

1.2.12 Francisellosis

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**(*Francisella*-like bacterium (FLB), *Piscirickettsia*-like organism (PLO),
Rickettsia-like organism, (RLO))**



Tilapia suffering an epizootic of Francisellosis in a Hawaiian lake. Photo courtesy of J. Block.

A. Name of Disease and Etiological Agent

1. Name of Disease

Francisellosis, *Piscirickettsia*-like organism (PLO), rickettsia-like organism (RLO), and chronic granulomatous disease.

2. Etiologic Agent

Francisella spp., suggested names, *Francisella piscicida*, *F. victoria*, *F. noatuensis*.

This non-motile, Gram-negative bacterium is a pleomorphic coccobacillus ranging from 0.5-1.5 µm in diameter. As a facultative intracellular pathogen, it replicates within membrane-bound intracytoplasmic vacuoles in infected cells similar to *Piscirickettsia salmonis*. Phylogenetic studies have placed this organism in the genus *Francisella*, as well as grouped it in the gamma subdivision of the proteobacteria. It is important to note that *Francisella* spp. causing disease in fish may be representatives of different species, sub-species or strains depending on the host species. This relationship has yet to be elucidated.

B. Known Geographical Range and Host Species

1. Geographic Range

Epizootics in tilapia have been reported in Taiwan, Hawaii, Latin America and the continental United States (Chen et al. 1994; Chern and Chao 1994; Hsieh et al 2006; Mauel et al. 2003; Mauel et al. 2005; Mauel et al.2007). In addition, similar bacteria have been reported in three-lined grunt in Japan (Fukuda et al. 2002; Kamaishi et al.2005), cod in Norway (Nylund et al. 2006; Olsen et al. 2006) and Atlantic salmon in Chile (Birkbeck et al. 2007).

2. Host Species

Isolated from tilapia (*Oreochromis* spp., *Tilapia* spp.), three-lined grunt *Parapristipoma trilineatum*, Atlantic salmon *Salmo salar* and Atlantic cod *Gadus morhua*. Identified by molecular sequencing in hybrid striped bass (*Morone chrysops* x *M. saxatilis*). Using PCR and *in situ* hybridization this bacteria has been detected in a number of ornamental fish all belonging to the Cichidae family; firebird *Aulonacara rubescens*, elegans *Pseudotropheus elegans*, zebra *Pseudotropheus zebra*, Rhodes's chilo *Chilotilapia rhoadesii*, Malawi eyebiter *Dimidiochromis compressiceps*, brown discus *Symphysodon aequifasciatus*, deep-water hap *Haplochromis electra*, electric blue hap *Sciaenochromis fryeri* blue-white labito *Labidochromis caeruleus*, *Placidochromis milomo*, and Fontosa cichlid *Cyphotilapia frontosa*.

C. Epizootiology

The life cycle of this pathogen has not been fully elucidated, but is probably similar to mammalian *Francisella* species, which appear to have a soil and waterborne reservoir. The bacterium is probably present in the water column since horizontal transmission has been demonstrated in tilapia. The disease has been reported in both fresh and marine fish. Strains of the bacterium from these different geographic locations and hosts may differ. The role of vectors or vertical transmission remains unknown. In populations with endemic chronic disease, the occurrence of mortalities is often associated with exposure to stressful conditions (e.g. poor water quality, rapid temperature changes or handling, etc.).

D. Disease Signs

Infected fish can display a variety of clinical signs and lesions (e.g. lethargy, inappetence, petechia, exophthalmia and abnormal swimming behavior). Multiple, variably-sized, white granulomas are generally detectable grossly in different organs (e.g. gills, spleen, kidney, testes, heart, ovaries and liver) (Figures 1, 3) with the spleen and kidney commonly enlarged (Figure 2). Mortalities can range from less than 1% to upwards of 90%.



Figure 1. Typical presentation of white nodules (granulomas) in spleen (A) and gills (B & C) of tilapia experiencing an epizootic of Francisellosis.

