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ORIGINAL ARTICLE

# Isolation and molecular detection of *Streptococcus agalactiae* from popped eye disease of cultured Tilapia and Vietnamese koi fishes in Bangladesh

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## ABSTRACT

**Objective:** Present research aims to isolate, identify, and determine the virulence of the *Streptococcus agalactiae* (group B *Streptococcus*; GBS), isolated from popped eye disease affected Tilapia and Vietnamese Koi (V. Koi) fishes.

**Materials and Methods:** A total of 330 fish samples were collected, of which Tilapia (*n* = 180) and V. Koi (*n* = 150), were collected from 35 affected ponds of four selected districts of Bangladesh. Isolation of the bacterium was done using different culture media (Nutrient broth, Plate count agar, Tryptic Soy Agar, and Blood agar), and identification by using various biochemical tests (conventional and using API 20 Strep kit) and polymerase chain reaction (PCR) using primers against *16S rRNA* gene of *S. agalactiae*. Antibiotic susceptibility of the bacteria was performed using seven different antibiotics disc (Tetracycline, Oxytetracycline, Chlortetracycline, Streptomycin, Ciprofloxacin, Gentamicin, and Neomycin). Virulence of the isolated *S. agalactiae* was determined by infecting healthy Tilapia and V. Koi fishes through experimental infection.

**Results:** Isolated bacteria were found Gram-positive paired and chained cocci, β-hemolytic and non-motile. Findings of biochemical and serological tests indicate that the isolated bacterium belongs to Group B *Streptococcus* of Lancefield classification. PCR result also confirmed that the bacteria were *S. agalactiae*. The bacterial isolates possessed resistance property against all the seven antibiotics used in this study. The isolated GBS was found highly virulent and showed 80%–90% mortality for Tilapia and V. Koi fishes in experimental infection within 1–6 days of post-infection.

**Conclusion:** From the findings of this study, it may be concluded that isolated GBS from the Tilapia and V. Koi fishes were highly virulent and possessed multidrug-resistance properties.

## ARTICLE HISTORY

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## KEYWORDS

Popped eye disease; *Streptococcus agalactiae*; GBS; PCR; antibiogram; pathogenicity



## Introduction

Bangladesh is one of the leading fish-producing countries in the world. In Fiscal Year 2017–18, about 42.77 lakh MT fish was produced, where aquaculture production contributes 56.24% of the total fish production. In 2018, Bangladesh ranked 3rd position in inland open water capture production and 5th in world aquaculture production according to FAO, the State of World Fisheries and Aquaculture report. Currently, Bangladesh has ranked 4th position in Tilapia production in the world and 3rd in Asia. In 2017–18, the Tilapia (*Oreochromis niloticus*) and Vietnamese Koi (V. Koi) (*Anabas testudineus*) Fish Production was 3,74,737 MT and 59,725 MT, respectively [1]. Because of easy cultivation, adaptation to a wide range of environmental conditions, faster growth, tolerant to high stocking density, Tilapia and V. Koi have been targeted as significant aquaculture efforts worldwide is relatively resistant to stress and parasitic and fungal diseases [2]. Tilapia and Koi fishes were first introduced to Bangladesh from Thailand in 1954 and 2002 [3]. Among the freshwater cultured fishes, Tilapia and V. Koi are considered highly consumed fin fishes in Bangladesh. These two fishes are highly popular among urban and rural

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people because of their availability and low cost [4]. About 18.5 million people are engaged in this sector, of which fish farmers are 13.86 million. Of the 13.86 million farmers, around 201,000 farmers are engaged in Tilapia and 23,000 in Koi cultivation [3]. There are 1.83 million ha total pond areas in Bangladesh, and the annual production of cultured fishes is 4.77 MT/ha [5].

Local fish farmers cultivate commercial Tilapia and V. Koi as good sources of income. However, infectious diseases have emerged as a significant threat to their growth and productivity. Streptococcosis is one of the most important bacterial infections for Tilapia and V. Koi worldwide. The causative agent of the disease has been attributed to *Streptococcus* spp. [6,7]. Stress is usually one of the predisposing factors resulting in Streptococcosis outbreaks, such as poor environmental conditions, rising environmental temperature, harvesting with lousy handling, transportation, and reduced water quality [8]. The disease manifestations of Streptococcosis include unilateral or bilateral exophthalmia, pale gills, corneal opacity, external hemorrhages, erratic swimming, enlarged spleen, and discoloration of the liver and ascites in the abdominal cavities [9]. It has been estimated that Streptococosis causes an annual loss equivalent to about 250 million US$ in intensive aquaculture [10].

The *Streptococcus agalactiae* infection of Tilapia and V. Koi was first noticed in the year 2014 in Bangladesh. Most of the fish farmers faced substantial financial losses due to the mass mortalities of Tilapia and V. Koi each year from 2016 until 2018. The newly emerged deadly bacterial disease of valued fin fishes Tilapia and V. Koi are known as popped eye disease. A massive outbreak of the disease has been reported from cultured ponds of four districts in Bangladesh. The aquaculture practice has been shifted from an extensive culture system to a semi-intensive and intensive culture system where highly valued fin fishes are cultivated at high stocking density using commercial fish feeds [11,12]. The faster expansion of the aquaculture industry leads to the increased chances of several pathogens outbreaks. Probably, these diseases are the major devastating threat to the intensive fish culture, resulting in substantial economic losses due to the high mortality of cultured fishes.

