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Development of a real-time PCR assay for identification and quantification of the fish pathogen *Francisella noatunensis* subsp. *orientalis*

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ABSTRACT: Members of the genus *Francisella* are small Gram-negative facultative intracellular bacteria that cause francisellosis in a wide variety of fish species worldwide. *F. noatunensis* subsp. *orientalis* has been recently described as a warm-water pathogen of tilapia *Oreochromis* spp. In this study, a quantitative real-time polymerase chain reaction (qPCR) TaqMan probe assay was developed to rapidly and accurately detect and quantify *F. noatunensis* subsp. *orientalis* from fish tissue. The target region of the assay was the *F. tularensis iglC* gene homologue previously found in *F. noatunensis* subsp. *orientalis*. Probe specificity was confirmed by the lack of signal and cross-reactivity with 12 common fish pathogens, 2 subspecies of *F. tularensis*, *F. noatunensis* subsp. *noatunensis*, and tilapia tissue. The range of linearity was determined to be 50 fg to 1.4 mg, and the lower limit of detection was 50 fg of DNA (equivalent to ~25 genome equivalents) per reaction. A similar sensitivity was observed with DNA extracted from a mixture of *F. noatunensis* subsp. *orientalis* and fish tissue. The assay was also able to detect and quantify *F. noatunensis* subsp. *orientalis* from the spleens of experimentally infected tilapia. No signal was observed in the control groups. In conclusion, we have developed a highly sensitive and specific assay that can be used for the specific identification and quantification of *F. noatunensis* subsp. *orientalis*.

KEY WORDS: *Francisella* · Tilapia · qPCR

INTRODUCTION

Francisellosis is an emergent disease in fish caused by Gram-negative facultative intracellular bacteria that are members of the genus *Francisella*. In tilapia the disease can be observed as an acute syndrome with few clinical signs and high mortality, or as a subacute to chronic syndrome with non-specific clinical signs like anorexia, exophthalmia, and anemia. Upon macroscopic and microscopic examination, internal organs are enlarged and contain widespread multifocal white nodules. Histological examination often reveals the presence of multifocal granulomatous lesions, with the presence of numerous small, pleomorphic, cocco-bacilli (Hsieh et al. 2006, Mauel et al. 2007, Soto et al. 2009a).

During the past 5 yr, bacteria of the genus *Francisella* have caused significant mortalities in cultured tilapia *Oreochromis* spp., Atlantic cod *Gadus morhua*, Atlantic salmon *Salmo salar* L., hybrid striped bass *Morone chrysops* × *M. saxatilis*, three-line grunt *Parapristipoma trilineatum*, and ornamental cichlids, both in warm- and cold-water environments (Kamaishi et al. 2005, Nylund et al. 2006, Olsen et al. 2006, Ostland et al. 2006, Birkbeck et al. 2007, Hsieh et al. 2007, Mauel et al. 2007, Mikalsen et al. 2007, Ottem et al. 2007, Soto et al. 2009a).

The identification and taxonomic characterization of the *Francisella* spp. identified as worldwide emerging pathogens of fish have been difficult owing to the fastidious nature of the bacteria and the small number of isolates recovered from fish (Soto et al. 2009a). In the majority of the cases, PCR and sequence comparison of

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the 16S rRNA have made it possible to place the organisms at 97% similarity to *F. tularensis,* 98% similarity to *F. philomiragia*, and 99% to other *Francisella* spp. strains isolated from fish species (Kamaishi et al. 2005, Hsieh et al. 2006, Ostland et al. 2006, Mauel et al. 2007, Mikalsen et al. 2007, Ottem et al. 2007, Soto et al. 2009a). The *Francisella* strain utilized in this research project at the Louisiana Aquatic Diagnostic Laboratory, LSU School of Veterinary Medicine, LADL 07-285A, isolated from tilapia *Oreochromis* spp. from Costa Rica, was confirmed by molecular analysis as *F. noatunensis* subsp. *orientalis* (Soto et al. 2009a) and exhibited 99% identity with *F. noatunensis* subsp. *noatunensis* isolated from diseased Atlantic cod in Norway (Euzéby 2009, Ottem et al. 2009), by sequence comparison of the 16S rRNA. The LADL 07-285A and 07-285B isolates used in this study also belong to the same group described as *F. asiatica* (Mikalsen & Colquhoun 2009); however, this name does not yet have valid taxonomic standing (J. Euzéby pers. comm.).

The diagnosis of this highly virulent fish pathogen has many constraints, including the fastidious nature of the bacterium and the lack of biochemical, molecular, and serological tests specific for this aquatic animal pathogen. Previous diagnosis of francisellosis in fish species has been made with the aid of histopathology, electron microscopy, conventional culture assays, conventional polymerase chain reaction (PCR) using *Francisella* sp. specific primers, 16S rRNA sequencing, and *in situ* hybridization (Kamaishi et al. 2005, Hsieh et al. 2006, Ostland et al. 2006, Mauel et al. 2007, Ottem et al. 2007, Soto et al. 2009a). However, the diagnosis of the pathogen remains a challenge, and some of the current techniques are difficult, time consuming, and expensive; some require specialized personnel and are prone to show false negatives because of low sensitivity, or false positives because of low specificity. Moreover, studies with *F. tularensis* have shown that diagnosis based on isolation by culture is prone to show false-negative results (Fujita et al. 2006).

