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Original Article

Large scale mortality in cultured Nile tilapia (*Oreochromis niloticus*): natural co-infection with *Aeromonas hydrophila* and *Streptococcus iniae*

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Abstract

Background: Nile tilapia is a highly valuable fish in the aquaculture sector. A culture farm has reported heavy mortalities of tilapia. **Aims:** The present study aimed to identify the etiological agent responsible for the heavy mortality in cage cultured tilapia. **Methods:** The moribund and freshly dead fishes were analyzed for clinical signs. Biochemical and molecular characterizations were performed to identify the etiological agents of the disease. Also, polymerase chain reaction (PCR) assay was used to detect the presence of the virulence genes. The susceptibility of the isolates to various antibiotics was tested by the disk diffusion method. **Results:** The results of the biochemical tests and PCR assay confirmed that co-infection with *Aeromonas hydrophila*, and *Streptococcus iniae* was responsible for the disease severity. Phylogenetic analysis of the *16S rRNA* gene showed that *A. hydrophila* and *S. iniae* isolates shared 99% and 98% sequence homology with *A. hydrophila* and *S. iniae* previously deposited in the Genbank database. The multiple antibiotic resistance (MAR) index of *A. hydrophila* was 0.16 and that of *S. iniae* was 0.71. The PCR test revealed that both pathogens harbored numerous virulence factors. The experimental infection study confirmed that the synergistic action of *A. hydrophila* and *S. iniae* led to increased mortality in tilapia. Histopathological changes were observed in the liver and spleen tissues of the co-infected fishes. **Conclusion:** These findings indicate that the disease outbreak in the tilapia culture farm occurred as a result of co-infection by *A. hydrophila* and *S. iniae*.

Key words: *A. hydrophila*, Cage culture, *S. iniae*, Tilapia, Virulence factors

Introduction

Nile tilapia (*Oreochromis niloticus*) is the world's third most farmed and commercially important freshwater fish species (FAO, 2020) with an annual production of 6.4 million metric tons (MT) valued at USD 9.8 billion (FAO, 2017). In India, tilapia farming is primarily done in ponds, cages, raceways, and tanks, with an estimated production of 18,000 tons in 2016 (Menaga and Fitzsimmons, 2017). Cage farming of tilapia in the river has expanded rapidly, allowing landless farmers to grow fish by utilizing available water resources. Fish cultured in open cages in rivers are naturally exposed to a wide range of pathogenic organisms, including bacteria, parasites, fungi, and viruses (Dong *et al*., 2015). Tilapia are highly susceptible to several bacterial diseases (Abdel-Latif *et al*., 2020), especially when grown in intensive farming conditions (El-Sayed, 2019). These environments harbor a variety of heterogeneous micro-organisms, leading to an increased probability of co-infection. Co-infections alter the disease course, severity, and duration of the infection (Abdel-Latif *et al*., 2020). Furthermore, due to synergistic interactions, the pathogenicity of one or more pathogens is often elevated (Abdel-Latif *et al*., 2020).

Notably, co-infections result in significantly higher mortality rates in farmed animals than single pathogen infections (Pakingking *et al*., 2003; Ma *et al*., 2019). Despite this, co-infections in aquatic animals have not been thoroughly studied and there is insufficient data on such infections. Because of a lack of understanding of co-infections, the dominant pathogen is treated while the co-infecting agent is ignored (Abdel-Latif *et al*., 2020).

The present study was carried out to identify the etiological agent responsible for the disease outbreak (with >30% cumulative mortality) of Nile tilapia in cage culture farms in Karnataka, India and to confirm its causative role by Koch's postulates. The bacteria were

identified by biochemical and molecular studies. Moreover, bacterial isolates were tested for the presence of virulence genes and susceptibility to antibiotics. This research can help draw attention to natural co-infection outbreaks and develop measures to safeguard tilapia from multiple infections.

Materials and Methods

Ethical statement

Not applicable.

Data availability statement

The data that supports the findings of this study have been included in the manuscript. The sequences generated in the present study were deposited in the NCBI GenBank data base under Accession No. MN442055 and MN206041.

Sample collection and examination

In January 2019, a disease outbreak in cultured Nile tilapia (*Oreochromis niloticus*) was reported from a cage farm located in the Tungabhadra River (Latitude: N15°21'12"; Longitude: E76°21'45"), Koppala district of Karnataka, India (Fig. 1). The farm contained a total of 48 cages with a stocking density of 50 No./m^3 per cage. The farmer noticed the first mortality in December 2018 and the mortality persisted on a daily basis since then. During one of the farm visits, moribund and recently deceased fish with clinical abnormalities and skin erosion were collected by random sampling and transported to the laboratory on ice to prevent further deterioration.

Fig. 1: A map from the Google earth shows the cage farm located in the Tungabhadra River at Koppala district of Karnataka, India

The fresh smears of gill and skin were observed for the presence of parasites under the microscope. Tissue samples of kidney, liver, and brain from five fish were pooled in L-15 (Leibovitz's) medium (HiMedia, India) and inoculated into a liver cell line from *O. niloticus* (OniL) (Swaminathan *et al*., 2018) for virus isolation. In addition, the fish samples were examined for post-mortem lesions in internal organs. Additionally, internal organs were used for RNA extraction using the Trizol reagent (RNA-XPress™, Himedia, India), and DNA was extracted as per previous protocol (Otta *et al*., 2003). PCR analysis was carried out using nucleic acids extracted from tissue samples and cell culture supernatant with suitable primers for TiLV (Eyngor *et al*., 2014), Iridovirus (Girisha *et al*., 2020), and Betanodavirus (Bigarre *et al*., 2009).

Bacteriological isolation and identification

For bacterial isolation, each fish was surface sterilized with 70% ethanol and swabs were taken from surface lesions, the kidneys, the spleen, the liver, and the gill. The samples were streaked onto tryptic soy agar (TSA) (HiMedia, India) and brain heart infusion agar (BHIA, HiMedia, India) plates under aseptic conditions followed by incubation at 30°C for 18-24 h. Based on colony morphology, the dominant colonies from the plates were selected and purified by continual streaking on fresh TSA plates (30°C for 18-24 h). A loopful of these pure cultures was preserved in Luria-Bertani (LB) broth (HiMedia, India) with 30% glycerol at -80°C until further characterization.

