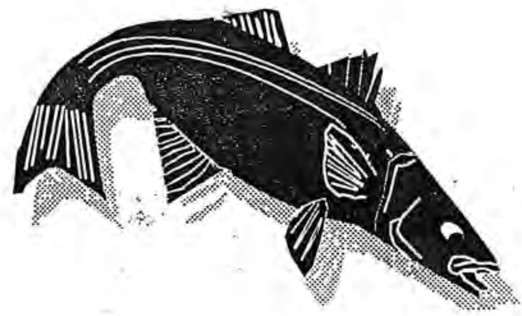
# BLUE BOOK

**Version 1**

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



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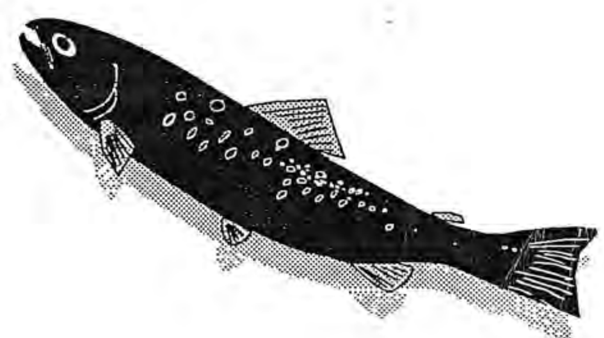

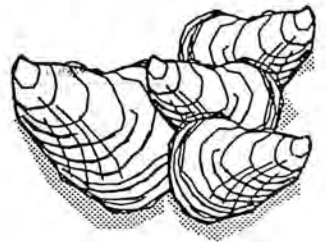

**John C. Thoesen**
**Editor**

Fourth Edition

1994

Fish Health Section

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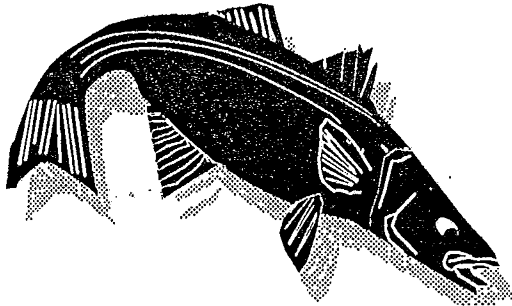

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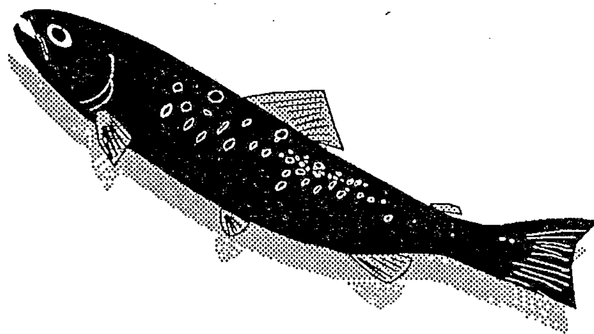

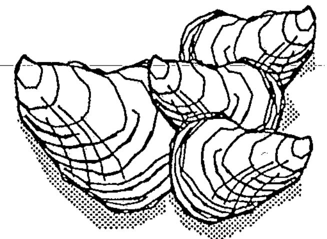

**John C. Thoesen**
**Editor**

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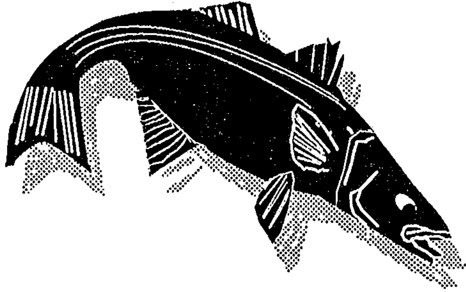

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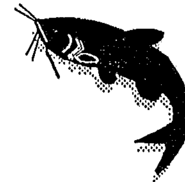

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# Preface

**As you will notice this 4th edition of the American Fisheries Society, Fish Health Section, Blue Book, uses an entirely different approach than in the past.**

Until now, previous Blue Book revisions meant reproduction of the complete volume, often leading to several years between updates. With the new loose-leaf format any section can be updated and distributed to Blue Book owners soon after being written.

One of the major concerns with this approach was the legality of such an edition. This concern was carefully researched and put before a lawyer who indicated that a loose-leaf edition is as legal in a courtroom as a bound one.

Getting revisions to Blue Book owners and the ability to reference new versions were other important concerns. The update method will be very simple and allow anyone to reference the correct Blue Book version. After a revision to the Blue Book has gone through the Blue Book Advisory Committee and been approved by the Technical Procedures Committee it will be ready to go to Blue Book owners. Each revision will have instructions on what sections to remove from the book and where to insert replacement sections. For convenience, a list of revisions will be filed in the back of the book allowing for a record of each revision. Each revision will also be dated at the bottom of each page to indicate that a particular section is an update. Each revision will also change the version of the Blue Book, therefore allowing it to be referenced accurately. For example, this first printing of the 4th edition is Version 1, the first revision will be Version 2, the second will be Version 3 etc. Each revision will be announced in the Fish Health Section Newsletter as well as American Fisheries Society Meetings. As professionals, it will also be our responsibility to keep up with revisions.

Another important concept of the fourth edition is the expansion to include shellfish diseases. For the first time, modern detection methods for both finfish and shellfish are assembled into one volume.

All these changes should prove to be a very useful method for keeping the increasingly changing field of fish health up to date.

# Acknowledgements

**I would like to take this opportunity to thank some very important people whose diligence and dedication helped make this edition of the Blue Book possible.**

First of all I would like to recognize and thank Diane Elliott, Jack Frimeth, Jack Ganzhorn, Chris Horsch, Scott LaPatra, and Steve Roberts, who made up the Blue Book Field Advisory Committee and who spent many hours in developing this edition.

I would like to thank all the authors who spent a great deal of time writing and rewriting the many drafts of their respective chapters.

I thank Ray Brunson, Dennis Anderson, Scott Foott, John Coll, Phyllis Barney, Dave Locke, John Schachte, Joe Marcino, Pete Walker, Kent Hauck, Emmett Shotts, Steve Kaattari, Ron Pascho, Frank Hetrick, Phil McAllister, Randy MacMillan, Doug Ramsey, Pete Taylor, John Cvitanich, and Mahesh Kumar, who took the time to review and comment on this document before publication.

Last but certainly not least I thank the Technical Proedures Committee made up of Rodney Horner, Tom Schwedler, John Schachte, and Kevin Amos for their review from a procedures and consistency approach. Also real effort was put forth by a committee appointed by Randy MacMillan to finalize the very controversial sampling chapter. This committee chaired by Paul Reno included Kevin Amos, Ron Goede, John Marcino and John Coll.

*My thanks and appreciation are extended to all of you.*

John C. Thoesen
Editor

# A Standard Inspection Procedure for Detection for Certain Pathogens in Salmonid Fish

## INTRODUCTION

Many Federal, Regional and State agencies require that prior to movement of salmonid fishes into or from a facility or watershed, overtly healthy fish must be inspected for the presence of certain pathogens. In addition, commercial aquaculture facilities are often interested in determining the health status of their stocks or stocks to be purchased. Natural resources agencies have a vested interest in determining the health status of feral stocks in watersheds within their purview. Most often the inspection process is limited to certain known pathogens of salmonids. The pathogens are those which currently, or in the past, have been known to be responsible for severe epizootics of disease and have not been found to be enzootic nationally. These pathogens are known to exist in a carrier state and therefore have the potential for generating epizootics of clinical disease under the appropriate conditions. Fortunately, these pathogens can be detected and identified in the absence of clinical signs of disease.

There is a well recognized group of pathogens included in this list. They are: infectious hematopoietic necrosis virus (IHNV), which is the etiologic agent of infectious hematopoietic necrosis; infectious pancreatic necrosis virus (IPNV), the agent of infectious pancreatic necrosis; the virus of hemorrhagic septicemia (VHS) responsible for egtved disease; *Aeromonas salmonicida* which causes furunculosis; *Yersinia ruckeri,* etiologic agent of enteric redmouth disease; *Renibacterium salmoninarum,* the bacterial kidney disease organism; and *Myxobolus cerebralis*; the agent of whirling disease. While other pathogens cause serious diseases in salmonids, they are often widely distributed and cannot be constrained or controlled because of their ubiquitous nature (such as the pathogenic *Flexibacter* species), and thus are not the focus of inspection procedures.

Various chapters of this manual describe accepted techniques for the isolation and/or identification of etiologic agents responsible for epizootics of salmonids and other fishes, as well as for invertebrates. For salmonid fishes, the methods for diagnosing IHN, IPN, VHS, furunculosis, enteric redmouth disease, and whirling disease are described in the appropriate chapters of this Manual (for bacterial pathogens: Chapters I, II, VII, IX, XIV; for viral pathogens: Chapters I, V, VI, VIII; for parasites: Chapters I and VI).

This Chapter describes a standardized procedure for the detection of specific pathogens in apparently healthy salmonids for the fish health inspection process. It is designed to provide

a single acceptible regime for the detection of the included pathogens and will promote standardization of techniques for fish health inspections.

While most of the techniques outlined in this chapter follow the procedures described in the other Chapters of this Manual which apply to the diagnosis of certain salmonid diseases, the fish health inspection format differs from disease diagnosis in a number of ways. In the case of inspections for pathogens in the absence of disease, far fewer numbers of the pathogen will be present than in diseased animals, and it is likely that the prevalence of infection will be lower than in enzootic or epizootic situations. Therefore, sampling and detection techniques must be used which maximize the chances of detecting these small numbers of organisms. In addition, the sampling process is different because there usually are no clinical signs to help narrow the search to certain pathogens. Thus, it was felt that a compilation of methods based on the diagnostic protocols outlined in this Manual would be helpful in aiding fish health professionals in carrying out fish health inspections.

When a report is issued describing the absence or presence of particular pathogens in a population of salmonids, it must be made clear which methods were used in the inspection process, since there are a number of methods and variations that are employed by fish health professionals. Those who are engaged in performing these inspections are urged to thoroughly understand the specific requirements of the jurisdiction or "customer" prior to starting the process of inspection. The use of this standard procedure may serve to allow comparison of results generated among different users.

# PART 1 : DEFINITIONS

## A. Pathogen Groups

Pathogens are divided into two basic groups

**Group 1** Consists of those fish pathogens most likely to be both vertically and horizontally transmitted or to be associated with distinct spawning populations, species and age groups as well as water supply. This group consists of the following:

Viruses: Virus causing hemorrhagic septicemia (VHSV), infectious hematopoietic necrosis virus (IHNV), and infectious pancreatic necrosis virus (IPNV),

Bacteria: *Renibacterium salmoninarum*

**Group 2** Consists of those pathogens most likely to be horizontally transmitted or to be associated with water sources. This group consists of the following:

Bacteria: *Aeromonas salmonicida, Yersinia ruckeri*

Parasite: *Myxobolus cerebralis*

## B. Size/Age Groups

Environmental and species differences can markedly affect the growth rate of salmonids. In addition, some pathogens are most readily detected at a certain size, whereas others are most readily detected at a certain age. Therefore, classifying fish for the purposes of sampling is difficult. For the purpose of fish health inspections, salmonids shall be assigned into four size groups for detection of viral and bacterial pathogens, and by age for *Myxobolus cerebralis*. The four size groups to be sampled for viruses and bacteria are as follows:

**Size Group 1** — Less than or equal to 4 cm (fry).

**Size Group 2** — Four to 6 cm (fingerlings)

**Size Group 3** — Non-brood fishes greater than 6 cm (yearlings/adults)

**Size Group 4** — Sexually mature fish used as brood stock.

For detection of *Myxobolus cerebralis*, young fish, approximately 4-12 months old are sampled. Fish continuously held in water at a temperature of ≥13°C may be sampled at 3 months of age; for fish held continuously in water at temperatures ≤ 12°C, fish older than 8 months are to be sampled. Sampling of fish older than 2 years is difficult since ossification of the skeleton may obscure the spores of the parasite.

## C. Definition of Lots

A sample lot for size group 1, 2 and 3 (non-brood facilities) is defined as a group of fish of the same species and age that originated from the same spawning stock and share a common water supply.

A sample lot for size group 4 is defined as a group of fish of the same species that originated from the same spawning stock and share a common water supply, but several age groups (e.g., 3,4, and 5 year old brood fish) may be combined to form a representative composite lot for lethal sampling.

## D. Sample Size

The number of fish selected for a fish health inspection shall be based upon an assumed prevalence level of infection of 5% for any of the listed pathogens. Table 1 indicates the number of animals required to detect at least one infected animal in a population with 95%

| Sample size based on an assumed prevalence level of 5% of the population | |
|---|---|
| **Lot Size (number of fish)** | **Number of fish required for sample** |
| 50 | 35 |
| 100 | 45 |
| 250 | 50 |
| 500 | 55 |
| ≥ 2,000 | 60 |

(More complete chart in FHL Procedures Manual)

confidence. If the population size is estimated to be between two grouping levels, sample from the next higher population class.

The sample of randomly selected animals to be collected shall include, in addition to the requisite number of fish listed above, fresh mortalities and moribund fish. Moribund fish or fresh mortalities shall be processed as for diagnosing clinically diseased fish as described in the appropriate chapters of this Manual. Fish without signs of disease must be alive when collected and shall be processed as soon as possible after collection, and in no case more than 48h post collection. If specimens are not maintained alive before processing, samples shall be stored on ice (0°C) **but not frozen.**

# PART II : PROCEDURES

## A. Necropsy Procedure

The appropriate chapters of this manual describe generally accepted techniques for the detection and identification of all of the pathogens for which inspections are usually performed (for bacterial pathogens: Chapters I, II, VII, IX, XIV; for viral pathogens: Chapters I, V, VI, VIII; for parasites: Chapters I and VI). In this section, a specific protocol for collecting appropriate samples for the various pathogens is described, in contrast to the more general descriptions of the methods for detection of clinically diseased individuals. There are four aseptic stringency levels for samples to be taken from each fish:

1. Samples for isolation of bacterial pathogens to be grown on bacteriological media, which must be collected **aseptically.**
2. Samples for virology are inoculated onto cell cultures, but decontaminated prior to application to the cells. These samples do not require aseptic handling, but should be collected as cleanly as possible to reduce the chance of contamination.
3. Samples for immunodetection of *Renibacterium salmoninarum* by fluorescent antibody testing need not be collected aseptically. These samples, however, shall be collected to prevent the potential cross contamination by immunocrossreactive bacteria in feed or intestinal contents.
4. Samples for parasite analysis, which are not collected aseptically.

This protocol is arranged into sections that approximately follow the time course of the fish health monitoring process itself: necropsy procedure in the field, then descriptions of the further processing which would occur in the laboratory. Once the samples from each individual animal have been collected and pooled as appropriate, they are then further processed by the methods described in **Sections B, C, and D** of this Chapter.

### 1. Preparation and selection of samples.

All fish must be first evaluated for the presence of gross external lesions, such as skin lesions, exophthalmos, hemorrhage, lordosis or scoliosis, ascites, darkening, etc., Fish with gross lesions shall be processed separately from the remainder of the samples for a lot. Isolation and confirmation of the etiologic agent, if any, responsible for the lesions shall be performed as described in the appropriate section of this

Manual (for bacterial pathogens: Chapters I, II, VII, IX, XIV; for viral pathogens: Chapters I, V, VI, VIII; for parasites: Chapters I and VI).

The samples for bacterial isolation shall be collected first, followed by samples for viral isolation, and finally for parasitological examination. Samples from individual fish are assayed for bacterial isolation or immunoidentification of *Renibacterium salmoninarum*, while samples for virology are grouped into pools of five fish or fewer for ease of assay. For parasitological examination tissues from individuals are pooled into appropriate weight groupings (see this Chapter, **Part II. A. 2. d.**, *M. cerebralis*).

The appropriate tissues to be processed for pathogen detection differ depending on the size of the fish to be examined.

a). Samples to be used from fish in size groups 1 and 2. The sampling of fish in size group 1 shall be for virus detection only, since the size of the kidney and other internal organs is not sufficient to allow collection of individual samples for bacterial isolation or for detection of *Renibacterium salmoninarum*, and *Myxobolus* spores would not have developed yet. Fish of size group 1 and 2 are sampled for the presence of viruses by two different methods. The tissues to be used for each are as follows:

1) Size group 1. Fish < 4–cm total length: entire fish shall be processed for virus isolation. Excise yolk sac if present on sac fry prior to processing for virus isolation. The head and tail may be removed from 3–4 cm fish prior to pooling and homogenization.

2) Size group 2. Fish 4–6 cm total length: Entire visceral mass, including scraping the dorsal surface of the peritoneal cavity containing the developing kidney, is excised and used for virus isolation.

b). Samples to be used from fish in size group 3 and 4. Fish from these size groups shall be sampled for viral, bacterial and parasitologic examination. Examinations for bacterial pathogens shall not take place within one month of cessation of application of antibiotics to fish to be examined. The specimens are collected according to the appropriate sampling schedule for the population and assumed prevalence level of 5% (see table in **Part I D.**). Samples of spleen, kidney, and skull cartilage are taken from size group 2 and 3 fish, and in addition, ovarian fluid samples are collected from size group 4 fish at spawning. Approximately 1.0 mL of ovarian fluid is needed from each fish: 0.5 mL for virology and 0.1-0.2 mL for *Renibacterium salmoninarum* detection.

**2. Necropsy procedure.**

a. Sampling tissues for bacterial isolation.

1. Fish are euthanized in 1mg/mL of tricaine methane sulfonate (MS-222), or placed directly on ice for transportation to the lab for dissection.

2. The ventral body surface is wiped with a paper towel to remove excess mucus, sprayed with 70 % ethanol using a spray bottle or squeeze bottle, and dried with a paper towel to sanitize the area of the ventral body surface to be dissected.

3. Using sterile scissors or scalpel flamed in alcohol and holding the pectoral fin aside with forceps, a cut is made through the skin and muscle in the median line of the ventral body wall extending from just posterior to the isthmus, proceeding posteriorly to the anus. The cut should move laterally at the anus to avoid cutting into the posterior intestine and permitting the intestinal contents to enter the visceral cavity. The most anterior cut (just posterior to the opercular opening) may be extended dorsally to the lateral line to make the entry to the interior of the body cavity easier and less prone to contamination.

4. With sterile forceps, lift the body wall and using the side of a sterile scalpel blade or handle (alcohol flamed if used for cutting the body wall), deflect the viscera. and gas bladder to expose the kidney.

5. Insert a sterile bacteriological loop (preferably a 1 µL disposable loop) through the membrane covering the kidney, either directly or via an aseptic incision, into the interior of the kidney, carefully avoiding touching the body wall or viscera. Streak for isolation a plate or slant containing either Tryptic Soy Agar (TSA) or Brain Heart Infusion Agar (BHIA). Streaks from four individual fish may be made on one 100 mm diameter plastic culture dish. The plates are incubated as described in **Part II B.1. a.** and putative pathogens are identified by the methods indicated in that section.

b. Sampling tissues for viral isolation.

After the sample for bacteriological analysis has been taken aseptically, tissue samples are taken for virology. Since these samples will be treated to inhibit bacterial/fungal growth, strict aseptic technique in collecting these samples is not necessary. However, sampling as aseptically as possible reduces the probability of contaminating inoculated cells.

The samples which are removed for virological examination are those which have proven to contain the target viruses most frequently and at the highest titer, in order to maximize the chances of detecting virus. The samples examined include kidney and spleen. The tissues from up to five fish may be pooled together for processing for viral pathogens.

1. Using a scalpel, a piece of midkidney is excised by cutting at both edges of the kidney parallel to the midline, then perpendicularly through the tissue to yield a piece approximately 0.5 $cm^2$.

2. The spleen is excised whole if it is smaller than 1 cc.

3. In sexually mature female fish (size group 4), ovarian fluid samples are also collected and processed separately from the visceral samples. Ovarian fluid samples may be taken by nonlethal sampling, if preferred, for repeat spawners. Approximately 1 mL of fluid from each of 5 fish is withdrawn from the eggs after spawning into a 10 cc syringe without needle and the pooled fluid is mixed by inversion several times. The syringe with fluid is capped and placed on ice and held for further processing.

4. The tissue is placed into a container (urine sample cups work well) containing Dulbecco's Phosphate Buffered Saline (PBS) without calcium or magnesium (0.15 M, pH 7.4-7.6) with either the antibiotics penicillin/streptomycin (100 IU or µg/mL, respectively) or gentamycin (200 µg/mL). Approximately four times the anticipated volume of all tissues taken from five fish (for 0.5g samples of each tissue, this would be 7.5 g of tissues per pool which would require 30 mL of PBS/antibiotics per pool) is needed as transport medium.
5. Pooled samples shall be held on ice until they are processed further for virus isolation, and the time between sampling and processing of the samples shall in no case be greater than 48 h. The tissues are further processed as described in **Section C.**

c. Sampling tissues for detection of *Renibacterium salmoninarum*.

After kidney, spleen and gill material has been removed for virological analysis, additional material from the kidney is collected for the immunodetection of *Renibacterium salmoninarum* by a fluorescent antibody test (direct: DFAT or indirect: IFAT).

1. Using the side of a scalpel blade, excise a small (2-5 $mm^3$) piece of remaining posterior kidney material and place on a labeled 1 x 3" microscope slide or other appropriate type of slide.
2. Using another clean slide, squeeze the kidney material between the two slides while moving the slide to spread the material over the surface until only a thin film remains. Extraneous material can be removed from the thin smear with the edge of the slide. This provides a duplicate slide for each sample which can be examined if the results of the primary sample are equivocal. The slides may be held for up to 24 h until fixation in methanol and further processing as described in **Part II B. 2.**
3. For FAT testing of ovarian fluid, a swab is dipped into the ovarian fluid prior to pooling for virology, and the material is spread thinly on a microscope slide.

d. Sampling tissues for parasite detection.

If all of the fish at a facility are on the same water supply and have been reared on site, or have been imported from areas which are known to be free of the pathogen, or have been imported as eggs, it is acceptable to consider them as a single lot. Any fish that do not meet these criteria must be examined separately. The samples at the 5% assumed prevalence level should be taken for each lot of fish. The sample should be designed so that the most susceptible species—brook trout and rainbow trout—are sampled preferentially to brown trout or Pacific salmon. Depending on the salmonid species to be tested and cultural conditions, fish ranging from 4 months to one year of age are in the optimal size range to detect spores. If the presence of the parasite is suspected, taking an extra 30 heads will allow confirmation of the identity of detected spores by histopathological examination.

Sampling for the parasite, *Myxobolus cerebralis* involves the dissection of the head cartilage for the detection of the characteristic spores. The entire head anterior to the opercular opening is severed from the body. This is the simplest method for smaller fish, and includes gill arch material as well as skull cartilage. The heads are placed in plastic bags and held on ice or frozen until further processed as described in **Part II D.**

## B. Bacterial isolation and identification

1. Detection and identification of *Aeromonas salmonicida* and *Yersinia ruckeri.*

a. Plates or slants inoculated from the kidney shall be inverted and incubated at 25°C for 48 h. Colonies which have formed after this time shall be characterized morphologically and the bacteria further characterized. The cultural characteristics of the two pathogenic organisms *Aeromonas salmonicida* and *Yersinia ruckeri* on tryptic soy agar (TSA) or brain heart infusion agar (BHIA) are as follows:

1). *A. salmonicida salmonicida*: round, entire, creamy colonies which secrete a diffusable brown pigment within 48 h at 25°C.

2). *A. salmonicida achromogenes*: punctiform, creamy, opaque, nonpigmented colonies which grow slowly and do not produce brown pigment.

3). *Yersinia ruckeri* : round, entire, creamy, translucent colonies.

b. Colonies which appear similar to the descriptions above shall be further characterized by the following tests, which are described in more detail in Chapter 2 of this Manual: General Procedures for Bacteriology in the section Bacterial Diseases of Fishes. A complete flow diagram for potential fish bacterial fish pathogens is included in Chapter 3 of that Section.

1. Gram stain reaction is performed by standard staining methods or by taking a bacteriological loopful of the colony and mixing in a drop of 10% KOH. After stirring for one minute, the presence of a viscous stringy mass when the loop is slowly pulled from the colony indicates a gram negative bacteria.

2. Cytochrome oxidase test: A plastic or platinum loop is touched to the edge of a colony where log phase growth is occurring, and applied to a piece of filter paper which contains a drop of freshly prepared 1% aqueous tetramethyl-p-phenylenediamine HCL (commercial cytochrome oxidase test strips may also be used). The development of a deep blue color within 10–15 sec indicates a cytochrome oxidase positive organism.

3. Motility test: Performed on log phase cultures with a wet mount prepared by taking a colony and emusifying in a drop of saline and covering with a coverslip, by the hanging drop technique, or by inoculating commercially available motility agar. A positive reaction in the hanging drop is indicated by movement of bacteria past one another and not by the random Brownian motion. In agar a positive response is indicated by growth (turbidity) away from the inoculation path.

c. Those colonies which are oxidase positive are further characterized using the listed tests for *A. salmonicida*, while those which are oxidase negative should be further tested for *Yersinia ruckeri*. A brief schematic table based on the scheme of Shotts (Chapter 3, Bacterial Diseases of Fishes, this volume) is shown below.

| Test | *A. salmonicida* | Yersinia ruckeri |
|---|---|---|
| Gram stain | - | - |
| Oxidase | + | - |
| Motility | - | |
| Glucose fermentation | + | NT |
| Lactose fermentation | NT | - |
| Inositol fermentation | - | NT |
| Phenylalanine deaminase | NT | - |
| Citrate utilization | NT | - |
| O/129 sensitivity | - | NT |
| Novobiocin sensitivity | - | NT |
| Triple Sugar Iron Agar | Acid slant/Acid butt | alKaline slant/Acid butt |
| Salicin | NT | - |
| Esculin | NT | - |
| NT = test not needed for identification. See Bacterial Diseases of Fishes, Section II for detailed scheme. | | |

d. Appropriate reactions in these physicochemical tests are presumptive evidence for the presence of the pathogen. Confirmation of the identity of the isolated organism as *A. salmonicida* or *Y. ruckeri* requires further biochemical/physiological characterization using tube tests or commercially available kits, or a positive reaction by reactions with specific antibody. Fluorescent antibody, agglutination, or enzyme immunoassays utilizing either polyclonal or monoclonal antibodies specific for the pathogen may be used for confirmation. Detailed methods are described in the following chapters of the Bacteriology Section of this Manual: Chapter I for Bacteriology, Chapter VII for *Yersinia ruckeri* or Chapter IX for *Aeromonas salmonicida*.

2. Immunodetection of *Renibacterium salmoninarum*

Detection and identification of *Renibacterium salmoninarum* from carrier fish is generally carried out by immunological tests since the agent is so difficult to grow on bacteriologic media. Either a direct (DFAT) or an indirect fluorescent antibody test (IFAT) can be used for detection of the pathogen in kidney tissues. The DFAT using polyclonal antibodies is currently in use more often than the IFAT, but the latter

is generally considered to be a more sensitive test. A brief description of the two techniques follows.

a. DFAT.

1. Smears prepared from kidney tissue or ovarian fluid as described in **Part II. A. 2. b.** are fixed in absolute methanol for 5 min and allowed to air dry. One set of the duplicate slides can then be stored and used to confirm the presence of low numbers of FAT + bacteria if there is a question of the validity of the test result.
2. A positive control consisting of a smear prepared from kidney tissue from an animal with clinical disease or negative tissue inoculated with bacteria from a pure culture of *Renibacterium salmoninarum* shall be prepared and **processed separately from the test samples.**
3. A negative control composed of a kidney smear from a *Renibacterium salmoninarum* negative animal shall also be prepared and included with the test samples.
4. The fixed smears are covered with 25 µL of an appropriate (varies from lot to lot as determined by the supplier) dilution of *Renibacterium salmoninarum*-specific antiserum, to which is coupled the FITC fluorochrome.
5. A 22 x 50 mm coverslip is placed over the smear to spread the antiserum over the surface of the smear and allow the use of small volumes of the antiserum.
6. The slide is incubated at room temperature in a humid atmosphere for one hour.
7. After incubation, the coverslip is pushed to the side and gently removed and the slide is placed in a slideholder with other slides and washed 3 x for 5 min each in PBS, pH 7.4.
8. The slide may be counterstained with 0.5% aqueous Evans blue, rhodamine B, or eriochrome black T for 5 min to reduce nonspecific background fluorescence, followed by a rinse in PBS prior to examination.
9. The smears are examined under ultraviolet light with excitation and emission bands set as appropriate for FITC fluorescence. At a magnification of 1,000 x., fifty fields are examined for each smear. The bacteria usually appear as small diplobacilli, often in clusters. A positive smear is considered to be one on which even a single appropriately-stained, appropriately-sized bacterium is detected.
10. When one or two bacteria are detected in 50 fields, confirmation by processing and examination of the duplicate slide is necessary. If no appropriately-stained, approriately-sized bacteria are detected on the replicate slide, the sample is considered negative.

b. IFAT

1. Steps 1-3 of the DFAT procedure (Section II B. 2 a.) are the same for IFAT.

2. The fixed smears are covered with 25 µL of an appropriate (varies from lot to lot as determined by the supplier) dilution of rabbit or goat *Renibacterium salmoninarum*-specific antiserum (primary antibody).

3. A 22 x 50 mm coverslip is placed over the smear to spread the antiserum over the surface of the smear and allow the use of small volumes of the antiserum.

4. The slide is incubated at room temperature in a humid atmosphere for one hour.

5. After incubation, the coverslip is pushed to the side and gently removed and the slide is placed in a slideholder with other slides and washed 3 x for 5 min each in PBS, pH 7.4.

6. The smears are covered with 25 µL of an appropriate (varies from lot to lot as determined by the supplier) dilution of secondary antibody, which is antiserum conjugated with FITC that is directed against rabbit or goat immunoglobulin, as appropriate.

7. A 22 x 50 mm coverslip is placed over the smear to spread the secondary antibody over the surface of the smear and allow the use of small volumes of the antiserum.

8. The slide is incubated at room temperature in a humid atmosphere for one hour.

9. After incubation, the coverslip is pushed to the side and gently removed and the slide is placed in a slideholder with other slides and washed 3 x for 5 min each in PBS, pH 7.4

10. The slide may be counterstained with 0.5% aqueous Evans blue, rhodamine B, or eriochrome black T for 5 min to reduce nonspecific background fluorescence, followed by a rinse in PBS prior to examination.

11. The smears are examined under ultraviolet light with excitation and emission bands set as appropriate for FITC fluorescence. At a magnification of 1,000 x., fifty fields are examined for each smear. The bacteria appear as small diplobacilli, often in clusters. A positive smear is considered to be one on which even a single appropriately-stained , appropriately-sized bacterium is detected.

12. In the case where a single or two bacteria are detected, confirmation by processing and examination of the duplicate slide is necessary as described above.

## C. Virus isolation and identification

1. Cell culture preparation

a. Samples are inoculated onto each of two cell lines. Mycoplasma-free (See Appendix 1, this Manual), continuous teleost cell lines CHSE-214 and EPC are prepared in 24 well multiwell tissue culture plates (MTP) within 48 h of anticipated inoculation with virus samples. The cells should have been tested and be known to exhibit characteristic cytopathogenic effect (cpe) when inoculated with IHNV, IPNV, VHSV at a concentration of $10^2$ $TCID_{50}$/ml. Cells should be tested at twice yearly intervals for their susceptbility to all three of these viruses, either by a

central laboratory, or by the laboratory carrying out the fish health inspection program.

b. The cells shall be 80-90% confluent at the time of inoculation. While it is appropriate to use antibiotics in the culture medium used for virus isolation, **it is never appropriate to use antibiotics for routine cultivation and maintenance of cell cultures.**

2. Sample preparation and application to cells.

   a. Pooled samples of the appropriate tissues or fluids from 5 fish collected as described in Part II A. 2. b. may be held on ice for up to 48 h until virological assay is performed.

   b. The tissues are then homogenized in a Stomacher™, a device that macerates tissue by compressing rapidly between paddles and a solid surface. The tissue sample is placed in a plastic bag (depending on volume, several containing a total of 80 mL of sample material can be homogenized simultaneously) and homogenized for one minute. The device requires no sanitizing between pools because the material does not come in contact with the interior of the device.

   c. The antibiotics penicillin (800 IU/mL) and streptomycin (800 µg/ml), or gentamycin (300 µg/mL), as well as the mycostatic agent nystatin (200 IU/ml) are added to the medium in which the tissue has been homogenized and the tissue homogenate is held at 15-18°C for 3 h, or overnight at 4°C prior to adding to cell cultures.

   d. The homogenate is then centrifuged at 1,500-2,000 x g for 10 min and the supernatant is applied to the cell cultures after a further 1:10 or 1:20 dilution in cell culture growth medium consisting of Eagle's minimum essential medium supplemented with 10% fetal bovine serum (MEM-10) with antibiotics at the concentrations described above.

   e. Samples of ovarian fluids need not be homogenized and can simply be diluted to 1:5 in growth medium with antibiotics as described above.

   f. Growth medium is decanted from CHSE-214 and EPC cells prepared in 24 well multiwell tissue culture plates less than 48 hours old (80-90% confluent). At least 100 µL of the diluted, pooled tissue or ovarian fluid sample is applied to each of two replicate wells (2.0 $cm^2$ area/well) of a 24 well MTP. If the cells are to be incubated in the 5% $CO_2$ atmosphere required for buffering of bicarbonate buffered growth medium to pH 7.4–7.8, the rigid polystyrene tops which come with the plates can be used to cover the wells. If they are to be incubated in ambient atmosphere, an adherent Mylar™ sheet should be placed on the top and sealed around all wells and the edges of the plate, or a cell culture medium compatible with ambient atmosphere, such as Liebowitz' L-15 with 10% fetal bovine serum or MEM–10 buffered with HEPES, should be used to maintain the pH in the 7.4–7.8 range during incubation period.

g. The cells shall be incubated at 15–18°C for 21 d. The cells shall be examined within 24 h of inoculation to determine if cytotoxic effects (CTE) generated by tissue products has occurred (unless high virus concentrations are present, virus-specific cytopathology (CPE) will not occur this rapidly, whereas nonspecific cytotoxicity will occur rapidly).

h. If cytotoxicity is noted, a 1:100 dilution of the cell supernatant is made, filtered through a 0.45 µm pore size filter, and reinoculated onto 4 wells of a 96 well MTP containing the same type of cells in which the cytotoxicity was noted.

i. The negative (uninoculated) controls consist of appropriately buffered cell culture medium and can be inoculated onto the same plates as the test samples.

j. If an apparent virus-specific CPE is noted, fresh cell cultures shall be prepared and supernatant fluid from the affected wells shall be diluted $10^{-3}$, filtered through a 0.45 µm pore size filter, and reinoculated onto 4 replicate wells of a 96 well MTP containing freshly prepared cells of the same type in which the CPE was noted, and incubated under the same conditions as the original sample for at least one week.

k. If CPE is again noted, identification of the replicating agent is necessary. At this point a plaque assay might be attempted to determine if the agent "plaques" and the physical characteristics of the plaque. Confirmatory identification of the agent shall be made by the virus neutralization test.

l. Supernatant material from the second passage is removed from the cell monolayer, diluted 1:100 and filtered as before. An aliquot of the filtered supernatant is removed and mixed with an equal volume of each of three specific polyclonal or monoclonal antibodies which are known to neutralize at least $10^3$ $TCID_{50}$/ml of IHNV, IPNV, or VHSV. Incubate for 1 h at 15–20°C.

m. Titrate the unneutralized virus in tenfold steps in 96 well MTP containing sensitive cells. A sample which is not mixed with antibody is used as a control to determine the original virus titer and is held at 15–20°C until all of the other samples have been inoculated. This untreated virus is titrated by $TCID_{50}$. A titer decrease in a sample mixed with antibody of 2 $\log_{10}$ indicates neutralization and confirms the identity of the virus. Other methods of serological confirmation of virus identification (ELISA/ immunodotblot assays) have been developed for each of the viruses, but are not preferred for confirmation of the viruses listed. Chapters V, VI and VIII can be consulted for further information.

n. If the virus does not react with any of the antisera to the known viruses, then a sample should be sent to a research laboratory which can perform further tests to identify the virus.

## D. Isolation and identification of *Myxobolus cerebralis*

A complete description of the methods used for processing tissue samples for the detection and identification of *M. cerebralis* is included in Chapter VI of the Section on

Parasitic Diseases of Fishes in this volume. The samples that have been taken must be first treated to remove noncartilaginous tissues and to avoid the potential of contamination with the closely related myxosporidian *M. neurobius*. This is generally accomplished by heating the tissues to simplify the removal of skin, muscle, and brain material. Once the samples are defleshed and cut into pieces, they are further processed to decrease the size of the cartilaginous pieces and to partially purify and concentrate the spores.

