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Pabitra Saharia
College of Fisheries Science, Raha Department of Aquatic Animal Health Management Assam Agricultural University, Assam, India

Hemanta Pokhrel
College of Fisheries Science, Raha Department of Aquatic Animal Health Management Assam Agricultural University, Assam, India

Binod Kalita
College of Fisheries Science, Raha Department of Aquatic Animal Health Management Assam Agricultural University, Assam, India

Inam Akhtar Hussain
College of Fisheries Science, Raha Department of Fish Processing Technology Assam Agricultural University, Assam, India

Saidul Islam
College of Veterinary Science, Khanapara Department of Parasitology Assam Agricultural University, Assam, India

Correspondence
Hemanta Pokhrel
College of Fisheries Science, Raha Department of Aquatic Animal Health Management Assam Agricultural University, Assam, India

Histopathological changes in Indian Major Carp, *Labeo rohita* (Hamilton), experimentally infected with *Aeromonas hydrophila* associated with hemorrhagic septicemia of Central Brahmaputra valley of Assam, India

Pabitra Saharia, Hemanta Pokhrel, Binod Kalita, Inam Akhtar Hussain and Saidul Islam

Abstract
High incidence of mortality in fish was recorded due to *Aeromonas* infection in carp culture system in the central valley of Brahmaputra region, Assam. Infected fish showed mostly gross ulcerative lesion on the skin with erosion of scales and fin and tail rot. *Aeromonas hydrophila* was dominant among the isolated bacteria from kidney, liver and skin of infected fish. Experimental infection in *L. rohita* with isolated *A. hydrophila* showed clinical signs with weakness, anorexia, swimming closer to surface, darkness in color with hyperemia and lysis of the fins. While, fluid accumulation in the abdomen, swollen liver, spleen and kidney with clear histopathological alterations were visible internally in moribund fishes. The study indicates that *A. hydrophila* is capable of causing pathological changes indifferent organs in *L. rohita* and cause mortality of affected fish in culture systems.

Keywords: *Aeromonas*, Pathogenecity, Histopathology, Ulcerative lesion

1. Introduction
Bacterial pathogens mainly *Aeromonas* spp. is causing a serious loss in aquaculture industry and pose a potential threat to human health. *A. hydrophila,* is a gram-negative aerobic and opportunistic, oxidase-positive motile bacterium that inhabits in aquatic environments. It is commonly found in fish, milk, red meat and poultry [20, 2]. It mainly causes disease and mortality in freshwater fishes, sometimes in marine fishes [2]. The bacteria also infect human beings and cause lesions ranging from gastroenteritis to septicaemia [21]. Therefore, the organism is considered a threat to the human health and fish. Prevalence of motile aeromonads in the freshwater ecosystem indicates their role in causing epizootics [6]. and causes secondary infections in association with EUS outbreak [20]. This pathogen is also responsible for 'Red – sore' disease [14].

Generally, *A. hydrophila* do not cause any problems in fish populations provided the fishes are under environmental or physiological stress or infected by other pathogens [12].Mortalities due to *A. hydrophila* infection were recorded in the South and South-East Asia farmed fish [19]; While [17] stated that bacterial isolates from sick freshwater ornamental fish from aquarium shops in Terengganu-Malaysia consist of 60 percent of *A. hydrophila* and causes diverse pathologic conditions such as dermal ulceration, rotting of the tails, fin haemorrhage, septiceamia, red sores, exophthalmia, erythro-dermatitis and scale protrusion especially in common carp, *Cyprinus carpio* [9, 3]. Chronic infections could lead to ulceration, inflammation and dermal lesions with focal haemorrhages [9] and during acute septicaemia, liver and kidney are the common target organs [14].A highly virulent strains of *A. hydrophila* was reported from the Indian Major Carps, *catla catla* from Andaman [22]. The present paper represents the results of pathogenecity and histopathological alterations of Indian Major Carps *L. rohita*, experimentally infected with *A. hydrophila*.

2. Material and Methods
2.1 Isolation and identification of bacteria
Infected fish with specific clinical signs were randomly collected from the outbreak cases and

was immediately brought to the fish disease diagnostic laboratory of College of Fisheries, Raha, covered in gel ice pack in insulated box. Samples from skin, fins, gills, kidney, liver, spleen and infected muscles were collected aseptically. Inoculation was done on Trypticase Soy Agar (TSA) and incubated aerobically at 28°C for 12 hrs. The plates were examined for bacterial growth. Dominant colonies were selected, re-streaked on Trypticase Soy Agar (TSA)and isolation and purification procedure was followed until pure colonies were obtained.