The bacterial strains in the present study were further identified to the genus level based on a battery of biochemical tests (Barrow and Feltham, 2004; Austin and Austin, 2012) including Gram's staining, catalase (3% hydrogen peroxide solution), cytochrome oxidase, Indole, and Vogues-Prauskauer (VP) test. The utilization of various sugars, as well as sensitivity to 0/129 were studied. Haemolytic activity testing was performed on LB agar (HiMedia, India) supplemented with 5% sheep blood (Swift *et al*., 1999). The identified bacterial isolates were stored in LB broth with 30% glycerol at -80°C until further use. The results of the biochemical test for the isolates were compared with *A. hydrophila* and *S. iniae* reference strains as reported previously (Table 1).

PCR analysis for the identification of bacterial isolates

DNA extraction

Genomic DNA extraction of isolates was performed by the cetyl trimethyl ammonium bromide (CTAB)-proteinase K method (Ausubel *et al*., 1990). Briefly, overnight grown bacterial cultures were pelleted and resuspended in extraction buffer (Tris-EDTA buffer, 10% SDA and 20 mg/ml proteinase K) and incubated at 45°C for 1 h. A CTAB/NaCl solution was then added to the mixture followed by incubation for 10 min at 65°C. DNA was extracted with phenol-chloroform and precipitated using ethanol. DNA pellet was dissolved in TE buffer. The purity and yield of extracted DNA was analyzed using a spectrophotometer (NanoDrop, Thermo Fisher Scientific, Canada).

Molecular confirmation of the isolates

The extracted DNA from the isolates was used for PCR amplification of *16S rRNA*, *gyr*B, and *ITS* genes Supplementary Table 1 (ST1). DNA amplifications were

Table 1: List of biochemical tests performed to identify the isolated bacteria

Test	*Aeromonas hydrophila*		*Streptococcus iniae*	
	Present study	Reference strain (Abdel-Latif *et al*., 2020)	Present study	Reference strain (Rahmatullah *et al*., 2017)
Gram's staining reaction	-	-	+	+
Cell morphology	rod	rod	Cocci	Cocci
Catalase	+	+	-	-
Oxidase	-	+	-	-
Hemolytic assay	β	β	α	β
O/F Test	F	NA	F	F
Indole	+	+	ND	NA
VP	+	+	-	+
Dextrose	+	NA	+	NA
Sucrose	+	+	+	NA
Starch	-	NA	+	NA
Raffinose	-	NA	-	-
Arabinose	-	+	-	-
Melibiose	-	-	+	NA
Salicin	-	NA	ND	NA
Mannitol	+	+	+	+
Sorbitol	-	-	-	-
Lactose	-	NA	-	-

+ Positive response, - Negative response, NA: Not available, and ND: Not done

Table 2: Antimicrobial susceptibility test of *A. hydrophila*

Antimicrobial agents	Disk concentration (µg)	Interpretative category and zone diameter			Zone of diameter (mm)	Result
		S	I	R		
Cephems						
Cefotaxime	30	≥26	23-25	≤22	28	S
Carbpenem						
Imipenem	10	≥23	20-22	≤19	25	S
Meropene	10	≥23	20-22	≤19	28	S
Tetracycline						
Tetracyclin	30	≥15	12-14	≤11	12	I
Quilanolone						
Ciprofloxaci	5	≥21	16-20	≤15	24	S
Phenicols						
Chloramphenico	30	≥18	13-17	≤12	10	R

R: Resistant, S: Sensitive, and I: Intermediary were designed as described by Table 3-CLSI-M45 (CLSI, 2016)

performed as per previous methods for detection of *16S rRNA* (Lane, 1991), the *gyrB* gene (Julia and Philip, 2011) and the *ITS* gene (Zhou *et al*., 2011). The *16S rRNA* amplicons were purified using the PCR Product Purification Kit (HiMedia, India) and sequenced using the Sanger method at Bioserve Labs Pvt. Ltd., Hyderabad, India. The resulting *16S rRNA* sequences were processed and compared with the sequences available in the GenBank database using the Basic Local Alignment Search Tool (BLAST). A phylogenetic analysis was conducted to compare the *16S rRNA* sequences from this study to those previously deposited in the Genbank database. The DNA sequence alignment was carried out using MUSCLE. The tree was generated by the maximum likelihood with a bootstrap value of 1000 replicates using MEGA X software (Kumar, 2018).

Antibiotic sensitivity test

Antibiotic susceptibility of *A. hydrophila* and *S. iniae* was determined using the Kirby-Bauer disc diffusion method (Bauer *et al*., 1966) on a bacterial lawn grown on Muller Hinton Agar (MHA) (HiMedia Laboratories Pvt. Ltd., India) as per the recommendations of the Clinical and Laboratory Standard Institute (CLSI, 2006; M24 and M100). Different groups of antibiotic discs were used in the study (Tables 2 and 3). The plates were incubated for 18-24 h at 30°C and examined for the appearance of a clear zone. The diameter of the inhibition zone was recorded. The sensitivity and resistance of each isolate were determined as per the manufacturer's instructions. The MAR index was calculated as the ratio of the number of antibiotics to which the isolate displayed resistance to the total number of antibiotics to which the isolate had been evaluated for susceptibility (Krumperman, 1983).

PCR assay for virulence genes

PCR assay was performed for the detection of virulence genes of *A. hydrophila* and *S. iniae* isolates. *A. hydrophila* was tested for the presence of virulence factors such as hemolysin (*ahh*1), aerolysin related cytotoxic enterotoxin (*act*), heat stable cytotonic enterotoxins (*ast*), exo enzymes elastase (*ela*), lipase (*lip*M), effector toxin (*aex*U), type IV pilus (*tap*D), and T6SS (*hcp*). Similarly, *S. iniae* was analyzed for the presence of virulence factors such as M-like protein

Table 3: Antimicrobial susceptibility testing of *S. iniae*

Antimicrobial agents	Disk concentration (µg)	Interpretative category and zone diameter			Zone of diameter (mm)	Result
		S	I	R		
Penicillin						
Penicillin	10	≥24	-	-	24	S
Ampicillin	10	≥24	-	-	17	R
Cephems						
Cefotaxime	30	≥24	-	-	28	S
Glycopeptide						
Vancomycin	30	≥17	-	-	12	R
Macrolides						
Erythroomycin	15	≥21	16-20	≤15	15	R
Tetracycline						
Tetracycline	30	≥23	19-22	≤18	18	R
Phenicols						
Chloramphenicol	30	≥21	18-20	≤17	17	R

R: Resistant, S: Sensitive, and I: Intermediary were designed as described by Table 2H1-CLSI-M100 (CLSI, 2017)

(*sim*A), C5 α peptidase (*scp*I), peptidoglycan decetylase (*pdi*), phospholgucomutase (*pgm*), capsular polysaccharide (*cps*D), and streptolysin S (*sag*A). The primers used for virulence gene investigations are listed in Supplementary Tables 2 and 3 (ST2 and ST3).