Real-time PCR is a well known molecular technique that is currently used in many laboratories for diagnosis of microbial pathogens, including the fastidious bacteria *Mycobacterium* spp., *Bacillus anthracis*, *Francisella tularensis*, and organisms that are non-culturable on cell-free media, the *Rickettsia* spp. and viruses (Bode et al. 2004, Kocagoz et al. 2005, Takahashi et al. 2007, Tomaso et al. 2007, Abril et al. 2008, Kidd et al. 2008). In recent years, fish disease diagnosticians have used this technique to identify and quantify bacterial, viral, and parasitic fish pathogens such as *Aeromonas salmonicida*, *Flavobacterium columnare*, *Renibacterium salmoninarum*, *Henneguya ictaluri*, largemouth bass virus, and recently *Francisella piscicida* in Norwegian cod (Balcázar et al. 2007, Getchell et al. 2007, Panangala et al. 2007, Suzuki & Sakai 2007, Griffin et al. 2008, Ottem et al. 2008). The high sensitivity, high specificity, and short turnaround time for results make this technique an attractive replacement method for conventional diagnostic techniques (Espy et al. 2006).

The genes of the intracellular growth locus (*iglA, iglB, iglC*, and *iglD*) are some of the most interesting genes identified in the genus *Francisella.* These genes are present as part of a 30 kb pathogenicity island described by Nano et al. (2004) and Barker & Klose (2007) in *Francisella tularensis.* The *Francisella* pathogenicity island (FPI), a cluster of 16 to 19 genes, has been found duplicated in some *F. tularensis* genomes, but as a single copy in the *F. philomiragia* subsp. *philomiragia* ATCC 25015 isolate, a close relative of the tilapia pathogen *F. noatunensis* subsp. *orientalis* strain LADL 07-285A (Nano & Schmerk 2007, Soto et al. 2009b).

The functions of the conserved proteins corresponding to the genes are elusive. Overall, Igl proteins appear to be essential for the ability of *Francisella tularensis* to survive inside the macrophage and cause disease (Golovliov et al. 1997, Lai et al. 2004, Lauriano et al. 2004, Nano et al. 2004, Santic et al. 2005, Brotcke et al. 2006, de Bruin et al. 2007). Homologues of the *F. tularensis iglABCD* genes in the tilapia pathogenic *F. noatunensis* subsp. *orientalis* strain LADL 07-285A were identified and described in a previous study (Soto et al. 2009b). The presence of a single *iglC* gene in the completely sequenced and closely related isolate *F. philomiragia* subsp. *philomiragia* ATCC 25017 suggests that the gene is also present in single copy in *F. noatunensis* subsp. *orientalis* strain LADL 07-285A. The presence of a single copy of the *iglC* gene in *F. noatunensis* subsp. *orientalis* makes the gene an excellent target for developing a highly specific diagnostic test and will provide a means to quantify with a high degree of confidence the amount of bacterial DNA present in a sample.

The aim of this study was to develop a quantitative real-time PCR assay using the previously described *iglC* gene of the fish pathogen *Francisella noatunensis* subsp. *orientalis* as a target. We describe a highly sensitive, specific, and reliable molecular diagnostic technique for identification and quantification of *F. noatunensis* subsp. *orientalis* from diseased fish.

MATERIALS AND METHODS

Bacterial species and strains. The bacterial strains used in this project were chosen because they represent common bacterial fish pathogens, or are members of the genus *Francisella.* Strain LADL 07-285A, isolated from diseased cultured tilapia *Oreochromis*

