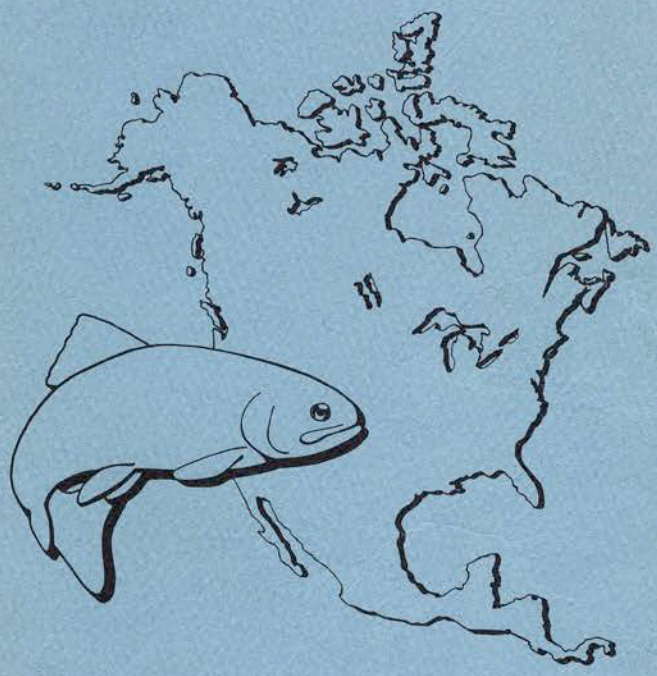
— Fish Health Blue Book —

PROCEDURES FOR THE DETECTION AND IDENTIFICATION OF CERTAIN FISH PATHOGENS



Fish Health Section
American Fisheries Society
AFS

AMERICAN FISHERIES SOCIETY

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PROCEDURES FOR THE DETECTION AND IDENTIFICATION OF CERTAIN FISH PATHOGENS

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EDITOR

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CONTENTS

SECTION

4 METHODS FOR THE DIAGNOSIS OF CERTAIN PARASITIC DISEASES

INTRODUCTION

Since the inception of the Fish Health Section of the American Fisheries Society, the development of standard methods has been a major goal. After due study by the Technical Procedures Committee to identify diseases for which standard methods were needed, a meeting of fish health specialists from the United States and several other countries was held in Denver, Colorado, in August 1974. The objective of the meeting was to decide which methods for diagnosis and detection of certain infectious diseases of fishes were to be proposed as standard and adopted by the Fish Health Section. The procedures given in the first edition of *Suggested Procedures for the Detection and Identification of Certain Infectious Diseases of Fishes*, published in 1975, were the methods considered to be the most reliable and sensitive.

The first edition was an unequivocal success and is known as the "Blue Book." The "Blue Book" is widely accepted in North America, Europe, and Japan, and it has resulted in the Fish Health Section becoming the lead organization in the fish health profession.

This edition is the first major revision and is in keeping with the original plans to revise the procedures as needed in order to assure that they reflect the state-of-the-art.

A comparison of the contents of this edition with the first edition shows that there have been new developments in the past few years. These changes are indicative of the dynamic nature of the fish health profession. We are pushing ahead to keep pace with the rapid growth of many aquacultural activities and increased interest in the epizootiology and health management of fish populations in nature and in husbandry.

The Executive Committee of the Fish Health Section wishes to express appreciation to all contributors of the revised text and to Mrs. Blanche Lawson, National Fish Health Research Laboratory, Kearneysville, West Virginia, for typing the revised edition.

David McDaniel
Editor

Section 1

GENERAL EXAMINATION PROCEDURES

I. Methods for Examination of Fish for Infectious Diseases

A. Sample only individual, carefully identified lots (species, age, source) of fish for examination. Use a sample size adequate to detect the infective agent (Table 1) in a carrier state or at the probability level requested.

Table 1. The minimum sample size for each lot will be in accordance with a plan which provides 95% confidence that an infected specimen will be included in the fish sampled, assuming a minimum incidence of infection 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

B. Samples should be collected from each affected tank or lot. Select any suspect fish, i.e. symptomatic or moribund specimens, as well as nonsymptomatic 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 6 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 before antiseptic or disinfectant procedures are applied. After the body has been opened using aseptic technique, tissues for bacterial cultures or tests should be collected before proceeding. An idealized necropsy procedure is outlined below.

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

2. Technique for Opening the Body Cavity

   a. 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.

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

3. Internal Examination. To reduce the risk of contamination, take bacterial inocula from the kidney and from any tissues that appear grossly abnormal immediately after opening the body cavity. Prepare and stain smears of these various materials. Record the appearance of the organs with respect to such features as: color and consistency, hemorrhage, inflammation,

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

4. 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.

Section 2

METHODS FOR THE DIAGNOSIS OF CERTAIN VIRAL 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. The use of two cell lines for each test is recommended to increase probability of virus isolation.

2. All cell culture stocks used for viral assays must be tested at three month intervals for presence of *Mycoplasma* spp. Testing may be done at qualified commercial or university laboratories. Alternatively new cell stocks may be obtained from the American Type Culture Collection (ATCC) or other sources of certified *Mycoplasma*-free cells.

3. All nonautoclavable media components must be tested and found free from *Mycoplasma* spp. and factors inhibitory to cell growth and/or virus replication. Certified *Mycoplasma* spp. free sera and other certified reagents need not be tested.

4. Only penicillin (100 IU/ml)-streptomycin (100 µg/ml) or gentamicin (100 µg/ml) and mycostatin (25 mg/ml) are permitted for routine cell culture work.

B. Virus Assay Controls

1. The positive control must demonstrate that the cells are sensitive to all fish viruses being assayed. If this is not possible, cells should periodically be sent to a laboratory to reaffirm 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 a multiplicity of infection of 0.1 $TCID_{50}$ per cell.

2. Uninoculated and 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 incubation period.

C. Sampling and Handling of Samples

1. During epizootics, a minimum of 10 fish in two pools (five fish each) shall be examined.

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.) The assumed minimum carrier incidence shall be 5% for fish tissue, kidney, and spleen samples. When examining ovarian fluid or seminal fluid, the assumed minimum carrier incidence shall be 2%.

3. Sample all fish populations by lot. A lot is defined as a group of fish of the same species and age that share 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 two complete inspections, with a 6-month interval, must be conducted on a hatchery before a disease status certificate is issued. An annual inspection is required to maintain a certified specific pathogen-free status.

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

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

6. Tissue examination 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 over 5 cm: Assay spleen and kidney only.

   d. Brood fish: 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. Samples may be pooled to keep numbers of virus assay units practical. No more than five fish shall be combined to form a pool.

8. Samples shall be assayed as soon as possible after collection; and must be stored or transported at 4°C. The samples should never be frozen nor stored for longer than 7 days prior to primary inoculation onto cell cultures.

9. If the samples must be transported or stored, and this time period exceeds 12 hours, the suspending medium shall be buffered saline (pH 7.0 to 7.8). At the discretion of the investigator, antibiotics may be used in the suspending medium to control growth of microbial contaminants. Only gentamicin, mycostatin, penicillin, and streptomycin shall be used, at concentrations no greater than those used for processing (Section C-11).

10. Tissues must be triturated by grinding in a mortar with pestle or homogenized by blender, homogenizer, or tissue grinder. Equipment used for homogenization within any given lot need not be sterilized between samples, unless the proportion of positive pools in the lot is being recorded. All equipment should be sterilized, preferably by autoclaving between sample lots.

11. The samples should be suspended in buffered saline (pH 7.0 to 7.8) and centrifuged at 2000 X g for 10 minutes and treated to retard microbial contaminants. Two methods for control of microbial contamination are available:

    a. Preferred method: Remove the supernatant and add an antibiotic mixture to obtain a final concentration of 100-2000 µg/ml gentamicin or 800 IU/ml of penicillin and 800 µg/ml streptomycin. Mycostatin may be added to give 400 units/ml. Incubate the mixture for 2 hours at room temperature or overnight at 4°C.

    b. Alternative method: Remove the supernatant and filter it through a 0.45 µm pore-diameter filter membrane. To avoid potential loss of virus due to filter adsorption, the maximum possible filtrate volume should be collected.

12. A minimum volume of each sample shall be inoculated onto appropriate cell cultures (Table 2). A minimum

of two cell culture units of each cell line shall be inoculated from each pool.

Table 2. Suggested inocula for various culture vessels

Assay vessel	Sample volume (ml)	Cell culture suspension (ml)
96 well plate	0.05 ml/well	0.10 ml/well
35 mm dish	0.10 ml/plate	monolayer
60 mm dish	0.30 ml/plate	monolayer

13. Ovarian fluid or seminal fluid samples shall be collected in five-fish pools with an approximately equal volume of fluid contributed by each fish. The sample can be clarified by centrifugation and treated with antibiotics as indicated in Section C-11.

14. Final dilution prior to inoculation onto cell cultures shall not exceed 1:200 (v/v). Final ovarian fluid or seminal fluid sample dilutions shall not exceed 1:10 (v/v).

15. Inoculated cell cultures will be incubated for 14 days. The incubation temperature will be dictated by the virus under consideration.

    a. IHN, IPN, or VHS viruses: 15°C.

    b. CCV: 27-30°C.

    c. *Herpesvirus salmonis:* 5-10°C.

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

16. 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, one or two blind passages are strongly advised. A blind passage is accomplished by removing fluid from inoculated cell cultures, diluting 1:100 (v/v) with buffered saline, and inoculating onto fresh cell cultures of the same type used for the first inoculation. Viral interference is known to occur with fish viruses.

D. Identification of Virus by Serum Neutralization

1. Select cell cultures showing typical virus CPE and

dilute fluid from these cultures to 1:1000 (10^{-3}) and 1:100,000 (10^{-5}).

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

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

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

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

6. Keep all mixtures at appropriate incubation temperature for 60 minutes, agitating each tube at 15 minute intervals.

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

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

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

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

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 101(2):267-269.

2. McAllister, P. E., and K. S. Pilcher. 1974. Autointerference in infectious hematopoietic necrosis virus of salmonid fish. Proceedings of the Society for Experimental Biology and Medicine 145:840-844.

3. Mulcahy, D. M., and J. L. Fryer. 1976. Double infection of rainbow trout fry with IHN and IPN viruses. Fish Health News 5(1):5-6.

4. Nicholson, B. L., and R. Dexter. 1975. Possible interference in the isolation of IPN virus from carrier fish. Journal of the Fisheries Research Board of Canada 32(8):1437-1439.

5. 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 30(9):1383-1384.

6. Schlotfeldt, H.-J., and J. W. Frost. 1975. Double infection of rainbow trout with viral haemorrhagic septicemia (VHS) and infectious pancreatic necrosis (IPN) viruses. Berliner und Muenchener Tieraerztliche Wochenschrift West Germany 88:454.

7. Wolf, K. 1970. Guidelines for virological examination of fishes. Pages 323-340 *in* S. F. Snieszko, editor. A symposium on diseases of fish and shellfish. American Fisheries Society Special Publication No. 5.

8. Wolf, K., and M. C. Quimby. 1969. Fish cell and tissue culture. Pages 253-305 *in* W. S. Hoar and D. J. Randall, editors. Fish physiology, vol. 3. Academic Press, New York.

9. Wolf, K., and M. C. Quimby. 1973. Towards a practical fail-safe system of managing poikilothermic vertebrate cell lines in culture. In Vitro 8(4): 316-321.

10. Wolf, K., and M. C. Quimby. 1973. Fish viruses: buffers and methods for plaquing eight agents under normal atmosphere. Applied Microbiology 25(4): 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*), eutthroat trout (*Salmo clarki*), lake trout (*Salvelinus namaycush*), Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), and chinook salmon (*Oncorhynchus tschawytscha*).

C. Clinical Signs of Diagnostic Significance

1. Infectious pancreatic necrosis is an acute disease causing mortality of fry and fingerlings, and occasionally of yearling trout and salmon. The largest and healthiest appearing fry or fingerlings usually are affected first. 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, exophthalmia, abdominal distension, and at times hemorrhages in ventral areas including bases of fins. Internally, multiple petechiae occur in the pyloric caecal area, and the liver and spleen are pale. The digestive tract is almost universally without food; accordingly, the stomach appears whitish. 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 clinical signs as described in Section II. C.

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. Histopathological observations may assist in diagnosing the disease. Isolation and identification of the virus as above accompanied by the demonstration of a high virus titer in the fish tissues (titer over 10^5 $TCID_{50}$/ml). A minimum of 10 fish in two pools is satisfactory for diagnosis provided that the sample is composed of fish showing clinical signs.

E. Procedures for Detecting Asymptomatic Infections

1. Follow the guideline in the general procedures section for sampling hatcheries according to lot. Kidney and spleen, whole fry, or visceral samples must be used for all assays except when dealing with situations where fish cannot be killed. In this case, ovarian and seminal fluid and feces can be used at the discretion of the investigator.

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

F. Procedures for Determining Prior Exposure to the 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 G. 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. Fish Disease Leaflet 27. 4 pp.

2. Jørgensen, P. E. V. 1973. Inactivation of IPN and egtved virus. Rivista Italiana Piscicoltura Ittiopatologia 8:107-108.

3. Wolf, K. 1972. Advances in fish virology: a review 1966-1971. Symposia of the Zoological Society of London 30:305-331.

