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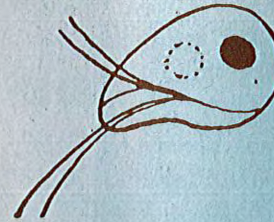

American Fisheries Society

Fish Health Section

Suggested Procedures for the Detection and Identification of Certain Infectious Diseases of Fishes

United States Department of the Interior
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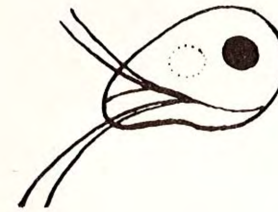

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Note

This manual has been assembled as a bound publication but it has also been punched to fit a three hole binder. Each section of the manual has been composed as a separate entity so that in updating of the manual, sections will be replaced as a unit. Such units can then be combined with this manual in a three ring binder.

SUGGESTED PROCEDURES FOR THE DETECTION AND IDENTIFICATION OF CERTAIN INFECTIOUS DISEASES OF FISHES

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Introduction

During the past twenty years intensive culture of freshwater and marine fish for sport or food has grown rapidly and continues to grow. The number, types, and severity of disease outbreaks caused by bacterial, parasitic, and viral agents among these propagated fishes have also increased. Rapid and accurate diagnosis and detection of these diseases is essential not only for control but also to better understand the epizootiology of the various diseases. Development of standard methods has been one of the major goals of the Fish Health Section of the American Fisheries Society since the establishment of the Section. After years of preliminary work by the Technical Procedures Committee to identify diseases for which standard methods were needed, a meeting was held in Denver, Colorado, in August 1974. The meeting was attended by fish health specialists from the United States and several other countries with the main objective being the selection of standard methods for diagnosis and detection of certain of the infectious diseases of fishes and the adoption of these methods by the Fish Health Section. The material in this report is the result of that meeting, and we believe that the procedures adopted are the most reliable and sensitive presently available. An attempt has been made not only to describe the suggested methods but also to provide information on numbers of fish to be examined and procedures for aseptic removal of tissues.

The diseases listed in this report require application of standardized methods to insure accurate diagnosis or detection. Many of the parasite diseases such as Ichthyophthirius and bacterial gill disease have not been described in detail. Although such diseases may cause substantial loss of fish, their diagnosis is easily accomplished using previously published information. References have been included in each section to direct the reader to methods of identification of these disease agents.

Finally, the Executive Committee of the Fish Health Section wishes to express its appreciation for the time and effort of all contributors, and to the Technical Procedures Committee for assembling and editing the report. Special thanks is also given to Dr. S. F. Snieszko of the Eastern Fish Disease Laboratory for his review.

Graham L. Bullock
President
Fish Health Section

Section 1

GENERAL EXAMINATION PROCEDURES

I. Methods for Examination of Fish for Infectious Diseases

A. Sample only individual, carefully identified lots of fish (species, age, source) for examination. Use a sample size adequate to detect disease (see Sample Size Table) in a carrier state or at the probability level requested.

B. Samples should be collected from each affected tank or lot. Select any suspect fish, i.e. symptomatic or moribund specimens, as well as non-symptomatic individuals.

C. Fish must be alive when collected, and it is desirable to have some fish alive at the time of laboratory examination. Examinations should begin within six hours after collection. If specimens cannot be maintained alive, the samples should be stored in sealed plastic bags and wet iced after collection.

D. 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 first before any 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.

E. 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 wet mounts of excised gill material, fins, and body scrapings for ectoparasites.

F. Technique for Opening the Body Cavity

1. Aseptic precautions. Sterilize instruments by dipping in 70% isopropyl alcohol (or 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 dorsad 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 thus obtained and loosen any adhesion between it and the underlying viscera with a blunt probe. Finally, starting near the vent, cut in a semi-circular route to complete excision of the body wall.

G. Internal Examination. To reduce the risk of contamination, take bacterial inocules 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, myxosporidian, and histological examinations.

H. 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.

II. Sample Size Table

The minimum sample size for each lot will be in accordance with a plan which provides 95% confidence of detecting a disease with an assumed incidence of infection at or greater than 2% or 5%. The minimum sample size for populations varying from 50 to infinity, for each inspection, is as follows:

Population or Lot Size	Incidence 2% Size of Sample	Incidence 5% Size of Sample
50	48	34
100	77	44
250	112	52
500	128	55
1,000	138	57
1,500	142	57
2,000	143	58
4,000	146	58
10,000	147	58
100,000 and larger	148	58

Section 2

METHODS FOR THE DIAGNOSIS OF CERTAIN VIRAL FISH DISEASES

I. General Procedures for Cell Culture and Virology

A. Quality Control

1. Susceptible, normal appearing and rapidly dividing cells shall be used for all virus assays. Cells less than 72 hours old and 80 to 90% confluent are preferred for sample inoculation.

2. All cell culture stocks must be tested for and found free from *Mycoplasma* sp. at three month intervals (suggested procedure in supplement). Only those stocks found free of *Mycoplasma* sp. shall be used in virus assays.

3. All non-autoclavable constituents of media must be tested and found free from *Mycoplasma* sp. (see procedure in supplement) and inhibition to cell growth and/or virus replication. Certified *Mycoplasma* free sera and other certified reagents need not be tested.

4. Only penicillin-streptomycin (100 units/ml) or gentamicin (100 µg/ml) and mycostatin (25 mg/ml) are allowed for routine cell culture work. Gentamicin is especially recommended for control of *Mycoplasma*.

B. Virus Assay Controls

1. The cells used in each virus assay must demonstrate sensitivity to at least one fish virus. It is preferred that cells demonstrate a sensitivity to all fish viruses being checked for by the assay. If this is not possible cells should periodically be sent to a laboratory which will check their sensitivity to the viruses of concern. Sensitivity shall be defined as the ability of the cells to show typical cytopathic effects (CPE) when exposed to 10^{-2} - 10^{-3} $TCID_{50}$ of virus.

2. Uninoculated or negative (inoculated with sterile saline or known uninfected fish tissue) controls must be incubated with each virus assay. These controls must remain free from CPE throughout the entire incubation period of the assay.

C. Sampling and Handling of Samples

1. During epizootics, a minimum of 10 fish in 2 pools shall be sampled.

2. When testing for virus in asymptomatic fish, sampling shall be according to the table of attribute sampling based upon a 95% level of confidence. (See table in Methods for Examination of Fish for Infectious Diseases.) When killed fish are used

as samples (kidney and spleen samples), the assumed minimum carrier incidence shall be 5%. In sampling of sex products (ovarian fluid or seminal fluid), the assumed minimum carrier incidence shall be 2%.

3. Within the above guidelines, sampling shall also be governed by the size and number of rearing units in which the fish (lot) are being held. If the fish are in more than one rearing unit, subsamples must be collected such that the subsamples will be approximately equal in size. If individual rearing units are larger than 0.5 acres, subsamples must be taken from at least 4 areas within the unit.

4. In any given sample or subsample, one should first select moribund fish, and then make up the remainder of the sample by random selection. Moribund fish are counted as part of the sample but should be processed separately from the randomly selected fish.

5. Sample all fish populations by lot. A lot is defined as a group of fish of the same species and age that share a a common water supply and originate from a discrete spawning population. When hatchery inspections for fish diseases are conducted, all lots of fish on the hatchery must be sampled. At least 2 complete inspections, with a six month interval, must be conducted on a hatchery before a disease status certificate is issued. An annual inspection is required to maintain a certified disease-free status.

6. Tissue sampling shall be according to the following plan:

   a. Sac Fry: Assay entire fry after removal of yolk sac.
   b. Fingerling to 5 cm: Assay entire viscera including kidney.
   c. Fish to 5 cm: Assay spleen and kidney only.
   d. Broodfish: Use ovarian fluid for as many of the samples as possible. If seminal fluid samples are used to make up the remainder of the required sample, do not combine ovarian and seminal fluids into same pools.

7. Pooling of samples is recommended to keep a practical number of virus assay units. In no case, however, shall the tissues from more than 5 fish be pooled to form a sample unit.

8. Samples shall be inoculated as soon as possible after collection. If inoculations cannot be made immediately, the samples will be stored or transported at 4°C. Samples must not be stored for any longer than 7 days prior to primary inoculation onto cell cultures.

9. If samples must be transported or stored, and this time period exceeds 12 hours, the transport medium shall be buffered saline (pH 7.0-7.8). At the discretion of the investigator, antibiotics may be used in the transport medium to control growth of microbial contaminants in the samples.

10. Tissue samples must be triturated by grinding in a mortar with pestle or homogenized by blender, homogenizer, or tissue grinder.

11. To control bacterial growth in inoculated cell cultures, samples will be processed as follows:

    a. Centrifuge samples at 2,000 X g for about 10 minutes.
    b. Add antibiotic mixture to the supernatant only to obtain a final concentration of 100-2,000 µg. Gentamicin and 400 units Mycostatin per milliliter of supernatant (penicillin-streptomycin at 800 units of penicillin and 800 µg streptomycin may be substituted for the gentamicin).
    c. Allow mixture to stand for at least 2 hours at 15-20°C. (Samples with antibiotics may be stored overnight at 5°C if inoculation cannot be made at the end of the 2 hour period.)
    d. Inoculate onto appropriate cell cultures according to other methods listed in this text. (As an alternate contamination control procedure, filtration of samples through a 0.45 µ filter can be used.)

12. Final organ sample dilution prior to primary inoculation onto cell culture shall not exceed 1:200 (1 gram of tissue in 199 cc diluent). Final ovarian fluid sample dilution shall not exceed 1:10.

13. All tests for virus detection will be incubated for a minimum of 14 days at 15-30°C (depending on virus being assayed for). A temperature of 15°C is recommended for IHNV, IPNV and VHSV. For CCV detection, 30°C is suggested. During this incubation period, the pH of cell cultures shall be maintained between 7.4 and 7.8, except in the case of the pH of cell cultures for IHNV and IPNV which shall be maintained between 7.0 and 7.8.

14. When assaying for virus in asymptomatic fish, and where circumstances indicate a likelihood that primary inoculation of cell cultures might not result in detection of virus, a blind passage should be considered by the investigator.

D. Identification of Virus by Serum Neutralization

1. Select cell cultures showing typical virus CPE and dilute fluid from these cultures to 1:1,000 (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).

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 10^2 - 10^3 $TCID_{50}$ per ml) instead of the diluted fluid from virus suspect cultures in step 1.

6. Keep all mixtures at room temperature for 60 minutes.

7. Inoculate 0.2 ml of each mixture onto duplicate cell cultures and keep two uninoculated cultures for controls.

8. Incubate all cultures at 15-30°C (depending on viral agent involved) for 24 to 96 hours. CPE should occur in the cells inoculated with control serum but not in the antiserum inoculated cells. Because of variable concentrations of virus in the cell culture fluid, and serological differences of some of the fish viruses, CPE may show up in the antiserum groups; however, this CPE is generally reduced and its appearance is delayed. Because of these variations, cell cultures should be observed daily.

E. References

1. Amend, D. F., and J. P. Pietsch. 1972. An improved method for isolating viruses from asymptomatic carrier fish. Transactions of the American Fisheries Society, vol. 101, no. 2, p. 267-269.

2. Ossiander, F. J. and G. Wedemeyer. 1973. Computer program for sample sizes required to determine disease incidence in fish populations. Journal of the Fisheries Research Board of Canada, vol. 30, p. 1383-1384.

3. Wolf, K. 1970. Guidelines for virological examination of fishes. In A symposium on diseases of fish and shellfish. American Fisheries Society Special Publication No. 5, p. 327-340.

4. Wolf, K., and M. C. Quimby. 1969. Fish cell and tissue culture. In Fish Physiology, vol. 3, W. S. Hoar and D. J. Randall, editors. Academic Press, New York, p. 253-305.

5. Wolf, K., and M. C. Quimby. 1973. Towards a practical fail-safe system of managing poikilothermic vertebrate cell lines in culture. In Vitro, vol. 8, p. 316-321.

6. Wolf, K., and M. C. Quimby. 1973. Fish Viruses: buffers and methods for plaquing eight agents under normal atmosphere. Applied Microbiology, vol. 25, p. 659-664.

II. Infectious Pancreatic Necrosis

A. Name of the Disease and Etiologic Agent. Infectious pancreatic necrosis (IPN), infectious pancreatic necrosis virus (IPNV).