1. Frozen samples may be used, but no formalin preserved material shall be used for this procedure. **All glassware and other equipment used in processing different batches of samples must be scrupulously cleaned to avoid cross contamination by spores.**
2. Deflesh heads by heating in water at 45-50°C for 1 h. Remove the brain and spinal cord and discard to prevent contamination of skull cartilage with spores of *M. neurobius* which is difficult to differentiate from *M. cerebralis*.
3. The plankton centrifuge method of O'Grodnick (1975) is used to concentrate any spores that are present in the skull cartilage. Up to 20 g of defleshed skull material may be pooled for further processing.
4. The samples are macerated in nine volumes of 10% buffered formalin in a blender set at high speed for 3 min.
5. The macerated material is filtered through a fine (0.25–0.5 mm) wire mesh screen or glass wool to remove large pieces of cartilage. Clogged filters may be rinsed with water and the rinse water is added to the filtrate.
6. The filtrate is placed in a separatory funnel with the outlet discharging into a plankton centrifuge. The filtrate is slowly added (a slow steady stream, but not dropwise) to the centrifuge which is set at high speed.
7. Centrifuge until all water has been removed; rinse throughly with one volume of distilled water in the separatory funnel.
8. Scrape the concentrated material from the wall of the cup contained within the centrifuge and place in a 50 mL disposable plastic centrifuge tube by rinsing with up to 30 mL of 10% buffered formalin.
9. Shake the sample until the material is uniformly suspended and place a drop of the suspension on a microscope slide.
10. Examine at least 50 fields of the slide at 450X magnification using phase contrast microscopy for the presence of *M. cerebralis* spores. The spores of this parasite appear oval, approximately 10 µm in length with two oval polar capsules in the anterior portion of the spore. Preliminary identification of *M. cerebralis* spores must be based on the morphological characters given by Lom and Hoffman (1971) since other myxosporidia appear morphologically similar to *M. cerebralis*.
11. Final confirmation of the identity of the spores must be carried out by resampling from the same lot of fish and confirming the presence of morphologically identical spores in cranial cartilage by histology. as described in Section E.2. of Chapter VI of the Parasitology section of this Manual.

# BACTERIAL DISEASES OF FISHES

I. GENERAL PROCEDURES FOR BACTERIOLOGY

II. FLOW CHART FOR THE PRESUMPTIVE IDENTIFICATION OF SELECTED BACTERIA FROM FISHES

## Gram-Negative Bacteria

III. BACTERIAL GILL DISEASE

IV. COLDWATER DISEASE

V. COLUMNARIS DISEASE

VI. *EDWARDSIELLA TARDA* SEPTICEMIA

VII. ENTERIC REDMOUTH DISEASE

VIII. ENTERIC SEPTICEMIA

IX. FURUNCULOSIS

X. OTHER DISEASES CAUSED BY *AEROMONAS SALMONICIDA*

XI. MOTILE AEROMONAS SEPTICEMIA

XII. VIBRIOSIS

XIII. COLDWATER VIBRIOSIS

## Gram-Positive Bacteria

XIV. BACTERIAL KIDNEY DISEASE

XV. PSEUDOKIDNEY DISEASE

XVI. STREPTOCOCCAL DISEASE

## Other

XVII. PISCIRICKETTSIAL DISEASE OF SALMONIDS

# I. General Procedures for Bacteriology

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## 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 General Sampling Procedures chapter). Fish that are found dead at the time of sampling are not acceptable for bacteriological examination, unless they are known to be very fresh (dead for less than 1/2 h). 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 h. Samples for culture should not be frozen. For the storage of samples intended for immunological tests, refer to the 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 frequently used medium for routine isolation and culture of many fish pathogens, and 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 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 48-50°C.
4. **Cytophaga agar.** This medium (Anacker and Ordal 1959) is used for the isolation of flexibacteria. 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 the growth of flexibacteria can be enhanced by the addition of 2-5% fetal bovine serum to this medium.

4. **Hsu-Shotts medium.** This medium (Bullock et al. 1986) is used for the isolation of flexibacteria; 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 (1984), Baron and Finegold (1990), and Balows et al. (1991).

## 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, and detailed instructions on their preparation and use are also included in bacteriology manuals. Miniaturized bacterial identification systems that include many of the tests are also available.

1. **Gram stain.** This test is important for the broad classification of bacteria, and should be done on young (log-phase) cultures. 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. For this test, a loopful of material from a bacterial colony is emulsified on a glass slide in a drop of 3% KOH. The suspension is stirred continuously for 60 seconds, and then the loop is slowly pulled from the suspension. 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.

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, multiply cross-linked fatty acids) from other gram-positive bacteria. Mycobacteria (and to a lesser extent, *Nocardia*) stain acid-fast.

3. **Motility.** For most bacteria, wet mounts 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 gives equivocal or negative results, motility can be evaluated further by stab-inoculating tubes of commercially available motility test medium (Difco Motility Test Medium or equivalent).

   For flexibacteria, an agar block motility test can be done by excising a 5-mm square block of agar supporting a suspected flexibacterial 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. The Kovacs method is a recommended procedure; reagents (and test kits) are available from several manufacturers. Material for testing should be taken from log-phase colonies by use of a sterile platinum or plastic loop (iron-containing loops can produce false positive reactions). A delayed reaction may occur if bacteria are taken from an acid environment (medium containing a utilizable carbohydrate).

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.

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

7. **Single substrate utilization tests.** Certain bacteria can be characterized by their ability to grow in the presence of a single compound. Common substrates that can fulfill this function and are useful for the differentiation of bacteria are citrate, malonate, and acetate. Growth on an agar slant containing the substrate of interest (with or without a pH indicator) is used as the end point of the test.

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.

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).

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).

11. **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.

12. **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. Certain organisms such as *Lactoba-*

*cillus*, *Carnobacterium*, and *Aerococcus* may appear catalase positive, but actually produce a "pseudocatalase."

13. **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. pneumonia*, which may have lost their active enzyme.
14. **Nitrate reduction test.** This test distinguishes between bacteria that cannot utilize nitrate ($NO_3$) as a nitrogen source, and those that can reduce nitrate to nitrite ($NO_2$) or a product beyond nitrite.
15. **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 20-22°C for 16-24 h, 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 performined 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.
16. **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.
17. **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: $\alpha$ (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 $\beta$ (beta), a clear, colorless zone around the bacterial colony, in which

the erythrocytes have undergone complete destruction; and γ (gamma), no hemolysis.

**18. CAMP test.** The CAMP test is used to identify Lancefield Group B streptococci (see Immunological Procedures below). Group B streptococci produce a proteinlike 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.

## 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. The reader is referred to chapters on specific pathogens for modifications of the FAT for use on fish tissue and body fluid samples. Following are general procedures for the confirmatory identification of bacteria obtained from cultures:

**1. Direct fluorescent antibody test (DFAT).** This procedure uses antibody that was prepared against the pathogen of interest, and then conjugated with a fluorescing dye, (fluorochrome), most commonly fluorescein isothiocyanate (FITC).

a. Make a smear of the "unknown" bacteria on a clean slide and dry at 60°C for 5 min. (Alternatively, the smear can be air-dried and then heat-fixed by passing it quickly through a flame so that the slide becomes warm, but not hot.) Smears for positive and negative controls should be prepared in the same manner; the former should contain bacteria known to be the species of interest, and the latter should contain bacteria known to be of another species.

b. Rehydrate the appropriate fluorochrome-conjugated purified immunoglobulin or antibody (if lyophilized) and dilute it according to the manufacturer's instructions. Filtration of the conjugated antibody (conjugate) through a 0.2 µm or 0.45 µm membrane filter before use helps to remove fluorescing particulate material. The

conjugate should be stored in accordance with the manufacturer's recommendations.

c. Place drops of the conjugate on slides to cover the smears. Allow the antibody to react with the smears for at least 5 min in a dark humid chamber at room temperature. Longer incubation may be necessary to attain adequate staining of heavy smears.

d. Rinse the slides with phosphate-buffered saline (PBS, pH 7.1-7.2), and wash in PBS for at least 2 min.

e. Air dry the slides, then add buffered glycerol mounting fluid (pH 9.0; available commercially), apply a cover glass, and examine with a microscope equipped for fluorescence microscopy. The filter set should be appropriate for the fluorochrome used.

f. Examine the smears under oil immersion (about 1000 X magnification) for bacteria showing specific apple-green fluorescence (positive reaction). Compare the fluorescence intensity and morphological characteristics with those of the bacteria on the positive control slide. Examine the negative control slide for fluorescence indicative of nonspecific reactivity or cross-reactivity.

g. Storage of stained smears for more than a few days is not recommended, because fluorescence may fade. If storage is necessary, slides should be stored at 4°C and protected from light.

2. **Indirect fluorescent antibody test (IFAT).** This is a double layer technique. For the IFAT, the first layer is unconjugated purified immunoglobulin or antibody prepared in one animal species (e.g. rabbit) against the bacterium of interest. The second layer is fluorochrome-conjugated antibody prepared in a second animal species (e.g. goat), and specific for immunoglobulins of the first animal species (e.g. goat anti-rabbit IgG). The advantage of this technique is that conjugated antibodies prepared against mammalian immunoglobulins are readily available from commercial sources.

a. Prepare "unknown" and control smears as for the DFAT.

b. Rehydrate both antisera (if lyophilized) and dilute as necessary.

c. Place drops of the unconjugated bacterium-specific first antibody on the slides to cover the smears. Allow the antibody to react with the smears for at least 5 min in a dark humid chamber at room temperature. Longer incubation may be necessary to attain adequate staining of heavy smears.

d. Rinse the slides with phosphate-buffered saline (PBS, pH 7.1-7.2), and wash in PBS for at least 2 min. Drain the slides.

e. Place drops of the conjugated second antibody on slides to cover the smears. Allow the antibody to react with the smears for at least 5 min in a dark humid chamber at room temperature. Longer incubation may be necessary to attain adequate staining of heavy smears.

f. Rinse and wash as in step d above.

g. Air dry the slides, apply mounting fluid and cover glasses, and examine as described for the DFAT. Reactions are interpreted in the same manner as for the DFAT.

## 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 insure 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 5 and 10 min. 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 2-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-labelled 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 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.

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# II. Flow Chart for the Presumptive Identification of Selected Bacteria from Fishes*

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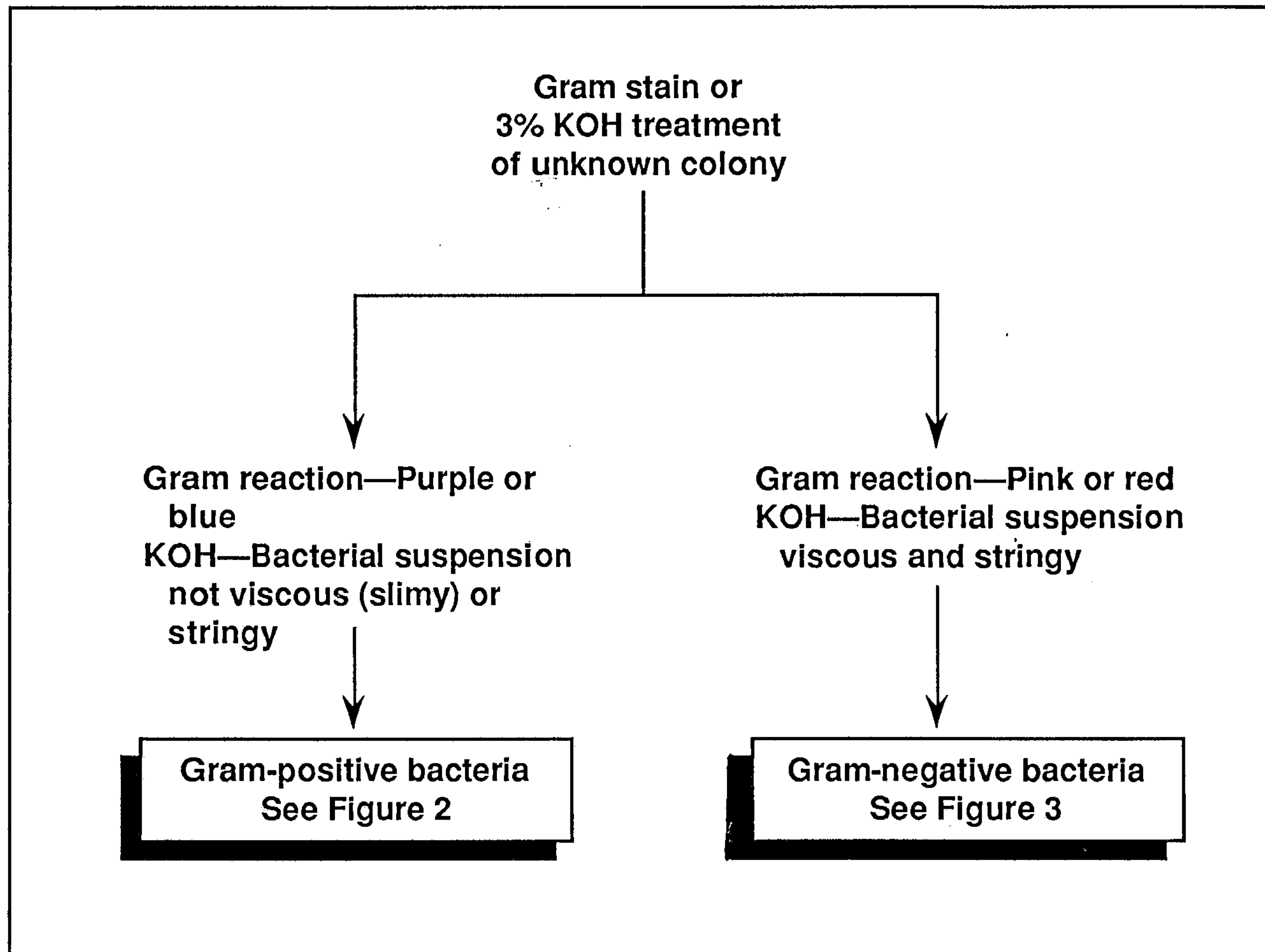


**Figure 1.** General identification of bacteria. For further description of the tests used, refer to the General Procedures for Bacteriology chapter in this book, chapters on specific bacteria, or a bacteriology manual (selected manuals are listed in the General Procedures for Bacteriology chapter).

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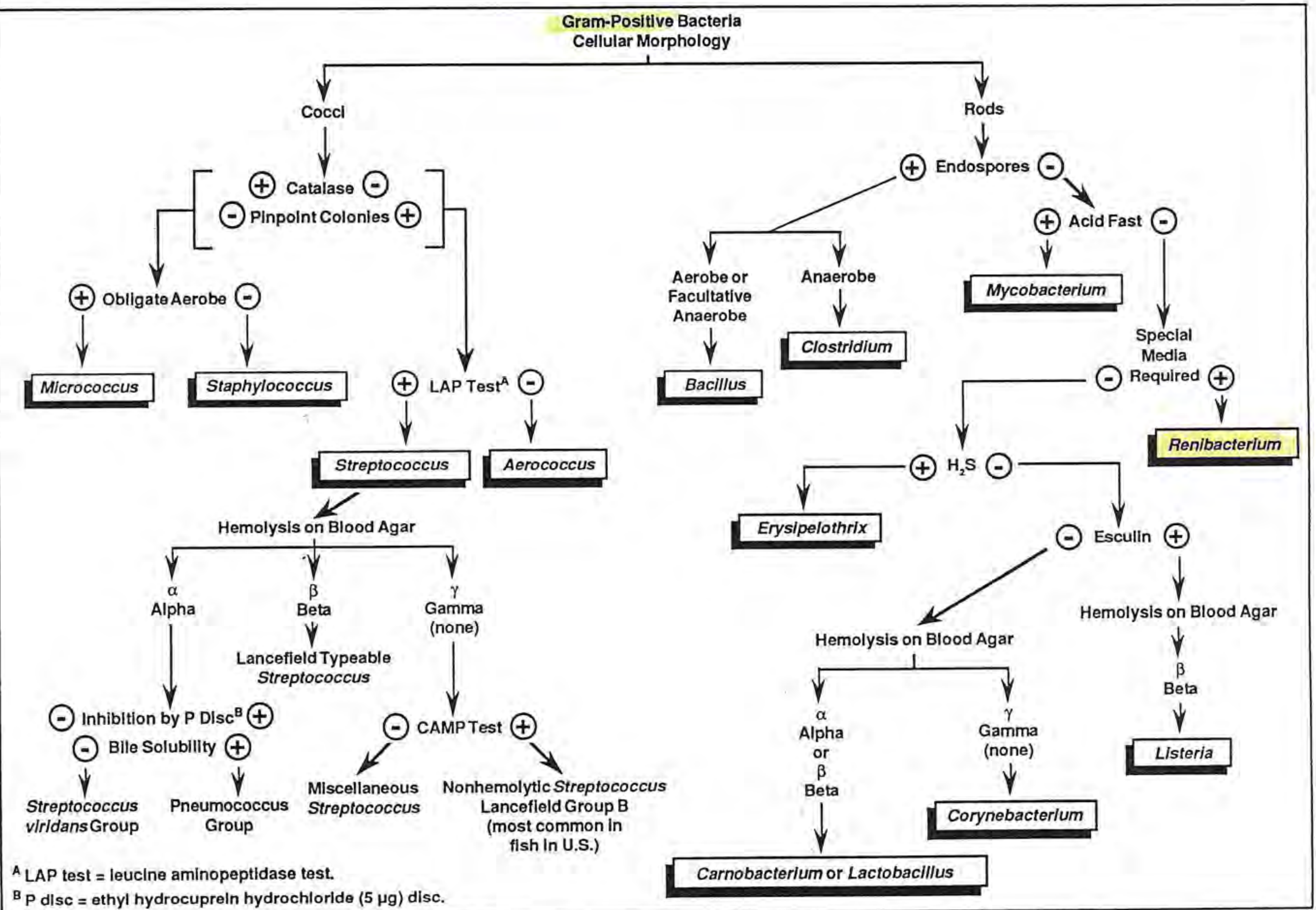
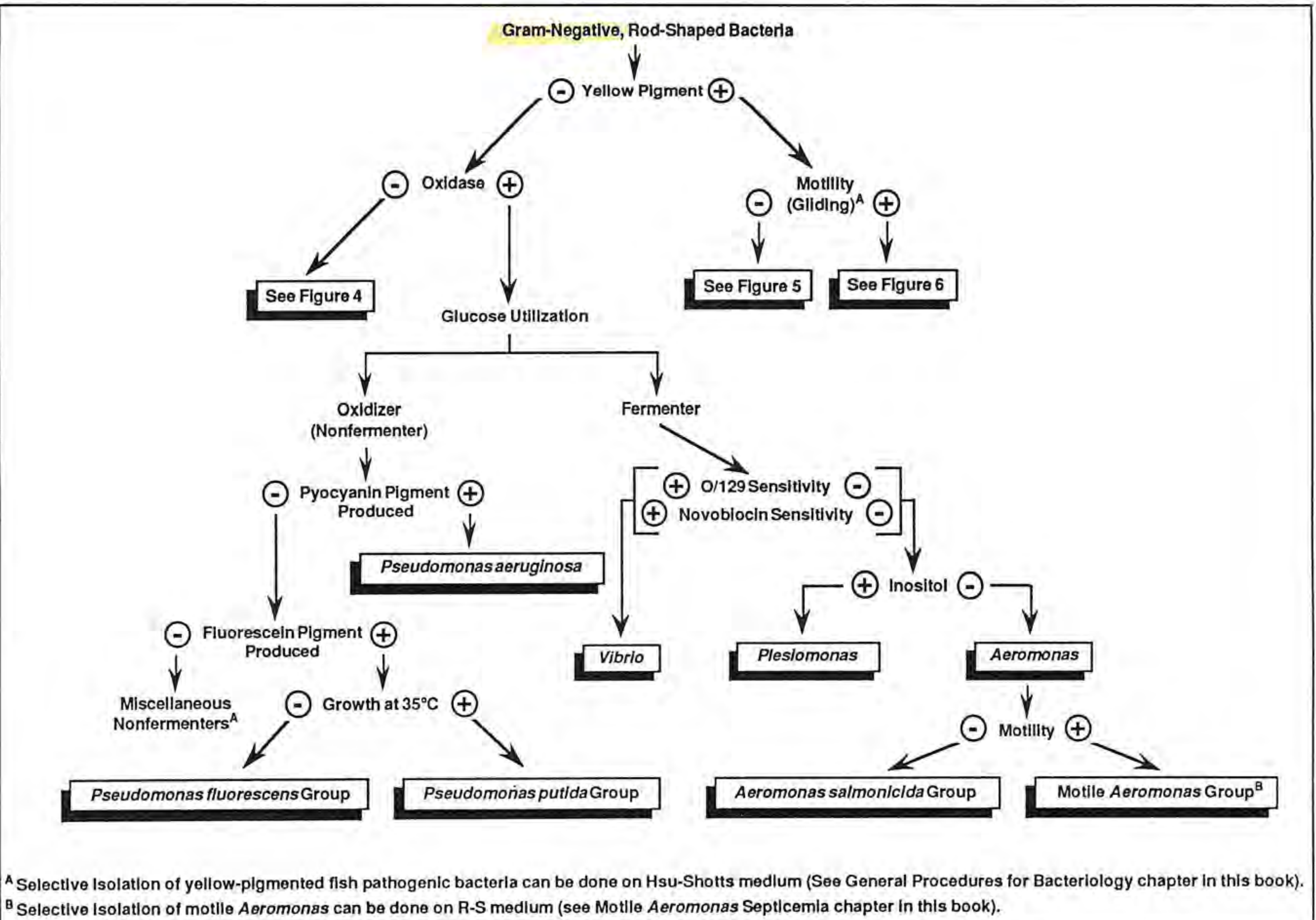
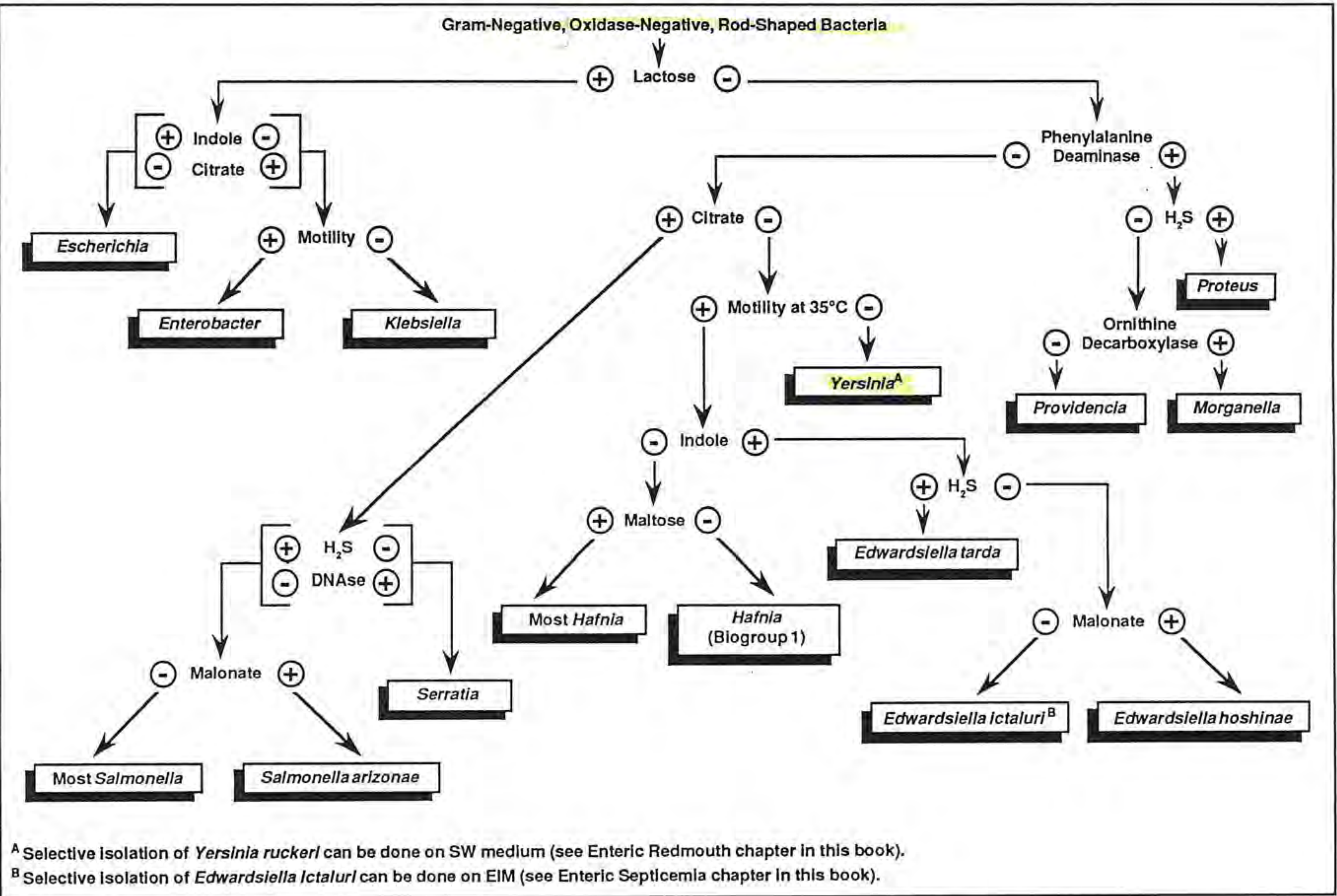



Figure 2. Tests for the identification of gram-positive bacteria.




[A] Selective isolation of yellow-pigmented fish pathogenic bacteria can be done on Hsu-Shotts medium (See General Procedures for Bacteriology chapter in this book).
[B] Selective isolation of motile *Aeromonas* can be done on R-S medium (see Motile *Aeromonas* Septicemia chapter in this book).

Figure 3. Tests for the identification of oxidase-positive, gram-negative, rod-shaped bacteria that do not produce yellow pigment.




[A] Selective isolation of *Yersinia ruckeri* can be done on SW medium (see Enteric Redmouth chapter in this book).

[B] Selective isolation of *Edwardsiella ictaluri* can be done on EIM (see Enteric Septicemia chapter in this book).

**Figure 4.** Tests for the identification of oxidase-negative, gram-negative, rod-shaped bacteria that do not produce yellow pigment.

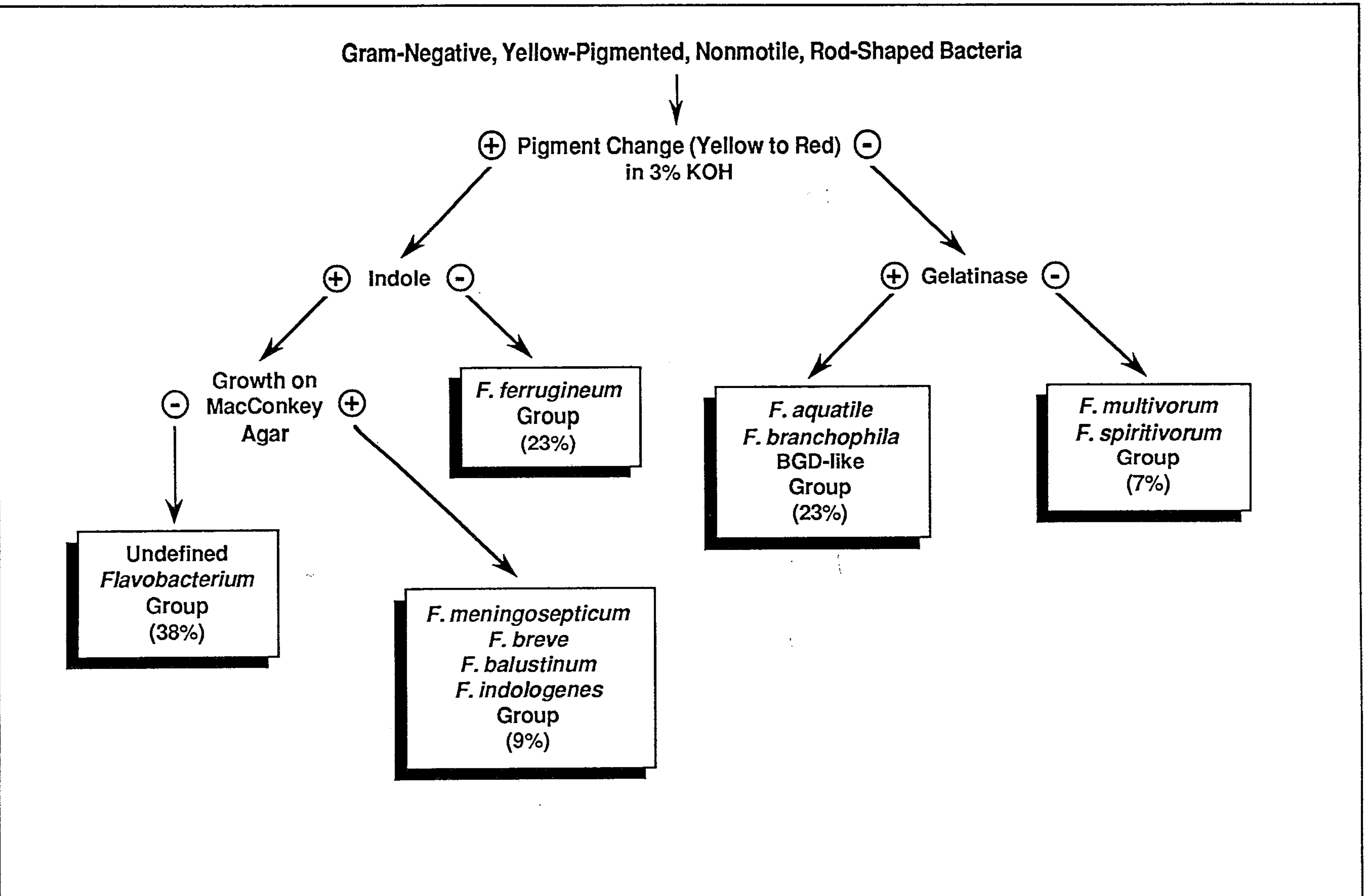



**Figure 5.** Tests for the identification of gram-negative, yellow-pigmented, nonmotile, rod-shaped bacteria (*Flavobacterium*). The numbers in parentheses are the percentages of isolates that fit into the various *Flavobacterium* groups, derived from a random recovery study of 74 isolates total (Shotts, E.B., Jr., and T.C. Hsu, unpublished data).

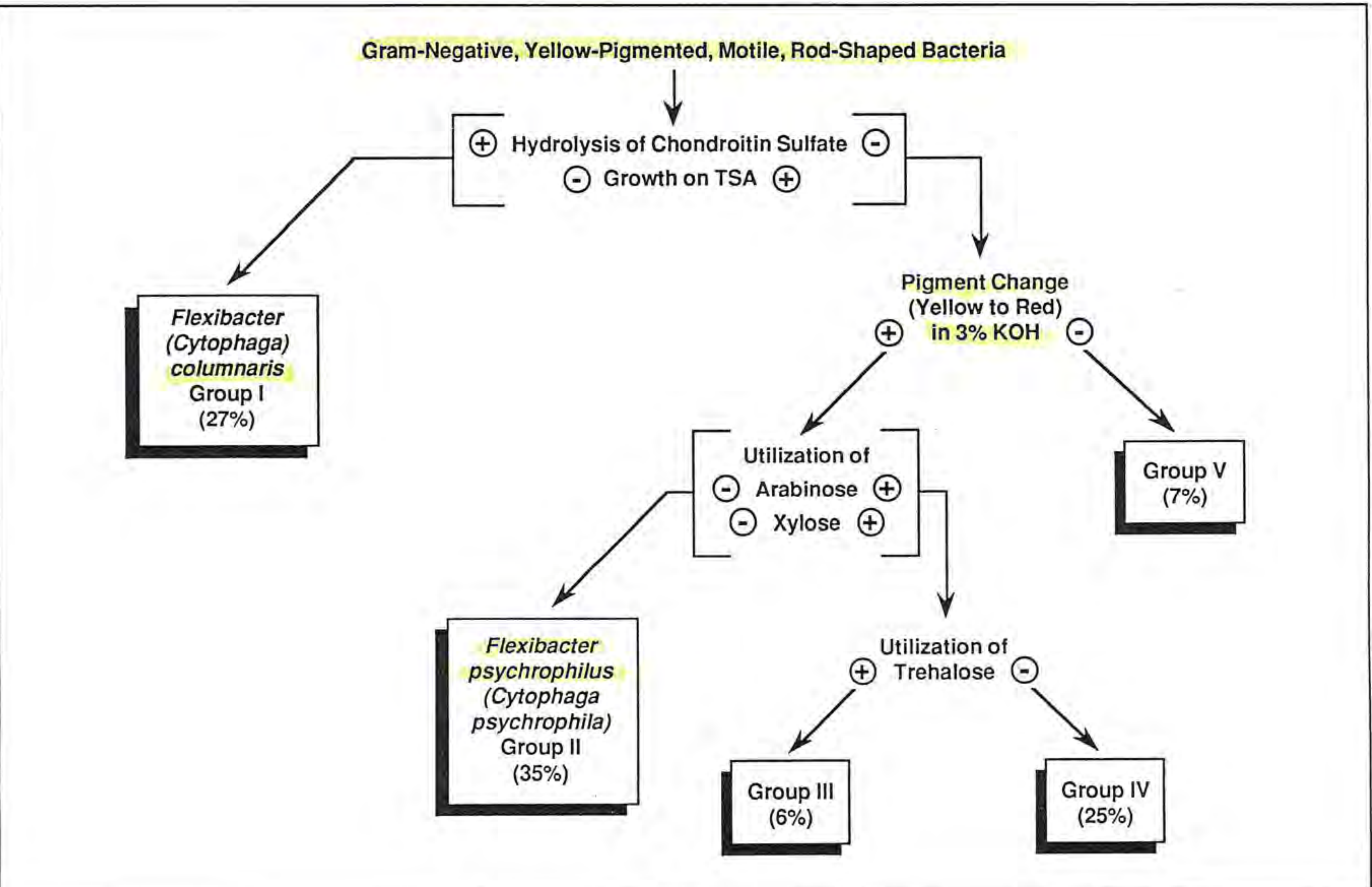



**Figure 6.** Tests for the identification of gram-negative, yellow-pigmented, rod-shaped bacteria that exhibit gliding motility. The numbers in parentheses are the percentages of isolates that fit into the various *Flexibacter* (*Cytophaga*) groups, derived from a random recovery study of 114 isolates total (Shotts, E.B., Jr., and T.C. Hsu, unpublished data).

# III. Bacterial Gill Disease

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## A. Name of Disease and Etiological Agent

Bacterial gill disease, *Flavobacterium branchiophila*. 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, and high ammonia concentrations. Bacterial gill disease has been observed in all fish life stages.

The source of *Flavobacterium branchiophila* 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 d 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 gill filaments.

Histologically, a proliferative hyperplasia of the gill lamellae epithelium 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).

## 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.

A FAT test has been developed for *Flavobacterium branchiophila* (Huh and Wakabayashi 1987) but is 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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## 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, *Flexibacter psychrophilus* is readily isolated from brain tissue. These two diseases condtions are most commonly observed in coho salmon.

## E. Disease Diagnostic Procedures

Diagnosis is based clinical signs along with isolation and identification of the etiological agent. Primary isolation should be made from lesions or the spleen on either cytophaga agar or one of the other media listed below. Cultures are incubated at 15-20°C for 3-6 d. 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 µm). In some cases, no external lesions are observed. Large numbers of rods can be observed in spleen tissue. The organism should produce moist, yellow, raised, convex colonies with or without a thin, spreading, smooth to irregular edge on cytophaga agar in 3-6 d at 15-20° C. The following media support good growth of this bacterium with more rapid appearance of colonies: Shieh (Shieh 1980), modified cytophaga agar (Wakabayashi and Egusa 1974), Hsu-Shotts (Baxa et al. 1986), TYE (Fugihara and Nakatani 1971) or TYES (Holt et al. 1989). Isolation of some strains is enhanced with addition of 1-5% fetal calf serum to cytophaga agar. Also, the addition of 0.5% casein or 0.5% bovine serum albumin (filter sterilized) to TYES (buffered with 0.03M Tris) is beneficial.

### 2. Confirmatory Diagnosis

The diagnosis is confirmed upon positive serological identification by either slide or microtiter agglutination test or indirect FAT. Different serotypes are reported, but common antigens have been observed to date in all isolates. Cross reaction among some serotypes may be weak.

## F. Procedures for Detecting Subclinical Infections

No procedures have been reported.

## 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, General Procedures for Bacteriology.

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# V. Columnaris Disease

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## A. Name of Disease and Etiological Agent

Columnaris disease, *Flexibacter (Cytophaga) columnaris*.

## 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 60-90% mortality common. The precise reservoirs of the pathogen are unclear, but it may occur naturally in the aquatic environment. Large numbers of *Flexibacter columnaris* 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. The foci may enlarge to be several centimeters in diameter, and skin in the affected area may be eroded,

resulting in shallow ulcers. 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. Often, the pathogen's yellow-pigmented cells may be present in large enough numbers to color the lesions yellow or orange. 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. 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.

## 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 3 d incubation at 20°C; cells are motile by gliding or flexing, but lack flagella; little or no disease is produced at 14°C or lower. The tendency of the columnaris bacterium to form mounds ("haystacks") or columns, as detected in wet mounts of diseased tissue, also aids diagnosis. The selective medium developed by Hsu and Shotts (Bullock et al. 1986) enhances isolation of *Flexibacter columnaris* from clinical specimens.

### 2. Confirmatory Diagnosis

a. The procedure of choice is an agglutination test using anti-*Flexibacter columnaris* serum. NOTE: Certain strains of *Flexibacter columnaris* agglutinate spontaneously in saline. The agglutination test can only be performed with such strains if their cell suspensions are first briefly sonicated or heated (5 min at 50°C) to prevent autoagglutination.

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 *Flexibacter columnaris*. Descriptions of *Flexibacter columnaris* are provided in several of the papers listed in the references.

## F. Procedures for Detecting Subclinical Infections

Because *Flexibacter columnaris* is considered ubiquitous in fresh water, a suitably sensitive detection procedure has not been actively pursued and may not be warranted.

## 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 I, General Procedures for Bacteriology.

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# VI. *Edwardsiella tarda* Septicemia

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## A. Name of Disease and Etiological Agent

Edwardsiella septicemia, *Edwardsiella tarda*

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical Range

Southeastern and southwestern United States, southeast Asia.

