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Pathological and microbiological characterisation of a streptococcosis outbreak in Genetically Improved Farmed Tilapia, reared in a brackish water cage farm, India

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Original Article

Abstract

Genetically Improved Farmed Tilapia (GIFT) is a scientifically validated species for brackish-water cage aquaculture. However, only limited information exists regarding the disease occurrences under cage farming conditions in India. The present study reports a disease outbreak incident in GIFT from a cage farm, six months post-stocking, resulting in a cumulative mortality of 45% over two weeks. Necropsy and microbiological analyses consistently isolated a common bacterium from different moribund fish. Experimental infection trials confirmed Koch's postulates, with an estimated LD_{50} of $6.5 \times 10^{6.65}$ CFU/fish via intramuscular injection. The pathogen was identified as *Streptococcus agalactiae,* and its Multiple Antibiotic Resistance (MAR) index was 0.08, indicating a low level of antibiotic resistance risk. Serum bactericidal assays revealed the pathogen's ability to proliferate in healthy tilapia serum, with significantly enhanced serum survival at elevated temperatures ($p \leq 0.05$). Histologically, infected fish tissues revealed mild congestion and haemorrhage in the brain, multifocal hemosiderin deposits, sub-capsular haemorrhage, capsular thickening and macrophage infiltration in spleen, primary lamellar congestion, secondary lamellar hyperplasia and fusion in gills, and necrotic changes of hepatic parenchyma. This report provides the first description of *S. agalactiae* infection in brackish-water cage-cultured GIFT in India, highlighting a potential health threat to tilapia aquaculture in such environments.

Keywords: *Streptococcosis, GIFT, cage farming, antibiotic resistance, lamellar hyperplasia*

Introduction

Nile tilapia (*Oreochromis niloticus*), the second-most farmed fish globally (FAO, 2020), has gained prominence due to its high productivity and value as a protein source and economic commodity (Vásquez-Machado *et al.,* 2019). The Genetically Improved Farmed Tilapia (GIFT) strain, developed through selective breeding in Malaysia and introduced to India via ICAR-Central Institute of Freshwater Aquaculture (ICAR-CIFA) and WorldFish (Singh and Lakra, 2011), is particularly suited for brackish-water cage farming due to its salinity tolerance (Imelda *et al.*, 2014). Recognised as a priority species for Indian aquaculture, GIFT is being promoted by ICAR-Central Marine Fisheries Research Institute (ICAR-CMFRI) as an alternative to shrimp farming during the freshwater influx of the southwest monsoon (Imelda *et al.,* 2014). However, intensified farming increases stress and disease susceptibility in fish (Klesius *et al.,* 2008), and limited data are available on diseases affecting GIFT in brackish-water cages.

Streptococcosis, a major disease in tilapia farming worldwide (Amal *et al.,* 2015; Delphino *et al.,* 2019). Economic losses due to streptococcosis in fish were estimated at USD 250 million annually (Klesius *et al.*, 2008). The infection is caused by a variety of streptococcal species, of which *S. agalactiae* and *S. iniae* are the most important (Evans *et al.,* 2002). *S. agalactiae* is particularly virulent, having been reported in at least 17 fish species globally, with outbreaks causing high

mortality and economic loss (Amal and Zamri-Saad, 2011). The economic losses associated with *S. agalactiae* have been estimated at USD 10 million in the USA, with USD 100 million globally (Bowater *et al.,* 2018). In India, *S. agalactiae* was first reported in freshwater pond-cultured tilapia (Adikesavalu *et al*., 2017), followed by another report from *cage-cultured Etroplus suratensis (*Girijan *et al*., 2023). The study further characterises the pathogen's microbiological and virulence traits and describes associated histopathological changes in various tissues of infected fish. This represents the third report of *S. agalactiae* in India and the first from brackish-water cage culture systems.

Material and methods

Background

Moderate mortalities were observed among GIFT fish (348 ± 60.5 g) reared in high-density polyethene floating cages (6 × 6 × 1.5 m) located in a brackish-water system at Poothotta, Kochi, Kerala (9°85′N; 76°38′E). The cages were stocked with 2200 juveniles of ~5 cm lengths in early March 2017 and fed commercial dry pellets (Growel India Pvt. Ltd, India) thrice daily. Mortalities began in September 2017, starting with 2 to 4 fish/day and escalating to 5 to 6% daily within three days by mid-September. Affected fish showed circling, surfacing, anorexia, lateral recumbency, and a cumulative mortality of ~45% over two weeks. Survivors gradually recovered after three weeks.

Disease investigation

Moribund fish showing circling behaviour were transported live to the fish health laboratory, ICAR-CMFRI, Kochi. Water parameters at the site were salinity: 6 ppt, temperature: 29 °C, and pH: 7.1. Gross lesions were inspected; skin and gill smears were examined for parasites. Blood sampling was through the caudal vasculature and inoculated directly onto Brain Heart Infusion Agar (BHIA). Further, necropsy was conducted under aseptic conditions and tissues from various internal organs, namely liver, brain, kidney, and spleen, were collected. The tissues were individually homogenised in sterile saline, streaked onto BHIA, and incubated at 28 °C for 24-48 h. Resulting colonies were purified and preserved on BHIA slants and as glycerol stocks.