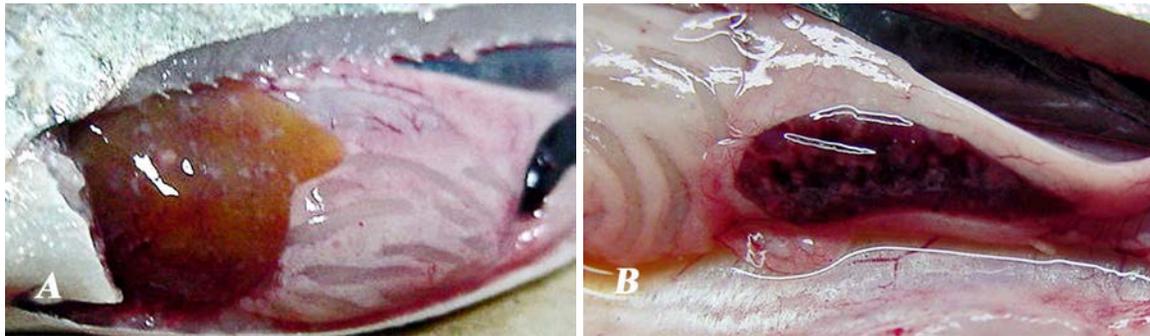


Figure 2. White foci present in the liver (A) and the spleen (B) of Atlantic salmon (*Salmo salar*) experiencing an epizootic of Francisellosis in a Chilean freshwater lake. Photographs courtesy of Harry Bohle, Aquatic Diagnostic Laboratory, Puerto Montt, Chile.



Figure 3. Muscle lesion observed in 0 – 20% of tilapia experiencing a Francisellosis epizootic.

E. Disease Diagnostic Procedures

Francisellosis can be diagnosed based on a variety of diagnostic parameters, including clinical signs, histopathology, bacterial isolation culture and PCR. Although not usually evident in high numbers, this gram-negative, intracellular pleomorphic bacterium, with approximate dimensions of 0.5 X 1.5 μm , can be demonstrated with special stains in either histologic sections or tissue impression smears. PCR amplification with species-specific, genus-specific and/or eubacterial-specific primers can be instrumental in reaching a definitive diagnosis. A fluorescent antibody test is also presently being investigated for commercial development as a possible serologic means of diagnosis.

1. Presumptive Diagnosis

Francisella sp. is a gram negative, highly fastidious, facultative intracellular bacterial pathogen similar in some ways to *Piscirickettsia salmonis*. The organism does not grow on general bacteriological media commonly used in fish diagnostic laboratories, but can be cultivated on selective cysteine-supplemented media. Alternatively, the organism may also be isolated using cell culture techniques. Samples of liver, kidney, spleen and blood are aseptically collected from diseased fish as described in Section 1, 1.1.1 General Procedures for Bacteriology. Tissues should be homogenized in antibiotic free HBSS, diluted and inoculated into the appropriate cell culture. Cod isolates are typically inoculated into salmon head kidney (SHK-1) cells or Atlantic salmon kidney (ASK) cells. Tilapia isolates have been cultured on Chinook salmon embryo (CHSE-214) cells and tilapia ovary (TO) cells. Specific host cell lines may be unavailable, but isolation may be attempted with available fish cell lines. Both artificial media and cell cultures should be incubated at 25°C for warm water isolations and at 15 - 20°C for cold water isolations. The isolated bacterium should be demonstrated to be Gram-negative, fastidious in its requirement for cysteine, non-motile, coccoid to coccobacillus, catalase negative, and cytochrome oxidase negative.



Figure 4. Typical granulomas observed in *Francisella* spp. infected tilapia spleen in a squash preparation.

Procedures for Presumptive Diagnosis

These techniques are recommended for isolating and/or identifying *Francisella* spp. from suspect fish displaying clinical disease signs and histologic lesions (e.g. granulomas with intracellular gram negative bacteria). The ability of these tests to detect sub-clinical infections is unknown at this time.

a.. Histopathology/cytology

Fish infected with *Francisella* spp. generally develop a disseminated granulomatous septicemia with multiple variably-sized granulomas present in a variety of tissues. Typically low numbers of gram-negative intracellular bacteria can be visualized within granulomas in histologic sections or tissue impression smears with special stains (e.g. Gram's and/or Giemsa stains). Often the *Francisella* sp. is difficult to observe with a Gram stain. Performing both Gram and Giemsa stains on comparable smears will allow the investigator to observe the bacteria on a Giemsa stain and determine it's Gram reaction by the presence of Gram positive or negative bacteria or by the absence of observable bacteria. Gram stain procedures are described in Section 2, 3.8.A.1 "Gram Stain." Tissue squash preparations can quickly demonstrate the presence of granulomas and when combined with the presence of typical *Francisella* sp. disease signs can be considered presumptive.

i. Cytology: Giemsa stain

Microscope slides with tissue culture supernatant smears or impressions of the kidney, liver, and spleen should be prepared, air-dried, and fixed for five minutes in absolute methanol.

