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Pathogenicity of *Aeromonas* spp. in carp polyculture systems in the central valley zone of Assam, North-east India

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Abstract

The present study was undertaken to assess the incidence of Motile *Aeromonas Septicemia* (MAS) in the freshwater aquaculture system in three districts *viz.* Nagaon, Morigaon and Sonitpur of Assam. A total of 293 ponds of varying size were surveyed during the disease outbreak and thirteen different diseases were recorded. For the first time, the study of severity of MAS in freshwater aquaculture system of Assam was undertaken and characterisation of Aeromonads was done through biochemical and molecular studies. *Aeromonas hydrophila, Aeromonas veronii* and *Aeromonas sobria* were detected from diseased fishes, with *A. hydrophila* being the most dominant species (51.64%) followed by *A. veronii* (21.97%) and *A. sobria* (18.68%). Pathogenicity studies through experimental infection of *Labeo rohita* with these isolates resulted in similar clinical signs as those exhibited by the diseased fishes collected from the farm during the outbreak. LD_{50} (lethal dose) doses estimated for *A. hydrophila, A. veronii* and *A. sobria* through probit analysis indicated that 50% mortality was noticed at $10^{-7.008}$, $10^{-8.034}$ and$10^{-8.213}$, respectively for *A. hydrophila, A. veronii* and *A. sobria* with 95% CI (class interval).

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Introduction

Findings from the previous reports suggest that one of the primary causes of fish infections is bacteria (Yesmin *et al.,* 2004). Vibrios in brackish and marine water systems and motile *Aeromonas* in freshwater habitats are the most prevalent pathogens connected to fish diseases in tropical settings (Capriano *et al.,* 2001; Otta *et al.,* 2003). The opportunistic pathogens, motile Aeromonas cause motile aeromonas septicemia (MAS), the most common of all the bacterial fish diseases. Ulcerative disorders and haemorrhagic septicemia may occur in wide range of aquatic and terrestrial life forms, including humans (Thune *et al.,* 1993; Austin and Adams, 2012). Diseases are often associated with *A. hydrophila* and other motile *Aeromonas* with different signs and symptoms as tiny exterior lesions, regional haemorrhages and septicemia, dropsy, exophthalmia and fin and tail rot in poikilotherms, particularly fish, like carps, eels, catfish, tilapia, trout, and ayu (Sahoo *et al.,* 1998; Austin and Austin, 2012).

The disease may infect any freshwater fish in the wild. It has been noted that as salinity increases, the prevalence of motile *Aeromonas* decreases, however they may still exist in brackishwater (Hazen *et al.,* 1978; Kaper *et al.,* 1981). Motile aeromonas infection (MAI) has been associated with many species of the freshwater-loving genus *Aeromonas*. *A. hydrophilla* (syn. *A. liquefaciens, A. formicans*) is presently the major fish pathogen; the members of this group are jointly known as the *A. hydrophilla* complex. Taxonomically, numerous additional species of *Aeromonas* have been reported, although only a limited number of Aeromonads are found associated with disease. This includes *A. allosaccharophila, A. sobria, A. janda, A. bestiarum, A. caviae,*

and *A. veronii* (Martinez-Murcia *et al.,* 1992), *A. sobria* (Toranzo *et al.,* 2005), *A. jandaei* (Esteve *et al.,* 2004) and *A. bestiarum, A. caviae* and *A. veronii* (Carnahan, 1993) may be considered as fish pathogens.

More than 80% of the mortality of cultured stocks in aquaculture is caused by bacterial infections, making them the most concerning source of disease (Austin *et al.,* 2005). By exploiting a breach in the fish's integument, they may be the primary or secondary invaders, weakening the immune system and potentially causing diseases in the host. Whether acting alone or in mixed infection with other organisms, the motile *Aeromonas* are responsible for significant financial losses annually.