Histopathology

Liver, spleen, gill, and brain tissues were fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin, sectioned at 4 µm, and stained with haematoxylin and eosin (Sumithra *et al*., 2019).

Phenotypic characterization

Morphological and biochemical tests (Table 1) were performed for preliminary pathogen identification (Collins *et al.,* 2004; Austin and Austin, 2012; Bergey *et al.,* 2009). Additional assays included lecithinase on egg yolk agar, haemolysis on ruminant blood agar, proteolysis on skim milk agar, siderophore production on Chrome azurol S (CAS) agar (Schwyn and Neilands, 1987), and capsule detection using India ink. Environmental tolerance was tested across temperatures (4 to 50 °C), salinities (0 to 7% or 0 to 70 ppt), and pH values (1 to 12).

Molecular characterization

Genomic DNA was extracted from the culture of one representative isolate of the outbreak (designated as CMFRI/SA-01) as per the established protocol (Wilson, 1987). Briefly, a single well-isolated colony was inoculated into 5 ml LB broth (Himedia) and incubated overnight at 30 °C in a shaking incubator. Post-incubation, the cell pellet was resuspended in 450 µl TEG buffer containing lysozyme (5 mg/ml), then vortexed. Subsequently, 50 µl of 10% SDS was added, mixed,

Table 1. Phenotypic characterisation of CMFRI/SA-01

Test	*S. agalactiae* CMFRI/SA-01	*S. agalactiae* from monosex Nile tilapia (Adikesavalu *et al*., 2017)
Gram staining	+	+
AustBauerMorphology	Cocci	Cocci
Oxidase	-	-
Catalase	-	-
Haemolysis	β	Γ
Esculin hydrolysis	-	-
Arginine dihydrolase	+	+
Urease	-	-
Glucose fermentation	+	+
Arabinose fermentation	-	-
Lactose fermentation	-	-
Sucrose fermentation	+	+
Sorbitol fermentation	-	-
D Mannitol fermentation	-	-
Mannose fermentation	+	+
D Raffinose fermentation	-	-
D Xylose fermentation	-	-
D sorbitol fermentation	-	-
Salicin fermentation	-	V
D Trehalose fermentation	+	+
D Galactose fermentation	-	V
Bacitracin resistance	-	-
0129 resistance	-	-
Novobiocin resistance	-	-
Optochin resistance	+	+
Growth in 6.4% NaCl	-	-

and the sample was placed at room temperature for 15 min, followed by at 60 °C for 10 min. Then, 10 µl of proteinase-K was added, vortexed, and incubated at 60 °C for 1 h. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, gently mixed, and centrifuged at 10,000 rpm for 15 min at 4 °C. To the aqueous phase, 1/10th volume of 3 M sodium acetate and 2.5 volumes of ice-cold absolute ethanol were added. The mixture was incubated at -20 °C overnight for DNA precipitation and centrifuged at 10,000 rpm for 15 min. The pellet was washed with 500 µl of 70% ethanol, centrifuged again, and air-dried. DNA was resuspended in 30 µl of elution buffer and stored at -20 °C. Subsequently, 16S rRNA gene amplification was performed using universal prokaryotic primers NP1F (5′-GAGTTTGATCCTGGCTCA-3′) and NP1R (5′-ACGGCTACCTTGTTACGACTT-3′) (Weisburg *et al.,* 1991). PCR was carried out in a 25 µl reaction mix containing 1 µl template DNA, 2.5 µl 1X Biolab *Taq* buffer, 0.5 µl dNTP mix, 0.5 µl each of forward and reverse primers, and 1.25 U Biolab *Taq* polymerase. Cycling conditions included initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 58 °C for 1 min, and 68 °C for 1.5 min, with a final extension at 68 °C for 5 min. The product was Sanger sequenced (Agrigenom, India), and the sequence was submitted to GenBank (Accession: MH916751). Phylogenetic analysis through ClustalW in 'MEGA7' (Kumar *et al.*, 2016) used the neighbour-joining method (Kimura 2-parameter), with *Staphylococcus aureus* as the outgroup. Rate variation among sites was modelled with a gamma distribution (shape parameter = 5). Bootstrap support was assessed using 1,000 replicates.

Experimental infection challenge

Healthy GIFT juveniles (*O. niloticus*) with a mean weight of 20 g (15 to 25 g) were quarantined for 2 weeks in oval-shaped Fibre-glass Reinforced Plastic (FRP) tanks holding 210 L of continuously aerated water having 6 ppt salinity. During experiments, the fish were maintained in glass tanks containing 40 L of filtered, continuously aerated water (salinity 6 ppt). Experiments were performed in duplicates to maintain the consistency of the results. The suspension was made in sterile PBS from the overnight growth of the bacteria in LB broth (representative isolate from infected fish was named as CMFRI/SA-01 strain). Experimental fish were randomly divided into four groups (two infected and two controls, six fish per tank) for testing the pathogenicity of CMFRI/SA-01. The first group was injected intramuscularly with 0.1 ml of the bacterial suspension containing 10^{10} CFU/ml. The second group was injected with 0.1 ml sterile PBS to form a control group for the first group. The third group was retained in glass aquaria containing 10 ml of bacterial suspension having a concentration of 10^{10} CFU/ml. Forth group was retained as the control for the third group. Fish were monitored for 10 days; moribund fish were collected, and blood, liver, and kidney samples were taken to confirm *S. agalactiae* by re-isolation (Suwannasang *et al.,* 2014).

