GENERAL AND SPECIALIZED MEDIA ROUTINELY EMPLOYED FOR PRIMARY ISOLATION OF BACTERIAL PATHOGENS OF FISHES

Author: Starliper, Clifford E.

Source: Journal of Wildlife Diseases, 44(1) : 121-132

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-44.1.121

Journal of Wildlife Diseases, 44(1), 2008, pp. 121–132

GENERAL AND SPECIALIZED MEDIA ROUTINELY EMPLOYED FOR PRIMARY ISOLATION OF BACTERIAL PATHOGENS OF FISHES

Clifford E. Starliper[1,2]

[1] US Geological Survey, Leetown Science Center, National Fish Health Research Laboratory, 11649 Leetown Road, Kearneysville, West Virginia 25430, USA
[2] Corresponding author (email: starliper@usgs.gov)

ABSTRACT: There are a number of significant diseases among cultured and free-ranging freshwater fishes that have a bacterial etiology; these represent a variety of gram-negative and gram-positive genera. Confirmatory diagnosis of these diseases involves primary isolation of the causative bacterium on bacteriologic media. Frequently used "general" bacteriologic media simply provide the essential nutrients for growth. For most of the major pathogens, however, there are differential and/or selective media that facilitate primary recovery. Some specialized media are available as "ready-to-use" from suppliers, while others must be prepared. Differential media employ various types of indicator systems, such as pH indicators, that allow diagnosticians to observe assimilation of selected substrates. An advantage to the use of differential media for primary isolation is that they hasten bacterial characterization by yielding the appropriate positive or negative result for a particular substrate, often leading to a presumptive identification. Selective media also incorporate agent(s) that inhibit the growth of contaminants typically encountered with samples from aquatic environments. Media that incorporate differential and/or selective components are ideally based on characters that are unique to the targeted bacterium, and their use can reduce the time associated with diagnosis and facilitate early intervention in affected fish populations. In this review, the concepts of general and differential/selective bacteriologic media and their use and development for fish pathogens are discussed. The media routinely employed for primary isolation of the significant bacterial pathogens of fishes are presented.

Key words: Bacteria, bacteriologic media, culture, fish, freshwater, primary isolation.

Diseases are a significant threat to the health of free-ranging and hatchery-reared populations of fishes, including a suite of important sport and restoration species. Prevention of pathogen introductions and disease transmissions is the most prudent and cost-effective means for management of fishery resources (Piper et al., 1982). Because fish reside in, or are reared in open-environment waters, and pathogens are transmitted horizontally via the water column, diseases are a recurring concern. Health management is a primary objective for fishery resource agencies, particularly the U.S. Fish and Wildlife Service's (FWS) regional Fish Health Centers and State Fishery Offices. The foundations for a program in fish health management include maintaining optimal husbandry, observing fish for development of disease signs or gross pathology, and providing periodic health/pathogen inspections and diagnostics. In the event of a disease outbreak, quick intervention with control and treatment measures is essential to preclude or minimize mortality and disease spread to other populations. With a bacterial etiologic agent, effective disease control can be dependent upon identification of the pathogen by primary bacterial culture. This approach offers a definitive diagnosis and allows for subsequent antimicrobial sensitivity testing against candidate therapeutic agents, which minimizes the development of antibiotic-resistant strains. It also provides a source of isolates from various hosts or geographic areas that can be archived and used in research.

Health and pathogen inspections at fish hatcheries conducted by FWS Fish Health Centers and State Agencies involve the evaluation of tissues from a defined number of individuals per lot (i.e., species and year-class) of fish (American Fisheries Society–Fish Health Section, 2004). During inspections of facilities that have no historic record of diseases and that have current resident fish that are apparently healthy, two or three general bacteriologic

media that can be used to culture a broad spectrum of potential pathogens, are typically used. On the other hand, specialized media (selective and/or differential) that target specific pathogens are often used at those facilities where a specific bacterium or disease has previously occurred, or where a bacterium is enzootic or deemed exotic. Specialized media have also been developed for fastidious bacterial pathogens and have been used to support surveillance programs, such as the FWS Wild Fish Health Survey (www.fws.gov/wildfishsurvey/), related to the detection of reportable pathogens in free-ranging fish.

General bacteriologic growth media are used to culture all bacteria present in tissues, including pathogenic and non-pathogenic organisms. Many of the fish pathogens do not require additional steps during sample processing for isolation, such as a pre-enrichment. However, mincing or homogenization of tissues facilitates recovery of some pathogens, including *Mycobacterium* spp. and *Renibacterium salmoninarum*. Aseptic techniques are employed when tissues are collected. Media in petri plates are typically streak-plate inoculated, or dilutions of tissues are made and volumes of each dilution are drop-plate inoculated. Two types of general media are used, which can be distinguished by their nutrient richness. Rich-nutrient general media that are frequently used include brain-heart infusion agar (BHIA), tryptic soy agar (TSA), and blood agar base with 5% sheep blood; these media are available as "ready-to-prepare" from suppliers (e.g., BD Difco [Becton, Dickinson and Co.], Sparks, Maryland, USA). Growth of some fish pathogens, such as *Flavobacterium psychrophilum* and *Flavobacterium columnare*, however, can be inhibited by high-nutrient media. For this reason, reduced-nutrient media also are used; the most common is cytophaga medium (Anacker and Ordal, 1959); R2A (BD Difco) is a good medium for isolation of bacteria from aquatic environments. Cytophaga medium is composed of 0.05% tryptone, 0.05% yeast extract, 0.02% beef extract, and 0.02% sodium acetate. Other pathogens have unique requirements for general growth. Media for *R. salmoninarum* (Sanders and Fryer, 1980) contain l-cysteine, marine source bacteria require salt supplementation (typically 1–1.5% NaCl), and Lowenstein or Middlebrook media are necessary for primary culture of *Mycobacterium* spp. A major difference in primary isolation of fish bacterial pathogens compared to isolation of bacteria in human or veterinary medicine is incubation temperature. With few exceptions, fish bacteria are inhibited by incubation temperatures above 30 C. Many fish disease diagnostic laboratories use packaged microbiologic identification systems as a tool for bacterial identifications. Caution should be exercised when using such systems because they are typically used for mammalian isolates, which have optimal incubation temperatures of 37 C. Because aquatic-origin isolates might not be included in manufacturers' databases, and assimilation of various substrates is often temperature dependant, fish diagnostic laboratories that rely on packaged systems need to develop their own databases and expected biocodes for the bacteria that they routinely encounter.

