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Unveiling the Mechanisms of *Aeromonas hydrophila* COF_AHE51 Induced Mortality in *Labeo rohita*: Hemato-biochemical and Immune-Pathological Perspectives

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ABSTRACT

Background: This study presents the successful isolation and comprehensive characterization of a selected virulent strain of *Aeromonas hydrophila*, denoted COF_AHE51, which was identified as the causative agent behind mass fish mortality in an aquaculture pond in Tripura.

Methods: The identification of this species was achieved through morphological, biochemical and molecular techniques. The pathogenicity was assessed by fulfilling Koch's postulate, determination of haemato-biochemical and immune-pathophysiological parameters and histopathological study. The antimicrobial resistance was examined by performing an antibiotic sensitivity test.

Result: Experimental infection of *Labeo rohita* with COF_AHE51 resulted in the development of hallmark clinical signs, such as haemorrhages, abdominal swelling, discoloration and tail and fin rot. The calculated LD_{50} of the pathogenic strain was 1.4×10^6 cfu/fish. In-depth hematological and immunological analyses, alongside histopathological examinations of affected tissues, revealed remarkable perturbations suggestive of systemic bacterial septicaemia. Furthermore, the strain was observed to be resistant to several commonly used antibiotics, including kanamycin, cefoxitin, cefotaxime and ampicillin, accentuating the considerable threat posed by *A. hydrophila* infections in aquaculture settings.

Key words: *Aeromonas hydrophila*, Antibiotic resistance, *Labeo rohita*, Pathogenicity, Septicaemia.

INTRODUCTION

Aeromonas spp. are widespread gram-negative bacteria found in various habitats, including soil, freshwater, brackish water, marine water and sewage and can act as opportunistic pathogens in organisms such as fish, amphibians and humans (Janda and Abbott, 2010). *A. hydrophila* is of particular interest due to its frequent association with infections in both fish and humans (Furmanek-Blaszk, 2014), causing severe disease manifestations in fish such as motile aeromonad septicemia (MAS), epizootic ulcerative syndrome and fin/tail rot (Das *et al*., 2015; Mallik *et al*., 2022). *A. hydrophila* is the most prevalent gram-negative pathogen responsible for extensive disease outbreaks and severe economic losses in global freshwater fish aquaculture (Aboyadak *et al*., 2015).

Rohu (*Labeo rohita*) is a primary Indian major carp widely used in carp polyculture systems due to its high growth potential and consumer appeal (Jena *et al*., 1998). It is a majorly cultured species in the aquaculture farms of Tripura, where fish is a crucial component of over 95% of the population's daily diet (Govt. of Tripura, 2023). Despite a per-capita fish consumption of 26.26 kg in 2020-2021, the sector faces challenges such as inadequate infrastructure, limited private entrepreneurship and a considerable gap between fish availability and demand (Debnath *et al*., 2018). Intensification practices have led to frequent disease outbreaks and the emergence of pathogens, posing a significant threat to the sustainability of the sector.

Identifying and characterizing pathogens causing disease outbreaks is crucial for effective diagnosis, management and control in aquaculture (Noga, 2010). To achieve this, every strain isolated from diseased fish or the environment should be evaluated for its pathogenicity, pathophysiology, histopathology and antimicrobial resistance patterns (Samayanpaulraj *et al*., 2019). In this study, we isolated and identified the pathogenic bacteria causing mass mortality in *L. rohita* and evaluated its virulence and antibiotic resistivity patterns.

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MATERIALS AND METHODS

Sample collection and isolation of bacteria from diseased fish

In November 2020, a bacterial disease outbreak occurred in a fish farm in West Tripura district, India (23°55′16.0″N, 91°18′45.0″E). Ten diseased *L. rohita* were collected for

diagnosis. Baseline data, behavioral and gross pathological signs were recorded. Water quality parameters of the farm were also analyzed using standard protocols (APHA, 2005). Kidney and liver samples were aseptically collected, homogenized, diluted and spread onto Nutrient Agar (NA) and Rimler-Shotts (RS) Agar plates. The plates were incubated at 29±1°C for 24 hours and a single pure isolates were obtained. The isolate COF_AHE51 was selected for this study. The study was conducted for a period of three years at College of Fisheries, Central Agricultural University, Lembucherra, Tripura.

