1.2.3 Columnaris Disease

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A. Name of Disease and Etiological Agent

Columnaris disease is caused by the Gram-negative bacterium *Flavobacterium columnare* (Bernardet et al. 1996). The bacterium has been reclassified several times and was formerly referred to as *Bacillus columnaris*, *Chondrococcus columnaris*, *Cytophaga columnaris*, and *Flexibacter columnaris*.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Columnaris disease has been reported in North and South America, Europe, Asia, Africa, and Australia. Given these reports, it is generally assumed that it has a worldwide distribution.

2. Host Species

Most freshwater fishes (cultured and wild) are considered susceptible to *F. columnare*.

C. Epizootiology

Columnaris disease can affect fish of all ages but is more prevalent in young fish. The severity and occurrence of columnaris disease is generally greater at warmer water temperatures (> 20 °C); however, the disease can occur in salmonids reared at 12-15°C. Columnaris disease can occur in fish without any predisposing conditions but outbreaks are commonly associated with stressful rearing conditions such as low dissolved oxygen, high ammonia and nitrite concentrations, and overcrowding. Additionally, handling and injuries to the skin/mucosa may predispose fish to columnaris disease. Mortality patterns can be acute, sub-acute, or chronic, depending on the particular conditions of the epizootic and the fish species involved. Mortality rates can be extremely high, with 60 to 90% mortality common. Columnaris disease is transmitted horizontally from fish to fish. The bacterium appears to be ubiquitous in the aquatic environment and studies have shown that fish can serve as reservoirs of the pathogen. Research has demonstrated that *F. columnare* can maintain viability in water for long periods of time

depending on the water chemistry. High levels of hardness (> 50 mg/L $CaCO_3$) and organic matter have been associated with good survival of the bacterium in water (Fijan 1968). Chowdhury and Wakabayashi (1988) demonstrated that formulated water containing 0.03% NaCl, 0.01% KCl, 0.002% $CaCl_2$ $2H_2O$, and 0.004% $MgCl_2$ $6H_2O$ enhanced the survival of *F. columnare* compared to distilled water and formulated water containing higher concentrations of sodium, potassium, calcium and magnesium.

D. Disease Signs

1. Behavioral Signs

Fish may become lethargic, exhibit a loss of appetite, and hang at the surface.

2. Gross Signs

In general, gross signs of columnaris disease are easily recognized and include frayed fins, depigmented lesions on the skin, and necrotic gill lesions. Skin lesions often begin around the dorsal fin and then increase in size and result in a gray to white lesion that has the appearance of a saddle (i.e., saddleback lesion) (Figure 1 and Figure 2). These lesions can also form on other portions of the caudal peduncle (Figure 2 and Figure 3). In some cases the margin of the lesion may be yellow in appearance due to the proliferation of the yellow-pigmented bacterium (Figure 4). Skin lesions can also occur initially as grayish-white cutaneous foci on the fins, head, and body (Figure 5). The foci may enlarge to be several centimeters in diameter, and skin in the affected area may be eroded, resulting in shallow ulcers (Figure 6). Gill tissue can exhibit severe necrosis and may appear white to brown and also yellowish due to the presence of large quantities of the bacterium (Figure 7, Figure 8, and Figure 9). Internal lesions are usually not present. Although these are the classical signs, in some cases diseased fish may die without any gross signs.

Figure 1. A bluehead chub *Nocomis leptocephalus* with multiple saddleback lesions. Picture by Andy Goodwin.

Figure 2. Rainbow trout *Oncorhynchus mykiss* exhibiting the characteristic saddleback lesion associated with columnaris disease. Pictures by Benjamin LaFrentz.

Figure 3. Hybrid tilapia *Oreochromis niloticus* × *O. aureus* exhibiting depigmented lesions on the caudal peduncle due to columnaris disease. Picture by Benjamin LaFrentz.

Figure 4. Skin lesions on channel catfish *Ictalurus punctatus* exhibiting a yellow color due to large numbers of *F. columnare* present. Picture by Andy Goodwin.

Figure 5. Focal skin lesion on the head of a channel catfish infected with *F. columnare*. Picture by Andy Goodwin.

Figure 6. Shallow skin ulcers in a channel catfish infected with *F. columnare*. Picture by Andy Goodwin.

