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[Article]

A Piscirickettsiosis-like Syndrome in Cultured Nile Tilapia in Latin America with *Francisella* spp. as the Pathogenic Agent

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Abstract.—In 2004, cultured Nile tilapia *Oreochromis niloticus* in several Latin America farms began to succumb to a disease similar to the piscirickettsiosis-like syndrome previously reported in tilapia in Taiwan and the United States. Mortality increased during 2005; reductions in tilapia biomass ranged from 5% to 80% in individual ponds and averaged 50% overall. All ages of fish have been involved. Clinical signs include lethargy, loss of appetite, petechia, exophthalmia, and abnormal swimming behavior. Gross lesions have included splenomegaly, renomegaly, and numerous white nodules observed in the spleen, kidney, testes, heart, ovaries, and occasionally the liver. A previously unreported black granulomatous lesion was reported in up to 30% of the fillets. Histologically, granulomatous infiltrates were observed in the kidney, spleen, liver, testes, ovary, and choroid gland, and rarely in the brain and heart. A small pleomorphic bacterium was observed in Giemsa-stained blood smears and spleen imprints. The bacterium did not grow on standard microbiological media and has not been isolated in cell culture. We obtained a near-complete 16S ribosomal DNA sequence with high similarity to *Francisella* spp. sequences previously identified in tilapias *Oreochromis* spp. (Taiwan), Atlantic cod *Gadus morhua* (Norway), and three-line grunts *Parapristipoma trilineatum* (Japan).

During the last decade, epizootics in tilapias *Oreochromis* spp. caused by fastidious intracellular bacteria have been reported in Taiwan, Hawaii, and the continental United States (Chen et al. 1994; Chern and Chao 1994; Mauel et al. 2003, 2005**;** Hsieh et al. 2006). In addition, similar bacteria have been reported in cultured European seabass *Dicentrarchus labrax* and white seabass *Atractoscion nobilis* (Comps et al. 1996; Athanassopoulou et al. 1999; Chen et al. 2000a), blue-eyed plecostomus *Panaque suttoni* (Khoo et al. 1995), three-line grunts *Parapristipoma trilineatum* (Fukuda et al. 2002; Kamaishi et al. 2005), the grouper *Epinephelus melanostigma* (Chen et al. 2000b), and Atlantic cod *Gadus morhua* (Nylund et al. 2006; Olsen et al. 2006). Such reports demonstrate the global distribution of these pathogenic bacteria, which occur in a variety of fresh- and saltwater fish species. Mortalities in these epizootics have ranged from less than 1% to upwards of 90%.

Clinically symptomatic tilapia are dark, have abnormal swimming behavior, and often have petechiae and exophthalmia. The gills exhibit epithelial hyperplasia and mild to severe consolidation of secondary lamellae. Two hallmarks of this syndrome in tilapia are splenomegaly (enlargement of 5–50-fold) and numerous granulomas present in the internal organs, especially the spleen and kidney. The liver and heart are exceptional in that few to no granulomas are present (Chen et al. 1994; Chern and Chao 1994**;** Mauel et al. 2003, 2005).

Horizontal transmission by waterborne or direct

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contact has been demonstrated by cohabitation of infected fish and uninfected fish (Chen et al. 1994; Chern and Chao 1994; Mauel et al. 2003). In temperature range experiments in Hawaii (Mauel et al. 2003), tilapia were maintained between 21.5°C and 26.3°C and the first mortalities occurred on day 15; mortality doubled almost daily thereafter. Tilapia maintained between 26.5°C and 29.2°C showed no mortality (Mauel et al. 2003).

In this report, we describe the clinical signs and gross and histological lesions associated with a piscirickettsiosis-like disease caused by *Francisella* spp. in farmed Nile tilapia *O. niloticus* in Latin America.

