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APPLIED STUDIES

Application of multiplex quantitative Polymerase chain reaction methods to detect common bacterial fish pathogens in Nile tilapia, *Oreochromis niloticus*, hatcheries in Costa Rica

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Edwardsiella spp., *Streptococcus* spp., and *Francisella noatunensis* subsp. *orientalis* are some of the most important fish pathogens affecting global tilapia, *Oreochromis* spp., aquaculture. In Costa Rica, the aquaculture industry is dominated by freshwater-cultured Nile tilapia, *Oreochromis niloticus*, which are raised in all seven national provinces. At present, little is known regarding the diversity of pathogens present in these facilities, and definitive identification of agents associated with disease outbreaks are rare. To evaluate the prevalence of common bacterial pathogens in these systems, this study used multiplex quantitative polymerase chain reaction (qPCR) assays targeting *Edwardsiella*, *Streptococcus*, and *Francisella* species as a diagnostic and surveillance tool. In 2017, seven different tilapia hatcheries were visited, and 350 fingerlings were subjected to necropsy and molecular diagnostic evaluation. Fish exhibiting gross signs of disease were subjected to histological and microbiological analysis. For the first time, *Edwardsiella anguillarum* was recovered and molecularly confirmed from diseased tilapia in Costa Rica. In addition, *F. noatunensis* subsp. *orientalis* was identified in a region of Costa Rica where it had not been previously reported.

KEYWORDS

Costa Rica, diagnosis, pathogen, tilapia

1 | INTRODUCTION

Tilapia, *Oreochromis* spp., is one of the most extensively cultivated fish worldwide (Food and Agriculture Organization of the United Nations [FAO], 2016; Huicab-Pech, Landeros-Sánchez, Castañeda-Chávez, Lango-Reynoso, & López-

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Collado, 2016). Global production was valued at $1.6 billion in 2000 but rapidly increased to $10 billion in 2014, representing over 4.85×10^6 m.t. of fish (Fitzsimmons, 2015). Latin American countries, including Costa Rica, have made an important contribution to national and international tilapia markets (FAO, 2016; Webster & Lim, 2006). Despite this, high feed costs and disease-related losses are significant obstacles limiting aquaculture production. Infectious diseases pose some of the greatest threats to profitability and are estimated to cost the industry millions of dollars annually in the form of lost feed days and overt mortality (Krkosek, 2010; Sommerset, Krossøy, Biering, & Frost, 2005). Streptococcosis, edwardsiellosis, and francisellosis are among the most important bacterial diseases associated with temperate and warm water–cultured fish, particularly tilapia (Agnew & Barnes, 2007; Figueiredo, Nobrega Netto, Leal, Pereira, & Mian, 2012; Griffin, Greenway, & Wise, 2017; Plump & Hanson, 2011; Soto, Hawke, Fernandez, & A. Morales, 2009).

Edwardsiella spp. are Gram-negative, rod-shaped bacteria belonging to the family *Enterobacteriaceae*. Historically, *Edwardsiella tarda* has been considered an economically important pathogen in finfish and eel aquaculture, including tilapia (Mohanty & Sahoo, 2007). Recently, it was determined that *E. tarda* actually represented three discrete genetic taxa (Abayneh, Colquhoun, & Sørum, 2013; Griffin et al., 2013). *E. tarda*, as it is currently defined, is genetically consistent with isolates previously deemed nonfish pathogenic *E. tarda*, which includes the strain (ATCC 15947) from humans (Griffin et al., 2017; Reichley et al., 2017). Isolates designated as fish pathogenic-typical and -atypical *E. tarda* now represent the newly described species *Edwardsiella piscicida* and *Edwardsiella anguillarum*, respectively (Abayneh et al., 2013; Griffin et al., 2017; Reichley et al., 2017; Shao et al., 2015; Yamada & Wakabayashi, 1999). Archived genetic sequence data have linked current *Edwardsiella* systematics to historical reports, suggesting that both *E. piscicida* and *E. anguillarum* pose significant risks to temperate and warm water fish (Buján et al., 2017; Griffin et al., 2014; Griffin et al., 2017; Reichley et al., 2017). *Edwardsiella ictaluri*, the etiological agent of enteric septicemia of catfish, is one of the most economically important diseases of cultured channel catfish in the southeastern United States (Hawke & Khoo, 2004; Hawke, McWhorter, Steigerwalt, & Brenner, 1981). Although historically considered a problem limited to catfish aquaculture in the United States, *E. ictaluri* strains have been recovered from disease outbreaks in other economically important fish species across the world (Crumlish, Dung, Turnbull, Ngoc, & Ferguson, 2002; Geng et al., 2013; Hawke et al., 2013; Hawke & Khoo, 2004; Phillips, Reichley, Ware, & Griffin, 2017; Ye, Li, Qiao, & Li, 2009) including Nile tilapia, *Oreochromis niloticus*, cultured in Costa Rica (Soto et al., 2012).

Francisella noatunensis subsp. *orientalis (Fno)* is a fastidious Gram-negative bacterium and the etiological agent of piscine francisellosis in tilapia (Colquhoun & Duodu, 2011). In recent years, *F. noatunensis* subspecies have caused disease outbreaks in different fish species from Asia and Europe, as well as North, South, and Central America (Soto et al., 2014). In Costa Rica, *Fno* has been documented as one of the most important pathogens affecting local tilapia aquaculture (Birkbeck, Feist, & Verner-Jeffreys, 2011; Sebastião, Pilarski, Kearney, & Soto, 2017; Soto et al., 2009). In addition, a novel *Francisella* species, *Francisella marina*, has been described from diseased spotted rose snapper, *Lutjanus guttatus*, in mariculture operations off the Pacific coast of Central America. Epidemiological factors related to outbreaks and host range, including tilapia, of the bacterium are not yet known (Soto et al., in press).