spp. was chosen as a representative of the warm-water strain of fish pathogenic *F. noatunensis* subsp. *orientalis*. The majority of the isolates tested were recovered by the Louisiana Aquatic Diagnostic Laboratory (LADL), in the School of Veterinary Medicine (LSU-SVM) at Louisiana State University, from diseased fish, while others were acquired from the American Type Culture Collection (ATCC). *F. tularensis* subsp. *novicida* U112 and *F. tularensis* subsp. *holarctica* (LVS isolate) DNA were kindly donated by Dr. Bernard Arulanandam and Dr. Jieh-Juen Yu, from the Department of Biology, University of Texas at San Antonio. *F. noatunensis* subsp. *noatunensis* is a recently described member of the genus *Francisella* isolated from farmed Atlantic cod displaying chronic granulomatous disease (Mikalsen et al. 2007, Mikalsen & Colquhoun 2009, Ottem et al. 2009) and was kindly donated by Dr. Anne-Berit Olsen, National Veterinary Institute, Bergen, Norway. *Francisella* isolates 1, 2, and 3 recovered from moribund hybrid striped bass (Ostland et al. 2006) and *F. victoria* recovered from tilapia (Kay et al. 2006) and showing >99% identity with *F. noatunensis* subsp. *orientalis* after 16S rDNA sequence comparison were kindly donated by Dr. John Hansen, Interdisciplinary Program in Pathology, University of Washington, Seattle, WA, USA. Following previously published molecular techniques and protocols (Soto et al. 2009b), PCR and sequence comparison of the *iglABCD* operon (Appendix 1, Figs. A1 & A2) and 16S rDNA sequences, as well as phenotypic characteristics, temperature requirements, and host range analysis, demonstrated that isolated *Francisella* sp. 1, *Francisella* sp. 2, *Francisella* sp. 3 (Ostland et al. 2006), and *F. victoria* (Kay et al. 2006) are in fact members of the recently described species *F. noatunensis* subsp. *orientalis* (Figs. A1 & A2) (Soto et al. 2009b). *F. noatunensis* subsp. *orientalis* strain LADL 07-285A was grown in cystine heart agar with hemoglobin (CHAH) supplemented as outlined by Soto et al. (2009a) for 48 h at 28°C. *F. noatunensis* subsp. *noatunensis* was grown in a similar manner but was incubated at 20°C for 5 d. *Flavobacterium columnare* was grown on dilute Mueller Hinton agar for 48 h at 28°C. *Mycobacterium marinum* and *Nocardia seriolae* were grown on Lowenstein Jensen slants for 1 wk at 28°C. All the other bacteria used in the study were grown on blood agar (BA) 5% sheep blood plates for 48 h at 28°C. The complete list of the isolates used in this study is shown in Table 1.

Template DNA preparation. Bacterial cultures grown on agar media were suspended in 1 ml of 1X phosphate-buffered saline (PBS), and 200 µl were used for nucleic acid isolation following the manufacturer's protocol in the High Pure PCR Template Preparation Kit (Roche Diagnostics). Nucleic acid was also extracted from a negative control consisting of 1X sterile PBS alongside of the unknowns to ensure no cross-contamination occurred during the extractions.

Table 1. Bacterial DNA used in the present study. qPCR: quantitative real-time PCR; a negative (–) threshold cycle (C_t) value represents no logarithmic amplification detected within 40 amplification cycles. ATCC: American Type Culture Collection; LADL: Louisiana Aquatic Diagnostic Laboratory, Louisiana State University School of Veterinary Medicine; NVI: National Veterinary Institute, Bergen, Norway; UTSA: Dept. of Biology, University of Texas at San Antonio; UW: University of Washington

Bacteria species	Source	qPCR result
Francisella noatunensis subsp. *orientalis* isolate LADL 07-285A	Tilapia *Oreochromis* spp., LADL	+
Francisella noatunensis subsp. *orientalis* isolate LADL 07-285B	Tilapia *Oreochromis* spp., LADL	+
Francisella noatunensis subsp. *orientalis* Strain 1	Hybrid striped bass *Morone chrysops* × *M. saxatilis*, UW	+
Francisella noatunensis subsp. *orientalis* Strain 2	Hybrid striped bass *Morone chrysops* × *M. saxatilis*, UW	+
Francisella noatunensis subsp. *orientalis* Strain 3	Hybrid striped bass *Morone chrysops* × *M. saxatilis*, UW	+
Francisella noatunensis subsp. *orientalis 'F. victoria'*	Tilapia nilotica *Oreochromis niloticus*, UW	+
Francisella noatunensis	Atlantic cod *Gadus morhua* L., NVI	–
Francisella tularensis subsp. *novicida* U112	UTSA	–
Francisella tularensis subsp. *holarctica* LVS	UTSA	–
Edwardsiella tarda	Tilapia *Oreochromis* spp., LADL	–
Edwardsiella ictaluri (ATCC 33202)	Channel catfish *Ictalurus punctatus*	–
Photobacterium damselae subsp. *piscicida* (ATCC 17911)	White perch *Roccus americanus*	–
Aeromonas hydrophila	LADL	–
Aeromonas salmonicida	LADL	–
Streptococcus iniae (ATTC 29177)	Amazon freshwater dolphin *Inia geoffresis*	–
Streptococcus agalactiae	Tilapia *Oreochromis* spp., LADL	–
Flavobacterium columnare (ATCC 23463)	Chinook salmon *Oncorynchus tshawytscha*	–
Vibrio anguillarum	Hybrid striped bass *Morone chrysops* × *M. saxatilis*, LADL	–
Vibrio mimicus	Crayfish *Procambarus clarkii*, LADL	–
Mycobacterium marinum	Florida pompano *Trachinotus carolinus*, LADL	–
Nocardia seriolae	Pompano *Trachinotus blochii*, LADL	–