In-vitro cytotoxicity assay

Bacterial secretory proteins from *A. hydrophila* and *S. iniae* were prepared according to Palang *et al*. (2020). Bacterial supernatants were filtered using a 0.22 µm filter (Millipore) before use. The tilapia liver cell line (OniL) (Swaminathan *et al*., 2018) provided by the National Repository of Fish Cell Line (NRFC), ICAR-NBFGR, Lucknow, India, was used in this study. OniL cells were maintained in L-15 Medium (Leibovitz's) (HiMedia, India) supplemented with 10% FBS (fetal bovine serum) (HiMedia, India). The cells grew in a T25 cell culture flask (CELLSTAR®, Greiner Bio-One, India) with 4 ml of the media and were incubated at 28°C. The flask containing OniL cells was inoculated with supernatant prepared from *A. hydrophila* and *S. iniae*. Similarly, sterile PBS was added to the cells, serving as a negative control. Further, the cells were incubated for 48 h and observed for any morphological changes under an inverted light microscope (OLYMPUS CKX41).

Pathogenicity study and histopathology

Healthy Nile tilapia (mean weight of 15 ± 4.2 g) were collected from the fish farm of the College of Fisheries, Mangalore, India, and acclimatized for 10 days before the infectivity test. A total of 40 fish were divided into experimental and control groups of 10 fish each. The fish were anaesthetized using quinaldine (100 mg/L) before the injection. The experimental fish were given an intraperitoneal injection of 0.1 ml of either an individual or mixed suspension of *A. hydrophila* (1.9×10^7 CFU ml) or *S. iniae* (1.6×10^7 CFU ml). Likewise, the fish in the control group were injected with 0.1 ml of sterile PBS. The clinical signs and mortality patterns were recorded daily.

For histopathological examination, tissue samples of the spleen and liver were collected from co-infection challenged fish. Specimens were fixed in a 10% buffered formalin solution for 48 h. The fixed samples were then embedded in paraffin wax, sectioned at 5 µm (Leica, Germany), stained with hematoxylin and eosin (H&E), and visualized under a light microscope (OLYMPUS).

Results

Fish epizootics

Naturally infected Nile tilapia exhibited clinical signs of a typical bacterial infection, such as lethargy, loss of appetite, unilateral or bilateral exophthalmia with corneal opacity, a dark discolouration of the body (melanosis), abnormal swimming, severe anaemia, petechiae on the operculum, loss of scales, severe skin ulceration, and heavy mortalities (Figs. 2A-D). During necropsy, infected fish were observed to have ascites in the peritoneal cavity and damaged internal organs. On the farm, there was a constant mortality rate of more than 30% from each cage. The water quality parameters during the outbreak were as follows: Water temperature (28.57 ± 1.23°C), pH (7.6 ± 0.36), and dissolved oxygen (6.3 ± 0.58 mg/L).

Fig. 2: Pathological characteristics of an infected Nile tilapia showing. (**A**) Dead/moribund fish floating at cage surface, (**B**) Unilateral or bilateral exophthalmia with corneal opacity, (**C**) Fin rot (black arrow) and scale loss (white arrow) with dark discoloration of the body (melanosis) (red arrow), and (**D**) Skin ulceration (black arrow)

The preliminary examination showed no evidence of parasitic infections in the fish samples. Similarly, infected fish did not display any signs of viral infection, which could be further confirmed by a lack of cytopathic effect (CPE) in tilapia liver cell lines (OniL) even after three blind passages.

Bacterial isolation and identification

Bacterial isolates from the infected Nile tilapia were putatively identified as motile *Aeromonas* and *Streptococcus* spp. using conventional biochemical tests. Motile rod-shaped Gram-negative *Aeromonas* isolated from the kidney and liver tissues of the infected fish produced smooth, convex, rounded, and β-hemolytic colonies on 5% sheep blood agar (Fig. 3A). It tested positive for catalase, cytochrome oxidase, Indole, VP, esculin hydrolysis, and it also showed resistance to antibiotic 0/129. Acid production was observed when sucrose was used as a source of carbon. The Gram-positive *Streptococcus* isolated from the sub-surface of infected fish grew in pairs and chains, producing transparent white pin-point colonies and displaying α-haemolysis on sheep blood agar (Fig. 3B). The isolate tested negative for catalase, cytochrome oxidase, and Indole/VP tests. Acid production was observed when dextrose, sucrose, starch, and mannitol were used as sources of carbon.

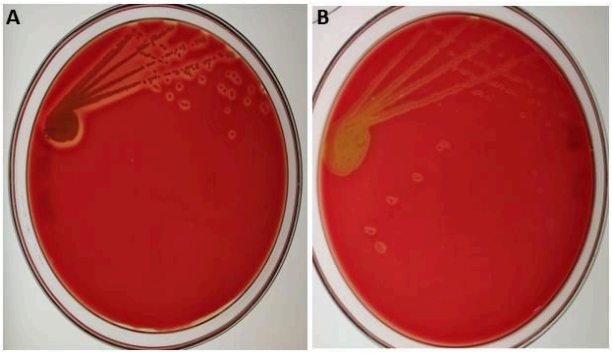

Fig. 3: Photographs showing haemolytic activity of bacterial isolates on 5% sheep blood agar. (**A**) β-haemolysis around *A. hydrophila* colonies, and (**B**) α-haemolysis around *S. iniae* colonies

Molecular confirmation of the isolates

The Gram-negative isolate was confirmed to be *Aeromonas* spp. By PCR testing for the *gyr*B gene (1124 bp), and the Gram-positive isolate was confirmed to be *Streptococcus* spp. By PCR testing for the *ITS* gene (377 bp). The PCR assay for the *16S rRNA* gene resulted in an anamplicon of 1485 bp for both isolates (Fig. 4). The BLAST analysis of *16S rRNA* sequences of the *Aeromonas* spp. and *Streptococcus* spp. Isolates showed sequence homology with previously reported *A. hydrophila* and *S. iniae*, respectively. The results of the phylogenetic tree indicated that *Aeromonas* spp. isolate in the present study clustered with the Chinese *A. hydrophila* strain (KY905657) (Fig. 5A) and *Streptococcus* spp. could be grouped with *S. iniae* isolates from Indonesia (KM209199) and Iran (MG912577) (Fig. 5B). The *16S rRNA* sequences of the *A. hydrophila* and *S. iniae* isolates from this study were deposited in GenBank under accession numbers MN442055 and MN206041, respectively.

