— *Fish Health Blue Book* —

PROCEDURES FOR THE DETECTION AND IDENTIFICATION OF CERTAIN FISH PATHOGENS

THIRD EDITION

Fish Health Section
American Fisheries Society

FISH HEALTH SECTION

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PROCEDURES FOR THE DETECTION AND IDENTIFICATION OF CERTAIN FISH PATHOGENS

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INTRODUCTION

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Standardized methodology for the inspection of fish for certain fish pathogens is an established need of the workers in the fish health community. The Fish Health Section, of the American Fisheries Society, recognized the need for a published protocol. Specialists from throughout the world met in Denver, Colorado in August, 1974 to discuss standardized methods for detection and
97 diagnosis of selected pathogens. The result of this meeting was the publication
98 in 1975 of Suggested Procedures for the Detection and Identification of Certain
98 Infectious Diseases of Fish. This manual soon became known as the "Blue Book".
99
101 As our understanding of pathogens and technology advanced, fish health specialists recognized the need to update the "Blue Book". Under the editorial pen of Dave McDaniel and many contributors, the second edition of the Fish Health "Blue Book" was published in 1979.

102
104 A few years after the publication of the revised edition, the Technical
111 Procedures Committee of the Fish Health Section determined a need to establish a protocol for conducting stock inspections, especially for "certification"
113 purposes, that would be sensitive, practical, efficient, and applicable to large numbers of samples. These new techniques were to be directed toward the detection of low numbers of specific pathogens, as found in carrier fish, and not toward detection of such pathogens in clinically ill specimens.

This third edition of the "Blue Book" is the result of a slow and careful process of revising the second edition and adding new material as deemed appropriate. Many of the methods are similar to those described in the old book, whereas others have changed significantly - much as a result of research to determine if the accepted methodologies were the best methods.

One will notice, as this manual is read, that writing styles and formats may differ slightly between sections. The reason for these differences is that several different authors contributed sections to this edition of the "Blue Book". The editor has not attempted to standardize linguistic styles used by each individual author.

Many people have contributed to this manual. Some are recognized in the appendix. If you have questions about any particular section, refer to the author appendix and therein you will find a name to whom you can direct your correspondence. For those not listed, as well as those who are, the Technical Procedures Committee would like to thank you for your effort and asks for your continued help and evaluation of the "Blue Book". Our special thanks go to Dr. Leo Margolis and the staff at the National Fish Health Center, Kearneysville, West Virginia, for their critical review of this document.

Kevin H. Amos
Chairman
Technical Procedures Committee

SECTION 1

GENERAL SAMPLING PROCEDURES

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GENERAL SAMPLING PROCEDURES

The success of an inspector in determining the status of certain pathogens in a population of fish is governed by proper sampling and testing procedures. This section will address the recommended sampling methods, whereas subsequent sections will address specific testing and isolation techniques.

Before the sampling parameters can be determined, the purpose of the work must be determined. If the goal is to isolate an agent from clinically ill fish, one particular method may be used, whereas if a population of healthy fish is being inspected for a specific agent(s), as in a certification procedure, another method may be used. It should be recognized that a disease certification requires a proper inspection but that an inspection does not imply certification. Both methods for sampling (i.e., inspection versus diagnostic) are included in Section 1.

When sampling and testing a population for a specific agent, a statistically significant number of specimens must be collected, but in no way does failure to isolate a certain pathogen from the sample guarantee the absence of that agent in the specimen examined or in the stock. This is particularly true of free-ranging or feral stocks of fish from which it is difficult to collect a representative and random sample. Other pertinent information, such as the disease history of the facility, watershed, and stock as well as the date and location of the samples, must be included in the inspection report and are essential in a certification procedure.

The Technical Procedures Committee determined that the necropsy procedure and sampling procedures are mutually exclusive and should be in separate sections. In the appendix, one can find a suggested necropsy procedure.

A. Sampling for the General Inspection of Fish for Pathogens

1. Samples must be collected on a lot-by-lot basis with each lot being maintained separately. A lot is defined as a group of fish of the same species and age that originated from the same discrete spawning population and that always have shared a common water supply. In the case of adult broodstock, various age groups may comprise the same lot, provided they meet the conditions above and have shared the same container(s) for one brood cycle.

2. In any given sample, or sub-sample, one should first select moribund fish and then make up the remainder of the sample with randomly selected live fish from all containers which represent the lot being examined. In the event the sample is being taken from adult broodstock of different ages which are sharing the same container, one should select for the older fish. Moribund fish are counted as part of the sample but should be processed separately from the randomly selected fish.

3. The minimum sample size for each lot must be in accordance with a plan that provides 95% confidence that infected specimens will be included in the fish sampled, assuming a minimum prevalence of infection equal to or greater than 2%, 5% or 10%, respectively. The minimum sample size for lots varying from 50 to infinity in size, for each inspection, is given in Table 1.

Table 1. Sample Size Based on Assumed Pathogen Prevalence in Lot.

Lot Size	At 2% Prevalence Size of Sample	At 5% Prevalence Size of Sample	At 10% Prevalence Size of Sample
50	50	35	20
100	75	45	23
250	110	50	25
500	130	55	26
1,000	140	55	27
1,500	140	55	27
2,000	145	60	27
4,000	145	60	27
10,000	145	60	27
100,000 or more	150	60	30

4. Fish must be alive when collected and should be processed as soon as possible after collection (refer to the specific etiological agents for the maximum allowable time between collection and processing). If specimens are not maintained alive before processing, samples should be stored in sealed, aseptic containers and kept on ice but not frozen.

5. Refer to the appropriate section in the "Blue Book" for procedures to test for the specific etiological agent(s).

B. Sampling of Moribund or Clinically Diseased Fish

1. Select at least ten (10) specimens that exhibit clinical signs typical of the disease affecting the fish in each lot to be tested.

2. Fish should be alive when collected and should be processed as soon as possible after collection (refer to the specific etiological agent(s) for the maximum allowable time between collection and processing). If specimens are not maintained alive before processing, samples should be stored in sealed, aseptic containers and kept on ice but not frozen.

3. Refer to the appropriate section of the "Blue Book" for procedures to test for the specific etiological agent(s).

SECTION 2

METHODS FOR THE DETECTION OF CERTAIN VIRAL AGENTS

Section 2

METHODS FOR THE DETECTION OF CERTAIN VIRAL AGENTS

I. General Procedures for Cell Culture

A. Quality Control

1. Susceptible, normal appearing, and rapidly dividing cells must be used for all virus assays.

2. Penicillin (100 IU/ml) - streptomycin (100 ug/ml) or gentamicin (100 ug/ml) and antifungal agents (Mycostatin R, 25 ug/ml; Fungizone R, 25 ug/ml) are permitted for media in routine cell culture work.

B. Virus Assay Controls

1. The positive controls must demonstrate that the cells are sensitive to all fish viruses being assayed.* Sensitivity is defined as the ability of the cells to show typical cytopathic effects (CPE) when exposed to a preparation of suspension or culture of stock virus at a level of 10^2 $TCID_{50}$/ml or equivalent. The positive control test must be conducted in a plate or vessel separate from the test assay.

2. Uninoculated controls must be incubated with each virus assay. These controls must remain free from CPE throughout the incubation period.

C. Sampling and Handling of Samples

1. To detect viral agents during an epizootic, a minimum of 10 fish in at least two pools (not to exceed five fish each) must be examined. The fish used should have recently died or be moribund and exhibit clinical signs typical of the disease.

2. Sampling asymptomatic fish must be done according to the Table of Attribute Sampling Based Upon a 95% Level of Confidence (see Table 1 in Section 1, General Sampling Procedures). The assumed minimum carrier prevalence shall be 5% or less. For the detection of various fish viruses, use the appropriate fish tissue listed in Table 2.

Table 2. Sample Material to be Used in Viral Tests of Asymptomatic Fish.

Virus	Sample Types
Infectious Hematopoietic Necrosis	Ovarian fluid, kidney - spleen
Infectious Pancreatic Necrosis	Kidney - spleen
Viral Hemorrhagic Septicemia	Kidney - spleen
Herpesvirus salmonis	Kidney - spleen
Channel Catfish Virus Disease	Kidney - spleen

*I.B.1 The Technical Procedures Committee recommends the establishment of a central laboratory, available to all fish virologists, which would test the sensitivity of cell lines to VHS and other viruses as required. The Committee also recommends a central supplier of cell lines (such as ATCC) so that all fish virologists would be using the same, standardized cell lines.

3. Tissue examinations shall be according to the following plan:

   (a) Fry and sac fry: Assay entire fry. If present, remove the yolk sac.

   (b) Fish 4-6 cm: Assay entire viscera including kidney.

   (c) Fish over 6 cm: Assay spleen and kidney.

   (d) Brood Fish: Ovarian fluid and a kidney-spleen sample.

4. No more than five fish shall be combined to form a pool.

5. Ovarian fluid samples shall be collected from each female prior to pooling of eggs and fluid to assure an approximately equal volume of fluid is contributed by each fish.

6. Samples shall be inoculated onto cells within 72 hours after collection and must be stored or transported refrigerated or on ice, but never frozen. For best results, samples should be inoculated onto cells within 48 hours after collection.

7. At the discretion of the pathologist, antibiotics and/or anti-fungal agents in a buffered tissue culture medium (pH 7.0-7.8) may be used on tissue samples (but not on ovarian fluid) to control the growth of microbial contaminants during long transportation periods. The concentration of the antibiotics or antifungals is not to exceed that used in processing (see C.9. below).

8. Samples must be kept below 15°C during processing and homogenization. Homogenization by sonication is not acceptable. Equipment used for homogenization within any given lot need not be sterilized between samples, unless the proportion of positive pools in the lot is being recorded. All equipment must be sterilized, preferably by autoclaving, between lots.

9. The samples should be suspended in a buffered tissue culture medium or base (pH 7.0 to 7.8) and centrifuged at 2000 x g (RCF) for ten minutes and may be treated to retard microbial contaminants by the following methods:

   (a) Combine supernatant from samples with an antibiotic - tissue culture solution mixture to obtain a final concentration of 100-2000 ug/ml gentamicin or 800 IU/ml of penicillin and 800 ug/ml streptomycin. Mycostatin ® or Fungizone ® may be added to obtain a final concentration of 400 IU/ml, respectively. Incubate the mixture for two hours at 15°C or overnight at 4°C.

   (b) As an alternative, filtration of supernatant through a .45 um membrane filter may be used. Absorption of certain viruses to membrane filters may occur and should be considered.

10. The dilution prior to inoculation of the tissue sample onto cell cultures must not exceed 1:100 (v/v). Ovarian fluid sample dilutions should not exceed 1:5 (v/v) and must not exceed 1:20 (v/v).

11. A minimum volume of each sample shall be inoculated onto appropriate cell cultures (see Table 3). A minimum of one cell culture unit (except microtiter, 96 well plate where four cell culture units will be used) for each cell line shall be inoculated from each sample pool. When using a microtiter system, the sample should be put in the wells first followed by the cells. When inoculating a monolayer, one should first aspirate off the growth media before adding the test sample. An incubation period of one hour should then be allowed prior to the addition of fresh growth medium.

Table 3. Suggested inocula for various culture vessels.

Assay Vessel	Minimum Sample Volume (ml)	Cell Culture Concentration
96 well plate	0.05 ml/well	Number sufficient to form monolayer within 24 hours, 80-90% for pre-formed
24 well plate	0.25 ml/well	80-90% confluent monolayer
12 well plate	0.5 ml/well	80-90% confluent monolayer
35 mm dish	0.10 ml/dish	80-90% confluent monolayer
60 mm dish	0.30 ml/dish	80-90% confluent monolayer

12. Inoculated cell cultures must be incubated for a minimum of 14 days, though incubation for 21 days is strongly recommended. At the discretion of the pathologist, blind passages may be desirable. The incubation temperature will be dictated by the virus under consideration:

    (a) IHN, IPN, or VHS viruses: 15°C

    (b) CCV: 17°-30°C

    (c) Herpesvirus salmonis: 10°-15°C

    During the incubation period, the pH of the cell culture must be maintained between 7.0 and 7.8, except for VHS virus assay where pH tolerances are 7.4 to 7.8.

Table 4. Sensitivity of Cell Culture Systems to Different Viruses (the cell lines listed below are the only test systems acceptable in testing for the indicated virus they are listed by).

Virus	Sensitivity of Cell Culture Systems (from most sensitive to least)
IPN	CHSE-214---BF-2---RTG-2
IHN	EPC---FHM---CHSE-214
VHS	EPC---FHM---CHSE-214---RTG-2
Herpes Virus	RTG-2---CHSE-214
CCV	CCO---BB

BF-2 = Bluegill fry -2; CHSE-214 = Chinook salmon embryo 214; EPC = Epitheliom papillosum cyprini; FHM - Fathead minnow; RTG-2 = Rainbow Trout Gonad-2; BB = Brown bullhead; CCO = Channel catfish ovary.

13. The cause of CPE in test systems requires identification by serum neutralization or further evaluation by subculturing. Levels of dilution for subcultures from original test systems shall not be less than 1:10 v/v or greater than 1:1000 v/v. Subcultures should be conducted as soon as possible after appearance of CPE in test systems but not more than seven (7) days after initial appearance.

D. Identification of Virus by Serum Neutralization

1. Select cell cultures showing typical virus CPE and dilute fluid from these cultures to 1:1000 (10^{-3}) and 1:100,000 (10^{-5}).

2. Dilute control serum (contains no antibody against fish viruses) and specific antiserum (contains antibody against a certain fish virus) as appropriate. Generally, the supplier of the serum will provide dilution information, but if this information is not available, dilute the serum with buffered saline (pH 7.0-7.8).

3. Mix equal portions of diluted cell culture fluid from Step 1 and diluted antiserum from Step 2.

4. Mix equal portions of diluted cell culture fluid from Step 1 and diluted control serum from Step 2.

5. Repeat Steps 3 and 4, except use known reference virus (suspension containing 500 $TCID_{50}$ per ml) instead of the diluted fluid from virus suspect cultures in Step 1.

6. Keep all mixtures at appropriate incubation temperature for 60 minutes, agitating each tube at 15-minute intervals (see C.12.).

7. Inoculate an appropriate amount (Table 2) of each mixture onto duplicate cell cultures. The same cell line used for primary isolation of the agent should be used for the neutralization test. Two cell cultures must be inoculated with antiserum diluted to be equivalent to the test sample-antiserum dilution. This serum control identifies serum toxicity. Two uninoculated cell cultures should be included as normal cell controls.

8. Incubate all cultures for 2 to 12 days, depending upon the viral agent, at the same temperature used for primary isolation.

9. Observe cell cultures daily. The appearance of CPE in the cultures inoculated with the control serum-virus mixture but not in the cultures inoculated with the antiserum-virus mixture identifies the agent as being related to the virus used for production of the antiserum. Because of serological variation known to occur with some viruses, CPE may appear in the cell cultures inoculated with the antiserum-virus mixtures, but this CPE is generally reduced and its appearance is delayed.

10. If the antiserum used does not neutralize or delay the infectivity, the test should be repeated using antiserum against another fish virus. If no single antiserum neutralizes the infectivity, a mixed virus infection, severe sample toxicity, or presence of a new agent should be suspected.

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II. Infectious Pancreatic Necrosis

A. Name of the Disease and Etiological Agent

Infectious pancreatic necrosis (IPN), infectious pancreatic necrosis virus (IPNV).

B. Known Geographic and Host Range of the Disease

1. Geographic Range

   North America, Europe, Japan, Korea, Taiwan, and Chile.

2. Host Range

   Brook trout (Salvelinus fontinalis), brown trout (Salmo trutta), rainbow trout (Salmo gairdneri), cutthroat trout (Salmo clarki), lake trout (Salvelinus namaycush), Atlantic salmon (Salmo salar), coho salmon (Oncorhynchus kisutch), chinook salmon (Oncorhynchus tschawytscha), Amago trout (Oncorhynchus rhodurus), and Arctic char (Salvelinis alpinus). Birnaviruses, a new taxonomic group that includes IPNV and IPNV-like viruses, have been isolated from numerous non-salmonid hosts including: European and Japanese eels, pike, striped bass, cod, white suckers, menhaden, tilapia, marine molluscs, and some crustaceans.

C. Clinical Signs of Diagnostic Significance

1. Infectious pancreatic necrosis is an acute disease causing mortality of fry and fingerlings, and occasionally of yearling trout and salmon. The largest and healthiest appearing fry or fingerlings usually are affected first. Twirling may occur when the mortality rate is high; swimming victims rotate around their longitudinal axis. When not otherwise obvious, alarming the fish by a sharp rap on the trough or other scare will often elicit the twirling response. Agonal behavior may alternate with quiescence during which victims lie on the trough bottom and respire weakly. Twirling is a terminal sign and death usually occurs within an hour or two.

2. Signs include overall darkening, exophthalmia, abdominal distension, and at times, hemorrhages in ventral areas including bases of fins. Internally, multiple petechiae occur in the pyloric caecal area, and the liver and spleen are pale. The digestive tract is almost universally without food; accordingly, the stomach appears whitish. Clear to milky mucus occurs in the stomach and anterior intestine.

D. Diagnostic Procedures for Disease Situations

1. Presumptive Diagnosis

   (a) The presence of typical CPE in cell culture.

   (b) A histological examination revealing pronounced pancreatic necrosis with both acinar and islet tissues affected with adjacent adipose tissue necrotic. Cytoplasmic inclusions in pancreatic cells near the edges of affected tissues.

   (c) The presence of clinical signs as described in Section II.C.

(d) A history of the hatchery or natural environment indicates a likelihood of IPNV infection.

2. Confirmatory Diagnosis

(a) Positive confirmation requires isolation and identification of IPNV by serum neutralization. The Birnaviruses from salmonid fish hosts appear to fall into three distinct serological groups (VR299, Ab and Sp) and serotype specific antiserum is required for confirmatory serum neutralization. Because there is evidence for divergent serotypes in non-salmonid hosts, more extensive serological tests may be required to identify isolates from those species. Histopathological observations may assist in diagnosing the disease. Isolation and identification of the virus as above, accompanied by the demonstration of a high virus titer in the fish tissues (titer over 10^5 $TCID_{50}$/gm). A minimum of 10 fish in two pools is satisfactory for diagnosis provided that the sample is composed of fish showing clinical signs.

E. Procedures for Detecting Asymptomatic Infections

1. Follow the guideline in the general procedures section for sampling hatcheries according to lot. Kidney and spleen, whole fry, or visceral samples must be used for all assays except when sufficient numbers of fish cannot be killed to meet a 2% or 5% prevalence. In these cases, a three-year sampling history of the progeny at the 2% or 5% prevalence level and holding of brood stock in isolation (no opportunity for contamination or infection from other fish) are required. If these conditions are met, tissue sampling of the brood at the 10% prevalence level is acceptable. Sex fluids or fecal samples are not satisfactory alternatives to tissue sampling for detection of asymptomatic IPNV carriers.

2. The procedures above are considered minimally acceptable and thus, no other procedures are described as less acceptable.

