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Study of bacterial haemorrhagic septicaemia causing mortality in Indian major carps from culture ponds of Kolleru lake, Andhra Pradesh, India

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ABSTRACT

Haemorrhagic septicaemia (HS) is a bacterial disease causing severe economic losses in freshwater aquaculture. The present study aimed to isolate, identify, and characterize its pathogen responsible for disease outbreaks and mortality in Indian major carps from ponds in the Kolleru Lake region, India through monthly sampling over eight months. Infected fish exhibited hemorrhages on the body, around the eyes, mouth, ventral surface, fins, and caudal region. Blood, kidney, liver, spleen, and gill tissues were processed for microbiological, biochemical, and molecular analysis. DNA from the isolates was amplified via PCR using primers specific to *Aeromonas hydrophila*, yielding a ~760 bp product, confirming the pathogen's presence. This finding establishes *A. hydrophila* as the causative agent of haemorrhagic septicaemia in *Labeo rohita* and *Catla catla*. The results underscore the frequent and significant mortalities in culture ponds caused by bacterial diseases such as haemorrhagic septicaemia and abdominal dropsy, highlighting the need for targeted disease management strategies in aquaculture.

Keywords: *Aeromonas hydrophila*, *C. catla*, Haemorrhagic septicaemia, Kolleru Lake, *L. rohita*

Aquaculture is a fastest-growing sector in animal food production, supplying approximately 46% of the global food fish demand and addressing rising protein needs. In India, the three Indian Major Carps (IMCs)-*Labeo rohita*, *Catla catla*, and *Cirrhinus mrigala*-constitute over 80% of total aquaculture output (CIFA 2004). Among bacterial pathogens in freshwater aquaculture, *Aeromonas hydrophila* is the most frequently encountered (Cipriano *et al.* 1984). Motile aeromonads cause a range of diseases, including motile bacterial haemorrhagic septicaemia (HS), aeromonad septicaemia, dropsy, ulceration, exophthalmia, and asymptomatic infections (Karunasagar *et al.* 1989, Swain *et al.* 2003, Austin and Austin 2007, Ozcan 2023). Reports of HS-related mortality and morbidity are documented worldwide, including in India (Bullock *et al.* 1971, Cipriano *et al.* 2001, Sahoo *et al.* 2008). Yambot (1998) isolated *A. hydrophila* from Nile tilapia (*Oreochromis niloticus*) showing haemorrhagic skin, scale loss, ulcerations, orbital haemorrhages, and exophthalmia. Abhishek *et al.* (2021) identified *A. hydrophila* and *A. jandaei* in *Anabas testudineus* with tail rot and ulcers, while Unver and Bakici (2021) reported morphological and anatomical damage from motile aeromonas septicaemia (MAS) in *Cyprinus carpio*.

Numerous studies have isolated *A. hydrophila* from wild and cultured freshwater fish, including rudd (*Scardinius erythrophthalmus hesperidicus*), chub (*Leuciscus cephalus albus*), tench (*Tinca tinca*), crucian carp (*Carassius carassius*), tilapia (*O. niloticus*), snakehead (*Channa punctatus*), and rainbow trout (*Oncorhynchus mykiss*), with symptoms such as anorexia, exophthalmia, skin haemorrhages, and acute or chronic MAS (Popovic *et al.* 2000, Nielsen *et al.* 2001, El-Ashram 2002, Yesmin *et al.* 2004, Kozinska 2007, Ahamad *et al.* 2013). Infections have also been reported in Russian sturgeon (*Acipenser gueldenstaedtii*), *Pangasius bocourti*, and both wild and cultured Nile tilapia, with signs of extensive haemorrhages, skin ulcerations, abdominal distension, gill swelling, and kidney congestion (Ibrahem *et al.* 2008, Timur *et al.* 2010, Bai *et al.* 2023). In India, Gopalakrishnan (1961) first reported HS as infectious dropsy caused by *A. hydrophila* in Indian carp from West Bengal. Karunasagar *et al.* (1986) described ulcerative infections in three carp species with loose scales, white snout lesions, and fin margin erosion, while Kumar and Dey (1986) reported bacterial septicaemia in silver carp (*Hypophthalmichthys molitrix*), with anaemia, weight loss, haemorrhages, dropsy, and mortality due to *A. hydrophila*. Later, Karunasagar *et al.* (1989) documented acute septicaemia outbreaks in IMC from West Godavari, Andhra Pradesh. Chandravanshi *et al.* (2020) identified *A. hydrophila* strains causing mortality in IMC from Ganjam district, Odisha. Other pathogenic

