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Francisella sp., an emerging pathogen of tilapia, *Oreochromis niloticus* (L.), in Costa Rica

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Abstract

Francisella sp. is an emergent bacterial pathogen that causes acute to chronic disease in warm and cold water cultured and wild fish species. During the past 3 years, the bacterium has been detected in tilapia, *Oreochromis niloticus*, cultured in Costa Rica. Infected fish presented non-specific clinical signs, such as erratic swimming, anorexia, anaemia, exophthalmia and high mortality. Upon macroscopic and microscopic examination, several internal organs (mainly spleen and kidney) were enlarged and contained white nodules. Histological examination revealed the presence of multifocal granulomatous lesions, with the presence of numerous small, pleomorphic, cocco-bacilli. The bacteria were isolated from infected tilapia on selective media and grown on several media with and without antibiotics. Specific PCR primers to the *Francisella* genus were used to confirm the preliminary diagnoses. In comparison with several bacterial 16S rRNA sequences, our isolate was found to share 99% identity with other *Fransicella* spp. isolated from fish, and more than 97% identity to the human pathogen *Francisella tularensis*. Koch's postulates were fulfilled after experimental intraperitoneal and gill exposure challenges.

Keywords: bacteria, Costa Rica, emergent fish pathogen, *Francisella*, granulomas, tilapia.

Introduction

Several emerging diseases have been described in the past 10 years causing significant mortalities and economic losses in a wide variety of freshwater and marine fish. According to Woolhouse & Dye (2001), 'an emerging pathogen can be defined as the causative agent of an infectious disease whose incidence is increasing following its appearance in a new host population or whose incidence is increasing in an existing host population as a result of long-term changes in its underlying epidemiology'. Some of the current emergent fish pathogens include organisms from different taxonomic groups and aetiology. Included among the most pathogenic are bacterial infections by *Lactococcus garvieae* and rickettsia-like organisms (RLO) including *Piscirickettsia* spp. and *Francisella* spp. in several cultured fish species worldwide (Chen, Tung, Chen, Tsai, Wang, Chen, Lin & Adams 1994; Chern & Chao 1994; Mauel & Miller 2002; Fryer & Hedrick 2003; Athanassopoulou, Groman, Prapas & Sabatakou 2004; Pereira, Ravelo, Toranzo & Romalde 2004; Corbeil, Hyatt & Crane 2005; Nylund, Ottem, Watanabe, Karlsbakk & Krossøy 2006; Hsieh, Wu, Tung & Tsai 2007). It is unclear in many of these cases if the increase in incidence is the result of spread of the pathogen to new hosts and geographic locations or the increased technology available to detect them.

Francisellosis is an acute to chronic disease caused by different *Francisella* species. It has been diagnosed in a wide variety of animals, including more than 200 mammals (including humans), as well as birds, reptiles, crustaceans and ticks. Members of the genus have also been found to be present in soil and water samples (Scoles 2004; Barns, Grow, Okinaka, Keim & Kuske 2005; Birkbeck, Bordevik, Frøystad & Baklien 2007; Keim, Johansson & Wagner 2007; Müller, Bocklisch, Schüler, Hotzel, Neubauer & Otto 2007). The most

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significant animal pathogen species belonging to this genus is the type species, *Francisella tularensis*, the causative agent of tularaemia. Besides being an important animal pathogen, *F. tularensis* is a zoonotic agent which has received considerable study as a potential bioterrorism agent. The organism has a high infectivity rate and multiple infectious routes (Keim *et al.*2007; Nano & Schmerk 2007).

The genus *Francisella* contains four recognized species, *F. tularensis*, *F. novicida*, *F. philomiragia* and, recently, *F. piscicida*, an isolate recovered from cod cultured in Norway (Thomas, Johansson, Neeson, Isherwood, Sjöstedt, Ellis & Titball 2003; Ottem, Nylund, Karlsbakk, Friis-Møller, Krossøy & Knappskog 2007b). Molecular diagnostic techniques and biochemical testing methods have been used to identify *Francisella* causing similar pathological lesions in fish species (Ostland, Stannard, Creek, Hedrick, Ferguson & Carlberg 2006; Mauel, Soto, Morales & Hawke 2007). The *Francisella* spp. identified as emerging pathogens of fish are poorly characterized because of the fastidious nature of the bacteria and the resulting difficulties in culturing the organisms from fish tissues. Sequence comparison of the 16S rDNA from pathogenic *Francisella* spp. has placed the fish isolates at 97–99% identity to other isolates from water and soil environmental samples and mammalian isolates of *F. tularensis* and *F. philomiragia* (Kamaishi, Fukuda, Nishiyama, Kawakami, Matsuyama & Yoshinaga 2005; Mailman & Schmidt 2005; Hsieh, Tung, Tu, Chang & Tsai 2006; Ostland *et al.* 2006; Birkbeck *et al.* 2007; Mauel *et al.* 2007; Mikalsen, Olsen, Tengs & Colquhoun 2007). From infections in fish, only on rare occasions have researchers been able to successfully isolate the bacterium (Kamaishi *et al.* 2005; Mikalsen *et al.* 2007).

