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Investigation of streptococcosis (*Streptococcus iniae*) as a cause of popeye and hemorrhagic disease in tilapia (*Oreochromis niloticus*) in the Mekong Delta, Vietnam

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Abstract. *Trung NB, Dung TT, Thi QVC. 2024. Investigation of streptococcosis (*Streptococcus iniae*) as a cause of popeye and hemorrhagic disease in tilapia (*Oreochromis niloticus*) in the Mekong Delta, Vietnam. Biodiversitas 25: 3032-3040. Streptococcus iniae*, is a dangerous pathogen that causes serious harm to several aquatic animals around the world. The current study aimed to isolate, characterize, and assess the pathogenicity of the *Streptococcus iniae* that causes popeye and hemorrhagic diseased nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) in the Mekong Delta, Vietnam. Infected tilapia showed disorienting or lethargic swimming, exophthalmia (unilateral or bilateral), corneal opacity, and hemorrhages on the body, the operculum, and the abdomen of the fish. Upon internal examination, the diseased fish were found to have a pale liver, swollen kidney, and white or yellow fluid accumulation in the abdominal cavity. With the use of the API 20Strep Kit, conventional techniques, and 16S rRNA gene sequencing, a total of 16 isolates were determined to be *S. iniae*. They are Gram-positive bacteria, cocci or streptococci, with β-hemolysis and small, white, opalescent colonies on BHIA medium after 24-36 hours of incubation at 28ºC. The obtained challenge tests showed that both isolates of RPBT02 and RPHG36 were highly virulent to fish, with LD_{50} values determined to be 2.34×10^4 and 1.81×10^4 CFU/mL, respectively. Interestingly, the two isolates of RPHG36 and RPBT02 in this study contained the virulence gene phosphoglucomutase (*pgm*), one of the factors that make *S. iniae* enhance antibiotic resistance and phagocytosis of the immune system by the host. These results of the study are important information for finding effective ways to control this bacterium in tilapia in the future.

Keywords: Streptococcal disease, phosphoglucomutase, *Streptococcus iniae*, tilapia, virulence

INTRODUCTION

Globally, the aquaculture business is expanding quickly (Barría et al. 2021). However, the expansion of the area along with intensive farming models with high density have caused infectious diseases to occur more often and cause more damage (Assefa and Abunna 2018). Besides, diseases caused by environmental (Fouad et al. 2022; Ndashe et al. 2023), nutritional (Admasu and Wakjira 2021), fungal (Mahbouba 2021), parasitic (Debnath et al. 2023), or viral factors (Surachetpong et al. 2017), bacterial diseases in tilapia are also frequently reported (Bwalya et al. 2020). In particular, streptococcosis is currently causing severe economic losses and affecting tilapia production worldwide (Legario et al. 2020). Both farmed and wild fish can contract streptococcosis in freshwater and saltwater (Van Doan et al. 2022) and cause annual losses of up to $250 million to aquaculture worldwide (Aboyadak et al. 2016). For example, *Streptococcus iniae* and *Streptococcus agalactiae* are the main bacterial pathogens affecting nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) farms, significantly impacting fish production globally (Khanzadeh et al. 2023; Khanzadeh et al. 2024).

Streptococcus iniae is a member of the Gram-positive cocci or streptococci group of bacteria. Until now, it has been discovered that *S. iniae* can affect at least 27 different species of fish that are both wild and cultured (Colussi et al. 2022), including rainbow trout (Tafi et al. 2020), olive flounder (Jeong et al. 2016), Amazon catfish (Tavares et al. 2018), Adriatic sturgeon (Colussi et al. 2022), European eel (Pirollo et al. 2023), and most commonly tilapia (Zheng et al. 2018). Diseased fish often exhibit typical clinical signs such as swimming disorientation, corneal opacity, dark skin, protruding eyes, meningitis, and hemorrhage. In Vietnam, black body illness in seabass (Hoa et al. 2018), and snakeskin gourami (Dung et al. 2024) have also been linked to *S. iniae* and *S. agalactiae*, respectively.

Presently, nile tilapia (*O. niloticus*) is the second most extensively cultivated fish globally, whose annual output is regularly rising. They can survive harsh environmental circumstances like low oxygen levels and elevated levels of organic materials within the water, as well as having a rapid rate of growth (Arguedas et al. 2017). As a result, tilapia can withstand large changes in temperature and salinity (Fajer-Ávila et al. 2017). Due to these characteristics, tilapia is one of the species that is most commonly grown worldwide, and by 2030, production is expected to reach 7.3 million tons (Abdel-Latif et al. 2020). However, the most common diseases in tilapia are currently caused by *Streptococcus* sp., *Aeromonas hydrophila*, *Flavobacterium columnare*, *Edwarsiella tarda*, *Ichthyophitirius multifillis*, *Gyrodactylus niloticus*, and *Tricodhina* sp. (Acosta-Pérez

et al. 2022; Haenen et al. 2023). In tilapia, the causative agent of streptococcosis has been identified with two main species, including *S. agalactiae* and *S. iniae* (Legario et al. 2020). To date, the majority of reports have shown that tilapia disease is mainly caused by *S. agalactiae* (Zhang 2021). Research by Phuoc et al. (2015), on the contrary, identified *S. iniae* and *S. agalactiae* as two bacterial species commonly occurring on diseased tilapia in the Mekong Delta.

