CHAPTER 5

Bacteriology

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I. Introduction

This section defines the procedures and techniques used to correctly identify the target bacterial pathogens identified for the Survey (*Yersinia ruckeri*, *Aeromonas salmonicida* and *Edwardsiella ictaluri*). Proper identification relies on bacterial growth characteristics, appropriate biochemical tests, and corroboration by serological techniques.

Pathogens of Regional Importance (PRIs) include: *Citrobacter freundii, Edwardsiella tarda, Flavobacterium columnare* and *Flavobacterium psychrophilum.* The later two bacteria have special requirements for culture and serological confirmation. Several excellent sources are listed in the Reference section for identification of PRIs and other bacteria that may be isolated from fish sampled for the Survey. Additional media formulas are also provided for PRIs in Appendix A.

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II. Media Preparation

A. PLATE MEDIA

1. Prepare media in stainless steel beakers or clean glassware according to manufacturer instructions. Check pH and adjust if necessary. Media must be boiled for one minute to completely dissolve agar. Common media recipes are given in Appendix A.

2. Cover beaker with foil, or pour into clean bottles being sure to leave lids loose. Sterilize according to manufacturer's instructions when given, or at 121°C for 15 minutes at 15 pounds pressure.

3. Cool media to 50°C.

4. Alternatively, media can be autoclaved and cooled to room temperature and refrigerated for later use. Store bottles labeled with media type, date and initials. When media is needed, boil, microwave or use a water bath to completely melt the agar. Cool to 50°C, then proceed to step 5.

5. Before pouring media, disinfect hood or counter thoroughly and place sterile petri dishes on the disinfected surface.

6. Label the plates or plate storage tins with the type of medium, preparers initials, and date made.

7. Remove bottle cap and pour plates or dispense with a Cornwall pipette, lifting each petri dish lid as you go. Pour approximately 15 to 20 mL per 100×15 mm petri dish. Replace lids as soon as the plate is poured.

8. Immediately after use, rinse the automatic pipettor in hot tap water followed by distilled water to remove all media and prevent clogging of the instrument.

9. Invert plates when the media has cooled completely (~ 30-60 minutes) to prevent excessive moisture and subsequent condensation on the plate lid. Do not use the UV light because it can denature the proteins in the media.

10. Allow plates to sit overnight at room temperature. Store plates upside down in the refrigerator in a tightly sealed plastic bag or plate storage tin.

11. Follow manufacturer's recommendation for storage period of prepared media.

B. TUBE MEDIA

1. Prepare media in stainless steel beakers or clean glassware according to manufacturer's instructions. If the medium contains agar, boil for one minute to completely dissolve the agar.

2. Media with indicators must be pH adjusted. This can be done when medium is at room temperature; otherwise compensation for temperature needs to be made.

3. Arrange test tubes in racks. Disposable screw cap tubes can be used for all tube media.

4. Use an automatic pipettor or Pipet-aid™ to dispense the medium. If using the Brewer or Cornwall pipette prime with deionized water, then pump the water out of the syringe prior to pipetting and discard the first few dispenses of medium. Dispense approximately 5 to 10 mL media in 16×125mm or 20×125mm tubes. Close caps loosely.

5. Immediately after use, rinse the automatic pipettor in hot tap water followed by distilled water to remove all media and prevent clogging of the instrument.

6. Follow manufacturer's recommendation for autoclave time and temperature.

7. If making slants, put tubes in slant racks after autoclaving. Adjust the slant angle to achieve the desired slant angle and butt length (i.e. short butt and long "fish-tail" slant for TSI or a standard slant over ¾ of the tube length for BHIA).

8. Tighten caps when tubes can be easily handled but still warm to the touch. Cool completely to room temperature in the slanted position.

9. Label the tubes or the tube rack with type of medium, preparers initials, and date made.

10. Store at 2-8°C, following manufacturer's recommendation for period of long-term storage.

III. Media Formulations

Numerous differential media and biochemical tests can be used to determine identification of bacterial cultures. Some common bacteriological media are listed in Appendix A, but this list is incomplete and a good bacteriology media reference such as Difco Manual, McFaddins Biochemical Media Used for Detection of Bacteria, or Atlas's Handbook of Microbiological Media should be used for additional information. The purpose and use of various media are described in the testing section.

IV. Bacterial Culture Isolation

A. Aseptically inoculate samples onto BHIA tubes or plates labeled with pertinent case history information.

B. Incubate aerobically for 24-48 hours at 20-24°C (room temperature). If no growth occurs at 24 and 48 hours, record this information on the data sheet. If no growth occurs after 96 hours, samples are discarded.

C. When growth does occur on field collection tubes or plates, use a sterile loop or needle to select a single colony to subculture onto fresh BHIA. If colonies are not well isolated, the plate will have to be re-inoculated on BHIA and thoroughly struck over the entire plate surface to achieve isolation of bacteria.

D. Incubate at 20-24°C for 24 hours to allow bacterial growth; all tests should be performed on 24-48 hour cultures.

    1. Using a sterile needle or small loop, pick individual distinct bacterial colonies. Use of a dissecting scope can aid in distinguishing between differing colony types. Assign an isolate number to each pure colony and record all colony characteristics on the data sheet.

    2. Begin initial testing to determine presumptive identification of pure strain bacterial cultures (CO, motility, catalase, and Gram stain).

    3. Based on preliminary tests, follow the Flowchart (Appendix D for major pathogens & E for PRIs) to determine which biochemical tests are needed to determine identification.

E. Inoculate biochemical tubes and label with pertinent case history information.

1. Follow the directions for interpretation of biochemical tests in the next section.

F. Treat all bacterial cultures as potential human pathogens. When testing is complete, either cryopreserve isolates of interest, or discard bacterial plates and biochemical tubes in a biohazard bag and autoclave.

V. Gram Stain

Gram stain pure strain cultures to determine whether Gram negative or Gram positive. Gram staining detects a fundamental difference in the cell wall composition of bacteria. (Kits are available commercially, or formulas for reagents are listed in Appendix B.)

A. Prepare a bacterial smear from a pure culture
   1. Put a drop of saline, distilled water, or PBS on a clean glass slide
   2. Using a sterile loop or needle touch an isolated colony and mix in the water drop.
   3. Mix until just slightly turbid (light inoculum is best, excess bacteria will not stain properly).
   4. Let air dry and heat fix. Do not overheat; slide should not be too hot to touch.
   5. Allow to cool.

B. Flood the slide with crystal violet, and allow to remain on the slide for 60 seconds

C. Wash off the crystal violet with running tap water.

D. Flood the slide with Gram's iodine, and allow to remain on the slide for 60 seconds.

E. Wash off with running tap water.

F. Decolorize with 95% alcohol and 5% acetone solution until the solvent flows colorless from the slide (approximate 5-10 seconds). Excessive decolorization should be avoided since it may result in a false gram-negative reading.

G. Rinse immediately with running tap water.

H. Counter stain with Safranin for 60 seconds.

I. Rinse with tap water and allow to air dry.

J. RESULTS:

   1. Gram negative: cells are decolorized by the alcohol-acetone solution and take on a pink to red color when counterstained with safranin.

2. Gram positive: cells retain the crystal violet and remain purple to dark blue.

K. QUALITY CONTROL: Use a known bacterial culture as a control for each case history to assess correct staining and interpretation (cultures can be obtained from ATCC® and maintained at 2-8°C for long term use). Additionally Fisher Scientific has gram control Slides (Catalog# 08-801)
   1. Positive: *Staphylococcus sp.*

   2. Negative: *Yersinia ruckeri*

VI. Alternative test for Gram Reaction – 3% Potassium Hydroxide

A. Add a heavy inoculum of pure culture of bacteria grown on a solid medium to a drop of 3% potassium hydroxide (KOH) solution (3 grams KOH per 100 mL distilled water) on a clean glass slide.

B. Stir for about one minute, occasionally lifting the loop to look for thickening and "stringing" of the slurry.

C. RESULTS:
   1. Gram positive bacteria will not appear to change the viscosity of the KOH solution.

   2. Gram negative bacteria will cause the KOH solution to become stringy or mucoid in appearance and consistency.

D. QUALITY CONTROL:
   1. Gram Positive: *Staphylococcus sp.* (ATCC® any isolate)

   2. Gram Negative: *Yersinia ruckeri*

VII. Presumptive Identification of Gram Negative Bacteria

Refer to Flow Chart in Appendix D and E then perform the following series of tests. All the following tests should be incubated aerobically unless stated otherwise.

A. **Carbohydrate Utilization (MacFaddin 1980)** - The following carbohydrates are utilized to aid in bacterial species identification: Arabinose, Rhamnose, Mannitol, Salicin, Sorbitol, and Sucrose (saccharose). The procedures to be followed for each of these media are identical.

   1. Inoculate carbohydrate tube (Appendix A "Carbohydrate Utilization media") with growth from an 18 to 24 hour pure culture.

2. Incubate with loosened cap 18 to 24 hours at 20-24oC. A prolonged incubation of up to four days may be necessary for some negative results.

3. RESULTS
   a) Positive - Acid is produced from fermentation, which turns media yellow.
   b) Negative - No fermentation of carbohydrate, media remains green.

   c) Aerogenic - Gas bubbles are present within the media.