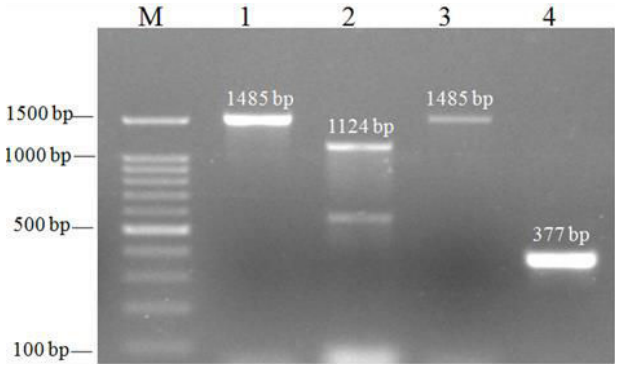

Fig. 4: PCR amplification of bacterial DNA using *16s rRNA* and specific primers. Lane M: 100 bp ladder. Lane 1: *A. hydrophila 16s rRNA* gene, Lane 2: *A. hydrophila gyrB* gene, Lane 3: *S. iniae 16s rRNA* gene, and Lane 4: *S. iniae ITS* gene

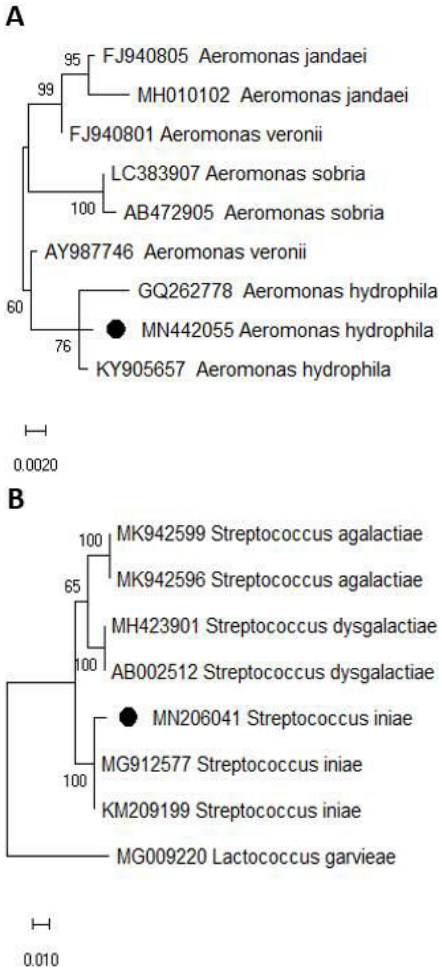

Fig. 5: Phylogenetic tree based on *16S rRNA* gene sequence showing the relationships of *A. hydrophila* and *S. iniae* with related species (**A** and **B**). Symbols (●) represent the isolates from the present study. The tree was generated by the Maximum Likelihood with a bootstrap value of 1000 replicates

Antibiotic sensitivity test

A. hydrophila isolated from tilapia was susceptible to 66.66% (4/6), resistant to 16.66% (1/6), and displayed intermediate susceptibility to 16.66% (1/6) of the antibiotics tested (Table 2). However, *S. iniae* was resistant to 71.4% (5/7) and susceptible to 28.6% (2/7) of the antibiotics (Table 3). *S. iniae* had a higher MAR index (0.71) in comparison to that of *A. hydrophila* (0.16).

PCR assay for virulence genes

Aeromonas hydrophila strain harboured 8 virulence genes including *Ahh*1, *act*, *ast*, *ela*, *lip*M, *aex*, *tap*D, and *hcp* (Fig. 6). On the other hand, *S. iniae* tested positive for 6 virulence genes, including *sim*A, *scp*I, *pdi*, *pgm*, *cps*D, and *sag*A (Fig. 7).

Cytotoxicity effect on tilapia cells

The tilapia cells incubated with the culture supernatant of bacteria displayed visible changes in cell morphology. The cells displayed a round shape with a vacuolated appearance after 48 h of incubation (Fig. 8A-C). These results indicated that the bacterial secretory proteins from *A. hydrophila* and *S. iniae* could bring about cytotoxic effects on the fish cell line.

Pathogenicity and histopathology

The clinical signs of experimental infection were similar to those of naturally infected fish in cages (Fig. 9A-B). The experimentally infected tilapia exhibited a mortality rate of 80% when challenged with a mixed culture of *A. hydrophila* and *S. iniae*, while only 20% and 30% mortality was observed in fish injected with either *A. hydrophila* or *S. iniae* (Fig. 10). Koch's postulates were confirmed by re-isolation of *A. hydrophila* and *S. iniae* from dead fish. Along with the experimental fish, the control fish were also tested for the presence of *A. hydrophila* or *S. iniae*, and they did not display mortality or any signs of infection. Further histological examination also excluded the disease in the control group fish. Histopathological changes were observed in the liver and spleen organs of the experimental co-infected Nile tilapia. Hyperactivation of melanomacrophage (MMC) centers with melanophores was observed in the spleen of the infected fish. Infected liver tissue displayed congestion of sinusoids, loss of hepatocytes, and vacuolar degeneration due to depletion of fat reserves (Figs. 11A-D).

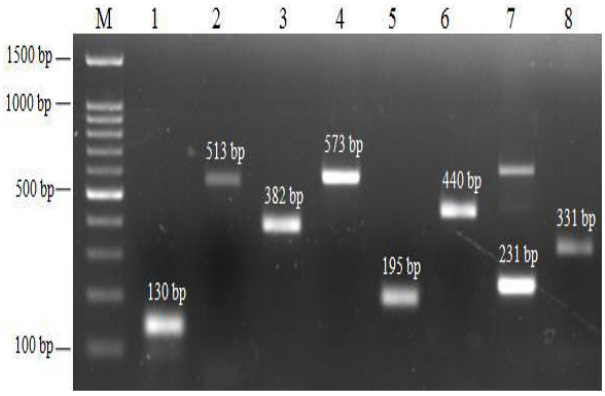

Fig. 6: PCR for detection of the *A. hydrophila* virulence genes. Lane M: 100 bp ladder. Lane 1: *ahh*1 gene, Lane 2: *ela* gene, Lane 3: *lip*M gene, Lane 4: *tap*D gene, Lane 5: *hcp* gene, Lane 6: *aex*U gene, Lane 7: *act* gene, and Lane 8: *ast* gene

