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Research article

Outbreaks of streptococcosis associated with *Streptococcus agalactiae* in farmed climbing perch *(Anabas testudineus)* in Vietnam

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Abstract

Streptococcus agalactiae causes serious economic damage to fish cultivation worldwide. The goal of this work was to isolate, identify, and evaluate the pathogenicity and susceptibility of the streptococci isolates to antibiotics recovered from climbing perch *(A. testudineus)* farmed in the southern part of Vietnam. Moribund and healthy fish were taken differently from intensive climbing perch farms. The diseased fish's clinical signs were observed, including darkened body color, ascites, hepatomegaly, an enlarged kidney, and splenomegaly. Based on the conventional method, the API 20 Strep test, and 16S rRNA gene partial sequencing, *S. agalactiae* was associated with disease. They have non β-hemolytic activity, gram-positive cocci, catalase, and oxidase-negative enzymes. The LD50 trial performance of *S. agalactiae* showed the virulence of this bacteria in climbing perch and fulfilled Koch's postulates with a value of 8.71 x 104 CFU/mL at day 7. Most of the challenged fish presented the same clinical signs as the natural infection. Hence, *S. agalactiae* were confirmed as the causative agents of the "dark body" disease. Antibiogram results demonstrated that *S. agalactiae* strains were completely susceptible to cefotaxime, doxycycline, and florfenicol. Interestingly, the results of this study found that *S. agalactiae* isolates are 100% resistant to sulfamethoxazole-trimethoprim. To the best of our knowledge, this is the first report of *S. agalactiae* as a pathogen of climbing perch.

Keywords: Antibiogram, Climbing perch, Dark body, LD_{50}, *Streptococcus agalactiae*

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INTRODUCTION

Since the first report of streptococcosis in cultured rainbow trout (*Oncorhynchus mykiss*) in Japan (Hoshina et al., 1958), these gram-positive cocci have caused large-scale epizootics throughout the world in a variety of fish species, particularly in cultivated warm-water fish (Romalde et al., 2008; El Aamri et al., 2010). Different species of the genus *Streptococcus* have been reported causing streptococcal infections, including *S. iniae* (Creeper and Buller, 2006), *S. dysgalactiae* (Yang and Li, 2009), *S. agalactiae* (Siti-Zahrah et al., 2008), *S. ictaluri* (Camus et al., 2008), *S. milleri* (Yanong and Francis-Floyd, 2006), *S. parauberis* (Doménech et al., 1996), and *S. phocae* (Romalde et al., 2008). Among these, however, *S. agalactiae* and *S. iniae* injections have increasingly been recognized as the two most serious pathogens to many marine and freshwater fish (Agnew and Barnes, 2007; Mian et al., 2009; Noga, 2010; Zamri-Saad et al., 2010; Geng et al., 2012), with globally estimated economic losses of more than US$150 million (Romalde et al., 2008).

The first isolation of *S. iniae* was reported from skin lesions of an Amazon freshwater dolphin (*Inia geoffrensis*) (Pier and Madin, 1976). Over the past few decades, the bacterium has become one of the most serious aquatic pathogens, with annual economic losses estimated in global aquaculture of over US$ 100 million (Shoemaker et al., 2001). At least 10 countries and 27 species of fish have been documented to have been affected by *S. iniae* (Agnew and Barnes, 2007; Noga, 2010). Likewise, Lancefield group B, *S. agalactiae,* recognized as a mammalian pathogen, has become common in aquaculture. This species has a broad range of hosts and has been shown to cause outbreaks in a variety of freshwater and saltwater fish species in Malaysia, China, Brazil, the USA, and Kuwait (Evans et al., 2002; Duremdez et al., 2004; Pasnik et al., 2009; Mian et al., 2009; Zamri-Saad et al., 2010; Geng et al., 2012). Both *Streptococcus* species have also been reported to be zoonotic pathogens in humans, mammalian animals, such as mice, cattle, dolphins, cats, and dogs, and poikilothermic animals, including frogs (Elliott et al., 1990; Lau et al., 2003; Facklam et al., 2005; Zappulli et al., 2005; Pereira et al., 2010).

Climbing perch (*A. testudineus*), a species of ray-finned fish, is indigenous to Vietnam. This is a widely distributed fish that can live in brackish bodies (Noinumsai et al., 2021). They are distributed to many countries in the world, such as Australia, India, China, the Philippines, Thailand, Laos, Cambodia, and many other Asian countries (Talwar and Jhingran, 1991). In Vietnam, climbing perch are being farmed in some provinces in the north and south. In the Mekong Delta, especially, fish are raised a lot in Hau Giang, Can Tho, Dong Thap, and Tien Giang provinces due to their high economic value, delicious meat quality, and few bones, which are suitable to the tastes of the majority of consumers (Alam et al., 2006). Additionally, this fish species is easy to raise, can be raised at a high stocking density, and is well adapted to the harsh conditions of the environment (Noinumsai et al., 2021). Intensive farming with high density along with environmental factors in ponds is the cause of many diseases, especially bacterial diseases (Haenen et al., 2023; Ndashe et al., 2023).

To date, climbing perch infected with many bacterial species, including *Aeromonas* spp., *Staphylococcus* spp., *Streptococcus* spp., *Salmonella* spp.,

and *Pseudomonas*, have been recorded in the world (Hossain et al., 2017; Chhanda et al., 2019). Ehsan et al. (2023) reported that *Aeromonas veronii was* obtained from *A. testudineus* affected by epizootic ulcerative syndrome (EUS). Another study by Mazumder et al. (2021) revealed that two virulent *Aeromonads* (*A. hydrophila* and *A. jandaei) were* associated with hemorrhagic septicemia and tail-rot disease in the farmed climbing perch *A. testudineus*. In the Mekong Delta and other aquaculture areas of Vietnam, a disease that has recently appeared in common climbing perch, known by fish farmers as the "dark body" disease, has caused losses of up to 50% (Dung et al., 2013). The etiological agent of this disease was determined to be the bacterium *S. iniae* (Dung et al., 2013). Previous research by Thinh et al. (2013) also showed the presence of *S. agalactiae* in black-body climbing perch. However, there is still little information about this bacterium in *A. testudineus* so far. Hence, the goal of this work was to isolate, identify, and evaluate the pathogenicity as well as susceptibility of *S. agalactiae* isolates to antibiotics recovered from climbing perch farmed in the southern part of Vietnam.