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species, including *A. veronii* and *Pseudomonas alcaligenes*, have been linked to abdominal dropsy and emaciation in *Barbonymus gonionotus* and *C. carpio* from the Andaman Islands (Jayasimhan *et al.* 2024). Multiple *A. hydrophila* strains have been reported in *C. catla* and *C. mrigala* with HS and EUS symptoms such as haemorrhages, blisters, abdominal dropsy, scale protrusion, abscesses, tail/fin rot, exophthalmia, and abdominal swelling (Nayak *et al.* 1999, Shome *et al.* 2005, Sahu *et al.* 2011). Dash *et al.* (2008) documented 400 dropsy cases in *C. mrigala*, *C. catla*, and *L. rohita*. Other reports confirmed *A. hydrophila* in diseased catfish (*C. carpio*), sturgeon (*Acipenser baerii*), and other species with haemorrhagic ulcers and abdominal lesions (Jayavignesh *et al.* 2011, Barde 2022, Serik *et al.* 2022).

Molecular detection methods have been widely applied for confirmation. Cascon *et al.* (1996) identified *A. hydrophila* Group 1 via PCR, while Swaminathan *et al*. (2004) developed PCR detection from aquatic samples. Yogananth *et al.* (2009) targeted aerolysin and haemolysin genes, and Mina *et al.* (2022) used PCR amplification of 16S rRNA and lipase genes to confirm *A. hydrophila* from infected carp organs in Iran. The present study is the first to document HS occurrence in *L. rohita* and *C. catla* from Kolleru Lake culture ponds, with a summary of global and Indian HS reports presented in Supplementary Tables 1 and 2.

MATERIALS AND METHODS

Study area: Kolleru Lake, India's largest freshwater lake and a wetland of international importance, serves as the main water source for surrounding culture ponds. For this study, the area was divided into two zones: Krishna–Kolleru basin and Godavari–Kolleru basin. Sampling was conducted in fish culture ponds located at Kaikaluru (Krishna district) and Bhimadolu (Godavari district), where the predominant species are Indian Major Carps (*L. rohita* and *C. catla*).

A preliminary survey identified ponds with high disease prevalence based on inputs from local fish farmers. Monthly sampling was carried out over eight months, focusing on early morning collections for 4–5 days, particularly during disease outbreaks. Live fish were first examined on-site; healthy individuals without visible symptoms were released immediately. Fish exhibiting clinical signs such as hemorrhages on the head, around the eyes, ventral body, pelvic and pectoral fins, as well as gill degeneration, were collected. Symptomatic fish, either alive or moribund, were carefully transported to the nearest fisheries laboratory for detailed microbiological examination (Fig. 1). This approach ensured the selection of representative diseased specimens from both study zones for pathogen identification and analysis.

Clinical examination of fish: Fish were thoroughly examined for external symptoms such as haemorrhages, scale loss, pigmentation, scale protrusion, and excessive mucus secretion, with observations recorded. For internal examination, fish were autopsied to assess organ condition. For microbiological studies, affected body areas were cleaned with 70% ethanol, and smears from these sites were aseptically collected using a sterile loop into nutrient broth. Wet mounts from internal organs (kidney, liver, spleen, gills) and external lesions were observed microscopically for pathogens. Blood, kidney, liver, gill, and spleen samples were aseptically inoculated into sterile nutrient broth, labeled, and incubated at 37°C for 24 h. Samples showing positive bacterial growth were selected for detailed microbiological analysis.

