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Isolation and molecular characterization of streptococcal species recovered from clinical infections in farmed Nile tilapia (*Oreochromis niloticus*) in the Philippines

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Abstract

Streptococcosis cause severe losses for global tilapia farming, especially in developing countries. The aim of this study was to identify and characterize streptococci recovered from Nile tilapia farmed in the Philippines. Moribund and apparently healthy fish were sampled from grow-out cages, ponds and hatcheries. Clinical signs observed included exophthalmia, eye opacity, ascites, lethargy, erratic swimming and haemorrhages. Results showed that both *Streptococcus iniae* and *Streptococcus agalactiae* were associated with disease in these sites. Consistent with global reports, including those from South-East Asia, *S. agalactiae* was more widespread than *S. iniae*. Molecular serotyping of the *S. agalactiae* isolates identified the serotype Ia and serotype Ib. Histopathological findings were meningitis, meningoencephalitis and septicaemia. Identical virulence profiles were found for all strains of *S. iniae*, while *S. agalactiae* strains were separated into virulence profile I and profile II. All strains were susceptible to the tested antibiotics and resistant to oxolinic acid. Only S. *agalactiae* serotype Ib showed resistance to sulphamethoxazole–trimethoprim. This is the first study from the Philippines to characterize the streptococci involved in disease outbreaks in tilapia aquaculture. Outputs from this study will promote the development of efficacious disease control strategies in tilapia farming for the Philippines and South-East Asia.

KEYWORDS

molecular typing, serotype Ia, serotype Ib, *Streptococcus agalactiae*, *Streptococcus iniae*, virulence genes

1 | INTRODUCTION

Streptococcosis outbreaks occur in a wide range of farmed fish species, globally (Mishra et al., 2018). Outbreaks in farmed freshwater tilapia continue to threaten global production, contributing to severe economic losses encountered worldwide (Amal & Zamri-Saad, 2011; Li et al., 2015; Liu et al., 2018; Mishra et al., 2018). Numerous incidences of streptococcosis have been reported in intensive tilapia culture systems in South-East Asia and have caused tremendous financial damage since tilapia farming is the most important, if not globally, in the region (Kayansamruaj, Areechon, & Unajak, 2020). While a range of bacterial aetiological agents have been identified from streptococcosis infections in fish, by far the greatest causes of these diseases are the Gram-positive, *Streptococcus agalactiae* and *S. iniae* (Agnew & Barnes, 2007; Mishra et al., 2018; Zhou et al., 2008). Understanding the pathogenesis in aquatic outbreaks is complex, as

both of these bacterial species possess and express an array of virulence genes and factors related to their pathogenicity. Identification of virulence gene patterns can be used to determine the genetic diversity of fish isolates belonging to streptococcal species and provide understanding of the evolutionary relationship between pathogen virulence and host adaptation (Godoy et al., 2013). Investigation of the genetic and epidemiological relationship of piscine streptococci is important in finding the appropriate health strategies, which may be unique depending on the geographical area. To support the tilapia farming sector, the "holy grail" is to produce highly efficacious prevention and control measures against fish loses due to streptococcal infections. Disease outbreaks from both of these bacterial pathogens often present with similar clinical signs and can be confused with other bacterial infections. In addition, concurrent infections with both *S. agalactiae* and *S. iniae* (Conroy, 2009) can be seen on single farms. These factors have contributed to a slower development of effective prevention and control strategies within tilapia farming systems globally and have resulted in more fragmented approaches. To reduce disease outbreaks, reliable data to discriminate between streptococcal species, their serotypes and genetic profiles are required. Uptake of molecular typing methods for *S. agalactiae* includes molecular typing by capsular polysaccharide (*cps*) gene, multi-locus typing (MLST) and genotyping of virulence genes present (Delannoy et al., 2013; Godoy et al., 2013; Kannika et al., 2017). Data from such studies have contributed towards improved knowledge on the aetiological identification, geographical distribution and molecular profile during disease outbreaks (LaFrentz et al., 2018). Currently, identification including molecular typing status of *S. agalactiae* and *S. iniae* affecting farmed tilapia in the Philippines is unknown. The objectives of the present study were to identify, characterize and profile streptococcal species recovered from clinically sick tilapia farmed in the Philippines, with a view to improving targeted disease prevention and control measures.

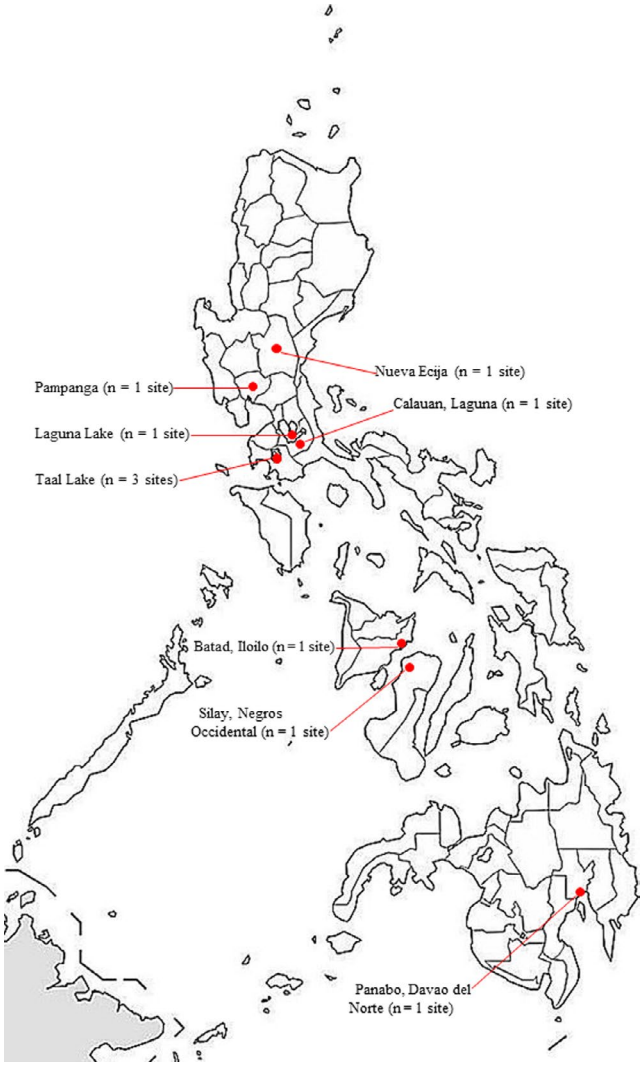

FIGURE 1 Sampling sites of the different tilapia farms in the Philippines (number indicates the number of farm/s sampled in the area) [Colour figure can be viewed at wileyonlinelibrary.com]

2 | MATERIALS AND METHODS

2.1 | Fish sampling

Fish were sampled from different tilapia farms that were currently suffering from a clinical disease outbreak. This was identified as morbidity/mortalities with external clinical signs as described in Table 3. Farmers were unable to provide exact data on mortalities, but the morbidities occurred all year. The sampling was performed in collaboration with the Fisheries Biotechnology Centre and Bureau of Fisheries and Aquatic Resources. A total of 106 fish were sampled during this study from 10 farms located in 3 geographical areas (Luzon, Visayas and Mindanao) of the Philippines (Figure 1). Of these farms, nine were grow-out and one was a hatchery where only broodstock fish were sampled. These farms were representative of the varied tilapia farming systems practised in the Philippines. At each cage or pond of a farm, a total of 7 fish were sampled for bacterial recovery and histopathology including a minimum of 2 apparently healthy fish. All tilapia sampled in this study were from natural clinical outbreaks with a body weight range of 30–500 g and exhibited various clinical signs of disease. These included lethargy and erratic swimming, petechiae, discoloration, exophthalmia (uni- and bilateral) (Figure 2) and corneal opacity. Internally, the moribund fish presented with enlarged spleen and kidney, ascites, gill pallor, brain haemorrhages and congested heart. The apparently healthy fish were judged as those that did not display any observable behavioural or clinical abnormalities. Samples of spleen, kidney and brain tissues from all fish were aseptically inoculated onto tryptone soya agar (TSA; Oxoid) and onto the selective Edward's medium (Oxoid) supplemented with colistin sulphate (5 mg/L). This medium is selective for the isolation of *S. agalactiae*. A laboratory-based pilot study was performed to confirm the specificity of Edward's medium for growth of Gram-positive *S. agalactiae* prior to use in the field study (data not presented). With the addition of liver and heart