Figure 7. Necrotic gills in a rainbow trout infected with *F. columnare*. Picture by Benjamin LaFrentz.

Figure 8. Necrotic gill lesions on a koi *Cyprinus carpio* infected with *F. columnare*. Picture by Andy Goodwin.

Figure 9. Necrotic gill lesions on a channel catfish infected with *F. columnare*. Picture by Andy Goodwin.

3. **Microscopic Signs**
   Wet mounts of gill tissue or skin lesions from diseased fish will reveal long slender rod shaped bacteria that often aggregate into columns or 'haystacks' of cells (Figure 10 and Figure 11), thus the name columnaris disease. Haystacks may vary in size, shape, and distribution (Figure 10 and Figure 11), but *F. columnare* cells on the haystack surface will be vigorously waving (Video 1). Haystacks may not always be obvious. Note that *F. columnare* cells float and can often be found against the underside of the cover glass where they may exhibit the "gliding motility" typical of this species (Video 2). Gliding motility is not always evident

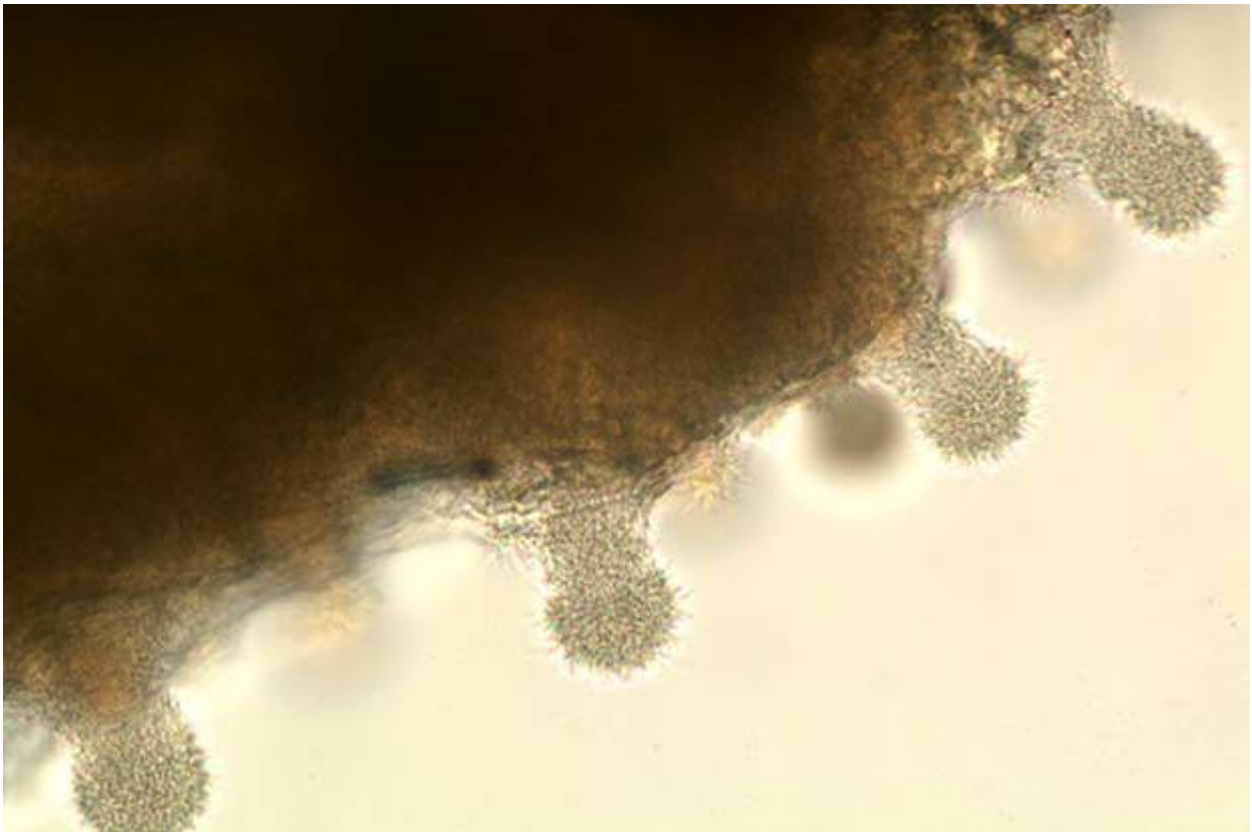

Figure 10. Wet mount of gill tissue from a koi infected with *F. columnare*. The cells have aggregated into 'haystacks'. Picture by Andy Goodwin.