Immerse slides in working solution of Giemsa stain for 30 minutes. Stock solution: 0.4 % Giemsa w/v in buffered methanol solution, pH 6.8 (Sigma Aldrich). Working solution: diluted 1:10 in phosphate buffer pH 6.0 (0.074M NaH₂PO₄, 0.009M Na₂HPO₄).

Destain with tap water.

Observe slides under oil immersion (1,000 X; 10 X eye piece and 100 X objectives). Examine a minimum of 50 fields. Tissue smears from infected organs show darkly stained coccoid to pleomorphic organisms, with dimensions of 0.5-1.5 μm . Visualization of organisms in smears is considered a PRESUMPTIVE positive result and the identification of *Francisella* spp. should be confirmed by an appropriate confirmatory method. (Figure 5)

ii. Tissue squash

Small tissue fragments are resected from the lesion of the tissue (spleen, kidney, ovaries, liver, etc) and placed on a microscope slide.

A cover slip or additional microscope slide is placed over the tissue and pressed down to gently crush the tissue.

Observe slides under low to medium magnification (10X – 40X) and note the presence or absence of granulomas. The presence of granulomas when combined with the presence of typical *Francisella* sp. disease signs can be considered presumptive. (Figure 4).

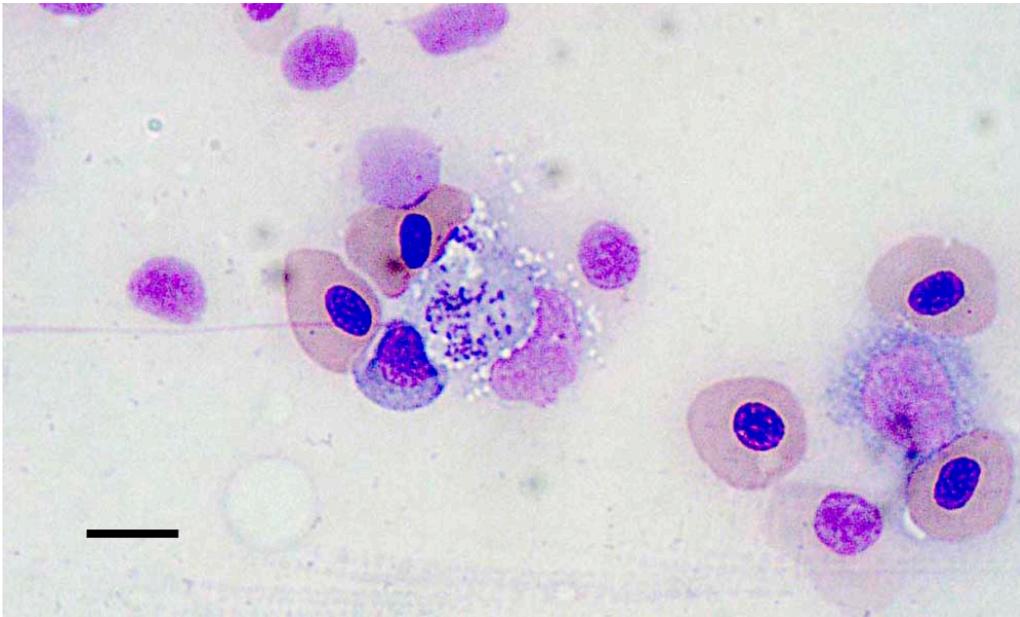


Figure 5. Giemsa stained tilapia blood smears demonstrating the *Francisella* bacterium in macrophages. Bar = 10 μm .

iii. Histopathology:

Granulomas with or without necrosis can be seen in infected tissues with H&E staining. See Figures 6-8. Tissue sections with suspect granulomas can be stained with Giemsa and/or Gram's stain. Bacterial numbers can be low and not readily apparent. Visualization of organisms in tissues is considered a PRESUMPTIVE positive result and the identification of *Francisella* spp. should be confirmed by an appropriate confirmatory method