For this reason, the present study was undertaken to isolate and identifies Group B *Streptococcus* (GBS) from popped eye diseased cultured Tilapia and V. Koi fishes associated with mass mortality in Bangladesh and the determination of its multi-drug resistance properties and pathogenicity in the healthy Tilapia and V. Koi by aquarium-based experimental infection.

## Materials and Methods

### *Ethical approval*

The experiments used in this study for the isolation of microorganisms and establishment of experimental infection purposes were approved by the Animal Welfare and Experimentation Ethics Committee (AWEEC) of Bangladesh Agricultural University, Mymensingh-2202 [AWEEC/BAU/2017(7)].

### *Sampling*

Dead Tilapia and V. Koi fishes were collected from 35 ponds of Gazipur (23° 59' 59.7876'' N and 90° 25' 12.9828'' E), Mymensingh (24°44''36.4128'' N and 90°23'54.1824'' E), Kishoreganj (24°22''–24°32'' N latitudes and 90°01''–91°01'' E longitudes) and Netrokona (24°47''–24°58'' N latitudes and 90°38''–90°50''E longitudes) districts during outbreaks starting from April to June of the year 2017, 2018, and 2019 (Fig. 1). A total of 330 fish samples, including Tilapia ($n$ = 180; body-weight ranging from 115 to 130 gm; Fig. 2A) and V. Koi ($n$ = 150; body-weight ranging from 100 to 120 gm; Fig. 2D), were collected from the affected ponds. During collection, symptoms, such as exophthalmia (popped eyes), erratic swimming, sluggish movement, swimming close to the surface layer of the water, lethargy, loss of appetite, and no escape reflex, were observed. The infected fishes were collected into sterile separate zipper plastic bags and transported to the Bacteriology


**Figure 1.** The filled red circle of the Map showing the fish (Tilapia and V. Koi) samples collected areas of the four different districts of Bangladesh.

Laboratory, Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh, maintaining a cool-chain.

### *Isolation of the S. agalactiae*

Liver, kidney, and brain samples were collected to isolate the virulent bacterium from Tilapia (Fig. 2B and C) and V. Koi (Fig. 2E and F) fishes by a post-mortem. The samples were characterized by encephalomalacia, enlargement of the liver, spleen, kidney, and hydro-peritoneum. The samples were inoculated initially into Tryptic Soya Broth and incubated at 37°C for 24–48 h. After that, the cultured broth was inoculated by streaking onto Tryptic Soy Agar (TSA) and incubated at 37°C for 24–48 h for the observation of colony morphology.

### *Morphological and biochemical characterization of the isolated S. agalactiae*

A loopful cultured broth and individual colony from the 24 h of incubated each sample (liver, kidney, and brain) was stained by Gram stain [13] and observed under 100× objectives for the visualization of the color, shape, and arrangement of the isolated bacteria. For biochemical tests, the bacterium was cultured in Brain Heart Infusion Broth (BHI), TSA, and Blood Agar (BA) and incubated at 37°C for 24 h. Broth and colonies of the isolated bacteria were used for a series of biochemical tests, *viz*., Voges–Proskauer (VP), Methyl Red (MR), Arginine Dihydrolase (ADH), Glucose, Oxidative-Fermentative (O/F), Catalase, Oxidase, Indole, Mannitol and Lactose, as per the methods described in the Bergey's Manual of Determinative Bacteriology [14]. Finally, API 20 Strep kit (bioMerieux Inc., Durham, NC) was used to identify *S. agalactiae*, as per the manufacturer's instruction. The test results were obtained at 4 and 24 h of incubation for the identification of *S. agalactiae* according to the profile index and table issued by the manufacturer [15].

### *Serotyping of the isolated bacteria by Lancefield grouping*

Using a commercial streptococcal grouping kit (Oxoid Ltd., Basingstoke, UK), the isolated bacteria were serologically confirmed as group B *Streptococcus* of the Lancefield grouping [16]. Methods followed for the serology were as per instructions of the manufacturer.

### *Molecular identification of the isolated bacteria using polymerase chain reaction PCR*

For the molecular detection of the isolated bacteria, seven representative isolates (four isolates from Tilapia and three isolates from V. Koi) were chosen to run the PCR assay. The isolates were cultivated in 5 ml of BHI broth. Centrifugation was done to collect the cell pellets and suspended them in 100 µl of Tris acetic acid EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The genomic deoxyribonucleic acid (DNA) was extracted using a commercial DNA extraction kit (Promega, Madison, WI). The oligonucleotide primers, F1 (5'-GAG TTT GAT CAT GGC TCA G-3') and R (Reverse)-(5'-ACC AAC ATG TGT TAA TTA CTC-3')