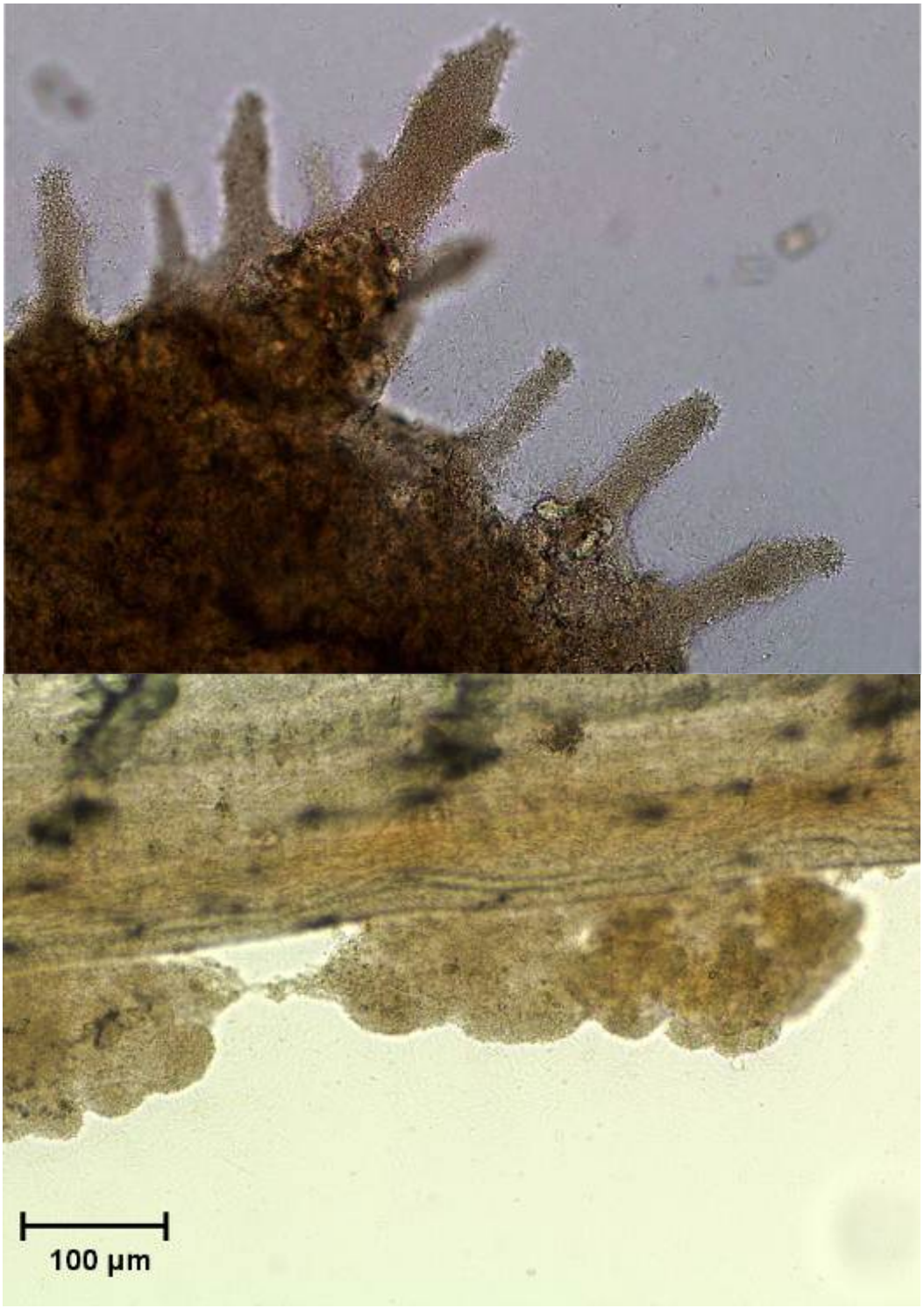

Figure 11. Variations in 'haystack' formations that may be observed in wet mounts. Pictures by Andy Goodwin.

