JJBS

Volume 15, Number 1,March 2022
ISSN 1995-6673
Pages 93 – 100
https://doi.org/10.54319/jjbs/150112

Jordan Journal of Biological Sciences

Histopathological Analysis of Striped Catfish, *Pangasianodon hypophthalmus* (Sauvage, 1878) Spontaneously Infected with *Aeromonas hydrophila*

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Received: November 27, 2020; Revised: March 19, 2021; Accepted: April 1, 2021

Abstract

Mass mortality of *Pangasianodon hypophthalmus* in cultured earthen pond was investigated. Infected *P. hypophthalmus* were characterized by number of clinical signs such as abnormal swimming behaviour, skin lesion, and local hemorrhages. Moribund samples were collected from Karnataka to investigate the etiological as well as histopathological changes. The causative agents of the diseased pangasius were identified as *Aeromonas hydrophila* using specific selective media, polymerase chain reaction (PCR) technique and through scanning electron microscopy (SEM). The amplified DNA product was 130bp for haemolysin genes of *A. hydrophila*. Scanning electron microscopy revealed straight rods with single polar flagellum of *A. hydrophila*. Histopathogical studies of different organs of diseased *P. hypothalamus* revealed cellular alteration such as necrosis and hemorrhages in the gill and liver, tubular degenerations in the kidneys, splenitis accompanied with hemosiderosis in spleen. Additionally, there was sloughed necrotic debris in intestinal lumen with increase in goblet cell number.

Keywords, Histopathology, *Aeromonas hydrophila*, *Pangasianodon hypophthalmus*, PCR, SEM

1. Introduction

Over the last few decades, aquaculture has played a vital and steadily rising role in food safety and monetary stability to the world (Houston *et al.,* 2020). Aquatic animals derived from aquaculture became the fundamental source of high-quality protein for human consumption. World fisheries production had reached at apex in 2017, producing 172.6 million tons with aquaculture contributing approximately fifty percent and the rest from capture (FAO, 2019). The modern technology has heighted the production of economically profitable fish species such as carps and catfishes, and thus the aquaculture system transformed from extensive to intensive and super intensive system. Striped catfish, *Pangasianodon hypophthalmus* (*P. hypophthalmus*) is *an* exotic fish species introduced in India from Bangladesh in 1997. Since its entry to India, pangasius has been playing a major role in many aspects: employment generation, cash flow, protein source, utilization of derelict ponds without disturbing the environment and indigenous fish species. Due to its unique fast growth capacity, pangasius become the third most cultivable fish species after rohu and catla and is highly desirable in export market (Singh and Lakra, 2012). In domestic market, pangasius has reasonable market price and possessed highly quality protein, essential amino acids, fatty acids, vitamins, and minerals in its flesh (Rahman *et al*., 2020). Recently, it has made its entry into ornamental fish markets in different parts of the world. The IUCN Red List has listed the pangasius catfish, *P. hypophthalmus*, of the family Siluriformes, order Pangasiidae, as an endangered freshwater fish species native to the Mekong delta (Vidthayanon and Hogan, 2013). Viet Nam has ranked top in pangasius production, generating nearly 11, 41000 tons (Nguyen, 2013). Though *P. hypophthalmus* has been regarded as highly disease resistant fish, recent report revealed that several diseases affect this fish in juvenile (Kumar *et al.,* 2018; Mamun *et al.,* 2020) as well as adult stages (Kumar *et al.,* 2013; Kumar *et al.,* 2015) causing a significant economic loss in Indian aquaculture.

Many bacterial infections threaten this industry including *Edwardsiella icataluri* (Yuasa *et al.,* 2003; Ferguson *et al.,* 2001) and *Aeromonas* spp. especially with *A. hydrophila* (Subagja *et al.,* 1999; Crumlish *et al*., 2010; Elgendy *et al*., 2017)) in East Asia and *Enterobacter cloacae* (Kumar *et al.,* 2013) and *Aeromonas jandaei* (Kumar *et al.,* 2015) in India. However, reports were scanty on *A. hydrophila* infection in pangasius catfish from cultured ponds. Usually in pond aquaculture, the water management is not well controlled, and in dry season sometimes water level reaches below the optimum level. In turn, fish get affected by stress, and this leads to the

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severe disease outbreak. High stocking density, irregular feeding, over feeding accompanied with lack of scientific knowledge may exacerbate the disease condition in such levels where the recovery would become impossible. Proper isolation and identification of bacterial infection can provide essential knowledge to the farmers for prevention and health management. Moreover, histopathological examination of tissue biopsies for the identification of aeromonads infection is extremely important. Therefore, present study was carried out for the isolation, identification, and histopathological studies of naturally infected pangasius catfish collected from natural pond of Karnataka.