Streptococcosis is a septicemic disease caused by Gram-positive members of the family Streptococcaceae (Jantrakajorn, Maisak, & Wongtavatchai, 2014). Although *Streptococcus dysgalactiae*, *Streptococcus milleri*, *Streptococcus phocae*, *Streptococcus parauberis*, *Streptococcus difficilis*, and *Streptococcus ictaluri* have been associated with disease in cultured fish (Austin & Austin, 2016), *Streptococcus iniae* and *Streptococcus agalactiae* are the predominant streptococcoal fish pathogens affecting tilapia culture (Evans et al., 2002; Hernandez, Figueroa, & Iregui, 2009; Iregui, Comas, Vasquez, & Verjan, 2014). Both have been associated with catastrophic mortalities in wild and cultured fish, often resulting in significant economic losses (Shoemaker, Xu, & Soto, 2017).

The diagnosis of these fish pathogens has many constraints, including the fastidious nature of some isolates, in addition to inconsistencies in biochemical, serological, and molecular tests. The diagnosis of these pathogens remains a challenge as current techniques can be laborious, time consuming and expensive, often requiring specialized personnel and equipment. Moreover, current techniques can be prone to false negatives because of low sensitivity or false positives attributed to low specificity. Quantitative polymerase chain reaction (qPCR) is a well-known molecular technique that is currently used in many laboratories for the diagnosis of microbial pathogens, including fastidious

bacteria. The high sensitivity, high specificity, and rapid turnaround time make this technique an attractive alternative to conventional diagnostic modalities (Espy et al., 2006). Given the importance of these pathogens to tilapia aquaculture, the current study investigated the utility of multiplex qPCR methods for the diagnosis of common bacterial pathogens in diseased tilapia and as a surveillance tool in apparently healthy fish collected from tilapia hatcheries in Costa Rica.

2 | MATERIALS AND METHODS

2.1 | Fish and tissue samples

Sample collection was carried out from January to May 2017. Seven different Nile tilapia aquaculture farms distributed throughout Costa Rica were sampled (Figure 1). Tilapia fingerlings were reared in a variety of freshwater systems (Table 1). The day of sampling, clinical history, and water quality parameters of the rearing systems were recorded. Fifty animals per farm were analyzed, giving priority to clinically affected fish observed or reported during the visit. Clinically affected fish included those presenting gross lesions, including exophthalmia and skin ulcers, or irregular behavior, including lethargy, anorexia, abnormal swimming, or struggling to maintain neutral buoyancy. Fish were euthanized with an overdose (175 mg/L) of AQUI-S® (AquaTactics Fish Health & Vaccines, Kirkland, WA), weighed, and necropsied. Splenic samples were collected aseptically from each animal, and the tissues were pooled (~20 mg; 5 spleens/pool) in sterile 1.5-mL microcentrifuge tubes containing 95% ethanol (ETOH) and then stored at 4°C. Selected tissues (gills, gut, spleen, liver and heart anterior, and posterior kidney) from fish with clinical signs of disease and gross lesions were fixed in 10% neutral buffered formalin for histological analysis and stained with hematoxylin and eosin (H&E), Giemsa, and Gram-Twort stains. In addition, spleen and posterior kidney samples from affected fish were inoculated onto Columbia agar plates supplemented with 5% sheep blood (Becton Dickinson, Sparks, MD). Aerobic cultures were incubated for 48–72 hr at 25°C. Bacterial colonies from primary isolation agar plates were inoculated on supplemented Columbia agar for purity of culture and stored cryogenically for later molecular identification (−80°C).

FIGURE 1 Map of Costa Rica indicating the Nile tilapia (*Oreochromis niloticus*) hatcheries sampled in this study

TABLE 1 Records summarized from Nile tilapia, *Oreochromis niloticus*, hatcheries sampled in this study (Figure 1). The total number of hatcheries sampled (n = 7) corresponds to those ones that were registered as fingerling producers in the government's national animal health service. The farm naming was performed according to chronological sampling

Farm	Average weight (grams)	Production system	Amount of production	Water temperature (°C) at collection	Number of sampled fish
A	19.1 (±7.6)	Recirculating system	2000	28	50
B	32.5 (±6.5)	Tanks (fiberglass, flowthrough)	180,000	26	50
C	15 (±8.6)	Tanks (concrete, flowthrough)	1,200,000	27.4	50
D	19.9 (±8.1)	Earth ponds	80,000	25	50
E	9.6 (±4.2)	Earth ponds	125,000	28	50
F	27.8 (±10.8)	Earth ponds	20,000	26.4	50
G	4.4 (±1.3)	Earth ponds	60,000	26	50

2.2 | DNA extraction

Ethanol was removed from spleen pools prior to homogenization in 500 μL of phosphate-buffered saline, and homogenized tissue was stored at −20°C. Total genomic DNA (gDNA) was isolated using the DNEasy Blood and Tissue Kit (QIAGEN, Germantown, MD) in accordance with the manufacturer's suggested protocol. Isolated gDNA was quantified using a Cytation™ 5 imaging reader and Gen3 software (BioTek, Winooski, VT). Total gDNA from each pool was then adjusted to 10 ng/μL with AE buffer (QIAGEN).

2.3 | *F. noatunensis* subsp. *orientalis* (*Fno*), *S. iniae*, and *S. agalactiae* multiplex qPCR assay

A multiplex qPCR using minor groove binder probes was designed for three bacterial strains commonly associated with disease outbreaks in cultured tilapia. Primer and probes were designed using Primer Express™ Software 3.0.1 (Thermo Fisher Scientific, Waltham, MA) targeting the *Fno sodB* (GenBank accession number CP018051), *S. iniae rpoB* (GenBank accession number CP017952), and *S. agalactiae gyrB* genes (GenBank accession number CP003919). Primers and probes (Table 2) were synthesized by Life Technologies (Grand Island, NY).