Phenotypic and molecular identification

Preliminary identification was performed using different phenotypic tests (listed in Table 1) following Tille (2017). Biofilm production test was performed following Freeman *et al.* (1989). The identity of the isolate was confirmed through the 16S rRNA gene amplification using a pair of universal oligonucleotide primers 27F (5′-AGAGTTT GATCCTGGCTCAG-3′) and 1492R (5′TACGGTTACCT TGTTACGACTT-3′) (Lane, 1991). The amplified PCR product was detected and purified using HiPurA™ PCR Product Purification Kit (Himedia, Mumbai, India) and sequencing was performed by the Sanger Sequencing method (Heather and Chain, 2016) in an automated DNA Analyzer (ABI 3730 (48 capillary) Sequencers, Applied Biosystems, Foster City, CA, United States). The identity of the bacterial isolate was assigned by comparing its DNA sequence with those available in the GenBank NCBI (National Center for Biotechnology Information) database using a BLAST (basic local alignment search tool) 2.13.0+ program (Zhang *et al*., 2000). The sequences were then aligned by pair wise alignment using clustal W and the phylogenetic tree was constructed using MEGA 11 software by the neighbour joining method (Saitou and Nei, 1987). The species was identified based on the lowest *E*-value and percentage similarity in BLAST.

Pathogenicity study

Healthy *Labeo rohita* fingerlings (10.38±3.2 cm, 14.8±3.68 g) were acclimatized for 14 days and randomly distributed into 500 L plastic tanks (six treatments and one control group in duplicate, with 10 fish per tank). The bacterial suspension was prepared by inoculating the selected bacterial isolate in nutrient broth (NB) for 48 hours at 29±1°C, centrifuged, washed and resuspended in 0.89% physiological saline. The concentration of bacteria in the stock solution was determined using a Neubauer cell counting chamber and six concentrations (10^4 to 10^9 cells/mL) were prepared by serial dilution. 10 fish in each tank were injected intraperitoneally with each concentration at 0.1 mL/fish, with a control group injected with physiological saline. The fish were fasted for 24 hours before the experiment and were anesthetized using clove oil (0.2 mL per liter) before inoculation. The experiment was conducted for 10 days and mortality rates and clinical signs were recorded. The fish were given mild aeration and were not fed during the study period. The LD_{50} was calculated using Reed and Muench (1938) method. The pathogenicity was confirmed by satisfying Koch's postulate.

Determination of haemato-biochemical and immune-pathophysiological parameters

After post infection study blood and serum were collected randomly from the treatments and control fish in triplicate drawn from the caudal vein. The haematological parameters, such as haemoglobin (Hb), packed cell volume (PCV), total RBCs and WBCs count were determined following Schaperclaus (1991). The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated using the formula given by Dacie and Lewis (2001).

$$MCV = \frac{PCV\ (\%)}{RBC\ count\ (In\ million\ mm^{-3})} \times 10$$

$$MCH = \frac{Haemoglobin\ (g/dl)}{RBC\ count\ (In\ million\ mm^{-3})} \times 10$$

$$MCHC = \frac{Haemoglobin\ (g/dl)}{PCV\ (\%)} \times 100$$

The biochemical parameters; glucose, alkaline phosphatase (ALP), serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), sodium (Na^+) and potassium (K^+) as well as the immunological parameters; total protein and albumin were determined using standard kits provided by Medsource Ozone Biomedicals Pvt. Ltd., India, according to the manufacturer's instructions. The globulin level was calculated by subtracting the albumin value from the total protein value. The respiratory burst activity was evaluated using the nitroblue tetrazolium (NBT) assay, following Anderson and Siwicki (1995). The antiprotease activity was determined using the procedure described by Zuo and Woo (1997) and calculated using the specified formula:

$$\text{Antiprotease activity} = \frac{\text{Proteolytic activity without antiprotease} - \text{Proteolytic activity with antiprotease}}{\text{Proteolytic activity without antiprotease}} \times 100\%$$