TaqMan primers and probe. The TaqMan primers and probe used in this study were designed on the basis of the nucleotide sequence comparison of the *iglC* gene of *Francisella tularensis* subsp. *novicida* U112 *iglC* (GenBank Accession number AY293579), *F. tularensis* subsp. *holarctica* FTNF002-00 *iglC* (GenBank Accession number CP000803), *F. tularensis* subsp. *mediasiatica* FSC147 *iglC* (GenBank Accession number CP000915), *F. philomiragia* ATCC 25017 *iglC* (GenBank Accession number CP000937), and *F. noatunensis* subsp. *orientalis* LADL 07-285A *iglC* (GenBank Accession number FJ386388) (Table 2). The primers and probe were designed following the real-time qPCR Assay Design Software (Biosearch Technologies). Primers and probe concentration were optimized at the beginning of the study to determine the minimum primer concentrations giving the maximum change in the normalized reporter dye fluorescence (ΔRn), and the minimum probe concentration that gave the minimum threshold cycle (C_t). The optimization was done according to the TaqMan Universal PCR Master Mix manufacturer (Applied Biosystems).

Real-time TaqMan PCR assays. The real-time PCR assays were conducted and analyzed within the Applied Biosystems 7500 Fast Real-Time PCR Systems (Applied Biosystems). The 25 µl reaction mixture consisted of a TaqMan Universal PCR Master Mix (Applied Biosystems), containing 10 pmol of each primer, 3 pmol of probe, and 5 µl of DNA extracted sample. Template controls containing PCR grade water and 7 serial dilutions of 100 ng µl^{-1} of *Francisella noatunensis* subsp. *orientalis* isolate LADL 07-285A diluted in PCR grade water and measured in a NanoDrop Spectrophotometer ND-1000 V3.5 (Nanodrop Technologies) were included in each run.

The unknown samples, as well as the diluted standards and negative controls, were run in triplicate. Cycling conditions were 2 min at 50°C, 15 min at 95°C followed by 40 cycles of 15 s at 95°C, and 60 s at 60°C.

Sensitivity of the real-time PCR assays: For sensitivity determination, the TaqMan assays were evaluated by 2 different independent methods. Three separate extractions of *Francisella noatunensis* subsp. *orientalis* DNA were adjusted to a concentration of 100 ng µl^{-1} NanoDrop Spectrophotometer ND-1000 V3.5 (Nanodrop Technologies), and 10-fold dilutions were made in PCR grade water until they reached a concentration of 1 fg µl^{-1}. Genome equivalent (GE) calculation was based on assuming a 2-MB genome size for *F. philomiragia* and several subspecies of *F. tularensis* (Takahashi et al. 2007, Tomaso et al. 2007, Abril et al. 2008, Ottem et al. 2008). For determination of colony-forming units (CFU), several isolated colonies of *F. noatunensis* subsp. *orientalis* were picked from a fresh CHAH culture and suspended in 1 ml of PBS at pH 7.2, until an optical density $(OD)_{600}$ of 0.75 was reached and measured in a DU-640 Spectrophotometer (Beckman Coulter). Ten-fold serial dilutions in PBS were made from this sample, and colony counts were performed on CHAH by the drop plate method to verify bacterial numbers. Extraction of DNA from 200 µl of each dilution was used for CFU quantification in the real-time PCR assay. Amplification efficiencies were determined, and all assays were run in triplicate.

Sensitivity of the real-time PCR assay in fish spleen: In order to determine the sensitivity limit of the assay, triplicate samples of 1 g of uninfected tilapia spleen (recently acquired fresh tissue) were homogenized with a Kontes PELLET PESTLE® Micro Grinder (A. Daigger) in a 4 ml suspension of early stationary phase *Francisella noatunensis* subsp. *orientalis* cells diluted in PBS to a final concentration of 2, 20, 200, 2×10^3, 2×10^4, 2×10^5, 2×10^6, 2×10^7 CFU g tissue^{-1}. A total of 200 µl of the homogenates containing approximately 50 mg of spleen were centrifuged at 12 000 × *g* for 1 min and DNA extracted following the manufacturer's protocol 'Isolation of Nucleic Acids from Mammalian Tissue', High Pure PCR Template Preparation Kit (Roche Diagnostics). Enumeration of *F. noatunensis* subsp. *orientalis* by real-time PCR was compared with plate count values, taking into account dilution/concentration factors due to volumes used in DNA extraction and final elution volumes. Amplification efficiencies were determined, and all assays were run in triplicate.

Experimental infectivity trial. The tilapia fingerlings used during the trial were obtained from a source with no history of *Francisella* infection, and a sub-sample of the population was confirmed as negative for *Francisella* bacteria by complete clinical, bacteriological, and molecular analysis as described by Soto et al. (2009a). Fish were maintained at 10 fish tank^{-1} and fed commercial tilapia feed daily (Burris Aquaculture Feeds,) at ~3% fish body wt d^{-1}. The mean weight of the fish was 9.1 g, and the mean length was 18 cm. Three tanks were used per treatment, and 1 tank was used as a control. Fish were immersed in 8 l of static water containing approximately 3.7×10^7

Table 2. TaqMan primers and probe used in the present study. FAM: 6-carboxy-fluorescein; BHQ-1: black hole quencher 1

Primers and probe	5′–3′ sequence	Melting temp. (°C)
iglC forward	gggcgtatctaaggatggtatgag	66.36
iglC reverse	agcacagcatacaggcaagcta	66.63
iglC probe	FAM atctattgatgggctcacaacttcacaa BHQ-1	68.34

CFU ml^{-1} in tank water for 3 h at 23 to 25°C, and then the volume of the tanks was adjusted to 20 l with clean oxygenated water. Control fish were treated in a similar manner but received sterile PBS.