F. Procedures for Determining Prior Exposure to the Etiological Agent

A serological procedure for determining prior exposure to IPNV is available, but since all known fish disease inspection programs at present require isolation and identification of the virus, no other procedure is described.

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

As described in the section on general procedures for cell culture virology.

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III. VIRAL HEMORRHAGIC SEPTICEMIA

A. Name of the Disease and Etiological Agent

Viral hemorrhagic septicemia (VHS) is a systemic infection, primarily of trout, caused by a rhabdovirus known as viral hemorrhagic septicemia virus (VHSV). The disease has also been known as Egtved disease and infectious kidney swelling and liver degeneration (INuL) disease, and the agent is Egtved virus.

B. Known Geographic and Host Range of the Disease

1. Geographic Range

   Most countries of continental Europe. To date (1985), outbreaks have not been reported elsewhere.

2. Host Range

   Rainbow trout (Salmo gairdneri) and brown trout (Salmo trutta), and to a lesser extent in northern pike (Esox lucius). Atlantic salmon (Salmo salar), brook trout (Salvelinus fontinalis), golden trout (Salmo aguabonita), rainbow trout X coho salmon (Oncorhynchus kisutch) hybrids, giebel (Carassius auratus gibelio), and goldfish (Carassius auratus) are susceptible by experimental challenge.

C. Clinical and Histopathologic Signs of Diagnostic Significance

1. A variety of clinical signs and histopathologic changes can be apparent in infected fish. Some fish can show profound clinical manifestations, whereas others appear to be nearly normal. The absence of clinical signs does not indicate that the fish are free of virus. Neither clinical signs nor histopathologic changes can be relied upon to distinguish VHS from other salmonid virus diseases.

2. Historically, the clinical manifestations of VHS have been categorized into acute, chronic, and nervous forms or stages. Such descriptions should be considered to represent degrees of serverity rather than progressive forms or stages of the disease.

3. Outbreaks of VHS can occur in all ages of fish and survivors of infection can become lifelong virus carriers. Overt disease can recur as a consequence of stress. External clinical signs can include exophthalmia, abdominal distension, darkened pigmentation, anemia, lethargy, hyperactivity, and hemorrhages in eyes, skin and gills, and at the base of fins. Internally, visceral mesenteries can show diffuse hemorrhages, the kidneys and liver can be hyperemic, swollen and discolored, and hemorrhages can occur in skeletal muscle. Histopathologic changes can occur in the liver, kidneys, spleen, and skeletal muscle. The hematopoietic areas of the kidneys and spleen are the initial foci of infection.

D. Diagnostic Procedures for Disease Situations

See methodology for virological examination in item F-1 to F-6 and Section 2.1 - General Procedures for cell culture.

E. Diagnostic Procedures for Detecting Asymptomatic Infections

See methodology for virological examination is item F-1 to F-6 and Section 2.1 - General Procedures for cell culture.

F. Diagnostic Procedures for Determining Prior Exposure to the Etiological Agent

1. United States regulations (Code of Federal Regulations, Title 50, Section 13.7, effective July 1, 1968) require that salmonids and salmonid eggs imported into the United States be certified free of VHS virus. The procedures outlined below conform to those described in the upcoming revision of Fish Disease Leaflet 9 entitled Approved Procedure for Determining Absence of Viral Hemorrhagic Septicemia and Whirling Disease in Certain Fish and Fish Products.

2. Because clinical signs and histopathologic changes associated with VHS are variable and cannot be used for presumptive or definitive diagnosis or certification, virological examination is required for certification that salmonids are free of VHS virus. Virological examination shall consist of the assay of prescribed samples in cell culture and, if virus is isolated, identification of the virus or viruses by serum neutralization.

3. Virological Examination: Sample Collection, Storage and Processing

See Section 2.1 - General Procedures for Cell Culture except for the following information which is specific for VHS:

(a) If fish are to be exported to the United States, the assay must be performed during the six weeks preceding importation.

(b) The size of sample assumes a minimum incidence of infection of 2% at a 95% confidence level (see Table 1).

(c) All handling and/or processing of specimens will be in a buffered tissue culture medium maintained at a pH of 7.4 to 7.8.

4. Virological Examination: Virus Assay in Cell Culture

Virus assay must be performed by using monolayer cell cultures of chinook salmon embryo (CHSE-214), epithelioma papillosum cyprini (EPC), fathead minnow (FHM), or rainbow trout gonad (RTG-2). The cell cultures should be 80%-90% confluent and no more than 72 hours old after being subcultured. Cultures should be inoculated at the rate of 0.1 mL of diluted sample per 10 cm^2 of cell sheet. At least 10 cm^2 must be inoculated for each sample. The culture medium must be removed from the monolayers before the sample is applied. The inoculated cultures are incubated at 15°C for 60 minutes to permit virus absorption. Overlay medium containing prescribed levels of antibiotics (see Section 2.1.C.) is then added. Either plaque or quantal assay can be performed. Cultures are incubated at 15°C and observed for 14 days or until cytopathologic change is evident. During the 14 day incubation, the pH of the cell culture medium must remain within the range of 7.4 to 7.8 or the assay must be repeated.

If no cytopathologic change is observed in the inoculated cultures, the sample must be blind-passaged once using dilutions prescribed in Section 2.1.C. If cytopathology is observed but is not of viral origin, the assay must be repeated. If cytopathologic change is observed and found to be of viral origin, the causative virus or viruses must be identified by serum neutralization assay.

5. Virological Examination: Serological Identification of VHS Virus

   (a) Since three serological types of VHS virus can be distinguished, antiserum to each serotype must be included in the serum neutralization assay.

   (b) The methodology for the serum neutralization assay is detailed in Section 2.1.D.; however, the cell cultures and conditions of incubation and pH control must be maintained as described in item 4 of this section.

6. Virological Examination: Cell Culture Susceptibility

   (a) To ensure that cells are susceptible to virus infection, a positive VHS virus control should be included in each population or lot assay and serum neutralization assay. The positive control cell cultures will be inoculated and incubated as indicated above; cells will be exposed to 50-500 $TCID_{50}$ or plaque forming units of VHS virus per 0.1 mL of inoculum. The positive control cell cultures must show cytopathologic change as evidence of susceptibility to VHS virus.

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IV. Infectious Hematopoietic Necrosis

A. Name of the Disease and Etiological Agent

Infectious hematopoietic necrosis (IHN), infectious hematopoietic necrosis virus (IHNV). Synonyms: the disease has also been referred to as Oregon sockeye disease or Sacramento River chinook disease. The viruses isolated from various host species are commonly referred to by the common name of the host species (i.e., chinook salmon, sockeye salmon, or rainbow trout strain).

B. Known Geographic and Host Range of the Disease

1. Geographic Range

Pacific Coast of North America from California to Alaska and Idaho. Epizootics have been reported from South Dakota, Minnesota, Idaho, West Virginia, Colorado, Montana, New York, Utah. The disease is widespread in Japan.

2. Host Range

Rainbow trout (Salmo gairdneri), including steelhead; chinook salmon (Oncorhynchus tshawytscha), sockeye salmon (O. nerka), cutthroat trout (S. clarki), and Atlantic Salmon (S. salar). Coho salmon (O. kisutch) have never been found to be naturally susceptible.

C. Clinical Signs of Diagnostic Significance

Typical external signs of the disease may include exopthalmia, anemia, hemorrhaging in the body musculature, lower jaw and at the base of fins, fecal casts may be present, abdominal swelling with ascites, and scoliosis or lordosis in survivors of epizootics. Internally, petechial hemorrhages in adipose tissues of the visceral cavity, body musculature, and mesenteries may be seen, the kidney and liver are edematous and pale, and subdermal hemorrhaging posterior to the cranium is common. Microscopically, there is extensive necrosis of the hematopoietic tissue of the spleen and anterior kidney. and focal necrosis in pancreatic and liver tissue is often observed. The above clinical signs are often associated with the disease but may not be observed in individual cases, and are totally absent in carrier fish.

D. Diagnostic Procedures for Disease Situations

1. Presumptive Diagnosis

(a) The isolation of the virus in cell culture with the development of typical cytopathology can be used for presumptive identification of the virus. Typical cytopathology includes nuclear chromatin margination and rounding of cells. This is best demonstrated by studying the plaque morphology.

(1) Cell Culture Methods

The epithelioma papillosum cyprini (EPC), chinook salmon embryo (CHSE-214), or fathead minnow (FHM) cell line must be used. Cell cultures must be incubated between 15° and 18°C for no less than 14 days. The pH

of the culture medium must be maintained between 7.0 and 7.8. The test must be repeated if the pH falls below 7.0 and no virus is isolated.

(2) One or two blind passages should be done when positive samples do not occur in a lot when the investigator feels there is a possibility that IHN virus is present.

(3) Viral Procedures. See section on General Procedures for Cell Culture. Alternatively, the plaque assay method of Burke and Mulcahy (1980) is acceptable and often provides more timely results than conventional cell culture in fluid medium. Ovarian, whole milt, and sperm-free seminal fluids should be absorbed raw, or diluted 1:2 (whole milt 1:10) in antibiotics [I.C.9.(a)], while a 1:20 dilution of kidney-spleen is recommended for absorption onto monolayered EPC cells. Cell cultures should be incubated at 15°-18°C for 10 days then examined microscopically for IHNV CPE. This is followed by fixing and staining of cell monolayers or subculture if virus is suspected. The following inoculum volume versus approximate cell culture vessel area should be used:

Inoculum Volume (ml)	Cell Culture Vessel Area (cm^2)
0.05 - 0.10	2.0
0.10 - 0.20	4.5
0.20 - 0.30	9.6 - 11
0.25 - 0.40	25

(b) Histopathological Changes

Typical necrosis of hematopoietic tissues in the kidneys and spleen, and necrosis of granular cells of the lamina propria can be used as a less acceptable method of presumptively identifying IHN disease. Selection and fixation of tissue for histological examination must be done according to accepted procedures.

(c) Clinical Signs

The presence of clinical signs and a history of IHNV can also be used for presumptive identification of IHN.

(d) Blood Smear Examination

Peripheral blood smears and kidney imprints taken from fish with frank IHN usually exhibit hematopathological changes. Sampling, fixing (in absolute methanol for at least 5 minutes), staining in Giemsa or Leishman-Giemsa for 10-15 minutes, and examination of slides may be done within 30 minutes. Pathological changes most frequently seen are necrobiotic bodies and monocytes (macrophages) with varying degrees of cytoplasmic vacuolation, giving a foamy appearance.

(e) Combination

Demonstration of all four of the above characteristics gives the strongest presumptive evidence for the presence of IHN diseases, but for positive identification, confirmatory procedures must be followed.

2. Confirmatory Diagnosis

(a) Isolation of virus with neutralization by specific IHN antiserum and associated with typical IHN histopathological changes positively identifies the disease as IHN. Serum neutralization identifies the virus; the presence of typical pathological changes must be present to diagnose the disease positively. In the absence of demonstrated histopathological changes, virus neutralization with specific antiserum and the presence of a high titer (10^5) infectious units or more) in the sample can be used to identify the disease positively. Procedures for virus neutralization are described in General Procedures for Cell Culture.

E. Procedures for Detecting Asymptomatic Infections

The only acceptable procedure for detecting IHN carriers is to test ovarian fluid for the presence of virus. A greater proportion of samples will be positive when samples are taken from post-spawning fish than from pre-spawning fish or samples taken during spawning. For detection, inoculated cell cultures must be observed for 14 days. It is strongly recommended that cell cultures be observed for 28 days or for 14 days, followed by a blind passage onto new cultures of the same cell line with incubation for a further 14 days. In all cases, fish should be sampled according to the section on General Procedures for Cell Culture. Cell culture and virological procedures are to be followed as described above under D.1.(a), and the identity of any isolated agent must be confirmed using the procedures described in Section D.2.(a), except no histopathological examination is needed.

F. Procedures for Determining Prior Exposure to the Etiological Agent

No serological tests are available at the present time.

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

These are described under General Procedures for Cell Culture.

H. References

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V. Channel Catfish Virus Disease

A. Name of the Disease and Etiological Agent

Channel catfish virus disease (CCVD), channel catfish virus (CCV).

B. Known Geographic and Host Range of the Disease

1. Geographic Range

Alabama, Arkansas, California, Colorado, Georgia, Iowa, Kansas, Kentucky, Mississippi, Nebraska, Oklahoma, Texas, West Virginia; and Honduras, Central America.

2. Host Range

Channel catfish (Ictalurus punctatus) is the primary host, and experimental infection of the blue catfish (I. furcatus) suggests that this species could be infected under natural conditions.

C. Clinical Signs of Diagnostic Significance

1. Epizootics are characterized by a variable rate of mortality in catfish that are less than six months old and less than 10 g in weight and when water temperatures exceed 25°C.

2. Infected fish swim erratically, sometimes rotating about the longitudinal axis and at times holding head up in the water.

3. Externally, diseased fish show abdominal distension, exophthalmia, pale or hemorrhagic gills, and petechiae at the base of fins and throughout the skin, particularly on the ventral surface.

4. Internally, the body-cavity is filled with a 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 states of disease. The gastrointestinal tract is filled with a mucoid secretion and it is void of food.

5. 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 renal 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.

D. Diagnostic Procedures for Disease Situations

1. Presumptive Diagnosis

Samples are processed as described in General Procedures for Cell Culture. 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. CCO cells are approximately 1 log (base

10) more sensitive than BB cells. Inoculated cultures are incubated for 14 days and observed for typical CPE. The presence of clinical signs during periods when water temperature exceeds 24°C can serve as a presumptive diagnosis.

2. Confirmatory Diagnosis

The virus must be isolated and its identity confirmed by serum neutralization or fluorescent antibody (FA) assay. The procedure for the serum neutralization assay is outlined in General Procedures for Cell Culture.

E. Procedures for Detecting Asymptomatic Infections

None are available.

F. Procedures for Determining Prior Exposure to the Etiological Agent

Positive results in the following tests do not mean that the fish are definitely carriers of CCV, nor do negative results mean that the fish are definitely free of CCV.

1. Serum samples from adult fish are heat inactivated at 45°C for 30 minutes. The serum is diluted 1:50 and reacted with known CCV at 100 $TCID_{50}$ or PFU/0.1 ml of serum/virus reactant mixture (tests conducted according to methods in General Procedures for Cell Culture). Sera demonstrating 50%+ plaque or $TCID_{50}$ reduction are considered "reactors" and can be considered to have come from fish that were exposed to CCV.

2. Brood fish sample size is determined by 2% prevalence level from the sample size table.

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

These are described under General Procedures for Cell Culture.

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VI. Viral Erythrocytic Necrosis

A. Name of the Disease and Etiological Agent

Viral erythrocytic necrosis (VEN), erythrocytic necrosis virus (ENV). This disease was originally designated as piscine erythrocytic necrosis (PEN).

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

B. Known Geographic and Host Range of the Disease

1. Geographic Range

Probably worldwide in the marine environment, but these viruses have been demonstrated in fish of the Atlantic and Pacific coasts of North America, the United Kingdom, Japan, Italy, and Chile. On the basis of light microscopy, VEN also occurs in Venezuela in one species of freshwater fish. Using electron microscopy, virus particles have been detected in the cytoplasm of erythrocytes of juvenile chum, coho and chinook salmon that have been reared exclusively in fresh water.

2. Host Range

The viruses have been demonstrated in the following anadromous and marine species: chum, pink, coho, chinook salmon (Oncorhynchus keta, O. gorbuscha, O. kisutch, and O. tshawytscha), steelhead trout (Salmo gairdneri), Atlantic and Pacific herring (Clupea harengus harengus and C. harengus pallasi), Atlantic cod (Gadus morhua), blenny (Blennius pholis), spot (Leiostomus xanthurus), and possibly the dogfish (Scyliorhinus canicula). Artificial infections have been accomplished in sockeye salmon (O. nerka), Atlantic salmon (S. salar) and in brown trout (S. trutta), rainbow trout (S. gairdneri), and brook trout (Salvelinus fontinalus). Demonstration of inclusions detected by light microscopy suggests that the following species may also suffer from VEN: freshwater electric eel (Electrophorus electricus), anadromous alewife (Alosa pseudoharengus), rainbow smelt (Osmerus mordax), marine Atlantic tomcod (Pollichthys oblongus), longhorn sculpin (Myxocephalus octodecemspoinosus), rock gunnel (Pholis gunnellus), sea raven (Hemitripterus americanus), seasnail (Liparis atlanticus), tautog (Tautoga onitis), and winter flounder (Pseudopleuronectes americanus).

C. Clinical Signs of Diagnostic Significance

The disease is usually characterized by the presence of a single, rounded, basophilic inclusion body, 1 to 4 mm in diameter, in the cytoplasm of affected erythrocytes. Also, there are reports of eosinophilic inclusion bodies and sometimes there is more than one inclusion per cell. The infection is best diagnosed by examining stained blood films. Severe infections, involving 90% to 100% of the erthrocytes, have been observed in feral fish, but the impact of the disease is unknown. The infection can be fatal in captive herring and in sea water-cultured chum and pink salmon, and high water temperatures are suspected of favoring the disease. Fatalities

follow massive destruction of the erythrocytes and the most striking gross sign in dying fish is a pallor due to anemia. In moribund chum and pink salmon, for instance, the gills and viscera (the spleen excepted) are bleached in appearance and hematocrit values of 2% to 10% are common. Mortalities in chum and pink salmon dying of uncomplicated VEN follow a chronic to subacute pattern. Also, VEN appears to predispose fish to other infections, and the mortality pattern is often complicated by more acute secondary (terminal) infections such as vibriosis in salt water or bacterial kidney disease or cold water disease in fresh water. These secondary infections may often cause VEN to be overlooked.

D. Diagnostic Procedures for Disease Situations

The viruses responsible for VEN have not yet been isolated. Consequently, diagnosis is based on a light microscopic examination of Giemsa or Wright stained blood films. Pinacyanol chloride has also been successfully used to stain smears. NOTE: If blood is unavailable for examination, stained smears prepared from blood-rich tissues such as kidney or spleen can be used.

1. Presumptive Diagnosis

A presumptive diagnosis is based on demonstrating that the erythrocytes contain cytoplasm inclusion bodies.

2. Confirmed Diagnosis

A confirmed diagnosis is based on demonstrating the presence of virions in the cytoplasm of erythrocytes using electron microscopy. In thin sections, the classically described virion outer coat is typically hexagonal in outline; there is usually a closely apposed inner coat that surrounds a centrally-located circular nucleoid. The diameters of the virions in teleosts range from 154 nm in the Atlantic herring to 330 nm in the Atlantic cod. A second type of virion has also been detected. This one from coho and chinook salmon reared exclusively in fresh water has a diameter of 50-70 nm.

E. Procedures for Detecting Asymptomatic Infections

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

F. Procedures for Determining Prior Exposure to the Etiological Agent

None are available.

G. 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.

H. References

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VII. Herpesvirus Disease of Salmonids

A. Name of the Disease and Etiological Agent

Herpesvirus disease of salmonids (HP). Herpesvirus salmonis (HPV).