2. Materials and Methods

2.1. Bacterial isolation from diseased fish

Moribund fish samples (n=10) were collected from the cultural pond of Mudigere (Lat.13°07′19″N; Long.75°37′38″E.) to the College of Fisheries, Mangalore, Karnataka, India in packed plastic bags containing 1/3rd of habitat water and 2/3rd oxygen. Moribund fishes displayed several clinical symptoms: numerous haemorrhagic spot all over the body, surface, rectal distension, and anemic body (Fig. 1a-c). Diseased fish were euthanized, and alcohol washed in order to avoid contamination. Swab samples of lesions, body cavity, and kidney were taken and streaked on the Rimler-Shotts (RS) media for the presumptive isolation of *A. hydrophila*. The pure colonies from RS agar plates were randomly picked, cultured on 1.5% Tryptone Soya Broth (TSB) and stored as streaks in Brain Heart Infusion (BHI) agar (Himedia, Mumbai) slants.

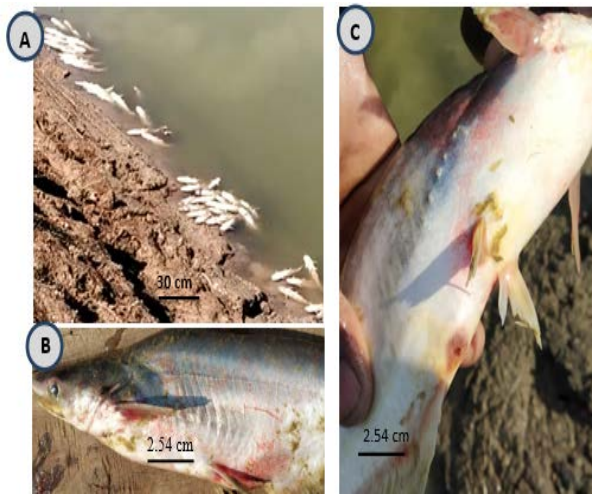

Figure 1. Mass mortality of pangasius in earthen pond of Karnataka **(A)** Diseased fish showing haemorrhages in the belly and fin bases **(B)**, rectal protrusion of *P. hypophthalmus* **(C)**

For DNA extraction, the bacterial isolates were grown on 1.5% tryoptone soya broth (Himedia) and harvested at OD 0.7 by centrifugation for 5 min at 4 °C at 5000 rpm.

Extraction of DNA was carried out by DNA-XPress™ Reagent (Himedia, Mumbai) and manufacturer's instruction were followed. Polymerase Chain Reaction (PCR) was performed in 0.2 ml micro-fuge tubes (Bio-rad, USA) in a programmable thermo cycler (C1000 Touch™ Bio-Rad, USA) using the haemolysinF: 5′-GCCGAGCGCCCAGAAGGTGAGTT-3′ and haemolysinR : 5′- GAGCGGCTGGATGCCGTTGT-3′ primers (Wang *et al.*, 2003). In PCR amplification, initial denaturation at 95°C for 5 min was maintained. Then, 39 cycles of denaturation, annealing, and extension steps were done at 95°C for 30 sec, 59°C for 30 sec and 72°C for 30 sec respectively. The final extension was carried out for 7 min at 72°C. The amplified PCR products were mixed with 6X-gel loading dye (Genei, Bangaluru) at 10:3 ratios and loaded onto 1.5% agarose gel and the product was electrophoresed at 130 V for 30 min. The amplicon was observed under U.V transilluminator (Major Science SmartView Pro Imager UVCI-2300, USA) and documented.

2.2. Field Emission Scanning Electron Microscopy

Bacterial species were characterized in FE-SEM according to the manufacturer's instruction. A small quantity of prepared bacterial broth was dispersed on a SEM specimen mount. Enough argon was maintained in the chamber so that the vacuum reads 0.08 mbar. Sample was coated with gold (Eiko 1B-3 at 0.15 torr) as appropriate for a specified period of time and current to obtain an acceptable coating. Finally, the sample was transferred back to the specimen box after coating for analyzing at Carl Zeiss Sigma VP Field Emission Scanning Electron Microscope in the Central Laboratory of DST-PURSE PROGRAMME, Mangalore University.