Following each challenge exposure, mortality was recorded every 12 h for 30 d. Prior to collection of spleen, moribund and survivor fish were euthanized with an overdose of MS-222. The spleens from dead, moribund, and survivor fish were collected aseptically in 1.5 microcentrifuge tubes (Fisherbrand, Fisher Scientific) and weighed, and DNA was extracted from ~20 mg of spleen following the manufacturer's protocol 'Isolation of Nucleic Acids from Mammalian Tissue', High Pure PCR Template Preparation Kit (Roche Diagnostics). The rest of the tissue was homogenized in ~50 µl PBS and plated on CHAH. The eluted DNA was stored at 4°C until used.

RESULTS

Specificity

The assay was found to be specific for the warm-water fish pathogen *Francisella noatunensis* subsp. *orientalis* (Table 1), and no evidence of cross-reactivity was detected (no significant elevated signal was observed with any of the other tested bacterial DNA) (Fig. 1).

Sensitivity

The sensitivity of the assay was determined using a triplicate dilution series from 0.5 fg reaction^{-1} to 1.4 mg reaction^{-1} of *Francisella noatunensis* subsp. *orientalis* genomic DNA. The lowest amount of detection was determined to be 50 fg DNA (equivalent to ~25 GE). C_t determined by TaqMan real-time PCR amplification of DNA, extracted from serial dilutions of pure *F. noatunensis* subsp. *orientalis* bacterial culture, showed a linear (R^2 = 0.994) relationship with log numbers of CFU from 2.5×10^7 to 2.5×10^1 CFU ml^{-1} based on plate counts (Fig. 2). Ten-fold serial dilutions of nucleic acid extracted from the initial dilutions of the pure bacterial culture also showed a linear relationship between the log amount of nucleic acid and the TaqMan real-time PCR C_t from 1.4 mg to 50 fg (Fig. 2). Linear detection of amplified product was also revealed in serially diluted *F. noatunensis* subsp. *orientalis* spiked spleen homogenates (R^2 = 0.985) (Fig. 2, Table 3). This indicates that the presence of tissue homogenate did not impede the sensitivity of the real-time PCR assay within this range of CFUs. Uninfected tilapia spleen and water controls showed no signal after 40 cycles.

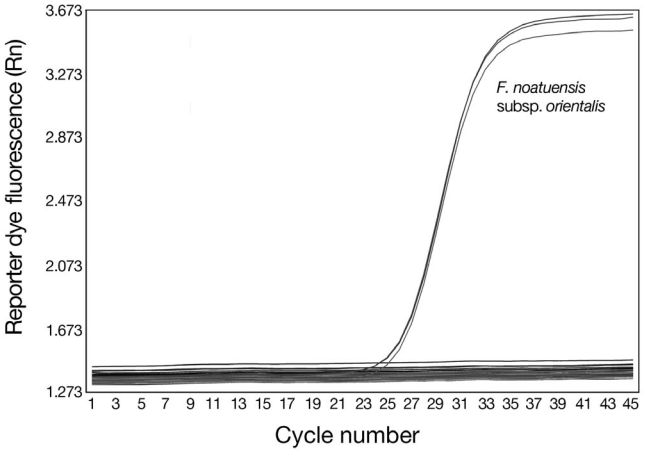

Fig. 1. Detection of the *iglC* gene by the TaqMan probe real-time PCR assay. A total of 500 pg per reaction of DNA from 21 different strains of fish pathogens or member of the genus *Francisella* sp. were used. The amplification plot displays normalized reporter dye fluorescence (Rn) as a function of cycle

Detection of *Francisella noatunensis* subsp. *orientalis* in experimentally infected fish

At 30 d following challenge, the mean mortality in the tanks was 56.6%. In order to test the ability of the *iglC* TaqMan assays to identify *Francisella noatunensis* subsp. *orientalis* in tilapia tissue, spleens from infected fish were analyzed. One hundred percent of the