Currently, there have been several studies on vaccines to control diseases caused by *Streptococcus* (Ramos-Espinoza et al. 2020; Wang et al. 2020). Vaccine research is based on an understanding of the pathogen, its pathogenic mechanisms, and virulence factors. Until now, many virulence factors originating from *S. iniae* have been reported, including β-haemolysin, M-like protein (*simA* gene), C5a peptidase (*scpI* gene), posphoglucomutase (*pgm* gene), the sag operon (*sagA* gene), polysaccharide deacetylase (*Pdi* gene), and CAMP factor-like (*cfi* gene) (Deng et al. 2017; Legario et al. 2020; Xu et al. 2024). Among them, in both Gram-positive and Gram-negative bacterial pathogens, the *pgm* gene is essential for the formation of polysaccharide capsules and pathogenicity (Buchanan et al. 2005). However, there have been very few studies so far on the presence of virulent genes in bacterial strains of *S. iniae*, a common pathogen in many important farmed fish species in Vietnam. Therefore, the research was conducted to isolate, identify, and evaluate the pathogenicity of *S. iniae* bacteria causing popeyed and hemorrhagic infections in tilapia (*O. niloticus*) cultured in the Mekong Delta. The data from this research can be used to find efficient alternatives for controlling *S. iniae* infection in tilapia farms.

MATERIALS AND METHODS

Fish samples for bacterial isolation

Diseased fish samples of *S. iniae* bacteria with symptoms of lethargy, protruding eyes, hemorrhage in the body, and fin angle were used to isolate *S. iniae*. While healthy/new fish, or moribunds, were used to isolate *S. iniae*. Fish weighing from 100 to 300 g/fish are raised in intensive cages in Ben Tre and An Giang (Figure 1). Fish are collected many times during the disease outbreaks, usually from February to June of the year. Each site was randomly selected from 3-6 cages, with 3-5 fish samples per cage.

Isolation of *S. iniae* bacteria

The isolation of *S. iniae* bacteria was performed according to the method of Rahmatullah et al. (2017). In brief, the fish is externally disinfected with 70% alcohol. The fish was then dissected to take microbiological samples from the internal organs, including the kidney, brain, liver, and spleen. Samples were inoculated on Brain Heart Infusion Agar medium (BHIA, Merck, Germany) and incubated at 28°C for 24-48 hours. The presumed colonies of *S. iniae* (round and small, slightly convex, and opalescent colonies) were subcultured on Tryptic Soy Agar (Merck, Germany). Pure bacterial isolates were stored in Brain Heart Infusion Broth (BHIB, Merck, Germany) supplemented with 20% glycerol (v/v) at -80°C for further experiments.