B. Known Geographic and Host Range of the Disease

1. Geographic range. Northern Hemisphere.

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 (*Onchorynchus kisutch*), and chinook salmon (*Onchorynchus tschawytscha*).

C. Clinical Signs of Diagnostic Significance

1. IPN 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. Whirling may occur when the mortality rate is high; swimming victims rotate about their long axis. When not otherwise obvious, alarming the fish by a sharp rap on the trough or other scare will often elicit the whirling response. Agonal behavior may alternate with quiescence during which victims lie on the trough bottom and respire weakly. Whirling is a terminal sign and death usually occurs within an hour or two.

2. Signs include overall darkening, exopthalmia, abdominal distention, 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. A clear to milky mucous occurs in the stomach and anterior intestine.

D. Diagnostic Procedures for Disease Situations

1. Presumptive diagnosis

a. Presence of typical CPE in cell culture.
b. Histological examination revealing pronounced pancreatic necrosis with both acinar and islet tissues affected. Adjacent adipose tissue necrotic. Cytoplasmic inclusions in pancreatic cells near the edges of affected tissues.
c. Presence of signs as described in C above.
d. History of the hatchery or natural environment indicates a likelihood of IPN infection.

2. Confirmatory diagnosis

a. Positive confirmation requires isolation and identification of the IPN agent by the methods described in general procedures. Histological confirmation is also required to ascertain that IPNV is the cause of the mortality.

b. 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 $TCID_{50}$/ml).

c. A minimum of 10 fish in 2 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 dealing with situations where fish cannot be killed. In this case, ovarian and seminal fluid can be used at the discretion of the investigator.

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

F. Procedures for Determining Prior Exposure to the Etiologic 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 Etiologic Agent. As described in the section on general procedures for cell culture virology.

H. References

1. Amend, D. F., and Gary Wedemeyer. 1970. Approved procedure for determining absence of infectious pancreatic necrosis (IPN) virus in certain fish and fish products. U.S. Bureau of Sport Fisheries and Wildlife, FDL-27. 4 p.

2. Jorgensen, P. E. V. 1973. Inactivation of IPN and Egtved virus. Rivista Italiana. Piscicoltura Ittiopatologia, vol. 8, p. 107-108.

3. Wolf, K. 1972. Advances in Fish Virology: A Review 1966-1971. Symposia of the Zoological Society of London, vol. 30, p. 305-331.

4. Wolf, K., and M. C. Quimby. 1971. Salmonid viruses: infectious pancreatic necrosis virus. Morphology, pathology, and serology of first European isolations. Archiv fur Gesamte Virusforschung, vol. 34, p. 144-156.

5. Wolf, K., S. F. Snieszko, C. E. Dunbar, and E. Pyle. 1960. Virus nature of infectious pancreatic necrosis in trout. Proceedings of the Society for Experimental Biology and Medicine, vol. 104, p. 105-108.

6. Yasutake, W. T. 1970. Comparative histopathology of epizootic salmonid virus diseases. American Fisheries Society Special Publication No. 5, p. 341-350.

III. Viral Hemorrhagic Septicemia

A. Name of the Disease and Etiologic Agent. Viral hemorrhagic septicemia (VHS), Egtved virus.

B. Known Geographic and Host Range of the Disease

1. Geographic range. VHS has been reported from a number of but not all European countries. There have been no reports of VHS outside of Europe.

2. Host range. Although several species of salmonids are experimentally susceptible to VHS by injection, epizootics have been reported only in rainbow trout.

C. Clinical Signs of Diagnostic Significance

1. Historically the clinical signs of fish infected with VHS have been categorized into the acute, chronic, and nervous forms. However, from a descriptive standpoint there is much overlap in the clinical signs observed with each form, and although the signs are associated with the disease, the signs in individual cases may not be observed. Therefore, the following clinical signs are not separated into three categories.

2. Typical external signs of the disease include exophthalmia, abdominal swelling with ascities, anemia, and perhaps some evidence of hemorrhaging at the base of fins. Internally visceral adipose tissue and peritoneal mesenteries show numerous diffuse petechial hemorrhages, the kidney is hyperemic and swollen, hemorrhages in the periocular connective tissues, multiple hemorrhages in the lateral skeletal muscles, and the liver is pale. Microscopically extensive necrosis of the hematopoietic tissue of the kidneys and spleen is typical. Also, focal necrosis in pancreatic and liver tissues are common, and hemorrhages in the skeletal muscles can be observed.

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 to presumptively identify VHS. Typical cytopathology includes rounding of cells and pyknosis of nuclei. Plaque morphology is very helpful in distinguishing VHS from IPN or IHN.

(1) Cell culture methods. The fathead minnow (FHM) cell line is preferred but other equally sensitive cell lines can be used. The rainbow trout gonad (RTG-2) cell line is less sensitive but can be used as an alternate. Cell cultures must be incubated between 13 and 15°C and the pH maintained between 7.4 and 7.8. The test must be repeated if the pH drops below 7.4 and no virus is isolated.

(2) Viral procedures. See general procedures for cell culture and virology.

b. Histopathological changes. Typical necrosis of kidney and spleen hematopoietic tissue with the absence of involvement of the granular cells of the *lamina propria* is a less acceptable method of presumptive diagnosis of VHS. Select and fixate tissue for biological examination by accepted procedures.

c. Clinical signs. The presence of clinical signs and a history of VHS can also be used for presumptive diagnosis of VHS.

d. Combination. Demonstration of all three of the above characteristics gives the strongest presumptive evidence for VHS, but for positive identification confirmatory procedures must be followed.

2. Confirmatory diagnosis

a. Most acceptable identification. Isolation of the virus with neutralization by specific VHS antiserum and associated with typical VHS histopathological changes positively identifies the disease. Fluorescent antibody (FA) techniques for identifying the Egtved virus has been reported and is an acceptable identification procedure. However, it is assumed that at this writing most investigators will not have FA capabilities; therefore, detailed procedures describing the FA technique are not discussed. Anyone using FA as a method of identifying the Egtved virus must provide data to show that the tests were properly controlled and the labeled antiserum is specific for the Egtved virus. Either FA or virus neutralization identifies the presence of virus, but histological changes typical of the VHS must be present to identify the disease. Procedures for virus neutralization are described in General Procedures for Cell Culture and Virology.

b. Less acceptable procedures. In the absence of demonstrated histopathological changes, virus neutralization with specific antiserum and a high titer (10^5 infectious units or above) of virus in the tissue can be used to positively identify the disease.

E. Procedures for Detecting Asymptomatic Infections. The procedures for detecting VHS carriers can be followed as described for IPNV. Sample each lot at the assumed 5% incidence level. Collect only kidney and spleen and do not process samples of tissue with more than 5 fish per pool. Follow cell culture and virological procedures as described above in D 1 a. Any isolated virus must be confirmed according to Section D 2 a above, except no histological examination is needed.

F. Procedures for Determining Prior Exposure to the Etiologic 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 Etiologic Agent. These are described under General Procedures for Cell Culture and Virology.

H. References

1. Campbell, J. B., and K. Wolf. 1969. Plaque assay and some characteristics of Egtved virus (virus of viral hemorrhagic septicemia of rainbow trout). Canadian Journal of Microbiology, vol. 15, p. 635-637.

2. Hoffman, G. L., S. F. Snieszko, and K. Wolf. 1970. Approved procedure for determining absence of viral hemorrhagic septicemia and whirling disease in certain fish and fish products. U.S. Bureau of Sport Fisheries and Wildlife Fish Disease Leaflet 9, p. 7.

3. Jorgensen, P. E. V. 1973. Inactivation of IPN and Egtved virus. Rivista Italiana. Piscicoltura Ittiopatologia, vol. 8, p. 107-108.

4. Wolf, K. 1972. Advances in fish virology: a review 1966-1971. Symposia of the Zoological Society of London, no. 30, p. 305-331.

5. Wolf, K. and P. E. V. Jorgensen. 1970. Salmonid viruses: double infection of RTG-2 cells with Egtved and infectious pancreatic necrosis viruses. Archiv fur Gesamte Virusforschung, vol. 29, p. 337-342.

6. Yasutake, W. T., and C. J. Rasmussen. 1968. Histopathogenesis of experimentally induced viral hemorrhagic septicemia in fingerling rainbow trout (Salmo gairdneri). Bulletin, Office International des Epizooties, vol. 69, p. 977-984.

IV. Infectious Hematopoietic Necrosis

A. Name of the Disease and Etiologic Agent. Infectious hematopoietic necrosis (IHN). (Synonyms: The disease has also been referred to as Oregon sockeye disease or Sacramento River chinook disease. The virus isolated from the various species is now more commonly referred to as the chinook salmon, sockeye salmon, or rainbow trout strain.)

B. Known Geographic and Host Range of the Disease

1. Geographic range. The initial geographic range of IHNV was the Pacific Coast of North America from California to Alaska; however, incidental outbreaks have been reported from South Dakota, Minnesota, Idaho, West Virginia, Colorado, Montana, and Hokkaido, Japan.

2. Host range. The natural host range includes rainbow trout (*Salmo gairdneri*) (including Steelhead), chinook salmon (*Oncorhynchus tshawytscha*), and sockeye salmon (*Onchorhynchus nerka*). So far as is known, the coho salmon (*Onchorhynchus kisutch*) appears to be resistant to IHNV.

C. Clinical Signs of Diagnostic Significance. Typical external signs of the disease may include exophthalmia, anemia, hemorrhaging at the base of fins, fecal casts, abdominal swelling with ascites, and scoliosis or lordosis in survivors of epizootics. Internally petechial hemorrhages in adipose tissues of the visceral cavity and mesenteries are often 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 necrosis of the granular cells of the intestinal *lamina propria* is of diagnostic value. 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 on cell culture with the development of typical cytopathology can be used to presumptively identify 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 fathead minnow (FHM) cell line is preferred, but other cell lines which are shown to be equally sensitive can be used. The rainbow trout gonad (RTG-2) cell line is less sensitive to IHNV but can be used as an alternate. Cell cultures are to be incubated between 15 and 18°C for no less than 10 days. The pH of the culture media is to 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) Viral procedures. See section on General Procedures for Cell Culture and Virology.

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 according to accepted procedures.

c. Clinical signs. The presence of clinical signs and a history of IHNV can also be used to presumptively identify IHN disease.

d. Combination. Demonstration of all three of the above characteristics gives the strongest presumptive evidence for the presence of IHN disease, but for positive identification confirmatory procedures must be followed.

2. Confirmatory diagnosis

a. Most acceptable identification. Isolation of virus with neutralization by specific IHN antiserum and associated with typical IHN histopathological changes positively identifies the disease as IHN. Serum neutralization only identifies the virus; the presence of typical pathological changes must be present to positively diagnose the disease. Procedures for virus neutralization are described in General Procedures for Cell Culture and Virology.

b. Less acceptable identification. In the absence of demonstrated histopathological changes, virus neutralization with specific antiserum and a high titer (10^5 infections units or above) of the sample can be used to positively identify the disease.

E. Procedures for Detecting Asymptomatic Infections. The only acceptable procedure for detecting IHN carriers is to test ovarian fluid for the presence of virus; however, in the case of rainbow trout either sex can be used. In all cases fish should be sampled according to section on General Procedures for Cell Culture and Virology. Samples may be collected and processed into 5 fish pools. Cell culture and virological procedures are to be followed as described above under D 1 a, and confirmation of the identity of any isolated agent must be followed as described above in D 2 a except no histopathological examination is needed.

F. Procedures for Determining Prior Exposure to the Etiologic 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 Etiologic Agent. These are described under General Procedures for Cell Culture and Virology.

H. References

1. Amend, D. F. 1970. Approved procedure for determining the absence of infectious hematopoietic necrosis (IHN) in salmonid fishes. U.S. Bureau of Sport Fisheries and Wildlife, Fish Disease Leaflet 31, 4 p.

2. Amend, D. F., and V. C. Chambers. 1970. Morphology of certain viruses of salmonid fish I. "In vitro" studies of some viruses causing hematopoietic necrosis. Journal of the Fisheries Research Board of Canada, vol. 27, p. 1285-1293.