2.2 Phenotypic characterization

Phenotypic characterization of *A. hydrophila* were carried out through biochemical analysis to species level by using the tests- gram staining, motility, catalase, Kovac's oxidase, indole typical growth reaction on Triple sugar iron agar, oxidation and fermentation, urease test, H_2S production, Methyl Red-Voges Proskauer (MR-VP), reduction of nitrate to nitrite, arginine dihydrolase and sugar fermentation test like sucrose, fructose, galactose, arabinose, maltose, cellobiose and raffinose.

2.3 Molecular charaterisation of *A. hydrophila*

The purified isolates were enriched in BHI broth and DNA extraction was carried out using phenol extraction method. Detection of *A. hydrophila* was done using PCR with specific primers (Table 1) with slight modifications to [23]. The PCR was performed in 50 µl reaction mixture containing 2.0 µl of template DNA, 1X assay buffer (10 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% Gelatin), 150 µl of 100 µM of each of the four dNTP's, 2 µl forward and reverse primers and 1.0 µl of *Taq* DNA polymerase (Sigma, USA). The PCR was performed using programmable thermocycler. The amplified DNA fragments were sequenced with an automated ABI 3100 Genetic analyzer using fluorescent label dye terminators (M/s Eurofins, Bangalore). The sequence information obtained in the present study was compared with all similar sequences available in the Gen Bank database.

Table 1: Primers used for PCR technique

Gene	Primer sequence (5' to 3')	Product size(bp)	Reference
AHH1 FR	GCCGAGCGCCCAGAAGGTGAGTT GAGCGGCTGGATGCGGTTGT	130	[23]

2.4 Fish

Clinically healthy rohu (*Labeo rohita*) was collected from nearby fish farm and immediately brought to the wet laboratory of College of Fisheries and acclimatization was done in aquariums having five fishes in each tank for 3 days. Fish were fed with a commercial pelleted diet at 3% body weight daily during the experiment. Water of the aquarium was exchanged partially daily to remove waste feed and faecal matter. The water temperature was maintained at 26 ± 1°C, pH 6·8 ± 0·2, and the oxygen concentration in the range of 5 to 6 mg/l.

2.5 Experimental infectivity

To study the appearance of clinical signs and sequential progress of histopathological changes in *L. rohita* were observed for a period of 10 days. Mean lethal dose (LD_{50}) was determined for *L. rohita* according to [18]. Each fish in the groups were challenged with the series of serial dilution against *A. hydrophila* isolate, grown over night on tryptic soya broth (TSB) at 37°C and cell suspension were prepared in phosphate buffer saline. Each fish was injected intraperitoneally with 0.1ml concentration of 10^5 CFU/ml. Control fish were injected with 0.1ml of phosphate buffered saline. Fish showing clinical symptoms were used for histopathological studies.

2.6 Histopathological sampling

For histopathological examination, tissue samples were obtained from skin, gills, liver, kidney and muscles and fixed in 10% Neutral Buffered Formalin (NBF). Dehydration and infiltration of tissues were done manually. Samples were embedded in paraffin and sectioned at 5µm using rotary microtome. (Leica, Germany). Sections were stained with hematoxylin and eosin (H&E) according to the method of [7].

3. Results

3.1Clinical findings

After 8 to 10 hours of the injection almost all the experimental fish except few were observed to be gathered in place followed by swelling in the injected areas. Acceptability of the feed was reduced and darkenening in the injected areas was visible after first day of post injection. On the third day, focal hyperemia of the skin over the pectoral fins was noticed; weakened fishes came closer to the surface and 20% mortality was recorded. By the fifth day, darkening of the skin was more obvious than earlier. By the seventh day, complete rejection of the feed, unstable swimming on the bottom of the aquarium, gross symptoms of the diseases such as gross ulcerative lesions on skin with erosion of scales and fin and tail rot, haemorrhagic skin, distended abdomen were noticed (Fig. 1)

3.2 Phenotypic characterization

The isolated bacteria which showed yellowish colonies on ASDAB medium (Agar starch DNA agar base, Hi media) were motile gram negative small rods (Fig. 2). The biochemical results for the isolates are given in the table 2.