### 2. Host Species

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*, and flounder *Paralichthys olivaceus*. Fish species that have shown susceptibility to laboratory challenge include; rainbow trout *Oncorhynchus mykiss*, yellowtail *Seriola quinquerdiata* and loach *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.

## C. Epizootiology

Edwardsiella septicemia in most species of fish appears to be favored by high water temperatures (30°C and above), and the presence of high levels of organic matter. The reservoir(s) of infection is (are) unclear; the bacterium has been associated with a variety of aquatic invertebrates and aquatic and terrestrial vertebrates. There is some speculation that *Edwardsiella tarda* may comprise a part of the normal microflora of the surfaces of certain fishes.

## D. Disease Signs

In channel catfish, the disease can occur as small cutaneous lesions that become large abscesses within the muscle; these abcesses are filled with malodorous gas and necrotic tissue. The most common manifestation in catfish and other species is generalized septicemia. In naturally diseased tilapia, clinical signs include loss of pigmentation, ascitic fluid in the abdominal cavity, a protruding hemorrhaged anus, and opaqueness in the eyes. Small white nodules may be present in the kidney, liver, spleen, and gills. In striped bass, eye disease has 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.

## 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 $30^{\circ}$- $35^{\circ}$C for 24-48 h.

### 1. Presumptive Diagnosis

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

### 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 or surface tissues of fish.

## 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 I, General Procedures for Bacteriology.

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# VII. Enteric Redmouth Disease

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## A. Name of Disease and Etiological Agent

Enteric redmouth, ERM, *Yersinia ruckeri*.

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical Range

North America, Europe.

### 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*). Certain aquatic invertebrates such as crayfish, and even mammals, notably muskrats, have been found to harbor large numbers of *Yersinia 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 6-8 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 d 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 the 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 h.

### 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. Identification of sorbitol-positive *Yersinia ruckeri* is best 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*.

## 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

No reliable procedures are available.

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

See See Section I, General Procedures for Bacteriology.

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# VIII. Enteric Septicemia

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## A. Name of Disease and Etiological Agent

Enteric septicemia, enteric septicemia of catfish, ESC, hole-in-the-head disease, *Edwardsiella ictaluri.*

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical range:

Contiguous United States, Thailand

### 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 *Ictalurus nebulosus*, danio *Danio devario*, walking catfish *Clarias batrachus*, green knifefish *Eigemannia virescens* and blue tilapia *Tilapia aurea.* Experimental infections have been established in salmonids.

## C. Epizootiology

Primarily fingerlings but also adult fish 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 8 d 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 on the fins. Pale gills, exophthalmia, and small ulcerations on the body are additional signs. Ulceration in the fontanelle of the frontal bones gives the disease its common name, hole in the head disease. Internally, petechiae are noted throughout the visceral mass and in the peritoneum and musculature. Ascites and enlargement of the liver, kidney and spleen are sometimes observed. 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. Danios infected with *Edwardsiella ictaluri* swim erratically in a spinning motion. Gross lesions have not been seen.

Histopathological examination of channel catfish with hole-in-the-head disease shows a diffuse granulomatous inflammation of the olfactory bulb and the telencephalon (olfactory lobe) of the brain. Fish with the enteric form of the disease exhibit granulomatous enteritis and hepatitis, and granulomatous inflammation with granulation tissue in the skeletal muscle of the jaw.

## 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 the 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°C for 2 to 4 d.

### 1. Presumptive diagnosis

The etiological agent is a short, gram-negative, cytochrome oxidase-negative rod. No indole is produced in tryptone broth. It grows slowly (5-7 d) and sparsely at 37°C. It is nonmotile or weakly motile and does not produce $H_2S$.

The TSI (triple sugar iron) reaction should be K/A (alkaline slant, acid butt).

### 2. Confirmatory Diagnosis

a. 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 Infections

Culture from the forebrain and hindgut can be attempted.

## 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 I, General Procedures for Bacteriology.

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# IX. Furunculosis

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## A. Name of Disease and Etiological Agent

Furunculosis, *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 4-12 d 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 8-9°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 2-3 d 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 -- More gradual in onset of mortality. Internal lesions are present but the fish also commonly have skin lesions.
4. Chronic -- Similar to subacute, but distinguished by evidence of healing around lesions.
5. Latent -- No mortality or clinical signs associated with *Aeromonas salmonicida* infections are evident.

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 20-25°C for 24-48 h. 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 I, General Procedures for Bacteriology.

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# X. Other Diseases Caused by *Aeromonas salmonicida*

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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.

### 1. 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.

### 2. Carp Erythrodermatitis

Carp erythrodermatitis (CE) is a subacute to chronic skin disease that occurs at 4-30°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.

### 3. Trout ulcer disease

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

Diagnostic procedures for atypical *Aeromonas salmonicida* infections are similar to those for furunculosis. However, the use of blood agar 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 (see e.g. 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 Furunculosis, Chapter IX).

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# XI. Motile *Aeromonas* Septicemia

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## A. Name of Disease and Etiological Agent

### 1. Name of disease

Motile Aeromonas septicemia (MAS), bacterial hemorrhagic septicemia (BHS), hemorrhagic septicemia, and other synonyms.

### 2. Etiologic agent

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

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical Range

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 diseases.

## 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. Histopathologically, the disease is similar to other generalized septicemic diseases caused by gram-negative rod-shaped bacteria.

## 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 20-25°C for 24-48 h 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 I, General Procedures for Bacteriology.

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# XII. Vibriosis

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## 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 1-1.5% 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 (0.5-3.0 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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Vibrio salmonicida

# XIII. Coldwater Vibriosis

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## 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 1-1.5% 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 $15^{o}$ 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 (0.5-3.0μm), 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 0/129 (2,4-diamino 6,7-diisopropyl pteridine phosphate) and novobiocin. Identification of the specific species *Vibrio salmonicida* is determined serologically, for example, the slide agglutination test or the fluorescent antibody test may be used.

## F. Procedures for Detecting Subclinical Infection

No procedures for detecting latent infections are routinely used.

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

No procedures for determining prior exposure are routinely used.

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

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

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Incub temp?
RT?

Biochem

add'l salt necessary?
API code avail?

Stock culture?

Copy of his paper?

# XIV. Bacterial Kidney Disease

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## A. Name of the Disease and Etiological Agent

Bacterial kidney disease (BKD), caused by *Renibacterium salmoninarum*

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical Range

North America, Chile, Scotland, England, France, Italy, Spain, Yugoslavia, Turkey, Iceland, Norway, Sweden, Finland, and Japan.

### 2. Host Species

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

## C. Epizootiology

*Renibacterium salmoninarum* may 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 (15.0-20.5°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 *Renibacterium 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. 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, abdominal distension, 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, and the dermatitis known as "spawning rash" in rainbow trout.

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 diffuse or discrete, but are rarely encapsulated. Central areas of necrosis are common. Gram staining of tissue sections reveals both extracellular bacteria and bacteria within macrophages.

## 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. The organism should fail to grow on TSA at 20°C, even when extended incubation periods (e.g., 2 weeks) are used.

### 2. Confirmatory diagnosis

In addition to bacteriological culture, several immunological tests to confirm the presence of cellular or soluble fractions 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), the 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). 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 the FAT in tissue smears are similar to those for identifying the bacteria from culture (see General Procedures for Bacteriology

chapter), with some modifications. The quality of the preparations and consequent sensitivity of the test may be enhanced by homogenization of kidney tissue samples prior to smear preparation, treating smears with a solvent (xylene or acetone) for 1-2 min followed by a tap water rinse) on the same day as staining, and increasing antibody staining time (30 min) (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 (Pascho and Mulcahy 1987, Pascho et al. 1987) and monoclonal antibodies (Turaga et al. 1987; Rockey et al. 1991) have been developed. Commercially prepared ELISA reagents are available for use in an ELISA procedure described by Pascho et al. (1991).

c. The kidney disease bacterium can be cultured from fresh tissues or body fluids, and then shown to be biochemically or immunologically identical to *Renibacterium salmoninarum.* The culture medium should include cysteine and serum (Evelyn 1977), although it has been reported that charcoal can be substituted for the serum component (Daly and Stevenson 1985). Antimicrobial agents can be added to the culture medium to inhibit the growth of other organisms present in the samples (Austin et al. 1983). Additional steps may be required to reduce the inhibitory effect of certain unknown elements of fish tissues on the growth of *Renibacterium salmoninarum* (Evelyn et al. 1981; Daly and Stevenson 1988). The optimal incubation temperature is 15°C, and extended incubations (up to 12 weeks) 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*.

## F. Procedures for Detecting Subclinical Infections

Monitoring of moribund fish in seemingly healthy stocks can be done by the FAT, by the ELISA, or by culture. In critical situations, the use of more than one technique for *Renibacterium salmoninarum* detection is recommended. The ELISA has proven sensitive enough to detect the presence of low levels of *Renibacterium salmoninarum* antigen in asymptomatic fish in a population (Pascho et al. 1987, Pascho et al. 1991). The viability of *Renibacterium salmoninarum* can only be determined by culture. Enhancement techniques such as the use of nurse cultures or supplementation of a medium with spent growth medium may be necessary to detect the bacterium in asymptomatic fish.

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 *Renibacterium 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). The filtration-FAT (Elliott and Barila 1987) is a sensitive technique for the enumeration *Renibacterium salmoninarum* in ovarian fluid.

## 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.

## 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 24 h. Samples for ELISA testing should be refrigerated if they are to be analyzed within 24 h 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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# Methods Appendix A

## Reagents required for the ELISA to detect *R. salmoninarum*

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

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

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

1. Store lypohilized preparation at 4°C. Stable for a minimum of one year.
2. Rehydrate lyophilized bacteria with 1.0 ml Nanopure water.
3. Prepare necessary dilutions, 1:100, 1:500, 1:1,000, and 1:5,000 (v/v), in PBS-T20 and store at -70°C.

**Coating buffer.** Sodium carbonate, pH 9.6, or a commercial coating solution.

1. Prepare fresh for each ELISA.
2. Sodium carbonate, pH 9.6. Store at room temperature and discard after 30 days. For 1 liter.

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

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

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

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

2. Rehydrate lyophilized coating antibody by first mixing 1.0 ml glycerol + 1.0 ml Nanopure water that contains 0.2% (2x) strength thimerosal, then transferring 1.0 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.

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

1. Prepare fresh for each ELISA.
2. PBS-T20. Prepare as described above.
3. Commercial wash solution concentrates. Store concentrate at 4°C after opening; usually stable for a minimum of one year. Dilute according to manufacturers instructions.

**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.

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

1. Store lypohilized preparation at 4°C. Stable for a minimum of one year.
2. Rehydrate lyophilized coating antibody by first mixing 1.0 ml glycerol + 1.0 ml Nanopure water that contains 0.2% (2x) strength thimerosal, then transferring 1.0 ml of the 50% glycerol solution to each product vial.
3. Rehydrate the contents of a sufficient number vials to test the anticipated number of fish that will be sampled for a given spawning season. Pool the contents of all vials, then dispense into several cryovials and store at -70°C.

**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.

**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.

# Methods Appendix B

## Checkerboard titration to determine the working concentrations of coating antibody and conjugate used in the ELISA to detect *R. salmoninarum*

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

This appendix describes a checkboard titration method for determining the working concentrations of the coating antibody and conjugate preparations. It is based on the standard ELISA protocol described in Appendix 2.

### Reagents and test samples

1. Coating antibody and conjugate

    a. Rehydrate several vials of each preparation according to instructions in Methods Appendix A.
    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 4 positive controls (1:100 (1), 1:1,000 (2), 1:2,000 (3), and 1:5,000 v/v (4) dilutions) described in Methods Appendix D.
    b. The buffer formulations and their uses are described in Appendix 2, and Methods Appendices A and D.

### 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 4 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 | | | | | | | | | | | | | |

**Checkerboard titration procedure**

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

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

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

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

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

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

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

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

3. Wash microplates to remove unbound immunoglobulin.

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. 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 3 h 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 for each dilution.

      Example 2. Calculation of the volume of a 1:2,000 dilution of the HRP-conjugate necessary when testing 3 concentrations of coating antibody; 3 microplates x 64 wells/plate = 192 wells.

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

      $1/2000 = X / 45\ mL$
      $X = 22.5\ \mu L$

      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 2 h at 25°C.

5. Wash microplates five times as described previously.

6. Substrate-chromogen reaction.

   a. Start the substrate-chromogen reaction

      1. 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.

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

      3. 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.

      4. 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

      1. While the substrate-chromogen reaction is proceeding at 37°C, prepare the stop solution. Each well will receive 50 µL.
      2. 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.
      3. Wipe any condensation off the bottom of the plate and immediately measure the absorbances at 405nm.

# Methods Appendix C

## Collection and processing of tissue or body fluid samples for the ELISA

### Sample Collection and Handling

1. General comments

   a. Field containers should be labeled, sterile, stomacher-type bags with a top closure, effecting 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, care should be exercised to avoid cross-contamination between fish and contamination of tissue sample from body fluids
   b. Collect a 2-to-5 gram sample; when sampling the kidney, it is recommended that this sample consist of a pool of small tissue pieces from the anterior, mid, and posterior kidney.
   c. For ovarian fluid, collect approximately 1 mL.
   d. Keep tissue and body fluid samples on ice during collection.

4. Sample collection from juvenile fish

   a. Juvenile fish are often collected as whole fish and dissected upon return to the laboratory.
   b. Remove the entire kidney and spleen from each fish. It is preferrable to test tissues from individual fish, but a tissue pool may be made if the fish are too small. Two-fish pools are recommended when an insufficient amount of tissue is recovered from an individual fish. *If you cannot adhere to these guidelines, then the fish are too small for testing.*
   c. As a minimum, need approximately 0.08 g of sample for testing
   d. Keep fish or tissues on ice during collection and processing.

### 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 min. and refrigerated overnight. Each sample must be centrifuged (8,000-10,000 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 min, 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 min, 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.

## References

Scottt, R., and N. Nesbitt. 1995. Efficacy of decontaminating tools for *Renibacterium salmoninarum* testing. Abstract 1995 Joint Meeting of the Fish Health Section American Fisheries Society and Eastern Fish Disease Workshop; Syracuse, New York, July 19-22, 1995.

## Methods Appendix D

### Description of controls used in the ELISA to detect *R. 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 min; and centrifuge 8,800 × g for 5 min.

# Methods Appendix E

## Method to detect *R. salmoninarum* by the ELISA

### Before the ELISA

1. Reagent preparation

   Methods Appendix A.

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 Methods Appendix B).
   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

   a. Prepare fish tissue or body fluid samples for the ELISA as described in Methods Appendix C. Heat each sample at 100°C for 15 min, then centrifuge 8,000 to 10,000 *x g* for 10 min. 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.

### ELISA Day 1

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

   a. General comments

      1. 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.
      3. 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.
      4. There are several controls that must be included in each ELISA. Their preparation and use are described in Methods Appendix D.

b. Procedure

1. 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.

2. 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 comphensate 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.

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

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

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

3. 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 d. Use water of reagent grade or equivalent.
4. 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.
5. 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:
6. 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 h.

**ELISA Day 2**

1. Wash microplates to remove unbound immunoglobulin.

   a. General comments

   1. 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.
   2. The wash buffer should remain at room temperature during the ELISA.

b. Procedure

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

2. Wash plates in numerical order.

3. 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 3 h at 25°C in a humid chamber.

3. Wash microplates five times as described previously.

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

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

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

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

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

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

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

c. Seal each plate with an adhesive plate sealer and incubate in a humid chamber for 2 h at 25°C.

5. Wash microplates five times as described previously.

6. Substrate-chromogen reaction.

a. General comments

1. The timing of the substrate-chromogen reaction is critical. The reaction must be stopped after exactly 20 minutes.
2. The volume of stop solution is 50 µL to insure that the wells are not over-filled. The SDS stop solution is prepared from a 5% (v/v) concentrate as follows:

   4 parts concentrate + 1 part water

b. Procedure to start the substrate (hydrogen peroxide) -chromogen (ABTS) reaction

1. 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.

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

3. Prepare the substrate-chromogen solution.

4. 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.

5. 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

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

2. 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.
3. Wipe any condensation off the bottom of the plate and immediately measure the absorbances at 405nm.

# XV. Pseudokidney Disease

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## A. Name of Disease and Etiological Agent

Pseudokidney disease, *Carnobacterium piscicola* (formerly classified as *Lactobacillus piscicola*).

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical Range

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 15-24°C for 24-72 h.

### 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 1.1-1.4 x 0.5-0.6 µm in size. Older cultures become gram variable. Other phenotypic traits include negative results for oxidase, catalase, urease, $H_2S$, nitrate 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, General Sampling Procedures for Bacteria.

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# XVI. Streptococcal Disease

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## A. Name of Disease and Etiological Agent

Streptococcal disease, caused by *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.

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical Range

United States, 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.

## 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 streptococccal types.

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

## D. Disease Signs

Disease signs are variable, but affected fish commonly have numerous raised, hemorrhagic, inflamed areas on the skin including the operclulum, around the mouth, at the bases of fins, and in general along the dorsolateral portions of the body. The abdomen is distended and the peritoneal cavity often contains bloody fluid. Exophthalmia is present with hemorrhage in the eye. 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 often swim in a tail chasing spiral.

Histological findings vary depending on the organisms and host species involved. Infections in the head often produce a granulomatous encephalitis and meningitis.

## 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 blood agar, or both, incubated at $25^{\circ}$-$35^{\circ}$C for 24-48 h. 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 blood agar, 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 I., General Procedures for Bacteriology.

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# XVII . Piscirickettsiosis

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## A. Name of Disease and Etiological Agent

**1. Name of disease**

Piscirickettsiosis, salmonid rickettsial septicemia, coho salmon septicemia, Huito disease

**2. Etiologic 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 2000).

## 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 6-12 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 (Figure 1).

## E. Disease Diagnostic Procedures

Piscirickettsiosis is diagnosed based on clinical signs, isolation of *P. salmonis* in cell culture, and detection in Geimsa stained tissue impressions or sections. It is pleomorphic, ranging from 0.5 to 1.5 µm in diameter, and frequently occurs pairs (Figure 2). Positive identification is confirmed using *P. salmonis*-specific antibodies in the IFAT.

### 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 the "General Procedures for Bacteriology" section. 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 - 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). 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 - 18°C for an additional 14 days.

### 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 6-12 hrs), 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 5 minutes in absolute methanol. Tissues smears to be examined by IFAT must be freshly prepared or stored at $\leq$-20°C.

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# VIRAL DISEASES OF FISHES

I. GENERAL PROCEDURES FOR VIROLOGY

II. CHANNEL CATFISH VIRUS DISEASE

III. ERYTHROCYTIC INCLUSION BODY SYNDROME

IV. HERPESVIRUS DISEASES OF SALMONIDS

V. INFECTIOUS HEMATOPOIETIC NECROSIS

VI. INFECTIOUS PANCREATIC NECROSIS

VII. VIRAL ERYTHROCYTIC NECROSIS

VIII. VIRAL HEMORRHAGIC SEPTICEMIA

IX. INFECTIOUS SALMON ANEMIA

APPENDIX 1. METHODS FOR TESTING FOR THE PRESENCE MYCOPLASMA IN STOCK CELL CULTURES

# I. General Procedures

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## I. Tissue and Fluid Sampling and Handling

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

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

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.

B. For storage of samples:

1. The samples should be maintained between 4 and $10^{\circ}$ C according to the virus(es) suspected. Samples should not be frozen.
2. The samples should not be stored longer than 48 h.
3. Tissues may be stored in a buffered solution that contains antibiotics, antifungals, or both. The pH should be maintained within 7.4 - 7.8 or within the range that the suspected virus(es) are stable.

## II. Preparing Samples for Virus Assays

The preparation of samples involves 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 antibiotics and antifungals or by filtration of the supernatant of centrifuged tissue samples.

A. 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 are acceptable:

| | |
|---|---|
| Gentamicin | 200 - 500 µg/mL |
| Penicillin | 800 IU/mL |
| Streptomycin | 800 µg/mL |
| Amphotericin B (Fungizone) | 20 µg/mL |
| Mycostatin (Nystatin) | 200 IU/mL |

B. The supernatant from centrifuged tissue samples can also be decontaminated by filtration through a 0.45 µm filter. Passing tissue culture medium supplemented with 10% serum through the filter before the sample is passed is recommended to minimize virus adherence to the filter.

## III. Inoculating the Samples

### A. 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 (Appendix 1). Penicillin(100 IU/mL), streptomycin (100 µg/mL), and antifungal agents such as Mycostatin/Nystatin (25 IU/mL) or Amphotericin B/Fungizone (2.5 µg/mL) can be used in media for cell culture and virus assay work.

### B. Inoculation

The cell cultures used for sample inoculation should be 80-90% confluent and not older than 48 h. A minimum of 50 µL of sample should be inoculated per 1.0 $cm^2$ of cell sheet . Uninoculated controls must be used. Dilution of original samples should not exceed 1:10 for fluids and 1:100 for tissue samples. Final antibiotic concentrations should not exceed 100 µg/mL gentamicin, 100 IU/mL penicillin, or 100 µg/mL of streptomycin and antifungal agent concentrations should not exceed 25 IU/mL of mycostatin (Nystatin) or 2.5 µg/mL or amphotericin B (Fungizone).

### C. Duration of Assay

The cell cultures should be incubated at 15°C and observed for cytopathological changes for a minimum of 14 d but 21 d 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 d is also recommended. The duration of the assay

may need to be longer depending on which viruses are suspected. When cytopathogical changes occur, the cultures should be subcultured or analyzed by serum neutralization or other confirmatory tests.

## IV. 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.

### A. Serum Neutralization Test

1. A dilution of neutralizing antiserum (polyclonal or monoclonal) should be used that allows neutralization of $10^2$ - $10^6$ plaque forming units (PFU) or 50% tissue culture infective dose ($TCID_{50}$) per mL of the homologous virus.
2. Dilute the suspect sample from $10^{-2}$ to $10^{-6}$ in a sterile balanced salts solution.
3. 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.
4. Inoculate onto the cell line in which the suspect virus was isolated. Incubate at 15° C for 14 d 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.

### B. Other Confirmatory Procedures

Other serological procedures that are routinely used for confirmatory virus identity include the fluorescent antibody test (FAT), dot blot methods, and the enzyme-linked immunosorbent assay (ELISA). Consult each virus section for references on alternative confirmatory methods.

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# II. Channel Catfish Virus Disease

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## 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

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 1-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, 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.

## 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 - 30°C at pH 7.2-7.4. Channel catfish ovary cells are approximately ten times more sensitive than BB cells. Inoculated cultures are incubated for 14 d and observed for typical CPE (cell fusion and synctium 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.

## 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 min. The serum is diluted 1:50 and reacted with known CCV at 100 $TCID_{50}$ or PFU/0.1 mL of the serum and virus mixture. Sera demonstrating greater than 50% plaque or $TCID_{50}$ reduction are considered to be from fish previously exposed to CCV. The CCV antibody titers in fish increase as water temperatures rise.

Prior exposure to CCV may also be indicated by detection of CCV DNA (Colyer et al. 1986, Bird, et al. 1988). Several reports describe the use of molecular cloning of channel

catfish virus genome and the use of nucleic acid probes to detect CCV genetic material in channel catfish. These techniques are not yet sufficiently refined for practical application.

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

See General Procedures chapter.

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# III. Erythrocytic Inclusion Body Syndrome

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## 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 which 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 5 months) or be more acute and are most often complicated with other pathogens, i.e. *Flexibacter 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 1-4% to slightly below normal values (25-30%).

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 0.8-2 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).

### Pinacyanol Chloride Staining and Light Microscopy

Air dried blood smears are fixed in 100% methanol for 5 min and then stained with pinacyanol chloride for 2 min. 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.

### Acridine Orange Staining and Fluorescent Microscopy

Methanol/ethanol (1:1) fixed smears which are rehydated in ethanol series of 100%, 70%, and 50% and stained with 0.1% aqueous acridine orange, (3,6-bis[dimethylamino] acridinium chloride hemi-[zinc chloride]) then washed in phosphate buffered saline 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

# IV. Herpesvirus Diseases of Salmonids

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## I. *Herpesvirus salmonis* Type 1

### A. Name of Disease and Etiological Agent

Herpesvirus disease of salmonids, *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 or coho salmon *Oncorhynchus kisutch.*

## C. Epizootiology

HPV-1 was isolated from 1971-1975 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, exophthalmos with hemorrhages in the orbital regions and the base of the fins. Mucoid fecal casts and pale gills are also evident. Ascites is evident as is a mottled liver, pale kidney, and empty anterior digestive tract. The liver, spleen, kidney, and heart 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 multi-nucleated giant cells with intranuclear Cowdry type A inclusion bodies. Samples containing very low levels of SHV or HPV will appear negative after 2-3 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 3 week initial incubation period followed by a blind passage and another 3 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 General Procedures chapter.

# II. *Herpesvirus salmonis* Type 2

## A. Name of Disease and Etiological Agent

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

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

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical Range

Japan

### 2. Host Range

OMV has been isolated from masu 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 1970-1974 at Towada 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 masu, chum and coho salmon and rainbow trout. Both vertical and horizontal transmission are likely. Susceptibility decreased with increasing age; by 8 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.

OMV--Fish experimentally infected with OMV became lethargic, anorexic and congregate at the water intake. Gross signs include exophthalmia and petechiation of the body surface. Naturally infected fish only showed a darkened body color. Experimentally challenged fish have mottled and swollen livers, swollen spleens and empty digestive tracts. Surviving fish may develop tumors on the external surfaces of the opercula, heads, mouths, eyes, and caudal fins. Microscopic examination of tissues from fish infected with OMV show necrosis and syncytia formation in the liver and hematopoietic tissue of the kidney and edema, and less severe necrosis of the spleen, pancreas, cardiac muscle and brain. One fish developed an epithelioma in the kidney.

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

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

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 NeTVA replicate at 10-20°C and virus replication at 15°C suggests that neither HPV or 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 General Procedures chapter.

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# V. Infectious Hematopoietic Necrosis

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## A. Name of Disease and Etiological Agent

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

## 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 virus is also considered to be enzootic to Hokkaido and Honshu Islands of Japan. The disease has been observed in other areas of the United States including Colorado, Minnesota, Montana, New York, South Dakota, West Virginia and Virginia. Recently, epizootics occurred in Taiwan, Italy, France, Switzerland, Korea, and the Peoples Republic of China, but it is not known if the virus has become established in these countries.

### 2. Host Species

Epizootics of IHN have commonly occurred in juvenile sockeye (kokanee) *Oncorhynchus nerka* and chinook salmon *Oncorhynchus tshawytscha* and steelhead and rainbow trout *Oncorhynchus mykiss*. The disease has also been reported in Atlantic salmon *Salmo salar*, 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.

## 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 12°C or less, but outbreaks have occurred at 15°C.

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. Degenerative changes in the same target organs occurs in older fish with IHN but granular cell involvement has not been observed.

## 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 h post-inoculation depending on the concentration of virus in the inoculum. The CPE also can be observed by studying plaque morphology. 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. A staphylococcal coagglutination assay has also been reported which can aid in a diagnosis if high virus concentrations are present (Bootland and Leong, 1992). 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). 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). Recently, 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. 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 possibly could be used for serological strain typing of IHNV isolates. A biotinylated DNA probe has also been developed (Deering et al. 1991).

## F. Procedures for Detecting Subclinical Infections

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

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

Hattenberger-Baudouy et al. (1989) found complement dependent neutralizing antibodies in juvenile and adult rainbow trout and suggested that this could be used as a tool for fish health monitoring. Techniques that have been used to detect antibodies to IHNV in fish serum include, neutralization (Hattenberger-Baudouy et al. 1989; Jorgensen, et al. 1991), fluorescent antibody and ELISA (Jorgensen et al. 1991).

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

See the General Procedures chapter.

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# VI. Infectious Pancreatic Necrosis

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## 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 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, 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, 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 sec 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 General Procedures chapter.

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# VII. Viral Erythrocytic Necrosis

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## 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

Probably 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 2-10%. 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 5 min 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%, 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 h of collection.

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# VIII. Viral Hemorrhagic Septicemia

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## 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 has also been isolated 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, giebel *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 longterm 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 papillosum 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 7.4-7.8) or cell culture medium (pH 7.4-7.8) 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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Way, K., and P.E. Dixon. 1988. Rapid detection of VHS and IHN viruses by the enzyme-linked immunosorbent assay (ELISA). Journal of Applied Ichthyology 4:182-189.

# IX. Infectious Salmon Anemia

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## 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: Hemorrhagic kidney syndrome (HKS)

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical Range

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

### 2. Host Species

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

## C. Epizootiology

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

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

## D. Disease Signs

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

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

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

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

## E. Disease Diagnostic Procedures

### 1. Presumptive Diagnosis

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

Kidney and spleen tissues (1cm$^3$ sample size) are collected, homogenized, and diluted 1/10 in phosphate buffered saline. Tissue homogenates are further diluted 1/10 in Minimum Essential Medium (MEM) with Hank's salts, L-glutamine, and 2% fetal bovine serum (FBS). Samples are inoculated onto either the chinook salmon embryo (CHSE-214) or salmon head kidney (SHK) cell line at a volume of 0.1 ml/well and adsorbed for 1 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).

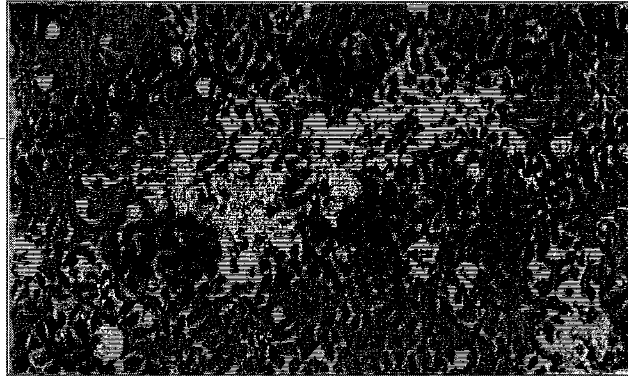

**Figure 1. Cytopathic effect on CHSE-214 cells**

**2. Confirmatory Diagnosis**

The routine method of confirmation of ISA virus is by RT-PCR (Appendix A). 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 Appendix A (ISAV RT-PCR) and Appendix B (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.

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## APPENDIX A

## REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR) FOR INFECTIOUS SALMON ANEMIA VIRUS (ISAV)

### 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.

### SPECIMEN

Kidney Tissue

A 30-50 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 70-90% ethanol or dipped sequentially in a 10,000 ppm bleach solution and DI-$H_2O$ between samples to prevent carryover contamination. The kidney is then submerged in five volumes (1 ml) of RNA Later™ in a 1.5 or 2.0 ml screw cap microfuge tube. The tissue can be stored in RNA Later™ for one day at 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.

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.

### MATERIALS

RNA Later™ (Ambion)
1.5 or 2.0 ml screw cap microfuge tubes
Sterile 1 and 10 ml pipets
Sterile plugged nuclease free 10µl, 100µl, and 1000µl 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 ì l, 10-100 ì l, 200-1000 ì l 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)
$\phi$x174 HAE III digested DNA size markers (Promega)
TAE buffer (See Appendix B)
Molecular biology grade agarose (Life Technologies)
Ethidium bromide
Electrophoresis equipment
UV transilluminator
Mastercycler Gradient thermocycler (Eppendorf/Fisher Scientific)
Microcentifuge (Eppendorf and IEC)
Photographic equipment with UV filter

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).

**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.

## 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.

**Kidney Tissue Homogenization**
Trizol Reagent

1. Remove kidney tissue to be extracted from RNA Later, place on a clean surface and trim a 20-30 mg piece using a scalpel flamed with 70-90% ethanol. Keep scalpel and forceps in 70-90% ethanol and flame between samples in order to prevent carryover to subsequent samples. Replace remaining kidney tissues in original microfuge tubes with RNA Later.
2. Place trimmed tissue in a 1.5 ml microfuge tube containing 500 µl of Trizol.
3. Grind tissue in the microfuge tube using a nuclease-free pellet pestle.
4. Pulse-centrifuge microfuge tube for a few seconds in order to pull down any remaining large pieces of tissue.

5. Transfer 250 µl of the supernatant to another microfuge tube containing 750 µl of Trizol for a total of 1 ml.

**RNA Extraction from Kidney Tissue**

Trizol Reagent

1. Phase separation
   Incubate supernatant/Trizol mixture at room temperature for 5 minutes. Add 200 µl chloroform to each tube using a new pipet tip for each sample. Shake for 15 seconds and incubate at room temperature for 5 minutes. Centrifuge cold at 12000 X g for 15 minutes in order to separate phases.
2. RNA precipitation
   Transfer clear aqueous phase to a new 1.5 ml microfuge tube (0.4-0.45ml). Avoid disturbing DNA in the interphase with the pipet tip. Add 500 µl of isopropyl alcohol. Incubate samples at room temperature for 10 minutes. Centrifuge cold at 12000 X g for 10 minutes.
3. 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 5 minutes.
4. Redissolving RNA
   Remove all of the supernatant. Add 20-40 µl Tris-EDTA buffer with Rnasin. Gently pipet up and down in order to dissolve RNA pellet.

**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.

**RT-PCR**

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

1. 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 |

2. Aliquot 49 µl of the master mix into each Ready-to-Go™ RT-PCR Beads tube.
3. Add 1 µl of each RNA sample to a Ready to Go™ RT-PCR Beads tube.
4. Place tubes in thermocycler and run RT-PCR59 program:
   Lid 105°C
   1. 42°C 15 min.
   2. 94°C 5min.
   3. 59°C 45 sec.
   4. 72°C 105 sec.
   5. 94°C 45 sec.
   6. 59°C 45 sec.
   7. 72°C 105 sec.
   8. GOTO 5 for 36 cycles

9. 72°C 7 min.
10. Hold 4°C

5. 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 1-1.5 hours.
6. Stain gel with ethidium bromide for 30-40 minutes. De-stain gel in DI-$H_2O$ for 10 minutes.
7. Photograph gel under UV illumination.

## 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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# APPENDIX B

# INDIRECT FLUORESCENT ANTIBODY TEST FOR INFECTIOUS SALMON ANEMIA VIRUS (ISAV)

## PRINCIPLE / PURPOSE

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

## 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.

## 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

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[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

## PROCEDURE

A. Sample preparation
   1. Kidney blot: Using sterile forceps and scalpel, remove a $1cm^3$ piece of tissue from the mid-kidney. Firmly touch one surface of the kidney chunk two times onto a clean slide. Each blot should cover approximately 1/2 of the slide surface. The first blot will contain more blood than the second, however avoid leaving a blot which is too thick or too thin.
   2. 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.
   3. Allow sample to dry for 30-60 minutes and fix by immersing in acetone for 10 minutes. Store slides at –20°C until stained.

B. Staining
   1. If slides are removed from –20°C, allow to reach room temperature before staining.

2. Trace each blot with the PAP pen. Allow to dry for a few minutes. Place 2-3 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.
3. 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 2-8°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.
4. Cover slides with bottom of tupperware container and allow incubate for 30 minutes. Do not allow slides to dry during the staining procedure.
5. 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 2-8 °C.
6. 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.
7. 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 6 minutes. Either metal slide racks or plastic specimen cups may be used during the washes.
8. The remainder of the staining procedure must be performed away from direct light.
9. 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 2-8 °C.
10. 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.
11. Repeat step 7.
12. 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 2-8°C until analysis.

C. Resulting stained slides

1. View slides under fluorescence with a wide green filter after mounting a coverslip with 1-2 drops of immersion oil.
2. The interpretive scoring method for IFAT/ISAV is as follows:
   - 1+ : sparse distribution of fluorescent grains or specks throughout
   - 2+ : more obvious distribution of grains (>10 field$^{-1}$ ) e.g: around cell membranes
   - 3+ : obvious distribution of grains e.g.,: broken lines around cell membranes
   - 4+ : broad fluorescence; three to four fully involved cells per field of view
3. An interpretive scoring of 1+ or 2+ is considered a suspect rating result for ISAV by IFAT.
4. An interpretive scoring of 3+ or 4+ is considered a positive rating result for ISAV by IFAT.

Appendix 1

# Methods for Testing for the Presence of Mycoplasma in Stock Cell Cultures

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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.

## 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 month intervals, for the presence of mycoplasmal contamination. Contaminated cultures should be discarded.
4. Mycoplasma-negative cultures must be handled before mycoplasma-positive cultures are handled in the laboratory. Separate media and solutions should be used for mycoplasma-negative and mycoplasma-positive cultures.
5. The antibiotics, penicillin (100 IU/mL) and streptomycin (100 μg/mL) or gentamicin (100μg/ml), and the antifungal agents, Mycostatin R (25μg/mL) or amphotericin B (2.5 μg/ml) are permitted for media in routine cell culture work.

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# I. General Procedures for Parasitology

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## 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, 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.

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 (ie. 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 swimbladder 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 5-10% 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 (8.5gm 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

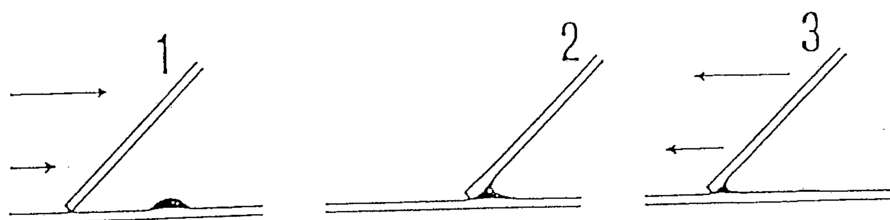


**Figure 1.** Preparation of a blood smear. (from Moller and Anders, 1986)

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).

## 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 slide 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 10-40X 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 (ie. 10% x 40% of stock = 4%).