All three assays were validated in both simplex and multiplex formats using both TaqMan Universal Master Mix (UMM; Thermofisher Scientific, Waltham, MA) and TaqMan Environmental Master Mix v2.0 (EMM; Thermofisher Scientific, Waltham, MA). The 12-ul qPCR simplex and multiplex reactions consisted of 6 μL of commercially available PCR Master Mix (UMM or EMM; Thermofisher Scientific, Waltham, MA), 2.5 pmols of each primer, 0.5 pmols of probe, 5 μL of gDNA, and nuclease-free water to volume. Reactions were run in a 384 well-plate and amplified in a 7900HT FAST qPCR system (Thermo Fisher Scientific) using the manufacturer's standard amplification conditions (2 min at 50°C, 10 min at 95°C, then 40 cycles of 15 s at 95°C and 60 s at 60°C). Fluorescent signals were collected following the anneal/extension step, and the quantification cycle (Cq) was calculated based on a threshold of 0.1 with the baseline fluorescence established from cycles 3 to 12.

The sensitivity and linear dynamic range of each assay was determined using serial dilutions of known quantities of genomic bacterial DNA isolated from each target organism (DNEasy Blood and Tissue Kit, QIAGEN, Germantown, MD). Each dilution series was run in triplicate on three separate occasions to assess repeatability and reproducibility of the assay (Figure 2). Only gDNA from the target bacteria was added to the reaction in simplex assay validation, whereas gDNA from all three target bacteria were combined in validation of multiplex reactions. To this end, each reaction mixture was supplemented with an equal mixture of ~1 ng of each nontarget bacterial gDNA to ensure that large quantities of nontarget DNA did not impair reaction efficiency (Figure 3). Standard curves were generated for each set of dilutions, and amplification efficiency (E) for all assays was calculated from the slope of the curve using the formula $E = 10^{1/-s}-1$. Samples with a mean Cq value of ≤40 (with fluorescence in all three wells) were

TABLE 2 Primers and probes used in multiplex quantitative polymerase chain reactions (qPCRs) for *Francisella noatunensis* subsp. *orientalis* (*Fno*), *Streptococcus agalactiae*, *Streptococcus iniae* and the *Edwardsiella* spp. melting temperature (T_m), amplicon length, amplicon guanine-cytosine content (GC-Content) and Genbank accession number are presented. The *Fno*, *S. agalactiae*, and *S. iniae* multiplex quantitative polymerase chain reaction (mPCR) was developed as part of the current study. Primer and probe sequences for the *Edwardsiella* spp. mPCR have been obtained from previous work (Bilodeau et al. 2003; Griffin et al. 2011; Reichley, Waldbieser, Lawrence, & Griffin, 2015,b; Reichley et al., 2017)

Primer/probe	Sequence (5′–3′)	T_m	Amplicon GC%	Amplicon length	Accession number
Fno			32	102 bp	CP018051
Fno-70f	GCTGCTTTCAATTGACTAGAAGGTT	58.0			
Fno-172r	CTGGCGTCATATTTAACAATTCAGC	58.0			
Fno-102p	FAM-ACTTAGAGTTAAACAATTCC-MGB-NFQ	68.0			
Streptococcus iniae			38	103 bp	CP017952
S. iniae-551f	GTGTCCGATTGAGACACCAGAG	58.0			
S. iniae-653r	GCGGTAAGGTGTTTGAATAAAGC	58.0			
S. iniae-583p	TET-TCGGTCTGATCAATAACTTA-MGB-NFQ	68.0			
Streptococcus agalactiae			39	104 bp	CP011328
S. agal-423f	CGGTGAATTAGATGGTATTTCAGTTG	58.0			
S. agal-527r	CCTCCTTCATGAGTGTGGATGTT	58.0			
S. agal-450p	VIC-AGTAGCAATGCAGTATAC-MGB-NFQ	68.0			
Edwardsiella anguillarum			50	131 bp	CP011516
EPL1583F	GATCGGGTACGCTGTCAT	56.9			
EPL1708R	AATTGCTCTATACGCACGC	56.6			
EPL1611P	TxRed-CCCGTGGCTAAATAGGACGCG-BHQ2	67.8			
Edwardsiella ictaluri			53	178 bp	NC_012779
EI481F	ACTTATCGCCCTCGCAACTC	61.7			
EI658R	CCTCTGATAAGTGGTTCTCG	55.0			
EI561P	HEX-CCTCACATATTGCTTCAGCGTCGAC-BHQ1	68.0			
Edwardsiella piscicida			52	131 bp	CP016044
EP14529F	CTTTGATCATGGTTGCGGAA	62.0			
EP14659R	CGGCGTTTTCTTTTCTCG	59.5			
EP14615P	FAM-CCGACTCCGCGCAGATAACG-BHQ1	68.3			
Edwardsiella tarda			54	115 bp	CP011359
ET3518F	CAGTGATAAAAAGGGGTGGA	57.5			
ET3632R	CTACACAGCAACGACAACG	56.4			
ET3559P	Cy5-AGACAACAGAGGACGGATGTGGC-BHQ2	67.0			

considered positive. Specificity of the assay was evaluated using previously identified bacterial pathogens recovered from aquatic animals (Table 3). These protocols were then used to evaluate gDNA isolated from collected spleen pools.