Methods

Nile tilapia in three aquaculture facilities in Central America began experiencing an epizootic in March 2004 that continues to the present time. The fish had been maintained in several pond types ranging from intensive ponds with four water changes per hour to extensive ponds with one water change per 10 d. Initially, the disease was noted in several intensive ponds on one farm but then spread throughout that farm and to the other two farms within a few months. Increases in mortality were observed after transfer of fish from pond to pond and during times of increased water turbidity and either decreasing or increasing water temperature. Temperatures in the water supply canal ranged from 21°C to 28°C; during times of shift, temperature could change by several degrees within a 24–48-h time period. Escaped Nile tilapia in water supply canals also experienced high levels of mortality during times of temperature change and high water turbidity.

Necropsy and bacteriology.—Three-hundred Nile tilapia were collected from the three facilities affected over a 1.5-year period and were subjected to routine necropsy procedures. After gross observations of the fish were made, gill clippings and skin scrapings were observed microscopically. Tissues (spleen, kidney, gill, brain, and blood) from Nile tilapia demonstrating signs of disease were collected aseptically and inoculated onto tryptic soy agar (TSA), TSA with 5% bovine blood, half-strength Mueller Hinton agar, and Columbia colistin–nalidixic acid (CNA) agar. Isolates were recultured on the appropriate media and obtained in pure culture. Bacteria were identified to the species level with an API-20E (bioMerieux, Inc., France) commercial identification kit used according to the manufacturer's protocols. Sensitivity of cultured isolates to three antibiotics (Romet, Terramycin, and florfenicol) was determined by use of the antimicrobial disk diffusion method (NCCLS 2003).

Histology.—The spleen, kidney, gill, brain, ovary, testis, muscle, liver, other internal organs, and occasionally the whole euthanatized fish (≤6 cm) were fixed in neutral buffered 10% formalin; embedded in paraffin; sectioned; stained with hematoxylin and eosin, Giemsa, Kinyoun's Acid Fast, or Brown and Brenn; and observed by light microscopy. Smears of blood taken from the caudal vein and impression smears of the kidney and spleen were air dried, fixed in acetone for 10 min, stained with Wright–Giemsa and observed by light microscopy.

Piscirickettsia salmonis-specific polymerase chain reaction and fluorescent antibody test.—Tissues were prepared, DNA extracted, and *Piscirickettsia salmonis*-specific polymerase chain reactions (PCRs) were performed as previously described (Mauel et al. 1996). Tissue sections and tissue presses were prepared, and a *Piscirickettsia salmonis*-specific fluorescent antibody test (FAT) was performed as described by Lannan et al. (1991). Anti-*Piscirickettsia salmonis* rabbit serum was provided by the late Dr. J. L. Fryer (Oregon State University, Corvallis).

16S ribosomal DNA sequencing.—Bacterial genomic DNA was isolated from aseptically collected tissues and blood from infected Nile tilapia by using DNA-Stat 60 (Tel-Tex, Inc., Friendswood, Texas, USA) according to the manufacturer's protocol. To amplify the 16S ribosomal DNA (rDNA) gene, 5 μL of the DNA was added to 45 μL of reaction mixture, which consisted of 5 μL Promega 10× PCR buffer B; 200 μmol of each deoxynucleotide triphosphate; 1 μmol of universal primer 27F (Lane 1991); 1 μmol of universal primer 1518R (Lane 1991); and 1.5 units of *Taq* DNA polymerase (Promega). The PCR amplification was accomplished by the following protocol: denaturing for 5 min at 94°C; 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min; and an extension period at 72°C for 7 min.

An aliquot (10 μL) of the PCR reaction mixture was size fractionated in a 1.5% agarose, 1× tris acetate–EDTA, pH 8.0 (40 mM tris acetate, 1 mM EDTA) gel containing 0.2 μg/mL ethidium bromide and was photographed under ultraviolet transillumination to confirm amplification of the correct product size and purity. The remaining PCR product was purified by using the PCRquick Kit (Qiagen, Valencia, California) according to the manufacturer's instructions. Three PCRs were performed with tissue from the kidneys of three separate Nile tilapia, and the amplicons were sequenced by using the PCR primers and the universal bacterial sequencing primers 519F, 519R, 1100F, and 1100R (Lane 1991) on an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, California). The generated sequence segments were assem-