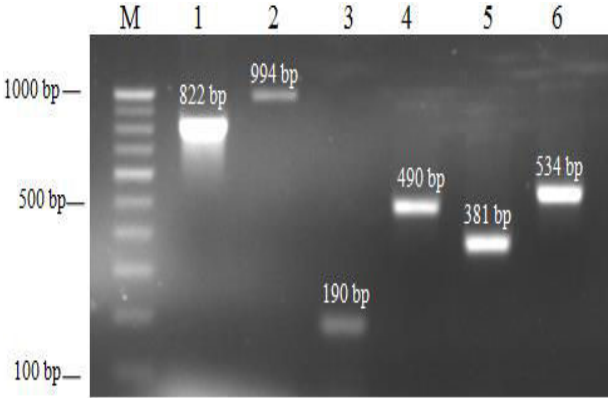

Fig. 7: PCR for detection of the *S. iniae* virulence genes. Lane M: 100 bp ladder. Lane 1: *scpl* gene, Lane 2: *sim*A gene, Lane 3: *sag*A gene, Lane 4: *pgm* gene, Lane 5: *pdi* gene, and Lane 6: *cps*D gene

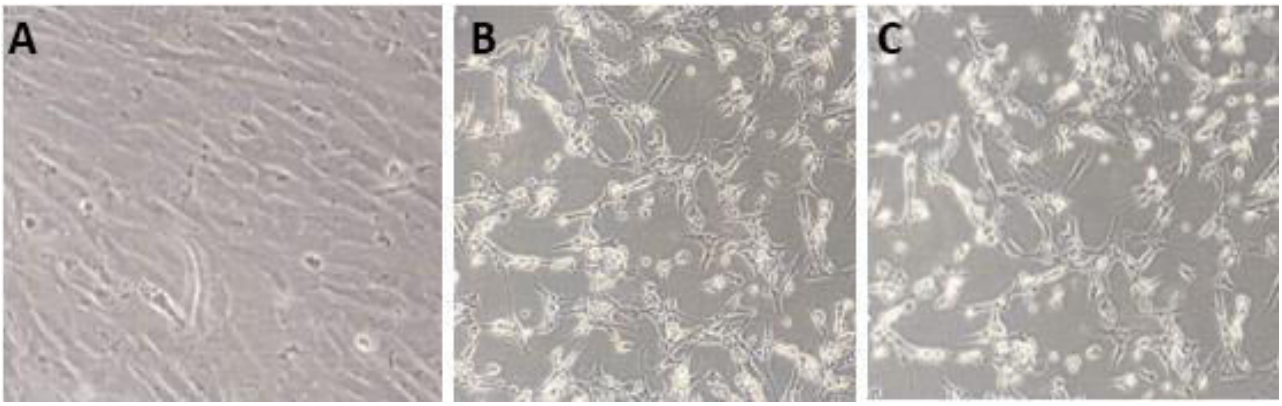

Fig. 8: Cytotoxicity of OniL cells. (**A**) Control cell lines, and (**B** and **C**) OniL cell lines incubated with culture supernatant of *A. hydrophila* and *S. iniae*, respectively, displaying a rounded and vacuolated appearance after 48 h incubation

Fig. 9: Photographs showing clinical signs in experimentally infected tilapia. (**A**) Hemorrhages at the base of operculum (black arrow) and fish body skin erosions (arrowhead), reddening (white arrow) and fin rot, and (**B**) Fish showing pale liver (black arrow) and fluid accumulation (white arrow)

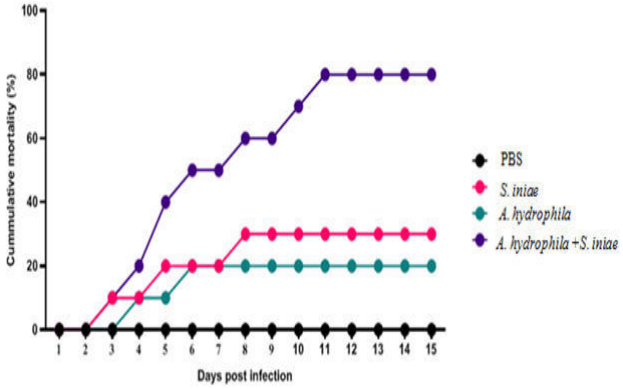

Fig. 10: Cumulative mortality percentage of tilapia challenged with *A. hydrophila* and *S. iniae*

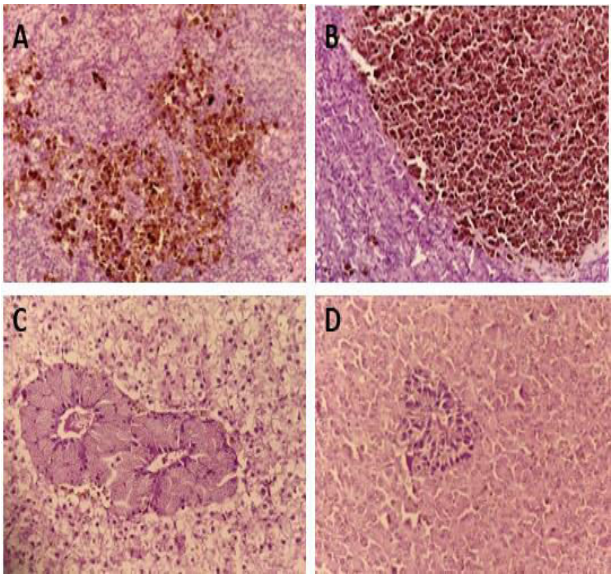

Fig. 11: Histopathology of spleen (**A**: Control and **B**: Infected) and liver (**C**: Control and **D**: Infected) of experimental infected tilapia. (**D**) Hyper activation of melanomacrophage centers in the infected spleen, and (**B**) Infected liver tissue showing congestion of sinusoids, loss of hepatocytes, and vacuolar degeneration due to depletion of fat reserves

Discussion

In this study, we report a disease outbreak associated with substantial mortality rates in Nile tilapia cultured in river-based cages in Karnataka, India in 2019. Tilapia fish cultured in floating cages are highly susceptible to disease outbreaks (Dong *et al*., 2015; Niu *et al*., 2020). *A. hydrophila* and *S. iniae* are the well-established etiological agents of disease outbreaks in cultured tilapia (Cutuli *et al*., 2015; Deng *et al*., 2015; Ortega *et al*., 2018; Abdel and Khafaga, 2020; Basri *et al*., 2020). *Aeromoniasis* and *Streptococcosis* outbreaks cause severe losses in tilapia culture (Rahmatullah *et al*., 2017; Monir *et al*., 2020; Niu *et al*., 2020; Pauzi *et al*., 2020). Environmental changes occurring as a result of human interventions, such as the transfer of organisms, environmental degradation, and agricultural practices, are the drivers of infectious diseases. Aquaculture has a significant impact on the ecology of fish and their pathogens. In addition, several variables in aquaculture can enhance pathogen virulence (Pulkkinen *et al*., 2010).