To the best of our knowledge, no studies have been conducted on the pathogenic *Aeromonas* species that cause MAS in any aquaculture systems of Assam, and there is no evidence that the prevalent fish diseases in the state have been sufficiently recorded. The present study intends to investigate the pathogenicity of *Aeromonas* spp. in the polyculture systems of Assam in light of all of these parameters. Finding and describing the infections responsible for the primary losses in polyculture systems as well as exploring their pathogenicity were the aims of the present study.

Materials and methods

Location

Three districts in the Central Brahmaputra Valley region of Assam, India, *viz.* Nagaon, Morigaon and Sonitpur were selected for the present investigation. These three districts were chosen as they enormously contribute to the state's overall fish yield.

Sample collection

For this investigation, 2659 infected fish with clinical signs and symptoms were gathered from the various fish farms of the experimental sites. Fish disease diagnostics facility at the College of Fisheries, Raha, Assam, received samples of moribund fishes, regardless of species, having clinical symptoms. When being transported from the site of sampling to the diagnostic laboratory for identification and detection of pathogens, fish samples exhibiting clinical signs and symptoms of ulcer, haemorrhage, red spot and dropsy were placed in sealed containers with gel ice packs.

Bacterial isolation, identification and characterisation

Tissue samples were aseptically collected from diseased fishes for isolation, identification and characterisation of *Aeromonas* species. The samples were dissected and liver, spleen and kidney were collected for bacteriological examination according to Noga (1996). Each sample comprised of 25 g of tissues that were aseptically collected and homogenised using a pestle and mortar. The homogenate was added to 225 ml of tryptone soy broth (TSB) for 18 to 24 h at 37ºC. The enriched samples were diluted in series, spread-plated on *Aeromonas* starch DNA agar base (ASDAB), treated with ampicillin and then cultured for a further 24 h at 37ºC. Yellow colonies that confirmed positive for oxidase were chosen for further examinations. By conducting a number of biochemical assays (Havelaar *et al.,*1987; Carnahan *et al.,* 1991; Kersters *et al.,*1996), the isolates under study were verified at the species level.

Pathogenicity studies

For pathogenicity tests, healthy juveniles of rohu (*Labeo rohita*) weighing 8 to 10 g were acclimatised for four days in a glass aquarium of 15 l capacity. Experimental fish were injected intramuscularly (i/m) with pre-prepared 0.1 ml of bacterial cell suspension. Experiments were carried out to determine the LD_{50} dose for each of the three *Aeromonas* species causing 50% mortality. Dilutions ranging from 10^{-1} to 10^{-9} were used for inoculating different batches of fishes and the LD_{50} value was calculated by employing the method of Reed and Muench (1938). Freshly prepared cultures of *A. hydrophila*, *A. veronii* and *A. sobria* were used for injecting juveniles of *L. rohita* and the mortalities were recorded every day. Necroscopy was done to obtain gross external and internal examinations of fish and then inoculation of bacteria from liver, kidney, gill, skin and muscle lesion were done on ASDAB plates. LD_{50} of test organisms were computed through probit analysis using SPSS.

Histopathological studies

Histopathological studies on naturally infected fishes with *Aeromonas* species was carried out in the fish diagnostic laboratory of College of Fisheries, Raha. Histopathological study was carried out to observe the histological changes due to *Aeromonas* spp. infection. Tissues of naturally infected fishes during disease outbreak were also processed for histopathological studies. Simultaneously, histopathological studies were carried out in the artificially infected fishes with fresh culture of *Aeromonas* species as per standard method (Bullock *et al*., 1971). For histopathological examination, tissue of liver, kidney, gill, skin and muscle of infected fishes (number of samples, n=3), were dissected out and fixed in neutral buffered formalin (NBF). Following paraffin wax embedding, tissue samples were trimmed into 5 µm sections using a Leica semi-automatic microtome and stained with hematoxylin and eosin (H&E). Following DPX mounting, the slides were viewed for any possible histopathological alterations using a compound microscope (Zeisis Pri-moster; Tuscen Cam, USB 2.0 H series).