MATERIALS AND METHODS

Ethical approval

The study protocol was approved by Regulations on Ethical Management in Animal Experiments (Decision No. 3965/QĐ-ĐHCT, October 15, 2021) of Can Tho University.

Fish sampling

Moribund and diseased fish with dark body signs were collected from 31 climbing perch intensive farms located in the southern provinces of Vietnam: Hau Giang, Can Tho, Tien Giang, and Dong Nai (Figure 1) during natural epidemics starting from January to September of the years 2021 and 2022. A total of 246 fish samples, including 210 diseased fish samples and 36 healthy fish samples as a control group with body weights ranging from 6 to 200 g, were sampled from the affected ponds. During collection, external symptoms, such as darkening of body color, listless swimming, anorexia and lethargy, ascites, bilateral or unilateral exophthalmia, clouding of the eyes, and hemorrhaged eyes, were observed (Figure 2). For bacterial isolation, the sick fish were assembled into zipper plastic bags and delivered to the laboratory.

Figure 1 Different sampling sites (light green circle) for *S. agalactiae* isolation.

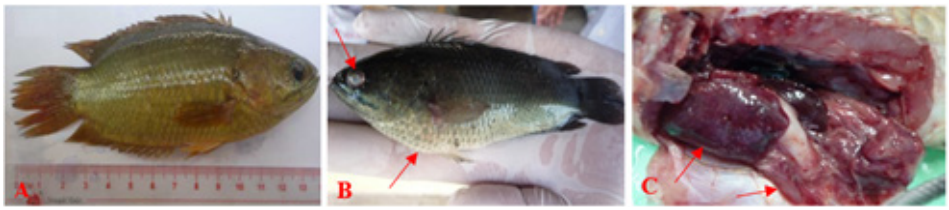

Figure 2 Clinical signs of "dark body" disease climbing perch.
A. Healthy climbing perch; B. Natural infected climbing perch showing "dark body", corneal opacity, and distended or swollen abdomen (arrow); C. Hypertrophy of liver and hemoperitoneum (arrow).

Isolation of S. agalactiae

Liver, kidney, spleen, brain, and blood samples were collected to isolate *S. agalactiae* from sick fish by post-mortem. The bacterial isolation was processed according to Frerichs and Millar (1993). Briefly, swabs of the liver, kidney, brain, and blood of each fish were taken aseptically, and streaked onto both 5% sheep blood agar (BA, Merck, Germany) and brain heart infusion agar (BHIA, Merck, Germany). The plates were then incubated at 28°C for 24–48 hours for the observation of colony morphology. The inspected colonies (white, round, and opaque) were subcultured on the same agar plates. Finally, pure isolates were kept at -80°C in BHI broth with 15% glycerol (v/v) for further studies.

Morphological, physiological, and biochemical characteristics

Bacteria grown on BA medium were checked for basic morphological, physiological, and biochemical properties such as Gram staining, mobility, oxidase, catalase, and the oxidation/fermentation (O/F) reaction. The ability of the isolates to grow on medium supplemented with 6.5% NaCl at pH 9.6 and 60°C was tested in BHI medium. Haemolytic activity testing was performed by growing bacteria in Blood Agar Base media supplemented with 5% (v/v) sheep blood and incubating for 18–24 hours at 37ºC. These features were performed as per the manuals of Frerichs and Millar (1993), and Buller (2004). Finally,

the API 20 Strep Kit (bioMerieux, France) was used to identify *S. agalactiae* according to the manufacturer's instructions.

Bacterial identification by PCR and gene sequencing

For the molecular identification, representative *S. agalactiae* isolates were used for the PCR reaction. Bacterial DNA was extracted as per Moore et al. (2004), with some modifications. In brief, bacteria were cultured in BHI broth for 24 hours at 28°C with agitation at 100 rpm. Cell suspensions were centrifuged at 4.500 rpm for 5 minutes at 4°C, and the obtained pellets were resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The suspension was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 15,000 rpm for 5 minutes. The DNA-containing upper aqueous phase was transferred into a separate 2 mL Eppendorf tube, and 0.7 volumes of isopropanol were added. The aqueous phase was recovered by centrifugation for 20 minutes, and genomic DNA was precipitated by ethanol. The extracted DNA was checked for purity and concentration at 260 and 280 nm using a spectrophotometer.

The specific primers F1: 5'-GAGTTTGATCATGGCTCAG-3' and IMOD: 5'-ACCAACATGTGTTAATTACTC-3' for *S. agalactiae* are expected to yield approximately 220 bp of the 16S rRNA gene (Martinez *et al.*, 2001). PCR reactions were performed with template DNA, 10X buffer, 1.5 mM MgCl2, 200 µM dNTPs, 2U Taq DNA polymerase, and 0.4 µM each of primers F1 and IMOD. The PCR performance of *S. agalactiae* was a cycle of pre-denaturation at 94°C for 4 minutes, 35 cycles of denaturation at 94°C for 1 minute, 35 cycles of annealing at 55°C for 1 minute, 35 cycles of extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The electrophoresis for the analysis of the amplification products was run with 2% agarose gel in TAE 1X. The electrophoresis result was read and photographed using BioRad UV 2000.

