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Duplex PCR assay and *in situ* hybridization for detection of *Francisella* spp. and *Francisella noatunensis* subsp. *orientalis* in red tilapia

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ABSTRACT: Conventional isolation and identification based on phenotypic characteristics is challenging with the highly fastidious, intracellular bacterium *Francisella noatunensis* subsp. *orientalis* (*Fno*). Here, we developed a duplex PCR method for simultaneous detection of the *Francisella* genus and *Fno* in one PCR reaction and an *in situ* hybridization method for paraffin section based diagnosis of *Fno*. The PCR results showed genus- and species-specific bands (1140 and 203 bp) from *Fno* but only one genus-specific band (1140 bp) from *F. noatunensis* subsp. *noatunensis*. Sensitivity of the duplex PCR assay revealed a detection limit of 20 to 200 fg genomic DNA (~10 to 100 genome equivalents) depending on DNA template extraction methods. The newly developed duplex PCR assay could be used to detect *Fno* from clinically sick fish exhibiting signs of visceral granulomas and would also be able to detect *Fno* infection in naturally diseased fish without symptoms of francisellosis, indicating potential application for diagnosis of field samples. The *in situ* hybridization assay using *Fno* species-specific probe revealed positive signals in multiple organs including the spleen, liver, kidney, gills and intestine of infected fish.

KEY WORDS: Molecular diagnostic assay · Francisellosis · ISH · Histology · Oreochromis · Aquaculture

INTRODUCTION

Francisellosis is a systemic infectious disease affecting intensive production of farmed tilapia globally. The disease is caused by *Francisella noatunensis* subsp. *orientalis* (*Fno*), a Gram-negative, intracellular bacterium (Soto et al. 2009, Nguyen et al. 2015). This bacteria has naturally or experimentally infected various warm freshwater fish, including threeline grunt *Parapristipoma trilineatum*, tilapia *Oreochromis* spp., hybrid striped bass *Morone chrysops* × *M. saxatilis*, and ornamental cichlids (Kamaishi et al. 2005, Hsieh et al. 2006, Ostland et al. 2006, Soto et al. 2009, Leal et al. 2014, Nguyen et al. 2015). The

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other closely related subspecies *Francisella noatunensis* subsp. *noatunensis* (*Fnn*) specifically affects coldwater fish, e.g. Atlantic cod *Gadus morhua* and Atlantic salmon *Salmo salar* (Olsen et al. 2006, Birkbeck et al. 2007). Both *Fno* and *Fnn* have been classified as highly fastidious intracellular bacteria that constitute a challenge for conventional diagnostic approaches (Birkbeck et al. 2007, Duodu et al. 2012, Nguyen et al. 2015). Some advanced diagnostic protocols have been previously developed, such as *Francisella*-specific PCR (Forsman et al. 1994), *in situ* hybridization for detection of *Francisella*-like bacteria (Hsieh et al. 2007), real-time PCR targeting an intracellular survival encoding gene or unique DNA sequences of *Francisella* (Soto et al. 2010, Duodu et al. 2012), and antibody-based immunohistochemistry (Soto et al. 2012). However, a conventional specific PCR method for identification and detection of *Fno* that most laboratories can easily access and apply has not yet been established. This study, therefore, aimed to develop (1) a conventional duplex PCR protocol targeting both the *Francisella* genus and *Fno* and (2) a DNA *in situ* hybridization method using a designated *Fno*-specific probe for paraffin section based diagnosis.