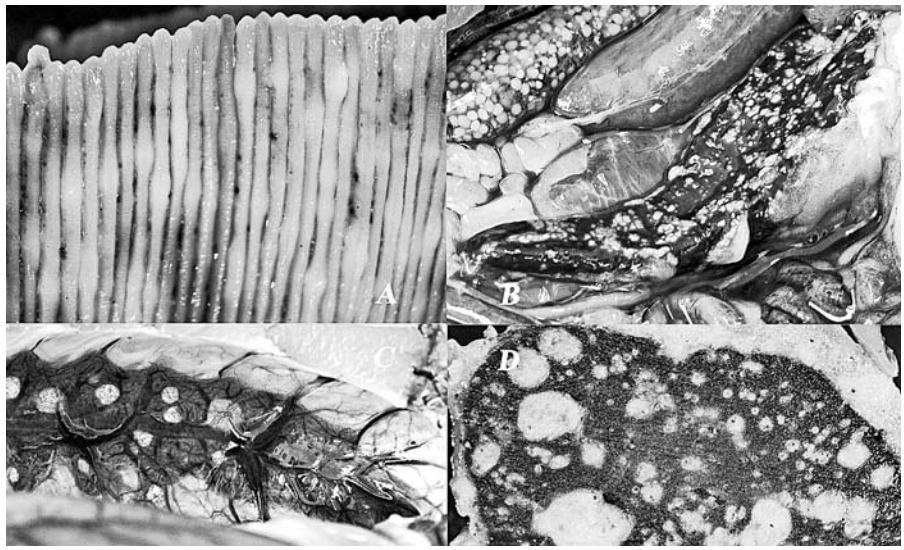

FIGURE 1.—Selected gross lesions observed in Nile tilapia affected by a piscirickettsiosis-like syndrome in Latin American fish farms: **(A)** white nodules in gills; **(B)** splenomegaly with white nodules; **(C)** renomegaly with white nodules; **(D)** individual to coalescing white nodules in cross-sectioned spleen.

bled, and a consensus sequence was generated from the three sequences by using Lasergene (DNASTAR, Inc., Madison, Wisconsin) software.

Phylogenetics.—The BLAST program was used to compare the consensus sequence with sequences from the National Center for Biotechnology Information nucleotide database; the most similar sequences were downloaded and aligned using the Clustal W application of the MEGA (Molecular Evolutionary Genetics Analysis) package (version 3.1; Kumar et al. 2004). The following bacteria sequences (with GenBank accession numbers) were obtained and used in the construction of phylogenic trees: *Francisella philomiragia* (AY243027); *F. tularensis* (AY243028); *Francisella* spp. isolates Ehime-1 (AB194068), AF-04–405 (DQ007455), AF-01–22 (DQ007456), CYH-2002 (AF385857), and Atlantic cod (DQ309246); tilapia parasite TPT-541 (AF206675); *Wolbachia persica* (M21292); *Piscirickettsia salmonis* isolates LF-89 (PSU36941), NOR-92 (PSU36942), EM-90 (PSU36940), ATL-4–91 (PSU36915), SCO-95A (AY498636), SCO-02A (AY498634), IRE-99D (AY498637), and IRE-91A (AY498633); uncultured *Piscirickettsia salmonis* Greece (AY542956); and *Rickettsiella grylli* (U97547). The alignment was used in maximum-parsimony and distance analysis within the MEGA package. Phylogenetic trees were calculated with the distance method (neighbor-joining algorithm) and *p*-distance model in the MEGA package; bootstrap analysis (1,000 replicates) was performed with the *p*-distance model in the MEGA package.

Results

Clinical Signs and Gross Pathology

Affected fish in all locations displayed similar clinical signs and lesions. Infected Nile tilapia were pale and sometimes emaciated, often swam erratically, and appeared to have difficulty staying at depth. At necropsy, the skin mucus was lower than normal and the skin exhibited focal areas of hemorrhage, erosions, loss of scales, and in severe cases, ulceration. Frequently, the lesions were infected with opportunistic pathogens, such as *Flavobacterium columnare, Epistylis*, and oomycetes (*Saprolegnia* spp.). Exophthalmia was common, as were enlarged spleens (~5–50 times normal) with multiple white granulomas (Figure 1). The gills had epithelial hyperplasia, occasional multifocal consolidation of secondary lamellae, and occasional necrosis with a patchy white appearance (Figure 1).

