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Real-time PCR assays targeting unique DNA sequences of fish-pathogenic *Francisella noatunensis* subspecies *noatunensis* and *orientalis*

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ABSTRACT: Specific identification and differentiation of the 2 subspecies of the fish pathogen *Francisella noatunensis*, namely, *F. noatunensis* subsp. *noatunensis* and *F. noatunensis* subsp. *orientalis*, remains a major diagnostic challenge. Following whole-genome sequencing and analysis of representatives of all major subclades of the genus *Francisella*, specific genomic regions were identified for each of the subspecies of this fish pathogen. Two specific real-time quantitative PCR assays, directed at hypothetical genes within these regions were developed. Specificity was confirmed by lack of signal and cross-reactivity with the closest relative, *F. philomiragia*, and other common bacterial fish pathogens. Both assays, used either as monoplex or multiplex, have a limit of detection of 10 genome equivalents. The quantitative sensitivity of the assays was not affected by the presence of kidney tissues or DNA from Atlantic cod *Gadus morhua* or tilapia *Oreochromis* sp.

KEY WORDS: *Francisella noatunensis* · Fish · DNA · qPCR

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INTRODUCTION

Francisellosis in fish is an emergent disease caused by Gram-negative facultative intracellular pathogens belonging to the genus *Francisella*. The disease is commonly characterized by the presence of multi-organ granuloma and high morbidity, with varying associated mortality levels (Colquhoun & Duodu 2011). A number of fish species are affected, including Atlantic cod *Gadus morhua* (Nylund et al. 2006, Olsen et al. 2006), tilapia *Oreochromis* sp. (Mauel et al. 2007, Jeffery et al. 2010), Atlantic salmon *Salmo salar* (Birkbeck et al. 2007), hybrid striped bass (white bass *Morone chrysops* × striped bass *M. saxatilis*) (Ostland et al. 2006) and three-line grunt *Parapristipoma trilinineatum* (Fukuda et al. 2002). At present, all recognized fish-pathogenic *Francisella* strains belong to either *F. noatunensis* subsp. *orientalis* or *F. noatunensis* subsp. *noatunensis* (Ottem et al. 2009). Both pathogens are highly infectious. Because of the similarity in the pathological and clinical manifestations of disease caused by both subspecies in different fish hosts, sensitive and specific tests are needed to identify the infective agent involved in any particular case.

Diagnosis of francisellosis in fish using conventional methodology can be challenging. These bacteria are highly fastidious in their growth requirements, are easily inhibited by a diverse range of bacteria and require culture on specialized agar over several days. They are biochemically unreactive and immunohistochemical analysis using polyclonal antibodies appears to be less sensitive than nucleic acid-based detection assays (Ottem et al. 2006, 2008). Currently,

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real-time PCR has become the preferred methodology for *Francisella* detection due to its sensitivity and rapid turnaround time. However, the high degree of genetic similarity between the fish-pathogenic strains and their almost ubiquitous close relative *F. philomiragia* (Barns et al. 2005, Berrada & Telford 2010, Duodu et al. 2012) has made development of specific assays challenging. Nevertheless, several PCR assays exhibiting varying degrees of specificity now exist for the detection of members of the genus *Francisella* in fish. Ottem et al. (2008) described a combination of 2 real-time PCR assays targeting the 16S rRNA gene and the outer membrane protein *fopA* gene, which enabled detection and differentiation of the 2 fish pathogens when used together in separate analyses. More recently, a real-time PCR for specific detection of *F. noatunensis* subsp. *orientalis* targeting the intracellular growth locus gene, *iglC*, has been developed (Soto et al. 2010). A common feature of these assays is the dependency on low levels of nucleotide variation between the recognized fish-pathogenic species, which conceivably may lead to cross-reaction and false identification. The high prevalence of closely related *Francisella* bacteria in the environment may also complicate PCR detection of these pathogens (Duodu et al. 2012).

Our objectives were, therefore, to develop real-time quantitative PCR (qPCR) assays based on unique sequences found only in respective target genes of *Francisella noatunensis* subspecies. Selection of target genes was achieved by comparison of whole genome sequences from all known subspecies of this fish pathogen and representatives of all major subclades of the *Francisella* genus. We present results showing that the 2 real-time qPCR assays developed in this study are highly sensitive and specifically detect *F. noatunensis* subsp. *noatunensis* and *F. noatunensis* subsp. *orientalis* strains in infected fish.