The recipes of differential media include at least one substrate that targets a particular characteristic (i.e., phenotype) that is unique to the bacterium. This character, or substrate, needs to be identified through extensive biochemical testing with a significant number of isolates of that species originating from a variety of geographic locations. Furthermore, the substrate must lend itself to inexpensive incorporation into a medium, and the bacterial action toward it must be observable with the aid of an indicator system that clearly differentiates between positive and negative reactions. It is not often that a bacterial species possesses a unique phenotype, so the goal is to select

a character that a minimal number of bacteria will share. An example of this is Coomassie brilliant blue (CBB) medium (Udey, 1982; Cipriano and Bertolini, 1988), which is an effective differential medium for field and laboratory work with *Aeromonas salmonicida*. Coomassie brilliant blue in the medium stains the A-layer protein on the cell surface of *A. salmonicida*, resulting in blue colonies. Occasionally, blue colonies are encountered on CBB that are not *A. salmonicida*, and this presumptive identification of *A. salmonicida* can be easily confirmed with additional biochemical tests. This approach reduces work as compared to systems using a nondifferential medium, where numerous colonies would have to be characterized. More important, a confirmatory disease diagnosis can be made more quickly. Sensitivity (i.e., reducing the number of presumptive colonies) can be enhanced by incorporating more than one differential substrate and/or by making the medium selective as well as differential; however, such media are more complicated to prepare. Various indicator systems are used in media to identify contrasting substrate assimilation results. One of the more frequently used is an acidic/basic response that utilizes one of numerous pH indicators (Difco Manual, 1998). For example, changes in pH are used to demonstrate assimilation of carbon source, such as carbohydrates, citrate, or malonate, or the decarboxylation/deamination of amino acids (i.e., arginine, lysine, ornithine). Depending on the medium, a pH change may be visualized as a colored bacterial colony against the contrasting color of the medium or a change to the color of the medium adjacent to colonial growth. Often, pH indicator systems are used in the same recipe along with other substrates and indicator systems. For example, triple sugar iron agar (BD Difco) has lactose, sucrose, and glucose along with phenol red as the pH indicator, and a hydrogen sulfide system, which uses thiosulfate as the sulfur source and a ferrous compound to form a visible (black) precipitate with hydrogen sulfide. Differential media may be prepared to detect enzymes that degrade a variety of substrates, including proteins (e.g., gelatin, casein, tyrosine, or elastin), a complex carbohydrate (starch), or a glycosaminoglycan (chondroitin). Media that contain these substrates are usually poured in petri plates where visible zones of clearing under or surrounding the bacterial growth indicate positive reactions. It should be noted that the percentage of the substrate used can affect the result. A high concentration of substrate may overwhelm weak positive enzymatic action, and care must be taken to use an appropriate (i.e., minimal) concentration to allow visualization of a zone of clearing, but not too high an amount as to yield false negative results. Differential media also can utilize protein stains, which can interact with bacterial cells during growth, resulting in colony staining.

Numerous environmental bacteria grow on both general and differential media used for work with fish. The flora varies with each locale, and it is generally believed that many of these bacterial species have not been characterized. Typically, there are a number of colonies that grow on differential media plates that may appear phenotypically similar to the target organism. A selective medium is developed in a similar manner to a differential medium, in that the selective agent allows for unimpeded growth by the target bacterium, while inhibiting as many nontarget organisms as possible. The advantage of a selective bacteriologic medium is that the growth of many of those confounding, presumptive colonies is inhibited. This effect is obvious when comparing results of paired plates with and without the selective agent(s). Selective media are particularly useful for isolation of relatively slow-growing fish pathogens such as *R. salmoninarum* and *F. columnare*. Examples of selective agents in-

corporated into media include antibacterial agents, antifungal agents, bile salts, crystal violet, sodium azide, phenlyethanol, selenite, and tellurite. For some of the pathogens, it is useful to take advantage of their unique characteristics and use nonspecific selective techniques, such as increased salt (NaCl), pH tolerance, nutrient richness of the medium, and incubation temperature. The goal for primary isolation of a specific bacterial etiology is to employ a combination or multifaceted differential and selective approach to maximize the sensitivity of culture and promote a quick diagnosis and treatment.

Yersinia ruckeri

Yersinia ruckeri is the cause of enteric redmouth disease in rainbow trout (*Oncorhynchus mykiss*), and BHIA and TSA are routinely used for field isolations and for laboratory studies. A differential medium (SW; Waltman and Shotts, 1984) has been developed for primary isolation of *Y. ruckeri*. It relies on two characters to increase selectivity, hydrolysis of Tween 80 and negative assimilation of mannitol. Colonies will be green (negative mannitol is indicated with the pH indicator bromthymol blue) with a halo of precipitate (Tween 80 hydrolysis with calcium chloride) adjacent to and surrounding each colony. Since the publication of SW medium, there have been important observations on its preparation and use. The original paper gave a percentage of 0.0003% for bromthymol blue, which was subsequently confirmed to be an error and should be 0.003% (Shotts, 1991). Even with the correct percentage, in most instances, the medium is pale yellow at the specified pH of 7.4. If sleeved plates stored in the refrigerator are greenish in color, they will quench toward yellow within a few days. This is caused by a chemical change in the Tween 80. A solution to this problem is to adjust the pH according to the color of the medium (green), which will be in the range of pH 8.0 to 9.5, and prepare and use the medium fresh. The increased pH is advantageous for primary isolation of *Y. ruckeri*, which grows well, while many environmental bacteria will not. However, the isolation of Tween 80 hydrolysis-negative strains from England has affected its usefulness for field isolations (Hastings and Bruno, 1985; Rodgers and Hudson, 1985; Davies and Frerichs, 1989). Rodgers (1992) developed a differential and selective medium (ROD) for *Y. ruckeri*. Colonies on ROD are yellow (maltose and ribose assimilation) on a red medium (pH 7.4) with yellow deposits in the medium (sodium desoxycholate) surrounding colonies. ROD also contains a hydrogen sulfide indicator system (*Y. ruckeri* does not produce hydrogen sulfide) and sodium dodecyl sulfate and sodium desoxycholate as antibacterials.