MATERIALS AND METHODS

Bacterial isolates and DNA extraction

The bacterial isolate *Francisella noatunensis* subsp. *orientalis* VMCU-FNO131, recovered from diseased red tilapia *Oreochromis* sp. and subsequently confirmed as the aetiological agent of francisellosis in Thai tilapia farms (Nguyen et al. 2015), was used in this study. Fourteen isolates of 7 other bacterial species previously recovered from diseased tilapia *Oreochromis* sp. and striped catfish *P. hypophthalmus* (Dong et al. 2015b,c) were also included to test for specificity of our duplex PCR protocol (Table 1). Bacterial growth conditions and boiling extraction of genomic DNA from all pure bacterial isolates used in this study were performed as previously described (Dong et al. 2015b,c, Nguyen et al. 2015). Additionally, genomic DNA of *Fno* isolate AL1104 and 2 isolates of *Fnn* (designated AL1106 and AL1108) kindly provided by Pharmaq Ltd. (Norway) (Table 1) were extracted by the QIAcube automated system using a DNeasy Blood & Tissue Mini kit (Qiagen). Except for the isolates VMCU06, VMCU11 and AL1108, bacterial species of all the tested isolates have been previously identified based on a combination of phenotypic characteristics and specific PCR or 16S rRNA sequence analysis (Table 1). The remaining 3 isolates were then subjected to 16S rRNA sequencing in this study. Approximately 1.5 kb 16S rRNA sequences of each isolate were obtained and deposited in GenBank under accession numbers KU860460, KU860461 and KU879058. Subsequently, VMCU06 and VMCU11 were identified as *Edwardsiella tarda* based on 99.8% sequence identity to 16S rRNA sequence of the *E. tarda* ATCC 15947 reference strain (accession no. NR118487), while AL1108 identified as *Fnn* with 100% identity to *Fnn* 2005/50/F292-6C (= LMG 23800 = NCIMB 14265, accession nos. DQ295795 and NR043696, respectively)

Table 1. Genomic DNA of bacterial isolates used for PCR assay in this study

Mark	Species	Strain designation	Origin	Reference(s)
F1	*Francisella noatunensis* subsp. *orientalis*	AL1104/PQ1104	Tilapia, Costa Rica	Duodu et al. (2012), Sjödin et al. (2012)
F2	*F. noatunensis* subsp. *noatunensis*	AL1106/PQ1106	Atlantic salmon, Chile	Duodu et al. (2012), Sjödin et al. (2012)
F3	*F. noatunensis* subsp. *noatunensis*	AL1108	Atlantic cod, Norway	This study
F4	*F. noatunensis* subsp. *orientalis*	VMCU-FNO131	Red tilapia, Thailand	Nguyen et al. (2015)
H1, H2	*Hahella chejuensis*	HN01, HN02	Tilapia, Thailand	Senapin et al. (2016)
FL1, FL2	*Flavobacterium columnare*	CF1, CF2	Striped catfish, Thailand	Dong et al. (2015c)
Et1	*Edwardsiella tarda*	VMCU06	Nile tilapia, Thailand	Laboratory strain
Et2	*Edwardsiella tarda*	VMCU11	Striped catfish, Thailand	Laboratory strain
Ei1, Ei2	*Edwardsiella ictaluri*	T1-1, T1-2	Striped catfish, Thailand	Dong et al. (2015c)
A1, A2	*Aeromonas veronii*	NK01, NK03	Nile tilapia, Thailand	Dong et al. (2015b)
P1, P2	*Plesiomonas shigelloides*	NK10, NK11	Nile tilapia, Thailand	Dong et al. (2015b)
S1, S2	*Streptococcus agalactiae*	NK13, NK14	Nile tilapia, Thailand	Dong et al. (2015b)

Table 2. Primers used in this study

Primer name	Sequence (5′ to 3′)	Target	Product size (bp)	Reference
F11	TACCAGTTGGAAACGACTGT	*Francisella* spp.	1140	Forsman et al. (1994)
R5	CCTTTTTGAGTTTCGCTCC			
FnoF1	GGCGTAACTCCTTTTAGCTTCC	*Francisella noatunensis* subsp. *orientalis*	203	This study
FnoR1	TTAGAGGAGCTTGGAAAAGCA			

Primer design

The *Francisella*-specific primers F11/R5 (Table 2) were obtained from Forsman et al. (1994). This primer set specifically targeted 16S rRNA sequences of bacterial species belonging to the *Francisella* genus. Since the 16S rRNA gene exhibited a very high identity (~99%) among species within the *Francisella* genus, another target DNA fragment was selected for the species-specific primer design. A uniquely identified sequence of a hypothetical protein gene of *Fno* species (GenBank accession no. JQ780323) has been employed as a target DNA in a real-time PCR detection and shown to be *Fno* species-specific (Duodu et al. 2012). The primer set FnoF1/FnoR1 (Table 2) was thus designed based on this region using Primer3 software (v. 0.4.0) (Untergasser et al. 2012).

