Vol. 158: 27–36, 2024
https://doi.org/10.3354/dao03780

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published April 25

Serotype distribution, virulence and antibiotic resistance of *Streptococcus agalactiae* isolated from cultured tilapia *Oreochromis niloticus* in Lake Volta, Ghana

Samuel Duodu[1,2,*], Angela N. A. Ayiku[1,2], Abigail A. Adelani[2], Derrick A. Daah[1,2], Enock K. Amoako[2], Mona D. Jansen[3], Kofitsyo S. Cudjoe[3]

[1]Department of Biochemistry, Cell and Molecular Biology, University of Ghana, LG54 Volta Rd, Legon-Accra, Ghana
[2]West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), College of Basic and Applied Sciences, University of Ghana, LG54 Volta Rd, Legon-Accra, Ghana
[3]Norwegian Veterinary Institute, Elizabeth Stephansons vei 1, 1433 Ås, Norway

ABSTRACT: *Streptococcus agalactiae* infection is one of the major factors limiting the expansion of tilapia farming globally. In this study, we investigated the serotype distribution, virulence and antimicrobial resistance of *S. agalactiae* isolates from tilapia farmed in Lake Volta, Ghana. Isolates from 300 moribund fish were characterised by Gram staining, MALDI-TOF/MS and 16S rRNA sequencing. Serotype identification was based on multiplex polymerase chain reaction (PCR) amplification of the capsular polysaccharide genes. Detection of virulence genes (*cfb, fbsA* and *cspA*) and histopathology were used to infer the pathogenicity of the isolates. The susceptibility of isolates to antibiotics was tested using the Kirby-Bauer disk diffusion assay. All 32 isolates identified as *S. agalactiae* were of serotype Ia. This was notably different from isolates previously collected from the farms in 2017, which belonged to serotype Ib, suggesting a possible serotype replacement. The prevalence of the pathogen was related to the scale of farm operation, with large-scale farms showing higher *S. agalactiae* positivity. Data from histopathological analysis and PCR amplification of targeted virulence genes confirmed the virulence potential and ability of the isolates to cause systemic infection in tilapia. Except for gentamicin, the majority of the isolates were less resistant to the tested antibiotics. All isolates were fully sensitive to oxytetracycline, erythromycin, florfenicol, enrofloxacin, ampicillin and amoxicillin. This study has improved our understanding of the specific *S. agalactiae* serotypes circulating in Lake Volta and demonstrates the need for continuous monitoring to guide the use of antimicrobials and vaccines against streptococcal infections in Ghanaian aquaculture systems.

KEY WORDS: *Streptococcus agalactiae* Ia · Tilapia · Antimicrobial susceptibility · Wild-type · Non-wild-type · Molecular serotyping · Ghanaian aquaculture · Multiplex PCR

1. INTRODUCTION

Streptococcus agalactiae, commonly known as Group B *Streptococcus* (GBS), is a Gram-positive bacterium that causes deadly bacterial infections. This bacterium is a well-known fish pathogen that causes severe disease in freshwater and marine fish species (Delannoy et al. 2013). It has zoonotic potential and can be transmitted to humans via contact with or consumption of undercooked fish or contaminated food products. Streptococcosis epidemics linked to *S. agalactiae* infection in cultured fish have been recorded

*Corresponding author: saduodu@ug.edu.gh

© The authors 2024. Open Access under Creative Commons by Attribution Licence. Use, distribution and reproduction are unrestricted. Authors and original publication must be credited.

Publisher: Inter-Research · www.int-res.com

globally. In tilapia *Oreochromis* spp., it is the main causal agent of bacterial infections, accounting for more than 50% of isolates from diseased farmed fish (Sheehan et al. 2009). Systemic infection typically presents with meningoencephalitis and septicaemia. The disease has high transmission and mortality rates, especially during high water temperatures approaching or exceeding 30°C (Mian et al. 2009).

S. agalactiae infection is a major challenge to the Ghanaian tilapia aquaculture industry. In 2017, *S. agalactiae* multilocus sequence type 261 (ST261), serotype Ib strain was identified as the dominant pathogen associated with mortalities in cage tilapia farms around Lake Volta in Ghana (Verner-Jeffreys et al. 2018). Before this study, some farmers had streptococcosis diagnosed on their farms through clinical investigations. A recent report also mentioned co-infection with *S. agalactiae* and other fish pathogens like the infectious spleen kidney necrosis virus (ISKNV) causing significant economic losses in the Ghanaian aquaculture industry (Ramírez-Paredes et al. 2021). Treatment with antibiotics and the use of autogenous vaccines are the main options by which streptococcosis is managed by tilapia farmers in Ghana. *S. agalactiae* has shown increasing resistance to antibiotics in many geographical locations across the globe where aquaculture is intensively practiced (Deng et al. 2019, Alazab et al. 2022). Aside from repeated uncontrolled usage of antibiotics promoting the emergence of resistant strains, outbreak of disease could also be influenced by the serotype and virulence of the infecting strain. Currently, 10 capsular serotypes of *S. agalactiae* are known (Cieslewicz et al. 2005, Slotved et al. 2007). These are Ia, Ib, II, III, IV, V, VI, VII, VIII and IX, classified based on specific capsular polysaccharide antigens, which are unique for each serotype. The most prevalent serotypes in fish infection outbreaks are Ia, Ib and III (Chideroli et al. 2017). So far, only serotype Ib has been confirmed in Ghana (Verner-Jeffreys et al. 2018, Ramírez-Paredes et al. 2021), yet farmers are already using a trivalent autogenous vaccine (Virbac) targeting serotypes Ia, Ib and III against *Streptococcus* infections. Serotype switching is becoming an increasingly important immune evasion mechanism that might seriously hamper vaccination efforts. It is therefore prudent to closely monitor for possible changes in serotype distribution while directing the development of vaccines against this pathogen.

More recently, traditional serotyping methods such as enzyme immunoassays, latex agglutination and co-agglutination have been replaced by polymerase chain reaction (PCR)-based assays. Both multiplex PCR and whole genome sequencing approaches have been reported to identify different serotypes of GBS with high accuracy based on sequence differences within the variable region (*cpsG*–*cpsK*) of the capsular gene cluster (*cps*) locus (Imperi et al. 2010, Tiruvayipati et al. 2021). In this study, molecular serotyping was adopted to serotype 32 *S. agalactiae* isolates recovered from farmed Nile tilapia in Ghana. The isolates were screened for antimicrobial resistance and further characterised by partial 16S rRNA sequencing and PCR detection of virulence genes.