Results

Bacterial isolation, identification and characterisation

Aeromonas species were isolated and described utilising *Aeromonas* starch DNA agar base (ASDAB) media as a selective medium. This medium displayed a great recovery rate for *Aeromonas* species and was exceedingly acceptable. The *Aeromonas* spp. were identified based on biochemical tests and the findings revealed that the species were *Aeromonas hydrophila, A. veronii* and *A. sobria* (Table 1).

Pathogenicity studies

Gross clinical features of infected fishes

Before experimental infection, all fishes were observed to be apparently healthy, bright and of good appearance. Post-challenge with *Aeromonas* species, the fishes lost their normal behaviour and appeared pale.

Table 1. Phenotypic and biochemical characteristics of different motile *Aeromonas* species isolated from infected fishes

Characteristics	*A. hydrophila*	*A. sobria*	*A. veronii*
Shape	Short rod	Short rod	Short rod
Gram staining	-	-	-
Motility	+	+	+
Cytochrome oxidase	+	+	+
Oxidative/Fermentative	F	F	F
Triple sugar iron agar test	-	+	-
Methyl red test	-	+	-
Esculin hydrolysis	+	-	+
Voges Proskauer test	+	+	+
Indole production	+	+	+
H_2S production	+	+	+
Gas from glucose	+	+	+
Arabinose fermentation	V	-	-
Glucose	+	+	+
Maltose	+	+	+
Sucrose fermentation	+	+	+
Ornithine decarboxylase	-	-	+
Gelatin liquefaction	+	+	+

= Fermentative, V= Variable

LD_{50} dose (lethal dose) was determined for each of the three pathogens causing 50% mortality in fish. Dilutions ranging from 10^{-1} to 10^{-9} were used for inoculating different batches of fishes. Juveniles of *L. rohita* were infected with freshly grown cultures of *A. hydrophila, A. veronii* and *A. sobria* and mortalities were recorded every 24 h for 14 days. All groups of fishes injected with the three strains of *Aeromonas* showed mortality and displayed clinical signs and symptoms that were equivalent to those of the infected fishes collected during the disease outbreak. Reisolating the bacteria enabled confirmation of the cause of death. A sigmoid pattern of the curve was noticed in all the cases (Fig. 1) and parallelism plots showed 50% mortality in the juveniles of *L. rohita* (Fig. 2). Data on percentage mortality and the curve showing log dilution at which 50% mortality occurred in juveniles of *L. rohita* are presented in Table 2, 3 and 4, for *A. hydrophila, A. veronii* and *A. sobria* respectively.

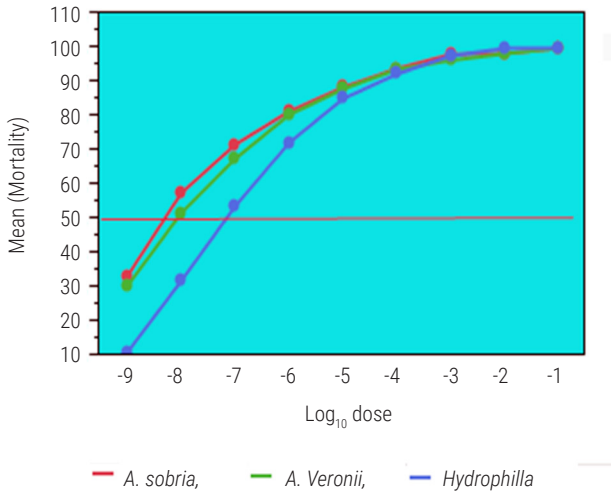


Fig. 1. Parallelism plots showing 50% mortality in the juveniles of *L. rohita* injected (i/m) with different doses of *A. hydrophila, A. veronii* and *A. sobria*

LD_{50} doses estimated for *A. hydrophila, A. veronii* and *A. sobria* through probit analysis using SPSS are provided in Table 5. The results indicated that 50% mortality was noticed at $10^{-7.008}$, $10^{-8.034}$ and $10^{-8.213}$ respectively for *A. hydrophila, A. veronii* and *A. sobria* with 95% CI.