FIGURE 2 Example of bilateral exophthalmia in a moribund tilapia [Colour figure can be viewed at wileyonlinelibrary.com]

samples, sections of the same tissues were also preserved in 10% (v/v) neutral buffered formalin for histopathology processing and screening. The inoculated agar plates were incubated at 28°C for 48 hr. Single colony subcultures were performed onto TSA to obtain pure isolates prior to identification. These were then stored using Protect Beads (Thermo Scientific, UK) at −70°C, and working stocks of 1 ml aliquots in 15% (v/v) glycerol were stored at −20°C.

2.2 | Histopathology

The tissues were fixed in 10% neutral buffered formalin for 24 hr before processing as described by Del-Pozo, Crumlish, Turnbull, and Ferguson (2010). Once embedded in paraffin, duplicate 5-μm-thick sections were cut and stained with haematoxylin and eosin and tissue Gram stain with crystal violet and counterstained with 1% neutral red, following routine laboratory methods. The sections were examined under light microscopy at up to 100× magnification and images captured using a digital slide scanner (ZEISS Axio Scan.Z1, ZEISS Germany).

2.3 | Bacterial identification

All pure cultures recovered were identified using traditional bacteriology methods including Gram stain, oxidase, catalase and motility (Barrow & Feltham, 2003). Additional tests included measuring haemolysis on 5% sheep blood agar (SBA), starch hydrolysis using the method of Cowan and Steel (Barrow & Feltham, 2003) and capsule formation following the protocol of Anthony in Cowan and Steel (Barrow & Feltham, 2003). Presumptive identification of the bacteria based on the above tests was accomplished following Buller (2004).

2.4 | Bacterial DNA extraction and identification using species-specific duplex PCR and 16S rRNA sequencing

DNA was extracted from single purified colonies following a crude boiling method with modification from Seward, Ehrenstein, Grundmann, and Towner (1997). The concentration of the DNA extracts was measured by NanoDrop (Thermo) spectrophotometer. The DNA samples were stored in sterile tubes in 20 μl aliquots at −20°C until required.

TABLE 1 Molecular serotyping primers for *Streptococcus agalactiae* isolates

Serotype	Target gene	Primer	Nucleotide (5′ → 3′)*	Target region (bp)
Ia	*cpsL*	*cpsL*-F	CAATCCTAAGTATTTTCGGTTCATT	688
		cpsL-R	TAGGAACATGTTCATTAACATAGC	
	cpsG	*cpsG*-F	ACATGAACAGCAGTTCAACCGT	272
		cpsG-R	ATGCTCTCCAAACTGTTCTTGT	
Ib	*cpsG*	*cpsG*-F	ACATGAACAGCAGTTCAACCGT	272
		cpsG-R	ATGCTCTCCAAACTGTTCTTGT	
	cpsJ	*cpsJ*-Ib-F	GCAATTCTTAACAGAATATTCAGTTG	621
		cpsJ-Ib-R	GCGTTTCTTTATCACATACTCTTG	
	cpsL	*cpsL*-F	CAATCCTAAGTATTTTCGGTTCATT	688
		cpsL-R	TAGGAACATGTTCATTAACATAGC	
III	*cpsG*	*cpsG*-F	ACATGAACAGCAGTTCAACCGT	352
		cpsG-2-3-6-R	TCCATCTACATCTTCAATCCAAGC	
	cpsL	*cpsL*-F	CAATCCTAAGTATTTTCGGTTCATT	688
		cpsL-R	TAGGAACATGTTCATTAACATAGC	

*Imperi et al. (2010)

TABLE 2 Primers used for the detection of virulence genes in *Streptococcus iniae* and *Streptococcus agalactiae*

Virulence factor	Target gene	Primer	Nucleotide (5′–3′)	Target region (bp)	Function
S. iniae					
M-like protein*	*simA*	simAfornew	AATTCGCTCAGCAGGTCTTG	994	Adhesin
		simArevnew	AACCATAACCGCGATAGCAC		
C5α peptidase*	*scpI*	scpIfor	GCAACGGGTTGTCAAAAATC	822	Immune evasin
		scpirev	GAGCAAAAGGAGTTGCTTGG		
Phosphoglucomutase*	*pgm*	pgmfor	TATTAGCTGCTCACGGCATC	490	Immune evasin
		pgmrev	TTAGGGTCTGCTTTGGCTTG		
Capsule*	*cpsD*	cpsDfor	TGGTGAAGGAAAGTCAACCAC	534	Immune evasin
		cpsDrev	TCTCCGTAGGAACCGTAAGC		
Polysaccharide deacetylase*	*pdi*	pdifor	TTTCGACGACAGCATGATTG	381	Adhesin
		pdirev	TTAGGGTCTGCTTTGGCTTG		
Cytolysin SLS*	*sagA*	sagAfor	AGGAGGTAAGCGTTATGTTAC	190	Invasin
		sagArev	AAGAAGTGAATTACTTTGG		
S. agalactiae					
CAMP factor**	*cfb*	cfb-F	GGATTCAACTGAACTCCAAC	600	Invasin
		cfb-R	GACAACTCCACAAGTGGTAA		
Serine protease**	*cspA*	cspA-F	CTGCTAAAGCACACCTAAAC	971	Immune evasin
		cspA-R	ATCAGTAGTGGTTCCTTTCC		
B-haemolysin/cytolysin***	*cylE*	cylE-F	CACTGCCAAGAGCAGTTGATT	558	Adhesin
		cylE-R	TTCCCCTTGGCGGATTTGGA		
Fibrinogen-binding protein A**	*fbsA*	fbsA-F	AACCGCAGCGACTTGTTA	278	Adhesin
		fbsA-R	AAACAAGAGCCAAGTAGGTC		
Hyaluronate lyase**	*hylB*	hylB-F	TCTATGCTGACGGTTCTTAC	323	Invasin
		hylB-R	GAGGTCTAAGTTTCGCTCTT		
Pilus-island 2B****	*PI-2b*	PI-2b-F	ACACGACTATGCCTCCTCATG	721	Immune evasin
		PI-2b-R	TCTCCTACTGGAATAATGACAG		
Penicillin-binding protein 1A**	*pbp1A/ponA*	pbp1A/ponA-F	AGGGGTAGTAGCATTACCAT	939	Immune evasin
		pbp1A/ponA-R	CAACTATATGACTGGGATCG		

*Baums et al. (2013),
**Kannika et al. (2017),
***Jiang et al. (2016),
****Chattopadhyay et al. (2011).