2. MATERIALS AND METHODS

2.1. Fish sampling

Moribund fish were sampled from 30 tilapia farms along Lake Volta in Ghana between August and November 2021, following ethical approval obtained from the University of Ghana Institutional Animal Care and Use Committee (UG-IACUC 007/20-21) and consent by farmers. The farms sampled included small- (n = 9), medium- (n = 12) and large-scale (n = 9) operations as defined by the Fisheries Commission of Ghana. On average, 10 biological samples of fry, fingerlings, grow-out adult fish and broodstocks were collected at each farm depending on the type of operational facility (i.e. grow-out and/or hatchery). Gross clinical symptoms were recorded for each fish. All fish sampled were euthanized with 0.20 ml of clove oil per 500 ml of water for 10 min. For bacteriological examination, the head kidney, brain and external lesions of the fish were streaked directly using sterile inoculation loops on 5% sheep blood agar (Oxoid), tryptone soya agar (Oxoid) and tryptone yeast extract agar supplemented with tobramycin at 5 µg ml^{-1} concentration. In addition, several fish tissue samples (brain, liver, kidney, spleen, heart, muscle, gills and pancreas) were collected and fixed in 10% buffered formalin for histopathological analysis. Epidemiological data on disease intervention measures and mortalities including the use of antimicrobials at the farm level was collated through interviews using a structured electronic questionnaire (see Text S1 in Supplement 1 at www.int-res.com/articles/suppl/d158p027_supp1.pdf).

2.2. Laboratory analysis

The bacterial cultures were incubated at ambient temperature (27–30°C) for up to 3 d and isolates were

sub-cultured for purity on brain heart infusion (BHI) agar. Bacteriological assessment was performed by a combination of phenotypic tests (bacterial colony morphology and Gram staining) and MALDI-TOF using the Bruker MALDI Biotyper Microflex LT and MBT Compass 4.1 reference library (version 2022) for the analysis. *Streptococcus agalactiae* isolates were further identified by Sanger sequencing of the 16S rRNA gene using the S-20 and A-18 primers described by Suau et al. (1999). A phylogenetic tree based on the 16S rRNA gene fragments (~500 bp) was constructed using the neighbour-joining method. For molecular serotyping, genomic DNA was amplified using Go Taq Green Master Mix (Promega) and *cps* primers (0.2 µM final concentration) targeting 3 serotypes (type Ia, Ib and III) in a multiplex PCR assay (Imperi et al. 2010). Detection of key virulence genes *cfb* (CAMP factor involved in host invasion), *fbsA* (the fibrinogen-binding protein FbsA for adhesion) and *cspA* (the serine protease cspA involved in immune evasion) were also multiplexed in a single PCR reaction using previously reported primers (Kayansamruaj et al. 2014, Kannika et al. 2017, Legario et al. 2020). The oligonucleotides and cyclic conditions used for PCR amplification of all genes included in this study are shown in Table S1.

For drug susceptibility testing, the *S. agalactiae* isolates were screened using the Kirby-Bauer disk diffusion assay on Mueller-Hinton agar (Oxoid) containing 5% sheep blood according to the Clinical Laboratory Standard Institute (CLSI) protocols for streptococci aquatic bacteria (CLSI 2020a,b). The following antibiotics were used: oxytetracycline (OXT; 30 µg), florfenicol (FLO; 30 µg), erythromycin (ERY; 15 µg), trimethoprim-sulfamethoxazole (SXT; 25 µg), enrofloxacin (ENR; 5 µg), gentamicin (CN; 30 µg), ampicillin (AMP; 10 µg) and amoxicillin (AML; 10 µg), supplied by Oxoid. The diameter of each zone of inhibition was determined after 24 and 48 h of incubation at 28°C. The testing was done in duplicate for each antibiotic and read by 2 different investigators. *S. pneumoniae* ATCC 49619 was used as the quality control (QC) strain. The *S. agalactiae* isolates were classified as wild-type (WT) and non-wild-type (NWT) based on their epidemiological cut-off (CO_{WT}) values generated using the normalized resistance interpretation (NRI) method (Kronvall & Smith 2016). WT refers to isolates whose susceptibility are indistinguishable from fully susceptible members of the group, with zone of inhibition values $\geqslant CO_{WT}$, while the NWT isolates are presumed to have acquired resistance characteristics and exhibit reduced susceptibility from the rest of the group, with zone of inhibition values $< CO_{WT.}$ Calculations of CO_{WT} using Excel (www.bioscand.se/nri/; accessed on 23 November 2023) were performed for the antibiotics (ENR, OXT, CN, FLO and AML) with no reference veterinary-specific or human medical breakpoints. Except for CN, each of these antibiotics were analysed with at least 50 observations from 'suspected' susceptible *S. agalactiae* isolates (n = 26) as recommended by Smith (2020). For AMP, SXT and ERY, the categorisation into susceptible, intermediate and resistant isolates was based on the breakpoints for *Streptococcus* spp. from humans, as recommended in CLSI (2020c).

Histopathological analysis of formalin-fixed tissue samples was performed following standard routine protocols (Suvarna et al. 2019). Briefly, paraffin-embedded tissues were sectioned at 2–2.5 µm thickness and stained with haematoxylin and eosin and Gram-Twort. The sections were examined under light microscope at up to 100× magnification for identification of bacteria. Images were captured using a Hamamatsu NanoZoomer S360 digital slide scanner together with the NDPview.2.7.25.0 (Hamamatsu Photonics K.K.) software program.

3. RESULTS

The *Streptococcus agalactiae* strains isolated from the brain, kidney and skin samples showed pinpointed whitish or creamy round colonies with smooth edges on BHI agar and were Gram-positive cocci, appearing in pairs or chains. Out of the 32 confirmed *S. agalactiae* isolates, 28 (87.5%) were β-haemolytic on blood agar. The overall farm-level prevalence of *S. agalactiae* was 23.3% (7 out of 30 farms) and the pathogen was widely distributed along the Eastern bank of Lake Volta (Fig. 1). It was detected both as single infection and in combination with other bacterial pathogens (*Aeromonas veronii*, *Edwardsiella tarda* and *Chryseobacterium* spp.) (Table S2). Data from positive farms, including type of culture systems, stocking density, water temperature and the accumulated mortality rate reported during major disease outbreaks, are presented in Table 1. Most positive farms were large-scale farms (71%, n = 5) and included a hatchery and combinations of hatchery, nursery and grow-out facilities, producing their own fingerlings from locally obtained broodstocks. Adult fish with a median weight of 300 g were most affected, which included some broodstocks. The infected fish presented with a range of gross clinical signs including haemorrhaging of skin and internal organs, distended abdomen (ascites), opaque eyes, exophthalmia, necrosis of viscera, mottled friable liver, enlarged gall bladder, congested kidney and