MATERIALS AND METHODS

Bacterial strains and DNA extraction

The bacterial strains used in this study are listed in Table 1. *Francisella noatunensis* subsp. *noatunensis* (NCIMB14265^T), isolated from diseased Atlantic cod in Norway (Olsen et al. 2006), and *F. noatunensis* subsp. *orientalis* (DSM21254^T), isolated from three-line grunt in Japan (Kamaishi et al. 2005), were chosen as representatives of the 2 subspecies. These were cultured and maintained on cysteine heart agar plates supplemented with 5% chocolatized sheep blood (CHAB) (Difco). Other strains of the genus *Francisella* and *Piscirickettsisa salmonis* were grown on the same CHAB media. Colonies from *Tenacibaculum maritimum* and mycobacteria were grown on marine agar (Difco Marine Agar 2216) and Middlebrook (Difco) agar, respectively. All other bacterial strains or species used in the study were cultured on blood agar (5% bovine/ovine blood) or blood agar supplemented with 1.5% NaCl. Total genomic DNA (gDNA) from bacterial cultures was extracted using the QIAamp DNA mini kit (Qiagen) following the manufacturer's instructions. DNA samples were stored at 4°C for use within 1 wk and at −20°C for longer storage.

Identification and description of unique DNA sequences

Sequences unique to *Francisella noatunensis* DSM21254^T (*F. noatunensis* subsp. *orientalis*) or NCIMB14265^T (*F. noatunensis* subsp. *noatunensis*) were identified using a basic search procedure utilising currently existing sequenced genomes for fish-pathogenic *Francisella* strains, as well as other environmental *Francisella* and human-pathogenic *F. tularensis* strains (Sjödin et al. 2012). Briefly, the procedure identifies specific genomic regions in a target genome by searching a reference database comprising sequences for all 'non-target' genomes included in the analysis. The criterion for selection was a lack of homology with genes in other members of the genus (to ensure no cross-reactivity) and that sequences for individual targets were conserved in all sequenced members of the target subspecies. As only 3 genome sequences for *F. noatunensis* subsp. *orientalis* are currently accessible, primers flanking the unique region were designed and used to amplify the selected locus from several *F. noatunensis* subsp. *orientalis* isolates listed in Table 1. The uniqueness of identified genomic regions was confirmed by additional basic local alignment search tool (BLAST) searches against the included genomes as well as against GenBank non-redundant nucleotide and protein databases. The specific loci for *F. noatunensis* subsp. *orientalis* (DSM21254^T) and *F. noatunensis* subsp. *noatunensis* (NCIMB14265^T) are deposited in GenBank under accession numbers JQ780323 and JQ780324, respectively. All genomes included in the analysis are listed in Table S1 in the supplement (available at www.int-res.com/articles/suppl/d101p225_supp.pdf).

Table 1. Specificity of the real-time PCR assays. Qualitative results of real-time PCR are indicated as positive (+) or negative (–)

Bacterial species (origin)	Strain	*F. noatunensis* subsp. *noatunensis* PCR	*F. noatunensis* subsp. *orientalis* PCR
Francisella noatunensis subsp. *noatunensis* (Norway)	NCIMB14265^T	+	–
F. noatunensis subsp. *noatunensis* (Chile)	PQ 1106	+	–
F. noatunensis subsp. *noatunensis* (Ireland)	NVI 7061	+	–
F. noatunensis subsp. *orientalis* (Japan)	DSM21254^T	–	+
F. noatunensis subsp. *orientalis* (Costa Rica)	PQ1104	–	+
F. noatunensis subsp. *orientalis* (Alajuela, Costa Rica)	LADL-07-285A	–	+
F. noatunensis subsp. *orientalis* (California, USA)	CAL 1	–	+
F. noatunensis subsp. *orientalis* (Midwest, USA)	F8	–	+
F. noatunensis subsp. *orientalis* (Latin America)	Victoria	–	+
F. noatunensis subsp. *orientalis* (California, USA)	CAL 2	–	+
F. noatunensis subsp. *orientalis* (Midwest, USA)	01100	–	+
F. noatunensis subsp. *orientalis* (Texas, USA)	LADL-11-060	–	+
F. philomiragia	CCUG 13404	–	–
F. philomiragia	CCUG 19701	–	–
F. philomiragia	ATCC 25015	–	–
Aeromonas salmonicida subsp. *salmonicida*	NCIMB 1102	–	–
A. salmonicida subsp. *achromogenes*	NCIMB 1110	–	–
A. salmonicida subsp. *salmonicida*	NCIMB 1103	–	–
A. salmonicida subsp. *smithia*	NCIMB 13210	–	–
Arthrobacter globiformis	NCIMB 8907	–	–
Bacillus cereus	NVI 148	–	–
Brochothrix thermosphacta	NCFB 1676	–	–
Carnobacterium piscicola	ATCC 1985	–	–
Escherichia coli	NCIMB 10000	–	–
Moritella viscosa	NVI 139	–	–
M. viscosa	NCIMB 2263	–	–
Mycobacterium avium subsp. *avium*	DSM 44156	–	–
Mycobacterium marinum	NVI 172	–	–
Nocardia asterioides	NVI 567	–	–
Pasteurella skyensis	Clinical isolate	–	–
Photobacterium phosphoreum	NCIMB 1953	–	–
Piscirickettsia salmonis	NVI 669	–	–
Proteus mirabilis	NCIMB 10823	–	–
Pseudomonas fluorescens	NCIMB 10067	–	–
Rhodococcus erythropolis	NVI 371	–	–
R. equi	Clinical isolate	–	–
Serratia marcescens	NCIMB 10351	–	–
Staphylococcus aureus subsp. *aureus*	NCIMB 11787	–	–
Streptococcus algalactiae	NCIMB(70)1334	–	–
Tenacibaculum maritimum lign.	NVI 2039	–	–
Vibrio anguillarum	NCIMB 1291	–	–
V. salmonicida	NCIMB 2262	–	–
V. splendidus	NVI 408	–	–
Yersinia ruckeri	NVI 2774	–	–