Challenge test

S. agalactiae S6FC3 isolate was chosen to perform the pathogenicity test. After being enriched red in BHI broth at 28°C for 18–24 hours, the culture was then centrifuged at 6.000 rpm at 4°C for 10 minutes. The bacterial concentration in sterile BHI broth was adjusted to an optical density (OD) of 1±0.02 at 620 nm, equivalent to 10^8 CFU/mL. The dilution method was used to reach the target of final concentrations of 10^3, 10^4, 10^5, and 10^6 CFU/mL for the challenge test.

Healthy fingerlings of climbing perch with an average weight of 6–10 g for the experiment were selected from various commercial farms in the Mekong Delta, Vietnam. The fish were acclimatized to experimental conditions for 7–14 days. Ten fingerlings were sacrificed randomly to examine the presence of any pathogens. For each treatment, ten fish were employed in three replications. Each fish received a 0.1 mL intraperitoneal injection of *S. agalactiae* S6FC3 suspension. An identical volume of sterile normal saline (0.85% NaCl) was injected into the control groups. Fish mortality and clinical signs were recorded daily until there were no deaths for 2 weeks. Newly dead or moribund fish were examined for disease signs and re-isolated on BA plates from the brain, liver, spleen, kidney, and blood. The median lethal dose (LD_{50}) was determined according to the formula of Reed and Muench (1938).

Antibiotic susceptibility assays

The susceptibility of *S. agalactiae* isolates to antibiotics was determined according to Bauer et al. (1966). The following antibiotics were tested: amoxycillin-clavulanic acid (25 µg), cefalexin (30 µg), cefotaxime (30 µg), colistin sulfate (5 µg), doxycycline (30 µg), erythromycin (5 µg), florfenicol (30 µg), flumequine (30 µg), gentamicin (30 µg), rifampicin (30 µg), streptomycin (10 µg), and sulfamethoxazole-trimethoprim (23,75/1,25 µg) (Oxoid, UK). Briefly, pure colonies are inoculated into 5 mL of BHI broth. The suspensions were then adjusted to 10^8 CFU/mL by comparison to the McFarland standard. The suspensions were spread on Mueller-Hinton Agar (MHA, Merck, Germany) containing 5% defibrinated sheep blood. *Escherichia coli* (ATCC 25922) was tested as quality control. Finally, the susceptibility and resistance of isolates were then determined according to the manufacturer's instructions and the criteria of the Clinical and Laboratory Standards Institute (CLSI, 2020).

Data analysis

Descriptive statistics were used to determine antimicrobial resistance and cumulative mortality. The BLASTn tool was used to compare the sequence similarity of bacterial strains with sequences in the NCBI database (National Center for Biotechnology Information). Using MEGA6 (Molecular Evolutionary Genetics Analysis) software and the neighbor-joining algorithm (Saitou and Nei, 1987), the phylogenetic tree illustrating the genetic links between bacterial strains was created (Tamura, 2013).

RESULTS

Clinical signs and post mortem

This study discovered a variation in the external signs observed in naturally affected fish. Some diseased climbing perches expose ascites, bilateral or unilateral exophthalmia, and clouding of the eyes (Figure 2). Meanwhile, others showed hemorrhaged eyes, hemorrhages, and lesions on the pectoral fins and anus. However, darkening of body color, listless swimming, anorexia, and lethargy were observed in all infected climbing perch. Internally, hemoperitoneum, hepatomegaly, splenomegaly, and an enlarged kidney were observed (Figure 2). During the outbreaks, cumulative mortality often reached 20–50%.

Bacterial isolation

A total of 30 bacterial isolates were collected from 210 samples of "dark body" climbing perch from Hau Giang, Can Tho, Tien Giang, and Dong Nai provinces (Table 1). Among them, 9/30 (30%) isolates originated from Hau Giang, 7/30 (23.33%) isolates from Can Tho, 8/30 (26.67%) from Tien Giang, and 6/30 (20%) isolates from Dong Nai province (Table 1). In general, bacteria were isolated on BA and BHIA nutrient agar from diseased fish internal organs, including the spleen, blood, liver, kidney, and brain.

Table 1 Isolation results of bacterial isolates from "dark body" disease climbing perch.

Sampling sites	Number of diseased fish	Number of *S. agalactiae isolates*
Hau Giang	62	9
Can Tho	53	7
Tien Giang	48	8
Dong Nai	47	6
Total	**210**	**30**

Morphological, physiological, and biochemical characteristics

Colonies obtained from the brain, liver, spleen, kidney, and blood of diseased fish grew well on BHIA and BA medium after incubation at 28°C for 24–48 hours. The colonies of isolated strains were 0.8–1 mm in diameter, white, round, and opaque (Figure 3A). Microscopic examination of Gram-stained smears from growth cultures and blood smears of affected fish revealed the presence of Gram-positive cocci in chains or pairs (Figures 3B and C). All isolates were catalase, oxidase, and O/F negative, and were able to grow in 6.5% NaCl but failed to survive at 60°C and pH 9.6 (Table 2). Furthermore, hemolytic examination showed that all isolates were non β-haemolytic (Figure 3D).

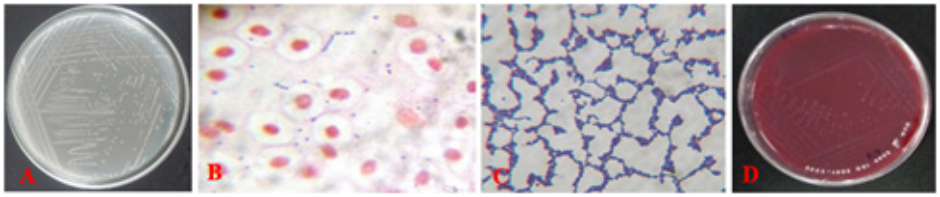

Figure 3 Biochemical characteristics of *S. agalactiae* isolates that originated from the "dark body" disease climbing perch.
A. The colony of *S. agalactiae* grows on BHIA; B. *S. agalactiae* is observed in the blood of infected climbing perch; C. Staining shows gram-positive; D. Non-hemolysis of *S. agalactiae* on BA plates.