### 1. Protozoan Smears/ Small Helminths

If a temporary preparation is required for protozoan smears or small helminths, this may be done in one of two ways. A preparation may be stored overnight as follows: a dampened paper towel is placed in the bottom of a Petri dish, the specimen slide placed on top of the paper towel, the lid placed on the dish, and then put into a 4°C refrigerator. A sample preparation may be retained for several days by adding a ring of nail polish or petroleum jelly around the perimeter of the cover slip to prevent dehydration and keeping this under refrigeration.

If any temporary preparation is to be fixed, the fixative can be added to one side of the cover slip. A dry paper towel is touched to the opposite side of the cover slip. This will cause the saline under the cover slip to be drawn into the paper and create a capillary action under the cover slip which will draw the fixative through the sample.

### 2. Helminths, Acanthocephalans, and Leeches

When necessary, living trematodes, cestodes, and leeches should be fixed under light pressure of a cover slip or glass slide. The saline is replaced slowly with warm fixative.

All helminth parasites should be cleaned with saline before fixation. This is accomplished by placing the parasite into an appropriate sized vial filled with saline, capping it and shaking it vigorously. Small parasites are best handled by sucking them up with

a Pasteur pipette to minimize damage, whereas larger parasites can be handled with forceps. The fluid is decanted and the procedure repeated as necessary. (Note: Decant into a jar, not over a drain.)

Monogeneans, trematodes, cestodes, acanthocephalans, and leeches can be fixed in 5 -10% neutral buffered formalin for 24 hours and transferred to 70% ethanol for storage and/or shipping. Many helminth parasites may be relaxed in fresh water before fixation. Some parasitologists suggest relaxing cestodes in 80°C tap water and then slowly replacing the water with fixative. Water or fixative that is too hot may result in blisters to the tegument or cuticle of certain parasites, therefore the use of warm (ca. 50°C) formalin to relax and fix live parasites is recommended.

Live acanthocephala should be left in distilled water overnight in the refrigerator to induce eversion of the proboscis before fixation in warm formalin. Large nematodes and acanthocephalans may have to have holes poked in them with fine dissecting needles. Entomology pins mounted onto the end of small wooden dowels are excellent for this purpose as well as for manipulating small, delicate parasites. Live nematodes and crustaceans are fixed for 24 hours in warm solution of 5 -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 labelled. 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 label is most important.

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# Ia. 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

# II. Ichthyobodiasis

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## A. Name of Disease and Etiological Agent

Ichthyobodiasis is caused by *Ichthyobodo* (*Costia*) spp. This is a protozoan flagellate belonging to the Class *Zoomastigophorea*, Order *Kinetoplastida*, Family *Bodonidae*. There are two species of *Ichthyobodo* commonly reported for fish: *I. pyriformis* and *I. necator*. A synonym for *Ichthyobodo*, is *Costia*. *Ichthyobodo* is sometimes spelled *Ictyobodo*, *Icthyobodo*, or *Ichtyobodo*.

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical Range

This parasite occurs throughout the world.

### 2. Host Species

This parasite has been reported from many fish species. It is reported that all species of freshwater fishes are susceptible to attack. It has been a problem in virtually all cultured fish ranging from tropical fish to salmonids.

## C. Epizootiology

*Ichthyobodo* is an obligate parasite that must have a fish host or it will die in a short time. It is attached to the host but adverse conditions may cause the parasite to encyst, either on the fish or free in the water. Electron microscopy has shown attachment to the host to be by a small disc with microtubules that extend into host cells. Young fish are reported to be more susceptible to *Ichthyobodo* than older fishes.

Temperature plays an important part in the infectivity of the parasite. Temperatures of 25° 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 5-20 µm in length. Reproduction takes place by simple transverse division, but the parasite is able to encyst after leaving the fish.

## 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.

### 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.

## 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 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.

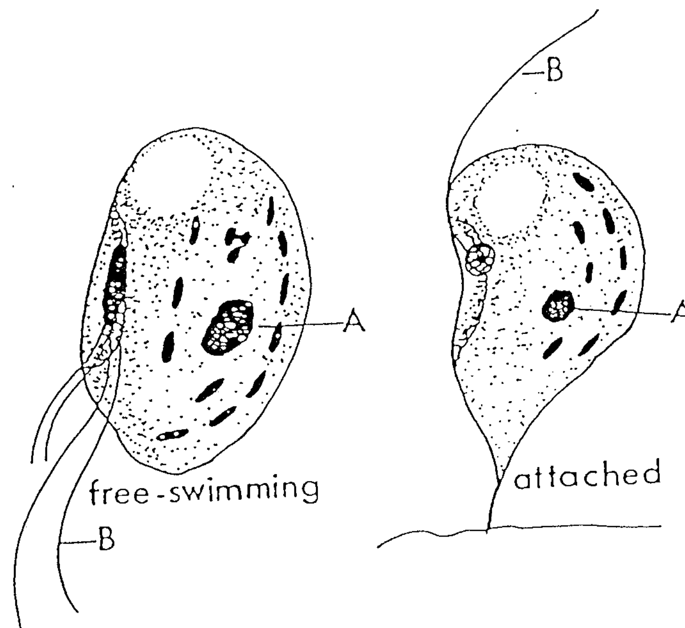


**Figure 1.** *Ichthyobodo.* Line drawing of free-swimming and attached form. A. Nucleus. B. flagella.

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# III. Hexamitiasis (Octomitosis) and Spironucleosis

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## A. Name of Disease and Etiological Agent

Hexamitiasis (Octomitosis) and Spironucleosis are infections by diplomonad flagellates *Hexamita* and *Spironucleus*, collectively called hexamitids (Family *Hexamitidae*, Subfamily *Hexamitinae* (Kulda and Nohynkova 1978)). In most studies, the genus identity of the hexamitid has not been confirmed, and some old literature incorrectly identified organisms as *Octomitus*. Species are poorly distinguished with the exceptions of *Hexamita salmonis*, *Spironucleus elegans* and *Spironucleus torosa* (Kulda and Lom 1964, Woo and Poynton in press).

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical Range

*Hexamita* and *Spironucleus* commonly infect freshwater and marine fish in many parts of the world including North America and Europe, and occur in wild, farm, and aquarium environments.

### 2. Host Species

Infections occur in members of the following families of fishes in coldwater environments: Acipenseridae, Anguillidae, Catostomidae, Centrarchidae, Cyprinidae, Cyprinodontidae, Gadidae, Gasterosteidae, Mugilidae, Percichthyidae, Percidae, Salmonidae, Siganidae, and Sparidae. The salmonids, such as rainbow trout *Oncorhynchus mykiss*, frequently carry *Hexamita salmonis* in their pyloric caeca and upper intestine. Gadids such as Atlantic cod *Gadus morhua* and haddock *Melanogrammus aeglefinus*, commonly carry *Spironucleus torosa* in their rectum. The best known warm water hosts of hexamitids are members of the Family *Cichlidae*.

## C. Epizootiology

### 1. Conditions Under Which Disease Occurs

Hexamitids are systemic invaders. Their life cycle is direct and transmission is usually by the ingestion of resistant cysts which have been voided with the host's feces. Hexamitids may change from commensals to pathogenic parasites, with the possibilities of systemic invasion, when the host's vitality suffers due to other factors such as poor water quality, temperature fluctuations, inadequate nutrition, and overcrowding. It is likely that pathological changes depend partly on the genus and species of the hexamitid present, and high density infections are not always accompanied by pathology. *Hexamita salmonis* has often been associated with moderate to severe mortalities, both acute and chronic in young farmed salmonids.

## D. Disease Signs

Although hexamitids are often associated with morbidity and mortality, particularly in young farmed salmonids, pathogenicity is disputed and poorly understood. Clinical signs of infection may vary. Systemic infections can occur in cyprinids, eels, cichlids, and salmonids (Mo et al. 1990).

### 1. Behavioral Changes Associated with the Disease

Fish may be anorexic, weak, excessively nervous or whirling.

### 2. External Gross Signs

Signs may include emaciation, dull and dark color, red vent, pale shiny feces, abdominal distension, and exophthalmia.

### 3. Internal Gross Signs

Gastrointestinal infections may be indicated by the presence of ascites, enteritis, yellow and watery or jelly-like gut contents, and a whitish plug between the stomach and intestine.

Systemic infections may include the presence of white, soft, necrotic areas in the kidney and liver (Mo et al. 1990).

### 4. Histopathological Changes Associated with the Disease

Gastrointestinal infections exhibit excess mucus, cytoplasmic blebbing, aptotic bodies in epithelial cells, and hepatocellular necrosis (Ferguson 1979, 1989).

Systemic infections exhibit necrotic areas in the liver and kidney, granuloma-like lesions in the kidney, and severe muscle degeneration (Mo et al. 1990).

## E. Disease Diagnostic Procedures

### 1. Presumptive Diagnosis

a. Isolation/detection of pathogen

Live trophozoites can be seen in fresh smears from the gastrointestinal tract. Detection of organisms may be improved if the preparation is diluted with tap or distilled water or 0.9% NaCl. Should it be difficult to take a sample of the contents of the gastrointestinal tract, as may be the case if the fish has not eaten for some time, the intestinal surface of the gastrointestinal tract may be moistened and wiped on a slide. Hexamitid trophozoites are motile, typically pyriform to elongate, 5-20 µm long, with six anterior and two posterior or recurrent flagella. They are usually visible in gastrointestinal smears and less commonly they may be in the bile, blood, internal organs or peritoneal exudate.

Smears or moist mounts, under a cover glass, are examined by using bright field phase contrast or Normarski illumination at 400X magnification, (N.B. While hexamitids can be detected *Hexamita* and *Spironucleus* can not be distinguished from each other in such an examination; see E2 below). Samples should be taken from a variety of sites along the length of the gastrointestinal tract. Unstained imprints of other tissues may be examined to detect the protozoa.

b. Clinical Signs

See D above.

c. Histopathological Examination

Tissues fixed with formalin or Bouin's may be stained using hematoxylin and eosin, or the Feulgen reaction (Lee et al. 1985) to show the paired nuclei of the flagellates. The flagellates may be free in the lumen of the gastrointestinal tract, aligned along the epithelium; or present in a variety of internal organs.

### 2. Confirmatory Diagnosis

Hexamitid trophozoites: To observe detailed structure of the flagellates, organisms in smears or wet mounts may be slowed down or immobilized by increasing the viscosity of the water via the addition of Protosol, methyl cellulose or Polyox resin WSR 310 (Lee et al. 1985). Trophozoites may also become less active at the margins of the cover glass as the preparation begins to dry out. Nuclei can be seen in Giemsa-stained smears (Ferguson 1989). Fibrillar structures such as flagella and microtubules, may be shown by protargol (silver protein) staining of Bouin's-fixed, wet smears (Lee et al. 1985).

Determination of genus: *Hexamita* and *Spironucleus* are well distinguished ultrastructurally (Lee 1985), principally by the position of the kinetosomes (flagellar bases) relative to the nuclei (Figure 1). Transmission electron microscopy shows that in *Hexamita*, kinetosomes are anterior of the cell (Brugerolle et al. 1973, Lee 1985). Other distinguishing features are (a) shape of nuclei: in *Hexamita* oval-spherical, in *Spironucleus* s-shaped with narrow anterior ends, (b) location of the cytostomal openings and shape of posterior end of body: in *Hexamita* openings are caudal at the

flattened end of the body, in *Spironucleus* openings are posterio-lateral, and the end of the body is more tapered (Kulda and Nohynkova 1978). The latter external features are most easily seen with scanning electron microscopy, although in larger hexamitids it may be possible to see them by light microscopy (100X, oil immersion). Samples for electron microscopy may be small pieces of gastrointestinal tissue (approximately 1mm), or a suspension of gastrointestinal contents, from heavily infected fish. Suitable fixatives include glutaraldehyde (Ferguson 1979) or a combined 4% paraformaldehyde/5% glutaraldehyde (Poynton and Morrison 1990).

Determination of species: Morphological and ultrastructural differences between *Hexamita salmonis*, *Spironucleus elegans*, and *Spironucleus torosa* are discussed by Woo and Poynton (in press).

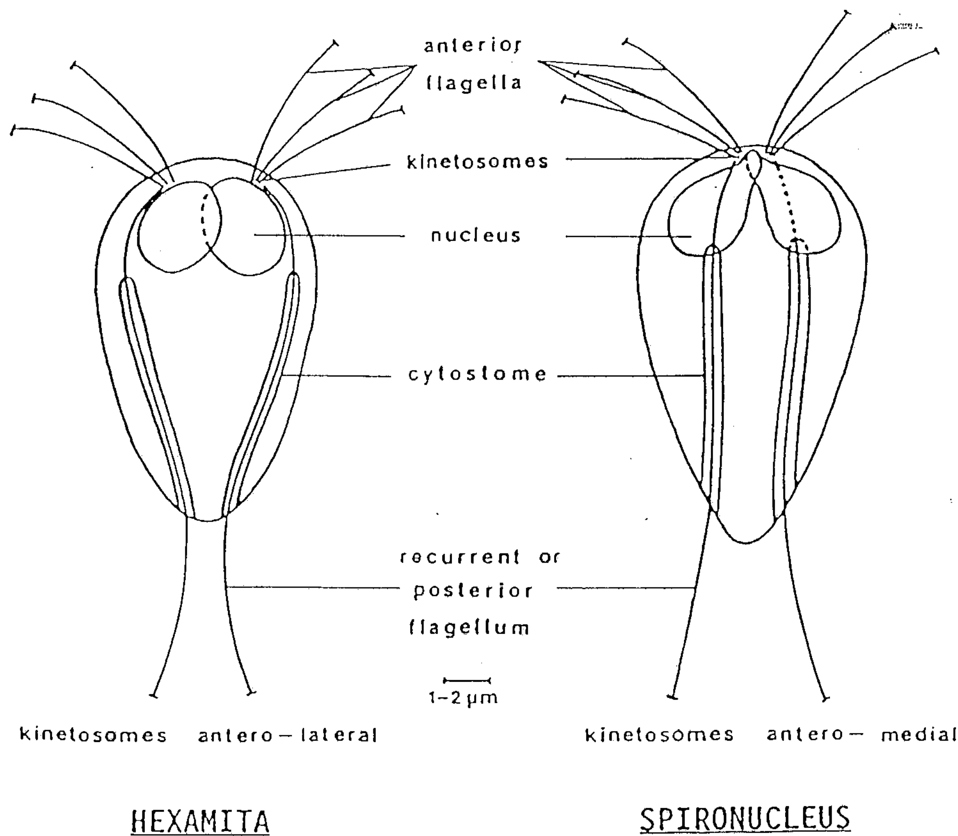


Figure 1. Morphology of *Hexamita* and *Spironucleus*. Note differences in four features between the genera: (i) position of kinetosomes (=flagellar bases): antero-lateral in *Hexamita*, antero-medial in *Spironucleus;* (ii) shape of nuclei; (iii) location of cytostome openings and (iv) shape of the posterior end. The shape of the body and the path of the recurrent flagella through the body are variable both within and between the species.. Figure based on those in Brugerolle 1974, Brugerolle et al.,

## F. Procedures for Detecting Subclinical Infections

Since the relationship between prevalence and density of infection and the disease are not well established (Woo and Poyton, in press) no recommendations are given.

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

No methods are currently available to detect previous infection with *Hexamita* or *Spironucleus.*

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

All samples must be obtained within minutes from a freshly killed fish. For preparation of fresh smears, fish should be transported live to the laboratory; trophozoites may survive for 15-30 min in freshly killed fish kept in the refrigerator. Samples for histology or electron microscopy must be fixed on site in accordance with those procedures described above in section E.

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# IV. Pleistophoriasis (Ovary Parasite of Golden Shiners)

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## A. Name of Disease or Etiological Agent

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

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical Range

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

### 2. Host Species

The type host for pleistophoriasis is the golden shiner and it has been reported once in fathead minnows, *Pimemphales promelas*. The parasite was not detected in goldfish *Carassius auratus* reared in the same hatchery ponds used for golden shiners. Efforts to experimentally transmit the parasite by intraperitoneal or intramuscular injection of spores from infected shiners to goldfish were unsuccessful (Nagel and Summerfelt 1977a). This technique has been used to infect goldfish and other cyprinid fish with *Pleistophora hypessobryconis*. *Pleistophora ovariae* was found in golden shiners from a creek and in shiners collected from a pond in the same watershed. It was not possible to ascertain whether these infected fish were feral or released from an angler's bait bucket. It is unlikely that a predatory fish would become infected by consuming an infected bait minnow. For example, *Pleistophora tahoenis*, which parasitize intramuscular connective tissue of the Piute sculpin *Cottus beldingi* (Summerfelt and Ebert 1969), has not been reported in lake trout *Salvelinus namaycush* in Lake Tahoe,

Nevada-California (Hoffman 1967, Canning and Lom 1986), even though the sculpin is their major prey.

## C. Epizootiology

*Pleistophora 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 *Pleistophora 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 *Pleistophora 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. *Pleistophora 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 *Pleistophora 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 1 oocyte. Egg development is described on a numerical scale of 1 to 7, where 7 is the mature ovum. The earliest stage of the parasite, the meront (schizont) is first seen in histological sections of stage 2 - 4 oocytes. Spores are present in stage 6 and 7 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 microsporidan spores in the ovary of golden shiners. However, presence of a microsporidian in fish ovaries should not be taken to imply the presence of *Pleistophora ovariae*.

Presumptive diagnosis may be facilitated by preparing a wet mount from fresh chilled, frozen, or preserved ovaries. Wet mounts are effective for finding spores, especially in fish before and after spawning. Spores are not present or they are rare in juveniles or adults from fall through the winter months.

To make a wet mount, remove one ovary and macerate it in a mortar with pestle - or thoroughly dice one ovary sample with a razor blade. Add saline (0.85%), drain off fluid, centrifuge the mixture to remove larger cellular debris (optional), then examine a drop of supernatant fluid from the centrifuged sample on a slide as a wet mount under a coverslip with brightfield, transmitted light microscopy at 950X magnification or with dark phase microscopy. Spores in wet mounts are most refractile when examined by dark phase microscopy. The characteristic morphology is also evident when spores in wet mounts are examined with brightfield transmitted light (Figure 1A and 1B). The ether separation method described by Landolt (1973), without enzyme digestion, is recommended for preparation of a relatively pure suspension of *Pleistophora 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 *Pleistophora* in a different host species.

In fresh material, the refractile portion of the spore is about 40% of the total spore length, followed by a less refractile or clear area occupying the rest of the spore. The coiled polar tube is visible in the clear area. The polar tube may be extruded by applying pressure to the coverslip (Figure 1D). Extrusion of the polar tube is a measure of spore viability.

Stained smears may be prepared from the suspension used for the wet mount. General procedures for the preparation and staining of protozoan smears are given by Kudo (1924). Canning and Lom (1986) describe some selective staining methods for microsporida. Leida (1978) described the staining response of spores in smears: 1. spores stained well with toluidine blue, the polar cap stained a dark blue-purple, the polar filament less intensively blue; 2. results with PAS were variable, at best the polar cap and polar filament stained faintly; 3. hematoxylin stained spores have a dark-blue or purple, girdle-like ring, sometimes with a dark nuclear spot (Figure 1C); and 4. Giemsa stained the polar cap intensely blue and the nucleus less intensely purple.

### 2. Confirmatory Diagnosis

Histological confirmation is required to establish "no infection" when wet mounts were used for preliminary screening. Histological sections are not needed when spores are found in wet mounts. For identification of life stages (meronts and sporoblasts) other than spores from late summer through spring, fix an ovary from a fresh specimen in 10% neutral (or buffered) formalin or Bouin's fixative and prepare histological sections by standard methods.

Histological sections should be prepared when wet mounts are negative to confirm the diagnosis. Paraffin sections should be prepared following standard histological procedures (Luna, 1968). Ovary may be sectioned longitudinally or transversely at 6 to 10 µm, with 3 to 5 sections per slide. Stain one slide with metachromatic blue stain containing 0.1% toluidine blue (Summerfelt and Warner 1970a), another with Mallory's aniline blue-collagen stain (Biological Staining Commission 1960:52-53), and a third with hematoxylin-eosin (H&E). The metachromatic blue stain was useful for a quick overview of a section to identify spores. Spores stain light to dark blue, with a dark band in the middle and dark blue at the end. This stain is poor for distinguishing cytological details of the developing oocytes. Mallory's stain is highly differential for primary and secondary yolk, connective tissue, nucleoli, and the follicle. It is also excellent for differentiating meronts (schizonts), sporoblasts and spores from the cytoplasm and yolk of the oocytes.

The intensity of infection can be estimated from the percentage of the cross-sectional area of the ovarian sections occupied by infected oocytes or atretic eggs.

## F. Procedures for Detecting Subclinical Infections

When examining a juvenile fish in the fall or winter, when the spores are uncommon, the only evidence of infection will be the presence of schizonts in histological sections. Schizonts can be seen in the cytoplasm of the stage 2-4 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 *Pleistophora 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.

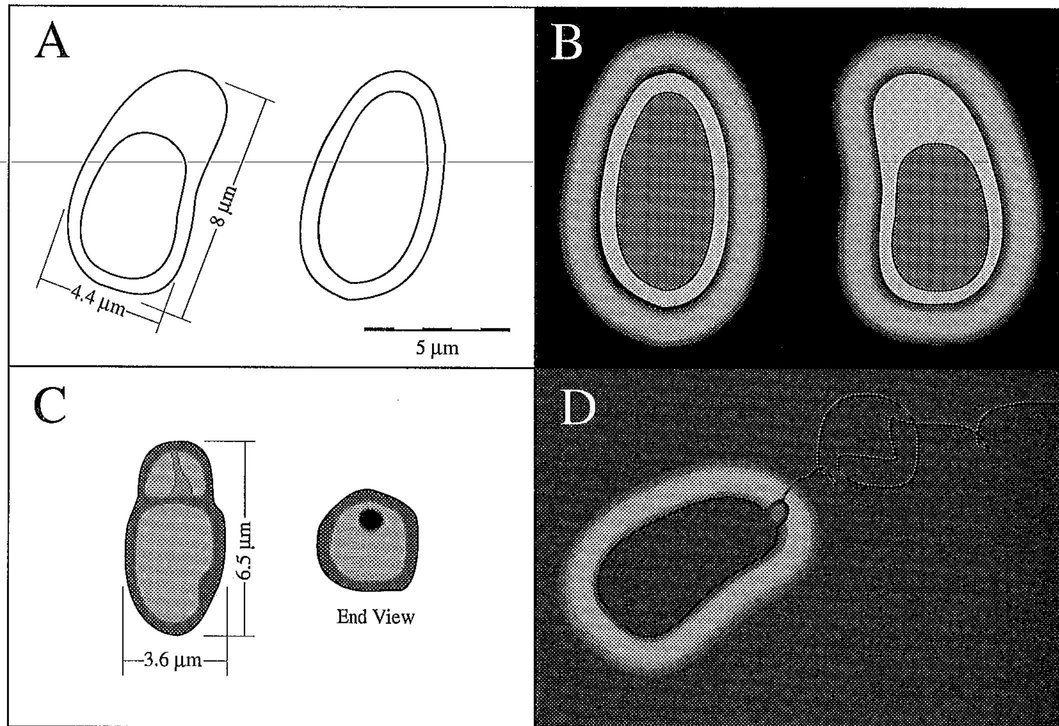


**Figure 1. Spores of *Pleistophora ovariae***

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 polar 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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# V. Salmonid Ceratomyxosis

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## A. Name of Disease and Etiological Agent

Salmonid ceratomyxosis is caused by *Ceratomyxa shasta* (Myxozoa: Myxosporea)

## B. Known Geographic Range and Host Species of the Disease

### 1. Geographical Range

The distribution of the infective stage of the parasite is restricted to the Pacific northwest of the United States and Canada. In the United States: Columbia, Cowlitz, Lewis (east fork), Washougal Rivers; LaCamas Lake; Snake River from its confluence with Columbia to about 430 miles upstream; Deschutes River System; Willamette River from mouth to about 100 miles upstream; Nehalem, Rogue, and Klamath Rivers; Klamath Lake; Sacramento, Mokelumne, Feather and Pitt River Systems. In Canada: Fraser River.

Anadromous salmon may come in contact with *C. shasta* during migration and infected juvenile and adult fish have been reported in freshwater and marine environments outside of the parasite's range.

### 2. Host Species

Natural infections of *Ceratomyxa shasta* are known to occur in the following: cutthroat trout *Oncorhynchus clarki*, pink salmon *Oncorhynchus gorbuscha*, chum salmon *Oncorhynchus keta*, coho salmon *Oncorhynchus kisutch*, rainbow trout and steelhead *Oncorhynchus mykiss*, sockeye salmon *Oncorhynchus nerka*, chinook salmon *Oncorhynchus tshawytscha*, Atlantic salmon *Salmo salar*, brown trout *Salmo trutta*, brook trout *Salvelinus fontinalis*.

Within a species, strains of salmonids originating from enzootic waters are often 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. Ceratomyxosis causes significant prespawning mortalities among infected adult fish.

Natural transmission of ceratomyxosis occurs when susceptible salmonids come in contact with water containing the infective stage. This water borne infectivity is seasonal and is normally limited to warmer water months. 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 does not appear to be attenuated.

## 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 (PMN's), fibroblasts, and macrophages. The epithelial lining necrotizes, fragments, and ultimately sloughs, and is replaced by fibrous connective tissue containing lost cells and trophozoites. The lumen may contain epithelial cells, epithelial cell fragments, PMN's, 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 400-440X magnification.

Presumptive diagnosis is based on identification of multicellular myxosporean trophozoites in salmonids showing signs of ceratomyxosis.

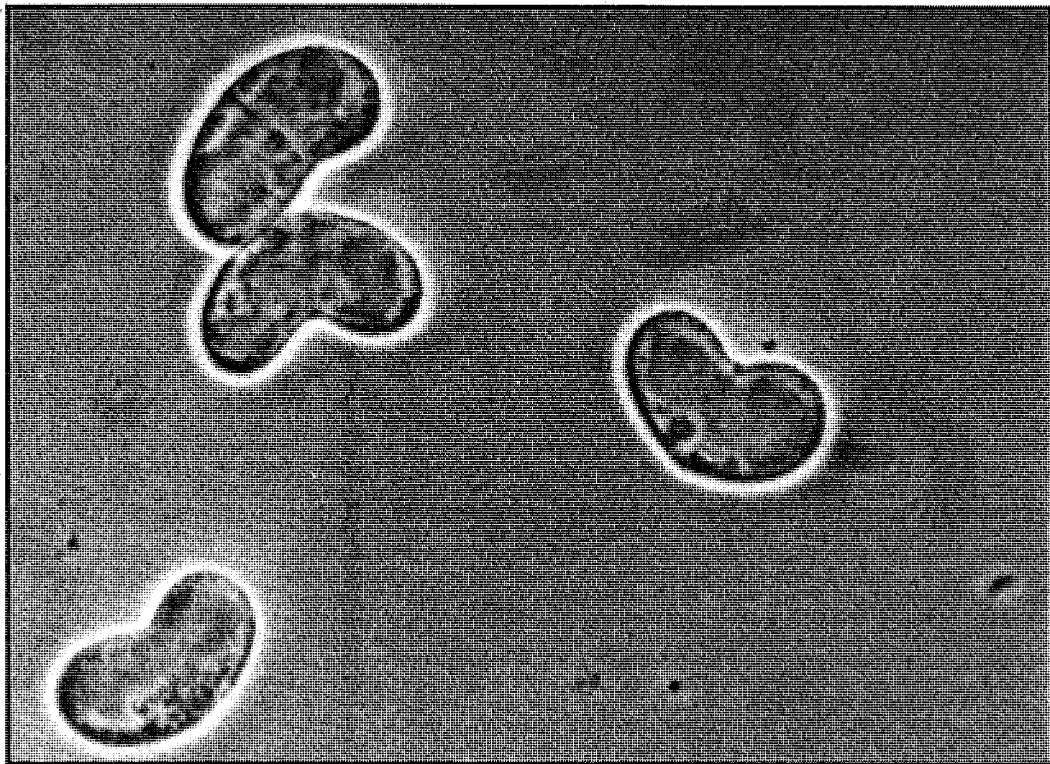

**Figure 1.** Mature *Ceratomyxa shasta* spores (bar equals 8 microns).

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 a light blue and the nuclei contain a dark-staining karyosome surrounded by a clear halo.

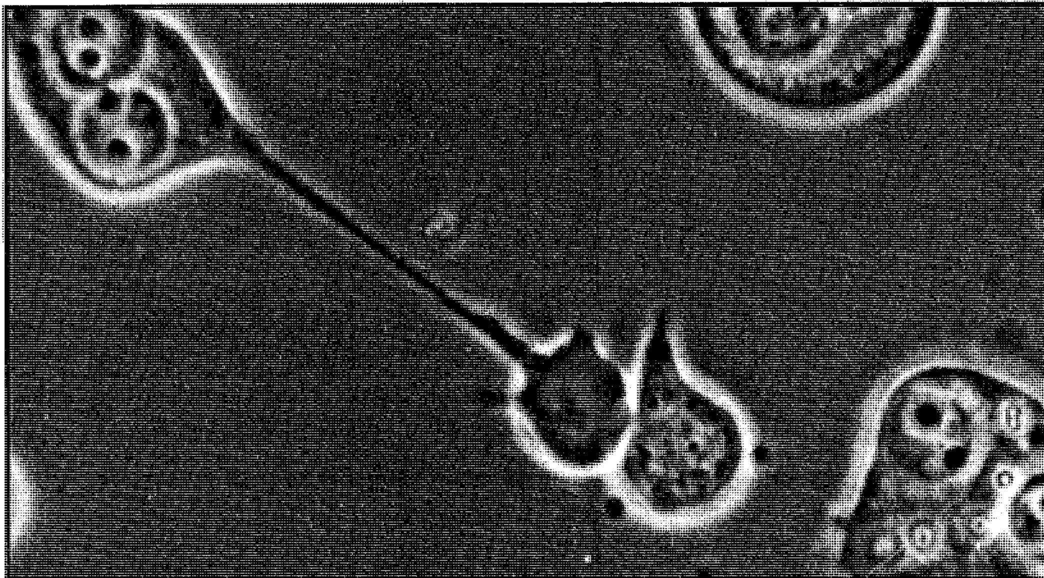

**Figure 2 .** *Ceratomyxa shasta* trophozoites (bar equals 8 microns).

## 2. Confirmatory Diagnosis

Prepare wet mounts and examine as described above.

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 14-23 µm long by 6-8 µm wide at the suture line.

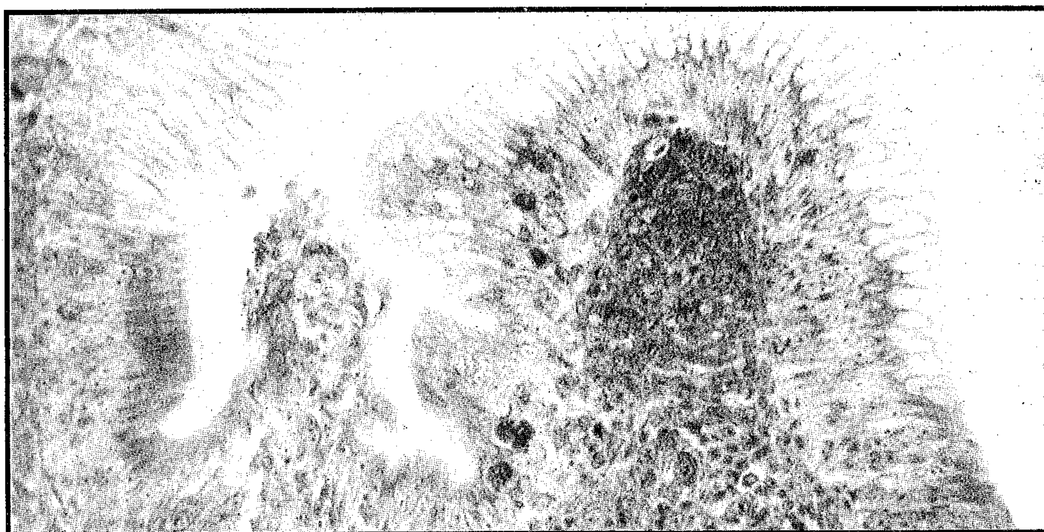

**Figure 3 .** Histological section of intestine from an infected rainbow trout, arrows indicate location of trophozoites.

Spores are most likely found in the posterior intestine, but sometimes can be found in other tissues, particularly the kidney, liver, and pyloric caeca.

Serological identification of *Ceratomyxa shasta* trophozoites can be accomplished using monoclonal antibodies (Bartholomew, et al. 1989b). Examination of intestinal scrapings is done by applying the sample material to gelatin-coated microscope slides (1 g gelatin/L hot distilled water, let cool, add 0.1 g chromic potassium sulfate), allowing them to air dry, and then fixing the material in acetone-xylene (1:1 V/V). Histological sections should also be mounted on gelatin-coated slides. Indirect fluorescent antibody staining of both wet mounts and deparaffinized, hydrated sections is as follows:

1. Apply monoclonal antibody (source: J. Bartholomew, National Fisheries Research Center, Bldg 204 Naval Station, Seattle, WA 98115) to the tissue and incubate for 15 min at room temperature in a humid chamber.
2. Gently wash with phosphate buffered saline (PBS) then let slides soak for 5 min and rinse again.
3. Incubate with fluorescein-conjugated anti-mouse antibody for 15 min at room temperature in a humid chamber.
4. Wash with PBS (5 min).
5. Counterstain with either Evan's blue (0.01% in PBS) or methyl green (1% in distilled water).
6. Rinse with PBS, blot dry and mount cover glass with buffered glycerol.

This procedure can be modified for bright light microscopy by substituting an alkaline phosphatase-conjugated second antibody for the fluorescein conjugate and localizing the enzyme with an insoluble substrate.

## F. Procedures for Detecting Subclinical Infections

Spore formation usually does not occur until late in an infection; therefore, diagnosis of ceratomyxosis in early or subclinical infections should rely on serological identification of trophozoites.

## 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 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. Preservation of samples in 10% neutral buffered formalin will eliminate infectivity.

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# VI. Whirling Disease of Salmonids

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## A. Name of Disease and Etiological Agent

Whirling disease is caused by the myxosporean parasite *Myxobolus (Myxosoma) cerebralis.* A synonyn for whirling disease is blacktail.

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical Range

California, Colorado, Connecticut, Idaho, Massachusetts, Michigan, Montana, Nevada, New Hampshire, New Jersey, Ohio, Oregon, Pennsylvania, Utah, Virginia, and West Virginia. The agent has also been found in Europe, United Kingdom, New Zealand, South Africa, and the USSR.

### 2. Host Species

All species of salmon, trout, char, and grayling are susceptible to infection. Coho salmon *Oncorhynchus kisutch,* and brown trout *Salmo trutta,* may show no signs of the disease and spores may be difficult to find even after heavy exposure at an early age. Brook trout *Salvelinus fontinalis,* and rainbow trout *Oncorhynchus mykiss,* are very susceptible. Intensity of exposure and fish age affect the severity of the disease.

## C. Epizootiology

Markiw and Wolf (1983) and Wolf and Markiw (1984) determined that the infective stage of whirling disease was an actinosporean. The life cycle involves tubificid worms, *Tubifex tubifex,* in the development of the infective stage (Wolf and Markiw 1985). Hamilton and Canning (1987a, b) have contested the life cycle proposed by Wolf and Markiw (1984): however they have more recently reported (1987b), that the transmission of whirling disease can occur when tubificids are added to test tanks with sterile mud and

*Myxobolus cerebralis* spores. In addition, El-Matbouli and Hoffman (1989) demonstrated with two *Myxobolus* species, one of which was *Myxobolus cerebralis*, that transmission via triactinomyxon formation in tubificid worms occurs. Regardless of the role of the tubificid, repeatable laboratory challenges have been developed to study various aspects of whirling disease in salmonids.

## D. Disease Signs

Frenzied, tail-chasing behavior, particularly when being fed or when startled. Whirling behavior usually occurs 2 to 3 months after infection and may last for up to a year in cold water situations. The posterior trunk and tail of young fingerlings may turn dark, especially in fish exposed at an early age, (blacktail). As the infected fish grows, the primary signs of the disease can be skeletal changes such as misshapen skulls and twisted spines. Loss rate is dose and age dependent; most serious losses occur in young, heavily infected fish.In histological sections cartilage may appear heavily plaqued depending on the degree of infection.

## E. Disease Diagnostic Procedures

### 1. Presumptive Diagnosis

Diagnosis of epizootic whiling disease depends upon the detection and identification *Myxosoma cerebralis* spores.

Remove the heads from five suspect fish and warm them in 45°C water for 1 to 3 minutes so the flesh will separate easily from the bone and cartilage.

Remove loose flesh and the brain to a waste container of 1:1 water and household bleach for disinfection.

Collect bone and cartilage samples from the brain case, otolith region, and gill arches.

Grind the sample with an equal volume of 10% formalin (to kill viable spores and prevent dissemination of the disease agent) in a mortar. If the fish are older than the desired 5 to 11 months of age, the skeletal parts should be softened with a volume of 1% hydrochloric acid sufficient to cover the sample for one to several hours.

Wash all grindings into a small beaker with water and allow the material to settle.

Sediment can be examined directly in wet mounts at 400X magnification. Alternatively, smears can be dried and stained according to the method of MacLean (1971). For this procedure, spread 5 to 10 drops of sediment onto a clean glass microscope slide and allow to air dry.

Stain the slide is with 1% aqueous malachite green for 5 min, rinse with tap water and destain by placing the slide for 30 s each into 70, 90, and 100% ethyl alcohol.