4. Wolf, K. 1976. Fish viral diseases in North America, 1971-75, and recent research of the Eastern Fish Disease Laboratory, U.S.A. Fish Pathology 10(2): 135-154.

5. 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 34:144-156.

6. 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 104:105-108.

7. Yasutake, W. T. 1970. Comparative histopathology of epizootic salmonid virus diseases. Pages 341-350 *in* S. F. Snieszko, editor. A symposium on diseases of fishes and shellfish. American Fisheries Society Special Publication 5.

III. Viral Hemorrhagic Septicemia

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

B. Known Geographic and Host Range of the Disease

1. Geographic range. Viral hemorrhagic septicemia 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. A rhabdovirus similar to Egtved virus has been isolated from moribund brown 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 ascites, 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 muscle 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

Plaques
VHS - well defined margin - interior w/ uniform distributed finely granular debris - cells in interior usually all killed.
IHN - ragged margin - cells round up and accumulate at plaque margin - interior contains rounded cells and discontinuous coarsely granular debris - cells near edge have marginated of chromatin
IPN - stellate plaque, cells somewhat pyknotic but interior contains live cells w/ minimal cellular debris. Plaque retains lace-like network of RTG-2 cells, though killed, retain normal shape but stain lightly w/ crystal violet.

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 fix 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. To confirm diagnosis, the VHS virus should be isolated and its identity confirmed by serum neutralization or fluorescent antibody assay. Histopathological changes typical of VHS may aid in diagnosing the disease. 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 identify the disease.

E. Procedures for Detecting Asymptomatic Infections. The procedures for detecting VHS carriers can be followed as described for IHNV. Sample each lot at the assumed 5% incidence level. Collect only kidney and spleen and do

not process samples of tissue with more than five fish per pool. Follow cell culture and virological procedures as described above in Section 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 15:635-637.

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. 7 pp.

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

4. Kinkelin, P. de, and M. Le Berre. 1977. Isolation of a rhabdovirus pathogenic for brown trout (*Salmo trutta*). Comptes Rendus de l'Academie de Science, Paris 284(D): 101-104.

5. Meier, W., and P. E. V. Jorgensen. 1975. A rapid and specific method for the diagnosis of viral haemorrhagic septicemia (VHS) of rainbow trout. Rivista Italiana Piscicoltura Ittiopathologia 10:11-16.

6. Wolf, K. 1972. Advances in fish virology: a review 1966-1971. Symposia of the Zoological Society of London 30:305-331.

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

8. Yasutake, W. T., and C. J. Rasmussen. 1968. Histopathogenesis of experimentally induced viral hemorrhagic septicemia in fingerling rainbow trout (*Salmo gairdneri*). Bulletin de l'Office International des Epizooties 69:977-984.

IV. Infectious Hematopoietic Necrosis

A. Name of the Disease and Etiologic Agent. Infectious hematopoietic necrosis (IHN), infectious hematopoietic necrosis virus (IHNV). (Synonyms: the disease has also been referred to as Oregon sockeye disease or Sacramento River chinook disease. The 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, New York, and Hokkaido, Japan.

2. Host range. The natural host range includes rainbow trout (*Salmo gairdneri*), including steelhead; chinook salmon (*Oncorhynchus tshawytscha*), and sockeye salmon (*O. nerka*). So far as is known, the coho salmon (*O. 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 in 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 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) One or two blind bassages should be done when positive samples do not occur in a lot when the investigator feels there is a possibility that IHN virus is present. Blind passages of negative samples are essential when carrier rate determinations are being done.

(3) 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. Isolation of virus with neutralization by specific IHN antiserum and associated with typical IHN histopathological changes positively identifies the disease as IHN. Serum neutralization identifies the virus; the presence of typical pathological changes must be present to positively diagnose the disease.

In the absence of demonstrated histopathological changes, virus neutralization with specific antiserum and a high titer (10^5 infectious units or above) of the sample can be used to positively identify the disease. Procedures for virus neutralization are described in General Procedures for Cell Culture and Virology.

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. A greater proportion of samples will be positive when samples are taken from post-spawning fish than from pre-spawning samples or samples taken during spawning. 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 five 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 in Section 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 pp.

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 27(7):1285-1293.

3. Amend, D. F., and L. Smith. 1974. Pathophysiology of infectious hematopoietic necrosis virus disease in rainbow trout (*Salmo gairdneri*): early changes in blood and aspects of the immune response after injec-

tion of IHN virus. Journal of the Fisheries Research Board of Canada 31(8):1371-1378.

4. 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 98(4):796-804.

5. 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 44:270-279.

6. 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 137:1042-1046.

7. Williams, I. V., and D. F. Amend. 1976. A natural epizootic of infectious hematopoietic necrosis in fry of sockeye salmon (*Oncorhynchus nerka*) at Chilko Lake, British Columbia. Journal of the Fisheries Research Board of Canada 33(7):1564-1567.

8. Yasutake, W. T., and D. F. Amend. 1972. Some aspects of pathogenesis of infectious hematopoietic necrosis (IHN). Journal of Fish Biology 4:261-264.

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

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

C. Clinical Signs of Diagnostic Significance

1. Epizootics are characterized by a 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, exophthalmia, 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 to yellowish fluid (ascites), and hemorrhages are evident throughout the musculature, liver, kidney, and spleen. The liver, kidney, stomach, and intestine may be pale in advanced states of disease. The gastrointestinal tract is filled with a mucoid secretion and it is void of food.

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

Cardiac tissue may become necrotic, and focal hemorrhages may occur in the cardiac musculature.

D. Diagnostic Procedures for Disease Situations

1. Presumptive diagnosis. Samples are processed as described in General Procedures for Cell Culture and Virology. Samples are inoculated onto brown bullhead (BB) cells (ATCC 59) and incubated at 25-30°C and at a pH of 7.2-7.4. Inoculated cultures are incubated for 14 days and observed for typical CPE. The presence of clinical signs during periods when water temperature exceeds 24°C can serve as presumptive diagnosis.

2. Confirmatory diagnosis. The virus must be isolated and its identity confirmed by serum neutralization or fluorescent antibody (FA) assay. The procedure for the serum neutralization assay is outlined in General Procedures for Cell Culture 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 possible prior exposure to CCV. Positive results do not mean that the fish are definitely carriers of CCV, nor do negative results mean that the fish are definitely free of CCV.

1. Serum samples from adult fish are heat inactivated at 56°C for 30 min. The serum is diluted 1:100 and reacted with known CCV at 100 $TCID_{50}$ or PFU/0.1 ml of serum/virus reactant mixture (tests conducted according to methods in General Procedures for Cell Culture and Virology. Sera demonstrating 50%+ plaque or $TCID_{50}$ reduction are considered "reactors" and can be considered to have come from fish that were weakly exposed to CCV.

2. Brood fish 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. Welborn, and J. P. Naftel. 1970. An acute viral disease of channel catfish. U.S. Bureau of Sport Fisheries and Wildlife. Technical Paper 43. 11 pp.

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 (*I. punctatus*) virus and antibody. Journal of the Fisheries Research Board of Canada 30(11):1641-1645.

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

4. Plumb, J. A. 1973. Effects of temperature change on mortality of fingerling channel catfish (*Ictalurus punctatus*) experimentally infected with channel catfish virus. Journal of the Fisheries Research Board of Canada 30(4):568-570.

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

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 35(3):170-172.

7. Wolf, K., and R. W. Darlington. 1971. Channel catfish virus: a new herpesvirus of ictalurid fish. Journal of Virology 8(4):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 29(2):149-150.

VI. Viral Erythrocytic Necrosis

A. Name of the Disease and Etiological Agent. Viral erythrocytic necrosis (VEN), originally piscine erythrocytic necrosis (PEN). Note: it appears that morphologically similar viruses of the icosahedral cytoplasmic deoxyribovirus group may be responsible for the disease in different classes of vertebrates, the virus involved depending on the host. Until they have been isolated and characterized it is recommended that they be designated by VEN followed by the name of the host, i.e. VEN of gadoid fishes, VEN of salmonid fishes.

B. Known Geographic and Host Range of the Disease

1. Geographic range. Probably worldwide in marine environment but, to date, the viruses have been demonstrated only in fish of the Atlantic and Pacific coasts of North America, the Welsh coast, and possibly the Italian coast. On the basis of light microscopy, VEN also occurs in Venezuela in one species of freshwater fish.

2. Host range. The viruses have been demonstrated in the following anadromous and marine species: chum and pink salmon (*Oncorhynchus keta* and *O. gorbuscha*), Atlantic and Pacific herring (*Clupea harengus harengus* and *C. harengus pallasi*), Atlantic cod (*Gadus morhua*), blenny (*Blennius pholis*), spot (*Leiostomus xanthurus*), and possibly the dogfish (*Scyliorhinus canicula*). Light microscopic evidence suggests that the following species may also suffer from VEN: the freshwater electric eel (*Electrophorus electricus*); the anadromous alewife (*Alosa pseudoharengus*) and rainbow smelt (*Osmerus mordax*); and the marine Atlantic tomcod (*Paralichthys oblongus*), longhorn sculpin (*Myxocephalus octodecemspinosus*), rock gunnel (*Pholis gunnellus*), sea raven (*Hemitripterus americanus*), seasnail (*Liparis atlanticus*), tautog (*Tautoga onitis*), and winter flounder (*Pseudopleuronectes americanus*).

C. Clinical Signs of Diagnostic Significance. The disease is characterized by the presence usually of a single, rounded, basophilic inclusion body, 1 to 4 microns in diameter, in the cytoplasm of affected erythrocytes. Apparently, only erythrocytes are infected, and the infection is best diagnosed by examining stained blood films. Severe infections, involving 90 to 100% of the erythrocytes, have been observed in feral fish, but the impact of the disease is unknown. The infection can be fatal in captive herring and in sea water-cultured chum and pink salmon, and high water temper-

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

D. Diagnostic Procedures for Disease Situations. The viruses responsible for VEN have not yet been isolated. Consequently, diagnosis is based on a light microscopic examination of Giemsa or Wright stained blood films. Note: If blood is unavailable for examination, stained smears prepared from blood-rich tissues such as kidney or spleen can be used.

1. Presumptive diagnosis. A presumptive diagnosis is based on demonstrating that the erythrocytes contain the characteristic ctyoplasmic inclusion bodies already mentioned.

2. Confirmed diagnosis. A confirmed diagnosis is based on demonstrating the presence of iridoviruslike virions in the cytoplasm of erythrocytes using electron microscopy. In thin sections, the virion outer coat is typically hexagonal in outline; there is usually a closely apposed inner coat that in turn surrounds a centrally-located circular nucleoid. The diameters of the virions in teleosts range from 154 nm in the Atlantic herring to 330 nm in the Atlantic cod.

E. Procedures for Detecting Asymptomatic Infections. None are available; however, low-grade infections may be detected by resorting to exhaustive examinations of stained blood films.

F. Procedures for Determining Prior Exposure to the Etiologic Agent. None are available.

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent. Samples are best transported in plastic bags on ice. If blood is to be examined by electron microscopy, 10 to 20 units of heparin must be added per ml of blood.

H. References

1. Arcay de Peraza, L., and M. T. de McLure. 1971. The "paranuclear corpuscles" in poikilothermal vertebrates. II. Description of a new species of *Toddia* in *Electrophorus electricus* (electric eel), with an expansion of the key to the species of the genus *Toddia* in poikilothermal vertebrates. Acta Biologica Venezuelica 7:201-209.

2. Evelyn, T. P. T., and G. S. Traxler. 1978. Viral erythrocytic necrosis: natural occurrence in Pacific salmon and experimental transmission. Journal of the Fisheries Research Board of Canada 35(6):903-907.

3. Johnston, M. R. L. 1975. Distribution of *Pirhemocyton* Chatton and Blanc and other, possibly related, infections of poikilotherms. Journal of Protozoology 22(4): 529-535.

4. Kelly, D. C., and J. S. Robertson. 1973. Icosahedral cytoplasmic deoxyriboviruses. Journal of General Virology 20(Suppl.):17-41.

5. Philippon, M., B. L. Nicholson, and S. W. Sherburne. 1977. Piscine erythrocytic necrosis (PEN) in Atlantic herring (*Clupea harengus harengus*): evidence for a viral infection. Fish Health News 6(1):6-10.

6. Sherburne, S. W. 1973. Erythrocyte degeneration in the Atlantic herring, *Clupea harengus harengus* L. Fishery Bulletin 71(1):125-134.

7. Sherburne, S. W. 1977. Occurrence of piscine erythrocytic necrosis (PEN) in the blood of anadromous alewife, *Alosa pseudoharengus*, from Maine coastal streams. Journal of the Fisheries Research Board of Canada 34(2): 281-286.

8. Stoltz, D. B. 1971. The structure of icosahedral cytoplasmic deoxyriboviruses. Journal of Ultrastructure Research 37:219-239.

9. Walker, R., and S. W. Sherburne. 1977. Piscine erythrocytic necrosis virus in Atlantic cod, *Gadus morhua*, and other fish: ultrastructure and distribution. Journal of the Fisheries Research Board of Canada 34(8):1188-1195.