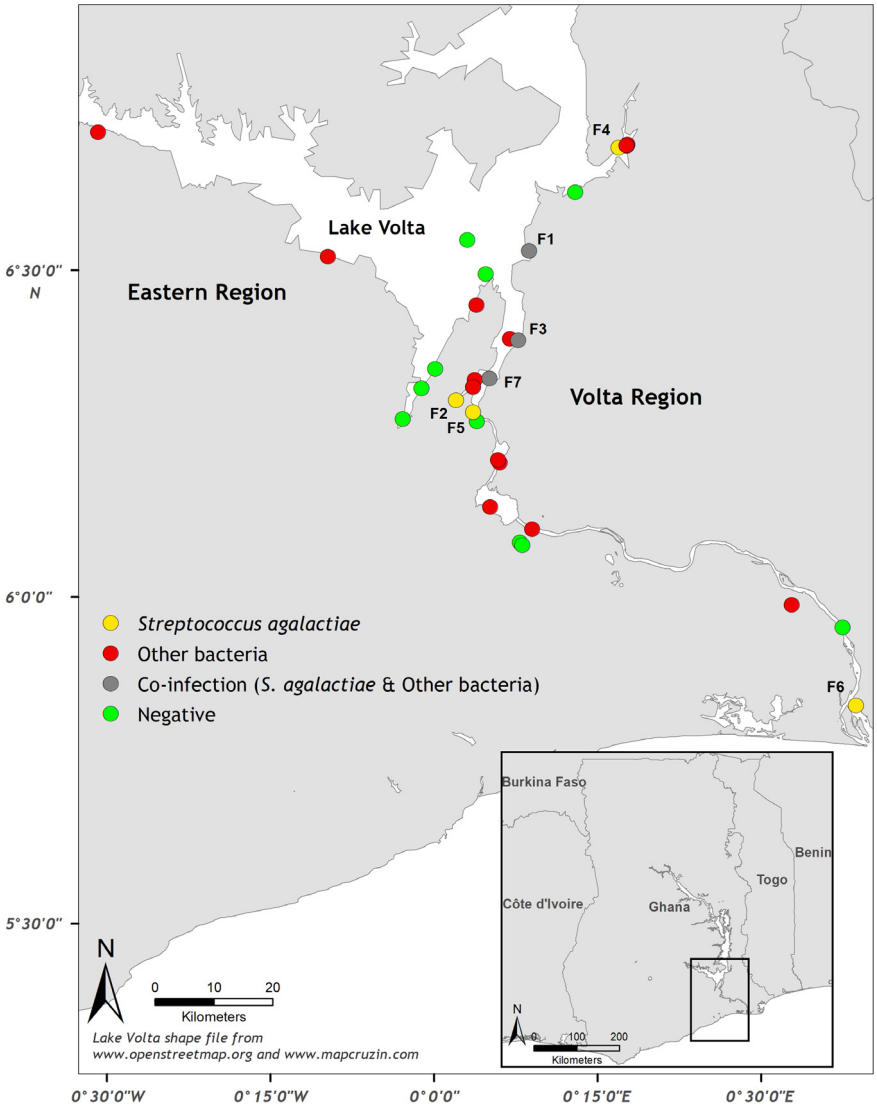

Fig. 1. Distribution of tilapia farms on Lake Volta with detection of *Streptococcus agalactiae* (yellow circles), other bacteria (red circles), co-infection of *S. agalactiae* with other bacteria (grey circles) and farms where bacteria were not detected (green circles). F1–F7 indicate the *S. agalactiae* positive farms. The maps were created using ArcGIS software (ESRI; www.esri.com)

splenomegaly (Table S2). Some fish found positive for *S. agalactiae* had no detectable gross signs. Molecular serotyping confirmed all isolates to be serotype Ia, amplifying the 688 and 272 bp DNA fragments of the *cpsL* and *cpsG* capsid genes, respectively (data not shown). Blast searches of the 500 bp 16S rRNA product sequences revealed significant similarity of 99.4–100%, with only *S. agalactiae* identified as a 'hit' based on the information obtained from GenBank (Table S3 in Supplement 2 at www.int-res.com/articles/suppl/d158p027_supp2.xlsx). Phylogenetic analysis of the 16S rDNA amplicons clustered the *S. agalactiae* isolates into 2 groups that were closely related to serotype Ia isolated from cultured tilapia in Asia (Fig. 2).

Histopathological analysis revealed multiple cases of bacterial infections with accompanying septicaemia. Fish that were positive for *S. agalactiae* by culture and PCR had bacteria in multiple organs, including the brain, spleen, liver, kidney, pancreas and heart (Fig. 3). Individual spleen and brain tissues contained large numbers of melanomacrophage centers with local fibrin deposits. These aggregates of highly pigmented phagocytes might reflect an ongoing immune response of the fish to an infection. All *S. agalactiae* isolated in this study carried the key virulence genes *fbsA*, *cfb* and *cspA* (related to host adhesion, invasion and immune evasion, respectively).

Table 1. Data on culture systems, stocking density, water temperature and accumulated mortality rates for *Streptococcus agalactiae*-positive tilapia farms. Cumulative mortality rate data are from 2019–2021

Farm	Type of holding facility	Stocking rate in cages (no. fish m^{-3})	Production scale	Water temperature (°C)	Cumulative mortality rate during major fish disease episodes (%)
1	Floating cages, tanks, and ponds	100–107	Large	29	80–90
2	Floating cages	16–20	Medium	28	> 50 (up to 90)
3	Floating cages	40–60	Large	28–30	80
4	Floating cages	20–40	Medium	23–29	80
5	Floating cages, tanks and ponds	16–24	Large	26–29	40
6	Floating cages, tanks ponds or reservoirs	40–92	Large	28–30	90
7	Floating cages	20–40	Large	28–29	90