Renibacterium salmoninarum

The cause of bacterial kidney disease in salmonid fish species, this gram-positive rod-shaped bacterium grows slowly, and media that support its growth are prepared containing l-cysteine HCl (typically 0.1%). Laboratory broth cultures take 10–14 days for log growth; a similar time is required for colonies to develop on primary plates to 2–3 mm. Because this bacterium is so slow to grow, field primary plates are very susceptible to contamination, which greatly confounds recovery and diagnosis. To eliminate this problem, selective kidney disease medium (SKDM; Austin et al., 1983) is an excellent choice for primary culture; this medium incorporates three antibacterials (cycloserine, polymyxin B, oxolinic acid) and an antifungal (cycloheximide). KDM2, the medium of Evelyn (1977), is used extensively for laboratory growth cultures by laboratories in geographic regions with endemic disease. Both SKDM and KDM2 include serum in their recipes: fetal bovine serum, fetal calf serum, or normal calf serum may be used. It was determined that sera from various sources suffice for bacterial

growth, but some differences may be encountered (e.g., fetal bovine sera result in a clearer medium in petri plates). Daly and Stevenson (1985) reported that activated charcoal may be used to substitute for serum. They found that serum was not providing growth nutrients but was acting as a detoxifier, and charcoal would perform this same function. Evelyn et al. (1989) observed satellitism in *R. salmoninarum*, whereby secreted growth products enhanced its own subsequent growth, and from this, metabolite supplementation was described (Evelyn et al., 1990). Metabolite is made simply by filter sterilizing a spent *R. salmoninarum* broth culture, then including this (usually at 1–5%) in future preparations of either broth or solid media. For primary isolation, it can be used to supplement KDM2 or SKDM. Metabolite does not result in an increase in colony-forming units/g (cfu/g) isolated, but it yields a more luxurious growth (i.e., larger, quicker) of colonies, and this allows for quicker diagnostic confirmation of disease.

Aeromonas spp.

The most significant pathogen of the genus *Aeromonas* to salmonid fish species is *A. salmonicida* subspecies *salmonicida*, the cause of furunculosis. Although it may be readily isolated from infected fish or grown in laboratory cultures using TSA, BHIA, or other general media, TSA supplemented with 0.01% CBB (Udey, 1982; Cipriano and Bertolini, 1988) offers an excellent presumptive identification. Suspect blue colonies are visible within 48 hr, and single colonies can be selected for easy and quick characterization with a few simple additional tests. Tryptic soy agar also aids in production of the brown water-soluble pigment, another presumptive character, in addition to the blue colony. Congo red agar (Ishiguro et al., 1985), a differential medium, is analogous to CBB in function, except that the A-layer cell protein binds congo red and colonies are red.

Motile *Aeromonas* spp. (e.g., *A. hydrophila*) are ubiquitous in untreated water supplies and are generally considered as secondary or opportunistic pathogens to cool- and cold-water fishes. These are not considered a significant primary cause of disease because diseases of salmonids are related to water temperature; salmonid fishes are reared at water temperatures between 10 C and 16 C, whereas the optimal temperatures for diseases from this group of bacteria are 22 C and above. Motile *Aeromonas* spp. are significant causes of diseases in warm-water fish species. These bacteria may be readily isolated from fish and are easily cultured in the laboratory on any of the routine general media, including TSA and BHIA. They grow to 5-mm-diameter, and larger, colony sizes within 24 hr. Selective and differential media are available to aid with primary isolation of *Aeromonas* spp. from fish, including Rimler-Shotts (RS; Shotts and Rimler, 1973) and SGAP-10C (starch-glutamate-ampicillin-penicillin, 10 μg/L C from glucose; Huguet and Ribas, 1991). SGAP-10C may also be used for isolation and quantification of the organisms from water (Jenkins and Taylor, 1995).

Edwardsiella spp.

Edwardsiella ictaluri, the cause of enteric septicemia of catfish (ESC; Hawke et al., 1981), is one of the leading causes of mortality to channel catfish aquaculture. This enteric bacterium can be isolated on general media including TSA blood agar or BHIA, or the *Edwardsiella* isolation medium of Shotts and Waltman (1990). Colistin is the selective agent in EIM, and, if necessary, the authors state that 0.5 μg/mL Amphotericin B (Fungizone®) could be included in the recipe for selection against fungal contamination.

Edwardsiella tarda is an enteric bacterium that has been reported as a cause of disease to channel catfish (Meyer and Bullock, 1973). Although it is not unusual to isolate *E. tarda*, it is not thought to be as significant as *E. ictaluri*. This bacterium is

common in reptiles, grows on routinely employed general media, and may also be isolated from fish on EIM. *Edwardsiella tarda* colonies grow quicker than *E. ictaluri* colonies and are easily differentiated by subsequent biochemical testing, such as the ability of *E. tarda* to produce hydrogen sulfide. Specific primary isolation of *E. tarda* is best accomplished using an enrichment step in either double-strength *Salmonella-Shigella* broth or selenite-cystine broth followed by direct plating onto *Salmonella-Shigella* agar (BD Difco; Wyatt et al., 1979; Muratori et al., 2000).

Flavobacterium spp.