The purpose of this study is to report and describe the clinical pathology, histopathology, microbiology and molecular characteristics of the emergent fish pathogen *Francisella* sp., affecting tilapia aquaculture in Costa Rica.

Materials and methods

Fish history

During August and September 2007, a farm in Alajuela, Costa Rica experienced increased morbidity and mortality in several freshwater-pond cultured tilapia. According to the producer, around 50–60% (~6000 fish) of the cultured tilapia died during this period, with mortalities as high as 200 fish/pond/day.

Approximately 50 tilapia, *Oreochromis niloticus* (L.), from the affected farm were received and analysed by the Pathology Service of the School of Veterinary Medicine of the Universidad Nacional de Costa Rica during August–October 2007. A complete necropsy showed consistent gross and microscopic lesions similar to those reported during high mortality events in cultured tilapia in Taiwan, Hawaii, the continental USA and Latin-America since 1994 (Chern & Chao 1994; Mauel, Miller, Frazier, Liggett, Styer & Montgomery-Brock 2003; Mauel, Miller, Styer, Pouder, Yanong & Goodwin 2005; Hsieh *et al.* 2006; Mauel *et al.* 2007).

Fifteen euthanized fish were sent to the Louisiana Aquatic Diagnostic Laboratory (LADL) at Louisiana State University – School of Veterinary Medicine (LSU-SVM) for further analysis.

Histological analysis

The gill, spleen, kidney, liver, heart, brain, ovary, testis and muscle were fixed in neutral buffered 10% formalin, processed by standard methods, stained with haematoxylin and eosin and Giemsa stain, and examined by light microscopy.

Isolation, media and growth conditions

Fish tissues (spleen, anterior kidney and liver) were aseptically collected and used for bacteriological analysis by streaking on different agar media. Commercially available media tested for primary recovery of bacteria from fish tissue smears included: trypticase soy agar (TSA) with 5% sheep blood, cystine heart agar (CHA) with rabbit blood and antibiotics, chocolate agar/improved Thayer-Martin biplate (Remel), chocolate II agar (GC II agar with haemoglobin and IsoVitaleX) and modified Thayer-Martin agar (BD BBL). Two types of agar plates used as primary isolation media were prepared in the media preparation laboratory at LSU-SVM: cystine heart agar supplemented with bovine haemoglobin solution (BD BBL) (CHAH) and Mueller-Hinton base supplemented with 3% foetal bovine serum, 1% glucose and 0.1% cystine. Polymixin B 100 units mL^{-1} and/or ampicillin 50 μg mL^{-1} were added to the media to select against secondary contaminants, since they are

widely use as selective agents for *Francisella* sp., and for fish disease diagnosis (Hawke & Thune 1992; Petersen, Schriefer, Gage, Montenieri, Carter, Stanley & Chu 2004).

Plates were incubated at 22–25 °C for 2–5 days. Colonies observed from primary isolation agar plates were re-plated for purity of culture under the same conditions. Once single colonies were observed and purity of the isolate determined, the isolate was re-suspended in liquid medium as reported by Baker, Hollis & Thornsberry (1985) with modifications. The liquid medium consisted of a modified Mueller-Hinton II cation adjusted broth supplemented with 2% IsoVitaleX (BD BBL) and 0.1% glucose (MMH). Broth cultures were grown overnight at 22 °C in a shaker at 175 rpm, and bacteria were frozen at −80 °C in the broth media containing 20% glycerol for later use.

