1.1.1 General Procedures for Bacteriology

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I. Sampling and Handling of Samples

For the detection and identification of bacterial pathogens in populations of fish showing disease signs, ideal samples are multiple (five or more) moribund fish or those showing clinical signs typical of the disease outbreak. For the detection of subclinical infections in populations of asymptomatic fish, larger sample numbers may be necessary (see Section 2, 2.2.D "Sample Number"). Fish that are found dead at the time of sampling are not acceptable for bacteriological examination, unless they are known to be very fresh. Contaminating bacteria can grow quickly in dead fish, particularly in warm water.

The selection of tissue samples for bacteriological assays varies depending on the pathogen suspected; the reader is referred to chapters on specific bacterial diseases. Organs most commonly tested include the kidneys and portions of any organ with visible lesions, although other organs such as the spleen or brain, and fluids such as blood plasma or ovarian fluid, are used for the detection of certain bacterial infections.

Samples for attempted culture of bacteria must be taken aseptically. If samples are to be taken from internal organs, disinfection of the body surface is recommended before incisions are made to expose the organs. Sterile tools must be used for making incisions and removing samples for culture. Samples intended for Gram stains or immunological tests also should be taken aseptically to prevent contamination with bacteria or antigens from extraneous sources. Homogenization of samples prior to culture or immunological testing may enhance the detection of bacteria or their antigens.

If samples cannot be inoculated immediately onto appropriate culture media, they may be stored on ice for up to 24 hours. Samples for culture should not be frozen. For the storage of samples intended for immunological tests, refer to the chapters on specific bacterial diseases.

II. Bacteriological Media and Test Reagents

A. Media

Specialized media for the detection of certain bacteria are referenced in the appropriate chapters. The following media commonly are used for the isolation of fish pathogens:

1. **Trypticase (or Tryptic) Soy Agar (TSA)**
   This is a commonly used medium for routine isolation and culture of many fish pathogens, and it is available from commercial sources.

2. **Brain-Heart Infusion Agar (BHIA)**
   This medium is used for routine isolation and culture of many fish pathogens, and it is available from commercial sources.

3. **Blood Agar (BA)**
   This medium is used for routine isolation and culture of many fish pathogens, and is also used for the visualization of hemolysis (B.20 – Characterization of Hemolysis). It is available from commercial sources, or can be prepared by the addition of sterile defibrinated blood (usually sheep, horse, or rabbit) at a concentration of 5% (volume/volume) to a sterile base medium such as TSA or BHIA, when the base medium has cooled to 48 to 50^{o}C.

4. **Tryptone Yeast Extract Salts Agar (TYES) Medium (Holt et al. 1989)**
   This medium is used for growth of *Flavobacterium psychrophilum*. This medium is a modification of one reported by Fujihara and Nakatani (1971) for growth of *F. columnare*.

Tryptone (Difco)	0.4%
Yeast extract (Difco)	0.04%
$MgSO_4.7H_2O$	0.05%
$CaCl_2.2H_2O$	0.05%
Agar	1.0%

   Adjust pH to 7.1-7.3.

   TYES plus skim milk agar is a recent modification that has the advantage of observing clearing in the medium around the *F. psychrophilum* colonies. For this medium sterile skim milk is added to TYES agar after the medium is cooled to 45 to 50°C just prior to pouring the plates. One mL of a 20% sterile skim milk solution is added for every 100 mL of TYES agar. The 20% skim milk is prepared by dissolving the skim milk powder in distilled water on a hot plate, then sterilizing the solution in 8 to 10 mL quantities.

5. **Cytophaga Agar**
   This medium (Anacker and Ordal 1959) is used for the isolation of flavobacteria. The formula is:

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

Agar	11.0 g
Distilled water to	1000.0 mL

Adjust pH to 7.2.

The growth of flavobacteria can be enhanced by the addition of 2 to 5% fetal bovine serum to this medium.