Determination of LD_{50}

Serial dilutions (10^5 to 10^{10} CFU/ml) of the bacterial suspension were injected intramuscularly (0.1 ml) into fish. Each tank contained six fish, with duplicate tanks for each dose. Control fish were injected with 0.1 ml of sterile PBS. Mortality was recorded for 10 days, and LD_{50} was calculated using the Reed and Muench (1938) method.

Serum survival tests

Fifty microliters of bacterial suspensions (10^7 CFU/ml) were incubated with 450 µl of healthy fish serum in duplicates at 25 °C, 28 °C, and 32 °C for 4 h as per the previously recommended protocol (Fouz *et al.,* 2002; Sumithra *et al.,* 2019). Controls (bacteria in PBS only) were incubated similarly. Viable counts were determined by the drop plating method.

Susceptibility to antibiotics

In-vitro susceptibility of CMFRI/SA-01 strain to 34 different antibiotics belonging to 14 different classes (Table 2) (HiMedia, India) was studied using disc diffusion test (Bauer *et al.*, 1966). The diameter of inhibition zones was measured for the interpretation of antimicrobial susceptibility. Multiple Antibiotic Resistance (MAR) index of the strain was calculated *via* the formula MAR index = X/Y, where 'X' denotes the number of antibiotics to which the isolate exhibited resistance and 'Y' represents the total number of antibiotics to which the isolate was assessed (Krumperman, 1983).

Statistical Analysis

Data were analysed using SPSS version 16.0 (SPSS Inc., IL, USA). One-way ANOVA followed by Tukey's HSD post-hoc test was used to compare mortality and serum survival at different temperatures. Statistical significance was set at $P \leq 0.05$.

Results

Clinical signs and gross lesions

Affected GIFT fish displayed lethargy, poor escape reflex, anorexia, circling, skin darkening (Fig. 1a), and excess mucus. Gross lesions included exophthalmia, focal cutaneous haemorrhages on belly, jaw, and fin bases (Fig. 1b, c), ascites, pale liver with congested vessels, and splenomegaly.

Table 2. Antibiotic susceptibility test results of CMFRI/SA-01

Sl. No	Class of antimicrobials	Antibiotics	Code (Number indicates concentration in μg per disc)	Result of antibiogram (R- Resistant I-Intermediate; S-Sensitive)
1	Penicillins	Penicillin	P^{10}	S
2		Ampicillin	AMP^{10}	S
3		Piperacillin	PI^{100}	S
4		Carbenicillin	CB^{100}	S
5		Amoxycillin/ Clavulanic acid	AMC^{30}	S
6	Cephalosporins	Cephalothin	CEP^{30}	S
7		Cefoxitin	CK^{30}	
8		Cefuroxime	CXM^{30}	S
9		Cefoperazone	CPZ^{75}	S
10	Carbapenems	Meropenem	MRP^{10}	S
11		Ertapenem	ETP^{10}	S
12		Imipenem	IPM^{10}	S
13	Aminoglycosides	Gentamicin	GEN^{10}	S
14		Streptomycin	S^{10}	R
15		Amikacin	AK^{30}	I
16	Macrolides	Erythromycin	E^{15}	S
17		Clindamycin	CD^{2}	S
18	Quinolones	Levofloxacin	LE^{5}	S
19		Ciprofloxacin	CF^{10}	S
20		Enrofloxacin	EX^{10}	S
21		Lomefloxacin	LOM^{10}	R
22		Norfloxacin	NX^{10}	S
23		Ofloxacin	OF^{5}	S
24		Nalidixic acid	NA^{30}	S
25	Tetracyclines	Tetracycline	TE^{30}	S
26		Doxycycline	DO^{30}	S
27	Polypeptides	Bacitrain	B10	S
28		Polymyxin-B	PB^{300}	S
29	Sulphonamides	Co-Trimoxazole (Sulpha/Trimethoprim)	$COT^{23.75/1.25}$	S
30	Amphenicols	Chloramphenicol	C^{30}	S
31	Rifamycins	Rifampicin	RIF^{5}	S
32	Nitrofurans	Nitrofurantoin	NIT^{300}	S
33	Aminocoumarin	Novobiocin	NV^{5}	S
34	Glycopeptide	Vancomycin	VA^{30}	S