B. Known Geographic and Host Range of the Disease

1. Geographic Range

Winthrop National Fish Hatchery, Washington, USA; Japan.

2. Host Range

Rainbow trout (Salmo gairdneri), kokanee (Oncorhynchus nerka).

Research indicates that fingerling Atlantic salmon (Salmo salar), brown trout (Salmo trutta), and brook trout (Salvelinus fontinalis) do not show signs of disease following injection (IP) of virus.

C. Clinical Signs of Diagnostic Significance

1. Herpesvirus salmonis was isolated from spawning rainbow trout at the Winthrop National Fish Hatchery, Washington in 1973, 1974 and 1975. The virus appeared to have caused unusually high loss in post-spawning fish. When the viral etiology was confirmed in 1975, the infected brood stock was phased out and the virus has not been reisolated since. A morphologically similar virus has been found in natural epizootics occurring annually since 1970 among fry of kokanee salmon on Honshu Island, Japan. The agent has also been isolated from moribund and dead adult kokanee on Hokkaido Island, Japan. However, it remains to be determined whether or not the virus causes death in adult salmonids. Most affected fry become lethargic as death approaches. During terminal stages some swim erratically while others are hyperactive.

2. Experimentally, signs include various degrees of abnormal darkening and exophthalmia, at times extreme. Hemorrhage may be present in the orbit of the eye of fish with severe exophthalmia. Abdominal distention and pale gills are common while other external signs are lacking. Ascitic fluid is abundant, and anemia and edema are evident in the visceral mass. The liver, spleen, and the digestive tract are flaccid and the vascular organs are mottled with areas of hyperemia. Kidneys are abnormally pale, though not noticeably swollen. The digestive tract is devoid of food.

D. Diagnostic Procedures for Disease Situations

1. Presumptive Diagnosis

(a) Typical CPE in susceptible cell cultures (RTG-2 and CHSE-214) occurs at 10°-12°C but not at 20°C. CPE consists of pyknosis and cell fusion (syncytial). Multinucleated giant cells are formed and atypical Cowdry type A inclusions can be demonstrated.

(b) Histopathological examination: blood in infected specimens contains abnormally high numbers of immature erythrocytes

and leucocytes. Kidney hematopoietic tissue is hypertrophied and mitotically hyperactive. Kidney tubules may be filled with serous material; renal tissue is edematous but where it occurs necrosis is focal and mild.

Serous deposits in the orbits undoubtedly contribute to exophthalmia. Skeletal muscle is edematous and shows accumulation of serous material. Cardiac muscle is similarly edematous, and hematopoietic activity is present in heart tissue, an organ in which blood cell formation does not normally occur.

The liver is probably the target organ for viral effects showing edema, fatty infiltration, and vascular stasis. Hepatic tissue shows areas of mild necrosis, as does pancreatic acinar tissue, the latter to a lesser degree. Pancreatic necrosis is never as severe or as extensive as it is in cases of infectious hematopoietic necrosis (IHN) and infectious pancreatic necrosis (IPN). The spleen lacks red pulp.

(c) Presence of signs as described in Section C above.

2. Confirmatory Diagnosis

Diagnosis and virus identification is based on internal signs, histopathological changes, presumptive tests, and ultimately on serum neutralization. There is reportedly no cross-reactivity with Oncorhynchus masou virus (OMV), a recently described herpesvirus causing tumors in certain salmonids in Japan.

The virus produces syncytia in RTG-2, RTF-1, and CHSE -214 cell lines. Fathead minnow (FHM) cells are refractory, or at least do not develop cytopathic effects. In fixed and stained cultures, the syncytia are readily visible. May-Brunwald-Giemsa staining reveals prominent cytoplasmic basophilia in the infected cells and intranuclear Cowdry type A inclusions.

The herpesvirus plaque type differs from those of the other salmonid viruses: cell rounding and syncytium development are readily recognizable, and also cellular debris is much reduced.

E. Procedures for Detecting Asymptomatic Infections

None are available.

F. Procedures for Determining Prior Exposure to the Etiological Agent

None are available.

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

Samples should be frozen for not more than seven (7) days.

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VIII. Herpesviruses-*Oncorhynchus Masou* Virus (OMV) and Yamame Tumor Virus (YTV)

A. Name of the Diseases and Etiological Agents

The diseases have not been given names, but both cause mortality in salmon fry and induce tumors in survivors. The agents have been named *Oncorhynchus masou* virus (OMV) and Yamame Tumor Virus (YTV) although they may be identical.

B. Known Geographic and Host Range of the Diseases

1. Geographic Range

Hokkaido and Honshu islands of Japan.

2. Host Range

(a) *Oncorhynchus masou*, common name yamame.

(b) Experimental infections have been demonstrated in chum salmon (*O. nerka*), coho salmon (*O. kisutch*) and rainbow trout (*Salmo gairdneri*).

C. Clinical Signs of Diagnostic Significance

1. OMV was first isolated from ovarian fluid of masou salmon with no abnormal external signs. YTV was first isolated from a mandibular tumor from the same species. Since virus has been isolated directly and from tissue cultures of tumors, it is suspected that both viruses are oncogenic. This is further supported by observation of tumor formation (particularly but not restricted to the mouth and head) in fish surviving artificial challenges with OMV.

2. Both YTV and OMV are pathogenic to fry of masou, chum, coho, and kokanee salmon and rainbow trout. Fish older than 240 days seem to be resistant to infection. Affected fish are anorexic, show exophthalmia and petechiation of the body surface, particularly under the jaw. In older fish that survive, induction of tumors beginning 130 days post-infection is noticed, primarily in the perioral maxilliary and mandibular regions.

D. Diagnostic Procedures for Disease Situations

1. Presumptive Diagnosis

(a) Typical CPE in susceptible cell cultures (RTG-2 and CHSE-214) at 15°C. CPE is characterized by massive syncitium and formation of multinucleate giant cells.

(b) Histopathological examination: The kidney of one-month-old salmon show typical syncitium formation with accompanying necrosis of the hematopoietic tissue. Glomeruli and tubules often are not affected. Epidermal cells of the mouth, jaw, and operculum showed necrosis and often contained numerous granules. Atrophy and necrosis of the liver are also observed. Necrosis of the pancreas and spleen are also found in moribund specimens. Similar but less severe changes are observed in older fish. In contrast to *Herpesvirus salmonis*, the target organ for OMV seems to be the kidney not the liver. Although in older fish infected with OMV, greater liver involvement is noticed.

2. Confirmatory Diagnosis

Diagnosis and virus identification is based on internal signs, histopathological changes, presumptive tests, and ultimately on neutralization with specific anti-OMV or anti-YTV serum. The serum neutralization test will distinguish between OMV and H. salmonis. YTV is related to NeVTA, a previously described herpesvirus from non-anadromous O. nerka (Sano, 1976). There are no reports of the serological relationships of YTV and OMV, although both induce similar changes and tumor formation in infected fry.

E. Procedures for Detecting Asymptomatic Infections

The presence of tumors of serum anti-OMV titers are indications of prior exposure to virus.

F. Procedures for Determining Prior Exposure to the Etiological Agent

Same as above.

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

Samples should be frozen for not more than seven (7) days. Ideally, samples should be transported on ice and processed within 24 hours.

H. References

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SECTION 3

METHODS FOR THE DETECTION OF CERTAIN BACTERIAL DISEASES

FOREWORD

In the following pages, methods are outlined for the diagnosis and identification (Figure 1) of the major bacterial fish diseases. Other bacterial diseases of concern have not been considered -- some because of their sporadic occurrence and others because of their uncertain etiology.

Inspections and certifications require routine procedures for detecting covert infections of bacterial pathogens. Section XII details a nonselective scheme for this.

Figure 1. Procedures for the presumptive identification of certain gram-negative bacteria associated with fish diseases.

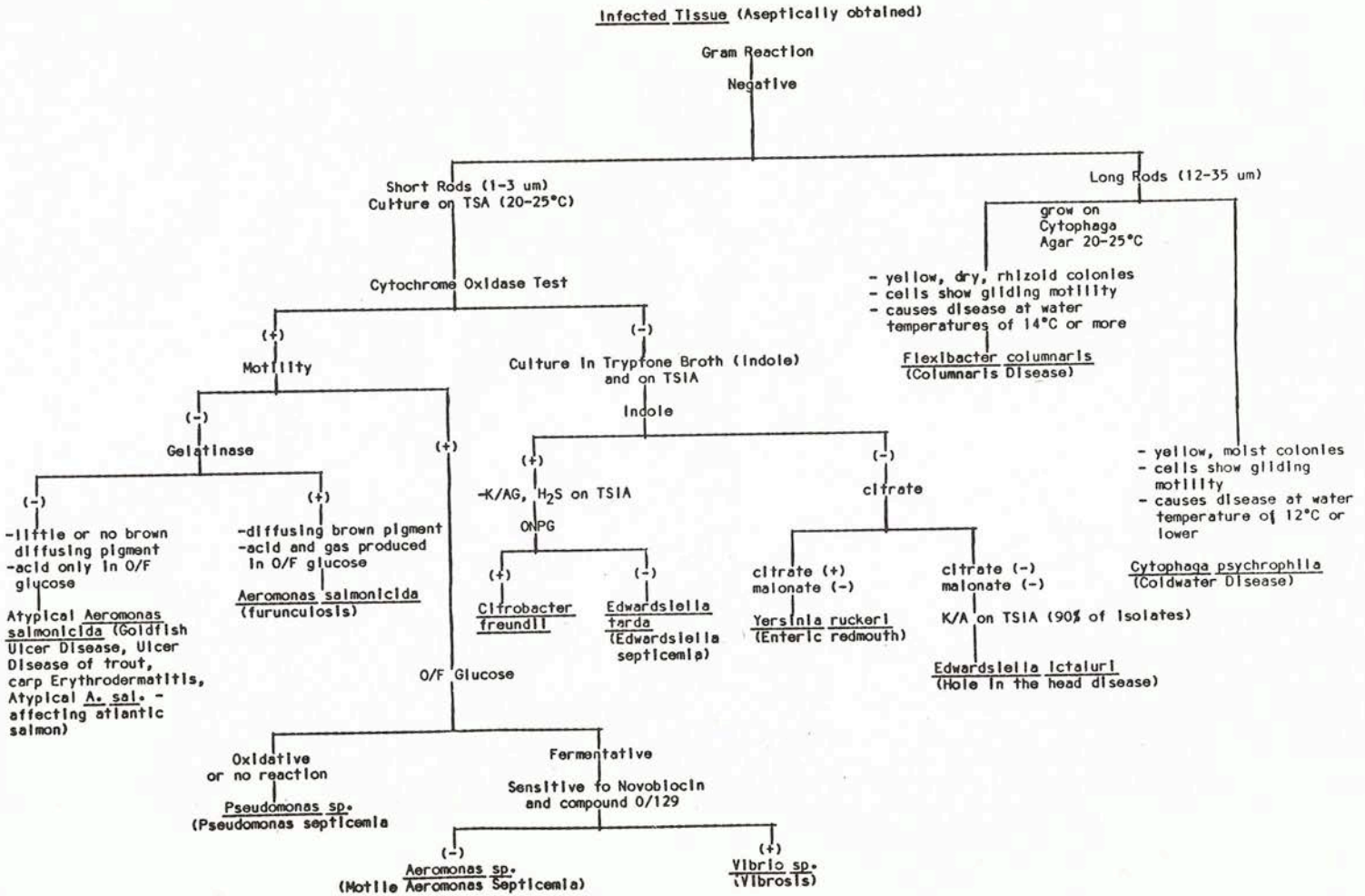
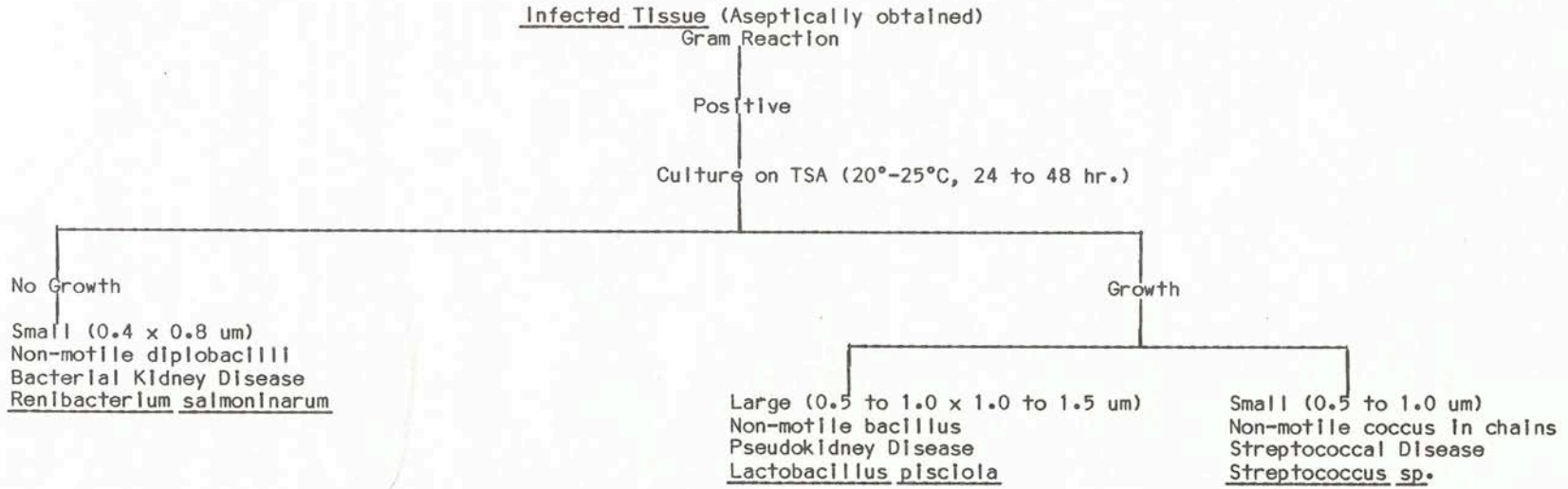


Figure 2. Procedures for the presumptive identification of certain gram-positive bacteria associated with fish diseases.



I. Furunculosis

A. Name of the Disease and Etiological Agent

Furunculosis (*Aeromonas salmonicida*).

B. Known Geographic and Host Range of the Disease

1. Geographic Range

Known to occur in North America, South America, Europe, Asia, and Africa. Reported predominantly in freshwater.

2. Host Range

All freshwater and marine fish are considered susceptible.

C. Clinical Signs of Diagnostic Significance

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

1. Peracute

Usually found among fingerlings. Fish darken and die readily. Internally, gross pathology resembles the Acute Form.

2. Acute (Acute septicemia) not size-specific

Generally, there are some indications of disease two-three days before die-off (darkening, going off feed). Internally, the viscera are hemorrhagic, the kidney tissue is very soft, the spleen is enlarged and the liver is very pale or mottled with petechiae.

3. Subacute

More pronounced and gradual onset of mortality. Rather than being solely a septicemic disease, skin lesions are present. Internally, there is intestinal inflamation and hemorrhaging in various organs.

4. Chronic

Similar to subacute, but distinguished by evidence of healing, especially around the lesions.

5. Latent

No mortality or clinical signs associated with *A. salmonicida* are evident.

D. Diagnostic Procedures for Disease Situations

Diagnosis is based on isolation and identification of the causative organism. Primary isolation should be made from the kidney on TSA or BHIA at 20° to 25°C for 24 to 48 hours.

1. Presumptive Diagnosis

The organism, when cultured as above, should be gram-negative, short (1-2 x .8 um) nonmotile and cytochrome oxidase-positive.

In culture, the organism has the shape or a cocco-bacillus and in tissues it is more rod-shaped. The majority of strains produce a brown diffusing pigment when grown on the above isolation media. Finally, although information on the reaction with glucose is not required in the definitive scheme outlined in Figure 1, it is probably worth noting that all strains of A. salmonicida ferment glucose. Some strains may show delayed and/or anaerogenic fermentation.

2. Confirmatory Diagnosis

(a) Confirmatory diagnosis is best accomplished serologically, by the direct FAT or agglutination test (microtiter or macroscopic slide). See Section XIV. NOTE: certain strains of A. salmonicida agglutinate spontaneously in saline.

(b) A more laborious confirmatory procedure is to show that the isolate is identical (or essentially identical) in its morphological, cultural and biochemical characteristics with A. salmonicida, various subspecies of which are described in the most recent edition of Bergey's Manual of Systematic Bacteriology (Holt et al. 1984).

(c) A sample of at least five moribund fish from each diseased holding unit should be examined to make a diagnosis. Freshly dead fish will suffice if moribund fish are unavailable. With this sampling procedure, it is possible to ascertain whether the same disease(s) is(are) involved in each of the diseased holding units.

E. Procedure for Detecting Asymptomatic Infections

Detection of latent furunculosis is more likely if an intestinal smear is cultured, with the kidney being the second organ of choice.

F. References

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1A. Other Diseases Caused by Aeromonas Salmonicida

Variants of A. salmonicida produce diseases other than furunculosis. These are ulcerative disease of goldfish, erythrodermatitis of carp, ulcer disease of trout, and systemic infections among several warmwater and marine species. The atypical strains vary in biochemical characteristics and may not produce water-soluble brown pigment.

Goldfish ulcer disease (GUD) causes serious losses in commercial goldfish farms, and because of extensive fish transfers, there are little 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 a fin and develops into large, open necrotic lesions. A bacteremia is generally not caused by A. salmonicida; if one does develop, it is usually caused by A. hydrophila.

Carp Erythrodermititis (CE) is a subacute to chronic skin disease that occurs at 4°-30°C and was originally associated with carp dropsy syndrome. Grass and silver carp 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.

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

References

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II. Motile Aeromonas Septicemia

A. Name of the Disease and Etiological Agent

Motile Aeromonas septicemia (MAS), Aeromonas hydrophila complex. (Synonyms -- bacterial hemorrhagic septicemia, BHS, hemorrhagic septicemia, and many others).

B. Known Geographic and Host Range of the Disease

1. Geographic Range

Worldwide in freshwater, but known to survive in saltwater of low salinity.

2. Host Range

All freshwater fish.

C. Clinical Signs of Diagnostic Significance

The disease occurs most frequently in warm waters with high content of organic matter following some stress or injury that results from handling, external parasites, low oxygen, or poor overwintering conditions. It is normally a generalized septicemia with clinical signs virtually indistinguishable from those of other septicemias caused by gram-negative bacteria. The disease may range in form from peracute (mortalities without gross lesions) and acute (hemorrhaging of gills, vent, and internal organs; blood-tinged fluid in the body cavity) to subacute and chronic. With the latter forms, abscesses and ulcers are evident externally.