3. Amend, D. F., W. T. Yasutake, and R. F. Mead. 1969. A hematopoietic virus disease of rainbow trout and sockeye salmon. Transactions of the American Fisheries Society, vol. 98, p. 796-804.

4. McAllister, P. E., J. L. Fryer, and K. S. Pilcher. 1974. Further characterization of infectious hematopoietic necrosis virus of salmonid fish (Oregon strain). Archiv fur Gesamte Virusforschung, vol. 44, p. 270-279.

5. McCain, B. B., J. L. Fryer, and K. S. Pilcher. 1971. Antigenic relationships in a group of three viruses of salmonid fish by cross neutralization. Proceedings of the Society for Experimental Biology and Medicine, vol. 137, p. 1042-1046.

V. Channel Catfish Virus Disease

A. Name of the Disease and Etiologic 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, Geogia, 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 blue catfish (*I. furcatus*) may be naturally infected and definitely experimentally infected.

C. Clinical Signs of Diagnostic Significance

1. Epizootics are characterized by a high rate of mortality in catfish that are less than 6 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 hanging head up in the water.

3. Externally, diseased fish have abdominal distension, exopthalmia, pale or hemorrhagic gills, 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, yellowish fluid, (ascites), hemorrhage 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 are present in hematopoietic tissue surrounding renal tubules. The liver has diffuse necrosis, edema and hemorrhage. Hemorrhage, edema, and possibly mucosal sloughing is present in the intestine. The spleen becomes congested, edematous and macrophages are laden with degenerate erythrocytes. Cardiac tissue may be necrotic and focal hemorrhage may also occur in the musculature.

D. Diagnostic Procedures for Disease Situations

1. Presumptive diagnosis. Samples are processed as described in General Procedures for Cell Culture and Virology. Assayed material is inoculated into brown bullhead (BB) cells (ATTC-59) and incubated at 25-30°C with a 7.2-7.4 pH. Inoculated cultures are incubated for 14 days and observed for typical CPE.

2. Confirmatory diagnosis. Confirmation of CCV is performed by the CCV-antiserum neutralization techniques outlined in General Procedures for Cell Culture and Virology.

E. Procedures for Detecting Asymptomatic Infections. None are available.

F. Procedures for Determining Prior Exposure to the Etiologic Agent. These procedures are for determining prior exposure to CCV only, and positive results do not mean that the fish are definitely carriers of CCV.

1. Serum samples from adult fish are heat inactivated at 45°C for 30 min. The serum is diluted 1:100 and assayed for neutralization activity against 100 $TCID_{50}$ or PFU/0.1 ml according to accepted methods. A positive sample (CCV neutralization) is indicated by a 1:100 serum dilution from which one obtains a 50% reduction of plaque forming units or $TCID_{50}$.

2. Broodfish sample size is determined by 2% incidence 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 and Virology.

H. References

1. Fijan, N. N., T. L. Wilborn, and J. P. Naftel. 1970. An acute viral disease of channel catfish. U.S. Bureau of Sport Fisheries and Wildlife, Technical Paper 43, 11 p.

2. Gratzek, J. B., M. H. McGlamery, D. L. Dawe, and S. Scott. 1973. Microcultures of brown bullhead (Ictalurus nebulosus) cells: their use in quantitation of channel catfish (Ictalurus punctatus) virus and antibody. Journal of the Fisheries Research Board of Canada, vol. 30, p. 1641-1645.

3. Plumb, J. A. 1971. Tissue distribution of channel catfish virus. Journal of Wildlife Diseases, vol. 7, p. 213-216.

4. Plumb, J. A. 1973. Effects of temperature change on mortality of fish infected with channel catfish virus. Journal of the Fisheries Research Board of Canada, vol. 30, p. 568-570.

5. Plumb, J. A. 1973. Neutralization of channel catfish virus by serum of channel catfish. Journal of Wildlife Diseases, vol. 9, p. 324-330.

6. Plumb, J. A., L. D. Wright, and V. L. Jones. 1973. Survival of channel catfish virus in chilled, frozen and decomposing channel catfish. The Progressive Fish Culturist, vol. 35, no. 3, p. 170-172.

7. Wolf K., and R. W. Darlington. 1971. Channel catfish virus: a new herpesvirus of ictalurid fish. Journal of Virology, vol. 8, p. 525-533.

8. Wolf, K., R. L. Herman, and C. Carlson. 1972. Fish Viruses: histopathologic changes associated with experimental channel catfish virus disease. Journal of the Fisheries Research Board of Canada, vol. 29, p. 149-150.

VI. Suggested Procedure for PPLO Detection - Modified Fabricant's Medium

A. Cells and sera and medium components should be checked for PPLO at least once every three months.

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

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

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

E. Modified Fabricant's Medium Formulation

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

2. Horse serum — 20%

3. Yeast extract (Hayflick and Chanock type). To prepare add 250 grams dried yeast (Fleishmann's active dry yeast or comparable product) to 1000 ml distilled water. Boil for 15 minutes (simmer), cool, centrifuge off yeast at 10,000 RPM for 10 minutes. Filter through Seitz EK - distribute in screw top tubes or bottles, sterilize by autoclaving. Store in freezer at -20°C. — 5%

4. The following solutions should be sterilized by autoclaving 20 min at 121°C.

   a. 0.2% solution of DNA (Na salt from thymus) — 1%

   b. 10% Na citrate — 1%

   c. Sterile molar potassium phosphate (K_2HPO_4) solution — 2%

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

5. Inhibitors

a. 100,000 units penicillin per 100 ml medium

b. 10 ml of 1% thallium acetate per 100 ml medium

F. 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.

Section 3

METHODS FOR THE DIAGNOSIS OF CERTAIN BACTERIAL FISH DISEASES

Foreword

In the following pages, methods are outlined for the diagnosis of 10 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. Certain of these diseases are mentioned in Section XIII and readers desiring more complete information on these and other unmentioned bacterial diseases are referred to the literature in Section XIV.

Several of the diseases have been renamed. It is hoped that the new names describe more effectively, both the nature of the condition and its etiology.

Finally, and most important, the methods described are best suited for diagnosing clinically diseased fish. Satisfactory procedures for the detection of covert bacterial fish infections are still lacking. Two approaches for detecting covert infections are being studied but both are still very much research topics. The first is an indirect method and is based on demonstrating pathogen-specific agglutinins in suspect fish; the second is direct and is based on demonstrating antigens of the pathogen in the fish tissues. The immunodiffusion and fluorescent antibody techniques that may be of value in the latter approach are briefly described in Section XII.

I. Furunculosis

A. Name of the Disease and Etiologic Agent. Furunculosis, *Aeromonas salmonicida*.

B. Known Geographic and Host Range of the Disease

1. Geographic range. Worldwide except possibly for Australia and New Zealand; reported predominantly in fresh water.

2. Host range. All fresh-water and marine fish are considered susceptible.

C. Clinical Signs of Diagnostic Significance. Furunculosis has been studied in salmonids in which it may take one of four clinical forms. The forms range from peracute (mortalities without gross lesions) and acute (gills hemorrhage readily), to subacute (the bodies darken) and chronic (bodies are dark and might show vesicles that contain blood-tinged fluid or that have broken to form ragged-edged ulcers). The acute forms of the disease are not specifically diagnostic for furunculosis but the signs associated with the chronic form of the disease, taken together with the source and history of the fish, may be of some diagnostic value.

D. Diagnostic Procedures for Disease Situations. Diagnosis is based on isolation and identification of the causative organism. Primary isolation should be made from kidney on tryptic (trypticase) soy or furunculosis agar at 20-25°C for 24-48 hours.

1. Presumptive diagnosis. The organism, when cultured as above, should be a Gram-negative, non-motile, coccoid rod (in tissues it is more distinctly rod-like); it should be cytochrome oxidase-positive and should produce a brown pigment that diffuses into the medium (an occasional strain may be incapable of producing the pigment).

2. Confirmatory diagnosis

a. The procedure of choice is the slide agglutination test using anti-*A. salmonicida* serum (see Section XII Serological Procedures). Note: Certain strains of *A. salmonicida* agglutinate spontaneously in saline; the slide agglutination test can only be carried out with such strains if the cell suspensions are first briefly sonicated to prevent autoagglutination.

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. Fairly detailed recent and early descriptions of A. salmonicida are contained in the two papers listed below.

c. A sample of at least five moribund fish from each diseased holding unit (e.g. tank, raceway, pond) should be used for making 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. Procedures for Detecting Asymptomatic Infections. No satisfactory procedure has been developed for routine use. For the time being, the likelihood of an asymptomatic infection occurring in a specific lot of fish is best based on the health history of the lot of fish in question and its source; at the moment, the best (most practical) way of developing the necessary health histories is by a regular monitoring of mortalities.

F. Procedures for Determining Prior Exposure to the Etiologic Agent. No satisfactory procedure is available, but the appearance of circulating anti-A. salmonicida agglutinins in trout serum following furunculosis outbreaks may form the basis of a serological method for determining prior exposure.

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiologic Agent. Samples are best transported in plastic bags on ice. If the samples cannot be examined immediately, freezing is permissible.

H. References

1. Evelyn, T. P. T. 1971. An aberrant strain of the bacterial fish pathogen Aeromonas salmonicida isolated from a marine host, the sablefish (Anoplopoma fimbria), and from two species of cultured Pacific salmon. Journal of the Fisheries Research Board of Canada, vol. 28, p. 1629-1634. (Note: typical strains of A. salmonicida are also described.)

2. Griffin, P. J., S. F. Snieszko, and S. B. Friddle. 1952. A more comprehensive description of Bacterium salmonicida. Transactions of the American Fisheries Society, vol. 82, p. 129-138.

II. Motile Aeromonas Septicemia

A. Name of the Disease and Etiologic Agent. Motile Aeromonas septicemia (MAS), Aeromonas hydrophila complex. (Synonyms--bacterial hemorrhagic septicemia, hemorrhagic septicemia, and many others.)

B. Known Geographic and Host Range of the Disease

1. Geographic range. Worldwide in fresh water.

2. Host range. Probably all fresh-water fish.

C. Clinical Signs of Diagnostic Significance. The disease occurs most frequently in warm waters of high organic matter content following some stress or injury such as might result 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. 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 isolation and identification of the etiologic agent. Primary isolation should be made from kidney on tryptic (trypticase) soy agar (TSA) incubated at 20-25°C for 24-48 hours. Note: If for some reason MAS is strongly suspected, kidney may, in addition, be inoculated onto the Rimler-Shotts (RS) medium which should then be incubated at 35°C. The RS medium provides for the very rapid detection (only 20-24 hours are required) of organisms in the A. hydrophila complex; it would, however, allow vibrios to go undetected (see Section on Media and Reagents).

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 is fermentative in glucose O/F medium.

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 shown to be insensitive to the vibriostatic agent 0/129 and novobiocin (see Section XII on Media and Reagents).

b. A confirmed MAS diagnosis is obtained on the RS medium if, by 20-24 hours, the organism produces yellow colonies that prove to be cytochrome oxidase-positive.

c. 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 item D2c in the section on furunculosis.

E. Procedures for Detecting Asymptomatic Infections. The remarks in item E of the furunculosis section apply here. Because organisms of the A. hydrophila complex are considered to be ubiquitous in fresh water, a search for a suitably sensitive detection procedure has not been actively pursued and may not be warranted.

F. Procedures for Determining Prior Exposure to the Etiologic Agent. At the present time there are no serological tests that have been developed.

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiologic Agent. The remarks in item G of the furunculosis section apply here.

III. Pseudomonas Septicemia

A. Name of the Disease and Etiologic Agent. Pseudomonas septicemia, *Pseudomonas* sp, particularly *P. fluorescens*, some outbreaks have been caused by a nonmotile capsulated *Pseudomonas*. (Synonyms--bacterial hemorrhagic septicemia, hemorrhagic septicemia.)

B. Known Geographic and Host Range of the Disease

1. Geographic range. Worldwide in fresh and sea water.

2. Host range. All species of fish are probably affected at one time or another.

C. Clinical Signs of Diagnostic Significance. The disease appears to be stress-mediated and occurs most frequently under warm-water conditions; it usually occurs as a generalized septicemia with clinical signs that vary according to the acuteness of the infection and which are very similar to those of other septicemias (see, for instance, motile Aeromonas septicemia).