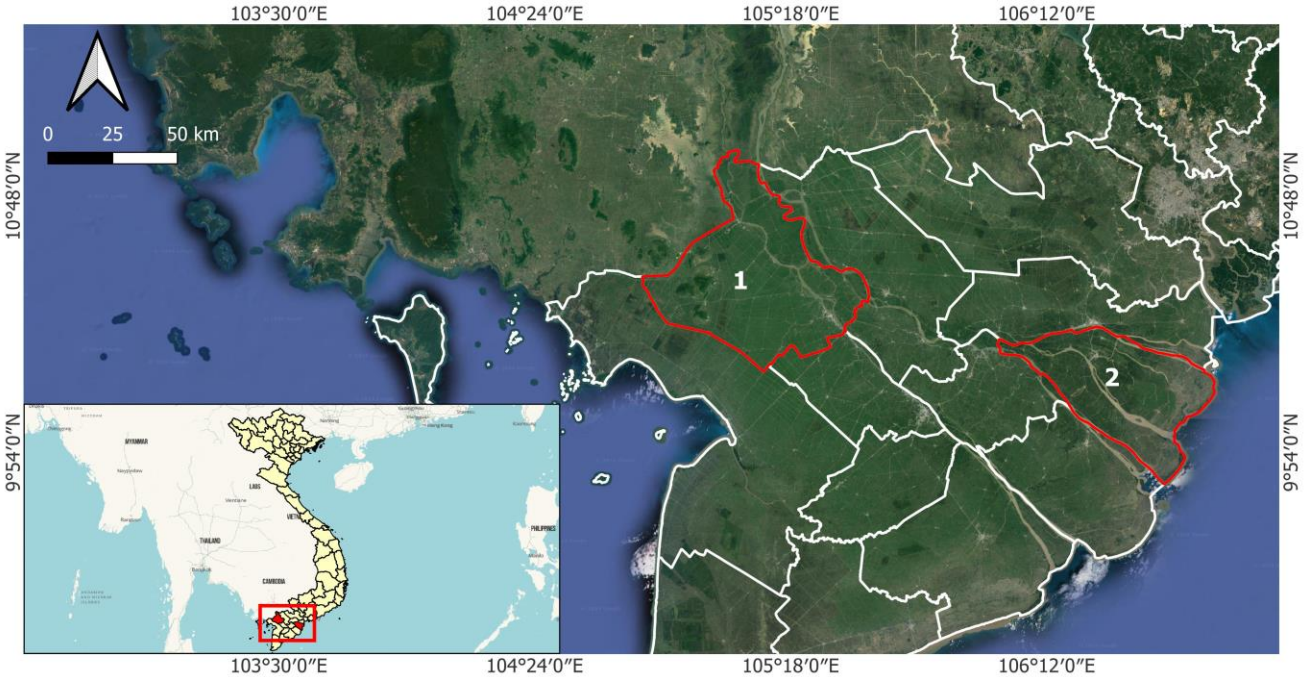


Figure 1. Location of fish samples for *Streptococcus iniae* isolation in the Mekong Delta, Vietnam. 1. An Giang Province; 2. Bến Tre Province

Identification of *S. iniae* using genotypic and phenotypic characteristics

The basic morphological, physiological, and biochemical characteristics of bacterial isolates, such as Gram staining, catalase reaction, oxidase, glucose oxidation/fermentation reaction (O/F), motility, capacity to grow in NaCl 6.5%, pH 9.6, at temperature values of 10°C, 28°C, 37°C, and 45°Cwere determined according to previous documents (Buller 2014). The hemolytic activity of the bacteria was tested on BA medium (Blood Agar) supplemented with 5% sheep blood (Xu et al. 2024). In addition, the study also used the commercial API 20 Strep kit (Microbank™, PRO-LAB Diagnostics, UK) to identify the bacterial isolates (Xu et al. 2024).

Identification of *S. iniae* by polymerase chain reaction

A PCR assay was utilized to identify bacterial isolates at the species level. *S. iniae* DNA was isolated according to Moore et al. (2004), with some modifications. Briefly, *S. iniae* were cultured in BHIB medium for 24 hours with 120 rpm. The collected pellets by centrifuging cell suspensions at 4,500 rpm for 5 minutes were then resuspended in Tris-EDTA (TE) buffer (20 mM Tris-HCl, 0.5 mM EDTA, pH 8.0). An equal volume of phenol, chloroform, and isoamyl alcohol (25:24:1) was added to the suspension, and it was centrifuged for five minutes at 15,000 rpm. The DNA-containing upper aqueous phase was transferred to a different 2 mL Eppendorf tube, and 0.7 volumes of isopropanol were added. The aqueous phase was recovered by centrifugation for 20 minutes, and the genomic DNA was precipitated using ethanol. Finally, using a spectrophotometer, the isolated DNA was examined for concentration and purity at 260 and 280 nm.

Sin-1: 5'-CTA GAG TAC ACA TGT AGC TA AG-3' and Sin-2: 5'-GGA TTT TCC ACT CCC ATT AC-3', species-specific primers that amplify a predicted 300 bp fragment of the 16S rRNA, were used to validate *S. iniae* (Saleh et al. 2017). Each PCR experiment was carried out in a 25 µL reaction volume with the following components: 10× PCR buffer, 2.0 mM $MgCl_2$, 250 µM dNTPs, 2U Taq DNA polymerase, 20 pmol each of the forward and reverse primers (Sin-1 and Sin-2), and 60 ng of *S. iniae* DNA samples. The conditions under which the PCR reactions were conducted were as follows: five minutes of initial denaturation at 94°C; thirty cycles of denaturation at 94°C for one minute; one minute of annealing at 55°C; one minute of extension at 72°C; and one final extension at 72°C for ten minutes. The PCR products were electrophoresed on a 1.5% agarose gel, and images were captured using a BioRad UV 2000 apparatus. Macrogen Company (Korea) performed the sequencing of the representative isolates of *S. iniae*.

Experimental challenge

Healthy *O. niloticus* fingerlings (with an average weight of weighing about 18±2 g/fish) were purchased from prestige hatcheries. Fish were acclimatized in a 500 L plastic tank for 1-2 weeks before the experiment. Fish were checked for parasites and bacteria before the experiment. Two representative strains of bacteria, RPBT02 and RPHG36, from Ben Tre and An Giang were selected to infect tilapia fingerlings. In brief, bacteria were grown in BHIB medium and shaken overnight at 120 rpm. The supernatant was then centrifuged at 4,000 rpm at 4°C for 15 minutes. Cell biomass was washed 2-3 times with sterile physiological saline (0.85% NaCl). Bacterial density was determined optically by a spectrophotometer at 600 nm with OD_{600}=0.67, corresponding to a bacterial density of 10^9 CFU/mL (Kannika et al. 2017). The bacterial broth was diluted with 0.85% NaCl to the required densities for the experimental challenge (Table 1).