Coat air-dried slides entirely with a thin film of low viscosity immersion oil and examine at about 200X magnification (not under oil immersion).

Scan the entire smear. Spores will appear as green ovals with dark green polar capsules against a nearly colorless background. At 200X magnification, there is less chance of missing spores and a larger area is covered at each pass over the slide than at higher magnifications required to find unstained spores..

Diagnosis of epizootic whirling disease depends upon the detection and identification of *Myxobolus cerebralis* spores.

*Myxobolus cerebralis* is the only species of *Myxobolus* found in the cartilage of salmonids. *Myxobolus squamalis* occurs in the scales of western U.S.A. salmonids, is about the same size as *Myxobolus cerebralis* (about 9µm), but possesses a narrow, but obvious, ridge that parallels either side of the sutural ridge. *Myxobolus kisutchi*, another western salmonid parasite, occurs in the central nervous system and is about the same size as *Myxobolus cerebralis*. *Myxobolus neurobius*, more widespread geographically, is also found in the central nervous system, but is larger (10 - 13 x 7.5 - 8 µm).

**2. Confirmatory Diagnosis**

a. Histological Confirmation

1. Preserve heads in 10% neutral buffered formalin (frozen heads may be preserved and processed but the sections are of poorer quality).
2. Decalcify for about 3 d.
3. Rinse for 4-5 h in running water.
4. Dehydrate in an ethanol-xylene series and embed in paraffin.
5. Cut into 5 to 7 µm sections.
6. Stain with a Geimsa stain (May-Grunwald Giemsa works well).
7. Scan section at 200X magnification for spores and trophozoites; the presence of spores, as described above,or trophozoites in association with cartilage lesions confirms diagnosis.

b. Serological Identification of *Myxobolus cerebralis*

This may be accomplished by the direct FAT described below. This test works best with fresh spores. Spores that have been stored in formalin for a week or more show reduced specific fluorescence, and older specimens show little or none (Wolf and Markiw 1985).

1. Transfer the suspect wet sediment from step E1 above, or from steps F1f, F2g, F2i or F2k below to a labelled centrifuge tube and concentrate the residues by centrifugation at 1200 x g for 10 minutes at room temperature. Decant the supernatant and, by the use of wet mounts, adjust the concentration of the residues with water to a volume that permits the best observation of spores among the debris.
2. Thoroughly clean FAT slides with detergent and deionized water and rinse well with acetone. Label slides for known positive *Myxobolus cerebralis* (positive

control), one or more other known sporozoan (negative control), and for material to be tested (suspect/unknown).

3. Lightly coat slides with a commercial tissue bonding agent or 50% egg albumin. (Egg albumin can be prepared by mincing egg white with scissors, filtering it through gauze and mixing with an equal volume of glycerin. Several drops of chloroform are added as a preservative; the albumin is stored in refrigerator).

4. Apply small drops of suspect/unknown and control material to the coated slides, dry the slide at 50°-60°C for 15 to 20 min. Fix slides in absolute methanol for 5 min and air dry.

5. Apply fluorescein isothiocyanate-conjugated rabbit anti-*Myxobolus cerebralis* serum to each slide. Allow serum to react for 30-60 min in the dark at room temperature.

6. Gently rinse conjugated antiserum from the slides with pH 9.0-9.5 buffer ($NaH_2CO_3$, 33.6 g; plus $Na_2CO_3$, 10.6 g in 1000 ml water) then soak slide in buffer, with gentle agitation for 5 min. Careless or too vigorous washing may lead to spore loss.

7. Remove slide and gently blot dry on clean absorbent paper. Add a drop of immersion oil to each test spot and examine at 400X or 1000X magnification on a fluorescence microscope. Positive identification of *Myxobolus cerebralis* depends upon the detection of spore and trophozoite stages which fluoresce apple green. Cartilage debris and spores may exhibit autofluoresce under UV light. However, this fluorescence is quite yellow.

## F. Procedures for Detecting Subclinical Infections

Samples should be weighted towards the most susceptible species and ages of fish available. For example, select brook and rainbow trout over brown trout or coho salmon if all are reared under the same conditions. Select fish about 5 months old if possible. However if fish are continuously exposed in water of 13°C or warmer, fish as young as 2 to 3 months of age may yield mature spores. In water below 12°C, fish may have to reach 8 to 10 months of age before mature spores can be found.

**The following procedures are acceptable for detection of infection in carrier fish (It is common practice in many diagnostic laboratories to only inspect the gills of larger fish.):**

**1. Plankton Centrifuge Method (O'Grodnick, 1975)**

a. Pool in 20 g batches, heads, dissected pieces of cranium, and all gill arches.

b. Thoroughly homogenize each batch in 200 ml of aqueous 10% formalin for 3 minutes in a high-speed blender.

c. Strain the homogenate through loose glass wool in a large funnel or through a fine screen or sieve. (Millipore XX40 047 04 support screen in an XX40 047 00 holder or Tyler sieves #60 {0.250 mm} and #80 {0.180 mm}).

d. Rinse any remaining sample through the glass wool or screen with water and save all washings. (Caution: Infective material may remain in discarded tissue and equipment).

e. Transfer the entire filtrate to a separatory feed line of a plankton centrifuge (026WA106 plankton centrifuge, Kahl Scientific Instrument Corp., PO Box 1166, El Cajon, CA 92022, or equivalent). Operate the plankton centrifuge on high speed and set the separatory funnel flow rate at the lowest level that gives a thin steady stream

f. Centrifuge until flow from the separatory funnel has been completed including at least one thorough rinse of the apparatus. The residues adhering to the inner walls of the centrifuge drum will contain spores and debris. With a rubber policeman, suspend this residue in the water that remains in the drum. Transfer this material to screw-capped, labelled tubes and store in a solution of 10% neutral buffered formalin until the sample can be examined.

g. Shake the sample well and transfer a drop of the suspension to a clean microscope slide and add a cover glass. Systematically search each test area for approximately 2-1/2 minutes or until spores are found.

**2. Digest Method (Markiw and Wolf 1974a)**

a. The following solutions are required:

1. Pepsin: to 1 L of distilled water add 5.0 g pepsin and 5 ml concentrated HCl.
2. Trypsin: to 1 L of distilled water add 0.2 g EDTA, 8.0 g NaCl, 0.2 g KCl, 0.2 g $KH_2PO_4$ and 5.0 g trypsin, 1.5 g $NaH_2PO_4$.

b. Remove the heads from fish to be analyzed. Heads may be frozen for future analysis.

c. Preserve at least five heads or half heads (cut longitudinally) for histology (if needed) in 10% buffered formalin.

d. Heat heads for approximately 10 min in 60°C water- The eyes will turn opaque when ready.

e. Deflesh heads and save bone and cartilage mainly from the cranial area. Samples from 5 fish may be pooled. For adult fish, homogenize bone and cartilage in an electric blender in a small amount of pepsin.

f. Place cleaned heads in a pepsin solution. Use about 20 ml of solution for each gram of head material. Stir at 37°C for 30 min.

g. Centrifuge pepsin digest at 1200 x g for 10 min. Dispose of the supernatant into disinfectant. Check for spores at 100 X and 400 X magnification at this step.

h. Add the trypsin solution. Use 20 ml for each g of undigested material. Adjust pH to 8.5 with NaOH. Stir at room temperature for at least 30 min.

i. Pour digested material through cheese cloth and save fluid. Centrifuge fluid at 1200 x g for 10 min. Resuspend the pellet in a small volume of buffered 10% formalin and examine for spores.

j. If no spores are found in the previous steps, layer a sample over a 55% glucose solution (1 cm depth of sample to 5 cm depth of glucose). Centrifuge in swinging bucket rotor at 1200 x g for 30 min. Aspirate off all liquid over the pellet.

k. Resuspend the pellet in a few drops of buffered formalin, mix and examine for spores.

**3. Core Method- Sampling Adults**

a. Sample fish with a borer similar to a cork borer (about 110 mm long and 19 mm in

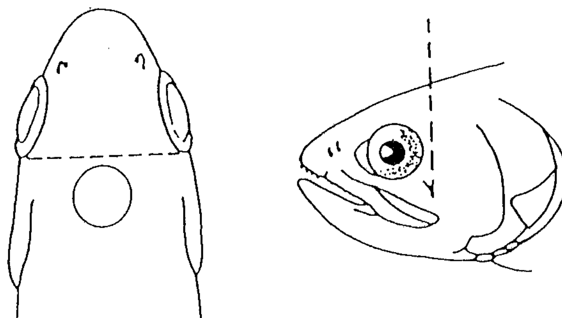

**Figure 1.** Dorsal and lateral views of an adult salmon head, indicating the location for obtaining a core sample for *Myxobolus cerebralis*.

diameter) by inserting the borer into the head, dorsally and perpendicular to the long axis of the body approximately behind the eyes and through the roof of the mouth (Figure 1). This sample should contain the semicircular canals, and the otoliths in smaller fish.

b. Remove the skin from the core.

c. Homogenize cores in small amount of pepsin (see F2a) in an electric blender.

d. Proceed with stirring step in procedure (F2f) in Digest procedure.

## G. Prodedures for Determining Prior Exposure

No procedures have been reported.

## H. 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. Preservation of samples in 10% buffered formalin will eliminate infectivity.

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STATE OF COLORADO
Roy Romer, Governor
DEPARTMENT OF NATURAL RESOURCES

**DIVISION OF WILDLIFE**

AN EQUAL OPPORTUNITY EMPLOYER

John W. Mumma, Director
6060 Broadway
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Telephone: (303) 297-1192

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APR 10 1997 BS
Northwest Indian
Fisheries Commission

April 2, 1997

To Whom It May Concern:

The Colorado Division of Wildlife Aquatic Animal Health Laboratory requests that all annual fish health inspections for *Myxobolus cerebralis* utilizing either the Plankton Centrifuge or Pepsin-Trypsin Digestion technique test **fresh** heads only. Minimum AFS Blue Book standards shall otherwise be adhered to. All annual inspections performed after May 1, 1997 must have used fresh heads or importation into the state of Colorado will be denied.

This request is based on experience gained in this laboratory with the comparison of fresh and frozen heads utilizing the Pepsin-Trypsin Digestion procedure. Results indicate the examination of fresh heads increases the sensitivity of detection of *M. cerebralis* spores.

If you have any questions or concerns regarding this request please contact us at the Aquatic Animal Health Laboratory. Thank-you.

Sincerely,

Linda A. Chitt
Peter G. Walker

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# VII. Proliferative Gill Disease

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## A. Name of Disease and Etiological Agent

Proliferative gill disease (PGD) is currently of uncertain etiology. It is most likely caused by a myxosporidean, possibly of the genus *Sphaerospora* or *Aurantiactinomyxon*. Initial reports that the condition was due to infections with the myxosporidean *Henneguya* were probably due to concomitant infections.

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical Range

The disease has been reported throughout the channel catfish growing region of the Southeastern United States and California.

### 2. Host Species

This disease has been described and observed only in the channel catfish, *Ictalurus punctatus*.

## C. Epizootiology

PGD produces clinical disease at temperatures between 16-26°C. In experimental exposure of fish to sediments from ponds with ongoing epizootics, clinical PGD was observed when fish were exposed at 19, 21 and 26°C but not when fish were exposed at 16°C. However fish exposed at 19 or 21°C and moved to 16°C developed clinical PGD. All sizes of fish are affected by PGD with mortality rates ranging from less than 1 to greater than 90% regardless of size. Poor water quality, such as low dissolved oxygen and high un-ionized ammonia may result in increased mortality rates. Current data suggest that the occurrence of PGD is associated with new ponds. Recurrence in previously affected ponds is rare and generally related to ponds that have been drained and refilled. Recent reports indicate that PGD can be experimentally induced in fish exposed to the oligochaete worm *Dero obtusa* infested with the triactinomyxid parasite *Aurantiactinomyxon*. However, as in natural epizootics and previous exposure studies, the parasite did not develop to a mature spore, indicating that the catfish may be an accidental host rather than an intermediate or final host involved in an indirect life cycle.

## D. Disease Signs

### 1. Behavioral Signs

Fish infested with the PGD organism become anorexic early in the development of the disease. Affected fish are extremely listless and exhibit increased susceptibility to moderately low dissolved oxygen levels.

### 2. Gross Signs

The principal external sign of PGD is massive degeneration of the primary filaments of the gill. In early stages the filaments appear pale and swollen, progressing to a state where filament breakage and loss are apparent.

### 3. Microscopic Signs

Light microscopy of early infections reveal extensive epithelial hyperplasia that, in combination with infiltrating inflammatory cells, results in occlusion of the laminar troughs. As the inflammation progresses, severely swollen nodules become apparent that contain characteristic parasitic cysts. Moderate to severe cartilaginous necrosis and hydropic degeneration and liquefactive necrosis of cells within the nodules are characteristic. The necrosis of the cartilaginous support rod ultimately results in breakage and loss of the filament. Histochemical stains and ultrastructural examination of infested tissue demonstrate intense concentrations of neutrophils surrounding the parasite within the nodules, and there is a significant neutrophilia (over 35%) in the circulatory blood. Although the primary pathological changes of PGD are associated with the gills, cysts have been detected in the liver, head kidney, trunk kidney, spleen, and brain. Severe inflammatory responses to the cysts in non-gill tissues is generally not apparent. In fish that survive, the final stage of infection is characterized by an absence of cysts in the gills and chondroplasia associated with regeneration of the filaments.

## E. Disease Diagnostic Procedures

### 1. Presumptive Diagnosis

PGD can be presumptively diagnosed by gross observation of pale, swollen, clubbed and broken gill filaments. In early stages, where filament breakage and loss have not occurred, microscopic examination of wet mounts will generally reveal focal areas of clearing of the cartilaginous support rod of the filament.

### 2. Confirmatory Diagnosis

Although cartilaginous necrosis is a strong indicator that the PGD organism is present, microscopic examination of histological sections for the characteristic cyst is required for confirmation. Cysts are generally not visible in necropsy wet mounts unless samples are examined from live fish within a few minutes of excision.

## F. Procedures for Detecting Subclinical Infections

Subclinical infestations have not been fully described, thus low level detection procedures have not been developed.

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

Procedures for determining prior exposure to PGD have not been developed.

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

Samples should be transported on ice, with care taken not to allow the gills to be immersed in ice water. Bouin's or formalin fixed tissue can be used for confirmation of PGD by histological procedures.

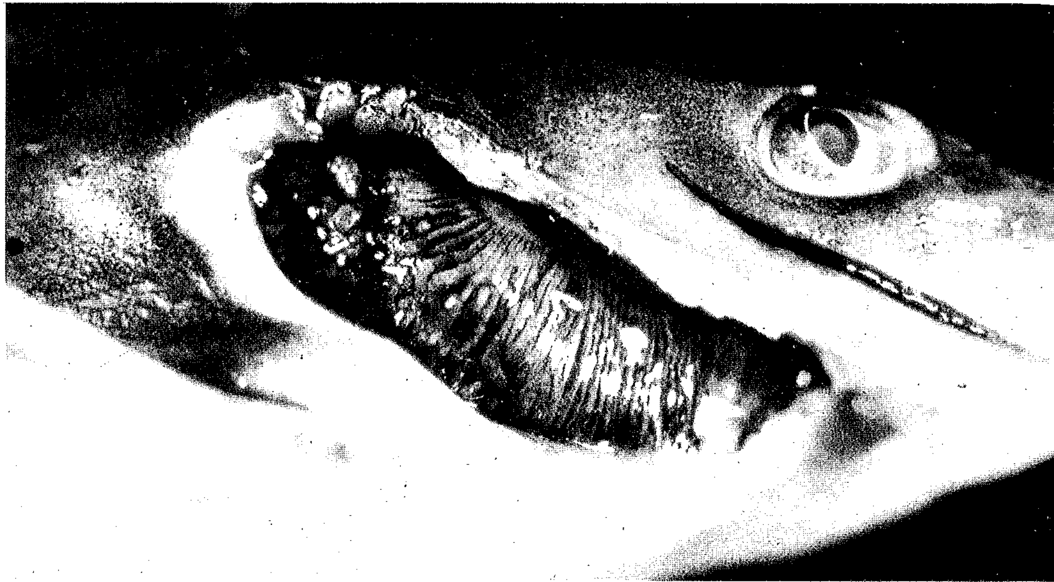

**Figure 1.** Gross photograph of channel catfish with proliferative gill disease (PGD). Note the extensive clubbing and breaks of the primary gill filamants.

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# VIII. Proliferative Kidney Disease

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## A. Name of Disease and Etiological Agent

Proliferative kidney disease (PKD) is caused by an unnamed myxosporean parasite (PKX).

## 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, Montana, and 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* have been experimentally infected.

## 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 1 month after exposure and clinical disease follows about 3-4 weeks later. Mortality is chronic and extremely variable (5-90%). High mortalities are often associated with secondary infections of *Aeromonas salmonicida*, *Flexibacter*

*columnaris*, or *Ichthyophthirius multifilis*. Recovery from PKD begins to occur in an infected population at about 12 weeks post-exposure and most fish that survive recover after 20 weeks.

## D. Disease Signs

### 1. Behavioral Changes Associated with the Disease

Fish with PKD are often lethargic and dark.

### 2. External Gross Signs

Affected fish exhibit exophthalmia, lateral body swelling, and a distended abdomen. Gills are pale due to anemia.

### 3. Internal Gross Signs

Internal gross signs are ascites and enlargement of the spleen and the posterior or entire kidney. The kidney is often grey and mottled, and uniformly enlarged, and the kidney capsule is often distended ventrally in a corrugated pattern.

### 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. 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.

## E. Disease Diagnostic Procedure

### 1. Presumptive Diagnosis

The PKX parasite can be presumptively identified in Giemsa-stained imprints of the posterior kidney or spleen. In these preparations, PKX parasites are round, 10-20 $\mu$m in diameter, and have a light staining, vacuolated cytoplasm. 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.

### 2. Confirmatory Diagnosis

Confirmatory diagnosis is based on histological examination of tissue sections stained with hematoxylin and eosin. The principal diagnostic feature of PKD is the presence of the PKX parasites in the renal interstitium. The PKX parasite is amoeboid, 5-20 $\mu$m in diameter, and has a foamy, eosinophilic cytoplasm. The primary cell contains 1-3 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

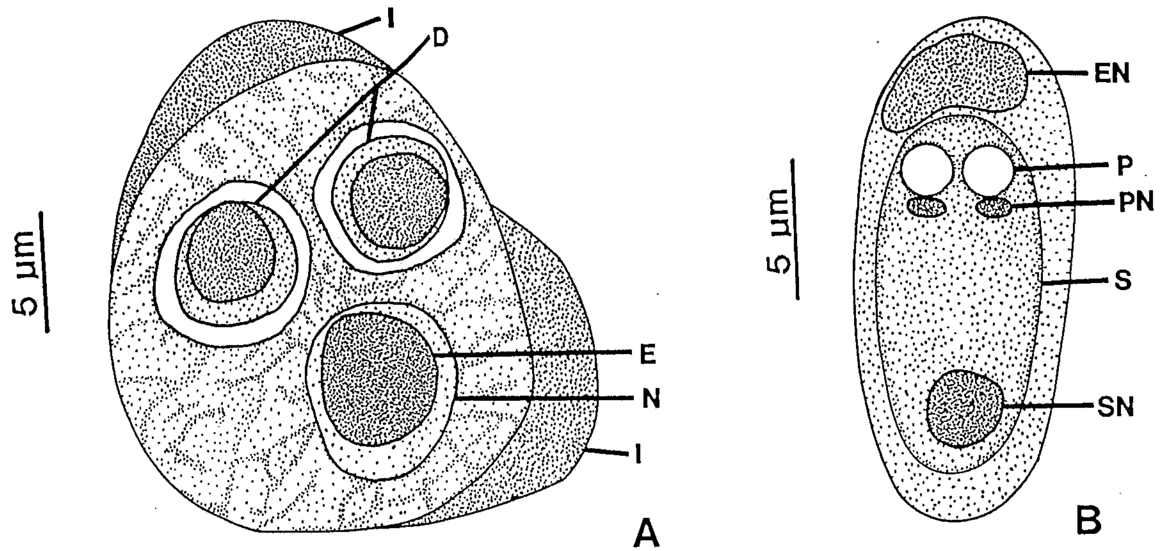


**Figure 1.** A) Typical PKX parasite found in the renal interstitium and blood vessels. The nucleus of the primary cell (N) contains a large, deep staining endosome (E). Daughter cells (D) are found in vacuoles in the primary cell. Inflammatory cells (I) surround the parasite.B) Plasmodia with monosporous sporoblast (S) found in the lumina of kidney tubules during recovery. P=polar capsule, EN=enveloping cell nucleus, SN=sporoplasm nucleus, PN=capsulogenic cell nucleus.

## 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 are also effective for detecting subclinical infection.

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

Sporogonic forms (pseudoplasmodia; Figure 1) 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. The sporoblast is pliable due to 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.

## 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.

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# IX. Ichthyophthiriasis

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## A. Name of Disease and Etiological Agent

Ichthyophthiriasis or "ich" is caused by the ciliate *Ichthyophthirius multifilis*, a hymenstomatid protozoan.

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical Range

*Ichthyophthirius multifilis* is worldwide in distribution.

### 2. Host Species

This parasite infects virtually every species of freshwater fish. Mosquito fish *Gambusia affinis*, grass carp *Ctenopharyngdon idella*, and sunfishes *Lepomis* spp.appear to be somewhat less susceptible to infection than other species.

## C. Epizootiology

The parasite is commonly found in small numbers on wild fish, and natural populations rarely experience heavy infections unless stressed. Disease outbreaks in cultured fish are common when naive fingerlings are brought in contact with older fish that may be carrying a few trophonts (the tissue-dwelling stage of the parasite), and the fish are crowded or otherwise stressed. 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. Estimates of the length of the life cycle in the literature vary a good deal. 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.

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. 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.

### 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 5 days duration (at 21°C). About 5 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.

In the gill, the growing parasites cause distortion of the lamellae. In heavy infections epithelial hyperplasia is common, as are hyperplasia and hypertrophy of chloride cells. Lamellae may be fused.

## 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. 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 Etiologic Agent

Infected live fish may be shipped in water or a mixture of water and ice.

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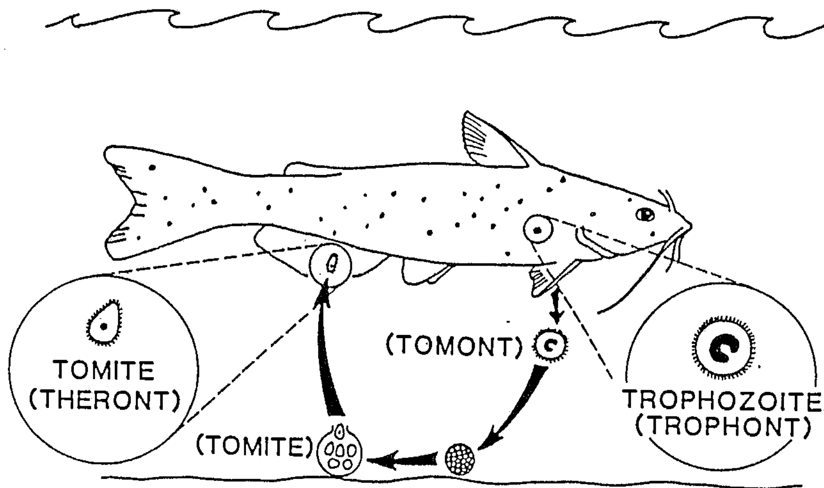


**Figure 1.** Life cycle of *Ichthyophthirius multifilis.* The trophont (tissue-dwelling stage) may be as small as 20 µm in diameter minutes after invasion and may grow to be as large as 1000 µm in diameter after feeding in the epithelium of the host. Size of the trophont is directly related to water temperature. The tomont stage typically swims free for no more than 1-2 hrs and secretes a cyst wall that attaches it to a substrate. Within the cyst wall, repeated mitotic divisions produce tomites that differentiate to form the infective stage, the theront. The theront must find a host within 36 h ( at 21°C) or die. Theronts have been reported to be 35-60 µm long and 15-22 µm wide. Size estimates for trophonts and theronts (and tomites) vary considerably in the literature.

# X. External Ciliated Parasite Infection

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The ciliated 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 live 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 are 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., *Trichodina* spp., *Tripartiella* spp.). (2) sessile Peritrichs(*Ambiphrya* spp., *Apiosoma* spp., *Epistylis* spp.). (3) motile Holotrichs(*Chilodonella* spp.). (4) sessile Suctoria (*Trichophrya* spp.).

## B. Known Geographic Range and Host Species of the Disease

1. Geographical Range

   These parasites are cosmopolitan and occur throughout the world.

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 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.

1. Motile peritrichs appear saucer-shaped and vary in diameter from 30µ to over 100*µm*. They possess a sclerotized denticular ring and a ring of cilia on their outer margins (Figure 1).
2. Sessile peritrichs may be barrel or urn-shaped and vary from 30µ to 150µ in length. Individually attached or in groups by a dichotomous stalk (Figure 2).
3. Motile holotrichs are heart-shaped varying in body length from 30µm to 150µm. A pharyngeal basket present and parallel rows of cilia on ventral surface (Figure 3).
4. Sessile suctoria have cilia present only on their larval stages whereas tentacles present on adult stages. The body orange to brown in color are from 50µm to 100µm in diameter. (Figure 4)

b. Clinical Signs - as in sections D-1 and D-2, above

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 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. This will sucessfully keep specimen fish for up to 10 hours. The least desirable method is to preserve the sample. Gill tissue and skin scrapings should be place in 5% buffered formalin for 24 hours and then transferred to 70% ethanol or AFA for shipment and/or storage.

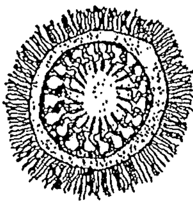
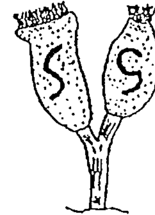


Figure 1. Trichodina
MOTILE PERITRICH



Apiosoma Ambiphrya Epistylis

Figure 2. SESSILE PERITRICH

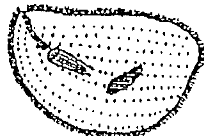


Figure 3. Chilodonella
MOTILE HOLOTRICH

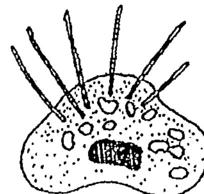


Figure 4. Trichophyra
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# XI. Monogenean Diseases

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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).

## 1. *Gyrodactylus* spp.

### A. Name of Disease and Etiological Agent

Gyrodactyliasis, *Gyrodactylus* spp. (*Platyhelminthes:Monogenea*).

### B. Known Geographical Range and Host Species of the Disease

#### 1. Geographical Range

*Gyrodactylus* spp. occur 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*.

Cyprinids including goldfish *Carassius auratus*, common carp *Cyprinus carpio*, and grass carp *Ctenopharyngodon idella*, and golden shiner *Notemigonus crysoleucas*.

## 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.

**Conditions under which disease occurs**

*Gyrodactylus* spp. are 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.

From the scanty evidence available it appears that on salmonid fishes, *Gyrodactylus* spp. are 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 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.

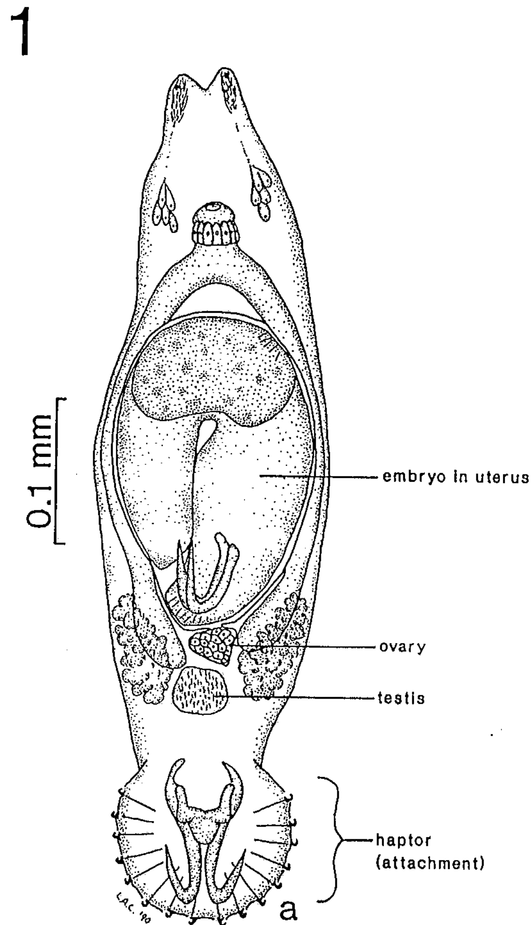
## E. Disease Diagnostic Procedures

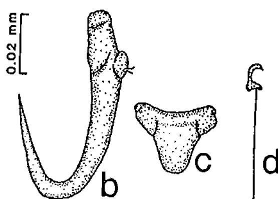
### 1. Presumptive Diagnosis

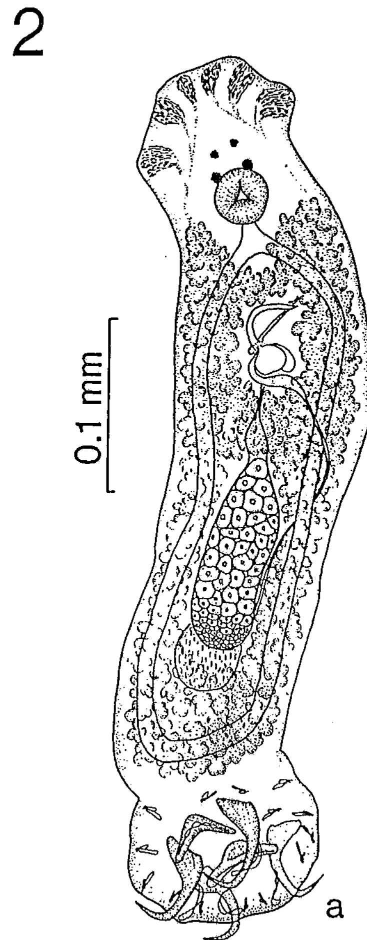
a. Isolation and detection of pathogen

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.



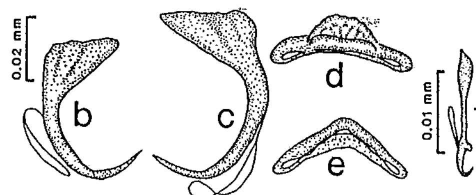




**Figure 1.** Generalized *Gyrodactylus* sp. (a ) entire animal, ventral

**Figure 2.** *Ligictaluridus* sp. (based on L.

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 - 45 minutes; shake the container vigorously for 2 or 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 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 4 liters of solution is appropriate) and the fish allowed to thrash about for 2 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 h 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.

# 2. 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

### 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, 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.

SECTIONS **E** (see Figure 2),**F, G, and H** are all similar to those for *Gyrodactylus* spp. above.

# 3. 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. occur on a wide variety of freshwater fishes throughout North America.

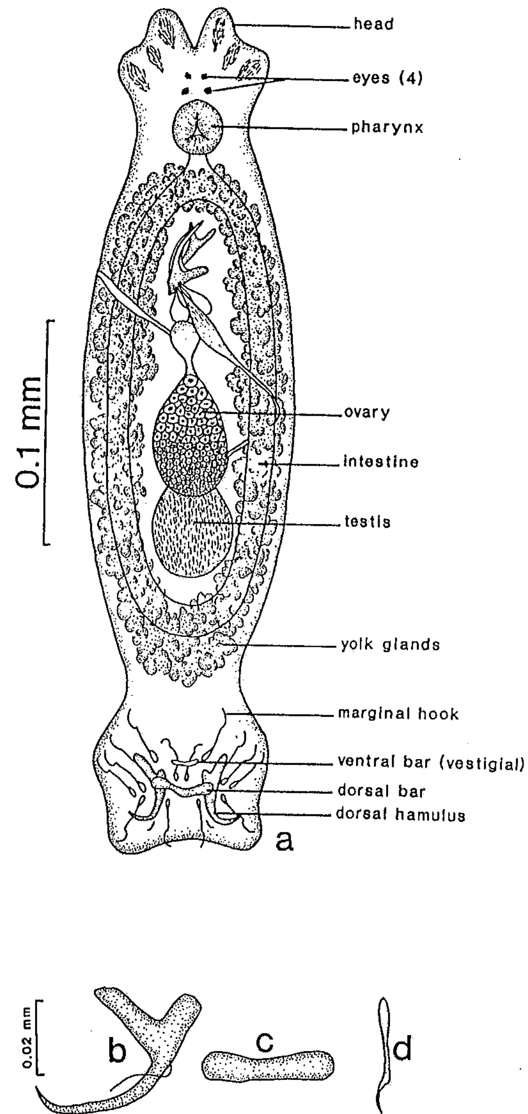



**Figure 3.** Generalized *Dactylogyrus* sp. (a) entire animal, ventral view, (b) dorsal hamulus, (c) dorsal transverse bar, (d) larval hook.

### 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.

### 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. 5-6 months at 1-2° C) than those in warmer areas (e.g. only a few days at 22-24° 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 and 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.

SECTIONS E (see Figure 3), F, G, and H are all similar to those for *Gyrodactylus* spp. above.

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# XII. Bothriocephalosis

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## 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*, *Bothriocephalus phoxini.*

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical Range

The Asian tapeworm has been reported in Asia, 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 New Hampshire and New York.

### 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: grass carp *Ctenopharygodon idella*, common carp *Cyprinus carpio*, mosquito fish *Gambusia affinis*, 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 squawfish *Ptychocheilus lucius*, and speckled dace *Rhinichthys osculus*.

## C. Epizootiology

Acute infection with the Asian tapeworm occurs when intensively cultured larval fish feed on copepods infested with high levels of *Bothriocephalus acheilognathi* procercoids. Up to 80% mortality among larval fish has been reported by fish producers; however, death as a direct result of tapeworm infection is not a common occurrence. In the United States, most problems associated with this worm involve reduced growth and the inability of infected fish to withstand harvesting procedures. The worm evidently shortens the life span and stunts the growth of feral fish.

Operculated eggs of the Asian tapeworm are shed with fish feces into the water. A motile coracidium emerges from these eggs and is eaten by cyclopoid copepods. Larval worm development proceeds within the copepod. Copepods are eaten by fish and the developing worm is released in the anterior portion of the alimentary canal. The worm attaches to the mucosal lining of the intestine and matures producing gravid segments which release eggs. Worms up to 60 cm have been reported; however, most mature worms measure between 5 and 8 cm.

The Asian tapeworm is a thermophile that prefers temperatures of 20-30°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 - 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 - 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 - 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 Changes Associated with the Disease

The only reported histopathological changes include thinning of the intestinal walls and damage to the villi.

## 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. The shape of the scolex is difficult to determine if the worm is dead, frozen, or preserved. Histological or clinical signs that are useful to the diagnosis of infected fish have not been demonstrated..

### 2. Confirmatory Diagnosis

Once tapeworms with pyramidal scolices are found, they must be distinguished from all others with similar shaped scolices. Some worms of the genera *Anoncocephalus*, *Atractolytocestus*, *Bathybothrium*, *Echinophallus*, *Eubothrioides*, *Eubothrium*, *Fistulicola*, *Glaridacris*, *Marsipometra*, *Proteocephalus*, *Ptychobothrium*, and *Schistocephalus* species have a pit viper or pyramid-shaped scolex. To aid in the differentiation of these tapeworms, the following definition and key for *Bothriochephalosis acheilognathi* is given.

*Bothriocephalus acheilognathi* is a complete and distinctly segmented, thin tapeworm that can reach a length of over 50 cm, but is usually less than 10. 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).

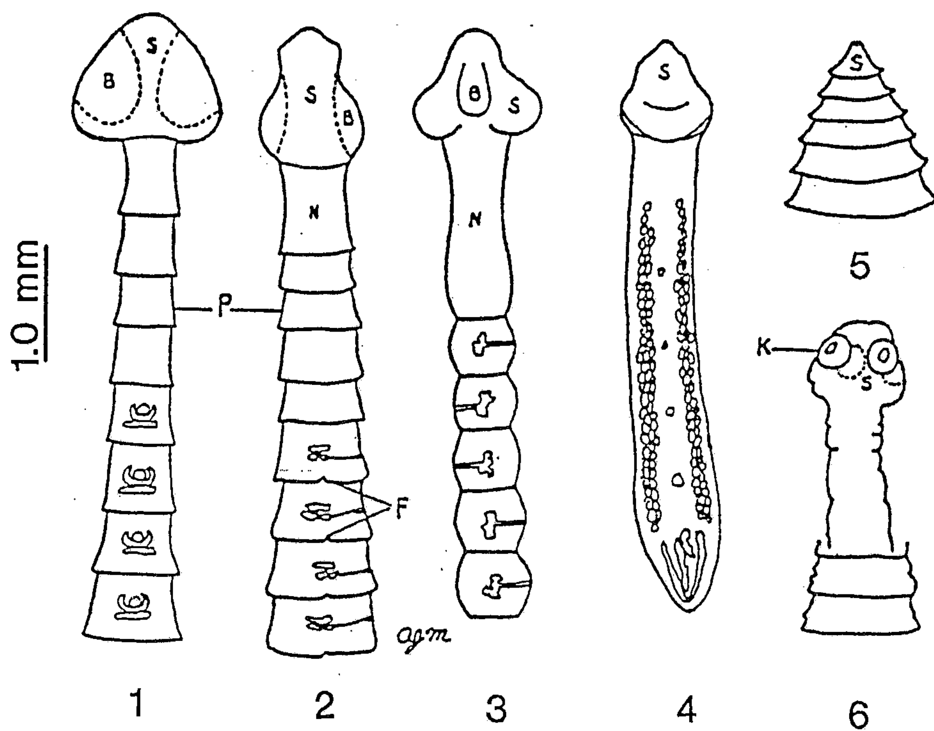


**Figure 1.** (1) *Bothriocephalus acheilognathi*; (2) *Eubothrium salvelini*; (3) *Marsipometra parva;* (4) *Glaridacris laruei* (unsegmented); (5) *Schistocephalus solidus*; (6) *Proteocephalus ambloplitis*; B - bothria; F - furrow; K - sucker; N - neck; P - proglottid; S-scolex.

