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Isolation, identification, and characterization of *Aeromonas hydrophila* from juvenile farmed pangasius (*Pangasianodon hypophthalmus*)

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Abstract
Bacterial samples had been isolated from clinically detected diseased juvenile Pangasius, collected from Mymensingh, Bangladesh. Primarily, the isolates were found as Gram-negative, motile, oxidase-positive, fermentative, and O/129 resistant *Aeromonas* bacteria. The species was exposed as *Aeromonas hydrophila* from esculin hydrolysis test. Ten isolates of *A. hydrophila* were identified from eye lesions, kidney, and liver of the infected fishes. Further characterization of *A. hydrophila* was accomplished using API-20E and antibiotic sensitivity test. Isolates were highly resistant to amoxyclav among ten different antibiotics. All isolates were found as immensely pathogenic to healthy fishes while intraperitoneal injection. Histopathologically, necrotic hematopoietic tissues with pyknotic nuclei, mild hemorrhage, and wide vacuolation in kidney, liver, and muscle were principally noticed due to Aeromonad infection. So far, this is the first full note on characterizing *A. hydrophila* from diseased farmed Pangasius in Bangladesh. The present findings will provide further direction to develop theranostic strategies of *A. hydrophila* infection.

Keywords: *Aeromonas hydrophila*, *Pangasianodon hypophthalmus*, pathogenicity, antibiotic sensitivity, histopathology.

1. Introduction
Aquaculture in Bangladesh has been expanded, diversified, and technologically advanced day by day. Bangladesh is ranked fifth in global aquaculture production [13]. The country produced 34, 10, 254 MT fish in 2012-2013, whereas 54.54% was contributed by the aquaculture sector [11]. Common aquaculture practices in Bangladesh have mainly been associated with the culture of carps, tilapias, catfishes, climbing perches, and shrimps. Nile tilapia (*Oreochromis niloticus*) and Pangasius (*Pangasianodon hypophthalmus*) are cultured mostly as the commercial basis by entrepreneurial farmers. Pangasius was introduced to Bangladesh in 1989 from Thailand, aimed to upsurge overall aquaculture production and to meet the increasing demand for food fish [4]. Presently, this fish is one of the important species in aquaculture of Bangladesh owing to its fast growth, year-round production, and high productivity.
Diseases are having an ever greater influence on fishes in a globally expanding and intensifying aquaculture system [26]. Faruk *et al.* [15] have reported that there were financial losses of ~15% of the actual production of rural fish farmers due to fish diseases. A global estimate of disease losses to aquaculture, according to World Bank reports in 1997 was US$ 3 billion per annum. Bacterial diseases are the most common infectious problem of commercial fish farms and ornamental fishes. Some pathogenic bacteria are responsible for fish diseases in captivity and responsible for kidney disease, dropsy, enteric red-mouth, tuberculosis, vibriosis, motile aeromonad septicemia, bacterial gill disease, mouth fungus, tail and fin rot, and columnaris diseases [5, 3]. The major bacterial pathogen *Aeromonas* spp. is responsible for hemorrhagic septicemia, a disease affecting a wide variety of freshwater and marine fish [25]. A*eromonas hydrophila* is the causative agent of MAS (motile *Aeromonas* septicemia) in both farmed and wild fishes. The disease is characterized by swollen abdomen, red mouth, hemorrhage in external surface and surrounding of the anus [1]. Epizootic of MAS have occurred since 1980 in Indonesia, followed by Malaysia, Thailand (1981), Burma and Philippines (1985), Sri Lanka (1987), Bangladesh, India, and Nepal (1988) [29, 21].

It is considered to an opportunistic pathogen that causes disease only when fish are under stress. *A. hydrophila* were frequently isolated from various lesions of epizootic ulcerative syndrome (EUS) of different fishes [37, 30, 17]. Rashid *et al.* [27] have identified *A. hydrophila* from epizootic ulcerative syndrome (EUS) affected shing, *Heteropneustes fossilis*.

Pangasius is highly tolerant to adverse environmental conditions such as low dissolved oxygen, pH, and fluctuations of turbidity [7]. However, with rapid expansion and intensification of farming systems, there is a growing concern regarding the health and incidence of disease problems in the culture of this species. Ferguson *et al.* [16] have described bacillary necrosis in Vietnamese *P. hypophthalmus* that later identified to be caused by *Edwardsiella ictaluri* by Crumlish *et al.* [10]. Currently in Bangladesh, disease incidence is one of the major problems restricting Pangasius farming, especially at their juvenile stage. However, due to lack of diagnostic support and appropriate therapeutants, farmers are suffering from severe financial losses. Consistently, there is a serious lack of information on the pathogen associated with the diseases of farmed Pangasius in Bangladesh. Therefore, objectives of the present study were isolation, identification, and characterization of bacterial pathogens (*A. hydrophila*) of diseased juvenile farmed Pangasius. We identified pathognomonic signs of the disease by inducing clinical infection to the healthy fish. Additionally, the histopathological study on the infected fishes was conducted and antibiotic sensitivity of the pathogen was observed.