Three different isolates (obtained from three different fish) were tested at different culture temperatures; 15, 20, 22, 25, 28, 30, 32, 35 and 37 °C on CHAH for a period of 7 days to find the *in vitro* optimal growth temperature of the bacteria.

DNA extraction

Two isolates (07-285A and 07-285B) recovered from fish cultured in different systems and submitted to the LADL LSU-SVM were used for molecular analysis. A loop of the bacterium was suspended in 400 μL of sterile water, washed and centrifuged at 3000 ***g*** for 5 min and re-suspended in 200 μL PBS. The bacterial suspension was subjected to DNA extraction and purification as per the manufacturer's protocol using the High Pure PCR Template Preparation Kit (Roche). DNA was stored at 4 °C until further use.

PCR and 16S rRNA gene sequence

Two different sets of primers were used during the study to amplify gene sequences important in identification of *Francisella*. The 50 μL *Francisella* sp.-specific PCR reaction was composed of 0.2 μM of each primer (F11, 5′-TAC CAG TTG GAA ACG ACTGT-3′ and F5, 5′-CCT TTT TGA GTT TCGCTC C-3′) developed by Forsman, Sandstrom & Sjostedt (1994), 0.2 mM of dNTPs, 2.5 mM $MgCl_2$, 5 U of *Taq* DNA polymerase (Applied Biosystems-Roche), 1× PCRx Amp buffer (Invitrogen), 1× PCRx Enhancer solution (Invitrogen) and approximately 200 ng of template DNA. Cycling conditions consisted of an initial denaturation step of 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 60 s at 60 °C and 60 s at 72 °C, with a final extension step of 5 min at 72 °C performed in a Perkin Elmer GeneAmp PCR System 2400 (PerkinElmer Life and Analytical Sciences, Inc.).

The 50 μL universal eubacterial 16S rRNA PCR reaction was composed of 0.5 μM of each primer (F1, 5′-GAG TTT GAT CCT GGC TCAG-3′ and R13,5′- AGA AAG GAG GTG ATC CAG CC-3′) (Dorsch & Stackebrandt 1992), 0.2 mM of dNTPs, 2.5 U of *Taq* DNA polymerase, 1× buffer H (Invitrogen) and approximately 200 ng of template DNA. Cycling conditions consisted of an initial denaturation step of 30 s at 94 °C, followed by 30 cycles of 30 s at 94 °C, 60 s at 58 °C and 90 s at 72 °C, with a final extension step of 7 min at 72 °C in a Perkin Elmer GeneAmp PCR System 2400. The PCR products were subjected to electrophoresis on a 1% agarose gel and stained with SYBR® Safe DNA gel stain (Invitrogen).

Amplicons for sequencing were purified with the QiaQuick PCR Cleanup Kit (Qiagen) as directed by the manufacturer and were sequenced on an Applied Biosystems 3130 Genetic Analyzer using PCR primers (F11 – F5) and (F1 – R13).

The sequence was compared with those stored in GenBank using the BLASTN program from the National Center for Biotechnology Information. Eubacterial 16S rRNA sequences of members of the genus *Francisella* and representative warm water fish pathogens were obtained and aligned using the Clustal W application of the Molecular Evolutionary Genetic Analysis (MEGA) package (version 4; Tamura, Dudley, Nei & Kumar 2007) and used in the construction of phylogenetic tree. The alignment was performed using neighbour-joining and distance analysis within the MEGA package. The evolutionary history in the phylogenetic tree was inferred using the neighbour-joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in $<50\%$ bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (complete

deletion option). There were a total of 1163 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

Experimental challenges

To fulfill Koch's postulates, experimental infections were performed by intraperitoneal injection (IP) and gill spraying (GS) with *Francisella* sp. Costa Rica isolate LADL07-285A. This isolate, recovered from cultured infected tilapia in Costa Rica was grown in CHAH at 25 °C for 72 h. Cells were harvested, suspended in 5 mL of MMH broth, and incubated in a shaking incubator overnight at 22 °C to obtain a final optical density at 600 nm (OD600) of 0.48. Enumeration of the bacteria was carried out by the drop plate method with 50 μL drops of each 10-fold dilution placed on cystine heart agar with haemoglobin. Resulting colony forming units per mL (CFU mL^{-1}) were determined.