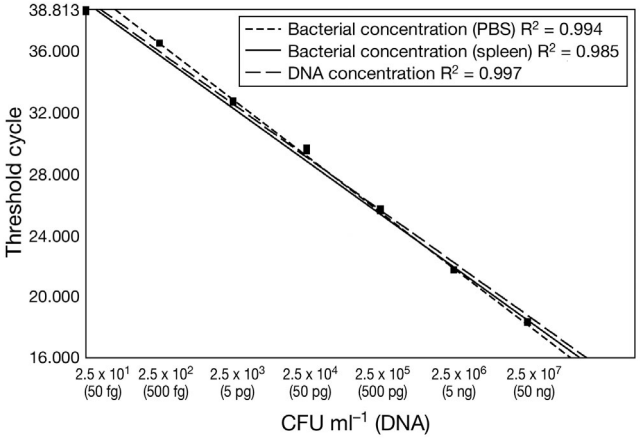

Fig. 2. *Francisella noatunensis* subsp. *orientalis*. Standard curve denoting the sensitivity limits of detection with 10-fold serial dilutions of *F. noatunensis* subsp. *orientalis* isolate LADL 07-285A DNA, colony-forming units (CFU) added to phosphate-buffered saline (PBS), and CFU added to spiked spleen homogenates. Threshold cycle values plotted against DNA or CFU added to PBS or spleen homogenates ranging from 2.5×10^1 to 2.5×10^7 CFU ml^{-1} show a linear correlation of R^2 = 0.994 for CFU in PBS (short-dashed line), R^2 = 0.985 for CFU in spleen homogenates (black line), and R^2 = 0.997 for DNA (long-dashed line). Results are from triplicate samples run simultaneously

Table 3. *Francisella noatunensis* subsp. *orientalis*. Quantification of the pathogen from inoculated phosphate-buffered saline (PBS) and spleen. Colony counts were determined by plate counts on threshold cycle (C_t) cystine heart agar with hemoglobin (CHAH) plates as described in the 'Materials and methods'. Real-time PCR determination of concentrations was based on the mean of triplicate samples. Concentrations were derived from a standard curve using the mean of triplicate C_t values of serial 10-fold dilutions of DNA extracted from known concentrations of bacteria. CFU: colony-forming units; qPCR: quantitative real-time polymerase chain reaction

Inoculum (log CFU ml^{-1})	Colony counts (log CFU ml^{-1} ± SD)		Extracted DNA qPCR (C_t ± SD)	
	PBS	Spleen homogenate	PBS	Spleen homogenate
7.3	7.59 ± 0.061	7.43 ± 0.13	18.35 ± 0.028	18.53 ± 0.228
6.3	6.62 ± 0.12	6.69 ± 0.12	25.70 ± 0.017	25.12 ± 0.117
5.3	5.52 ± 0.13	5.46 ± 0.15	29.65 ± 0.059	29.33 ± 0.125
4.3	4.86 ± 0.12	4.88 ± 0.12	32.80 ± 0.112	32.1 ± 0.134
3.3	3.59 ± 0.33	3.5 ± 0.0.39	36.54 ± 0.083	36.74 ± 0.121
2.3	2.43 ± 0.19	2.58 ± 0.2	38.74 ± 0.016	38.32 ± 0.026
1.3	0.33 ± 0.35	1.03 ± 0.43	25.70 ± 0.118	26.12 ± 0.418

morbid and survivor (challenged) fish were positive by the assay, and all non-challenged fish were negative. Detection of the bacteria by culture on CHAH agar media was possible in 58% of the dead fish and 38% of the survivors. The mean amount of *F. noatunensis* subsp. *orientalis* GE detected in spleens from dead fish analyzed by real-time PCR was 1.8×10^5 GE mg^{-1} of spleen tissue, while surviving fish presented a mean amount of 1.5×10^3 GE mg^{-1}of spleen tissue.

DISCUSSION

In the present study, we developed a TaqMan real-time quantitative PCR assay for the rapid identification and quantification of the emergent fish pathogen *Francisella noatunensis* subsp. *orientalis*. The development of this highly sensitive diagnostic method will enhance the diagnosis of this fastidious organism, which could be present at low levels in fish tissue and may require specialized media in which to grow; this organism may possibly be overgrown by secondary contaminants following attempts at primary isolation. Molecular diagnostic tests have been used to alleviate some of these problems and have been used along with DNA sequencing to give definitive diagnosis in complicated cases (Hsieh et al. 2006, Mauel et al. 2007, Soto et al. 2009a). As with many other diagnostic techniques, conventional PCR has certain limitations. It is time consuming, the results are based on band size discrimination and are measured at End-Point (plateau), and there is often low sensitivity, low resolution, and no quantification. On the other hand, real-time PCR collects data in the exponential phase, has increased dynamic range of detection, and has reduced time in post PCR processing (Espy et al. 2006). It is not surprising that the technique has increasingly been used in the past 5 yr for the detection and quantification of important human and veterinary fastidious bacterial pathogens like *Mycobacterium tuberculosis, F. tularensis, Bacillus anthracis*, etc. (Bode et al. 2004, Kocagoz et al. 2005, Takahashi et al. 2007, Tomaso et al. 2007, Abril et al. 2008). The disadvantages of real-time PCR include the cost of the assay, which is substantially higher than that of either microscopy or conventional PCR, and the need for specialized real-time PCR analyzers, which are currently beyond the means of many laboratories. Also, the presence of specific bacterial DNA in a tissue sample may not always indicate disease or viable bacteria. Thus, it is important to correlate the findings of this molecular technique with the history and macro- and microscopic findings of the clinical isolate (Bode et al. 2004, Espy et al. 2006, Kocagoz et al. 2005, Takahashi et al. 2007, Tomaso et al. 2007, Abril et al. 2008).