A duplex PCR was developed to differentiate between *S. agalactiae* and *S. iniae* using species-specific primers: LOX-1 (5′-AAGGGGAAATCGCAAGTGCC-3′) and LOX-2 (5′-ATATCTGATTGGGCCGTCTAA-3′) for target gene *lctO* of *S. iniae* which amplifies approximately 870 bp of the gene (Mata, Blanco, Dominguez, Fernandez-Garayzabal, & Gibello, 2004), and STRA-AgI (5′-AAGGAAACCTGCCATTTG-3′) and STRA-AgII (5′-TTAACCTAGTTTCTTTAAAACTAGAA-3′) which targets the *16-23S* intergenic spacer region of *S.agalactiae* which is about 270 bp (Phuektes, Browning, Anderson, & Mansell, 2003). Optimization of the duplex PCR was done by doing specificity and sensitivity tests. Each PCR was performed in a 25 μl reaction mixture consisting of 12.5 μl of 2X HS MyTaq MasterMix (Bioline, UK), 1.5 μl of 10 pmol of each oligonucleotide primer (Eurofins MWG Operon, Germany), 2.0 μl of DNA template and Milli-Q water to volume. The amplification profile consisted of an initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 25 s, with a final extension of 72°C for 10 min. The PCR products were obtained by electrophoresis in a 1% (w/v) agarose gel prepared in 0.5X TAE (Tris–acetate–EDTA) buffer with ethidium bromide (0.5 μg/ml), visualized on a UV transilluminator. Positive control isolates were used which included type strain of *S. agalactiae* NCIMB 701348 and *S. iniae* ATCC 29178.

TABLE 3 *Streptococci* isolates recovered and identified from different tilapia farms in the Philippines from 2018 to 2019

Farm No.	Location	Farm description	Fish No.	Clinical signs	Organ bacteria recovered*	Bacteria
1	Taal Lake Site 1	Freshwater grow-out floating cage 1	4	Gill pallor, ascites	K	*S. iniae*
			5	One eye absent, ascites	K, S	*S. iniae*
		Freshwater grow-out floating cage 2	1	Redness, ascites, discoloration, hepatosplenomegaly	K, S and B	*S. agalactiae* Ib
			3	Gill pallor, hepatosplenomegaly	K, S and B	*S. agalactiae* Ia
			4	Ascites, hepatosplenomegaly	K, S and B	*S. agalactiae* Ia
			5	Gill pallor, eyes missing, hepatosplenomegaly	K, S and B	*S. agalactiae* Ia
		Freshwater grow-out floating cage 3	1	Bilateral exophthalmia, ascites hepatosplenomegaly	K and B	*S. agalactiae* Ia
			2	Loose scales, discoloration, gill pallor	B	*S. agalactiae* Ia
			3	Loose scales, gill pallor, ascites	S	*S. agalactiae* Ia
			4	Corneal opacity, fin rot, ascites	K and B	*S. agalactiae* Ia
			5	Gill pallor, corneal opacity	S and B	*S. agalactiae* Ia
2	Taal Lake Site 2	Freshwater grow-out floating cage 1	3	Gill pallor, ascites	K	*S. iniae*
			2	Gill pallor, ascites	K	*S. iniae*
		Freshwater grow-out floating cage 2	1	Corneal opacity, ascites, hepatosplenomegaly	B	*S. agalactiae* Ib
			3	Ascites, hepatosplenomegaly	K & S	*S. agalactiae* Ia
			4	Ascites, redness, hepatosplenomegaly, corneal opacity, fin rot	S B	*S. agalactiae* Ia *S. agalactiae* Ib
3	Taal Lake Site 3	Freshwater grow-out floating cage 1	1	Discoloration, corneal opacity, ascites	K	*S. iniae*
			3	Discoloration, corneal opacity, ascites	S	*S. iniae*
		Freshwater grow-out floating cage 2	1	Anus swollen, petechiae, ascites, gill pallor, ascites	K	*S. iniae*
			3	Gill pallor, redness, ascites, hepatosplenomegaly	K & B	*S. agalactiae* Ia
			4	Anus swollen, ascites, hepatosplenomegaly	K	*S. agalactiae* Ia
			5	Anus swollen, ascites, hepatosplenomegaly	S	*S. agalactiae* Ia
4	Calauan, Laguna	Freshwater earthen pond hatchery 3	2	Redness of body, petechiae operculum and head	S and B	*S. agalactiae* Ia
6	Nueva Ecija	Freshwater grow-out earthen pond	1	Bilateral exophthalmia	K	*S. agalactiae* Ia

*K = kidney, S = spleen, B = brain.

To complement the duplex-PCR results, all *S. iniae* isolates identified from the duplex PCR as *S. iniae* ($n = 7$) and *S. agalactiae* ($n = 2$) were randomly selected and processed for 16S rRNA PCR sequence analysis. The two *S. agalactiae* selected were *S. agalactiae* NFFTC and *S. agalactiae* BSMF1. The 16S rRNA gene was PCR-amplified using universal primers 20F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1500R (5′-CGGTTACCTTGTTACGACTT-3′) (Weisburg, Barns, & Lane, 1991) which amplifies approximately 1501-bp region of the gene. Sequences were aligned with ClustalW algorithm against phylogenetically related organisms available in GenBank in the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

2.5 | Molecular serotyping of *Streptococcus agalactiae* isolates

Molecular serotype based on the *cps* gene was determined using a multiplex PCR described by Imperi et al. (2010) with minor modifications. Instead of 19 primers, seven primers (Table 1) were used to amplify serotypes Ia, Ib, II and III as these were found to be the main serotypes infecting fishes (Delannoy et al., 2016). The *S. agalactiae* (STIR-CD-17) serotype Ib and Milli-Q water were used as positive and negative controls, respectively. The PCR contained 12.5 μl of 2X HS MyTaq MasterMix, 1.0 μl of 10 pmol of each oligonucleotide primer, 5.0 μl of 20 ng gDNA and 0.5 μl of Milli-Q water. PCR amplification was performed in Biometra thermal cycler (Analytik Jena) using a cycling protocol of 1 cycle of 1 min at 95°C; 15 cycles of 60 s at 95°C, 60 s at 54°C and 2 min at 72°C; 25 cycles of 60 s at 95°C, 60 s at 56°C and 2 min at 72°C; and final cycle of 10 min at 72°C. The PCR products were then separated by electrophoresis in 1.5% (w/v) agarose gel and visualized under UV light.

2.6 | Antibiotic susceptibility assay

All the isolates were tested for antibiotic sensitivity to amoxicillin (AML 10 μg), oxytetracycline (OT 30 μg), florfenicol (FFC 30 μg), sulphamethoxazole–trimethoprim (SXT 25 μg), oxolinic acid (OA 2 μg) and enrofloxacin (ENR 5 μg) (Oxoid UK). The antibiotics selected were those routinely screened in the diagnostic laboratory at the Institute of Aquaculture, University of Stirling, and representative of antibiotics licensed for use in global aquaculture. The Kirby–Bauer method was applied: spread plates were produced on TSA by aseptically removing 2–3 single colonies per strain from a single colony subculture plate and emulsifying in 5 ml of sterile saline (0.85% NaCl) which gave a turbidity equal to McFarland standard of 1, and gave an approximate bacterial concentration of 3.0×10^8 CFU/ml. A bacterial lawn was produced and the antibiotic discs applied using an Oxoid ™ antimicrobial susceptibility disc dispenser and incubated at 28°C for 72 hr. Results were interpreted as sensitive (≥16 mm), partially sensitive (12–15 mm) or resistant (≤11 mm) based on the diameter zone of inhibitions (Crumlish, Dung, Turnbull, Ngoc, & Ferguson, 2002).

2.7 | Detection of virulence genes

Isolates were screened for a total of 6 and 7 virulence genes for the *S. iniae* and *S. agalactiae*, respectively (Table 2) using conventional PCR. *S. iniae* ATCC 29178 and *S. agalactiae* STIR-CD-17 were used as positive controls, while Milli-Q water was used as the negative control. The PCR conditions followed those cited in the literature (Table 2) with minor modifications to the time for denaturation (15 s), annealing (15 s) and extension (30 s). Temperatures for each stage were followed as described in the cited publication.