6. Hsu-Shotts Medium.

This medium (Bullock et al. 1986) is used for the isolation of flavobacteria; the addition of neomycin sulfate enhances the isolation of these bacteria by suppressing the growth of many other bacteria. The formula is:

Tryptone	2.0 g
Yeast extract	0.5 g
Gelatin	3.0 g
Agar	15.0 g
Distilled water to	1000.0 mL

After it is autoclaved, the medium is cooled to 45^{o}C, and filter-decontaminated neomycin sulfate is added to a final concentration of 4.0 mg/L.

Formulations for routine bacteriological media can be found in manuals on bacteriological methods such as MacFaddin (1985), Difco Laboratories (1998), Baron and Finegold (1990), and Murray (1999).

B. Test Reagents and Procedures for Phenotypic Characterization of Bacteria

The following are some tests commonly employed in the presumptive identification of bacteria isolated from fish. Commercially prepared media and reagents are available for biochemical tests. Detailed instructions on their preparation and use are also included in bacteriology manuals, and some descriptions are included in Section 2, 3.7 Reagents, Media, and Media Preparation and Section 2, 3.8 Bacterial Identification Techniques. Miniaturized bacterial identification systems that include many of the tests are also available commercially; their use for fish pathogen identification is further discussed in Section 2, 3.8.D.2 “Commercial Identification System.”

1. Gram Stain

This test is important for the broad classification of bacteria, and should be done on young (log-phase) cultures. Gram stain procedures are described in Section 2, 3.8.A.1 “Gram Stain.” Nonstain methods are available to aid in the determination of the true Gram stain reaction of problem organisms. One of these is the potassium hydroxide (KOH) test. The cell walls of gram-negative bacteria are broken down by KOH, and viscid chromosomal material is released. This causes the suspension to become thick and stringy. Procedures for the KOH test are described in Section 2, 3.8.A.2 “3% Potassium Hydroxide.” Fluorescent stains for differentiating gram-positive and gram-negative bacteria can also be used and are available from commercial sources.

2. Acid-Fast Stain

This test is used to distinguish gram-positive (or gram-variable) bacteria with cell walls that contain mycolic acids (long-chain, multiple cross-linked fatty acids) from other gram-positive bacteria. Mycobacteria (and to a lesser extent, *Nocardia*) stain acid fast.

3. Motility

For most bacteria (i.e., those motile by flagella), wet mounts (hanging drops) are prepared from log-phase cultures with TSB (trypticase soy broth or tryptic soy broth) as the suspending medium. If microscopic examination of wet mounts (hanging drops) gives equivocal or negative results, motility can be evaluated further by stab-inoculating tubes of commercially available semi-solid motility test medium (Difco Motility Test Medium or equivalent). The use of the hanging drop method and motility test medium for motility evaluations are described in Section 2, 3.8.C "Motility."

For flavobacteria, an agar block motility test can be done by excising a 5-mm square block of agar supporting a suspected flavobacterial colony, placing the block (colony side up) on a glass slide, and covering the block with a cover glass. The margin of the colony is examined with a microscope at about 400X magnification for evidence of gliding or creeping motility.

4. Cytochrome Oxidase Test

This test indicates the presence of the enzyme cytochrome oxidase, an iron-containing porphyrin enzyme that participates in the electron transport mechanism and in the nitrate metabolic pathways of some bacteria. The test is used to initially characterize gram-negative bacilli. Cytochrome oxidase procedures are discussed in Section 2, 3.8.B "Cytochrome Oxidase."

5. Carbohydrate Utilization Tests

Preparations of basal media to which specific carbohydrates are added are used to differentiate bacteria according to their patterns of carbohydrate utilization. Utilization of a specific carbohydrate causes a pH change, which is detected by a pH indicator in the medium. To determine if organisms metabolize carbohydrates oxidatively or fermentatively, OF medium is used. Gas production can be detected in some media. Further discussions of OF basal medium and certain carbohydrate utilization tests are included in Section 2, 3.7.B.4 "Oxidation/Fermentation (OF) Medium," Section 2, 3.8.D.1.a "Glucose Fermentation," and Section 2, 3.8.D.1.e "Carbohydrate Utilization (MacFaddin 1980)."