Histopathological studies

Tissues of liver, kidney, gill, skin and muscles were processed and analysed for pathological changes. Similarly, tissues of artificially infected fishes with clinical symptoms of MAS and tissues of healthy fishes were also processed for histopathological studies to compare the major histopathological changes due to MAS and presented in Figs. 3, 4, 5 and 6.

Liver

Aeromonas infected fishes exhibited rupture of congested portal vessel, pyknosis, mild necrosis and vacuolation of hepatocytes (Fig. 3b). Haemorrhage was also observed in the hepatocytes due to discharge of blood cells, focal necrosis and haemorrhage in the liver. Whereas in the control fish, normal structure and regular appearance of hepatocytes were observed (Fig. 3a).

Kidney

Histological observation of kidney in *Aeromonas* infected fish revealed glomerular atrophy and tubular epithelial cell vacuolation. Additional alterations included renal tubular epithelial cell degeneration and necrosis in some tubules, epithelial cell vacuolation, sloughing off of cells from the subcutaneous membrane and total necrosis of some renal tubules (Fig. 4b), polymorphonuclear cell infiltration resulting in a massive widening of the intertubular area (Fig. 4c), mild to moderate blood vessel congestion with mild haemorrhage in some areas and vacuole enlargement (Fig. 4d). Whereas, in kidney tissue of control group, normal renal tubules were observed (Fig. 4a).

Table 2. Percentage mortality of *L. rohita* injected (i/m) with varying doses of *A. hydrophila*

Dilution	Mortality	Survival	Cumulative value		Mortality	
			Total mortality	Total survived	Ratio	Percentage
10^{-1}	10	0	66	0	66/66	100
10^{-2}	10	0	56	0	56/56	100
10^{-3}	9	1	46	1	46/47	97.8
10^{-4}	8	2	37	3	37/40	92.5
10^{-5}	8	2	29	5	29/34	85.2
10^{-6}	7	3	21	8	21/29	72.4
10^{-7}	6	4	14	12	14/26	53.8
10^{-8}	5	5	8	17	8/25	32.0
10^{-9}	3	7	3	24	3/27	11.1

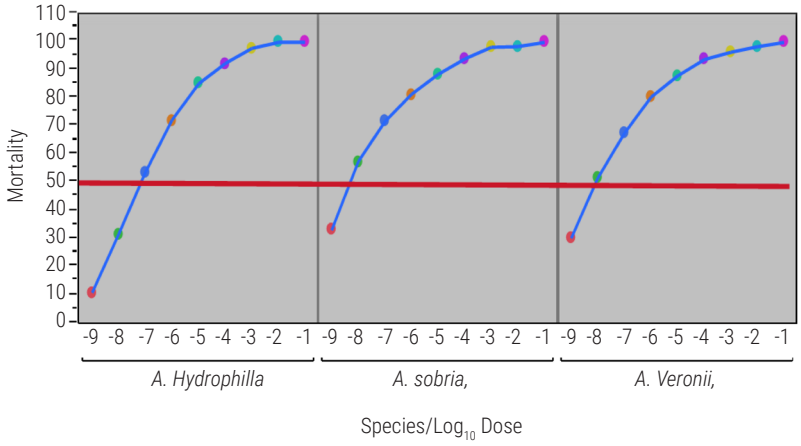
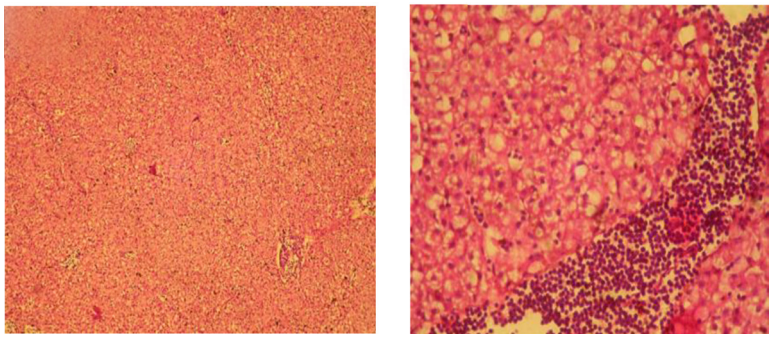