D. Diagnostic Procedures for Disease Situations. Diagnosis is based on isolation and identification of the etiologic agent. Primary isolation should be made from kidney on tryptic (trypticase) soy agar at 20-25°C for 24-48 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.

2. Confirmatory diagnosis

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

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 item D2c in the section on furunculosis.

E. Procedures for Detecting Asymptomatic Infections. The remarks in item E of the furunculosis section apply here. Because *Pseudomonas* is considered to be ubiquitous in water, a search for a suitably sensitive detection procedure has not been actively pursued and may not be warranted.

F. Procedures for Determining Prior Exposure to the Etiologic 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 Etiologic Agent. The remarks in item G of the furunculosis section apply here.

IV. Vibriosis

A. Name of the Disease and Etiologic Agent. Vibriosis, *Vibrio* sp., *V. anguillarum* or other species or varieties which also may be pathogenic to fish. (Synonyms--boil disease, ulcer disease, salt water furunculosis, red pest or red boil of eels, and others.)

B. Known Geographic and Host Range of the Disease

1. Geographic range. Worldwide, principally in marine situations, but some outbreaks have occurred in fresh water.

2. Host range. All marine and fresh water 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 in which the disease is frequently seen it 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). Marine fish may exhibit one or more of the foregoing signs; fresh water Ictalurids apparently suffer from a non-systemic form of the disease in which there is superficial erosion of the caudal peduncle.

D. Diagnostic Procedures for Disease Situations. Diagnosis is based on isolation and identification of the etiologic agent. Primary isolation should be made from kidney (and lesion material, where necessary) on tryptic (trypticase) soy agar incubated at 20-25°C for 24-48 hours.

1. Presumptive diagnosis. Criteria for a presumptive diagnosis are satisfied if the TSA isolate is a short, motile, cytochrome oxidase-positive, Gram-negative, usually curved rod that is fermentative in glucose O/F medium.

2. Confirmatory diagnosis

a. Criteria for a confirmed diagnosis are satisfied if the TSA isolate ferments glucose anaerogenically and is sensitive to the vibriostatic agent 0/129 and novobiocin.

b. For making a diagnosis, a sample of five moribund fish from each affected holding unit (e.g. tank, raceway,

pond, enclosure) is recommended. For amplification, see item D2c in the furunculosis section.

E. Procedures for Detecting Asymptomatic Infections. The remarks in item E of the furunculosis section apply here. Because *Vibrio* sp. is considered ubiquitous (at least, in sea water), a search for a suitably sensitive detection procedure has not been actively pursued and may not be warranted.

F. Procedures for Determining Prior Exposure to the Etiologic 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 Etiologic Agent. The remarks in item G of the furunculosis section apply here.

V. Enteric Redmouth

A. Name of the Disease and Etiologic Agent. Enteric redmouth (ERM), RM bacterium. (Synonyms--Hagerman redmouth disease, redmouth disease.)

B. Known Geographic and Host Range of the Disease

1. Geographic range. Limited to North America thus far. Confirmed isolations have been made in Alaska, Arizona, California, Colorado, Idaho, Montana, Nevada, Ohio, Oregon, Saskatchewan, Tennessee, Utah, and Washington.

2. Host range. Potentially all salmonids. (Isolations from non-salmonids not yet reported.) Confirmed isolations have been made from Atlantic salmon (*Salmo salar*), brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*Onchorhynchus kisutch*), cutthroat trout (*Salmo clarki*), rainbow trout (*Salmo gairdneri*), and sockeye salmon (*Onchorhynchus nerka*).

C. Clinical Signs of Diagnostic Significance. The disease may occur as a peracute, acute, or subacute to chronic condition. The clinical signs of the acute forms of the disease are very similar to those seen in other bacterial septicemias. In the more chronic infections, the clinical signs are somewhat more diagnostic for ERM and, when considered in conjunction with the origin and history of the fish (the disease has a rather restricted distribution), can provide valuable clues as to the identity of the disease. In chronic infections the fish are dark, lethargic, and commonly show bilateral exophthalmia 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 tryptic (trypticase) soy agar incubated at 20-25°C for 24-48 hours.

1. Presumptive diagnosis. For presumptive identification, the organism should at least be shown to be a Gram-negative, cytochrome oxidase-negative rod that fails to produce indole in tryptone broth and produces an acid (only) reaction on the slant and in the butt of slanted triple sugar iron agar (see Fig. 1). Additional characteristics that might also be verified for the organism in presumptive

testing are its ability to ferment glucose (acid, but no gas, is produced in O/F glucose medium) and its motility.

2. Confirmatory diagnosis

a. The procedure of choice is the slide agglutination test using anti-RM bacterium serum (see Section XII on Serological Procedures).

b. If for some reason the diagnostic antiserum is not available, the isolate must be shown to be morphologically, culturally, and biochemically identical (or essentially identical) with the RM bacterium, a detailed description of which has been published (see the reference below).

c. 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 item D2c in the section on furunculosis.

E. Procedures for Detecting Asymptomatic Infections. The remarks in item E of the furunculosis section apply here.

F. Procedures for Determining Prior Exposure to the Etiologic Agent. No satisfactory procedure is available, but the appearance of circulating anti-RM bacterium agglutinins in rainbow trout following outbreaks of ERM may form the basis of a serological procedure for determining prior exposure.

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiologic Agent. The remarks in item G of the furunculosis section apply here.

H. References.
Ross, A. J., R. R. Rucker, and W. H. Ewing. 1966. Description of a bacterium associated with redmouth disease of a rainbow trout (*Salmo gairdneri*). Canadian Journal of Microbiology, vol. 12, p. 763-770.

VI. Edwardsiella Septicemia

A. Name of the Disease and Etiologic Agent. Edwardsiella septicemia, *Edwardsiella tarda*. (Synonym--emphysematous putrefactive disease of catfish.)

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 (*Carasius auratus*), and fresh water eels (*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 in which it has been well documented, it initially manifests itself as small, cutaneous lesions located posteriolaterally 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.

D. Diagnostic Procedures for Disease Situations. Diagnosis is based on isolation and identification of the etiologic agent. Primary isolation should be made from kidney (and from other lesion material, if necessary) on to tryptic (trypticase) soy agar incubated at 20-25°C (preferably at 25°C) for 2-4 days.

1. Presumptive diagnosis. For presumptive identification, the etiologic agent should at least be shown to be a short, Gram-negative, cytochrome oxidase-negative rod that produces indole in tryptone broth and produces the following reactions on slanted triple sugar iron agar: an alkaline slant, and a butt showing acid and gas as well as hydrogen sulfide production (see Fig. 1). Additional characteristics that might also be verified for the organism in presumptive testing are its motility and its ability to ferment glucose (both acid and gas are produced in glucose O/F medium).

2. Confirmatory diagnosis

a. A diagnostic antiserum is not available for *E. tarda*. For a confirmed diagnosis the isolate must be shown to be urease negative in addition to possessing the characteristics listed in the preceding paragraph. The urease reaction serves to distinguish *E. tarda* from closely related organisms with which it might otherwise be confused.

b. For making a diagnosis, a sample of five moribund fish from each affected holding unit (e.g. tank, pond) is recommended. For amplification, see item D2c in the section on furunculosis.

E. Procedures for Detecting Asymptomatic Infections. The remarks in item E of the furunculosis section apply here.

F. Procedures for Determining Prior Exposure to the Etiologic 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 Etiologic Agent. The remarks in item G of the furunculosis section apply here.

H. References

1. Meyer, F. P., and G. L. Bullock. 1973. *Edwardsiella tarda*, a new pathogen of channel catfish (*Ictalurus punctatus*). Applied Microbiology, vol. 25, p. 155-156.

2. Wakabayashi, H., and S. Egusa. 1973. *Edwardsiella tarda* (*Paracolobactrum anguillimortiferum*) associated with pond-cultured eel disease. Bulletin of the Japanese Society of Scientific Fisheries, vol. 39, p. 931-936.

VII. Columnaris Disease

A. Name of the Disease and Etiologic Agent. Columnaris disease, *Flexibacter columnaris*. (Synonyms--*Chondrococcus columnaris*, cotton wool disease, mouth fungus.)

B. Known Geographic and Host Range of the Disease

1. Geographic range. Probably worldwide.

2. Host range. All fresh-water fishes are considered susceptible.

C. Clinical Signs of Diagnostic Significance. The disease affects fish of all ages and is favored by warm-water 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 or unremarkable even though the pathogen may be present.) External lesions may occur on the body surface, on the gills, or on both. On the fins, head and trunk, they occur initially as greyish-white cutaneous foci. The foci may enlarge to be several centimeters in diameter and the skin in the affected area may be eroded so that shallow ulcers are produced. 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.

D. Diagnostic Procedures in Disease Situations

1. Presumptive diagnosis. Presumptive diagnosis is based on showing that the lesions contain long, thin (5 to 12 microns by 0.75 micron), Gram-negative rods; it should produce a dry, rhizoid, yellowish colony on cytophaga agar within 3 days at 20°C, and should be motile by a gliding or flexing motion on solid surfaces; it should seldom cause disease at temperatures below 14°C.

2. Confirmatory diagnosis

a. The procedure of choice is the slide agglutination test using anti-*F. columnaris* serum. Because certain strains of *F. columnaris* agglutinate spontaneously in saline, the slide 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 the three papers listed below.

c. 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 item D2c in the section on furunculosis.

E. Procedures for Detecting Asymptomatic Infections. The remarks in item E of the furunculosis section apply here. Because *F. columnaris* is considered ubiquitous in fresh water, a search for a suitably sensitive procedure has not been actively pursued and may not be warranted.

F. Procedures for Determining Prior Exposure to the Etiologic Agent. No routine procedure is available, but the seasonal appearance of circulating anti-*F. columnaris* agglutinins in Columbia River fish has been interpreted as evidence of prior exposure to the pathogen.

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiologic Agent. Specimens should be placed in individual plastic bags with precautions taken to see that external lesions are not contaminated during handling and transport. The bagged specimens should be transported on ice. Freezing should be avoided.

H. References

1. Ordal, E. J., and R. R. Rucker. 1944. Pathogenic myxobacteria. Proceedings of the Society for Experimental Biology and Medicine, vol. 56, p. 15-18.

2. Pacha, R. E., and S. Porter. 1968. Characteristics of myxobacteria isolated from the surface of fresh-water fish. Applied Microbiology, vol. 16, p. 1901-1906.

3. Pacha, R. E., and E. J. Ordal. 1970. Myxobacterial diseases of salmonids. *In* A Symposium on Diseases of fishes and Shellfishes, S. F. Snieszko, editor. American Fisheries Society Special Publication No. 5, p. 243-257.

VIII. Coldwater Disease

A. Name of the Disease and Etiologic Agent. Coldwater disease, *Cytophaga psychrophila*. *C. psychrophila* is not recognized as a species in the current edition of Burgey'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 from the continental United States, predominantly from the northwestern United States.

2. Host range. All salmonids are probably affected but juvenile coho (*Oncorhynchus kisutch*) and fall chinook (*O. tshawytscha*) salmon are particularly susceptible.

C. Clincial Signs of Diagnostic Significance. The disease is a fresh-water 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; these are frequently 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 Procedures for Disease Situations

1. Presumptive diagnosis. The disease may be considered presumptively diagnosed if it occurs at water temperatures of 12°C or below; if the lesions contain a long, thin (3.5 to 7.5 microns by 0.75 micron), Gram-negative rod; it 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. Confirmatory diagnosis

a. The procedure of choice is the slide agglutination test using anti-*C. psychrophila* serum (see section XII on Serological Procedures).

b. If the diagnostic antiserum is for some reason unavailable, a more laborious confirmatory procedure is to show that the isolate is morphologically, culturally, and biochemically identical (or essentially identical) with *C. psychrophila*, descriptions of which are given in the three works listed at the end of this section.

c. 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 item D2c in the section on furunculosis.

E. Procedures for Detecting Asymptomatic Infections. The remarks in item E in the furunculosis section apply here. It should be emphasized, however, that the pathogen is considered to be far more widespread in fresh water than is indicated above, and for this reason, a suitably sensitive detection procedure may not be warranted.

F. Procedures for Determining Prior Exposure to the Etiologic Agent. At the present no serological tests have been developed.

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiologic Agent. Specimens should be placed in plastic bags and shipped on ice. Freezing should be avoided.