2.3. Histopathology of diseased P. hypophthalmus

For histopathological study, moribund fish were euthanized, and different organs such as gills, liver, kidney, spleen and intestine were cut-off, washed with physiological saline and fixed in 10% buffered formalin for 72 h. For each sample, 3 fish were taken. For intestine, up to 10 villi per slide and no less than five were considered in the present study. Following the fixation, dehydration was done in series of alcoholic solutions in lower to higher concentration. Tissue was embedded with paraffin wax and sectioned at 5-6 µm (Thermo Scientific, Microm HM-325, USA). The counter stains, haematoxylin and eosin, were used in this study. All the histological procedures were followed as detailed by Bullock (1989). The tissues sections were photographed with the light microscope (Olympus, BX3-25ND25, Japan).

2.4. Challenge with A. hydrophila

Isolated *A. hydrophila* were revived from -40 ºC and grown on to 1.5% TSB. The bacterial broth was harvested at OD 0.7 to achieve 10^7 CFU/ml. Juvenile pangasius catfish weighing 20.5 ±5.35 were used for challenge studies. Each juvenile fish (n=10) was challenged (intramuscular) with 0.2ml of bacterial broth (*A. hydrophila*) and for control (n=10) 0.2 ml PBS were injected. Challenged fish were monitored for 10 days to check the clinical symptoms. Fish were sacrificed prior to the approval of the animal ethics committee, College of Fisheries, Mangalore.

3. Results

3.1. Presumptive and molecular identification of A. hydrophila

Yellow colonies were grown on the RS medium. The DNA amplified product of the desired size was 130bp (Fig. 2).

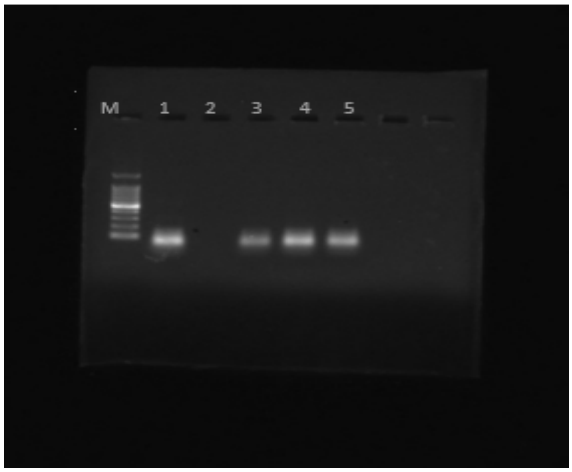

Figure 2. Detection of *Aeromonas hydrophila* by PCR. Lane: M, 1000 bp ladder Marker; Lane 1: Positive control (A. hydrophila MTCC 1739), Lane 2: Negative control (*Vibrio parahaemolyticus* ATCC AQ4037 isolates) Lane 3-5: Samples.

3.2. Scanning Electron Microscopy (SEM)

The scanning electron microscopy showed straight rods with rounded ends having single polar flagellum (Fig. 3A-B)

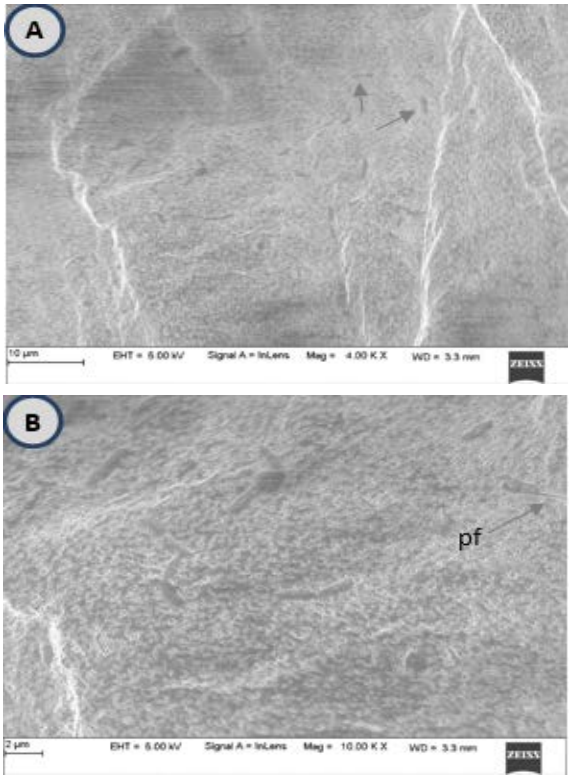

Figure 3. Feild emission scanning electron microscopy revealed **(A:4000X)** rod shapped *A. hydrophila* (arrows) **(B: 10000X)** with polar flagellum (pf)