Each PCR was performed in a 25 μl reaction mixture consisting of 12.5 μl of 2X HS MyTaq MasterMix (Bioline, UK), 1.5 μl of 10 pmol of each oligonucleotide primer (Eurofins MWG Operon, Germany), 2.0 μl of DNA template and Milli-Q water to volume. The PCR products were obtained by electrophoresis in a 1% (w/v) agarose gel prepared in 0.5X TAE buffer with ethidium bromide (0.5 μg/ml), visualized on a UV transilluminator.

3 | RESULTS

3.1 | Fish sampling

A total of 106 tilapia were sampled during this study which represented 68 moribund fish presenting with a range of clinical signs of disease and 38 were observed as apparently normal as judged by naked eye. A total of 25 Streptococcal isolates were recovered from 24 (35%) moribund tilapia which presented a wide range of clinical signs, typical of streptococcosis in fish (Table 3). No streptococcal species were isolated from apparently healthy tilapia from any of the farms visited. The streptococcal species were recovered from 7 grow-out floating cages, 1 hatchery and 1 earthen pond, all freshwater, and included in 3 sites in Taal Lake, 1 site in Calauan, Laguna, and 1 site in Nueva Ecija, Philippines (Table 3). Only *S. agalactiae* were recovered from the brain samples of the fish (Table 3), and only in 1 site, both bacterial species were recovered (Table 3). A total of 7 *S. iniae* and 18 *S. agalactiae* were isolated from the moribund tilapia within this study (Table 3). From a single moribund tilapia in Taal Lake Site 2 cage 2, we recovered *S. agalactiae* serotypes Ia and Ib from the spleen and brain tissue, respectively (Table 3). From the remaining 53 fish sampled in this study, either no bacteria were recovered (apparently normal) or only Gram-negative or bacteria were isolated. These were not included further in this study.

3.2 | Histopathology

A range of histopathology changes were observed from the moribund tilapia infected with both *S. iniae* and *S. agalactiae*. By far, the greatest histological changes were meningoencephalitis (Figure 3a) or meningitis (Figure 3b) which was observed in almost 80% of the diseased fish with streptococcosis. Other histological changes included myonecrosis, endocarditis, myocarditis and pericarditis.

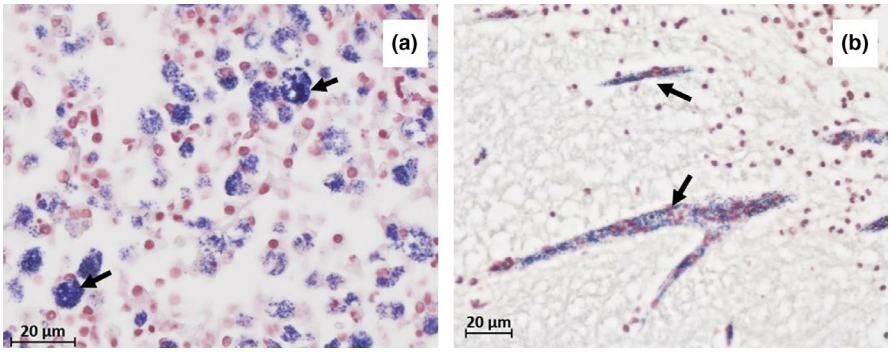

FIGURE 3 Gram-stained sections of (a) brain of tilapia with meningoencephalitis due to *Streptococcus iniae* showing macrophages (arrows) filled with bacteria; (b) meningitis in tilapia infected with *Streptococcus agalactiae* showing bacterial infiltration (arrow) in the brain capillaries. The purple-stained cells are streptococci, while the pink bodies are the nuclei of brain cells [Colour figure can be viewed at wileyonlinelibrary.com]

Histiocytosis and increased melanomacrophage centres were noted in the spleen, and glomerulonephritis, interstitial necrosis and tubular degeneration were observed in the kidneys. The liver presented separation of hepatocytes, perivascular inflammation, necrosis and vacuolation. Mononuclear and bacterial infiltration was seen in most of the sampled organs. These changes were similar to previous descriptions of clinical streptococcosis outbreaks in farmed tilapia (Asencios et al., 2016; Ortega et al., 2017).

Histological analysis of the brain tissues of clinically sicked tilapia showed subtle differences between *S. iniae*, *S. agalactiae* Ia and *S. agalactiae* Ib. Both *S. iniae* and *S. agalactiae* Ib showed meningoencephalitis with bacteria-filled macrophages in brain cortex (Figure 3a). For *S. agalactiae* Ia, although a large amount of bacteria was present in the brain capillaries (Figure 3b), there was mild meningitis of the brain and absence of meningoencephalitis. However, *S. agalactiae* Ia presented high number of bacteria *n* the sampled internal organs most notably in the atrium of the heart.

3.3 | Phenotypic observations and identification of streptococci

Bacterial recovery on TSA plates showed colonies were small, whitish-grey and smooth-edged, while in Edward's medium the colonies were small, blue to blueish colour with a white smooth edge. This was typical appearance on the agar types. Three isolates on TSA media produced very small colony variants (SCVs) that did not increase in size even after incubation for 72 hr. Irrespective of colony morphology, all strains were Gram-positive, oxidase and catalase-negative cocci. They were all non-motile as determined by hanging drop method, fermentative on oxidation/fermentation reactions and positive for the presence of a capsule. All of the 7 *S. iniae* strains were positive for hydrolysis of starch, while *S. agalactiae* strains are negative. Only three strains of *S. agalactiae* were negative for haemolysis, BSMF1, BSMF4-2 and SMF1, and the rest were haemolytic (Table 4).

3.4 | Molecular identification and virulence profile of Streptococcal species

The species-specific duplex PCR discriminated *S. iniae* from *S. agalactiae*, where a single band was observed at 870 bp for *S. iniae* and at 270 bp for *S. agalactiae* (Figure 4, Table 5). Good correlation was found between the 16S rRNA sequence results and the duplex-PCR results for *S. iniae* and *S. agalactiae strains* (Table 4).

All six virulence genes (*simA*, *scpI*, *pgm*, *cpsD*, *pdi* and *sagA*) (Figure 5a) were detected for all *S. iniae* strains ($n = 7$). For *S. agalactiae* isolates, all virulence factors (Figure 5b) were detected in 15 strains. Three strains of *S. agalactiae* (BSMF1, BSMF-4 and SMF1) were absent for gene *cylE*. This led to 2 virulence profiles for the *S. agalactiae* strains: profile I (all virulence genes present) and profile II (one virulence gene absent).

3.5 | Molecular serotyping of *Streptococcus agalactiae* isolates

From the 18 isolates identified as *S. agalactiae*, 15 (83%) isolates were identified as serotype Ia, while the remaining 3 (17%) were identified as serotype Ib. Serotype Ia was indicated by the presence of *cpsL* (688bp) and *cpsG* (272 bp), while serotype Ib by *cpsL* (688bp), *cpsJ* (621 bp) and *cpsG* (272 bp) (Figure 6, Table 6). Serotype Ia and Ib shares the presence of *cpsL* gene. No other serotype was detected in this study.

3.6 | Antibiotic susceptibility

The antibiotic susceptibility showed that 100% ($n = 7$) of *S. iniae* isolates were resistant only to oxolinic acid only and susceptible to all other antibiotics tested. For the *S. agalactiae* isolates, 100% ($n = 18$) were resistant to oxolinic acid and 17% ($n = 18$) were resistant to sulphamethoxazole trimethoprim. They were susceptible to all other antibiotics tested.

3.7 | *Streptococcus agalactiae* typing profile

Based on the phenotypic and molecular characterization, a typing scheme (Table 7) was applied for the *S. agalactiae* isolates, separated into genotype I (capsular serotype Ia, β-haemolytic, *cylE*-positive and SXT-susceptible) and genotype II (capsular serotype Ib, non-haemolytic, *cylE*-negative and SXT-resistant). The genotype I was more widely distributed, while genotype II was only found within a single geographical region, Taal Lake.