Selection and optimization of primers and TaqMan MGB probes

PCR primers and TaqMan MGB probes (Table 2) were designed for selected unique sequences using the Primer Express software v. 2.0 (PE Applied Biosystems). Probes were 5′-labeled with fluorescent reporter dyes: 6-carboxy-fluorescein (FAM) and VIC® for DSM21254^T and NCIMB14265^T specific targets, respectively. The 3′ ends of the probes were tagged with a non-fluorescent quencher dye to reduce background fluorescence. Both primers and probes were synthesized by Applied Biosystems (PE Applied Biosystems) and stored at –20°C before use. Primers were initially selected based on cycle threshold (C_q) values and melting curve analyses of PCR products following a SYBR-Green I real-time PCR protocol (Duodu & Colquhoun 2010). The concentra-

Table 2. PCR primers and probes used in the study. GenBank accession number of target gene is shown in parentheses. F: forward primer; R: reverse primer; P: probe

Target	Sequence (5'–3')	Amplicon length (bp)	Source
Francisella noatunensis subsp. *noatunensis* (JQ780324)	TGA GTT GGT AAC CAT TGA TTG TAC ATA GT (F) CGA GTA CCT GGT GGG AGA AAG A (R) TTG TTT TTC AAG GAG TAT AGG TGC A (P)	97	Present study
F. noatunensis subsp. *orientalis* (JQ780323)	CAT GGG AAA CAA ATT CAA AAG GA (F) GGA GAG ATT TCT TTT TTA GAG GAG CT (R) AAT GCC AAA ATG AAT ATG CT (P)	85	Present study
Atlantic cod tissue (EF-1α)	CGG TAT CCT CAA GCC CAA CA (F) GTC AGA GAC TCG TGG TGC ATC T (R)	93	Olsvik et al. (2011)
Tilapia tissue (EF-1α)	TGA CTG CGC TGT GCT GAT C (F) CTT GGA GAT ACC AGC CTC GAA (R)	61	Speers-Roesch et al. (2010)

tion of primer–probe combinations were then optimized for best signal output (ΔR_n) in both singleplex and multiplex PCR reactions.

Real-time PCRs

Real-time PCR assays were performed using a Stratagene Mx3005P thermal cycler (Stratagene). Reactions for the TaqMan assays were carried out in a final volume of 25 µl, consisting of 12.5 µl 2× TaqMan Universal PCR master mix, 300 nM each primer, 100 nM fluorogenic probe and 1 µl DNA template. The PCR cycling conditions were as follows: 50°C for 2 min for uracil-DNA glycosylase (UNG) enzyme activity, 95°C for 10 min to simultaneously denature the UNG enzyme and activate the polymerase, and 45 cycles at 95°C for 15 s and 60°C for 1 min. Multiplex TaqMan probe-based amplification was carried out using the Qiagen multiplex PCR kit (Qiagen) in a 25 µl reaction containing 300 nM of each primer. Probe concentrations were optimized to yield minimal spectral overlap between fluorescence levels of the reporter dyes for each target. These were 100 and 300 nM for the VIC and FAM labeled probes, respectively. The thermal cycling conditions for the multiplex were as follows: 95°C for 15 min for enzyme activation, 45 cycles of amplification at 95°C for 30 s and annealing at 60°C for 1 min. Melting-curve analysis consisting of 1 cycle at 95°C for 30 s, 55°C for 30 s and 95°C for 30 s was also performed after SYBR-Green I PCR to check the specificity of amplification products. The primer concentrations and reaction conditions for both singleplex and multiplex SYBR-Green PCRs were the same as previously described (Duodu & Colquhoun 2010). Each PCR run included a negative (no-DNA template) control of ultrapure Milli-Q water. All samples and controls were analyzed in triplicate. The data were collected during each elongation step and analysis was carried out using MxPro 4.10 software (Stratagene), applying the default calculation of the threshold fluorescence.