## KEY FOR THE SEPARATION OF ADULT* BOTHRIOCEPHALUS ACHEILOGNATHI FROM OTHER TAPEWORMS WITH PYRAMIDAL OR ARROWHEAD-SHAPED SCOLICES

1a. Non-segmented or segmentation incomplete or indistinct ........ *Atractolytocestus*, *Glaridacris*, Ptychobo*thrium*

1b. Segmentation complete and distinct ........ 2 .

2a. Scolex with 4 or 5 suckers ........ *Proteocephalus*

2b. Scolex with 2 bothria no suckers ........ 3

3a. Tapeworms of marine fish ........ *Anoncocephalus*, *Echinophallus*, *Eubothrioides*, *Fistulicola*

3b. Tapeworms of freshwater fish ........ 4

4a Dorsal-ventral median furrow ........ *Bathybothrium*, *Eubothrium*

4b. No dorsal-ventral median furrow ........ 5

5a. Neck ........ *Marsipometra*

5b. No neck ........ 6.

6a Wide portion of scolex (constricted) is the same or less than the width.of the anterior segments ........ *Schistocephalus*

6b. Solex clearly wider than anterior segments. ........ *Bothriocephalus acheilo*

* Scolex characters are usually found in juvenile worms in fish.

## 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 General Sampling Procedures).

Sever the head of the fish just behind the opercular flap. 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. 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 (2-4 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 s. 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

Not 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 5 minutes and then transfer to 10% formalin.

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# XIII. Lernaeid Parasitism

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## 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 then 1.8 parts per thousand (Hoffman 1976).

## D. Disease Signs

### 1. Behavioral Signs

Behavioral changes are similar to infections of other external parasites (flashing, listlessness, and eventual morbidity).

### 2. External Gross Signs

If there is opportunity to observe fishes closely or in hand, the parasites are readily apparent. They appear as bristle-like projections usually up to 6.5 mm long attached to the body surface. Usual attachment sites are fin bases and the oral cavity, but any exterior surface may be parasitized. Irritation by the parasites and local microbial infection at attachment sites will usually show some degree of inflammation and ulceration. The presence of several parasites does not equate to morbidity unless they provide opportunity for a secondary infection by microbes or unless fishes are particularly small.

### 3. Internal Gross Signs

Internal signs are not evident unless the anchors penetrate thc 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 5-10% 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

Not 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.

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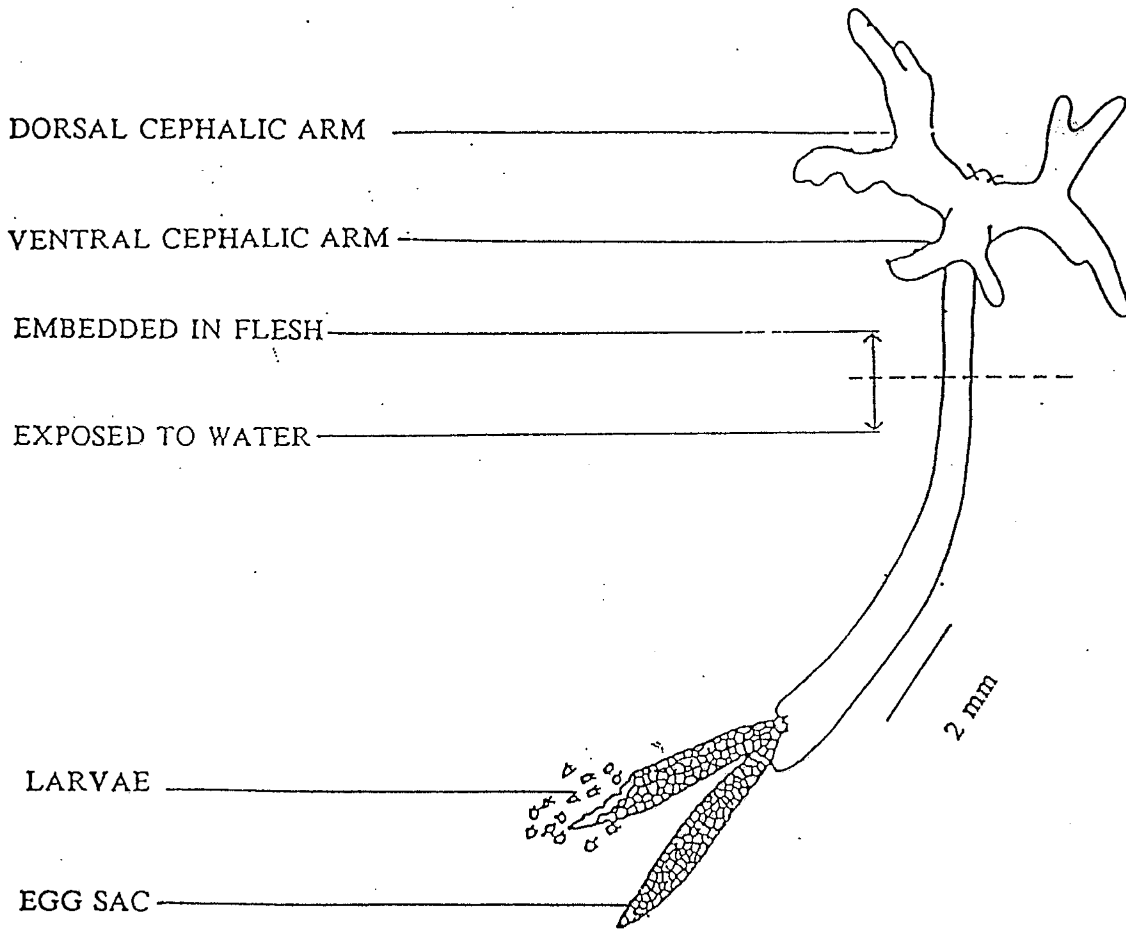


**Figure 1.** Diagnostic features of *Lernaea cyprinacea*, a species commonly associated with a wide variety of hosts.

# XIV. Gill Maggot Disease

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## A. Name of Disease and Etiological Agent

Gill maggot of salmon and trout, also called fish louse or gill louse.

*Salmincola salmoneus*.

*Salmincola californiensis* - [Synonyms: *Salmincola bicauliculata*; *Salmincola carpenteri*; *Salmincola falculata*].

*Salmincola edwardsii* - [Synonyms: *Lernaeopoda edwardsii*; *Lernaeopoda arcturi*; *Salmincola exsanguinata*].

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical Range

Circumpolar, in freshwater habitats of the northern hemisphere.

*Salmincola salmoneus*. Natural distribution straddles Atlantic Ocean, found in North America and Europe.

*Salmincola californiensis*. Natural distribution straddles Pacific Ocean, found in North America and Asia.

*Salmincola edwardsii*. Natural distribution includes eastern and central North America and Europe.

### 2. Host Species

Fishes of the Family *Salmonidae*.

*Salmincola salmoneus* - Atlantic salmon *Salmo salar*.

*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*.

*Salmincola edwardsii* - Reported on brook trout *Salvelinus fontinalis*, arctic char *Salvelinus alpinus*, lake trout, dolly varden and the mountain whitefish. Fingerlings of Atlantic salmon, 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

Infestations 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 (ie. 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 infested 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 - 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 infestations. Infested 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 (eg. Kabata 1988). For expert diagnosis to the species level, remove the copepods from fish and place in a glass or plastic vial with 70% alcohol or 5% formalin and forward to a fish parasitologist.

## F. Procedures for Detecting Subclinical Infections

Sampling of fish populations or lots using a sample size adequate to detect a 5% level of prevalence (see Ossiander and Wedemeyer 1973). The infective stage (copepodid) and juvenile stages (chalimus I-IV) may be detected by using a dissecting microscope with objectives 6-12X.

## 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 infestations. Frontal filaments (of juveniles) and bullae (of adults) may sometimes be distinguished from surrounding tissue. Gill tissues may recover after a light to moderate infestation 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.

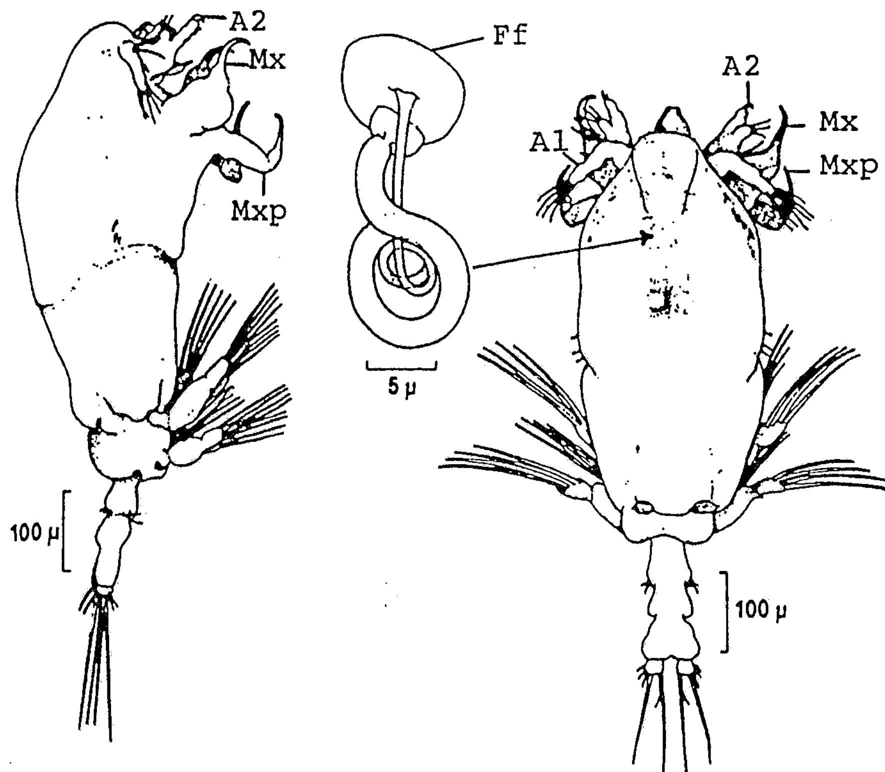


**Figure 1.** *Salmincola*, copepodid (infective stage). A1, antennule. A2, antenna. Mx, Maxilla. Mxp, maxilliped. Ff, frontal filament. (From Kabata and Cousins 1973).

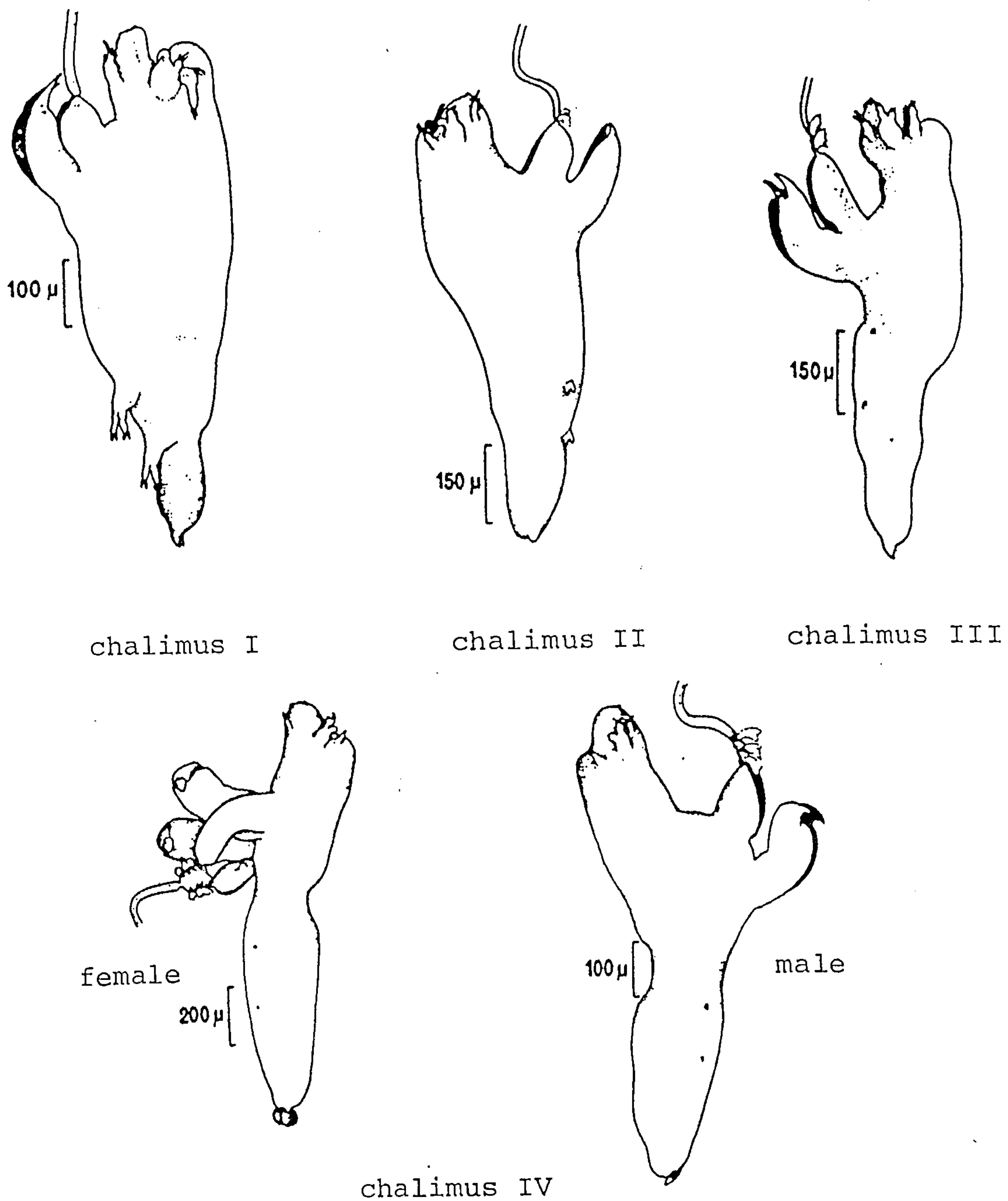


**Figure 2.** *Salmincola*, chalimus stages (attached by frontal filament). (From Kabata and Cousins 1973).

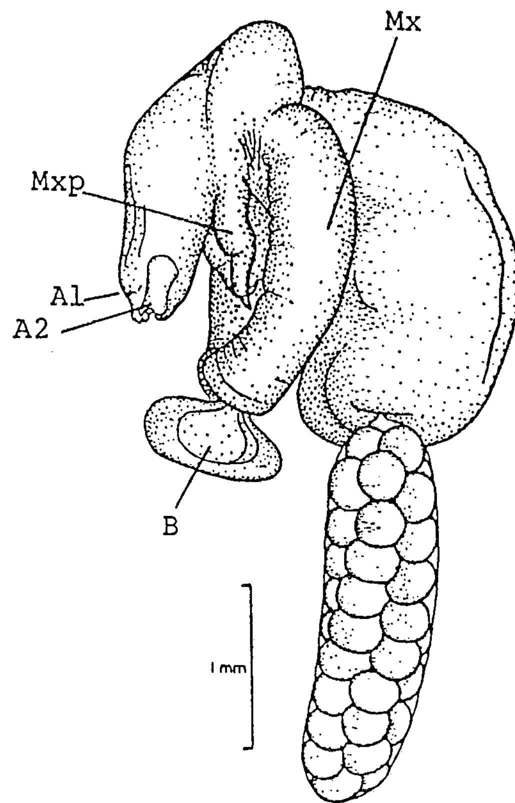


**Figure 3.** Salmincola, adult female. Al, antennule. A2, antenna. Mx, maxilla. Mxp, maxilliped. B, bulla. (From Fryer, G. 1981)

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# XV. Sea Lice

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## 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: salmon louse, salmon lice, sea louse, white spot, 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:

*Caligus clemensi*: Pacific coast of Canada (British Columbia) and probably the northwest coast of the United States (Washington).

*Caligus curtus*: Atlantic coast of Canada (Bay of Fundy).

*Caligus elongatus*: Atlantic coast of Canada, Ireland, and Scotland.

*Lepeophtheirus salmonis*: 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.

*Caligus clemensi*

Salmonid hosts: 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: Pacific herring *Clupea harengus pallasi*, three spine stickleback *Gasterosteus aculeatus*, greenling *Hexagrammos* sp., Pacific ratfish *Hydrolagus colliei*, copper rockfish *Sebastes caurinus*, and walleye pollack *Theragra chalcogramma*.

*Caligus curtus*

Salmonid hosts: Atlantic salmon, *Salmo salar*.

Other hosts: Generally considered a parasite of gadid fishes, but has a broad host range, including more than 35 species of teleosts and elasmobranchs.

*Caligus elongatus*

Salmonid hosts: rainbow trout, brown trout *Salmo trutta*, Atlantic salmon, and brook trout *Salvelinus fontinalis*.

Other hosts: Very broad host range, with more than eighty species of teleost and elasmobranch hosts reported.

*Lepeophtheirus salmonis*

Salmonid hosts: pink, chum, coho, sockeye, chinook, Atlantic, cherry or masu salmon, *Oncorhynchus masou*, rainbow trout, coastal cutthroat trout *Oncorhynchus clarki*, and brook trout.

Other hosts: 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 ten 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 net pen 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 5 weeks at 10 - 13°C. The generation time of *Lepeophtheirus salmonis* is approximately 6 weeks at 9 - 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 net pen 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 (1, 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 4 setae in *Caligus curtus* and 5 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.

**The following key serves to distinguish the adults of the four species of sea lice reported from sea-farmed salmon and trout.**

**Key to adult sea lice of 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 elongatus* (Figures 1G and 1H) (Atlantic)

## 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.

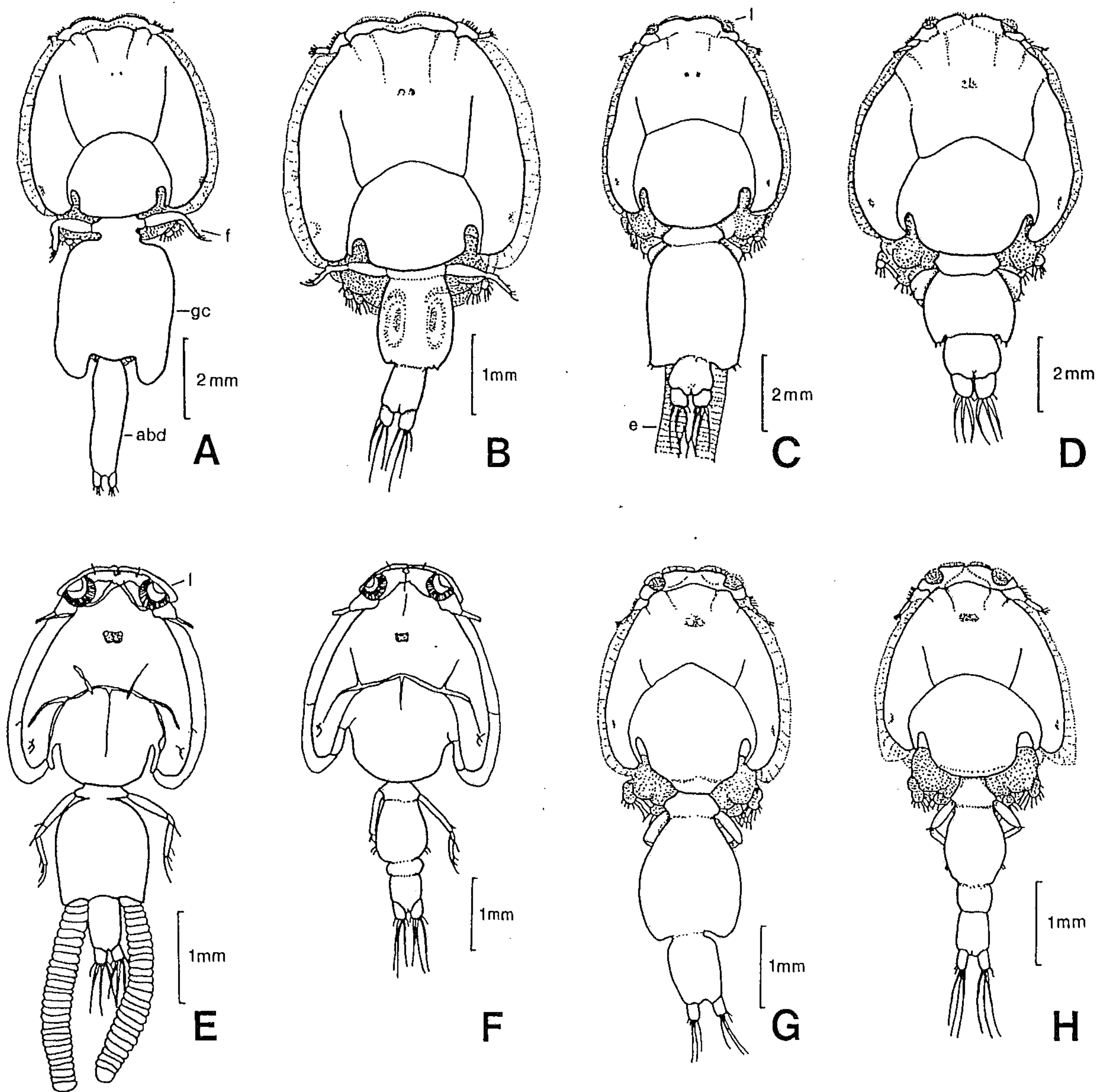


**Figure 1.** Adult stages of sea lice: (A) *Lepeophtheirus salmonis*, female; (B) same, male; (C) *Caligus curtus*, female; (D) same, male; (E) *Caligus clemensi*, female; (F) same, male; (G) *Caligus* elongatus, female; (H) same, male (A-D, G, H, redrawn

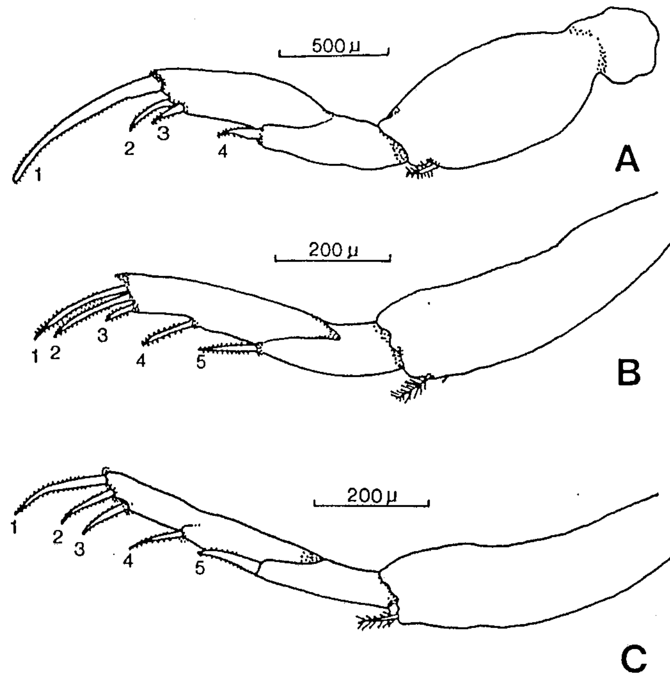


**Figure 2.** Structure of the fourth leg: (A) *Caligus curtus*; (B) *Caligus clemensi;* (C) *Caligus elongatus* (A, redrawn from Kabata 1979; B, redrawn from Kabata 1972; C, original).

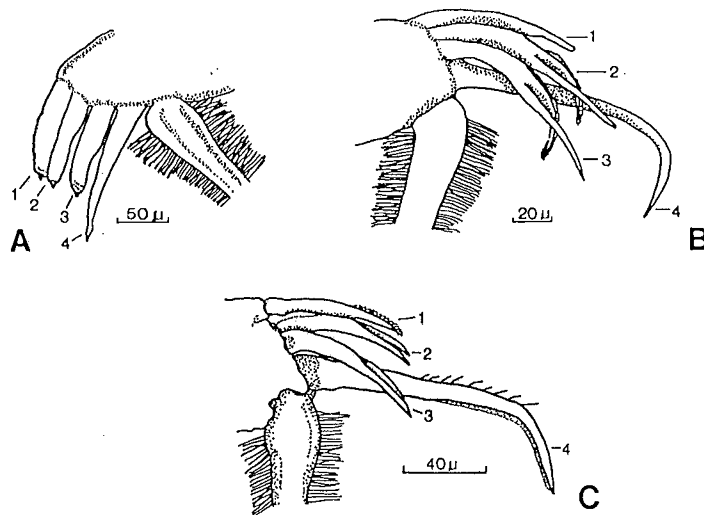


**Figure 3.** Distal margin of the exopod of the first leg: (A) *Caligus curtus;* (B) *Caligus clemensi;* (C) *Caligus elongatus* (A,C, modified from Kabata 1979; B, modified

## 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 2 weeks in 10% formalin and then transfer to 10% glycerine alcohol (10 ml glycerine in 90 ml of 70% ethyl alcohol).

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# XVI. Branchiuran Fish Louse Disease

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## A. Name of Disease and Etiological Agent

Fish louse or fish lice

*Argulus* . Over 120 species reported from freshwater and marine fishes. The genus is in poor taxonomic condition. Many species presently documented are based on inadequate original descriptions and are probably synonomous with the more common and established species.

List of Common or Economically Important Species of Argulus

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, Argulus versicolor* .

Eurasia: *Argulus coregoni, Argulus indicus, Argulus japonicus, Argulus foliaceus, Argulus scutiformis, Argulus viridis.*

Africa: *Argulus africanus, Argulus amblopites, Argulus brachypeltis, Argulus exiguus Argulus jollymanni, Argulus rhipidophorus, Argulus striatus.*

South America: *Argulus chromidis, Argulus juparensis, Argulus nattereri, Argulus pestifer, 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 (eg: *Argulus flavescens* has been reported from 18 species of freshwater fishes in North America). A single species may infect fishes from several different orders and families. The argulids can be considered opportunistic parasites in most reported cases, particularly on cultured fishes.

## C. Epizootiology

The fertilized female leaves the host skin or gills and lays several hundred eggs on submerged objects. A free-swimming second copepodid larva hatches after 48 - 72 h, and must find a host within 48-98 h or die.

Larvae are positively phototaxic, and development occurs more rapidly in bright illumination. Several molts of copepodid and sub-adult stages occur on the host. The adult stage is reached in approximately 15-40 d, 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 23-28°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* cause 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, 5-20 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 (1-3mm) and are not easily seen with the unaided eye. The larval stages of most species are unknown.

b. Clinical signs

Presence of the parasites on the host skin, fins,or gills. Recognition of external tissue pathology 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.

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# Parasitic Diseases of Fishes


I. GENERAL PROCEDURES FOR PARASITOLOGY

Ia. KEY TO MAJOR TAXA OF ADULT PARASITES OF FISHES

II. ICHTHYOBODIASIS (COSTIASIS)

III. HEXAMITIASIS (OCTOMITOSIS) AND SPIRONUCLEOSIS

IV. PLEISTOPHORIASIS (OVARY PARASITE OF GOLDEN SHINERS)

V. SALMONID CERATOMYXOSIS

VI. WHIRLING DISEASE OF SALMONIDS

VII. PROLIFERATIVE GILL DISEASE

VIII. PROLIFERATIVE KIDNEY DISEASE

IX. ICHTHYOPHTHIRIASIS

X. EXTERNAL CILIATED PARASITE INFECTION

XI. MONOGENEAN DISEASES

XII. BOTHRIOCEPHALOSIS

XIII. LERNAEID PARASITISM

XIV. GILL MAGGOT DISEASE

XV. SEA LICE

XVI. BRANCHIURAN FISH LOUSE DISEASE

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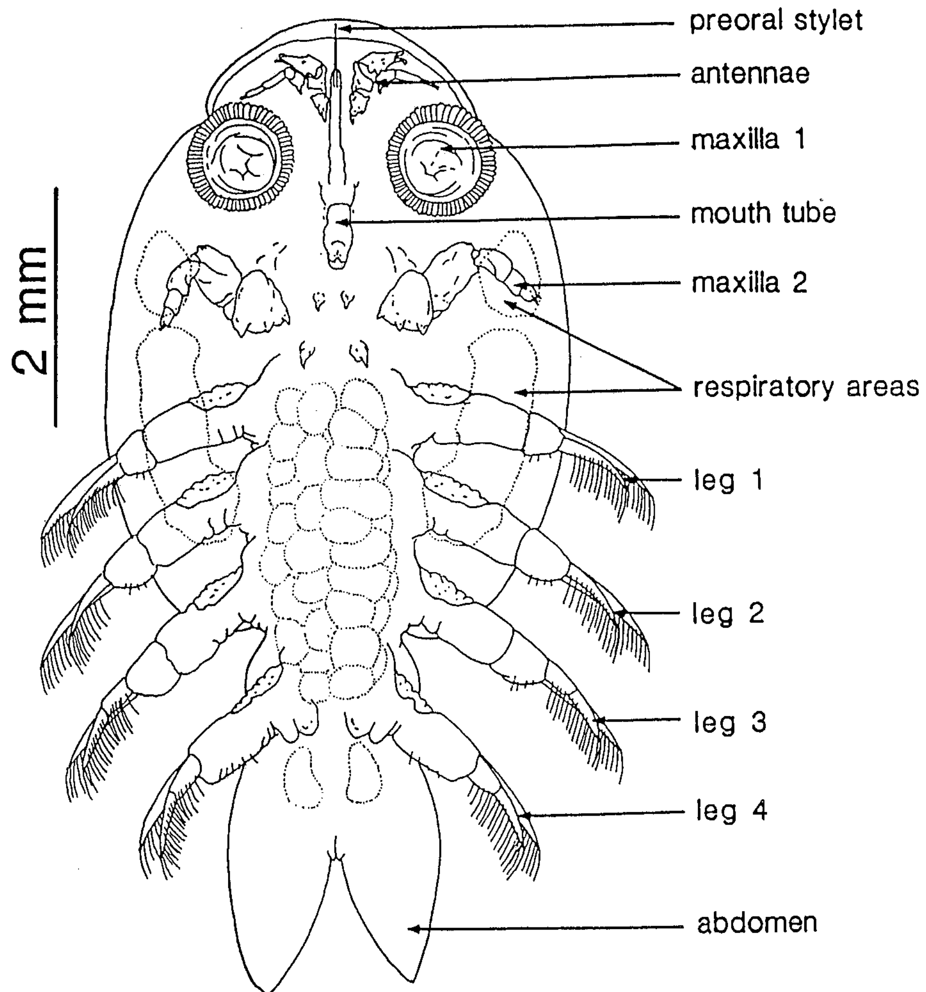


**Figure 1.** Ventral view of *Argulus alosae* showing pertinent diagnostic features (redrawn from Kabata 1988).

# Mycotic Diseases of Fishes

I. GENERAL PROCEDURES FOR MYCOLOGY

II. BRANCHIOMYCES

III. ICHTHYOPHONUS DISEASE

IV. SAPROLEGNIA

V. MISCELLANEOUS MYCOTIC DISEASES

# I. General Procedures for Mycology

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## 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 dorsolateral 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

Simple 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 General Procedure for Parasitology.

# II. Branchiomyces

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## A. Name of Disease and Etiological Agent

Branchiomycosis (Gill Rot) is caused by *Branchiomyces sanguinis* and *B. demingrans.*

## B. Known Geographical Range and Host Species of the Disease

### 1. Geographical Range

Branchiomycosis has been reported from Eastern Europe and from the United States in Alabama, Arkansas, Florida, Illinois, Georgia, Missouri, Ohio, Rhode Island, and Wisconsin (Piper et al. 1982).

### 2. Host Species

*Branchiomyces* is a major problem in Eastern European fish culture (Meyer 1973) where it infects pike *Esox lucius*, tench *Tinca tinca*, and common carp *Cyprinis carpio*. In the United States *Branchiomyces* has been found in rainbow trout *Oncorhynchus mykiss*, largemouth bass *Micropterus salmoides*, smallmouth bass *Micropterus dolomieu*, striped bass *Morone saxatilis*, northern pike *Esox lucius*, walleye *Stizotedion vitreum* , pumpkinseed *Lepomis gibbosus*, and guppies *Poecuilia reticulata.*

## C. Epizootiology

*Branchiomyces* invades the gill blood vessels and is most prevalent in fish raised in warm water with high organic content. The fungus can be recognized in gill filament wet mounts. Non-septate, branched hyphae filled with protoplasmic mass, which divides into granular "spherical spores", can often be seen in gill lamellae (See micrograph, pp 41 Hoffman and Meyer 1974).

## D. Disease Signs

External signs for branchiomycosis include pale, whitish gills with accompanying necrosis, fish gasping at the surface, and high mortality.

## E. Disease Diagnostic Procedures

Examine wet gill tissue microscopically (100X or 400X magnification). Observe non-septate 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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# III. Ichthyophonus Disease

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## A. Name of Disease and Etiological Agent

Ichthyophonus disease is caused by the intrusion of endospore bodies of *Ichthyphonus hoferi* into new hosts or within the body of an already infected host. The disease is sometimes referred to as "swinging disease". Swinging disease of trout is the abnormal swimming motion due to lordosis and scoliosis caused by *Ichthyophonus*.

## B. Known Geographical Range and Host Species of the Disease.

Cultured trout in the Western United States, Australia, Europe, Japan, Taiwan. Marine fish and aquarium fishes worldwide.

## C. Epizootiology

*Ichthyophonus* is an internal and obligate fungal parasite with low host specificity. Similar organisms have been reported from Amphibia and Copepoda, but it is uncertain whether these organisms are the same as occur in fish. *Ichthyophonus* has occurred in carp, trout, and salmon, and could occur whenever the flesh of raw fish is fed to cultured fishes or the spores allowed to accumulate in ponds. No sexual cycle has been determined. The proposed life cycle involves the subdivision of multinucleate spores forming small, motile, endospore bodies (with or without prior formation of hyphae and terminal hyphal bodies). These enter new hosts or are distributed within the body of an already infected host, and subsequently grow into large multinucleate spores. The major infection sites are the blood-rich organs (kidney, heart, spleen, and liver).

## D. Disease Signs.

Infected fish cease feeding and become lethargic. In acute infections, trout develop a gross lumpy appearance. Behavorial changes may also occur due to deformities. Spinal deformities may develop; nodules may develop in the kidney, liver, or muscle. Spores also may occur in 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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# IV. Saprolegnia

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## A. Name of Disease and Etiological Agent

Saprolegniasis is caused by the attachment of the swimming zoospores (etiological agent) of common water molds to the gills, skin, or fins.

The water molds belong to the Subdivision Diplomastigomycotina and the Class Oomycetes.

The most prevalent species associated with infections are *Saprolegnia diclina* type 1 and *Saprolegnia ferax*. Other genera of importance within this family include *Achyla*, *Aphanomyces*, *Dictyuchus*, *Leptolegnia*, *Leptomitus*, and *Phythium*. One or several representatives from the above genera can be found at the same infection site.

## B. Known Geographical Range and Host Species of the Disease.

### 1. Geographical Range

Water molds are ubiquitous and inhabit all freshwater. These organisms are universally distributed and can even be found in brackish water with salinities up to 2.8 percent.

### 2. Host Species

The water molds are parasitic to insects, amphibians, and fish and fish eggs of both warmwater and coldwater species.

## C. Epizootiology

In fish propagation, handling injuries, malnutrition, temperature shock, external parasitism, and spawning increase the susceptibility of fish to infection by water molds.

Whenever fungal zoospores are present in excess of 23,000 spores/liter, there is potential for infection. If these infections are left unchecked, high mortality can result.

## D. Disease Signs

The skin or other surfaces of infected fish and the surface of fish eggs become covered

with white, cottony tufts of non-septate filamentous hyphae.

## E. Disease Diagnostic Procedures.

### 1. Isolation

Although there are numerous media available for isolating and culturing aquatic fungi, the two simplest media to use are Corn Meal Agar (CMA) or Saboraud's Agar (SAB) (Fuller 1978). Isolates grow well at room temperature (20 to 25°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 General Procedure Section) 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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# V. Miscellaneous Mycotic Diseases

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### Deuteromycetes

Deuteromycosis is caused by imperfect fungi found in the class Deuteromycetes.

Representative genera and reported hosts:

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.

### 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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# Diseases of Shellfish

## Molluscan Diseases

I. PERKINSUS MARINUS INFECTION OF AMERICAN OYSTERS

II. HAPLOSPORIDIOSIS OF AMERICAN OYSTERS

III. OYSTER SEASIDE HAPLOSPORIDIOSIS

IV. VELAR VIRUS DISEASE OF PACIFIC OYSTERS

V. DENMAN ISLAND DISEASE

VI. NOCARDIOSIS OF PACIFIC OYSTERS

VII. BONAMIASIS OF OSTREID OYSTERS

VIII. HEXAMITIASIS OF OYSTERS

IX. MARTEILIASIS OF *OSTREA EDULIS*

X. SHELL DISEASE OF OYSTERS

XI. HEMATOPOIETIC NEOPLASM OF BIVALVE MOLLUSCS

XII. MISCELLANEOUS DISEASES OF MOLLUSCS

RICKETTSIA AND CHLAMYDIA

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VIBRIOSIS

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HINGE LIGAMENT DISEASE OF JUVENILE BIVALVE MOLLUSCS

## Crustacean Diseases

I. *LAGENIDIUM* OF DECAPOD CRUSTACEANS

# INTRODUCTION

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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.