TABLE 4 Phenotypic and biochemical characteristics of recovered *Streptococci* strains

Streptococcal species and ID No.	Growth in Edward's medium	Gram stain	Oxidase	Catalase	Capsule	O/F	Starch hydrolysis	Haemolysis
Streptococcus iniae (7)	+	**G + cocci**	−	−	+	+	+	+
Streptococcus agalactiae ($n = 15$)	+	**G + cocci**	−	−	+	+	−	+
S. agalactiae ($n = 3$) (BSMF1, BSMF4-2, SMF1)	+	**G + cocci**	−	−	+	+	−	−

4 | DISCUSSION

The work reported here identified streptococcosis as a cause of infectious diseases in farmed tilapia in the Philippines, occurring in both grow-out farms and hatcheries. The data presented showed that *S. agalactiae* was the more prevalent and geographically widespread aetiological agent, while *S. iniae* appeared to be confined to a specific area. While we acknowledge the limited sample sizes, these findings are in agreement with reports from neighbouring South-East Asia (SEA) countries (Anshary, Kurniawan, Sriwulan, Ramil, & Baxa, 2014; Barkham et al., 2019; Jantrakajorn, Maisak, & Wongtavatchai, 2014; Kayansamruaj et al., 2019; Syuhada et al., 2020) and in China (Li et al., 2014; Su et al., 2019) where *S. agalactiae* is the primary streptococci species associated with fish disease. This observed prevalence of *S. agalactiae* over *S. iniae* is similar to reports worldwide, especially in the major tilapia-producing regions in Asia and Latin America (Liu et al., 2016). Over the last 20 years, tilapia production in the Philippines has declined, and several contributory factors have been identified (Guerrero, 2019). The results from this study would confirm that infectious disease outbreaks are a contributing factor to the decline in tilapia production in the Philippines.

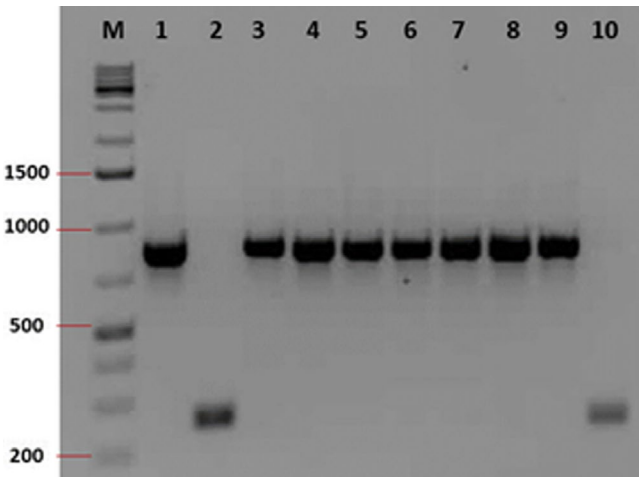

FIGURE 4 Duplex-PCR amplification of the LOX and STRA-Ag genes for the identification of *S. iniae* and *Streptococcus agalactiae* isolates from clinically affected tilapia in the Philippines. Lane M: 1 kb molecular marker; Lane 1: *S. iniae* ATCC 29178; Lane 2: *S. agalactiae* NCIMB 701348; Lanes 3–9 are the band size for *Streptococci iniae* (870 bp); Lane 10 is the band size for *S. agalactiae* (270 bp); and Lane 11: negative control (Milli-Q water) [Colour figure can be viewed at wileyonlinelibrary.com]

A wide range of identification methods have been applied to differentiate different streptococcal species and confirm serotypes in *S. agalactiae* populations occurring in farmed tilapia (Barkham et al., 2019; Mishra et al., 2018). In this study, we found excellent agreement between the duplex species-specific PCR and the 16S rRNA sequencing in identification of the bacterial species. The duplex-PCR method is relatively simple and cheap and can be performed within most laboratories with limited training. Uptake of the duplex PCR could be incorporated within either active or passive surveillance screening programmes within fish disease studies would be beneficial to differentiate between the cause of the disease at the time of infection and screening changes in aetiology over time. This method does not replace the need for more advanced molecular epidemiology methods, for example WGS, but it could be a sensible first step in understanding the aetiology and efficacy of any biosecurity or health management programmes.

It would appear that 2 serotypes of *S. agalactiae* are circulating in the Philippine tilapia farming systems and type Ia appears to be more prevalent. This serotype was more frequently recovered from the clinically sick grow-out and broodstock tilapia, while serotype Ib was only isolated from grow-out fish samples. Serotyping of *S. agalactiae* isolates is important when considering vaccine development as cross-protection between the different bio/serotypes does not occur in fish (Munang'andu, Paul, & Evensen, 2016). The results from the Philippines are in agreement with data from surrounding SEA countries and China which all reported *S. agalactiae* serotype Ia as the most prevalent and widespread cause of streptococcosis in freshwater tilapia aquaculture (Kannika et al., 2017; Kayansamruaj et al., 2019; Lusiastuti, Textor, Seeger, Akineden, & Zschock, 2014; Su et al., 2019; Syuhada et al., 2020). In this study, *cps* gene serotyping was applied which is used widely by others as a reliable way to serotype *S. agalactiae* (Kannika et al., 2017). While we recognize the limited sample size in this study, our findings were similar to surrounding SEA countries, but further characterization is needed to determine the sequence types, genetic and proteomic profiles, and exposure/transmission routes of the serotypes which will be vital for future vaccination programmes in the Philippines.

Histopathological changes in this study were similar to previous reports for streptococcal infections (Ferguson, 2006). In this study, it was not possible to differentiate histologically between *S. iniae* and *S. agalactiae* Ib. However, there were non-significant

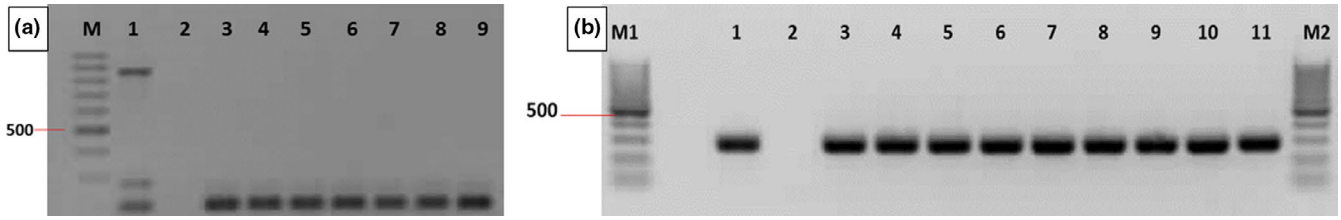

FIGURE 5 PCR amplification of (a) cytolysin SLS (*sagA*) gene in *Streptococcus iniae* isolates. Lane M is the DNA ladder of 100 bp; Lane 1 is the positive control (*S. iniae* ATCC 29178); Lane 2 negative control (Milli-Q water); Lanes 3–9 are the band size for saga gene (190 bp); (b) fibrinogen-binding protein A (*fbsA*) gene in *Streptococcus agalactiae* isolates. Lanes M1 and M2 are DNA ladder of 100 bp; Lane 1 is the positive control (*S. agalactiae* STIR-CD-17); Lane 2 negative control (Milli-Q water); and Lanes 3–11 are the band size for *fbsA* gene (278 bp) [Colour figure can be viewed at wileyonlinelibrary.com]

trends in the pathology associated with the two bacteria. In moribund fish infected with *S. iniae* and *S. agalactiae* serotype Ib, there was inflammation of the internal organs and meningitis with a thickened meninges and infiltration of lymphocytes and macrophages in the brain. Although no attempt was made to quantify the bacteria, there were apparently fewer bacteria in the infected fish. In the tilapia infected with *S. agalactiae* serotype Ia, a high number of bacteria were observed in the internal organs, notably in the atrium of the heart and in capillaries of the optic lobes of the brain; although a mild meningitis was consistently seen, there was no meningoencephalitis. Our data are consistent with previous histological studies on streptococcal infections in tilapia (Chen, Chao, & Bowser, 2007; Ferguson, 2006). The histopathology results obtained in this study support the highly invasive ability of *S. agalactiae* serotype Ia and its ability to cause acute infection compared with *S. iniae* which is more chronic (Chen et al., 2007; Ferguson, 2006). While a comparative or sequential pathology study was not performed, the histological results provided support the need for a more refined understanding of the complex pathogenesis of streptococcal infections in fish.