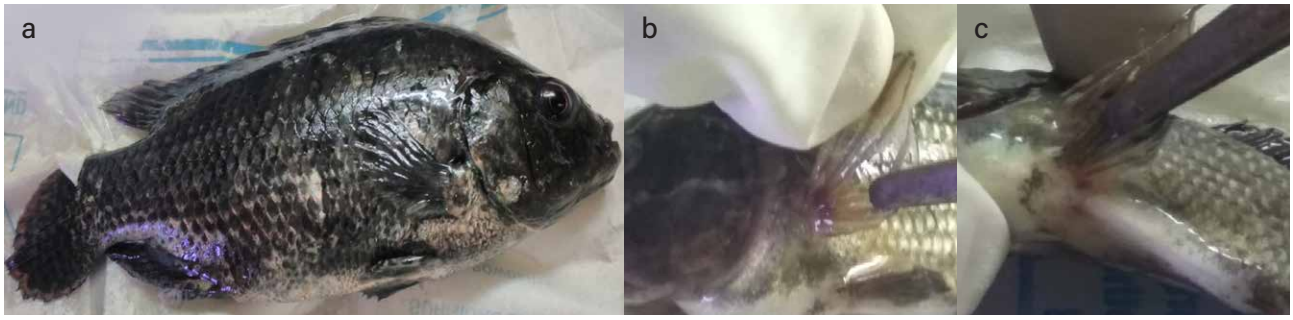

Fig. 1. Clinical signs noticed during disease outbreak, a) Darkened skin; b and c) Focal cutaneous haemorrhages at the base of fins

Screening for pathogens

No parasites were found. Pure bacterial colonies (representative isolate was designated CMFRI/SA-01) grew on BHIA from all the analysed samples, *viz*., peripheral blood, brain, kidney, liver and spleen tissues of moribund fish after 48 h incubation.

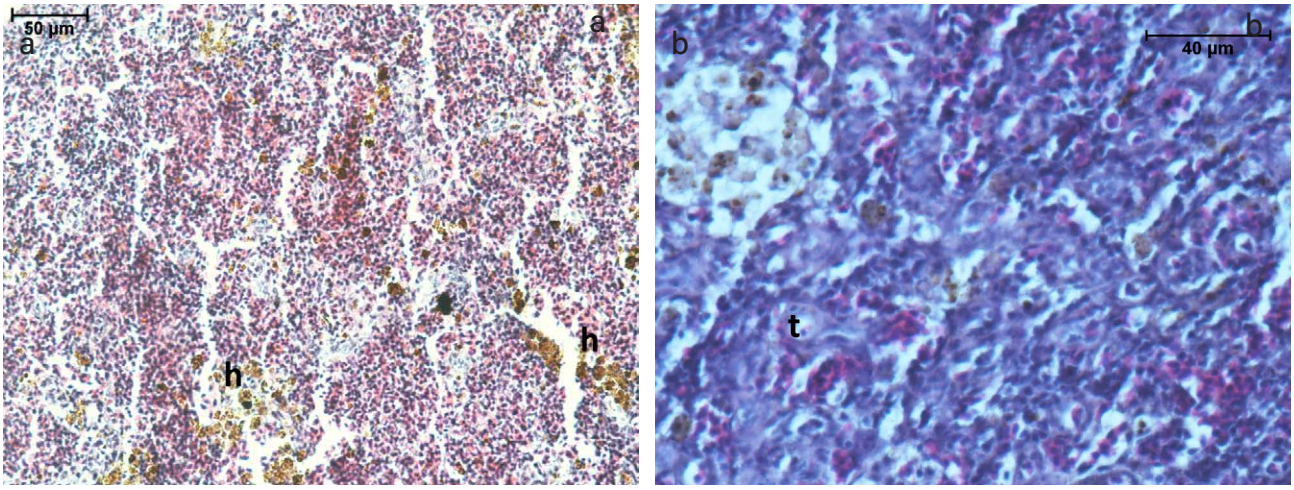

Fig. 2. Histopathology of spleen, a) Multifocal hemosiderin deposits, b) thrombosis. (h) in splenic parenchyma H & E Bar = 50 µM; (t) in splenic blood vessels H & E Bar = 40 µM