Table 2: Phenotypic characteristics of *A. hydrophila* isolate

Biochemical test	Results	Sugar test	Results
Gram staining	-	Arabinose	+
Cytochrome oxidase	+	Fructose	+
Catalase	+	Galactose	+
Indole	+	Maltose	+
Voges proskauer	-	Sucrose	+
Methyl red	+	Cellobiose	-
Motility	+	Raffinose	-

3.3 Molecular charaterisation of *A. hydrophila*

Positive result indicated with a band size of 130bp for haemolysin gene by bacterial isolates, which confirms the isolated bacteria to be *A. hydrophila* (Fig. 3).

3.4 Histopathological changes

Major histopathological changes of the experimental infection in *L. rohita* were mainly observed in liver, kidney, skin and gills. Other changes observed in fish muscles are necrosis with separation of muscle fibres due to edema and focal areas

of inflammatory cells infiltrating between the muscles fibres of the dermis.

3.5 Liver
Experimenatlly infected fishes in the present study showed rupture of congested portal vessel, pyknosis, mild necrosis and vacuolation of hepatocytes along with appearance of vacuoles in the heptocytes of fish due to release of blood cells (Fig. 4)

3.6 Kidney
Histopathological changes in kidney were observed chiefly the vacuolation of the tubular epithelial cells and glomerular atrophy (Fig. 5, 6). Other changes observed were degeneration of renal tubular epithelial cells, vacuolation of the epithelial cells, sloughing off cells from the basement membrane and complete necrosis of renal tubules (Fig. 7). Infiltration of polymorphonuclear cells leading to the massive widening of the inter tubular areas were also seen (Fig. 8). Moreover, mild to moderate congestion of the blood vessels with mild haemorrhage at certain places and atrophic changes in glomerular indicated by the enlargement of bovine space.

3.7 Skin
Changes observed in the skin due to infection of *A. hydrophila* were necrosis and denudation of the epidermal cells (Fig. 9) and infiltration of inflammatory cells, lymphocyte and other plasma cells (Fig 10). Colonization of bacteria and infiltration of inflammatory cell in focal area in dermal layers were most significant (Fig. 11).

3.8 Gills
Histological alterations occurred in the gills of *L. rohita* exhibited clubbing and fusion of gill filaments and dilatation of the central venous sinus (Fig. 12); while excessive mucous secretion, fusion of secondary gill lamella, hypertrophy of epithelial cells and unilateral hyperplasia in the gill lamella were also observed (Fig.13)

4. Discussion
Phylogenetic microbial and molecular charaterisation of the bacterial isolate associated with septicaemic disease of carps in the present study is the *Aeromonas hydrophila*. Motile aeromonads are a compose part of normal intestinal microflora of healthy fish, which cause diverse pathologic conditions that include dermal ulceration, tail or fin rot, ocular ulcerations, erythrodermatitis, hemorrhagic septicemia, red sore disease, red rot disease, and scale protrusion disease [10]. The gross signs of disease after infection on fish produces abdominal distension, focal hemorrhagic necrosis, dropsy, fin and tail rot in this study were similar to those observations made earlier by [4].

The target organs for acute infection of *A. hydrophila* in fish is mainly found in liver and kidney reported by [5]. Rupture of the congested portal vessel, mild necrosis and vacoulation of hepatocytes was observed in liver of *L. rohita* during the study which has the similarity with the observations made by [1], where necrosis and hemorrhage in the kidney, liver, pancreas and intestine were mostly visible in fish. [11] reported diffused necrosis in the kidney and histopathological alterations in the liver of *Channa punctatus* due to the impact of *A. hydrophila;* while, [15] observed haemorrhages with focal necrosis and vaculation in the hepatocytes, necrosis of sheathed arteries in the spleen and necrosis of renal tubules and glomeruli. Presence of histopathological changes like vacuolation of the tubular epithelial cells, glomerular atrophy, degeneration of renal tubular epithelial cells and necrosis in tubules are clearly documented during the study. [14] observed that the dermis and epidermis became eroded and the underlying musculature became severely necrotic. Eel (*Anguilla japonica*) infected with *A. hydrophila* also indicated liquefacient necrosis of muscle bundles in addition to septic hemorrhage, spleen damage, fatty liver, renal hematopoietic tissue atrophy, and necrosis in nephron [8].The present study also documented the necrosis and denudation of the epidermal cells and infiltration of inflammatory cells, lymphocyte and other plasma cells, colonization of bacteria and infiltration of inflammatory cell in focal area in dermal layers were most significant. [13, 16] reported the adherence of bacteria to intestine and skin followed by invasion of the intestine and skin which has the conformity with the present report.