2. Materials and Methods

2.1 Field investigation, collection of fish and water sample

Pangasius showing clinical signs of disease were collected from a private fish farm of Muktagacha, Mymensingh, Bangladesh (24.7583° N, 90.2667° E). The farm contained seven stocking ponds each with an average area and depth of 30485.55 ft^2 and 2 ft respectively with well treated and a good water exchange system. The farm owner had stocked 0.5 inches sized 10-12 days old pangasius fry at a high density of about 37850 fries per decimal (~9 lac fry per hectare). The higher mortality of the reared fish was noticed at winter season. The infected fish were brought to the Fish Disease Laboratory of the Department of Aquaculture, Bangladesh Agricultural University (BAU) for bacterial and histopathological analysis. Water samples were also taken from the diseased ponds to the laboratory for the determination of water quality parameters ―pH, dissolved oxygen (DO), ammonia (NH_3), ammonium (NH_4), phosphate (PO_4), nitrite ($N0_2$)― using commercially available testing kit (Sera®, GmbH, Germany).

2.2 Isolation of pathogenic bacterial isolates

The collected fishes were dissected under sterile conditions and bacterial swabs were taken aseptically using a sterile loop from kidney, liver, spleen, skin, and eye lesions. For isolation of bacteria, tryptic soy agar (TSA) medium (Difco™, Becton, Dickinson and Company, NJ, USA) was used. The inoculated plate was incubated at 24 °C for 48 h. A total of ten bacterial isolates were selected from the pure cultures and were maintained on TSA plates. The glycerol stock cultures were stored at -80 °C for long term preservation.

2.3 Primary characterization and identification of isolates

The experimental layout for the identification and characterization of collected isolates is depicted in Fig. 1. Freshly recovered pure isolates were used for primary characterization. The morphological characteristics of bacterial colonies including shape, size, and color were investigated on the TSA plates. Simultaneously, physiological characteristics were studied by observing the growth of each isolated colony at various temperatures (4, 24, 37, and 40 °C) and different NaCl condition (0, 1, 2, 3 and 4%). Biochemical identification of the isolates was performed using Gram's staining, motility, oxidase, Oxidative-Fermentative (O-F), and O/129 sensitivity tests using standard methods described earlier [35]. Further characterizations of the isolates were conducted using the API-20E kit [6]. Esculin hydrolysis test was also performed for species determination of the Aeromonad group.

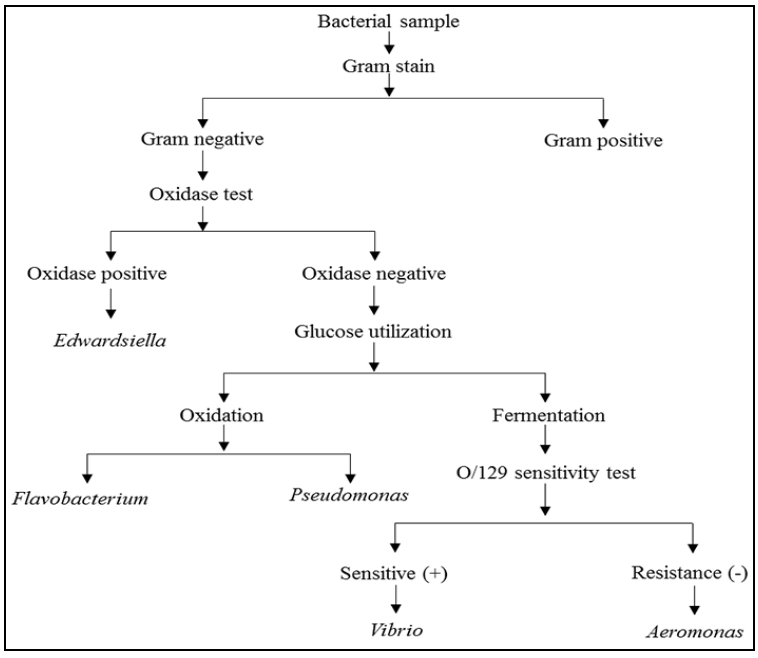

Fig 1: Sketch of primary identification of bacterial isolates. The original image from Tonguthai *et al.* [35] has been modified.

2.3.1 Gram's staining
Gram's staining was performed on a glass slide from a single bacterial colony by crystal violet solution (1 min), iodine solution (1 min), and safranine (2 min). The slide was washed properly by running tap water before the start of the next step. The slide image analyses were performed using a light microscope (Nikon Co., Tokyo, Japan).

2.3.2 Motility test
Motility test was performed using "hanging drop" method from the bacteria cultured in both agar and broth media. Briefly, a drop of bacterial suspension was taken on a glass slide. The slide was then carefully reversed to allow hanging of the culture drop. Finally, the drop was observed under light microscope (Nikon Co., Tokyo, Japan).

2.3.3 Oxidase test
Oxidase test was performed using commercially available oxidase detection strips (Oxoid, Thermo Fisher Scientific Inc., Waltham, MA, USA). In briefly, a few amount of bacteria was placed on the strips containing oxidase reagents (N, N-dimethyl-1,4-phenylene diammonium chloride) and the color was observed after 10 seconds.

2.3.4 O-F test
Oxidative or fermentative glucose metabolism was examined using O-F basal medium containing bromothymol blue (Difco™, Becton, Dickinson and Company, NJ, USA) with 10% (w/v) dextrose, 10% (w/v) lactose and 10% (w/v) saccharose. Briefly, a single bacterial colony was stabbed into freshly prepared tubes of O-F medium in both anaerobic (with liquid paraffin) and aerobic (without paraffin) conditions. The color of the medium was examined after incubating the tubes at 24°C for 24 h.

2.3.5 O/129 sensitivity test
The test was performed using commercially available diagnostic discs (Oxoid, Thermo Fisher Scientific Inc., Waltham, MA, USA). Briefly, the suspended bacterial culture in sterile distilled water was carefully spread over the TSA plates (containing 1.5% NaCl) and let it dry for one minute. Then both 10g and the 150g discs of O/129 was placed over the plate and pressed firmly downwards. The plate was inverted and incubated at 24°C for 24 h.