Histopathological study

Liver and kidney were obtained from freshly dead/moribund infected fish and immediately fixed in 10% neutral buffered formalin. The specimens were processed and stained according to the method described by Slaoui and Fiette (2011). Pre-embedding was carried out using an automated tissue processor (Thermo Scientific-Shandon, Citadel 2000, USA) and embedding was performed using a tissue embedder (Shandon Histocentre 3, USA). The embedded sections were then cut into 4-5 µm thick ribbons using a rotary microtome (Leica RM2245, Germany). The tissue sections were stained with haematoxylin and eosin (H&E), mounted in DPX and examined using a 40× trinocular microscope (Carl Zeiss Research- PRIMOSTAR-3).

Antibiotic sensitivity test

The antibiotic sensitivity test was performed by spreading a freshly prepared 0.5 McFarland Standard bacterial suspension on Mueller-Hinton Agar plates. Standard antibiotic disks (HiMedia, India) were dispensed and incubated at 35±2°C for 16 to 18 hours. The antibiotic sensitivity was determined by measuring the zone of inhibition using a ruler and compared with the interpretive chart of zone diameter to determine susceptibility, intermediate, or resistance according to the CLSI Performance Standards for Antimicrobial Susceptibility Testing (Wayne, 2022).

Statistical analysis

Data were analyzed using SPSS (SPSS Inc., Chicago IL, USA). Results were presented as a mean±standard error. Comparisons of the mean values were determined by One-way ANOVA and Duncan's test. A probability level of 0.05 was used to find out the significance in all cases.

RESULTS AND DISCUSSION

Disease outbreak farm investigation

Aquaculture diseases result from a complex interplay between the host, environment and pathogen. Intensification of aquaculture practices has led to the emergence of numerous diseases due to a lack of understanding of the balance between these factors (Snieszko, 1974). In this study, the disease outbreak farm was a poorly managed 0.25-hectare perennial grow-out pond with a high stocking density (4,500-5,000 fish). Diseased fish showed lethargy, haemorrhages (red patches), discoloration and tail/fin rot (Fig 1 A-B), resulting in a high mortality rate. Water quality parameters (value in mean±standard deviation) revealed decreased pH (6.5±0.3), alkalinity (21±0.46 mg/L) and hardness (26±0.5 mg/L) and elevated ammonia concentration (0.15±0.2 mg/L), with temperature (25±0.5°C) and dissolved oxygen (7.6±0.6 mg/L) within acceptable ranges. Fish in suboptimal environmental conditions are more susceptible to *A. hydrophila* infection (Harikrishnan and Balasundaram, 2005) and the current disease outbreak was likely predisposed by exceeding the pond's carrying capacity and failing to observe strict biosecurity measures, resulting in poor water quality.

Isolation, phenotypic and molecular identification

The colonies on NA and RS plates showed a uniform predominantly white, round, convex and smooth colonies with diameters ranging from 1-2 mm. The selected pure isolate COF_AHE51 was gram-negative, motile, facultative anaerobic and rod-shaped bacteria. Biochemical test showed positive reactions for all tests, except for the methyl red, urea hydrolysis, lactose and rhamnose fermentation tests (Table 1). Based on these test results, the isolated bacteria was preliminarily identified as *Aeromonas* species. The results were consistence with similar studies on *Aeromonas* sp. by Abbott *et al.* (2003) and Mazumder *et al.* (2021) with variation observed in lactose fermentation and lysine decarboxylation.

The 16S rRNA gene sequence analysis using BLAST revealed a high degree of similarity (98.98%) with the reference *Aeromonas hydrophila* strain TCS1 (GenBank accession number MN650222). The phylogenetic tree showed that COF_AHE51 was grouped with a cluster of known *A. hydrophila* strains (Fig 2). Identification of *A. hydrophila* species through the 16S rRNA gene was also employed by Mazumder *et al.* (2021). The sequence was deposited in GenBank and assigned the accession number OQ244496.