Experimental infection of naïve *O. niloticus* (average length ~9.0 cm and average weight ~18.9 g) was tested by the IP and GS exposure routes. The fish were obtained from a source considered to be free of *Francisella* infection and were found to be negative for francisellosis by culture of spleen and head-kidney smears and by PCR, prior to use in the study. Fish were maintained in three different tanks (10 fish per tank), representing the two different challenge methods and a control tank at 23–25 °C. Prior to challenge, all fish were anaesthetized with MS-222 (100 mg L^{-1}). The IP challenge fish received a 0.1-mL injection of the bacterial suspension (~10^7 CFU $fish^{-1}$). The GS challenge fish were sprayed with 0.1–0.2 mL of the bacterial suspension, and left out of the water for approximately 15 s. Control fish were treated in a similar manner, but received 0.1 mL of sterile MMH broth.

Following each challenge exposure, the fish were placed in the respective tanks and mortality was recorded every 12 h for 10 days. Dead and moribund fish were subjected to a complete clinical, bacteriological and histopathological examination. The identity of isolated bacteria was confirmed by PCR.

Results

Clinical signs and histopathology

Affected tilapia fingerlings ranged between 6.5–9.0 cm in length and 13–20 g in weight. Except for lethargy, abnormal swimming behaviour, rare exophthalmia, and anorexia, no clear external clinical signs were observed in the fish. Examination of the gills and skin in wet mounts revealed the presence of light *Ichthyobodo*, *Ambiphyra*, monogenean and heavy *Trichodina* sp. infections. Internally, the most significant gross pathological change observed was the presence of widespread, multifocal white nodules dispersed in the anterior kidney, posterior kidney and spleen (Fig. 1). Also, fish had marked splenomegaly and renomegaly. In some cases, white nodules were observed in gills, liver, choroid gland, and sporadically in the gastrointestinal walls and mesenteric fat.

Histopathologically, the most severe changes involved the gills, spleen and kidneys, but pathologic changes were also present in the liver, heart, eye, central nervous system and gastrointestinal tract. Granulomatous inflammation was present in almost every organ, with large numbers of macrophages containing small pleomorphic coccobacilli. In addition to the granulomatous response, the gills exhibited primary and secondary lamellar fusion because of epithelial hyperplasia (Fig. 2a). In severe cases, a widespread cellular infiltrate and presence of granulomas were observed in pericardium and myocardium (Fig. 2b). Granuloma formation was not observed in the brain; instead a massive macrophagic inflammatory infiltrate was found in severely infected fish. When special stains (Giemsa) were used small, pleomorphic coccobacilli were visible inside and outside the cells (Fig. 3).

Media and growth conditions

Cystine heart agar supplemented with bovine haemoglobin solution and antibiotics, the modified

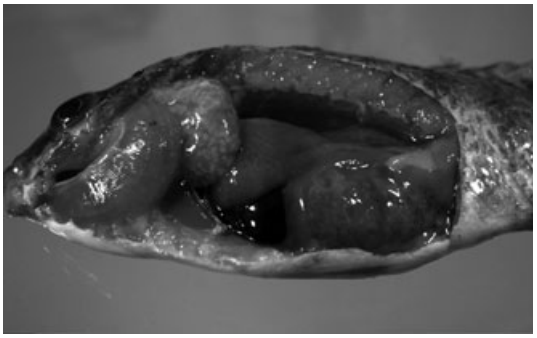

Figure 1 Splenomegaly and renomegaly with widespread multifocal white nodules in *Francisella*-infected Nile tilapia (*Oreochromis niloticus*).