Specificity and sensitivity of duplex PCR assay

Several duplex PCR mixtures and thermocycler conditions were trialled in this study (data not shown) in order to determine optimal PCR conditions, which were finally set up as follows. The PCR reaction mixture was composed of 12.5 µl Master Mix (GoTaq®Green, Promega USA), 0.8 µM of each primer F11 and R5, 0.2 µM of each primer FnoF1 and FnoR1, 4 µl of DNA template (150–200 ng) and nuclease-free water in a final volume of 25 µl. Thermocycler conditions were performed as follows: denaturation at 94°C for 3 min; 35 cycles of amplification at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min; final extension at 72°C for 5 min. PCR products were then electrophoresed with 1% agarose gel and visualized under UV light The specificity of the duplex PCR assay was tested with DNA extracted from 14 bacterial isolates recovered from diseased fish as mentioned above (Table 1). Sensitivity testing of the PCR assay was performed with 10-fold serial dilutions (200 ng to 0.2 fg) of genomic DNA from the 2 bacterial isolates VMCU-FNO131 and AL1104 (Table 1). DNA concentration of each bacterial isolate was quantified using Colibri Spectrometer (Titertek Berthold, Germany) and adjusted to the desired concentrations mentioned above.

Sequence analysis of PCR products

Two amplified amplicons of 1140 and 203 bp in size, generated by genus- and species-specific primers, respectively, were purified and cloned into pPrime cloning vector (5PRIME). Recombinant plasmid was sequenced by 1st BASE Pte Ltd. (Malaysia). A BLAST search of NCBI was performed with those sequences to confirm accuracy of amplified products.

Detection of *Fno* in fish samples

A group of 8 specimens of naturally diseased red tilapia (group NG1, Table 3) exhibiting typical granulomas in the internal organs were collected from affected farms in Kanchanaburi province in Thailand during a disease outbreak in 2013 and subjected to the duplex PCR assay. Additionally, a group of 10 experimentally infected red tilapia (EG1, Table 3), which had been intraperitoneally injected with ~10^6 CFU $fish^{-1}$ of *Fno* VMCU-FNO131 and exhibited similar clinical signs to the naturally infected fish, were also used. These samples were previously tested positive by genus-specific PCR assay (Nguyen et al. 2015). Samples from natural disease outbreaks exhibiting columnaris disease (N1 to N4, Table 3) and one set of samples collected in 2012 from naturally diseased red tilapia with unknown clinical signs (N5, Table 3) were also investigated for the presence of *Fno.* A mixture of the internal organs (liver, kidney and spleen) was aseptically taken from each fish and preserved in absolute alcohol prior to DNA extraction. Fish DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Germany) according to the manufacturer's instructions. Extracted DNA was kept at −20°C until used. Genomic DNA of *Fno* VMCU-FNO131 and of AL1104 was used as positive controls while nuclease-free water without DNA

Table 3. Fish samples used for duplex PCR assay. ND, not determined; +ve, PCR positive test; –ve, PCR negative test

Group	No. of fish	Fish species	Origin	Clinical features	+ve/–ve
NG1	8	Red tilapia	Kanchanaburi, Sep 2013	Naturally diseased fish exhibiting visceral granulomas	8/8
EG1	10	Red tilapia	ND, 2014	Experimentally diseased fish exhibiting visceral granulomas	10/10
N1	5	Red tilapia	Kanchanaburi, Feb 2013	Naturally diseased fish exhibiting clinical signs of columnaris disease but no visceral granulomas observed	5/5
N2	5	Red tilapia	Kanchanaburi, Feb 2013		0/5
N3	4	Red tilapia	Kanchanaburi, Sep 2013		0/4
N4	5	Red tilapia	Kanchanaburi, Sep 2013		0/5
N5	7	Red tilapia	ND, 2012	Naturally diseased fish with unknown clinical signs	5/7

templates was used as the negative control. Duplex PCR reactions were performed as described above.

Histological assessment and *in situ* hybridization

The internal organs (spleen, liver, kidney and intestine) and the gills of the 3 naturally *Fno* infected fish and 3 experimentally infected fish were preserved in 10% neutral buffered formalin for histological assessment and DNA *in situ* hybridization assay. Paraffin sectioning was conducted following standard protocols. Sections were stained with hematoxylin and eosin (H&E) for histological examination. The slides exhibited the presence of granulomas, a typical pathological feature of francisellosis, and were selected for *in situ* hybridization assay. *Fno* species-specific PCR product of 203 bp (Fig. 1) was employed as a template for digoxygenin (DIG)-labeled probe preparation using a commercial PCR DIG-labeling mix (Roche Molecular Biochemicals). The probe was purified using a Favorgen Gel/PCR Purification Kit (Taiwan) and used in standard *in situ* hybridization assays with fish tissue sections as previously described (Dong et al. 2015c, Senapin et al. 2016). The same kind of tissue collected from 2 healthy tilapia that had tested negative by *Francisella*-specific PCR assay served as negative control for *in situ* hybridization assay.