2.4 | *E. tarda*, *E. piscicida*, *E. anguillarum*, and *E. ictaluri* multiplex quantitative polymerase chain reaction assay

As described above, gDNA from all spleen pools were subjected to a multiplex real-time PCR assay targeting the four *Edwardsiella* spp. known to infect fish (Reichley et al., 2017). Each sample was run in triplicate on a C1000 Touch™ thermal cycler equipped with a CFX96™ optical reaction module (BioRad, Hercules, CA) and analyzed using the End Point utility of the CFX Manager™ version 3.1 software (End Cycles to Average: 5; Percent of Range: 10.0, BioRad,

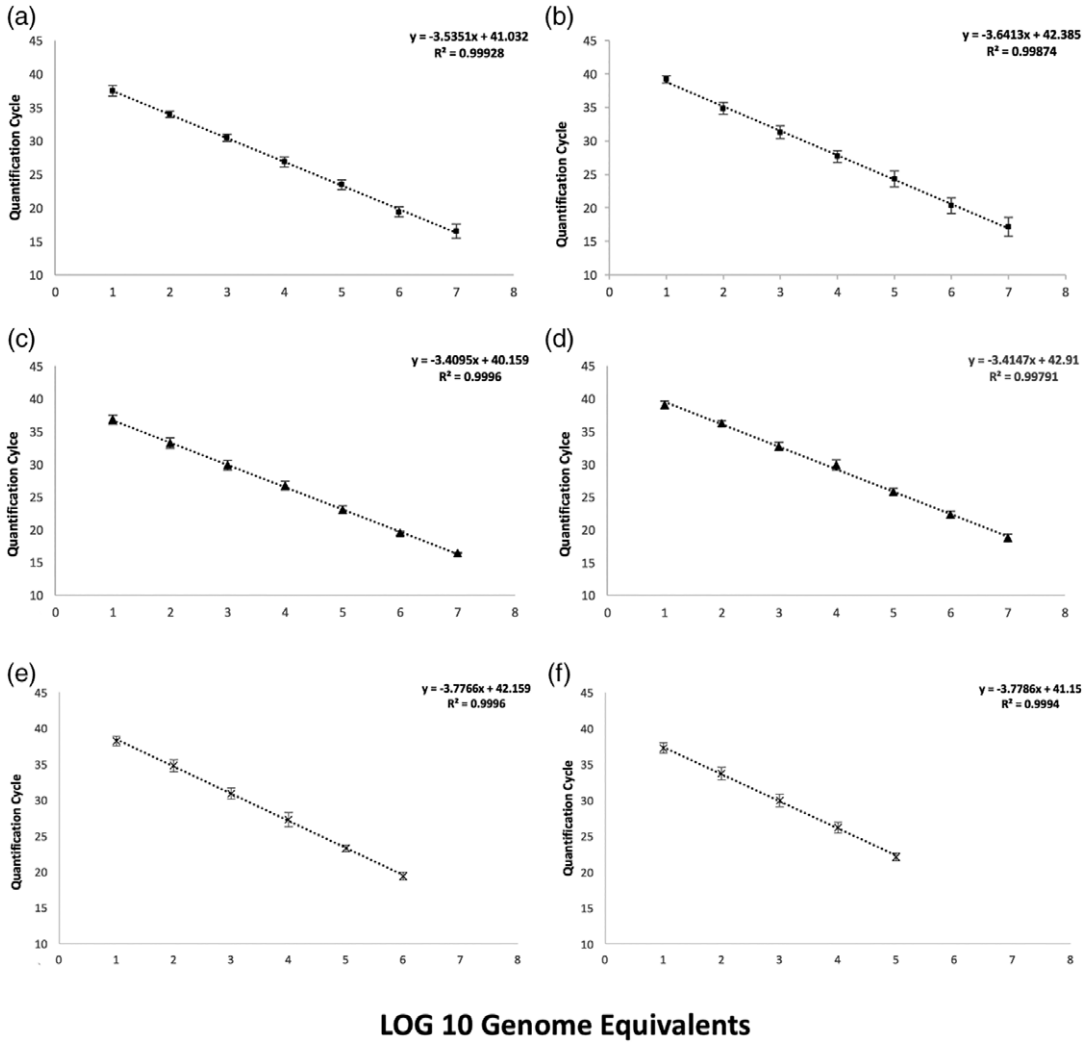

FIGURE 2 Mean quantification cycles for known quantities of *Francisella noatunensis* subsp. *orientalis* (*Fno*) (a, b), *Streptococcus iniae* (c, d), *Streptococcus agalactiae* (e, f) target DNA using universal (a, c, e) or environmental master mix (b, d, f) (applied biosystems). A 10-fold dilution series of quantified bacterial DNA was analyzed for each assay. Error bars indicate *SD*s generated from samples ran in triplicate on three separate plates. Reaction efficiencies for each assay were calculated from the slope of the log-linear portion of concurrently run standards (PCR efficiency = $10^{1/-s}-1$). PCR, polymerase chain reaction

Hercules, CA). Primers and probes were synthesized commercially (Eurofins MWG; Louisville, KY). Each probe was labeled with a fluorescent reporter dye (*E. anguillarum*, Texas Red; *E. ictaluri*, 5-HEX; *E. piscicida*, 6-FAM; *E. tarda*, Cy5) on the 5′ end and with appropriate quencher dye (black hole quencher-1 for HEX and 6-FAM; black hole quencher-2 for Texas Red and Cy5) on the 3′ end. Primer and probe sequences are provided in Table 2.