In this study, all three functional categories of virulence genes namely adhesins, invasins and immune evasins were present in all isolates revealing their pathogenic and invasive abilities. Identical virulence gene profiles were found for all the *S. iniae* isolates recovered from infected fish which may suggest that they have arisen from a single clone as these strains were all recovered from a single geographical location. In the *S. agalactiae* isolates, only serotype Ib lacked the *cylE* gene. This is the structural gene involved in β-haemolysis/cytolysis of the red blood cells (Pritzlaff et al., 2001). The lack of *cylE* gene was confirmed by the lack of haemolysis expressed in the 3 non-haemolytic *S. agalactiae* isolates tested when grown on sheep blood agar. The serotype Ia isolates from Thailand and Vietnam (Kayansamruaj et al., 2019) share the same patterns of virulence genes with the Philippine isolates. Studies have shown that β-haemolysin is a virulence factor that influences *S. agalactiae* survival in macrophages (Doran, Liu, & Nizet, 2003; Sagar et al., 2013) and promotes infection of the less or non-haemolytic strains by their ability to evade the host immune response and remain dormant inside macrophages until suitable conditions for their reactivation. The presence of *cylE* gene in *S. agalactiae* serotype Ia is believed to promote invasiveness (Chu et al., 2016) supporting rapid spread in the bloodstream and organs of the infected host, while its absence is considered a factor in the development of more chronic infection in fish (Li et al., 2014). For the non-haemolytic *S. agalactiae* serotype Ib isolates, the observed numerous bacteria-filled macrophage cells in the brain resulting in meningoencephalitis were indicative of a more chronic type infection.

In the Philippines, the use of antibiotics in tilapia aquaculture is not common because of the prevailing view that tilapia is resistant to

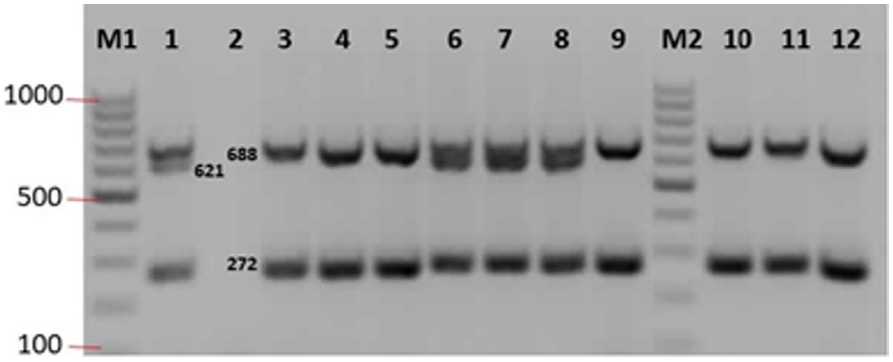

FIGURE 6 Multiplex PCR amplification of *cps* genes of *Streptococcus agalactiae*. Lanes M1 and M2 are the DNA ladder of 100 bp; Lane 1 is the positive control (*S. agalactiae* STIR-CD-17); Lane 2 is the negative control (Milli-Q water); Lanes 3, 4, 5, 9, 10, 11 and 12 are the band size for serotype Ia (272 bp and 688 bp); and Lanes 6, 7 and 8 are the band size for serotype Ib (272 bp, 621 bp and 688 bp) (BSMF1, BSMF4-2 and SMF1) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 5 Correlation of duplex PCR and 16S rRNA gene sequencing in identification of Streptococci

Bacterial species and isolate ID number	Duplex-PCR results		16S rRNA sequence result (Per. Ident.)
	lctO (870 bp) (*Streptococcus iniae*)	*16-23S* (270 bp) (*Streptococcus agalactiae*)	
S. iniae (Si1-Si7)	+	−	≥98% *S. iniae* ATCC 29178
S. agalactiae NFFTC	−	+	100.0% *S. agalactiae* ATCC 51487
S. agalactiae BSMF1	−	+	99.86% *S. agalactiae* ATCC 700208

TABLE 6 Molecular serotyping results of *Streptococcus agalactiae* strains

Bacterial isolate	*cpsL* (688 bp)	*cpsG* (272 bp)	*cpsG* (352 bp)	*cpsJ* (621 bp)	Serotype
S. agalactiae ($n = 15$)	+	+	−	−	**Ia**
S. agalactiae ($n = 3$) (BSMF1, BSMF4-2, SMF1)	+	+	−	+	**Ib**

TABLE 7 Typing profile of the *Streptococcus agalactiae* isolates affecting farmed tilapia in the Philippines

Bacterial species/isolate	Capsular serotype	Haemolysis	*cylE*	Susceptibility to SXT
S. agalactiae ($n = 15$)	**Ia**	**β**	+	**S**
S. agalactiae ($n = 3$) (BSMF1, BSMF4-2, SMF1)	**Ib**	**Non-haemolytic**	−	**R**

Note: S = sensitive, R = resistant, β = beta-haemolytic.

diseases. However, recent episodes of disease outbreaks leading to mass mortality have led to some fish farmers starting to use antibiotics particularly amoxicillin which is administered orally mixed with feed. The antibiotic susceptibility profile of the Philippine strains was similar to previous reports in Thailand (Dangwetngam, Suanyuk, Kong, & Phromkunthong, 2016; Jantrakajorn et al., 2014; Kannika et al., 2017; Klingklib & Suanyuk, 2017).

While in general the results from this study are consistent with previous studies for SEA countries, this is the first report of the appearance of small colony variants (SCVs) from fish *S. agalactiae* strains. Small colony variants are a slow-growing subpopulation of bacteria with distinctive phenotypic and pathogenic traits that are involved in chronic and recurrent infections (Proctor et al., 2006). In this study, after second subculture of *S. agalactiae* three isolates (BSMF1, BSMF4-2 and SMF1) exhibited very slow growth and pinpoint colonies that did not change in size, which were considered as SCV. These SCVs all belonged to serotype Ib, were non-haemolytic, *cylE* gene-deficient and SXT-resistant strains. Currently, there have been no reports of SCV appearance of piscine *S. agalactiae*, but in non-fish *S. agalactiae* strains reporting the appearance of SCV morphology, they are associated with subacute, recurrent and chronic infections, reduced antibiotic susceptibility and resistance to oxidative burst (Banno et al., 2014; Painter, Hall, Ha, & Edwards, 2017; Proctor et al., 2006). The piscine *S. agalactiae* SCV strains in this study are similar to human *S. agalactiae* SCV strains in that they both are non-haemolytic or have reduced haemolytic activity, small pinpoint colonies and resistance SXT (Banno et al., 2017). A change from wild-type phenotype to SCV could be a survival strategy for *S. agalactiae* serotype Ib fish strains similar to strategies used by SCV of human *S. aureus* and *Salmonella* sp. (Proctor et al., 2006).

It is clear that as with other SEA countries, streptococcal infections cause disease and fish losses within the tilapia production systems in the Philippines. Similar to previous reports from neighbouring countries, both *S. iniae* and *S. agalactiae* are present, able to cause disease with clinical signs similar to those previously reported, and *S. agalactiae* type Ia was the most prevalent pathogen. This study is the first to confirm that a range of streptococcal species is causing disease outbreaks in tilapia farms in the Philippines and that uptake of these data will better inform the disease prevention and control strategies for the Philippine sector and contribute towards a geographically distinct vaccine.