4. QUALITY CONTROL

Carbohydrate	**Positive Control Isolate**	**Negative Control Isolate**
Arabinose	*Escherichia coli* (ATCC® 25922)	*Yersinia ruckeri*
Sorbitol	*Escherichia coli* (ATCC® 25922)	*Y. ruckeri* Type I
Rhamnose	*Enterobacter aerogenes* (ATCC® 13048)	*Yersinia ruckeri*
Salicin	*Enterobacter aerogenes* (ATCC® 13048)	*Yersinia ruckeri*

5. PRECAUTIONS
   a) Difficulty in interpreting test results may occur with slow growing bacteria. Prolonged incubation may be required.

   b) Heavy bacterial growth throughout the media can offset the color of a negative (green) reaction, giving the appearance of a weakly positive (yellow) reaction. This is especially true with yellow-pigmented bacteria. These tubes should be retested if a true yellow color is not noted within several days.

B. **Catalase** – this test determines bacterial production of catalase enzymes.

1. Place a drop of hydrogen peroxide (3% H_2O_2 - reagent grade) on a microscope slide or in the concave surface of a hanging drop slide.

2. With a sterile loop, collect a sample of 18-24 hour old pure bacterial culture.

3. Place the loop in the hydrogen peroxide.

4. If the test is positive, there will be immediate bubbling or foaming, and liberation of O_2 gas.

5. Record results.

C. **Cytochrome oxidase** – this test determines the presence of cytochrome oxidase enzymes. The use of an iron-containing metal inoculation loop can lead to a false-positive reaction. Use only plastic or platinum loops for this test.

1. Add an inoculum of pure 18 -24 hour old bacterial culture to the test strip impregnated with reagent.

2. RESULTS:
   a) Positive: purple color within 5-10 seconds (reactions that occur after 10 seconds are negative).
   b) Negative: no purple color.

3. QUALITY CONTROL:
   a) Positive: *Pseudomonas aerugenosa* (ATCC® 10145)
   b) Negative: *Yersinia sp.*

D. **Decarboxylase Test (Lysine and Ornithine) -** A determination of bacterial enzymatic capability to decarboxylate an amino acid to form an amine with resultant alkalinity.

1. For each isolate to be tested, it is necessary to inoculate a decarboxylase control tube and lysine or ornithine test tube (Appendix A "Decarboxylase Medium Base"). Use light inoculum from 18 to 24 hour pure culture.

2. Add 1 to 2 mL oil overlay to each tube.

3. Incubate 24 hours at 20-24°C. A prolonged incubation of up to four days may be necessary.

4. RESULTS

Test Result	**Lysine or Ornathine Tube**	**Control Tube**
Positive	Turbid to faded purple (glucose fermented, decarboxylase produced)	Yellow (glucose fermented)
Negative	Yellow (glucose fermented, decarboxylase not produced)	Yellow (glucose fermented)
Negative	Purple (glucose not fermented, decarboxylase not produced)	Purple (glucose not fermented)

5. QUALITY CONTROL
   a) Positive - *Enterobacter aerogenes* (ATCC® 13048)

   b) Negative - Proteus vulgaris (ATCC® 13315)

6. PRECAUTIONS
    a) At the end of incubation, the lysine tube might show a layer of purple over yellow. Gently shake the tube before interpreting the result.

    b) An indistinct yellow-purple color may be difficult to interpret. Use the control tube for comparison. Any trace of purple color after a 24-hour incubation in the amino acid tube denotes a positive result.

    c) Do not interpret tests prior to 18 to 24 hours. During the first 12 hours, only glucose is fermented which produces a yellow color. Decarboxylase enzymes do not form until the acidic environment is established by the fermentation of glucose.

E. Esculin Test - To determine the ability of an organism to hydrolyze the glycoside esculin (aesculin) to esculetin (aesculetin) and glucose in the presence of bile (10 to 40%).

1. Inoculate the surface of the bile esculin slant (Appendix A "Bile Esculin Agar") with inoculum from an 18 to 24 hour old pure culture.

2. Incubate 20-24°C for 24 to 48 hours.

3. RESULTS
    a) Positive - Presence of a black to dark brown color on the slant.

    b) Negative - No blackening of the medium.

4. QUALITY CONTROL
    a) Positive - *Enterobacter aerogenes* (ATCC® 13048)

    b) Negative - *Yersinia ruckeri*

5. PRECAUTIONS - False positives may occur with hydrogen sulfide producing organisms, such as S*hewanella putrefaciens.* Neither of the target organisms for these protocols will, however, produce hydrogen sulfide.

F. **Gelatinase** – A test to determine bacterial production of gelatinase enzymes that liquefy gelatin.
1. Inoculate by stabbing ½ to 1 inch deep into the nutrient gelatin media (Appendix A "Nutrient Gelatin") with a heavy inoculum from an 18 to 24 hour pure culture.

2. Inocubate 18 to 24 hours at 20-24°C.

3. RESULTS
    a) Positive – Media is liquefied. Weak results can be visualized by rapping the tube against the palm of the hand to dislodge droplets of liquid from the media. Any drops seen are considered positive.

    b) Negative – No liquefaction occurs in media.

4. QUALITY CONTROL
    a) Positive - Proteus vulgaris (ATCC® 8427)

    b) Negative - Escherichia coli (ATCC® 25922)

5. PRECAUTIONS
    a) The liquid will generally appear turbid due to bacterial growth.

    b) Nutrient gelatin softens at temperatures above 20oC. Keep refrigerated until ready to inoculate, and do not let tubes reach room temperature or warmer. This will make interpretation of results difficult. Tests, which are incubated at 35^{o}C, should be refrigerated prior to recording results.

G. **Indole Test** - A test to determine bacterial ability to split indole from the tryptophan molecule. Certain bacteria are able to oxidize the amino acid, tryptophan, with tryptophanase enzymes to form three indolic metabolites - indole, skatole (methyl indole), and indoleacetate. Indole, pyruvic acid, ammonia, and energy are principle degradation products of tryptophan. Indole, when split from the tryptophan molecule, can be detected with the addition of Kovac's reagent. The reagent is not a dye or stain, but reacts with indole to produce an AZO dye.

1. Inoculate tryptone broth (Appendix A"Tryptone Broth") with a light inoculum from an 18 to 24 hour pure culture.

2. Incubate 24 to 48 hours at 20-24^{o}C

3. At the end of 24 hours incubation do the following:

    a) Aseptically remove 2 mL of media and place in an empty sterile test tube. Save extra tube for 48-hour incubation, if necessary.

    b) Add about 5 drops of Kovac's reagent (Appendix B "Kovac's Indole Reagent") to one of the tubes and agitate tube.

    c) If a positive reaction is observed, the test is complete.

d) If the 24 hour incubated sample is negative, incubate the remaining tube for an additional 24 hours, and test again for the presence of indole with Kovac's reagent.

4. RESULTS
   a) Positive - Within 1 to 2 minutes, a cherry red ring will form at the surface of the media.

   b) Negative - No color formation is observed at the surface; the color remains that of the reagent – yellow.

   c) Variable - An orange color may develop. This indicates the presence of skatole, which may be a precursor of indole formation.

5. QUALITY CONTROL
   a) Positive - Escherichia coli (ATCC® 25922)

   b) Negative - *Pseudomonas aerugenosa* (ATCC® 27853)

6. PRECAUTIONS
   a) Avoid inhaling fumes of Kovac's. Wear gloves to avoid skin contact.

   b) Tests for indole should be conducted after both 24 and 48 hours of incubation before a test can be declared negative. Split the broth culture prior to performing the 24-hour test. If negative, incubate the untested tube (without Kovac's) for another day and try again.

   c) Do not eliminate the 24-hour test, because some organisms may have produced indole by 24 hours, but have broken it down by 48 hours. DO BOTH!

   d) Kovac's reagent should be fresh. A color change from yellow to brown indicates aging and results in reduced sensitivity of the test.

   e) The procedure described here produces more reliable results than those obtained from MIO (motility-indole-ornithine) medium.

H. **Malonate Test -** A method to establish if a bacterial isolate is able to utilize sodium malonate as its only source of carbon.

1. Inoculate malonate media (Appendix A "Malonate Broth") with a light inoculum from an 18 to 24 hour pure culture.

2. Incubate 24 to 48 hours at 20-24^{o}C.

3. RESULTS
    a) Positive - Light blue to deep blue color throughout the media.

    b) Negative - Color remains the same as un-inoculated tube - green.

4. QUALITY CONTROL

    a) Positive - *Enterobacter aerogenes* (ATCC® 13048)

    b) Negative - *Yersinia ruckeri*

5. PRECAUTIONS - The test tube must be incubated for at least 48 hours before it may be called negative. Since some bacteria produce only slight alkalinity, it is useful to compare the test to an un-inoculated tube. Any trace of blue color denotes a positive reaction.

I. **Motility** – this test determines if a bacterial isolate is motile by means of flagella.

1. Place a drop of distilled water or sterile PBS onto the center of a clean microscope cover glass. Place an additional tiny drop in one corner of the cover glass (to adhere the cover glass to the depression slide when it is inverted). Inoculate the center drop from a pure strain culture that is 24-48 hours old using a sterile loop. Carefully invert the cover glass and place over the concave portion of a hanging drop slide. Observe for motility using phase contrast at 400× magnification on a compound microscope. Care should be taken to not interpret "drift" or "Brownian motion" as motility. Record results as motile or non-motile.

2. If this method fails to show motility then:
    a) Inoculate a nutrient broth with the isolate and incubate at room temperature until growth is obtained, usually 24 hours. After incubation use a sterile loop or sterile dropper and place a drop on a clean cover glass. Place a tiny drop of distilled water in one corner of the same cover glass. Continue as above.
    b) Semi-solid motility test medium can also be used. Stab the medium with a small amount of inoculum. Incubate overnight at room temperature. If the bacterial species is motile, the medium will become turbid with growth that radiates from the line of inoculum. If the bacterial species is non-motile, only the stab line will have visible bacterial growth.