The spleen and kidney were the primary organs affected. The spleen exhibited splenomegaly with multiple white nodules (1–5 mm). In severe cases, the spleen was observed to adherence to other organs, such as the liver and intestine. The lesions in spleen were present in the capsule as well as in the splenic parenchyma. The kidney had renomegaly with multiple white nodules and focal areas of necrosis.

Black pinpoint to large (≥3 cm), granulomatous

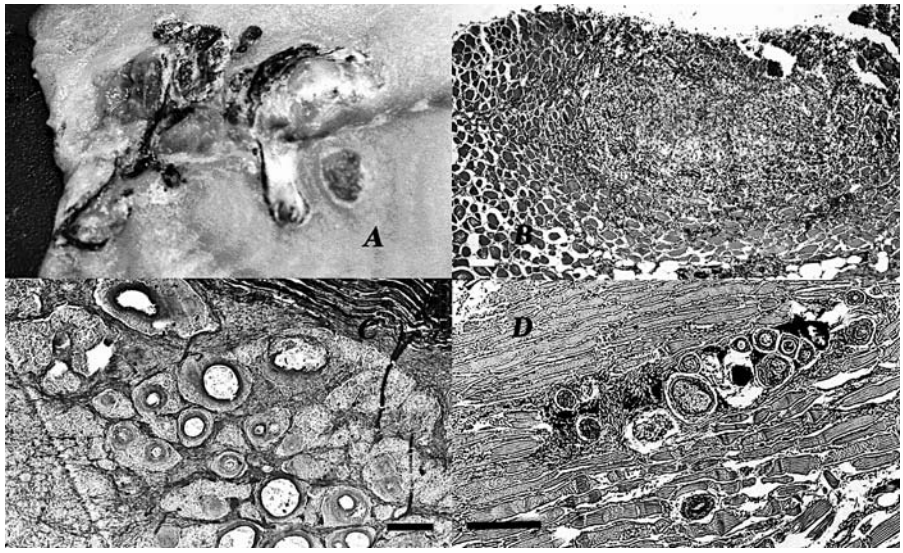

FIGURE 2.—Gross and histological lesions in the muscle of Nile tilapia affected by a piscirickettsiosis-like syndrome in Latin American fish farms: **(A)** gross lesion; **(B)** granulomatous lesion; **(C)** granulomas; **(D)** granulomas observed in small black lesions. Scale bar (B–D) is equal to 50 μm.

lesions were observed in skeletal muscle (Figure 2). The lesions varied from small white–tan nodules with a soft texture to 1–3-cm lesions with wide areas of necrosis and, occasionally, black pigmentation. The lesions in muscle were present only in adult fish (>1 kg). The percentage of fish with muscle lesions in a pond varied from nearly 0% to 14%. In the heart, small white nodules were occasionally observed grossly in the pericardium.

Flavobacterium columnare was infrequently observed microscopically, associated with necrotic gill tissue. All of the fish examined had light to moderate levels of *Trichodina* on the skin and gills.

Bacteriology

Aeromonas sobria, *Aeromonas hydrophila, Edwardsiella tarda, Serratia* spp., *Pseudomonas* spp., and *Plesiomonas shigelloides* were rarely and inconsistently isolated from the tissues of fish. All tissues cultured negative on Columbia CNA for streptococci. *Flavobacterium columnare* was occasionally isolated from gill and skin. The aeromonads and columnaris isolates were susceptible to Romet, Terramycin, and florfenicol.

Histopathology

Granulomas and mixed inflammatory infiltrates composed of mononuclear cells and neutrophils were observed in multiple tissues (Figures 1, 2). The granulomas consisted of large, foamy, vacuolated macrophages encircled by thin fibrous capsules and small cuffs of lymphocytes with fewer neutrophils. The centers of the granulomas were often completely necrotic or had small areas of necrosis that, when stained with Giemsa, revealed numerous magenta-colored bodies. These bodies were also found free in the tissue infiltrates (Figure 3). With a Brown and Brenn tissue Gram stain, the bodies stained Gram negative. Acid Fast bacterial organisms were not detected.