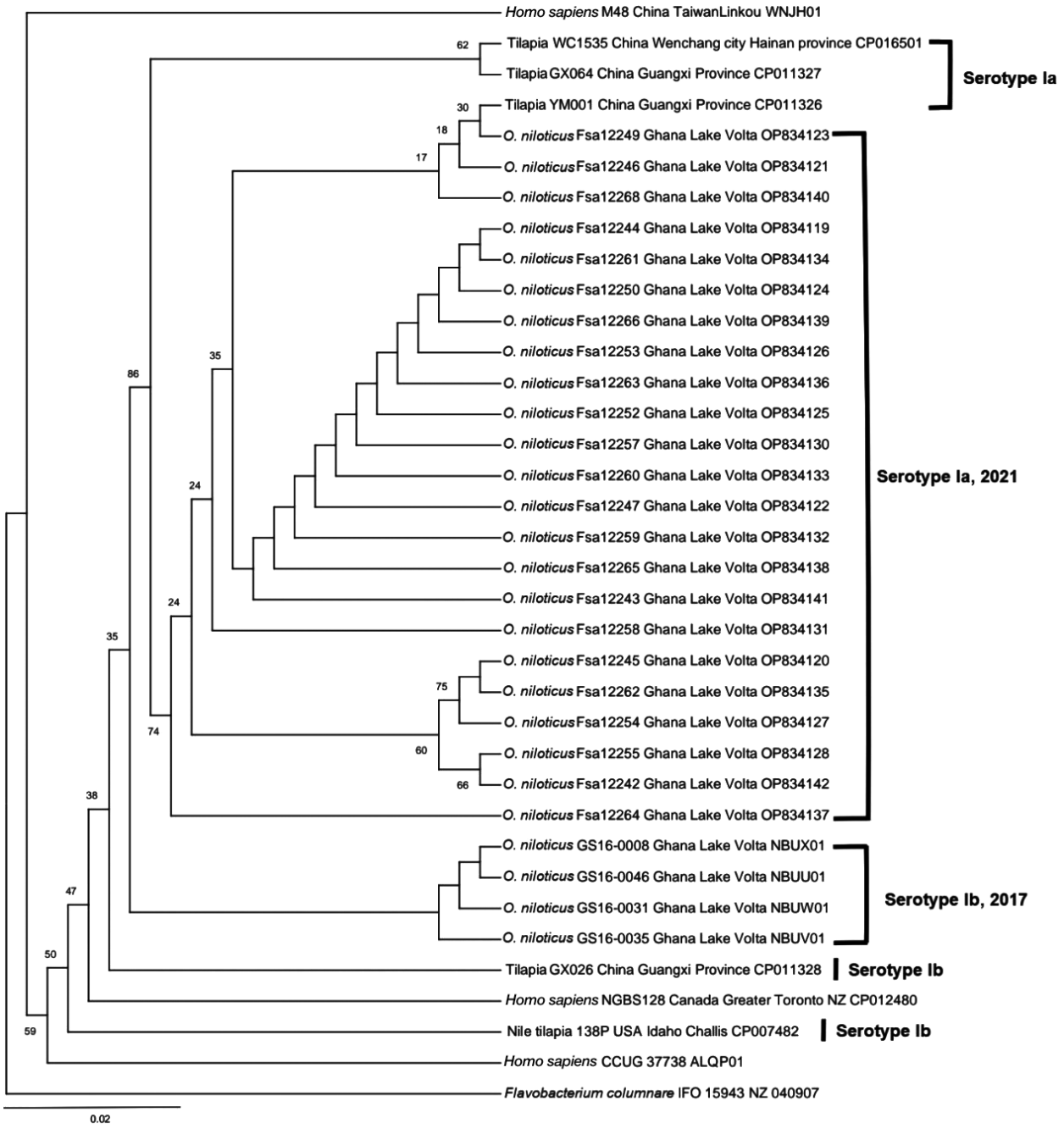

Fig. 2. Phylogenetic tree based on 16S rDNA sequences of *Streptococcus agalactiae* isolates from farmed tilapia in Lake Volta. The tree was inferred by neighbour-joining analysis using Unipro UGENE v.44.0 on fragments of ~500 aligned nucleotide positions. Sequences from this study are labelled as 1a, 2021, corresponding to the serotype and year of isolation. Reference sequences from related studies are labelled as 1b, 2017, corresponding to the serotype and year of isolation (Verner-Jeffreys et al. 2018). Bootstrap values (%) represent 1000 replicates. Accession numbers of all sequences included in the analysis are indicated

For antimicrobial susceptibility testing, the isolates were generally classified based on the CO_{WT} values or using human breakpoints of the targeted antibiotics (Tables 2 & 3). A histogram displaying the distribution of zone sizes using the NRI method is presented in Fig. S1. The distribution of zone sizes for nearly all tested antibiotics was symmetrical around the mean except for CN, where the observations were clustered around the left tail of the distribution curve. The non-normality of the CN observations does not satisfy the NRI assumption of fully susceptible WT strains, and therefore the CO_{WT} for this drug could not be estab-