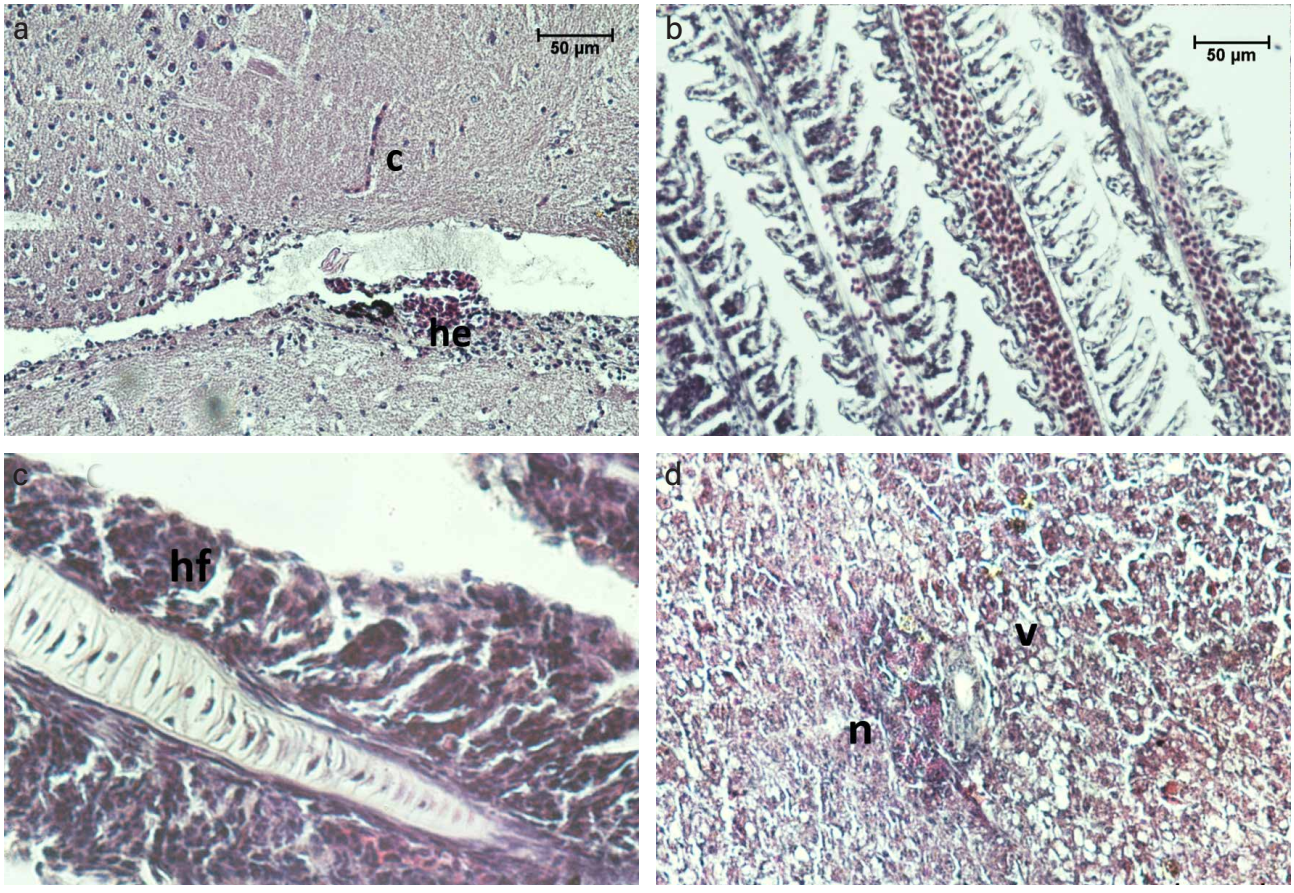

Fig. 3. Histopathology of visceral organs, a) Hemorrhage (he) and congestion (c) in brain H & E Bar = 50 µM; b) Primary lamellar congestion (c) in gills H & E Bar = 50 µM; c) Secondary lamellar hyperplasia and fusion (hf) in gills H & E Bar = 50 µM; d) Vacuolation (v), degenerative and necrotic (n) changes of hepatocytes H & E Bar = 50 µM (Abbreviations used for marking the lesions; c: congestion; he: haemorrhage; hf: hyperplasia and fusion; v: vacuolation; n: necrosis)

Histopathology

Microscopic lesions were found in the brain, spleen, liver and gills, with conspicuous changes in the spleen. Spleen showed multifocal hemosiderin deposits (Fig. 2a), enlarged germinal centres, capsular thickening with sub-capsular haemorrhage (Fig. 2b), thrombosis (Fig. 2c), macrophage infiltration and vacuolation (Fig. 2d). Brain showed mild

congestion and haemorrhage (Fig. 3a). Gills revealed primary lamellar congestion (Fig. 3b), secondary lamellar hyperplasia and fusion (Fig. 3c). Liver displayed vascular congestion, vacuolation, degeneration, and necrosis (Fig. 3d).

Phenotypic characterisation of the isolate

The isolate formed pinpoint whitish-grey colonies on BHIA plates and produced β-hemolysis on blood agar (Table 1). It was positive for protease, siderophore, capsule, and lecithinase production. Growth occurred at 25 to 45 °C, pH 7 to 10, and salinity up to 1% (10 ppt).

Molecular characterisation of the isolate

The sequence obtained for 16S rRNA gene (GenBank: MH916751) confirmed identity as *S. agalactiae* (100% homology). Percentage homology with 16SrRNA gene sequence of other closely related *Streptococcus* sp., such as *S. dysgalactiae* subsp. *equisimilis* strain CIP 105120 (NR 043661.1), *S. pyogenes* strain JCM 5674 (NR 112088.1), *S. iniae* strain ATCC 29178 (NR 025148.1), *S. urinalis* strain 2285-97 (NR 115738.1), *S. ictaluri* strain 707-05 (NR 115802.1), *S. hongkongensis* strain HKU30 (NR 117974.1) and *S. canis* strain ATCC 43496 (NR 115729.1) were 98%, 97%, 97%, 96%, 96%, 96% and 96% respectively. Comparative phylogenetic analysis showed monophyletic clustering with other *S. agalactiae* strains (Fig. 4), regardless of host origin.