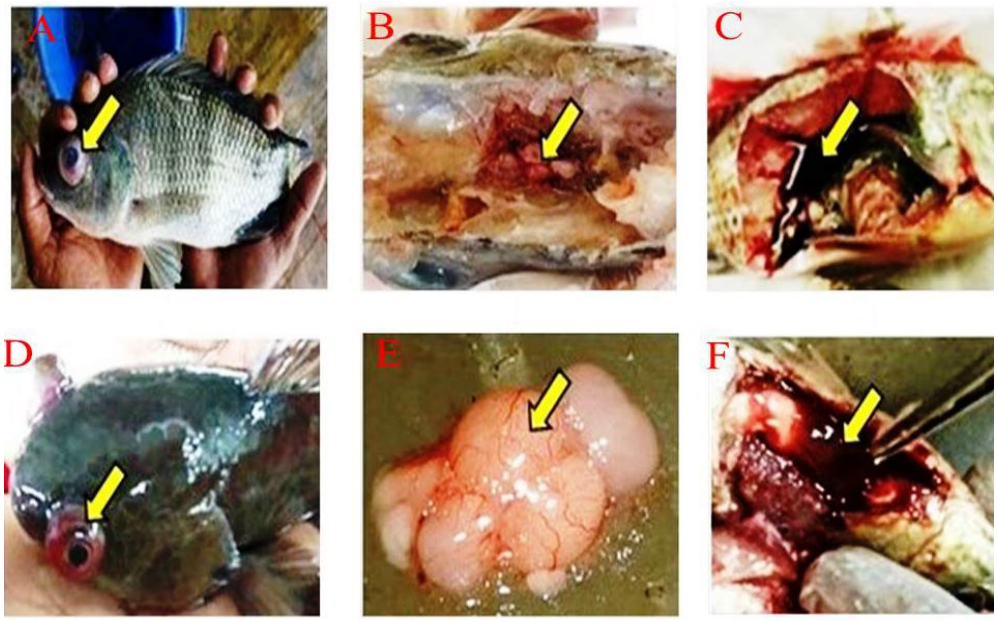


**Figure 2.** Various lesions in different organs of dead Tilapia and V. Koi fishes infected naturally by *S. agalactiae* (GBS) image (A) showing exophthalmia, (B) hyperemia of meninges and (C) enlargement of the liver in the dead Tilapia fish; image (D) showing exophthalmia, (E) hyperemia of meninges, and (F) enlargement of the liver in the dead V. Koi fish.

were used for amplifying a 220-bp amplicon from the *16S rRNA* gene of *S. agalactiae* [17]. The amplification of target DNA was done in a thermal cycler (Mastercyclear, Eppendorf, Germany). The reaction mixture composed of genomic DNA, primers F1 and R (0.2 pM of each primer), 1 × AmpliTaq DNA polymerase buffer, 100 pM of each dNTP, and 1.25 U of AmpliTaq DNA polymerase. The cycle settings included an initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 60 sec, primer annealing at 55°C for 60 sec, and extension at 72°C for 60 sec, with the final extension at 72°C for 10 min. Finally, the PCR products were checked for expected size in agarose gel electrophoresis (Biometra, Germany) and UV transilluminator [18]. After confirmation of the isolates using morphological, biochemical, serological, and PCR, the pure culture was stored at −86°C by keeping it into a mixture of 50% glycerol and BHI broth for future analysis.

### *Antibiotic sensitivity pattern of isolated S. agalactiae*

Antimicrobial susceptibility was analyzed according to Kirby–Bauer' method [19]. The isolated bacteria (*S. agalactiae)* were propagated on BHI agar and incubated at 30°C for 24 h. The bacteria were harvested and diluted the turbidity, which was equivalent to a MacFarland No. 0.5 standard solution, followed by spreading onto triplicate Mueller-Hinton Agar (Oxoid Ltd, Basingstoke, UK). Tetracycline (30 µg/disc), Oxytetracycline (30 µg/disc), Chlortetracycline (25 µg/disc), Ciprofloxacin (5 µg/disc), Streptomycin (10 µg/disc), Gentamicin (10 µg/disc), and Neomycin (30 µg/disc) were used for antibiotic sensitivity determination. After setting the antimicrobial discs on the freshly inoculated Mueller-Hinton agar, the plates were incubated at 37°C for 24–48 h. After incubation, the zone of inhibition diameter was calculated and interpreted as per the recommendation of CLSI [20].

### *Determination of pathogenicity of the isolated S. agalactiae*

#### *Selection and management of Tilapia and V. Koi fishes*

Each forty-five adult Tilapia (average body-weight 220 gm) and V. Koi (average body-weight 135 gm) were obtained from two commercial ponds. Fishes were collected randomly, and performing microbiological examinations to confirm that they have no disease or abnormality. The fishes were maintained in a separate glass aquarium (120 l) at the Bangladesh Fisheries Research Institute (BFRI), Mymensingh, for 3 weeks. They were kept at least 1 week for adaptation to the environment. The fishes were fed twice daily with commercial fish feed at 2% of their body weight until completion during the adaptation period. The fishes were starved for 24 h before the introduction of infection. The water was checked continuously and changed with 50% fresh water daily. The parameters of water quality were measured using Yellow Spring Instruments (YSI) 85 (pH 7.2 ± 0.3, temperature 29°C ± 1.2°C, DO 5.8 ± 1.2 mg/l and ammonia 0.3 ± 0.1 mg/l) [21].

#### *Preparation of bacterial inoculum*

The isolated and purified stock of GBS was allowed to grow onto the BHI agar at 30°C for 24 h. Several identical colonies of the bacteria were inoculated into 10 ml BHI broth for 24 h at 30°C. After incubation, the broth was centrifuged at 15,000 rpm for 15 min at 4°C, and the pellet was obtained. The phosphate-buffered saline (PBS) was used to wash the pelleted bacteria [22]. The bacterial suspensions were diluted with sterile saline solution (0.85%) to reach the concentration at $7.0 \times 10^7$ colony forming unit (CFU)/ml [23] and $1.0 \times 10^7$ CFU/ml by a 10-fold serial dilution [24].

