1.2.2 Coldwater Disease

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A. Name of the Disease and Etiological Agent

Coldwater disease is also referred to as bacterial coldwater disease, rainbow trout fry syndrome, fry mortality syndrome, peduncle disease, or low temperature disease and is caused by the Gram negative bacterium, *Flavobacterium psychrophilum*. Coldwater disease and its causative agent have been reviewed by Cipriano and Holt (2005) and Barnes and Brown (2011).

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

The disease appears to occur in temperate regions worldwide with confirmed detections in the United States, Canada, France, Denmark, Sweden, Finland, Estonia, Spain, Japan, Chile, Germany, Korea, Northern Ireland, Norway, Peru, Scotland, Spain, Switzerland, Turkey, United Kingdom, and Australia.

2. Host Species

All salmonids are probably affected, but juvenile coho salmon *Oncorhynchus kisutch* and rainbow and steelhead trout *Oncorhynchus mykiss* are particularly susceptible. Also reported in Chinook salmon *Oncorhynchus tshawytscha*, sockeye salmon *Oncorhynchus nerka*, chum salmon *Oncorhynchus keta*, Arctic char *Salvelinus alpinus*, grayling *Thymallus thymallus*, sea trout *Salvelinus trutta*, whitefish *Coregonus muksun*, amago salmon *Oncorhynchus rhodurus*, iwana

salmon *Salvelinus leucomaenis pluvius*, lake trout *Salvelinus namaycush*, and pink salmon *Oncorhynchus gorbuscha*. Serious losses also occur in cutthroat trout *Oncorhynchus clarki*, brook trout *Salvelinus fontinalis*, brown trout *Salmo trutta*, and Atlantic salmon *Salmo salar*. *F. psychrophilum* has been reported in non-salmonid species including eel *Anguilla anguilla*, common carp *Cyprinus carpio*, tench *Tinca tinca*, crucian carp *Carassius carassius*, sea lamprey *Petromyzon marinus* L., perch *Perca fluviatilis* L., roach *Rutilus rutilus*, Japanese eel *Anguilla japonica*, Japanese dace Ugui *Tribolodon hakonensis*, pale chub *Zacco platypus*, Japanese crucian carp funbuna *Carassius auratus langsdorfii*, two species of goby *Chaenogobius urotaenia* and *Rhinogobius brunneus*, white sturgeon *Acipenser transmontanus*, goldfish *Carassius auratus*, and ayu *Pleoglossus altivelis*.

C. Epizootiology

Juvenile fish are primarily affected; infections also occur in yearlings and smolts. Epizootics frequently occur in fish held in protected water supplies and there is substantial evidence for vertical transmission of *F. psychrophilum* even when eggs are disinfected with iodophor. This pathogen has been isolated from mature adult coho and Chinook salmon from spleen, kidney, milt and ovarian fluid, the surface of eggs, and the contents of both unfertilized and eyed eggs (Baliarda et al. 2002; Brown et al. 1997; Chen et al. 2008; Cipriano 2005; Ekman et al. 1999; Holt et al. 1993; Kumagai and Nawata 2011; Lindstrom et al. 2009; Madetoja et al. 2002; Madsen et al. 2005; Madsen and Dalsgaard 2008; Taylor 2004; Vatsos et al. 2001; Vatsos et al. 2006). Vertical transmission of the bacterium appears to primarily be a concern for salmonid species as *F. psychrophilum* has not been found in ovarian fluid, milt, and unfertilized eggs from ayu (Kumagai et al. 2004).

The actual mode of entry into the egg is unknown. It may enter during oogenesis or vitellogenesis, similar to *Renibacterium salmoninarum* (Bruno and Munro 1986). It is also possible that *F. psychrophilum* enters the egg during the post-ovulation, pre-spawning period. Kumagai et al. (2000) and Kumagai and Nawata (2010) determined that *F. psychrophilum* is re-isolated only from eggs immersed in high concentrations of bacteria prior to water hardening. However, if eggs are exposed post-water hardening, the bacterium is not re-isolated. These results indicate that high concentrations of *F. psychrophilum* in the ovarian fluid can lead to uptake of the bacterium by the egg. Finally, the bacterium may attach to the surface of the egg and passively enter during the water hardening process. Regardless of the mode of entry, the bacterium is believed to localize in the perivitelline space (Kumagai et al. 1998; Kumagai et al. 2000; Kumagai & Nawata, 2010; Vatsos et al. 2006).