2.3.6 API-20E microbiological identification
API-20E microbial identification kit (BioMérieux Marcy-l'Étoile, France) was used to perform 21 biochemical tests at a time. The sample was prepared by suspending a single colony from a fresh culture into 5 ml of sterile saline solution. Then the suspension was carefully inoculated in all the tube sections according to the manufacturer instructions. The cupule sections of |CIT|, |VP| and |GEL| were completely filled up with the sample. For the ADH, LDC, ODC, H_2S and URE tests, the cupule sections were filled with sterile mineral oil to make an anaerobic condition. The prepared strip was covered with a lid and incubated for 2 days at 24°C. After incubation, all reactions were recorded.

2.3.7 Esculin hydrolysis
For esculin hydrolysis test, bile esculin agar medium (Difco™, Becton, Dickinson and Company, NJ, USA) was used to identify the Aeromonad species. Sterile tubes were used for slant preparation of the medium. Isolates were inoculated on the bile esculin agar slants and left them for incubation at 24 °C for 24 h. The reaction color was recorded after incubation.

2.4 Antibiotic sensitivity test
The antibiotic susceptibility of *Aeromonas* sp. was determined by the disc diffusion method. Ten different discs of antibiotic were chloramphenicol (30µg/disc), oxytetracycline (30µg/disc), trimethoprim (25µg/disc), erythromycin (15µg/disc), streptomycin (25µg/disc), chlortetracycline (30µg/disc), nitrofurantoin (300µg/disc), amoxyclav (30µg/disc), doxycycline hydrochloride (30µg/disc), and azithromycine (15µg/disc) (Oxoid, Thermo Fisher Scientific Inc., Waltham, MA, USA). Briefly, 200 µl of suspended culture in sterile distilled water was taken and spread over the surface of Mueller-Hinton agar (Thermo Fisher Scientific Inc., Waltham, MA, USA) plate. After drying, the antibiotic discs were placed over the plates and incubated at 24 °C for 24 h. The degree of sensitivity of *A. hydrophila* isolates to ten antibiotics was determined by measuring the zone of clearance around the antibiotic discs.

2.5 Determination of colony forming unit (cfu ml^{-1})
Colony forming units (cfu ml^{-1}) were determined according to the drop count method described previously [23]. Several replicated drops (20 µl $drop^{-1}$) of 10-fold seven times diluted bacterial suspension was put on TSA plates and estimated the cfu ml^{-1} using following formula: cfu ml^{-1} = number of colonies × 20(volume added) × dilution factor × 50

2.6 Determination of pathogenicity
For pathogenicity test, 8 to 10 g weighed known healthy stocks of *P. hypoththalmus* was acclimatized for 4 days in 15-litre capacity in a well-labelled glass aquarium. Fish were injected either intramuscularly (IM) or intraperitoneally (IP) with pre-prepared 0.1 ml of bacterial suspension contains 1.6 $x10^8$ cfu ml^{-1}. Control groups were injected either IM or IP with 0.1 ml of sterile saline. The each group (n = 10) of fish were monitored for 14 days with daily replacement of water (one-third), removal of dead fish and debris. The mortality and morbidity were recorded on daily basis. Necroscopy was involved gross external and internal examinations of fish and inoculation of bacteria from kidney on TSA plate were done.

2.7 Histological procedure
Samples (1 cm^3) from muscle with skin, gills, liver, and kidney were collected aseptically from the infected fish and were fixed in 10% buffered formalin. The samples were then trimmed and processed in an automatic tissue processor (Shandon Citadel 1000, GMI, USA) for dehydration, clearing, and infiltration for 21 hours. Then the samples were embedded, trimmed, sectioned (5 µm thick ribbon) and stained with hematoxylin and eosin [28]. Finally observed them under a compound microscope (Olympus) and photomicrographs of the stained sections were obtained by using a photomicroscope (Olympus-CH-2, New York Microscope Company, Inc.).

3. Results
Maximum precaution has been taken to minimize the suffering of the fish and to protect other persons and animals.

3.1 Water quality of selected ponds and clinical signs of diseased fish

The temperature, pH, dissolved oxygen (DO), ammonia (NH_3), ammonium (NH_4), phosphate (PO_4), and nitrite (NO_2) content of water collected from two ponds of the selected area were 18°C, 7.5-8.0, 4-6 ppm, 0.05-0.1 ppm, 0 ppm, 0-1.0 ppm, and 0-0.1 ppm, respectively. The diseased fishes collected for the experiments were 10-15 g weight and 12-14 cm length. Grossly, periorbital oedema and corneal opacity were the prominent eye lesions. Clinically, there was bilateral exophthalmia with superficial reddening around the eyes and mouth (Fig. 2A). Hemorrhagic ulceration at the base of the fins was also noticed (Fig. 2B). Kidney, spleen, and liver were hemorrhagic, enlarged and the presence of congested blood vessels was noted (Fig. 2C).

Fig 2: Clinical signs of diseased farmed Pangasius (*P. hypophthalmus*). (A) Bilateral exophthalmia with superficial reddening around the eyes and mouth. (B) Hemorrhagic ulceration at the base of the fins. (C) Swollen and enlarged liver (white arrow), and kidney (red arrow).

