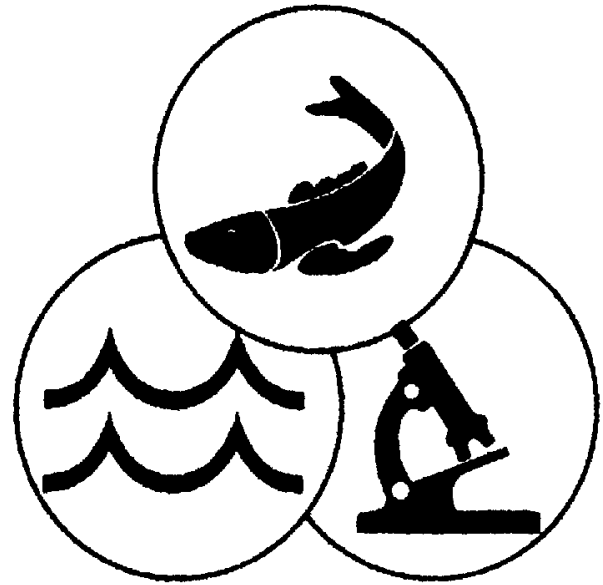
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Section 2

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 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/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 pipettors, 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 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.
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.

9. Primers

Newly received primer batches should first be tested on known positive/negative controls.

10. 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°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 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°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	100 mg
	DI water	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 - <u>Working Solution</u> (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:

<u>Primer Length</u>	<u>ng of Primer Equal to 10 pmol</u>
15	50
16	53
17	56
18	59
19	63
20	66
23	78
24	80

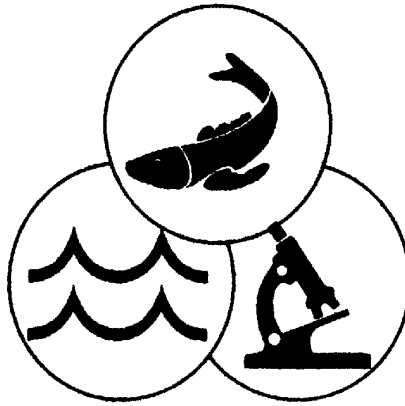
6.6 References

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](#)**

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 affiliation 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 eight 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 Fish 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).
2. The committee will be co-chaired by one individual from each organization.
3. The terms of these committee members shall be three consecutive years.
 - a. Terms shall begin two months prior to the individuals first annual meeting (July).
 - b. Terms shall conclude 10 months after the individuals 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 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 new 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.** 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 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.
- B.** 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.
- C.** 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.
- D.** 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.