The results from the API 20 Strep Kit revealed 90% S6FC3 isolate similarity to *S. agalactiae*. This isolate exhibited positive results for VP (Voges-Proskauer), HIP (hydrolysis of hippurate), βGUR (β-glucuronidase), ALP (alkaline phosphatase), LAP (leucine aminopeptidase), and ADH (arginine dihydrolase), the rest were negative, including ESC (esculin), PYRA (pyrrolidonyl arylamidase), GAL α (α-galactosidase), GAL β (β-galactosidase), RIB (D-ribose), ARA (L-arabinose), MAN (D-mannitol), SOR (D-sorbitol), LAC (D-lactose), TRE (D-trehalose), INU (inulin), RAF (D-raffinose), AMD (starch), and GLYG (glycogen) (Table 2).

Table 2 Morphological, physiological, and biochemical features of the *S. agalactiae* S6FC3 isolate.

Reaction	*S. agalactiae* S6FC3 isolate	*S. agalactiae**
Gram stain	+	+
Cell morphology	cocci, chain	cocci, chain
Colony diameter	0.28 mm	
Growth day	48 hours	
Hemolysis	Non-haemolytic	Non-haemolytic
Catalase	-	-
Oxidase	-	-
O/F	-/-	
Motility	-	-
Growth in 6.5% NaCl	+	+
Growth at pH 9.6	-	-
Growth at 10°C	-	-
Growth at 45°C	-	-
Voges–Proskauer	+	+
Hydrolysis of Hippurate	+	+
Esculin	-	-
Pyrrolidonyl arylamidase	-	-
α-galactosidase	-	-
β-glucuronidase	+	-
β-galactosidase	-	-
Alkaline phosphatase	+	+
Leucine aminopeptidase	+	+
Arginine dihydrolase	+	+
D-ribose	-	+
L-arabinose	-	-
D-mannitol	-	-
D-sorbitol	-	-
D-lactose	-	-
D-trehalose	-	+
Inulin	-	-
D-raffinose	-	-
Starch	-	-
Glycogen	-	-

Notes: (+) positive reaction; (-) negative reaction; * Amal et al. (2012) and Niu et al. (2020)

Identification of S. agalactiae by PCR

The PCR result showed that the tested bacterial isolates were positive for a DNA band at the 220 bp position (Figure 4). The Blast results showed that the bacterial strain *S. agalactiae* S6FC3 isolate had 100% homology to *S. agalactiae* strain G_35 (MT626753.1), *S. agalactiae* strain G_27 (MT626748.1), *S. agalactiae* strain Israel1 (MK517599.1), and *S. agalactiae* strain ATCC 51487 (KU561093.1).

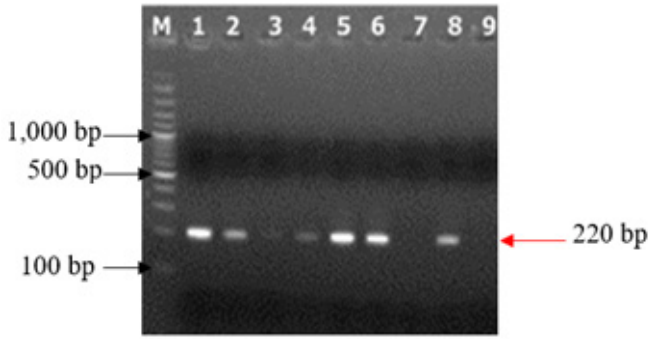

Figure 4 Detection of *S. agalactiae* isolates by PCR reaction.
M: 100 bp ladder; Lane 1–8: isolates S6FC3, R21, R27, R39, R59G, R44, and R62M, respectively; Lane 9: Negative control.

Based on the 16S rDNA sequences of the S6FC3 isolate and the homologous sequences of other strains of *S. agalactiae* and *S. iniae* (outgroup) in the GenBank, a phylogenetic tree was constructed (Figure 5). In this study, the S6FC3 strains investigated, together with *S. agalactiae* (MT626753.1), *S. agalactiae* (MT626748.1), *S. agalactiae* (MK517599.1), and *S. agalactiae* (KU561093.1), formed a closed cluster (Figure 5). To sum up, the S6FC3 strain was determined to be *S. agalactiae* based on morphology, API 20 Strep tests, and 16S rRNA sequence analysis results.

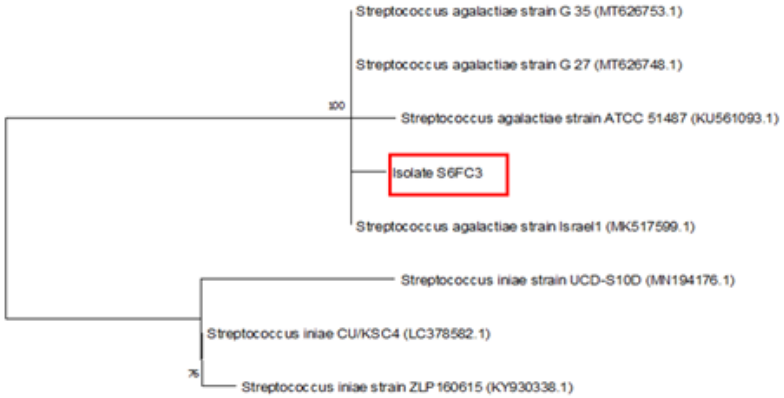

Figure 5 Phylogenetic analysis of S. agalactiae recovered from climbing perch in this research and isolates retrieved from GenBank. Bootstraps of 1,000 replicates were performed.