The 25-μL reaction contained 12 μL of PCR master mix (TaqMan Environmental Mastermix 2.0, Applied Biosystems, Foster City, CA), 5 pmols of each primer, 0.5 pmols of each probe, 5 μL of gDNA (10 ng/μL) template, and nuclease-free water to volume. Amplifications were performed with the following temperature profile: 1 cycle of 95°C for 15 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Data collection occurred following the 60°C annealing/extension step at the end of each cycle. Genomic DNA isolated from the spleen of naive blue (n = 2)

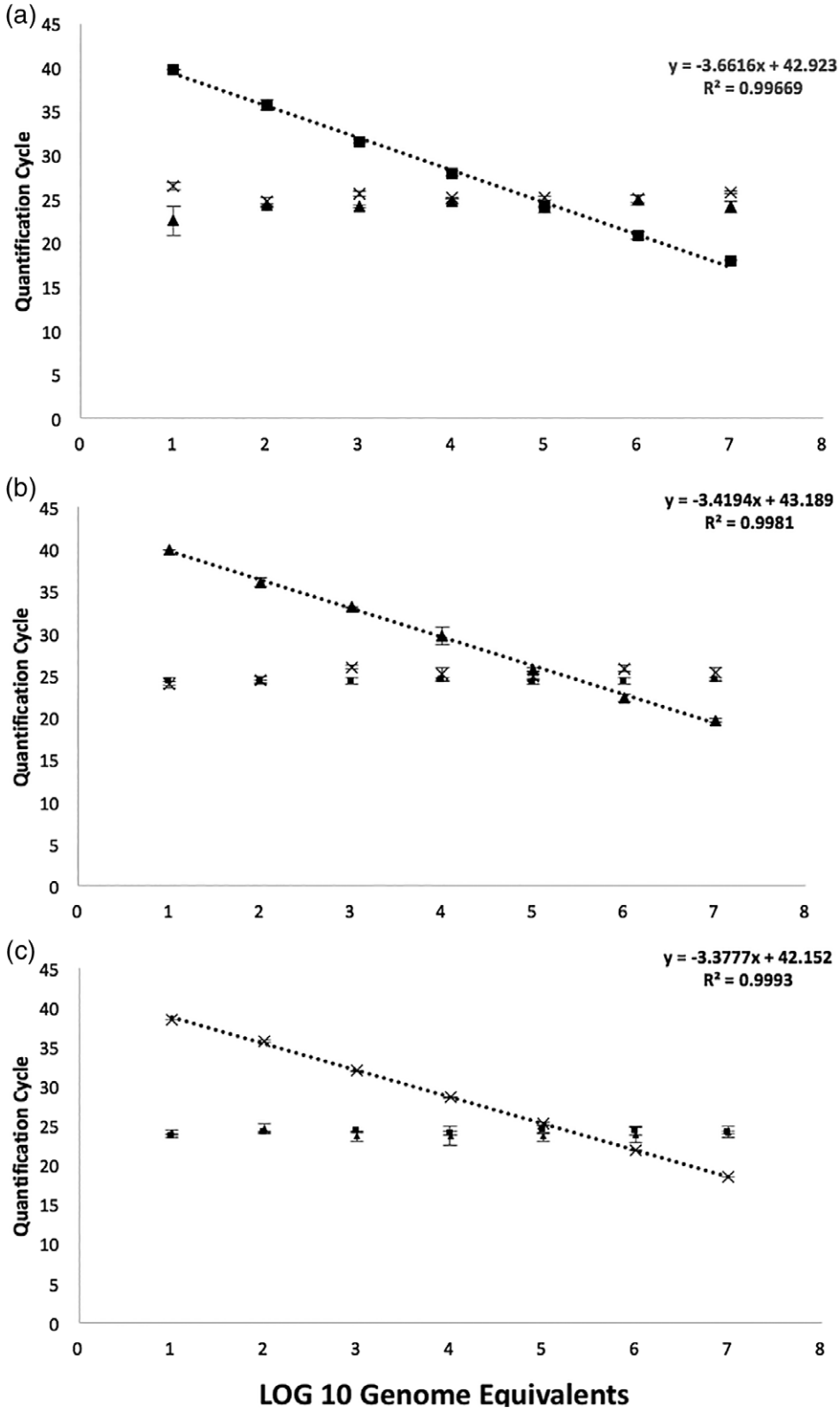

FIGURE 3 Legend on next page.

and Nile (n = 2) tilapia was analyzed concurrently, with all samples serving as true negative controls. Positive controls consisting of an equimolar pool of *E. anguillarum* LADL 05–105 (Griffin et al., 2014; Reichley, Waldbieser, Lawrence, & Griffin, 2015), *E. ictaluri* S97-773 (Griffin et al., 2014), *E. piscicida* S11-285 (Griffin et al., 2014; Reichley, Waldbieser, Tekedar, Lawrence, & Griffin, 2016), and *E. tarda* (Griffin et al., 2014; Reichley, Waldbieser, Tekedar, Lawrence, & Griffin, 2015) gDNA, as well as no template controls, were included with each plate.

2.5 | Results

Regardless of the PCR supermix used, the newly designed multiplex qPCR assay targeting *Fno*, *S. iniae*, and *S. agalactiae* was found to be repeatable and reproducible, with a linear dynamic range covering at least five orders of magnitude. Based on estimated genome sizes of 1.9 Mb for *Fno* (Figueiredo et al., 2016), 2 Mb for *S. iniae* (Gong et al., 2017), and 2 Mb for *S. agalactiae* (Mainardi et al., 2016), the limit of sensitivity for each assay was approximately 20, 8, and 20 genome equivalents, respectively. Each assay was specific to its respective target, with no evidence of cross-reactivity from nontarget organisms (Figure 3; Table 3).