3.3. Histopathological studies of infected organs

Several histopathological abnormalities in gill tissues, such as loss of secondary gill lamellae, lamellar clubbing, cellular necrosis and hypertrophy were observed (Fig. 4A-B). The liver histopathology displayed hepatocyte necrosis, pyknotic nuclei and formation of Kupffer cells (Fig. 5A-B). Loss of hematopoietic tissues, destruction of Bowman's space, tubule degeneration, glomerular necrosis, tubular necrosis, infiltration of leukocyte cells was detected in kidney section (Fig. 6A-B). Histopathology of spleen showed macrophages and lymphocytes aggregated around ellipsoids accompanied with hemosiderosis (Fig. 7A-B). Though intestinal architecture was not altered in infected pangasius, sloughed necrotic debris in lumen increased goblet cells was noted (Fig. 8A-B).

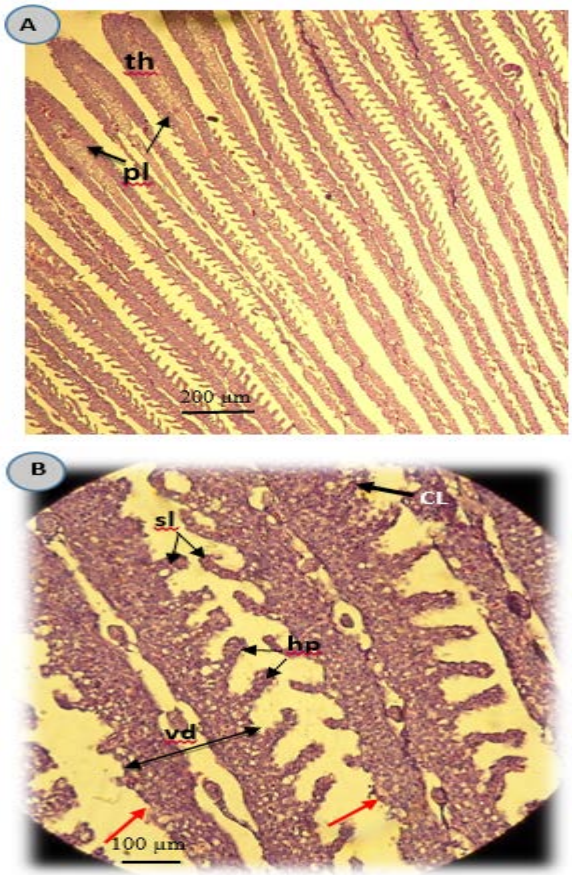

Figure 4. Photomicrographs of the gill of infected *P. hypophthalmus* (**A: 100x**), showing thickening (th) of primary gill lamellae (pl) **(B: 400x, H & E) C**lubbing, vasodialation (vd) and loss of secondary gill lamellae (sl, red arrows) accompanied with hypertrophy and hyperplasia