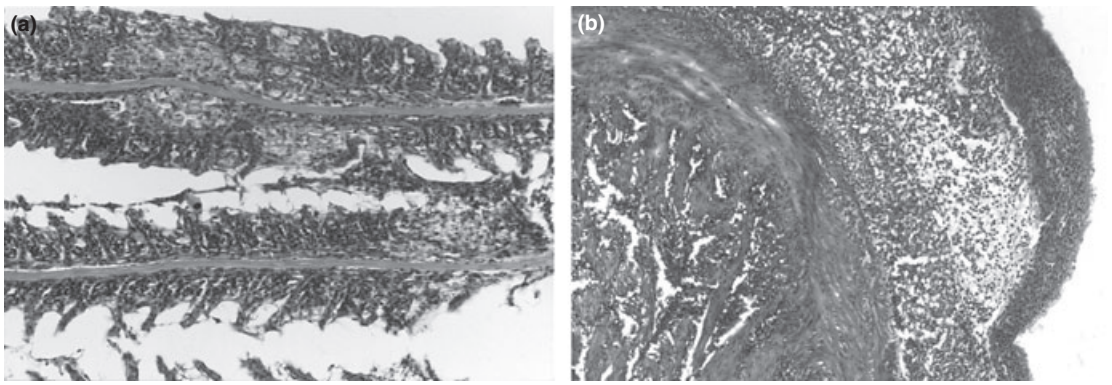

Figure 2 Histopathological findings of francisellosis in Nile tilapia. (a) Hyperplasic and hypertrophic primary and secondary gill lamellae, (b) pericarditis with granulomatous cellular infiltrate.

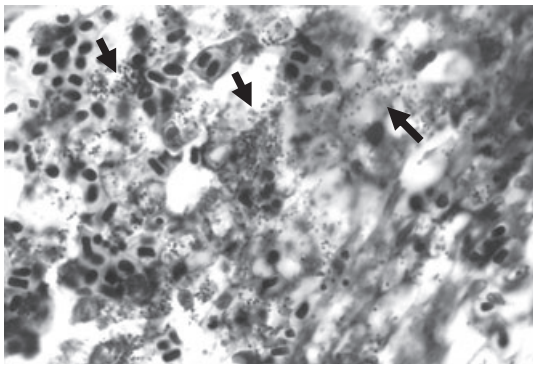

Figure 3 *Francisella* sp. bacteria seen intra and extracellularly (arrows) (Giemsa, ×100).

Thayer-Martin agar, and CHA with rabbit blood and antibiotics were useful for the primary isolation of *Francisella* sp. from the spleen and kidneys of diseased fish.

The chocolate agar/improved Thayer-Martin biplate, chocolate II agar, and the Mueller-Hinton base supplemented with 3% foetal bovine serum, 1% glucose and 0.1% cystine were not suitable for primary isolation, although sub-culture could be successfully performed on these agars. The *Francisella* sp. failed to grow on TSA agar with 5% sheep blood, however, other secondary and contaminating organisms, including *Plesiomonas shigelloides*, *Aeromonas* sp. and *Pseudomonas* sp. were isolated. The strains of *Francisella* sp. isolated from tilapia from Costa Rica by the LADL were designated as strains LADL07-285A and LADL07-285B.

Growth of *Francisella* sp. was visible on CHAH, 36- to 48-h post-inoculation and colonies were grey, smooth and convex. Optimal growth of *Francisella* sp. occurred at 28–30 °C, but growth was present from 20 to 28 °C after 4 days of incubation. Growth at 22–25 °C was slower than at 28 °C, and no growth was observed at 15 °C or at 33 °C. By light microscopy, the morphology of the bacterium was extremely pleomorphic, non-motile and very small in size (~0.5–1 μm wide).

Molecular analysis

The isolates recovered from the infected spleen and kidneys yielded the appropriately amplified PCR products of 1150 bp using the *Francisella* genus-specific primers F11 and F5 (Fig. 4a). When using the universal eubacterial 16S rRNA primers F1 and R13, a 1384 bp product was amplified from LADL07-285A and LADL07-285B. The sequence for isolate LADL07-285A was deposited in GenBank under the accession number EU672884 (Fig. 4b).

The 16S rRNA sequence obtained was compared with those stored in GenBank using the BLASTN program. The Clustal W program was used to create a neighbour-joining tree based on the 1384 bp sequence of 16S ribosomal RNA and shows the phylogenetic relationship of *Francisella* sp. LADL07-285 (Alajuela, Costa Rica) with other *Francisella* sp., and other fish pathogens (Fig. 5).

A 97–99% identity was shown between the isolate recovered from tilapia in Alajuela, Costa Rica and other *Francisella* species sequences stored in GenBank. Interestingly, all the *Francisella* sp. isolated from fish shared around 99% identity to our isolate, while the mammalian pathogens *F. tularensis* and *F. philomiragia* shared around 97% and 98% homology with our isolate. Estimates of evolutionary divergence between isolate LADL07-285 16S rRNA