Bacteria of this genus can be difficult to isolate from fish because they are often associated with skin lesions that can harbor heavy loads of other nonpathogenic bacteria. Two to three days of incubation are necessary for most *Flavobacterium* spp., and in general, a longer incubation time increases the chance for overgrowth by contaminants. Fish pathogenic Flavobacteria are long (up to about 5 μM), thin, gram-negative rods that are cultured on reduced-nutrient media, and most researchers and diagnosticians prepare and use media according to published recipes. A general medium that is often used is cytophaga medium (Anacker and Ordal, 1959). *Flavobacterium columnare* (formerly *Flexibacter columnaris* and *Cytophaga columnaris*), which causes columnaris disease in many cool- and warm-water free-ranging and cultured fish species, is the most frequently isolated pathogen. Hawke and Thune (1992) described a selective primary isolation medium (SCA; selective cytophaga agar) for *F. columnare*; it is prepared by supplementing cytophaga medium with 5 μg neomycin/ml and 200 IU polymyxin B/ml of medium. A differential and selective medium for *F. columnare* was described by Bullock et al. (1986) that incorporated 4 μg neomycin sulfate/ml media as a selective antibacterial and gelatin for a differential character. *Flavobacterium columnare* is gelatinase positive, and on SCA, a clear zone surrounding the colonies is visible after incubation. Shieh medium supplemented with 1 μg tobramycin/ml is another selective medium that has been reported for primary isolation from diseased fish (Decostere et al., 1997).

Flavobacterium psychrophilum (formerly *Flexibacter psychrophilus* and *Cytophaga psychrophila*), is the cause of bacterial cold-water disease and fry syndrome to cultured and wild rainbow trout and salmon species. Like *F. columnare*, the most commonly used medium for primary isolation and laboratory growth cultures is cytophaga medium (Anacker and Ordal, 1959). Other media have been described in the literature but have yet to be frequently used by fish diagnosticians. For the most part, all of the described media for *F. psychrophilum* are general growth media and are not differential or selective. Optimal growth temperature for this bacterium is 15–16 C, which is cool enough to retard some but not all contaminant growth during primary isolation, especially from lesions. Kidney tissue can afford a more aseptic sample for primary isolation from clinically diseased fish. Several of the other reported media (Anderson and Conroy, 1969; Holt, 1987; Rangdale et al., 1997) are variations to the recipe of cytophaga medium, while others contrast with the generally accepted requirement for low nutrient concentration. For example, serum (up to 5%) has been incorporated (Lorenzen, 1993; Brown et al., 1997). Daskalov et al. (1999) supplemented cytophaga medium with galactose, glucose, rhamnose, and skimmed milk. A significant improvement in growth of *F. psychrophilum* on cytophaga medium was reported by Rangdale et al. (1997) by increasing tryptone tenfold (to 0.5%) and increasing beef extract from 0.02% to 0.05%.

Less common fish bacterial pathogens

Many diseases of fishes with a bacterial etiology are currently limited to certain

hosts and geographic origins but may result in high mortality given optimal circumstances. Like other animals, stressed or immunocompromised fish often are more susceptible to opportunistic pathogens. Low dissolved oxygen and temperatures, for example, in addition to representing common stressors, may serve to enhance pathogen growth. Such environmental stressors along with opportunistic pathogens can result in mortality events with a combined and related noninfectious and infectious etiology. These types of problems are more common to cultured fishes and are sometimes a consequence of rearing conditions.

Carnobacterium maltaromicus (formerly *C. piscicola* and *Lactobacillus piscicola*; Mora et al., 2003), a gram-positive rod, is the causal agent of a disease that has been primarily associated with hatchery-reared rainbow trout brood stock at spawning or postspawning (Herman et al., 1985; Starliper et al., 1992). During spawning season, fish can be stressed by gamete development and through handling as part of the husbandry of spawning. Primary isolation of *C. maltaromicus* can be accomplished using general bacteriologic media, such as TSA, BHIA, or blood agar. Two selective media for gram-positives are Rogosa SL (for *Lactobacillus* spp.) and azide blood agar (Difco Manual, 1998). Although neither of these has been extensively field tested for primary isolation from fish, they could be useful for *C. maltaromicus*. Starliper and Morrison (2000) used Rogosa SL agar and azide blood agar (Difco Manual, 1998) during a general surveillance for fish pathogens, including *Lactobacillus* and related gram-positive genera from freshwater mussels; however, *C. maltaromicus* was not isolated.

Streptococcus iniae is a gram-positive cocci that causes diseases in both freshwater and marine water fishes. Disease in tilapia (*Oreochromomis niloticus*) often is associated with fish reared under intensive culture, recycle systems. Water quality has been noted as a predisposing factor, and improving water quality has been demonstrated to be pivotal in alleviating disease and mortality. General media, BHIA, or blood agar, may be used for primary isolation; however, increased sensitivity is reported with media that have a selective capability for *S. iniae*. Thallium acetate-oxolinic acid (TAOA) agar medium and TAOA with 3% horse blood, which allows for the detection of hemolysis, have been reported to greatly improve primary isolations of *S. iniae* from Japanese flounder (*Paralichthys olivaceus*), and from water and sediment from culture systems having infected fish (Nguyen and Kanai, 1999; Nguyen et al., 2002). In the United States, Columbia CNA agar (BD Difco) is routinely employed in diagnostic cases where *S. iniae* is the suspected cause (J. Hawke, Louisiana State University, pers. comm.). This medium is prepared with 5% blood to detect hemolysin production, and it contains colistin sulfate and nalidixic acid as selective antibacterial agents.

There are many non-glucose-fermenting bacteria that are ubiquitous in freshwater aquatic environments and are frequently isolated from healthy and diseased fishes. Among the common genera isolated are *Pseudomonas*, *Acintobacter*, *Moraxella*, *Shewanella*, *Alcaligenes*, and *Branhamella*. These bacteria grow on any of the previously described high- and reduced-nutrient general bacteriologic media. On streak plating from external sites such as mucus and gills or from internal sites such as kidney tissues, it is common to culture up to 10–20 total colonies per plate from apparently healthy fish. Members of these genera are only occasionally identified as causes of diseases and are generally considered opportunistic; disease problems associated with these agents probably relate to underlying stress-induced problems in culture conditions and one or more of these bacteria might be present in relatively higher numbers as secondary infections. *Pseudomonas fluorescens* and *Shewanella putrifaciens* are two examples of species that have been identified as occasional

causes of disease and mortality. Both are readily culturable from fish external lesions or kidneys using general media. Selective media have been described for *Pseudomonas* spp., including *Pseudomonas* isolation agar (BD Difco) and the media of Gould et al. (1985) for primary isolation of fluorescent Pseudomonads (e.g., *P. fluorescens*).