3.2 Bacteriological observation and morphological characteristics of isolates

A total 10 bacterial isolates were recovered from diseased fish (Table 1), and *A. hydrophila* was identified based on the morphological, conventional, and biochemical analysis as described in methods section. Morphologically the isolated colonies showed yellowish opaque, round, convex, smooth edged, and semi-translucent colonies on TSA plates (Fig. 3A and 3B). They were gram-negative, rod-shaped bacteria (Fig. 3C and 3D).

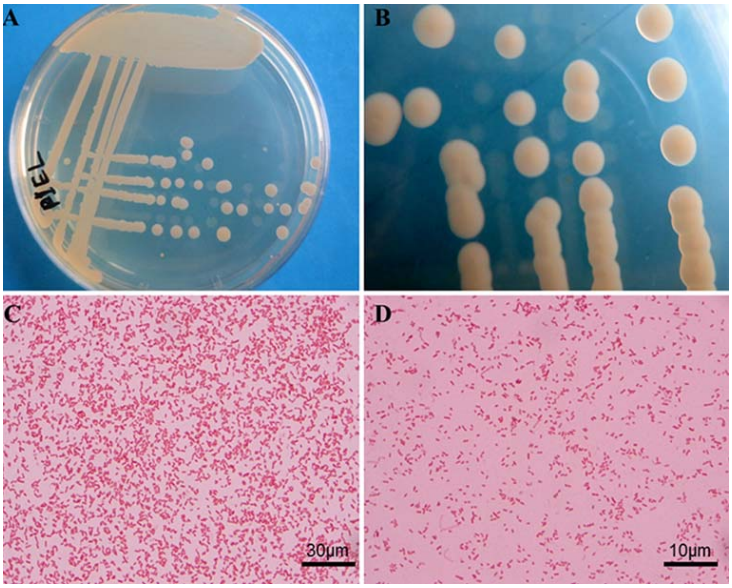

Fig 3: Morphological characteristics of *A. hydrophila*. (A-B) Single colonies of P1EL. (B-C) Gram's stained bacteria under light microscope.

Table 1: List of the freshly collected bacterial isolates from pangasius

Fish host	Recovery site	Laboratory Code
Pangasius *(Pangasianodon hypophthanmus)*	Eye lesion	P1EL
		P2EL
		P3EL
	Kidney	P1K
		P2K
		P3K
	Liver	P1L
		P2L
		P3L
		P4L

3.3 Biochemical characterization

The biochemical characteristics of the isolates those measured by conventional methods were summarized in Table 2. All isolates were oxidase-positive, fermentative, motile, O/129 resistant, and hydrolyze esculin. The bacteria were capable of producing acid from arabinose, whereas acid and gas from different sugar media such as maltose, sucrose, and dextrose. Consequently, the isolates showed positive growth at 37 °C with the optimum at 24 °C but no growth found at 4°C and 40 °C. Additionally, they were capable of growing in 0-1% NaCl, however, no growth was noted in 2-4% NaCl media.

Table 2. Biochemical characteristics of *A. hydrophila* isolates determined by conventional methods

Characteristics		Response of *A. hydrophila* isolates									
		P1EL	P2EL	P3EL	P1K	P2K	P3K	P1L	P2L	P3L	P4L
Gram reaction		-	-	-	-	-	-	-	-	-	-
Shape		Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Motility		+	+	+	+	+	+	+	+	+	+
Oxidase		+	+	+	+	+	+	+	+	+	+
O/129 sensitivity		-	-	-	-	-	-	-	-	-	-
O/F		F	F	F	F	F	F	F	F	F	F
Esculin hydrolysis		+	+	+	+	+	+	+	+	+	+
TSI		+	+	+	+	+	+	+	+	+	+
Acid from arabinose		+	+	+	+	+	+	+	+	+	+
Acid and gas production	Maltose	+	+	+	+	+	+	+	+	+	+
	Sucrose	+	+	+	+	+	+	+	+	+	+
	Dextrose	+	+	+	+	+	+	+	+	+	+
Growth at different temp.	4 °C	-	-	-	-	-	-	-	-	-	-
	37 °C	+	+	+	+	+	+	+	+	+	+
	40 °C	-	-	-	-	-	-	-	-	-	-
Growth in NaCl	0%	+	+	+	+	+	+	+	+	+	+
	1%	+	+	+	+	+	+	+	+	+	+
	2%	-	-	-	-	-	-	-	-	-	-
	3%	-	-	-	-	-	-	-	-	-	-
	4%	-	-	-	-	-	-	-	-	-	-

F: Fermentative; (+): Positive response; (-): Negative response

3.4 API-20E microbiological identification

Twenty-one biochemical tests were performed using the commercially available API-20E microbiological kit as described in methods. The positive or negative reactions of all isolates were recorded and summarized in Table 3. All isolates gave positive results by reacting with beta-galactosidase, arginine dihydrolase, lysine decarboxylase, sodium citrate, urea, tryptophan deaminase, tryptophane, sodium pyruvate, gelatinase, glucose, mannitol, sucrose, melibiose, amygdalin, and arabinose. However, negative reactions were shown for ornithine decarboxylase, H_2S production, inositol, sorbitol, and rhamnose (Table 3).

Table 3: Biochemical characteristics of isolates determined through the API 20E kit.