Specificity evaluation of the real-time PCR assays

To test the specificity of primer and probes, gDNA extracted from close phylogenetic neighbors (*Francisella philomiragia*) and other bacterial fish pathogens were tested (Table 1). We also checked for cross-reactivity between the primers and primer–probe combinations designed for the individual target subspecies. The same TaqMan and SYBR-Green PCR conditions were applied as described above and each assay was performed by testing 2 ng of gDNA as template.

Limit of detection and efficiency of the real-time PCR assays

The sensitivities of the assays were established using 2 separate methodologies. Initial analyses were performed on 10-fold serial dilutions of gDNA extracted from pure bacterial cultures of NCIMB 14265^T and $DSM21254^T$ strains. DNA measured with a NanoDrop spectrophotometer ND-100 (NanoDrop Technologies) was diluted in deionized water (Qiagen), and each assay was tested using amounts ranging from 2 ng to 2 fg DNA per PCR (3 extractions in triplicate PCR runs). To determine the limit of detection (LOD) in fish matrices, bacterial cultures of the 2 strains were grown separately for 3 d at 22°C in Eugon broth (Difco) with shaking (150 rpm) to late

exponential phase and serially diluted 10-fold in phosphate-buffered saline (PBS). Aliquots of dilutions (200 µl) were individually mixed with equal amounts of fish tissue homogenate generated from approximately 0.5 g of head kidney tissues of Atlantic cod (NCIMB14265^T) and tilapia (DSM21254^T) homogenized in 2 ml PBS. These fish were initially screened by PCR and culturing to ensure that they were free from *Francisella* infection. The resulting bacterial cells in PBS and fish homogenates were enumerated on CHAB agar plates and 300 µl of these samples were concurrently subjected to total DNA extraction as described above. The numbers of colony-forming units (CFUs) were compared with the C_q value following real-time PCR amplification. Assay sensitivity was determined using DNA obtained from the calculated numbers of *Francisella* cells in PBS and fish homogenates ranging from 1.0 to 2.0 × 10^6 to 1.0 to 2.0 × 10^1 CFU ml^{-1}. To assess the linear relationship of the assays, the square regression coefficients (r^2) were calculated based on the standard curve generated by plotting C_q values against number of CFUs or genome equivalents (GEs). The efficiencies (E) of each assay were calculated as $E = 10^{-1/x} - 1$, where x is the slope of the regression curve.

Diagnostic validation of the real-time PCR assays

To determine the diagnostic sensitivity and specificity of the assays, tests were performed on experimentally infected fish. Tilapia (mean weight = 150 g) were obtained from a farm with no previous history of fish francisellosis. Fish were acclimated for a minimum of 10 d in recirculating systems at 25°C under optimum water quality conditions. Prior to challenge exposure, 5 to 10 fish were analyzed for evidence of *Francisella* by clinical examination, bacteriological isolation and by PCR (Soto et al. 2009a). Infectivity challenges in tilapia were made following a bath challenge model previously described (Soto et al. 2009b). Briefly, tilapia were maintained in 2 different 40 l tanks (6 fish per tank) and challenged with *F. noatunensis* subsp. *orientalis* strain LADL 07-285A. Bacterial suspension in PBS was added to water to a final concentration of 10^4 CFU ml^{-1}. The water temperature was maintained at 25°C throughout the experiment. The negative controls inoculated with PBS were similarly handled but were not exposed to *F. noatunensis* subsp. *orientalis*. In addition, cod intraperitoneally injected with *F. noatunensis* subsp. *noatunensis* strain NCIMB14265^T (7 d post-infection and maintained at 15°C) were also analyzed (fish kindly provided by Dr. M. Seppola, Nofima Marin, Norway). DNA was extracted from ~20 mg of kidney tissue derived from both infected and control fish using either QIAamp DNA Mini Kit (Qiagen) or the High Pure PCR Template Preparation Kit (Roche Diagnostics) following the manufacturer's protocol.