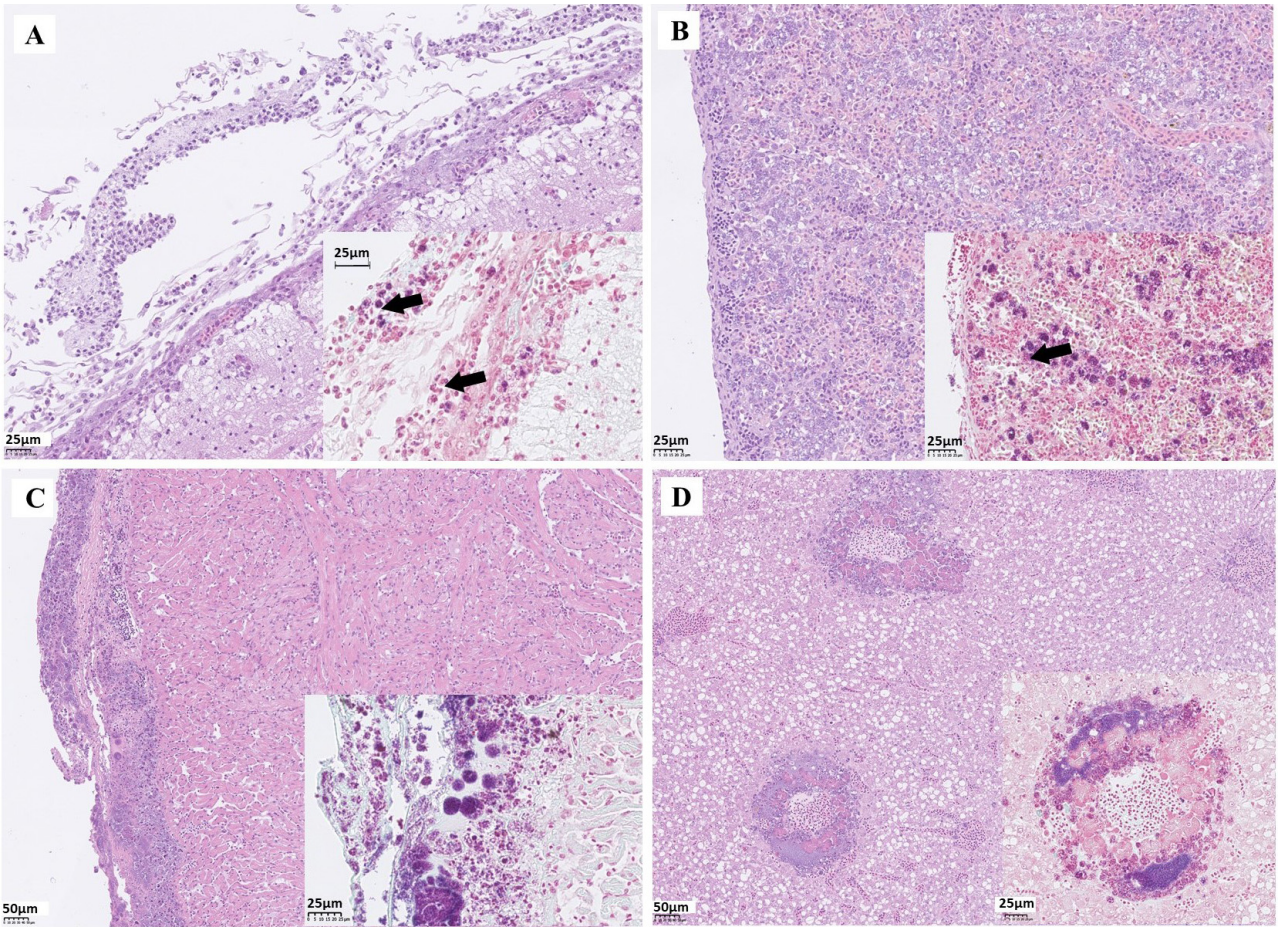

Fig. 3. Histopathological sections of tissues from tilapia *Oreochromis niloticus* that tested positive for *Streptococcus agalactiae* (culture and PCR). All sections were stained with H&E and Gram-Twort. (A) Meningoencephalitis of the brain showing thickened, cell-rich meningi with fibrin deposits and spread occurrence of coccoid bacteria. Note the presence of phagocytosed Gram-positive bacteria in inflammatory cells (inset arrows). (B) Hyperemic spleen tissue with numerous phagocytic cells in the ellipsoids containing myriad intracytoplasmic Gram-positive cocci bacteria (inset arrows). (C) Epicarditis of the heart cardiac ventricle showing inflammatory cells and local fibrin deposits. Note the numerous Gram-positive cocci bacteria in phagocytic cells and as well as those freely present in the extracellular space (inset). (D) Hepatopancreas with multifocal necrotic zones surrounding bacterial infiltrates that are centred on remaining exocrine pancreas tissue. Lipid droplets dominate the hepatocellular cytoplasm, consistent with reduced food intake. Inset shows Gram-positive bacterial colonies and bacteria spreading into the surrounding hepatic parenchyma

lished with accurate precision (Kronvall & Smith 2016). All isolates were categorized as WT for ENR, OXT, FLO and AML (Table 2). For CN, the results were difficult to interpret because the CO_{WT} required to provide the basis for differentiating the WT from the NWT population was not determined. However, the majority of the analysed isolates (n = 25 out of 26, 96%) showed relatively smaller zone sizes in the range of 9–12 mm with only one putative WT isolate (zone diameter: 25 mm) identified (Table 2). Only 3 isolates (11.5%) demonstrated resistance to SXT, with a single isolate showing an intermediate susceptibility phenotype (Table 3). Moreover, all isolates were found to be susceptible to AMP and ERY. Although we did obtain the zone of inhibition values for the QC strain (Table S4), these were not used in the assessment to determine the susceptibility or resistance of the isolates.

Based on the farm interview responses, the use of antibiotics was relatively higher among small-scale farmers compared to medium- and large-scale farms (Fig. 4A). Typically, OXT and AML were the most commonly administered drugs. Aside from antibiotics, other management interventions adopted by farmers for disease control included vaccination (mainly autogenous vaccines), heat-shock treatment, salt bathing, chemicals (hydrogen peroxide, H_2O_2; potassium permanganate, $KMnO_4$), probiotics (e.g. rhodomax), local herbs (cactus, rosemary, bitterleaf,

Table 2. Antibiotic susceptibility pattern of *Streptococcus agalactiae* isolates from tilapia. Disk diffusion zone of inhibition diameter of each isolate was measured after culturing on Muller-Hinton agar supplemented with 5% sheep blood at 28°C for 48 h. WT (wild-type): susceptible strains; NWT (non-wild-type): resistant strains. ENR: enrofloxacin (5 µg); OXT: oxytetracycline (30 µg); CN: gentamicin (30 µg); FLO: florfenicol (30 µg); AML: amoxicillin (10 µg). Note that the designation of most CN values as NWT was not based on the epidemiological cut-off (CO_{WT}) value, but rather on presumed resistance. CO_{WT} values were determined using mean − 2.5 SD. ND: not determined

Isolate sample ID	Antibiotic zone of inhibition (mm) ENR	OXT	CN	FLO	AML
Fsa12242	20 (WT)	25 (WT)	9 (NWT)	26 (WT)	28 (WT)
Fsa12243	25 (WT)	28 (WT)	11 (NWT)	27 (WT)	30 (WT)
Fsa12244	20 (WT)	23 (WT)	10 (NWT)	25 (WT)	28 (WT)
Fsa12245	21 (WT)	28 (WT)	9 (NWT)	25 (WT)	29 (WT)
Fsa12246	23 (WT)	25 (WT)	11 (NWT)	25 (WT)	29 (WT)
Fsa12248	21 (WT)	26 (WT)	10 (NWT)	26 (WT)	30 (WT)
Fsa12249	22 (WT)	24 (WT)	10 (NWT)	25 (WT)	29 (WT)
Fsa12250	25 (WT)	25 (WT)	10 (NWT)	27 (WT)	31 (WT)
Fsa12253	21 (WT)	25 (WT)	10 (NWT)	26 (WT)	28 (WT)
Fsa12254	24 (WT)	31 (WT)	10 (NWT)	28 (WT)	31 (WT)
Fsa12255	20 (WT)	23 (WT)	12 (NWT)	25 (WT)	26 (WT)
Fsa12256	19 (WT)	27 (WT)	9 (NWT)	27 (WT)	30 (WT)
Fsa12257	21 (WT)	26 (WT)	11 (NWT)	26 (WT)	31 (WT)
Fsa12258	22 (WT)	28 (WT)	11 (NWT)	26 (WT)	29 (WT)
Fsa12259	21 (WT)	23 (WT)	9 (NWT)	27 (WT)	30 (WT)
Fsa12260	21 (WT)	26 (WT)	10 (NWT)	26 (WT)	28 (WT)
Fsa12261	25 (WT)	27 (WT)	10 (NWT)	28 (WT)	29 (WT)
Fsa12262	19 (WT)	22 (WT)	10 (NWT)	26 (WT)	29 (WT)
Fsa12263	20 (WT)	22 (WT)	11 (NWT)	26 (WT)	27 (WT)
Fsa12264	20 (WT)	27 (WT)	12 (NWT)	25 (WT)	28 (WT)
Fsa12265	23 (WT)	29 (WT)	10 (NWT)	30 (WT)	31 (WT)
Fsa12266	20 (WT)	25 (WT)	9 (NWT)	21 (WT)	29 (WT)
Fsa12267	21 (WT)	25 (WT)	10 (NWT)	25 (WT)	29 (WT)
Fsa12268	20 (WT)	23 (WT)	10 (NWT)	28 (WT)	29 (WT)
Fsa12269	24 (WT)	31 (WT)	25 (WT)	30 (WT)	30 (WT)
Fsa12270	20 (WT)	21 (WT)	10 (NWT)	27 (WT)	29 (WT)
Epidemiological cut-off values (mm)					
WT mean	21	25	ND	26	29.45
WT SD	2.66	3.34	ND	2.05	1.17
CO_{WT}	14	16	ND	21	26

Table 3. Antibiotic susceptibility pattern of *Streptococcus agalactiae* isolates from tilapia. Disk diffusion zone of inhibition diameter of each isolate was measured after culturing on Muller-Hinton agar supplemented with 5% sheep blood at 28°C for 48 h. Isolates were categorized as resistant (R), intermediate (I) and susceptible (S) based on human breakpoints and interpretive criteria established for *Streptococcus* spp. (CLSI 2020c). AMP: ampicillin (10 µg); SXT: trimethoprim-sulfamethoxazole (25 µg); ERY: erythromycin (15 µg)