Granulomatous proliferative responses were often present at the base of the gill arch. The gills had large

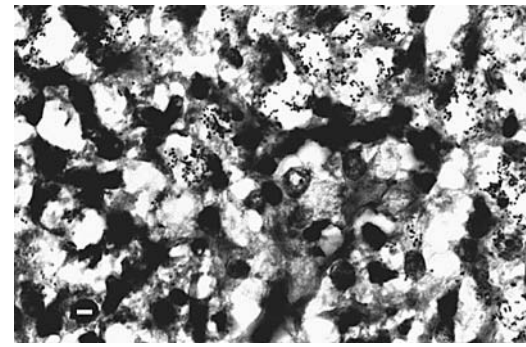

FIGURE 3.—Photomicrograph of granulomatous inflammation in the meninges of Nile tilapia affected by a piscirickettsiosis-like syndrome in Latin American fish farms; presence of Giemsa-stained bacteria is shown. Scale bar is equal to 10 μm.

TABLE 1.—Presence of granulomas in the organs of Nile tilapia ($n = 28$) affected by a piscirickettsiosis-like syndrome in Latin American fish farms.

Organ	Number (%) of fish with granulomas
Muscle	3 (10.7)
Gills	10 (35.7)
Spleen	23 (82.1)
Kidney	26 (92.8)
Liver	9 (32.1)
Heart	8 (28.5)
Testis	4 (14.2)

numbers of mixed inflammatory cells and mononuclear inflammatory cells that often contained numerous intracytoplasmic basophilic bodies. Impression smears of the gills stained with Giemsa showed these intracellular bodies as magenta-colored, irregular spheres. Cytological examination of Giemsa-stained blood smears revealed pleomorphic bacteria both in macrophages and free in the serum.

Focal to diffuse necrosis and necrotizing vasculitis were common in affected organs, especially in kidney and spleen, where intravascular coagulation resulting in fibrin thrombi in the vessels was observed. This necrosis was accompanied by a chronic inflammatory infiltrate of mononuclear cells and the formation of granulomas. In the spleen and kidney, the granulomas and histiocytic inflammation were often confluent to diffuse. Granulomatous infiltrates were also observed in choroid gland, testes, ovary tissues, liver, heart, and brain (Table 1).

The lesions present in the liver showed granuloma formation in the hepatic parenchyma. In the acute phase, the infection is characterized by the accumulation of macrophages and connective tissue around the lesion. During the chronic stages, granulomas were observed. Hepatic lipidosis was universally observed.

In the heart, the lesions were characterized by a severe infiltration of mononuclear cells, mainly macrophages with granuloma formation in the pericardium. Some granulomas were observed in the myocardium as well as activation of the macrophages. Pathological findings in the heart included epicarditis and endocarditis with an inflammatory infiltrate and fragmentation of some myocardial fibers.

In affected fish that demonstrated a severe systemic infection, the meninges presented a severe macrophage infiltration with the formation of granulomas. Only in sporadic cases was the formation of granulomas observed in the neural tissue. Lesions observed in the eye were mainly in the choroid gland, which showed severe macrophage infiltration and granuloma formation. Chronic inflammatory cells were observed in the lamina propia and submucosa of the stomach and intestine. Only in a few cases were granulomas observed in the serosa of the stomach and intestines.

Granulomas and granulomatous infiltrates were also found in the small, black and large, dark muscle lesions (Figure 2). Fillet lesions showed a lost of epidermis, necrosis, and mixed inflammatory infiltration by neutrophils and mononuclear cells. Muscle fiber necrosis was observed with a severe infiltration by macrophages and, to a lesser degree, by lymphocytes, neutrophils, and giant cells in sporadic cases. The formation of granulomas was a common finding. They were characterized by a central necrotic area with infiltration of macrophages and connective tissue proliferation around the lesion.