Characteristics	Response to isolates									
	P1EL	P2EL	P3EL	P1K	P2K	P3K	P1L	P2L	P3L	P4L
Beta-galactosidase	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+	+	+	+	+
Lysine Decarboxylase	+	+	+	+	+	+	+	+	+	+
Ornithine Decarboxylase	-	-	-	-	-	-	-	-	-	-
Citrate utilisation	+	+	+	+	+	+	+	+	+	+
H_2S production	-	-	-	-	-	-	-	-	-	-
Urease production	-	-	-	-	-	-	-	-	-	-
Tryptophane deaminase	+	+	+	+	+	+	+	+	+	+
Indole production	+	+	+	+	+	+	+	+	+	+
Acetoin production	+	+	+	+	+	+	+	+	+	+
Gelatinase	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	+	+	+
Amygdalin	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+	+

3.5 Antibiotic sensitivity test

Eight isolates were found to be sensitive to doxycycline, erythromycin, chloramphenicol, azithromyc in, chlortetracycline, streptomycin, trimethoprim, oxytetracycline, and nitrofurantoin whereas resistant to amoxyclav. Isolates showed highest cumulative sensitivity to chloramphenicol and lowest to amoxyclav and erythromycin. Other two isolates (P1EL and P4L) showed sensitivity to all of the antibacterial agents (Fig. 4A and B).

3.6 Pathogenicity test

The percent cumulative mortality (PCM) obtained upon termination of the trial (14 days post-challenge) were ranged from 40% to 100% for different isolates using infection through IM and IP routes. Simultaneously, IP route was found comparatively more effective for infecting juvenile *P. hypophthalmus* than IM route. Isolates P1EL and P2EL were found more pathogenic, followed by P1K and P4L isolates. Isolated P1EL and P2EL were showed 100% PCM in IP infection (Fig. 4D). However, in IM infection, the PCM was

found 70 and 80%, respectively (Fig. 4C). No deaths occurred in the control group over the course of the trial. Additionally we found that the injected fishes showed the same clinical signs as the diseased fish.

3.7 Histopathological observation

Histopathologically, kidney, liver and muscle of *P. hypophthalmus* were found affected due to aeromonad infection. Degeneration and loss of renal tubules were evident in the kidney section of affected fish. Necrotic hemopoietic tissues with many pyknotic nuclei, mild hemorrhage, and wide vacuolation were also noted in the kidney (Fig. 5A and B). Necrotic hepatocytes, pyknosis, and vacuolation were observed in liver (Fig. 5C and D). In addition, the epidermis and dermis of the skin of the infected fish were almost lost and colonies of bacteria were found in myotomes of muscle (Fig. 5E and F).