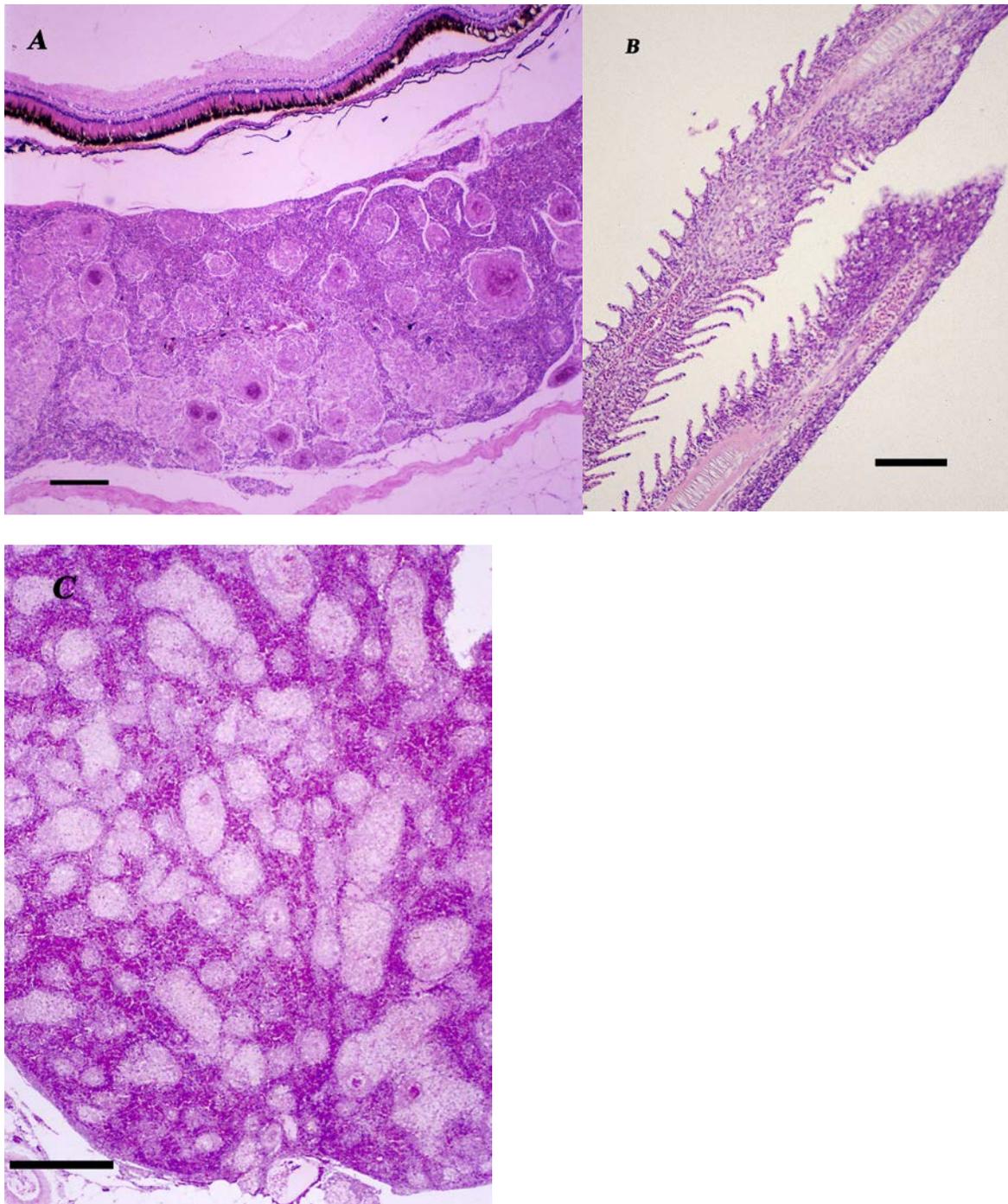


Figure 6. Numerous granulomas in H&E stained tilapia tissues. A. choroid gland, Bar = 25 μm ; B. gills, Bar = 50 μm ; C. spleen, Bar = 50 μm .