3. QUALITY CONTROL
    a) Positive: *Escherichia coli* (ATCC® 25922)
    b) Negative: *Aeromonas salmonicida*

J. Nitrate Reduction – To determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) or further reduced products.

1. Inoculate the broth (Appendix A "Nitrate Broth") with 18 to 24 hour old pure culture.

2. Incubate 24-48 hours at 20-24^{o}C aerobically.

3. After incubation add about 5 drops of *a*-naphthylamine and sulfanilic acid (Appendix B *"a*-naphthylamine", "sulfanilic acid") to the medium and shake gently to mix reagents..

4. If there is no color development after addition of *a*-naphthylamine and sulfanilic acid, add a small amount of zinc dust.

5. RESULTS
   a) Positive – Formation of a pink or red color in the medium within 1-2 minutes following the addition of *a*-naphthylamine and sulfanilic acid. or no color development within 5-10 minutes after adding zinc dust.
   b) Negative – No pink or red color development within 1-2 minutes following the addition of *a*-naphthylamine and sulfanilic acid. or red color development within 5-10 minutes after adding zinc dust..

6. QUALITY CONTROL
   a) Positive – *Escherichia coli* (ATCC® 25922)

   b) Negative – *Acinetobacter sp.* (ATCC® 33304)

7. PRECAUTIONS – Make sure to watch for the color change as the color may fade quickly. Before final determination of results are made be sure to add a small amount of zinc dust. To much zinc dust can reduce the nitrate to quickly resulting in a false negative reaction. Strongly reducing bacteria may exhibit a brown precipitate.

K. **Glucose Fermentation (OF Basal with 1% Glucose)** – Bacteria metabolize carbohydrates by oxidative and/or fermentative pathways. Oxidation occurs in the presence of atmospheric oxygen (aerobic), whereas fermentation takes place in an anaerobic environment. Metabolism of the carbohydrate dextrose by either an aerobic or anaerobic pathway results in acid production. The resulting acidic environment causes the Brom Thymol blue pH indicator in the medium to turn from green to yellow. The presence of bubbles in the tube indicates gas production (aerogenic). If no reaction occurs, the medium can remain unchanged or become alkaline (blue at the surface).

1. A deep butt tube (~7 mL in 16 × 125mm) is used for this test.

2. With a sterile needle take a small inoculums from an isolated colony and stab to the bottom of the tube.

3. Incubate at 20-24°C for 24-48 hours. Check tubes at 24 hr for acid and/or gas production.

4. RESULTS: A = acid(yellow); AG = acid + gas, N = no change or alkaline

	Top of Tube	Bottom of Tube
Oxidative	A	N
Fermentative	AG or A	AG or A
Non-reactive	N	N

5. QUALITY CONTROL: Fermentative: *Aeromonas* sp.
Oxidative: *Pseudomonas* sp.

L. **Simmons Citrate –** Organisms that are able to use citrate as the sole source for metabolism and growth are able to grow on Simmons citrate agar. By metabolizing citrate by the bacteria alkaline conditions are formed in the medium. The pH indicator in Simmons citrate agar, bromothymol blue, will turn from green from acidic conditions to a royal blue when the medium becomes alkaline.

1. Inoculate the agar (Appendix A "Simmons Citrate Agar") by making a streak onto the surface of the slant with a 18 to 24 hour old pure culture.

2. Incubate for up to 4 days at 20-24^{o}C.

3. RESULTS:
   a) Positive – growth and medium color change to a blue-green or royal blue.

   b) Negative – little or no growth and no color change in the medium, remaining dark green.

4. QUALITY CONTROL:
   a) Positive – *Enterobacter aerogenes* (ATCC® 13048)

   b) Negative – *Escherichia coli* (ATCC® 25922)

5. PRECAUTIONS – Inoculation with a large amount of bacteria may produce a yellow to tan color on the slant. This does not signify a reaction.

M. **Triple Sugar Iron (TSI) -** This medium can determine the ability of an organism to utilize specific carbohydrates incorporated in a basal growth medium, with or without the production of gas, along with the determination of hydrogen sulfide (H_2S) production. TSI agar contains the three sugars in varying concentrations: glucose (1X), lactose (10X), and sucrose (10X). It also contains the pH indicator phenol red. If sugar fermentation occurs, glucose will be initially used and the butt of the tube will be acidic

(yellow). After glucose utilization the organism may continue to ferment the remaining sugars. If this occurs the entire tube will become acidic. Certain bacteria are unable to utilize any sugars and will breakdown the peptone present. Peptone utilization causes an alkaline (red) shift in the medium that causes a color change from orange to red. Blackened medium is caused by hydrogen sulfide production, which changes ferrous sulfate to ferrous sulfide. In addition, splitting of the medium or presence of bubbles in the butt of the tube can determine gas production.

1. With a sterile needle inoculate the TSI slant by stabbing to the bottom of the tube and then streaking the surface of the slant as the needle is drawn out of the tube. Screw the cap on loosely.

2. Incubate at 20-24°C. Read after 18-24 hours.

3. RESULTS:

   A = Acid; K = Alkaline; H2S = Hydrogen sulfide produced; N =No change

Slant/Butt	Color/Reaction	Interpretation
K/N or K/A	red/orange (oxidative) or red/yellow (fermentative)	only peptone utilized or only glucose-fermented
A/A	yellow/yellow and/or sucrose-fermented	glucose, plus lactose
gas	splitting or bubbles	gas production
H_2S	black butt	Hydrogen sulfide produced

4. QUALITY CONTROL:
   A/A: ± gas: *Aeromonas* spp., *Escherichia coli* (ATCC® 25922)
   K/A w/gas H_2S: *Edwardsiella tarda, Salmonella typhimurium*
   K/A: *Proteus spp., Shigella flexneri* (ATCC® 12022)
   K/N: *Pseudomonas spp.*
   K/N w/H_2S: S*hewanella putrefaciens*

N. **O/129 Discs** – this test determines the sensitivity of a bacterial organism to the vibriostatic agent 2,4-diamino-6,7 di-isopropylpteridine (O/129).

1. Suspend bacteria in sterile saline or PBS (Appendix B “Saline Solution”or “Phosphate Buffered Saline Solution”).

2. Streak suspension on plate in three planes with a cotton swab.

3. Aseptically place sensitivity disc in the center of inoculum.

4. Incubate at 20-24°C for 24 hours.

5. This test can be done on the same plate as the antibiotic sensitivity test.

6. RESULTS: Sensitive: Zone of inhibition around disc
   Resistant: Growth adjacent to disc

7. QUALITY CONTROL: Positive: *Vibrio anguillarum*
   Negative: *Aeromonas hydrophila*

O. **Commercial Identification Systems** – Several commercial test strips or kits are available for biochemical testing of bacteria. Bear in mind that these kits are designed for human and/or animal testing and the manufacturer's recommended incubation temperature is 37°C. The decreased incubation temperature (22°C- room temperature) required for most fish pathogens results in slightly different reactions and longer incubation periods. Therefore, test results may not follow the manufacturer's identification profiles exactly. A good approach to this problem is to develop a library of known fish pathogen profiles based on serological testing. Referring to this information will assist with interpretation when these commercial test strips are used at room temperature (see Appendix E for some common API profiles based on known bacterial isolates).

1. API 20E™ – The API 20E™ system is a standardized, miniaturized version of conventional procedures for the identification of *Enterobacteriaceae* and other Gram-negative bacteria. It is a ready-to-use, microtube system designed for the performance of 23 standard biochemical tests from isolated colonies on plating medium. The test strip consists of microtubes containing dehydrated substances which are reconstituted by adding a bacterial suspension, incubated so that the organisms react with the contents of the tubes, and read when the various indicator systems are affected by the metabolites or added reagents, generally after 18-24 hours of incubation. Refer to the instructions enclosed with each kit for more detailed information. The API system™ is available from bioMérieux (1-800-638-4835, catalog #20-109/20-179).

2. Bionor™ – There are three Bionor Aqua™ rapid agglutination tests that are useful in fish pathology labs; Mono-As™ for *Aeromonas salmonicida* (product # DD 020), Mono-Va™ for *Vibrio anguillarum* (product # DE 020), and Mono-Yr™ for *Yersinia ruckeri* Type I (product # DC 020). These kits contain a test reagent and a control reagent. The test reagents consist of mono-dispersed particles coated with antibodies that form a granular particle agglutination pattern when mixed with the homologous bacteria. When the bacterial isolate is mixed with the control reagent, no agglutination will appear. See instructions enclosed with each test for complete directions. These tests appear to be very specific. For example, the Mono-As will not agglutinate with *Aeromonas hydrophila* and the Mono-Yr™ is type I specific. The test kits can be purchased from Bionor, Strømdaljordet 4, P.O. Box 1868 Gulset, N-3701 Skien, Norway.

3. Biolog – MicroLog™ is a microbial identification system able to identify and characterize a wide variety of organisms based on carbon source utilization. The

system has identification databases that contain over 1400 different species/genera of aerobic and anaerobic bacteria and yeasts. The identification databases include a wide variety of organism including animal, plant, and water pathogens. The system also allows the user the capability to build customized organism databases. All organisms can be identified/characterized using one of four standardized MicroPlates™; GN2-MicroPlate™ for gram-negative bacteria; GP2- MicroPlate™ for gram-positive bacteria; AN-MicroPlate™ for anaerobic bacteria; and YT-MicroPlate™ for yeasts. Each of these MicroPlates™ contains a pre-selected group of carbon sources as well as an indicator dye. When the appropriate MicroPlate™ is inoculated and incubated, a characteristic pattern (fingerprint) of the organism develops based on which carbon sources the organism can utilize. The resulting pattern can be read either visually or with Biolog's MicroStation Reader™. The results are then compared by the system software to the extensive organism database for final identification. Biolog sells both manual and automated versions of its popular identification/characterization system. Products are available directly from Biolog, 3938 Trust Way, Hayward, CA. 94545 (1-510-785-2564 or website www.biolog.com).