Isolate Sample ID	Antibiotic zone of inhibition (mm) AMP	SXT	ERY
Fsa12242	28 (S)	21 (S)	27 (S)
Fsa12243	30 (S)	25 (S)	30 (S)
Fsa12244	28 (S)	21 (S)	25 (S)
Fsa12245	29 (S)	24 (S)	30 (S)
Fsa12246	30 (S)	20 (S)	24 (S)
Fsa12248	31 (S)	21 (S)	30 (S)
Fsa12249	28 (S)	23 (S)	27 (S)
Fsa12250	30 (S)	21 (S)	30 (S)
Fsa12253	28 (S)	0 (R)	27 (S)
Fsa12254	32 (S)	21 (S)	30 (S)
Fsa12255	30 (S)	22 (S)	27 (S)
Fsa12256	31 (S)	18 (I)	29 (S)
Fsa12257	25 (S)	24 (S)	26 (S)
Fsa12258	29 (S)	25 (S)	28 (S)
Fsa12259	33 (S)	0 (R)	26 (S)
Fsa12260	29 (S)	21 (S)	26 (S)
Fsa12261	31 (S)	24 (S)	30 (S)
Fsa12262	30 (S)	21 (S)	27 (S)
Fsa12263	27 (S)	21 (S)	26 (S)
Fsa12264	30 (S)	23 (S)	26 (S)
Fsa12265	29 (S)	19 (S)	31 (S)
Fsa12266	27 (S)	20 (S)	26 (S)
Fsa12267	28 (S)	20 (S)	27 (S)
Fsa12268	29 (S)	24 (S)	27 (S)
Fsa12269	33 (S)	0 (R)	29 (S)
Fsa12270	31 (S)	21 (S)	28 (S)

neem leaves, garlic) and other management practices (less feeding, refluxing, reduced stocking density and removal of dead and diseased fish) (Fig. 4B).

4. DISCUSSION

Streptococcus agalactiae remains a major concern to the global tilapia aquaculture industry and poses potential health risks to workers and consumers. It affects most production systems and can result in high levels of mortality. This bacterium has been previously associated with severe mortality events in farmed tilapia in Ghana (Verner-Jeffreys et al. 2018, Ramírez-Paredes et al. 2021). In addition, it was the predominant bacterial pathogen found in this study. Of the 32 *S. agalactiae* isolates recovered from 300 moribund and diseased fish, 100% were found to be serotype Ia. This was confirmed by 16S rDNA sequencing and PCR amplification of 688 and 272 bp fragments, which corresponded to the *cps* genes *cpsL* and *cpsG*, respectively (Imperi et al. 2010). Surprisingly, no other serotype was detected in this study, even though the sampling coverage included tilapia farms where *S. agalactiae* serotype Ib had been previously identified (Verner-Jeffreys et al. 2018). Serotype Ib is an adapted fish lineage with low growth fitness in culture medium compared to multi-host serotypes such as Ia and III (Leal et al. 2019). Thus, it is possible that the pathogen might still circulate on farms but not have been detected by the limitation of microbiological cultivation, as Ia can grow and overlap

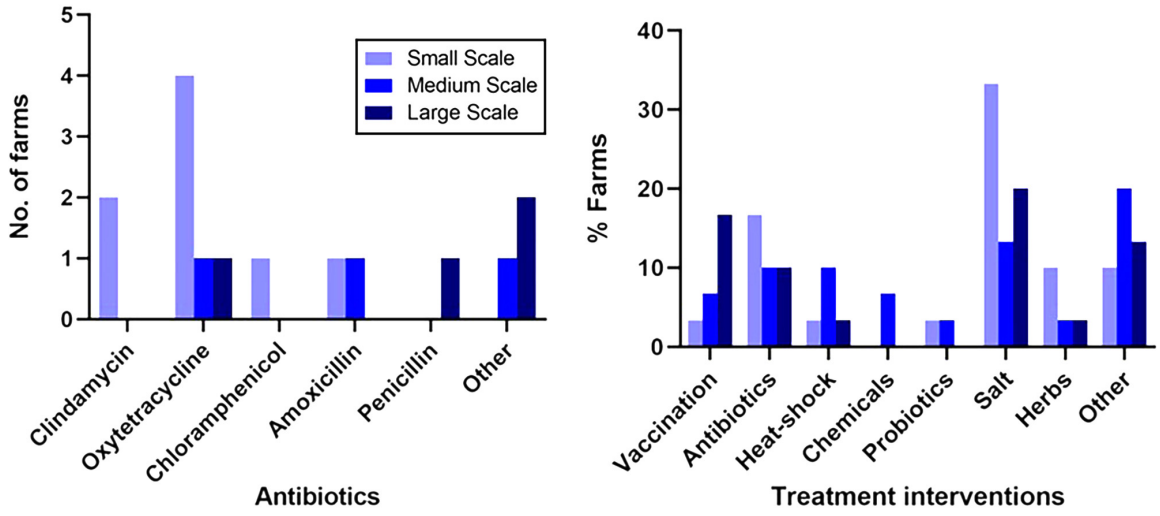

Fig. 4. Farmer interview responses to (A) antibiotic use and (B) specific treatments or interventions adopted at different scales of tilapia farm operation (small, medium and large). Data were generated using a structured questionnaire and by interviews. The 'other' antibiotics include tetracycline, penicillin and amoxiclav; interventions not listed were less feeding, refluxing, reduced stocking density and removal of dead and diseased fish

the growth of Ib. In addition, some farmers are already using a conjugate autogenous vaccine targeting 3 serotypes (type Ia, Ib and III) to treat or manage *Streptococcus* infection in Lake Volta, which might have replaced or controlled the presence of serotype Ib on the farm. The vaccine works best in healthy fish and provides herd immunity at normal growth temperatures of 27–30°C. Although the water temperatures varied across positive farms (23–30°C; Table 1), no outbreaks have been reported from farms that had received autogenous vaccines against streptococcal infection. Nevertheless, it is quite worrisome that antigens against serotype III are included in the vaccine formulation despite its apparent absence in Ghana.

Serotype switching and cross-boundary species transmission may contribute to the emergence of new serotypes within confined geographical areas. Based on the 16S rDNA sequencing data, most of the isolates were closely related to the Chinese tilapia isolates YM001 (GenBank accession no. CPO11326) and GX064 (GenBank accession no. CPO11327), all from the Guangxi Province (Fig. 2). Several reports from Southeast Asian countries including the Philippines have implicated serotype Ia as the most prevalent and widespread cause of streptococcosis in freshwater tilapia aquaculture systems (Lusiastuti et al. 2014, Kannika et al. 2017, Kayansamruaj et al. 2019, Legario et al. 2020, Syuhada et al. 2020). While introduction of infected fish may be the most common mode of *S. agalactiae* transmission, the pathogen can also be transmitted vertically (Pradeep et al. 2016). Its detection in broodstocks in this study highlights the importance of monitoring and controlling potential local spread through the sale and movement of fingerlings from infected hatcheries. Detailed genomic characterization and further epidemiological information may be required to support tracing of the origin of *S. agalactiae* Ia in Ghanaian tilapia cultures.