Infection experiment

Infected climbing perch started to die 5 hours after injection with the *S. agalactiae* S6FC3 isolate at a concentration of 10^5 and 10^6 CFU/mL (Figure 6). Cumulative mortality rates were recorded at 60, 80, and 100% at doses of 10^5, 10^6, and 10^7 CFU/mL, respectively. The LD_{50} values of the *S. agalactiae* S6FC3 isolate were determined at 8.71×10^4 CFU/mL on day 7. All dead fish showed typical signs, including anorexia, lethargy, a black body, an enlarged abdomen, enlarged kidneys, and hemorrhage similar to those of wild-infected fish. In the control treatment, no mortality or pathological signs were recorded. The experiment with the isolate S6FC3 ended after 12 days of injection. Isolate S6FC3 was re-isolated and re-identified from liver, kidney, brain, spleen, and blood samples of lethargic and dead fish after infection. Besides, during the infection experiment, the water temperature and pH were stably maintained, fluctuating between 28±2oC and 7.8±3, respectively.

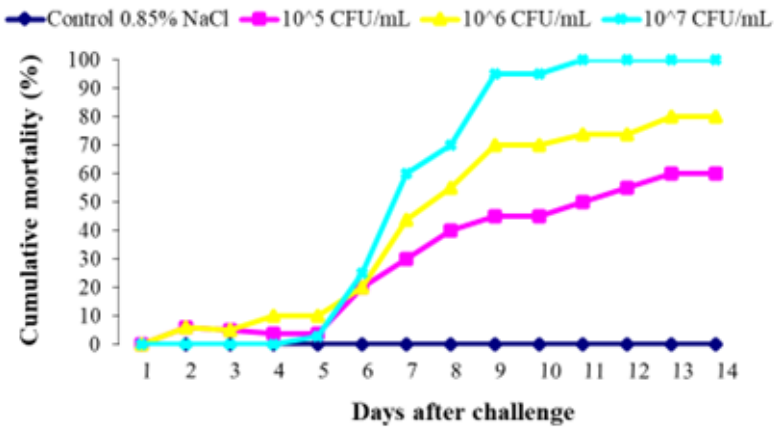

Figure 6 Cumulative mortality (%) of *S. agalactiae* S6FC3 isolate.

Antibiogram

Antibiogram results showed that *S. agalactiae* isolates exhibited high sensitivity to many antibiotics (Table 3). *S. agalactiae* isolates were completely susceptible to cefotaxime, doxycycline, and florfenicol. They show reduced sensitivity to erythromycin (66.7%), are highly resistant to streptomycin (81.5%), and are completely resistant to colistin sulfate. In particular, all *S. agalactiae* isolates were completely resistant to the antibiotic sulfamethoxazole-trimethoprim in this study. The percentages of susceptibility and resistance of isolated *S. agalactiae* isolates are presented in Table 3.

Table 3 Antibiotic susceptibility of thirty *S. agalactiae* strains from dark body disease climbing perch.

Antibiotics	Disk content (µg)	Resistance (%)	Susceptible (%)
Amoxycillin-clavulanic acid	25	33.3	66.7
Cefalexin	30	33.3	66.7
Cefotaxime	30	0	100
Colistin sulfate	5	100	0
Doxycycline	30	0	100
Erythromycin	5	33.3	66.7
Florfenicol	30	0	100
Flumequine	30	66.7	33.3
Gentamycin	30	3.7	96.3
Rifampicin	30	33.3	66.7
Streptomycin	10	85.1	14.9
Sulfamethoxazole-trimethoprim	23,75/1,25	100	0

DISCUSSION

Streptococcosis is a serious disease of a variety of cultured and wild fish species, especially tilapia (Haenen et al., 2023). In the current study, a total of 30 isolates originated from diseased climbing perch were confirmed as *S. agalactiae* based on basic morphological, physiological, and biochemical properties, the API 20 Strep kit, and the 16S rRNA sequence. In general, the bacterial isolates obtained from sick climbing perch had characteristics in common with *S. agalactiae* described by previous studies (Buller, 2004; Liu

et al., 2014). According to Al-Harbi (2016), *S. agalactiae* isolates obtained from the diseased hybrid tilapia (*O. niloticus* x *O. aureus*) in Saudi Arabia were gram-positive cocci, catalase-negative, and oxidase-negative. A similar result was also found in the research of Liu et al. (2014), who reported that *S. agalactiae* collected from barcoo grunter (*Scortum barcoo*) in China appeared whitish and smooth-edged on BHI agar plates. Importantly, *S. agalactiae* S6FC3 in the current study showed no hemolytic activity on sheep blood plates. The findings are in accordance with the research by Al-Harbi (2016), which showed that *S. agalactiae* isolates from diseased hybrid tilapia were non-hemolytic. On the other hand, Suhermanto et al. (2019) revealed that two biotypes of *S. agalactiae* strains related to the outbreaks in tilapia culture in Indonesia were β-hemolytics and non-hemolytics. According to Sudpraseart et al. (2021), **the β-hemolysis *S. agalactiae* strains showed a greater mortality rate than the γ-hemolysis strains, despite being from the same cluster by pulsed-field gel electrophoresis (PFGE), year, and locale.** Furthermore, the *S. agalactiae* S6FC3 isolate was able to develop in 6.5% NaCl but failed to survive at 10°C, 45°C, and pH 9.6 (Table 2). This analysis differs from that of Duremdez et al. (2004), who found that all *S. agalactiae* isolates from silver pomfrets (*Pampus argenteus*) in Kuwait could grow between 20 and 42°C but not at 5, 10, 15, or 45°C. Besides, the isolates from Duremdez et al. (2004) can grow in concentrations of 0.5, 3.0, 4.0, 4.5, 5.0, 5.5, and 6.0% NaCl, but there was no growth seen at pH 9.6 and 6.5% NaCl. Another research, Wang et al. (2013) indicated that *S. agalactiae* obtained from the outbreak of epidemically diseased tilapias in China could live on media supplemented with 6.5% NaCl at pH 9.6, a temperature of 10°C, and 45°C. Identification results by the API 20 Strep kit showed that strain S6FC3 had similar characteristics to *S. agalactiae* in the study of Amal et al. (2012), with the exception of negative reactions for β-glucuronidase, D-ribose, and D-trehalose. Niu et al. (2020) speculate that the difference in phenotype may be caused by *S. agalactiae* isolates from various locations and periods. The results presented that sequencing the 16S rRNA gene can be used in combination with morphological, physiological, and biochemical characteristics to precisely identify *S. agalactiae*.