RESULTS

The selected loci were unique for each target strain and corresponded to hypothetical genes with no homologs according to NCBI BLASTP searches. Both unique regions were conserved in all isolates of individual subspecies. The designed primer–probe sequences demonstrated no significant similarity using a BLASTN search with other sequences in NCBI database. Both PCR assays demonstrated specific amplification of their respective individual DNA targets and displayed single peaks in the melting curve analysis using the SYBR-Green PCR protocol. The melting temperatures (T_m) of the generated PCR products for *F. noatunensis* subsp. *noatunensis* and *F. noatunensis* subsp. *orientalis* specific assays were 75.2 and 72.2°C, respectively. When the specificity of the assays, defined as the ability to discriminate between target (inclusivity) and non-target bacteria (exclusivity), was tested against a panel of DNA templates from several bacterial strains (Table 1), only the target strains gave the expected PCR signal specific for each assay, resulting in 100% inclusivity and exclusivity. The absence of amplification signal from non-targeted bacterial strains was confirmed using both TaqMan and SYBR-Green detection chemistries. The LOD of each assay towards purified gDNA from each target bacterium was determined. The LOD was defined as the lowest amount of DNA at which all 9 replicate runs from 3 different extractions gave a positive PCR signal. For both assays, this was determined to be 20 fg DNA (~10 GEs) per PCR (Table 3). To determine the LOD in a fish matrix, standard curves were constructed using triplicate extractions of DNA obtained from serial dilutions of *Francisella* cells spiked into cod (*F. noatunensis* subsp. *noatunensis*) and tilapia (*F. noatunensis* subsp. *orientalis*) homogenates. The linear regression analysis of mean C_q values against CFU detected from fish homogenates yielded r^2 values and PCR efficiencies, which were directly comparable with those obtained with DNA from bacterial cultures suspended in PBS (Table 4). Both assays exhibited detection of 1.0 ×

Table 3. *Francisella noatunensis.* TaqMan and SYBR-Green PCR performances based on serial dilutions of genomic DNA from 2 subspecies. C_q: threshold cycle number; variance = SD^2

Amount of DNA per PCR	Signal ratios (no. of PCR positives)	TaqMan C_q Mean	TaqMan C_q Variance of mean	SYBR-Green C_q Mean	SYBR-Green C_q Variance of mean
F. noatunensis* subsp. *noatunensis					
2 ng	9/9	20.08	0.17	18.23	0.04
200 pg	9/9	23.53	0.39	22.2	0.17
20 pg	9/9	27.12	0.39	26.13	0.27
2 pg	9/9	30.43	0.36	29.69	0.65
200 fg	9/9	34.21	0.66	33.26	0.09
20 fg	9/9	37.60	0.65	36.45	0.43
2 fg	3/9	39.29	0.05	38.16	0.74
Regression equation:		$y = -3.5129x + 41.123$ $r^2 = 0.9998$		$y = -3.6526x + 40.444$ $r^2 = 0.9985$	
F. noatunensis* subsp. *orientalis					
2 ng	9/9	20.16	0.44	18.15	0.49
200 pg	9/9	23.54	0.50	22.03	0.10
20 pg	9/9	27.20	0.22	25.63	0.11
2 pg	9/9	30.67	0.39	29.97	1.52
200 fg	9/9	34.38	0.47	33.41	1.30
20 fg	9/9	37.29	0.33	36.27	0.71
2 fg	3/9	40.38	0.65	33.89	1.26
Regression equation:		$y = -3.4756x + 41.037$ $r^2 = 0.9991$		$y = -3.688x + 40.485$ $r^2 = 0.997$	

Table 4. *Francisella noatunensis.* Standard curve parameters for *F. noatunensis* subsp. *noatunensis* and *F. noatunensis* subsp. *orientalis* based on TaqMan and SYBR-Green analyses in PBS and Atlantic cod and tilapia tissues (*F. noatunenesis* subsp. *noatunensis* was used in cod and *F. noatunenesis* subsp. *orientalis* was used in tilapia)

qPCR/template	*F. noatunensis* subsp. *noatunensis*			*F. noatunensis* subsp. *orientalis*		
	r^2	Slope	Efficiency	r^2	Slope	Efficiency
TaqMan						
PBS	0.996	–3.46	0.945	0.998	–3.25	1.031
Fish tissue (cod/tilapia)	0.997	–3.20	1.054	0.999	–3.13	1.087
SYBR-Green						
PBS	0.997	–3.18	1.063	0.993	–3.47	0.942
Fish tissue (cod/tilapia)	0.995	–3.66	0.876	0.997	–3.15	1.077