Fig. 2. Graph showing the log dilution at which 50% of mortality occurred in the juveniles of *L. rohita* injected (i/m) with different doses of *A. hydrophila, A. veronii* and *A. sobria*



(a) (b)

Fig. 3. Representative photomicrographs of the liver tissue of *L. rohita* (H&E, x400). (a). Normal liver showing systemic arrangement of hepatocytes; (b) Congested portal vessel and vacuolated hepatocytes in fishes infected by *Aeromonas* spp.

Table 3. Percentage mortality of *L. rohita* injected (i/m) with varying doses of *A. veronii*

Dilution	Mortality	Survival	Cumulative value		Mortality	
			Total mortality	Total survival	Ratio	Percentage
10^{-1}	10	0	74	0	74/74	100
10^{-2}	9	1	64	1	64/65	98.4
10^{-3}	9	1	55	2	55/57	96.4
10^{-4}	9	1	46	3	46/49	93.8
10^{-5}	8	2	37	5	37/42	88.0
10^{-6}	8	2	29	7	29/36	80.55
10^{-7}	7	3	21	10	21/31	67.74
10^{-8}	7	3	14	13	14/27	51.85
10^{-9}	7	3	7	16	7/23	30.43

Table 4. Percentage mortality of *L. rohita* injected (i/m) with varying doses of *A. sobria*

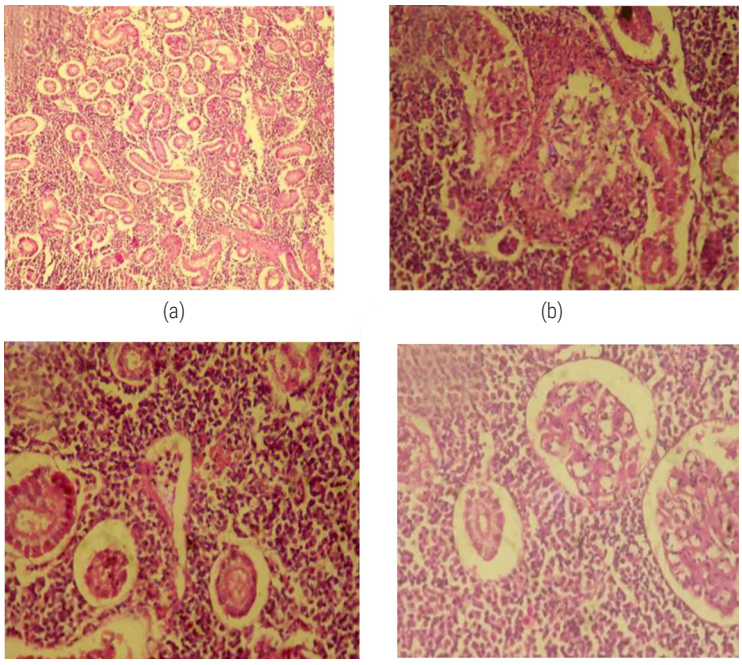
Dilution	Mortality	Survival	Cumulative value		Mortality	
			Total mortality	Total survival	Ratio	Percentage
10^{-1}	10	0	76	0	76/76	100
10^{-2}	9	1	66	1	66/67	98.5
10^{-3}	9	1	57	2	57/58	98.2
10^{-4}	9	1	48	3	48/51	94.1
10^{-5}	8	2	39	5	39/44	86.6
10^{-6}	8	2	31	7	31/38	81.5
10^{-7}	8	2	23	9	23/32	71.8
10^{-8}	8	2	15	11	15/26	57.6
10^{-9}	7	3	7	14	7/21	33.3

Gills

Histological changes in the gills of infected fish included gill filament clubbing and fusion, central venous sinus dilatation (Fig. 5a), excessive mucus secretion, secondary gill lamella fusion, epithelial cell hypertrophy and unilateral gill lamella hyperplasia (Fig. 5b).