Mycobacterium spp. cause chronic diseases in both freshwater and marine fishes, and infected fish typically demonstrate classical signs such as lethargy, wasting, and poor growth; external lesions may be present as well as granulomas in internal tissues. Primary isolation is commonly made using Lowenstein and Middlebrook media (BD Difco). Rhodes et al. (2004) developed a tissue preparation and media inoculation procedure that enhances the recovery of *Mycobacterium* spp. from fish (i.e., striped bass *M. saxatilis*) using Middlebrook 7H9 medium supplemented with antimicrobials (Teska et al., 1997) to reduce overgrowth by contaminants and thereby increase recovery of the pathogen.

Photobacterium damselae subspecies *piscicida* (formerly *Pasturella piscicida*), which causes fish pasturellosis, has been isolated from freshwater and saltwater fishes. Primary isolation of the bacterium is accomplished on TSA, TSA with 5% sheep blood, BHIA, or blood agar base containing 5% sheep blood. This bacterium produces a systemic infection and can be readily isolated from kidney tissues. Primary isolation from fish from marine environments may be enhanced by supplementation of any of the aforementioned media with 1% NaCl.

Intracellular bacterial pathogens cause significant diseases and mortality to a variety of fish species. *Piscirickettsia salmonis*, cause of piscirickettsiosis (salmonid rickettsial septicemia), and *Francisella* sp. (francisellosis) are two gram-negative pathogens that can be difficult to recover from diseased specimens. For both of these diseases, various authors report that tissue processing or homogenization prior to inoculation of media or cell lines greatly enhances culture success.

At present, there are no reports of bacteriologic media that allow for successful primary isolation or laboratory cultures of *P. salmonis*. However, this bacterium has been isolated using cell-culture techniques, usually with Chinook salmon embryo (CHSE-214) cells (Fryer et al., 1990, 1992; Gaggero et al., 1995; Palmer et al., 1996; Skarmeta et al., 2000; Fryer and Hedrick, 2003; Birkbeck et al., 2004a), epithelioma papulosum ciprini cells (Fryer and Hedrick, 2003), or brown bullhead catfish (*Ictalurus nebulosus*) cells, (Almendras et al., 1997). In the brown bullhead catfish cells, cytopathic effect (CPE) associated with the type strain of *P. salmonis* (ATCC VR 1361; Fryer et al., 1992) was not observed until 45 days following inoculation. Birkbeck et al. (2004b) showed approximately 100-times greater cell yield of *P. salmonis* grown in Sf21 insect cells (from the fall armyworm [*Spodoptera frugiperda*]), compared to CHSE-214 cells. Fryer et al. (1990) isolated *P. salmonis* using the CHSE-214 cell line; although the isolate could be subcultured in five of seven cell lines on passage, it could not be subsequently grown on media. These reports indicate an optimum incubation temperature ranging from 15 C to 18 C, and antimicrobials should not be used in cell-culture media or any tissue transport or processing buffers or media.

Nyland et al. (2006) isolated *Francisella* sp. from Atlantic cod (*Gadus morhua* L.) on blood agar plates supplemented with 0.1% cysteine and 1% glucose, and the primary culture demonstrated excellent growth in salmon head kidney cells with medium B1817. This media consists of marine broth, fetal bovine serum, yeastolate, l-cysteine HCl, and glucose (Ottem et al., 2007). Olsen et al. (2006) were not successful at primary isolation of *Francisella* sp. from Atlantic cod kidney, heart, and spleen on blood agar (Oxoid; Basingstoke, UK) with 5% bovine blood or blood

TABLE 1. Summary of suggested bacteriologic media and incubation temperatures for primary isolation of fish bacterial pathogens.

Pathogen	Bacteriologic media	Incubation temperature range
Gram-negative bacteria; luxuriant growth typically within 1–4 days:		
Aeromonas salmonicida	Tryptic soy agar (TSA), Coomassie brilliant blue, Congo red agar	18–20 C
Motile *Aeromonas* spp.	Brain-heart infusion agar (BHIA), TSA, Starch-glutamate-ampicillin-penicillin-10 μg/L (SGAP-10C;), Rimler-Shotts	20–25 C
Edwardsiella ictaluri	BHIA, TSA, *Edwardsiella* isolation medium (EIM)	30 C
Edwardsiella tarda	BHIA, TSA, *Salmonella–Shigella* (SS), EIM	30–35 C
Flavobacterium columnare	Selective cytophaga agar, Cytophaga agar, the medium of Bullock et al. (1986) or Decostere et al. (1997)	25–30 C
Flavobacterium psychrophilum	Cytophaga medium of Rangdale et al. (1997)	15–16 C
Photobacterium damselae subsp. *piscicida*	Blood agar (BA), BHIA, or TSA with or without 5% sheep blood	25–28 C
Yersinia ruckeri	BHIA, TSA, Ribose-ornithine-desoxycholate (ROD), Shotts-Waltman (SW)	20–25 C
Other common aquatic bacteria (i.e., *Pseudomonas* spp., *Acintobacter* spp., etc.)	BHIA, TSA, BA, Cytophaga agar	18–25 C
Gram-negative bacteria; fastidious and slow growing:		
Francisella sp. (spp.)	BA plus 0.1% cysteine and 1% dextrose, Cystine heart agar plus 1% hemoglobin, Cystine heart agar plus 5% sheep blood, Thayer-Martin, Eugon medium with 1% hemoglobin	20–25 C
Mycobacterium spp.	Middlebrook 7H9, Middlebrook 7H10, Lowenstein	20–25 C
Gram-positive bacteria:		
Carnobacterium maltaromicus	BHIA, TSA, BA	28–32 C
Renibacterium salmoninarum	Selective kidney disease medium (SKDM), KDM2; either may be supplemented with (~1%) metabolite	15–16 C
Streptococcus iniae	TSA plus 5% blood, Columbia CNA plus 5% blood, BA, BHIA	25–30 C