10^1 to 2.0×10^1 CFU in 25 mg of kidney tissue homogenate (Fig. 1). Amplification of elongation factor 1 (EF-1α) genes for cod and tilapia resulted in low PCR signals (C_q values ≈ 21) indicating a high concentration of fish DNA within the samples analyzed. Multiplexing the 2 assays in a single tube did not influence the PCR efficiency or the limit of detection of the individual DNA targets (Fig. 2A). Specific melting peaks with mean (±SD) T_m of 75.18 ± 0.194°C and 72.35 ± 0.273°C were generated from amplicons obtained by co-amplifying the 2 target strains in a wide range of bacterial gDNA from 2 ng to 2 fg (Fig. 2B). Analysis of kidney tissue samples from experimentally infected fish 7 d post-injection (p.i.) resulted in 100% detection of individual target subspecies in their respective fish host. In all, 12 and 15 individual head kidney samples from tilapia and cod, respectively, were analyzed with C_q values in the range of 23.1 to 34.5 and 21.3 to 26.1, respectively. The detection specificity was confirmed by both TaqMan and SYBR-Green assays. Non-challenged fish were all real-time PCR negative. A cut-off C_q value of 37 was established as a positive diagnostic threshold for both subspecies based on titration of DNA from pure bacterial cultures and spiked fish tissues.

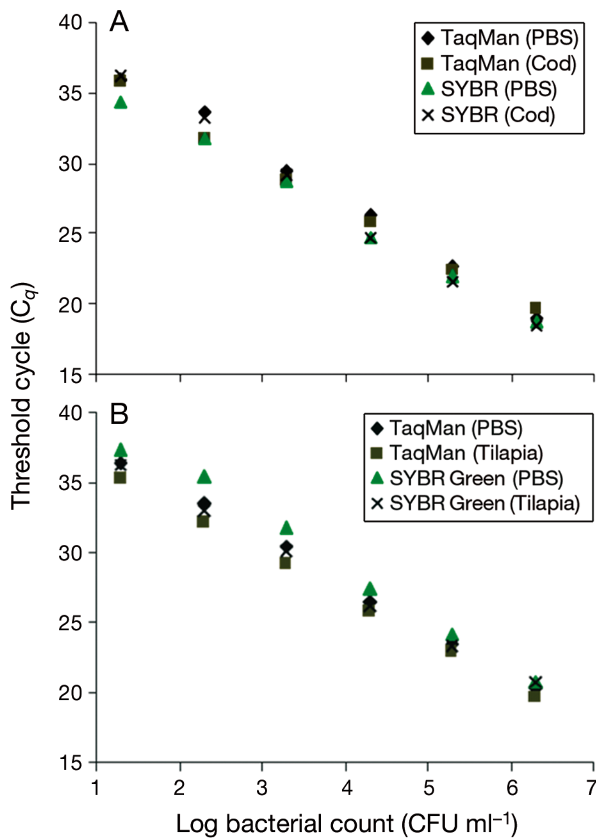

Fig. 1. *Francisella noatunensis.* Standard curves for (A) *F. noatunensis* subsp. *noatunensis* and (B) *F. noatunensis* subsp. *orientalis* showing the relationship between 10-fold serial dilutions of CFU versus C_q for both TaqMan and SYBR-Green assays. The calculated square regression coefficients (r^2), slope and amplification efficiencies are shown in Table 4. Results are from triplicate samples run in triplicate