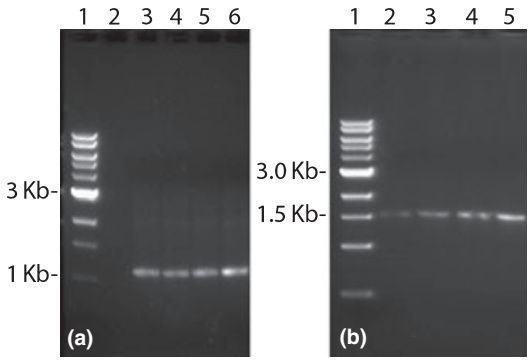

Figure 4 PCR amplification of *Francisella* sp. isolates recovered from Nile tilapia. (a) Amplification using genus-specific primers. Lane 1, 1 kb ladder. Lane 2, negative control. Lane 3, isolate 07-285A wild type recovered from cultured tilapia. Lane 4, isolate 07-285B wild type recovered from cultured tilapia. Lane 5, isolate 07-285A recovered from intraperitoneal challenge. Lane 6, isolate 07-285A recovered from gill exposure challenge. (b) Amplification using eubacterial 16 s rRNA primers. Lane 1, 1 kb ladder. Lane 2, isolate 07-285A wild type recovered from cultured tilapia. Lane 3, isolate 07-285B wild type recovered from cultured tilapia. Lane 4, isolate 07-285A recovered from intraperitoneal challenge. Lane 5, isolate 07-285A recovered from gill exposure challenge.

with other *Francisella* sp., and other fish pathogens are given in Table 1.

Experimental challenge

Intraperitoneal injection of $\sim 10^7$ CFU fish^{-1} caused 100% mortality in naïve tilapia by 72-h post-inoculation. Tilapia exposed to bacteria by gill immersion also exhibited high mortality (80%), but this occurred gradually over the duration of the study (10 days). The clinical signs presented in the experimentally challenged fish were consistent with those found in the naturally infected cases. In the IP injection group, a more acute onset of the disease was seen and most fish died in a short period of time (< 48-h post-challenge). The clinical signs in the acutely infected fish were bloody ascites, slight swelling of the spleen and kidney, with increased number and size of melanomacrophage centres but no granulomas were seen. Numerous small coccobacilli were present both intracellularly and extracellularly in the tissues. Fish exposed by gill immersion presented with a more subacute to chronic form of the disease, showing signs of anorexia and erratic swimming behaviour. At necropsy, splenomegaly and renomegaly were pronounced and granulomas were numerous in both organs. Numerous intra and extracellular bacteria were observed microscopically in gills, spleen and anterior and posterior kidney. *Francisella* sp. was re-isolated from both challenged groups by inoculating homogenates of spleen and posterior kidney on CHA supplemented with bovine haemoglobin solution and antibiotics. The isolates were confirmed by PCR as members of the genus *Francisella*. At the completion of the experimental challenge, all control fish were alive and no bacterial infection was detected by bacteriological, histopathological or molecular analysis.

Discussion

Since the first diagnosis of 'rickettsia-like' bacteria (*Piscirickettsia salmonis*) in the 1980s, different cultured freshwater and marine fish worldwide have been described with intracellular micro-organisms causing high mortalities and granulomatous lesions (Fryer, Lannan, Giovannoni & Wood 1992; Chen *et al.* 1994; Hsieh *et al.* 2006; Mauel *et al.* 2007; Ottem, Nylund, Karlsbakk, Friis-Møller & Krossøy 2007a). Diagnosis in many of these cases has been challenging because of various issues, namely, highly fastidious micro-organisms, low numbers of bacteria, non-culturable micro-organisms, multiple infectious agents, antibiotic treated hosts and/or emergent diseases in the same case. PCR and sequencing of 16S rRNA has been used for definitive diagnosis of controversial and problematic cases (Ostland *et al.* 2006; Mauel *et al.* 2007).

During the past 5 years, the use of molecular techniques has helped to clarify the presence of an emerging group of fish pathogens that are members of the genus *Francisella*, family *Francisellaceae*, Gamma subclass of the class Proteobacteria. This group of pathogens causes high mortalities in important cultured fish like cod, *Gadus morhua* L., in Norway, three line grunt, *Parapristipoma trilineatum* (Thunberg), in Japan, hybrid striped bass (*Morone chrysops* x *M. saxatilis*) in the USA, Atlantic salmon, *Salmo salar* L., in Chile, and tilapia, *Oreochromis* sp., in Latin America, Taiwan and now Costa Rica (Kamaishi *et al.* 2005; Hsieh *et al.* 2006; Olsen, Mikalsen, Rode, Alfjorde, Hoel, Straum-Lie, Haldorsen & Colquhoun 2006; Ostland *et al.* 2006; Birkbeck *et al.* 2007; Mikalsen *et al.* 2007). The existence of one or more species or subspecies in this group is still in question and will have to be addressed by taxonomists in the future. Certainly, phenotypic and genetic differences exist