H. References

1. Borg, A. F. 1960. Studies on myxobacteria associated with diseases in salmonid fishes. Wildlife Disease, vol. 8, p. 1-85, 2 microcards.

2. Pacha, R. E. 1968. Characteristics of *Cytophaga psychrophila* (Borg) isolated during outbreaks of bacterial cold-water disease. Applied Microbiology, vol. 16, p. 97-101.

3. Pacha, R. E., and E. J. Ordal. 1970. Myxobacterial diseases of salmonids. *In* A Symposium on Diseases of Fishes and Shellfishes, S. F. Snieszko, editor. American Fisheries Society Special Publication No. 5, p. 243-257.

IX. Saltwater Myxobacteriosis

A. Name of the Disease and Etiologic Agent. Saltwater myxobacteriosis, *Sporocytophaga*. (The taxonomic significance of *Sporocytophaga* is problematic at this time.)

B. Known Geographic and Host Range of the Disease

1. Geographic range. Reports to date have been from the northwestern United States and Scotland.

2. Host range. Immature salmonids in sea water.

C. Clinical Signs of Diagnostic Significance. The disease appears to be a cutaneous (non-systemic) condition with lesions occurring most frequently on the flanks and ventral surface of the fish; lesions on the gills have not been reported. The lesions, which can be very extensive, appear like surface abrasions. Mortalities are usually quite low and are likely due to loss of body fluids.

D. Diagnostic Procedures for Disease Situations

1. Presumptive diagnosis. The affected fish should show shallow external lesions that contain large numbers of a long relatively thick Gram-negative rod; in smears the rods are often bent into curious configurations (walking sticks and horseshoes); no growth occurs on cytophaga agar but on cytophaga agar supplemented with a 1.5-2.0% sea salts, yellow colonies are produced in several days at 20-22°C; microcysts are often present among the vegetative cells of older colonies.

2. Confirmatory diagnosis

a. No confirmatory serological techniques are available.

b. For making a diagnosis, a sample of five moribund fish from each affected holding unit (e.g. tank, raceway, pond, enclosure) is recommended. For amplification, see item D2c in the section on furunculosis.

E. Procedures for Detecting Asymptomatic Infections. Asymptomatic carriers are not likely to be of great significance because the organism is likely to be carried on the surface of the fish in which position it may be washed off or effectively treated; in addition, the organism is thought to be far more widespread in sea water than is indicated above. A suitably sensitive detection procedure may therefore be unnecessary.

F. Procedures for Determining Prior Exposure to the Etiologic 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 Etiologic Agent. Specimens should be placed in individual plastic bags and then shipped on ice. Freezing, drying, and low salinity (as from melting ice) should be avoided.

X. Bacterial Kidney Disease

A. Name of the Disease and Etiologic Agent. Bacterial kidney disease, *Corynebacterium* sp. (Synonyms--kidney disease, corynebacterial kidney disease, Dee disease.)

B. Known Geographic and Host Range of the Disease

1. Geographic range. North America, Scotland, and Japan.

2. Host range. Confirmed isolations have been from salmonids to date; 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 occurs in fish less than 6 months old. The chronic disease is characterized internally by an enlarged, edematous kidney that may appear grey 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. A 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: exophthalmia, skin petechiation, vesicles in the skin.

D. Diagnostic Procedures for Disease Situations

1. Presumptive diagnosis. Smears of infected tissue should contain numerous small, Gram-positive, non-acidfast diplobacilli that occur both intra- and extra-cellularly; the organism should fail to grow on tryptic (trypticase) soy agar at 20°C, even when extended incubation periods (e.g. 2 weeks) are used.

2. Confirmatory diagnosis

a. The procedure of choice is the immunodiffusion test using antiserum prepared against the causative bacterium and homogenates of infected fish tissue (see section XII on Serological Procedures). A positive immunodiffusion test without demonstration of the bacteria in a smear should be considered a presumptive diagnosis only.

b. A more time-consuming and laborious procedure is to isolate the causative bacterium and show that it is identical (or essentially identical) with the kidney disease bacterium. (Descriptions of the kidney disease

bacterium are provided in the three papers listed below.) Isolation may be accomplished at 15-20°C on a blood- and -cysteine-enriched medium such as the one described by Ordal and Earp (1956) in the paper below, or still, on nutrient agar (Difco) containing -cysteine·HCl (1 mg/ml) and calf serum proteins (5 mg/ml), adjusted to pH 6.5 (T. Evelyn, unpublished data).

c. For making a diagnosis, a sample of five moribund fish from each affected holding unit (e.g. tank, raceway, pond, enclosure) is recommended. For amplification, see item D2c in the section on furunculosis.

E. Procedures for Detecting Asymptomatic Infections. The remarks in item E in the furunculosis section apply here. Monitoring of mortalities in seemingly healthy stocks should be done by immunodiffusion and culture. It should also be borne 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. Procedures for Determining Prior Exposure to the Etiologic Agent. None has been developed for routine use but studies based on circulating agglutinins are being conducted which may provide evidence of prior exposure.

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiologic Agent. Specimens are best packed in plastic bags and shipped on ice if they are destined for culture. However, freezing is acceptable for culture and for immunodiffusion testing.

H. References

1. Bullock, G. L., H. M. Stuckey, and P. K. Chen. 1974. Corynebacterial kidney disease of salmonids: growth and serological studies on the causative bacterium. Applied Microbiology, vol. 28, p. 811-814.

2. Evelyn, T. P. T., G. E. Hoskins, and G. R. Bell. 1973. First record of bacterial kidney disease in an apparently wild salmonid in British Columbia. Journal of the Fisheries Research Board of Canada, vol. 30, p. 1578-1580.

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

4. Smith, I. W. 1964. The occurrence and pathology of Dee disease. Scotland Department of Agriculture and Fisheries, Freshwater Salmon Fisheries Research Series No. 34. 12 p.

XI. Media and Reagents

A. Media. Incubation temperatures appropriate for the bacterium under test should be used.

1. Trypticase Soy Agar (BBL)
   Tryptic Soy Agar (Difco)

2. Oxidation/Fermentation (O/F) Basal Medium (BBL, Difco)--This medium is used with 1% glucose as the test carbohydrate. Instructions on the use of the medium and the interpretation of results are given in the BBL Manual of Products and Laboratory Procedures (5th ed. 1968), p. 129-130 and in Difco Supplementary Literature (Oct. 1968) p. 255. Note: a marine version of this medium is also available from Difco.

3. Triple Sugar Iron Agar (BBL; Difco)--Instructions on the use of this medium and the interpretation of results are given in the BBL Manual of Products and Laboratory Procedures (5th ed. 1968), p. 148-149, and in the Difco Manual (9th ed. 1953), p. 166-168.

4. Tryptone Broth--Use for testing for indole production. The medium should contain 1% tryptone and 0.5% NaCl. Methods and reagents for testing for indole are given in the Difco Manual (9th ed. 1953), p. 53-54.

5. Urea Agar or Urea Agar Base (BBL, Difco)--Use for determining urease activity. Medium may be obtained ready-for-use in slanted tubes (as Urea Agar), or it may be compounded in the laboratory using Urea Agar Base plus Agar. Instructions for preparing the medium and reading the results are given in the BBL Manual of Products and Procedures and in Difco Supplementary Literature.

6. Rimler-Shotts (RS) Medium--Use for detecting *Aeromonas liquefaciens* complex organisms. Preparation of the medium and interpretation of results are given in the following paper: Shotts, E. B., Jr., and R. Rimler. 1973. Medium for the isolation of *Aeromonas hydrophila*. Appl. Microbiol. 26: 550-553.

7. Cytophaga Agar--For the composition of this medium, see: Anacker, R. L., and E. J. Ordal. 1959. Studies on the myxobacterium *Chondrococcus columnaris*. 1. Serological typing, J. Bacteriol. 78: 25-32. Note: Synthetic sea water salts are added to this medium to grow marine myxobacteria; these salts are commercially available.

8. Media for motility testing

   a. Motility of the short rods is best determined by the hanging drop method using log phase cultures in tryptone broth (medium in 4 above) or by using one of the several motility test media available from BBL and Difco.

   b. Motility of the long rods (i.e. myxobacteria) is most reliably determined by microscopically observing cells at the margins of young colonies on cytophaga agar (agar block method).

B. Reagents

1. Gram stain and acid-fast stains--Reagents for the above stains are commercially available from a number of sources. Instructions on the use of the stains and on the interpretation of results are given in: Society of American Bacteriologists Manual of Microbiological Methods. 1957. (McGraw-Hill Book Co. Inc., New York, Toronto, and London.)

2. Cytochrome oxidase test--Paper test strips, impregnated with the appropriate chemicals, are available from the Patho Tec. Co., General Diagnostics Division of Warner Chilcott, Morris Plains, N. J., U.S.A.; instructions on the use of the test strips are provided in a pamphlet "Pathotec Test Papers in Diagnostic Microbiology" published by the company.

3. Vibriostatic agent 0/129--This compound is available commercially from Calbiochem. Use 20.0 micrograms of the compound per disc. Note: Prepare a 0.1% w/v solution of the compound in acetone and apply 0.02 ml/disc. Dry at 37°C to evaporate the acetone, and store discs in a tightly sealed bottle at 4°C.

4. Novobiocin sensitivity discs (Difco)--Use the 5 micrograms novobiocin per disc level. Note: the sensitivity tests with the vibriostatic agent 0/129 and novobiocin may be carried out on the same agar plate. The sensitivity test is described (p. 341) in Difco Supplementary Literature (Oct. 1968) and may be carried out on tryptic soy agar (p. 423).

C. Figure 1 shows procedures for the differentiation of certain gram-negative bacteria associated with fish diseases.

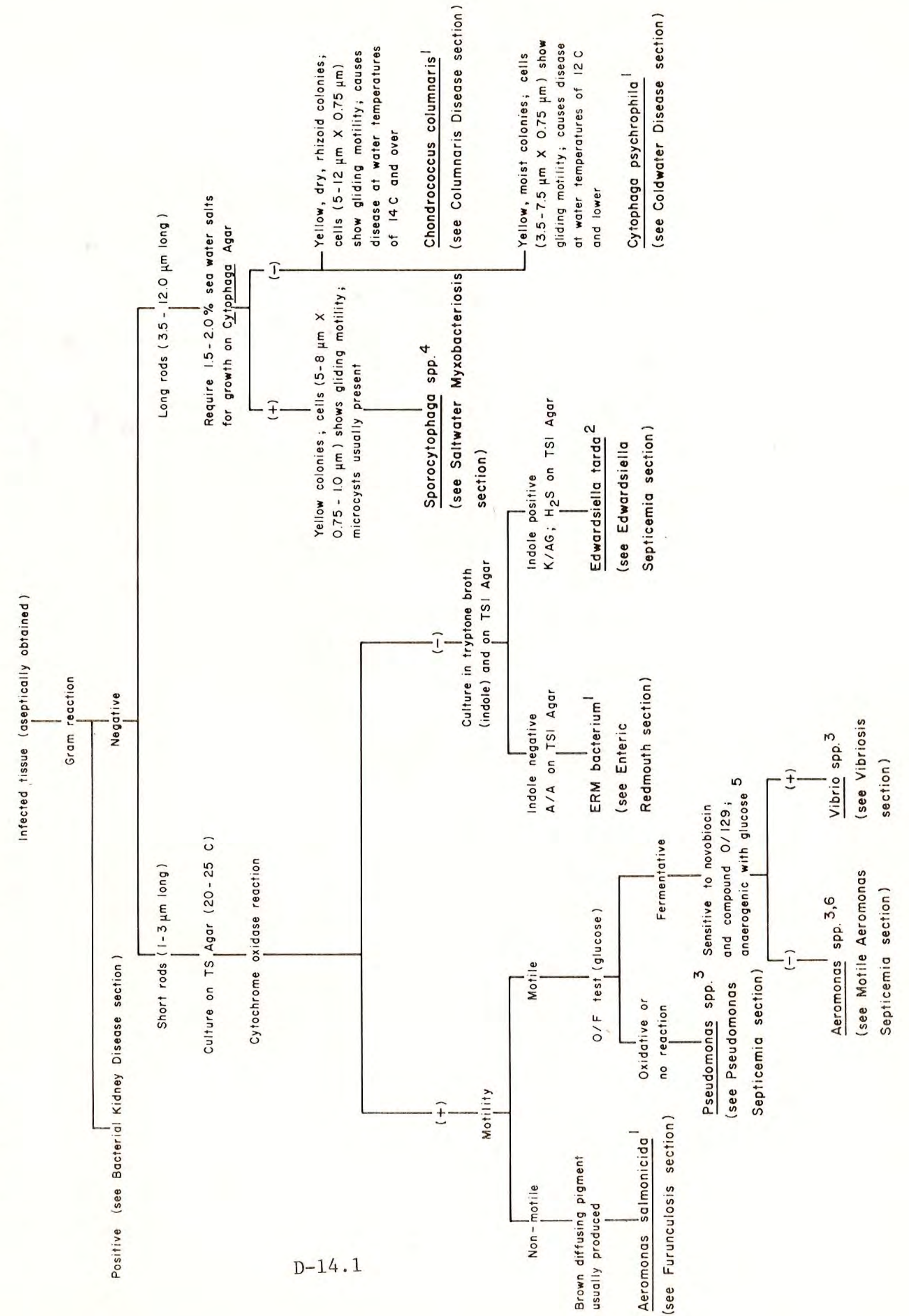

Figure 1. Procedures for the Differentiation of Certain Gram-Negative Bacteria Associated with Fish Diseases*

* Abbreviations: TS Agar = tryptic (trypticase) soy agar; O/F test (glucose) = test to determine whether glucose is attacked oxidatively or fermentatively; TSI Agar = triple sugar iron agar slants; A/A = acid slant/acid butt; K/AG, H_2S = alkaline slant/acid and gas, as well as hydrogen sulfide, in butt.