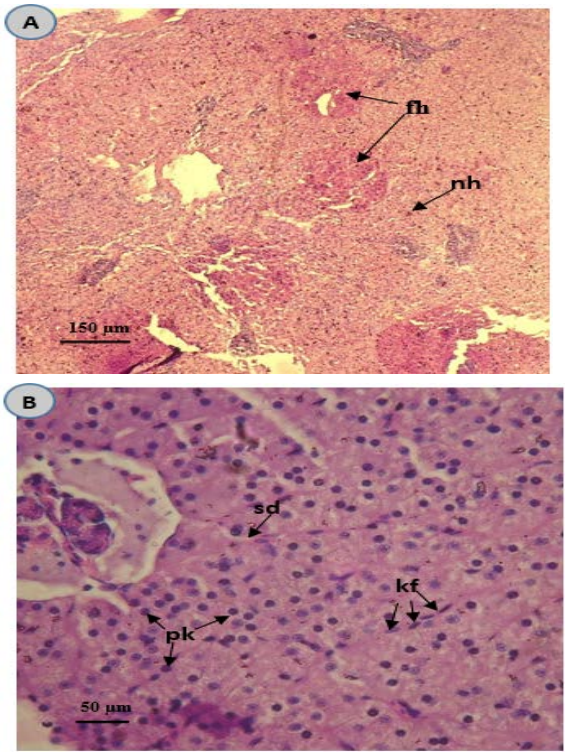

Figure 5. Liver histological section of naturally infected pangasius, display (**A: 100x**), multifocal haemorrhages (fh) with necrotized hepatocytes (nh) (**B: 400x)** Numerous pyknotic nuclei (pk), aggregated Kupffer cells (kf)

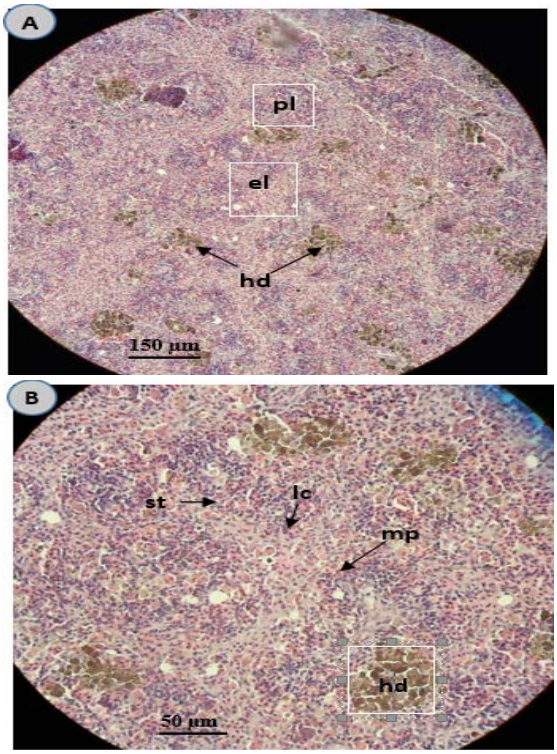

Figure 7. Photomicrographs of the infected spleen, **(A: 100x)** red and white pulp (pl) and ellipsoids (el) and numerous hemosiderosis (hd) **(B: 400x)** splenitis (st) with macrophages and lymphocytes aggregated around ellipsoids accompanied with hemosiderosis

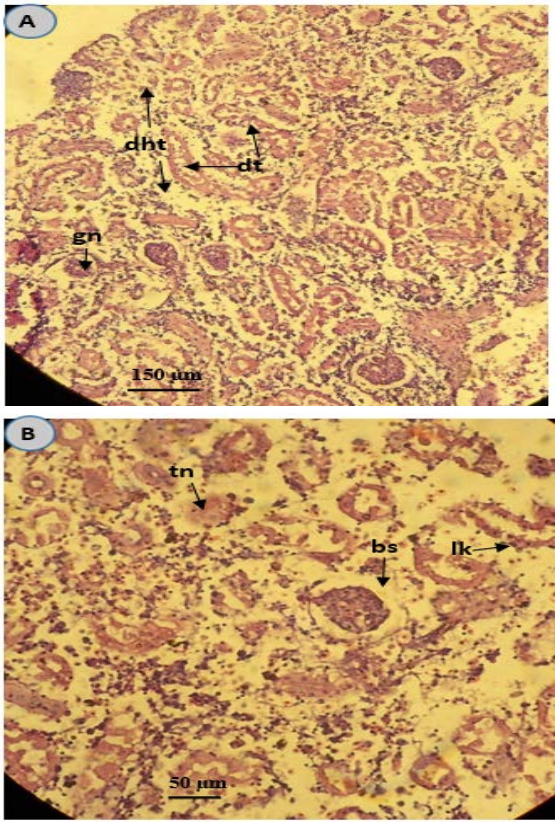

Figure 6. Kidney histopathological changes of infected P. hypophthalmus showing (A: 100x), acute degeneration of hematopoietic tissues (dht), degeneration of both distal and proximal tubules (dt) glomerular necrosis (gn) and (B: 400x) destruction of Bowman's space (bs), tubular necrosis (tn), necrotic tubules (lk).