D. Diagnostic Procedures for Disease Situations

Diagnosis is based on the isolation and identification of the etiological agent. Primary isolation should be made from kidney on TSA incubated at 20°-25°C for 24-48 hours. NOTE: If, for some reason, MAS is strongly suspected, the kidney may be inoculated onto Rimler-Shotts (RS) medium which should then be incubated at 35°C. The RS medium facilitates the rapid identification of organisms in the A. hydrophila complex. Colonies are yellow (as opposed to green) with no black center and are detectable within 18 to 24 hours. Due to the acid reaction produced on the RS medium by organisms in the A. hydrophila complex, their identification using the acid-sensitive cytochrome oxidase test cannot be accomplished on this medium. Instead, the cytochrome oxidase test must be performed on growth taken from TSA.

1. Presumptive Diagnosis

Criteria for a presumptive diagnosis are satisfied if the TSA isolate proves to be a short, motile, cytochrome oxidase-positive, gram-negative, usually straight rod that ferments glucose.

2. Confirmatory Diagnosis

(a) A confirmed diagnosis is obtained if the TSA isolate produces gas during the fermentation of glucose in addition to having the characteristics already listed. If the isolate proves to be an anaerogenic glucose fermenter, a

confirmed MAS diagnosis then requires that the isolate be resistant to the vibriostatic agent 0/129 and novobiocin (see Section XIII).

(b) Serological identification is very difficult because this bacteria has a wide range of antigenic properties.

(c) To make a diagnosis, examination of a sample of five moribund fish from each affected holding unit is recommended. For amplification, see item I.D.2(c).

E. Procedures for Detecting Asymptomatic Infections

Because A. hydrophila is considered normal flora of fish intestinal tract, a detection procedure is not warranted.

F. References

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III. Pseudomonas Septicemia

A. Name of the Disease and Etiological Agent

Pseudomonas septicemia, *Pseudomonas* sp. particularly *P. fluorescens*, some outbreaks have been caused by nonmotile capsulated *Pseudomonas*. (Synonyms -- bacterial hemorrhagic septicemia, hemorrhagic septicemia.).

B. Known Geographic and Host Range of the Disease

1. Geographic Range

Fish-pathogenic strains occur worldwide in fresh and sea water.

2. Host Range

All species of fish are considered susceptible.

C. Clinical Signs of Diagnostic Significance

The disease is stress-mediated and occurs most frequently under warm water conditions. The disease usually occurs as a generalized septicemia. Clinical signs vary and depend upon the severity of the infection (see motile *Aeromonas* septicemia).

D. Diagnostic Procedures for Disease Situations

Diagnosis is based on isolation and identification of the etiological agent. Primary isolation should be made from kidney on TSA at 20° to 25°C for 24 to 48 hours.

1. Presumptive Diagnosis

Criteria for a presumptive diagnosis are satisfied if the isolate is a short, motile, cytochrome oxidase-positive, gram-negative rod that is oxidative or inactive with glucose (in glucose O/F medium) and frequently produces a fluorescent pigment. Also, a greenish to yellow-green colony on RS media indicates *Pseudomonas* sp.

2. Confirmatory Diagnosis

(a) The criteria are the same as those described above for the presumptive diagnosis.

(b) To make a diagnosis, examination of a sample of five moribund fish from each affected holding unit is recommended. For amplification, see Section I.D.2.(c).

E. Procedures for Detecting Asymptomatic Infections

Because *Pseudomonas* is ubiquitous in water, a detection procedure has not been actively pursued and may not be warranted.

F. References

1. Bullock, G. L. 1965. Characteristics and pathogenicity of a capsulated *Pseudomonas* isolated from goldfish. Applied Microbiology 13:89-92.

2. Bullock, G. L., S. F. Snieszko, and C. E. Dunbar. 1965. Characteristics and identification of oxidative pseudomonads isolated from diseased fish. Journal of General Microbiology 38:1-7.

3. Kusuda, R., and T. Toyoshima. 1976. Characteristics of a pathogenic Pseudomonas isolated from cultured yellowtail. Fish Pathology 11:133-139.

4. Shotts, E. B., Jr., and G. L. Bullock. 1975. Bacterial diseases of fishes: diagnostic procedures for gram-negative pathogens. Journal of the Fisheries Research Board of Canada 32:1243-1247.

IV. Vibriosis

A. Name of the Disease and Etiological Agent

Vibriosis, *Vibrio* sp., *V. anguillarum*, and *V. ordalii*.

B. Known Geographic and Host Range of the Disease

1. Geographic Range

   Worldwide, principally in marine environments, but sporadic outbreaks have occurred in freshwater.

2. Host Range

   All marine and freshwater fish are considered susceptible.

C. Clinical Signs of Diagnostic Significance

The disease is normally a generalized septicemia with clinical signs virtually indistinguishable from those of other septicemias. In salmonids, the disease ranges in form from peracute (mortalities without gross lesions) and acute (hemorrhaging of the eyes, gills, vent, skin and internal organs, blood-tinged fluid in the body cavity) to subacute and chronic (hemorrhagic ulcerations of the skin and underlying muscle).

D. Diagnostic Procedures for Disease Situations

Diagnosis is based on isolation and identification of the etiological agent. Primary isolation should be made from kidney on TSA or BHIA with 1% NaCl incubated at 20°-25°C for 24 to 48 hours.

1. Presumptive Diagnosis

   Criteria for a presumptive diagnosis are satisfied if the isolate is a short, motile, cytochrome oxidase-positive, gram-negative usually curved rod that produces acid but no gas in glucose O/F medium.

2. Confirmatory Diagnosis

   (a) Criteria for a confirmed diagnosis are satisfied if, in addition to having the characteristics mentioned above, the TSA isolate is sensitive to novobiocin and the vibriostatic compound O/129 (NOTE: Because of the presence of novobiocin in the RS medium, vibrios will fail to grow).

   (b) Antisera are available for identifying both *V. anguillarum* and *V. ordalii*. The recommended diagnostic procedure is the macroscopic slide or microtiter agglutination test.

   (c) To make a diagnosis, examination of a sample of five moribund fish from each holding unit is recommended. For amplification, see Section I.D.2.(c).

E. References

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V. Enteric Redmouth

A. Name of the Disease and Etiological Agent

Enteric redmouth, ERM, Yersinia ruckeri.

B. Known Geographic and Host Range of the Disease

1. Geographic Range

North America, Europe.

2. Host Range

Potentially all salmonids. Confirmed isolations have been made from Atlantic salmon (Salmo salar), brook trout (Salvelinus fontinalis), brown trout (Salmo trutta), chinook salmon (Oncorhynchus tshawytscha), coho salmon (Oncorhynchus kisutch), cutthroat trout (Salmo clarki), rainbow trout and steelhead (Salmo gairdneri) and sockeye salmon (Oncorhynchus nerka). Isolations have also been made in goldfish (Carassius auratus), cisco (Coregonus sp.), and largemouth bass (Micropterus salmoides).

C. Clinical Signs of Diagnostic Significance

The disease may occur as peracute, acute, or in the subacute to chronic condition. The clinical signs of the acute forms of the disease are very similar to those seen in other bacterial septicemias; however, the frequent presence of a reddening in the mouth, hemorrhages in the lower intestine, 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 exopthalmia which may have progressed 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.

D. Diagnostic Procedures for Disease Situations

Diagnosis is based on isolation and identification of the causative organism. Primary isolation should be made from the kidney on TSA incubated at 20° to 25°C for 24 to 48 hours.

1. Presumptive Diagnosis

The organism should be a gram-negative, cytochrome oxidase-negative, motile rod. It does not produce indole in tryptone broth but produces an alkaline slant and an acid (only) butt on TSI (triple sugar iron) agar. Also, the organism is an anaerogenic glucose fermenter. NOTE: If ERM is strongly suspected, kidney may, in addition, be inoculated onto Shotts-Waltman (SW) medium which should then be incubated at 20°-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 A. hydrophila will grow on SW, but they will produce large, yellow colonies).

2. Confirmatory Diagnosis

(a) Confirmatory testing of the Hagerman Strain (Type I) is best accomplished serologically, using the direct FAT. See

Section XIV. Type I antisera should be used. However the O'Leary Strain (sorbitol positive, Type 2) cannot be confirmed reliably using this method.

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

(c) To make a diagnosis, a sample of five moribund fish from each affected holding unit is recommended. For amplification, see Section I.D.2.(c).

E. Procedures for Detecting Asymptomatic 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.

F. References

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VI. Edwardsiella Septicemias

VIA. *E. tarda*

A. Name of the Disease and Etiological Agent

Edwardsiella septicemia, *Edwardsiella tarda*.

B. Known Geographic and Host Range of the Disease

1. Geographic Range

Southeastern and southwestern United States; southeast Asia.

2. Host Range

Channel catfish (*Ictalurus punctatus*), goldfish (*Carassius auratus*), largemouth bass (*Microperus salmoides*), brown bullhead (*Ictalurus nebulosus*), "wild mullet", chinook salmon (*Oncorhynchus tshawytscha*), striped bass (*Morone saxitalis*), and freshwater eel (*Anguilla japonicus*). The organism has also been found in a variety of other animals including seals, sea lions, turtles, alligators, and snakes, and has been implicated as a pathogen in certain diseases of humans, cattle, pigs, and birds.

C. Clinical Signs of Diagnostic Significance

The disease is favored by high water temperatures (30°C and above) and, in channel catfish, it initially manifests itself as small, cutaneous lesions located posterolaterally on the fish. Later, abscesses may develop within the muscles of the flank and caudal peduncle. Large cavities filled with a malodorous gas and necrotic tissue may be produced. The lesions may be visible externally as swellings (if enough gas has been produced in the underlying lesions) or as bleached areas. In the freshwater eel, the disease shares many signs in common with other bacterial septicemias but it may be distinguished from these by the nature of the putrefactive lesions produced in the kidney and liver. The lesions are essentially cavities filled with a foul-smelling, purulent material. The disease may also occur in peracute form showing none of the above pathology.

D. Diagnostic Procedures for Disease Situations

Diagnosis is based on isolation and identification of the etiological agent. Primary isolation should be made from kidney onto TSA incubated at 25°C for two to four days.

1. Presumptive Diagnosis

For presumptive identification, the etiological agent should be a short, gram-negative, cytochrome oxidase-negative rod that produces indole in tryptone broth. TSI (triple sugar iron) agar reaction should be alkaline slant, acid butt, with gas and H_2 production. In addition, the organism is motile and ferments glucose with both acid and gas produced.

2. Confirmatory Diagnosis

(a) A confirmed diagnosis is accomplished if the isolate is agglutinated in the slide or microtiter agglutination test with *E. tarda* antiserum. Caution should be used in this

test as there are many serotypes and confirmation may fail with the use of a monovalent antiserum.

(b) If for some reason the diagnostic antiserum is not immediately available, the identity of the presumptively identified isolate is confirmed if it proves to be urease-negative and produces lysine decarboxylase.

(c) To make a diagnosis, examination of a sample of five moribund fish from each affected holding unit is recommended. For amplification, see Section I.D.2.(c).

E. References

1. Egusa, S. 1976. Some bacterial diseases of freshwater fishes in Japan. Fish Pathology 10:103-114.

2. Ewing, W. H., A. C. McWhorter, M. R. Escobar, and A. H. Lubin. 1965. Edwardsiella, a new genus of Enterobacteriaceae based on a new species, E. tarda. International Bulletin of Bacteriological Nomenclature and Taxonomy 15:33-38.

3. Meyer, F. P., and G. L. Bullock. 1973. Edwardsiella tarda, a new pathogen of channel catfish (Ictalurus punctatus). Applied Bacteriology 25:155-156.

VIB. *E. ictaluri*

A. Name of the Disease and Etiological Agent

Edwardsiella septicemia of catfish, "Hole in the Head" disease, *Edwardsiella ictaluri*.

B. Known Geographic and Host Range of the Disease

1. Geographic Range

First recognized in the late 1970's in Columbus, Georgia. Southeastern United States.

2. Host Range

Channel catfish (*Ictalurus punctatus*), white catfish (*Ictalurus catus*), black bullhead (*Ictalurus melas*), yellow bullhead (*Ictalurus natalis*), brown bullhead (*Ictalurus nebulosus*), and danio (*Danio devario*).

C. Clinical Signs for Disease Situations

When water temperatures are at 24°C and above, *E. ictaluri* affects fingerlings and adult fish, with a low grade infection in brain tissue; externally there are white areas, blebs, and open lesions, hence, the name "hole in the head" disease. The fish have ascites, petechial hemorrhages in the musculature, and a flaccid gut with bloody fluid. Post-infection, the bacteria overwinters in carrier fish in the forebrain and hindgut, at very low prevalences (1% or less of the population).

D. Diagnostic Procedure for Disease Situations

Diagnosis is based on isolation and identification of the etiological agent. Primary isolation should be made from the kidney onto TSA incubated at 35°C for two to four days.

1. Presumptive Diagnosis

For presumptive identification, the etiological agent should be a short, gram-negative, cytochrome oxidase-negative rod. No indole is produced in tryptone broth. It grows slowly (5-7 days) and sparsely at 37°C. It is non-motile and does not produce H_2S. TSIA reaction should be K/A (alkaline slant, acid butt).

2. Confirmatory Diagnosis

(a) A confirmed diagnosis is accomplished if the isolate is agglutinated in the slide or microtiter agglutination test with *E. ictaluri* antiserum.

(b) To make a diagnosis, examination of a sample of five moribund fish from each affected holding unit is recommended. For amplification, see Section I.D.2.(c).

E. References

1. Hawke, J. P. 1979. A bacterium associated with diseases of pond-cultured channel catfish. Journal of the Fisheries Research Board of Canada 36:1508-1512.

2. Hawke, J. P., A. C. McWhorter, A. G. Steigerwalt, and D. J. Brenner. 1981. Edwardsiella ictaluri sp. nov. the causative agent of enteric septicemia of catfish. International Journal of Systematic Bacteriology 31:396-400.

3. Waltman, W. D., E. B. Shotts, and V. S. Blazer. 1985. Recovery of Edwardsiella ictaluri from danio (Danio devario). Aquaculture (accepted).

VII. Columnaris Disease

A. Name of Disease and Etiological Agent

Columnaris disease, Flexibacter columnaris. Flexibacter columnaris is not recognized as a species in the current edition of Bergey's Manual.

B. Known Geographic and Host Range of the Disease

1. Geographic Range

   Probably worldwide.

2. Host Range

   All freshwater fishes are considered susceptible.

C. Clinical Signs of Diagnostic Significance

The disease affects fish of all ages and is favored by warmwater conditions (14°C and over). When highly virulent strains of the pathogen are involved, the fish may die without any gross signs of pathology 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 tissues become 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.

D. Diagnostic Procedures for Disease Situations

1. Presumptive Diagnosis

   The following criteria provide a basis for presumptive diagnosis: long, thin (5 to 17 X .7 um) gram-negative rods in lesions; dry rhizoid, yellowish colonies on cytophaga agar (3 days incubation at 20°C); cells mobile (gliding or flexing), but no flagella; little or no disease produced at 14°C or lower. The tendency of the columnaris bacterium to form mounds or columns, as detected in wet mounts of diseased tissue, also aids diagnosis.

2. Confirmatory Diagnosis

   (a) The procedure of choice is an agglutination test using anti-F. columnaris serum. NOTE: Certain strains of F. 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 minutes at 50°C) to prevent autoagglutination.

(b) If the diagnostic antiserum is, for some reason, unavailable, a more laborious confirmatory procedure is to show that the isolate is identical in its morphological, cultural, and biochemical features with F. columnaris. Descriptions of F. columnaris are provided in three of the papers listed in the references.

(c) To make a diagnosis, a sample of five moribund fish from each affected holding unit (e.g., tank, raceway, pond) is recommended. For amplification, see Section I.D.2.(c).

E. Procedures for Detecting Asymptomatic Infections

Because F. columnaris is considered ubiquitous in freshwater, a search for a suitably sensitive procedure has not been actively pursued and may not be warranted.

F. References

1. Amend, D. F. 1983. Columnaris (Flexibacter columnaris) disease of freshwater fishes and a brief review of other flexibacterial disease of fish. Pages 139-151 In D. P. Anderson, M. M. Dorson, and P. Dubourget, editors. Antigens of Fish Pathogens Collection. Foundation Merieux, Lyon, France.

2. Bootsma, R., and J. P. M. Clerx. 1976. Columnaris disease of cultured carp, Cyprinus carprio L. Characterization of the causative agent. Aquaculture 7:371-384.

3. Fujihara, M. P., and R. E. Nakatani. 1971. Antibody production and immune response of rainbow trout and coho salmon to Chondrococcus columnaris. Journal of the Fisheries Research Board of Canada 28:1253-1258.

4. Garnjobst, L. 1945. Cytophaga columnaris (Davis) in pure culture: a myxobacterium pathogenic to fish. Journal of Bacteriology 49:113-128.

5. Pacha, R. E., and E. J. Ordal. 1970. Myxobacterial disease of salmonids. Pages 243-257 In S. F. Snieszko, editor. A symposium on diseases of fishes and shellfishes. American Fisheries Society Special Publication No. 5.

6. Snieszko, S. F., and G. L. Bullock. 1976. Columnaris disease of salmonids. U.S. Fish and Wildlife Service. Fish Disease Leaflet 45. 10 pp.

7. Wakabayashi, H., S. Egusa, and J. L. Fryer. 1980. Characteristics of filamentous bacteria isolated from a gill disease of salmonids. Canadian Journal of Fisheries and Aquatic Sciences 37:1499-1504.

VIII. Coldwater Disease

A. Name of Disease and Etiological Agent

Coldwater disease, Cytophaga psychrophila. Cytophaga psychrophila is not recognized as a species in the current edition of Bergey's Manual. (Synonyms - peduncle disease, low temperature disease.)

B. Known Geographic and Host Range of the Disease

1. Geographic Range

To date, the disease has been reported only in the continental United States, predominantly from the northwestern United States.

2. Host Range

All salmonids are probably affected, but juvenile coho salmon (Oncorhynchus kisutch) are particularly susceptible. Fingerling fall chinook (O. tshawytscha) are occasionally infected.

C. Clinical Signs of Diagnostic Significance

The disease is a freshwater condition that normally occurs when the water temperatures are 12°C or below. Juvenile fish are primarily affected, the causative organism being recoverable from both the external lesions and the internal organs. In alevins, the ventral surface of the yolk sac becomes eroded and the sac may rupture, releasing its contents. In fingerlings with the acute form of the disease, the fish may darken in the peduncle region and die without any surface lesions appearing. More commonly, however, superficial lesions occur, which frequently are first observed in the peduncle area but may also occur on other areas of the trunk and head. The lesions may enlarge and the underlying tissues may be extensively eroded. If the fish survives long enough, it may suffer a loss of its caudal fin and the vertebral column in the caudal peduncle may eventually be exposed. In the chronic form of the disease, the fish may exhibit lordosis and scoliosis.

D. Diagnostic Procedure for Disease Situations

1. Presumptive Diagnosis

The disease may be presumptively diagnosed if it occurs at water temperatures of 12°C or below and the lesions contain long, thin (3.5 to 7.4 x 0.75 um) gram-negative rods. The organism should produce a moist, yellow, spreading colony on cytophaga agar within 3 days at 20°C and should exhibit a gliding motility on solid surfaces.