The gross pathology of *S. agalactiae*-infected fish in this study revealed many features typical of septicemic disease (Zamri-Saad et al. 2010, Li et al. 2014). Meningoencephalitis was evident, with fish displaying thickened meninges, infiltration of a large number of mixed inflammatory cells and spread occurrence of coccoid bacteria, consistent with previous histological studies on streptococcal infections in tilapia (Ferguson 2006, Chen et al. 2007). These pathological changes may reflect the highly invasive nature of *S. agalactiae* serotype Ia and its ability to cause acute infection as also suggested by other investigators (Legario et al. 2020). Indeed, all *S. agalactiae* isolates recovered from infected fish in this study harboured the *fbsA*, *cfb* and *cspA* genes with a 100% positivity rate. These genes encode for major virulence factors known to play significant roles in the pathogenesis of streptococcal infection (Lin et al. 2011). We found most of the isolates (87.5%) to be β-hemolytic. Although non-haemolytic *S. agalactiae* strains can be extremely virulent (Mian et al. 2009), there are cases where β-haemolytic serotype Ia strains were associated with higher mortality rates than strains showing no haemolysis (Sudpraseart et al. 2021). As previously reported from Ghana (Verner-Jeffreys et al. 2018, Ramírez-Paredes et al. 2021), *S. agalactiae* co-infection

with other fish pathogens was also evident in this study (Table S2). However, the extent to which such polymicrobial infections may influence the pathogenesis of *S. agalactiae* is unknown and therefore worth further investigation.

Interestingly, high susceptibility to antimicrobials was generally recorded in this study. This could be expected, given that *S. agalactiae* was highly prevalent in large-scale farms where antibiotic usage was relatively low (Fig. 4A). A recent report has shown that drug susceptibility profiles of fish GBS isolates are influenced by their serotype (Leal et al. 2023). Serotype Ia and Ib isolates are largely antibiotic-sensitive compared to serotype III (Sapugahawatte et al. 2022, Leal et al. 2023). *S. agalactiae* CN susceptibility in tilapia has been reported with mixed results. While some reported higher resistance in consistent with the present findings (Laith et al. 2017, Alazab et al. 2022), other studies found lower levels of resistance (Abuseliana et al. 2010, Deng et al. 2019). These differences could also be attributed to serotype variability or to the environmental conditions and frequency of antimicrobial use in different fish culture systems. Although GBS strains may possess intrinsic resistance to aminoglycosides, resistance to CN can be transferred both chromosomally and extrachromosomally due to horizontal acquisition of transposons or plasmids (Sendi et al. 2016). Thus, the presence of high-level CN resistance in *S. agalactiae* is of great concern since it could spread to other aquatic organisms as well as to humans and prevent or delay effective therapy (Creti et al. 2022). In Ghana, FLO is the only approved drug for use in aquaculture. As indicated in this study, none of the isolates were resistant to this antibiotic, which has been proven to be effective and relatively safe to use (Kosoff et al. 2009). Currently, farmers have restricted access to FLO, and the drug is only prescribed by a few certified fish health veterinarians. Nevertheless, the recent emergence of FLO-resistant GBS strains in Brazilian fish farms (Leal et al. 2023) should be a caution to guide future strategic deployment of the antibiotic in Ghanaian aquaculture.

It is generally recommended that susceptibility testing should be performed under standard conditions with the appropriate QC requirements (Watts et al. 2018). Yet no acceptable QC ranges have been established for testing streptococcal fish pathogens at 28°C (CLSI 2020a,b). Given the reproducible zone diameter readings obtained for *S. pneumoniae* ATCC 49619 in this study, variations with the test performance are expected to be minimal. Thus, the data generated could be expanded for use in future QC testing of streptococcal aquatic pathogens. Nevertheless, the QC data presented should be treated as 'local' and only be applied to the test performed in the present study.

Overall, our data has provided evidence to show that *S. agalactiae* serotype Ia might be the predominant circulating serotype in cultured tilapia in Lake Volta, thereby expanding the global spread of this pathogen in aquaculture. This is critical baseline information for targeted autogenous vaccine development and deployment plans. Moreover, appropriate surveillance systems are required to identify the transmission routes of the pathogen and to monitor possible serotype replacement or the emergence of new variants. This should inform effective vaccine and therapeutic intervention strategies for streptococcal disease management in Ghanaian tilapia aquaculture systems.

Acknowledgements. We thank all the participating farmers and the Fisheries Commission, Ghana for their consent and cooperation in the field sampling. We are extremely grateful to Duncan Colquhoun, Raoul Valentin Kuiper, Toni Erkinharju, Geir Bornø, Attila Tarpai, Tone Mathisen Fagereng and Hannah Segbefia for their support with the laboratory work and data analysis. The financial support by NORAD through the Fish for Development Ghana project (gha-17/0005) and WACCBIP, University of Ghana, which receives funding from World Bank African Centre of Excellence (WACCBIP + NCDs, Awandare) is duly acknowledged.

LITERATURE CITED

Abuseliana A, Daud H, Aziz SA, Bejo SK, Alsaid M (2010) *Streptococcus agalactiae* the etiological agent of mass mortality in farmed red tilapia (*Oreochromis* sp.). J Anim Vet Adv 9:2640–2646

Alazab A, Sadat A, Younis G (2022) Prevalence, antimicrobial susceptibility, and genotyping of *Streptococcus agalactiae* in tilapia fish (*Oreochromis niloticus*) in Egypt. J Adv Vet Anim Res 9:95–103

Chen CY, Chao CB, Bowser PR (2007) Comparative histopathology of *Streptococcus iniae* and *Streptococcus agalactiae*-infected tilapia. Bull Eur Assoc Fish Pathol 27:2–9

Chideroli RT, Amoroso N, Mainardi RM, Suphoronski SA and others (2017) Emergence of a new multidrug-resistant and highly virulent serotype of *Streptococcus agalactiae* in fish farms from Brazil. Aquaculture 479:45–51

Cieslewicz MJ, Chaffin D, Glusman G, Kasper D and others (2005) Structural and genetic diversity of group B *Streptococcus* capsular polysaccharides. Infect Immun 73: 3096–3103

CLSI (Clinical and Laboratory Standards Institute) (2020a) Performance standards for antimicrobial susceptibility testing of bacteria isolated from aquatic animals, 3rd edn. CLSI supplement VET03. Clinical and Laboratory Standards Institute, Wyne, PA

CLSI (2020b) Performance standards for antimicrobial susceptibility testing of bacteria isolated from aquatic animals, 3rd edn. CLSI supplement VET04. Clinical and Laboratory Standards Institute, Wyne, PA