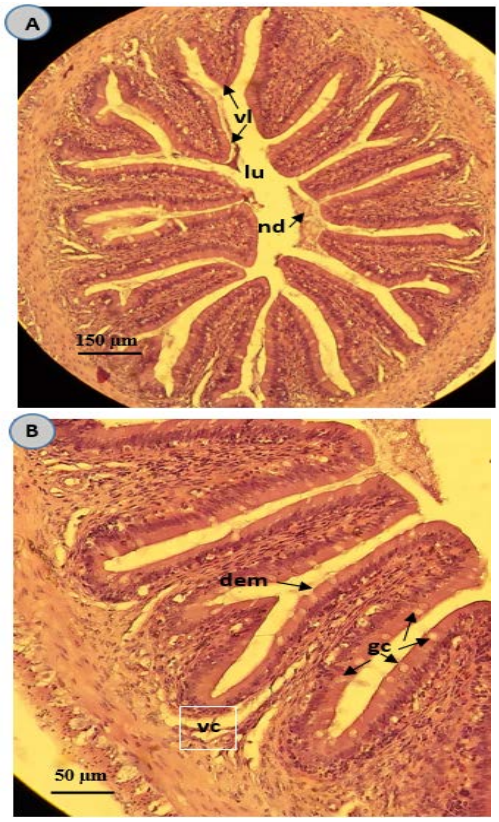

Figure 8. Intestinal histopathology of spontaneously infected pangasius, **(A:100x)** normal architecture of villi (vl) with sloughed necrotic debris (nd) in its lumen (lu) **(B: 400x)** vacuolation (vc), damage of enterocytes (dem) and increased aggregated goblet cells (gc).

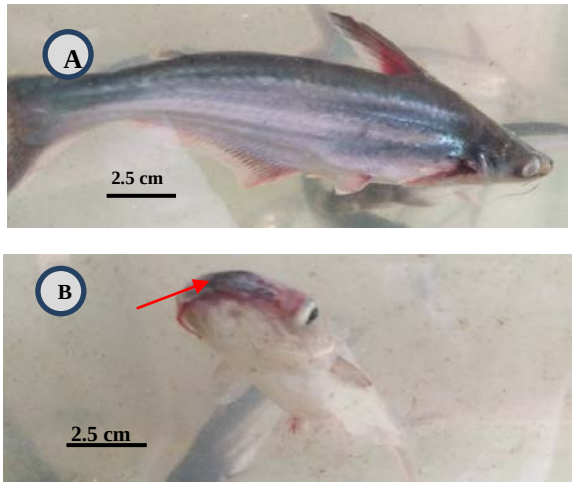

Figure 9. Juvenile pangasius were challenged with isolated *A. hydrophila* from naturally infected pangasius showing the (**A)** hemorrhagic fin bases **(B)** Reddish head with grayish (arrow) cotton wool patches on the tip of mouth and rectal protrusion.

3.4. Challenged studies

In challenged studies juvenile of pangasius showing the similar clinical signs (Fig. 9A-B) compared to the spontaneously infected adult *P. hypophthalmus* collected from earthen pond.

4. Discussion

Bacterial diseases are often regarded as the vicious circle in fish health management, in which administration of drug leads to many negative effects on living biota and may be exacerbated with the change of climate (Houston *et al.*, 2020). Among the infectious diseases, bacterial diseases accounted for more than half (54.59%) routine outbreaks of diseases in finfish aquaculture (Dhar *et al.*, 2014). Gram negative *A. hydrophila* infection known as 'motile aeromonas septicemia' is an opportunistic invader associated with epizootic ulcerative syndrome (EUS) and/or in stressful water environment (Roberts, 1993; Pathiratne *et al.*, 1994; Lio-Po *et al.*, 1998). Nowadays, traditional farmers are usually culturing pangasius catfish in an intensive system with frequent malicious aeromonads infection. This study was aimed to detect the causative agent and histopathological studies involved in the mass mortality of the pangasius in earthen pond of Karnataka, India.

There has been an increasing trend to use Rimler-Shotts (R-S) selective medium for the isolation pathogenic *A. hydrophila*. In the present study, yellow colonies were grown in overnight incubation of inoculated agar media. Rimlar-Shotts agar media supplemented with an antibiotic, novobiocin showed growth of yellow colonies on agar plates for the isolation of *A. hydrophila* from *Oreochromis niloticus* (Aboyadak *et al.*, 2017; AlYahya *et al.*, 2018), rohu (Siriyappagouder *et al.*, 2014), gold fish (Mamun *et al.*, 2019a), water samples (Davis and Sizemore 1981; Arcos *et al.*, 1988), frozen fish (Yogananth *et al.*, 2009) and from human (Khalaf *et al.*, 2005). Isolation of A. *hydrophila* through R-S medium was 94% valid for presumptive recognition in humans (Shotts and Rimler, 1973).