Biochemical analysis

Isolation and colony characteristics of bacterial pathogen: Blood and tissue samples (liver, kidney, gills, spleen) were aseptically inoculated into nutrient broth and incubated at 37°C. Bacterial growth was assessed after 24–48 h by broth turbidity. Turbid cultures were pour-plated on nutrient agar, TSA, and RS Medium (HiMedia). Colony features such as shape, size, and color, were recorded, and dominant morphotypes were isolated by repeated streaking to obtain pure cultures. These were preserved on slants at –20°C in vials for long-term storage.

Biochemical characterization: Isolates were phenotypically characterized by Gram staining and

Fig. 1. (A) Fish sampling and (B) *L. rohita* showing haemorrhages on the body

microscopic observation. Biochemical traits of suspected *Aeromonas hydrophila* were evaluated using standard methods (Cowan and Steel 1993, Bergey's Manual 1984). A reference strain, *A. hydrophila* (MTCC 646), served as a positive control in both phenotypic and biochemical analyses.

Molecular studies

Bacterial DNA extraction for PCR: DNA from bacterial isolates was extracted via the Phenol–Chloroform technique (Sambrook and Russell 1985) and verified on a 1.5% agarose gel prior to storage at 4°C. PCR primers specific to the 1952 bp lipase gene of Aeromonas hydrophila were chosen from GenBank (Cascon *et al*. 1996): forward 5'-AACCTGGTTCCGCTCAAGCCGTTG-3' and reverse 5'-TTGCCTCGCCTCGGCCCAGCAGCT-3'. PCR was conducted employing Kapa ready-mix (Kapa Biosystems, USA) in a 25 µL reaction including approximately 100 ng of DNA, 2.5 µL of buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM primers, 1 U of Taq polymerase, and sterile water. Cycling parameters: initial denaturation at 94°C for 4 min; 40 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 1.5 min; final extension at 72°C for 5 min. Amplified products were analyzed using a 1.5% agarose gel.

RESULTS AND DISCUSSION

Over eight months, 947 fish (*L. rohita* and *C. catla*) were collected from culture ponds in Krishna and Godavari regions of Kolleru Lake. Of these, 229 were infected, giving an overall prevalence of 24%. Among various mixed infections involving monogeneans, protozoans, copepods, and bacteria, 106 fish showed symptoms of HS, with a prevalence of 46%. Infection rates were higher in the Krishna–Kolleru region than in the Godavari–Kolleru region.

Clinical signs of HS in L. rohita and C. catla: HS-affected fish exhibited hemorrhages on the body, mouth, eyes, ventral surface, pelvic and pectoral fins, and caudal region. Advanced cases showed degeneration of internal organs, scale edema, liver and gall bladder enlargement, erythema, and splenomegaly. Both *L. rohita* and *C. catla* were equally affected, with no noticeable difference in infection severity between species (Fig. 2).

Isolation and colony characteristics: A total of 530 tissue samples from liver, blood, kidney, spleen, and gills of infected *Labeo rohita* and *Catla catla* were cultured on nutrient agar, TSA, and RS medium. Colonies were small, smooth, circular, and convex on nutrient agar; smooth, convex, tan to buff on TSA; and golden yellow on RS medium.

Biochemical characterization: Motile, yellow, rod-shaped isolates (1–4 µm × 0.3–1 µm) were observed microscopically, occurring singly, in pairs, or in chains. They were Gram-negative and positive for oxidase and catalase. Biochemical tests showed positive results for OF, MOF, arginine dihydrolase, lysine decarboxylase, TSI, indole, MR, VP, citrate utilization, and acid production from glucose, sucrose, maltose, lactose, and arabinose, with growth in 0% and 3% NaCl. Negative reactions were recorded for ornithine decarboxylase, H_2S, gelatin hydrolysis, nitrate reduction, and gas from glucose, sucrose, maltose, and lactose; results for Simmon's citrate were variable.