VIII. Characteristics of Target Bacterial Pathogens

A. *Aeromonas salmonicida*: Gram-negative small rod, non-motile, brown diffusible pigment* on TSA or BHIA, cytochrome oxidase positive**, ferments OF basal glucose. Additional biochemical testing on API and corroboration by serological methods (agglutination or FAT) is recommended.

B. *Edwardsiella ictaluri*: Gram-negative small rod, motile, cytochrome oxidase negative, ferments OF basal glucose, produces alkaline slant and acid butt with gas (K/Ag) on TSI. A differential medium, *Edwarsiella ictiluri* medium (Appendix A), is also available to aid in identification. Further biochemical testing on API, and serological testing for corroboration is recommended.

C. *Yersinia ruckeri*: Gram-negative small rod, motile, cytochrome oxidase negative, alkaline/acid (K/A) on TSI, ferments OF basal glucose. Biochemical tests with API and corroboration by serological testing are recommended.

D. *Renibacterium salmoninarum*: Gram-positive rod, extremely slow growing (4-6 weeks) on KDM-2 or other types of specialized growth agar. Identify with FAT or ELISA. Corroboration with PCR.

* Some strains of *A.salmonicida* do not produce brown diffusible pigment, or pigment production is delayed.

** An Oxidase-negative *A.salmonicida* has been reported (Chapman *et al.,* 1991)

IX. Characteristics of Bacterial Pathogens of Regional Importance

A. *Citrobacter freundii:* Gram-negative, rod shaped (1.0µm x 2.0-6.0 µm), motile, cytochrome oxidase negative, ferments OF basil glucose, alkaline slant and acid butt with gas (K/Ag) or the entire tube can become acidic with gas production on TSI., and also produces H_2S on TSI, and lysine and ornathine decarboxylase negative. Further biochemical testing on API, and serological testing for corroboration is recommended.

B. *Edwarsiella tarda*: Gram-negative, small rods (1.0 µm x 2.0-3.0 µm),motile cytochrome oxidase negative ferments OF basil glucose, alkaline slant and acid butt with gas (K/Ag) or the entire tube can become acidic with gas production on TSI., and also produces H_2S on TSI, and lysine and ornathine decarboxylase positive. Further biochemical testing on API, and serological testing for corroboration is recommended.

C. *Flavobacterium columnare:* Gram-negative, gliding motility, long rods, yellow pigmented bacteria. For additional characteristics see Appendix F

D. *Flavobacterium psychrophilum:* Gram-negative, gliding motility, long rods, yellow, yellow pigmented. For additional characteristics see Appendix F

X. Corroboration Methods

In most cases corroboration is performed by either direct or indirect FAT. However antisera is not always available for all bacterial pathogens. If antisera is not available, there has been recent developments of PCR methods to aid in bacterial identification. See Bibliography associated with those pathogens for additional corroboration methods. In particular the methods for corroboration of PRIs are not covered in this chapter. Refer to the AFS-FHS Blue Book (2004) for additional information on PRIs. In the case that antisera or PCR is not available for corroboration, determination of bacterial identification can be made on biochemical and morphology characteristics alone.

A. Slide Agglutination Test - Slide, or serum agglutination, test confirms bacterial identification by agglutination of a pure bacterial culture with its specific antiserum (e.g., *Aeromonas salmonicida*, *Yersinia ruckeri, Edwardsiella ictaluri* as well as PRIs such as *Flavobacterium psychrophilum*, and *Flavobacterium columnare*).

1. Procedure**:**

a) Put 0.5-1.0 mL PBS into a 12x75mm tube. (**A. salmonicida* may work better with deionized water.)
b) Sterilize a loop and collect a sample of a 24-48 hour pure bacterial culture.
c) Suspend the bacteria in the PBS by thoroughly vortexing.
d) Heat *F. columnare* and *F. psychrophilum* cultures 5 minutes at 50-55°C to prevent auto agglutination.
e) Using a ring plate, put one drop of bacterial suspension onto each of three wells.

f) Negative Control - on the first well, place a drop of PBS (*or water).
g) On the second well, place a drop of antiserum for the appropriate bacterium, and on the third well, place a drop of normal rabbit serum (NRS).
h) Mix the drops together by gently rocking the plate back and forth. You can also use a rotating plate set on slow speed.
i) Allow 5-10 minutes for agglutination to occur. It may be helpful to observe the plate on a light stand or with an illuminated magnifier to see agglutination of bacteria and antibody.
j) Interpretation: Agglutination in the well with bacteria and antiserum is a positive test; it is often referred to as a somewhat flaky 'dissolved aspirin' appearance. Negative controls should appear turbid, but without agglutination.

B. Fluorescent Antibody Technique (FAT) for bacteria. The Fluorescent Antibody Test is one serological method for corroboration testing of Gram-negative bacterial isolates such as *Aeromonas salmonicida, Citrobacter freundii, Edwarsiella tarda,* and *Yersinia ruckeri*. It is also often used for the Gram-positive bacterium *Renibacterium salmoninarum* (Rs), the causative agent of bacterial kidney disease (BKD) in very small fish where the quantity of kidney tissue is inadequate for ELISA.
In general, there are two types of staining procedures that utilize fluorescent antibodies; the indirect (IFAT) and direct (DFAT) techniques. The principle and techniques are similar, however the indirect utilizes a second antibody, which is often biotinylated for increased sensitivity. The direct FAT is used more commonly for bacterial corroboration testing and the indirect method, IFAT, is most often used for corroboration testing of viral isolates by staining cell cultures infected with virus. This section describes the DFAT only; see Chapter 12- Corroboration Testing of Viral Isolates, for the IFAT protocol.

There are three basic steps for DFAT: preparing and fixing bacterial cultures or kidney tissue on glass slides; staining the slides with antibody reagents; reading and interpreting the slides.

1. Preparing the slides (2 methods described here for pure bacteria or tissues):

a) Pure Bacterial Cultures (corroboration testing of pure isolates of *Aeromonas salmonicida, Citrobacter freundii, Edwarsiella tarda,* or *Yersinia ruckeri*). Pure isolates of bacteria are diluted in sterile PBS and applied to a FAT slide. Air-dry and fix in absolute methanol for 5-10 minutes. Proceed to step #2.

b) Kidney Smears from small fish - DFAT for *Renibacterium salmoninarum.* Kidney samples for FAT most often are collected in the field during necropsy. Kidney tissue is collected from each fish using sterile tools between lots to avoid cross-contamination. The kidney tissue is squashed onto the slide in a manner to provide a thin smear (thick smears tend to adhere poorly to the slide and are difficult to view microscopically).

c) Ovarian Fluid pellet smears – DFAT for *Renibacterium salmoninarum*, In the case of sampling wild fish that are too valuable to sacrifice for lethal sampling or will only be used as brood fish, then ovarian fluid is collected.

i. Ovarian fluid sample is collected in individually or pooled (up to five fish) using approximately 1 mL per fish.

ii. Transfer two 1.5 mL aliquots from each original pool of ovarian fluid (or 0.5 mL per fish) to sterile, labeled 1.5 mL microcentrifuge tubes (see **Note)**. Freeze the remainder of the sample at -20°C for PCR corroboration.

iii. Centrifuge the 1.5 mL aliquots at 10,000 X g for 15 minutes (see **Note**).

iv. The pellet is carefully removed with a small amount of supernatant using sterile pipette and a thin smear is prepared on a glass slide. Pellets originating from the same ovarian fluid sample tube may be combined on one slide.

Note: Elliot and Mckibben (1997) document the importance of using a minimum of 10,00 X g for sufficient sedimentation of *R. salmoninarum* from 0.5 mL of ovarian fluid collected from individual fish. Enough ovarian fluid must be transferred from each pool to represent 0.5 mL for each fish represented in the pool (i.e. 2.5 mL from a 5-fish-pool). Most 15 mL polystyrene centrifuge tubes used for collection and processing of ovarian fluid samples cannot be centrifuged at a relative centrifugal for (rcf) as high a 10,000g. It is, therefore, necessary to use polypropylene tubes or aliquot the appropriate amount of liquid into microcentrifuge tube suitable for the required rcf.

d) After the tissue has completely dried (air dried or heat fixed), slides are fixed in acetone for approximately 5-10 minutes.

e) At the lab (or receiving facility), the slides are checked for completeness of labeling (case number and fish identification) and then stained immediately or refrigerated for staining on a subsequent day.

2. Staining - Once slides are prepared, the staining procedure is the same regardless of the sample type (bacterial isolate or kidney tissue), however a counter stain is usually not required for bacterial cultures.

a) Positive and Negative Controls: Slides containing both a non-cross-reacting bacterial species and a known positive control can be prepared in quantity and stored refrigerated for use as controls for DFAT staining.