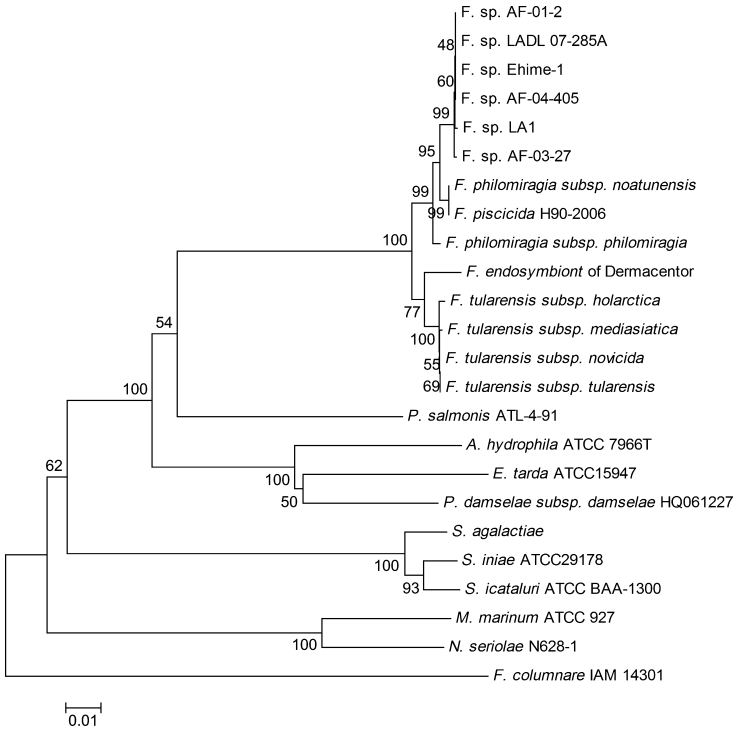

Figure 5 Evolutionary relationships of 24 taxa based on partial 16S rRNA sequences of members of the genus *Francisella* and representative warm water fish pathogens.

between the cool water *Francisella* pathogens of marine fish such as the cod and warm water pathogens of freshwater fish species such as the tilapia.

The clinical signs, gross pathological changes and histopathological findings described from the affected tilapia from Alajuela, Costa Rica are in agreement with previous reports by Hsieh *et al.* (2007) and Mauel *et al.* (2007) in cultured tilapia. Interestingly, during this event, the predominant clinical sign was the high mortality present among the fingerlings. According to farmers, affected fish will swim erratically for 5–10 min and then succumb and die. This clinical sign may be related to the amount of granulomatous inflammatory cellular infiltration present in the central nervous system, as the most severely affected fish are those that exhibit this behaviour.

Only rarely have fish health diagnosticians been able to recover the causative bacterium from affected fish and culture it in bacteriological media (Kamaishi *et al.* 2005; Hsieh *et al.* 2006; Olsen *et al.* 2006; Birkbeck *et al.* 2007). We strongly recommend the use of a selective medium such as CHAH with the addition of Ampicillin and Polymixin B when *Francisella* sp. is suspected as the causative agent of disease. This proved to be the most reliable medium of all those evaluated in this study, providing good growth of the pathogen and inhibiting secondary and contaminating bacteria. In an *in-vitro* growth temperature comparison, we found that isolate LADL07-285A from tilapia differs in optimal temperature from the *Francisella* sp. diagnosed from farmed Atlantic cod and recently re-named *F. philomiragia* subsp. *noatunensis* (Mikalsen *et al.* 2007). The optimal temperature for the tilapia isolate was found to be 28–30 °C, whereas that for the cod isolate was 22 °C. These results are consistent with the environmental temperatures at which the fish are cultured. Further research is under way to determine the optimal temperature for disease development in tilapia (Soto, E. & Hawke, J. unpublished data).