PCR based diagnosis are the confirmatory test for the identification of particular bacterial pathogens. In this study, we used specific primers for the haemolysin genes to confirm *A. hydrophila*. Haemolysin is the virulent factor of *A. hydrophila* (Gonzalez-Serrano *et al.*, 2002; Castro-Escarpulli *et al.*, 2003). Also, an amplified DNA product of the expected size of 130bp was obtained by PCR. Identifying *A. hydrophila* isolates through molecular technique (PCR) which targets the haemolysin gene is more efficient, reliable, and faster compared to biochemical test (Wang *et al.*, 2003) and has also been found extremely effective (Stratev *et al.*, 2016). Several studies were conducted on the identification of pathogenic *A. hydrophila* targeting virulent gene such as haemolysin, lip and aerolysin (Xia *et al.*, 2004; Kingombe *et al.*, 1999; Yogananth *et al.*, 2009). The scanning electron microscopy revealed straight rods with rounded ends having single polar flagellum. Our results are corroborated with Roberts et al (2012) who reported similar morphological features of *A. hydrophila*.

Harikrishnan and Balasundaram (2005) described many clinical symptoms such as reddish spot, abdominal dropsy, exophthalmia caused by aeromoniasis. In other studies, moribund fish samples exhibited rail and fin rots (Austin and Austin 1993), dermal ulceration and muscle necrosis (Bullock *et al.*, 1971) and hemorrhagic bacteremia (Leung *et al.*, 1995), is also evident in the present study. Motile aeromonas septicemia also known as hemorrhagic septicemia in aeromonads infection and diagnosed by several external symptoms such as red spot on the whole body, hemorrhagic fin bases, deep ulceration, rectal protrusion, peritoneal dropsy, sloughed off epidermis, pale gills, abrasive muscle and often bled in bottom of the dorsal fin (Harikrishnan and Balasundaram, 2005). The most prominent internal signs are swollen abdomen, anemic body and excess formation of body fluid which cause anal bulge resulting dysfunction to the inner organs (Jhingran and Das 1990). *A. hydrophila* were isolated and identified from diseased pangas (Parven *et al.*, 2020; Nahar *et al.*, 2016), stinging catfish (Goni *et al.*, 2020, Rashid *et al.*, 2008), Nile tilapia (Hamom *et al.*, 2020), Indian major carp (Lakshmanan *et al.*, 1986)

Histopathological section of gill tissue in the present study displayed several pathologies such as clubbing, disappearance of secondary gill lamellae, substantial hypertrophy, and hyperplasia at the nib of primary gill lamellae. Thickening of primary lamellae and vasodialation in approximately 90% of the area with congestion and lamellar joining in secondary gill lamellae was observed. Cellular necrosis, blood cells, congestion, hypertrophied and hyperplastic secondary gill lamellae were also seen from diseased pangasius. Gills are the first line of defense in fish, therefore recurrent bacterial infections resulted in significant changes in gill architecture and physiology. Channel catfish gills showed similar histopathological alteration such as fusion and thickening of gill lamellae after experimentally infected with virulent *A. hydrophila* (Abdelhamed *et al.*, 2017). Similarly, authors of a recent study revealed secondary gill lamellar loss in addition to clubbing and hyperplasia from farmed pangasius in Maharashtra, India (Kumar *et al.*, 2015). Histological changes offer a quick method to determine the effects of irritants, in various tissues and organs (Shraideh and Najjar, 2011).