1 Confirmed identifications are achieved in slide agglutination tests using specific antisera.

2 A confirmed identification is obtained by showing the organism to be urease-negative on Urea Agar; this test serves to separate _E. tarda_ from other enteric organisms with which it might be confused.

3 Tests outlined, provide satisfactory confirmatory identification of the organisms in these genera.

4 Confirmed identification not yet possible.

5 An occasional motile _Aeromonas_ isolate may be anaerogenic.

6 If a motile _Aeromonas_ sp. is suspected, a rapid diagnosis may be obtained by use of the Rimler-Shotts medium (see the Motile Aeromonas Septicemia section).

XII. Serological Procedures

A. Slide Agglutination Test

1. Definition. A procedure for rapid confirmation of a presumptively identified bacterial isolate. The test is based upon agglutination of the isolate in saline suspension when mixed with specific antiserum.

2. Materials

   a. Glass slide. An alcohol-cleaned microscope slide or glass plate marked off into three 2.0 cm circular areas with a grease pencil.

   b. Test antigen. Log phase culture of the presumptively identified bacterial isolate to be confirmed.

   c. Diagnostic antiserum. A standardized specific and polyvalent antiserum prepared against a reference antigen. This reagent is available for many of the recognized bacterial fish pathogens from the Eastern Fish Disease Laboratory (EFDL*) and should be stored at 4°C with the addition of aqueous thimerosal to 1:10,000.

   d. Reference antigen. A standard control antigenic preparation capable of reacting specifically with the diagnostic antiserum. This reagent may be made from a confirmed positive isolate, or better still, should be obtained from EFDL* and stored as specified.

   e. Diluent. A physiological (0.90%) sodium chloride solution, autoclaved, and stored at room temperature.

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* Eastern Fish Disease Laboratory
U. S. Fish and Wildlife Service
Route 1, Box 17A
Kearneysville, West Virginia, U.S.A.
25430

3. Test procedure. Test reagents are added to the marked glass slide in the following manner:

Reagent	Test Area #1	#2	#3
Diluent	1-2 drops	1-2 drops	-
Test antigen	Suspend a loop full of material from a selected colony to homogeneity in the diluent		-
Reference antigen	-	-	2-3 drops of a homogeneous suspension
Diagnostic antiserum	1-2 drops	-	1-2 drops

The slide is then rotated gently for 30 seconds to achieve mixing. Agglutination is indicated by clumping beginning at the periphery of the suspension within 2 minutes. Absence of agglutination at the end of 2 minutes may be confirmed by noting homogeneity of the suspension under the microscope at low power.

4. Results and their interpretation. The following patterns of results in the three test areas may occur:

a. (+ - +). A confirmed identification is indicated by agglutination in test areas 1 and 3 and lack of agglutination in test area 2.

b. (+++ or ++-). A false positive result due to auto-agglutination is suggested by any detectable agglutination in area 2. The test should be rerun following heat treatment of the test antigen at 60°C for 1 hour or light sonication. These treatments often prevent auto-agglutination.

c. (--- or +--). A rare occurrence is the appearance of a false negative as suggested by the failure of the reference antigen to agglutinate in area 3. This result may be due to inactive diagnostic antiserum or to a mistake in reagent choice. The test should be rerun with correct reagents including fresh diagnostic antiserum.

d. (--+). A true negative result (proving that the isolate is different from the known pathogen) is indicated by the lack of agglutination in areas 1 and 2 and agglutination in area 3.

B. Double Immunodiffusion Test

1. Definition. This particular test is a rapid, specific, confirmatory procedure for visualizing the presence of diffusible antigens of fish pathogens while they are still in the host tissues. This test is specifically devised for application to bacterial kidney disease.

2. Materials

a. Medium. Prepare 0.5-0.75% agarose in pH 7.2 to 7.4 0.1 M phosphate buffered 0.90% physiological saline containing 0.2% sodium azide. Boil and pour into culture plates to a depth of 3-4 mm. Refrigerate to solidify and cut a well pattern such as the one shown in Figure 2. The wells should be 5.0 mm in diameter and 5.0 mm apart when measured from closest edges. The well pattern is best obtained using a commercial well-cutter or a cork-borer and template. Commercial Ouchterlony double diffusion plates designed for the purpose are available from: ICN Diagnostic Products
P. O. Box 3932
Portland, Oregon, U. S. A.
97708

b. Diagnostic antiserum. A standardized specific and polyvalent antiserum prepared against a reference antigen and available from EFDL.

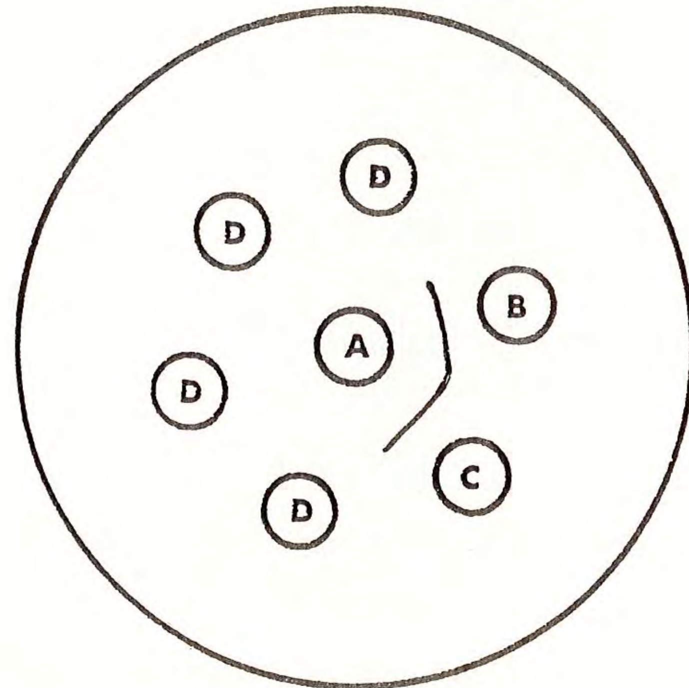
c. Reference antigen. A standardized specific antigenic preparation available from EFDL.

d. Test antigen. A portion of tissue (usually kidney) which is homogenized with an equal portion of sterile 0.9% physiological saline.

3. Test procedure. Diagnostic antiserum is pipetted into the central well of the diffusion plate, being careful not to overfill and flood the well. Reference and test antigens are pipetted into peripheral wells. Following 24 hours of incubation at room temperature in a humidified chamber the plate is examined under oblique lighting against a dark background.

Figure 2

Immunodiffusion plate showing continuous precipitin band formed by KD positive test antigen and KD suspect test antigen



A= Antiserum well
B= Test antigen well
C= Control antigen well
D= Additional test antigen wells as required

G. L. Bullock
H. M. Stuckey

4. Results and their interpretation. Positive confirmatory results are indicated by the presence of one or more precipitin bands in the agar space between the central well and the peripheral well containing test antigen and between the central well and the peripheral well containing the reference antigen. If a precipitin band forms only between the central well and the reference antigen, this constitutes a negative result (no, or insufficient, test antigen is present).

C. Indirect Fluorescent Antibody (IFA) Test

1. Definition. The IFA technique is designed to detect the presence of specific bacterial agents in suspect materials. The test involves two successive antigen-antibody reactions. The first reaction is between the bacterial agent and a diagnostic antiserum prepared against it. The second reaction is between the antiserum and fluorescing globulin prepared against the antiserum.

2. Materials

a. Fluorescent microscope. A special microscope and ultraviolet light source with a cardiod type darkfield condenser capable of dry field and oil immersion scanning. The appropriate filter combination for the specific fluorescent globulin conjugate should be used (i.e., fluorescein isothiocyanate works well with a BG-12 and OG-1 filter combination).

b. FA slides. Acid cleaned FA quality microscope slides and coverslips which are commercially available.

c. PBS. pH 7.6 0.1 M phosphate buffered 0.9% physiological saline. (Bicarbonate buffered saline with a pH of 10-12 may also be used in place of the PBS to enhance fluorescence as noted later.)

d. FA mounting medium. A special non-fluorescing mounting medium, available from commercial sources.

e. Diagnostic antiserum. A standardized, specific, and polyvalent antiserum prepared against a reference antigen. This reagent is available for many of the recognized bacterial fish pathogens from EFDL.

f. *Fluorescent globulin conjugate*. A standardized preparation of species-specific antiglobulin, conjugated with a fluorescent dye, and designed to immunologically combine with the diagnostic antiserum. This product is readily available from commercial sources, with a choice of fluorescent dyes such as fluorescein isothiocyanate or rhodamine.

g. *Reference antigen*. A saline suspension of a log phase broth culture of the reference bacterium. This is to be used as a positive control.

3. *Test procedure*. Two alcohol-cleaned FA slides are appropriately labeled to designate the sample and positions 1,2,3, and 4. A drop of the test antigen is applied to each of the positions 1,3, and 4. A drop of the reference antigen is applied to position 2. The slides are air dried at room temperature and fixed for 30 minutes in either methanol or acetone at $-20^{o}C$. The slides are again dried and may be stored at this time if necessary. The fixed preparations are gently washed in PBS for 3-5 minutes and gently blotted. Diagnostic antiserum is dropped on positions 1 and 2. Specific unconjugated antiglobulin is dropped on position 3. The slides are incubated at room temperature in a humidified chamber for 30 minutes. The slides are gently washed in three changes of PBS (5 minutes per wash). Fluorescent globulin conjugate is placed on all positions and the preparations incubated as above. The slides are again washed with PBS or a more alkaline bicarbonate buffered physiological saline may be used at this point to enhance fluorescence. The preparations are washed again in distilled water, blotted gently, and mounted with FA mounting medium.

4. *Results and their interpretation*. When the mounted preparations are examined by ultraviolet light microscopy, a positive confirmative test is indicated by the presence of bright fluorescing bacteria in positions 1 and 2 and the lack of similar fluorescence in control positions 3 and 4. Negative results are indicated by the lack of fluorescence in positions 1, 3, and 4 and the presence of bright fluorescing bacteria in position 2. For the test to be meaningful, position 2 should always fluoresce while positions 3 and 4 never should.

XIII. *Miscellaneous Bacterial Fish Diseases*. The following bacterial fish diseases have not been included in the foregoing work because of their uncertain or mixed etiology (disease A) or because of their infrequent occurrence (diseases B-H). Diseases B-H have not been diagnosed in wild or propagated fish within the last several years. Additional information on these diseases can be obtained in the references listed at the end of this section.

A. *Bacterial Gill Disease*. A peracute to acute non-systemic disease chiefly affecting propagated juvenile salmonids and ictalurids. Causes significant losses among juvenile salmonids.