Examination of the spleens of 187 Nile tilapia of various ages showed a progression in the presence of granulomas according to the age of the fish: 15% in fingerling fish (1–10 g; $n = 80$), 75% in market-size fish (500–750 g; $n = 83$), and 100% in broodstock ($\geq$1,000 g; $n = 24$).

Piscirickettsia salmonis-specific PCR and FAT

No *Piscirickettsia salmonis*-specific PCR amplification product was obtained from the infected Nile tilapia tissues tested. No fluorescence was observed in Nile tilapia tissues analyzed with the *Piscirickettsia salmonis*-specific FAT.

16S rDNA Phylogenic Analysis

An almost complete 16S rDNA sequence was obtained from infected kidney and was deposited in GenBank (DQ473646). Using the maximum-parsimony and neighbor-joining analysis within the MEGA package, we found that the *Francisella* spp. LA1 organism did not appear to belong to *Piscirickettsia* but was a member of the *Francisella* genus (Figure 4). Using the Kimura two-parameter model with a pairwise distance calculation (MEGA), we found that *Francisella* spp. LA1 was 0.001% distant from *Francisella* spp. AF-01–22, CYH-2002, and Ehime-1 and tilapia parasite TPT-541 (Taiwan); 0.002% distant from *Francisella* spp. AF-04–405; 0.008% distant from *Francisella* spp. Atlantic cod; and 0.007% distant from *F. philomiragia*. The sequences *Francisella* spp. AF-01–22 and CYH-2002 and TPT-541 were derived from agents of Nile tilapia in Taiwan, and the sequence *Francisella* spp. Ehime-1 was derived from an agent in three-line grunts from Japan.

Discussion

This report details a piscirickettsiosis-like syndrome similar to epizootics in tilapias (Chen et al. 1994; Chern and Chao 1994; Mauel et al. 2003, 2005; Hsieh