#### *Experimental design for infection*

Tilapia and V. Koi fishes were divided into two separate experimental groups. Three trial experiments were designed as 1st, 2nd, and 3rd for two species of fishes. Each experimental trial group was further divided into two sub-groups based on the route of infection, *viz*., oral and intraperitoneal (IP), including one control. Each group comprised five fishes kept in a 120-l glass aquarium. The oral group was inoculated with 0.1 ml of $7.0 \times 10^7$ CFU/ml of *S. agalactiae,* according to Iregui et al*.* [23], and the IP group inoculated with 0.1 ml of $1.0 \times 10^7$ CFU/ml of *S. agalactiae,* according to Pereira et al*.* [24]. Simultaneously, the control group was injected with 0.2 ml of PBS as a mock control. All fishes were observed twice daily to record any clinical signs, including the abnormal behavior or mortalities, from the inoculation of bacteria until day-14 of post-infection (p.i.).

#### *Re-isolation of GBS from the experimentally infected dead fishes*

Tissue samples (liver, kidney, and brain) were collected from the experimentally infected Tilapia and V. Koi fishes immediately after their death. Re-isolation of GBS from dead fishes was done using various bacteriological media as used previously for field samples. Bacterial isolates from experimentally infected Tilapia and V. koi fishes were subjected to identification using various biochemical tests and molecular assays.

## Results

### *Percent mortality of Tilapia and V. Koi fishes of different outbreak years*

Percent mortality of cultured Tilapia and V. Koi fishes of four districts of the outbreak years from 2016, 2017, and 2018 were recorded. The highest rate of mortality of Tilapia fishes

of the year 2016 was recorded in Gazipur (93%), followed by Kishoreganj (89%), Mymensingh (81%), and Netrokona districts (73%). In 2017, the mortality rate was 90%, 87%, 79%, and 66% in Gazipur, Kishoreganj, Mymensingh, and Netrokona districts, respectively. On the other hand, the fish mortality rate in the year 2018 was recorded as 90%, 87%, 82%, and 80% in Mymensingh, Gazipur, Netrokona, and Kishoreganj districts, respectively (Fig. 3).

On the contrary, the highest rate of mortality of V. Koi fish of the year 2016 was recorded in Mymensingh (96%), followed by Gazipur (87%), Netrokona (79%), and Kishoreganj districts (77%). In 2017, the mortality rate was 91%, 86%, 82%, and 72% in Mymensingh, Gazipur, Kishoreganj, and Netrokona districts, respectively. In contrast, in 2018, the mortality rate was 79%, 78%, 73%, and 60% in Gazipur, Netrokona, Mymensingh, and Kishoreganj districts, respectively (Fig. 3).

### *Total viable count (TVC) and total streptococcus count (TSC) of Tilapia and V. Koi*

Total viable count (TVC) calculated from the liver, kidney, and brain tissue of Tilapia fishes was $7.9 \times 10^7$ CFU/gm, $6.6 \times 10^7$ CFU/gm, and $5.9 \times 10^4$ CFU/gm, respectively. Similarly, TSC was $7.4 \times 10^6$ CFU/gm, $5.5 \times 10^5$ CFU/gm, and $2.8 \times 10^4$ CFU/gm, respectively. On the other hand, TVC calculated from the liver, kidney, and brain tissue of V. Koi was $4.4 \times 10^6$ CFU/gm, $4.1 \times 10^5$ CFU/gm, and $8.4 \times 10^2$ CFU/gm, respectively, whereas the TSC was $3.2 \times 10^5$ CFU/gm, $2.2 \times 10^4$ CFU/gm, and $3.5 \times 10^2$ CFU/gm, respectively (Table 1).

### *Morphological and biochemical characteristics of the isolated S. agalactiae*

The bacteria isolated from both the Tilapia and V. Koi fishes on TSA have shown pen-headed, white opaque color, circular, entire raised edges, glistening colony and found as Gram-positive, paired, and chain-forming coccus. The isolated *S. agalactiae* developed small translucent colonies with a clear thin/broad zone of β-hemolysis on BA media. Biochemical tests indicated that the isolates were positive to VP, MR, ADH, Glucose, and Oxidative–Fermentative tests, whereas negative to Catalase, Oxidase, Indole, Mannitol, and Lactose tests. The bacteria also manifested similar results by the API 20 Strep kit.