Once infection is established, moribund and dead fish serve as a potent source for horizontal transmission. Handling, high loading densities and high organic loads are thought to increase susceptibility to infection.

Although the reservoirs for the bacterium are not clear, it should be emphasized that *Flavobacterium* sp. are common inhabitants of aquatic ecosystems.

The disease often occurs when water temperatures are 12°C or below. However, many outbreaks occur or persist at temperatures up to 16°C, and coldwater disease is now common in many trout rearing facilities with constant 15°C temperatures.

D. Disease Signs

A variety of disease signs are exhibited. Erosive skin and muscle lesions are most common. These are frequently observed first in the peduncle area, but they may also occur on other areas of the body surface. The lesions may enlarge and the underlying tissue may be extensively eroded. If the fish survives long enough, it may suffer loss of its caudal fin and the vertebral column in the caudal peduncle may be exposed. Pale gills are also commonly observed. In later stages of an epizootic, or in acute infections, fish may darken either in the peduncle region or entirely and die with no external lesions. In trout, the bacterium can occur on the eroded caudal fin, and it may be present in internal organs. Exophthalmia may be observed. On rare occasions this bacterium is isolated from lesions on the gills of yearling rainbow trout. In coho salmon alevins, the ventral surface of the yolk sac becomes eroded and the sac may rupture.

Following a severe epizootic, at least two disease conditions may be observed. In the first, fish appear lethargic and are found swimming near the outlet screens. Thereafter, spinal deformities (scoliosis and lordosis) and chronic mortality occur. In the second, fish display a spiral swimming behavior, a dorsal swelling posterior to skull, and dark pigmentation on one side of the body. In the latter case, *F. psychrophilum* is readily isolated from brain tissue. These two disease conditions are most commonly observed in coho salmon. However, these signs are also frequently observed in steelhead and rainbow trout. *Flavobacterium psychrophilum* is often present as a secondary pathogen when fish populations are experiencing viral infections of erythrocytic inclusion body syndrome (EIBS), infectious hematopoietic necrosis (IHN), and infectious pancreatic necrosis. When yearling coho or Chinook salmon are found to have coldwater disease infections it is advisable to examine blood for presence of EIBS inclusions. It is also advisable to test for IHN virus in *F. psychrophilum* infected steelhead and rainbow trout that are undergoing severe loss.

Figure 1. Juvenile coho salmon: upper fish is uninfected and lower fish has coldwater disease with severe infection and erosion of the caudal fin and peduncle. Photo provided by Rich Holt, Oregon State University (OSU).

Figures 2 and 3. Juvenile coho salmon with coldwater disease lesions of the lower jaw, belly, and peduncle area. Photos provided by Rich Holt, OSU.

Figure 4. Juvenile steelhead trout with hemorrhaging on the body near location of the spleen. Photo provided by Craig Banner, Oregon Dept. of Fish and Wildlife (ODFW).

Figure 5. Juvenile steelhead trout with swollen, hemorrhaged deep muscle lesion containing coldwater disease bacteria. Photo provided by Rich Holt, OSU.