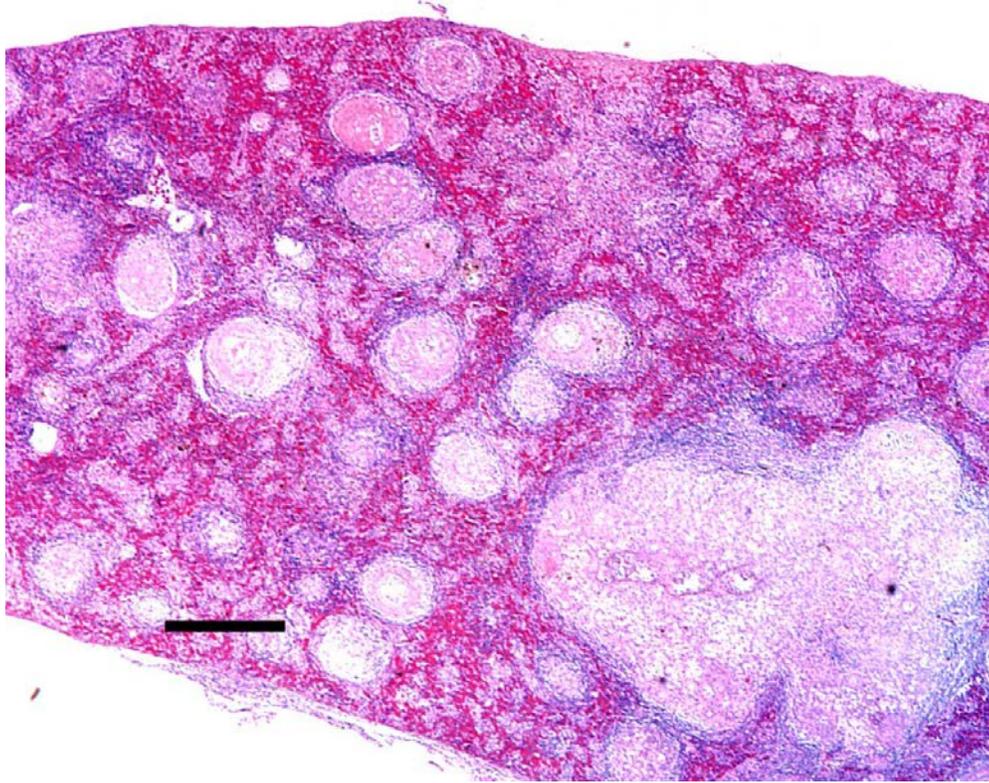


Figure 7. Tilapia spleen H&E stained tissue section demonstrating numerous granulomas and areas of necrosis. Bar = 50 μ m.



Figure 8. Tilapia muscle H&E stained section demonstrating granulomas as fine dark lesions grossly visible in muscle tissue. Bar = 50 μ m

b. Isolation and Culture of *Francisella* spp.

Primary isolation is made on enriched blood agar plates with added 0.1% cysteine and 1% glucose (BCG), cysteine heart agar with 1% hemoglobin (CHAH) or Thayer-Martin media.

- i. Aseptically collect the appropriate tissue samples from diseased fish and inoculate media prepared in tubes or agar plates as described in Section 2, 2.2 Sampling. Appropriate tissues would include any tissues containing the characteristic white nodules such as the spleen, kidney, and liver. Blood should also be cultured since a bacteremia is often present.
- ii. Incubate the primary isolation plates for up to 30 days at 15 - 20°C for cold water species (e.g. cod), or at 25°C for warm water species (e.g. tilapia).

Francisella spp. colonies develop slowly and, are smooth and white/grayish in color. Colonies are grossly visible as early as 3-6 days. (Figure 9)

If no growth occurs after 30 days, cultures are discarded and reported as negative for *Francisella* spp. by the bacteriological culture method. Since this is a slow growing organism, there is the potential problem that inoculated plates may be overgrown with contaminating organisms and produce a false negative. Further diagnostic efforts should include culturing in fish cell lines and/or non-culture identification methods such as PCR.

- iii. Use a sterile loop or needle to select a single colony to subculture onto fresh media for further testing. If colonies are not well isolated, a new plate should be streaked to achieve single colonies.
- iv. Incubate at the appropriate temperature for 3-6 days.
- v. Assign an isolate number to each pure colony and record all colony characteristics on the data sheet.
- vi. Begin initial identification of pure strain bacterial cultures (Section 2, 3.A1 Laboratory Reference Flow Chart Appendix 1).

Gram Determination (Section 2, 3.8.A “Gram Reaction”)

Francisella spp. are Gram-negative. Gram-positive isolates may be reported as negative for *Francisella* spp.

Presence of Cytochrome Oxidase (CO) (Section 2, 3.8.B “Cytochrome Oxidase”)

Francisella spp. is oxidase negative. CO positive isolates may be reported negative for *Francisella* spp.

Motility (Section 2, 3.8C “Motility”)

Francisella spp. is non-motile. Motile isolates may be reported as negative for *Francisella* spp.

- vii. Perform biochemical testing on each isolate (Section 2, 3.A1 Laboratory Reference flow chart Appendix 1).