Sample collection occurred from January to May, a period of volatile weather patterns, seasonal changes, and the transition from the dry to rainy season. This is also the period when disease outbreaks typically occur in Costa Rica. Sampling occurred on the seven largest Nile tilapia aquaculture farms in Costa Rica, all of which possessed on-site hatcheries. Farms A, D, E, F, and G did not report any disease-related mortality before or at the time of the site visit, and no pathogenic bacteria were detected by qPCR in spleen pools collected from these operations (Table 4). Farm B had experienced 30–40% mortality in affected systems 6 weeks prior to the visit. Water temperature at the time of sampling ranged from 24 to 26°C. Gross pathological findings in this group were typical of piscine francisellosis, including splenomegaly, renomegaly, and visceral adhesions. White nodules, corresponding microscopically to granulomas and foci of necrosis, were variably present in the spleen, kidney, liver, myocardium, pericardium, and gills. Histologically, multifocal, variably sized granulomas composed of central areas of necrosis surrounded by mantles of epithelioid macrophages were consistently observed. Vacuolated macrophages, containing small pleomorphic coccobacilli and scattered lymphocytes, were present on the periphery (Figure 4a). More chronic cases were characterized by granulomas containing larger areas of free and phagocytized necrotic debris, but there were no identifiable bacteria in H&E- or Giemsa-stained sections. Extensive fibroplasia was associated with some locations, such as the pericardial cavity. All 10 spleen pools recovered from this hatchery were positive for *Fno* (32.85 $\pm$ 1.7), while the qPCR was negative for *Streptococcus* spp. and *Edwardsiella* spp. No bacteria were cultured on Columbia blood agar from any of the sampled fish. However, special media, such as Thayer Martin, are required for the isolation of *Fno*.

Farm C was experiencing low mortalities (6–8%) during the visit at which time water temperatures ranged from 26 to 29°C. Diseased fish were lethargic, externally darkened, and swimming erratically. Necropsy demonstrated bilateral exophthalmia, hepatomegaly, splenomegaly, and renomegaly. Again, white nodules consistent with foci of necrosis and granulomas were widespread in kidneys and spleens. Microscopic lesions varied from small foci of parenchymal coagulative necrosis and hemorrhage to organized granulomas, both containing variable numbers of free and phagocytized bacterial rods. Necrotic foci were frequently surrounded by mixed mononuclear inflammatory infiltrates, while granulomas had large hypereosinophilic cores of cellular debris surrounded by epithelioid macrophages and, often, a capsule of fibrous connective tissue (Figure 4b). Multifocal basophilic mineral deposits, consistent with mild nephrocalcinosis, were present in the renal interstitium and tubular lumens of some fish. No significant

FIGURE 3 Mean quantification cycles for serial 10-fold dilutions of known quantities of *Francisella noatunensis* subsp. *orientalis* (*Fno*) (a), *Streptococcus iniae* (b), *Streptococcus agalactiae* (c) target DNA using environmental master mix (applied biosystems). A dilution series for each assay was performed in the presence of an equal mixture of ~1 ng of each nontarget bacterial gDNA. Error bars indicate *SD*s generated from samples run in triplicate on three separate plates. Reaction efficiencies for each assay were calculated from the slope of the log-linear portion of concurrently run standards (PCR efficiency = $10^{1/-s}-1$). PCR, polymerase chain reaction

TABLE 3 Isolates used in this study to validate the *Francisella noatunensis* subsp. *orientalis* (*Fno*), *Streptococcus iniae*, and *Streptococcus agalactiae* qPCR assay

Isolate	Year of isolation	Location	Fish	Reference
Fno 1	2001	Japan	Three line grunt	Kamaishi et al., 2005
Fno 2	2011	Texas, United States	Tilapia	Unpublished
Fno 3	2012	Costa Rica	Nile tilapia	Unpublished
Fno 4	2012	Hawaii, United States	Tilapia	Soto, McGovern-Hopkins, et al., 2013
Fno 5	2013	Florida, United States	French grunt	Soto et al., 2014
S. iniae	1994	IL, United States	Tilapia	Chou et al. 2014
S. iniae	1994	MA, United States	Hybrid striped bass	Chou et al. 2014
S. iniae	2015	CA, United States	Mossambique tilapia	Soto et al. 2017
S. iniae	2015	CA, United States	White sturgeon	Soto et al. 2017
S. iniae	2015	CA, United States	White sturgeon	Soto et al. 2017
S. iniae	2001	MN, United States	Nile tilapia	Chou et al. 2014
S. iniae	2003	FL, United States	Albino rainbow shark	Chou et al. 2014
S. iniae	1999	Southern Caribbean	Reef fish	Ferguson et al. 2000
S. iniae	2008	West Indies	Reef fish	Keirstead et al. 2014
S. agalactiae	2015	Costa Rica	Nile tilapia	Soto et al. 2016
S. agalactiae	2005	Honduras	Tilapia	Soto et al. 2015
S. agalactiae	2009	CA, United States	Tilapia	Unpublished
S. agalactiae	2009	CA, United States	Tilapia	Unpublished
S. agalactiae	2009	CA, United States	Tilapia	Unpublished
S. agalactiae	2009	CA, United States	Tilapia	Unpublished
S. agalactiae	2009	CA, United States	Tilapia	Unpublished
S. agalactiae	2017	CA, United States	Tilapia	Unpublished
S. agalactiae	2017	CA, United States	Tilapia	Unpublished
S. agalactiae	2017	CA, United States	Tilapia	Unpublished
S. agalactiae	2017	CA, United States	Tilapia	Unpublished
Flavobacterium columnare	2017	CA, United States	Rainbow trout	Unpublished
Flavobacterium psychrophilum	2017	CA, United States	Rainbow trout	Unpublished
Mycobacterium marinum	2017	CA, United States	Zebrafish	Unpublished
Mycobacterium chelonae	2017	CA, United States	Zebrafish	Unpublished
Mycobacterium abbsecuss	2017	CA, United States	Zebrafish	Unpublished
Nocardia sp.	2017	CA, United States	Marine mammal	Unpublished
Edwardsiella ictaluri	2012	Costa Rica	Tilapia	Unpublished
Edwardsiella anguillarum	2017	Costa Rica	Tilapia	Current study
Aeromonas sp.	2017	CA, United States	Tilapia	Unpublished
Aeromonas sp.	2017	CA, United States	Koi	Unpublished
Aeromonas sp.	2017	CA, United States	Tilapia	Unpublished