# I. *Perkinsus marinus* Infection of American Oysters

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## 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 etiologic 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 known to occur only 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 30-50% 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 (18-30°C). The disease is known to increase dramatically

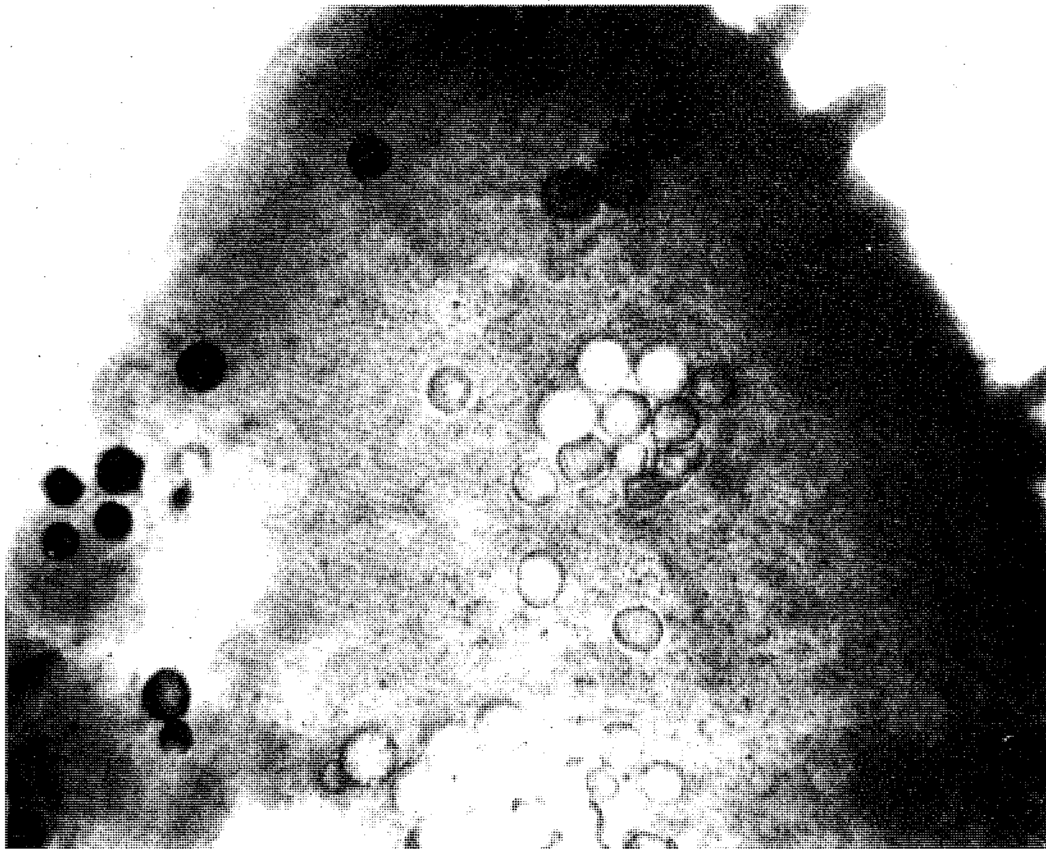

**Figure 1.** Mantle tissue of American oyster infected with *Perkinsus marinus* incubated in FTG medium and stained with Lugol's stain. The enlarged aplanospores near the periphery of the tissue stain blue to brown and appear dark in the photomicrograph while those deeper in the tissue are unstained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

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. Non-specific signs include emaciation, gaping, and pale digestive gland.

## E. Disease Diagnostic Procedures

### 1. Presumptive Diagnosis

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

### 2. Confirmatory Diagnosis

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

1. 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
2. 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).
3. 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.
4. 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.
5. 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.
6. 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 5-10 µm diameter, rarely to 20 µm or larger,

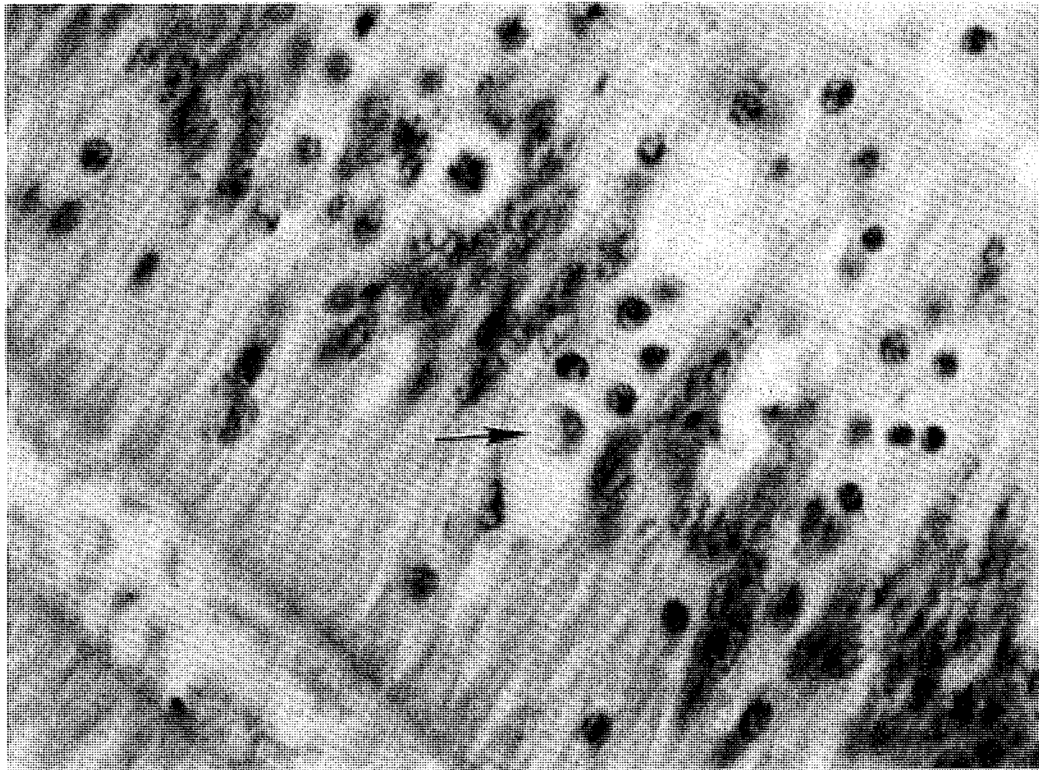

**Figure 2.** Arrow shows typical uninucleate aplanospore ("hypnospore") of *Perkinsus marinus* in the intestinal epithelium. The eccentric vacuole giving the signet ring appearance is characteristic of the microorganism in tissue, 1800X.

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.

## 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°-20°C for up to 24 hours during transport to the laboratory for examination.

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# II. Haplosporidiosis of American Oysters

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## A. Name of Disease and Etiological Agent

This disease is caused by *Haplosporidium nelsoni* (=*Minchinia nelsoni*, Acetospora). 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

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

### 2. Host Species

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 4-9% 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. Mortalites 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 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

Mantle recession occurs in heavily infected oysters, with heavy fouling along periphery of 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

Demonstration of plasmodial forms (described below) in hemacytological 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 4-30 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: **Initial** stage in which relatively small plasmodia are confined to the gill and palp epithelium. **Intermediate** stage characterized by widespread focal infections and hemocytic infiltration. **Advanced** stage with massive parasite proliferation in most tissues. **Terminal** stage characterized by necrosis of host and parasite tissues and **Remission** stage in which hemocyte 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 is described above.

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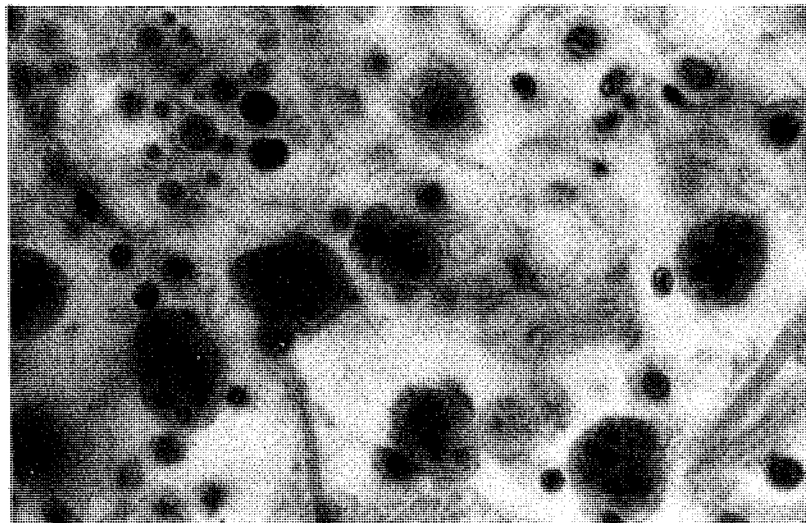

**Figure 1.** Plasmodia of *Haplosporidium nelsoni* in tissues of American oyster. Note the multiple nuclei with endosomes, 1125X. Photomicrograph courtesy of Susan Ford.

# III. Oyster Seaside Haplosporidiosis

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## 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

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.

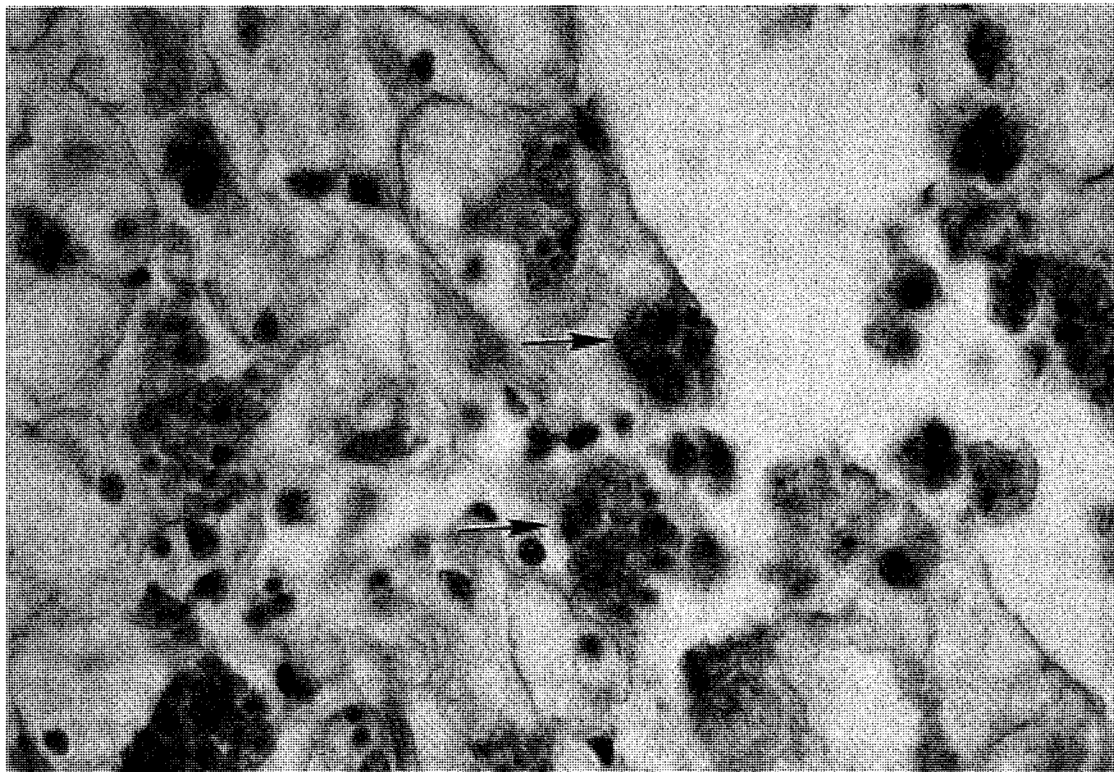

**Figure 1.** Sporulation of *Haplosporidium costale* in an American oyster (arrows), 1125X.

## 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 µ m 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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# IV. Velar Virus Disease of Pacific Oysters

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## A. Name of Disease and Etiological Agent

Oyster velar virus disease (OVVD) is also referred to as "blisters" by oyster hatchery biologists. Morphologically, the etiologic 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 etiologic 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 - 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 Figures 1, 2).

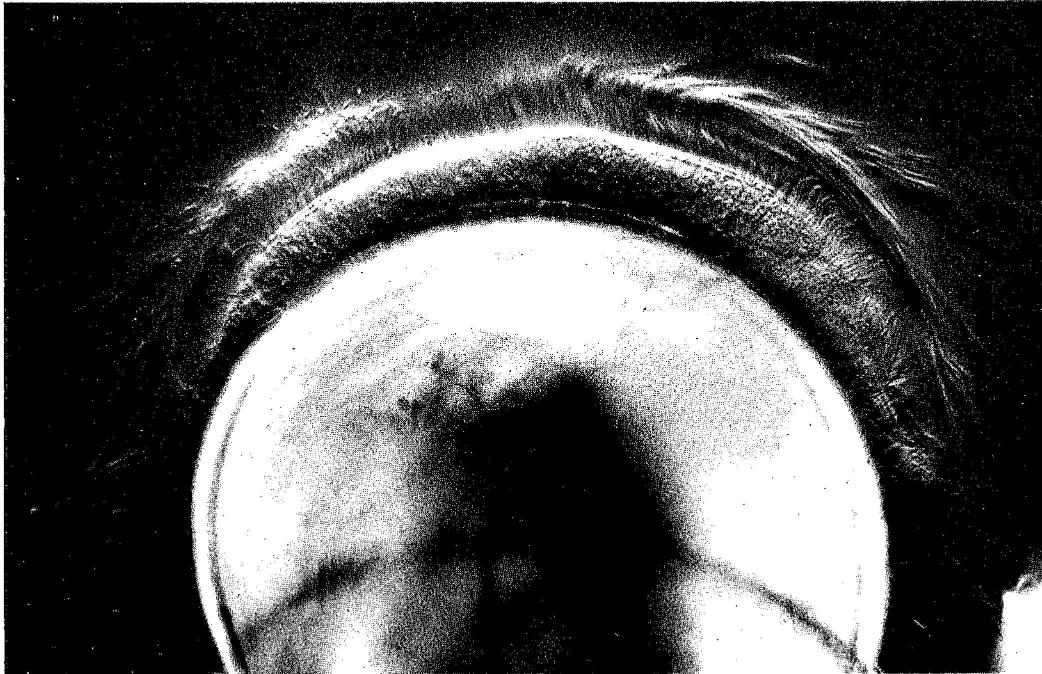

**Figure 1.** Wet mount of normal Pacific oyster larvae showing the extended intact ciliated velum, 383X. From Elston and Wilkinson 1985, with permission.

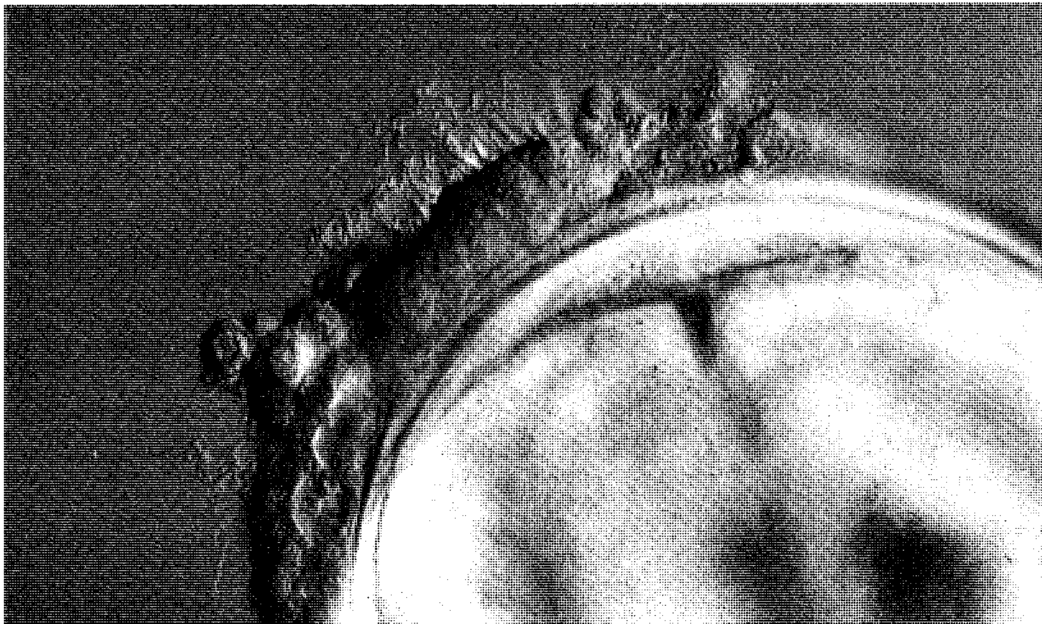

**Figure 2.** Wet mount of OVVD infected Pacific oyster larva showing velar degeneration including loss of cilia and loss of epithelial cells. As the cells round up and are in the process of detaching from the velum, they form the characteristic blisters

## 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.

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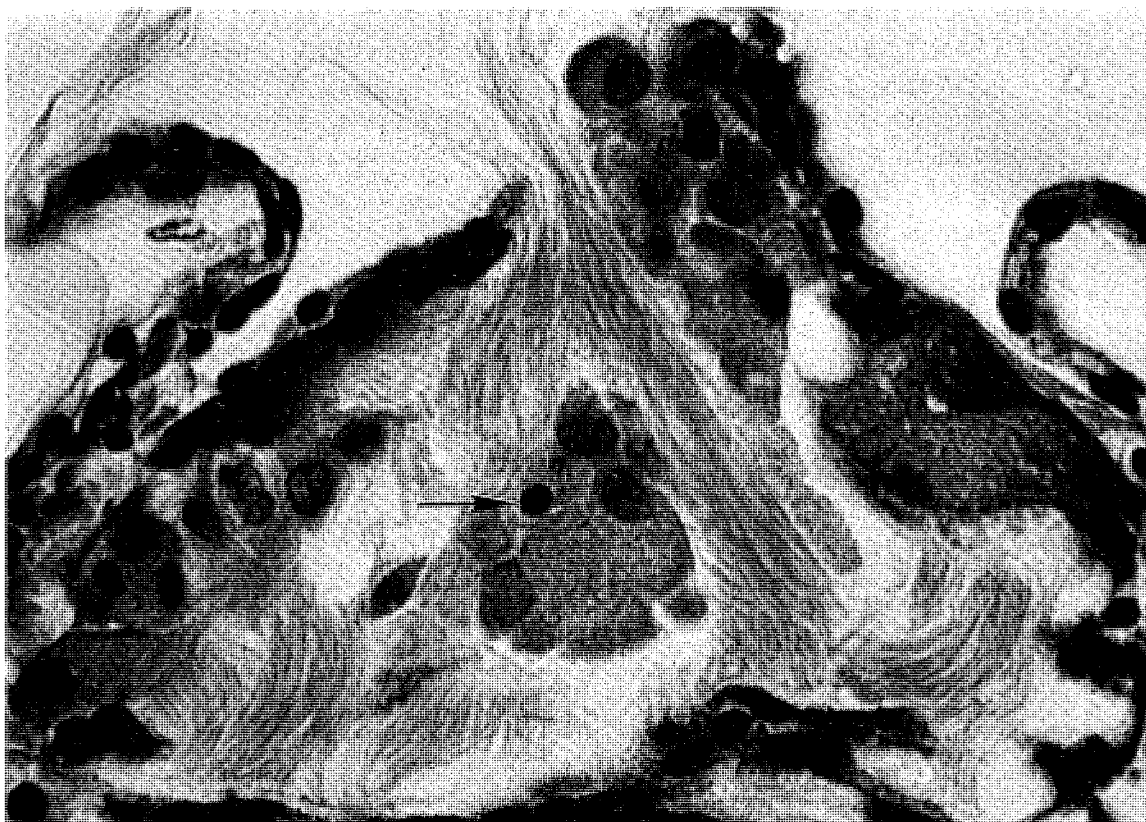

**Figure 3.** The intracytoplasmic inclusion body in detached velar epithelial cell (arrow) of *Crassostrea gigas* is characteristic of oyster velar viral disease, 2225X.

# V. Denman Island Disease

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## 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 5 to 7 years old oysters were affected while 2 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 5 to 7 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

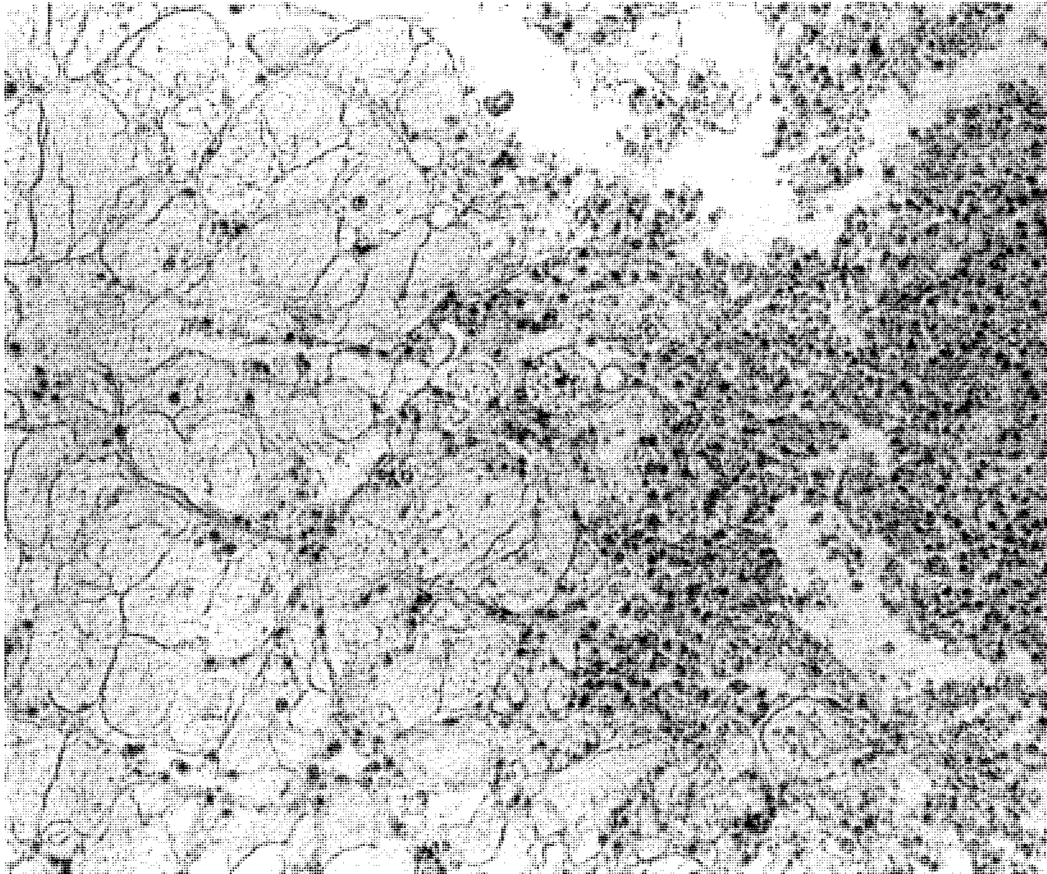

**Figure 1.** Histological section showing normal vesicular tissue of the Pacific oyster on the left and vesicular tissue infected with *Mikrocytos mackeni* on the right, 310X.

# VI. Nocardiosis of Pacific Oysters

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## 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

Matsushima Bay, Japan; discontinuously on the west coast of North America from Tomales Bay, California to British Columbia, Canada, including oyster culture areas in Willapa Bay and Puget Sound, Washington.

### 2. Host Species

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

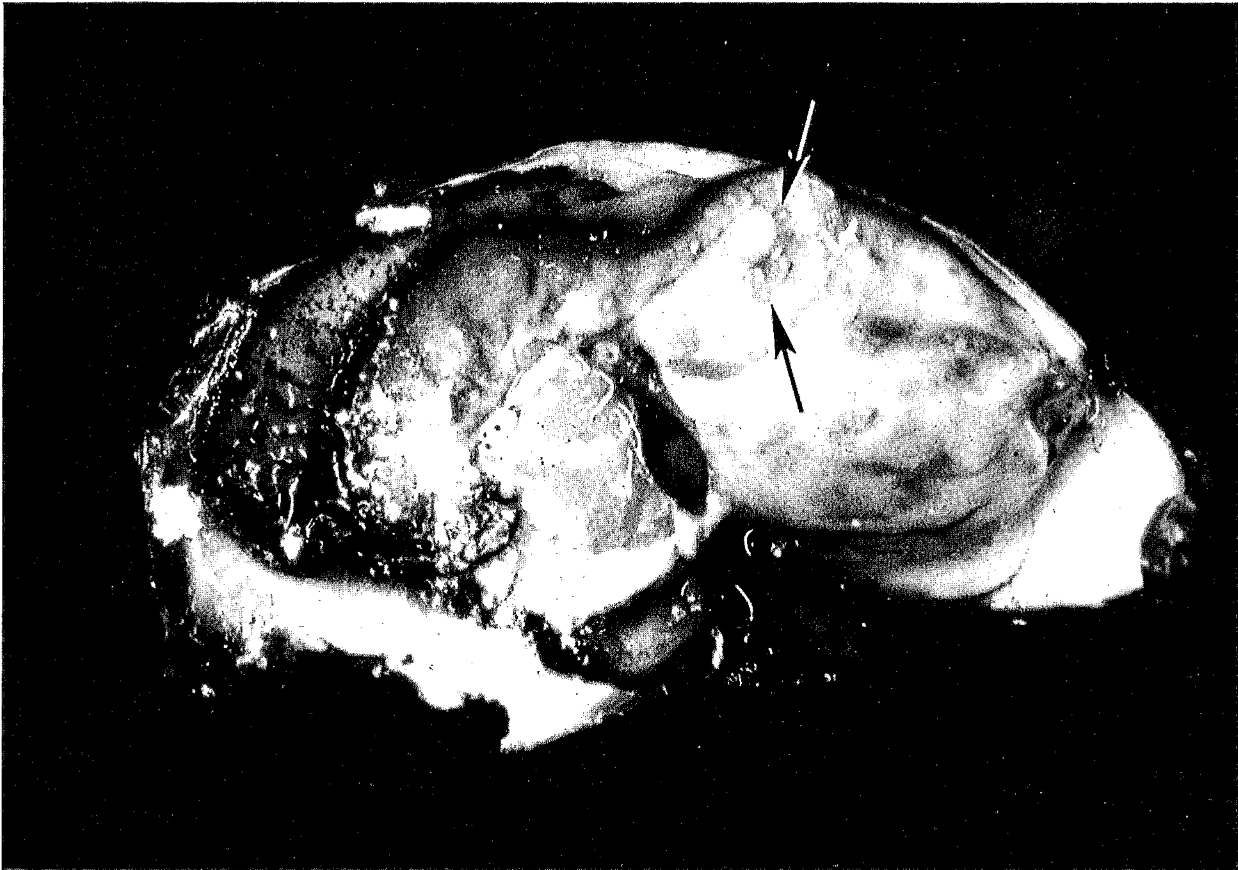

**Figure 1.** Pacific oysters with nocardiosis. This disease as well as others such as Denman Island disease is characterized by the formation of yellow to green pustules on the mantle and other tissue surfaces as shown here (arrows). From Elston et al. 1987, with permission.

# VI. Nocardiosis of Pacific Oysters

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## 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

Matsushima Bay, Japan; discontinuously on the west coast of North America from Tomales Bay, California to British Columbia, Canada, including oyster culture areas in Willapa Bay and Puget Sound, Washington.

### 2. Host Species

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

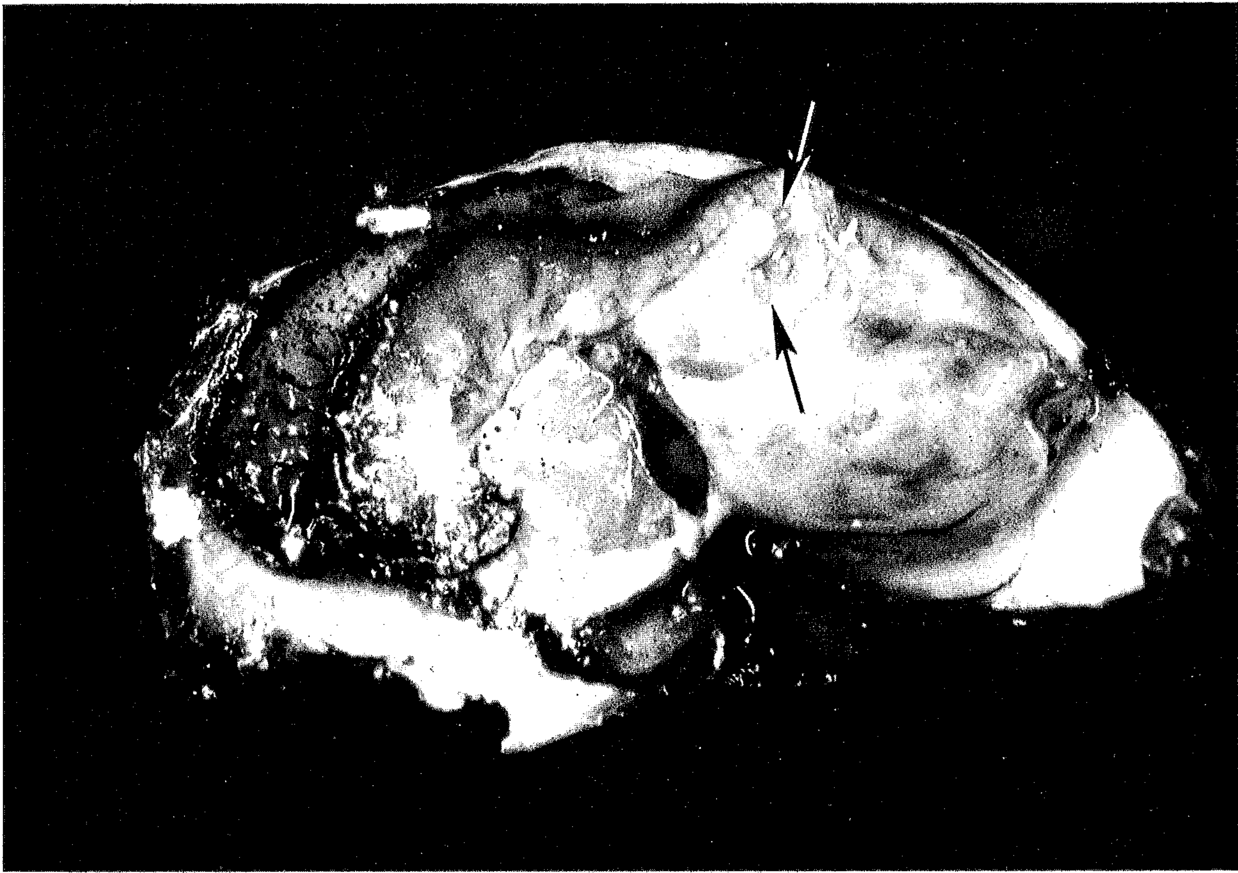

**Figure 1.** Pacific oysters with nocardiosis. This disease as well as others such as Denman Island disease is characterized by the formation of yellow to green pustules on the mantle and other tissue surfaces as shown here (arrows). From Elston et al. 1987, with permission.

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.

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**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. From Elston et al. 1987, with permission.

# VII. Bonamiasis of Ostreid Oysters

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## 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

*Bonamia ostreae*:

Europe: Atlantic and Mediterranean France and Spain, The Netherlands, England, Ireland, Denmark.

North America: California and Washington.

*Bonamia* sp.

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. Epizootiolgy

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 2-, 3- and 4-year-old infected oysters. The disease can

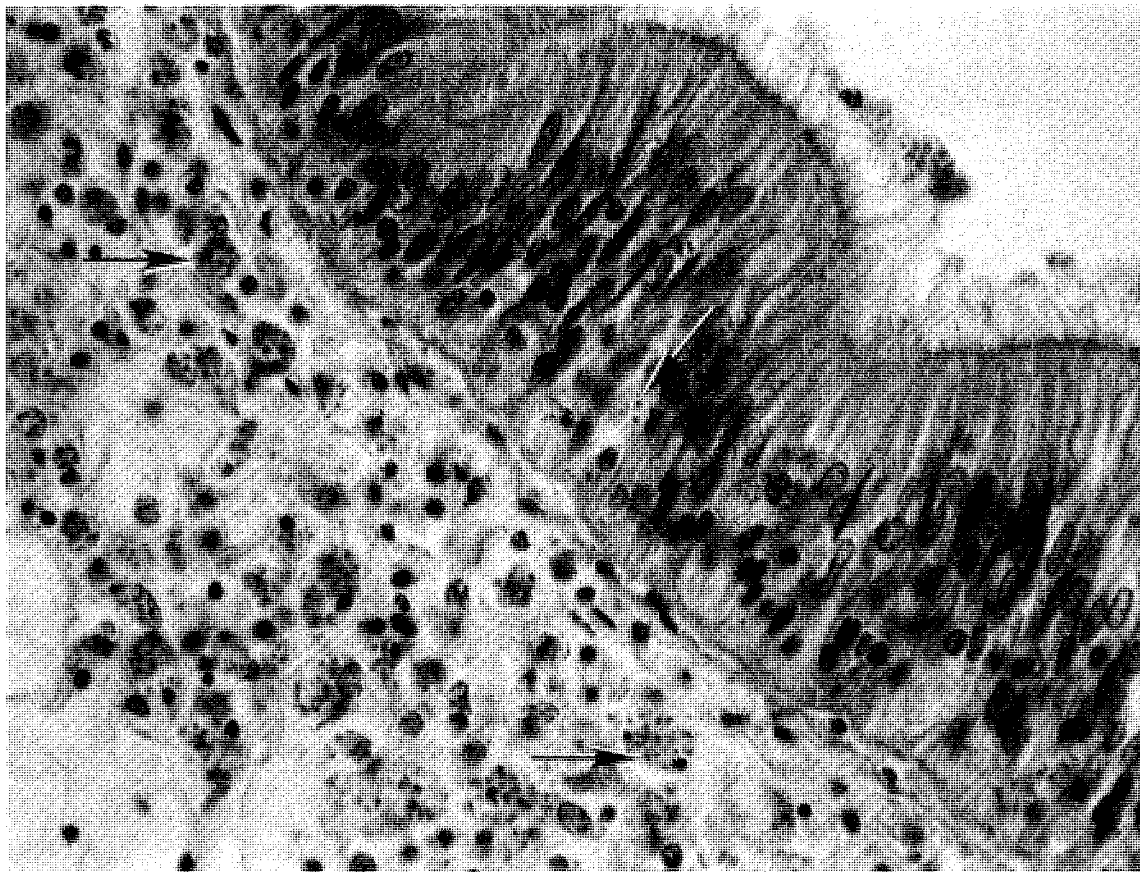

**Figure 1.** A relatively light infection of *Bonamia ostreae* in the European flat oyster. Arrow indicates a hemocyte containing the 2-3 µm organisms with eccentric nuclei, 1200X.

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.

## 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

### 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 2-3 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, N.Y. 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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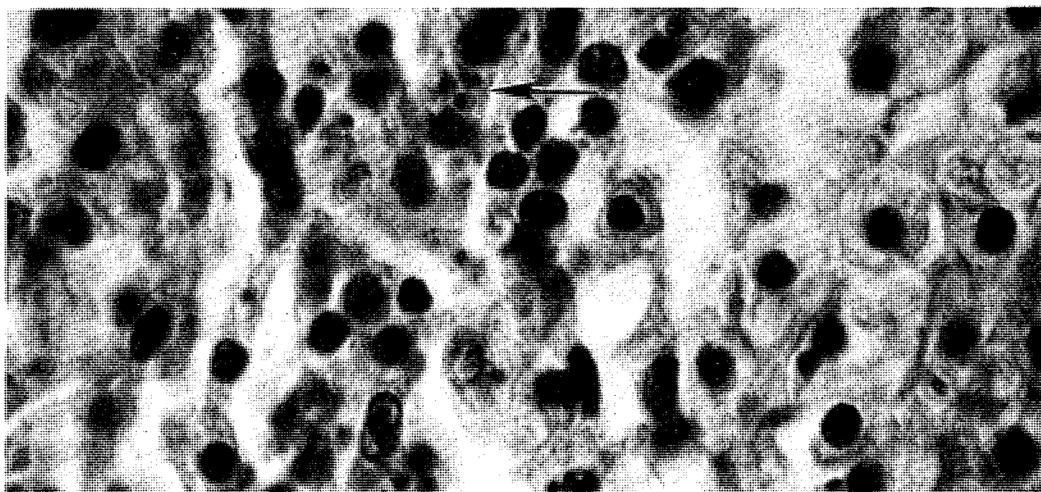

**Figure 2.** Histological section of *Ostrea edulis* heavily infected with *Bonamia ostreae*. Hemocytes infected with multiple organisms are shown at the arrows in the vascular sinus underlying the stomach epithelium. Organisms in hemocytes between epithelial cells are also evident (arrow), 480X.

# VIII. Hexamitiasis of Oysters

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## A. Name of Disease and Etiological Agent

*Hexamita nelsoni* of the phylum Sarcomastigophora is the reputed etiologic 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 waterborne 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:

*Saccostrea commercialis* (Australian rock oyster), Australia.

*Crassostrea gigas* (Pacific oyster), Pacific Northwestern United States.

*Crassostrea virginica* (American oyster), Prince Edward Island, Canada.