6. Triple Sugar Iron (TSI) Agar

Triple sugar iron agar is frequently used during the initial identification of gram-negative bacilli, particularly members of the Enterobacteriaceae. This medium can detect three primary characteristics of a bacterium: the ability to produce gas from the fermentation of sugars, the production of large amounts of hydrogen sulfide (H_2S) gas, and the ability to ferment glucose, lactose and sucrose. The interpretation of results is described in Section 2, 3.8.D.1.b "Triple Sugar Iron (TSI)." Lead acetate paper, a more sensitive indicator of H_2S production, is sometimes used in conjunction with TSI or other media containing available sulfur compounds.

7. Single Substrate Utilization Tests

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

of the test. The malonate test is described in and Section 2, 3.8.D.1.g "Malonate Test," respectively.

8. Indole Test

This is a test for the enzyme tryptophanase, and is used in the characterization of gram-negative bacteria, particularly Enterobacteriaceae. Bacteria that produce this enzyme can degrade the amino acid tryptophan into pyruvic acid, ammonia, and indole. Indole is detected by its combination with an indicator aldehyde to form a colored product. The indole test is described in Section 2, 3.8.D.1.d "Indole Test."

9. Decarboxylase/Dihydrolase Reactions

These are tests for enzymes that degrade certain amino acids by decarboxylation or dihydrolation. Common tests used in the identification of fish pathogenic bacteria include those for arginine dihydrolase/decarboxylase (ADH), lysine decarboxylase (LDC), and ornithine decarboxylase (ODC). The lysine decarboxylase test is described in Section 2, 3.8.D.1.f "Decarboxylase Test (Lysine)."

10. Hydrolysis Tests

These tests are used to differentiate bacterial species according to their abilities to produce certain hydrolyzing enzymes. The products of hydrolysis are detected by some visual reaction. Substrates commonly used in the characterization of fish pathogenic bacteria are urea (for urease activity), gelatin (for gelatinase activity), esculin (for esculin hydrolysis), and starch (for starch hydrolysis). The gelatinase test and esculin test are described in Section 2, 3.8.D.1.c "Gelatinase" and Section 2, 3.8.D.1.h "Esculin Test," respectively.

11. DNAse Test

Extracellular nucleases are produced primarily by the same bacterial species that produce the protease gelatinase. Commercially produced agar media are available for this test.

12. Phenylalanine Deaminase Test

This test determines the ability of an organism to deaminate the amino acid phenylalanine to yield indolepyruvic acid, and is used for grouping Enterobacteriaceae.

13. Catalase Test

This test is used to detect the presence of the enzyme catalase, which catalyzes the liberation of oxygen and water from hydrogen peroxide. The test reagent (3% hydrogen peroxide) is readily available. Because red blood cells in blood agar media contain catalase, weak false positive reactions can be obtained with colonies taken from these media. It is recommended that control catalase tests be performed with a small loopful of blood-containing agar on the same slide with the organism; a strong positive reaction from the organism can be distinguished from a weak positive reaction from the medium alone.

14. Bile Solubility Test

Streptococcus pneumoniae can be distinguished from other alpha hemolytic streptococci (B.20 – Characterization of Hemolysis) by this test. *Streptococcus pneumoniae* possesses an autocatalytic enzyme that normally functions to lyse the cell wall during cell division; colonies of this organism also will autocatalyze within 30 minutes after exposure to the surfactant sodium deoxycholate, one of the major components of bile. Other alpha hemolytic streptococci lack such an active enzyme and will not dissolve in bile. The bile solubility test may not work with old colonies of *S. pneumoniae*, which may have lost their active enzyme.