Fig 1: Fish showing clinical signs.

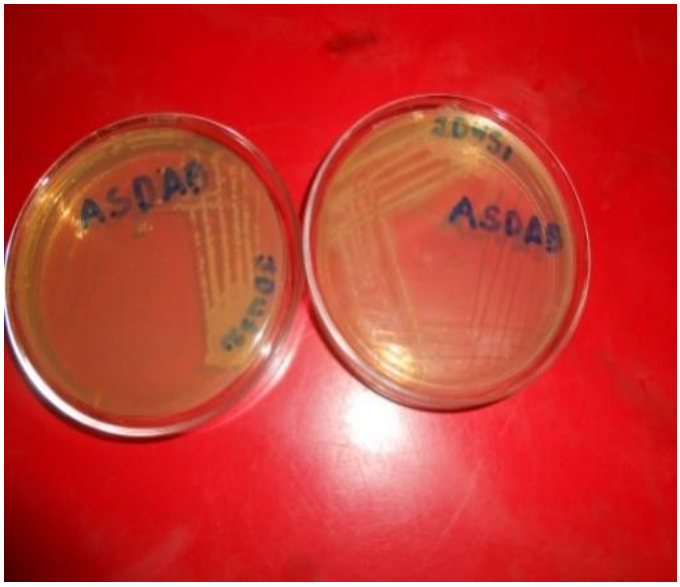
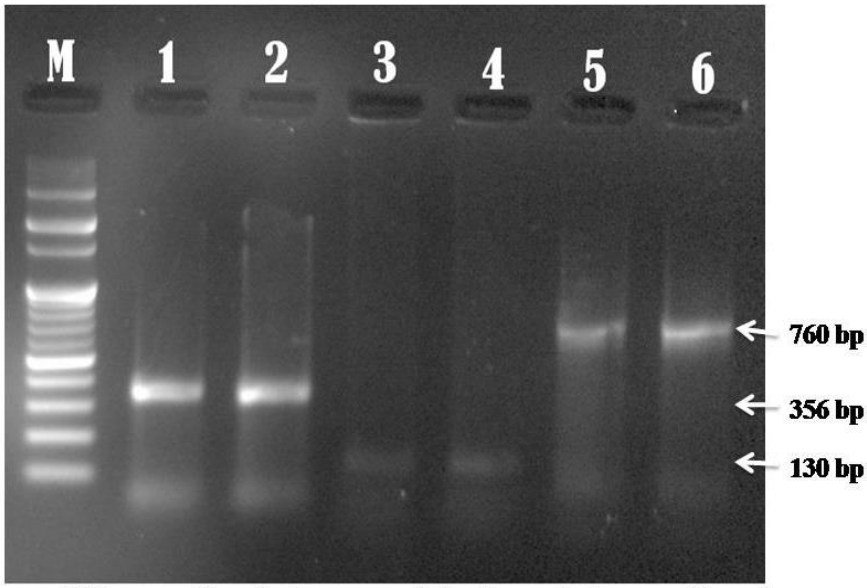


Fig 2: *A. hydrophila* isolates showing yellowish colonies on ASDAB



2% Ethidium Bromide stained Agarose gel showing results for some diseased fish samples (*DF1 & DF2*) screened for *Aeromonas hydrophila* infection in fishes; M: 100 bp DNA ladder; 1 & 2 : PCR amplicons showing positive results for *Aeromonas* spp. 16SrRNA gene (*A16S*) for *DF1 & DF2*; 3 & 4: PCR amplicons showing positive for *Aeromonas hydrophila* specific haemolysin gene(*ahh1*) for *DF1 & DF2*: 5 & 6: PCR amplicons showing positive results for *Aeromonas* spp. lipase gene (*Alip*) for *DF1 & DF2*.