E. Disease Diagnostic Procedures

Diagnosis is based on clinical signs characteristic of columnaris disease and isolation/identification of the etiological agent.

1. Presumptive Diagnosis

a. Observation of gross signs of disease
Moribund fish should exhibit the gross signs characteristic of columnaris disease as described in Section D, Disease Signs.

b. Presence of long, thin Gram-negative rods in lesions
Wet mounts of gill and/or skin lesions prepared on microscope slides should exhibit *F. columnare* as described in Section D.3., Microscopic Signs.

c. Isolation of putative *F. columnare* colonies
Primary isolation of *F. columnare* should be from the gills, lesions, and/or internal organs. The head kidney and spleen are good organs to target for isolation of *F. columnare* and it may be easier to obtain pure cultures from these. Specialized low nutrient microbiological media are needed to support the growth of *F. columnare*, such as tryptone yeast extract salts agar (TYES), cytophaga agar, Hsu-Shotts agar (see formulation for these media in Section 1, 1.1.1 General Procedures for Bacteriology), or modified Shieh agar (LaFrentz & Klesius 2009). Cultures should be incubated at 20-30°C for 1 to 3 days. The addition of tobramycin at a concentration of 1 μg/mL to the aforementioned media enhances the isolation of *F. columnare* from clinical specimens by reducing the growth of environmental bacteria (Decostere et al. 1997). Additionally, the selective medium developed by Hsu and Shotts also enhances isolation. *Flavobacterium columnare* produces flat, rhizoid, yellow colonies with irregular margins that tightly adhere to the agar (Figure 12 and Figure 13). Colony morphologies and adherence to agar can vary depending on the isolate and moisture content of agar plates used. Other colony morphologies can occur and may include round and smooth yellow colonies (Figure 14), and slightly rhizoid to non-rhizoid colonies (Figure 15). In Gram-stains, cells of *F. columnare* are Gram-negative rods and typically 3 to 10 μm long by 0.3 to 0.7 μm wide (Figure 16). Longer cells may occur with lengths of up to 20 μm.

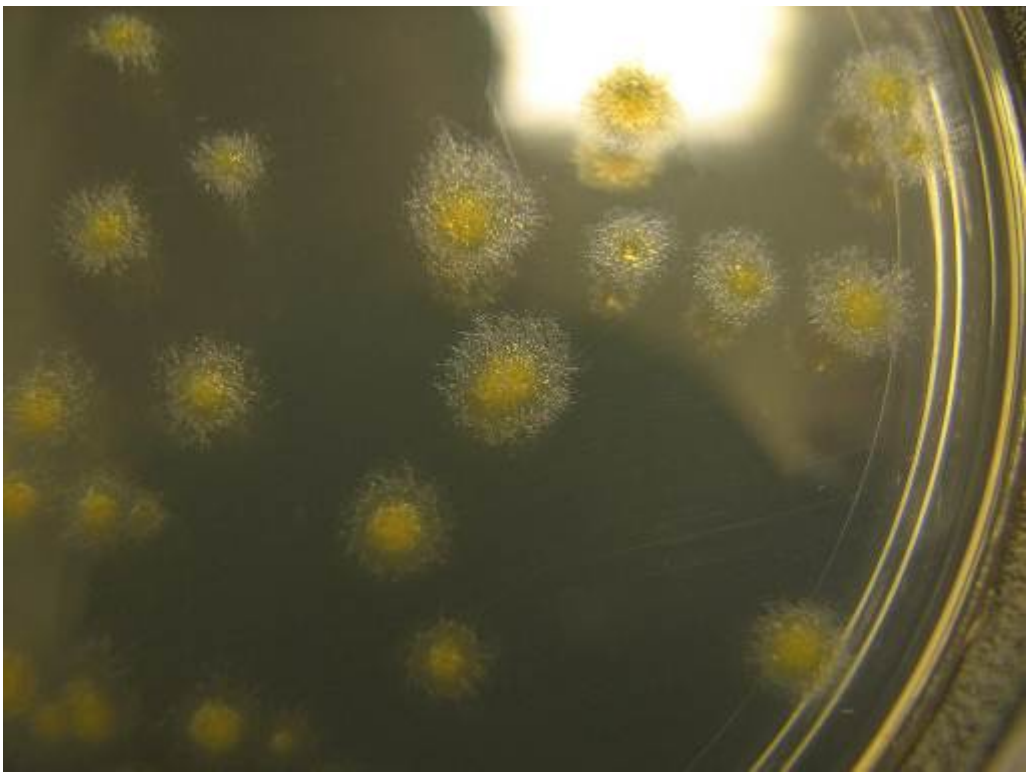

Figure 12. *Flavobacterium columnare* colonies exhibiting the characteristic yellow, flat, and rhizoid morphology with irregular margins. Picture by Benjamin LaFrentz.