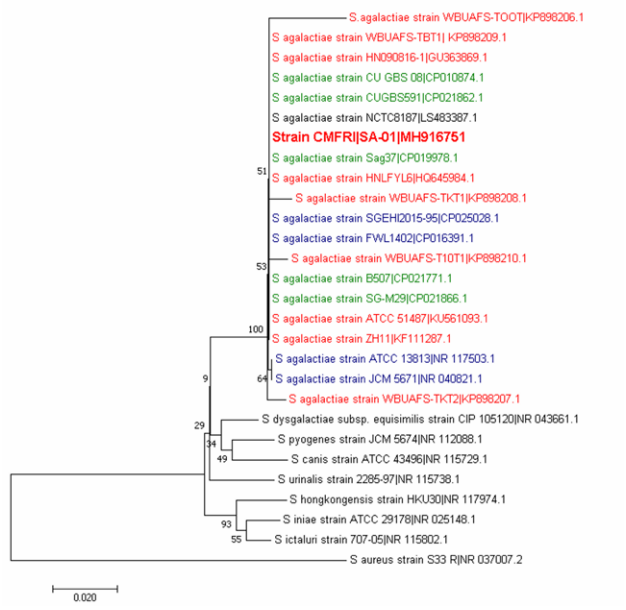

Fig. 4. Phylogenetic tree showing the position of *S. agalactiae* CMFRI/SA-01 strain in relation to other *S. agalactiae* strains and members from the genus based on 16SrRNA gene sequence. Names of each bacteria including strain ID are followed by corresponding GenBank accession numbers. Tilapia strains, human strains and strains from other host species (Bovine, marine fish, frog) are depicted in red, green and blue colour respectively

Experimental infection challenge

Intramuscular challenge with a higher dose (10^9 CFU/fish) caused 100% mortality within 24-72 h. Infected fish exhibited reduced appetite, surfacing, imbalance, less activity, exophthalmia, ascites, and focal cutaneous haemorrhages on the belly, lower jaw and petichae at the base of fins, while no mortality and disease symptoms were observed in control fish. The pathogen could be re-isolated from the moribund fish (blood, liver and kidney). However, the strain was unable to cause mortality by immersion challenge even at 10^{10} CFU/ml.

Determination of LD_{50} through injection challenge

LD_{50} was $6.5 \times 10^{6.65}$ CFU/fish through injection challenge. Significant differences in cumulative mortality were observed among dose groups ($P < 0.05$). However, there was no statistically significant difference between the groups exposed to CFU higher than 6.5×10^8 CFU/fish and CFU lower than 6.5×10^5 CFU/fish ($P > 0.05$) (Fig. 5).

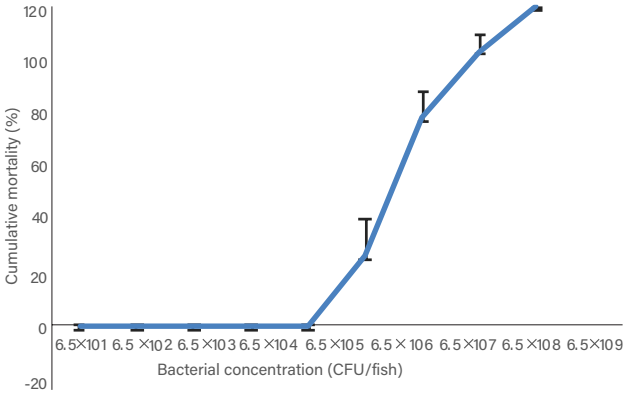

Fig. 5. Cumulative mortality percentage of GIFT juveniles at different CFUs of CMFRI/SA-01 strain following intramuscular challenge. Error bars represent the standard error

Serum survival tests

The pathogen (CMFRI/SA-01) survived and multiplied efficiently in serum samples of healthy GIFT fish. There was a statistically significant difference in serum survival ratio between the three different temperatures as a whole, and survival was statistically significantly higher at 32 °C than at 25 °C and 28 °C ($P < 0.05$). No significant difference was found between 25 °C and 28 °C ($P = 0.999$; Fig. 6).

Susceptibility to antibiotics

The strain was resistant to two antibiotics, namely Streptomycin and Lomefloxacin, and showed intermediate resistance to Amikacin (Table 2). MAR index was 0.08.