2. Confirmtory Diagnosis

The diagnosis is confirmed upon positive serological identification using either slide or microtiter agglutination or indirect FAT.

3. For making a diagnosis, a sample of five moribund fish from each affected holding unit is recommended. For amplification, see Section I.D.2.(c).

E. References

1. Bullock, G. L. 1972. Studies on selected myxobacteria pathogenic for fishes and on bacterial gill disease in hatchery-reared salmonids. U.S. Fish and Wildlife Service, Technical Paper of the Bureau of Sport Fisheries and Wildlife, No. 60. 30 pp.

2. Bullock, G. L., and S. F. Snieszko. 1970. Fin rot, coldwater disease, and peduncle disease of salmonid fishes. U.S. Fish and Wildlife Service, Fish Disease Leaflet 25. 3 pp.

3. Pacha, R. E. 1968. Characteristics of Cytophaga psychrophila (Borg) isolated during outbreaks of bacterial coldwater disease. Applied Microbiology 16:97-101.

4. Wood, J. W. 1974. Diseases of Pacific salmon; their prevention and treatment, pp. 22-24. Washington State Department of Fisheries, Olympia, Washington.

IX. Bacterial Gill Disease

A. Name of the Disease and Etiological Agent

Bacterial gill disease (Eastern gill disease). Although Flexibacteria have been traditionally implicated as etiological agents, a recently isolated organism tentatively classified as a Flavobacterium has been demonstrated to be an etiological agent of bacterial gill disease in Japan. However, other bacteria are also seen in some outbreaks.

B. Known Geographic and Host Range of the Disease

1. Geographic Range

   Worldwide, usually seen in hatchery-reared fishes.

2. Host Range

   Principally salmonids; may occur in any warmwater fish.

C. Clinical Signs of Diagnostic Significance

Bacterial gill disease occurs primarily among fingerling salmonids and is associated with poor environmental conditions. Factors such as elevated ammonia levels, low dissolved oxygen, crowding, and an excess of particulate matter in water may trigger an outbreak. Affected fish stop feeding, ride "high" in the water, and will often line up facing the current to force water over gill surfaces. Epizootics may be explosive with losses exceeding 50% in 24 hours.

D. Diagnostic Procedures for Disease Situations

Diagnosis is accomplished by examination of gills for the presence of clubbing of lamellae and accumulation of masses of long, thin, gram-negative bacteria on the tips of the lamellae. Bacteria may be seen on stained smears of gill tissue.

E. References

1. Bullock, G. L. 1972. Studies on selected myxobacteria pathogenic for fishes and on bacterial gill disease in hatchery-reared salmonids. U.S. Fish and Wildlife Service, Technical Paper of the Bureau of Sport Fisheries and Wildlife No. 60. 30 pp.

2. Snieszko, S. F. 1981. Bacterial gill disease of freshwater fish. U.S. Fish and Wildlife Service, Fish Disease Leaflet 62. 11 pp.

3. Wakabayashi, H., S. Egusa, and J. F. Fryer. 1980. Characteristics of filamentous bacteria isolated from a gill disease of salmonids. Canadian Journal of Fisheries and Aquatic Sciences 37:1499-1504.

X. Bacterial Kidney Disease

A. Name of the Disease and Etiological Agent

Bacterial kidney disease, Renibacterium salmoninarum.

B. Known Geographic and Host Range of the Disease

1. Geographic Range

North America, Scotland, France, Japan, England, Iceland, Italy, Spain, Yugoslavia, Germany, and Chile.

2. Host Range

All salmonids are considered susceptible.

C. Clinical Signs of Diagnostic Significance

Acute and subacute forms of the disease occur only sporadically. More typically, the disease is a chronic one that seldom shows clinical signs in fish less than six (6) months old. The chronic disease is characterized internally by an enlarged edematous kidney that may appear gray and corrugated. The kidney usually exhibits off-white lesions that vary in size and number. These lesions sometimes occur in other organs, chiefly the liver and spleen. The turbid fluid is often present in the abdominal and pericardial cavities, especially in older fish. Externally, the clinical signs are of less diagnostic value. Fish may appear normal or they may show one or more of the following signs: exopthalmia, skin petechiation, vesicles in the skin.

D. Diagnostic Procedures for Disease Situations

1. Presumptive Diagnosis

Smears of infected tissue shoud contain numerous small, gram-positive, non-acidfast diplobacilli that occur both intra- and extra-cellularly. 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

(a) Kidney disease may be diagnosed using an indirect or direct fluorescent antibody test (FAT) applied to fresh, formalin fixed, or frozen infected tissues. The FAT is described in Section XIV.

(b) Immunodiffusion in which material from kidneys or lesions from infected fish and antisera are used, can provide definitive diagnosis within 24 hours. Coagglutination can also be used (Kimura 1978).

(c) A more laborious procedure is to isolate the causative bacterium and show that it is identical (or essentially identical) to the KD bacterium. Descriptions of the kidney disease bacterium are provided in papers listed in the references. Mediums commonly used are those described by Ordal and Earp (1956), Evelyn (1977) and Austin (1983). Optimal temperature is 15°-18°C and growth is slow (may require up to six weeks incubation).

(d) To make a diagnosis, a sample of five moribund fish from each affected holding unit is recommended. For amplification, see Section I.D.2.(c).

E. Procedures for Detecting Asymptomatic Infections

Monitoring of mortalities in seemingly healthy stocks should be done by FAT. Bear in mind that the chances of detecting the pathogen appear to be increased if trout are examined in fall and salmon are examined in spring.

F. References

1. Austin, B., T. M. Embley, and M. Goodfellow. 1983. Selective isolation of Renibacterium salmoninarum. FEMS Microbiology Letters. 17(1983)111-114.

2. Bullock, G. L. 1980. Bacterial kidney disease of salmonid fishes caused by Reinbacterium salmoninarum. U.S. Fish and Wildlife Service, Fish Disease Leaflet 60. 10 pp.

3. Chen, P. K., G. L. Bullock, H. M. Stuckey, and A. C. Bullock. 1974. Corynebacterial kidney disease of salmonids: rapid serological diagnosis of clinical cases. Journal of the Fisheries Research Board of Canada 31:1939-1940.

4. Evelyn, T. P. T. 1977. An improved growth medium for the kidney disease bacterium and some notes on using the medium. Bulletin de L'Office International des Epizooties 87:511-513.

5. Kimura, T. 1978. Bacterial kidney disease of salmonids. Fish Pathology 13:43-52.

6. Kimura, T., Y. Ezura, and K. Tajima. 1978. Serological diagnosis of bacterial kidney disease (BKD), immunodiffusion test by heat stable antigen extracted from infected kidney. Fish Pathology 13:103-108.

7. Ordal, E. J., and B. J. Earp. 1956. Cultivation and transmission of etiological agent of kidney disease in salmonid fishes. Society of the Proceedings for Experimental Biology and Medicine 92:85-88.

8. Sanders, J. E., and J. L. Fryer. 1980. Renibacterium salmoninarum gen. nov., sp. nov., the causative agent of bacterial kidney disease. International Journal of Systematic Bacteriology 30:496-502.

XI. Pseudokidney Disease

A. Name of the Disease and Etiological Agent

Pseudokidney disease, Lactobacillus piscicola. (Synonym - big kidney disease.)

B. Known Geographic and Host Range of the Disease

1. Geographic Range

Reports, to date, have been from the United States and Canada. Possibly worldwide since lactobacilli are part of the normal flora of both marine and freshwater fish.

2. Host Range

Potentially all freshwater and marine fish are susceptible. Confirmed isolations from coho (Oncorhynchus kisutch) and chinook salmon (Oncorhynchus tshawytscha) and rainbow trout (Salmo gairdneri) and cutthroat trout (Salmo clarki).

C. Clinical Signs of Diagnostic Significance

The disease is most often observed in fish one year old or older, which have been stressed, such as that associated with spawning or handling. Pathological signs attributed to this disease are varied and include septicemia, abdominal distention, splenomegaly, ascites fluid accumulation, internal hemorrhaging, muscular abscesses and blood cavities or blisters under the skin. Chronically infected trout may have various combinations of these disease signs.

D. Diagnostic Procedures for Disease Situations

Diagnosis is based on isolation and identification of the causative organism. Primary isolation should be made from the kidney (or from lesion material, if present) onto tryptic (trypticase) soy agar incubated at 15° to 22°C for 24 to 72 hours.

1. Presumptive Diagnosis

Colonies are pinpoint, white, convex, circular entire and nonpigmented when grown on trypticase soy agar. The organism is a nonmotile, nonsporeforming gram-positive rod which becomes gram-variable within 24 hours on culture media. Short chains of two or three cells are often observed. All isolates are catalase- and oxidase-negative, do not reduce nitrates or produce hydrogen sulfide on triple sugar iron slants.

2. Confirmatory Diagnosis

(a) If more detailed tests are required to compare the identity of a isolate to L. piscicola, refer to the paper by Hiu et al. listed in the references.

(b) For making a diagnosis, a sample of five moribund fish from each affected holding unit (e.g., tank, raceway, pond) is recommended. For amplification, see Section I.D.2.(c).

E. Procedures for Detecting Asymptomatic Infections

No procedures have been reported for detection of carriers.

F. Procedures for Determining Prior Exposure to the Etiological Agent

At present, no serological tests have been developed.

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

See Section I, General Sampling Procedures.

H. References

1. Cone, D. K. 1982. A Lactobacillus species from diseased female rainbow trout, Salmo gairdneri Richardson, in Newfoundland. Canadian Journal of Fish Diseases 5:479-485.

2. Evelyn, T. P. T., and L. A. McDermott. 1961. Bacteriological studies of freshwater fish. Canadian Journal of Microbiology 7:375-382.

3. Hiu, S. F., R. A. Holt, N. Sriranganathan, R. J. Seidler, and J. L. Fryer. 1984. Lactobacillus piscicola, a new species from salmonid fish. International Journal of Systematic Bacteriology 34:393-400.

4. Ross, A. J., and R. J. Toth. 1974. Lactobacillus - a new fish pathogen? Progressive Fish-Culturist 36:191.

5. Rucker, R. R., B. J. Earp, and E. J. Ordal. 1953. Infectious diseases of Pacific salmon. Transactions American Fisheries Society 83:297-312.

XII. Streptococcal Disease

A. Name of the Disease and Etiological Agent

Streptococcal disease, nonhemolytic, Lancefield Group B streptoccocci (unclassified).

B. Known Geographic and Host Range of the Disease

1. Geographic Range

United States, Republic of China, Japan; primarily from brackish or freshwater environments.

2. Host Range

Freshwater and marine fish as well as frogs and eels are considered susceptible.

C. Clinical Signs of Diagnostic Significance

Affected fish have numerous raised hemorrhagic inflamed areas on the skin including the opercle, around the mouth, base of fins and in general along the dorsolateral portions of the body. Abdomen is distended and the peritoneal cavity often contains bloody fluid. Exophthalmia is present. The liver is usually pale and the spleen dark red. Kidneys appear normal. A hemorrhagic enteritis with bloody fluid present in the intestinal lumen is also seen.

D. Diagnostic Procedure for Disease Situations

Diagnosis is based on isolation and identification of the causative organism. Primary isolation should be made from fish tissue on TSA and/or blood agar (BAP) at 25°-35°C for 24-48 hours.

1. Presumptive Diagnosis

The cultured organism should be a gram-positive, nonmotile, cytochrome oxidase-negative, catalase-negative coccus in chains. The colonies are pinpoint to pinhead in size and convex. In liquid culture, broth may appear noticeably viscous.

2. Confirmatory Diagnosis

Isolation of a nonhemolytic, catalase-negative gram-positive coccus in chains which is typed by Lancefield grouping as Group B.

3. Detection of Asymptomatic Infections

Development of methodology to detect carriers has not been developed since outbreaks have been infrequent. Infection appears to be very communicable as long as infected fish are present. Fish stress enhances probability of infection.

E. Comment

There are reports in the literature, particularly from Japan, where other Lancefield Groups of streptococci have caused fish and eel mortality. The most common Lancefield group noted is D. Water salinity in brackish environments may exert some selectivity since streptococci grow in concentrations up to 6.5% without difficulty.

F. References

1. Plumb, J. A., J. H. Schachte, J. C. Gaines, W. Peltier, and B. Carroll. 1974. Streptococcus sp. from marine fishes along the Alabama and northwest Florida coast of the Gulf of Mexico. Transactions of the American Fisheries Society 103:358-361.

2. Wilkinnson, H. W., L. G. Thacker, and R. R. Facklam. 1973. Nonhemolytic Group B Streptococci of human, bovine and ichthyic origin. Infection and Immunity 7:496-498.

XIII. Selected Additional Reading on Bacterial Fish Diseases

These books and manuals are some of the resources available for more detailed information on procedures and on the pathogens themselves:

Amlacher, E. 1970. Textbook of fish diseases (translated and updated by D. A. Conroy and R. L. Herman). T.F.H. Publications, Inc., Neptune City, New Jersey. 302 pp.

Anderson, D. P. 1974. Fish immunology. In: S. F. Snieszko and H. R. Axelrod, editors. Book 4 of Disease of Fishes. T.F.H. Publications, Inc., Neptune City, New Jersey. 239 pp.

Brown, E. E., and J. B. Gratzek. 1980. Fish farming handbook - food, bait, tropicals, and goldfish. AVI Publishing Co., Inc., Westport, Connecticut.

Bullock, G. L. 1971. Identification of fish pathogenic bacteria. In: S. F. Snieszko and H. R. Axelrod, editors. Book 2B of Diseases of Fishes. T.F.H. Publications, Inc., Neptune City, New Jersey. 41 pp.

Bullock, G. L., D. A. Conroy, and S. F. Snieszko. 1971. Bacterial diseases of fishes. In: S. F. Snieszko and H. R. Axelrod, editors. Book 2A of Diseases of Fishes. T.F.H. Publications, Inc., Neptune City, New Jersey. 151 pp.

Kabata, Z. 1985. Parasites and diseases of fish cultured in the tropics. Taylor and Francis. Philadelphia, Pennsylvania.

McFadden, J. F. 1980. Biochemical tests for identification of medical bacteria. 2nd Edition. Williams and Wilkens Pub., Baltimore, Maryland.

Plumb, J. A., and P. R. Bowser. 1983. Microbial Fish Disease Laboratory Manual. Brown Printing Co., Montgomery, Alabama. 95 pp.

Post, G. W. 1983. Textbook of fish health. T.F.H. Publications, Inc., Neptune, New Jersey. 256 pp.

Roberts, R. J. (Editor). 1978. Fish Pathology. Bailliere Tindall, London, England. 318 pp.

Roberts, R. F., and C. J. Sheperd. 1975. Handbook of trout and salmon diseases. Fishing News (books) Ltd., West Byfleet, Surrey, England. 168 pp.

Snieszko, S. F. (Editor). 1970. A symposium on diseases of fishes and shellfishes. Publication No. 5, American Fisheries Society, Washington, D.C. 526 pp.

XIV. Bacteriological Methods for Asymptomatic Carriers

A. Target Organisms

Yersinia ruckeri, *Aeromonas salmonicida*, and *Renibacterium salmoninarum*.

Collection and number of fish shall be in accordance with Section 1 of the sample collection methods.

B. Sample Storage and Incubation

The hindgut is the site of choice for sensitivity. If the hindgut is atrophied, as in many returning anadromous salmonids, kidneys may be sampled. If gross lesions resembling those of bacterial kidney disease or furunculosis are present, samples must include materials from the periphery of the lesions.

C. Sample Storage and Incubation

Samples must be inoculated onto TSA or media of equal growth supportive value for *Y. ruckeri* and *A. salmonicida*. TSA cultures shall be incubated at 20°-25°C as soon as possible, but if this cannot be accomplished within 24 hours, the cultures should be cooled to 10°C or lower but shall not be maintained at freezing temperatures. Cultures must be placed in incubators within 72 hours after collection. Samples collected for *R. salmoninarum* must consist of hindgut/feces or kidney tissues (as allowed above) and may be spread onto slides on site or preserved and returned to the laboratory.

D. Identification Methods

1. *R. salmoninarum*

Fluorescent antibody techniques (direct or indirect) must be employed for examining samples. A minimum of 50 fields using 1000X magnification, must be examined for the typical *R. salmoninarum* organism.

2. *Y. ruckeri* and *A. salmonicida*

Detection and identification of these gram-negative bacteria must be accomplished by isolating and characterizing the organisms in culture. Presumptive identification should consist of cultural, morphological, and biochemical characterization, followed by serological confirmation. If biochemical tests alone are employed to identify these bacteria, an optimized battery of tests with good separatory values should be used. A suggested battery for *Y. ruckeri* would be testing nonmotile organisms (motile at less than 37°C) giving an alkaline/acid TSI reaction and cytochrome oxidase-negative as follows:

Arabinose	-	Sucrose	-
Rhamnose	-	Malonate	-
Trehalose	+	Phenylalamine deaminase	-
Mannitol	+	Lysine decarboxylase	+

Refer to the most current edition of Bergey's Manual of Determinative Bacteriology for the characteristics of *A. salmonicida*. Some variations in the biochemical characters may occur and interpretation may be necessary.

Sampling of hindgut material increases the number of confounding organisms and some screening method may be necessary. The schema presented in Figures 1 and 2, Page 36 and 37, is appropriate for the presumptive identification of observed bacteria.

For confirmatory identification of the bacteria of interest, refer to the appropriate section in the description of each agent.

XV. Test Media, Reagents, and Methods

A. Media

1. TSA (Trypticase or Tryptic Soy Agar) or BHIA (Brain Heart Infusion Agar)

These are used for routine isolation and culture of most fish pathogens.

2. O/F (Oxidation/fementation) Basal Medium

This medium is used with 1% glucose to indicate how carbohydrates are utilized. A form for marine organisms is also available.

3. TSIA (Triple Sugar Iron Agar)

This medium is used for differentiating gram-negative bacteria.

4. Tryptone Broth

Tryptone Broth is used to test for indole production.

5. Urea Agar (or urea agar base)

Urea Agar is used for determining urease activity.

6. Rimler-Shotts (RS)

See Section II.F.4.

7. Cytophaga Agar

Cytophaga Agar is used for culturing flexibacteria:

Tryptone	0.5 g
Yeast Extract	0.5 g
Sodium Acetate	0.2 g
Beef Extract	0.2 g
Agar	11.0 g
H_2O to	1000.0 ml
pH	7.2

8. Shotts-Waltman (SW)

See Section V.F.7.

For instructions on preparing these media and interpreting the results, consult the supplementary literature prepared by the manufacturer or one of the references listed in Section XI.

B. Reagents, Methods

1. Gram Stain, Acid-fast Stain

These are commercially available. Detailed instructions for staining fish pathogenic bacteria are given in several of the references listed in Section XI.

2. Cytochrome Oxidase Test

Use either a commercially impregnated strip or make one: put a few drops of 1% aqueous solution of dimethyl-p-phenylenediamine oxalate on No. 1 filter paper. Positive is indicated by a change to blue color, negative - no change.