The assay developed in this study is directed against the previously identified *iglC* gene in *Francisella noatunensis* subsp. *orientalis* isolate LADL 07-285A (Soto et al. 2009b). The homologous gene in *F. tularensis* subspecies has been found to be upregulated *in vivo* and *in vitro* when the bacterium is infecting and colonizing macrophages and has been found to be essential for the survival of the bacterium inside the cells (Nano et al. 2004, Santic et al. 2005).

The specificity of the TaqMan probe real-time *iglC* PCR assay was assessed with other strains of the genus *Francisella* (*F. tularensis* subsp. *novicida* U112 and *F. tularensis* subsp. *holarctica* LVS), clinically relevant cold- and warm-water fish pathogens (*F. noatunensis* subsp. *noatunensis*, *Streptococcus* spp., *Edwardsiella* spp., *Aeromonas* spp., *Vibrio* spp., *Mycobacterium* spp., *Photobacterium* spp., etc.), and non-infected tilapia splenic tissue. After 40 cycles, DNA samples from these strains failed to show amplification using the real-time PCR assay, and the assay showed no

cross reaction of the chosen primers and probe with fish tissue or opportunistic fish pathogens listed above. This is particularly important with francisellosis, because moribund and dead fish are commonly found with secondary infections, and attempts to isolate *Francisella* spp. can be very difficult owing to the fastidious nature of the organism. There is evidence that the presence of other bacteria in clinical specimens may inhibit the growth of *Francisella* spp. and may impair the ability to isolate *F. tularensis* (Petersen & Schriefer 2005). The high specificity achieved by the TaqMan real-time PCR assay did not amplify the closely related cold-water pathogen *F. noatunensis* subsp. *noatunensis,* but it did amplify representative *F. noatunensis* subsp. *orientalis* isolates recovered from warm-water cultured tilapia and striped bass. We have previously described that after comparison of the 16S ribosomal RNA sequence, the *F. noatunensis* subsp. *orientalis* LADL 07-285A and *F. noatunensis* subsp. *noatunensis* shared more than 99% homology, but when the *iglC* genes were compared, the cod and the tilapia isolates shared only ~90% homology, making this gene a more specific target to differentiate between similar fish pathogens (Soto et al. 2009b). The differences found between the *iglC* sequences make the present assay specific for the *F. noatunensis* subsp. *orientalis* isolates.

The sensitivity limit of the assay was found to be ~50 fg of DNA (equivalent to ~25 GE or CFU) of *Francisella noatunensis* subsp. *orientalis*. In this study we used different approaches to verify that our DNA extraction methodology and the real-time PCR assay did not interfere with the results obtained in the assay. After suspending viable live bacteria in tilapia tissue homogenates and in PBS, performing CFU counts in CHAH, extracting the DNA under the same conditions, and running the assay, we found that fish tissue did not negatively affect the real-time PCR detection or quantification of *F. noatunensis* subsp. *orientalis*.

When experimentally infected tilapia were used to simulate wild epizootics, the real-time PCR assay enabled detection of the bacterium in all the dead, moribund, and surviving fish 30 d post challenge, whereas it was possible to isolate the bacteria by conventional culturing on agar plates in only 58.8% (10 of 17) of dead and moribund fish, and in 38% (5 of 13) of the survivor fish 30 d post challenge. The presence of secondary contaminants like *Aeromonas* spp. was greatly reduced by the use of selective media (CHAH with addition of polymixin B and ampicillin) when plating tissue from autolytic fish at necropsy. The TaqMan probe real-time PCR assay also allowed us to quantify the amount of GE of the bacterium in infected tilapia spleen, thus revealing a 2-fold higher amount of GE in dead tilapia spleen than in survivors.

In conclusion, we have developed an *iglC*-based TaqMan real-time PCR assay with high sensitivity and specificity for the detection and quantification of the emergent warm-water fish pathogen *Francisella noatunensis* subsp. *orientalis*. The assay can be used not only as a rapid diagnostic test for francisellosis but also as a research tool for bacterial persistence, drug therapy efficacy, epidemiological studies, screening of broodstock fish, and detection of reservoirs for infection.