GGCGTAACTCCTTTTAGCTTCCTAGAACATCAATCTCAGTTA
GAACGAAAAGATTATACAATAACAATAACATTTCTTGGAGAA
AATTATTCGCAAATAGCTCGTACAGGAACAGCCAAATTAACA
CATATCTCATGGGAAACAAATTCAAAAGGAATTATATTTAAA
TGCCAAAATGAATATGCTTTTCCAAGCTCCTCTAA

Fig. 1. Sequence of *Fno*-specific probe for *in situ* hybridization in this study. Underlines represent FnoF1 and FnoR1 primer positions

RESULTS

Specificity and sensitivity of duplex PCR protocol

The duplex PCR assay was designed to simultaneously detect *Francisella* bacteria and *F. noatunensis* subsp. *orientalis* species (*Fno*) in a single reaction. A published primer pair based on 16S rDNA (1140 bp) for the specific detection of *Francisella* (Forsman et al. 1994) in combination with newly designed primers targeting a 203 bp fragment of a unique sequence from *Fno* (Duodu et al. 2012) were employed. The results shown in Fig. 2 revealed that 2 target amplicons were obtained from isolates of *Fno* (AL1104 and VMCU-FNO131 (F1 and F4, respectively, in Fig. 2 and Table 1) whereas only one genus-specific amplicon was yielded from isolates of *Fnn* (AL1106 and AL1108) (F2 and F3, Fig. 2 and Table 1). The amplified amplicons from positive samples were subjected to DNA sequencing and confirmed successful amplification of each target by respective primer pairs. No cross-reactivity was observed with DNA from other fish bacterial pathogens (Fig. 2). Sensitivity of duplex PCR assay using 10-fold serial dilutions of genomic DNA of *Fno* AL1104 and VMCU-FNO131 isolates were 20 fg and 200 fg per reaction, respectively (Fig. 3). Note that the difference in the detection limit of the assays might be due to the differences in the DNA extraction protocols (see first section of 'Materials and methods').

Detection of *Franciscella* and *F. noatunensis* subsp. *orientalis* in fish samples

Duplex PCR assay targeting 2 different DNA fragments revealed 100% positive results in both natu-

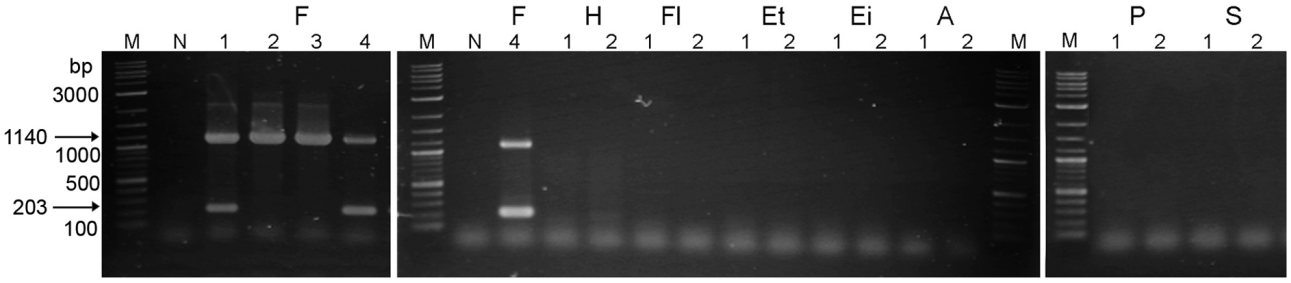

Fig. 2. Specificity test of duplex PCR assay yielding dual amplicons of 1140 and 203 bp from *Fno* isolates (Lanes F1 and F4) and a single amplicon of 1140 bp from *Fnn* isolates (Lanes F2 and F3) . No amplicon was generated with remaining non-*Francisella* bacteria. M, DNA marker; N, negative control; F, *Francisella*; H, *Hahella chejuensis*; Fl, *Flavobacterium columnare*; Et, *Edwardsiella tarda*; Ei, *Edwardsiella ictaluri*; A, *Aeromonas veronii*; P, *Plesiomonas shigheloides*, S, *Streptococcus agalactiae*. Numbers represent individual isolates listed in Table 1. Arrows indicate amplicons generated by duplex PCR assay