3. Vibriostatic Agent 0/129

The sensitivity disks are prepared as follows: saturate antibiotic assay filter paper disks with 0.1% (wt./vol.) solution of the compound in acetone. Dry at 37°C to evaporate the acetone, and store the disks in a tightly sealed bottle at 4°C. 0/129 (2,4-diamino-6, 7 diisopropylpteridine) is available from Calbiochem. Use a TSA plate.

4. Novobiocin Sensitivity Disks

Available commercially, use the 5 mcg per disk level. NOTE: The sensitivity tests for 0/129 and Novobiocin can be carried out on the same plate.

5. Motility

Examine log-phase cultures in wet mount preparations. If this method gives doubtful results, check by stab-inoculating tubes of motility test medium.

XVI. Serological Procedures

Confirmation of diagnosis is usually based on serology. At this time, FA (Fluorescent antibody) and slide, microtiter, or test tube agglutination are the tests most often employed. New techniques for rapid detection of fish pathogens are being developed currently. There will, no doubt, be introduced more efficient and sensitive methods than those discussed here in the near future.

A. Fluorescent Antibody Test (FAT)

Direct FITC-labeled conjugates have been prepared, standardized, and lyophilized for *R. salmoninarum*, *A. salmonicida*, *E. tarda*, *V. anguillarum*, and *Y. ruckeri*. These are available from the Biologics Section, National Fish Health Research laboratory. Antisera for these pathogens are also made at many private and government laboratories around the country, and are generally available upon request.

1. Direct FAT

(a) Place bacterial smear or tissue smear on a clean slide and dry at 60°C for 5 minutes. Also, set up positive and negative controls.

(b) Rehydrate conjugate, dilute as necessary. Drop dilutions on smears: unknown sample, known positive sample, and negative control sample.

(c) Drop dilutions of bacteria-specific antiserum on smears: unknown sample, known positive sample, and negative control sample.

(d) Allow antisera to react with smear for 5 minutes or longer in a dark humid chamber.

(e) Rinse slide with PBS (pH 7.2) and wash in PBS for at least 2 minutes.

(f) Air dry, add pH 9.0 mounting fluid and coverslip. Examine under oil immersion.

Apple-green fluorescence gives positive identification of bacteria. If storage is necessary, use 4°C and protect the slides from light.

2. Indirect FAT

This technique makes use of FITC-labeled goat antirabbit serum and bacteria-specific antiserum from rabbits.

(a) Place bacterial smear or tissue smear on a clean slide and dry at 60°C for 5 minutes. Also, set up positive and negative controls.

(b) Rehydrate both antisera, dilute as necessary.

(c) Drop dilutions of bacteria-specific antiserum on smears: unknown sample, known positive sample, and negative control sample.

(d) Allow antisera to react with smear for 5 minutes or more.

(e) Rinse slide with PBS (pH 7.2), then wash in PBS for 2 minutes and blot dry.

(f) Overlay smears with FITC-labeled goat antirabbit serum. Allow reaction 5 minutes or more.

(g) Rinse and wash as in Step (e) above.

(h) Add pH 9.0 mounting fluid and coverslip. Examine under oil immersion. Apple-green fluorescence gives positive identification of bacteria. If storage is necessary, use 4°C and protect the slides from light.

B. Macroscopic Slide Agglutination Test

1. Clean a microscope slide and mark circular divisions with a wax crayon.

2. The dilutions of all antigen suspensions (unknown and controls) should be standardized to a known concentration of particulate materials; i.e., McFarland standard #3 or a spectrophotometer reading of 40% T, at 645 nm. However, a "cloudy" suspension is sufficient in some cases.

   Add one drop of each bacterial suspension (unknown and controls) to 3 previously labeled spots (6 total). If using bacteria from a solid medium, emulsify in saline solution on the slide and examine to ascertain that the bacteria are well separated and not in visible clumps.

3. Add a drop of the specific antiserum for the suspected bacteria to two drops of the antigen suspensions. Mix gently with an applicator stick.

4. Add a drop of normal serum to two drops of the antigen suspensions. Mix gently with an applicator stick.

5. Observe reactions immediately; then 5 and 10 minutes later. Agglutination or clumping of the test bacteria and the positive control (but not the bacteria only or normal serum) is a positive test.

C. Quantitative Agglutination Tests

1. Test Tube Agglutination Test

   This test will give the antibody titer of the antiserum used, in addition to positive confirmation of the diagnosis. It is based on the same technique of mixing bacterial suspensions with antiserum. The antisera are doubly diluted serially.

2. Microtiter Agglutination Test

   Basically, this is the test tube agglutination test on a very small scale. Using special microtiter plates, microdiluters, and pipette droppers, the antisera are serially double diluted and a specific amount of particulate antigen is added. In addition to antibody titer, cross reactivity of antigens and antisera can be demonstrated.

D. References

Details on these serological techniques and others are in the references previously cited. In addition, one can find information in the following draft publication: Fish Biologics Guide. Regimens and protocols for the production and use of antisera, antigens, and other reagents for fish disease serodiagnostics. Authors are D. P. Anderson and O. W. Dixon at the National Fish Health Research Laboratory, Kearneysville, West Virginia.

SECTION 4

METHODS FOR THE DETECTION OF CERTAIN PARASITIC DISEASES

Section 4

Methods for the Detection of Certain Parasitic Diseases

I. General Procedures for Parasitology

A. External Parasites

1. Visible to the Naked Eye

Examine all body surfaces and gills carefully; some ectoparasites can be seen with the naked eye.

2. Visible at 10 X Magnification (Dissection Microscope)

Many parasites which cannot be seen with the naked eye can be seen at 10 X if the light is adjusted properly. This magnification offers the advantage of scanning a large surface area and finding parasites that might be missed at higher magnification. Larger protozoa such as Ichthyophthirius, some trichodinids, Ambiphrya, Epistylis, and Chilodonella can be seen at 10 X.

3. Visible at 100 X to 500 X

Prepare wet mounts of gill filaments and mucus scraped from the dorsolateral surface of the fish. All motile ectoparasites can be seen at 100 X, but frequently the use of higher magnification is helpful.

B. Internal Parasites

1. Visible to the Naked Eye

After the fish has been opened and bacteriological samples collected, examine all organs carefully for the presence of cysts, worms, or unusual appearance. Examine the eyes and brain and place them in separate dishes of water or 0.85% saline.

2. Visible at 10 X

Examine the viscera, the gills, excised eyes (externally and internally), and the brain under the dissection scope. In salmonids, from the western United States, gill arches and larger kidney blood vessels should be stripped with forceps and observed at 10 X for Sanguinicola. The alimentary canal should be opened in water and examine carefully. If there is too much fecal material, use the procedure for Bothriocephalus achellognathi (IV.E.1.).

3. Visible at 100 X to 500 X

Wet mount preparations should be made of intestinal contents, urinary and gall bladders, kidneys, liver, brain, and any suspect cysts. All motile parasites can be detected at 100 X, some spores can be seen at 200 X, but others can be found only at 500 X.

C. Procedures for Killing and Preserving Some Parasites

1. Protozoa

If parasites are numerous, simple killing and preservation in 10% formalin is satisfactory if further study is needed. Dried blood smears containing Cryptobia (synonym Trypanoplasma) or other haemoflagellates may be stored for several months. If species identification of trichodinids, Tetrahymena, and Chilodonella is desired, smears should be made and air-dried. Prepared slides can be stored dry for several months but should be stained by Klein's silver nitrate method for examination.

2. Monogenea and Digenetic Trematodes

Kill in 90°C, 10% formalin and preserve in 10% formalin. Encysted forms should be freed before killing.

3. Tapeworms

Free encysted worms and kill in 80°C fresh water; preserve in 10% formalin.

4. Nematodes

Free encysted worms, kill in 70°C, 70% alcohol. (FIRE HAZARD: place vial of alcohol in a small beaker of water for heating.)

5. Acanthocephala

When the proboscis is extended, drop the worm in 90°C, 10% formalin. If the proboscis is not extended, hold in refrigerator overnight in distilled water.

6. Parasitic Copepods and Argulus

Kill and preserve in 70% alcohol. Also include juvenile and larval forms if present on fish. NOTE: Alternative methods for killing, fixing, and preserving parasites are provided in: Pritchard, M. H., and G. O. W. Kruse. 1982. The collection and preservation of animal parasites. Technical Bulletin No. 1. The Harold W. Manter Laboratory, University of Nebraska Press, Lincoln, Nebraska. 141 pages.

D. Staining, Processing and Mounting of Specimens for Study

See Meyer, M. C., and O. W. Olson. 1975. Essentials of parasitology. Pages 263-270. Wm. C. Brown Co., Dubuque, Iowa; Pritchard and Kruse, 1982. (See reference citation above.); or similar publications.

II. Whirling Disease of Salmonids

A. Name of the Disease and Etiological Agent

Whirling disease is caused by the sporozoan parasite Myxosoma cerebralis. Synonym: Blacktail.

B. Known Geographic and Host Range of the Disease

1. Geographic Range

California, Connecticut, Massachusetts, Michigan, Nevada, New Jersey, Ohio, Pennsylvania, Virginia, and West Virginia. The agent has also been found in Europe, United Kingdom, New Zealand, South Africa, and the USSR. – Oregon (1987)

2. Host Range

All species of salmon, trout, 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, thereby creating the possibility for accidental transfer of asymptomatic carriers. Brook trout (Salvelinus fontinalis) and rainbow trout (Salmo gairdneri) are very susceptible. Intensity of exposure and the age of fish when exposed affects the severity of the disease (O'Grodnick 1979).

C. Clinical Signs of Diagnostic Significance

1. Whirling

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.

2. Blacktail

The posterior trunk and tail of young fingerlings may turn dark, especially in fish exposed at an early age.

3. Skeletal Deformities

As the fish grow, the primary signs of the disease in infected fish are skeletal changes such as misshapen skulls and twisted spines.

4. Mortality

Loss rate is dose and age dependent; most serious losses occur in young, heavily infected fish.

D. Diagnostic Procedures for Disease Situations

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 sample with an equal volume of 10% formalin (to kill

viable spores to 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 one percent hydrochloric acid sufficient to cover the sample for one to several hours. Wash all grindings into a small beaker with water and allow material to settle. Sediment can be examined directly in wet mounts at 400 X or, using the method of MacLean (1971), spread 5 to 10 drops of sediment onto a clean glass microscope slide and allow to air dry. The slide is stained with 1% aqueous malachite green for 5 minutes, rinsed in tap water and destained by placing for 30 seconds each into 70, 90, and 100% ethyl alcohol. Air-dried slides are coated entirely with a thin film of low viscosity immersion oil and examined with the 20 X objective lens and 10 X eye pieces (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 200 X, 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 Myxosoma cerebralis spores.

Myxosoma cerebralis is the only species of Myxosoma found in the cartilage of salmonids (the genus Myxosoma can be verified by the absence of an iodinophilous vacuole, which is present in Myxobolus). Myxosoma squamalis occurs in the scales of western U.S.A. salmonids, is about the same size as M. cerebralis (about 9 um), 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, is about the same size as M. cerebralis, but possesses an iodinophilous vacuole. Myxobolus neurobius, more widespread geographically, also is found in the central nervous system, but is larger -- 10 to 13 X 7.5 to 8 um. If there is any confusion, contact a fish parasitologist or a fish histopathologist qualified to contribute to the identification of Myxosoma cerebralis infection. If serological identification is needed, proceed as in "F" below.

E. Procedures for Detecting Asymptomatic Infections

Select a sample size that provides 95% confidence of detecting M. cerebralis in a population having a 5% prevalence of infection (see Table 1, Section 1, General Sampling Procedures). 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 fingerlings about 5 months old. 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:

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 5% 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 entire filtrate to a separatory funnel which discharges by gravity into the central feed line of a plankton centrifuge (026WA106 plankton centrifuge, Kahl Scientific Instrument Corp., P.O. Box 1166, El Cajon, California 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 refrigerate 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 slip. Systematically search each test area for approximately 2-1/2 minutes or until spores are found.

NOTE: Provisional method for detecting Myxosoma cerebralis spores in adult salmon and trout. (Proposed by Rich Holt, Oregon Department of Fish and Wildlife, Department of Microbiology, Oregon State University, Corvallis, Oregon 97331).

(1) Pool together two gill arches from each of six adult fish. Do not exceed 20 g per pool.

(2) Briefly heat to 60°C and strip away soft tissues.

(3) Transfer to 10 volumes of Ebner's solution (8% hydrochloric acid in 3% aqueous sodium chloride) and soak overnight to soften heavy cartilage.

(4) Rinse with water and proceed as in "E.1.b." above.

2. Digest Method

(a) Remove heads from the fish that have been sampled. If necessary, refrigerate at 5°C. This method works only with fresh or frozen material - not with tissues that have been fixed in formalin.

(b) Heat heads in 50°C water for 5 to 10 minutes. Remove and discard lower jaw, eyes, skin, and soft tissues, but save gill arches and cranial cartilage or bone or both. Weigh to nearest gram.

(c) Mincing of cranial elements of young fish is optional. Bony heads of older fish should be ground in a food chopper. Mix

well and examine a small sample for spores; if none are found, proceed with step "d" below. If it is desired that efficiency of spore recovery be quantified, spores that have been stained with silver nitrate may be added prior to mincing.

(d) Add 20 to 25 volumes of pepsin solution* and digest at 37°C. Thirty minutes digestion time will usually suffice for material from young fish. Up to 4 hours may be required for reduction of bone from adult fish. Centrifuge digest at 1200 X g for 10 minutes at 20° to 15°C. Discard the supernatant fluid.

(e) Add 15 to 20 volumes trypsin solution** to the pepsin residue from step "d" above, and adjust pH to 8.0 to 8.5. Digest at 20° to 25°C with stirring for 30 minutes.

(f) Stop digestion by adding one-tenth volume of serum or one-tenth volume of bovine serum albumin 10% solution. Remove undigested residues by filtration through a pad of glass wool or fine mesh material.

(g) Centrifuge suspension at 1200 X g for 10 minutes at 20° to 25°C. Discard supernatant. Resuspend pellet in 8 to 10 volumes of water or balanced salt solution. Examine for spores, if none are found, proceed with step "h" below.

(h) To centrifuge tube(s) or bottle of appropriate size, add about 5 cm of depth of 55% aqueous dextrose solution; then carefully layer onto the dextrose about 1 cm of depth of tryptic digest residue. Do not mix. Centrifuge at 20° to 25°C at 1200 X g for about 30 minutes in a swinging bucket rotor. Aspirate and discard all of the liquid material over the pellet.

(i) Resuspend the pellet in 2 to 4 volumes of water and examine microscopically for spores.

F. Serological Identification of Myxosoma cerebralis

This may be accomplished by the direct fluorescent antibody technique (FAT) that follows. This test works best with fresh spores. Spores that have been stored in formalin for a week or more show reduced

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*0.5% pepsin solution, pH 1.8 to 1.9, in 0.5% HCl. Dissolve 5 g powdered pepsin in 1000 ml water and add 5 ml concentrated HCl. Solution may be refrigerated for up to 1 month or frozen.

**0.25% trypsin in Rinaldini's solution. Make a paste of 2.5 g trypsin powder and Rinaldini's solution then dilute to 1 liter.

Rinaldini's solution:

NaCl	8.0 g
KCl	0.2 g
$C_6H_5Ha_3O_7 \cdot 2H_2O$ (sodium citrate)	1.0 g
$NaH_2PO_4 \cdot H_2O$ (sodium phosphate, monobasic)	0.05 g
$NaHCO_3$	1.0 g
Glucose	1.0 g
Distilled or deionized water	1.0 l
Phenol red, 1% solution	1.0 ml

Frozen storage only.

specific fluorescence, and older specimens show little or none (Wolf and Markiw, 1985).

NOTE: FITC conjugated-rabbit and anti-*Myxosoma cerebralis* antisera, known positive, and known negative control materials are available in small quantities upon written request to the Biologics Section, National Fish Health Research Laboratory, Route 2, Box 50, Kearneysville, West Virginia 25430.

1. Transfer the suspect suspension from step "g' above to a labelled centrifuge tube and concentrate the residues by centrifugation at 1200 X g for 10 minutes at room temperature. Decant the supernate and, by the use of wet mounts, adjust the concentration of the residues with water to a volume that permits best observation of spores among the debris.

2. Thoroughly clean a FAT slide with detergent and deionized water and rinse well with acetone. Label one or more circular areas for known positive *Myxosoma cerebralis* (+ control) or other known sporozoan (- control), and for material to be tested (unknown).

3. Lightly coat slide with Tissue Bond (Harleo) or 50% egg albumin (egg white minced with scissors and filtered through gauze and mixed with an equal volume of glycerin -- add several drops of chloroform to preserve and store in refrigerator).

4. Apply small drops of unknown and control material to appropriate spots on the coated slide and dry the slide at 50° to 60°C for 15 to 20 minutes. Fix slide in absolute methanol for 5 minutes and air dry.

5. Apply fluorescein isothiocyanate conjugated rabbit anti-*Myxosoma cerebralis* serum to each test spot. Allow serum to react for 30 to 60 minutes in the dark at room temperature.

6. Gently rinse conjugated antiserum from slides with pH 9.0 to 9.5 buffer ($NaHCO_3$: 33.6 g, plus Na_2CO_3; 10.6 g in 1000 ml water) then soak slides in buffer, with gentle agitation for 5 minutes. 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 1000 X on a fluorescent microscope.

CAUTION: Cartilage debris and spores may auto-fluoresce under UV light. This fluorescence is quite yellow. Positive identification of *Myxosoma* cerebralis depends upon the detection of spore and prespore stages which fluoresce "apple green."

G. Disinfection

From the outset, an awareness should be maintained that whirling disease spores may be present if fish samples which, if untreated, could result in the accidental dissemination of the disease. All equipment, expendable supplies, toweling, and dissected tissues should be thoroughly disinfected with 10% formalin or a 1:1 solution of household liquid bleach and water. Hot smoking (66°C for 40 minutes) kills the spores (Wolf and Markiw, 1982) so heat disinfection should be effective. If infected fish samples are to be retained, they should be preserved in 10% formalin. In the event of

work with positive fish, it is probable that there will be mechanical carry-over of spores from sample to sample on equipment used in the procedure. While this may enhance detection, it can be avoided by taking the following steps:

1. Expendable items (microscope slides, cover-slips, pipettes, toweling, etc.) should be disinfected, cleaned, and discarded or incinerated.

2. Reusable equipment should be thoroughly disinfected, for containment purposes, and rigorously cleaned before reuse.

H. Procedures for Determining Prior Exposure to the Etiological Agent

Griffin and Davis (1978) have described a serodiagnostic test for detecting circulating antibodies indicating Myxosoma cerebralis infection in rainbow trout. Still to be developed, however, is an analysis of cross-reactivity with other myxosporeans, knowledge of the age of the fish when antibody is detectable, temperature relationships, and "an understanding of the predictive value of specific antibody as an indicator of the presence or absence of infection." The method has not been field tested.