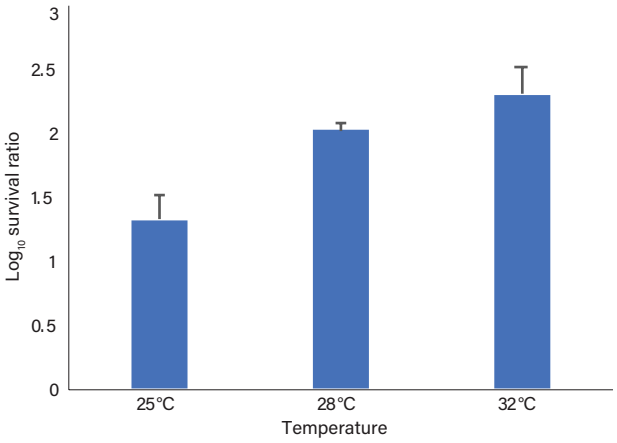

Fig. 6. Survival ratio of CMFRI/SA-01 strain after incubation for 4 h in healthy GIFT fish serum. Log_{10} of the survival ratio is depicted on Y axis. Error bars represent the standard error

Discussion

Streptococcosis is a significant global disease in cultured and wild fish, caused by various *Streptococcus* species (Delphino *et al.*, 2019). Among various streptococcal species, *S. agalactiae* (Group B Streptococcus; GBS) is an emerging fish pathogen causing high morbidity and mortality (Amal and Zamri-Saad, 2011). In India, reports are limited, with the only two known cases from pond-cultured monosex Nile tilapia in West Bengal (Adikesavalu *et al.*, 2017) and cage-farmed Etroplus suratensis in Kerala (Girijan *et al.*, 2023). This study marks the first report of GBS infection in brackishwater cage-farmed GIFT tilapia in India, detailing the characteristics of the pathogen.

The outbreak occurred in a brackish water cage farm off Kochi, Kerala, India, with a cumulative mortality rate of 45% within two weeks. Bacterial isolation attempts from different diseased fish gave morphologically similar isolates, indicating a common bacterial aetiology in the stock. Experimental infection of GIFT juveniles demonstrated that the isolate was pathogenic to fish. The challenged fish showed signs and lesions similar to those occurred in natural outbreak and the pathogen could be re-isolated from the moribund fish (blood, liver, brain, kidney and spleen) confirming Koch postulates. The infective bacterium was tentatively identified as Group B Streptococci (GBS) based on conventional microbiological methods (Bergey *et al.*, 2009), which was later confirmed by molecular characterization. Clinical signs, gross and histological lesions resembled those reported from other streptococcosis outbreaks (Salvador *et al.*, 2005; Zamri-Saad *et al.*, 2010; Adikesavalu *et al.*, 2017). The major gross lesions observed during necropsy included exophthalmia, skin darkening, focal cutaneous haemorrhages on the abdomen, anal pore, jaw, and fin bases, along with ascites, pale liver with congested blood vessels, and splenomegaly. These findings were consistent with those reported in naturally infected tilapia by previous researchers (Figueiredo *et al.*, 2006; Amal and Zamri-Saad, 2011). The organs affected, namely the brain, spleen, and liver, also corresponded with earlier observations (Alsaid *et al.*, 2013). Histopathological examination further confirmed the septicaemic nature of *S. agalactiae*. In the spleen, lesions such as congestion, necrotic foci, and an increased number of melano macrophage centres were evident. Key histopathological features included multifocal hemosiderin deposition, enlarged germinal centres, capsular thickening with subcapsular haemorrhage, thrombosis, macrophage infiltration, and vacuolation, findings similar to those described in red tilapia (Alsaid *et al.*, 2013). Other studies have also documented increased macrophage infiltration in the spleen during bacterial infections (Asencios *et al.*, 2016). Notably, the infiltration of mononuclear cells, predominantly macrophages, observed in the spleen in the present study had been reported in various organs during *S. agalactiae* infection (Filho *et al.*, 2009; Iregui *et al.*, 2014). Hepatic lesions included vascular congestion, cytoplasmic vacuolation, cellular degeneration and necrosis, which are in agreement with previous reports of experimentally infected tilapia with *Streptococcus* spp. (Mohamed *et al.*, 2014).

The higher stocking density, poor water quality, and water temperature less than 31°C can cause higher mortality rates in streptococcosis (Evans *et al.*, 2006; Amal *et al.*, 2015). The comparatively lower cumulative mortality rate than earlier Indian reports (55 to 70%) could be attributed to differences in stocking density and water quality, although data for direct comparison were unavailable.

The isolated strain (CMFRI/SA-01) shared typical phenotypic traits with previously described Indian *S. agalactiae* isolates (Adikesavalu *et al.*, 2017), except for its β-haemolytic activity. Non-haemolytic strains, reported by Adikesavalu *et al.* (2017) and others (Wang *et al.*, 2013), may represent fish host-restricted clonal complexes (Delannoy *et al.*, 2013), while haemolytic variants like CMFRI/SA-01 are widely reported across hosts including humans. It is important to note that during phenotypic characterisation, the isolate was able to survive up to 1% (10 ppt) salinities. This supports its involvement in the disease outbreak, as the affected water had a salinity of 6 ppt (0.6%). Additionally, the findings align with the comparative biochemical characterisation reported by Adikesavalu *et al.* (2017), where a similar Indian *S. agalactiae* isolate from monosex Nile tilapia failed to grow at 6.5% NaCl. These results indicate that *S. agalactiae* is more likely to be involved in disease outbreaks in freshwater or brackishwater aquaculture systems, rather than in marine environments. Molecular identification

via 16S rRNA gene sequencing confirmed 100% identity with known *S. agalactiae* strains. Phylogenetic analysis suggested the presence of multiple genotypes in India, as originally suggested by Adikesavalu *et al.* (2017). Nevertheless, there was no clustering of isolates based on their host origin, indicating the potential for interspecies transmission in GBS. In concordance with the present results, potential for infection between homothermic hosts and poikilothermic animals has been demonstrated (Evans *et al.*, 2009).