Pathogenicity study

The cumulative mortality rate and mortality curve (Fig 3) showed high mortality of *L. rohita* within 3-4 days of experimental infection study and a significant variation with

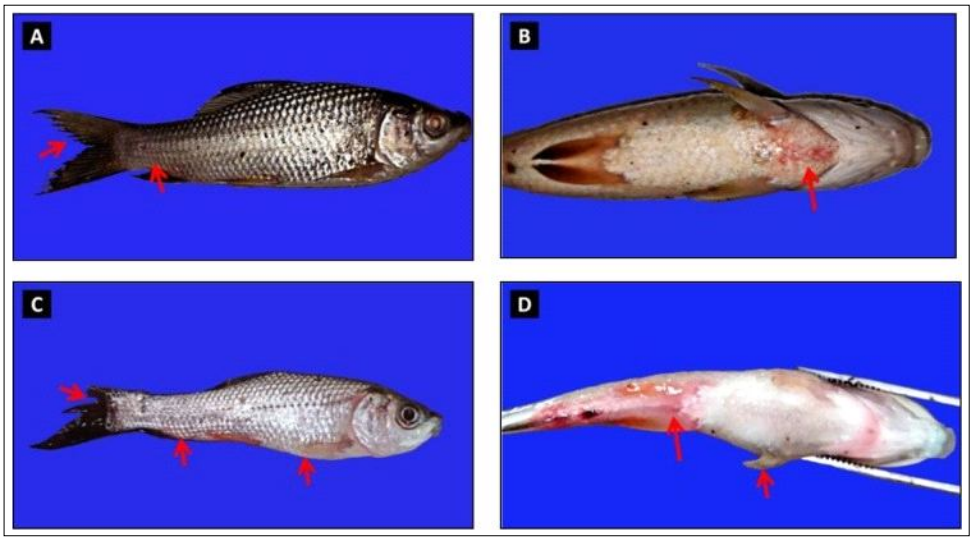
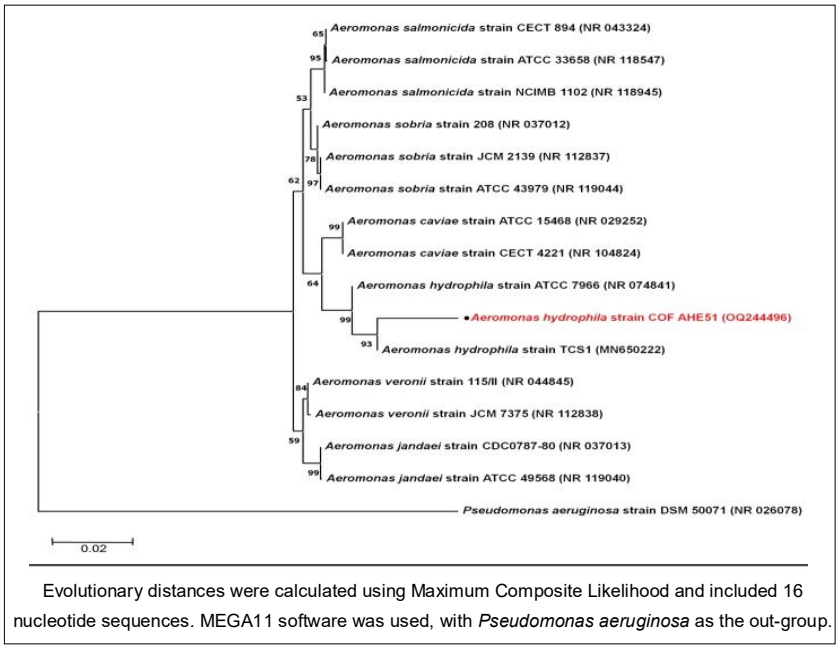