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

Samples collected from apparently normal, moribund, or dead fish should be packed on ice for shipment. Heads should be "salted" with powdered antibiotics (outdated drugs are satisfactory) to prevent the rapid growth of normal microbial flora. Infected fish, mud, or water should not be transported to areas free of whirling disease without taking effective disease containment precautions. Fish or tissues preserved in 10% formalin can, if necessary, be processed through the blender and plankton centrifuge.

J. Procedures to be Followed When Salmonids or Salmon Eggs to be Imported Come From a Geographic Area Where Myxosoma cerebralis has Never Been Found and Where There is No Reason to Suspect Introduction of the Parasite (Olson and Holt, 1982)

1. Importation of Juvenile Salmonids Between 4 and 12 Months of Age

Follow standard methods suggested above and examine 60 heads from stock to be imported.

2. Importation of Eggs or Fingerling Salmonids under 4 Months of Age

Using above techniques, examine area of the posterior skull containing the semicircular canals from each of 60 parent salmonids or if broodstock in limited supply, examine 60 heads from 4- to 12-month-old salmonids from the same water supply.

3. Disinfect eggs and/or water in which fish are shipped with an iodophor (100 ppm for 10 minutes).

K. Procedures to be Followed When Salmonids or Salmonid Eggs to be Imported Come From a Geographic Area Where Myxosoma cerebralis has Been Reported (Olson and Holt, 1982)

1. No salmonids shall be imported from watersheds where M. cerebralis has been reported; salmonid eggs shall not be imported from these watersheds unless the parasite has been absent in

yearly samples of 4- to 12-month-old salmonids for a period of 5 years.

2. Salmonids from watersheds where *M. cerebralis* has not been reported, but where its presence is considered to be a strong possibility due to proximity to geographic areas where infected salmonids have been found, will require the following examinations:

   (a) Importation of juvenile salmonids between 4 and 12 months of age: Examine 60 heads from stock to be imported using methods described above and provide documentation that *M. cerebralis* has been absent at the origination site for the previous 2 years.

   (b) Importation of eggs or fingerling salmonids under 4 months of age: Using above techniques, examine 60 heads from 4- to 12-month-old salmonids from the same water supply and provide documentation that *M. cerebralis* has been absent at the originating site for the previous 2 years.

   (c) Disinfect eggs and/or water in which fish are shipped, with an iodophor (100 ppm for 10 minutes).

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III. Salmonid Ceratomyxosis

A. Name of the Disease and Etiological Agent

Salmonid ceratomyxosis, Ceratomyxa shasta.

B. Known Geographic and Host Range of the Disease

1. Geographic Range

(a) Areas where C. shasta can be transmitted to susceptible fish:

(1) Oregon

Deschutes River System, Columbia River below its confluence with the Deschutes River, Willamette River from its mouth to approximately 100 miles upstream, Nehalem River and Rogue River.

(2) Washington

LaCamas Creek and LaCamas Lake, and the Cowlitz River.

(3) California

Pitt, Feather, Sacramento, and the Klamath River (including Klamath Lake in Oregon) systems.

(4) British Columbia

Fraser River

(b) Areas where infected adult fish have been found:

(1) Oregon

Widely distributed through the Columbia River Basin, Nehalem River, Rogue River, and the Trask River.

(2) California

Widely distributed through the Sacramento and Klamath River Basins.

(3) Washington

Widely distributed through the Columbia River Basin.

(4) Idaho

Found in salmon returning to Columbia River Basin tributaries originating in this state.

(5) British Columbia

Reported in salmon at several widely scattered locations.

(6) Pacific Ocean

Occasionally observed in ocean-caught salmon.

2. Host Range

Coho salmon (Oncorhynchus kisutch), chinook salmon (O. tshawytscha), sockeye salmon (O. nerka), chum salmon (O. keta), rainbow and steelhead trout (Salmo gairdneri), cutthroat trout (S. clarki), brown trout (S. trutta), Atlantic salmon (S. salar), and brook trout (Salvelinus fontinalis). Differences in susceptibility among strains are known to exist and should be considered when stocking salmonids within the endemic range of C. shasta.

C. Clinical Signs of Diagnostic Significance

1. Juvenile Salmonids

(a) Development of the infective stage of C. shasta is retarded when water temperatures are below 10°C, accounting for the seasonal occurrence of this disease. Above this temperature, some or all of the following signs may be observed:

(1) Cessation of feeding.

(2) Abdomen becomes distended and filled with ascitic fluid.

(3) Exophthalmia.

(4) Hemorrhagic areas develop in intestine, rectum may prolapse.

(5) Thickening of intestinal wall and lesions in various internal organs and body muscle.

(6) Mortality may become catastrophic.

2. Adult Salmonids

(a) Swollen and hemorrhagic areas may develop in the intestine; perforation of the intestinal wall may also occur with a resultant peritonitis.

(b) Spores can usually be found in the intestine and gall bladder. On occasion, spores may be recovered from lesions in other visceral organs.

D. Diagnostic Procedures for Disease Situations

1. Examination of Dead Specimens

(a) Prepare wet mounts of material from the lower intestinal wall or ascitic fluid if present. Contents of the gall bladder and lesions present in any tissue should also be examined. Use phase contrast or bright field light microscopy (440 X) for examination of wet mounts.

(b) Dried smears may be stained by the Ziehl-Nelsen method but without heating. By this method, the polar capsules stain red against a bluish sporoplasm and background.

(c) Permanent preparations can be obtained from smears fixed in Schaudin's fixative and stained with Heidenhain's Iron Hematoxylin.

2. Examination of Live Specimens

(a) An intestinal lavage technique (Coley, 1979) can be used for collection of Ceratomyxa shasta spores. The equipment used in the lavage technique is a 10 cc glass syringe with approximately 15 cm of 6.35 mm diameter (outside) Tygon tubing attached to the end of the syringe. The free end of the tubing is beveled to eliminate sharp edges and to aid in entering the anus of the fish. Fish to be examined are first anesthetized, then 5-6 cc of physiological saline is dispensed from the syringe into the rectum. The tubing is to be inserted approximately 5-7 cm or until it occupies about one-half the length of the lower intestine. Following injection of the saline, the plunger is slowly retracted while simultaneously withdrawing the tube. The slow withdrawl of the tupe while collecting the lavage fluid is to prevent the collapse of the intestinal wall and/or blockage of the tube by the intestinal wall. Following the collection of 3-5 cc of fluid from the intestine, the tube can be removed. The samples are then dispensed into screw-cap vials and held at 4°C or on ice until processing. The samples are then centrifuged at 320 X g for five minutes to concentrate spores and other stages of the parasite. Smears are made from the concentrated material and stained with a modified Ziehl-Nelson method.

E. Procedures for Detecting Asymptomatic Infections

1. The plankton centrifuge as used for whirling disease has proven useful, but by using intestine instead of skeletal parts (W. G. Taylor, USFWS, personal communication).

2. See diagnostic procedures given in "D" above.

3. See discussion of sampling procedures for Myxosoma cerebralis. Ceratomyxa shasta may be considered to be absent from a population when a sample size sufficient to give a 95% confidence level of detection (assuming a carrier rate of 5%) is examined and found negative (See Table 1, Section 1, General Sampling Procedure).

F. Procedures for Determining Prior Exposure to the Etiological Agent

At the present time, no serological tests have been developed.

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

Samples may consist of living, moribund, or dead fish which may be frozen prior to examination. To avoid the spread of C. shasta, it is recommended that fish, mud, or water not be transported away from the known geographic range of the parasite.

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IV. Bothriocephalus achellognathi (Asian tapeworm)

A. Name of the Disease and Etiological Agent

Bothriocephalosis. Bothriocephalus achellognathi Yamaguti (Cestoda: Pseudophyllidea). Synonyms: B. gowkongensis Yea, 1955; B. opsariichthydis Yamaguti, 1934; B. phoxini Molnar and Murai, 1983.

B. Known Geographic and Host Range of the Disease

1. Geographic Range

Southeastern United States, Southern Europe, Southern Asia.

2. Host Range in United States

Golden shiner (Notemigonus crysoleucas), fathead minnow (Pimephales promelas), grass carp (Ctenopharyngodon idella), mosquito fish (Gambusia affinis).

3. Host Range in Europe and Asia

B. achellognathi has been found in 23 species including Ctenopharyngodon idella, Cyprinus carpio, Aristichthys nobilis, Silurus ganis (catfish), Lucioperca lucioperca, Perca fluviatilis, and Gambusia affinis (Osmanov, 1971).

C. Clinical Signs of Diagnostic Significance

High intensity infection in young fish: intestinal blockage, occasionally intestinal perforation, emaciation, death. Low intensity infection: no obvious signs.

D. Diagnostic Procedures for Disease Situations

1. Presumptive Diagnosis

Presence of tapeworms in the intestine of any of the above hosts.

2. Confirmatory Diagnosis

Bothriocephalus achellognathi (previously also called B. opsariichthydis and B. gowkongensis) is a segmented tapeworm which has a flattened scolex (head) with 2 bothria (lateral, deep, elongated, sucking grooves), no hooks and no tentacles (see Figure 1). In dorsal or ventral view (normal viewing position of the worm), the scolex takes a strong pit viper or arrow head appearance when extended and a balled or fist-shaped appearance when contracted. In lateral view, the scolex is rectangular when extended and balled when contracted. The posterior section of the scolex in the dorsal or ventral view is about 3-3 1/2 times the width of the first proglottid (segment) in both extended and contracted positions. Marsipometra and Eubothrium spp., tapeworms which can also have arrow shaped scolices, usually have scolices less than 3 times the width of the first segment. B. opsariichthydis have no neck. The neck which is present and very obvious on Marsipometra species and sometimes present in Eubothrium species is a non-segmented area, posterior to scolex, anterior to the first obvious segment and is 2 or more times the average length of the anterior segments (see Figure 1). B. opsariichthydis also has no dorsal and ventral medium furrow.

This furrow, present in Eubothrium spp., is not always clearly visible for the full length of the worm, but with careful viewing, short sections of the furrow are evident. The furrow may appear as a small indentation on the posterior portion of several proglottids (see Figure 1).

Other characters can be used for identification, but are much less clear or involve staining and mounting of specimens. Two are mentioned here (see Figure 2). The eggs of B. opsariichthydis are operculated whereas neither are from Marsipometra or Eubothrium. To see the operculum, one needs to observe several eggs (10-15) at about 400 power, vary the light intensity and the operculum may show up on some of the eggs. The operculum occurs on the more pointed end of the eggs. Specimens can be also fixed, stained, and mounted so that the internal structure is visible. B. opsariichthydis has a median genital pore (Figure 2) whereas the pore is marginal for Marsipometra and Eubothrium. It may be quite difficult to see even when stained well.

In case of doubt, a sample of tapeworms should be killed in 80°C water, preserved in 10% formalin, and mailed to a parasitologist. In some cases, mature segments must be stained and studied to achieve a correct identification.

E. Procedures for Detecting Asymptomatic Infections

Sample size should be adequate to detect the presence of infected fish at a 5% level of prevalence (see Table 1, Section 1, General Sampling Methods).

1. Procedures to be Followed When Fish are Less Than 4 Inches in Total Length

   (a) Starve fish for 48 hours and squash the intestinal tract between two microscope slides. View under 30-100 X magnification using reflected or transmitted light. For positive identification of the parasite, see part D.2. of this section.

   (b) Alternatively, starve fish for 48 hours and then scrape the opened intestinal tract with a glass slide and examine the contents for the parasite. This latter method can be used with pooled samples (Johnson, 1982, personal communication). If there is too much fecal material for examination, pour intestinal contents into a bottle (about 5 by 15 cm), fill with water, shake vigorously, allow to stand 15 minutes, aspirate most of the fluid carefully and examine sediment at 10 X.

2. Procedures to be Followed When Fish Larger Than 4 Inches in Total Length

   (a) Prepare as above, but pool in groups of 5 fish (the number to be used depends on the size and amount of intestinal contents). With larger fish, flush the excised alimentary canal with water before slitting.

F. Procedures for Determining Prior Exposure to the Etiological Agent

At the present time, no serological or other tests have been developed for use in detection of Bothriocephalus acheilognathi.

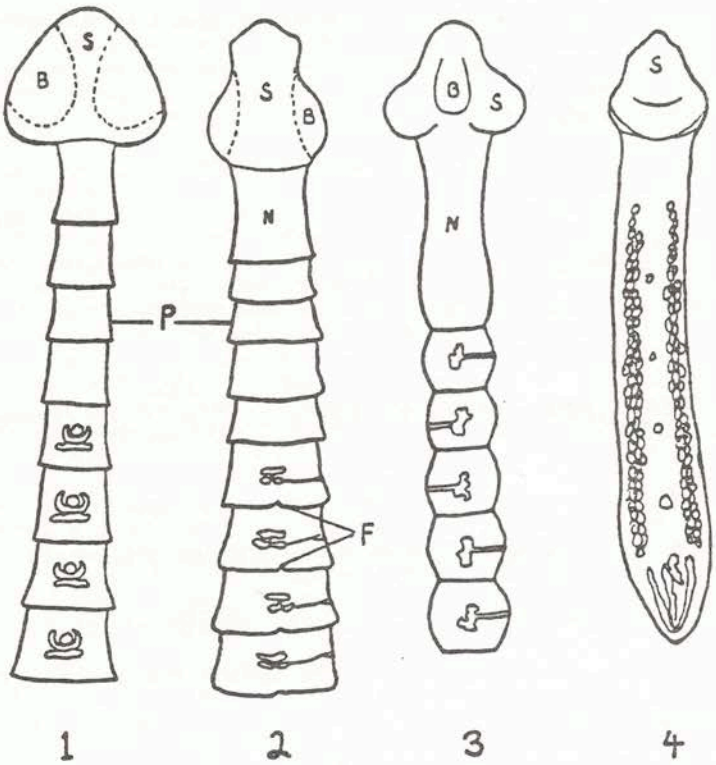

Figure 1. (1) Bothriocephalus acheilognathi; (2) Eubothrium salvelini; (3) Marsipometra parva; (4) Glaridacris laruei (unsegmented); B - bothria; F - furrow; N - neck; P - proglottid; S - scolex. (Drawing by Drew Mitchell).

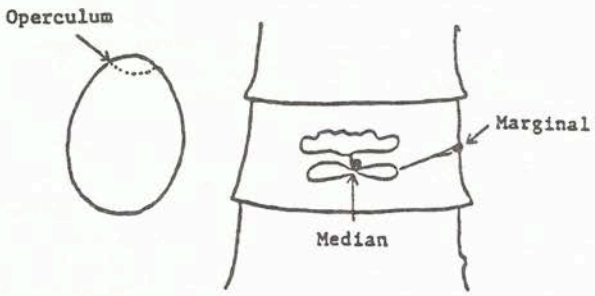

Figure 2.

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

Submitting live fish is best; if this is not possible, kill a sample of the tapeworms in 80°C water and transfer to 10% formalin.

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V. Proliferative Kidney Disease (PKD)

A. Name of the Disease and the Etiological Agent

Proliferative kidney disease, the etiological agent of which is currently unnamed. It is often referred to in the literature as "PKX". It is thought to be a myxosporan.

B. Known Geographic and Host Range of the Disease

1. Geographic Range

The disease has been reported to occur throughout the major rainbow trout producing countries of Europe. It has also been reported to occur in California, Idaho, Washington, and in British Columbia.

2. Host Range

The disease is known to occur in rainbow trout and steelhead trout (Salmo gairdneri), brown trout (S. trutta), cutthroat trout (S. clarki), Atlantic salmon (S. salar), chinook salmon (Oncorhynchus tshawytscha), coho salmon (O. kisutch), grayling (Thymallus arcticus), pike (Esox lucius). It has been observed in both confined; i.e., hatchery and free-living populations of fish.

C. Clinical Signs of Diagnostic Significance

Clinical PKD is usually manifested by lethargy, generalized melanosis, hypoplastic anemia, distended abdomen, bilateral exophthalmia. The posterior kidney and spleen are often enlarged to several times their usual size and may contain grey granulomata, especially in long-standing cases. The clinical course of the disease is characterized as chronic. The mortality pattern is low, but persistent. Cumulative mortalities range from 5% to more than 90%. The clinical course usually abates spontaneously in mid- to late September through mid-October.

D. Diagnostic Procedures for Disease Situations

1. Differential Diagnosis

Clinically, the following diseases have similar manifestations: IHN, bacterial kidney disease (BKD), sanguincolliasis (Sanguincola klamathensis), nephrocalcinosis, and low-grade copper toxicity.

2. Presumptive Diagnosis

The presence of lightly staining extra- and intramacrophage protozoa containing 1-7 "daughter cells" in stained imprints of posterior kidney and spleen. The "parasitized" macrophages are often surrounded by small lymphocytes, the reported "satellite" condition.

3. Confirmatory Diagnosis

With transmitted electron microscopy (TEM), the primary cell contains multivesicular bodies, limpid bodies, mitochondria, and electro-dense bodies ("haplosporosomes") which contain an electron-lucent bar. With light microscopy, the organism is PAS-positive. In the kidney, particularly the posterior kidney,

there is marked lymphocytic hyperplasia to the point that the nephrons are often compressed. Organisms are often seen in the renal tubules and blood vessels. In the spleen, there is a marked diminution of the erythrocytic elements due to the lymphocytic hyperplasia.

E. Procedures for Detecting Asymptomatic Infections

Sample the suspect population in accordance with the method to provide a 5% prevalence detection level (see Table 1, Section 1, General Sampling Procedures). Collect acetone-fixed imprints of posterior kidney and 10% neutral-buffered formalin-fixed samples of posterior and mid-kidney, spleen and gastrointestinal tract. Early in the "PKD season"; i.e., mid-March to mid-May, acetone-fixed smears of pyloric caecal and large intestinal mucosa scrapings should be examined. The acetone-fixed imprints and smears may be stained using either the methylene blue technique or the Leishman-Giemsa method. The formalin-fixed material, after sectioning, may be stained using the PAS and/or the H&E techniques.

F. Procedures for Determining Prior Exposure to the Etiological Agent

No methods are currently available to detect previous infections with the PKD-causing organism.

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

All samples must be fixed on site in accordance with the procedure described in "E" above. The organism will deteriorate very rapidly in iced samples - often to the point that it becomes unrecognizable.

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SECTION 5

METHODS FOR THE DETECTION OF CERTAIN MYCOTIC DISEASES

Section 5

METHODS FOR THE DETECTION OF CERTAIN MYCOTIC DISEASES

I. Introduction

Previous editions of the "Blue Book" included mycotic diseases with other parasitic diseases.

The distinct nature of fungal infections requires that they be dealt with in their own section. The authors recognize that the methods used to detect parasitic infections are generally the same ones to be followed in this section. We also note that the listings in this section are certainly not exhaustive; however, we expect in future editions of the "Blue Book" this section on mycotic diseases will be more extensive and will continue to be given its own identity.

II. General Procedures For Mycology

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 at 10 X, 100 X, and 500 X 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 lense or dissecting microscope for the presence of fungi and lesions associated with them. 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 10 X to 500 X.