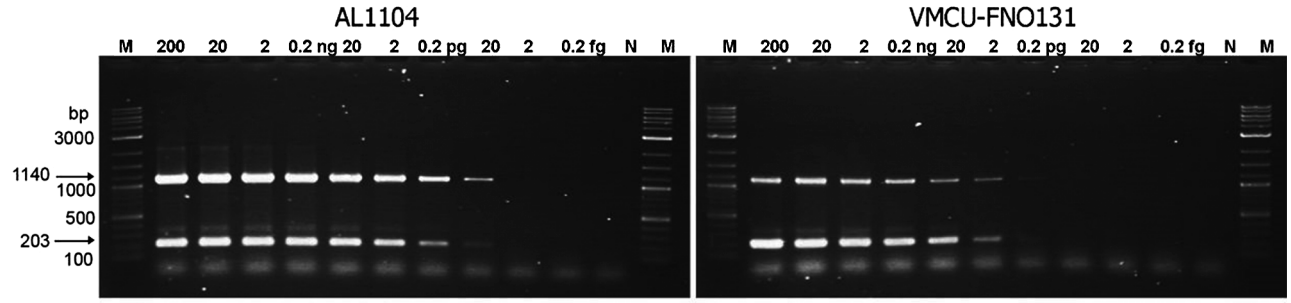

Fig. 3. Sensitivity of duplex PCR assay using 10-fold serial dilution of genomic DNA of *Fno* AL1104 and VMCU-FNO131 (20 fg and 200 fg per reaction, respectively). M, DNA marker; N, negative control. Arrows indicate amplicons (1140 and 203 bp) generated by duplex PCR assay

rally (n = 8) and experimentally (n = 10) diseased fish exhibiting clinical signs of visceral granulomas (Fig. A1 in the Appendix, NG1 and EG1 in Table 3). Interestingly, positive test results were also found in 5/5 fish in one group (N1 in Table 3; no figure shown) among 4 groups (N1 to N4) of naturally diseased fish exhibiting clinical signs of columnaris disease, but no granulomatus lesion was observed. The set of the samples with unknown clinical signs (N5, Table 3) also gave positive tests in 5 out of 7 tested samples (no figure shown).

Histological assessment of francisellosis

The presence of granulomas, previously described as a typical histological feature of francisellosis, was observed in multiple organs including the kidney and liver, but predominantly in the spleen of both naturally and experimentally diseased fish (Fig. 4). Different stages of granulomas were distinctively observed in the spleen of the infected fish (Fig. 4). An interaction between melanin-containing macrophages and bacteria in melanomacrophage centers (MMC) was observed in the early stages of granulomas (Fig. 4A–C). Melanin-containing cells and other inflammatory cells surrounded the infected area and centralized epithelioid cells, while the necrotic core was separated from surrounding epithelioid cells and eosinophilic spindle-shaped cells (Fig.4B,C). In later stages, the size of the necrotic core and epithelioid cell layer was first significantly reduced (Fig. 4D,E) and then disappeared (Fig. 4F). No abnormal histological changes were noted in the examined tissues of healthy fish (not shown).

In situ hybridization assay

An *in situ* hybridization assay was carried out in the tissues of both diseased and healthy tilapia using an *Fno* species-specific probe. Positive signals occurred in numerous organs of both naturally and experimentally infected fish, including the spleen (Fig. 5A), kidney (Fig. 5B), pancreas tubule cells in the liver (Fig. 5C), both primary and secondary gill filaments (Fig. 5D) and epithelial cells in the intestine (Fig. 5E). Strong reactive signals were also found in the necrotic core of early stages of granulomas in the kidney or spleen (Fig. 5F) and weak positive signals