DISCUSSION

Molecular detection of bacterial pathogens has become an integral part in clinical diagnosis of many fish diseases. In the present study, 2 real-time qPCR assays were developed for sensitive detection and discrimination of an emerging group of fish pathogens belonging to the genus *Francisella*. The assays demonstrated high analytical sensitivity and specificity. As both primers and probes are derived from sequences unique to each target subspecies the likelihood of false positives or cross reaction is low. Both TaqMan and SYBR-Green real-time PCR showed very high specificity for each assay, resulting in 100% inclusivity and exclusivity. The absence of non-target bacterial DNA amplification is particularly important as secondary infections with other bacteria commonly occur. Such mixed infections are most probably due to the chronic nature, i.e. low mortality and high morbidity, of the disease and consequential weakening of the immune system in affected fish. Experiences in our own laboratory confirm that isolation of *F. noatunensis* subsp. *noatunensis* from Atlantic cod is readily inhibited in the presence of a wide range of bacteria, both fish-pathogenic and environmental, making non-culture-based detection very relevant. The LOD of each assay using pure gDNA was determined to be ~20 fg for both TaqMan and SYBR-Green detection assays. Considering a genome size of approximately 1.9 Mbp for *F. noatunensis*, the LOD observed here corresponds to ~10 GEs or CFUs, which compares favourably with those reported previously for assays based on single copy gene targets (Ottem et al. 2008, Soto et al. 2010). The comparable LODs between gDNA and CFU ml^{-1} in inoculated fish tissues suggest that the analytical sensitivity of the assay is not negatively influenced by the presence of host fish DNA. Given the high degree of sensitivity and linearity (r^2) over the wide range of DNA template concentrations provided by both TaqMan and SYBR assays, our methods may be used for quantitative evaluation of *Francisella* cells from fish tissues at different stages of disease development. The assays allow multiplexing in a single reaction without compromising the PCR amplification efficiency. The 2 subspecies could also be clearly differentiated simultaneously based on the unique T_m of their specific amplicons following SYBR-Green PCR amplification. While use of SYBR-Green chemistry reduces the cost of analysis, it also constitutes a practical and robust tool for rapid screening and initial diagnosis of disease. As both *F. noatunensis* subsp. *noatunensis* and *F. noatunensis* subsp. *orientalis* have been identified from tilapia and Atlantic cod on and around the British Isles (Jeffery et al. 2010, Zerihun et al. 2011), the described assays may prove valuable diagnostic tools in this area. Simulation of wild epizootics using experimentally infected tilapia and cod resulted in high diagnostic sensitivity and specificity. Although the detection of individual subspecies was directed at early phase infections (7 d p.i.) the fish were asymptomatic and had no severe clinical signs, which is characteristic of carrier- and chronically infected populations.

Until recently, non-culture discrimination of fish-pathogenic *Francisella* strains directly in fish tissues has been almost impossible. Although multiple locus variable tandem repeat analysis (MLVA) has recently been used for typing and discrimination of isolates to the subspecies level (Brevik et al. 2011), the technique requires culture and additional steps, including gel/capillary electrophoresis after PCR amplification, which makes it impractical for rapid