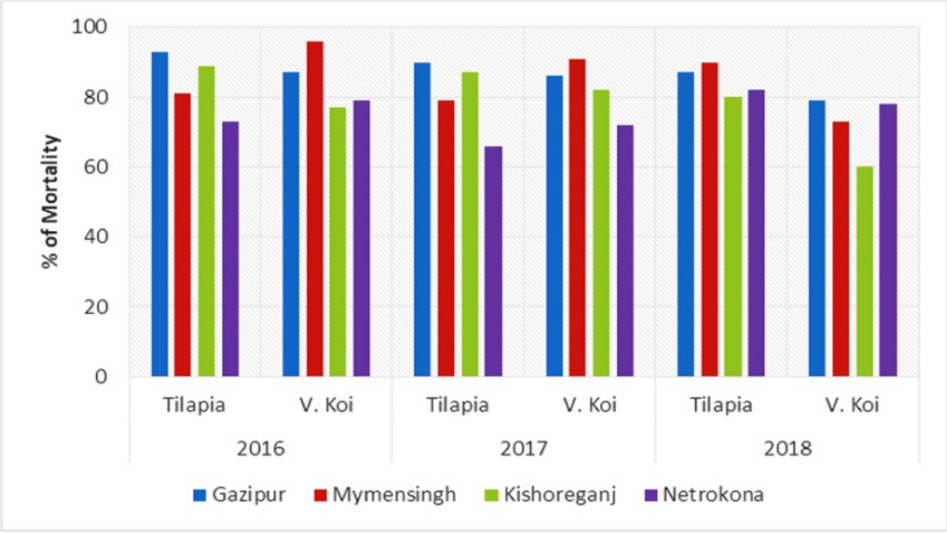


**Figure 3.** Showing mortality percent of Tilapia and V. Koi fishes of four districts during outbreak years, 2016–2018.

**Table 1.** TVC and TSC from different organs of naturally infected dead Tilapia and V. Koi fishes.

| Organ of the fishes | Tilapia | | V. Koi | |
|---|---|---|---|---|
| | TVC (CFU/gm of sample) | TSC (CFU/gm of sample) | TVC (CFU/gm of sample) | TSC (CFU/gm of sample) |
| Liver | $7.9 \times 10^7$ | $7.4 \times 10^6$ | $4.4 \times 10^6$ | $3.2 \times 10^5$ |
| Kidney | $6.6 \times 10^7$ | $5.5 \times 10^5$ | $4.1 \times 10^5$ | $2.2 \times 10^4$ |
| Brain | $5.9 \times 10^4$ | $2.8 \times 10^4$ | $8.4 \times 10^2$ | $3.5 \times 10^2$ |

### *Serotyping of the isolated S. agalactiae by Lancefield grouping*

Serotyping of all the *S. agalactiae* isolated from different organs (liver, kidney, and brain) of the dead Tilapia and V. Koi fishes showed clear agglutination reactions with the group B specific antiserum of streptococcal antigen grouping kit, indicating that the bacteria were group B *Streptococcus* (GBS) (Table 2).

### *Molecular detection of S. agalactiae*

The isolated organisms were subjected to PCR using gene-specific primers against *16S rRNA* gene and confirmed that all the isolates were *S. agalactiae* (Fig. 4). A similar PCR result was found in the re-isolated samples from both the Tilapia and V. Koi in an aquarium-based experimentally induced infection (Fig. 5).

### *Antibiotic susceptibility pattern of the isolated bacteria*

The antibiogram profile of the bacteria (GBS) isolated from Tilapia and V. Koi fishes were found resistant against all the seven conventional and latest generations of antibiotics used in this study.

### *Pathogenicity results of the isolated S. agalactiae*

Development of clinical signs, like unilateral or bilateral exophthalmia, erratic swimming, pale gills, corneal opacity, external hemorrhages, and ascites, was started in Tilapia and V. Koi fishes of each (1st, 2nd, and 3rd) trial group, infected experimentally with GBS either through the oral or IP route of infection within day 1 of p.i., and continue dying until day-6 of p.i. In contrast, the fishes of mock (PBS) control groups did not die. They even failed to show single signs of infection as manifested by GBS-infected fishes during the entire period (14 days) of observation.

In this study, the highest mortality (100%) of the Tilapia fishes of all the trial groups (1st-3rd trial groups) was recorded between days 1th and 6th of p.i. through the IP route with the GBS. The mortality rate of Tilapia fishes of similar trial groups (1st–3rd trial groups) was

**Table 2.** Latex agglutination test for identifying *Streptococcal* groups (A, B, C, D, F, and G) of bacteria isolated from Tilapia and V. Koi fishes.

| Species of fishes | Type of tissues selected for the isolation of *Streptococcus* spp. | Lancefield grouping of *Streptococcus* spp using streptococcal antigen grouping kit | | | | | | Interpretation of serotyping of the fish isolates of *Streptococcus* spp |
|---|---|---|---|---|---|---|---|---|
| | | GroupA | Group B | Group C | Group D | Group F | Group G | |
| Tilapia | Liver | – | + | – | – | – | – | GBS |
| | Kidney | – | + | – | – | – | – | GBS |
| | Brain | – | + | – | – | – | – | GBS |
| V. Koi | Liver | – | + | – | – | – | – | GBS |
| | Kidney | – | + | – | – | – | – | GBS |
| | Brain | – | + | – | – | – | – | GBS |

+ indicates positive agglutination; – indicates negative agglutination; GBS indicate group B *Streptococcus*.

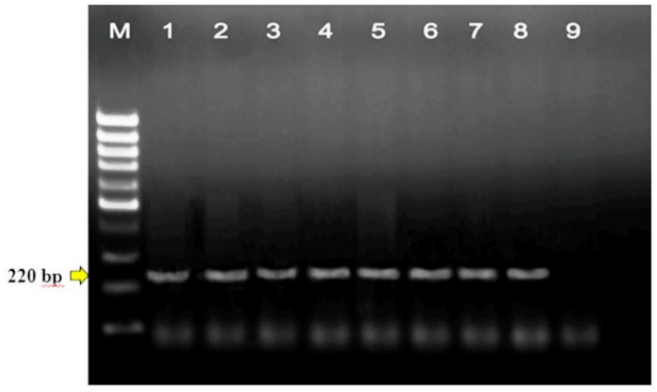


**Figure 4.** Image of 2% agarose gel electrophoresis showing the PCR amplification product of the 16SrRNA of *S. agalactiae* (GBS) isolated from naturally infected diseased Tilapia and V. Koi. M indicates 100-bp DNA ladder; Lane 1 is positive control; Lane 2–5 are positive isolates of *S. agalactiae* from Tilapia; Lane 6–8 are positive isolates of *S. agalactiae* from V. Koi, and Lane-9 is a negative control.