Bacterial concentrations of 10^3, 10^4, 10^5, and 10^6 CFU/mL were used in challenge tests (Table 1). Each injection concentration was repeated three times with a density of 10 fish per tank. A 100 µL bacterial solution was injected into the base of the pectoral fins of the fish. Injecting the same volume of physiological saline (0.85% NaCl) was the control treatment. Fish were observed daily and continuously for 14 days after infection. Freshly dead fish, or moribunds, were re-isolated on BHIA medium and identified by PCR reaction. The LD_{50} (lethal dose) value of bacterial isolates was determined according to the formula of Reed and Muench (1938).

Determination of virulence genes

The phosphoglucomutase virulence gene (*pgm* gene) of isolated *S. iniae* isolates was determined by specific primer pairs PGM-F: 5'-GAA CTA GCT AGT TAC TTT TGT AAC TG-3' and reverse primer PGM-R: 5 '-CTA ATT CAC AAA AGT GTT GAT TTC AG-3' with an amplified product size of 1,865 bp (Buchanan et al. 2005). The protocol for the PCR assay was performed as described above.

Data analysis

The cumulative mortality was calculated using descriptive statistics. Using the BLASTn tool, the sequences from the isolates were compared to sequences from GenBank. The CLUSTALW program was then used to multialign the sequences. The phylogenetic tree was created using the neighbor-joining approach with a bootstrap value of 1,000 replicates and MEGA X software. The MEGA X software was used to draw the graphs.

Table 1. Treatment groups and bacterial concentrations used in this study

Treatment group	Number of fish	Isolates	Challenge dose (CFU/mL)
1	10	*S. iniae* RPBT02	10^3
2	10		10^4
3	10		10^5
4	10		10^6
5	10	*S. iniae* RPHG36	10^3
6	10		10^4
7	10		10^5
8	10		10^6
9	10	Control (0.9% NaCl solution)	No bacteria

CFU: Colony-Forming Units

RESULTS AND DISCUSSION

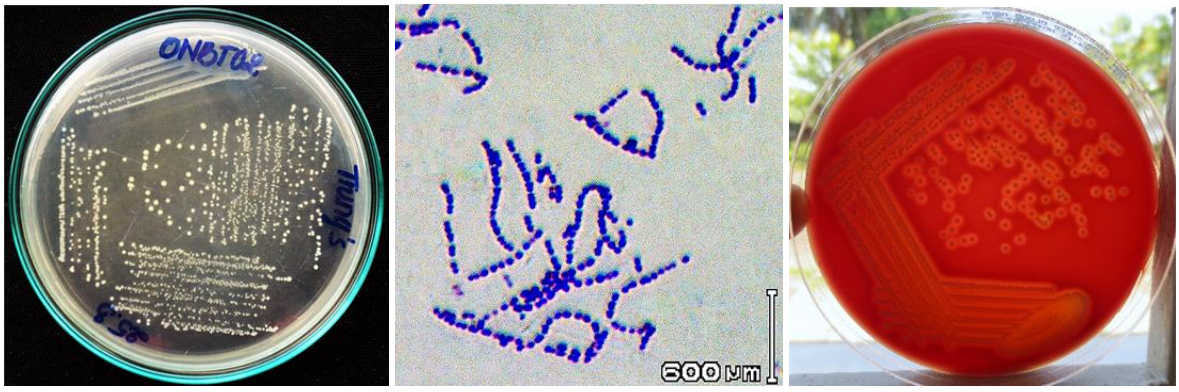
Clinical signs of naturally diseased fish

The results showed that diseased fish in the wild showed disorienting or lethargic swimming, protruding and corneal opacity, sometimes loss of eyes, hemorrhages on the body surface, in the opercula, and at the base of fins (Figure 2). The internal clinical signs of the fish included a pale liver, a swollen kidney, a swollen spleen, and bleeding, sometimes white or yellow fluid, in the abdominal cavity (Figure 2).

Bacterial isolation

The results of this study isolated 16 strains of *S. iniae* on BHIA medium from organs such as the spleen, liver, kidney, and brain of tilapia with protrusion and hemorrhage in the two provinces of An Giang and Ben Tre. The obtained results showed that the isolates had pint-point and opaque colonies (Figure 3.A), Gram-positive bacteria (Figure 3.B), cocci, or streptococci when grown on nutrient medium. In addition, they are nonmotile, negative for catalase and oxidase tests, and incapable of oxidizing and fermenting glucose (O/F), but they have β-hemolytic activity on BA medium supplemented with 5% sheep blood (Figure 3.C). Bacteria can grow well at 10ºC but not at 6.5% NaCl, pH 9.6, or 45ºC (Table 2).