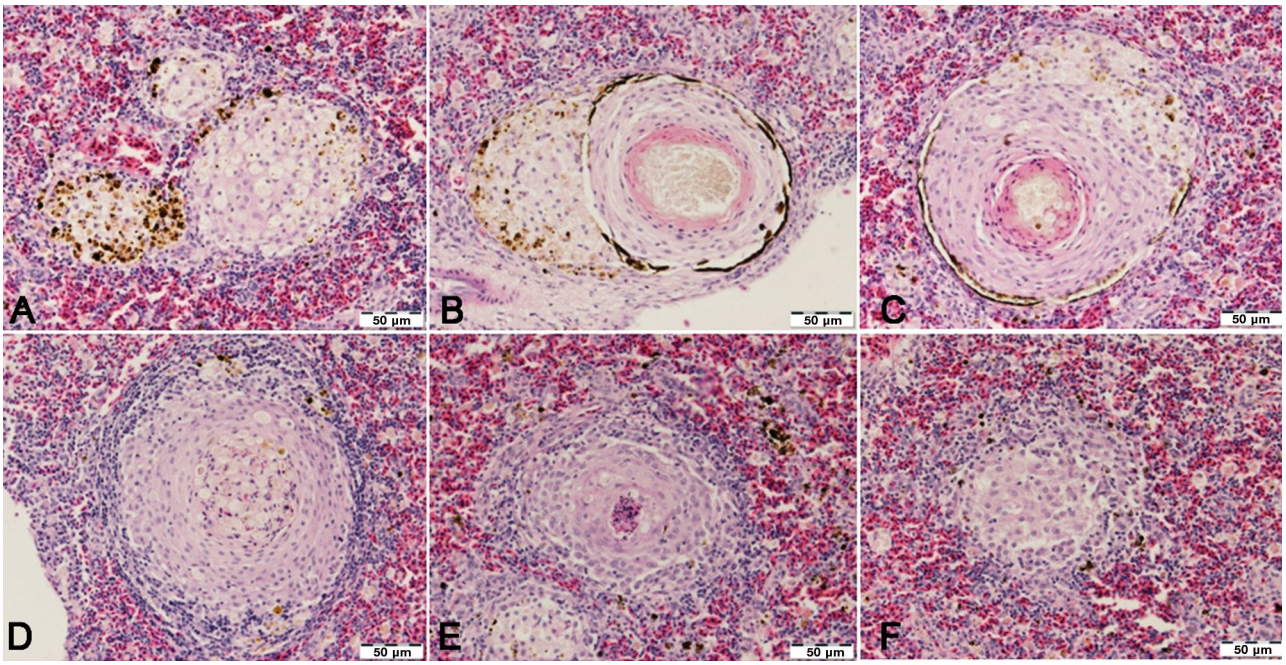

Fig. 4. Different stages of granulomas in the spleen of *Fno* infected fish. (A–C) Interaction between melanin-containing cells (dark brown) and bacteria in melanomacrophage centers was observed in the early stages of granulomas. (D–F) The necrotic core and epithelioid cell layer were significantly reduced in late stages