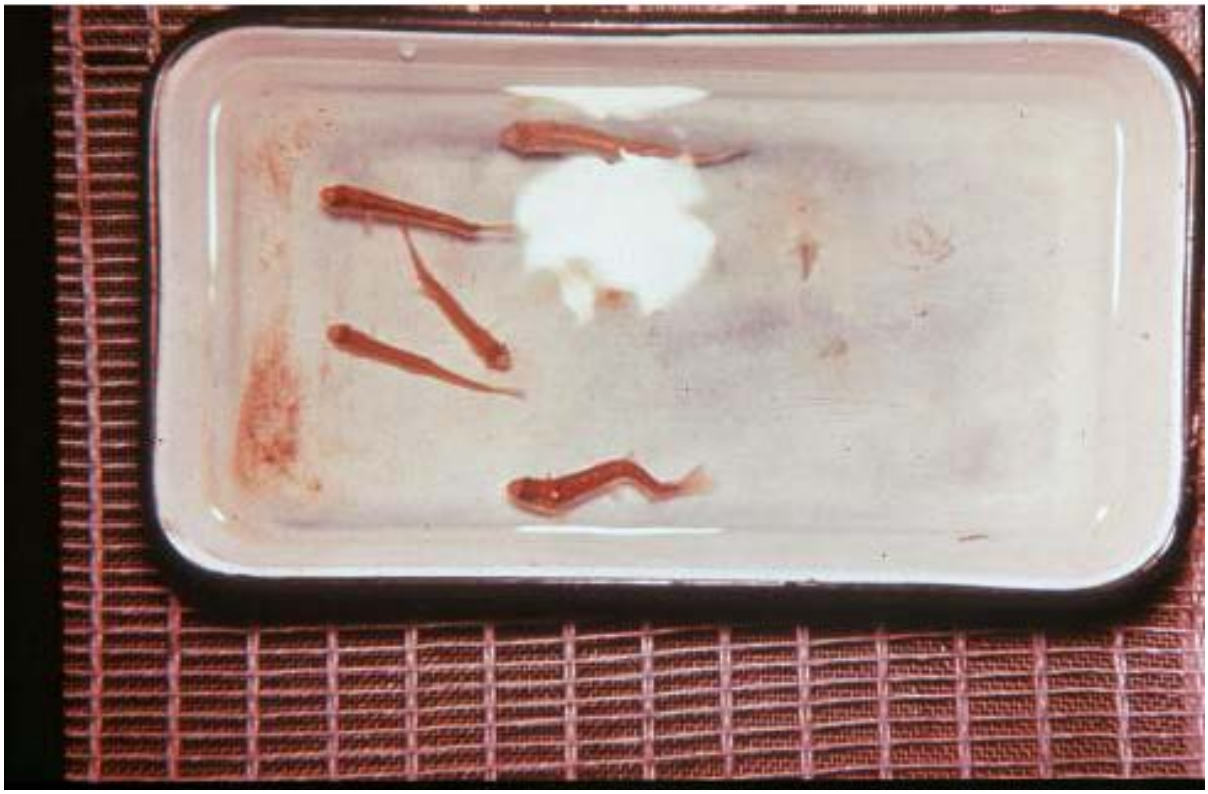

Figure 6. (Top Photo) Coho salmon juveniles with *F. psychrophilum* infections of the brain, meninges, spinal cord area. **Figure 7. (Bottom Photo)** Spinal deformed "survivors" of a coldwater disease epizootic. Photos provided by Rich Holt, OSU.

E. Disease Diagnostic Procedures

Diagnosis is based on clinical signs along with isolation and identification of the etiological agent. Primary isolation should be made from lesions, kidney, or the spleen on either TYES agar (Holt et al. 1989) (see formulation for this medium in Section 1, 1.1.1 General Procedures for Bacteriology) or one of the other media listed below. Cultures are incubated at 15 to 20°C for 3 to 6 days. The brain is the organ of choice for culture under certain conditions as described above.

1. Presumptive Diagnosis

Skin and muscle lesions contain long, thin, gram-negative rods (0.5-0.7 x 2-7 µm). In some cases, no external lesions are observed. Large numbers of rods can sometimes be observed in spleen tissue. Bacteria from lesions or tissues can be observed in wet mounts or in Gram or safranin stained, air-dried imprints (Figure 9). The organism should produce moist, golden-yellow, raised, convex colonies with or without a thin, spreading, smooth to irregular edge on tryptone yeast extract salts (TYES) agar in 3 to 6 days at 15 to 20°C. The following media support good growth of this bacterium: cytophaga agar (Anacker and Ordal 1959), Shieh (Shieh 1980), modified cytophaga agar (Wakabayashi and Egusa 1974), Hsu-Shotts (Baxa et al. 1986), TYE (Fujihara and Nakatani 1971), or TYES. Isolation of some strains is enhanced with addition of 1 to 5% fetal calf serum to cytophaga agar. Also, the addition of 0.2 to 0.5% sterile skim milk (added just prior to pouring plates) or 0.5% bovine serum albumin (filter sterilized) to TYES may promote heavier and more rapid growth. Growth and maintenance of *F. psychrophilum* has been recently reviewed (Cain and LaFrentz 2007).