S. agalactiae has a broad temperature range and is pathogenic to humans and animals (Evans et al., 2009). Spiral swimming, anorexia, ocular opacity, unilateral or bilateral exophthalmia, and skin ulcers and hemorrhages were common signs of disease in fish (Laith et al., 2017). Affected organs, such as the eye, liver, kidney, and brain, show gross pathological changes, enlarging and showing hemorrhagic and inflammation signs (Pretto-Giordano et al., 2010). The dark-body diseased climbing perch in this study presented a variation of external signs, including ascites, bilateral or unilateral exophthalmia, and clouding of the eyes (Figure 2). Additionally, darkening of body color, listless swimming, anorexia, and lethargy were also observed in all infected climbing perch. Interestingly, hemoperitoneum, hepatomegaly, splenomegaly, and an enlarged kidney were internally observed (Figure 2). In this study, generally, clinical signs induced by *S. agalactiae* were consistent with those reported in rabbitfish (*Siganus canaliculatus*) (Yuasa et al., 1999), gilthead seabream (*Sparus auratus*) and mullet (*Liza klunzingeri*) (Evans et al., 2002), silver pomfret (*Pampus argenteus*) (Duremdez et al., 2004), tilapias (*Oreochromis* spp.) (Abuseliana et al., 2011), Asian sea bass (*Lates calcarifer*) (Suanyuk et al., 2010), red porgy (*Pagrus pagrus*) (El Aamri et al., 2010) and Ya-Fish

(*Schizothorax prenanti*) (Geng et al., 2012). Interestingly, dark bodies due to *S. agalactiae* in this study have been found to be consistent with dark body diseases caused by *S. iniae* in the research of Dung et al. (2013). In order to better control the disease in the future, it will be crucial to ascertain whether isolates of *S. iniae* and *S. agalactiae* from climbing perch exhibit different clinical symptoms or infect different farming ponds.

In this study, experimental challenges with the *S. agalactiae* S6FC3 isolate exhibited high mortality (60–100%), which demonstrated that this isolate was pathogenic to climbing perch (Figure 6). Many previous studies have revealed that *S. agalactiae* causes high mortality in fish (Garcia et al., 2008; Al-Harbi, 2016). Particularly, many previous findings have demonstrated that β-haemolytic *S. agalactiae* were not more virulent than non-haemolytic bacteria (Al Harbi, 2016). *S. agalactiae* (group B Streptococcus; GBS), isolated from popped eye disease-affected tilapia and Vietnamese koi fishes, was found to be highly virulent and showed 80%–90% mortality for tilapia and V. Koi fishes in experimental infection, according to Rahman et al. (2021). Similar results were recorded in the study of Pereira et al. (2010), who revealed that the intraperitoneal route illustrated 100% mortality in tilapia fish. In the current study, the LD_{50} value of the *S. agalactiae* S6FC3 isolate was determined at 8.71 × 10^4 CFU/mL on day 7. The LD_{50} value of *S. agalactiae*-injected climbing perch was similar to that determined by El Aamri et al. (2010) in red porgy (*Pagrus pagrus*). In general, this value is higher than those of Syuhada et al. (2020), who reported that the LD50–240 hours of *S. agalactiae* serotypes Ia ST7 and III ST283, which originated from outbreaks of streptococcosis in red hybrid tilapia in Malaysia, were determined at 8.7 × 10^3 CFU/mL and 6.3 × 10^3 CFU/mL, respectively. This also means that the *S. agalactiae* S6FC3 isolate in the study has lower virulence than the two *S. agalactiae* serotypes in the study of Syuhada et al. (2020). Based on the LD_{50} value, the *S. agalactiae* S6FC3 isolate in this study demonstrated lower virulence than those experimentally infecting Nile tilapia (6.14×$10^{1.17}$ CFU/mL) (Mian et al., 2009) and red tilapia (1.65×10^5 CFU/mL) (Abuseliana et al., 2011). On the contrary, the virulence *of the S. agalactiae* S6FC3 isolate is higher than that reported by Suwannasang et al. (2014), who reported that the LD_{50} of *S. agalactiae* serotypes Ia and III was 1.58 × 10^6 and 2.10 × 10^8 CFU/fish, respectively. According to the virulence of the isolate, route of infection, host species affected, fish age, environmental, and water quality parameters, disease progression in fish can vary somewhat (Agnew and Barnes, 2007). According to Al-Harbi (2016), *S. agalactiae*, the etiological agent of streptococcosis in hybrid tilapia (*O. niloticus* × *O. aureus),* occurred, and mortality rates ranged from 40–80% when water temperatures were above 28°C, in combination with high fish stocking density and poor water quality.

Antibiotics known as beta-lactams have a variety of therapeutic actions and few negative side effects. They disrupt the formation of peptidoglycan, a crucial component of bacterial cell walls, and compromise the strength of the cell walls, leading to cell lysis (Pandey and Cascella, 2022). Antibiogram results of this investigation showed that bacterial strains were highly sensitive to β-lactam antibiotics such as amoxycillin-clavulanic acid (66.7%), cefalexin (66.7%), and cefotaxime (100%) (Table 3). These findings are in line with the study of Legario et al. (2020), which revealed that *S. agalactiae* isolated

from farmed Nile tilapia (*O. niloticus*) in the Philippines were susceptible to amoxicillin. Similarly, the research of Osman et al. (2017) showed that the 17 *Streptococcus* isolates recovered from Nile tilapia presenting septicemia in aquaculture and wild sites in Egypt were sensitive to amoxicillin-clavulanic acid. On the contrary, the research of Abedin et al. (2020) illustrated that *Streptococcus* spp. isolated from climbing perch (*A. testudineus*) in Bangladesh was highly resistant to amoxicillin.