The clinical signs of infection displayed by tilapia in this study were bilateral exophthalmia, dark discoloration of the body, irregular swimming, loss of scales, and severe skin ulceration, which were consistent with those of prior studies (Aboyadak *et al*., 2015; Deng *et al*., 2015; Ortega *et al*., 2018; Shoemaker *et al*., 2000; Nguyen *et al*., 2001).

Antibiotic susceptibility tests indicated that *A. hydrophila* had a low MAR index (0.16), whereas *S. iniae* had a higher MAR index (0.71). A low MAR index (<0.20) indicates that the strain came from a low-risk source, whereas a high MAR index (>0.25) indicates that the strain came from a high-risk source that is constantly exposed to antibiotics (Krumperman, 1983). The indiscriminate use of antibiotics against a bacterial pathogen can result in the development of antibiotic resistance, posing a threat to the aquatic environment and human health (Smith *et al*., 1994; Aisyhah *et al*., 2015). Understanding the pathogenesis requires research on the virulence factors involved in disease progression. In this study, *A. hydrophila* was found to harbour several virulence genes (*Ahh*1, *act*, *as*t, *ela*, *lip*M, *aex*U, *tap*D, and *hcp*). Previous investigations reported that the pathogenesis of *A. hydrophila* is associated with the production of haemolysin, enterotoxins, aerolysin, exoenzyme, and lipase genes (Asao *et al*., 1984; Stehr *et al*., 2003; Wang *et al*., 2003). Several virulence genes (*sim*A, *scp*I, *pdi*, *pgm*, *cps*D, and *sag*A) were identified in *S. iniae* isolated from tilapia in the present study.

Major virulence factors that contribute to the pathogenesis of *S. iniae* have been previously identified as phosphoglucomutase enzyme, exopolysaccharide, M proteins, capsular polysaccharides, and cytolysin streptolysin S (Baiano and Barnes, 2009). Similarly, Deng *et al*. (2015) reported the presence of six virulence genes in *S. iniae* isolated from Siberian sturgeon. Higher infectivity, tissue-damaging capacity, and faster growth are all signs of enhanced virulence (Pulkkinen *et al*., 2010).

The cytotoxic effect of secretory products of *A. hydrophila* and *S. iniae* on tilapia cells was tested on the tilapia liver (OniL) cell culture. Rounding, cytoplasmic vacuolization, and detachment from the surface were observed in cells treated with the supernatant. This indicates that *A. hydrophila* and *S. iniae* release virulence components capable of inducing cytotoxicity on OniL cells. Extracellular proteins from *A. veronii* and *A. hydrophila* have previously been shown to have similar cytotoxic effects on OniL and DRG cells, respectively (Raj *et al*., 2019; Nithin *et al*., 2021). Similarly, cytotoxicity has been observed in TK-1 cells when they were exposed to supernatant from *S. agalactiae* culture (Palang *et al*., 2020).

An experimental challenge study was performed to confirm the pathogenicity of the isolated strains and to reproduce the disease in healthy fish. In this study, co-infection challenge resulted in higher mortality in comparison to infection with individual strains. Similar findings have been reported by several other researchers (Assis *et al*., 2017; Dong *et al*., 2017). This study confirmed that a synergistic impact of *A. hydrophila* and *S. iniae* infection led to increased disease severity and mortality in cultured tilapia.

The histopathological examinations in the present study revealed the presence of hyper activated melanomacrophage centers in the spleen, and this indicates that the bacterial infection stimulated an immune response in the infected fish (Salah *et al*., 2012). Similarly, the increased necrosis of hepatocytes in the liver tissue indicates systemic bacterial infection in the liver, which is also in accordance with previous reports (Ray and Homechaudhuri, 2014).

In conclusion, this study shows that the natural co-infection of *A. hydrophila* and *S. iniae* led to heavy mortalities in cage cultured Nile tilapia in Karnataka, India. This study provides baseline information on the existence of highly virulent pathogenic strains affecting tilapia cage culture. This highlights the need to expand the list of bacterial pathogens to be tested in the routine surveillance program and to improve the management practices.

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Conflict of interest

The authors declare that there is no known conflict of interest.

References

Abdel-Latif, H; Dawood, M; Menanteau-Ledouble, S and El-Matbouli, M (2020). The nature and consequences of co-infections in tilapia: A review. J. Fish Dis., 43: 651-664. https://doi.org/10.1111/jfd.13164.

Abdel-Latif, H and Khafaga, AF (2020). Natural co-infection of cultured Nile tilapia *Oreochromis niloticus* with *Aeromonas hydrophila* and *Gyrodactylus cichlidarum* experiencing high mortality during summer. Aquac. Res., 51: 1880-1892.

Aboyadak, IM; Ali, NGM; Goda, AMAS; Aboelgalagel, WH and Salam, AME (2015). Molecular detection of *Aeromonas hydrophila* as the main cause of outbreak in tilapia farms in Egypt. J. Aquac. Mar. Biol., 2: 2-5. doi: 10.15406/jamb.2015.02.00045.

Aisyhah, MAS; Amal, MNA; Zamri-Saad, M; Siti-Zahrah, A and Shaqinah, NN (2015). *Streptococcus agalactiae* isolates from cultured fishes in Malaysia manifesting low resistance pattern towards selected antibiotics. J. Fish Dis., 38: 1093-1098.

Asao, T; Kinoshita, Y; Kozaki, S; Uemura, T and Sakaguchi, G (1984). Purification and some properties of *Aeromonas hydrophila* hemolysin. Infect. Immun., 46: 122-127.

Assis, G; Tavares, G; Pereira, F; Figueiredo, H and Leal, C (2017). Natural coinfection by *Streptococcus agalactiae* and *Francisella noatunensis* subsp. *orientalis* in farmed Nile tilapia (*Oreochromis niloticus* L.). J. Fish Dis., 40: 51-63.

Ausuhel, FM; Brent, R; Kington, RE; Moore. DD; Seidman. JG; Smith, JA and Struhl, K (1990). Current Protocols in Molecular Biology. 1: Greene Publishing Associates, Inc. and John Wiley and Sons, Inc., NY.

Baiano, JC and Barnes, AC (2009). Towards control of Streptococcus iniae. Emerg. Infect Dis., 15: 1891-1896. doi: 10.3201/eid1512.090232. PMID: 19961667; PMCID: PMC3044519.