The liver tissue sections of diseased pangasius revealed multiple hemorrhages on the hepatic tissues along with

numerous pyknotic nuclei, dilated sinusoids, vacuolization of cytoplasm, cellular necrosis of hepatocytes and aggregated Kupffer cells. Supplementation of lead acetate caused hepatic necrosis and degenerative liver cell as well as upregulated the Kupffer cells numbers in other vertebrates (Albishtue *et al*., 2020). Mamun *et al.,* (2019b) reported histopathological abnormalities in gill, liver, and kidney in pangasius fed *A. hydrophila* as oral vaccine. AlYahya *et al.* (2000) reported severe aggregation blood cells and accruement of pyknotic nuclei in the hepatopancreas of Blue nile tilapia artificially infected with *A. hydr*ophila. Focal necrosis with lymphocyte infiltration, presence of melanomacrophage centres accompanied with necrotic hepatocyte (Faruk *et al.,* 2012) indicated the cytotoxicity by *A. hydrophila* toxins (Donta and Haddow, 1978) could lead to the primary organ failure (kidney and liver). Histopathology of liver, kidney and spleen of natural infected barramundi was validated with severe pathologies such as macrophage aggregation with hepatitis, degeneration of hematopoietic tissues and granulomatous splenitis (Loach *et al.,* 2017).

Renal histopathology revealed acute degeneration of hematopoietic tissues, and destruction of Bowman's space, glomerular necrosis, tubular necrosis, infiltration of leukocytes was detected. Our findings were corroborated with the symptoms reported in other teleosts (Islam *et al.,* 2008; Kumar *et al.,* 2016; AlYahya *et al.,* 2000; Fergason *et al.,* 2001; Abdelhamed *et al.,* 2017). In the present study, among all organs the kidney become the most affected organ, and virtually lost its original anatomy. Fish kidney is a commonly targeted organ during bacterial sepsis due to the toxins released by aeromonads (Kumar *et al.,* 2015). Severe histopathological changes were noticed in kidney such as degeneration of renal tubule with alteration of renal corpuscle, necrotic capillaries and loss of Bowman's capsule when fish were exposed to polluted water (Takashima and Hibiya 1995). Invasion and multiplication of pathogenic bacteria can damage the kidney and lead to the glomerulonephritis (Prasad *et al.,* 2018).

Spleen is regarded as one of the vital organs that respond to fight microbial infection through innate and adaptive immune system. Extended ellipsoid with white and red pulp were observed in the present study. However, spleen section showed splenitis with aggregated macrophage cells and numerous hemosiderosis. During bacterial infection, macrophages congregate, resulting in erythrocyte phagocytosis in the spleen and iron deposition (hemosiderin), which leads to splenic hemosiderosis (Wang *et al.,* 2010). Spleen histopathology of experimentally infected channel catfish revealed degeneration of endothelial and reticular cells; in addition to pyknosis, cytolysis and accumulation of hemosiderosis (Abdelhamed *et al*., 2017) is also evident in our study.

The gut morphology of the intestinal section did not alter in this study. However, several histological changes such as damage of enterocytes, vaculoation, necrotic enterocytes and infiltration of leukocyte cells were observed. Abundant goblet cells in the present study indicated excess production of mucus. The goblet cells are also known as mucous cells playing an important role in defense mechanism of host (Rathore *et al.,* 2019). It secrets the mucus to keep the gut environment healthy and protective (Cerezuela *et al.,* 2012). The goblet cells along with enterocytes can evoke the nonspecific immune system to enhance the phagocytosis process for the elimination of pathogenic bacteria from intestinal surfaces. Compared to the present findings, similar pathology such as vacuolation, necrosis, loss of villi and hemorrhages in mucosa and sub mucosal layers of experimentally infected *Heteropneustes fossilis* (Islam *et al.,* 2008) were also delineated. Similarly, histomorphology of gut of channel catfish revealed necrotized mucosal and enterocytes surface accompanied with bacterial accretion and enteritis (Abdelhamed *et al.,* 2017).

5. Conclusion

Aeromoniasis, known as motile aeromonas septicemia or red spot diseases, is one of the notorious problems in modern and traditional aquaculture farms in India. Acute *A. hydrophila* infection can cause a farmer to lose their entire crop. Early detection could provide the necessary clue for the prevention and disease management. The ubiquitous opportunistic pathogen, *A. hydrophila* has been potential threat to the pangasius farming, and care must be taken in order to control this disease with proper medications and prophylactic measures. Good aquaculture practices including effective water management in dry season can reduce the bacterial infection in traditional (earthen) pond aquaculture. The best weapon a farmer has to be follow is the basic biosecurity principles and adopting sustainable management practices towards maximum yield.

Acknowledgments

First two authors gratefully acknowledge the funding received towards their Ph.D. from the Indian Council of Agricultural Research for awarded of Netaji Subhas-ICAR International fellowship 2015-16.

Conflict of Interests

Authors have no conflict of interests to declare.

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