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CONFLICT OF INTEREST

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership or other equity interest; and expert testimony or patent-licensing arrangements) or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

DATA AVAILABILITY STATEMENT

No supporting data have been shared in any public repositories.

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REFERENCES

Agnew, W., & Barnes, A. C. (2007). *Streptococcus iniae*: An aquatic pathogen of global veterinary significance and a challenging candidate for reliable vaccination. *Veterinary Microbiology*, *122*, 1–15.

Amal, M. N. A., & Zamri-Saad, M. (2011). Streptococcosis in Tilapia (*Oreochromis niloticus*): A review. *Pertanika Journal of Tropical Agricultural Science*, *34*(2), 195–206.

Anshary, H., Kurniawan, R., Sriwulan, S., Ramil, R., & Baxa, D. V. (2014). Isolation and molecular identification of the etiological agents of streptococcosis in Nile tilapia (*Oreochromis niloticus*) cultured in net cages in Lake Sentani, Papua, Indonesia. *Springerplus*, *3*, 627.

Banno, H., Kimura, K., Tanaka, Y., Kitanaka, H., Jin, W., Wachino, J., … Arakawa, Y. (2014). Characterization of multidrug-resistant group B streptococci with reduced penicillin susceptibility forming small non-beta-hemolytic colonies on sheep blood agar plates. *Journal of Clinical Microbiology*, *52*(6), 2169–2171.

Banno, H., Kimura, K., Tanaka, Y., Sekizuka, T., Kuroda, M., Jin, W., … Arakawa, Y. (2017). Analysis of multidrug resistant group B streptococci with reduced penicillin susceptibility forming small, less hemolyitc colonies. *PLoS One*, *12*(8), e0183453.

Barkham, T., Zadoks, R. N., Azmai, M. N. A., Baker, S., Bich, V. T. N., Chalker, V., … Chen, S. L. (2019). One hypervirulent clone, sequence type 283, accounts for a large proportion of invasive *Streptococcus agalactiae* isolated from humans and diseased tilapia in Southeast Asia. *PLoS Neglected Tropical Diseases*, *13*(6), e0007421. https://doi.org/10.1371/journal.pntd.0007421

Barrow, G. I., & Feltham, R. K. A. (2003). *Cowan and steel's manual for the identification of the medical bacteria* (3rd ed.). Cambridge, UK: Cambridge University Press.

Baums, C. G., Hermeyer, K., Leimbach, S., Adamek, M., Czerny, C.-P., Horstgen-Schwark, G., … Steinhagen, D. (2013). Establishment of a Model of Streptococcus iniae Meningoencephalitis in Nile Tilapia (*Oreochromis niloticus*). *Journal of Comparative Pathology*, *149*, 94–102. https://doi.org/10.1016/j.jcpa.2012.10.003

Buller, N. B. (2004). *Bacteria from fish and other aquatic animals: A practical identification manual*. Oxfordshire, UK: CABI Publishing.

Chattopadhyay, D., Carey, A. J., Caliot, E., Webb, R. I., Layton, J. R., Wang, Y., … Ulett, G. C. (2011). Phylogenetic lineage and pilus protein Spb/SAN1518 affect opsonin-independent phagocytosis and intracellular survival of Group B Streptococcus. *Microbes and Infection*, *13*, 369–382.

Chen, C. Y., Chao, C. B., & Bowser, P. R. (2007). Comparative histopathology of *Streptococcus iniae* and *Streptococcus agalactiae*-infected tilapia. *Bulletin-European Association of Fish Pathologists*, *27*(1), 2–9.

Chu, C., Huang, P., Chen, H., Wang, Y., Tsai, I., Lu, C., & Chen, C. (2016). Genetic and pathogenic difference between *Streptococcus agalactiae* serotype Ia fish and human isolates. *BMC Microbiology*, *16*, 175. https://doi.org/10.1186/s12866-016-0794-4

Conroy, G. (2009). Estreptocococis en tilapia: prevalencia de las especies de *Streptococcus* en América Latina y sus manifestaciones patológicas. *En: Memorias De Streptococcus en Peces De Aguas Cálidas*, *3*, 15–20.

Crumlish, M., Dung, T. T., Turnbull, J. F., Ngoc, N. T. N., & Ferguson, H. W. (2002). Identification of *Edwardsiella ictaluri* from diseased freshwater catfish, *Pangasius hypophthalmus* (Sauvage), cultured in the Mekong Delta, Vietnam. *Journal of Fish Diseases*, *25*, 733–736. https://doi.org/10.1046/j.1365-2761.2002.00412.x

Dangwetngam, M., Suanyuk, N., Kong, F., & Phromkunthong, W. (2016). Serotype distribution and antimicrobial susceptibilities of *Streptococcus agalactiae* from infected cultured tilapia (*Oreochromis niloticus*) in Thailand: Nine-year Perspective. *Journal of Medical Microbiology*, *65*, 247–254.

Delannoy, C. M., Crumlish, M., Fontaine, M. C., Pollock, J., Foster, G., Dagleish, M. P., … Zadoks, R. N. (2013). Human *Streptococcus agalactiae* strains in aquatic mammals and fish. *BMC Microbiology*, *13*, 41.

Delannoy, C. M., Zadoks, R. N., Crumlish, M., Rodgers, D., Lainson, F. A., Ferguson, H. W., … Fontaine, M. C. (2016). Genomic comparison of virulent and non-virulent *Streptococcus agalactiae* in fish. *Journal of Fish Diseases*, *39*, 13–29.

Del-Pozo, J., Crumlish, M., Turnbull, J. F., & Ferguson, H. W. (2010). Histopathology and ultrastructure of segmented filamentous bacteria-associated rainbow trout gastroenteritis. *Veterinary Pathology*, *47*(2), 220–230.

Doran, K. S., Liu, G. Y., & Nizet, V. (2003). Group B streptococcal beta-hemolysin-cytolysin activates neutrophil signalling pathways in brain endothelium and contributes to development of meningitis. *The Journal of Clinical Investigation*, *112*, 736–744.

Ferguson, H. W. (ed.). (2006). *Systemic pathology of fish: A text and atlas of normal tissues in teleosts and their responses in disease* (2nd ed., 366 pp). London, UK: Scotian Press.

Godoy, D. T., Carvalho-Castro, G. A., Leal, C. A., Pereira, U. P., Leite, R. C., & Figueiredo, H. C. (2013). Genetic diversity and new genotyping scheme for fish pathogenic *Streptococcus agalactiae*. *Letters in Applied Microbiology*, *57*(6), 476–483.

Guerrero, R. D., III, (2019). Farmed tilapia production in the Philippines is declining: What has happened and what can be done. *Philippine Journal of Science*, *148*(2), xi–xv.

Imperi, M., Pataracchia, M., Alfarone, G., Baldassarri, L., Orefici, G., & Creti, R. (2010). A multiplex PCR assay for the direct identification of the capsular type (Ia to IX) of *Streptococcus agalactiae*. *Journal of Microbiol Methods*, *80*, 212–214.

Jantrakajorn, S., Maisak, H., & Wongtavatchai, J. (2014). Comprehensive investigation of Streptococcosis outbreak in cultured Nile tilapia, *Oreochromis niloticus*, and Red Tilapia, *Oreochromis* sp., of Thailand. *Journal of the World Aquaculture Society*, *45*(4), 392–402.