Barrow, GI and Feltham, RKA (2004). Cowan and Steel's manual for the identification of medical bacteria. 3rd Edn., Cambridge University Press. P: 329.

Basri, L; Roslindawani, MN; Salleh, A; Yasin, ISM; Saad, MZ; Rahaman, NYA; Barkham, T and Amal, MNA (2020). Co-infections of tilapia lake virus, *Aeromonas hydrophila* and *Streptococcus agalactiae* in farmed red hybrid tilapia. Animals. 10: 2141. doi: 10.3390/ ani10112141.

Baums, CG; Hermeyer, K; Leimbach, S; Adamek, M; Czerny, CP; Schwark, GH; Weigand, VP; Baumgєartner, W and Steinhagen, D (2013). Establishment of a model of *Streptococcus iniae* Meningoencephalitis in Nile Tilapia (*Oreochromis niloticus*). J. Comp. Pathol., 149: 94-102.

Bigarre, L; Cabon, J; Baud, M; Heimann, M; Body, A; Lieffrig, F and Castric, J (2009). Outbreak of betanodavirus infection in tilapia, *Oreochromis niloticus* (L.), in fresh water. J. Fish Dis., 32: 667-673.

Clinical and Laboratory Standards Institute (CLSI) (2006). *Performance standards for antimicrobial disk susceptibility tests*. Approved Standard, 9th Edn., IN Clinical and Laboratory Standards Institute document M2-A9 (ISBN 1-

56238-586-0). Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania, 19087-1898, USA.

Clinical and Laboratory Standards Institute (CLSI) (2016). *Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria*. 3rd Edn., CLSI guideline M45. Wayne, PA: Clinical and Laboratory Standards Institute.

Clinical and Laboratory Standards Institute (CLSI) (2017). *Performance standards for antimicrobial susceptibility testing*. 27th Edn., CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute.

Cutuli, MT; Gibello, A; Rodriguez-Bertos, A; Blanco, MM; Villarroel, M; Giraldo, A and Guarro, J (2015). Skin and subcutaneous mycoses in tilapia (*Oreochromis niloticus*) caused by *Fusarium oxysporum* in coinfection with *Aeromonas hydrophila*. Med. Mycol. Case Rep., 9: 7-11.

Deng, ML; Yu, ZH; Geng, Y; Wang, KY; Chen, DF; Huang, XL; Ou, Y; Chen, ZL; Zhong, ZJ and Lai, WM (2015). Outbreaks of Streptococcosis associated with *Streptococcus iniae* in Siberian sturgeon (*Acipenser baerii*) in China. Aquac. Res., 48: 1-11. doi: 10.1111/are.12934.

Dong, HT; Nguyen, VV; Le, HD; Sangsuriya, P; Jitrakorn, S; Saksmerprome, V; Senapin, S and Rodkhum, C (2015). Naturally concurrent infections of bacterial and viral pathogens in disease outbreaks in cultured Nile tilapia (*Oreochromis niloticus*) farms. Aquaculture. 448: 427-435. doi: 10.1016/j.aquaculture.2015.06.027.

Dong, HT; Techatanakitarnan, C; Jindakittikul, P; Thaiprayoon, A; Taengphu, S; Charoensapsri, W; Khunrae, P; Rattanarojpong, T and Senapin, S (2017). *Aeromonas jandaei* and *Aeromonas veronii* caused disease and mortality in Nile tilapia. *Oreochromis niloticus* (L.). J. Fish Dis., doi: 10.1111/jfd.12617.

El-Sayed, AFM (2019). *Tilapia culture*. 2nd Edn., Cambridge, MA: Academic Press. PP: 1-275.

Eyngor, M; Zamostiano, R; Tsofack, JEK; Berkowitz, A; Bercovier, H; Tinman, S and Eldar, A (2014). Identification of a novel RNA virus lethal to tilapia. J. Clin. Microbiol., 52: 4137-4146.

FAO (2017). Statistics and Information Branch, Fisheries and Aquaculture Department/FAO. Fishery and Aquaculture Statistics. Global Production by Production Source 1950-2015 (FishstatJ). FAO Fisheries and Aquaculture Department (online), Rome.

FAO (2020). The state of world fisheries and aquaculture, 2020. Sustainability in Action. Food and Agriculture Organization of the United Nations, Rome. https://doi.org/10.4060/ca9229en.

Girisha, SK; Puneeth, TG; Nithin, MS; Kumar, BN; Ajay, SK; Vinay, TN; Suresh, T; Venugopal, MN and Ramesh, KS (2020). Red sea bream iridovirus disease (RSIVD) outbreak in Asian seabass (*Lates calcarifer*) cultured in open estuarine cages along the west coast of India: First report. Aquaculture. 520: 734712. https://doi.org/10.1016/j.aquaculture.2019.734712.

Grim, CJ; Kozlova, EV; Sha, J; Fitts, EC; van Lier, CJ; Kirtley, ML and Shak, JR (2013). Characterization of *Aeromonas hydrophila* wound pathotypes by comparative genomic and functional analyses of virulence genes. mBio., 4: e00064-e13. https://doi.org/10.1128/mBio.00064-13e00064-00013.

Julia, WP and Phillip, HK (2011). Molecular identification and virulence of three *Aeromonas hydrophila* isolates cultured from infected channel catfish during a disease outbreak in west Alabama (USA) in 2009. Dis. Aquat. Org., 94: 249-253.

Krumperman, PH (1983). Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. Appl. Environ. Microbiol., 46: 165-170.

Kumar, S; Stecher, G; Li, M; Knyaz, C and Tamura, K (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol., 35: 1547-1549.

Lane, DJ (1991). 16S/23S rRNA sequencing. In: Stackebrandt, E and Goodfellow, M (Eds.), *Nucleic acid techniques in bacterial systematic*. New York, John Wiley and Sons. PP: 115-175.

Ma, J; Bruce, TJ; Oliver, L and Cain, KD (2019). Co-infection of rainbow trout (*Oncorhynchus mykiss*) with infectious haematopoietic necrosis virus and *Flavobacterium psychrophilum*. J. Fish Dis., 42: 1065-1076. https://doIorg/10.1111/jfd.13012.

Menaga, M and Fitzsimmons, K (2017). Growth of tilapia industry in India. J. World Aquac. Soc., 48: 49-52.

Monir, MS; Yuso, SM; Mohamad, A and Ina-Salwany, MY (2020). Vaccination of tilapia against Motile Aeromonas Septicemia: A review. J. Aquat. Anim. Health. 32: 65-76.