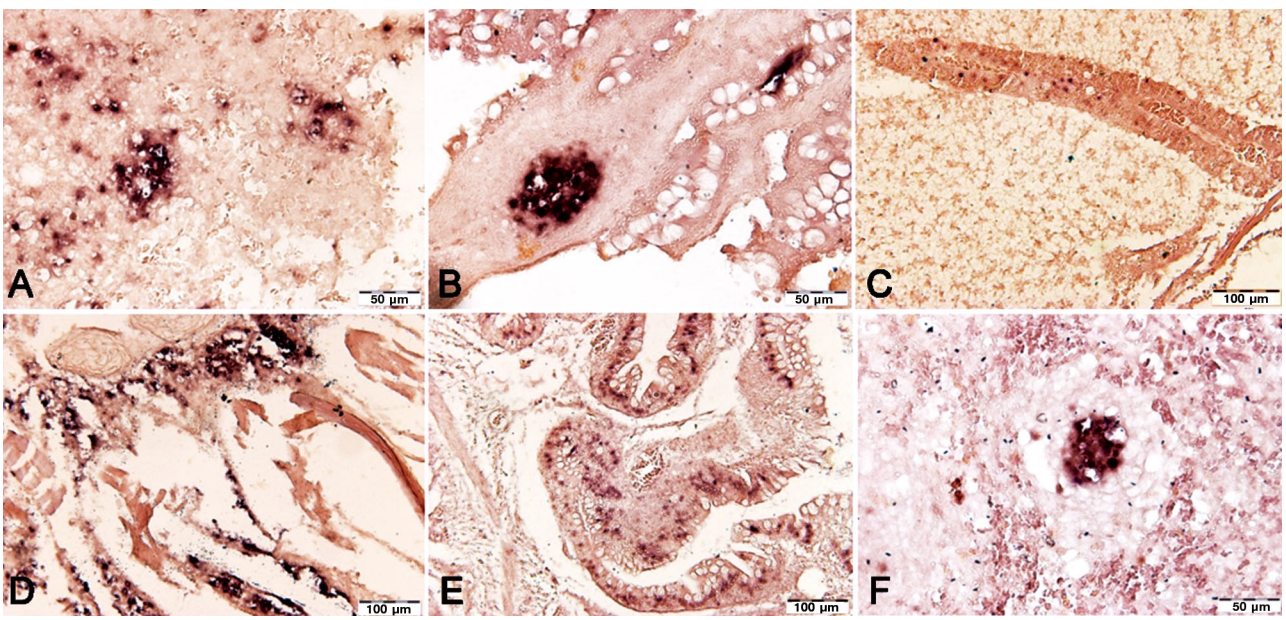

Fig. 5. *In situ* hybridization of *Fno* species-specific probe in *Fno* infected fish. Positive signals were observed in different fish organs including (A) spleen, (B) kidney, (C) pancreas tubule cells in the liver, (D) both primary and secondary gill filaments and (E) epithelial cells in the intestine. (F) Positive hybridization reactions were also found in the core of typical granuloma in the kidney or spleen

were observed in the later stages (Fig. A2 in the Appendix). However, the examined organs of healthy fish did not show positive signals using the same probe for *in situ* hybridization assay (no figure shown).

DISCUSSION

Nucleic acid-based detection methods have been widely applied for clinical diagnosis not only of human diseases, but also in other mammals and

aquatic animals, due to their high specificity and sensitivity compared to other methods. In the case of *F. noatunensis* subsp. *orientalis* (*Fno*) infection in fish, PCR and *in situ* hybridization detection methods are currently preferred approaches since *Fno* is a fastidious intracellular bacterium that is difficult to grow on conventional agar plates and biochemical test media. The *Francisella* genus-based PCR method (Forsman et al. 1994) has been most commonly used in fish disease laboratories (Hsieh et al. 2007, Soto et al. 2009, Nguyen et al. 2015). Some primer sets were later designed for quantitative real-time PCR assays but found not suitable for conventional PCR assay due to short target DNA fragments (Soto et al. 2010, Duodu et al. 2012). Therefore, an *Fno* species-specific primer set was newly designed in this study. Additionally, the duplex PCR approach simultaneously targeting both *Francisella* genus and *Fno* species in a single reaction has been developed. The duplex PCR detection limit was 20–200 fg of total genomic DNA, approximately equivalent to ~10–100 genomes based on an *Fno* genome size of 1.86 Mbp (NCBI accession nos. NZ_CP011923, NZ_CP012153, CP006875, CP011921 and CP003402) and an assumption that a target gene exists as a single copy per genome. The specificity of our duplex PCR assay was proven by a lack of cross-binding to genomic DNA of common fish bacterial pathogens. However, the target sequence used for *Fno* species-specific primer design and *in situ* hybridization probe showed relatively high similarity (93–95%) to the genome sequence of newly published strains of *Francisella philomiragia* (accession nos. CP009440, CP009442 and CP009444) isolated from humans (Johnson et al. 2015). *F. philomiragia* was previously known as a human pathogen until a new subspecies, *F. philomiragia* subsp. *noatunensis*, isolated from Atlantic cod *Gadus morhua* was proposed by Mikalsen et al. (2007). A later study reclassified *F. philomiragia* subsp. *noatunensis* as *Francisella noatunensis* (Ottem et al. 2009). Therefore, the use of a duplex PCR assay and an *in situ* hybridization protocol for fish samples is still practical for disease diagnosis.

When applied to fish samples, the newly developed duplex PCR protocol gave 100% positive results for previously known *Fno* infected fish (both naturally and experimentally diseased fish) exhibiting clinical signs of granulomas in the internal organs. Additionally, the detection assay revealed *Fno*-infected samples from naturally diseased fish without symptoms of francisellosis, indicating a practical ability of the duplex PCR method in diagnosis of field samples. Interestingly, the set of *Fno*-positive fish (N1, Table 3) was detected in the group exhibiting gross signs of columnaris disease in which *Flavobacterium columnare* was previously recovered (Dong et al. 2015a). This finding indicates that natural co-infections of *Fno* and *F. columnare* were found in this fish farm, supporting our recent hypothesis that multiple bacterial infections are the cause of major losses in intensively cultured Nile tilapia (*Oreochromis niloticus*) farms (Dong et al. 2015b).