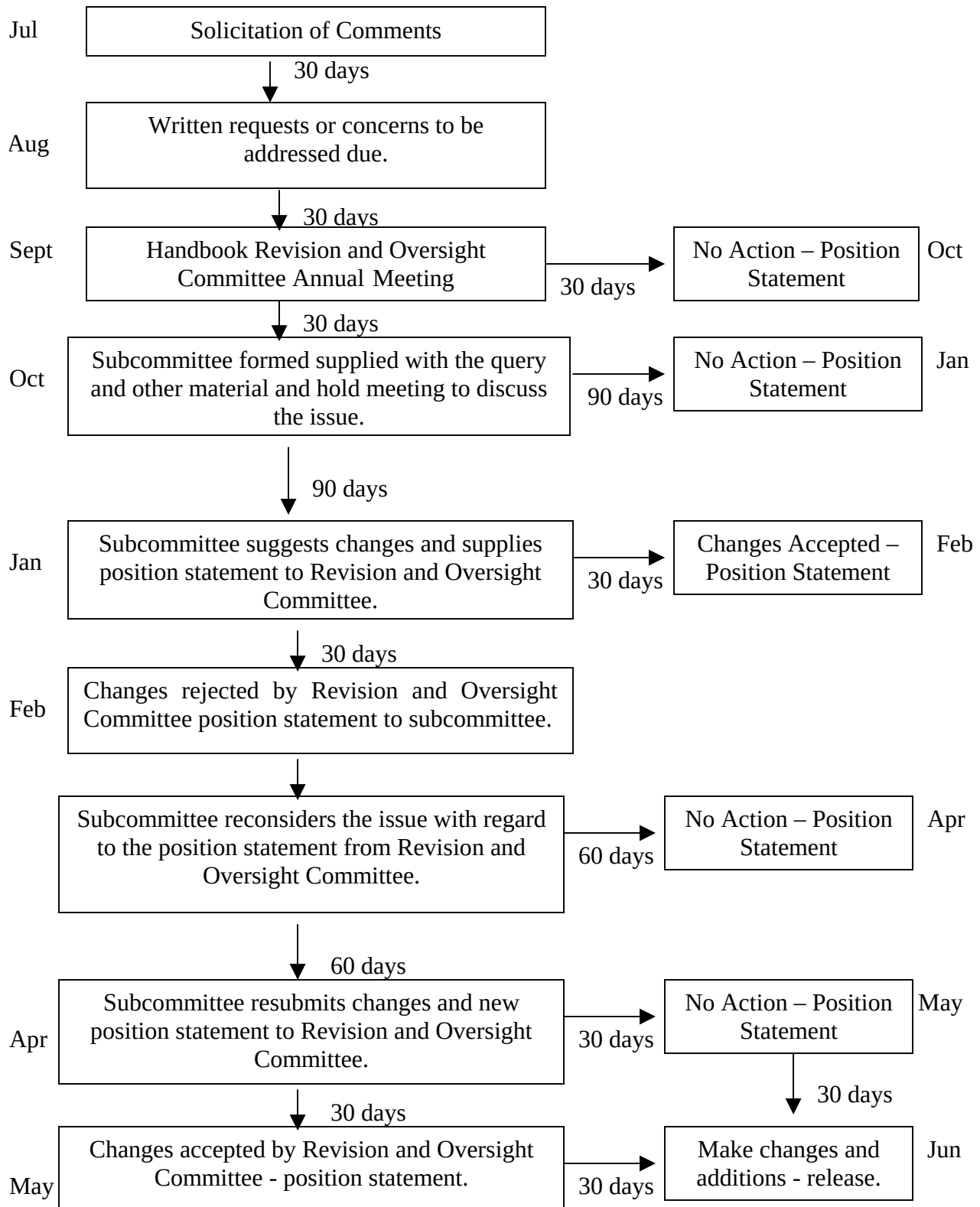
A1.3 Process for Amending the Handbook - 2

- 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.
- E.** 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 (February) 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.

A1.3 Process for Amending the Handbook - 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

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.

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.

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 led 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 (immunofluorescence 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 too long, but at this time there is no available information to make a change in the incubation time.

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 sensitive but not necessarily specific 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.

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.

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.

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.

Citation Guidelines

A. Chapters in Section 1 of this document may be cited as follows:

Bartholomew, J. L. 2003. Salmonid ceratomyxosis. *In: Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens*. Blue Book 5th Edition, 2003, Fish Health Section, American Fisheries Society.

Johnson, K. 1994. Lemaeid parasitism. *In: Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens*. Blue Book 5th Edition, 2003, Fish Health Section, American Fisheries Society.

B. Section 2 of this document may be cited as:

USFWS and AFS-FHS. 2003. Standard Procedures for Aquatic Animal Health Inspections. *In: Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens*. Blue Book 5th Edition, 2003, Fish Health Section, American Fisheries Society.

C. The document in its entirety may be cited as:

AFS-FHS Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens. Blue Book 5th Edition, 2003, Fish Health Section, American Fisheries Society.



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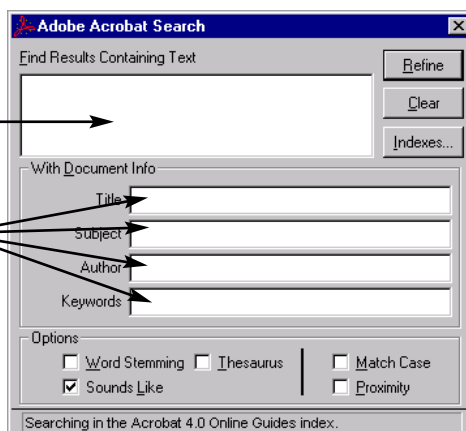
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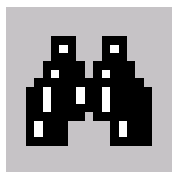
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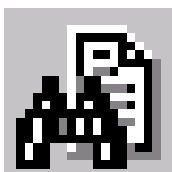




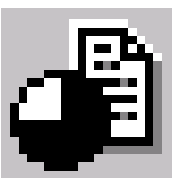
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