Nguyen, HT; Kanai, K and Yoshikoshi, K (2001). Experimental *Streptococcus iniae* infection in Japanese flounder *Paralichthys olivaceus*. Fish Pathol., 36: 40-41.

Nithin, MS; Girisha, SK; Kushala, KB; Chandan, DV; Puneeth, TG; Naveen Kumar, BT; Vinay, TN; Suresh, T; Sahoo, L and Ramesha, KS (2021). Novel lytic bacteriophages (AhFM4 & AhFM5) as bio-control measures against multidrug resistant biofilm producing *Aeromonas hydrophila* (AhZ1K). Aquaculture. 544: 737106. https://doi.org/10.1016/j.aquaculture.2021.737106.

Niu, G; Khattiya, R; Zhang, T; Boonyayatra, S and Wongsathein, D (2020). Phenotypic and genotypic characterization of *Streptococcus* spp. isolated from tilapia (*Oreochromis* spp.) cultured in river-based cage and earthen ponds in Northern Thailand. J. Fish Dis., 43: 391-398. https://doi.org/10.1111/jfd.13137.

Ortega, C; Garcıa, I; Irgang, R; Fajardo, R; Tapia-Cammas, D; Acosta, J and Avendāno-Herrera, R (2018). First identification and characterization of *Streptococcus iniae* obtained from tilapia (*Oreochromis aureus*) farmed in Mexico. J. Fish Dis., 41: 773-782. https://doi.org/10.1111/jfd.12775.

Pakingking, RJ; Takano, R; Nishizawa, T; Mori, KI; Iida, Y; Arimoto, M and Muroga, K (2003). Experimental coinfection with aquabirnavirus and viral hemorrhagic septicemia virus (VHSV), *Edwardsiella tarda* or *Streptococcus iniae* in Japanese flounder *Paralichthys olivaceus*. Fish Pathol., 38: 15-21.

Palang, I; Hirono, I; Senapin, S; Sirimanapong, W; Withyachumnarnkul, B and Vanichviriyakit, R (2020). Cytotoxicity of *Streptococcus agalactiae* secretory protein on tilapia cultured cells. J. Fish Dis., 43: 1229-1236. https://doi.org/10.1111/jfd.13230.

Pauzi, NA; Mohamad, N; Azzam-Sayuti, M; Ina-Salwany, MD; Zamri-Saad, M; Nasruddin, NS and Amal, MNA (2020). Antibiotic susceptibility and pathogenicity of *Aeromonas hydrophila* isolated from red hybrid tilapia (*Oreochromis niloticus* - *Oreochromis mossambicus*) in Malaysia. Vet. World. 13: 2166-2171.

Perretta, A; Antúnez, K and Zunino, P (2018). Phenotypic, molecular and pathological characterization of motile aeromonads isolated from diseased fishes cultured in Uruguay. J. Fish Dis., 41: 1559-1569.

Pulkkinen, K; Suomalainen, LR; Read, AF; Ebert, D;

Rintamäki, P and Valtonen, ET (2010). Intensive fish farming and the evolution of pathogen virulence: the case of columnaris disease in Finland. Proc. Biol. Sci., 277: 593-600. https://doi.org/10.1098/rspb.2009.1659.

Rahmatullah, M; Ariff, M; Kahieshesfandiari, M; Daud, HM; Zamri-Saad, M; Sabri, MY and Ina-Salwany, MY (2017). Isolation and pathogenicity of *Streptococcus iniae* in cultured red hybrid Tilapia in Malaysia. J. Aquat. Anim. Health. 29: 208-213. https://doi.org/10.1080/08997659.2017.1360411.

Raj, NS; Swaminathan, TR; Dharmaratnam, A; Raja, SA; Ramraj, D and Lal, KK (2019). *Aeromonas veronii* caused bilateral exophthalmia and mass mortality in cultured Nile tilapia, *Oreochromis niloticus* (L.) in India. Aquaculture. 512: 734278. doi: 10.1016/j.aquaculture.2019.734278.

Ray, SD and Homechaudhuri, S (2014). Morphological and functional characterization of hepatic cells in Indian major carp, *Cirrhinus mrigala* against *Aeromonas hydrophila* infection. J. Environ. Biol., 35: 253-258.

Salah, MA; Wael, GN; Mounir, M and Salem-Bekhi, MM (2012). Bacteriological and histopathological studies on Enterobacteriacea in Nile tilapia *Oreochromis Niloticus*. J. Pharm. Biomed., 2: 94-104.

Sen, K and Rodgers, M (2004). Distribution of six virulence factors in Aeromonas species isolated from US drinking water utilities: a PCR identification. J. Appl. Microbiol., 97: 1077-1086. https://doi.org/10.1111/j.1365-2672.2004.02398.x.

Shoemaker, CA; Evans, JJ and Klesius, PH (2000). Density and dose: Factors affecting mortality of *Streptococcus iniae* infected tilapia (*Oreochromis niloticus*). Aquaculture. 188: 229-235.

Smith, P; Hiney, MP and Samuelesen, OB (1994). Bacterial resistance to antimicrobial agent used in fish farming: a critical evaluation of method and meaning. Annu. Rev. Fish Dis., 4: 273-313.

Stehr, F; Kretschmar, M; Kroger, C; Hube, B and Schafer, W (2003). Microbial lipases as virulence factors. J. Mol. Catal. BEnzym., 22: 347-355. doi: 10.1016/S1381-1177(03)00049-3.

Swaminathan, TR; Ravi, C; Kumar, R; Dharmaratnam, A; Basheer, VS; Pradhan, PK and Sood, N (2018). Derivation of two tilapia (*Oreochromis niloticus*) cell lines for efficient propagation of Tilapia Lake Virus (TiLV). Aquaculture. 492: 206-214.

Swift, S; Lynch, JM; Fish, L; Kirke, DF; Tomas, JM; Stewart, GS and Williams, P (1999). Quarum sensing-dependent regulation and blockade of exoprotease production in *Aeromonas hydrophila*. Infect. Immun., 67: 5192-5199.

Wang, GH; Clark, CG; Liu, CY; Pucknell, C; Munro, CK and Kruk, TM (2003). Detection and characterization of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas* sobria by multiplex PCR. J. Clin. Microbiol., 41: 1048-1054. doi: 10.1128/JCM.41.3.1048-1054.2003.

Zhou, SM; Fan, Y; Zhu, XQ; Xie, MQ and Li, AX (2011). Rapid identification of *Streptococcus iniae* by specific PCR assay utilizing genetic markers in ITS rDNA. J. Fish Dis., 34: 265-271. https://doi.org/10.1111/j.1365-2761.2010.01233.x.

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