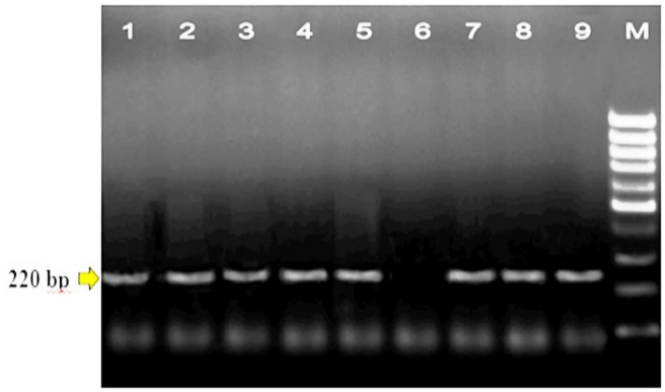


**Figure 5.** Image of 2% agarose gel electrophoresis showing the PCR amplification product of the *16SrRNA* of *S. agalactiae* (GBS) re-isolated from experimentally infected Tilapia and V. Koi; Lane 1 is positive control; Lane 2–5 are positive isolates of *S. agalactiae* from Tilapia; Lane 6 is a negative control, and Lane 7–9 are positive isolates of *S. agalactiae* from V. Koi; M indicates 100 bp DNA ladder.

80%–100%. The fishes started to die from day 1 to 6 of p.i. when infected through the oral route of infection (Table 3).

On the contrary, the highest mortality rate (100%) of the V. Koi fish was noticed in all the trial groups (1st–3rd trial groups) within 1–6 days of p.i. through the IP route with a similar GBS. However, the rate of mortality of V. Koi fishes of similar trial groups (1st–3rd trial groups) varied from 80% to 100% when infected through the oral route of infection (Table 3). The remaining 20% survived-fishes of either species (Tilapia and V. Koi); those showed mild symptoms of diseases in experimental infection in three trial groups 1st–3rd recovered gradually within 7–14 days p.i. (Table 3).

Post-mortem findings of the dead Tilapia (Fig. 6 A–C) and V. Koi (Fig. 6 D–F) fishes of experimental infection revealed similar characteristic symptoms as the fishes of field outbreak like exophthalmia, encephalomalacia, and pathological lesions in the internal organs like enlargement of liver, spleen, kidney, and hydro-peritoneum.

## Discussion

Streptococcosis is one of the leading causes of death of cultured Tilapia and V. Koi fishes globally, caused by *S. agalactiae* (GBS). In Bangladesh, this disease (popped eye disease) has appeared as an emerging threat for cultured Tilapia and V. Koi fishes since 2016–2018, particularly in four districts of Bangladesh, namely Gazipur, Mymensingh, Kishoreganj, and Netrokona. The nature of the disease and the rate of fish mortality were beyond control during the outbreak years.

In this study, we isolated and identified the causal agent and determined the virulence of the newly emerged pathogen of Tilapia and V. Koi fishes' popped eye diseases. The pathogenicity of isolated bacteria was determined by infecting healthy Tilapia and V. Koi fishes by experimental infection.

Dead fishes (Tilapia and V. Koi) were collected from the affected ponds showing characteristics of clinical symptoms including lethargy, unilateral or bilateral exophthalmia, corneal opacity, and distension of the abdomen. The distension of the abdomen is caused due to the excessive accumulation of water in the abdomen. Post-mortem pathognomonic lesions of Streptococcosis included severe congestion in the brain, enlargement of the liver, spleen, kidney, and hydro-peritoneum. The dead Tilapia and V. Koi fishes showed characteristics clinical symptoms, and post-mortem lesions in their internal organs of the present study have similarities with the signs and lesions manifested by the two species of cultured fishes [9].

Isolation of bacteria from organs (liver, kidney, and brain) of dead Tilapia and V. Koi fishes indicated that the TVC was found relatively higher ($7.9 \times 10^7$ CFU/gm for Tilapia and $4.4 \times 10^6$ CFU/gm for V. Koi) in the liver compared to that of the kidney ($6.6 \times 10^7$ CFU/gm for Tilapia and $4.1 \times 10^5$ CFU/gm for V. Koi) and brain ($5.9 \times 10^4$ CFU/gm for Tilapia and $8.4 \times 10^2$ CFU/gm for V. Koi). Isolation of TSC from the liver, kidney, and brain of dead Tilapia and V. Koi fishes were also found higher ($7.4 \times 10^6$ CFU/gm for Tilapia and $3.2 \times 10^5$ CFU/gm for V. Koi) in the liver compared to that of the kidney ($5.5 \times 10^5$ CFU/gm for Tilapia