Biochemical profiles of the isolates were compared with the reference strain *Aeromonas hydrophila* (MTCC 646) using Bergey's Manual (1984), showing close similarity and confirming their identity. These findings align with earlier reports (Nayak *et al.* 1999, Al-Dughaym 2000, Abbott *et al.* 2003, Sabur 2006, Mostafa *et al.* 2008, Sahu *et al.* 2011, Chandravanshi *et al.* 2020, Abhishek *et al.* 2021, Serik *et al.* 2022) (Supplementary Table 3).

DNA extraction produced clear bands on 1.5% agarose gel, matching the 100 bp DNA ladder (100–3000 bp) (Fig. 3).

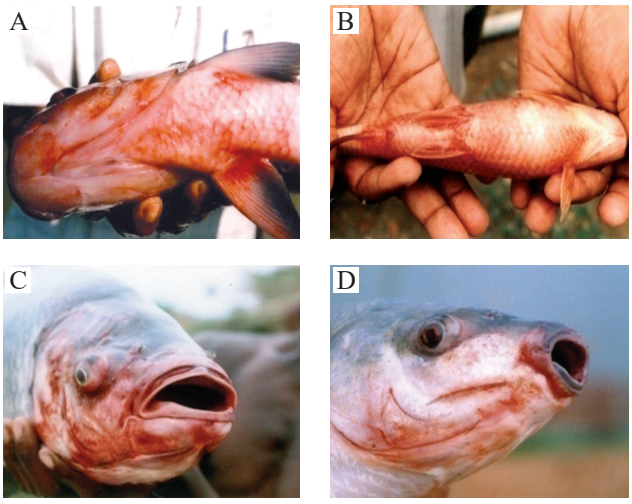

Fig. 2. (A) and (B) *L. rohita* showing haemorrhages on the ventral surface of the body and along the sides of the body, and (C) and (D) *C. catla* showing haemorrhages around the mouth and surrounding the eyes.

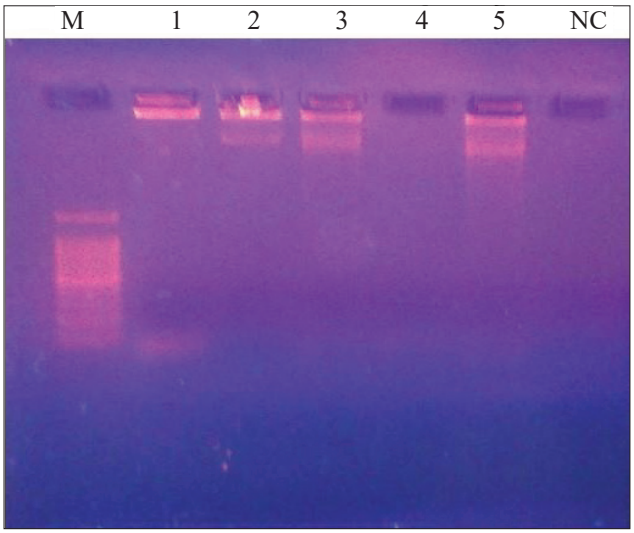

Fig. 3. Agarose gel electrophoresis of genomic DNA from *A. hydrophila* isolates. Lane M: 3 kb DNA ladder, Lanes 1–5: *A. hydrophila* isolates, Lane NC: Negative control.

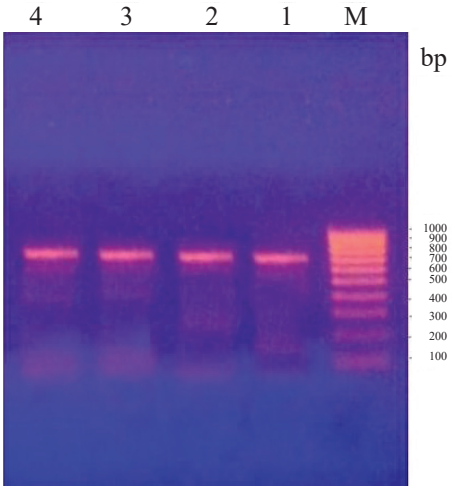

Fig. 4. PCR amplification of *A. hydrophila* DNA samples. Lane M: 1 kb DNA ladder, Lanes 1–4: *A. hydrophila* isolates. Molecular weight markers are shown on the right side of the gel.