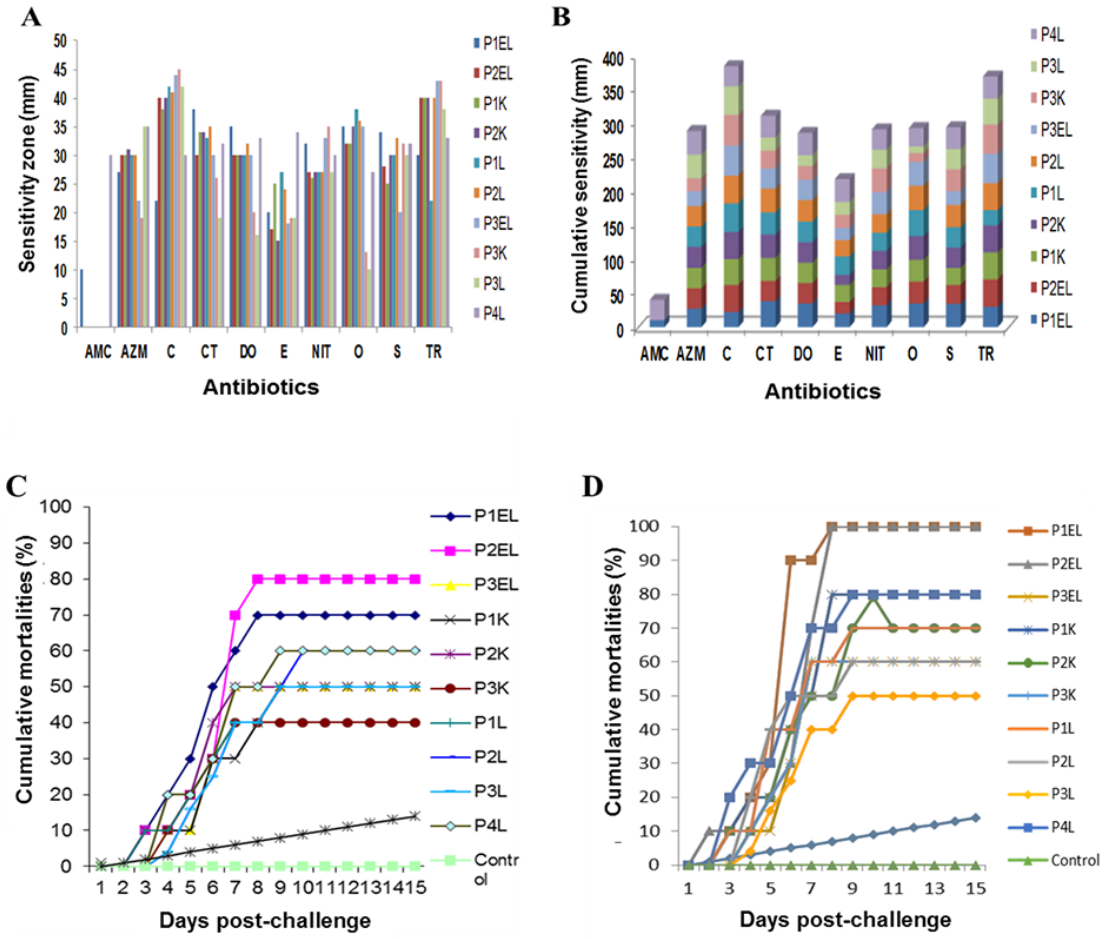

Fig 4: Antibiotic sensitivity and pathogenicity test. (A) Sensitivities of *A. hydrophila* isolates to different antibiotics (AMC: Amoxyclav, AZM: Azithromycin, C: Chloramphenicol, CT: Chlortetracycline, DO: Doxycycline, E: Erythromycin, O: Oxytetracycline, S: Streptomycin, NIT: Nitrofurantoin, TR: Trimethoprim, R: Resistance). (B) Cumulative sensitivities of *A. hydrophila* isolates to different antibiotics. (C). Percent cumulative mortality of fish by intramuscular infection with A. hydrophila at 14 days post-challenge. (D). Percent cumulative mortality by intra-peritoneal infection at 14 days post-challenge.

4. Discussion

A. hydrophila sp. is a ubiquitous pathogenic bacterium in fresh water that causes significant losses to the aquaculture industry. The present study examined the occurrence of *Aeromonas* sp. infection in juvenile *P. hypophthalmus*. Ten strains of *A. hydrophila* were isolated, identified, and characterized from clinically diseased fish. An intensive search in literature reported that several pathogenic bacterial genera-*Aeromonas*, *Micrococcus*, *Edwardsiella*, *Pseudomonas*, *Coryneformes*, *Flavobacterium*, *Enterobacteriaceae*, *Acinetobacter*, *Achoromobacter*, and *Alcaligenes*- were reported in farmed Thai pangas in Bangladesh [32]. However, we showed the first comprehensive report for the isolation and characterization of *A. hydrophila* from exotic farmed pangasius in Bangladesh. Previously, Mamnur Rashid *et al.* [22] have isolated / identified the same bacterial species from naturally infected farmed silver carp in Bangladesh.

Clinical signs associated with the Aeromonad infection observed in the present study were bilateral exophthalmia with superficial reddening around the eyes and mouth (Fig. 2A). Hemorrhagic ulceration was also noticed at the base of the fins (Fig. 2B). Internally, the kidney and liver were swollen and rounded enlarged spleen was observed (Fig. 2C). Consistent with the current findings, hemorrhagic septicemia was also reported in fish (Milkfish, *Chanos chanos*) due to *A. hydrophila* infection, especially when fish are under stress [34, 8]. Sindermann [33] has stated that the opportunistic pathogen (such as *A. hydrophila*) causes infection only when the host resistance has been lowered by environmental stress factors, such as high organic load, overcrowding, and sub-lethal oxygen levels. Therefore, an

extremely higher stocking density of the fish (about 37850 fries per decimal) observed in the current investigation may be a major risk factor of the disease susceptibility. In addition, the low water depth (2ft in average) in the selected ponds with sudden fall of temperature (from 25° to 18 °C) could be other contributing factors for the infection.

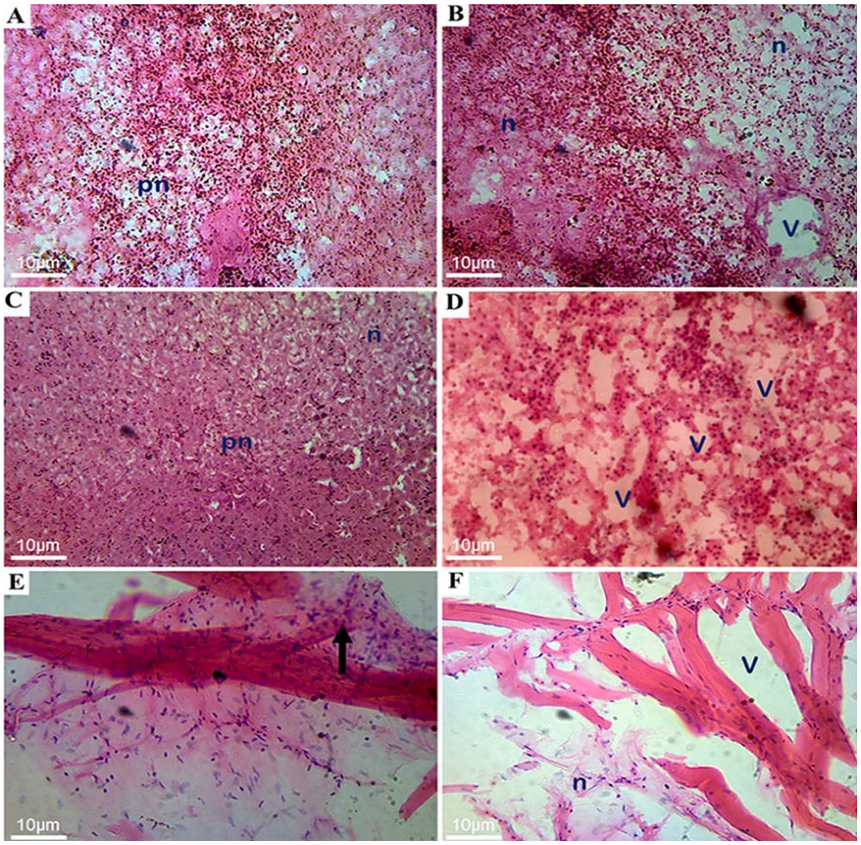

Fig 5: Cross section of kidney, liver, and muscle of *P. hypophthanmus.* (A) Degenerated kidney tubules along with necrotic hemopoietic tissues (n), pyknotic nuclei (pn). (B) Necrotic hemopoietic tissues (n), and wide vacuum (v). (C) Necrotized hepatocyte (n) with pyknotic nuclei (pn). (D) Massive necrosis of hepatocyte with wide vacuum (v). (E) Loss of myotomes in muscle due to bacterial cells. (F) Highly necrotic (n) myotomes with wide vacuum (v) due to bacterial cell. Hematoxylin and eosin × 120.