Fig 1: (A-B) Diseased *L. rohita* collected from the farm, showing tail rot, discolouration and haemorrhage on the base of fins and skin. (C-D) Experimental infected *L. rohita*, showing tail and fin rot, abdominal swelling and hemorrhage on the base of fins and skin.

an increase in bacterial concentration over 10-days. LD_{50} of *A. hydrophila* strain COF_AHE51 was 1.4×10^6 cfu/fish and infected fish exhibited lethargy, hemorrhages, abdominal swelling and tail/fin rot (Fig 1 C-D), indicating its virulence. LD_{50} values of 4.53×10^6 to 1.319×10^9 cfu/fish and $10^{5.4}$-$10^{7.5}$ cfu/fish have been reported in gourami (*Osphronemus gouramy*) by Rozi *et al.* (2018) and in European eels (*Anguilla anguilla*) by Esteve *et al.* (1993) infected with different strains of *A. hydrophila*.

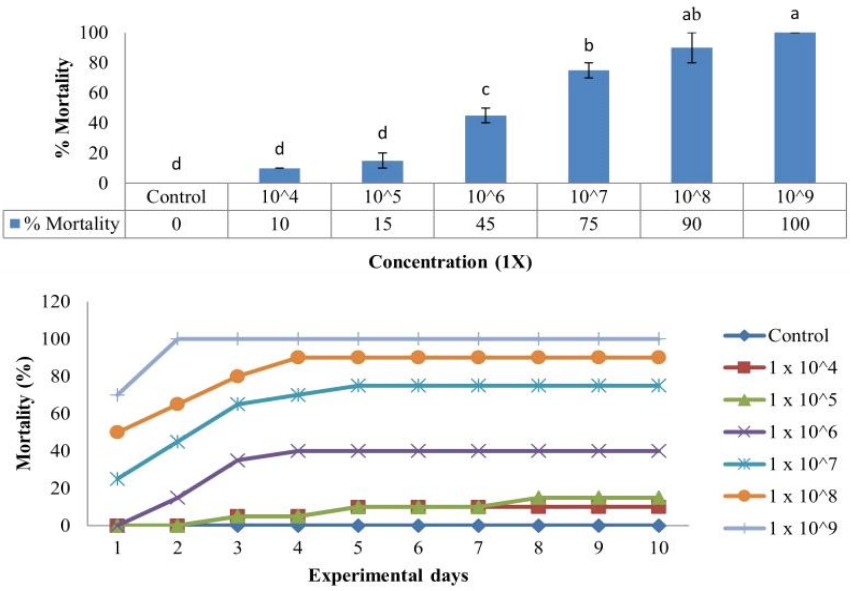
Haemato-biochemical and immune-pathophysiological parameters

Table 2 presents haemato-biochemical and immune-pathophysiological parameters of control and infected rohu



Evolutionary distances were calculated using Maximum Composite Likelihood and included 16 nucleotide sequences. MEGA11 software was used, with *Pseudomonas aeruginosa* as the out-group.

Fig 2: A phylogenetic tree of *Aeromonas* spp. constructed from 16S rRNA sequences using the Neighbor-Joining method with bootstrap (1000 replicates).



Means without a common superscript letter differ (p<0.05) as analyzed by one-way ANOVA and the DUNCAN test.

Fig 3: Cumulative mortality rate and mortality curve of *L. rohita* infected with *A. hydrophila* strain COF_AHE51.

Table 1: Phenotypic identification of the selected isolated bacteria strain COF_AHE51.