Tetracyclines are one of the most popular bacteriostatic drugs used in aquaculture (Bondad-Reantaso et al., 2023). By attaching to the cell's ribosomal 30S subunit, tetracyclines prevent the synthesis of bacterial proteins. In this study, antibiogram results indicated that *S. agalactiae* strains were fully susceptible to doxycycline (Table 3). The results are different from the study of Osman et al. (2017), which revealed that *Streptococcus* species had the highest resistance to tetracycline (94.1%). Similar results obtained from Sherif et al. (2022) indicated that *S. agalactiae* and Nile tilapia (*O. niloticus*) showed full resistance to doxycycline. In a study, Alazab et al. (2022) revealed that *S. agalactiae* obtained from tilapia fish (*O. niloticus*) in Egypt were resistant to tetracycline at a rate of 66%. However, the findings line up with the research of Klingklib and Suanyuk (2017), who reported that all *S. agalactiae* strains derived from the infected climbing perch were 100% sensitive to oxytetracycline. Meanwhile, Deng et al. (2019) revealed that *S. agalactiae* strains from diseased farmed fish in China were resistant to doxycycline (7.14%).

Quinolones are bactericidal antibiotics with a broad spectrum that share a bicyclic core structure with 4-quinolones. They stop the action of enzymes needed for bacterial DNA replication (Fàbrega et al., 2009). In this work, bacterial isolates are still highly sensitive to erythromycin (sensitivity rate is over 60%) (Table 3). The study of Alazab et al. (2022) indicated that 95% of isolated strains were resistant to erythromycin. Additionally, Deng et al. (2019) reported that 42.9% (12/28) of the *S. agalactiae* strains from diseased farmed fish in China were resistant to erythromycin. Tawab et al. (2022), who reported that 33.3% of *S. iniae* isolates derived from *O. niloticus* in Egypt were resistant to streptomycin. In a study, Colussi et al. (2022) showed that *S. iniae* from Adriatic sturgeon (*Acipenser naccarii*) in Northern Italy is susceptible to erythromycin. Klingklib and Suanyuk (2017) reported that all the isolated strains obtained from the infected climbing perch were 100% susceptible to erythromycin. Pulpipat et al. (2023) presented that *S. agalactiae* serotype VII from snakeskin gourami (*Trichogaster pectoralis*) in Thailand was sensitive to amoxycillin and erythromycin.

In aquaculture, the flumequine synthetic fluoroquinolone is efficient against gram-negative bacteria (Lai and Lin, 2009). Similar to erythromycin, all *S. agalactiae* strains in the finding were susceptible to flumequine (Table 3). The finding is in accordance with Laith et al. (2017), who showed that *S. agalactiae* isolated from natural infections in hybrid tilapia (*O. niloticus*) in Malaysia was 100% susceptible to flumequine. A similar result from Musa et al. (2009) revealed that most of *S. agalactiae* strains recovered from *O. niloticus* ponds in Malaysia were found to be sensitive to flumequine. According to Trung et al. (2013), *S. iniae* isolated from seabass (*L. calcarifer*) was also reported to be resistant to flumequine.

A series of broad-spectrum antibiotics known as amphenicols inhibit the synthesis of proteins by microorganisms by attaching to the peptidyl

transferase enzyme at the 50S subunit of the 70S bacterial ribosome, having bacteriostatic effects in the process (Bondad-Reantaso et al., 2023). Florfenicol is an antibiotic commonly used in aquaculture in many countries around the world (Hieu and Trang, 2021). In this study, 100% of the bacteria exhibited sensitivity to florfenicol (Table 3). This investigation is similar to the research of Deng et al. (2019), who presented that all *S. agalactiae* isolates from diseased farmed fish in China were susceptible to florfenicol. Similar study was gained by Sherif et al. (2022), who indicated that *S. agalactiae and* the Nile tilapia (*O. niloticus*) were highly sensitive to florfenicol and ciprofloxacin. In the Philippines, according to Legario et al. (2020), both *S. agalactiae* and *S. iniae* derived from farmed Nile tilapia were susceptible to florfenicol. Another study by Colussi et al. (2022) revealed that *S. iniae* in Adriatic sturgeon (*Acipenser naccarii*) in Northern Italy is susceptible to florfenicol. Similarly, Tawab et al. (2022) reported that *S. iniae* strains collected from *O. niloticus* in Egypt were completely susceptible to florfenicol.

Aminoglycosides are bactericidal, broad-spectrum antibiotics that attach to the ribosome's 30S subunit and prevent bacteria from synthesizing proteins (Krause et al., 2016). In the study, *S. agalactiae* was 96.3% sensitive to gentamycin (Table 3). Research by Alazab et al. (2022) showed that *S. agalactiae* resistance to gentamycin was 38%, while the sensitivity rate was 62%. In China, Deng et al. (2019) reported that 28.6% (8/28) of the *S. agalactiae* strains from diseased farmed fish were resistant to gentamicin. The study of Colussi et al. (2022) showed that *S. iniae* in Adriatic sturgeon (*Acipenser naccarii*) in Northern Italy is susceptible to gentamycin. From popped eye disease-affected tilapia and V. Koi fish in Bangladesh, Rahman et al. (2021) demonstrated that the isolated *S. agalactiae* isolates had resistance properties against all seven antibiotics: gentamycin, oxytetracycline, tetracycline, chlortetracycline, ciprofloxacin, streptomycin, and neomycin. In Egypt, Osman et al. (2017) revealed that *Streptococcus* species, pathogens for Nile tilapia cultivation, had the lowest resistance to gentamicin, streptomycin, and sulfamethaxozole-trimethoprim.