Figure 2. Clinical signs of diseased tilapia: A. Net cages for tilapia cultivation; B. Diseased tilapia; C. Eyes with corneal opacity and exophthalmia on nile tilapia; D. Pale liver, congested kidney, and spleen in tilapia (*arrow*)



A **B** **C**

Figure 3. *Streptococcus iniae* isolates originated from diseased tilapia: A. Colony of an isolated *S. iniae* strain on BHIA medium; B. Gram staining (100×); C. Hemolytic activity (β) of an isolated *S. iniae* strain

Table 2. Phenotypic and genotypic characteristics of isolated *Streptococcus iniae* isolates

Reaction	*S. iniae* RPBT02 isolate	*S. iniae* RPHG36 isolate	*S. iniae*[*]
Gram stain	+	+	+
Cell morphology	Cocci, chain	Cocci, chain	Cocci, chain
Colony diameter	0.5-1 mm	0.5-1 mm	1 mm
Growth day	36-48 hours	36-48 hours	24-48 hours
Motility	-	-	-
Catalase	-	-	-
Oxidase	-	-	-
O/F reaction	-/-	-/-	+/+
Hemolysis	β	β	α/β
Growth in 6.5% NaCl	-	-	-
Growth at pH 9.6	-	-	-
Growth at 10°C	-	-	-
Growth at 28°C	+	+	+
Growth at 37°C	+	+	+
Growth at 45°C	-	-	-
Voges–Proskaeur	+	+	-
Hydrolysis of Hippurate	+	+	-
Esculin degradation	+	+	+
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; [*] Buller (2014)

Identification of *S. iniae* by the API 20 Strep Kit

Identification results by API 20Strep kit revealed that *S. iniae* bacteria were negative for Voges-Proskauer reaction, hippurate hydrolysis, pyrrolidonyl arylamidase, α-galactosidase, β-galactosidase, L-arabinose, D-mannitol, D-sorbitol, D-lactose, inulin, and D-raffinose and positive for esculine degradation, β-Glucuronidase, alkaline phosphatase, leucine aminopeptidase, arginine dihydrolase, D-ribose, D-trehalose, starch and glycogen (Table 2).

Identification of *S. iniae* by PCR reaction

The results of electrophoresis demonstrated that the PCR product amplified the specific gene fragment of all bacteria, with the DNA band appearing at a position of 300 bp in size compared to the standard marker (Figure 4). The blast results showed that six bacterial strains isolated from infected tilapia showed 99-100% similarity to *S. iniae* on GenBank.

A phylogenetic tree showing the genetic relationships between isolates of *S. iniae* and strains from NCBI was constructed (Figure 5). The obtained findings indicated that these bacterial strains in the research were distributed into two separate groups, A and B (Figure 5). For the A cluster, the RPBT07, RPBT01, RPBT02, and RPHG36 strains are distributed in the same group and have a close genetic relationship to *S. iniae* strains with accession numbers of KF815728.1, KF826094.1, KM209199.1, FJ870987.1, KF555594.1, and KC708484.1 on GenBank. On the contrary, the RPHG47 and RPDN49 strains belong to the same group in the B cluster.

Experimental challenge

For strain RPHG36, the experimental results showed that the mortality of experimental fish was noted after 2 days at a concentration of 10^6 CFU/mL, while mortality in the other dosage groups started on day 3. Notably, the majority of deaths occurred between days five and ten post-infection (Figure 6). Interestingly, at concentrations of 10^6 and 10^5 CFU/mL, strain RPHG36 caused high mortality rates of 93% and 77%, respectively. Meanwhile, cumulative mortality in the remaining treatments was 47% and 40%, respectively. No mortality was observed in the control treatment throughout the 14 days of the experimental challenge (Figure 6).

For strain RPBT02, the highest mortality rate observed was 97% at a concentration of 10^6 CFU/mL, with the lowest being 33% at 10^3 CFU/mL. The mortality rates for the 10^5 and 10^4 CFU/mL treatments were 67% and 40%, respectively. Similar to strain RPHG36, no mortality was recorded in the control groups (Figure 6). The LD_{50} values for *S. iniae* strains RPHG36 and RPBT02 were calculated to be 1.81×10^4 and 2.34×10^4 CFU/mL, respectively. Additionally, the infected nile tilapia exhibited clinical signs consistent with those observed in farmed diseased fish, including erratic swimming, loss of orientation, exophthalmia (unilateral or bilateral), corneal opacity, and hemorrhages on the body surface, operculum, and abdomen. Internal examination revealed a pale liver, swollen kidney, and white or yellow fluid in the abdominal cavity. The bacteria were successfully re-isolated from diseased fish in all experimental groups on BHIA medium and confirmed via PCR assay.