15. Nitrate Reduction Test

This test distinguishes between bacteria that cannot utilize nitrate (NO_3) as a nitrogen source, and those that can reduce nitrate to nitrite (NO_2) or a product beyond nitrite.

16. Antimicrobial Sensitivity Tests

Patterns of sensitivity to various antimicrobial reagents are sometimes used in the characterization of fish pathogenic bacteria. Most commonly, sensitivity to novobiocin is used to distinguish *Vibrio* spp. from motile *Aeromonas* spp. The novobiocin disk (5 µg concentration) is applied to a TSA plate that has been surface-seeded uniformly with the organism under test. After incubation at 20 to 22°C for 16 to 24 hours, a sensitive organism shows a clear zone of inhibition around the disk. Because TSA is widely used for the primary isolation of many fish pathogenic bacteria, this is the medium generally used for novobiocin sensitivity tests performed to differentiate *Vibrio* spp. from motile *Aeromonas* spp. However, disk diffusion tests performed to determine the sensitivity patterns of bacterial isolates to a battery of compounds are done on Mueller-Hinton agar (the para-aminobenzoic acid present in TSA interferes with the action of sulfonamides). Procedures for the conduct and interpretation of disk diffusion antimicrobial susceptibility tests are described in more detail in manuals on bacteriological procedures.

17. Vibriostatic Agent 0/129 Sensitivity Test

Sensitivity to the vibriostatic agent 0/129 (2,4-diamino-6,7-diisopropylpteridine phosphate, available from Sigma Chemical Co.) is used to differentiate *Vibrio* spp. from motile *Aeromonas* spp. The sensitivity disks are prepared as follows: Dissolve 0/129 in acetone at 0.1% (weight/volume). Saturate Whatman antibiotic filter paper disks (6 mm) with the 0/129 solution, and drain off the excess. Dry the disks at 37°C, and store them in a tightly sealed bottle at 4°C. Control disks (saturated with acetone only) also should be prepared. For 0/129 sensitivity tests, TSA plates are surface-seeded with bacteria, test and control disks are applied to the plates, the plates are incubated, and results are interpreted in the same manner as for novobiocin sensitivity tests. Novobiocin sensitivity and 0/129 sensitivity tests can be conducted on the same plate.

18. P Disk Test

The P disk (optochin resistance) test is used to identify strains of *Streptococcus pneumoniae*. Disks impregnated with 5 µg of optochin (ethyl hydrocupreine) are placed on inoculated blood agar plates. *Streptococcus pneumoniae* is not optochin resistant and a zone of inhibition will develop around the disk. The isolate is resistant to optochin if the zone of inhibition is greater than 14 mm for a 6-mm disc or 16 mm for a 10-mm disk. Correct interpretation of the optochin zone requires a confluent lawn of bacterial growth. If the growth is too light, an erroneously large zone (false susceptibility) may be observed.

19. LAP Test

The LAP test detects the presence of leucine aminopeptidase (LAP). Among the LAP-positive bacteria are the lactococci, enterococci, and several streptococci, but the ß-hemolytic streptococci and aerococci are LAP-negative. The test is performed using commercially available discs that are impregnated with leucine-ß-naphthylamide. The LAP enzymatic activity releases ß-naphthylamine, which turns red on the addition of p-dimethylaminocinnamaldehyde.

20. Characterization of Hemolysis

Patterns of hemolytic action of bacteria on erythrocytes in blood-containing media (usually

BA) are used in the identification of some bacteria, principally streptococci. The major types of hemolysis are: α (alpha), consisting of an indistinct zone of incomplete lysis of erythrocytes around the bacterial colony, accompanied by a greenish to brownish discoloration of the medium; and β (beta), a clear, colorless zone around the bacterial colony, in which the erythrocytes have undergone complete destruction; and γ (gamma), no hemolysis.