agar with 1.5% NaCl at 22 C and 15 C, respectively. However, they recovered isolates through cell cultures, and at an incubation temperature of 22 C, heavy growth was demonstrated on cystine heart agar (BD Difco). Growth was limited to the initial streak lines; single colonies did not develop. On cystine heart agar with 5% sheep blood, small colonies were noted at 3 days and enlarged to 2–3 mm in diameter with an extended incubation time. Isolates did not grow on buffered charcoal yeast or Bacto chocolate agar enriched (BD Difco) media. Kamaishi et al. (2005) isolated *Francisella* sp. from three-line grunt (*Parapristipoma trilineatum*) on cystine-heart agar (BD Difco) supplemented with 1% hemoglobin after two weeks at 25 C. Eugon agar (BD BBL, Franklin Lakes, New Jersey, USA) with 1% hemoglobin and Eugon broth (BD BBL) with 2 mM $FeCl_3$ also supported bacterial culture growth; the latter was also good for large-scale cultivation (Kamaishi et al., 2005). Hsieh et al. (2006) isolated *Francisella* sp. from tilapia (*Oreochromis* spp.) on Thayer-Martin medium in a 5% CO_2 incubator at 23 C;

colonies took 3–6 days to develop. They also recovered the bacterium in CHSE-214 cells, noting CPE at 7 days. However, they did not recover the bacterium from fish using blood agar, MacConkey, BHIA with 5% sheep red blood cells, blood cystine-glucose agar, or in 6% NaCl broth. *Francisella* sp. causing disease in hybrid striped bass (*Morone chrysops* × *M. saxatilis*) was identified through phylogenetic analyses of bacterial DNA from kidney tissues (Ostland et al., 2006). Ostland et al. (2006) were unsuccessful in their attempts at primary isolation using the following bacteriologic media: TSA with 5% sheep blood, BHIA, BHIA with 5% sheep blood, BHIA with 1% NaCl, MacConkey, Mueller-Hinton with 5% sheep blood, Middlebrook 7H10, Lowenstein-Jensen (LJ), LJ–Gruft modification, and chocolate and SP4 agars. In addition, they did not culture the causative bacterium in CHSE-214 incubated at 15 C.

Suggested media for the fish bacterial pathogens discussed in this paper and guidelines for incubation temperatures are summarized in Table 1.

LITERATURE CITED

ALMENDRAS, F. E., S. R. JONES, C. FUENTEALBA, AND G. M. WRIGHT. 1997. In vitro infection of a cell line from *Ictalurus nebulosus* with *Piscirickettsia salmonis*. Canadian Journal of Veterinary Research 61: 66–68.

AMERICAN FISHERIES SOCIETY (AFS)–FISH HEALTH SECTION (FHS). 2004. Fish Health Section, blue book: Suggested procedures for the detection and identification of certain finfish and shellfish pathogens. 2004 Edition. AFS-FHS, Bethesda, Maryland, USA, CD-ROM.

ANACKER, R. L., AND E. J. ORDAL. 1959. Studies on the Myxobacterium *Chondrococcus columnaris*. I. Serological typing. Journal of Bacteriology 78: 25–32.

ANDERSON, J. I. W., AND D. A. CONROY. 1969. The pathogenic myxobacteria with special reference to fish diseases. Journal of Applied Bacteriology 32: 30–39.

AUSTIN, B., T. M. EMBLEY, AND M. GOODFELLOW. 1983. Selective isolation of *Renibacterium salmoninarum*. FEMS Microbiology Letters 17: 111–114.

BIRKBECK, T. H., S. RENNIE, D. HUNTER, L. A. LAIDLER, AND S. WADSWORTH. 2004a. Infectivity of a Scottish isolate of *Piscirickettsia salmonis* for Atlantic salmon *Salmo salar* and immune response of salmon to this agent. Diseases of Aquatic Organisms 60: 97–103.

———, A. A. GRIFFEN, H. I. REID, L. A. LAIDLER, AND S. WADSWORTH. 2004b. Growth of *Piscirickettsia salmonis* to high titers in insect tissue culture cells. Infection and Immunity 72(6): 3693–3694.

BROWN, L. L., W. T. COX, AND R. P. LEVINE. 1997. Evidence that the causal agent of bacterial cold-water disease *Flavobacterium psychrophilum* is transmitted within salmonid eggs. Diseases of Aquatic Organisms 29: 213–218.

BULLOCK, G. L., E. B. SHOTTS, JR., AND T. C. HSU. 1986. Columnaris disease of fishes. Fish Disease Leaflet 72. US Fish and Wildlife Service, Washington, D.C., 9 pp.

CIPRIANO, R. C., AND J. BERTOLINI. 1988. Selection for virulence in the fish pathogen *Aeromonas salmonicida*, using Coomassie brilliant blue agar. Journal of Wildlife Diseases 24: 672–678.

DALY, J. G., AND R. M. W. STEVENSON. 1985. Charcoal agar, a new growth medium for the fish disease bacterium *Renibacterium salmoninarum*. Applied and Environmental Microbiology 50: 868–871.

DASKALOV, H., D. A. AUSTIN, AND B. AUSTIN. 1999. An improved growth medium for *Flavobacterium psychrophilum*. Letters in Applied Microbiology 28: 297–299.

DAVIES, R. L., AND G. N. FRERICHS. 1989. Morphological and biochemical differences among isolates of *Yersinia ruckeri* obtained from wide geographical areas. Journal of Fish Diseases 12: 357–365.

DECOSTERE, A., F. HAESEBROUCK, AND L. A. DEVRIESE. 1997. Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare* (*Flexibacter columnaris*) from diseased fish. Journal of Clinical Microbiology 35: 322–324.