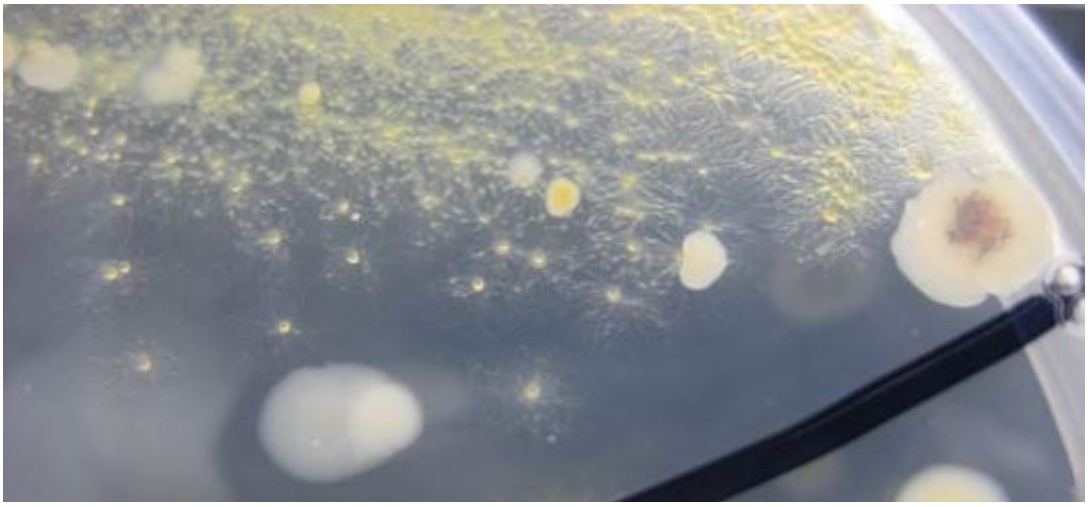

Figure 13. *Flavobacterium columnare* isolated from a skin lesion of a walleye *Sander vitreus* exhibiting columnaris disease. A portion of the skin lesion was scraped with an inoculating loop and plated onto modified Shieh agar containing tobramycin (1 µg/mL). Picture by Benjamin LaFrentz.

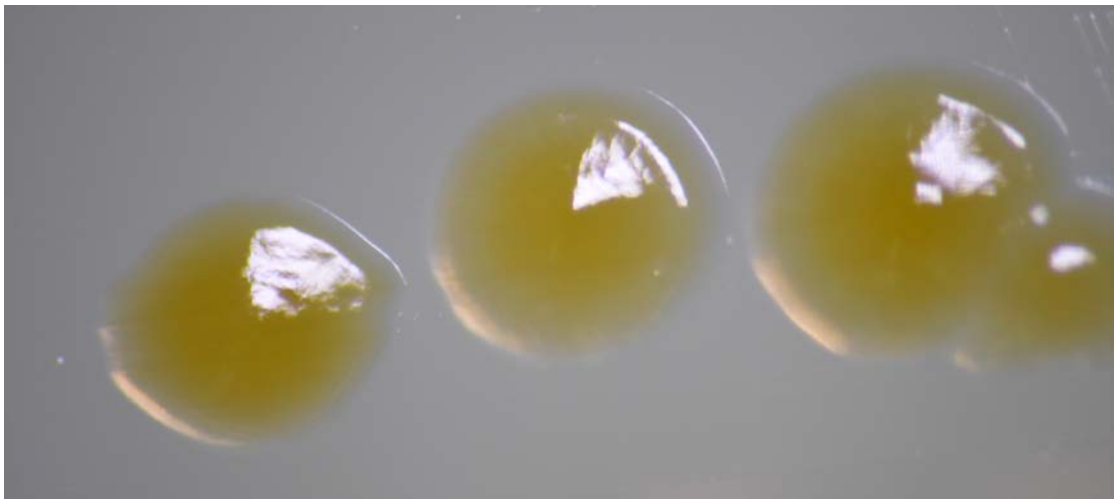

Figure 14. Round and smooth yellow colonies produced by some isolates of *F. columnare*. Picture by Benjamin LaFrentz.