B. *Ulcer Disease*. A subacute to chronic systemic bacterial disease of salmonids caused by *Hemophilus piscium*. Disease is still present in Northeastern United States.

C. *Flavobacteriosis*. An acute to chronic systemic bacterial disease of fresh water and marine fishes caused by several members of the genus *Flavobacterium*.

D. *Streptococcae septicemia*. An acute systemic bacterial disease of warmwater fishes caused by *Streptococcus* sp.

E. *Mycobacteriosis*. A chronic systemic granulomatous bacterial disease of all fishes caused by *Mycobacterium fortuitum*.

F. *Streptomycosis*. A chronic systemic bacterial disease of salmonids and certain other fresh water fish caused by members of the genus *Streptomyces*.

G. *Nocardiosis*. A chronic systemic bacterial disease of all fish caused by *Nocardia asteroides*.

H. *Pasteurellosis*. An acute systemic disease of marine fish caused by *Pasteurella piscicida*.

XIV. Selected Additional Reading on Bacterial Fish Diseases (1950-1974). The books and manuals in this bibliography contain much detailed and valuable information on bacterial fish diseases and are listed here for those wishing additional information on this topic.

A. Amlacher, Erwin. 1970. "Textbook of Fishes Diseases" Translated by Conroy and Herman TFH Publications.

B. Anonymous. 1971. "Fish Disease Manual, Region 3" Bureau of Sport Fisheries and Wildlife.

C. Davis, H. S. 1956. "Culture and Diseases of Game Fish" University of California Press.

D. Klontz, G. W. 1973. "Syllabus of Fish Health Management" Texas A & M University.

E. Leitritz, Earl. 1962. "Trout and Salmon Culture" Fish Bulletin No. 107, State of California Department of Fish and Game.

F. Mawdesley-Thomas, L. (Ed.). 1972. "Diseases of Fish" Symposia of the Zoological Society of London, No. 30 Academic Press.

G. Reichenbach-Klinke, H. and Elkan. 1965. "The Principal Diseases of Lower Vertebrates" Academic Press.

H. Sindermann, Carl. 1969. "Principal Diseases of Marine Fish and Shellfish" Academic Press.

I. Snieszko, S. (Ed.). 1970. "A Symposium on Diseases of Fishes and Shellfish" American Fisheries Society, Special Publication No. 5.

J. Snieszko, S. F. and Axelrod, H. R. (Eds.). 1971-1974. "Diseases of Fishes--Vols. I-IV" TFH Publications.

K. Wood, J. W. 1968. "Diseases of Pacific Salmon. Their Prevention and Treatment" Washington Department of Fisheries.

Section 4

METHODS FOR THE DIAGNOSIS OF CERTAIN PARASITIC FISH DISEASES

Foreword

The following methods were established by the Fish Health Section of the American Fisheries Society at its meeting in Denver, Colorado, August 12-15, 1974. Due to the rapid increase in interest in the area of aquaculture, it has become imperative that nationally accepted procedures be established for the detection of the more serious fish parasites.

The authors realize that the parasite list is not all inclusive; therefore, additional lists of parasite diseases have been compiled.

I. Whirling Disease

A. Name of the Disease and Etiologic Agent. Whirling disease, *Myxosoma cerebralis*. (Synonym: blacktail.)

B. Known Geographic and Host Range of the Disease

1. Geographic range. California, Connecticut, Massachusetts, Michigan, Nevada, Ohio, Pennsylvania, Virginia, West Virginia. The agent has also been found in continental Europe, England, New Zealand, South Africa, USSR, and South America.

2. Host range. All species of salmon, trout, and grayling.

C. Clinical Signs of Diagnostic Significance

1. Whirling. Mad, tail-chasing behavior, particularly at feeding time or when startled. This usually occurs at two to eight months of age.

2. Skeletal deformities. Signs include sunken heads and spinal curvatures.

3. Blacktail. The posterior of the fish may be dark and nearly black at two to eight months of age.

4. Mortality occurs, although not catastrophically.

D. Diagnostic Procedures for Disease Situations. Remove the head, deflesh where feasible, and cut lengthwise. Grind the cranium and gill arches in a mortar, add about 20 ml of water, stir thoroughly and examine two or more drops thoroughly for spores at high-dry magnification (430X). Staining with fast green will increase ease of detecting spores. As an alternate method, remove the head and cut lengthwise, scrape the cut surface with a scalpel, add a little water, mix and examine two drops as above. Diagnosis of an epizootic is considered positive if all of the following criteria have been satisfied:

1. Determine the presence of typical spores which do not contain iodinophilous vacuoles.

2. Determine that the size of fresh or preserved spores are 8-10 microns.

3. Use histological sections to verify the presence of spores in cartilagenous tissue.

E. Procedures for Detecting Asymptomatic Infections. Use a sampling pattern to provide detection at a 5% level of incidence according to the sample size table. Larger samples are recommended in cases where there is a past history of whirling disease. The following two procedures are equally acceptable for the detection of carrier fish:

1. Digest method

a. Collect heads from salmonids 4 to 12 months of age in batches of 10 to 20 fish per lot. Refrigerate at 5°C for storage.

b. Heat heads in 50°C water for 5 to 10 minutes. Using forceps, pick heads free of pigmented tissue, eyes and musculature. Save gill arches and leave brain in place. Weigh defleshed heads to nearest gram.

c. Mince heads into 3-5 mm (1/4 inch) pieces with scissors.

d. Prepare 0.5% pepsin and 0.5% HCl by adding 5.0 ml of concentrated HCl and 5.0 grams of pepsin (Difco) to one liter of deionized water. Pepsin-acid solution may be refrigerated for up to two weeks or frozen at -20°C for prolonged storage.

e. Using 25 ml of pepsin solution per gram of tissue, calculate the total pepsin solution required for digestion. Digest heads in one-half of the calculated volume of pepsin solution; 30 minutes for small heads, up to 3 to 4 hours for large heads. Digest at 37°C for the above mentioned times. Centrifuge digest at 1200 G (full speed on clinical centrifuge with six 30 ml tubes) for 10 minutes and discard the supernatant. Transfer the sediment to the remaining pepsin solution and complete digestion at 37°C.

f. Centrifuge digest as before, measure the precipitate to the nearest ml, and then raise the pH to 8.0 to 8.5 with 0.1N NaOH (1 gm NaOH in 250 ml distilled water).

g. Prepare an alkaline (pH 8.0) trypsin solution by adding 5.0 grams of trypsin (Difco) to 1 liter of Rinaldini's saline which is composed of the following:

Sodium chloride		8.0 grams
Potassium chloride		0.2
Sodium citrate		1.0
Sodium hydrogen phosphate		0.05
Sodium bicarbonate		1.0
Glucose		1.0
Distilled deionized water	qs to:	1.0 liter

Grind trypsin in mortar, add 10 ml of Rinaldini's saline solution from above, mix and dilute to a liter. Add 1 ml of 1% phenol red, if desired. Filter through glass wool fiber (Corning 3950) and then through coarse filter paper or Millipore pre-filter.

h. Add sufficient trypsin solution (pH 8.0) to double the volume of the tissue suspension from step f above. Digest further at 22°C with stirring for 30 minutes.

i. Halt digestion by adding out-dated serum to make a final concentration of 1:4 (serum to digestive mixture). Filter suspension through glass wool fiber.

j. Centrifuge suspension at 1,200 G for 10 minutes. Discard supernatant. Re-suspend pellet in 10 ml or half-strength Balanced Salts Solution with 20% fetal calf serum.

k. Place 3 ml of 55% (w/v) aqueous dextrose solution in each of a pair of 12 ml conical centrifuge tubes. Carefully layer 5.0 ml of the re-suspended digest over the surface of the dextrose in each tube without mixing. Centrifuge at 1,200 G for 30 minutes.

l. Collect the pelleted material with a Pasteur pipet. Place 2-3 drops from each tube on a clean slide, place coverslip and examine at 430X for the presence of *M. cerebralis* spores.

m. Systematically search each slide for approximately 2½ minutes or until spores are found. Confirmation by another qualified fish disease specialist should be sought following a diagnosis of *M. cerebralis*.

n. After use all glassware used in the above procedure should be soaked for a minimum of 30 minutes in a solution containing 166 ml per liter of liquid bleach (Clorox, Purex, Hylex) before washing with soap and water. Instruments and tube caps should be boiled for 15 minutes.

2. Plankton centrifuge method

a. Take five to ten heads from 8 to 15 cm fish or one to five heads from larger fish and homogenize in a blender for 5 minutes in 175 to 200 ml of water. If infective or viable spores are desired, chlorinated water should be avoided.

b. Remove the homogenized material and vacuum filter the entire volume through bacteriological filter using only the wire mesh pre-filter. The filter may clog and need to be cleaned with water, but the rinse water must be saved and combined with the filtrate. This step of the procedure removes fish scales and other coarse material which can be discarded.

c. Place the entire filtrate in a separatory funnel so located to discharge into the plankton centrifuge. Start a low rate of flow from the separatory funnel into the centrifuge and run centrifuge at full speed.

d. Centrifuge until all water has been removed. The residue adhering to the walls of the revolving drum will contain spores and some fish tissue. Scrape all residue from the wall using a rubber policeman.

e. Place the residue and suspended material from the drum into a 27 ml bottle and fill completely with distilled water. Place the cap on the bottle and shake until the material is uniformly suspended (about 2 minutes).

f. Place a drop of the suspension on a slide and add coverslip. Scan the slide for spores at 430X magnification. A hema-cytometer may be used if quantitative results are desired. Four 1mm squares are counted in each chamber with each drop of suspension.

3. Formula for determining spore counts

$$\frac{\text{Number of spores}}{1 \text{ ml}} = \frac{\text{Total number of spores counted} \times 10^4}{\text{Number of 1 mm squares counted}}$$

27 ml (Suspended Volume) x number of spores in 1 ml = total number of spores present

4. All equipment and tissues associated with examinations for M. cerebralis should be disinfected with a 10% formalin solution or a 1:1 solution of clorox and water; the disinfected tissue should then be incinerated. Selected non-processed fish from all positive cases should be preserved in 10% formalin for future reference. It is probable that there will be mechanical carry-over of spores on equipment used in the test. To avoid false positive findings resulting from carry-over, the following steps should be considered:

a. Less costly items (microscope slides, coverslips, Pasteur pipettes) should be discarded.

b. All other equipment must be rigorously cleaned and disinfected before it is re-used.

F. Procedures for Determining Prior Exposure to the Etiologic Agent. At the present time there are no serological tests developed for use in detection of whirling disease.

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiologic Agent. Samples may consist of living, moribund, or dead fish which may be frozen prior to examination. To avoid the spread of M. cerebralis, it is not recommended that fish, mud, or water be transported away from the known geographic range of the parasite.

H. References

1. Contos, N., and H. Rothenbacker. 1973 An efficient concentration and purification method for spores of Myxosoma cerebralis. Progressive Fish-Culturist. In Press.

2. Hoffman, G. L., C. E. Dunbar, and A. Bradford. 1969. Whirling disease of trouts caused by Myxosoma cerebralis in the United States. U.S. Department of the Interior, Special Sceintific Report--Fisheries No. 427 (reprinted with additions). 15 p.

3. Hoffman, G. L., S. F. Snieszko, and K. Wolf. 1968. Approved procedure for determining absence of viral hemorrhagic septicemia and whirling disease in certain fish and fish products. U.S. Fish and Wildlife Service, Fish Disease Leaflet No. 9. 7 p.

4. Landolt, M. 1973. Myxosoma cerebralis: isolation and concentration from fish skeletal elements--trypsin digestion method. Journal of the Fisheries Research Board of Canada, vol. 30, p. 1713-1716.

5. Markiw, M. E., and K. Wolf. 1974. Myxosoma cerebralis: isolation and concentration from fish skeletal elements--sequential enzymatic digestions and purification by differential centrifugation. Journal of the Fisheries Research Board of Canada, vol. 31, p. 14-20.

6. Parsher, J. B., W. M. Tidd, and R. A. Tubb. 1971. Techniques for extracting and quantitatively studying the spore stage of the protozoan parasite Myxosoma cerebralis. Progressive Fish-Culturist, vol. 33, p. 193-196.