21. CAMP Test

The CAMP test is used to identify Lancefield Group B streptococci (see Immunological Procedures below). Group B streptococci produce a protein-like substance called the "CAMP factor," which can act synergistically with the beta toxin produced by certain strains of *Staphylococcus aureus*, resulting in an enhanced zone of hemolysis around the streptococci. The test is performed with an appropriate strain of *S. aureus*, or with commercially available CAMP factor-impregnated filter paper disks.

22. Simmons Citrate Test

Organisms that are able to use citrate as the sole source of carbon for metabolism and growth are able to grow on simmons citrate agar. In the process of metabolizing citrate, bacteria produce alkaline conditions in the medium. Simmons citrate agar contains the pH indicator bromothymol blue, which is green under acidic conditions, and royal blue when the medium becomes alkaline.

Magnesium sulfate	0.20 g
Monoammonium phosphate (NH4H2PO4)	1.00
Dipotassium phosphate (K2HPO4)	1.00
Sodium citrate	2.00
Sodium chloride	5.00
Agar	15.00
Bromthymol blue	0.08
Final pH 6.9 ± 0.2 at 25°C	

Heat gently with occasional agitation. Boil 1 to 2 minutes until agar dissolves. Fill 13 x 100 or 16 x 150 mm screw-cap tubes 1/3 full. Autoclave 15 minutes at 121°C. Before medium solidifies, incline tubes to obtain 4 to 5 cm slants and 2 to 3 cm butts. Final pH, 6.8 ± 0.2.

III. Immunological Procedures

Confirmation of the identity of most fish pathogenic bacteria is based on serological tests. Most common among these are the fluorescent antibody test and the slide or microtiter agglutination test. These and certain other techniques developed for the identification of bacteria in culture, or for the detection and identification of bacteria directly in fish tissues or body fluids, are discussed briefly below. The specificity of these and other immunological tests for the identification of fish pathogens will depend on the specificity of the antibodies used.

A. Fluorescent Antibody Test (FAT)

Both direct and indirect FATs have been developed for the identification of bacterial fish pathogens. Antibodies for specific bacteria are available from commercial sources, or from

certain research laboratories. Fluorochrome-conjugated antibodies for the indirect FAT are readily available from commercial sources. Most commonly, the FAT is used to confirm the identity of bacteria isolated in culture. For some pathogens, notably *Renibacterium salmoninarum*, the FAT is used for the detection and identification of bacteria directly in fish tissues or body fluids. Descriptions of direct and indirect FAT procedures are included in Section 2, 3.8.E "Fluorescent Antibody Test (FAT)." The reader is also referred to chapters on specific pathogens for modifications of the FAT for use on fish tissue and body fluid samples.

B. Agglutination Tests

The identity of many bacterial fish pathogens can be confirmed by agglutination reactions with specific antisera. Certain bacterial species exhibit a tendency to autoaggregate spontaneously and are therefore difficult to examine by agglutination tests. Such bacteria may be subjected to pretreatment procedures to prevent autoaggregation, or they may be tested by alternative procedures such as latex bead agglutination or staphylococcal coagglutination. The reader is referred to chapters on specific bacterial pathogens for modifications to agglutination tests. Principles and procedures for some agglutination techniques are also discussed by Roberson (1990).