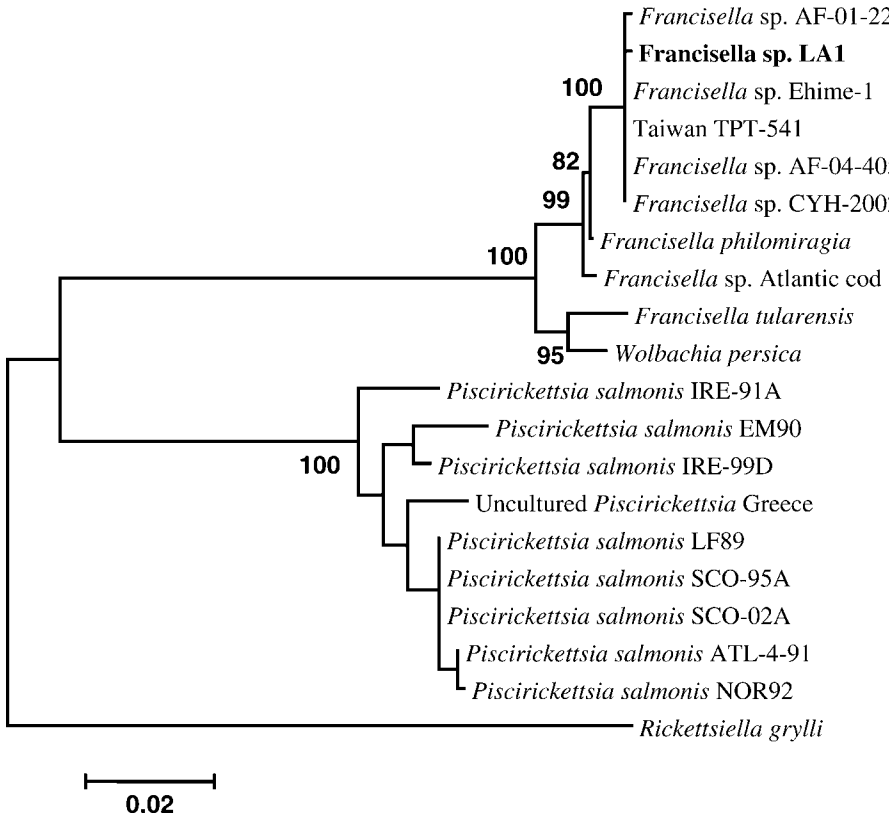

FIGURE 4.—Neighbor-joining tree, based on 1,121 base pairs of the 16S ribosomal DNA (rDNA) sequence, indicating the phylogenetic relationships of *Francisella* spp. LA1 with its nearest relatives based on GenBank 16S rDNA sequences. Numbers at the branch nodes represent bootstrap values as percentages of 1,000 replications. Scale bar indicates *p* distances.

et al. 2006), three-line grunts (Fukuda et al. 2002; Kamaishi et al. 2005), and Atlantic cod (Nylund et al. 2006; Olsen et al. 2006). The gross lesions were similar in these epizootics; occasional skin ulcerations were noted in tilapias but not in Atlantic cod. All reports noted white nodules of multifocal distribution in most organs. In Atlantic cod, the swelling of the spleen was less than that observed in tilapias. The small and large, dark lesions in the muscle observed in this study were not reported in the earlier epizootics. The muscle lesions required the processing plant to hire additional workers to monitor each fillet on a light table to check for the lesions. The large lesions were cut out of the fillets, often leading to the production of Nile tilapia nuggets or strips and the loss of significant value. The small, pinpoint muscle lesions were found to be near the skin of the fillets, and the processing plant was able to excise the majority by removing additional muscle with the skin. This led to fillets of smaller weight and lower value.

The agent of the epizootic reported here has a 99.9% similarity to the GenBank 16S rDNA sequences of agents found in tilapia from Taiwan and three-line grunts from Japan. The 16S rDNA sequences of the agent in this report and the Taiwanese and Japanese agents are 99.3% similar to *F. philomiragia* and 99.2% similar to the *Francisella* spp. from Atlantic cod.

Francisella philomiragia are Gram-negative, small cocci that have been associated with animal and human disease and have been isolated from streams, rivers, and lakes. The principal disease reservoirs are voles and water rats. There is a history of saltwater exposure in human patients with *F. philomiragia* infection; in one study, 12 of 14 infected patients lived within 50 mi (~83 km) of a saltwater coastline (Wenger et al. 1989). Fifty percent of patients with *F. philomiragia* had chronic granulomatous disease. Many patients with *F. philomiragia* had exhibited recent episodes of near-drowning in salt water. No human cases related to the handling of the *Francisella* spp.–infected fish by fish farm or processing plant workers were identified by medical personnel monitoring the outbreak. We

speculate that the Nile tilapia pathogen is a new *Francisella* species that is closely related to *F. philomiragia*; studies are currently ongoing to determine the relationship.

Another common observation between this study and the previous reports was the presence of pleomorphic intracellular bacteria in Giemsa- or Gram-stained tissue presses, blood smears, and early granulomatous tissues. The bacteria were present in the early stages of the granuloma formation. As the granulomas matured, the bacteria were no longer present and cells at the center of the granulomas were replaced by a clear zone.

An anomaly of this epizootic is the duration. From published reports and conversations with researchers and aquatic diagnosticians who dealt with previous disease outbreaks, most have described a single, severe mortality event or a severe mortality event followed by reduced severity over time. In this case, the fluctuating mortality has remained higher than normal for a couple of years and has involved several peaks of high mortality. This pattern may be the result of environmental conditions not understood at this time, virulence factors of the agent, or possibly the strain of Nile tilapia being cultured.

The need for gross and microscopic observation of Nile tilapia tissues, especially the spleen, before transfer of the fish either within a facility or to a new facility is apparent. Also, development of a specific diagnostic test is greatly needed. The identification of the *Francisella* agent in these farms and its close relationship to agents in previously reported epizootics in tilapias and other fish species from widely separated regions of the world suggest that this is an emerging group of fish pathogens. Because the number of recent reports of these agents has grown, investigations into the pathobiology of these agents and the development of control strategies are important for the future of fresh and marine fish culture.

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