Jiang, H., Chen, M., Li, T., Liu, H., Gong, Y., & Li, M. (2016). Molecular Characterization of *Streptococcus agalactiae* Causing Community-and

Hospital-Acquired Infections in Shanghai, China. *Frontiers in Microbiology*, *7*, 1308. https://doi.org/10.3389/fmicb.2016.01308

Kannika, K., Pisuttharachai, D., Srisapoome, P., Wongtavatchai, J., Kondo, H., Hirono, I., … Areechon, N. (2017). Molecular serotyping, virulence gene profiling and pathogenicity of *Streptococcus agalactiae* isolated from tilapia farms in Thailand by multiplex PCR. *Journal of Applied Microbiology*, *122*(6), 1497–1507.

Kayansamruaj, P., Areechon, N., & Unajak, S. (2020). Development of fish vaccine in Southeast Asia: A challenge for sustainability of SE Asia aquaculture. *Fish & Shellfish Immunology*, *103*, 73–87.

Kayansamruaj, P., Soontara, C., Unajak, S., Dong, H. T., Rodkhum, C., Kondo, H., … Areechon, N. (2019). Comparative genomics inferred two distinct populations of piscine pathogenic *Streptococcus agalactiae*, Serotype Ia ST7 and Serotype III ST 283, in Thailand and Vietnam. *Genomics*, *111*, 1657–1667.

Klingklib, C., & Suanyuk, N. (2017). *Streptococcus agalactiae* serotype Ib, an emerging pathogen affecting climbing perch (*Anabas testudineus*) and Gunther's walking catfish (*Clarias macrocephalus*) polycultured in Southern Thailand. *The Thai Journal of Veterinary Medicine*, *47*(2), 183–197.

LaFrentz, B. R., Garcia, J. C., Waldbieser, G. C., Evenhuis, J. P., Loch, T. P., Liles, M. R., … Chang, S. F. (2018). Identification of four distinct phylogenetic groups in *Flavobacterium columnare* with fish host associations. *Frontiers in Microbiology*, *9*, 452.

Li, L., Wang, R., Liang, W., Huang, T., Huang, Y., Luo, F., … Gan, X. (2015). Development of live attenuated *Streptococcus agalactiae* vaccine for tilapia via continuous passage *in vitro*. *Fish & Shellfish Immunology*, *45*, 955–963.

Li, Y., Liu, L., Huang, P. R., Fang, W., Luo, Z. P., Peng, H. L., … Li, A. X. (2014). Chronic streptococcosis in Nile tilapia, *Oreochromis niloticus* (L.), caused by *Streptococcus agalactiae*. *Journal of Fish Diseases*, *37*(8), 757–763.

Liu, C., Feng, J., Zhang, D., Xie, Y., Li, A., Wang, J., & Su, Y. (2018). Clustering analysis of antibiograms and antibiogram types of *Streptococcus agalactiae* strain from tilapia in China. *Microbial Drug Resistance*, *24*(9), 1431–1439.

Liu, G., Zhu, J., Chen, K., Gao, T., Yao, H., Liu, Y., … Lu, C. (2016). Development of *Streptococcus agalactiae* vaccines for tilapia. *Diseases of Aquatic Organisms*, *21*(122), 163–170.

Lusiastuti, A. M., Textor, M., Seeger, H., Akineden, O., & Zschock, M. (2014). The occurrence of *Streptococcus agalactiae* sequence type 261 from fish disease outbreaks of tilapia *Oreochromis niloticus* in Indonesia. *Aquaculture Research*, *45*, 1260–1263.

Mata, A. I., Blanco, M. M., Dominguez, L., Fernandez-Garayzabal, J. F., & Gibello, A. (2004). Development of a PCR assay for *Streptococcus iniae* based on the lactate oxidase (*lctO*) gene with potential diagnostic value. *Veterinary Microbiology*, *101*, 109–116. https://doi.org/10.1016/j.vetmic.2004.03.012

Mishra, A., Nam, G., Gim, G., Lee, H., Jo, A., & Kim, H. (2018). Current challenges of streptococcus infection and effective molecular, cellular, and environmental control methods in aquaculture. *Molecules and Cells*, *41*(6), 495–505.

Munang'andu, H. M., Paul, J., & Evensen, Ø. (2016). An overview of vaccination strategies and antigen delivery systems for *Streptococcus agalactiae* vaccines in Nile Tilapia (*Oreochromis niloticus*). *Vaccines*, *4*(4), 48. https://doi.org/10.3390/vaccines4040048

Ortega Asencios, Y., Barreiro Sánchez, F., Bueno Mendizábal, H., Huancaré Pusari, K., Ostos Alfonso, H., Manchego Sayán, A., … Sandoval Chaupe, N. (2016). First report of Streptococcus agalactiae isolated from *Oreochromis niloticus* in Piura, Peru: Molecular identification and histopathological lesions. *Aquaculture Reports*, *4*, 74–79. https://doi.org/10.1016/j.aqrep.2016.06.002

Ortega, C., Garcia, I., Irgang, R., Fajardo, R., Tapia-Cammas, D., Acosta, J., & Avendaño-Herrera, R. (2017). First identification and characterization of *Streptococcus iniae* obtained from tilapia (*Oreochromis aureus*) farmed in Mexico. *Journal of Fish Diseases*, *41*, 773–782.

Painter, K. L., Hall, A., Ha, K. P., & Edwards, A. M. (2017). The electron transport chain sensitizes *Staphylococcus aureus* and *Enterococcus faecalis* to oxidative burst. *Infection and Immunity*, *85*(12), e00659–e717.

Phuektes, P., Browning, G. R., Anderson, G., & Mansell, P. D. (2003). Multiplex polymerase chain reaction as a mastitis screening test for assay for *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Streptococcus uberis*. *Journal of Dairy Research*, *70*, 149–155.

Pritzlaff, C. A., Chang, J. C., Kuo, S. P., Tamura, G. S., Rubens, C. E., & Nizet, V. (2001). Genetic basis for the beta-haemolytic/cytolytic activity of group B Streptococcus. *Molecular Microbiology*, *39*(2), 236–247.

Proctor, R. A., von Eiff, C., Kahl, B. C., Becker, K., McNamara, P., Herrmann, M., & Peters, G. (2006). Small colony variants: A pathogenic form of bacteria that facilitates persistent and recurrent infection. *Nature Reviews/Microbiology*, *4*, 295–305.

Sagar, A., Klemm, C., Hartjes, L., Mauerer, S., Zandbergen, G., & Spelleberg, B. (2013). The β-Hemolysin and intracellular survival of *Streptococcus agalactiae* in human macrophages. *PLoS One*, *8*(4), e60160. https://doi.org/10.1371/journal.pone.0060160

Seward, R. J., Ehrenstein, B., Grundmann, H. J., & Towner, K. J. (1997). Direct comparison of two commercially available computer programs for analysing DNA fingerprinting gels. *Journal of Medical Microbiology*, *46*, 314–320. https://doi.org/10.1099/00222615-46-4-314

Su, Y., Liu, C., Deng, Y., Cheng, C., Ma, H., Guo, Z., & Feng, J. (2019). Molecular typing of *S. agalactiae* isolates of serotype Ia from tilapia in Southern China. *FEMS Microbiology Letters*, *366*(13), fnz154.

Syuhada, R., Zamri-Saad, M., Ina-Salwanay, M. Y., Mustafa, M., Nasruddin, N. N., Desa, M. N. M., … Amal, M. N. A. (2020). Molecular characterization and pathogenicity of *Streptococcus agalactiae* serotypes Ia ST7 and III ST283 isolated from cultured red hybrid tilapia in Malaysia. *Aquaculture*, *515*, 7354543. https://doi.org/10.1016/j.aquaculture.2019.734543

Weisburg, W. G., Barns, S. M., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, *173*(2), 697–703.

Zhou, S. M., Xie, M. Q., Zhu, X. Q., Ma, Y., Tan, Z. L., & Li, A. X. (2008). Identification and genetic characterization of *Streptococcus iniae* strains isolated from diseased fish in China. *Journal of Fish Diseases*, *31*, 869–875.

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