The API-20E microbiological kits are effectively used to identify Enterobacteriaceae, which has now been also adapted for characterizing Vibrionaceae [31]. It was found that the API-20E system could be used to identify the *Aeromonas* strains under certain conditions [25, 36]. In the current study API-20E kits were used to identify *A. hydrophila* along with several conventional analyses. According to the results from the API-20E system, it has been showed the evidence of Aeromonad group (Table 3). In addition, the Gram-negative, motile, oxidative, acid forming (from arabinose), fermentative, and O/129 resistance potentiality of the causal organism also conclusively proved the Aeromonad bacterial infestation (Table 2). Simultaneously, a positive result from Esculin hydrolysis test was indicated the *A. hydrophila* infections that consistence with the finding of earlier researchers (Table 2) [12].

In our current study isolated *A. hydrophila* also showed variable sensitivity to the ten antibacterial agents, whereas eight of them were sensitive to doxycycline, erythromycin, chloramphenicol, azithromycin, chlortetracycline, streptomycin, trimethoprim, oxytetracycline and nitrofurantoin (Fig. 4A and 4B). Chowdhury [9] has reported that higher percentage of the *Aeromonas* bacteria represent strong resistant to the erythromycin. However, the majority of them showed very low resistant against oxolinic acid and sulfamethoxazole. The main drawback of the antibiotic treatment of *A. hydrophila* infection is the high potentiality of the organism to develop resistance against the antibiotic [24]. In the current study, except two bacterial isolates (*A. hydrophila*) all of them were resistant to amoxyclav (Fig. 4A and 4B). Since amoxyclav as it is one of the most common antibiotic used for aquaculture in Bangladesh, excessive use of amoxyclav might predispose current findings. Consistent with our hypothesis, Jongjareanjai *et al.* [20] have reported that *A. hydrophila* showed considerable levels of resistance against chloramphenicol, penicillin, amoxicillin, metronidazole, sulphamethoxazole-trimetroprim and amikacin because of indiscriminate use of these antibiotics in the aquatic environment.

The number, route, mode of transmission, and stability of an infectious agent outside to the host determines the severity of the infection. In the present study we showed that IP route was found more effective (PCM 100% in 14 days post-challenge) for infecting and reproducing disease in healthy fish compare to that the IM (PCM 40%) (Fig. 4C and 4D). Higher virulence's of *Aeromonas* infection also has been demonstrated by several earlier studies following IP route of infection in different fish species [2, 18, 32]. On the other hand, we also conducted a pathogenicity study of the isolated *Aeromonad* strain. Our results demonstrated the isolates were capable of producing clinical lesions in healthy fish similar to the bacterial infection. The onset and development of

hemorrhagic eye lesions demonstrated in the current study indicated an acute effect on the fish. Moralities in the experimental fish were most likely as a consequence of septicemia, since high numbers of pure cultures of the inoculated (aeromonad strains) were noticed in the kidney of the infected fish (Fig. 5A and 5B). Colonies of the bacteria were found in the myotome of muscles of the infected fish (Fig. 5C and 5D). Similar lesion also has been reported by another study due the aeromonad infection in with diseased grown up pangasius [19, 14].

5. Conclusion

A. hydrophila is a widely distributed pathogenic bacterium especially in warm water aquaculture that causes great economic losses to the aquaculture industry. To best of our knowledge, we showed the initial evidence of the isolation, identification, and characterization of *A. hydrophila* from the diseased juvenile pangasius in Bangladesh. We hypothesized that the bacterium could be one of the major pathogens of pangasius farming in Bangladesh. Our current findings provide a new horizon to develop possible theranostic strategies of *A. hydrophila* infection. However, further studies are needed to demonstrate the molecular and serological characterization of the organism. In addition, identification of virulence factors of *A. hydrophila* from pangasius also needs to be studied.

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7. Conflict of Interest

The authors declare that they have no conflict of interest.