Figure 15. Non-rhizoid (left) and slightly rhizoid (right) colonies produced by some isolates of *F. columnare*. Pictures by Benjamin LaFrentz.

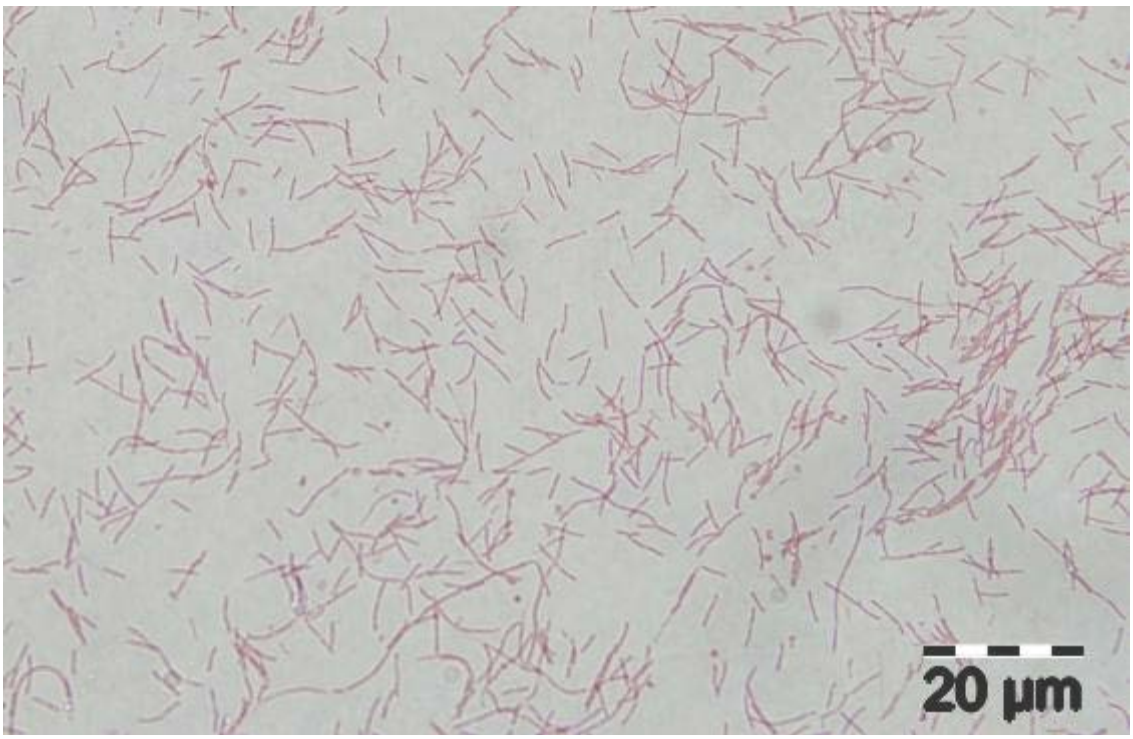

Figure 16. Cell morphology of *F. columnare* following Gram staining and light microscopy. Cells were obtained following culture in TYES broth. Picture by Benjamin LaFrentz.

2. Confirmatory Diagnosis

a. Griffin Screen

Confirmation of a putative *F. columnare* isolate may be accomplished by using the Griffin screen (Griffin 1992). The Griffin screen takes advantage of five characteristics that in combination are unique to *F. columnare*: (1) the ability to grow in the presence of neomycin sulfate and polymyxin B, (2) color and colony morphology consistent with *F. columnare*, (3) production of a diffusible, gelatin-degrading enzyme, (4) binding of Congo red dye in the surface secretions of the colony, and (5) production of a diffusible enzyme that degrades chondroitin sulfate A.