Pathogenicity was confirmed via intramuscular challenge, reproducing disease symptoms and yielding an LD_{50} of $6.5 \times 10^{6.65}$ CFU/fish, a similar value reported for GBS serotype Ia and lower than that for the GBS serotype III strain (2.10×10^{8} CFU/fish) (Suwannasang *et al.*, 2014). Initially, a higher dose was used during the experimental infection to assess the pathogenicity of the isolate obtained from diseased fish. Only after confirming the virulence of the isolate through this initial challenge the LD_{50} was determined, to establish the actual dose required to cause mortality. Since LD_{50} estimation requires the use of a large number of fish, the preliminary confirmation of pathogenicity was essential to minimise unnecessary animal use (Sumithra *et al.*, 2019). The strain was non-virulent during the experimental infection to assess the pathogenicity via immersion. Accordingly, LD_{50} experiments by immersion were avoided. In concordance to our observation, the inability of *Streptococcus* sp., to infect tilapia fish unless skin is scarified prior to bath challenge was reported (Chang and Plumb, 1996). Serum survival assays revealed the isolate could replicate in healthy GIFT serum, reaching a greater cell number than control. Resistance to fish serum is indispensable for dissemination of septicaemic pathogen to vital body organs (Amaro *et al.*, 1997). Therefore, serum survival results were in agreement with the isolation of bacteria from various vital organs in both natural outbreak and experimental infection. Notably, survival was significantly higher at 32 °C, which might be a reason for the higher mortality in GBS infection of tilapia during water temperature less than 31 °C (Evans *et al.*, 2006; Amal *et al.*, 2015). Antibiotic susceptibility testing showed resistance or intermediate resistance to only three antibiotics (Streptomycin, Amikacin, and Lomefloxacin) with a low MAR index (0.08). The resistance to aminoglycoside (streptomycin and amikacin) was expected based on previous studies and this aminoglycoside resistance features are thought to be intrinsic to *S. agalactiae* without being a result of antibiotic usage in fish farms (Amal *et al.*, 2012; Aisyhah *et al.*, 2015). The MAR index (≤ 0.2) indicated that the strain expresses only a low-level risk antibiotic resistance (Paul *et al.*, 1997). However, use of antibiotics should be discouraged in cage farms due to the risk of resistance development (Monteiro *et al.*, 2016). Instead, controlling bacterial diseases in cage farms requires an integrated approach that combines good husbandry practices, early disease detection, and targeted therapeutic interventions. Maintaining optimal water quality through regular cleaning of nets, optimal stocking densities, and feeding practices is critical. Regular health monitoring, isolation of the infected fish, biosecurity measures such as controlling the movement of fish, disinfecting equipment, and quarantine of newly purchased fish before releasing into cages, and vaccination against specific bacterial pathogens are the major strategies (Klesius *et al.*, 2008).

In conclusion, this study reports a natural epizootic caused by *S. agalactiae*, resulting in a cumulative mortality of 45% within two weeks among GIFT (*O. niloticus* L.) reared in a brackishwater cage farm in India. Experimental challenge studies confirmed the etiological role of the pathogen, with an LD_{50} of $6.5 \times 10^{6.65}$ CFU/fish. However, the pathogen exhibited no virulence in immersion challenge studies. The pathogen shared typical phenotypic traits with previously described Indian *S. agalactiae* isolates except for its β-haemolytic activity. Virulence assessment *via* serum survival assays confirmed the septicaemic nature of the pathogen. The observation of increased serum survival at higher temperatures may explain the higher incidence of infections under elevated thermal conditions. These findings underscore the urgent need for effective vaccines, alternative disease control measures, and stringent biosecurity protocols to mitigate the impact of this emerging pathogen in Indian aquaculture.

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Author contributions

Conceptualisation: TGS, KJR; Methodology: TGS, VNA, KJR, GS, APV, SP; Data collection: TGS, VNA; Data analysis: TGS, KSSR; Writing original draft: TGS; Writing review and editing: KSSR, KJR; Supervision: TGS, KSSR

Conflicts of interest

The authors declare that they have no conflict of financial or non-financial interests that could have influenced the outcome or interpretation of the results

Data availability

The data are available and can be requested from the corresponding author.

Ethical statement

The study was conducted following the ethical standards and guidelines established by the "EU Directive 2010/63/EU for animal experiments,", "U.K. Animals Scientific Procedures Act," 1986. All procedures involving live fish were approved by the ICAR-CMFRI (MBT/HLT/23).

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