C. Procedures for Killing and Preserving Fungi

Simple killing and preservation in 10% formalin is satisfactory if further study is needed.

D. Staining, Processing, and Mounting of Specimens for Study

See Section I.D., General Procedures for Parasitology.

III. Ichthyophonus Disease

A. Name of the Disease and Etiological Agent

Ichthyophonus disease, Ichthyoponus hoferi (Ichthyosporidium hoferi).

B. Known Geographic and Host Range of the Disease

1. Geographic Range

Cultured trout in western United States, marine fish, aquarium fishes, worldwide.

2. Host Range

Complete lack of host specificity. Similar organisms have been reported from Amphibia, but it is unknown if these are the same as the forms occurring in fish. Ichthyophonus has occurred in carp, trout, and salmon and it could occur whenever raw fish flesh is fed to cultured fishes or the spores are allowed to accumulate in ponds.

C. Clinical Signs of Diagnostic Significance

Infected fish cease feeding and become lethargic. In acute infections, trout develop a gross lumpy appearance. 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.

D. Diagnostic Procedures for Disease Situations

Examine wet mounts of fresh kidney tissue or other suspect tissue for spherical bodies of various sizes ranging from 10 um to 100 um using a lower power (100 X) microscope. Observe spheres closely for hyphal protrusions. These germ tubes are diagnostically significant.

E. Procedures for Detecting Asymptomatic Infections

Sample size should be adequate to detect a 5% level of prevalence (see Section 1, Table 1, General Methods of Sampling). 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.

F. Procedures for Determining Prior Exposure to the Etiological Agent

At the present time, there are no serological or other tests developed for use in detection of Ichthyophonus.

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

Live or fresh fish are preferred. Infected tissue can also be stored frozen with no apparent adverse effect on spore morphology, but viability is unknown.

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IV. Miscellaneous Mycotic Diseases

A. External

Species of the phycomycete genera Saprolegnia, Achlya, Aphanomyces, Leptomitus, and Pythium seldom attack the skin of healthy fish; however, they commonly attack fish that have suffered stress from temperature shock, spawning injury, wounding, and lesions of other diseases. Fungi must be cultured for species identification.

B. Internal

1. Branchiomyces

Invades the gill blood vessels, most prevalent in fish raised in warm water of high organic content. The fungus can be recognized in gill filament wet mounts in which the "beaded" spores can often be seen in gill lamellae.

2. Other Internal Fungi

Several systemic fungal infections have been described. The most common are: Exophiala salmonis of channel catfish occurring in brain tissue, Scolecobasidum humicola and Phoma herbarum of salmonids, Basidiobolus of trout and turbot, and Fusarium of carp (Richards, 1978). The filaments and spores can be demonstrated in simple wet mounts of affected tissues. These fungi can be pathogenic and should be searched for.

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Appendix 1

Suggested Necropsy Procedure

The necropsy procedure assumes that the same fish will serve as the source of tissues for the various (bacterial, viral, parasitic) tests. A modified procedure may be required when working with very small fish. Material for use in examining for external parasites must be taken before antiseptic or disinfectant procedures are applied. After the body has been opened using aseptic technique, tissues for bacterial cultures or tests should be collected before proceeding. An idealized necropsy procedure is outlined below.

A. External Examination

Note and record all gross abnormalities such as unusual body color, presence of opaque films, exophthalmia, raised scales, eroded opercula, hemorrhagic areas, inflammation, ulcers, body swellings, and clubbed or abraded gills. Inoculate appropriate media with material from these lesions. Prepare and stain smears from these lesions. If a flexibacterial infection is indicated, prepare wet mounts of lesion scrapings and examine microscopically for typical forms. Examine small fish in water under a dissection microscope at 10 X; lift operculum to observe gills. With larger fish, use pieces of fins and gills--Monogenea and large Protozoa can be seen with properly adjusted light. Examine wet mounts of excised gill material, fins, and body scrapings for ectoparasites.

B. Technique for Opening the Body Cavity

1. Aseptic Precautions

Sterilize instruments by dipping in 70% ethyl alcohol, then ignite the alcohol before use. Disinfect the surface of the fish by wiping it with cotton or cellulose tissues soaked in 70% alcohol. Adequate disinfection has been achieved when the surface of the fish turns slightly opaque.

2. Incision and Cuts

With the fish placed on its right side, remove the left pectoral fin with scissors. Using a scalpel, make an incision in the body wall on the mid-ventral line opposite the posterior edge of the stump of the removed fin. Insert the blade of another pair of scissors in the incision and cut dorsally until resistance indicates that the upper extremity of the cavity has been reached. Then, taking care not to puncture the intestine, cut along the mid-ventral line until just short of the vent. Lift the flap of tissue thus obtained and loosen, with a blunt probe, any adhesion between it and the underlying viscera. Finally, starting near the vent, cut in a semicircular route to complete excision of the body wall.

C. Internal Examination

To reduce the risk of contamination, take bacterial inocula from the kidney and from any tissues that appear grossly abnormal immediately after opening the body cavity. Prepare and stain smears of these various materials. Record the appearance of the organs with respect to such features as: color and consistency, hemorrhage, inflammation, pustules, nodules or growths, and the presence or absence of food or mucus in the

stomach and intestine. Following this, remove tissues for other purposes, such as virological, myxosporean, and histological examinations. Important parasites may be found in organ systems of fish. With the aid of the dissection microscope, tease apart samples from all organs, including muscle and brain. Wet mounts should be made of kidney, urinary bladder, intestinal contents, liver, brain, blood, and any other suspect material.

D. Disposal of Samples

The receiving laboratory should handle and dispose of samples and other items liable to be infectious in a manner that precludes the dissemination of disease agents. All material, such as fish carcasses or tissues, transport containers and water, microbial cultures, and contaminated equipment should therefore be autoclaved, incinerated, or otherwise disinfected before being discarded.

Appendix 2

Additional Parasitic Diseases of Fishes

The following parasites may be very destructive to fish. Infected fish should not be transferred to fisheries facilities where such parasites do not exist. Highlights of host range, diagnosis, and other pertinent facts for these diseases are given below.

A. Protozoa

1. Ichthyophthirius multifiliis (Ich).

No host specificity but some fish such as golden shiners (Notemigonus crysoleucas) are not as vulnerable as salmonids, catfish, perch, and eels. Ich can be verified by its large size, presence of cilia, plasticity of form, and horseshoe-shaped macronucleus when mature. Sometimes Hemiophrys is confused with Ich, but a simple nuclear stain (methyl green 0.25% in 1% acetic acid) will demonstrate the 2 macronuclei of Hemiophrys. Inapparent infections are common and transfer of such fish presents a real danger to uninfected stocks.

2. Ichthyobodo necator (Costia necatrix).

Knows no fish host bounds and is even found on salamanders. Diagnosis is based on recognition of small (oval in face view, cupped in side view) forms or the attached form which is pear-shaped, found on gills and body (Becker, 1977).

3. Cryptobia salmositica (Trypanoplasma salmositica)

Occurs in blood of salmonids in the western United States and Canada and can be recognized by its motion in wet blood smears diluted in equal volumes of saline. Discovery during examination of excised gill filaments is not uncommon. Two flagella, one anterior and one posterior, can be seen (Becker, 1977).

4. Chilodonella cyprini

Is not host-specific, but is a problem only at 5°-10°C. Oval in face view, extremely flat in side view, about 60 um long, has 10-12 ciliary rows on each side; is most dangerous to warmwater fishes in early spring, but can be a problem anywhere the temperature is low (Hoffman et al., 1979).

5. Chilodonella hexasticha

Similar to the warmwater Chilodonella cyprini, has been a problem at 19°-20°C, it is smaller (41-48 um) and has fewer ciliary rows (usually 6-8). This protozoan may cause problems in pond culture of catfish and baitfishes (Hoffman et al., 1979).

6. Trichodina, Tripartiella, Trichodinella

Nearly all fish are vulnerable but there are many species of these parasites, some of which are specific for certain fishes or groups of fishes. Attempts should be made to identify troublesome species so that proper avoidance can be practiced (Hoffman, 1978).

7. Eimeria aurati (coccidiosis of goldfish).

Can be identified by demonstrating oocysts containing four spores which are found in sediment from fecal washes. Potentially dangerous parasite in goldfish culture (Hoffman, 1965).

8. Myxobolus insidiosus of Pacific salmon

This myxosporean heavily infects the musculature of cultured chinook and coho salmon at certain stream-fed hatcheries in Oregon and Washington. Fish become infected in March and April. Heavy infection may cause muscular damage resulting in swimming difficulty, white patches on the skin, and increased mortalities.

9. Pleistophora ovariae

Ovarian parasite of golden shiners and fathead minnows. Oocytes filled with spores are more opaque than healthy oocytes and can be seen with the naked eye. Spores are large for Microsporida (about 6.6 um long) which must be verified microscopically (see Summerfelt and Warner, 1970). These methods are the only ones available until the state of the art is improved. According to Mitchell (1982, personal communication), he may be close to developing a statistically valid method of isolating spores from ovarian tissue, but it will be expensive.

10. Pleistophora salmonae

Small cysts in gills of salmonids. Spores about 4.5 um long. Work in progress by Carol Morrison, Halifax, Nova Scotia, may prove this to be Nosema or Glugea.

11. Glugea hertwigi

Microsporidan causing huge cysts in the viscera of rainbow smelt in Lake Erie, Northeastern United States and Canada. Many juvenile smelt are killed during natural spring epizootics (Dechtiar, 1965; Nepszy et al., 1978).

12. Dermocystidium salmonis

Small cysts in gills and fins of salmonids. Small, spherical spores that always possess a very large vacuole (Davis 1947; Pauley, 1967).

13. Henneguya sp. of Channel Catfish (H. postexilir and H. longicauds)

(a) Gill filaments from at least 60 fish should be removed and examined microscopically for the presence of cysts between secondary lamellar filaments. Fresh or preserved material may be examined.

(b) Cysts of H. longicauda will appear polysporous, whitish in fresh material, grayish-brown in preserved material; circular to oblong (130-370 X 110-120 um). Cyst walls smooth, thin without protrusions of host tissue; propagative cells near edge of cyst with mature spores and more advanced stages more central; monosporous sporoblast. Cysts of H. postexilis will appear polysporous, circular to oblong ranging 12 X 12-80 X 75 um; small dense cysts with no mature spores as well as large loosely packed cysts with developmental stages and mature spores present; early developmental stages near outer edges, mature spores and

advanced stages more central; surface of cysts smooth without protrusions of host tissue; cyst wall approximately 1 um; monsporous sporoblast. Larger cysts up to 1,110 um should not be mistaken for the above species, as they may be caused by H. exilis.

(c) Once cysts are identified, they should be excized, placed into distilled water, teased apart and air-dried onto slides. Slides can then be stained with 1% basic fuchsin for 10 minutes, dipped 2-3 times in 2 changes of 95% and 100% ethanol, then in 2 changes of xylene and mounted in permount. Stained spores are definitely identified by microscopic examination under oil emersion, using guidelines described by Minchew (1977).

C. Monogenea (gill and body flukes)

1. Gyrodactylus

Many species, usually species-specific for fish host. Possesses two large posterior anchors, no eye spots, and an embryo with anchors and hooks in utero.

2. Dactylogyrus (gill flukes)

Many species, usually on cyprinids. Goldfish Dactylogyrus is probably the most important. Possesses two large posterior hooks, four anterior eye spots, and egg(s) in utero.

3. Cleidodiscus (gill flukes)

Many species, on many fishes. Catfish Cleidodiscus can be dangerous to fry. Possesses four large posterior anchors, four anterior eye spots, and egg(s) in utero (Hoffman, 1979).

D. Digenetic trematodes

Adults or metacercariae in fish.

1. Diplostomulum spathaceum (eye lens fluke)

Found in many fishes. The metacercaria is not encysted, possesses forebody and hindbody, oral sucker, ventral sucker, ventral holdfast organ, and two anterolateral pseudosuckers. May cause blindness. (Hoffman, 1979)

2. Ornithodiplostomum ptychocheilus (brain fluke of fathead minnow)

Found in the viscera of many cyprinids, but in the cranial cavity and brain of Pimephales promelas, Notropis heterolepis, and Notropis cornutus. Can be serious in cultured fatheads. Is encysted, has forebody and hindbody, anterior oral sucker, ventral sucker, and ventral holdfast (Hoffman, 1960).

3. Clinostomum complanatum (Clinostomum marginatum, yellow grub)

Unsightly, usually yellowish grub. Body with large ventral sucker and anterior "shoulders." Encysted; large, 1-2 mm long.

4. Nanophyetus salmincola (salmon poisoning fluke)

Cysts may be numerous in viscera, particularly kidney, of salmonids in Western United States. Cycst can be seen in wet squashes of kidney (Baldwin et al., 1967).

5. Sanguinicola sp.

(a) Adult Worms

Sanguinicola davisi, fully developed individuals are flattened and spindleshaped flukes, having a length of 8.5 mm and a width of 0.21 mm. There is no oral sucker, acetabulum or pharynx. The mouth is subterminal and leads into a long slender esophagus that ends in an X-shaped cecum with four short, rounded lobes. The single testis is large, irregular in shape and approximately equatorial in position; the sperm duct leads to a crook like thick-walled cirrus sac. The bilobed ovary located at the posterior margin of the testis opens by means of a short oviduct into a gourd-shaped ootype. Genital pores open separately near the posterior end of the body with the female pore being anterior to the male pore. Yolk glands fill the entire body. Eggs are oval, nonoperculate, and measure 63 by 35 um (Chacko, 1980, personal communication). Major gill arteries, dorsal aorta, and heart chambers from at least 60 fish should be examined for blood flukes fitting the above description. Squeeze contents from blood vessels, splitting large ones if necessary.

(b) S. klamathensis

This is a closely related, but much smaller, species occurring in trout of the same general area with S. davisi. Immature flukes as well as adults are seen in gill arteries, heart, liver, and kidney. The life history and histopathology of this parasite have been worked out by Evans and Heckmann (1973). For both S. davisi and S. klamathensis see also Hoffman (1967).

(c) Miracidia

Gill filaments from at least 60 fish should be removed and examined for the presence of eggs and developing miracidia. Miracidia are readily identified by their single dark pigmented eye spot. Again, refer to descriptions by Evans and Heckman (1973).

E. Cestodes (tapeworms)

Adults in intestine, plerocercoids in viscera and musculature.

1. Proteocephalus ambloplitis (bass tapeworm)

Adult in intestine of Micropterus spp., plerocercoids in viscera of bass and small fish. Globular scolex has four suckers and an anterior vestigial apical sucker. The eggs are dumbell-shaped (Esch et al., 1975).

2. Eubothrium spp. of trout and salmon

In intestine and pyloric ceca. Importance is controversial. Scolex is heart-shaped with dorsal and ventral bothria (grooves).

3. Diphyllobothrium sebago

Plerocercoids in viscera of salmonids in Northeastern United States. Migration of larvae through liver and heart may be fatal. Scolex has no suckers, only precursors of bothrial grooves. When fixed, the body wrinkles giving appearance of false segmentation (Meyer and Robinson, 1963).

4. Diphlobothrium cordiceps

Plerocercoids in muscle and viscera of cutthroat trout in Western United States. Somewhat similar to Diphylobothrium sebago.

F. Nematodes (roundworms)

Goezia, a spined nematode, produces modules in the intestine of Micropterus salmoides in the Southeastern United States. Eustrongylides sp. cause swollen abdomen in tropical fish and small bait fish in extreme Southwestern and Southeastern United States.

G. Acanthocephala (thorny headed worms)

1. Acanthocephalus jacksoni

Acanthocephalus jacksoni of trout in Northeastern United States (Bullock, 1962) may cause concern among fishermen finding them in lower intestine of fish.

H. Copepods

1. Lernaea elegans (L. cyprinacea, anchor parasite)

No host specificity. Adult female with large Y-shaped dorsal arms (Hoffman, 1976).

2. Salmincola californiensis (the western Samincola)

Attacks gills of trout (Salmo sp.) and salmon (Oncorhyncus sp.). This copepod has been found as far east as West Virginia (G. L. Hoffman, personal observation).

3. Salmincola edwardsi (the brook trout Salmincola)

Attacks fins and gills primarily of eastern brook trout (Salvelinus fontinatlis).

4. Ergasilus spp. (hay fork claspers)

On gills of many fish. Ergasilus labracis of striped bass (Morone saxatilis) can be lethal.

I. Branchiura

Argulus (fish louse). Occasionally causes problems. Try to avoid Asian Argulus japonicus and European Argulus foliaceus and Argulus giordanii.

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Appendix 3

Suggested Procedure for PPLO Detection

A. Modified Fabricant's Medium

1. Cells, sera, and medium components may be checked for PPLO using the method below.

2. To accomplish this check, an aliquot of 1 ml of cell suspension is planted in 16 x 125 mm screw cap tubes. After 24 hours, the cells are scraped off and 0.5 ml of cells and tissue culture fluid (TCF) are planted in each of two PPLO tubes containing modified Fabricant's medium. One is incubated aerobically at room temperature. The other is placed in a candle jar (10% CO_2 at 37°C). The tests are observed for 21 days.

3. Whenever growth is apparent, subcultures are made on plates to obtain the typical fried egg colonies.

4. For best results, Fabricant's medium should be prepared in quantities to last about 1 month (store at 4°C).

5. Modified Fabricant's Medium Formulation

(a) PPLO broth without crystal violet (Difco) 70%
(To make plates, substitute Difco PPLO agar for the PPLO broth without crystal violet.)

(b) Horse serum 20%

(c) Yeast extract (Hayflick and Chanock type) 5%
(To prepare: add 250 grams dried yeast [Fleishmann's active dry yeast or comparable product] to 1,000 ml distilled water. Bring to boil, reduce heat, simmer 15 minutes, cool, centrifuge off yeast at 10,000 RPM for 10 minutes. Filter through Seitz EL--distribute in screw top tubes or bottles, sterilize by autoclaving. Store in freezer at 20°C.)

(d) 0.2% solution of DNA (Na+ salt from thymus) 1%

(e) 10% Na citrate 1%

(f) Sterile molar potassium phosphate (K_2HPO_4) solution 2%

(The above solutions d, e, f, should be sterilized by autoclaving 20 minutes at 121°C.)

(g) 0.4 ml of 0.5% phenol red per 100 ml medium (.002% phenol red) 1%

(h) Inhibitors

(1) 100,000 units penicillin per 100 ml medium

(2) 10 ml of 1% thallium acetate per 100 ml medium

6. The prepared medium is aseptically dispensed to sterile tubes containing 2 ml 2% agar prepared in advance, autoclaved and allowed to solidify before addition of Fabricant's medium.

B. Cultures of cell lines used for virus detection may be sent to commercial laboratories for testing.

C. The detection of mycoplasma contamination of cell cultures may be done by measurement of uridine phosphorylase activity. The process consists of incubation of radioactive uridine with culture lysates and subsequent chromatographic separation of uridine from the reaction product, uracil.

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Appendix 4

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