Determination of the virulence gene

In this study, the presence of the phosphoglucomutase (*pgm*) gene was detected in five out of sixteen (31.25%) isolates of *Streptococcus iniae*. The *pgm*-positive isolates included RPBT02, RPBT07, RPHG36, RPHG47, and RPTG70, with PCR amplification producing a product of 1,865 base pairs (Figure 7).

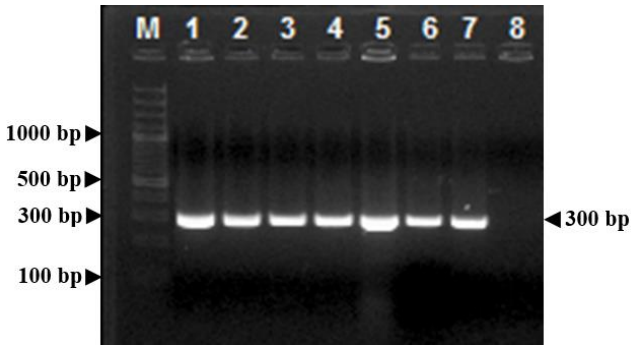


Figure 4. Results of electrophoresis of PCR products identifying *Streptococcus iniae*: M: 100 bp standard marker; Lanes 1-7: isolates RPHG36, RPDN49, RPTB01, RPBT02, RPBT07, RPAG01, and RPAG04, respectively; Lane 8: Negative control (H_2O)

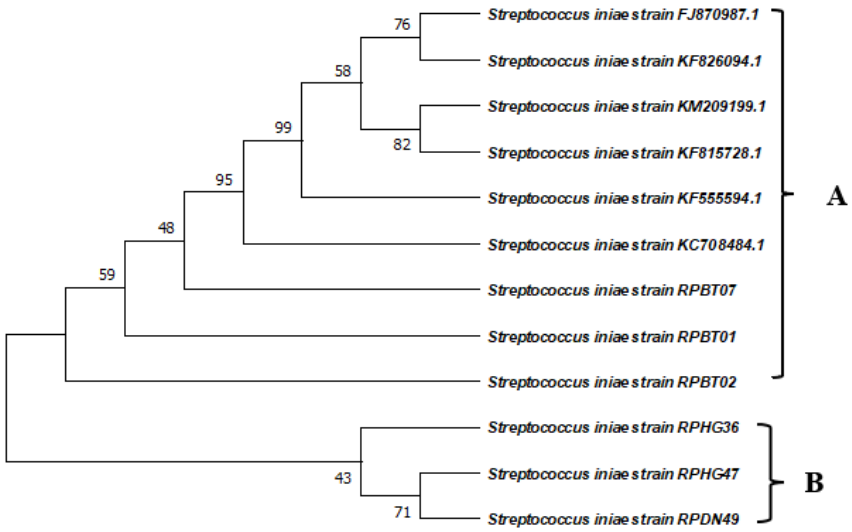


Figure 5. Phylogenetic tree showing genetic relationships of *Streptococcus iniae* strains based on 16S rRNA gene fragments (numbers in branches are bootstrap values with 1,000 replicates)

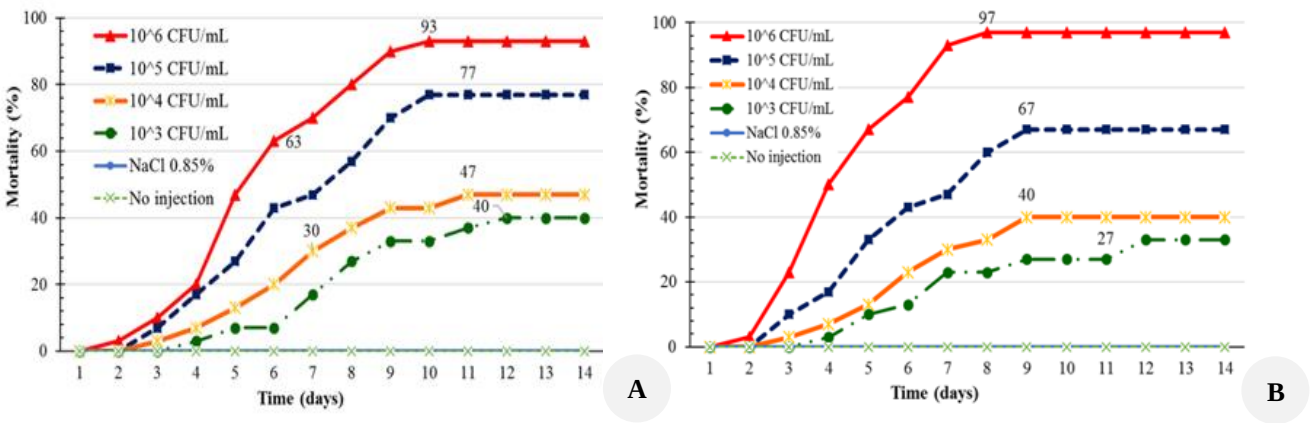


Figure 6. Cumulative mortality (%) infected with *Streptococcus iniae* strains RPHG36 and RPBT02: A. Isolate RPHG36; B. Isolate RPBTO2

