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.

**Key words**
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* (Agniew & 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.

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
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

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Target gene</th>
<th>Primer</th>
<th>Nucleotide (5′ → 3′)</th>
<th>Target region (bp)</th>
</tr>
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<tbody>
<tr>
<td>Ia</td>
<td>cpsL</td>
<td>cpsL-F</td>
<td>CAATCCTAAGTATTTTCGGGTTCATT</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>cpsL-R</td>
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<td></td>
<td>cpsG</td>
<td>cpsG-F</td>
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<td>272</td>
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<td></td>
<td>cpsG-R</td>
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<td>cpsG-F</td>
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</tr>
<tr>
<td></td>
<td>cpsJ</td>
<td>cpsJ-F</td>
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<td></td>
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<tr>
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<td>CAATCCTAAGTATTTTCGGGTTCATT</td>
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<td></td>
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<td>cpsL-F</td>
<td>CAATCCTAAGTATTTTCGGGTTCATT</td>
<td>688</td>
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<tr>
<td></td>
<td></td>
<td>cpsL-R</td>
<td>TAGGACATGTTCTTACATTACTG</td>
<td></td>
</tr>
</tbody>
</table>

*Imperi et al. (2010)
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’-TTAACCTAGTTTCTTTAAAAACTAGAAG-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 2 Primers used for the detection of virulence genes in *Streptococcus iniae* and *Streptococcus agalactiae*

<table>
<thead>
<tr>
<th>Virulence factor</th>
<th>Target gene</th>
<th>Primer</th>
<th>Nucleotide (5’-3’)</th>
<th>Target region (bp)</th>
<th>Function</th>
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<td><em>S. iniae</em></td>
<td>simA</td>
<td>simAfornew</td>
<td>AATTCGCTACGAGGCTCTTG</td>
<td>994</td>
<td>Adhesin</td>
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<td></td>
<td>simArevnew</td>
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<tr>
<td></td>
<td>scpI</td>
<td>scpIfornew</td>
<td>GCAAAGGCTGTGAAAAATC</td>
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<td>Immune evasin</td>
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<td></td>
<td>pgm</td>
<td>pgmfor</td>
<td>TATTAAGGCTCATCAGGCTATC</td>
<td>534</td>
<td>Immune evasin</td>
</tr>
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<td></td>
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<tr>
<td></td>
<td>pdi</td>
<td>pdiFo</td>
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<tr>
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<td>cfbF</td>
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<td>hyIL</td>
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<td>Invasin</td>
</tr>
<tr>
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<td>hyILR</td>
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<tr>
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*Baums et al. (2013),
**Kannika et al. (2017),
***Jiang et al. (2016),
****Chattopadhyay et al. (2011).
### TABLE 3
Streptococci isolates recovered and identified from different tilapia farms in the Philippines from 2018 to 2019

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

*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 \((5'-\text{AGAGTTTGATCATGGCTCAG-3'}\)) and 1500R \((5'-\text{CGGTTACCTTGTTACGACTT-3'}\) (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 \(\mu\)l of 2X HS MyTaq MasterMix, 1.0 \(\mu\)l of 10 pmol of each oligonucleotide primer, 5.0 \(\mu\)l of 20 ng gDNA and 0.5 \(\mu\)l 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 \(\mu\)g), oxytetracycline (OT 30 \(\mu\)g), florfenicol (FFC 30 \(\mu\)g), sulphonamethoxazole–trimethoprim (SXT 25 \(\mu\)g), oxolinic acid (OA 2 \(\mu\)g) and enrofloxacin (ENR 5 \(\mu\)g) (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 \(3.0 \times 10^6\) 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 (\(\geq 16\) mm), partially sensitive (12–15 mm) or resistant (\(\leq 11\) 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 \(\mu\)l reaction mixture consisting of 12.5 \(\mu\)l of 2X HS MyTaq MasterMix (Bioline, UK), 1.5 \(\mu\)l of 10 pmol of each oligonucleotide primer (Eurofins MWG Operon, Germany), 2.0 \(\mu\)l 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 \(\mu\)g/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 histopathological 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.
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 in 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 (*sim*Á, *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 Ib, while the remaining 3 (17%) were identified as serotype Ia. 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 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.
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.

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...
trends in the pathology associated with the two bacteria. In mori-
bund 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 macro-
phages 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 re-
sults 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 sup-
port 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 in-
volved 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 iso-
lates 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 iso-
lates. 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 invasive-
ness (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 iso-
lates, 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 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]
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, 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
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DATA AVAILABILITY STATEMENT
No supporting data have been shared in any public repositories.

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