Phenotypic test	COF_AHE51 strain
Gram reaction	Gram negative
Colony shape	Round
Colony size	1-2 mm
Elevation	Convex
Margin	Entire
Texture	Smooth
Colour	White/Cream
Motility	Positive
Catalase	Positive
Oxidase	Positive
Indole	Positive
Methyl-Red	Negative
Voges-Proskauer	Positive
Citrate utilization	Positive
Gas formation	Positive
H_2S production	Positive
Nitrate reduction	Positive
Oxidation-Fermentation	Fermentative
Urea hydrolysis	Negative
Gelatin hydrolysis	Positive
Starch hydrolysis	Positive
Lipid hydrolysis	Positive
Casein hydrolysis	Positive
DNA hydrolysis	Positive
Biofilm production	Positive
Fermentation of glucose	Positive
Fermentation of lactose	Negative
Fermentation of sucrose	Positive
Fermentation of mannitol	Positive
Fermentation of rhamnose	Negative
Arginine dihydrolase	Positive
Lysine decarboxylation	Positive
Identified genus/species	*Aeromonas* sp.

after 10 days of experimental infection study. Haematological, biochemical and immunological parameters are crucial indicators of an animal's health (Laltlanmawia *et al*., 2019). Infected fish had decreased Hb, PCV and total RBC count, while total WBC count, MCV and MCH values were increased. Significant decreased in these parameters can be correlated to the hemolytic activity of *A. hydrophila* and due to the destruction of hemopoitic tissue (Tiwari and Pandey, 2014). The increase in WBC count suggests that infected fish may be mounting an immune response and variation in MCV and MCH indicates anaemic conditions, these changes are consistent with previous study (Vignesh *et al*., 2022).

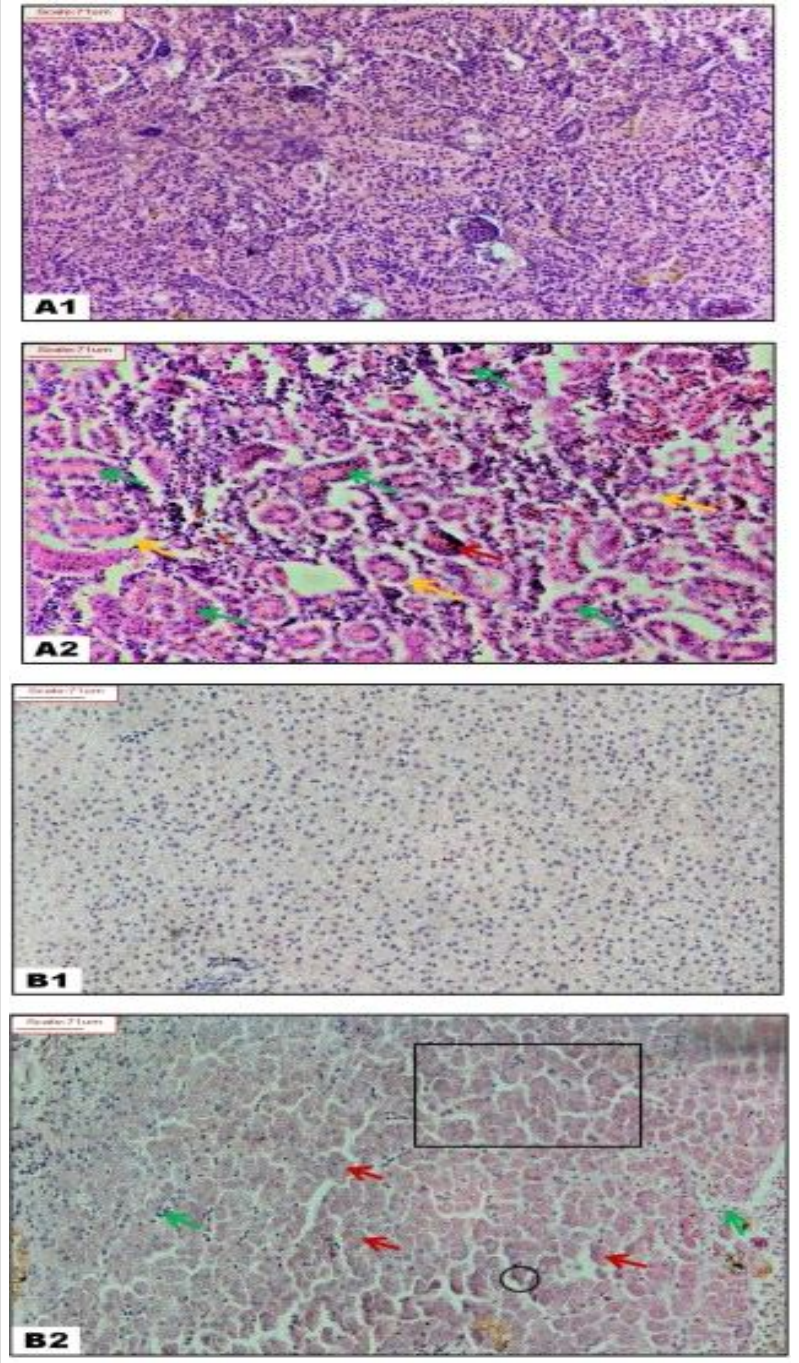
Biochemical analysis showed significantly higher levels of ALP, SGPT, SGOT and K^+ in infected fish and decreased glucose and Na^+ levels. ALP, SGOT and SGPT are crucial liver-specific enzymes widely used as biomarkers for assessing liver damage and diagnosing diseases (Kim *et al*., 2008). Elevated levels of these enzymes in this study indicate liver injury and stress. A similar alteration was also observed by Samayanpaulraj *et al*. (2019) in fish infected with *A. hydrophila*. Decreased glucose levels may be due to rapid liver glycogen depletion in the initial stage and fasting during the infection study. Serum electrolyte imbalances, such as decreased sodium and increased potassium, may be due to poor renal function or impairment caused by the pathogen (Ighodaro and Omole, 2010).