PCR amplification from genomic DNA of targeted isolates yielded ~760 bp amplicons, confirming the presence of *A. hydrophila* (Fig. 4).

Kumar and Dey (1986) reported bacterial septicaemia in silver carp (*Hypophthalmichthys molitrix*) in Bhubaneswar, Orissa, identifying *Aeromonas hydrophila* as the etiological agent. Karunasagar *et al.* (1986) similarly reported an ulcerative condition in Catla catla characterized by white skin lesions on the snout, scale loss, and fin erosion. Shome *et al.* (1996) and Shome and Shome (1999) documented significant outbreaks of infectious dropsy, including a chronic variant, in *Cirrhinus mrigala* and *C. catla* from South Andaman, associated with a highly virulent strain of *A. hydrophila*. Dash *et al.* (2008) investigated infectious dropsy in IMCs in Andhra Pradesh, West Bengal, and Orissa, identifying C. catla as the most susceptible species, followed by *Labeo rohita* and *C. mrigala*. Ahamad *et al.* (2013) identified *A. hydrophila* as the etiological agent in carp outbreaks exhibiting pronounced clinical manifestations of HS.

Pathogenesis involves multiple virulence factors, including structural components such as outer membrane proteins, flagella, pili, lipopolysaccharides, and the type III secretion system (T3SS), as well as extracellular toxins and enzymes. Key toxins include aerolysin (*aerA*), hemolysin (*hlyA*), and cytolytic enterotoxin (*act*), while enzymes such as lipases, proteases, and nucleases contribute to pathogenicity (Pollard *et al.* 1990, Cascon *et al.* 1996, Yogananth *et al.* 2009, Mina *et al.* 2022). The substantial genetic, biochemical, and antigenic diversity within aeromonads makes disease attribution complex, and the group is considered a complex of pathogens causing HS and ulcerative conditions (Cipriano *et al.* 2001).

In the present study, *A. hydrophila* was isolated from diseased *L. rohita* and *C. catla* from Kolleru Lake culture ponds. Infected fish showed haemorrhages over the body, skin lesions, scale loss and protrusion, and occasional exophthalmia. Gross lesions were typically septicaemic, including splenomegaly, gall bladder enlargement, and ascitic fluid accumulation causing abdominal distension. The clinical signs aligned with previous reports in various fish species, including IMCs (Chandravanshi *et al.* 2020), *Oreochromis niloticus* (Yambot 1998), *Leuciscus cephalus albus*, *Scardinius erythrophthalmus hesperidicus*, *Tinca tinca* (Popovic *et al.* 2000), *Carassius carassius*, *Megalobrama amblycephala* (Nielsen *et al.* 2001), *O. niloticus* (El-Ashram 2002), *Channa punctatus* (Yesmin *et al.* 2004), *Oncorhynchus mykiss* (Kozinska 2007), *O. niloticus* (Ibrahem *et al.* 2008), *Acipenser gueldenstaedtii* (Timur *et al.* 2010), catfish (Jayavignesh *et al.* 2011, Sarkar and Rashid 2012), and other carp species (Ye *et al.* 2013).