1. Slide Agglutination Test

This test is used for rapid confirmatory identification of bacteria grown in culture.

a. Clean glass microscope slides and mark circular divisions (two circles per slide) with a wax crayon.

b. Prepare a suspension of one or more colonies of the unknown bacterium in a small amount of physiological saline. To ensure a uniform suspension without clumps, it is best prepared in a small test tube rather than directly on the slide. The bacterial suspension should be standardized to a known concentration of particulate materials; i.e. a McFarland standard #3 or a spectrophotometer reading of 40% T at 645 nm. If this cannot be done, preparation of a cloudy suspension approximating the color and density of skimmed milk is generally acceptable. Suspensions of positive controls (bacteria known to be the species of interest) and negative controls (known bacteria of an unrelated species) can be prepared in the same manner.

c. Place a drop of the unknown bacterial suspension in each of the two circles on a slide. Add a drop of the appropriate specific antiserum to one circle on the slide. Add a drop of normal serum to the other circle on the slide. Mix gently with an applicator stick, or rock the slide gently. Follow the same procedure for the control bacteria.

d. Observe the reactions immediately for agglutination (clumping), then incubate the slides at room temperature and observe after five and 10 minutes. Results can be checked with a microscope at low magnification.

For positive identification of the unknown bacterium, the following reactions should be observed:

Circle with unknown bacterium plus antiserum: agglutination

Circle with unknown bacterium plus normal serum: no agglutination

Circle with positive control bacterium plus antiserum: agglutination

Circle with positive control bacterium plus normal serum: no agglutination

Circle with negative control bacterium plus antiserum: no agglutination

Circle with negative control bacterium plus normal serum: no agglutination

2. **Microtiter agglutination test.** This test gives the titer of the antiserum used, in addition to confirmatory identification of the bacterium being tested. The test is done in 96-well microtiter plates. A constant bacterial concentration, but two-fold serial dilutions of antiserum are used. The procedure is described by Roberson (1990).

C. Lancefield Grouping of Streptococci

Most beta-hemolytic streptococci and some alpha-hemolytic or nonhemolytic streptococci possess specific carbohydrate cell wall antigens. These carbohydrate antigens are called streptococcal group antigens or Lancefield group antigens. In the past, serological typing of cell wall components was used to separate streptococci into species. Although recent DNA homology studies have shown that this is not possible, serological typing is still a useful aid for the identification of isolates. Group-specific sera (precipitating, agglutinating, and fluorescent-antibody sera) are commercially available for use with extracts, cell suspensions, and spent broth media. Beta-hemolytic streptococci are characteristically tested with group A, B, C, D, and F antisera. Alpha-hemolytic or nonhemolytic streptococci are usually tested with antisera to groups B, D, and N. Procedures for streptococcal antigen extraction and serotyping are described in bacteriological procedures manuals.

D. Other Immunological Tests

In addition to the tests described above, other immunological tests have been developed for the identification of bacteria in culture, or for the detection and identification of specific bacteria in fish tissues or body fluids. Included among these are such techniques as staphylococcal coagglutination, latex bead agglutination, counterimmunoelectrophoresis, and enzyme immunoassays. Of the enzyme immunoassays, the enzyme-linked immunosorbent assay (ELISA) has perhaps gained the widest use. An ELISA is designed to detect a specific substance in a complex mixture by binding that substance to an antigen- or antibody-coated surface. After binding has occurred, an enzyme-labeled antibody specific for the bound substance is applied. With the addition of appropriate reagents, the enzyme catalyzes a reaction that yields a colored end product, thus allowing detection and quantification of the bound substance. Various ELISAs have been developed for the detection of bacteria or bacterial products as well as viruses, drugs, hormones, toxins, carcinogens, and antibodies. The reader is referred to chapters on specific pathogens for further discussions of the ELISA and other immunological techniques.

IV. Nucleic Acid-based Identification Procedures

Nucleic-acid-based tests are now gaining favor for identification of certain bacterial fish pathogens. Among the early tests developed were nucleic acid probes designed to detect specific unique segments of DNA or RNA of the target bacterium. These techniques have been largely supplanted by a plethora of molecular tools known collectively under the name polymerase chain reaction (PCR). The PCR procedures are considered to have a greater potential for improving both the sensitivity and specificity for detection and identification of a target bacterium because of the enzymatic amplification steps incorporated in the tests. The reader is referred to chapters on specific bacteria for discussions of PCR procedures.

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