In addition to colony morphology and the morphology of cultured bacterial cells, characteristics helpful for distinguishing *F. psychrophilum* from other commonly encountered aquatic, yellow-pigmented bacteria include: failure to grow on brain heart infusion agar and failure to grow at 30°C. Also, when a portion of a *F. psychrophilum* colony is swirled in a drop of 20% potassium hydroxide, the cell material turns red or reddish-brown indicating the presence of flexirubin-type pigments. This test is specific for *F. psychrophilum* when other growth characteristics are met. A specific fluorescent antibody test (FAT) is available to rapidly identify *F. psychrophilum* from slides of tissue imprints or cultured bacteria (see confirmatory diagnosis section).

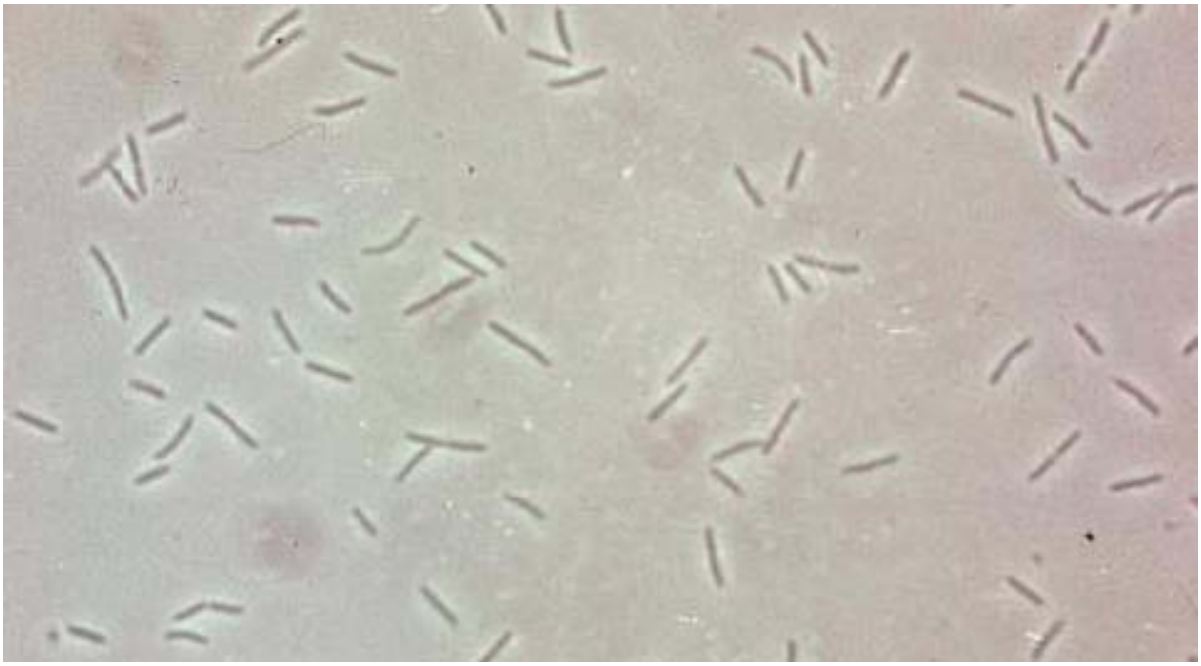

Figure 8. Cells of *F. psychrophilum* in wet mount preparation observed at 1000X . Photo provided by Rich Holt, OSU.