Similar to gentamycin, *S. agalactiae* isolates were also highly resistant to streptomycin in the study, with a rate of 85.1% (Table 3). From the cultured Nile tilapia in Egypt, Tawab et al. (2022) reported that 26.7% of *S. iniae* strains were resistant to streptomycin. Research by Colussi et al. (2022) revealed *S. iniae* in Adriatic sturgeon (*Acipenser naccarii*) in Northern Italy as resistant to streptomycin. In Bangladesh, Rahman et al. (2021) demonstrated that the *S. agalactiae* isolates originated from tilapia and V. Koi fish causing popped eye disease and were resistant to streptomycin and other antibiotics, including oxytetracycline, tetracycline, chlortetracycline, gentamicin, ciprofloxacin, and neomycin. *Streptococcus* species, a serious pathogen for Nile tilapia in Egypt, exhibited the least amount of resistance to streptomycin, besides gentamicin, and sulfamethoxazole-trimethoprim, according to Osman et al. (2017). Sherif et al. (2022) presented that *S. agalactiae* in *O. niloticus* exhibited complete resistance to streptomycin, as well as amoxicillin, cefotaxime, lincomycin, doxycycline, spiramycin, and cephradine.

Sulfonamides are a group of artificial bacteriostatic antibiotics that prevent bacteria from synthesizing DNA, purines, and folic acid (Ovung and Bhattacharyya, 2021). In the study, *S. agalactiae* was 100% resistant to sulfamethoxazole-trimethoprim (Table 3). Research by Alazab et al. (2022)

showed that *S. agalactiae* resistance to sulfamethoxazole-trimethoprim was 76%, while the sensitivity rate was 24%. In Egypt, Tawab et al. (2022) reported that 13.3% of *S. iniae* strains from *O. niloticus* were resistant to sulfamethoxazole-trimethoprim. Similarly, Legario et al. (2020) presented that only *S. agalactiae* serotype Ib from farmed Nile tilapia (*O. niloticus*) exhibited resistance to sulfamethoxazole-trimethoprim. The findings of Colussi et al. (2022) indicated that *S. iniae* in Adriatic sturgeon (*Acipenser naccarii*) was susceptible to sulfamethoxazole-trimethoprim. From the infected climbing perch, Klingklib and Suanyuk (2017) reported that 100% of the *S. agalactiae* strains displayed complete resistance to sulfamethoxazole-trimethoprim. Osman et al. (2017) revealed that *Streptococcus* species had the lowest resistance to sulfamethoxazole-trimethoprim, together with streptomycin and gentamicin. Sherif et al. (2022) indicated that *S. agalactiae* from Nile tilapia exhibited intermediate resistance to clindamycin, amoxicillin clavulanate, and sulfamethoxazole-trimethoprim. Pulpipat et al. (2023) presented that only 45.54% of *S. agalactiae strains* from *T. pectoralis* in Thailand displayed sensitivity to sulfamethoxazole-trimethoprim.

The antibiotic colistin, sometimes referred to as polymyxin E, is a member of the polymyxin family and has a notable inhibitory impact on gram-negative bacterial infections (Mohapatra et al., 2021). In Vietnam, colistin production and business in the aquaculture sector are limited. However, the use of this antibiotic is still applied in the culture of catfish (*Pangasianodon hypophthamus*) and red tilapia (*Oreochromis* sp.) in the Mekong Delta (Phu et al., 2017) and may select for colistin resistance in aquatic animals. In the study, *S. agalactiae* isolates were completely resistant to colistin (Table 3). Osman et al. (2017) revealed that *Streptococcus* species from Nile tilapia aquaculture in Egypt exhibited total susceptibility to colistin. On the other hand, research by Laith et al. (2017) illustrated that *S. agalactiae* isolated from natural infections in hybrid tilapia (*O. niloticus*) in Malaysia was sensitive to colistin.

Rifampicin is an antibiotic that prevents RNA synthesis in bacteria by impeding DNA-dependent RNA polymerase (Mosaei and Harbottle, 2019). In the study, *S. agalactiae* isolates were highly sensitive to rifampicin (Table 3), with a rate of 66.7%. Research by Suhermanto et al. (2019) showed that almost all *S. agalactiae* isolates derived from *O. niloticus* cultures in Indonesia were resistant to rifampicin. In an investigation, Trung and Dung (2018) also recorded 62.5% rifampicin resistance in bacteria isolated from cage-raising cobia (*Rachycentron canadum*) in Kien Giang province of the Mekong Delta. Deng et al. (2015) revealed that *S. iniae*, a serious infectious disease characterized by body ulcers, internal organ hemorrhage, and nodules in *Acipenser baerii* in China, was sensitive to amoxicillin, doxycycline, and florfenicol but resistant to novobiocin, ofloxacin, and rifampicin. According to Trung et al. (2013), *S. iniae* isolated from seabass (*L. calcarifer*) was also reported to be resistant to colistin sulfate and flumequine.

CONCLUSIONS

The present study confirmed *S. agalactiae* as a new host of dark-body climbing perch in the southern provinces of Vietnam based on phenotypic and genotypic properties, the API 20Strep kit, and species-specific primers of the 16S rRNA sequence. Sick fish with typical signs observed in natural and experimental infections, including darkened body color, ascites, acute meningitis, hepatomegaly, an enlarged kidney, and splenomegaly. The findings found that the *S. agalactiae* S6FC3 isolate exhibits high mortality; the LD_{50} value was determined at 8.71 x 10^4 CFU/mL. In this investigation, *S. agalactiae* isolates were completely sensitive to cefotaxime, doxycycline, and florfenicol. Interestingly, the results of this study found that *S. agalactiae* isolates are still fully resistant to sulfamethoxazole-trimethoprim. The results of this study will encourage the creation of disease prevention techniques that are effective for climbing perch farming in the future.

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AUTHOR CONTRIBUTIONS

Tu Thanh Dung: conceptualization, interpretation of the study, and writing manuscript.
Quach Van Cao Thi: Conceptualization, performed experiments, draft, and editing preparation, and wrote the manuscript.
Nguyen Bao Trung: performed experiments, sample collection, statistic analysis, writing, and editing of the manuscript.

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