changes were present in the gastrointestinal tract, liver, heart, or brain. Six spleen pool samples from this hatchery were qPCR positive for *E. anguillarum*, while the qPCR was negative for all other pathogens tested (Table 4). Two pure bacterial isolates, morphologically consistent with an *Edwardsiella* spp., were obtained from affected fingerlings and later molecularly confirmed as *E. anguillarum* based on previously established methods (Griffin et al., 2014; Reichley et al., 2017).

TABLE 4 Molecular diagnosis of *Francisella noatunensis* subsp. *orientalis* (*Fno*), *Streptococcus iniae*, *Streptococcus agalactiae*, *Edwardsiella tarda*, *Edwardsiella piscicida*, *Edwardsiella anguillarum*, and *Edwardsiella ictaluri* using multiplex quantitative polymerase chain reaction (qPCR). Seven Nile tilapia hatcheries were sampled. Fifty splenic samples were collected from each farm and subjected to molecular diagnosis. Ten pools (n = 5 animals) per farm were analyzed

Hatchery	*Fno*	*E. anguillarum*	*E. tarda*	*E. piscicida*	*E. ictaluri*	*S. agalactiae*	*S. iniae*
A	–	–	–	–	–	–	–
B	(10/10)$^{+}$	–	–	–	–	–	–
C	–	(6/10)$^{+}$	–	–	–	–	–
D	–	–	–	–	–	–	–
E	–	–	–	–	–	–	–
F	–	–	–	–	–	–	–
G	–	–	–	–	–	–	–

$^{+}$Positive pools per farm.

3 | DISCUSSION

Disease impacts on aquaculture production extend beyond direct losses associated with mortality. Disease also results in arrested production and growth, additional labor and energy costs, and increased treatment expenditures. Furthermore, the release of pathogens into the environment poses a potential risk to wild fish populations and humans. Delays in diagnosis and the initiation of antimicrobial treatment in response to bacterial pathogens can result in therapeutic failures, especially with the use of medicated feeds, as inappetence often increases as an outbreak progresses in a population (Reimschuessel, Miller, & Gieseker, 2013). As a result, rapid diagnostics play a key role in health management and improved production efficiencies in aquaculture.

In the current project, a novel molecular assay demonstrated the presence of *Fno* DNA in southeast Costa Rica (farm B), the first report of the pathogen in this region. Although no mortalities were occurring in the hatchery at the time of sampling, fingerlings from the farm exhibited internal gross and histopathological lesions consistent with piscine francisellosis (Colquhoun & Duodu, 2011; Soto et al., 2013; Soto et al., 2013), and *Fno* DNA was confirmed in all samples. A mortality event 6 weeks earlier had gone undiagnosed following the introduction of broodstock from regions where *Fno* is endemic. According to Peeler and Taylor (2011), the movement of fish between hatcheries poses a high-risk factor for the dissemination of bacterial pathogens to previously unaffected farms and/or new geographical areas. This is particularly important with diseases such as francisellosis, where the role of asymptomatic carriers in disease transmission is not well established. This case highlights the need for best management practices, including pathogen surveillance, implementation of biosecurity protocols, and the purchase of certified pathogen-free stock to prevent or minimize the introduction of disease agents into hatcheries and farms and to limit their spread on production operations (Wise, Camus, Schwedler, & Terhune, 2004; Plump & Hanson, 2011).

This is also the first report of the recently described *E. anguillarum* (Shao et al., 2015) affecting tilapia culture in Costa Rica. It is unknown whether this finding indicates a newly emerging pathogen in Costa Rica or if the organism has been present but has gone previously undiagnosed or misdiagnosed given that the lesions are similar to those of *Fno* and *E. ictaluri* infections in tilapia (Soto et al., 2009; Soto et al., 2012). While *E. ictaluri* is common on catfish operations in the United States, causing annual outbreaks at permissible temperatures (Hawke & Khoo, 2004; Klesius, 1992), similar information regarding *E. anguillarum* is not yet available. Further studies focusing on phylogenetics, virulence, and antimicrobial susceptibility are needed to gain a better understanding of *E. anguillarum* and its potential role in disease outbreaks in tilapia aquaculture in Central America.

No other *Edwardsiella* spp. were identified in this study. Furthermore, neither *S. agalactiae* nor *S. iniae* were detected in any of the spleen pools submitted for molecular analysis. However, previous reports have shown that *Streptococcus* spp. are endemic to Costa Rican tilapia farms, particularly in operations where water temperatures