Tube Method (Section 2, 3.8.D.1 “Tube Method”)

Carbohydrate Utilization (Section 2, 3.8.D.1.e “Carbohydrate Utilization”)

Francisella spp. ferment sucrose. Isolates yielding negative results for sucrose may be reported as negative for *Francisella* spp.

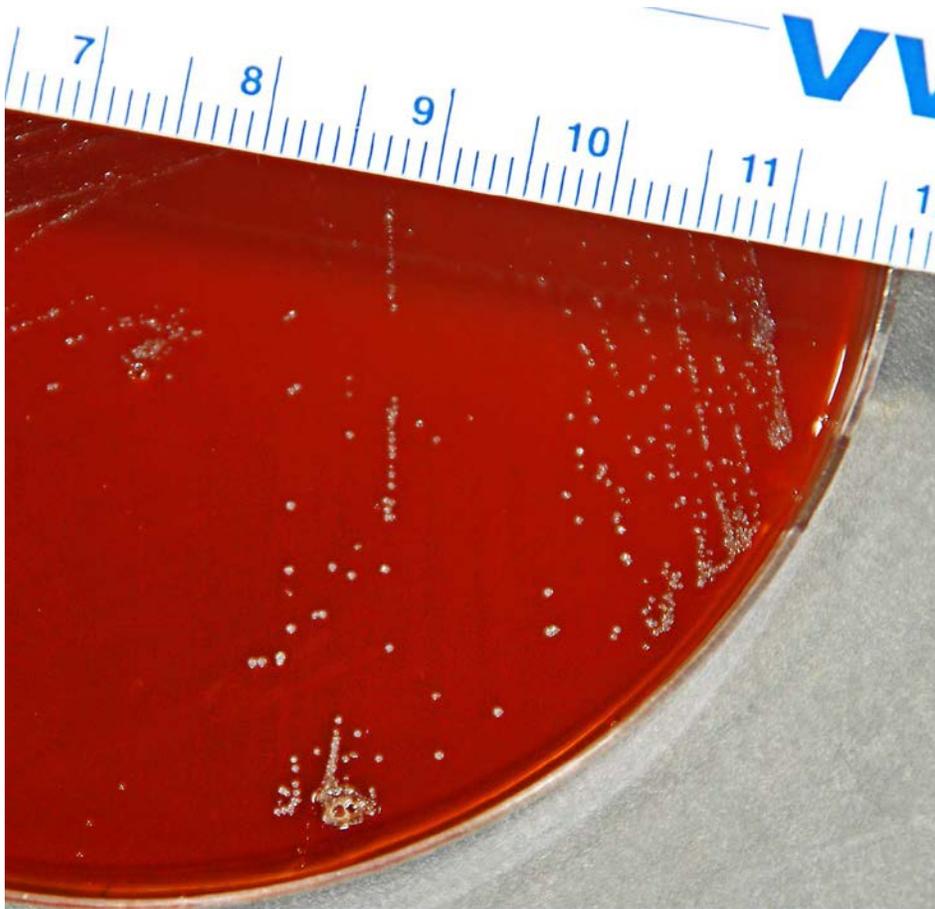


Figure 9. *Francisella* spp. isolated from tilapia and cultured on enriched blood agar after 2 weeks incubation at 16°C. Ruler - cm.

- c. Culture of Intracellular Bacteria in Cell Culture (Lannan and Fryer 1991)
 - i. Samples of kidney, spleen, liver and blood are aseptically collected from diseased fish during either overt or covert infections as described in Section 2, 2.2.E.3 “Collection of Tissues for the Detection of Viral Agents.” DUE TO POSSIBLE SENSITIVITY OF *Francisella* spp. TO ANTIBIOTICS *IN VITRO*, ANTIBIOTICS SHOULD NOT BE USED DURING COLLECTION OF TISSUES OR IN CELL CULTURE MEDIA. Because no antibiotics will be used, stringent aseptic techniques must be used to collect tissues, and during processing to inoculate cell cultures.
 - ii. Tissue should be homogenized in 1:20 (w/v) sterile antibiotic-free HBSS. Do not centrifuge. *Francisella* spp. cells may be bound in membranes and centrifugation will remove the bacteria from the supernatant. The homogenate should be diluted 1/5 and 1/50 in antibiotic-free HBSS. These final two dilutions of the homogenate are the inocula for cell cultures, and are 1:100 and 1:1,000 dilutions of the original tissue.
 - iii. The diluted homogenate can be inoculated directly (0.1 mL/ per well of a 24-well plate) into the antibiotic-free culture medium overlaying the cell monolayer (suggested cell

lines are: Chinook salmon embryo (CHSE-214), salmon head kidney (SHK-1), and Atlantic salmon kidney (ASK) cells (refer to Section 2, 4.3 Cell Culture). Inoculation onto cell lines developed from the affected species may also be attempted.