VII. Herpesvirus Disease of Salmonids

A. Name of the Disease and Etiologic Agent. Herpesvirus disease of salmonids (HP). *Herpesvirus salmonis* (HPV).

B. Known Geographic and Host Range of the Disease

1. Geographic range. Winthrop National Fish Hatchery, Washington, USA; Japan.

2. Host range. Rainbow trout (*Salmo gairdneri*), kokanee (*Oncorhynchus nerka*).

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

C. Clinical Signs of Diagnostic Significance

1. *Herpesvirus salmonis* has been isolated from adult rainbow trout at the Winthrop National Fish Hatchery, Washington. A morphologically similar virus has been found in natural epizootics occurring annually since 1970 among fry of kokanee salmon on Honshu Island, Japan. The agent has also been isolated from moribund and dead adult kokanee on Hokkaido Island, Japan. However it remains to be determined whether or not the virus causes death in adult salmonids. Most affected fry become lethargic as death approaches. During terminal stages some swim erratically while others are hyperactive.

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

D. Diagnostic Procedures for Disease Situations

1. Presumptive diagnosis.

a. Typical CPE in susceptible cell cultures (RTG-2) occurs at 10°C but not at 20°C. CPE consists of pyknosis and cell fusion. Multinucleated giant cells

are formed as in no other salmonid virus presently known. Atypical Cowdry type A inclusions can be demonstrated.

b. Histopathological examination: Blood in infected specimens contains abnormally high numbers of immature erythrocytes and leucocytes. Kidney hematopoietic tissue is hypertrophied and mitotically hyperactive. Kidney tubules may be filled with serous material; renal tissue is edematous but where it occurs necrosis is focal and mild.

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

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

Pancreatic syncytia pathognomonic

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

2. Confirmatory diagnosis. Diagnosis and virus identification is based on internal signs, histopathological changes, presumptive tests and ultimately on serum neutralization.

The virus is the only salmonid pathogen demonstrably capable of inducing syncytium formation in rainbow trout gonad cells (RTG-2). The rainbow trout fry (RTF-1) cell line is similarly susceptible; but fathead minnow (FHM) cells are refractory, or at least do not develop cytopathic effects. In fixed and stained cultures, the syncytia are readily visible. May-Grunwald-Giemsa staining reveals prominent cytoplasmic basophilia in the infected cells and intranuclear Cowdry type A inclusions.

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

Herpesvirus involvement in infected tissues or cell cultures, as revealed by electron microscopy, is also presumptive identification of the agent.

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

F. Procedures for Determining Prior Exposure to the Etiologic Agent. None are available.

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiologic Agent. Samples should be frozen for not more than 7 days.

H. References

1. Sano, T. 1976. Viral diseases of cultured fishes in Japan. Fish Pathology 10(2):221-226.

2. Wolf, K. 1976. Fish viral diseases in North America, and research of the Eastern Fish Disease Laboratory. Fish Pathology 10(2):135-154.

3. Wolf, K., and W. G. Taylor. 1975. Salmonid viruses: a syncytium-forming agent from rainbow trout. Fish Health News 4(2):3.

4. Wolf, K., T. Nagabayashi, and M. C. Quimby. 1975. *Herpesvirus salmonis:* tests of species susceptibility, suggestions for isolation, geographic distribution, and control measures. Fish Health News 4(4):6.

5. Wolf, K., R. L. Herman, R. W. Darlington, and W. G. Taylor. 1975. Salmonid viruses: effects of *Herpesvirus salmonis* in rainbow trout. Fish Health News 4(3):8.

6. Wolf, K., and M. C. Quimby. 1976. *Herpesvirus salmonis:* growth characteristics in vitro. Fish Health News 5(1):9.

7. Wolf, K., R. W. Darlington, T. Nagabayashi, and M. C. Quimby. 1976. *Herpesvirus salmonis:* characterization of a new pathogen from rainbow trout (*Salmo gairdneri*). Abstracts of the Annual Meeting of the American Society for Microbiology. p. 235.

VIII. Suggested Procedure for PPLO Detection

A. Modified Fabricant's Medium

1. Cells, sera, and medium components should be checked for PPLO at least once every 3 months.

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

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

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

5. Modified Fabricant's medium formulation.

a.	PPLO broth without crystal violet (Difco) (To make plates, substitute Difco PPLO agar for the PPLO broth without crystal violet.)	70%
b.	Horse serum	20%
c.	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. Bring to boil, reduce heat, simmer 15 minutes, 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%
d.	0.2% solution of DNA (Na^+ salt from thymus)	1%
e.	10% Na citrate	1%
f.	Sterile molar potassium phosphate (K_2HPO_4) solution	2%

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

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

h. Inhibitors

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

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

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

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

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

D. References

1. Levine, E. M. 1974. A simplified method for the detection of mycoplasma. Methods in Cell Biology 8: 229-248.

2. McGarrity, G. J. 1976. Spread and control of mycoplasmal infection of cell cultures. In Vitro 12: 643-648.

Section 3

METHODS FOR THE DIAGNOSIS OF CERTAIN BACTERIAL DISEASES

Foreword

In the following pages, methods are outlined for the diagnosis and identification (Fig. 1) of the major bacterial fish diseases. Other bacterial diseases of concern have not been considered--some because of their sporadic occurrence and others because of their uncertain etiology. 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 are best suited for diagnosing overt infections. Satisfactory procedures for the routine detection of covert infections or for establishing that prior contact with a pathogen has occurred are still lacking. Progress in this area is being made and the last few years have seen some promising detection methods developed: these include a survey procedure for serum antibodies against enteric redmouth disease, a fluorescent antibody technique that may prove sensitive enough to detect low-grade infections with the enteric redmouth and kidney disease bacteria, and a procedure for eliciting overt furunculosis in carrier fish. These procedures may well prove suitable for the detection of other covert infections.

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 freshwater.

2. Host range. All freshwater 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 to 25°C for 24 to 48 hours.

1. Presumptive diagnosis. The organism, when cultured as above should be a gram-negative, nonmotile, cytochrome oxidase-positive, short (coccoid) rod (in tissues, it is more distinctly rod-shaped). Although the existence of achromogenic strains is well recognized, the majority of strains can be expected to produce a brown diffusing pigment when grown on the above isolation media. Finally, although information on the reaction with glucose is not required in the differentiative scheme outlined in Fig. 1, it is probably worth noting that all strains of *A. salmonicida* ferment glucose; with some strains the reaction may be delayed and/or anaerogenic.

2. Confirmatory diagnosis.

a. Confirmatory diagnosis is best accomplished serologically using specific anti-*A. salmonicida* serum.

The most widely used procedure is the slide agglutination test (see Section XVI. 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*, various subspecies of which are described in the 8th edition of Bergey's Manual of Determinative Bacteriology.

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 simple 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.

Notwithstanding the foregoing, where it is especially important to do so, the detection of covert infections with *A. salmonicida* may be facilitated by holding suspect fish at elevated temperatures and by injecting them with an immunosuppressor. A combination of the above treatments has been successfully used to elicit overt furunculosis in carrier trout.

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. In addition, if the prior exposure has resulted in covert infections, these infections might be detected using the heat stress-immunosuppressor technique just mentioned.

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. Buchanan, R. E., and N. E. Gibbons. 1974. Bergey's manual of determinative bacteriology, 8th edition. Williams and Wilkins Co., Baltimore, Maryland. 1268 pages.

2. Bullock, G. L., and H. M. Stuckey. 1975. *Aeromonas salmonicida*: detection of asymptomatically infected trout. Progressive Fish-Culturist 37(4):237-239.

3. 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 28(10):1629-1634.

4. Krantz, G. E., and C. E. Heist. 1970. Prevalence of naturally acquired agglutinating antibodies against *Aeromonas salmonicida* in hatchery trout in central Pennsylvania. Journal of the Fisheries Research Board of Canada 27(5):969-973.

5. McCarthy, D. H. 1975. Fish furunculosis, caused by *Aeromonas salmonicida* var *achromogenes*. Journal of Wildlife Diseases 11:489-493.

6. Snieszko, S. F., and G. L. Bullock. 1975. Fish furunculosis. U.S. Bureau of Sport Fisheries and Wildlife. Fish Disease Leaflet 43. 10 pp.

IA. Ulcerative Furunculosis of Goldfish

A phenotypic varient of *Aeromonas salmonicida* was found to be the cause of extensive losses of goldfish in commercial hatcheries. Older (3 to 8 year) brood stock were primarily affected, mortality ranged from 45 to 99%. A study of 45 isolates, from the United States, England, and Japan, showed that goldfish *A. salmonicida* varied from typical *A. salmonicida* in its slow pigment production and failure to liquefy gelatin, and to ferment arabinose, glycerol, salicin, and trehalose. Unlike typical furunculosis, ulcerative furunculosis is almost always an external infection characterized in the advanced state by large, open necrotic lesions. Diagnostic procedures include isolation of the causative bacterium from the leading edge of the ulcer and identification by cultural and biochemical tests. Serological confirmation is accomplished with rabbit anti-*A. salmonicida* using agglutination or fluorescent antibody tests. At present there are no established treatment procedures.

An organism similar to the goldfish *A. salmonicida* was found to be the cause of carp erythrodermatitis. Additional data are needed to establish whether the organism from carp is identical to the goldfish pathogen.

A. References

1. Bootsma, R., N. Fijan, and J. Blommaert. 1977. Isolation and preliminary identification of the causative agent of carp erythrodermatitis. (Izdvajanje i prethodna identifikacija uzrocnika eritrodermatitisa sarana.) Veterinarski Arhiv 47(6):291-302.

2. Elliott, D. G., and E. B. Shotts, Jr. 1978. Diagnosis and experimental induction of an ulcerative disease in goldfish. Pages 18-20 *in* Proceedings of the Joint Third Biennial Fish Health Section/AFS and Ninth Annual Midwest Fish Disease Workshops at Kansas City, Missouri. 15-18 August 1978.

3. Leteux, F. J. 1978. Decrease productivity and increased costs in commercial goldfish hatcheries caused by goldfish ulcer disease. Page 17 *in* Proceedings of the Joint Third Biennial Fish Health Section/AFS and Ninth Annual Midwest Fish Disease Workshops at Kansas City, Missouri. 15-18 August 1978.

4. McCarthy, D. H., and C. T. Rawle. 1975. The rapid serological diagnosis of fish furunculosis caused by "smooth" and "rough" strains of *Aeromonas salmonicida*. Journal of General Microbiology 86(1):185-187.

5. Shotts, E. B., D. G. Elliott, D. G. McCarthy, and F. D. Talkington. 1978. Characterization of the causative agent of ulcerative furunculosis in goldfish. Pages 21-24 *in* Proceedings of the Joint Third Biennial Fish Health Section/AFS and Ninth Annual Midwest Fish Disease Workshops at Kansas City, Missouri. 15-18 August 1978.

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 freshwater.

2. Host range. Probably in all freshwater 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 the isolation and identification of the etiologic agent. Primary isolation should be made from kidney on tryptic (trypticase) soy agar (TSA) incubated at 20 to 25°C for 24 to 48 hours. Note: If, for some reason, MAS is strongly suspected, kidney may--in addition--be inoculated onto Rimler-Shotts (RS) medium which should then be incubated at 35°C. The RS medium facilitates the rapid identification of organisms in the *A. hydrophila* complex. Colonies of the organisms are detectable within 18 to 24 hours, and only an additional 24 hours for subculturing on TSA to permit their identification using the cytochrome oxidase test are required. (Due to the acid reaction produced on the RS medium by organisms in the *A. hydrophila* complex, their identification using the acid-sensitive cytochrome oxidase test cannot be accomplished on this medium. Instead, the cytochrome oxidase test must be performed on growth taken from TSA.)

1. Presumptive diagnosis. Criteria for a presumptive diagnosis are satisfied if the TSA isolate proves to be a short, motile, cytochrome oxidase-positive, gram-negative, usually straight rod that 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 insensitive to the vibriostatic agent 0/129 and novobiocin (see Section XV. Media and Reagents).

b. A confirmed MAS diagnosis is obtained if the organism on RS medium produces yellow colonies within 18 to 24 hours, and if these colonies, subcultured on TSA, prove to be cytochrome oxidase-positive. Note: As indicated before, the cytochrome oxidase test should not be performed on growth taken directly from the RS medium.

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 D. 2. c. in the section on furunculosis.

E. Procedures for Detecting Asymptomatic Infections. The remarks in item E. of the furunculosis section regarding the lack of a simple detection procedure apply here. Because organisms of the *A. hydrophila* complex are considered to be ubiquitous in freshwater, 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.

H. References

1. Bain, N., and J. M. Shewan. 1968. Identification of *Aeromonas, Vibrio* and related organisms. Pages 77-84 *in* B. M. Gibbs, and D. A. Shapton, editors. Identification methods for microbiologists, part B. Academic Press, Inc., London and New York.

2. McCarthy, D. H. 1975. The bacteriology and taxonomy of *Aeromonas liquefaciens*. Ministry of Agriculture, Fisheries, and Food, Fish Disease Laboratory. Technical Report Series No. 2. Weymouth, Dorset. 107 pp.

3. Shotts, E. B., Jr., and G. L. Bullock. 1975. Bacterial diseases of fishes: diagnostic procedures for gram-negative pathogens. Journal of the Fisheries Research Board of Canada 32(8):1243-1247.