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Appendix 1. Evolutionary relationships of the genus *Francisella* on partial *iglABCD* and *iglC* sequences

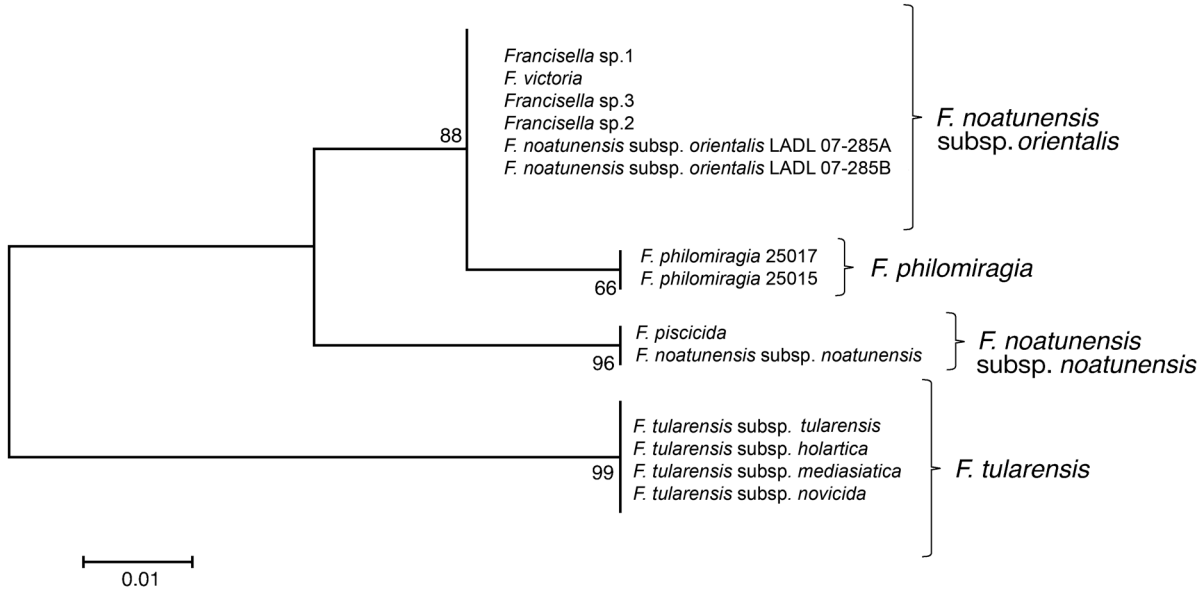

Fig. A1. Evolutionary relationships of 14 members of the genus *Francisella* based on partial *iglABCD* sequences. The evolutionary history was inferred using the neighbor-joining method (Saitou & Nei 1987). The optimal tree with the sum of branch length = 0.14285714 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the data set (complete deletion option). There were a total of 70 positions in the final data set. Phylogenetic analyses were conducted in MEGA 4 (Tamura et al. 2007)

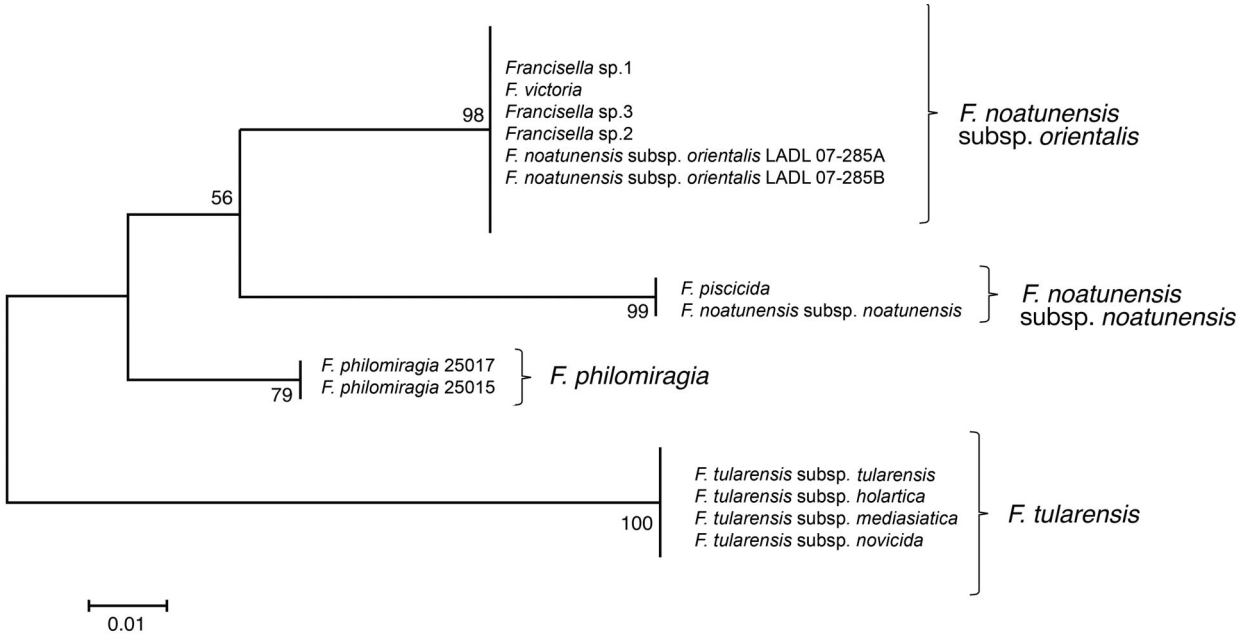

Fig. A2. Evolutionary relationships of 14 members of the genus *Francisella* based on partial *iglC* sequences. The optimal tree with the sum of branch length = 0.23571429 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). Other details as in Fig. A1

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