When comparing the 1384 bp partial 16S rRNA sequence of isolate LADL07-285A to other fish and mammalian pathogens, a high identity (more than 99%) was found between all the fish *Francisella* sp. sequences. Interestingly, a different tree branch can be observed amongst the warm water cultured fish species showing nearly 100% similarities and the marine cold water isolates from cod (Fig. 5). Recent comparison of the 16S rRNA gene sequences, six partial housekeeping gene sequences (*groEL*, *shdA*, *rpoA*, *rpoB*, *pgm* and *atpA*), and DNA–DNA

Table 1 Estimates of evolutionary divergence between 16S rRNA sequences of members of the genus *Francisella* and representative warm water fish pathogens and isolate LADL 07-285A

Strain	GeneBank accession no.	% Distance from LADL 07-285A	% Identity with LADL 07-285A
Francisella sp. AF-01-2	AY928388	0	99
Francisella sp. Ehime-1	AB194068	0	99
Francisella sp. AF-04-405	DQ007455	0	99
Francisella sp. LA1	DQ473646	0.001	99
Francisella sp. AF-03-27	AY928393	0.001	99
Francisella philomiragia subsp. *noatunensis* strain 2006/09/130	EF490217	0.007	99
Francisella piscicida isolate H90-2006	EF685350	0.007	99
Francisella philomiragia subsp. *philomiragia* ATCC 25015	AJ698862	0.009	99
Francisella tularensis subsp. *novicida* strain FSC 040	AY968237	0.020	97
Francisella tularensis subsp. *tularensis* strain FSC 199	AY968225	0.020	97
Francisella tularensis subsp. *mediasiatica* strain FSC 148	AY968235	0.021	97
Francisella tularensis subsp. *holarctica* strain UT01-1901	AY968232	0.021	97
Francisella endosymbiont of *Dermacentor variabilis* strain 2040460	AY805306	0.028	96
Piscirickettsia salmonis ATL-4-91	U36915	0.144	84
Photobacterium damselae subsp. *damselae* HQ061227	EF635307	0.164	82
Aeromonas hydrophila ATCC 7966T	X74677	0.176	81
Edwardsiella tarda	EF467289	0.187	82
Streptococcus agalactiae strain ATCC 13813	DQ303183	0.217	79
Streptococcus iniae 29178	DQ303187	0.221	79
Streptococcus ictaluri BAA-1300	DQ462421	0.222	79
Mycobacterium marinum ATCC 927	AF456240	0.233	78
Nocardia seriolae N628-1	EF513204	0.233	82
Flavobacterium columnare IAM 14301	AB010951	0.261	75

The number of base differences per site from analysis between sequences is shown. All results are based on the *p*-distance parameter model with a pairwise distance calculation.

hybridization gave enough evidence to name the isolate from cod *F. philomiragia* subps. *noatunensis* subsp. nov. (Mikalsen *et al.* 2007). Kay, Petersen, Duus, Perry & Vinogradov (2006) characterized the lipopolysaccharide and β-glucan from an isolate recovered from moribund tilapia by chemical and spectroscopy methods and confirmed the isolate as a relative of *F. tularensis*, with enough similarities to be placed in the same genus. Whether this isolate and the isolates from other fish species worldwide are the same is still to be determined.

To begin understanding the causal relationship of the micro-organism and the disease, Koch's postulates were fulfilled with the isolate recovered from Costa Rican tilapia. High mortality rates were observed over a period of 10 days in fish challenged by intraperitoneal injection and gill exposure, causing both acute and chronic presentations of disease, respectively. Acute disease caused by IP injection was characterized by rapid mortality with no clearly observable clinical signs or gross pathological changes. Chronic disease followed gill immersion exposure and was typified by the presence of multiple white nodules in various organs. After clinical, histopathological, bacteriological and molecular analysis, the *Francisella* sp. isolate was recovered and shown to be the responsible agent for the disease.

In conclusion, the causative agent of a high mortality episode in cultured tilapia in Costa Rica has been identified as a member of the genus *Francisella* by bacteriological, histopathological and molecular analysis. Research in the areas of diagnostic tools, virulence mechanisms and virulent factors, immune response and vaccines, prevention methods and treatments are urgent and necessary for aquaculture industries to be able to prevent and control this emergent pathogen.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Estimates of evolutionary divergence between 16S rRNA sequences of members of the genus *Francisella* and representative warm water fish pathogens. The number of base differences per site from analysis between sequences is shown. All results are based on the *p*-distance parameter model with a pairwise distance calculation

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