4. Snieszko, S. F., and G. L. Bullock. 1974. Diseases of fishes caused by bacteria of the genera *Aeromonas*, *Pseudomonas*, and *Vibrio*. U.S. Bureau of Sport Fisheries and Wildlife. Fish Disease Leaflet 40. 10 pp.

III. *Pseudomonas* septicemia

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

B. Known Geographic and Host Range of the Disease

1. Geographic range. Fish-pathogenic strains occur worldwide in fresh and seawater.

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 warmwater 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 to 25°C for 24 to 48 hours.

1. Presumptive diagnosis. Criteria for a presumptive diagnosis are satisfied if the isolate is a short, motile, cytochrome oxidase-positive, gram-negative rod that is oxidative or inactive with glucose (in glucose O/F medium) and frequently produces a fluorescent pigment.

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 D. 2. c. in the section on furunculosis.

E. Procedures for Detecting Asymptomatic Infections. The remarks in item E. of the furunculosis section regarding the lack of a simple detection procedure 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.

H. References

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

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

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

4. Shotts, E. B., Jr., and G. L. Bullock. 1975. Bacterial diseases of fishes: diagnostic procedures for gram-negative pathogens. Journal of the Fisheries Research Board of Canada 32(8):1243-1247.

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, saltwater 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 environments, but some outbreaks have occurred in freshwater.

2. Host range. All marine and freshwater fish are considered susceptible.

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

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, when necessary) on tryptic (trypticase) soy agar containing 1% NaCl incubated at 20 to 25°C for 24 to 48 hours.

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

2. Confirmatory diagnosis.

a. Criteria for a confirmed diagnosis are satisfied if, in addition to having the foregoing characteristics, the TSA isolate ferments glucose vibriostatic agent O/129 and novobiocin. (Note: Because of the presence of novobiocin in the RS medium, vibrios fail to grow on this medium.)

b. At present, serologic diagnosis of vibriosis may not always be possible because of a polyvalent antiserum covering all of the presently recognized serotypes of the fish-pathogenic vibrios is not yet available.

Notwithstanding this, antisera are available for identifying the two serotypes most often responsible for outbreaks of vibriosis in salmonids in North America. The recommended diagnostic procedure is the slide agglutination test (see Section XVI. Serological Procedures). Note: The problem of autoagglutination in saline has only rarely been encountered with the fish-pathogenic vibrios.

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 D. 2. c. in the furunculosis section.

E. Procedures for Detecting Asymptomatic Infections. The remarks in item E. of the furunculosis section regarding the lack of a simple detection procedure apply here. Because the fish pathogenic vibrios are considered ubiquitous (at least in seawater), 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.

H. References

1. Bain, N., and J. M. Shewan. 1968. Identification of *Aeromonas, Vibrio* and related organisms. Pages 77-84 *in* B. M. Gibbs, and D. A. Shapton, editors. Identification methods for microbiologists, part B. Academic Press, Inc., London and New York.

2. Bullock, G. L. 1977. Vibriosis in fish. U.S. Fish and Wildlife Service. Fish Disease Leaflet 50. 11 pp.

3. Conroy, D. A., and G. C. Withnell. 1974. The use of a slide agglutination test as an aid in the diagnosis of vibrio disease in fish. Rivista Italiana di Piscicoltura Ittiopatologia 9(3):69-74.

4. Harrell, L. W., A. J. Novotny, M. H. Schiewe, and H. O. Hodgins. 1976. Isolation and description of two vibrios pathogenic to Pacific salmon in Puget Sound, Washington. Fishery Bulletin 74:447-449.

5. Muroga, K., and S. Egusa. 1975. Studies on *Vibrio anguillarum* isolated from salt-water and freshwater fishes. Fish Pathology 8(1):10-25.

6. Skoge Johnsen, G. 1977. Immunological studies on *Vibrio anguillarum*. Aquaculture 10:221-230.

V. Enteric Redmouth

A. Name of the Disease and Etiologic Agent. Enteric redmouth (ERM), ERM bacterium, the proper name is *Yersinia ruckeri* sp. nov. (Synonyms--Hagerman redmouth disease, redmouth disease.)

B. Known Geographic and Host Range of the Disease

1. Geographic range. Until quite recently, the disease was thought to be limited to North America where confirmed isolations have been made in the following states/provinces: Alaska, Arizona, Arkansas, British Columbia, California, Colorado, Idaho, Indiana, Maine, Missouri, Nebraska, Nevada, New Mexico, North Carolina, Nova Scotia, Ohio, Oregon, Saskatchewan, Tennessee, Utah, Washington, and West Virginia. It now appears, however, that the disease may also occur in Australia. A recent study of long-frozen cultures isolated from diseased rainbow trout in Australia indicates the cultures to be the ERM bacterium.

2. Host range. Potentially all salmonids. Confirmed isolations have been made from Atlantic salmon (*Salmo salar*), brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*Oncorhynchus kisutch*), cutthroat trout (*Salmo clarki*), rainbow trout and steelhead (*Salmo gairdneri*), and sockeye salmon (*Oncorhynchus nerka*).

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; however, the frequent presence of reddening in the mouth, hemorrhages in the lower intestine, and a yellow discharge from the vent may be of some diagnostic value. In the more chronic infections, the clinical signs are somewhat more diagnostic of ERM and, when considered in conjunction with the origin and history of the fish, 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 to 25°C for 24 to 48 hours.

1. Presumptive diagnosis. For presumptive identification, the organism should be a gram-negative, cytochrome oxidase-negative rod that fails to produce indole in tryptone broth and produces an alkaline slant and an acid (only) butt on 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 glucose O/F medium) and its motility.

2. Confirmatory diagnosis.

a. Confirmatory tests are best accomplished serologically, using the slide agglutination test or the indirect fluorescent antibody technique. (see Section XVI. Serological Procedures). An advantage of the latter technique is that it can be applied directly to diseased tissue, thus facilitating an early diagnosis of ERM. Note: Two serotypes of the ERM bacterium are now recognized, one of them having been discovered only very recently.

b. If for some reason the appropriate diagnostic antiserum is not available, the isolate must be morphologically, culturally, and biochemically identical (or essentially identical) with the ERM bacterium (see pertinent references 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 D. 2. c. in the section on furunculosis.

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

F. Procedures for Determining Prior Exposure to the Etiologic Agent. The appearance of circulating anti-ERM bacterium antibodies in salmonids following outbreaks of ERM may form the basis for determining prior exposure to the pathogen. A simple and sensitive serological procedure--inert particle

agglutination--has been developed for surveying fish stocks for these antibodies.

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. Bullock, G. L., H. M. Stuckey, and E. B. Shotts, Jr. 1977. Early records of North American and Australian outbreaks of enteric redmouth disease. Fish Health News 6(2):96-97.

2. Bullock, G. L., and S. F. Snieszko. 1979. Enteric redmouth disease of salmonids. U.S. Fish and Wildlife Service. Fish Disease Leaflet 57. 7 pp.

3. Busch, R. A., and A. J. Lingg. 1975. Establishment of an asymptomatic carrier state infection of enteric redmouth disease in rainbow trout (*Salmo gairdneri*). Journal of the Fisheries Research Board of Canada 32(12):2429-2432.

4. Dulin, M. P., T. Huddleston, R. E. Larson, and G. W. Klontz. 1976. Enteric redmouth disease. University of Idaho, College of Forestry, Wildlife, and Range Science, Bulletin No. 8. Moscow. 15 pp.

5. Hansen, C. B., and A. J. Lingg. 1976. Inert particle agglutination tests for detection of antibody to enteric redmouth bacterium. Journal of the Fisheries Research Board of Canada 33(12):2857-2860.

6. Johnson, G. R., F. Wobeser, and B. T. Rouse. 1974. Indirect fluorescent antibody technique for detection of RM bacterium of rainbow trout (*Salmo gairdneri*). Journal of the Fisheries Research Board of Canada 31(12):1957-1959.

7. O'Leary, P. J. 1977. Enteric redmouth of salmonids: a biochemical and serological comparison of selected isolates. M.Sc. Thesis. Oregon State University. Corvallis. 93 pp.

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

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 (*Carassius auratus*), largemouth bass (*Micropterus salmoides*), brown bullhead (*Ictalurus nebulosus*), "wild mullet" and fresh water eel (*Anguilla japonicus*). The organism has also been found in a variety of other animals including seals, sea lions, turtles, alligators, and snakes, and has been implicated as a pathogen in certain diseases of humans, cattle, pigs, and birds.

C. Clinical Signs of Diagnostic Significance. The disease is favored by high water temperatures (30°C and above) and, in channel catfish it initially manifests itself as small, cutaneous lesions located 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. In the fresh water eel, the disease shares many signs in common with other bacterial septicemias but it may be distinguished from these by the nature of the putrefactive lesions produced in the kidney and liver. The lesions are essentially cavities filled with a foul-smelling, purulent material. The disease also may occur in peracute form showing none of the above pathology.

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

1. Presumptive diagnosis. For presumptive identification, the etiologic agent should 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 confirmed diagnosis is accomplished if the isolate is agglutinated in the slide agglutination test with anti-*E. tarda* serum (see Section XVI. Serological Procedures).

   b. If for some reason the diagnostic antiserum is not immediately available, the identity of the presumptively identified isolate is confirmed if it proves to be urease-negative and produces lysine decarboxylase. (The urease reaction serves to distinguish *E. tarda* from closely related organisms with which it might otherwise be confused.)

   c. 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 D. 2. c. in the section on furunculosis.

E. Procedures for Detecting Asymptomatic Infections. The remarks in item E. of the furunculosis section regarding the lack of a simple detection procedure 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. Egusa, S. 1976. Some bacterial diseases of freshwater fishes in Japan. Fish Pathology 10(2):103-114.

2. Ewing, W. H., A. C. McWhorter, M. R. Escobar, and A. H. Lubin. 1965. *Edwardsiella*, a new genus of

Enterobacteriaceae based on a new species, *E. tarda*. International Bulletin of Bacteriological Nomenclature and Taxonomy 15:33-38.

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

4. Wakabayashi, H., and S. Egusa. 1973. *Edwardsiella tarda* (*Paracolobactrum anguillimortiferum*) associated with pond-cultured eel disease. Bulletin of the Japanese Society of Scientific Fisheries 39(9):931-936. (In Japanese.)

5. White, F. H., C. F. Simpson, and L. E. Williams, Jr. 1973. Isolation of *Edwardsiella tarda* from aquatic animal species and surface waters in Florida. Journal of Wildlife Diseases 9:204-208.

VII. Pasteurellosis

A. Name of the Disease and Etiologic Agent. Pasteurellosis, *Pasteurella piscicida*. Some of the members of the genus *Pasteurella* and *P. plecoglosacida* n. sp. have been placed into the genus *Yersinia*. Whether *P. piscicida* should be moved to *Yersinia* is not clear and until a decision is made it will be left as *P. piscicida*. (Synonyms--white perch pasteurellosis, pseudotuberculosis, bacterial tuberculoidosis.)

B. Known Geographic and Host Range of the Disease

1. Geographic range. United States and Japan.

2. Host range. Occurs in marine and estuarine fishes including cultured yellowtail (*Seriola quinqueradiata*), sea bream (*Chrysophrys major*), white perch (*Morone americanus*), striped bass (*Morone saxatilis*), and cultured ayu (*Plecoglossus altivelis*).

C. Clinical Signs of Diagnostic Significance. Acutely infected fishes rarely show gross pathological changes. Slight dropsy, darkening, and anemia may occur in yellowtail just prior to death. Miliary lesions are common in kidneys and spleen. Histopathologically, the lesions contain masses of bacteria, epithelial cells, and fibroblasts. The causative bacterium occurs throughout the internal organs.

D. Diagnositc Procedures for Disease Situations. Diagnosis is based on isolation and identification of the causative bacteria. Primary isolation should be made at 20 to 25°C on brain heart infusion agar or tryptic soy agar containing 1 to 2% NaCl or seawater.

1. Presumptive diagnosis. For presumptive diagnosis the organism should be a gram-negative, nonpigmenting, nonmotile rod (1-2 µm long X 0.5-7.5 µm wide) which stains bi-polarly. Gelatinase is not produced, and acid but no gas is produced in O/F glucose.

2. Confirmatory diagnosis. Strains of *P. piscicida* have been shown to possess common antigens and confirmatory diagnosis can be accomplished by simple slide agglutination of isolated cultures or by direct fluorescent antibody test on infected tissues. The serological procedures will also separate *P. piscicida* from achromogenic *Aeromonas salmonicida*.

E. Procedures for Detecting Asymptomatic Infections. No procedures have been reported for detection of carriers.

F. Procedures for Determining Prior Exposure to the Etiologic Agent. None reported.

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

H. References

1. Bullock, G. L. 1978. Pasteurellosis of fishes. U.S. Fish and Wildlife Service. Fish Disease Leaflet 54. 7 pp.

2. Janssen, W. A., and M. T. Surgalla. 1968. Morphology, physiology, and serology of a *Pasteurella* species pathogenic for white perch (*Roccus americanus*). Journal of Bacteriology 96(5):1606-1610.

3. Kitao, T., and M. Kimura. 1974. Rapid diagnosis of pseudotuberculosis in yellowtail by means of the fluorescent antibody technique. Bulletin of the Japanese Society of Scientific Fisheries 40(9): 889-893.