DIFCO MANUAL. 1998. Difco Manual. 11th Edition. Difco Laboratories, Division of Becton Dickinson and Company, Sparks, Maryland, 862 pp.

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

———, G. R. BELL, L. PROSPERI-PORTA, AND J. E. KETCHESON. 1989. A simple technique for accelerating the growth of the kidney disease bacterium *Renibacterium salmoninarum* on a commonly used culture medium (KDM2). Diseases of Aquatic Organisms 7: 231–234.

———, L. PROSPERI-PORTA, AND J. E. KETCHESON. 1990. Two new techniques for obtaining consistent results when growing *Renibacterium salmoninarum* on KDM2 culture medium. Diseases of Aquatic Organisms 9: 209–212.

FRYER, J. L., AND R. P. HEDRICK. 2003. *Piscirickettsia salmonis*: A gram-negative intracellular bacterial pathogen of fish. Journal of Fish Diseases 26: 251–262.

———, C. N. LANNAN, L. H. GARCÉS, J. J. LARENAS, AND P. A. SMITH. 1990. Isolation of a Rickettsiales-like organism from diseased Coho salmon (*Oncorhynchus kisutch*) in Chile. Fish Pathology 25(2): 107–114.

———, ———, S. J. GIOVANNONI, AND N. D. WOOD. 1992. *Piscirickettsia salmonis* gen. nov., sp. nov., the causative agent of an epizootic disease in salmonid fishes. International Journal of Systematic Bacteriology 42(1): 120–126.

GAGGERO, A., H. CASTRO, AND A. M. SANDINO. 1995. First isolation of *Piscirickettsia salmonis* from Coho salmon, *Oncorhynchus kisutch* (Walbaum), and rainbow trout, *Oncorhynchus mykiss* (Walbaum), during the freshwater stage of their life cycle. Journal of Fish Diseases 18: 277–279.

GOULD, W. D., C. HAGEDORN, T. R. BARDINELLI, AND R. M. ZABLOTOWICZ. 1985. New selective media for enumeration and recovery of fluorescent Pseudomonads from various habitats. Applied and Environmental Microbiology 49: 28–32.

HASTINGS, T. S., AND D. W. BRUNO. 1985. Enteric redmouth disease: Survey in Scotland and evaluation of a new medium, Shotts-Waltman, for differentiating *Yersinia ruckeri*. Bulletin of the European Association of Fish Pathologists 5: 32–35.

HAWKE, J. P., AND R. L. THUNE. 1992. Systemic isolation and antimicrobial susceptibility of *Cytophaga columnaris* from commercially reared channel catfish. Journal of Aquatic Animal Health 4: 109–113.

———, A. C. MCWHORTER, A. G. STEIGERWALT, AND D. J. BRENNER. 1981. *Edwardsiella ictaluri* sp. nov. causative agent of enteric septicemia of catfish. International Journal of Systematic Bacteriology 31: 396–400.

HERMAN, R. L., K. MCALLISTER, G. L. BULLOCK, AND E. B. SHOTTS, JR. 1985. Postspawning mortality of rainbow trout (*Salmo gairdneri*) associated with *Lactobacillus*. Journal of Wildlife Diseases 21: 358–360.

HOLT, R. A. 1987. *Cytophaga psychrophila*, the causative agent of bacterial cold water disease in salmonid fish. PhD Thesis, Oregon State University, Corvallis, Oregon, 166 pp.

HSIEH, C. Y., M. C. TUNG, C. TU, C. D. CHANG, AND S. S. TSAI. 2006. Enzootics of visceral granulomas associated with *Francisella*-like organism infection in tilapia (*Oreochromis* spp.). Aquaculture 254: 129–138.

HUGUET, J. M., AND F. RIBAS. 1991. SGAP-10C agar for the isolation and quantification of *Aeromonas* from water. Journal of Applied Bacteriology 70: 81–88.

ISHIGURO, E. E., T. AINSWORTH, T. J. TRUST, AND W. W. KAY. 1985. Congo red agar, a differential medium for *Aeromonas salmonicida*, detects the presence of the cell surface protein array involved with virulence. Journal of Bacteriology 164: 1233–1237.

JENKINS, J. A., AND P. W. TAYLOR. 1995. An alternative bacteriological medium for the isolation of *Aeromonas* spp. Journal of Wildlife Diseases 31: 272–275.

KAMAISHI, T., Y. FUKUDA, M. NISHIYAMA, H. KAWAKAMI, T. MATSUYAMA, T. YOSHINAGA, AND N. OSEKO. 2005. Identification and pathogenicity of intracellular *Francisella* bacterium in threeline grunt *Parapristipoma trilineatum*. Fish Pathology 40: 67–71.

LORENZEN, E. 1993. The importance of the brand of the beef extract in relation to the growth of *Flexibacter psychrophilus* in Anacker and Ordals medium. Bulletin of the European Association of Fish Pathologists 13: 64–65.

MEYER, F. P., AND G. L. BULLOCK. 1973. *Edwardsiella tarda*, a new pathogen of channel catfish (*Ictalurus punctatus*). Applied Microbiology 25: 155–156.

MORA, D., M. SCARPELLINI, L. FRANZETTI, S. COLOMBO, AND A. GALLI. 2003. Reclassification of *Lactobacillus maltaromicus* (Miller et al. 1974) DSM 20342^T and DSM 20344 and *Carnobacterium piscicola* (Collins et al. 1987) DSM 20730^T and DSM 20722 as *Carnobacterium maltaromaticum* comb. nov. International Journal of Systematic and Evolutionary Microbiology 53: 675–678.

MURATORI, M. C. S., A. L. DE OLIVEIRA, L. P. RIBEIRO, R. C. LEITE, A. P. R. COSTA, AND M. C. C. DA SILVA. 2000. *Edwardsiella tarda* isolated in integrated fish farming. Aquaculture Research 31: 481–483.