CLSI (2020c) Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from

animals, 5th edn. CLSI supplement VET01S. Clinical and Laboratory Standards Institute, Wyne, PA
Creti R, Imperi M, Berardi A, Angeletti S, Gherardi G (2022) Laboratory breakpoints for assessing high level gentamicin resistance in *Streptococcus agalactiae*: it is the time for a consensus. Clin Microbiol Infect 28:1050–1052
Delannoy CM, Crumlish M, Fontaine MC, Pollock J and others (2013) Human *Streptococcus agalactiae* strains in aquatic mammals and fish. BMC Microbiol 13:41
Deng L, Li Y, Geng Y, Zheng L and others (2019) Molecular serotyping and antimicrobial susceptibility of *Streptococcus agalactiae* isolated from fish in China. Aquaculture 510:84–89
Ferguson HW (ed) (2006) Systemic pathology of fish: a text and atlas of normal tissues in teleosts and their responses in disease, 2nd edn. Scotian Press, London
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.* J Microbiol Methods 80:212–214
Kannika K, Pisuttharachai D, Srisapoome P, Wongtavatchai J and others (2017) Molecular serotyping, virulence gene profiling and pathogenicity of *Streptococcus agalactiae* isolated from tilapia farms in Thailand by multiplex PCR. J Appl Microbiol 122:1497–1507
Kayansamruaj P, Pirarat N, Katagiri T, Hirono I, Rodkhum C (2014) Molecular characterization and virulence gene profiling of pathogenic *Streptococcus agalactiae* populations from tilapia (*Oreochromis* sp.) farms in Thailand. J Vet Diagn Invest 26:488–495
Kayansamruaj P, Soontara C, Unajak S, Dong HT and others (2019) Comparative genomics inferred two distinct populations of piscine pathogenic *Streptococcus agalactiae*, serotype Ia ST7 and serotype III ST283, in Thailand and Vietnam. Genomics 111:1657–1667
Kosoff RE, Chen CY, Wooster GA, Getchell RG and others (2009) Florfenicol residues in three species of fish after 10-day oral dosing in feed. J Aquat Anim Health 21:8–13
Kronvall G, Smith P (2016) Normalized resistance interpretation, the NRI method: review of NRI disc test applications and guide to calculations. APMIS 124:1023–1030
Laith AA, Ambak MA, Hassan M, Sheriff SM and others (2017) Molecular identification and histopathological study of natural *Streptococcus agalactiae* infection in hybrid tilapia (*Oreochromis niloticus*). Vet World 10: 101–111
Leal CAG, Queiroz GA, Pereira FL, Tavares GC, Figueiredo HCP (2019) *Streptococcus agalactiae* sequence type 283 in farmed fish, Brazil. Emerg Infect Dis 25:776–779
Leal CAG, Silva BA, Colombo SA (2023) Susceptibility profile and epidemiological cut-off values are influenced by serotype in fish pathogenic *Streptococcus agalactiae*. Antibiotics (Basel) 12:1726
Legario FS, Casiano H, Turnbull CJF, Crumlish M (2020) Isolation and molecular characterization of streptococcal species recovered from clinical infections in farmed Nile tilapia (*Oreochromis niloticus*) in the Philippines. J Fish Dis 43:1431–1442
Li YW, Liu L, Wang PR, Luo ZP, Peng HL, Wang YX, Li AX (2014) Chronic streptococcosis in Nile tilapia, *Oreochromis niloticus* (L.), caused by *Streptococcus agalactiae.* J Fish Dis 37:757–763
Lin FP, Lan R, Sintchenko V, Gilbert GL, Kong F, Coiera E (2011) Computational bacterial genome-wide analysis of phylogenetic profiles reveals potential virulence genes of *Streptococcus agalactiae.* PLOS ONE 6:e17964
Lusiastuti AM, 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. Aquacult Res 45: 1260–1263
Mian GF, Godoy DT, Leal CA, Yuhara TY, Costa GM, Figueiredo HC (2009) Aspects of the natural history and virulence of *S. agalactiae* infection in Nile tilapia. Vet Microbiol 136:180–183
Pradeep PJ, Suebsing R, Sirthammajak S, Kampeera J and others (2016) Evidence of vertical transmission and tissue tropism of *Streptococcosis* from naturally infected red tilapia (*Oreochromis* spp.). Aquacult Rep 3:58–66
Ramírez-Paredes JG, Paley RK, Hunt W, Feist SW and others (2021) First detection of infectious spleen and kidney necrosis virus (ISKNV) associated with massive mortalities in farmed tilapia in Africa. Transbound Emerg Dis 68: 1550–1563
Sapugahawatte DN, Li C, Dharmaratne P, Zhu C and others (2022) Prevalence and characteristics of *Streptococcus agalactiae* from freshwater fish and pork in Hong Kong wet markets. Antibiotics (Basel) 11:397
Sendi P, Furitsch M, Mauerer S, Florindo C and others (2016) Chromosomally and extrachromosomally mediated high-level gentamicin resistance in *Streptococcus agalactiae.* Antimicrob Agents Chemother 60:1702–1707
Sheehan B, Labrie L, Lee YS, Wong FS and others (2009) Streptococcosis in tilapia — vaccination effective against main strep species. Glob Aquacult Advocate 5:72–74
Slotved HC, Kong F, Lambertsen L, Sauer S, Gilbert GL (2007) Serotype IX, a proposed new *Streptococcus agalactiae* serotype. J Clin Microbiol 45:2929–2936
Smith P (2020) Eight rules for improving the quality of papers on the antimicrobial susceptibility of bacteria isolated from aquatic animals. Dis Aquat Org 139:87–92
Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, Doré J (1999) Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl Environ Microbiol 65:4799–4807
Sudpraseart C, Wang PC, Chen SC (2021) Phenotype, genotype and pathogenicity of *Streptococcus agalactiae* isolated from cultured tilapia (*Oreochromis* spp.) in Taiwan. J Fish Dis 44:747–756
Suvarna SK, Layton C, Bancroft JD (2019) Bancroft's theory and practice of histological techniques, 8th edn. Elsevier, Amsterdam
Syuhada R, Zamri-Saad M, Ina-Salwanay MY, Mustafa M and others (2020) Molecular characterization and pathogenicity of *Streptococcus agalactiae* serotypes Ia ST7 and III ST283 isolated from cultured red hybrid tilapia in Malaysia. Aquaculture 515:734543
Tiruvayipati S, Tang WY, Barkham TMS, Chen SL (2021) GBS-SBG — GBS serotyping by genome sequencing. Microb Genom 7:000688
Verner-Jeffreys DW, Wallis TJ, Cejas IC, Ryder D and others (2018) *Streptococcus agalactiae* multi locus sequence type 261 is associated with mortalities in the emerging Ghanaian tilapia industry. J Fish Dis 41:175–179
Watts JL, Sweeney MT, Lubbers BV (2018) Antimicrobial susceptibility testing of bacteria of veterinary origin. Microbiol Spectr 6:1–16
Zamri-Saad M, Amal MNA, Siti-Zahrah A (2010) Pathological changes in red tilapias (*Oreochromis* spp.) naturally infected by *Streptococcus agalactiae.* J Comp Pathol 143: 227–229

Editorial responsibility: Alicia Toranzo, Santiago de Compostela, Spain
Reviewed by: C. A. Gomes Leal and P. Barato

Submitted: July 12, 2023
Accepted: February 8, 2024
Proofs received from author(s): April 9, 2024