**Table 3.** Determination of pathogenicity of the isolated *S. agalactiae* (GBS) in the healthy Tilapia and V. Koi fishes by aquarium-based experimentally induced infection.

| Experimental groups | Hosts | No. of fishes used per trial | Bacterial isolate | Routes and doses of inoculated bacteria and PBS | | | Number and % mortality of fishes (Day 1-day 6 of p.i.) | | | Number and % of sick/ recovered fishes (Day 7-day 14) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Oral (0.1 ml/dose of $7 \times 10^7$ CFU/ml) | IP (0.1 ml/dose of $1 \times 10^7$ CFU/ml) | Mock control group IP injection of PBS (0.2 ml/ dose) | Oral | IP | Mock control group (PBS) | Oral | IP | Mock control group (PBS) |
| 1st trial | for Tilapia | 15 | *S.agalactiae* (GBS) (field isolate) | 5 | 5 | 5 | 4 (80%) | 5 (100%) | 0 | 1 (20%) | 0 (0%) | 0 |
| 2nd trial | | 15 | | 5 | 5 | 5 | 5 (100%) | 5 (100%) | 0 | 0 (0%) | 0 (0%) | 0 |
| 3rd trial | | 15 | | 5 | 5 | 5 | 4 (80%) | 5 (100%) | 0 | 1 (20%) | 0 (0%) | 0 |
| 1st trial | for V. Koi | 15 | | 5 | 5 | 5 | 4 (80%) | 5 (100%) | 0 | 1 (20%) | 0 (0%) | 0 |
| 2nd trial | | 15 | | 5 | 5 | 5 | 4 (80%) | 5 (100%) | 0 | 1 (20%) | 0 (0%) | 0 |
| 3rd trial | | 15 | | 5 | 5 | 5 | 5 (100%) | 5 (100%) | 0 | 0 (0%) | 0 (0%) | 0 |

IP indicates the IP route of inoculation; p.i. Indicates post-infection; CFU indicates colony-forming unit; The routes and doses of bacterial (GBS) inoculum used in the experimental infection of this study were followed as per the routes and doses used by Iregui et al. [22] and Pereira et al. [24].

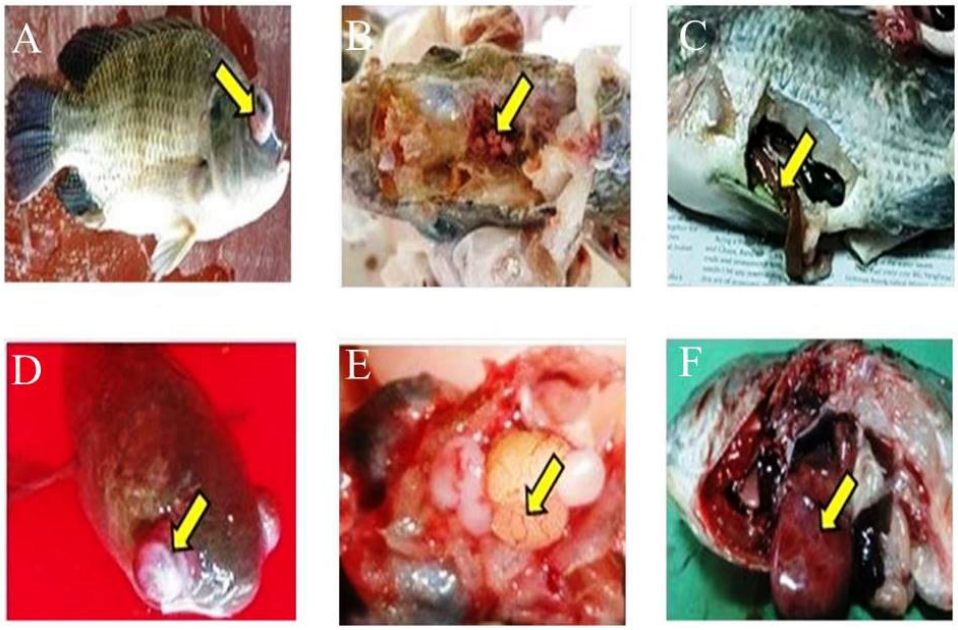


**Figure 6.** Various lesions are manifested by Tilapia and V. Koi fishes when infected experimentally with *S. agalactiae* (GBS) image (A) showing exophthalmia, (B) hyperemia of meninges and (C) enlargement of the liver of Tilapia fish, and the image (D) showing exophthalmia, (E) hyperemia of meninges and (F) enlargement of the liver of the V. Koi fish.

and $2.2 \times 10^4$ CFU/gm for V. Koi) and brain ($2.8 \times 10^4$ CFU/gm for Tilapia and $3.5 \times 10^2$ CFU/gm for V. Koi) in this study. The isolation of *S. agalactiae* indicates that the bacterial load was higher in the liver than that of the kidney and brain. The lower concentration of GBS in the brain than that of both fishes' liver and kidney might be due to the blood-brain barrier or the blood-cerebrospinal fluid barrier [25]. The presence of *S. agalactiae* in the brain of both species of fishes indicated that the bacteria were highly pathogenic and possessed multi-organ invasive power.

The GBS isolated from Tilapia and V. Koi fishes was Gram-positive cocci tending to form pairs and chains and possessing β-hemolytic and non-motile properties. The Lancefield sero-grouping classification indicated that the isolated *Streptococcus* spp. was group B *Streptococcus* [26], as supported by several other reports [27,28]. However, they identified the GBS from popped eye disease within a few days after the disease onset. Similar to our finding, other researchers also did PCR-confirmation of the GBS [17,18].