The duplex PCR assay was also performed with DNA samples extracted from the testis and ovaries collected from 20 apparently normal male and female Nile tilapia broodstocks (data not presented). All samples exhibited negative results but a non-specific PCR product (~320 bp) was generated in some tested samples. The kidney, liver and spleen are the main target organs of *Fno* (Soto et al. 2009, 2013, Nguyen et al. 2015). Thus, these organs are also recommended for use in the duplex PCR protocol reported in this study.

The presence of granulomas in the internal organs of infected fish is a remarkable histological feature of francisellosis (Soto et al. 2009, Jeffery et al. 2010, Nguyen et al. 2015). In this study, we further described different stages of granuloma formation in which host–pathogen interactions occurred within melanomacrophage centers (MMCs) at a histological level. *In situ* hybridization using the *Fno* species-specific probe revealed the target tissues and location of *Fno* including the core of a typical granuloma. The presence of *Fno* in epithelial cells of the intestine and gills suggested that these 2 organs may serve as portals of entry for *Fno* infection in fish. MMCs are macrophage aggregates or a group of pigment-containing cells which are usually located in lymphoid tissue such as the kidney and spleen and are also found in the liver. MMCs have been suggested to be primitive analogues of the germinal centres of lymph nodes in mammals, which play an important role in the response to infectious agents. Development of granulomas in MMCs may be associated with chronic infections of a resistant intracellular bacterium (Agius & Roberts 2003).

In conclusion, duplex PCR assay and/or *in situ* hybridization using the species-specific probe described in this study may be helpful in the confirmation of a diagnosis of *Fno* infection in fish.

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Appendix

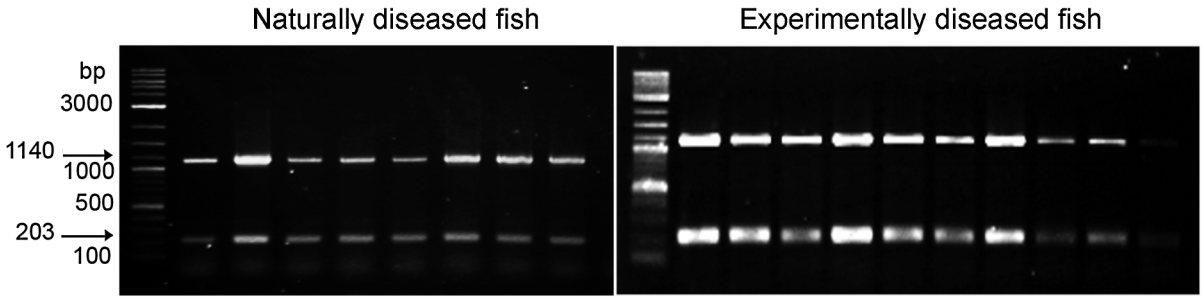

Fig. A1. Detection of *Francisella* and *Fno* in clinical fish samples. Details of naturally diseased fish (left) and experimentally diseased fish (right) are listed in Table 3. Arrows indicate amplicons (1140 and 203 bp) generated by duplex PCR assay

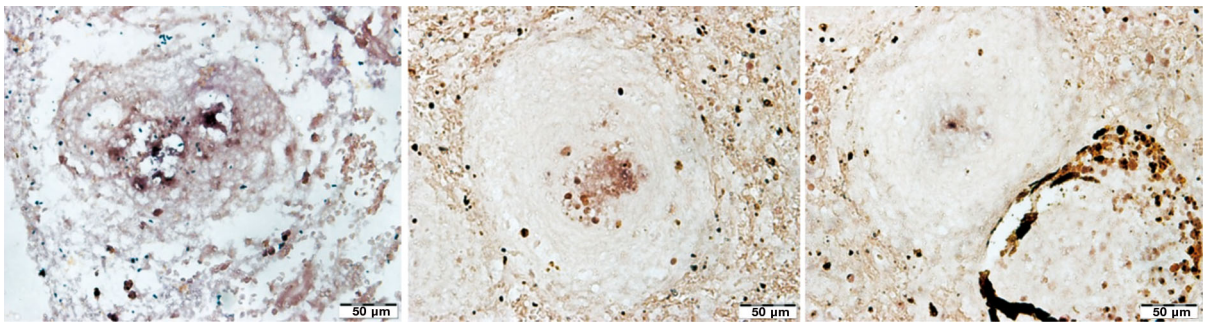

Fig. A2. *In situ* hybridization by *Fno* species-specific probe showing light positive signals in late stages of granuloma of *Fno*-infected fish

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