- iv. Incubate at 15-20°C for cold water fish samples and 25°C for warm water fish samples for 28 days and observe for cytopathic effect (CPE). CPE may first be observed 7 days post inoculation at 23°C and 11 days at 10°C. With time, the CPE will progress until the entire cell sheet is destroyed. A drop of the suspect cell culture supernatant can be dried on a glass slide, fixed with methanol and stained with Giemsa stain (described below). Examine for presence of the bacteria.
- v. If CPE does not occur (except in positive control) cultures should be incubated for an additional 14 days.
- vi. If CPE is absent after the 42 day combined incubation period, samples are reported as negative by the cell culture method and may be discarded using the proper decontamination procedures. If CPE associated with the bacteria occurs at any time during this assay, it is considered a PRESUMPTIVE positive and the identification of *Francisella* spp. should be confirmed by the appropriate method.

2. Confirmatory tests

a. Serological methods

Presently, a serological test to confirm *Francisella* spp. is not available.

b. Polymerase Chain Reaction (PCR)

A species-specific PCR for the *Francisella* spp., infecting fish in Taiwan, has been developed and is described below (Hsieh et al 2006, 2007). In addition, the 16S rRNA gene can be amplified using universal eubacterial primers with the amplicons subsequently sequenced to confirm the identity of the isolated pathogen. Also, a *Francisella* genus-specific PCR can be performed using primers developed by Forsman et al 1994.

i. Preparation of Infected Cell Culture Supernatant or Tissue

Use of a commercially available spin column to purify DNA from cell culture supernatant or tissue is recommended for PCR sample preparation. In addition to following the manufacturer's instructions on use of the columns, initial digestion of the sample in lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, 4 mg/mL Proteinase K at 37°C for 30 minutes is suggested (refer to Section 2, 3.5.B.2 "Nested Polymerase Chain Reaction (PCR) for Confirmation of *R. salmoninarum* DNA").

ii. The cell culture supernatant preparation:

Triturate the overlying cell culture media to disrupt the suspect cell culture, as if you were preparing a re-inoculation in a virology assay.

Transfer 0.2 mL of the suspension to a clean sterile 1.5 mL microcentrifuge tube and centrifuge at 4°C.

Discard the supernatant and treat the pellet as a tissue sample in the extraction procedure.

- iii. Amplification using *Francisella* species-specific primers, universal eubacterial 16S rRNA primers or *Francisella* genus-specific primers:

General QA/QC considerations must be reviewed before performing PCR (see Section 2, Chapter 6 Polymerase Chain Reaction (PCR) for more specific QA/QC considerations for PCR).

Procedures for the initial round:

- a. Using Section 2, 3.A3.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.
- b. Using Section 1.2.12.1–Initial Amplification of Nucleic Acid by PCR for the Confirmation of *Francisella* spp., record the date of assay and calculate the amount of each reagent to go into the “Master Mix” (MM) according to the number of samples to be processed and the amount of MM needed per reaction (45 μ L). Add four to the number of samples so that there is enough to run controls.
- c. Working in a sterile hood, add PCR reagents except for sample DNA to the MM tube in the order listed in Section 1.2.12.2 - 2nd Amplification of Nucleic Acid by PCR for the Confirmation of *Francisella* spp., adding water first and Taq last. Keep all reagents cold during mixing, and return them to the freezer immediately after use. Do not expose enzymes, primers, or dNTP’s to UV light.

Water to make a 45 μ L total volume per reaction.

1X PCR buffer
1.5 mM MgCl₂.
dNTP mix (0.2 μ M).

Primers (1 μ M each)

Francisella species-specific primers

FLB16S180f 5'-GCG-GAT-TAA-AGG-TGG-CCT-TTG-C-3'
FLB16S465r 5'-CCT-GCA-AGC-TAT-TAA-CTC-ACA-GG-3'

or

Universal Bacterial 16S rDNA PCR

EubA (1522R) 5'-AAG-GAG-GTG-ATC-CAN-CCR-CA-3'
EubB (27F) 5'-AGA-GTT-TGA-TCM-TGG-CTC-AG-3'

or

Francisella genus-specific PCR

F11 5'-TAC-CAG-TTG-GAA-ACG-ACT-GT-3'
F5 5'-CCT-TTT-TGA-GTT-TCG-CTC-C-3'.