Fig 3: *A. hydrophila* isolates showing positive result for hemolysin gene at 130 bp

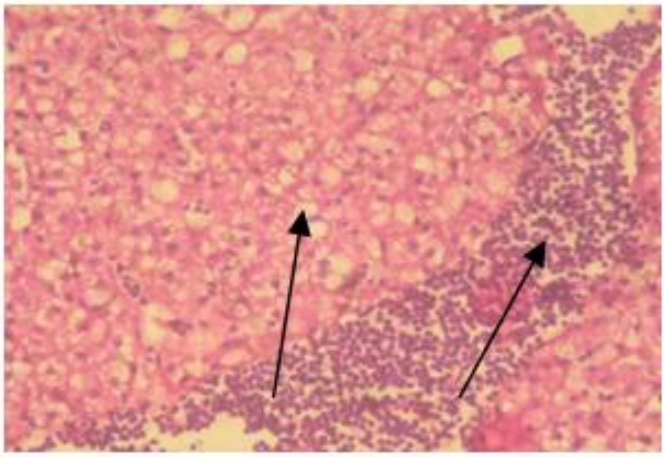


Fig 4: Photomicrograph of liver of *labeo rohita* showing Congested portal vessel and vacuolated hepatocytes. X40

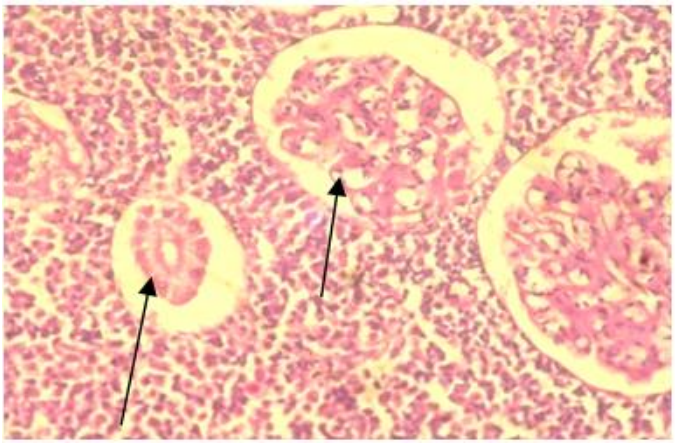


Fig 6: Photomicrograph of kidney of *labeo rohita* showing glomerular atrophy. X40

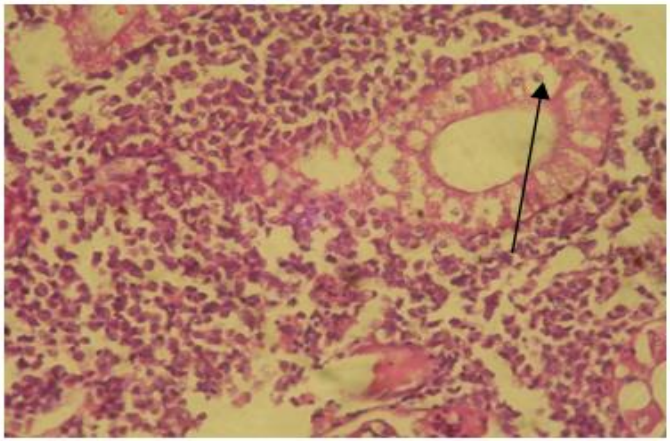


Fig 5: Photomicrograph of kidney of *labeo rohita* showing vacuolation of the tubular epithelial cells. X40

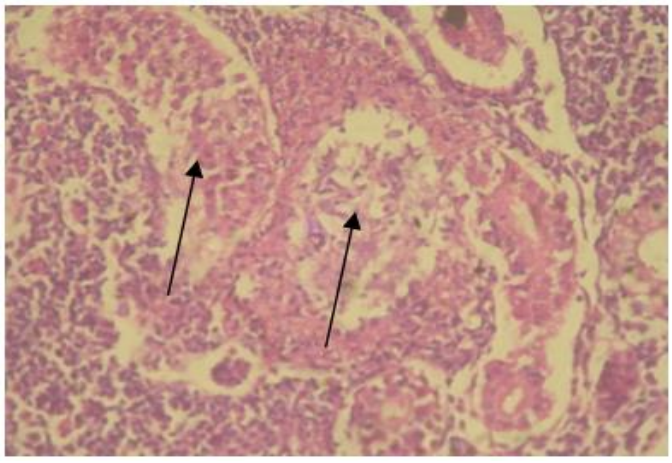


Fig 7: Photomicrograph of kidney of *labeo rohita* showing complete tubular necrosis. X40