Chandravanshi *et al.* (2020) examined moribund *L. rohita*, *C. catla*, *C. mrigala*, *L. calbasu*, and *Cyprinus carpio* with haemorrhages, ulcers, abdominal distension, and fin erosion, confirming *A. hydrophila* by microbiological, biochemical, and molecular methods. Unver and Bakici (2021) reported motile aeromonad septicaemia with inflammation, skin eruptions, tissue necrosis, fin ray degeneration, exophthalmia, and pus-like fluid in the abdominal cavity. Abhishek *et al.* (2021) isolated *A. hydrophila* from *Anabas testudineus* with tail rot and ulcers. Serik *et al.* (2022) characterized *A. hydrophila* from *Acipenser baerii*, with surface ulcers, abdominal haemorrhages, pale gills, congested kidneys, and haemorrhagic liver lesions.

In all these cases, *A. hydrophila* was the dominant HS pathogen, identified using culture, morphology, biochemical, and molecular methods (Nayak *et al.* 1999). In the present study, biochemical identification to genus and species level followed Cowan and Steel (1993) and Bergey's Manual (1984) and was consistent with reports from India and abroad. Abhishek *et al.* (2021) noted circular, yellowish colonies (2–3 mm) on nutrient agar, motility, Gram-negative staining, and oxidase and catalase positivity. Present isolates showed slightly convex, mucoid, yellowish colonies on RS agar, and smooth, rounded, convex tan-to-buff colonies on TSA, similar to Abbott *et al.* (2003) and Chandravanshi *et al.* (2020).

Biochemical analysis revealed positive reactions for methyl red, VP, lysine decarboxylase, indole, and acid from lactose, with some differences from previous reports—e.g., Sabur (2006) and Mostafa *et al.* (2008) reported MR and lysine decarboxylase negativity, Serik *et al.* (2022) reported indole negativity, and Nayak *et al.* (1999) reported no acid from lactose. Present isolates also differed from Sabur (2006), Mostafa *et al.* (2008), Abhishek *et al.* (2021), and Serik *et al.* (2022) by showing hydrogen sulphite (H_2S) production from glucose, and gelatin hydrolysis. The strain described by Serik *et al.* (2022) showed positive methyl red, oxidase, arginine dihydrolase, VP, H_2S, lysine decarboxylase, and sucrose fermentation-similar to present findings. Overall, basic biochemical characteristics matched Bergey's Manual and earlier reports (Nayak *et al.* 1999, Abbott *et al.* 2003, Sabur 2006, Mostafa *et al.* 2008,

Al-Dughaym 2000, Sahu *et al.* 2011, Chandravanshi *et al.* 2020, Abhishek *et al.* 2021, Serik *et al.* 2022), with minor differences likely due to strain variation.

Molecular detection of *A. hydrophila* has been achieved through PCR targeting virulence genes. Pollard *et al.* (1990) amplified a 209 bp *aerA* fragment encoding aerolysin. Cascon *et al.* (1996) evaluated 50 bacterial strains and found a 760 bp lipase (*lip*) gene fragment specific to *A. hydrophila*. Swaminathan *et al.* (2004) confirmed this specificity, amplifying a 760 bp product only from *A. hydrophila*. Yogananth *et al.* (2009) amplified *aerA* (416 bp) and *hlyH* (597 bp) genes. Mina *et al.* (2022) confirmed *A. hydrophila* using 16S rRNA (685 bp) and *lip* (763 bp) PCR products. In the present study, the targeted *lip* gene amplification produced the expected ~760 bp product from genomic DNA of *A. hydrophila* isolates, with no amplification in negative controls, confirming species identity.

HS emerged as the most prevalent bacterial infection in IMCs from Kolleru Lake, affecting both *L. rohita* and *C. catla*, with slightly higher incidence in *L. rohita*. Seasonal variation was significant, with peak prevalence in summer. The present study is the first documented report of *A. hydrophila* from culture ponds in the Kolleru Lake region. By combining morphological, biochemical, and molecular identification, it confirms *A. hydrophila* as the dominant bacterial pathogen causing HS in IMCs in this area. These findings contribute valuable data to the epidemiology of fish bacterial diseases in India and highlight the urgent need for improved disease management and biosecurity practices to reduce economic losses in aquaculture.

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CONFLICTS OF INTEREST

There is no conflict of interest among authors.

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