Possessing the GBS's multidrug-resistance property revealed in this study indicated that there might have inappropriate use of antibiotics for Tilapia and V. Koi's aquaculture. The antibiogram profiles found in this study were agreed with several other reports [29–31]. In another study, Ali et al. [29] reported that *S. agalactiae* isolated from red Tilapia in Malaysia possessed resistance property against Neomycin and Gentamicin. Geng et al. [30] also stated that the bacteria (*S. agalactiae*) isolated from Tilapia showed Gentamicin resistance, whereas Hardi [31] reported that *S. agalactiae* isolated from Tilapia showed resistance to Tetracycline as well.

Most of the Tilapia and V. Koi fishes infected through the IP route showed the highest mortality rate (100%) within 1–6 days of p.i. The results of fish mortality in the experimental infection through the IP route agreed with another study's finding [24]. Pereira et al. [24] reported that the IP route showed 100% mortality after 1–6 days of p.i. in Tilapia fish. The result of mortality of Tilapia and V. Koi fishes by GBS through oral route infection was 86.6%–90% within 1–6 days of p.i., respectively. The rate of mortality of either species of fishes in the experimental condition of the present study agrees with the findings of Iregui et al. [23]. Their studies stated that the oral route of infection of healthy red Tilapia by GBS showed 80%–90% mortality within 1–6 days of p.i.

The clinical manifestations observed in the experimentally infected Tilapia and V. Koi fishes supported other researchers' reports [32,33]. They reported that the experimentally-infected Nile Tilapia with GBS resulted in clinical signs like unilateral or bilateral exophthalmia, corneal opacity, and hydro peritoneum. The post-mortem findings of the dead Tilapia and V. Koi fishes of the experimental infection revealed characteristic pathological signs of Streptococcosis like encephalomalacia, enlargement of the liver, spleen, kidney, and hydro peritoneum. The post-mortem findings observed in this study are similar to the previous reports [34].

The death and survivability patterns of the Tilapia and V. Koi fishes in the experimental infection through two routes of infection by GBS were interesting. Around 15%–20% of fishes of either species (Tilapia and V. Koi) showed mild symptoms of diseases in the experimental condition in each of the three trial groups recovered gradually within 7–14 days of p.i. However, the reasons for survivability of the 15%–20% of both the Tilapia and V. Koi fishes in an experimental infection with virulent GBS are not very clear yet. It is assumed that some factors are involved in their survivability, like possessing strong innate immunity, the gradual development of adaptive immunity, better nutrition status, and cross-immunity by other species. The results of this study highly aline with the findings of the earlier study. The clinical signs and occurrence of tissue damage leading to death tend to appear slower for the oral route of infection than that of IP infection by GBS [35]. The reasons for the variation of fish mortality through two routes of infection by GBS in the experimental condition were not clear. However, it can be speculated that the non-specific mucosal immunity of skin and gut might play essential roles in the survivability of 15%–20% mild infected gradually recovered fishes in the experimental infection [36,37]. More studies are needed on the circulation of *S. agalactiae* in Tilapia and V. Koi fishes to allow selection of therapeutics that can be more effective. The present study's findings would help us with adequate and suitable measures to prevent and control future disease outbreaks. There might be another approach of controlling popped eye diseases in cultured Tilapia and V. Koi fishes by using an effective vaccine prepared with the locally circulating strain of *S. agalactiae*.

## Conclusions

The pathogenic bacterial isolate recovered from dead Tilapia and V. Koi of almost all ponds have been confirmed as *S. agalactiae*. The isolated bacteria contain multidrug-resistance properties. The GBS isolated from Tilapia and V. Koi is equally pathogenic and virulent in aquarium-based experimental infection of healthy Tilapia and V. Koi fishes. The death pattern and the rate of morbidity and mortality of either fish species in experimental infection are similar to that of natural disease caused by *S. agalactiae.* From the present study's findings, it may be recommended that abuse of antimicrobial agents should be stopped in controlling infectious diseases of cultured fishes. Otherwise, the prevalence of multidrug-resistant bacteria will be increased and drain out to the environment, which is extremely harmful to humans and other living individual. The best way of controlling popped eye diseases of cultured Tilapia and V. Koi fishes caused by *S. agalactiae* is to start to use an effective vaccine prepared with the locally circulating field isolate of the bacteria.

## List of abbreviations

V. koi = Vietnamese koi, GBS = group B *Streptococcus*, CFU = Colony Forming Unit, h = hour, min = minutes, sec = seconds, DNA = Deoxyribonucleic acid, PCR = Polymerase Chain Reaction, BFRI = Bangladesh Fisheries Research Institute, MT = Metric ton, FAO = Food and Agricultural Organization, CLSI = Clinical and Laboratory Standards Institute, DO = Dissolved oxygen.


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## Conflict of interest

The authors declare that they have no conflict of interest.

## Authors' contribution

MMR designed methodology, formal analysis, design concept investigation, and writing an original draft. MAR, MSM, and MEH also design concept, writing-review, and Editing. AKMK and MTH contribute to reviewing and editing the manuscript. MPS, MTR, and MAI were involved in the study design, supervision, resources, validation, review and writing, and project administration.

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