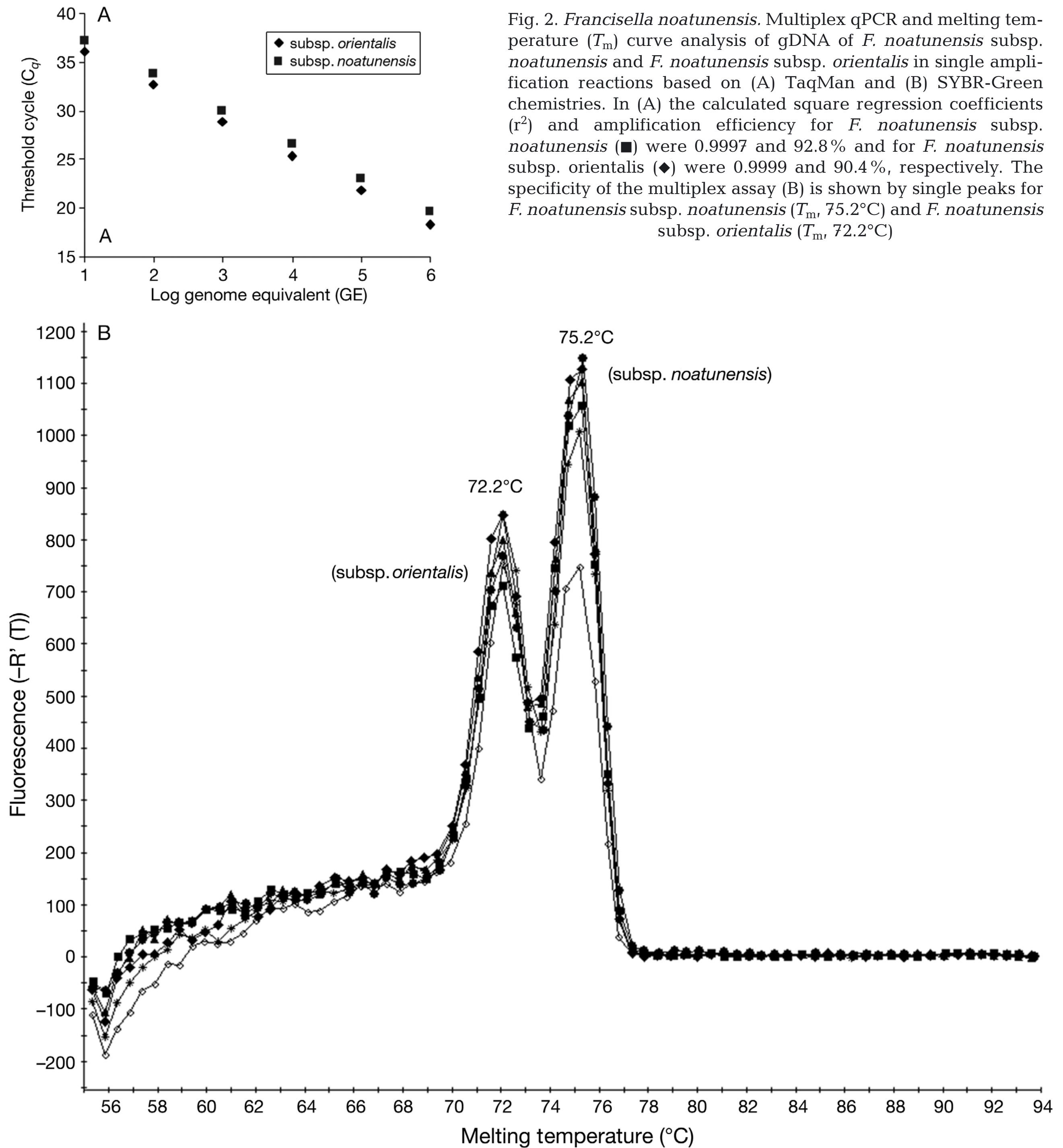

Fig. 2. *Francisella noatunensis.* Multiplex qPCR and melting temperature (T_m) curve analysis of gDNA of *F. noatunensis* subsp. *noatunensis* and *F. noatunensis* subsp. *orientalis* in single amplification reactions based on (A) TaqMan and (B) SYBR-Green chemistries. In (A) the calculated square regression coefficients (r^2) and amplification efficiency for *F. noatunensis* subsp. *noatunensis* (■) were 0.9997 and 92.8% and for *F. noatunensis* subsp. orientalis (◆) were 0.9999 and 90.4%, respectively. The specificity of the multiplex assay (B) is shown by single peaks for *F. noatunensis* subsp. *noatunensis* (T_m, 75.2°C) and *F. noatunensis* subsp. *orientalis* (T_m, 72.2°C)

identification of the targeted strains. A real-time qPCR assay designed for specific detection of *F. noatunensis* subsp. *noatunensis* targeting the 16S rRNA gene demonstrated excellent sensitivity with a detection limit of ~2 to 3 GEs, but failed to distinguish between the 2 subspecies (Ottem et al. 2008). In the same study, using *fopA* as the gene target, Ottem et al. (2008) could not distinguish between *F. philomiragia* and the subspecies *F. philomiragia* subsp. *noatunensis*. More recently, however, Soto et al. (2010) described a TaqMan real-time PCR assay for specific detection of *F. noatunensis* subsp. *orientalis* targeting the *iglC* gene, with a detection level of approximately 25 GEs. Although genetic variation is

evident in the *iglC* sequences (~90% identity), development of a specific assay for *F. noatunensis* subsp. *noatunensis* based on this gene has not been reported. In another study using conventional PCR, the *groEL* gene was identified as a suitable target for specific detection of *F. noatunensis* subsp. *noatunensis*, but its specificity against *F. noatunensis* subsp. *orientalis* and other close relatives was not tested (Kulkarni et al. 2011). Nevertheless, the presence of large numbers of closely related *Francisella* species in the environment (Barns et al. 2005, Berrada & Telford 2010, Duodu et al. 2012) should be borne in mind, particularly when identifying infection in species of fish that have been previously unexamined. More recently, Duodu et al. (2012) identified environmental sequences matching both primers and probes used in previous detection of *F. noatunensis* subsp. *noatunensis* from wild fish species and other invertebrate aquatic hosts (Ottem et al. 2008). This finding suggests the possibility of false positives among previously published PCR-based environmental *F. noatuanensis* detections. As the distribution range and varieties of fish species affected by francisellosis remain unknown, specific identification of the individual subspecies is important. The assays reported in this study therefore represent a major diagnostic improvement over existing assays as they allow specific detection and differentiation of the 2 currently known subspecies of this fish pathogen.

In conclusion, we have developed specific real-time PCR assays for individual detection of *Francisella noatunensis* subsp. *noatunensis* and *F. noatunensis* subsp. *orientalis* that can utilize either TaqMan or SYBR-Green detection chemistries and be multiplexed. Although there always remains the possibility that uncharacterized strain variants may exist or new variants may arise that do not possess a particular target sequence, both assays demonstrate specific and sensitive identification of the currently known fish-pathogenic *Francisella* strains.

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