4. Matsusato, T. 1975. Bacterial tuberculoidosis of culture yellowtail. Pages 115-118 *in* Proceedings of the Third U.S.-Japan Meeting on Aquaculture at Tokyo, Japan, 15-16 October 1974. Special Publication of Fishery Agency, Japanese Government and Japan Sea Regional Fisheries Research Laboratory.

5. McCarthy, D. H. 1975. Detection of *Aeromonas salmonicida* antigen in diseased fish tissue. Journal of General Microbiology 88(Pt 2):384-386.

6. McCarthy, D. H., and C. T. Rawle. 1975. The rapid serological diagnosis of fish furunculosis caused by "smooth" and "rough" strains of *Aeromonas salmonicida*. Journal of General Microbiology 86(Pt 1): 185-187.

VIII. Columnaris Disease

A. Name of the Disease and Etiologic Agent. Columnaris disease, *Flexibacter columnaris* (formerly *Chondrococcus columnaris*). (Synonyms--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 even though the pathogen may be present.) External lesions may occur on the body surface, on the gills, or on both. Lesions occur initially as greyish-white cutaneous foci on the fins, head, and trunk. The foci may enlarge to be several centimeters in diameter, 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. A peracute form has been noted in small catfish.

D. Diagnostic Procedures for Disease Situations

1. Presumptive diagnosis. The following criteria provide a basis for presumptive diagnosis: long, thin (5 to 17 X .7 μm) gram-negative rods in lesions; dry rhizoid, yellowish colonies on cytophaga agar (3 days incubation at 20°C); cells mobile (gliding or flexing), but no flagella; little or no disease produced at 14°C or lower.

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 auto-agglutination.

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

c. 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 D. 2. c. in the section on furunculosis.

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

F. 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. Becker, C. D., and M. P. Fujihara. 1978. The bacterial pathogen *Flexibacter columnaris* and its epizootiology among Columbia River fish. American Fisheries Society Monograph 2. 92 pp.

2. Fujihara, M. P., and F. P. Hungate. 1971. *Chondrococcus columnaris* disease of fishes: influence of Columbia River ladders. Journal of the Fisheries Research Board of Canada 28(4):533-536.

3. Fujihara, M. P., and R. E. Nakatani. 1971. Antibody production and immune responses of rainbow trout and coho salmon to *Chondrococcus columnaris*. Journal of the Fisheries Research Board of Canada 28(9):1253-1258.

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

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

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

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

IX. Coldwater Disease

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

B. Known Geographic and Host Range of the Disease

1. Geographic range. To date the disease has been reported only 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. Clinical Signs of Diagnostic Significance. The disease is a freshwater condition that normally occurs when the water temperatures are 12°C or below; juvenile fish are primarily affected, the causative organism being recoverable from both the external lesions and the internal organs. In alevins, the ventral surface of the yolk sac becomes eroded and the sac may rupture releasing its contents. In fingerlings with the acute form of the disease, the fish may darken in the peduncle region and die without any surface lesions appearing. More commonly, however, superficial lesions occur; 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 X 0.75 μm), 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. phychrophila* serum (see Section XVI. 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 references.

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 D. 2. c. 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 freshwater 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 Diseases 8:1-85. (2 microcards.)

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

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

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

X. 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 and northeastern United States and Scotland.

2. Host range. Immature salmonids in seawater.

C. Clinical Signs of Diagnostic Significance. The disease appears to be a cutaneous (nonsystemic) 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 may be significant among salmonids intensively cultured in seawater.

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 to 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 D. 2. c. 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 and may be washed off or effectively treated; in addition, the organism is thought to be far more widespread in seawater 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.

H. References

1. Anderson, J. I. W., and D. A. Conroy. 1969. The pathogenic myxobacteria with special reference to fish diseases. Journal of Applied Bacteriology 32(1):30-39.

2. Sawyer, E. S. 1976. An outbreak of myxobacterial disease in coho salmon (*Oncorhynchus kisutch*) reared in a Maine estuary. Journal of Wildlife Diseases 12:575-578.

XI. Bacterial Gill Disease

A. Name of the Disease and Etiologic Agent. Bacterial gill disease (Eastern gill disease). Although Flexibacteria have been traditionally implicated as etiologic agents a recently isolated organism tentatively classified as a *Flavobacterium* has been demonstrated to be the etiologic agent of bacterial gill disease in Japan.

B. Known Geographic and Host Range of the Disease

1. Geographic range. Worldwide, usually seen in hatchery reared fishes.

2. Host range. Principally salmonids, may occur in any warmwater fishes.

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

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

E. Procedures for Detecting Asymptomatic Carriers. Not applicable since etiologic agent is probably ubiquitous.

F. Procedures for Determining Prior Exposure to the Etiologic Agent. At present there is no standard procedure, agglutinins have been found in trout from previous outbreaks.

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiologic Agent. Living moribund specimens are preferred, but freshly dead, refrigerated specimens are probably satisfactory. Freezing should be avoided since its effect on the etiologic agent is not known.

H. References

1. Kimura, N., H. Wakabayashi, and S. Kudo. 1978. Studies on bacterial gill disease in salmonids--I. Selection of bacterium transmitting gill diseases. Fish Pathology 12(4):233-242. (in Japanese)

XII. Bacterial Kidney Disease

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

B. Known Geographic and Host Range of the Disease

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

2. Host range. Confirmed isolations have been from salmonids to date; all salmonids are considered susceptible. 6 mo or older

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., abdominal distension

/ fibrinous peritonitis
X cystic cavitation of body musculature

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. Kidney disease may be diagnosed using an indirect or direct fluorescent antibody test (FAT) applied to fresh, formalin fixed, or frozen infected tissues. Since the FAT is more sensitive than gram stain for detecting kidney disease bacteria, the procedure may be used in place of gram stain in presumptive diagnosis. The FAT is described in Section XVI. Serological Procedures.

b. An immunodiffusion procedure employing antiserum, fresh or frozen infected tissue, and one of the nonnutritive media described in Section XIV. may also be used to provide a definitive diagnosis.

c. 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 papers listed in the references.) 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), or still, on nutrient agar (Difco) containing cysteine·HCl (1 mg/ml) and calf serum proteins (5 mg/ml), adjusted to pH 6.5.

d. 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 D. 2. c. 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 FAT 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., and H. M. Stuckey. 1975. Fluorescent antibody identification and detection of the *Corynebacterium* causing kidney disease of salmonids. Journal of the Fisheries Research Board of Canada 32(11):2224-2227.

2. Bullock, G. L., H. M. Stuckey, K. E. Wolf, and P. K. Chen. 1975. Bacterial kidney disease of salmonid fishes. U.S. Fish and Wildlife Service. Fish Disease Leaflet 41. 7 pp.

3. Chen, P. K., G. L. Bullock, H. M. Stuckey, and A. C. Bullock. 1974. Serological diagnosis of corynebacterial disease of salmonids. Journal of the Fisheries Research Board of Canada 31(12):1939-1940.

4. Evelyn, T. P. T. 1977. An improved growth medium for the kidney disease bacterium and some notes on using the medium. Bulletin de l'Office International des Epizooties 87(5-6):511-513.

5. 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 30(10):1578-1580.

6. 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 92:85-88.

7. Smith, I. W. 1964. The occurrence and pathology of Dee disease. Scotland Department of Agriculture and Fisheries, Freshwater Salmon Fisheries Research Series 34:1-12.

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). These diseases are closely associated with poor water quality and/or management practices. Additional information may be obtained in the references listed at the end of this section.

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

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

C. Streptococci septicemia. An acute systemic bacterial disease of warmwater and marine fishes caused by *Streptococcus* sp.

D. Mycobacteriosis. A chronic systemic granulomatous bacterial disease of all fishes caused by *Mycobacterium fortuitum*, *M. marinum*.

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

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

G. Anaerobic Pathogens. Systemic infections of several marine species were reported to be caused by *Catenabacter* sp. and *Eubacterium tarantellus*.

XIV. Selected Additional Reading on Bacterial Fish Diseases.
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, E. 1970. Textbook on fish diseases. (Translated and updated by D. A. Conroy and R. L. Herman.) T.F.H. Publications, Inc., Neptune City, New Jersey. 302 pp.

B. Anderson, D. P. 1974. Fish immunology, Book 4 (239 pp) *in* S. F. Snieszko and H. R. Axelrod, editors. Diseases of fishes. T.F.H. Publications, Inc., Neptune City, New Jersey.

C. Bullock, G. L. 1971. Identification of fish pathogenic bacteria, Book 2B (41 pp) *in* S. F. Snieszko and H. R. Alexrod, editors. Diseases of fishes. T.F.H. Publications, Inc., Neptune City, New Jersey.

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

E. Mawdesley-Thomas, L., editor. 1972. Diseases of fish. Symposium of the Zoological Society of London, No. 30. Academic Press, London, New York. 380 pp.

F. Reichenbach-Klinke, H. H., and M. Landolt. 1973. Reichenbach-Klinke's fish pathology. T.F.H. Publications, Inc., Neptune City, New Jersey. 512 pp.

H. Snieszko, S. F., editor. 1970. A symposium on diseases of fishes and shellfish. American Fisheries Society, Special Publication No. 5. 526 pp.

I. Wedemeyer, G. A., F. P. Meyer, and L. Smith. 1976. Environmental stress and fish diseases, Book 5 (192 pp) *in* S. F. Snieszko and H. R. Axelrod, editors. Diseases of fishes. T.F.H. Publications, Inc., Neptune City, New Jersey.

J. Wood, J. W. 1974. Diseases of Pacific Salmon. Their prevention and treatment. 2d ed. Washington Department of Fisheries, Seattle. 82 pp.

XV.

XV. Media and Reagents

A. Media. Incubation temperatures should be from 10-30°C, whichever is appropriate for the bacterium under test should be used. Unless otherwise stated, media are commercially available from several companies.

1. Trypticase or tryptic soy agar; brain heart infusion agar--these media are used for routine isolation and culture of most fish pathogens.

2. Oxidation/Fermentation (O/F) Basal Medium--This medium is used with 1% glucose to indicate method by which carbohydrates are attacked. Note: a marine version of this medium is also available.

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--Used 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)--Used 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 hydrophila* 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--Used for culturing Flexibacteria. The composition is:

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

Agar	11.0 g
	1000.0 ml
pH	7.2

8. Media for motility testing

   a. Motility by means of flagella 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. Gliding motility (i.e. flexibacteria) 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.)

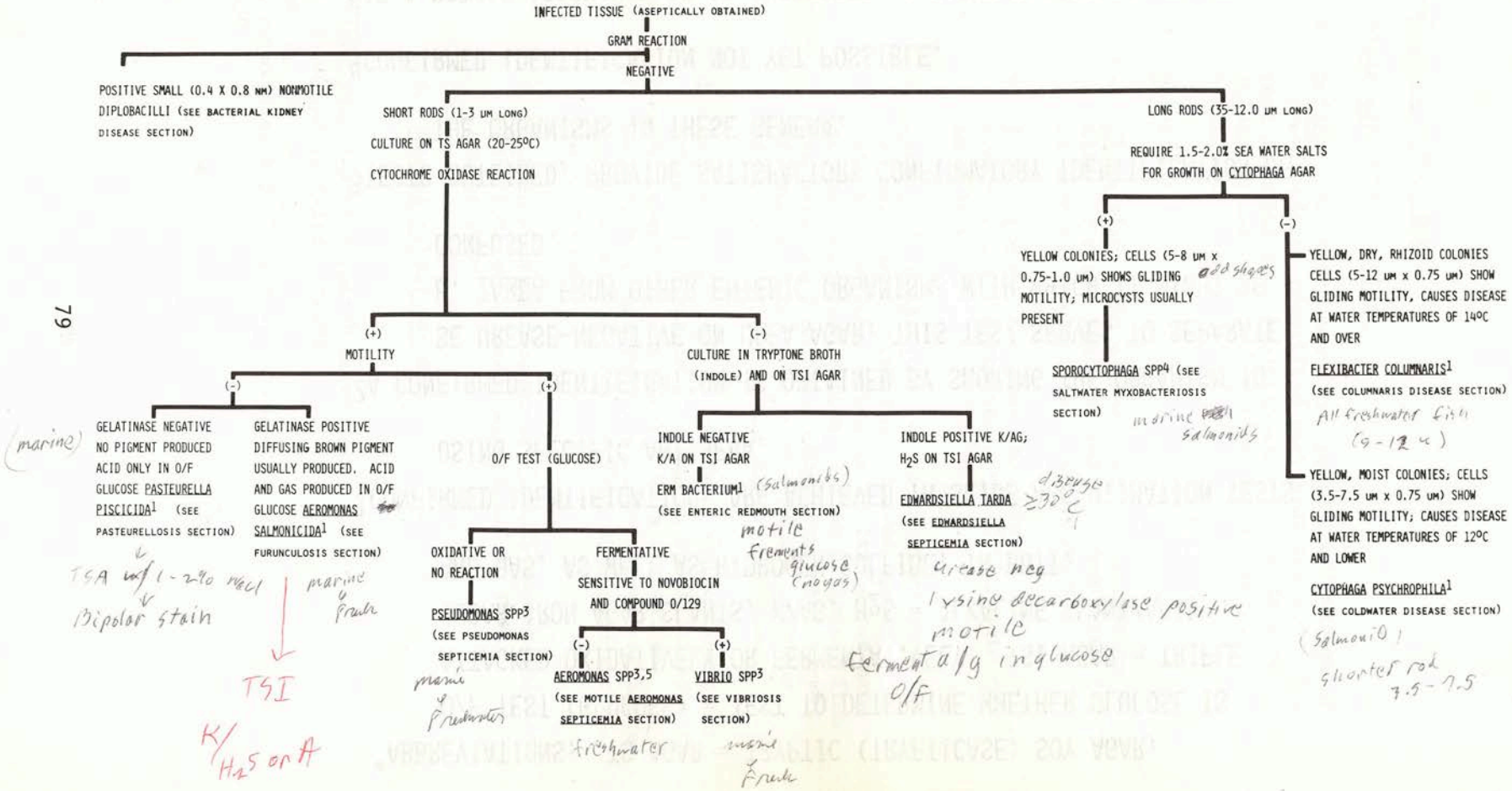
2. Cytochrome oxidase test--Paper test strips, impregnated with the appropriate chemicals, are available from the Patho Tec. Co., General Diagnostics Division of Warner Chilcott, Morris Plains, N. J., U.S.A.; instructions on the use of the test strips are provided in a pamphlet "Pathotec Test Papers 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. 425).

pg 335

FIGURE 1. PROCEDURES FOR THE PRESUMPTIVE IDENTIFICATION OF CERTAIN 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; 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]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).