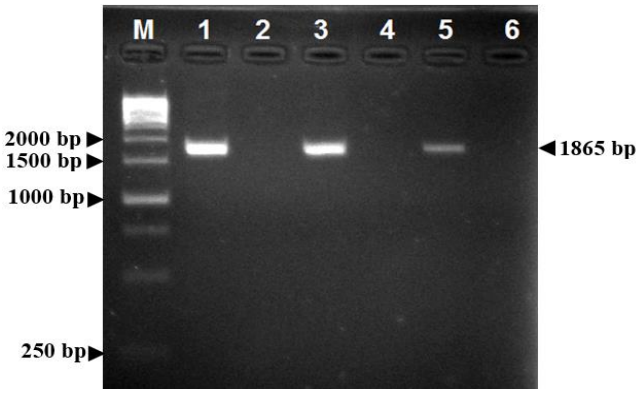


Figure 7. Results of electrophoresis of PCR products to detect *pgm* genes in *Streptococcus iniae* isolates: M: 100 bp standard marker; Lane 1: isolate RPHG36; Lane 3: isolate RPTB02; Lane 5: isolate RPBT07; Lane 6: negative control (H_2O)

Discussions

In tilapia, streptoccosis is mainly caused by two main agents, *S. agalactiae* and *S. iniae* (Shoemaker et al. 2017). The current work isolated and identified 16 *S. iniae* strains based on phenotypic features, the API 20Strep Kit, and 16S rRNA gene sequencing. Generally, the phenotypic and genotypic traits of the bacterial isolates in this investigation were similar to those of *S. iniae* previously reported (Rahmatullah et al. 2017). Specifically, they are Gram-positive, spherical, streptococci with pin-point and opaque colonies, nonmotile, catalase- and oxidase-negative (Table 2). However, the study found some differences in the physiological and biochemical characteristics of isolated *S. iniae* compared with reported *S. iniae*. For instance, the study showed that all isolates of *S. iniae* have β-type hemolytic activity, while many previous reports showed that this bacterium has two common (α or β) forms of hemolysis (Anshary et al. 2014). The isolates were not capable of oxidizing and fermenting glucose. Meanwhile, Buller (2014) described that *S. iniae* bacteria can oxidize and ferment glucose. Identification results by the API 20Strep Kit revealed that all isolates of *S. iniae* had similar characteristics to those of *S. iniae* described by Ortega et al. (2018). Interestingly, this investigation found differences in the biochemical characteristics of isolated bacterial isolates in comparison to previous studies. Specifically, isolated bacterial isolates were positive for arginine dihydrolase, β-glucuronidase, arginine dihydrolase, and glycogen but negative for pyrrolidonyl arylamidase and mannitol. However, the study of Buller (2014) showed that *S. iniae* can react positively or negatively to these criteria.

In the current investigation, the observed findings indicated that the clinical signs of the infected fish in the study were consistent with the diseased fish in the wild, with characteristic features such as disorienting or lethargic swimming, hemorrhages on the body, gill area, and also in the abdomen, pale liver, swollen spleen, and white or yellow fluid in the abdominal cavity (Figure 2). Previously, studies showed that *S. iniae*-infected fish displayed clinical signs such as popeye, corneal opacity, brain inflammation, swimming disorientation, hemorrhage, anorexia, dark skin, and abdominal fluid (Xu et al. 2024). In tilapia, as per Maulu et al. (2021), streptoccosis occurs in almost all stages of cultured fish, especially in the grow-out stage, causing heavy losses to the aquaculture industry. The main agents are *S. agalactiae* and *S. iniae*, but there is no clinical difference between these two species. Therefore, the diagnosis of the disease needs to be made based on the combination of phenotypic, and molecular features (Figure 4) for precise identification of *S. iniae* from popeye diseased tilapia. In addition, further studies need to be done to elucidate the differences or similarities in pathological characteristics between the two species of bacteria, *S. agalactiae* and *S. iniae*, in tilapia.