Positive controls are always used in corroboration testing to correctly identify morphology and fluorescence of the bacteria in question. The negative control is important in determining overall staining technique, background debris, and non-specific fluorescence.

b) Place slides in dark, humidified chamber, and place one drop of specific FITC conjugate on each well of the sample slide and control slides.
c) Incubate for 30-60 minutes at room temperature, according to manufacturer's recommendation.
d) Using a squirt bottle or transfer pipette, GENTLY rinse the slides with PBS (or FTA Buffer) by flooding the solution over all wells.
e) If Rhodamine counter stain has not been incorporated in the FAT stain, place the slides in a staining rack and GENTLY rinse in the following order:
    i. PBS for 30 seconds
    ii. Evan's blue counter stain for 3-4 minutes for *Renibacterium salmoninarum*. (A counter stain is not necessary for corroboration testing of pure bacterial cultures, Rhodamine can also be used if desired.)
    iii. Final rinse/soak in PBS or FTA buffer for 5-10 minutes.
f) Air-dry completely.
g) Add a very small drop of FA Mounting Fluid, pH 9 (Difco #3340-57) to each well or target area. Place a 24x50 mm cover glass over the slide using care not to trap air bubbles.
h) Spread the mounting fluid by gently pressing the cover glass with the blunt end of a pen or lab marker evenly over the target area. The fluid should just cover the target area (If the mounting fluid spreads out from under the cover glass, too much mounting fluid was applied).
i) Add one drop of immersion oil to the cover glass over every other well and examine at 1000X using the epiflourescent filter.

3. Reading and Rating: Slides are read at 1000x on a compound fluorescence microscope (refer to the microscope manufacturer for correct wavelength and filters required for FITC epifluoresence microscopy). The positive and negative control slides are read first. This has two purposes: (1) quality assurance for the staining process (positive control should have myriad numbers of fluorescing bacteria, the negative control should have no fluorescence); and (2) to familiarize the reader with the correct bacterial size, shape, and magnitude of the fluorescent halo of bacteria in the positive control. The reader can refer back to the positive control as a reference, if needed, to confirm suspect bacteria in the sample wells.

    a) Bacterial Corroboration Testing: Positive bacterial isolates will fluoresce strongly and have the same morphology and size as the positive control.

b) Kidney and Ovarian pellet smears tested for *Renibacterium salmoninarum* - bacterium will have a distinctly **apple-green fluorescent cell wall**; be the appropriate **size - 1 micron long by 0.5 micron**; and be the **proper shape (bean shaped or pear shaped with one end appearing slightly pinched)**. Compare any suspect bacteria to the control slide to be sure all three of the above criteria are met for *Renibacterium salmoninarum*.

4. Hints for Good Results:

a) Use FITC conjugates at optimum working dilution. Follow manufacturer's recommendation to test for optimum working concentration (see page 23).
b) Filter all conjugated antibody reagents (.45um filter) prior to use to reduce background debris that fluoresce nonspecifically and cause difficulty in reading and interpreting the slides.
c) Prepare thin smears; thick smears will not stain or fix properly and are more easily washed off during the staining process, and thick slides require frequent focusing to observe multiple focal planes.
d) Evenly distribute the kidney material in PBS, or use a very light inoculum of pure bacterial culture (excess bacteria will stain poorly).
e) Heat-fix slides prior to fixing in acetone. If there is not an adequate way available to heat-fix the slides they can be air-dried and sent to the lab without fixation.
f) Control slides should be rinsed in separate Coplin jars to avoid any potential for cross contamination during the staining process.
g) Use acetone to fix slides; the acetone reduces the lipid content of the preparation (de-fatting) increasing the overall fluorescence quality and intensity.

5. FAT Material Suppliers

a) Kirkegaard and Perry Laboratories, Inc
2 Cessna Court
Gaithersburg, MD 20879-4145 USA
Phone: 800/638-3167, 301/948-7755
Web Site: http://www.kpl.com

Antibodies available: Polyclonal antibodies available in FITC-conjugated and other preparations for *Renibacterium salmoninarum* only. KPL also provides positive control material for FAT.

b) Microtek International, LTD (Bayotek)
6761 Kirkpatrick Crescent
Saanichton, B.C.,CA

Phone: 250-652-4482
Web Site: http://microtek-intl.com

6. Reagents

Phosphate buffered saline, pH 7.2 (PBS)

NaCl (sodium chloride, MW 58.44)	7.20 g
Na_2HPO_4 (sodium phosphate, anhydrous dibasic, MW 141.96)	1.48 g
KH_2PO_4 (potassium phosphate, anhydrous, monobasic, MW 136.1)	0.43 g

Bring components to 1 L with distilled water. Adjust pH to 7.2 with 1 M NaOH or HCl.

7. Determination of Antiserum and Conjugate Working Dilutions for FAT.

In most cases commercially prepared antisera and conjugates are lyophilized in a concentrated state. Each should be reconstituted according to the manufacturer's instructions. Aliquots of 0.5 mL can be frozen for later dilution into a working solution of the reagent. Reagents are more stable if frozen as a stock solution. The proper working dilution is the highest dilution that still maintains maximum brightness of the fluorochrome. Generally the manufacturer will recommend between 1:20 to 1:50 using a suitable buffer (PBS) as the diluent. However, in all cases each laboratory must establish the proper working dilution by starting with the manufacturer's recommendation and bracketing, or testing dilutions on either side of the recommended concentration. The following example shows preparation of a direct FAT conjugate where the manufacturer recommends a working dilution of 1:40.

a) Using the stock solution dilute the antiserum at 1:20, 1:30, 1:40, 1:50, 1:60 using PBS or another buffer as recommended.
b) The FAT is performed on replicates of a known positive control, each replicate using a different dilution of the conjugated antiserum. In this way the working dilution can be determined as the endpoint of optimum fluorescence (the highest dilution that still provides a bright specific fluorescence with little or no background staining).

XI. Antibiotic Sensitivity Testing

The antibiotic sensitivity test determines the sensitivity or resistance of a bacterial isolate to specific antibiotics. Filter paper discs, each saturated with a different antibiotic, are evenly spaced on an agar plate surface inoculated with a lawn of the bacterial isolate to be tested. The antibiotics diffuse into the surrounding medium, and create a decreasing gradient of the antibiotic concentration. If sensitive, a zone of bacterial growth inhibition (clear zone) will be present around the antibiotic disc. The following antibiotics can be routinely tested: oxytetracycline, sulfadimethoxine with ormetaprim (Romet 30), erythromycin, penicillin, polymyxin, O/129, and novobiocin.

1. Suspend cells from a pure bacterial culture in log phase (24-48 hour culture) in sterile saline to obtain a turbidity equivalent to a 0.5 McFarland standard.

2. Streak a Mueller-Hinton agar plate with a sterile cotton swab soaked with the bacterial suspension. Swab the plate in three separate planes.

3. Aseptically place antibiotic discs to be tested onto a freshly inoculated plate. Press onto agar surface lightly.

4. Invert the plates and incubate at 25°C for 24-48 hours. Observe and record results by measuring the diameters of the zone of inhibition around each disc.

5. Results: The following table lists zone of inhibition for each compound

   Sensitive: A specific diameter zone of inhibition around the disc.
   Resistant: Bacterial growth within the zone of inhibition or adjacent to the disc.

Zone Diameter Standards

Antimicrobial	Disc Content	Resistant	Intermediate	Sensitive
Erythromycin [1]	15ug	No zone	< 15 mm	≥ 15 mm
Novobiocin [2]	30 ug	No zone	< 10 mm	≥ 10mm
Oxolinic Acid [1]	2 ug	No zone	< 15 mm	≥ 15 mm
Oxytetracycline [1]	30 ug	No zone	< 15 mm	≥ 15 mm
Penicillin G [3]	10 U	≤ 11 mm	12-21 mm	≥ 22 mm
Romet 30 [1]	25 ug	No zone	< 15 mm	≥ 15 mm
O/129 [2]	0.1% (W/V)	No zone	< 7 mm	≥ 7 mm

[1] Model Comprehensive Fish Health Protection Program. Pacific Northwest Fish Health Protection Committee, September 1989.

[2] CFDM pathology case materials

[3] "Performance Standards for Antimicrobial Disc Susceptibility Tests," NCCLS 1981.

6. QUALITY CONTROL

Control cultures should be included with each sensitivity test. Cultures are available from American Type Culture Collection (ATCC®) or National Collection of Industrial, Food and Marine Bacteria (NCIMB) of the United Kingdom (www/ncimb.co.uk.)

An excellent reference for antibiotic sensitivity testing is the Provisional Antimicrobial Susceptibility Testing Protocols – 27 December 1998, produced by the Workshop on MIC Methodologies in Aquaculture. Jerry Tjernagel (www/gtbugs@mnic.net) of MicroBiologics is attempting to make all the NCIMB strains available in the United States for use by fish health professionals. Also contact Tom Bell, Bozeman INAD office for a copy of the Provisional Antimicrobial Susceptibility Testing Protocols or further information.

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Appendix 5.A - Media Formulations

A. COMMON GROWTH MEDIA

Brain Heart Infusion Agar (BHIA) - A basic agar for most bacterial cultures, better for warm water fish.

- Suspend 52 g of dehydrated medium (Difco #0418) in 1 L distilled water and heat to boiling. Boil for one minute to completely dissolve agar. Sterilize and dispense as required.

Tryptic Soy Agar (TSA) – A basic agar for most bacterial cultures.

- Suspend 40 g of dehydrated medium (Difco #0369) in 1L distilled water and heat to boiling. Boil for one minute to completely dissolve agar. Sterilize and dispense as required. Store at 2-8°C. Final pH = 7.3±0.2 at 25°C.