Immunological parameters showed significant increases in total serum protein and globulin levels and reductions in albumin, respiratory burst and antiprotease activity. The increase in total protein and globulin levels indicates activation of the fish's humoral immune response to infection by producing acute-phase proteins, including certain types of globulins, which contribute to the overall increase in total protein levels in the blood (Werner and Reavill, 1999). Albumin is an essential protein that regulates various physiological functions in fish (Tothova *et al*., 2016). Decreased albumin levels in the infected fish can be

Table 2: Haemato-biochemical and immune-pathophysiological parameters of infected and control *L. rohita*.

Haematological parameters	Hb (g/dL)	PCV (%)	Total RBC ($\times 10^6$ mm^{-1})	Total WBC ($\times 10^4$ mm^{-1})	MCV (fL)	MCH (pg)	MCHC (%)
Control	5.63±0.07^a	22.3±1.5^a	3.9±0.09^a	3.98±0.24^b	57.2±2.6^b	14.5±0.16^b	25.4±1.4
Treatment	4.4±0.12^b	13.3±0.9^b	1.62±0.04^b	5.58±0.1^a	82.6±7.1^a	27.2±0.035^a	33.4±2.7
Biochemical parameters	**Glucose (mg/dL)**	**ALP (U/L)**	**SGPT (U/L)**	**SGOT (U/L)**		**Na^+ (mmol/L)**	**K^+ (mmol/L)**
Control	83±1^a	72.3±3^b	7.46±1^b	4.66±0.8^b		47.9±0.4^a	4.01±0.2^b
Treatment	73±2^b	86.5±3^a	18.3±2^a	10.8±0.6^a		38±1^b	6.33±0.1^a
Immunological parameters	**Total protein (g/dL)**	**Albumin (g/dL)**	**Globulin (g/dL)**	**NBT (OD-540 nm)**			**Antiprotease (%)**
Control	4.51±0.2^b	1.62±0.04^a	2.89±0.2^b	0.795±0.02^a			50.2±0.12^a
Treatment	6.45±0.2^a	1.42±0.03^b	5.03±0.1^a	0.589±0.04^b			49.6±0.19^b

Values are means±SEM, n=3 per treatment group. Means in a column without a common superscript letter differ (P<0.05) as analyzed by one-way ANOVA and the DUNCAN test.



Progressive focal necrosis, tissue structural loss and vacuolar degeneration (black rectangle), nuclear loss and peripheral margination of nucleus in degenerating hepatocyte (black circle), swollen hepatic cells with degeneration and necrosis (red arrow), infiltration of sinusoids with leukocytes (green arrow) (H&E).