XVI. 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 is available for many of the recognized bacterial fish pathogens from the National Fish Health Research Laboratory* (NFHRL) and should be stored at 4°C with the addition of 1:5000 sodium azide.

   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 NFHRL and stored as specified.

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

   f. Normal rabbit serum.

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* National Fish Health Research Laboratory
U.S. Fish and Wildlife Service
Route 3, Box 50
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
Test antigen	1-2 drops	1-2 drops	---
Reference antigen	---	---	1-2 drops
Specific antiserum	1-2 drops	---	1-2 drops
Normal serum	---	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 autoagglutination 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 slight sonication. These treatments often prevent autoagglutination.

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 serological confirmation of overt kidney disease. Materials and Methods are described in the paper by Chen et al. 1974. J. Fish. Res. Board Can. 31: 1939-1940.

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 that agent; for example rabbit anti-KD serum. The second reaction is between the specific antiserum and fluorescing globulin prepared against the antiserum; for example goat anti-rabbit IGg conjugated with fluorescein isothiocyanate.

2. Uses. At present there are published IFA procedures for identification of the KD bacterium from infected fish tissue and ERM cultures. Results of laboratory trials indicate that the bacteria causing furunculosis, vibriosis, and ERM may be presumptively identified from diseased fish tissues.

3. Materials

a. Fluorescent microscope. The development of incident (reflected) fluorescence microscopy has greatly enhanced the IFA procedure for identification of bacteria. Since there is no darkfield condenser used in this system, and the objective lens serves as condenser, there is ample light for examination of specimens with the 100 X objective. Also, since vertical illumination for incident light fluorescence microscopy are available for about $2000.00, IFA is a practical diagnostic procedure for most laboratories. Appropriate filters for fluorescein isothiocyanate should be used, such as BG-12 exciter filter and OG 515 barrier filter.

b. FA slides. Coated FA slides which have 12 test areas are commercially available from Shandon

Southern Instruments, Inc., 515 Broad Street, Sewickley, Pennsylvania 15143. Before use slides are either acid cleaned or lightly coated with Bon Ami which is then wiped off with a paper towel.

c. Buffers. The following buffers are used in the IFA procedure:

PBS - commercially available from Difco Labs Inc., or may be prepared as follows:

NaCl	12.0	g
$NaH_2 PO_4 H_2O$	2.07	g
40% NaOH (w/v)	1.0	ml
Water up to pH 6.8-7.2	1500	ml

d. FA mounting fluid. A special non-fluorescing mounting medium, pH 9.0, is available from commercial sources.

e. Diagnostic antiserum. A standardized, specific, and polyvalent antiserum prepared against a reference antigen, available for many of the recognized bacterial fish pathogens from the National Fish Health Research Laboratory.

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. If all diagnostic antisera are prepared in rabbits, then only one conjugate is required. Goat or sheep anti-rabbit globulin conjugated with fluorescein isothiocyanate is commercially available.

g. Proper antisera and conjugate dilution. Working strength of either diagnostic antisera or fluorescent globulin conjugate must be determined by dilution for each system. The antiserum or conjugate is serially diluted in PBS and applied to replicate positive control slides. The next to last dilution showing bright fluorescence is used as a working solution. With the bacterial pathogens a 1:40 dilution of diagnostic antiserum and 1:50 conjugate have worked well. In addition a 1:50-1:00 dilution of Rhodamine (Difco Labs Inc.) incorporated in the conjugate has been used as a counterstain.

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

4. Test procedure. For identification of isolated cultures or a specific disease such as KD proceed as follows:

a. Label a cleaned slide 1, 2, and 3. Place a drop of test antigen (culture or tissue suspension) in 1 and 2 and a drop of reference antigen in 3.

b. Fix in a drying oven at 60°C for 5 minutes.

c. Place a drop of specific antiserum dilution on 1 and 3 and a drop of normal rabbit (diluted 1:40 in PBS) on 2. Incubate in a moist chamber for 30 minutes.

d. Rinse off sera with PBS, wash 10 minutes in PBS and gently blot dry.

e. Add 1:50 dilution of conjugate to all test spots and incubate in a moist chamber for 30 minutes.

f. Rinse with PBS then wash 10 minutes and blot dry.

g. Mount specimens with mounting fluid, coverslip, and examine slide under oil immersion. A positive test is indicated by bright apple green fluorescence while negative cells should be stained red with the counterstain. Bacteria in position 3 should always be positive while those in 2 (normal serum) should always be negative. If bacteria in position 1 fluoresce confirmation identification of the culture is obtained.

If one is attempting to presumptively diagnose furunculosis, vibriosis, or ERM proceed as follows:

a. Prepare a suspension of infected fish tissues (usually kidney) and make a thin smear in five test spots on a cleaned FA slide.

b. Dry and fix as above. Prepare (working) dilutions of the following rabbit antisera: *A. salmonicida*, ERM, *V. anguillarum* Manchester, *V. anguillarum* 1669,* and a comparable dilution of normal rabbit serum.

* There are two serotypes of *V. anguillarum*.

c. Add a drop of each antiserum and normal serum to one test spot on the FA slide and incubate 30 minutes in a moist chamber.

d. Wash in PBS and proceed with conjugate staining; wash again in PBS, mount and examine.

e. Interpretation of results is as follows: the spot receiving normal serum and conjugate should always be negative. If bacteria in the test spot receiving the *A. salmonicida* antiserum fluoresce then a presumptive diagnosis of furunculosis may be made, while presumptive ERM or vibriosis diagnosis is made by observing fluorescing bacteria in test spots stained with ERM or *V. anguillarum*. If more than one of the test smears show fluorescence, it may be indicative of a mixed infection or cross-reaction. Bacteria should be isolated and identified before a confirmatory diagnosis is made.

D. Direct Fluorescent Antibody Test (FAT)

Direct FITC conjugates for *A. salmonicida*, *C. columnaris*, *E. tarda*, *V. anguillarum*, and *Y. ruckeri* have been prepared, standardized, and lyophilized for use in a rapid direct FAT procedure. The conjugates are available upon request from the Biologics Section, National Fish Health Research Laboratory, Route 3, Box 50, Kearneysville, West Virginia 25430. The suggested procedure is:

1. Prepare smear from culture or infected tissue, and dry at 60°C for 5 minutes.

2. Add conjugate, diluted as indicated, to smear and allow to react 5 minutes. If desired, Difco rhodamine may be added to conjugate at a concentration of 1:100 or 1:200.

3. Rinse with pH 7.2 PBS and wash in PBS 2 minutes.

4. Air dry, add pH 9.0 mounting fluid and coverslip, and examine under oil immersion.

Positive controls consist of known cultures for each of the conjugates, while the use of two or more unrelated conjugates serve as negative controls. The direct *C. salmoninus* conjugate is used for routine diagnosis and detection of KD, while conjugates for the gram-negative pathogens are used for presumptive identification. Additional field testing is required to establish the use of direct FAT alone for diagnosis.

Renibacterium salmoninarum

Section 4

METHODS FOR THE DIAGNOSIS OF CERTAIN PARASITIC DISEASES

I. General Procedures for Parasitology

A. External Parasites

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

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

3. Visible at 100 X to 500 X. Prepare wet mounts of gill filaments and mucus scraped from the dorso-lateral surface of the fish. All motile ectoparasites can be seen at 100 X but frequently the use of higher magnification is helpful.

B. Internal Parasites

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

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

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

C. Procedures for Killing and Preserving some Parasites

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

2. Monogenea and digenetic trematodes. Kill in 90°C 10% formalin and preserve in 10% formalin. Encysted forms should be freed before killing.

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

4. Nematodes. Free encysted worms, kill in 90°C 70% alcohol. (FIRE HAZARD: place vial of alcohol in a small beaker of water for heating.)

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

6. Parasitic copepods and Argulus. Kill and preserve in 70% alcohol. Also include juvenile and larval forms if present on fish.

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

1. See Meyer, M. C., and O. W. Olson. 1975. Essentials of parasitology. Pages 263-270. Wm. C. Brown Co., Dubuque, Iowa; or similar publications.

II. Whirling Disease of Salmonids

A. Name of the Disease and Etiologic Agent. Whirling disease caused by the sporozoan parasite *Myxosoma cerebralis*. (Synonym: Blacktail)

B. Known Geographic and Host Range of the Disease

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

2. Host range. All species of salmon, trout, and grayling are susceptible. Coho salmon and brown trout may show no signs of the disease and spores may be difficult to find even after heavy exposure at an early age thereby creating the possibility for accidentral transfer of asymptomatic carriers. Brook trout and rainbow trout are very susceptible. Intensity of exposure and the age of fish when exposed affects the severity of the disease (O'Grodnick 1979).

C. Clinical Signs of Diagnostic Significance

1. Whirling. Frenzied, tail-chasing behavior, particularly when being fed or when startled. Whirling behavior usually occurs 2 to 3 months after infection and may last for up to a year in cold water situations.

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

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

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

D. Diagnostic Procedures for Disease Situations. Remove the heads from five suspect fish and warm them in 45°C water for 1 to 3 minutes so the flesh will separate easily from the bone and cartilage. Remove loose flesh and the brain to a waste container of 1:1 water and household bleach. Collect bone and cartilage samples from the brain case, otolith region, and gill arches. Grind sample with and equal volume of 10% formalin (to kill viable spores

to prevent dissemination of the disease agent) in a mortar. Wash all grindings into a small beaker with water and allow material to settle. Sediment can be examined directly in wet mounts at 400 X or, using the method of MacLean (1971) spread 5 to 10 drops of sediment onto a clean glass microscope slide and allow to air dry. The slide is stained with 1% aqueous malachite green for 5 minutes, rinsed in tap water and destained by dripping for 30 seconds into 70, 90, and 100% ethyl alcohol. Air dried slides are coated entirely with a thin film of low viscosity immersion oil and examined with the 20 X objective lens and 10 X eye pieces (not under oil immersion). Scan the entire smear. Spores will appear as green ovals with dark green polar capsules against a nearly colorless background. At 200 X there is less chance of missing spores and a larger area is covered at each pass over the slide than at higher magnifications required to find unstained spores. Diagnosis of epizootic whirling disease depends upon the detection and identification of *Myxosoma cerebralis* spores which can be accomplished as described below in E. h.

E. Procedures for Detecting Asymptomatic Infections. Use a sampling pattern which provides the capability of disease detection at the 5% level of incidence according to the sample size table. Samples should be weighted towards the most susceptible species and ages of fish available. For example, select brook and rainbow trout over brown trout or coho salmon if all are reared under the same conditions. Select fingerlings about 5 months old; if fish are continuously exposed in water of 13°C or warmer, fish as young as 2-3 months of age may yield mature spores. In water below 12°C fish may have to reach 8-10 months of age before mature spores can be found.

The following procedures are acceptable for detection of carrier fish.

1. Digest method.

   a. Remove heads from the fish that have been sampled. If necessary refrigerate at 5°C.

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

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

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

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

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

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

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

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

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

Rinaldini's solution:	
NaCl	8.0 g
KCl	0.2 g
$C_6H_5Na_3O_7 \cdot 2H_2O$ (sodium citrate)	1.0 g
$NaH_2PO_4 \cdot H_2O$ (sodium phosphate, monobasic)	0.05 g
$NaHCO_3$	1.0 g
Glucose	1.0 g
Distilled or deionized water	1.0 l
Phenol red, 1% solution	1.0 ml

Frozen storage only.

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

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

2. Plankton centrifuge method.

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

b. Thoroughly homogenize each batch in 200 ml of aqueous 5% formalin for 3 minutes in a high speed blendor.

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

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

e. Transfer entire filtrate to a separatory funnel which discharges by gravity into the central feed line of a plankton centrifuge. (026WA106 plankton centrifuge, Kahl Scientific Instrument Corp., P.O. Box 1166, El Cajon, Calif. 92022, or equal.) Operate the plankton centrifuge on high speed and set the separatory funnel flow rate at the lowest level that gives a thin steady stream.