*Ostrea edulis* (European flat oyster), Holland; Maritime region, Eastern Canada.

*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 2-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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# IX. Marteiliasis of *Ostrea edulis*

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## 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 2- and 3-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.

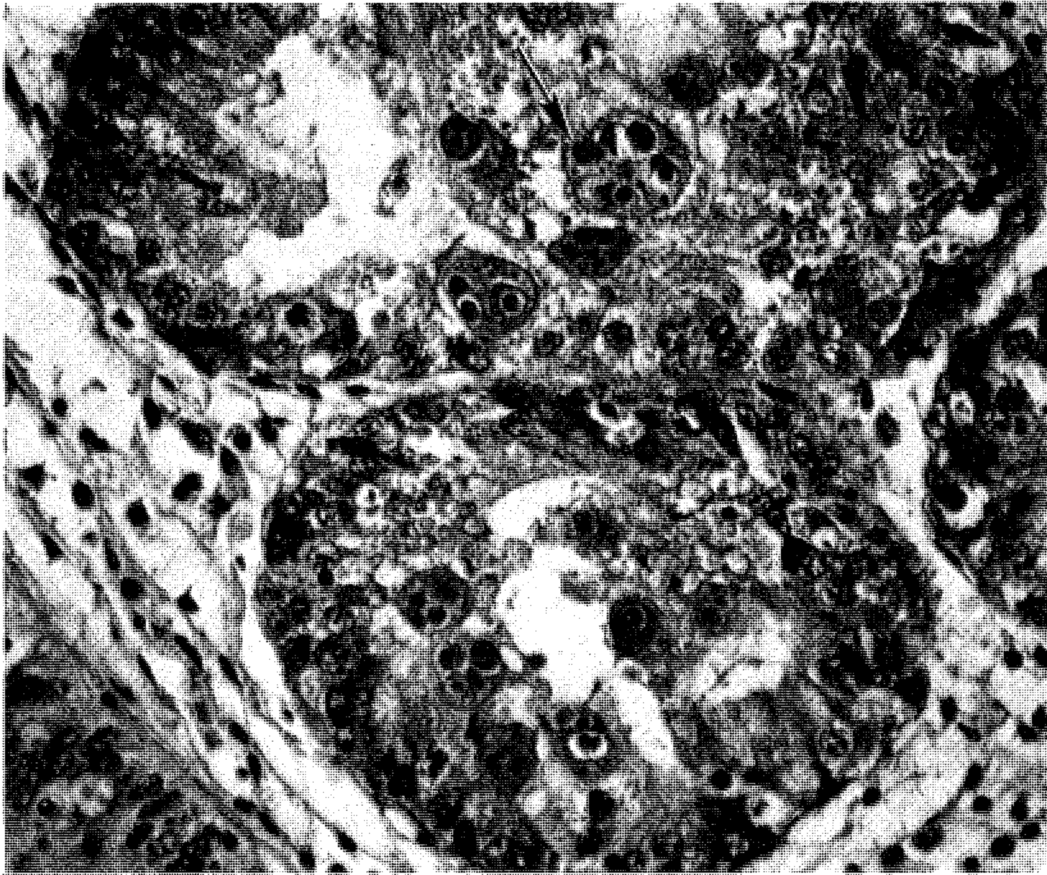

**Figure 1.** Digestive tubules of the European flat oyster infected with *Marteilia refringens*. Several sporangiosori are evident within the tubular epithelial cells. The characteristic birefringent material resulting from sporulation is shown at the arrow, 600X.

## 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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# X. Shell Disease of Oysters

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## A. Name of Disease and Etiological Agent

Shell disease is caused by the fungus *Ostracoblabe implexa* and can thus be properly referred to as oyster ostracoblabiasis. The disease has also been referred to as "maladie du pied" and "maladie de la charniere" in reference to the effects of disease on the adductor muscle (incorrectly referred to as the foot) and the hinge ligament.

## B. Known Geographical Range and Host Species of the Disease

The disease is well documented in the European flat oyster *Ostrea edulis* and the Portuguese oyster *Crassostrea angulata* in continental Europe (France and The Netherlands) and the United Kingdom and in *Crassostrea gryphoides and C. cucullata* in India. It has been reported in Atlantic Canada in *Ostrea edulis* and in the American oyster *Crassostrea virginica* on the Atlantic seaboard of the United States. The disease rarely progresses beyond the white spot stage in *Crassostrea.*

## C. Epizootiology

Shell disease may have caused massive mortalities of *Ostrea edulis* in The Netherlands at various times and has also been claimed to be associated with severe oyster kills in France. Definitive proof that the disease is responsible for the oyster kills is lacking. Oysters are infected above 20°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

**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. Photograph courtesy of David J. Alderman.

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.

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

# XI. Hematopoietic Neoplasm of Bivalve Molluscs

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## 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 etiologic 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:

*Adule californica:* west coast of North America.

*Arctica islandica* (mahogany quahog): Rhode Island Sound, eastern North America.

*Cerastoderma edule* (common cockle): south coast of Ireland near Cork and the Brittany region of France.

*Saccostrea commercialis* (Australia rock oyster): Australia.

*Crassostrea gigas* (Japanese or Pacific oyster): Matsushima Bay, Japan and Breton, France.

*Crassostrea rhizophorae:* Brazil.

*Crassostrea virginica* (Eastern or American oyster): discontinuously on the Atlantic and Gulf coasts of North America.

*Macoma balthica* (duck clam): Chesapeake Bay, eastern North America and Finland.

*Macoma calcarea*: Baffin Island, Canada.

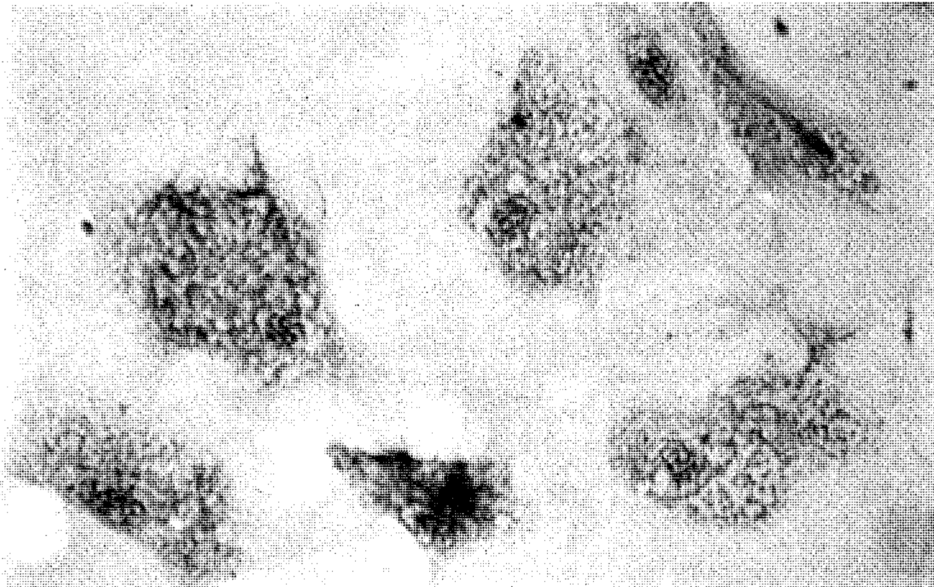

**Figure 1.** Hemocytological preparation of normal blood cells from *Mytilus*.

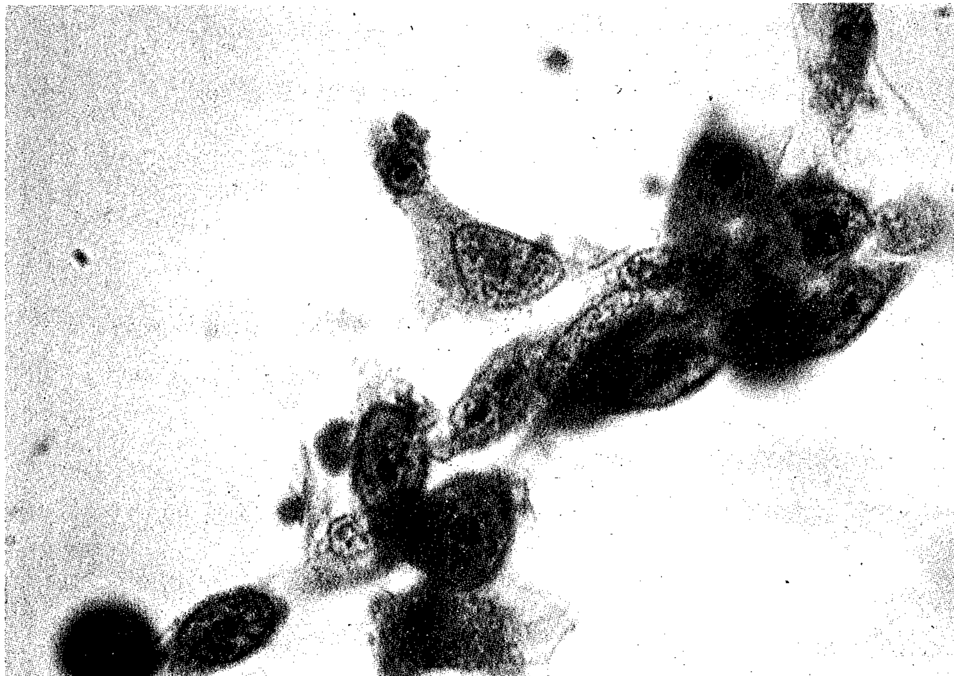

**Figure 2.** Hemocytological preparation of affected blood cells of *Mytilus* with hemic neoplasia. These cells are characterized by enlarged pleomorphic nuclei with prominent nucleoli, but the cells are still attached and well spread out on the glass

*Macoma nasuta*: Yaquina Bay, Oregon.

*Macoma irus*: Yaquina Bay, Oregon.

*Mya arenaria* (soft shell clam): Atlantic coast of North America, discontinuously from Chesapeake Bay to Nova Scotia.

*Mya truncata*: Baffin Island, Canada.

*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.

*Ostrea chilensis* (Chilean oyster): Chiloe, Chile.

*Ostrea lurida* (*Ostreola conchaphila*),(Olympia oyster): Yaquina Bay, Oregon.

*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.

## 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.

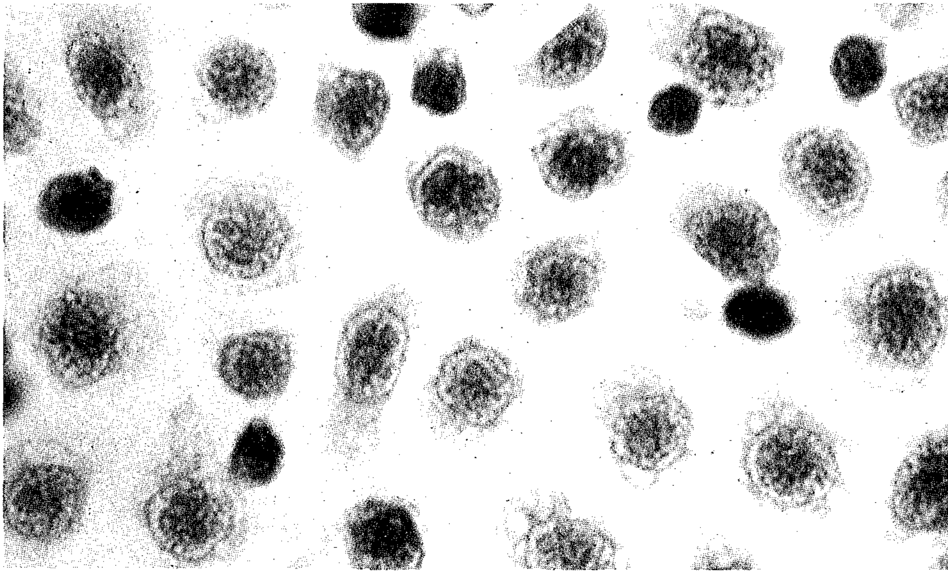

**Figure 3.** Hemocytological preparation of affected blood cells of *Mytilus* with hemic neoplasia. This mussel was in the terminal stages of the disease. The blood contains predominantly neoplastic blood cells that poorly attach or spread on the glass substrate. Blood cells exhibit a high nucleus to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

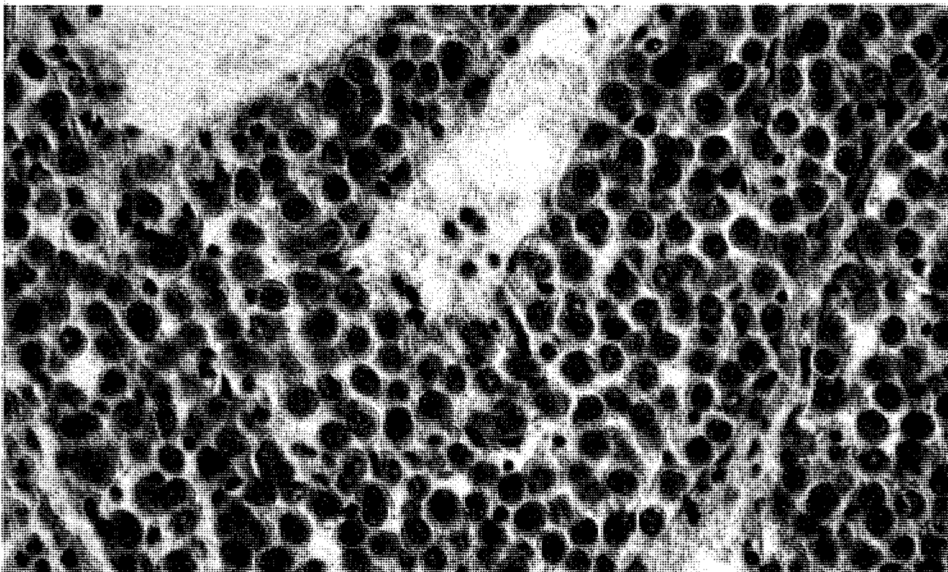

**Figure 4.** Histological section of *Mytilus* with hemic neoplasia showing the massive proliferation of neoplastic cells in the vascular spaces, 400X.

## E. Disease Diagnostic Procedures

### 1. Presumptive Diagnosis

Presence of large (typically 6-8 µm, but often up to 10% 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 (Figures 2 and 3) in hemocytological preparations with large (6% and larger compared with normal fixed nuclear diameters of 3 to 5% ), 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 (Figures 4 and 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.

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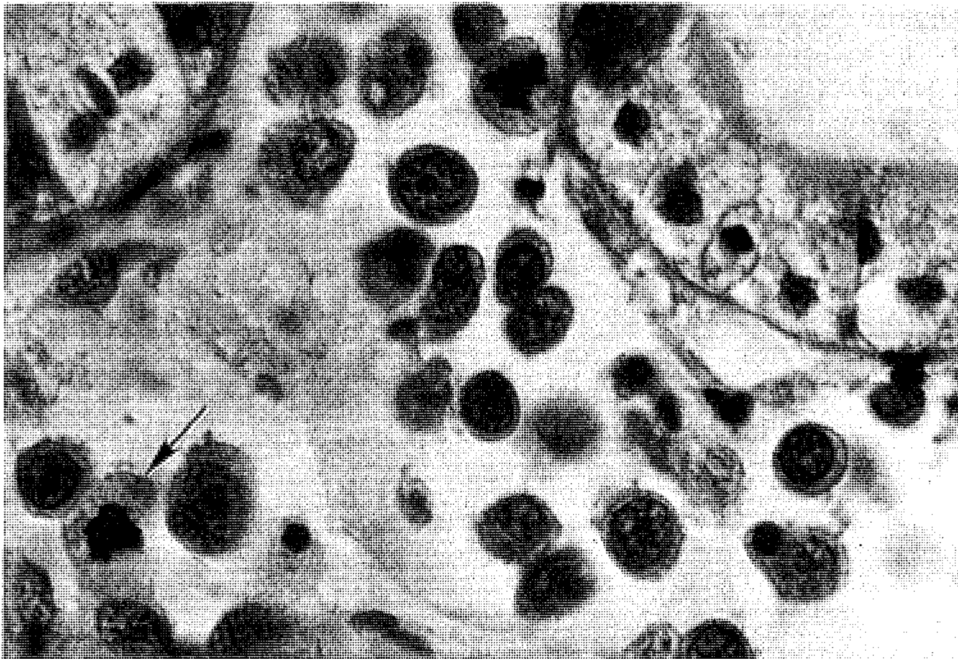

**Figure 5.** Histological section of *Mytilus* with hemic neoplasia showing typical enlarged hemocytes with large nuclei and scant cytoplasm. Two such cells in mitosis are shown at the arrows, 1200X.

# XII. Miscellaneous Diseases of Molluscs

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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 (Figures 1 and 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*

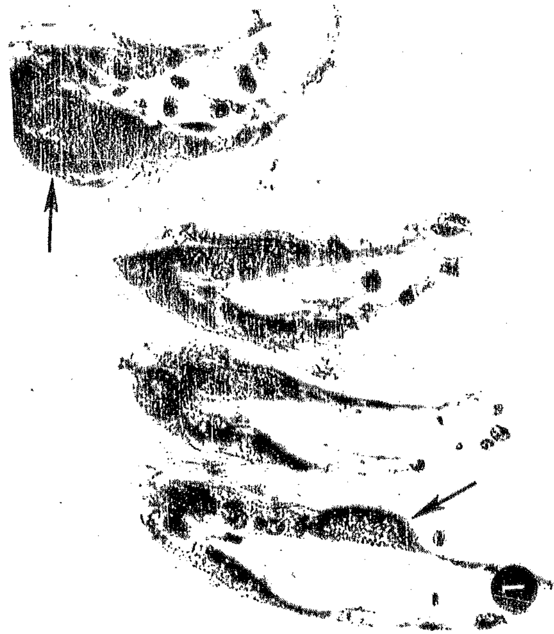

**Figure 1.** Rickettsia-like organisms in the branchial epithelium of the Japanese scallop (arrows) *Patinopecten yessoensis*, 580X. From Elston 1986, with permission.

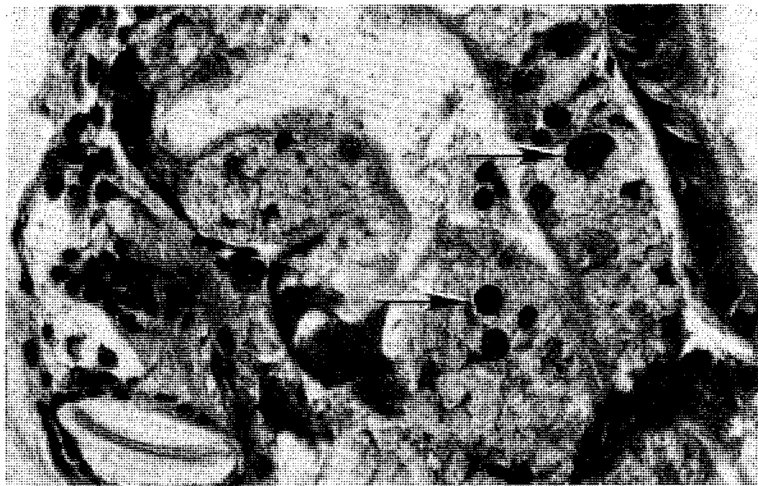

**Figure 2.** Rickettsia-like organisms in the digestive tubular epithelium of the Pacific razor clam *Siliqua patula* (examples at arrows), 880X. From Elston and Peacock 1984, with permission.

*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.

## 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 1930's. 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-

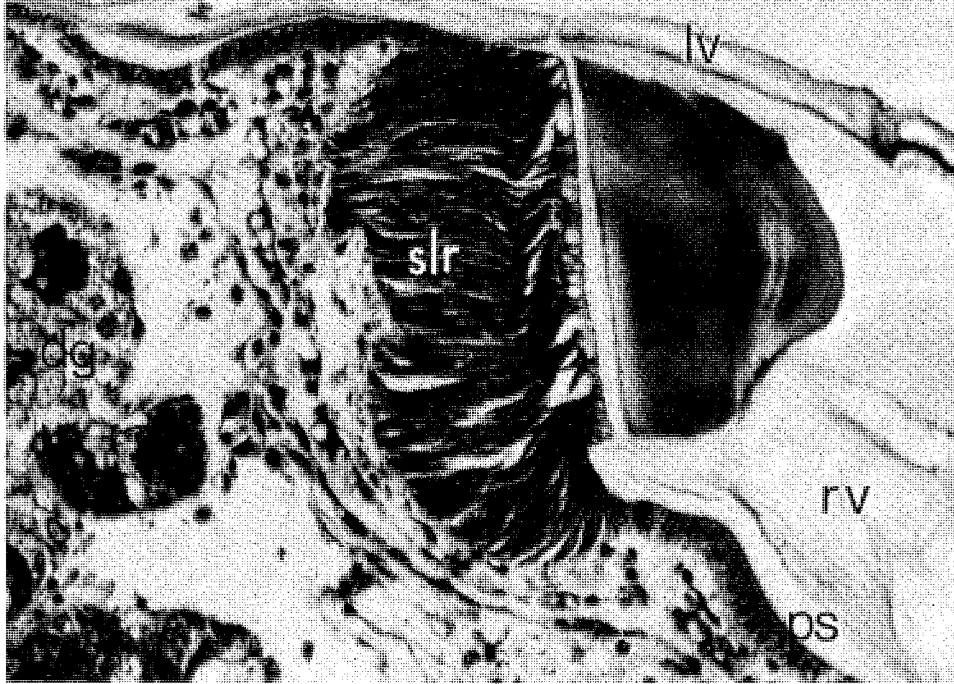


**Figure 3.** Normal hinge ligament of oyster showing the intact resilium (lr) and other adjacent organs (dg, digestive gland; slr, sub-ligamental ridge; lv, rv, left and right valves; ps, pallial space), 160X From Dungan and Elston 1988, with permission.

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 (Figures 3 and 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 etiologic agents. The presumptive causative organism can be cultivated by careful excision of the ligament and dilution on sea-water cytophaga agar (see reference).

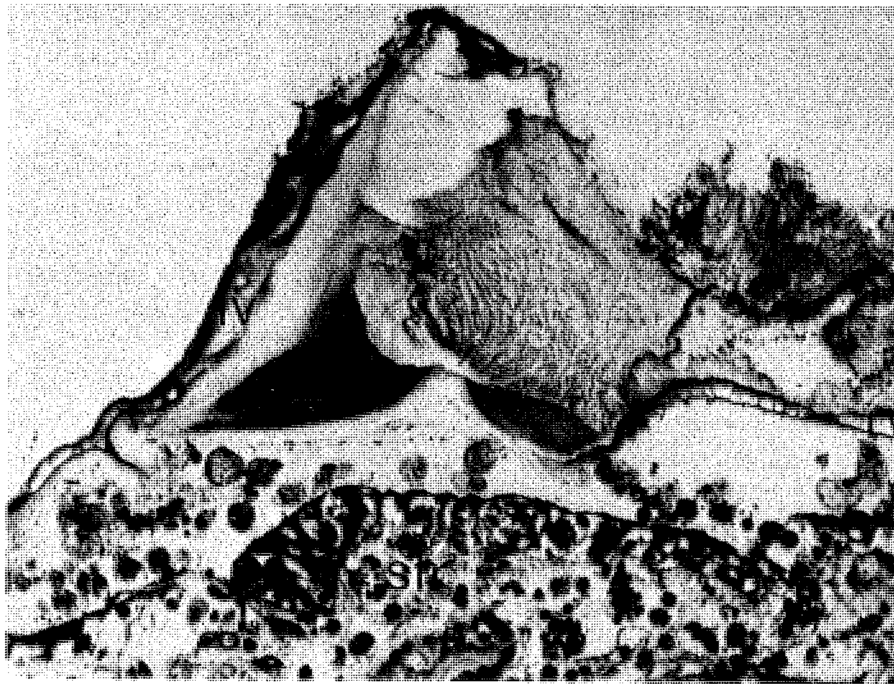

**Figure 4.** Hinge ligament eroded with cytophaga-like bacteria. The bacteria appear as fibers oriented at right angles to the eroding resilium margin (arrow). The sub-ligamental ridge appears necrotic and is separated from the internal resilium surface by a wide pallial space containing bacteria and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

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# I. *Lagenidium* of Decapod Crustaceans

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## 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

Virginia, Texas, Florida, Oregon, California, Mexico, Tahiti, and Honduras.

### 2. Host Species

Ova of the blue crab *Callinectes sapidus*, cultured shrimp, Dungeness crab, and American lobster *Homerus americanus.*

## C. Epizootiology

Although the complete life cycle for *Lagenidium* has not been worked out, it is most likely the motile zoospore stage which serves as the etiological agent. Mortality can reach 100%.

## D. Disease signs.

*Lagenidium* is an internal parasite of ova and juvenile marine crustacea held under artificial or experimental rearing systems. Septate, irregular hyphae (5-12 µm diameter) fill the egg or body of the young crab. Zoosporangia form and penetrate the host wall serving as discharge tubes. The fungus is invasive and causes massive tissue destruction. The only tissue reaction reported is melanized areas associated with the hyphae.

## E. Disease Diagnostic Procedures

### 1. Presumptive diagnosis

Microscopic observation of stout hyphae inside egg or discharge tubes with vesicles, which protrude the surface of eggs or the gills of juvenile crabs. In sterile sea water,

observe the release of biflagellate zoospores from vesicles.

### 2. Confirmatory Diagnosis

Remove infected ova or gill tissue and place in Cantino's PYG agar or broth prepared in sea water (30 ppt salinity). Hyphae are branched, septate, irregular, and have a diameter of 5-12 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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# V. Salmonid Ceratomyxosis

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## A. Name of Disease and Etiological Agent

Salmonid ceratomyxosis is caused by *Ceratomyxa shasta* (Myxozoa:Myxosporea)

## B. Known Geographic Range and Host Species of the Disease

### 1. Geographical Range

The distribution of the infective stage of the parasite is restricted to the Pacific northwest of the United States and Canada. In the United States: Columbia, Cowlitz, Lewis (east fork) and Washougal rivers; LaCamas Lake; Snake River from its confluence with the Columbia River to about 440 miles upstream; Deschutes River including Davis, Odell, Crescent and Suttle lakes; Willamette River from the mouth to about 100 miles upstream; Nehalem, Rogue, and Klamath rivers; Klamath Lake; Sacramento, Mokelumne, Feather and Pitt River systems. In Canada: Fraser River.

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 range.

### 2. Host Species

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.*

Resistance to *Ceratomyxa shasta* infection varies between species and may also vary between strains of a species. 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.

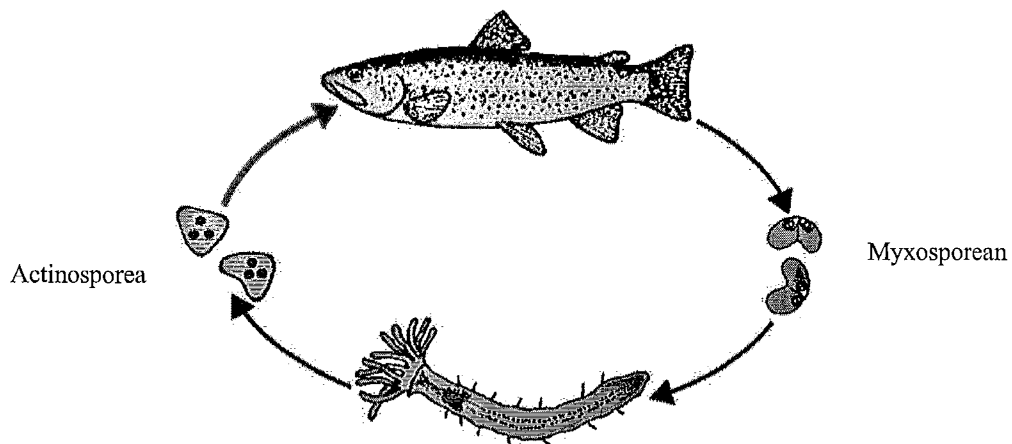


**Figure 1.** Life cycle of *Ceratomyxa shasta* showing salmonid and polychaete host and alternating myxosporean and actinosporean life stages.

## D. Disease Signs

Clinical signs of ceratomyxosis vary among fish species. In most cases, at least some of the following will be seen: anorexia, lethargy, marked darkening (especially in rainbow trout/steelhead), distended abdomen, exopthalmia, a swollen and hemorrhagic vent, and emaciation. In juvenile salmonids, the digestive tract may be grossly swollen, necrotic and hemorrhagic with mucoid contents. The intestine and pyloric caeca may be lined with caseous material.

Additional characteristics may include ascites, kidney lesions (fluid filled blebs/pustules to firm creamy white nodules) and hemorrhaging and (or) necrosis of liver, gall bladder, spleen, gonads, kidney, heart, gills, and skeletal musculature.

In adult salmonids the walls of the intestine and pyloric caeca may be thickened and hemorrhagic. Nodular lesions may develop in the intestinal wall perforating the intestine in chinook salmon.

Gross lesions (which may abscess) can occur in liver, kidney, spleen, or musculature. Abscesses of the body musculature are particularly common in coho salmon.

Depending on a number of factors (e.g. host species), a variety of organs may be affected. However, *Ceratomyxa shasta* has a predilection for the digestive tract (especially posterior intestine and pyloric caeca) and, secondarily, the kidney.

Development of *Ceratomyxa shasta* infections in the posterior intestine typically triggers acute inflammation involving polymorphonuclear leukocytes (PMN's), 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, PMN's, fibroblasts, trophozoites, pansporoblasts, and spores in later stages.

Pathological changes are less pronounced in the pyloric caeca. Trophozoites are often abundant between epithelial cells and in the muscularis externa. There may be separation of muscle layers due to the large number of trophozoites, but muscle necrosis is normally not severe.

The kidney is more severely affected in salmon than in trout. In chum salmon, kidney necrosis is severe, affecting both renal and hematopoietic elements. All normal tissue may be destroyed and replaced by developing parasites. Focal lesions are also common in the liver.

## E. Disease Diagnostic Procedures

### 1. Presumptive Diagnosis

Wet mounts can be prepared form the wall of the posterior intestine or of ascites if present. Material obtained via intestinal lavage is acceptable (Coley et al. 1983). Lesions present in any tissue should also be examined. Wet mounts should be scanned in a systematic manner under phase contrast or brightfield microscopy at 250-400X 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 containing a dark-staining karyosome surrounded by a clear halo.

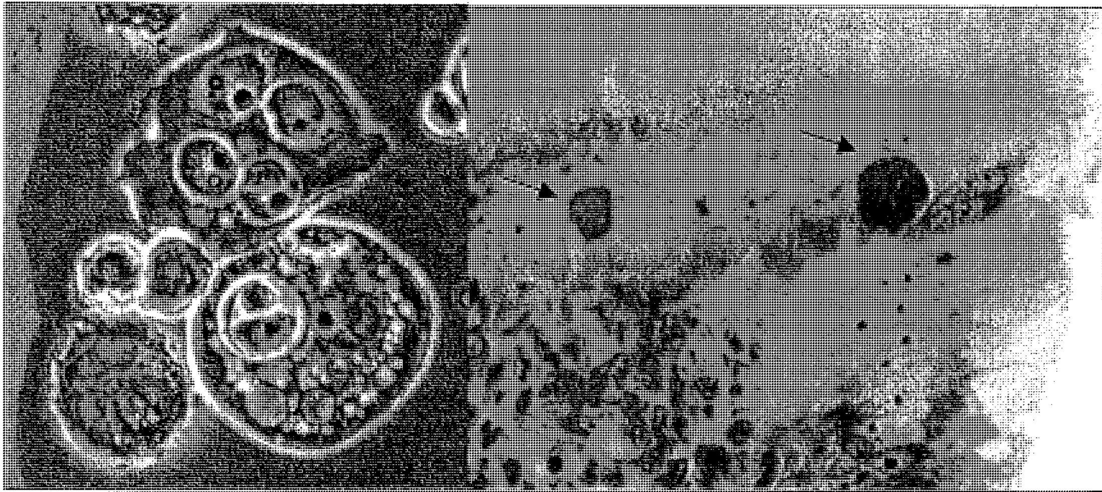

**Figure 2.** *Ceratomyxa shasta* trophozoites.

**Figure 3.** Histological section of intestine from an infected rainbow trout, arrows indicate location of trophozoites

## 2. Confirmatory Diagnosis

Confirmatory diagnosis of ceratomyxosis is based on detecting the characteristic kidney bean-shaped mature spores of *Ceratomyxa shasta* in wet mounts or histological sections. Spores observed in wet mounts are about 14-23 µm long by 6-8 µm wide at the suture line (Figure 4). 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.

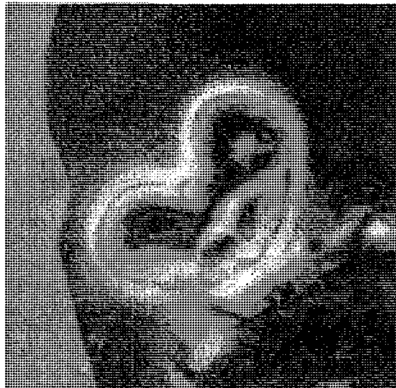

**Figure 4.** Mature *Ceratomyxa shasta* spore

## 3. Other Diagnostic Methods

Serological identification of *Ceratomyxa shasta* trophozoites can be accomplished using monoclonal antibodies [Bartholomew et al. 1989b; Appendix A].

Molecular diagnosis of *Ceratomyxa shasta* can be accomplished using parasite-specific primers in a polymerase chain reaction assay [PCR; Palenzuela et al. 1999; Appendix B].

## 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 freshly 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.

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## Methods Appendix A

### Fluorescent Antibody Technique for *Ceratomyxa shasta* (Bartholomew et al. 1989b)

The fluorescent antibody technique can be used on wet mounts of intestinal scrapings which have been air dried and fixed in acetone-xylene (1:1V/V) on gelatin-coated slides (1 g gelatin/L hot distilled water, let cool, add 0.1 g chromic potassium sulfate). However, the procedure works best on histological sections mounted on slides coated for tissue adherence. Indirect fluorescent antibody staining of tissues prepared of wet mounts and of deparaffinized, hydrated sections is as follows:

1. Apply monoclonal antibody (source: J. Bartholomew, Dept. of Microbiology, Oregon State University) to the fixed tissue and incubate for 15 min at room temperature in a humid chamber.
2. Gently wash with phosphate buffered saline (PBS) then let slides soak in PBS for 10 min and rinse again.
3. Incubate with fluorescein-conjugated anti-mouse antibody for 15 min at room temperature in a humid chamber.
4. Wash with PBS as above.
5. Counterstain briefly with either Evan's blue (0.01% in PBS) or methyl green (1% in distilled water).
6. Rinse with PBS, blot dry and mount cover glass with buffered glycerol.

This procedure can be modified for bright light microscopy by substituting an alkaline phosphatase-conjugated second antibody for the fluorescein conjugate and localizing the enzyme with an insoluble substrate.

## Methods Appendix B

## Molecular Detection of *Ceratomyxa shasta* Using the Polymerase Chain Reaction Assay (Palenzuela et al. 1999)

### 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:

- 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.
- 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).
- Cut a small piece of the gut (25-100 mg, about 2 -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 500µl DNA extraction buffer (see next section for recipe). Conventional dissecting tools can be used, but only if they are completely decontaminated between samples.
- 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:

-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.

-First wipe off any pieces of tissue, blood or mucus from the tools, then immerse them in 10% bleach (Clorox) solution for ***at least*** one minute. Rinse before use with distilled water from a wash bottle. This concentration of bleach is corrosive for metals after long exposures, but this is not a problem if the exposure is limited to the sampling, and tools are rinsed well in distilled water before storage. We normally have two containers (per person) with bleach during sampling, each containing a set of dissecting tools. After use, the tool is wiped clean with a piece of paper and put back in the bleach solution. The next time we use a tool, we get if from the other set and so on.

## PROTOCOL FOR THE DIAGNOSIS OF *Ceratomyxa shasta* IN FISH INTESTINES BY PCR

### 1. DNA EXTRACTION

Reagents:

1. 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.

2. Protëinase K.

Obtain comerciallyas 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

3. RNAse A.

A 10 mg $ml^{-1}$ solution can be purchased as a 50% glycerol solution that is liquid at -20 °C.

Methods:

Special care must be taken during DNA, as carry-over of DNA from other samples occurs easily. Use new blades and/or tools to take and handle the tissues. Samples can be fresh or frozen. There is no need to clean the tissues from fat or adherent tissue.

a. Dissect intestine on a clean surface as described above. Cut a small piece (25-75 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.

b. Add 5 µl of proteinase K to a final concentration of 200 $\mu g.ml^{-1}$ (from a liquid stock at 20 mg ml). Incubate at 37° 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).

c. 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.

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d. 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:

1. 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 |

2. PCR Master mix

*Taq polymerase and template (sample DNA) are not included in the master mix, but the concentrations of the reagents are calculated for 20 µl reactions with 1 µl of sample and 0.2 µl of Taq polymerase per reaction:

For **<u>10 ml</u>** of Cs 1-3 master mix:

| | |
|---|---|
| Sterile water | 7.5 ml |
| Taq 10x PCR Buffer (Promega) | 1 ml |
| $MgCl_2$ 25 mM (Promega) | 800 µl |
| dNTPs (at 100mM; Promega) | 80 µl (20 µl each A,T,G,C) |
| Primer F (CS1 at 500 µM) | 10 µl |
| Primer R (CS3 at 500 µM | 10 µl |
| **(*) Taq Polymerase** | **[100 µl]*** |
| **(*) Sample** | **[500 µl]*** |

Make the mix on ice and under nuclease-free conditions. Aliquot and store at -20° 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 1-3 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.