b. Polymerase Chain Reaction

Several *F. columnare* specific PCR protocols have been developed, but it should be noted that some were designed using a *F. johnsoniae* DNA sequence that was misidentified as *F. columnare* (see Darwish et al. 2004). The PCR protocols developed by Triyanto et al. (1999), Darwish et al. (2004), and Welker et al. (2005) are appropriately designed. The PCR developed by Welker et al. (2005) has been tested and used for amplification of *F. columnare* DNA in tissue samples from diseased fish as well as from pure bacterial cultures. The sensitivity of the protocol, determined by spiking fish tissues with *F. columnare*, was reported to range from 30 to 59 colony-forming units per mg of tissue. The protocol for this PCR is described below.

1. DNA Extraction From Fish Tissues Or Cultured Cells

DNA is extracted from fish tissues (gill, skin lesions, internal organs) or bacterial cells using a commercially available kit (eg., DNeasy Blood and Tissue kit, Qiagen, Valencia,

CA, USA) following the manufacturer's protocol. The concentration of the extracted DNA is determined by using a spectrophotometer.

2. **PCR Conditions**
The PCR conditions below were adapted from Welker et al. (2005) and use a Hotstar Taq plus Master Mix kit (Qiagen). **NOTE:** If a different Taq polymerase is used, the Master Mix cocktail will need to be modified according to manufacturer's directions.

Master Mix Cocktail: For a 20 µL reaction, combine 10 µL of 2X Master Mix, 0.8 µL of the forward and reverse primers (final concentration of 0.4 µM each), 2.0 µL of Coral Load (10X), 3.9 µL of water, and 2.5 µL of template DNA (approximately 50 ng).

Forward Primer (FCISRFL): 5'- TGCGGCTGGATCACCTCCTTTCTAGAGACA -3'

Reverse Primer (FCISRR1): 5'- TAATYRCTAAAGATGTTCTTTCTACTTGTTTG -3'

Controls: Proper controls should be used for every PCR. Negative controls may include no template, DNA extracted from normal healthy tissues, and/or DNA extracted from bacteria other than *F. columnare*. Positive controls may include DNA extracted from normal healthy tissues spiked with *F. columnare*, DNA extracted from fish tissues infected with *F. columnare*, and/or DNA extracted from *F. columnare* cells.

Cycling Conditions: The thermocycler conditions consists of: 5 min at 95°C; 40 cycles of 30 s at 94°C, 45 s at 55°C, and 60 s at 72°C; final cycle of 10 min at 72°C.

3. **Interpreting the Results**
Following PCR and agarose electrophoresis, positive results are indicated by a specific PCR product with a molecular weight of 500-550 base pairs. PCR amplification of a few unique *F. columnare* isolates using this protocol has resulted in two PCR products with sizes of approximately 450 and 475 base pairs (Welker et al. 2005). The negative control reactions should contain no specific PCR products.

F. Procedures for Detecting Subclinical Infections

A quantitative PCR assay has been developed for *F. columnare* (Panangala et al. 2007) and tested for sensitivity in bacterial cultures and experimentally infected fish. It may be possible to detect subclinical infections using this assay, but it has not been tested for this purpose.

G. Procedures for Determining Prior Exposure to the Etiological Agent

Enzyme-linked immunosorbent assays (ELISA) have been developed for detecting serum antibodies specific for *F. columnare* in channel catfish (Shoemaker et al. 2003) and tilapia (Grabowski et al. 2004). Assays for determining agglutinating antibody titers have also been developed in rainbow trout and coho salmon (Fujihara and Nakatani 1971).

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

See Section 1, 1.1.1 General Procedures for Bacteriology for general information. Fresh moribund fish should be used for presumptive diagnosis and re-isolation of *F. columnare*. If fish cannot be processed immediately, specimens may be held at 4°C. Fresh tissues should be used for DNA extraction. If tissues cannot be processed immediately, they may be held at 4°C. It may be possible to store tissues in 95% ethanol or frozen for later DNA extraction. However, some research has suggested that freezing tissues can make detection by PCR difficult (Suomalainen et al. 2006). *Flavobacterium columnare* cultures on agar plates tend to lose viability over the course of 3 to 6 days, and care must be taken to properly maintain these. Specific protocols for the laboratory maintenance of *F. columnare* are available (Cain and LaFrentz 2007).

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