7. Rydlo, M. 1971. Evidence of Myxosoma cerebralis spores in various organs of rainbow trout afflicted with whirling disease. Osterreichs Fisch., vol. 24, p. 97-99 (in German).

8. Tidd, Wilber M., R. A. Tubb, and V. Wright. 1973. Modification of Myxosoma cerebralis spore extraction technique. Progressive Fish-Culturist, vol. 35, p. 227-228.

II. Salmonid Ceratomyxosis

A. Name of the Disease and Etiologic 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 uninfected 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.

(2) Washington. LaCamas Creek and LaCamas Lake; and the Cowlitz River.

(3) California. Pitt, Feather, Sacramento, and San Joaquin River Systems and the Klamath River (Iron Gate Hatchery).

b. Areas where infected adult fish have been found:

(1) Oregon. Widely distributed through the Columbia River Basin; Nehalem River; and the Trask River.

(2) Pacific Ocean. Occassionally observed in ocean caught salmon.

2. Host range. Coho salmon (Onchorhynchus kisutch), chinook salmon (O. tshcawytscha), sockeye salmon (O. nerka), rainbow 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 C. shasta is retarded when water temperatures are below 10°C, but the following clinical signs may develop in juvenile salmonids when water temperature is approximately 19°C or higher:

(1) Cessation of feeding approximately 13 to 16 days after infection.

(2) Mortality begins approximately 21 days after infection and may become catastrophic.

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

(4) Hemorrhagic areas develop in intesting; rectum may prolapse.

(5) Exophthalmia may occur.

(6) Spores may be found in ascitic fluid, gall bladder, and intestine.

2. Adult salmonids

a. Perforation of the intestine may occur with a resultant peritonitis, and swollen areas may develop in intestine.

b. Spores can usually be found in the intestine and gall bladder. On occasion, spores may be recovered from liver tissue where they occur in discolored areas.

D. Diagnostic Procedures for Disease Situations

1. Examine wet mounts from suspected tissue using phase contrast or bright field light microscopy (440X).

2. When using acid-fast staining of dried smears, the polar capsules will be acid-fast. (Do not use heat in acid fast stain.)

3. Permanent preparations can be prepared from smears fixed in Schaudin's fixative and stained with Heidenhains Iron Hematoxylin.

4. Examine 25 to 30 fields (440X) for the presence of spores.

E. Procedures for Detecting Asymptomatic Infections

1. See diagnostic procedures given in D.

2. 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 (assuming a carrier rate of 5%) is examined and found negative.

F. Procedures for Determining Prior Exposure to the Etiologic Agent. At the present time there are no serological tests that have been developed.

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiologic 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 not recommended that fish, mud, or water be transported away from the known geographic range of the parasite.

H. References

1. American Public Health Association. 1965. Standard methods for the examination of water and wastewater. 12th edition. New York. 769 p.

2. American Water Works Association. 1953. AWWA standard for filtering material. B100-53. New York. 16 p.

3. Bedell, G. W. 1971. Eradicating *Ceratomyxa shasta* from infected water by chlorination and ultraviolet irradiation. Progressive Fish-Culturist, vol. 33, no. 1, p. 51-54.

4. Burrows, R. E., and B. D. Combs. 1968. Controlled environments for salmon propagation. Progressive Fish-Culturist, vol. 30, no. 3, p. 123-136.

5. Conrad, J., and M. DeCew. 1966. First report of *Ceratomyxa* in juvenile salmonids in Oregon. Progressive Fish-Culturist, vol. 28, no. 4, p. 238.

6. Huff, C. B., H. F. Smith, W. D. Boring, and N. A. Clarke. 1965. Study of ultraviolet disinfection of water and factors in treatment efficiency. U.S. Public Health Service, Public Health Reports, vol. 80, no. 8, p. 695-704.

7. Leith, D. A., and K. D. Moore. 1967. Pelton Pilot Hatchery progress report, November 1966 through October 1967. Oregon Fish Commission, Research Division. Processed. 50 p.

8. Nagy, R. 1964. Application and measurement of ultraviolet irradiation. American Industrial Hygiene Association Journal, vol. 25, p. 274-281.

9. Noble, E. R. 1950. On a myxosporidian (protozoan) parasite of California trout. Journal of Parasitology, vol. 36, p. 457-460.

10. Sanders, J. E., J. L. Fryer, and R. W. Gould. 1970. Occurrence of the myxosporidian parasite Ceratomyxa shasta, in salmonid fish from the Columbia River Basin and Oregon coastal streams. A Symposium on Diseases of Fish and Shellfishes. American Fisheries Society Special Publication No. 5, p. 133-141.

11. Schafer, W. E. 1968. Studies on the epizootiology of the myxosporidian Ceratomyxa shasta Noble. California Fish and Game, vol. 54, p. 90-99.

12. Vlasenko, M. I. 1969. Ultraviolet rays as a method for the control of diseases of fish eggs and young fishes. Problems of Ichthyology, vol. 9, no. 5, p. 697-705.

III. Branchiomycosis

A. Name of the Disease and Etiologic Agent. Branchiomycosis, fungi of the genus *Branchiomyces*. (Synonyms: European gill rot.)

B. Known Geographic and Host Range of the Disease

1. Geographic range. Alabama, Arkansas, Florida, Georgia, Missouri, Ohio, Rhode Island, Wisconsin, Continental Europe, and USSR.

2. Host range. Largemouth bass (*Micropterus salmoides*), smallmouth bass (*Micropterus dolomieui*), striped bass (*Morone saxatilis*), guppy (*Poecilia reticulastus*), pumpkinseed (*Lepomis gibbosus*), northern pike (*Esox lucius*), and rainbow trout (*Salmo gairdneri*).

C. Clinical Signs of Diagnostic Significance. Anoxia, high level mortality, and localized gill necrosis, but these are not specifically diagnostic.

D. Diagnostic Procedures for Disease Situations

1. Presumptive diagnosis

a. Prepare wet mounts (tissue squash) of affected gill tissue.

b. Examine under 100X and 440X for non-septate hyphae and spores, usually present in lamellar capillaries.

2. Confirmatory diagnosis

a. Prepare standard histological sections.

b. Stain with H & E; iron haematoxylin; or PAS reaction.

c. Examine for non-septate hyphae and spores.

d. Compare morphology of hyphae and spores with characteristics given by Meyer and Robinson. (1973)

E. Procedures for Detecting Asymptomatic Infections. Sample adequate to detect a 5% level of infection or until a confirmed positive is found.

F. Procedures for Determining Prior Exposure to the Etiologic 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 Etiologic Agent. Ship suspect tissue containing live organism in unchlorinated tap water in screw-cap plastic container. Add an antibiotic such as gentamycin, streptomycin, or chloramphenicol as a bacteriostat. If such a shipment is not possible, ship tissue preserved in 10% formalin.

H. References

1. Amlacher, E. 1970. Textbook of Fish Diseases. Translated by D. A. Conroy and R. L. Herman. TFH Publications. New Jersey. p. 172-175.

2. Reichenbach-Klinke, H. H. 1973. Fish Pathology. TFH Publications. New Jersey. p. 135-138.

3. Meyer, F. P., and J. A. Robinson. 1973. Branchiomycosis: a new fungal disease of North American fishes. Progressive Fish-Culturist, vol. 35, no. 2, p. 74-77.

IV. Ichthyophonus Disease

A. Name of the Disease and Etiologic Agent. Ichthyophonus disease, *Ichthyophonus hoferi*, (Ichthyosporidium) hoferi.

B. Known Geographic and Host Range of the Disease

1. Geographic range. Trout culture in Western United States, marine fish, aquarium fishes.

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 is likely to occur whenever raw fish flesh is fed to cultured fishes.

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

1. Presumptive diagnosis. Examine wet squash of fresh kidney tissue or other suspect tissue under low power (100X) for spherical bodies of various sizes ranging from 10 to 100 microns. Observe spheres closely for hyphal protrusions.

2. Confirmatory diagnosis. Prepare histological sections stained with H & E. Refer to Reichenbach-Klinke (1973). (Chapter 3, page 102-130) for taxonomic details and morphology. Sectioned spores will have an amorphous appearance.

E. Procedures for Detecting Asymptomatic Infections. Histological sections of kidney tissues or other suspect tissues. Sample size should be adequate to detect 5% level of incidence.

F. Procedures for Determining Prior Exposure to the Etiologic Agent. At the present time there are no serological tests developed for use in detection of *Ichthyophonus*.

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiologic Agent. Fresh fish are preferred but one can detect this disease using tissue preserved in 10% formalin. Infected tissue can also be stored frozen with no ill effect.

H. References

1. Erickson, J. D. 1965. Report on the problem of Ichthyosporidium in rainbow trout. Progressive Fish-Culturist, vol. 27, no. 4, p. 179-184.

2. Fischthal. 1944. Observations on a sporozoan parasite of the eelpout, Zoarces anguillaris, with an evaluation of candling methods for its detection. Journal of Parasitology, vol. 30, no. 1, p. 35-36.

3. Ghiltino, P. 1970. Piscicoltura Ittiopatologia. Edizioni Rivista di zovtecnia. Italy. vol. 2, p. 166-173.

4. Gustafson, Paul V., and R. R. Rucker. 1956. Studies on an Ichthyosporidium infection in fish; transmission and host specificity. U.S. Department of the Interior, Fish and Wildlife Service Special Scientific Report-Fisheries No. 166.

5. Reichenbach-Klinke, H. H. 1973. Fish Pathology. TFH Publications. New Jersey. p. 102-123.

6. Ross, A. J., and Parisot. 1958. Record of the fungus Ichthyosporidium Caullery and Nesnil, 1905, in Idaho. Journal of Parasitology, vol. 44, no. 4, p. 453-454.

7. Rucker, R. R., and P. V. Gustafson. 1953. An epizootic among rainbow trout. Progressive Fish-Culturist, vol. 15, no. 4, p. 179-181.

8. Sindermann, Carl J., and L. W. Scattergood. 1954. Diseases of fishes of the Western North Atlantic. II. Ichthyosporidium disease of the sea herring (Clupea haerengus). Department of Sea and Shore Fisheries Research Bulletin No. 19. Maine.

V. Diseases of Concern

A. The following diseases are considered to be potentially dangerous, but due to limited knowledge at the present time concerning their impact on the resource, only concern about their occurrence and spread can be expressed. Detailed diagnostic methods have not been given for these diseases. Diagnosis is based on identification of the parasite which usually may be accomplished by referring to: Hoffman, G. L., 1967. Parasites of North American Freshwater Fishes. University of California Press, Berkeley and Los Angeles.

1. Microsporidia
   a. Neon tetra disease-Pleistophora hypessobryconis
   b. Pleistophora salmonae
   c. Glugea hertwigi
2. Myxosporidia. Interlamellar form of Henneguya exilis on channel catfish.
3. Digenea
   a. Sanguinicola klamathensi
   b. Sanguinicola davisi
4. Mycoses
   a. Systemic mycosis of catfish and trout
   b. Swimbladder fungus of salmonids
   c. Dermocystidium
5. Nematodes
   a. Goezia
   b. Philometra of the eye of centrarchids
6. Copepoda. Salminicola.

B. The following list contains parasites that cause significant losses each year but are of less concern than the preceding parasites. Most are widespread, but they should not be shipped to areas where they do not already exist. Important research needs are indicated.

1. Ichthyophthirius multifiliis (Ich, white spot). Although easy to detect in epizootics, the "immune" carriers are a continual threat in fisheries commerce. More research is needed on Xenodiagnosis (bioassay) and immunological methods. Cosmopolitan distribution.

2. Lernaea cyprinacea (anchor worm). Detection of juvenile forms in low incidence is difficult. Widespread in most areas of USA. Transfer to non-endemic areas should be avoided.

3. Miscellaneous external parasites. Some species of Trichodina, Epistylis, Chilodonella, Costia, Saprolegnia, Achlya, Gyrodactylus, and Dactylogyrus.

4. Eustrongylides. This large, unsightly, red nematode larva is widespread and pathogenic.

5. Myxidium of eels. One or more of these species is pathogenic to cultured eels. The genus is readily recognized but species identification is difficult.

6. Eimeria of eels and other fishes. These have frequently been overlooked. The eel form is pathogenic.

7. Pleistophora ovariae may destory up to 90% of the developing eggs in the ovaries of golden shiners.

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