At least 27 species of farmed and wild fish are fatally affected by *S. iniae* bacteria, leading to significant losses in freshwater and marine finfish (Song et al. 2017; Pirollo et al. 2023). In this study, the challenge results illustrated a relatively high mortality rate in two bacterial strains, *S. iniae* RPHG36 and *S. iniae* RPBT02 (Figure 6). Similarly, the cumulative mortality in *Lates calcarifer* Bloch, 1790 reached up to 76.7% and 80% when injected with *S. iniae* strains TA1 and HTA3 at a density of 10^6 CFU/mL on day 8 post-challenge. In the present study, the LD_{50} values of two strains, RPHG36 and *S. iniae* RPBT02, were identified to be 1.81×10^4 and 2.82×10^4 CFU/mL, respectively. These results are lower than the previous reports. In China, research by Cai et al. (2016) revealed that the LD_{50} values of *S. iniae* in golden pompano, *Trachinotus ovatus* Linnaeus, 1758 was 1.47×10^7 CFU/mL. Another study by Mmanda et al. (2014), presented that the LD_{50} value of *S. iniae* causing disease in seabass was 9.65×10^6 CFU per fish. Additionally, according to research by Rahmatullah et al. (2017), the $LD_{50\text{-}336h}$ obtained from *S. iniae*-affected red hybrid tilapia was 3×10^2 CFU/mL. In Vietnam, many experiments have also been conducted to determine the virulence value of *S. iniae*. Dung et al. (2013) revealed that the virulence of *S. iniae* on climbing perch (*Anabas testudineus* Bloch, 1792) was determined with an LD_{50} value ranging from 10^3-10^5 CFU/mL after 5-6 days of infection. The bacterial virulence may be affected by many other factors, such as changing water temperature or bad environmental conditions, that will weaken the immune system of fish when pathogens enter the body to cause damage and disease (Guijarro et al. 2015; Zhang et al. 2023).

According to Sharma et al. (2017), virulence factors are primarily linked to a pathogenic microorganism's capacity to cause any disease condition in a susceptible host. Therefore, the discovery of genes related to virulence can provide a solid justification for the possible pathogenicity of *S. iniae*. In the current work, the *pgm* genes were detected in five out of sixteen strains of *S. iniae* isolated from diseased tilapia (Figure 7). Woo and Park (2014) also identified the size of phosphoglucomutase gene segments at 3,037 bp on isolated *Streptococcus parauberis* from *Paralichthys olivaceus* Temminck and Schlegel, 1846. The enzyme phosphoglucomutase can transfer the phosphate group of the simple sugar α-D-glucose from the 1' to the 6' position or vice versa from the 6' to the 1' position. The enzyme phosphoglucomutase facilitates glucose-6-phosphate and glucose-1-phosphate, plays an important role in the

production of polysaccharide envelopes, and is toxic in some pathogens, including Gram-positive and Gram-negative bacteria. By impairing the fish's immune response, avoiding phagocytosis, and resisting the lysozyme death mechanism, the aforementioned genes were in charge of *S. iniae*'s pathogenicity. The virulence factors, on the other hand, aid *S. iniae* in adhering to and invading host cells as well as hemolyzing RBCs and phagocytes (El-Tawab et al. 2022). Virulence studies have shown that *S. iniae* can adhere, penetrate, and be resistant to the host immune system (Juárez-Cortés et al. 2024). Some of the major virulence factors against *S. iniae* have been described as phosphoglucomutase, an enzyme that contributes to bacterial cell wall formation as well as antibiotic resistance (Buchanan et al. 2005) and reduces the phagocytosis of macrophages by the polysaccharide envelope on the bacterial cell surface (Gao et al. 2024). Research by Buchanan et al. (2005) described phenotypic changes in conformation, production of polysaccharide membranes, and sensitivity to the immune system of fish in the absence of phosphoglucomutase of *S. iniae* that resulted in a significant decrease in bacterial virulence. Presently, phosphoglucomutase-deficient *S. iniae* is used as an effective attenuated vaccine to protect fish during culture against infection with the deadly *S. iniae* bacteria (Buchanan et al. 2005). Thus, the presence of the phosphoglucomutase gene in two bacterial strains, *S. iniae* RPHG36 and *S. iniae* RPBT02, in the study has contributed to a better understanding of the molecular nature of virulence in bacteria. Further research on this gene and other *S. iniae*'s virulence genes is required in the future to lead to the production of vaccines based on virulence genes to control the disease in tilapia in Vietnam effectively.

In conlusion, this study has identified the bacterial strains as *S. iniae*, the pathogen responsible for popeye and hemorrhagic disease in nile tilapia (*O. niloticus*), using a combination of morphological, physiological, biochemical, and 16S rRNA gene sequencing. The results of bacterial infection satisfy Koch's postulate. *S. iniae*-infected fish exhibited signs of disease similar to those found in wild fish. The bacterial strain in this study was relatively virulent, with LD_{50} values determined to be 1.81×10^4 and 2.82×10^4 CFU/mL, respectively. In the study, two strains of bacteria, *S. iniae* RPHG36 and *S. iniae* RPBT02, both have phosphoglucomutase virulence genes, one of the factors that help *S. iniae* enhance antibiotic resistance and phagocytosis of the host immune system. These findings are important scientific information for finding effective ways to control bacteria on tilapia in the future.

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