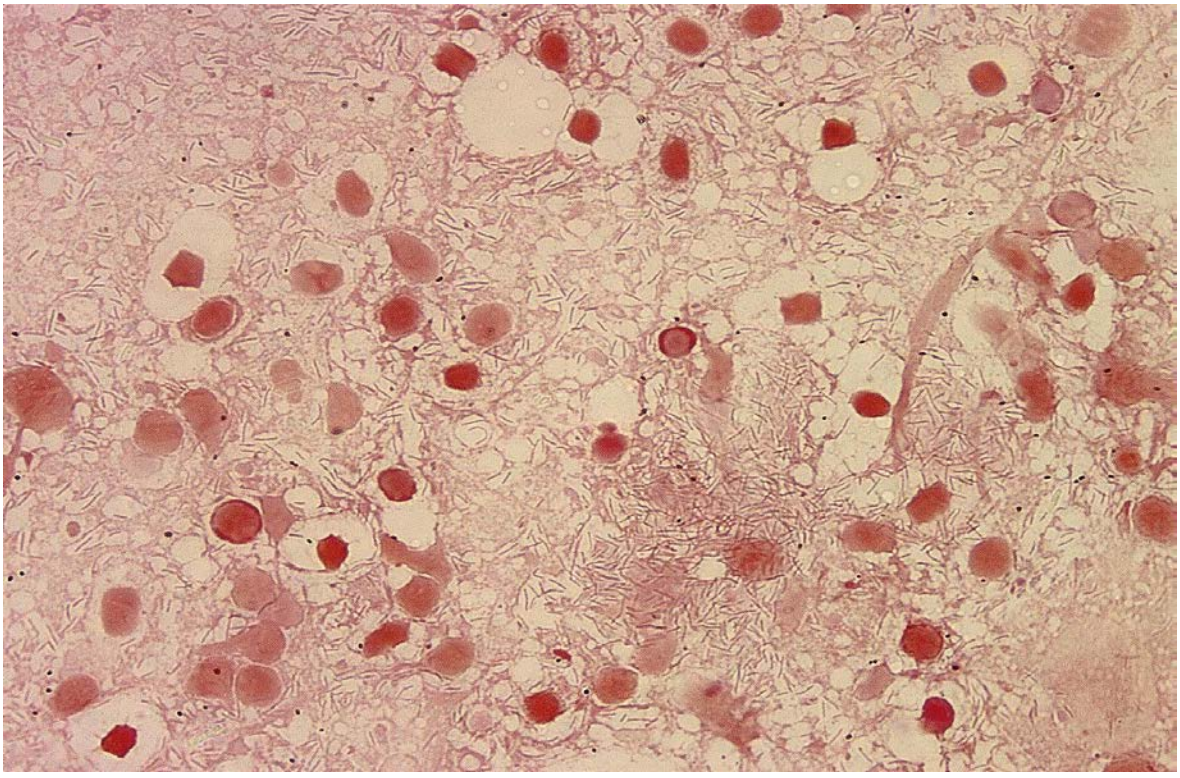

Figure 9. Gram stained *F. psychrophilum* cells. A smear prepared from the spleen of an infected fish. Photo provided by Craig Banner, ODFW.

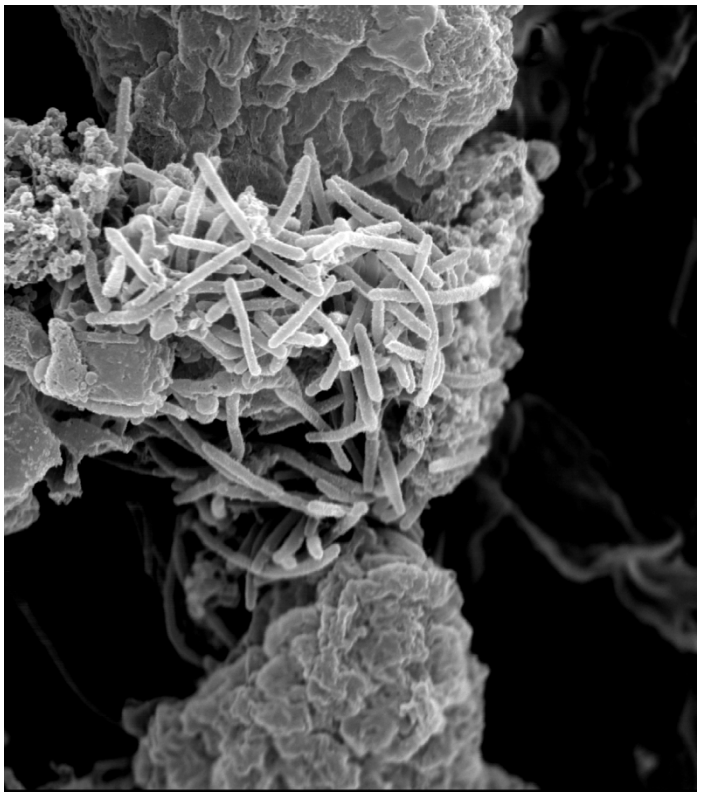

Figure 10. Scanning EM of a skin sample from a rainbow trout injected with *F. psychrophilum* CSF 259-93. Photo provided by N. Lindstrom, University of Idaho.