Taq polymerase (2.5 units per reaction).

- d. Place 45 μ L of MM into each 0.2 mL PCR tube and close the caps tightly. Move PCR tubes to sample loading area.

- e. In sample loading area, load 5 μ L of each sample DNA to the appropriately labeled PCR tubes. Close caps tightly.
- f. Load the sample tubes into the thermocycler wells.

The thermocycler should be programmed for the following regime if utilizing:
***Francisella* species-specific primers:**

Denature the mixture at 94°C for four minutes.
Amplify by 35 cycles of:
 Denaturing at 94°C for 30 seconds.
 Annealing at 58°C for 30 seconds.
 Extending at 72°C for 30 seconds.
Post-dwell at 4 to 15°C after cycling is complete.

Continue with protocols described in step iv. below.

The thermocycler should be programmed for the following regime if utilizing the **16S rDNA universal primers:**

Denature the mixture at 94°C for four minutes.

Amplify by 30 cycles of:
 Denaturing at 94°C for 30 seconds.
 Annealing at 60°C for 1 minute.
 Extending at 72°C for 1 minute.
Post-dwell at 4 to 15°C after cycling is complete.

Continue with protocols described in step iv. below.

The thermocycler should be programmed for the following regime if utilizing the
***Francisella* genus-specific primers:**

Denature the mixture at 94°C for four minutes.

Amplify by 35 cycles of:
 Denaturing at 94°C for 1 minute.
 Annealing at 65°C for 1 minute.
 Extending at 72°C for one minute.
 Post-dwell at 4 to 15°C after cycling is complete.

Continue with protocols described in iv. below.

- iv. Visualization of PCR product by electrophoresis (see Section 2, Chapter 6 Polymerase Chain Reaction (PCR) for general procedures).

Visualization of amplified products resulting from PCR for detection of *Francisella* spp. DNA is best accomplished after electrophoresis on a 2% agarose gel (Section 2, 6.3C “Detection of Product”).

- a. Using Section 2, 3.A3.G Worksheet G – Photodocumentation of the PCR Product Gel, record location of each sample on the agarose gel at the time samples are loaded.
- b. After electrophoresis, stain gel with ethidium bromide and visualize on an UV transilluminator.
- c. Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at 1540 bp. Note any unusual band occurrences. Negative controls should not have any bands. Suspicion of contamination indicates that PCR should be re-run on samples from the extracted DNA tube.
- d. Document the electrophoresis results (Section 2, 6.3.G “Visualize the DNA”). Photograph all gels and attach the photo to Section 2, 3.A3.G Photodocumentation of the PCR Product Gel. Attach to case history information.

Amplified PCR products of the proper size ~1540 bp (Universal PCR), or ~1140 bp (*Francisella* genus-specific PCR), or 286 bp (*Francisella* species-specific PCR) can then be submitted to a commercial or academic laboratory for sequencing. Derived sequences can be compared to known 16S rDNA sequences by performing a Blast search through the National Center for Biotechnology Information (NCBI) site, <http://www.ncbi.nlm.nih.gov/BLAST/>.

3. In situ hybridization

In situ hybridization (ISH) utilizing a Digoxigenin-labeled probe, generated by PCR using the *Francisella* spp-specific primer set, has been found to be useful for confirmation of suspect histologic lesions or positive PCR findings (Hsieh et al 2006, 2007). Tissues that have been examined include the brain, eye, gill, kidney, spleen, liver, heart and digestive tract.

F. Procedures for Detecting Subclinical Infections

In *Francisella*-suspect populations from endemic geographic regions or hatcheries with past histories of infection, any or all of the previously-described diagnostic protocols may be used to detect suspected subclinical infections. Since clinical expression of this disease appears to be stress-related, the aquaculturist may want to stress a small subsample of a suspect fish population in order to induce disease expression. In Hawaii, clinical disease was successfully induced in a sample of fish from a suspect population exposed to suboptimal water temperatures (Mauel et al 2003). Considering the high assay sensitivity and their ability to detect bacteria in low numbers, the genus-specific *Francisella* spp PCR or *in situ* hybridization, using the *Francisella* spp-specific PCR amplicon as the probe assay, could also be attempted on tissues and blood from suspect fish.

G. Procedures for Determining Prior Exposure to Etiological Agent

No procedures have been reported.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of Etiological Agent

See Section 1, 1.1.1 General Procedures for Bacteriology.

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