Fig 4: A1: Control fish kidney tissue; A2: Infected fish kidney tissue. Glomerular atrophy (red arrow), renal tubule enlargement, degeneration and necrosis (green arrow), diffused necrosis with dissociation of basement membrane and karyolysis (yellow arrow). B1: Control fish liver tissue. B2: Infected fish liver tissue.

Table 3: Antibiogram of *A. hydrophila* strain COF_AHE51.

List of antibiotics	*A. hydrophila* (COF_AHE51)
Amikacin (AK30)	S
Gentamicin (GEN10)	S
Kanamycin (K30)	R
Tobramycin (TOB10)	S
Streptomycin (S10)	S
Cephalothin (CEP30)	S
Cefoxitin (C×30)	R
Cefotaxime (CT×30)	R
Azithromycin (AZM15)	S
Erythromycin (E15)	S
Nitrofurantoin (NIT300)	S
Amoxicillin (AMC30)	S
Ampicillin (AMP10)	R
Oxacillin (O×1)	S
Ticarcillin (TI75)	I
Tetracyclin (TE30)	S
Chloramphenicol (C30)	S
Trimethoprim (TR5)	S

S= Susceptible; I= Intermediate; R= Resistance.

attributed to reduced synthesis due to liver failure, or protein depletion due to hemodilution (Lee, 2012). Similar finding was reported by Maqsood *et al.* (2009) in *Cyprinus carpio* infected with *A. hydrophila*. The reduction in respiratory burst and anti-protease activity suggests an immune-suppressive effect of the pathogen on the host's immune response. These findings are consistent with a previous study by Laltlanmawia *et al*. (2023) on fish infected with pathogenic bacteria.

Histopathological study

The kidney and liver tissue of infected *Labeo rohita* exhibited significant pathological changes, including focal necrosis, swollen cells, atrophy, structural loss and cell degeneration, as depicted in Fig 4 A2 and B2. These alterations are consistent with a previous study on fish infected with *A. hydrophila* (Rozi *et al*., 2018; Devadason, 2023). The kidney is an important organ for fish hematopoiesis (Davidson and Zon, 2004), while the liver plays an essential role in plasma protein synthesis and the regulation of various biochemicals in the blood (Mitra and Metcalf, 2012). Impairments in kidney function may account for a reduction in hematological parameters, whereas hepatic degeneration may explain the observed changes in immunological and biochemical parameters.

Antimicrobial sensitivity test

Antimicrobial resistance (AMR) is a major public health concern that hinders the effective treatment of diseases caused by pathogenic microorganisms. *A. hydrophila* strain COF_AHE51 was resistance to 21.1%, susceptibility to 73.6% and an intermediate response to 5.3% of the total antibiotics tested (Table 3). The isolate exhibited resistance to kanamycin, cefoxitin, cefotaxime and ampicillin. An intermediate response was observed for ticarcillin, whereas all the other antibiotics were effective against the isolate. Ramadan *et al*. (2018) reported similar resistance patterns in *A. hydrophila* strains to kanamycin, cefoxitin and cefotaxime. Mazumder *et al*. (2021) also found resistance to ampicillin. These resistance patterns could be attributed to various resistance mechanisms employed by *A. hydrophila*, including enzyme inactivation, gene mutation and active efflux, as reported by Guo *et al*. (2022).

CONCLUSION

In this study, *A. hydrophila* strain COF_AHE51 was identified as the cause of significant fish mortality in Tripura, India. This virulent strain induced septicemia and organ damage while also exhibiting resistance to multiple antibiotics. Nonetheless, further research is necessary to elucidate the precise genetic mechanisms and specific virulence factors underlying its pathogenesis and antibiotic resistance.

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Ethics statement

This study was reviewed and approved by the Institutional Animal Ethics Committee (IAEC), CAU-CF/48/IAEC/2018/03-21, dated 1st October 2021, of the College of Fisheries, Central Agricultural University, Imphal.

Conflict of interest: None.

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