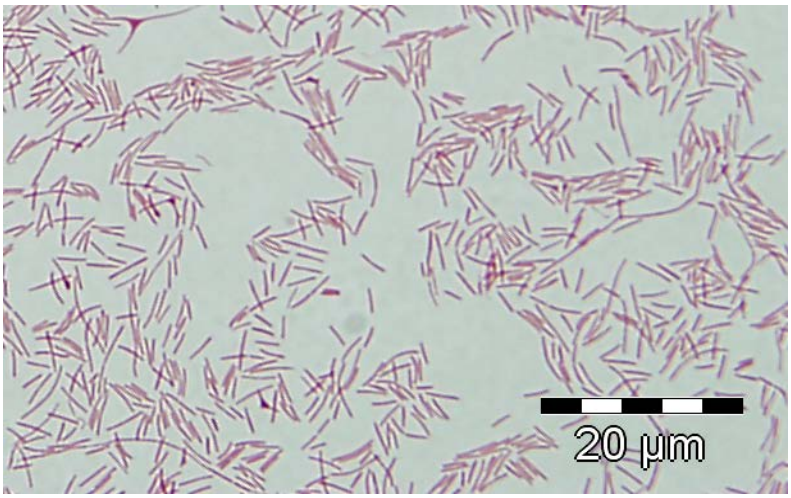

Figure 11. Cell morphology of *F. psychrophilum* following Gram staining and light microscopy. Photo provided by Ken Cain, University of Idaho.