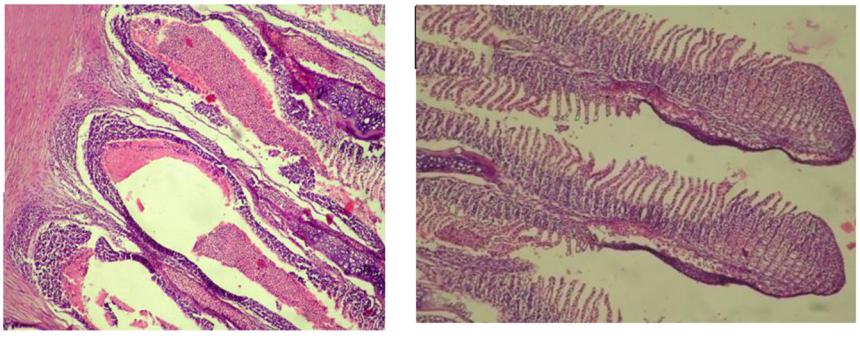
Skin

Colonisation of bacteria in the epidermis of skin provides evidence of probable infection caused by *A. hydrophila*, which are further confirmed by the appearance of denudation and necrosis of the epidermal cells (Fig. 6a) as well as the infiltration of lymphocytes, plasma cells and inflammatory cells (Fig. 6b).



(a) (b) (c) (d)

Fig. 4. Representative photomicrographs of kidney tissue of *L. rohita* (H&E, x400). (a) Normal kidney showing normal distribution of bowman's capsule and renal tubules. (b) Complete tubular necrosis; (c) Infiltration in the inter tubular area. (d) Glomerular atrophy in fishes infected by *Aeromonas* spp.



(a) (b)

Fig. 5. Representative photomicrographs of the gill tissue of *L. rohita* (H&E, x400). (a) Dilation of central venous sinus; (b) Unilateral hyperplasia in the gill lamella in fishes infected by *Aeromonas* spp.

Muscle

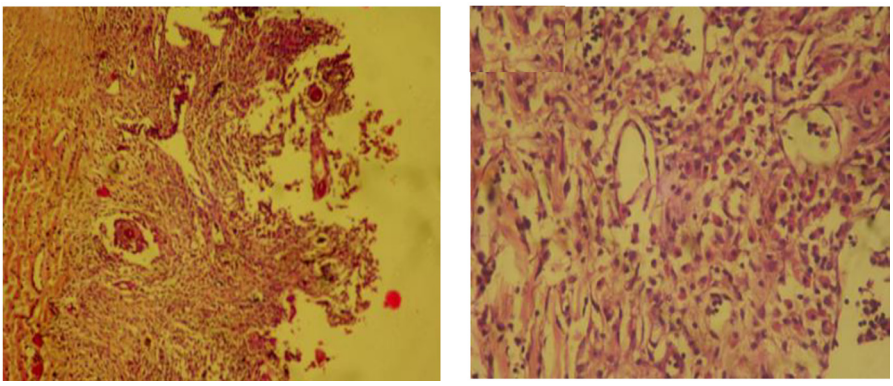
Prominent histopathological changes observed in the fish muscle tissue were necrosis of muscle fibers and separation of the fibres due to edema. Moreover, focal area of inflammatory cells in between muscle bundles was also observed (Fig. 7b).