NGUYEN, H. T., AND K. KANAI. 1999. Selective agars for the isolation of *Streptococcus iniae* from Japanese flounder, *Paralichthys olivaceus*, and its cultural environment. Journal of Applied Microbiology 86: 769–776.

———, ———, AND K. YOSHIKOSHI. 2002. Ecological investigation of *Streptococcus iniae* in cultured Japanese flounder (*Paralichthys olivaceus*) using selective isolation procedures. Aquaculture 205: 7–17.

NYLAND, A., K. F. OTTEM, K. WATANABE, E. KARLSBAKK, AND B. KROSSØY. 2006. *Francisella* sp. (Family Francisellaceae) causing mortality in Norwegian cod (*Gadus morhua*) farming. Archives of Microbiology 185(5): 383–392.

OLSEN, A. B., J. MIKALSEN, M. RODE, A. ALFJORDEN, E. HOEL, K. STRAUM-LIE, R. HALDORSEN, AND D. J. COLQUHOUN. 2006. A novel systemic granulomatous inflammatory disease in farmed Atlantic cod, *Gadus morhua* L., associated with a bacterium belonging to the genus *Francisella*. Journal of Fish Diseases 29: 307–311.

OSTLAND, V. E., J. A. STANNARD, J. J. CREEK, R. P. HEDRICK, H. W. FERGUSON, J. M. CARLBERG, AND M. E. WESTERMAN. 2006. Aquatic *Francisella*-like bacterium associated with mortality of intensively cultured hybrid striped bass *Morone chrysops* × *M. saxatilis*. Diseases of Aquatic Organisms 72: 135–145.

OTTEM, K. F., A. NYLAND, E. KARLSBAKK, A. FRIIS-MØLLER, AND B. KROSSØY. 2007. Characterization of *Francisella* sp., GM2212, the first *Francisella* isolate from marine fish, Atlantic cod (*Gadus morhua*). Archives of Microbiology 187: 343–350.

PALMER, R., M. RUTTLEDGE, K. CALLANAN, AND E. DRINAN. 1996. A Piscirickettsiosis-like disease in farmed Atlantic salmon in Ireland—Isolation of the agent. Bulletin of the European Association of Fish Pathologists 17(2): 68–72.

PIPER, R. G., I. B. MCELWAIN, L. E. ORME, J. P. MCCRAREN, L. G. FOWLER, AND J. R. LEONARD. 1982. Fish hatchery management. US Department of the Interior, Fish and Wildlife Service, Washington, D.C., 517 pp.

RANGDALE, R. E., R. E. RICHARDS, AND D. J. ALDERMAN. 1997. Minimum inhibitory concentrations of selected antimicrobial compounds against *Flavobacterium psychrophilum* the causal agent of rainbow trout fry syndrome (RTFS). Aquaculture 158: 193–201.

RHODES, M. W., H. KATOR, I. KAATTARI, D. GAUTHIER, W. VOGELBEIN, AND C. A. OTTINGER. 2004. Isolation and characterization of mycobacteria from striped bass *Morone saxatilis* from the Chesapeake Bay. Diseases of Aquatic Organisms 61: 41–51.

RODGERS, C. J. 1992. Development of a selective-differential medium for the isolation of *Yersinia ruckeri* and its application in epidemiological studies. Journal of Fish Diseases 15: 243–254.

———, AND E. B. HUDSON. 1985. A comparison of two methods for isolation of *Yersinia ruckeri* from rainbow trout (*Salmo gairdneri*). Bulletin of the European Association of Fish Pathologists 5: 92–93.

SANDERS, J. E., AND J. L. FRYER. 1980. *Renibacterium salmoninarum* gen. nov., sp. nov., the causative agent of bacterial kidney disease in salmonid fishes. International Journal of Systematic Bacteriology 30: 496–502.

SHOTTS, E. B. 1991. Selective isolation methods for fish pathogens. Journal of Applied Bacteriology, Symposium Supplement 70: 75S–80S.

———, AND R. RIMLER. 1973. Medium for the isolation of *Aeromonas hydrophila*. Applied Microbiology 26: 550–553.

———, AND W. D. WALTMAN, II. 1990. A medium for the selective isolation of *Edwardsiella ictaluri*. Journal of Wildlife Diseases 26: 214–218.

SKARMETA, A. M., V. HENRÍQUEZ, M. ZAHR, C. ORREGO, AND S. H. MARSHALL. 2000. Isolation of a virulent *Piscirickettsia salmonis* from the brain of naturally infected Coho salmon. Bulletin of the European Association of Fish Pathologists 20(6): 261–264.

STARLIPER, C. E., AND P. MORRISON. 2000. Bacterial pathogen contagion studies among freshwater bivalves and salmonid fishes Journal of Shellfish Research 19: 251–258.

———, E. B. SHOTTS, AND J. BROWN. 1992. Isolation of *Carnobacterium piscicola* and an unidentified gram-positive bacillus from sexually mature and post-spawning rainbow trout *Oncorhynchus mykiss*. Diseases of Aquatic Organisms 13: 181–187.

TESKA, J. D., L. E. TWERDOK, J. BEAMAN, M. CURRY, AND R. A. FINCH. 1997. Isolation of *Mycobacterium abscessus* from Japanese medaka. Journal of Aquatic Animal Health 9: 234–238.

UDEY, L. 1982. A differential medium for distinguishing Alr+ from Alr– phenotypes in *Aeromonas salmonicida*. *In* Proceedings of the 13th annual conference and 7th Eastern Fish Health Workshop. International Association for Aquatic Animal Medicine, Baltimore, Maryland, May 9–13, 41 pp.

WALTMAN, W. D., AND E. B. SHOTTS, JR. 1984. A medium for the isolation and differentiation of *Yersinia ruckeri*. Canadian Journal of Fisheries and Aquatic Science 41: 804–806.

WYATT, L. E., R. NICKELSON, II., AND C. VANDERZANT. 1979. *Edwardsiella tarda* in freshwater catfish and their environment. Applied and Environmental Microbiology 38: 710–714.

Received for publication 27 September 2006.