Tryptone Yeast Extract Salt Agar (TYES) – A nutrient poor agar to help select for *Flavobacterium.*

Tryptone	5.0g
Yeast Extract	0.4g
$MgSO_4 \cdot 7H_2O$	0.5g
$CaCl_2 \cdot 2H_2O$	0.2g
Agar	10.0g
d-H_2O	1 L

Mix ingredients and heat to dissolve completely. Sterilize and dispense as required. Store at 2.8°C. Final pH = 7.1±0.2.

Cytophaga Agar – Another medium for isolating Flavobacterium. Differences in cell and colony morphology aid in distinguishing the numerous bacteria that grow on this partially selective agar. Streak for isolation on two plates, incubate one plate at 20°C and the other at 25°C for 3-5 days.

RESULTS:

i. *Flavobacterium psychrophilum* – No growth at 25°C. Growth of bright yellow colonies at 20°C with convex center, and spreading periphery.
ii. *Flexibacter* (marine) – Orange or yellow colonies with uneven edges.
iii. *Flavobacterium columnare* – Light or no growth at 20°C. Greater growth of yellow convoluted centered colonies with rhizoid edges at 25°C.
iv. *Flavobacterium sp*. – Growth range 10-25°C, with best growth at 18°C. Light yellow round colonies, transparent and smooth.

FORMULA:

Tryptone	0.5g
Yeast Extract	0.5g
Sodium Acetate	0.2g
Beef Extract	0.2g
Agar	10.0g
d-H_2O	1 L

Suspend the above ingredients in distilled water and heat to dissolve. Adjust pH to 7.2-7.4. Sterilize and dispense as required. Store at 2-8°C.

Nutrient Broth - To use when a liquid growth medium is preferred.

- Dissolve 8 g of the dehydrated medium (Difco #0003) in 1 L of distilled water. Dispense 5-7 mL/tube and autoclave for 15 minutes at 15 pounds pressure. Final pH = 6.8 at 25°C. Store at 2-8°C.

B. COMMONLY USED MEDIA TO IDENTIFY GROWTH AND BIOCHEMICAL CHARACTERISTICS

Bile Esculin Agar

- A commercially prepared dehydrated media (Difco #0879) used for the esculin test. The esculin agar is prepared according to manufacturer's recommendations.

Carbohydrate Utilization media

- A basal media for carbohydrate utilization tests, available in a commercially prepared dehydrated powder (DF #0688). The OF basal is prepared according to manufacturer's recommendations prior to the addition of individual carbohydrates as described below:

- To prepare final medium aseptically add 10 mL of a filter-sterilized (0.45 μm) 10% carbohydrate solution to autoclaved and cooled (50°C) media resulting in a 1% final concentration, with the exception of salicin, which should be made as a 5% solution resulting in a 0.5% final concentration (see below). Only one carbohydrate is added to the basal medium for each test to be run.

  10% Arabinose (1 g Arabinose to 10 mL in d-H_2O)
  10% Rhamnose (1 g Rhamnose to 10 mL in d-H_2O)
  10% Sucrose (1 g Sucrose to 10 mL in d-H_2O)
  10% Sorbitol (1 g Sorbitol to 10 mL in d-H_2O)*
  5% Salicin (0.5 g Salicin to 10 mL in d-H_2O)

- Mix flask thoroughly and aseptically dispense into sterile tubes. Store at 2 to 8°C. Final pH = 6.8 $\pm$ 0.2 at 25°C.

  *A sorbitol utilization slant media can also be prepared and utilized as described in Cipriano and Pyle (1985).

Cytochrome Oxidase Spot Test

- Individual test strips can be purchased from Remel (1-800-255-6730, catalog #38-191), or can be prepared in the laboratory with the following procedure.
- Cut Whatman #1 filter paper into strips and autoclave
- Spread filter paper on a glass petri dish.
- Put a few drops of oxidase reagent on each strip to saturate it.

- Oxidase reagent: Tetramethyl-p-phenylenediamine dihydrochloride (Eastman Organic Chemicals) Prepare 1% aqueous solution.
- Dry completely, then store in brown bottle. Freeze surplus until needed.

Decarboxylase Medium Base

- A commercially prepared dehydrated basal media (Difco #0872) for use in Lysine and Ornithine test. The decarboxylase medium base is prepared according to manufacturer's recommendations. The basal media, without addition of Lysine or Ornithine, serves as the control.

- To make L-Lysine media add 5 g L-Lysine to 1 liter of prepare basal decarboxylase media. Dispense into tubes. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool. Final pH = 6.8 $\pm$ 0.2 at 25°C. Store at 2 to 8°C.
- To make L-Ornithine media add 5 g L-Ornithine to 1 liter of prepare basal decarboxylase media. Dispense into tubes. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool. Final pH = 6.8 $\pm$ 0.2 at 25°C. Store at 2 to 8°C.

Glucose Fermentation medium

- Suspend 9.4 g of dehydrated medium (Difco #0688) in 1 L of distilled water and heat to boiling. Distribute in 100-mL amounts, autoclave for 15 minutes at 15 pounds pressure (121°C). To prepare final medium aseptically add 10 mL of a filter-sterilized (0.45µm) 10% glucose solution. Mix flask thoroughly and aseptically dispense 5.0 mL into sterile tubes. Store at 2-8°C. Final pH = 6.8 $\pm$ 0.2 at 25°C.

MacConkey agar (for use with API)

- Suspend 50 g of the dehydrated medium (Difco #0075) in 1 L distilled water and heat to boiling. Dispense into bottles and autoclave at 15 pounds pressure for 15 minutes. Pour into petri plates. Final pH = 7.1 ± 0.2. Store at 2-8°C.

Malonate Broth

- A commercially prepared dehydrated media (Difco #0395) used for the malonate test. The malonate broth is prepared according to manufacturer's recommendations.

Motility test medium

- Suspend 20 g of dehydrated medium (Difco #0105) in 1 L distilled water and heat to boiling. Dispense 5 mL/tube and autoclave at 15 pounds pressure for 15 minutes. Cool medium in an upright position in a cold water bath. Final pH = 7.2 $\pm$ 0.2 at 25°C. Store at 2-8°C.

Mueller-Hinton agar

- Suspend 38 g of dehydrated medium (Difco #0252) into 1 L distilled water and heat to boiling to dissolve completely. Autoclave for 15 minutes at 15 pounds

pressure (121°C). Aseptically dispense into sterile petri dishes. Store at 2-8°C. Final pH = 7.3 ± 0.1 at 25°C.

Nitrate Broth

- A dehydrated medium, available commercially (Difco #0268, (Remel® 061532) for use in testing nitrate reduction. The nitrate broth is prepared according to manufacturer's recommendations.

Nutrient Gelatin

- A dehydrated medium, available commercially (Difco #0011) for use in testing presence of Gelatinase. The Nutrient Gelatin is prepared according to manufacturer's recommendations.

Simmons Citrate agar

- A dehydrated medium, available commercially (Difco #0091, Remel® #060496) for use in testing. The Simmons citrate agar is prepared according to manufacturer's recommendations.

Triple sugar iron agar (TSI)

- Suspend 65 g of the dehydrated medium (Difco #0265) in 1 L of distilled water and heat to boiling. Dispense 7 mL/tube. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool in slanted position. Store at 2-8°C. Final pH = 7.4 at 25°C.

C. DIFFERENTIAL AND SELECTIVE MEDIA USED TO IDENTIFY BACTERIA PATHOGENIC TO FISH.

***Edwarsiella ictiluri* Medium** *Shotts and Waltman (1990)

- A differential medium used to help in identification of *Edwarsiella ictiluri, Edwardsiella tarda, Aeromonas hydrophila* and other species of bacteria.

- To make mix the following ingredient in a two-liter flask:

  1 L deionized water
  17 g agar (omit for enrichment medium)
  10 g Bacto-tryptone
  10 g yeast extract
  3.5 g mannitol
  1.25 g phenylalanine
  1.2 g ferric ammonium citrate
  5 g sodium chloride
  1 g bile salts
  0.03 g bromothymol blue

- Adjust pH to 7.0 – 7.2 with 1.5 – 2.0 mL 3.0N NaOH. Autoclave media without

removing stirring bar. Cool to 48 - 45˚C while mixing the following in a small beaker:

0.010 g colistin
0.5 mg (2 mL of 250 µg/mL) fungizone

- Swirl to dissolve colistin, pour into 5-mL syringe, and filter sterilize solution through a 0.45 µm syringe filter directly into autoclaved medium. Add 4 mL deionizer water to syringe, filter sterilize into medium, recover flask, and place on stirring platform for a few minutes. Ideally, agar will be 46 - 48˚C and then can be aliquoted into petri dishes. Once inoculated, plates should be incubated at 30˚C for 36 h.

- Results:

Edwarsiella ictiluri colonies appear light green, 0.5 – 1.5 mm.
Edwarsiella tarda colonies appear light green with dark green (H_2S) centers, 1.5 – 2.5 mm.
Proteus sp. colonies appear brown-green and may swarm.
Serratia marcescens colonies appear red, 1.0 – 2.0 mm.
Aeromonas hydrophila colonies resistant to inhibitors appear yellow-green, 0.5 – 5.0 mm.

Appendix 5.B - Reagents

A. Gram stain reagents

These stains can be ordered as a complete kit from VWR (#l5204-004) or can be reconstituted as follows:

Crystal violet

crystal violet (90% dye content)	20.0 g
ethanol (95%)	200 mL
ammonium oxalate	8.0 g
dH_20	800 mL

Mix first two ingredients and let sit overnight or until dye goes into solution. Add remaining ingredients and filter before use.