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

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

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

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

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

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

(4) Rinse with water and proceed as in E. 2. b. above.

h. In the event that 7-10 μ spores indistinguishable from *Myxosoma cerebralis* are found and circumstances warrant confirmation, the following procedures should be followed:

(1) Contact a fish parasitologist or a fish histopathologist qualified to contribute to the identification of *Myxosoma cerebralis* and to verify the presence of whirling disease pathology. Arrange for sample shipment as directed.

(2) Serological identification of *Myxosoma cerebralis* may be accomplished by direct fluorescent antibody technique (FAT) as follows:

NOTE: FITC conjugated-rabbit anti-*Myxosoma cerebralis* antisera, known positive and known negative control materials are available in small quantities upon written request to the Biologics Section, National Fish Health Research Laboratory, Route 3, Box 50, Kearneysville, West Virginia 25430.

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

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

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

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

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

(f) Gently rinse conjugated antiserum from slides with pH 9.0-9.5 buffer ($NaHCO_3$: 33.6 g, plus Na_2CO_3; 10.6 g in 1000 ml water) then soak slides in buffer, with gentle agitation for 5 minutes. Careless or too vigorous washing may lead to spore loss.

(g) Remove slide and gently blot dry on clean absorbent paper. Add a drop of immersion oil to each test spot and examine at 1000 X on a fluorescent microscope.

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

i. From the outset an awareness should be maintained that whirling disease spores may be present in fish samples which, if untreated, could result in the accidental dissemination of the disease. All equipment, expendable supplies, toweling, and dissected tissues should be thoroughly disinfected with 10% formalin or a 1:1 solution of household liquid bleach and water. If infected fish samples are to be retained, they should be preserved in 10% formalin. In the event of work with positive fish, it is probable that there will be mechanical carry-over of spores from sample to sample on equipment used in the procedure. While this may enhance detection, it can be avoided by taking the following steps:

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

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

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

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

H. References

1. Contos, N., and H. Rothenbacker. 1974. An efficient concentration and purification method for the spores of *Myxosoma cerebralis*. Progressive Fish-Culturist 36(2):101-102.

2. Griffin, B. R., and E. M. Davis. 1978. *Myxosoma cerebralis*: detection of circulating antibodies in infected rainbow trout (*Salmo gairdneri*). Journal of the Fisheries Research Board of Canada 35(9): 1186-1190.

3. Halliday, M. M. 1976. The biology of *Myxosoma cerebralis*: the causative organism of whirling disease of salmonids. Journal of Fish Biology 9(4):339-357.

4. Hoffman, G. 1976. Whirling disease of trout. U.S. Fish and Wildlife Service. Fish Disease Leaflet 47. 10 pp.

5. Hoffman, G. L., C. E. Dunbar, and A. Bradford. 1969. Whirling disease of trouts caused by *Myxosoma cerebralis* in the United States. U.S. Fish and Wildlife Service. Special Scientific Report-Fisheries 427. 15 pp.

6. Landolt, M. 1973. *Myxosoma cerebralis*: isolation and concentration from fish skeletal elements--trypsin digestion method. Journal of the Fisheries Research Board of Canada 30(11):1713-1716.

7. Lom, J., and G. L. Hoffman. 1971. Morphology of the spores of *Myxosoma cerebralis* (Hofer, 1903) and *M. cartilaginis* (Hoffman, Putz, and Dunbar, 1965). The Journal of Parasitology 57(6):1302-1308.

8. MacLean, D. G. 1971. A simplified procedure for detecting *Myxosoma cerebralis* (whirling disease) spores in large lots of fish. Progressive Fish-Culturist 33(4):203.

9. Markiw, M. E., and K. Wolf. 1978. *Myxosoma cerebralis*: fluorescent antibody techniques for antigen recognition. Journal of the Fisheries Research Board of Canada 35(6):828-832.

10. Markiw, M. E., and K. Wolf. 1974a. *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 31(1):14-20.

11. Markiw, M. E., and K. Wolf. 1974b. *Myxosoma cerebralis*: comparative sensitivity of spore detection methods. Journal of the Fisheries Research Board of Canada 31(10):1597-1600.

12. O'Grodnick, J. J. 1979. Susceptibility of various salmonids to whirling disease (*Myxosoma cerebralis*). Transactions of the American Fisheries Society (in press).

13. O'Grodnick, J. J. 1975. Whirling disease (*Myxosoma cerebralis*) spore concentration using the continuous plankton centrifuge. Journal of Wildlife Diseases 11(1):54-57.

14. Prasher, J. B., W. Tidd, and R. Tubb. 1971. Techniques for extracting and quantitatively studying the spore stage of the protozoan parasite *Myxosoma cerebralis*. Progressive Fish-Culturist 33(4):193-196.

15. Rydlo, M. 1971. Evidence of *Myxosoma cerebralis* spores in various organs of rainbow trout afflicted with whirling disease. Osterreichs Fischerei 24:97-99. (in German)

16. Tidd, W., R. Tubb, and V. Wright. 1973. Modification of *Myxosoma cerebralis* spore extraction technique. Progressive Fish-Culturist 35(4):227-228.

17. Wolf, K., and M. E. Markiw. 1979. *Myxosoma cerebralis*: a method for staining spores and other stages with silver nitrate. Journal of the Fisheries Research Board of Canada 36(1):88-89.

III. 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 susceptible fish:

(1) Oregon. Deschutes River System, Columbia River below its confluence with the Deschutes River, Williamette River from its mouth to approximately 100 miles upstream, Nehalem River and Rogue River.

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

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

b. Areas where infected adult fish have been found:

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

(2) California. Widely distributed through the Sacramento and Klamath River Basins.

(3) Washington. Widely distributed through the Columbia River Basin.

(4) Idaho. Found in salmon returning to Columbia River basin tributaries originating in this state.

(5) British Columbia. Reported in salmon at several widely scattered locations.

(6) Pacific Ocean. Occasionally observed in ocean caught salmon.

2. Host range. Coho salmon (*Oncorhynchus kisutch*), chinook salmon (*O. tshawytscha*), sockeye salmon (*O. nerka*), chum salmon (*O. keta*), rainbow and steelhead trout (*Salmo gairdneri*), cutthroat trout (*S. clarki*),

brown trout (*S. trutta*), Atlantic salmon (*S. salar*), and brook trout (*Salvelinus fontinalis*). Differences in susceptibility among strains are known to exist and should be considered when stocking salmonids within the endemic range of *C. shasta*.

C. Clinical Signs of Diagnostic Significance

1. Juvenile salmonids.

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

(1) Cessation of feeding

(2) Abdomen becomes distended and filled with ascitic fluid

(3) Exophthalmia

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

(5) Spores may be found in the intestinal wall, other internal organs, and ascitic fluid

(6) Mortality may become catastrophic.

2. Adult salmonids.

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

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

D. Diagnostic Procedures for Disease Situations

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

2. Dried smears may be stained by the Ziehl-Neilsen method but without heating. By this method the polar

capsules stain red against a bluish sporoplasm and background.

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

4. Examine 25 to 30 fields (440 X) 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 no serological tests 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. Bedell, G. W. 1971. Eradicating *Ceratomyxa shasta* from infected water by chlorination and ultraviolet irradiation. Progressive Fish-Culturist 33(1):51-54.

2. Burrows, R. E., and B. D. Combs. 1968. Controlled environments for salmon propagation. Progressive Fish-Culturist 30(3):123-136.

3. Conrad, J., and M. DeCew. 1966. First report of *Ceratomyxa* in juvenile salmonids in Oregon. Progressive Fish-Culturist 28(4):238.

4. Margolis, L., and T. P. T. Evelyn. 1975. *Ceratomyxa shasta* (Myxosporida) disease in chum salmon (*Oncorhynchus keta*) in British Columbia. Journal of the Fisheries Research Board of Canada 32(9):1640-1643.

5. Noble, E. R. 1950. On a myxosporidan (protozoan) parasite of California Trout. Journal of Parasitology 36:457-460.

6. Sanders, J. E., J. L. Fryer, and R. W. Gould. 1970. Occurrence of the myxosporidan parasite *Ceratomyxa shasta*, in salmonid fish from the Columbia River Basin and Oregon coastal streams. Pages 133-141 *in* S. F. Snieszko, editor. A symposium on diseases of fishes and shellfish. American Fisheries Society Special Publication 5.

7. Sanders, J. E., J. L. Fryer, D. A. Leith, and K. D. Moore. 1972. Control of the infectious protozoan *Ceratomyxa shasta* by treating hatchery water supplies. Progressive Fish-Culturist 34(1): 13-17.

8. Schafer, W. E. 1968. Studies on the epizootiology of the myxosporidan *Ceratomyxa shasta* Noble. California Fish and Game 54(2):90-99.

9. Udey, L. R., J. L. Fryer, and K. S. Pilcher. 1975. Relation of water temperature to ceratomyxosis in rainbow trout (*Salmo gairdneri*) and coho salmon (*Oncorhynchus kistuch*). Journal of the Fisheries Research Board of Canada 32(9):1545-1551.

10. Vlasenko, M. I. 1969. Ultraviolet rays as a method for the control of diseases of fish eggs and young fishes. Problems of Ichthyology 9(5):697-705.

11. Wales, J. H., and H. Wolf. 1955. Three protozoan diseases of trout in California. California Fish and Game. 41:183-187.

12. Wood, J. W. 1974. Diseases of Pacific salmon, their prevention and treatment. State of Washington, Department of Fisheries, Hatchery Division, 2nd edition. 82 pp.

13. Zinn, J. L., K. A. Johnson, J. E. Sanders, and J. L. Fryer. 1977. Susceptibility of salmonid species and hatchery strains of chinook salmon (*Oncorhynchus tshawytscha*) to infections by *Ceratomyxa shasta*. Journal of the Fisheries Research Board of Canada 34(7):933-936.

IV. *Bothriocephalus acheilognathi* (Asian tapeworm)

A. Name of the Disease and Etiologic Agent. *Bothriocephalus acheilognathi* Yamaguti (Cestoda: Pseudophyllidea). (Synonyms--*B. gowkongensis* Yea, 1955; *B. opsariichthydis* Yamaguti, 1934; *B. phoxini* Molnar and Murai, 1973. Corrections by Molnar 1977).

B. Known Geographic and Host Range of the Disease

1. Geographic range. Southeast U.S.A., Southern Europe, Southern Asia.

2. Host range in U.S.A.. Golden shiner (*Notemigonus crysoleucas*), fathead minnow (*Pimephales promelas*), grass carp (*Ctenopharyngodon idella*), mosquito fish (*Gambusia affinis*).

3. Host range in Europe and Asia. *B. acheilognathi* has been found in 23 species including *Ctenopharyngodom idella, Cyprinus carpio, Aristichthys nobilis, Silurus glanis* (catfish), *Lucioperca lucioperca, Perca fluviatilis,* and *Gambusia affinis* (Osmanov 1971).

C. Clinical Signs of Diagnostic Significance. High intensity infection in young fish: intestinal blockage, occasionally intestinal perforation, emaciation, death. Low intensity infection: no obvious signs.

D. Diagnostic Procedures for Disease Situations

1. Presumptive diagnosis. Presence of tapeworms in the intestine of any of the above hosts.

2. Confirmatory diagnosis. *Bothriocephalus acheilognathi* can be recognized by its "pit-viper" shaped scolex. Many of this tapeworm's freshly laid eggs will contain developed larvae, especially in warmwater situations.

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

E. Procedures for Detecting Asymptomatic Infections. Sample size should be adequate to detect a 5% level of incidence.

1. Small fish (to 4"). Remove the intestines of fish in groups of 10, slit the intestines and examine at 10 X under a dissection microscope. If there is too much

fecal material for accurate examination, pour above material into a bottle about 5 by 15 cm, fill with water, shake vigorously, allow to stand 15 minutes, aspirate most of the fluid carefully and examine sediment at 10 X.

2. Large fish (larger than 4"). Prepare as above but pool in groups of 5 fish (the number to be used depends on the size and amount of intestinal contents). With larger fish, flush the excised alimentary canal with water before slitting.

F. Procedures for Determining Prior Exposure to the Etiologic Agent. At the present time no serological or other tests have been developed for use in detection of *Bothriocephalus acheilognathi*.

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiologic Agent. Submitting live fish is best; if not possible, kill a sample of the tapeworms in 80°C water and transfer to 10% formalin.

H. References

1. Bauer, O. N., V. A. Musselius, and Yu. A. Strelkov. 1969. Diseases of pond fishes (Bolezni prudovykh ryb) Izdatel'stvo "Kolos," Moskva, 1969 (English translation TT72-50070, U.S. Department of Commerce, National Technical Information Service, Springfield Virginia. 220 pp.)

2. Hoffman, G. L. 1976. The Asian tapeworm, *Bothriocephalus gowkongensis*, in the United States, and research needs in fish parasitology. Pages 84-90 *in* Proceedings of the 1976 Fish Farming Conference and Annual Convention of Catfish Farmers of Texas. Texas A&M University, College Station, Texas.

3. Molnar, K. 1977. On the synonyms of *Bothriocephalus acheilognathi*, Yamaguti, 1934. Parasitologia Hungarica 10:61-62.

4. Osmanov, S. O. 1971. Parasites of fishes of Uzbekistan (Russian). Tashkent. 532 pp.

5. Scott, A. L., and J. M. Grizzle. 1979. Pathology of cyprinid fishes caused by *Bothriocephalus gowkongensis* Yea, 1955 (Cestoda: Pseudophyllidea). Journal of Fish Diseases 2(1):69-73.