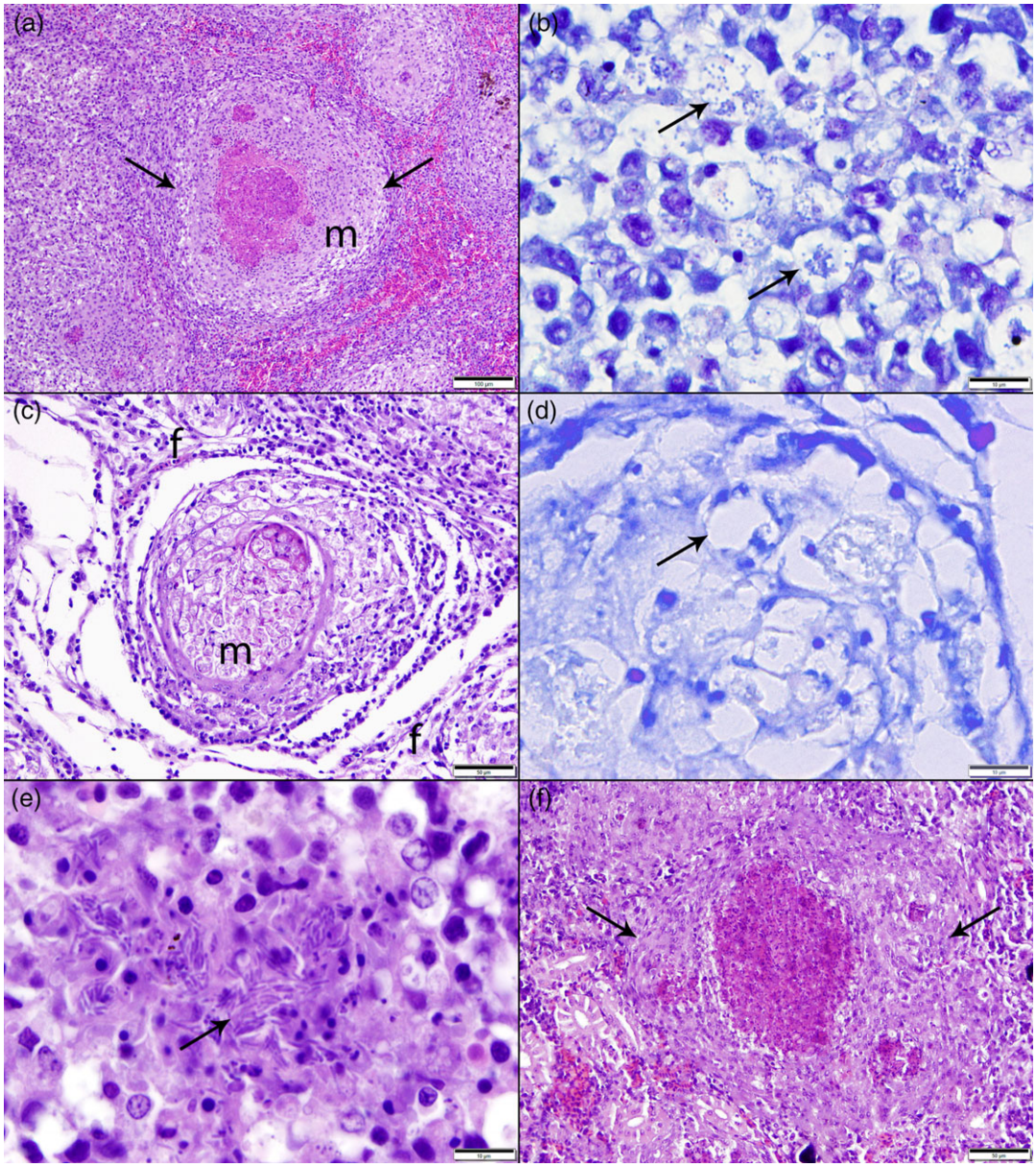

FIGURE 4 *Francisella noatunensis* subsp. *orientalis* (*Fno*) and *Edwardsiella anguillarum* lesions in Nile tilapia fingerlings. (a) Early granuloma development (arrows) in head kidney of a tilapia experimentally challenged with *Fno*. There is central necrosis surrounded by a zone of epithelioid macrophages, many with cytoplasmic vacuoles (H&E stain, bar = 100 μm). (b) During the early stages of infection, coccoid bacteria (arrows) typical of *Fno* are present within cytoplasmic vacuoles of macrophages (Giemsa stain, bar = 10 μm). (c) Nile tilapia from a Costa Rican hatchery chronically infected with *Fno*. A granuloma in the pericardial cavity is surrounded by fibroplasia (f) and scattered lymphocytes. A thin wall of epithelioid cells envelops a large central region of vacuolated macrophages (m) laden with granular cell debris (H&E stain, bar = 50 μm). (d) In these chronic lesions, bacteria could not be visualized in vacuoles (arrow) despite positive PCR results (Giemsa stain, bar = 10 μm). (e) *E. anguillarum* bacterial rods (arrow) within an area of necrosis in a naturally infected tilapia from Brazil (H&E stain, bar = 10 μm). (e) Granuloma formation in a tilapia from Costa Rica. In the absence of bacteria, the granuloma (arrows) cannot be readily distinguished from that induced by *Fno* in (a) above (H&E stain, bar = 50 μm). PCR, polymerase chain reaction

exceed 30°C (Barquero-Calvo, unpublished data). Further studies are necessary on the impacts of streptococcosis on tilapia hatcheries in Costa Rica.

Although this study was limited in scope, it demonstrates the value of using molecular techniques to obtain accurate diagnostic results in hatchery situations, both among symptomatic and asymptomatic populations of fish. This is particularly evident with diseases such as francisellosis and edwardsiellosis that produce similar gross and microscopic lesions. While the two conditions are readily differentiated microscopically when bacteria can be visualized, small coccoid bacteria versus rods, respectively, with chronicity bacterial numbers can be low, complicating histological interpretation (Soto, Kidd, et al., 2013). Molecular-based methods also circumvent diagnostic failures resulting from a lack of specialized media to culture more fastidious organisms, such as *Fno*. The methods developed and results obtained are applicable to the design of future surveillance programs for fish diseases, decreasing the chances of disseminating bacterial pathogens between hatcheries, farms, and the environment. Furthermore, this study highlights the crucial need for the establishment of a national disease surveillance program to prevent the introduction of disease agents, as well as limit their impacts and spread on Costa Rican aquaculture facilities.

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