Gram's iodine

iodine crystals	1.0 g
potassium iodide	2.0 g
dH_20	300 mL

Decolorizer

acetone	40 mL
ethanol (95%)	60 mL

Safranin

safranin O	2.5 g
95% ethanol	100 mL
dH_20	900 mL

Filter safranin solution before use.

B. Kovac's Indole Reagent

Isoamyl alcohol	30 mL
p-Dimethyl aminobenzaldehyde	2 g
Hydrochloric acid (HCl)	10mL

Dissolve the aldehyde in the alcohol. Slowly add the acid to the mixture. Store solution at 2 to 8°C in amber dropper bottle.

C. *a*-naphthylamine Reagent (Remel® "Nitrate Reagent A TI No. 21242)

N-N-Dimethyl-1-naphthylamine	6 mL
Glacial Acetic Acid	286 mL
Deionized Water	714.0 mL

D. Oxidase reagent

Tetramethyl-P-Phenylenediamine Dihydrochloride	1.0g
$d\text{-}H_2O$	100 mL

E. O/129 Discs

0.1% solution of pteridine dissolved in acetone

OR

0.1% solution of pteridine phosphate dissolved in sterile distilled water.

Autoclave at 15 pounds per square inch (psi) at 121°C for 15 minutes. Place sterile blank discs (Difco, Bacto Concentration Discs ¼") in pteridine or pteridine phosphate solution, remove and dry at 37°C. Store at 2-8°C in a dark screw-cap bottle. Use on agar plates (usually Mueller-Hinton) to test the sensitivity of cultures to this vibrio-static agent.

F. Phosphate Buffered Saline Solution

A dehydrated saline reagent, available commercially (Difco # DF2314). The Phosphate Buffered Saline Solution is prepared according to manufacturer's recommendations.

Standard 1X concentration formula (0.15 M NaCl, 0.01 M phosphate; make 1 L)

NaCl	8.50 g
Na_2HPO4 (anhydrous)	1.07 g
$NaH_2PO_4 \bullet H_2O$ (monohydrate)	0.34 g
d-H_2O	to 1 L

Adjust pH to 7.1 with 1 N hydrochloric acid or 0.1 N sodium hydroxide.

G. Saline Solution (0.85%) - sterile

Sodium Chloride	8.5 g
Distilled water	1000 mL

Mix the above thoroughly and then aliquot about 7 mL into glass culture tubes (16×125mm or 20×125mm tubes). Loosely place lids on the tubes and autoclave for 15 minutes at 15 pounds pressure at 121°C. After the tubes have been autoclaved allow the tubes to cool and then tighten the caps.

H. Sulfanilic acid (Remel® "Nitrate Reagent A TI No. 21239)

Sulfanilic acid	8 g
Glacial Acetic acid	286.0 mL
Demineralized Water	714.0 mL

Appendix 5.C –
Profiles Obtained with API-20E for Known Fish Pathogens

A. Yersinia ruckeri

The following table represents API20E profiles for *Yersinia ruckeri* when cultures were tested at 22°C rather than the manufacturer's recommended incubation temperature of 35-37°C. All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT. Based on the profile submitted to API, bacterial identification is given in order of probability, then remarks as to the likelihood of the profile are provided when profiles are poorly matched to the manufacturer's database.

Bacterial Isolate ID	API PROFILE*	API Manual or Computer Identification
1. Eastern Fishery Disease Laboratory (EFDL) Positive Control #1- Type II (11.29) 2. Nisqually Fall chinook (3/88)	**5307500**	1.) *Serratia mercescens* 2.) *Serratia liquefaciens* 3.) *Hafnia alvei*
1. Eastern Fishery Disease Laboratory (EFDL) Positive Control #2- Type II (11.29) 2. Fall Chinook, Suquamish R, WA (3/88) 3. Spring Chinook, Skookum Creek, WA (2/88)	**5107500**	(Same ID as 5307500 above) 1.) *Serratia mercescens* 2.) *Serratia liquefaciens* 3.) *Hafnia alvei*
Unknown source – Isolate confirmed by biochemical and serological testing.	**5144100**	1.) *Escherichia coli* 2.) *Yersinia ruckeri*
Eastern Fishery Disease Laboratory (EFDL) Positive Control - Type I (11.4)	**5107100**	"Unacceptable profile"
Coho, Quinault River, WA	**5106100**	"Questionable ID"
Late Fall Chinook, Battle Creek, CA (11/94)	**5105100**	1.) *Hafnia alvei* "Acceptable ID"
Notes from ERM archived files – previous testing	**5104500**	"Questionable ID"

Bacterial Isolate ID	API PROFILE*	API Manual or Computer Identification
1. Hagerman – Type I (11.4)	**5104100**	1.) *Yersinia ruckeri* "Very good ID"
Coho, Quilcene R., WA (11/88)	**5104000**	1.) *Yersinia ruckeri* "Very good ID"
Unknown source	**5100100**	1.) *Yersinia ruckeri* "Excellent ID"
Unknown source	**4105100**	1.) *Hafnia alvei*
Unknown source	**4104100**	1) *Yersinia ruckeri* 2) *Salmonella gallinarum*
Unknown source	**4104000**	1.) *Yersinia ruckeri* "Acceptable ID"
Unknown source	**0104100**	1.) *Yersinia ruckeri* "Acceptable ID"

*** *Yersinia ruckeri* generally fails to produce a positive citrate reaction when incubated at room temperature (22-25C). Refer to the API Manual for specific biochemical tests and interpretation of API20E™ profiles. Also see references listed on page 5-39.**

B. ***Aeromonas salmonicida***

The following represents API20E profiles for *Aeromonas salmonicida* isolates following manufacturer's instructions but incubating test strips at room temperature (22°C). All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT.

Bacterial Isolate ID (collection date)	**API PROFILE***	**API Manual or Computer Identification**
spp not identified, Makah NFH, WA (8/88)	**0006104**	1.) *Pseudomonas pseudomaleae* "Acceptable ID"
Winter Steelhead, Makah NFH, WA (1/89) Chum, Makah NFH , WA (12/89)	**0006104**	Same as above
Winter Steelhead, Quinault NFH, WA (1/89)	**0006104**	Same as above
Spring Chinook, Entiat NFH, WA (8/89)	**0006104**	Same as above
Spring Chinook, Quilcene NFH, WA (3/91)	**2006104**	1.) *Aeromonas salmonicida*
Profiles given in **API MANUAL** for *Aeromonas salmonicida*	**6006104** **6006504** **4006104** **2006104**	*Aeromonas salmonicida* "Good to Excellent ID"

***Aeromonas salmonicida* generally fails to produce positive relations for ONPG, ADH, and LDC when incubated at room temperature (22-25C).**

Profiles provided in the API Manual are based on positive reactions for some or all of these first 3 biochemical tests, therefore the first digit of the "acceptable" profiles for A.sal include the values 2, 4, or 6. More often, a zero value is obtained after 24-48 hours incubation at room temperature. Longer incubation periods are required for these tests.

C. ***Edwarsiella ictiluri***

The following represents a API20E profile for *Edwarsiella ictiluri* isolates following manufacturer's instructions but incubating test strips at room temperature (25°C). All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT.

Bacterial Isolate ID	API PROFILE*	API Manual or Computer Identification
Catfish	**4004000**	"unreliable identification"

***Edwarsiella ictiluri* generally fails to produce a positive lysine reaction when incubated at room temperature (22-25C). Refer to the API Manual for specific biochemical tests and interpretation of API20E™ profiles.**

D. ***Edwarsiella tarda***

The following represents a API20E profile for *Edwarsiella tarda* isolates following manufacturer's instructions but incubating test strips at room temperature (25°C). All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT.

Bacterial Isolate ID (collection date)	API PROFILE*	API Manual or Computer Identification
Catfish	**4544000**	*Edwarsiella tarda* "Excellent Identification"
Catfish	**4144000**	*Edwarsiella tarda* "very good ID"
Catfish	**4744000**	*Edwarsiella tarda* "very good ID"
Catfish	**4145000**	"unacceptable profile"

E. ***Citrobacter freundii***

The following represents API20E profile for *Citrobacter freundii* isolates listed by the manufacturer.

Bacterial Isolate ID (collection date)	API PROFILE*	API Manual or Computer Identification
Profiles given in **API MANUAL** for *Citrobacter freundii*	**160457257** **160457357** **160477357** **160477257** **360477357** **360457357** **360457257**	*Citrobacter freundii* "Good to Excellent ID"

References and Bibliography:

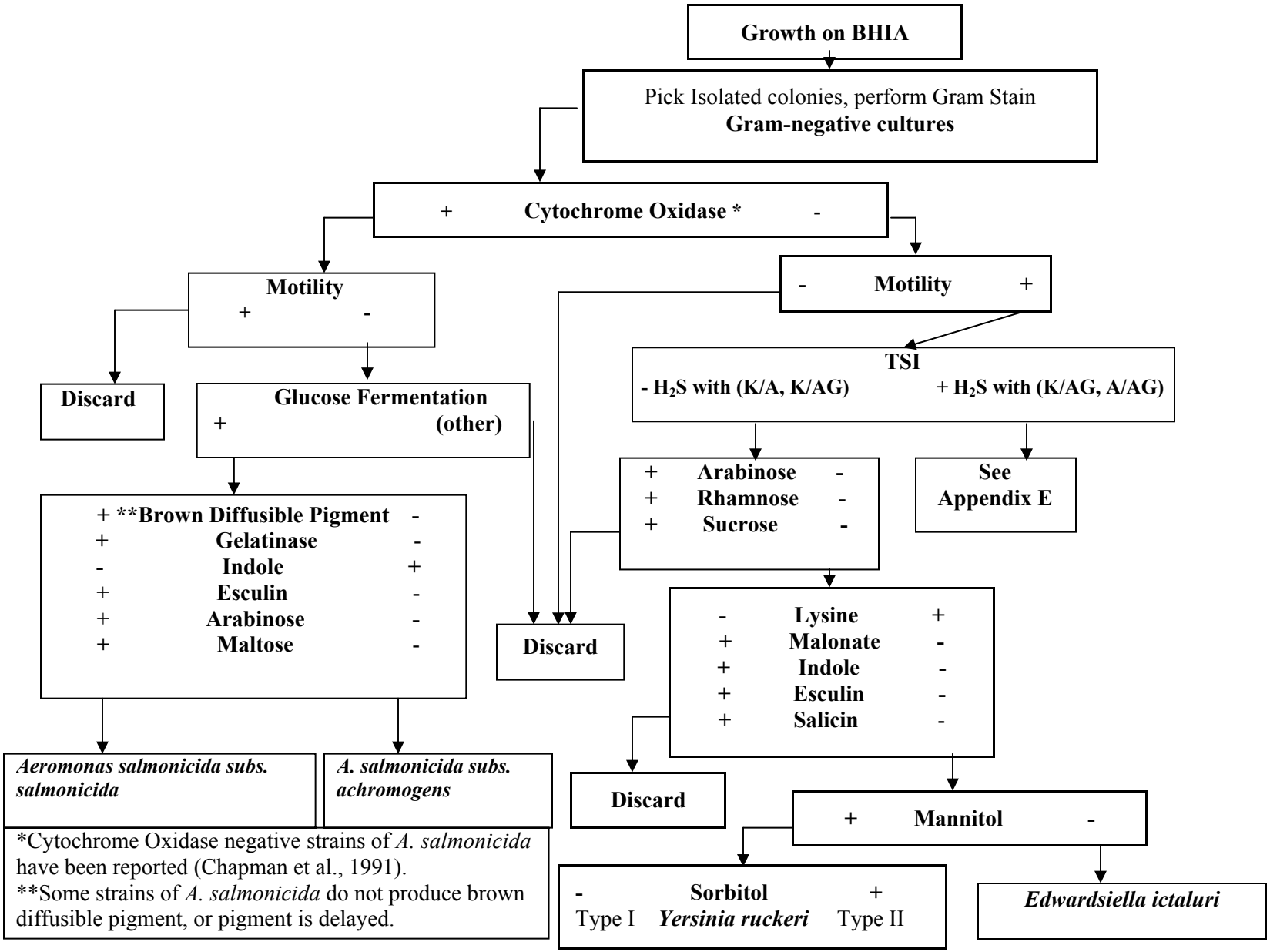
Romalde, J.L., and A.E. Toranzo. 1991. Evaluation of the API-20E system for the routine identification of the enteric redmouth disease. Bull. Eur. Ass. Pathol. 11(4), 147.

Shotts E.B., and W.D. Waltman. II 1990. A medium for selective isolation of *Edwarsiella ictiluri.* Journal of Wildlife Disease 26(2): 214-218.

Kent, M.L. 1982. Characteristics and identification of Pastuerella and Vibrio species pathogenic to fishes using API-20E (Analytab Products) multitube test strips. Can. J. Fish. Aquat. Sci., 39. 1725-1729.

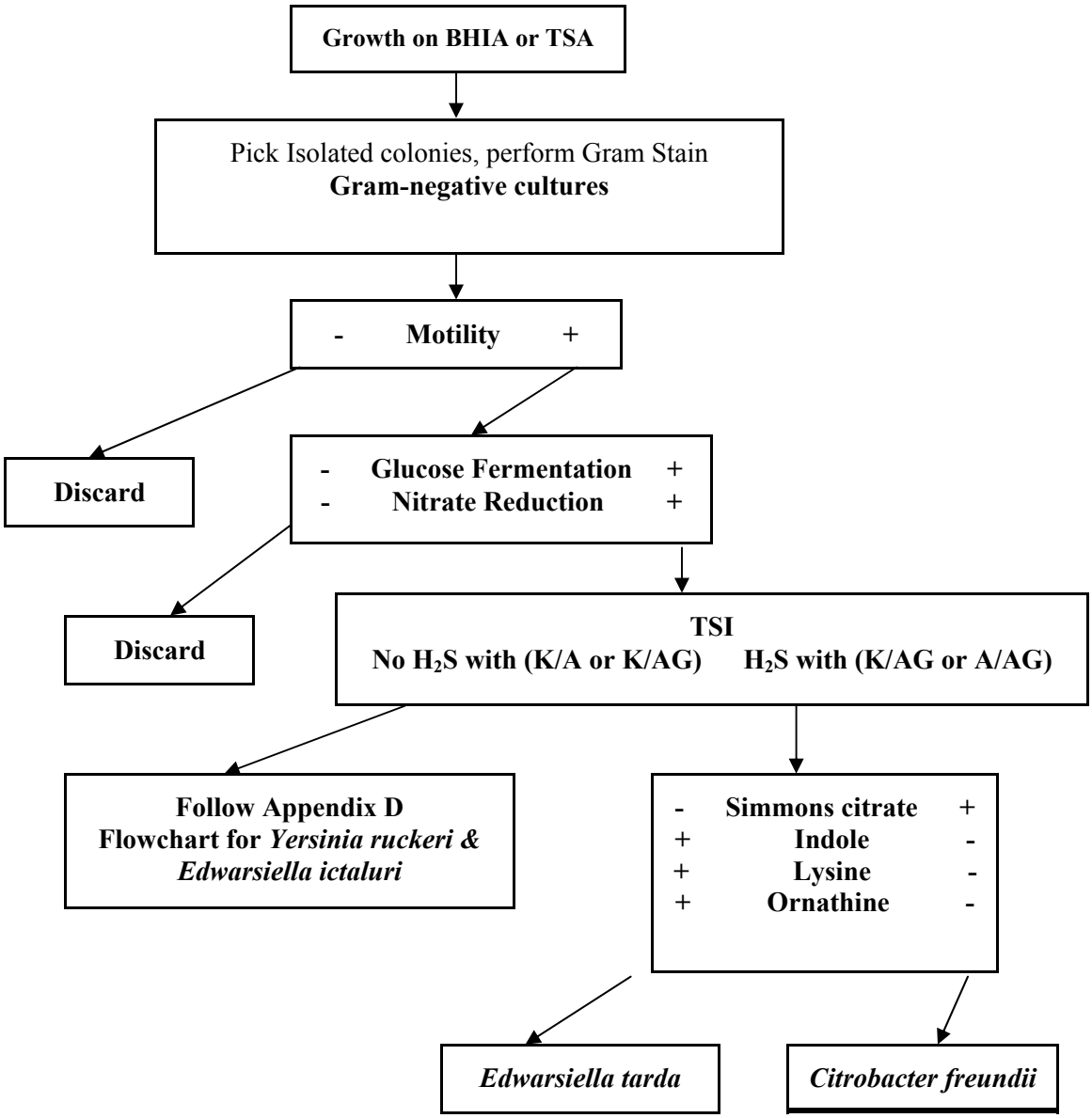
Toranzo, A.E., Y.Santos, T.P. Nieto and J.L. Barja. 1986. Evaluation of different assay systems for identification of environmental Aeromonas strains. Appl. Environ. Micorbiol., 51:652-656.

Appendix 5.D – Flowchart for Targeted Major Gram-Negative Fish Pathogens



*Cytochrome Oxidase negative strains of *A. salmonicida* have been reported (Chapman et al., 1991).
**Some strains of *A. salmonicida* do not produce brown diffusible pigment, or pigment is delayed.

Appendix 5.E – Flowchart for Targeted Pathogens of Regional Importance Gram-Negative Fish Pathogens



Appendix 5.F – Some Characteristics of Long Gram-negative Bacteria

Flavobacterium columnare	*Flavobacterium psychrophilum*
Morphology and Growth Characteristics	
Growth on TYE or Cytophaga agar	Growth on TYE or Cytophaga agar
Little or no growth at 20°C, optimum growth at 25°C.	No growth at 30°C; optimum growth at 15-18°C.
Yellow dry colonies, rhizoid margin, spreading, adhere to agar.	Moist yellow colonies, entire margin, spreading slowly.
Thin gram-negative rods 0.4 x 2-20 um	Thin rods, 0.5 x 2-7um
Motility by gliding, or flexing motion: forms "haystacks" of aggregate cells on wet mount.	Gliding motility

Additional information on the characterization of *Flavobacterium columnare* and *Flavobacterium psychrophilum* can be found in the AFS-FHS Blue Book (2004).

Biochemical Reactions

No acid from simple or complex carbohydrates (glucose, lactose, galactose and sucrose)

Does not reduce nitrates, produces hydrogen sulfide

Weak catalase positive

Proteolytic for gelatin, casein, albumin

Flexirubin pigments present; colonies turn orange-brown on addition of 20% KOH.

Corroboration Test

Serum agglutination with specific antisera. If antisera is not available there are PCR methods available for aiding identification of *Flavobacterium psychrophilum* (Taylor and Winton 2002).

Bibliography:

AFS-FHS Suggested procedures for the detection and identification of certain finfish and shellfish pathogens. Blue Book 6th Edition, 2004, Fish Health Section, American Fisheries Society.

Taylor P.W. and J.R. Winton. 2002. Optimization of Nested Polymerase chain Reaction Assays for Identification of *Aeromonas salmonicida, Yersinia ruckeri*, and *Flavobacterium psychrophilum.* Journal of Aquatic Animal Health 14:216-224.