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

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

C. Clinical Signs of Diagnostic Significance. Infected fish cease feeding and become lethargic. In acute infections, trout develop a gross lumpy appearance. Spinal deformities may develop; nodules may develop in the kidney, liver, or muscle. Spores also may occur in spleen and brain. In chronic infections, no visible signs may develop.

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

E. Procedures for Detecting Asymptomatic Infections. Sample size should be adequate to detect 5% level of incidence. Smears of kidney tissue which are air dried and stained with methylene blue have been successfully used in some laboratories as a screening procedure. The statistical reliability of this method is unknown.

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

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiologic Agent. Live or fresh fish are preferred. Infected tissue can also be stored frozen with no apparent ill effect on spore morphology, but viability is unknown.

H. References

1. Erickson, J. D. 1965. Report on the problem of *Ichthyosporidium* in rainbow trout. Progressive Fish-Culturist 27(4):179-184.

2. Fischthal, J. 1944. Observations on a sporozoan parasite of the eelpout, *Zoarces anguillaris*, with an evaluation of candling methods for its detection. Journal of Parasitology 30(1):35-36.

3. Ghittino, P. 1970. Piscicoltura Ittiopatologia. Edizioni Rivista di Zovtecnia. Italy. 2:166-173.

4. Gustafson, P.V., and R. R. Rucker. 1956. Studies on an *Ichthyosporidium* infection in fish; transmission and host specificity. U.S. Fish and Wildlife Service. Special Scientific Report-Fisheries 166. 8 pp.

5. Reichenbach-Klinke, H. H., and M. Landolt. 1973. Fungi and algae as fish parasites. Pages 102-123 *in* Christa Ahrens, translator. Fish Pathology. T.F.H. Publications, Inc. Neptune, New Jersey.

6. Ross, A. J., and T. Parisot. 1958. Record of the fungus *Ichthyosporidium* Caullery and Nesnil, 1905, in Idaho. Journal of Parasitology 44(4):453-454.

7. Rucker, R. R., and P. V. Gustafson. 1953. An epizootic among rainbow trout. Progressive Fish-Culturist 15(4):179-181.

8. Sinderman, C. J. 1970. Principle diseases of marine fish. Academic Press, New York. 369 pp.

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

A. Fungi

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

2. Internal.

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

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

B. Protozoa

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

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

3. *Trypanoplasma salmositica* (*Cryptobia salmositica*). Occurs in blood of salmonids in western U.S. and can be recognized by its motion in wet blood smears diluted in equal volumes of saline. Discovery during examination of excised gill filaments is not uncommon. Two flagella, one anterior and one posterior can be seen (Becker 1977).

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

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

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

7. *Eimeria aurati* (coccidiosis of goldfish). Can be identified by demonstrating oocysts containing four spores which are found in sediment from fecal washes. Potentially dangerous parasite in goldfish culture (Hoffman 1965).

8. *Henneguya postexilis* and *Henneguya longicauda* of channel catfish. Both of these are responsible for "interlamellar" *Henneguya*. When cysts are present, they are small (up to 370 μ). *Henneguya exilis* produces larger cysts (up to 1110 μ) in the gills. For species identification see Minchew (1977).

9. *Myxobolus insidiosus* of Pacific salmon. This myxosporidian heavily infects the musculature of cultured

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

10. *Pleistophora ovariae*. Ovarian parasite of golden shiners and fathead minnows. Oocytes filled with spores are more opaque than healthy oocytes and can be seen with the naked eye. Spores are large for Microsporida (about 6.5 μ long) which must be verified microscopically. See Summerfelt and Warner (1970).

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

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

13. *Dermocystidium salmonis*. Small cysts in gills and fins of salmonids. Small, spherical spores which always possess a very large vacuole (Davis 1947, Pauley 1967).

C. Monogenea (gill and body flukes).

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

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

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

D. Digenetic trematodes. Adults or metacercariae in fish.

1. *Sanguinicola davisi* and *Sanguinicola klamathensis* (*Cardicola* spp., blood flukes). Nondescript, flapping

adults can be expressed from main blood vessels of the gill arch and kidney in trout in Western United States. More often the presence of eggs containing developing miracidia with a spectacular "eye spot" can be seen in gill lamellae (Evans and Heckmann 1973).

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

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

4. *Clinostomum complanatum* (*Clinostomum marginatum*, yellow grub). Unsightly, usually yellowish grub. Body with large ventral sucker and anterior "shoulders." Encysted; large, 1-2 mm long.

5. *Nanophyetus salmincola* (salmon poisoning fluke). Cysts may be numerous in viscera, particularly liver, of salmonids in Western United States. Cysts can be seen in wet squashes of kidney (Baldwin et al. 1967).

E. Cestodes (tapeworms). Adults in intestine, plerocercoids in viscera and musculature.

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

2. *Eubothrium* spp. of trout and salmon. In intestine and pyloric ceca. Importance is controversial. Scolex is heart-shaped with dorsal and ventral bothria (grooves).

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

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

F. Nematodes (roundworms). *Goezia*, a spined nematode, produces dangerous nodules in the intestine of *Micropterus salmoides* in the Southeastern United States. *Eustrongylides* sp. causing swollen abdomen in tropical fish and small bait fish in extreme Southwestern and Southeastern United States should be studied.

G. Acanthocephala (thorny headed worms)

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

H. Copepods

1. *Lernaea elegans* (*L. cyprinacea*, anchor parasite). No host specificity but may attack golden shiners and buffalo fish to a greater degree. Adult female with large Y-shaped dorsal arms (Hoffman 1976).

2. *Salmincola californiensis* (the western *Salmincola*). Attacks gills of rainbow and cutthroat trout and salmon. This copepod has been found as far east as West Virginia (G. L. Hoffman, personal observation).

3. *Salmincola edwardsi* (the brook trout *Salmincola*). Attacks fins and gills primarily of eastern brook trout.

4. *Ergasilus* spp. (hay-fork claspers). On gills of many fish. *Ergasilus labracis* of striped bass can be lethal.

I. Branchiura

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

J. References

1. Baldwin, N. L., R. E. Millemann, and S. E. Knapp. 1967. "Salmon poisoning" disease. III. Effect of experimental *Nanophyetus salmincola* infection on the fish host. Journal of Parasitology 53(3): 556-564.

2. Becker, C. D. 1977. Flagellate parasites of fish. Chapter 10 *in* J. P. Kreier, editor. Parasitic protozoa, vol. 1. Academic Press, New York. 441 pp.

3. Bullock, W. L. 1962. A new species of *Acanthocephalus* from New England fishes, with observations on variability. Journal of Parasitology 48(3): 442-451.

4. Bykhovskaya-Pavlovskaya, I. E., et al. 1962. Key to parasites of freshwater fish of the USSR. Zoological Institute, Academy of Science. USSR. (English translation TT64-11040, OTS, Department of Commerce, Washington, D. C. 919 pp.)

5. Davis, H. S. 1947. Studies of the protozoan parasites of freshwater fishes. U.S. Fish and Wildlife Service. Fishery Bulletin 51(41):1-29.

6. Dechtiar, A. O. 1965. Preliminary observations on *Glugea hertwigi*, Weissenberg, 1911 (Microsporidia: Glugeidae) in American smelt, *Osmerus mordax* (Mitchell) from Lake Erie. Canadian Fish Culturist 34:35-38.

7. Esch, G. W., W. C. Johnson, and J. R. Coggins. 1975. Studies on the population biology of *Proteocephalus ambloplites* (Cestoda) in the smallmouth bass. Proceedings of the Oklahoma Academy of Science 55:122-127.

8. Evans, W. A., and R. A. Heckmann. 1973. The life history of *Sanguinicola klamathensis*. Life Sciences (Great Britain) 13:1285-1291.

9. Hoffman, G. L. 1960. Synopsis of Strigeoidea (Trematoda) of fishes and their life cycles. U.S. Fish and Wildlife Service. Fishery Bulletin 175. 60:439-469.

10. Hoffman, G. L. 1965. *Eimeria aurati* n. sp. (Protozoa: Eimeriidae) from goldfish (*Carassius auratus*) in North America. Journal of Protozoology 12(2):273-275.

11. Hoffman, G. L. 1967. Parasites of North American freshwater fishes. University of California Press. Berkeley, California. 486 pp.

12. Hoffman, G. L. 1969. Fungi (*Saprolegnia* and relatives) of fish and fish eggs. U.S. Fish and Wildlife Service. Fish Disease Leaflet 21. 6 pp.

13. Hoffman, G. L. 1976. The anchor parasite (*Lernaea elegans*) and related species. U.S. Fish and Wildlife Service. Fish Disease Leaflet 46. 8 pp.

14. Hoffman, G. L. 1977. Copepod parasites of freshwater fish: *Ergasilus, Achtheres,* and *Salmincola*. U.S. Fish and Wildlife Service. Fish Disease Leaflet 48. 10 pp.

15. Hoffman, G. L. 1977. *Argulus*, a branchiuran parasite of freshwater fishes. U.S. Fish and Wildlife Service. Fish Disease Leaflet 49. 9 pp.

16. Hoffman, G. L. 1978. Ciliates of freshwater fishes. Chapter 8 *in* J. P. Kreier, editor. Parasitic protozoa, vol. II. Academic Press, New York. 730 pp.

17. Hoffman, G. L. 1979. Worms (helminths and leeches). *in* J. Plumb, editor. Principle diseases of farm-raised catfish. Southern Cooperative Series 225. Agricultural Experimental Station, Auburn, Alabama. 92 pp.

18. Hoffman, G. L., S. K. Kazubski, A. J. Mitchell, and C. E. Smith. 1979. *Chilodonella hexasticha* (Kiernik 1979) (Protozoa, Ciliata) from North American warmwater fish. Journal of Fish Disease 2(2):153-157.

19. Holland, M. 1971. *Saprolegnia* life cycle. Carolina Tips 34(1):1-4.

20. Lucký, Z. 1977. Methods for the diagnosis of fish diseases (Metodicki Navody k Diagnostice Nemoci Ryb.) English Translation TT75-58005. U.S. Department of Commerce, National Technical Information Service. Springfield, Virginia 22161. 140 pp.

21. Meyer, F. P. 1974. Parasites of freshwater fishes; II, Protozoa 3. (*Ichthyophthirius multifiliis*). U.S. Fish and Wildlife Service. Fish Disease Leaflet 2. 5 pp.

22. Meyer, M. C., and O. W. Olsen. 1975. Essentials of parasitology. Wm. and Brown Company, Dubuque, Iowa. 303 pp.

23. Meyer, M. C., and E. S. Robinson. 1963. Description and occurrence of *Diphyllobothrium sebago* (Ward, 1910). Journal of Parasitology 49(6):969-973.

24. Minchew, C. D. 1977. Five new species of *Henneguya* (Protozoa: Myxosporida) from ictalurid fishes. Journal of Protozoology 24(2):213-220.

25. Mitchell, L. G. 1977. Myxosporida. Chapter 4 *in* J. P. Kreier, editor. Parasitoc protozoa, vol IV. Academic Press, New York. 386 pp.

26. Neish, G. A., and Hughes (no date). Fungal diseases of fishes. T.F.H. Publications, Inc. Neptune City, New Jersey. (in preparation.)

27. Nepszy, S. J., J. Budd, and A. O. Dechtiar. 1978. Mortality of young-of-the-year rainbow smelt (*Osmerus mordax*) in Lake Erie associated with the occurrence of *Glugea hertwigi*. Journal of Wildlife Diseases 14(2):233-239.

28. Pauley, G. B. 1967. Prespawning adult salmon mortality associated with a fungus of the genus *Dermocystidium*. Journal of the Fisheries Research Board of Canada 24(4):843-848.

29. Richards, R. H. 1978. Mycology of teleosts. Chapter 9 *in* R. Roberts, editor. Fish Pathology. Lea and Febiger, Philadelphia, Pennsylvania, or Bailliere-Tindall, London. 318 pp.

30. Roberts, R. J., editor. 1978. Fish pathology. Lea and Febiger, Philadelphia, Pennsylvania, or Bailliere-Tindall, London. 318 pp.

31. Schubert, G. 1974. Cure and recognize aquarium fish diseases. T.F.H. Publications, Inc., Neptune City, New Jersey. 127 pp.

32. Scott, W. W., and A. H. O'Bier. 1962. Aquatic fungi associated with diseased fish and fish eggs. Progressive Fish-Culturist 24(1):3-15.

33. Seymour, R. L. 1970. The genus *Saprolegnia*. Verlag-Von J. Cramer, Kampstein, West Germany. 124 pp.

34. Summerfelt, R. C., and M. C. Warner. 1970. Incidence and intensity of infection of *Plistophora ovariae*, a microsporidian parasite of the golden shiner, *Notemigonus crysoleucas*. Pages 142-160 *in* S. F. Snieszko, editor. A symposium on diseases of fishes and shellfishes. American Fisheries Society, Special Publication 5.

35. Wellborn, T. L. 1970. Life history and pathology of the eye nematode, *Philometra intraoculus* sp. n. of the bluegill, *Lepomis macrochirus*. Ph.D. Thesis. Auburn University, Auburn, Alabama. 81 pp.