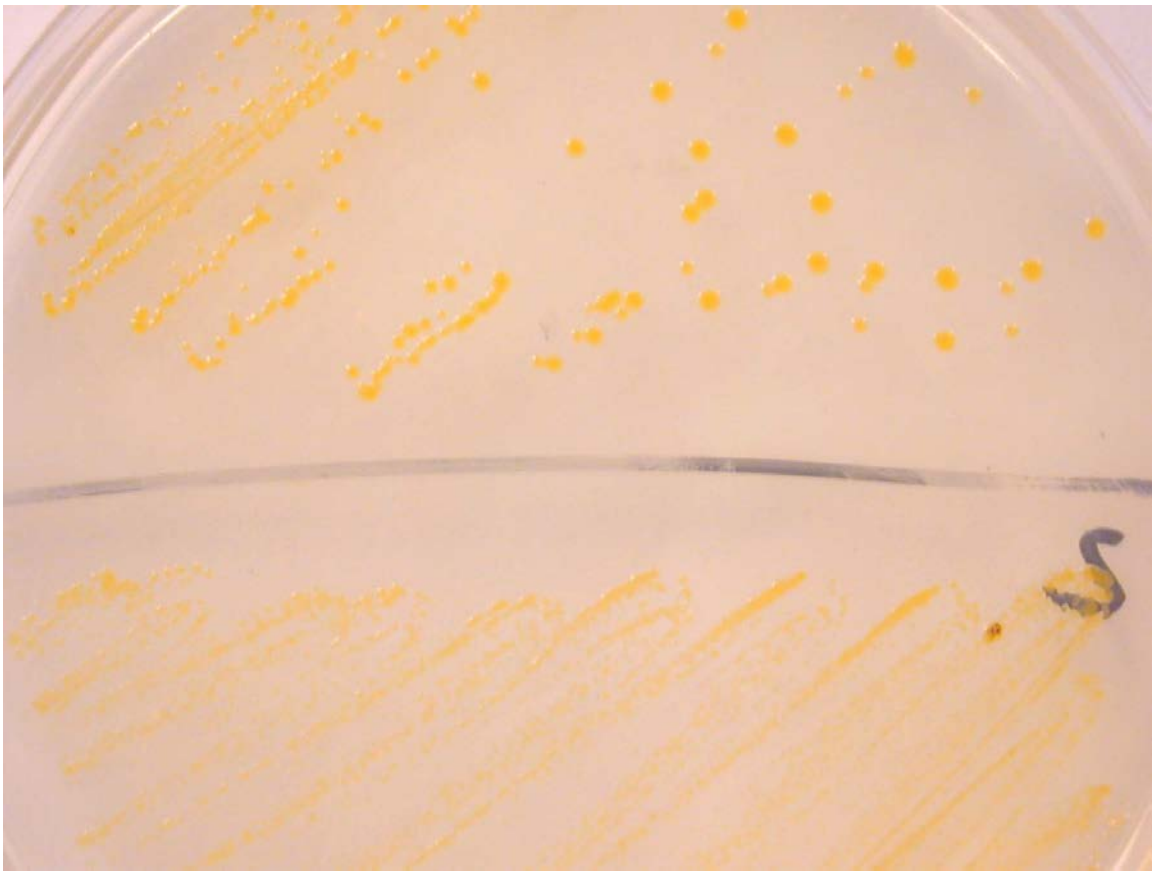

Figure 12. *Flavobacterium psychrophilum* colonies on TYES. Photo provided by Rich Holt, OSU.

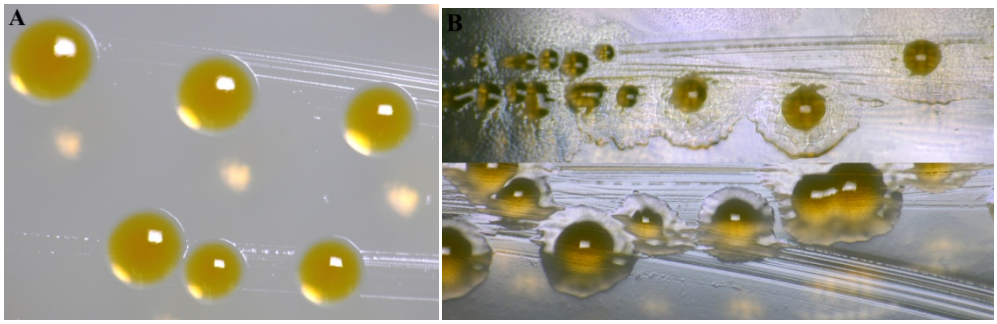

Figure 13. Morphology of *F. psychrophilum* colonies exhibiting an entire margin, *F. psychrophilum* colonies exhibiting a thin spreading margin ("fried egg") morphology (B). Photos provided by Ken Cain, University of Idaho.

2. Confirmatory Diagnosis

The diagnosis is confirmed upon positive serological identification by either slide or microtiter agglutination test or indirect FAT using polyclonal antiserum. However, such antiserum is currently limited in availability and cross-reactivity to *Flavobacterium columnare* has been reported. In addition, a small percentage of isolates meet all the criteria for a presumptive diagnosis, but fail to react with the polyclonal *F. psychrophilum* antiserum. Recent development of a monoclonal antibody (MAb FL43) that is specific to an outer membrane protein on the exterior of *F. psychrophilum* has allowed for development of new confirmatory assays. The monoclonal antibody has been tested against 66 *F. psychrophilum* strains from various sources

and all were reactive, but it was not reactive to five common *Flavobacterium* spp. (Lindstrom et al. 2009). Confirmatory identification of putative *F. psychrophilum* isolates can be made with a FAT using the FITC labeled MAb FL43. Conjugated antibody is available commercially (see below) and the protocol for the FAT procedure can be found at www.immunoprecise.com/datasheets/IPA116-FAT.pdf.

A polymerase chain reaction assay (PCR) is also recommended for confirmation of these isolates and is available as an alternative to confirmation with serological tests. Numerous PCR assays have been published for *F. psychrophilum* in recent years. Primers specific to the 16S ribosomal gene (Toyoma et al. 1994; Urdaci et al. 1998) or the *gyrB* gene (Izumi and Wakabayashi 1997) have been used successfully in single-round, multiplex (Taylor and Winton 2002), nested (Izumi & Wakabayashi 1997; Taylor 2004; Wiklund et al. 2000), and quantitative PCR (Orieux et al. 2011). Using these assays, *F. psychrophilum* can be detected in tissue samples (Orieux et al. 2011; Wiklund et al. 2000), formalin fixed samples (Crumlish et al. 2007), ovarian fluid (Baliarda et al. 2002; Long et al. 2012; Taylor 2004), and water (Madetoja et al. 2002; Wiklund et al. 2000). Depending on sample type, these assays are fairly sensitive. Protocols for PCR assays can be found in the cited literature.