Table 5. LD_{50} values of *Aeromonas* isolates during challenge studies in *L. rohita*

Bacterial pathogen	Log_{10} LD_{50}; 95% CI
A. hydrophila	$Log_{10}LD_{50}$=-7.008; 95% CI: 6.096-8.676
A. veronii	Log_{10} LD_{50}= -8.034;95% CI: 5.990-13.086
A. sobria	Log_{10} LD_{50}=-8.293;95% CI: 5.944-14.963

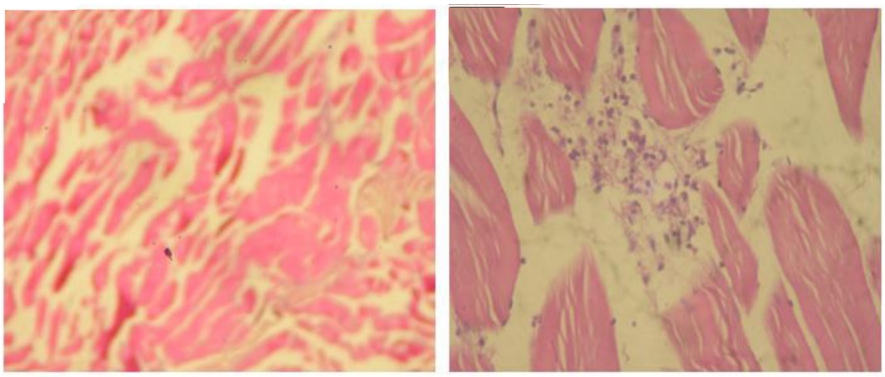
Discussion

The outcomes of the current investigation demonstrates that *A. hydrophila* is more virulent in nature having a high LD_{50} compared to that of *A. veronii* and *A. sobria*. This infers that the isolated motile Aeromonads are pathogenic to fishes with varying degree of pathogenicity. Based on the LD_{50} values, Santosh *et al.* (1988) grouped bacterial isolates as virulent when the LD_{50} value lies between 10^3-10^5, weakly virulent when the value is between 10^6-$10^7$and as avirulent when it is more than 10^8 cfu ml^{-1}. Based on their categorisation, the bacterial isolates of *A. hydrophila, A. veronii, A. sobria* can be defined as virulent or pathogenic, as their LD_{50} values fall in the range determined for each type. During current investigations, pathogenicity study revealed



(a) (b)

Fig. 6. Representative photomicrographs of skin tissue sections of *L. rohita* (H & E, x400). (a) Necrosis and denudation of the epidermal cells; (b) Infiltration of inflammatory cells in fishes infected by *Aeromonas* spp.



(a) (b)

Fig. 7. Representative photomicrographs of muscle tissues of *L. rohita* (H & E, x400). (a) Necrosis of muscle fibers; (b) Separation of muscle fibres due to edema in fishes infected by *Aeromonas* spp.

that the LD_{50} on normal healthy *L. rohita* juveniles was at$10^{-7.008}$, $10^{-8.034}$ cfu ml^{-1} and $1\times10^{-8.293}$ cfu ml^{-1} for *A. hydrophila, A veronii* and *A. sobria* respectively. Angka (1990) also reported that walking catfish when injected intraperitoneally with 10^{7} cfu ml^{-1} of *A. hydrophila* found to be pathogenic. Shome *et al*. (1996) found *A. hydrophila is* pathogenic to *C. carpio* at a dose of $1.5x10^{9}$ cfu ml^{-1}. Karunasagar *et al*. (1989) reported that the LD_{50} of *A. hydrophila* isolates from carp fingerlings ranged from 10^{5}-10^{6} cfu ml^{-1}.

During the Aeromoniasis epidemic in an organised composite carp culture farm in Odisha, Mohanty *et al*. (2008) also assessed the LD_{50} of experimentally infected *L. rohita* with the isolates of *A. hydrophila.* The LD_{50} dose was found to be $1.15x10^{6}$ cfu per fish which suggests that *A. hydrophila* is an important bacterium causing MAS in pond aquaculture. Aeromonads were the primary cause of necrosis of haemopoietic tissues with pyknotic nuclei, moderate haemorrhage and broad vacuolation in the kidney, liver and muscle. Similar results were also recorded by Faruk (2010) where foulder (*Paralichthyso livaceus*) was challenged with *Edwardsiella tarda*. Mammur (1997) also tried artificial infection in carp, catfishes and kawoi with $3.2\text{-}5.4x10^{7}$ cfu ml^{-1} of *A. hydrophila* and observed that the histopathological signs and symptoms in the experimental fish were similar to those of natural motile *Aeromonas* septicaemia.