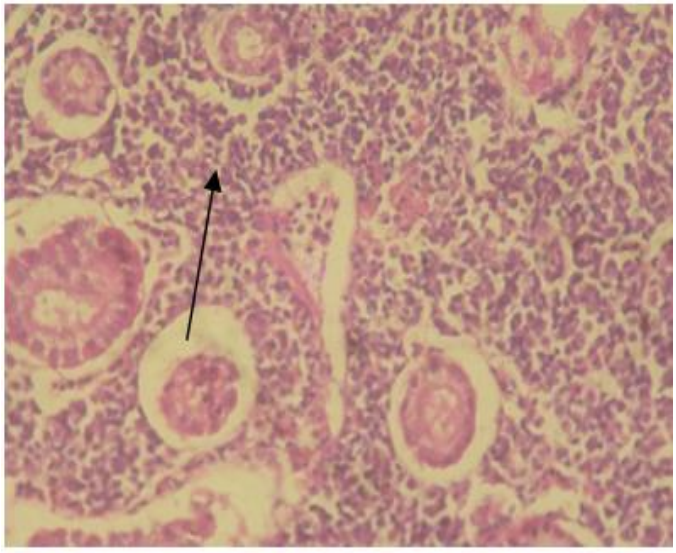


Fig 8: Photomicrograph of kidney of *labeo rohita* showing Infiltration in the intertubular area. X40

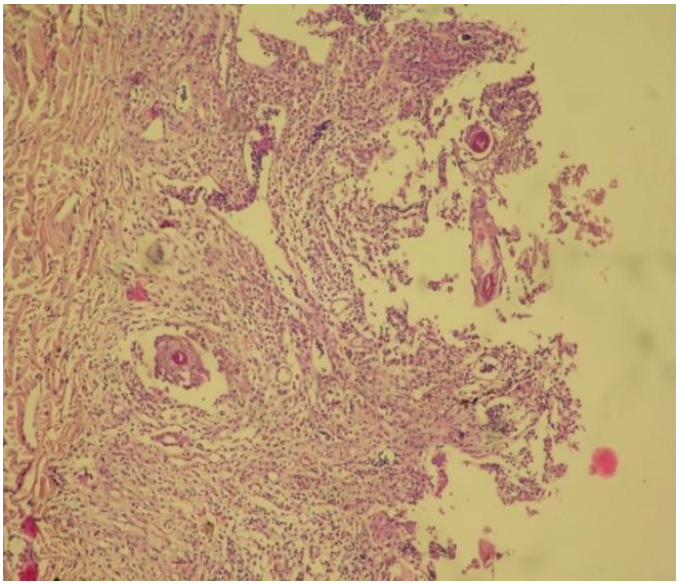


Fig 9: Photomicrograph of skin of *labeo rohita* showing Necrosis and denudation of the epidermal cells Skin. X40

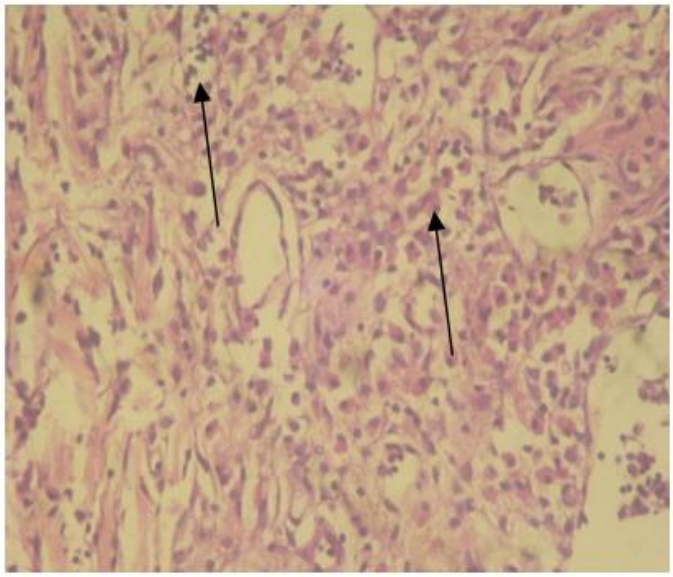


Fig 10: Photomicrograph of skin of *labeo rohita* showing Infiltration of inflammatory cells, lymphocyte and other plasma cells. X40