Current source for anti-*F. psychrophilum* serological reagents (MAb FL 43):

ImmunoPrecise
Unit 3204-4464 Markham Street
Victoria, BC V8Z 7X8
CANADA
Tel: 1-250-483-0308
Fax: 1-250-483-0309
http://www.immunoprecise.com/products_monoclonal.php

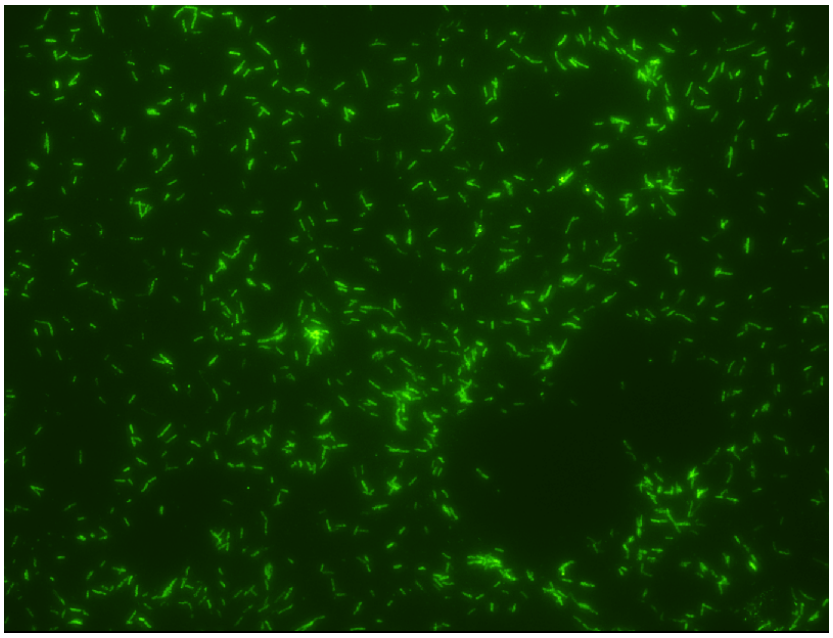

Figure 14. *Flavobacterium psychrophilum* cells labeled with MAb FL43 and viewed using an epifluorescent microscope and a fluorescein isothiocyanate filter (60X magnification). Cells were smeared on a glass slide and fixed with acetone prior to labeling. Photo provided by A. Long, University of Idaho.

F. Procedures for Detecting Subclinical Infections

Monoclonal antibody FL43 (described above) has also been used to develop an ELISA that can detect subclinical infections in fish and be used to potentially screen broodstock for infection severity (Lindstrom et al. 2009). This ELISA has been validated (Long et al. 2012) and both diagnostic sensitivity and specificity are high (>0.97). Laboratory experiments have shown that the assay is able to detect moderate to high levels of *F. psychrophilum* in challenged fish not exhibiting clinical disease symptoms (Long et al. 2012). Both the unconjugated FL 43 and the HRP-conjugated antibody can be purchased from ImmunoPrecise. The protocol for the assay can be found at http://immunoprecise.com/datasheets/IPA116-ELISA.pdf.

G. Procedures for Determining Prior Exposure to Etiological Agent

Due to the ubiquitous nature of *F. psychrophilum*, it is likely that most salmonids are routinely exposed to this pathogen at low levels depending on the rearing environment and water source. It is possible that carrier status could be determined by PCR and/or culture methods, but if *F. psychrophilum* has cleared the fish's system, determining prior exposure would only be possible using immunosurveillance methods. An ELISA is available that tests for anti-*F. psychrophilum* antibodies in the serum of fish (LaFrentz et al. 2002). If fish have elevated antibody titers to this pathogen, then it would provide an indication of prior exposure.

H. Procedures for Transportation and Storage of Samples

Flavobacterium psychrophilum can be prepared for storage or transport by culturing for 72 h in TYES medium containing 0.18% agar. Cultures can be shipped on ice or stored for weeks refrigerated at 4°C. For longer storage, growth should be homogenized with a pipet and then aliquotted and frozen at -80°C. Alternatively, the bacterium can be cryopreserved in glycerol as described by Cain and LaFrentz (2007).

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