During the current investigation, low to moderate histological alterations were recorded both in natural and artificially infected fishes. *A. hydrophila*, produces toxins and extracellular toxins including haemolysin, protease and elastase, that were linked to the liver's blocked portal channel and vacuolated hepatocytes, which turned out to be equivalent to prior studies by Paperna (1996). Observations of vacuolated hepatocytes in the affected liver by Nieto *et al.* (1984) and Rodriguez *et al*. (1992) are also congruent with the present observations. In the kidney, vascular, degenerative, necrotic and inflammatory changes of the tubular epithelium were found to be in accordance with previous study by Erer (1981). Additional changes include vacuolation of cells of the epithelial layer, sloughing off cells from the bottom membrane, degeneration and necrosis of renal tubular epithelial cells in some tubules and total necrosis of certain renal tubules. There were mild to severe haemorrhages in blood vessels. In post-experimental infection of *L. rohita,* liver showed minor necrosis, vacuolation of hepatocytes and rupture of the clogged portal vessel. While, Alagappan (2009) detected haemorrhages accompanied by localised necrosis and vacuolation in the hepatocytes, splenic sheathed artery necrosis and renal tubule and glomerulus necrosis. According to Angka (1990) and El-Barbbary (2010), *A. hydrophila* causes histological

alterations in the liver as well as widespread necrosis in the kidney of *Channa punctata*.

According to Laith (2013), histopathological changes in the skin encompassed oedema, focal hyaline degeneration in muscles, vacuolar degeneration in hepatocytes, hyperplasia of spleen lymph follicles, necrosis, hyperplasia in the secondary lamellae of the gill and degenerative changes in the kidney's glomerular epithelium. Whereas, Nahar *et al*. (2016), observed that the major cause of the histological abnormalities, that involved necrotic hematopoietic tissues with pyknotic nuclei, mild haemorrhage and widespread vacuolation in the kidney, liver and muscle, was *Aeromonas* infection. The kidney, liver, skin and muscles of the experimentally infected fish in the current investigation all revealed equivalent histological alterations. These organs appear to be targeted by bacterial toxins, which leads to loss of structural integrity. Chein and Chein (1994) also observed that Japanese eels (*Anguilla japonica*) infected with *A. hydrophila* demonstrated necrosis of muscle bundles in addition to haemorrhagic septicemia, spleen damage, fatty liver, renal hematopoietic tissue atrophy and nephron necrosis.

Additionally, the current investigation reported bacterial colonisation, inflammatory cell infiltration, lymphocyte and other plasma cell infiltration and necrosis and denudation of the epidermal cells in the dermal layers' focus region. According to Horne and Baxendale (1983) and Kanno and Murugo (1989), germs attach to the skin and intestines before invading them. The present histopathological investigation was carried out on experimentally infected fish which revealed that there was no significant difference between histopathological changes attributed to natural MAS infection and artificially caused infection. Moreover, it may be concluded that the histopathological symptoms of natural and artificial MAS are same. The study also confirmed that *A. hydrophila* and other motile aeromonads though opportunistic pathogens, are serious concern for pond aquaculture.

It was confirmed that *Aeromonas* species were able to develop infections as well as cause mortality in the experimental fish. Therefore, to combat the *Aeromonas* septicemia outbreak in carp polyculture, it is advised to use appropriate management techniques and adopt a proper preventative approach. The present study provides a comprehensive overview of Aeromoniasis in the districts under study, highlighting the existence of critically potent disease-causing pathogens within the culture environment, which may cause severe losses to the current production potential. However, further investigations on the significance of *Aeromonas* in the other parts of the state may be carried out along with in depth studies on isolation and characterisation of other pathogenic bacteria of relevance to aquaculture.

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