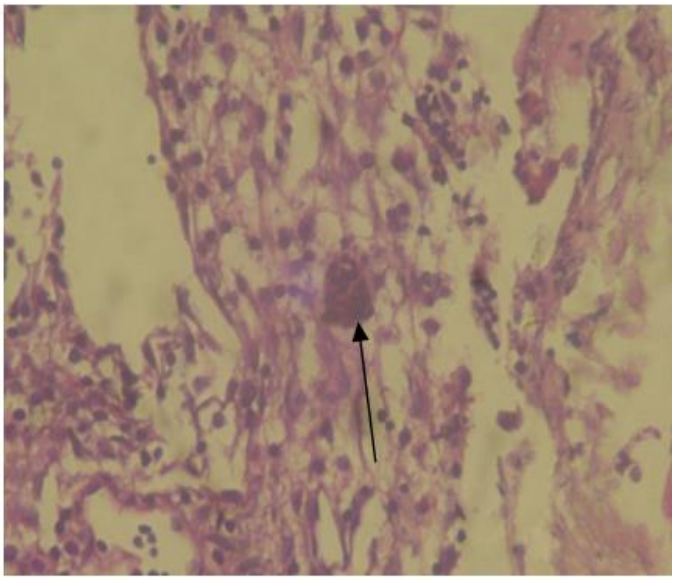


Fig 11: Photomicrograph of skin of *labeo rohita* showing bacterial colonisation. X40

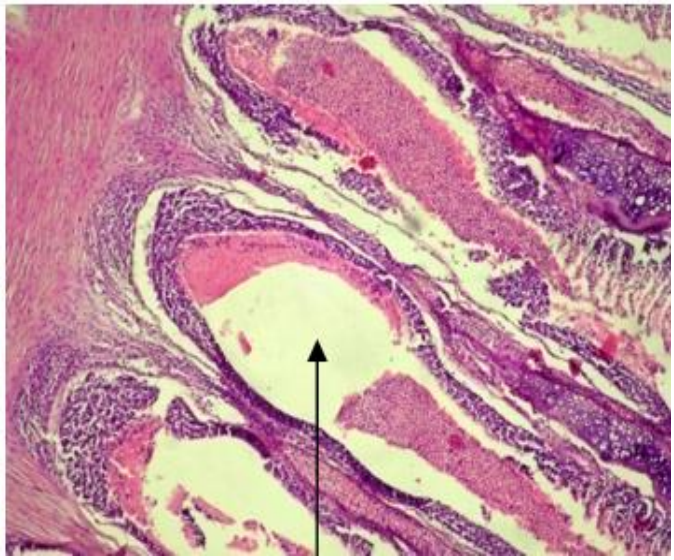


Fig 12: Photomicrograph of Gills of *labeo rohita* showing Dilatation of the central venous sinus H&E. X40

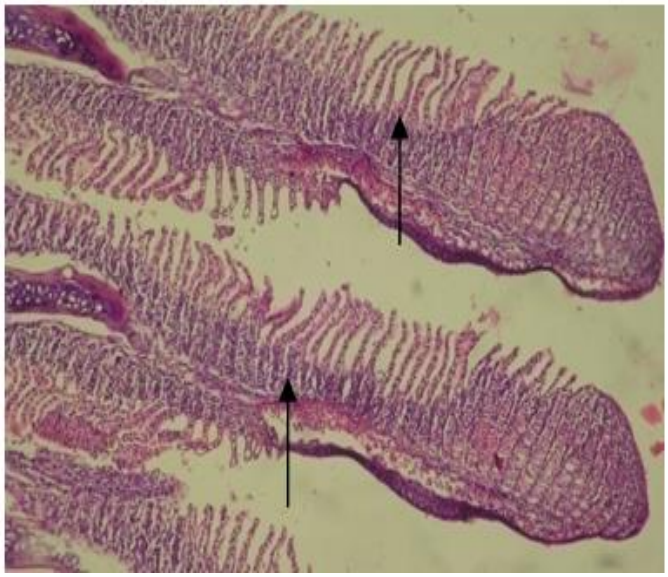


Fig 13: Photomicrograph of Gills of *labeo rohita* showing unilateral hyperplasia in the gill lamella H&E. X40

5. Conclusion

The pathogenecity of *A. hydrophila* in various freshwater fishes are well documented. The results of present study led to the contention that *A. hydrophila* may be a primary pathogen associated with hemorrhagic septicemia of the freshwater fish. From the present study it is also clear that *A hydrophila* progressively showed the development of clinical characteristics features that led to the histopathological changes during the infectivity and confirms that histopathological alterations are good biomarkers for field assessment, in particular in tropical areas that are naturally subject to a multiplicity of environmental conditions.

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