8. References

1. Alain K. Isolation of *Aeromonas hydrophila* from naturally diseased thai pangas *Pangasius hypophthalmus.* MSc thesis, Bangladesh Agricultural University, Mymensingh, Bangladesh, 2009.
2. Angka SL, Lam TJ, Sin YM. Some virulence characteristics of *Aeromonas hydrophila* in walking catfish (*Clarias gariepinus*) Aquaculture 1999; 130:103-112.
3. Austin B, Austin DA. Bacterial Fish Pathogens: Diseases of Farmed and Wild Fishes. Edn 3, Springer-Praxis, Chichester, UK, 1999.
4. Banglapedia. Exotic Fish. Banglapedia, National Encyclopedia of Bangladesh, 2015. http://www.banglapedia.org/ 2 March, 2015.
5. Banu GR. Studies on the bacteria *Aeromonas* spp. in farmed fish and water in Mymensingh region. MSc. thesis, Bangladesh Agricultural University, Mymensingh, Bangladesh, 1996.
6. Barrow GI, Feltham RKM. *Cowan* and Stell's Manual for the Identification of Medical Bacteria. 3rd edition. Cambridge University Press, UK, 1993, 311.
7. Belton B, Haque MM, Little DC, Sinth LX. Certifying catfish in Vietnam and Bangladesh: Who will make grade and will it matter? Food Policy 2011; 36:289-299.
8. Chiou S, Chang M. Correlation between extracellular enzymes and virulence of *Aeromonas hydrophila,* Journal of Fisheries Society Taiwan 1994; 21:369-379.
9. Chowdhury MBR. Involvement of aeromonads and pseudomonads in diseases of farmed fish in Bangladesh Fish Pathology 1998; 33(4):247-254.
10. Crumlish M, Dung TT, Turnbull JF, Ngoc NTN, Ferguson HW. Identification of *Edwardsiella ictaluri* from diseased freshwater catfish, *Pangasius hypophthalmus* (Sauvage), cultured in the Mekong Delta, Vietnam, Journal of Fish Diseases. 2002; 25(12):733-736.
11. DOF, Fisheries statistical year book of Bangladesh Fisheries resource survey system, Department of Fisheries, Ministry of Fisheries and Livestock, Dhaka. 2012-2013. http://document.bdfish.org/2015/08/fisheries-statistical-yearbook-of-bangladesh-2012-2013/ 2 March 2014.
12. Erdem B, Karipta E, Çil E, Işik K. Biochemical identification and numerical taxonomy of *Aeromonas* spp. isolated from food samples in Turkey, Turkish Journal of Biology. 2009; 35:463-472.
13. FAO (Fisheries and Aquaculture Department). Global Aquaculture Production Statistics for the year, 2011. ftp://ftp.fao.org/FI/news/GlobalAquacultureProductionStatistics2011.pdf 2 March 2014.
14. Faruk MAR, Patwary ZP, Hasan MM. Clinical and histopathological investigations in exotic catfish *Pangasianodon hypophthalmus* (Sauvage, 1878) under culture condition, Indian Journal of Fisheries. 2012; 59(4):183-185.
15. Faruk MAR, Sarker MMR, Alam MJ, Kabir MB. Economic loss from fish diseases on rural freshwater aquaculture of Bangladesh, Pakistan Journal of Biological Sciences. 2004; 7(12):2086-2091.
16. Ferguson HW, Turnbull JF, Shinn A, Thompson K, Dung TT, Crumlish M. Bacillary necrosis in farmed *Pangasius hypophthalmus* (Sauvage) from the Mekong Delta, Vietnam, Journal of Fish Diseases. 2001; 24(9):509-513.
17. Hasan MA. Pathogenicity of *Aeromonas hydrophila* in EUS like disease affected *Heteropneustes fossilis*. MSc thesis, Bangladesh Agricultural University, Mymensingh, Bangladesh, 2007.
18. Iqbal MM, Tajima K, Sawabe T, Nakano K, Ezura Y. Phenotypic and genotypic identification of *Aeromonas* species isolated from the epizootic ulcerative syndrome fishes in South East Asian countries Fish Pathology 1998; 33(4):255-263.
19. Islam MT. Histopathological studies of shing (*Heteropneustes fossilis*) experimentally infected with the bacterial pathogen *Aeromonas hydrophila*. MSc thesis, Bangladesh Agricultural University, Mymensingh, Bangladesh, 2006.
20. Jongjareanjai M, Assawawongkasem N, Chansue N. *In vitro* antibiotic susceptibility of *Aeromonas hydrophila* isolated from disease ornamental fish, Journal of Veterinary and Medicine. 2009; 39(3):225-229.
21. Lio-Po GD, Albright LJ, Alapide-Tendencia EV. *Aeromonas hydrophila* in the Epizootic Ulcerative Syndrome EUS of snakehead (*Ophiocephalus striatus*) and catfish (*Clarias batrachus*): Quantitative estimation in natural infection and experimental induction of dermomuscular necrotic lesion. In: M Shariff, RP Subasinghe and JR Arthur (eds). Diseases in Asian Aquaculture I Fish Health Section, Asian Fisheries Society, Manila, 1992, 461-474.

22. Mamnur Rashid M, Hossain MS, Ali MF. Isolation and identification of *A. hydrophila* from silver carp and its culture environment from Mymensingh region, Journal of Bangladesh Agricultural University. 2013; 11(2):373-376.
23. Miles AA, Misra SS, Irwin JO**.** The estimation of the bactericidal power of blood. Journal of Hygiene. 1938; 38(6):732-749.
24. Mitchell AJ, Plumb JA. Toxicity and efficacy of furanace on channel catfish *Ictalurus punctatus* (Rafinesque) infected experimentally with *Aeromonas hydrophila*, Journal of Fish Diseases. 1980; 3:93-99.
25. Paniagua C, Revero O, Anguita J, Naharro G. Pathogenicity factors and virulence for rainbow trout (*Salmo garidneri*) of motile *Aeromonas* spp. isolated from a river, Journal of Clinical Microbiology. 1990; 28:350-355.
26. Plumb JA. Trends in Fresh water Fish Disease Research. In: TW Flegel and IH MacRae (eds). Diseases in Asian Aquaculture III. Fish Health Section, Asian Fisheries Society, Manila, 1997, 35-47.
27. Rashid MM, Hasan MA, Mostafa K, Islam MA. Isolation of *Aeromonas hydrophila* from EUS affected shing *Heteropneustes fossilis* from a fish farm in Mymensingh Progressive Agriculture 2008; 19(1):117-124.
28. Roberts RJ. Fish pathology. Eds 3. WB Saunders, London, UK, 2001.
29. Roberts RJ, Frerichs GN, Miller SD. Epizootic Ulcerative Syndrome: the current position. In: M Shariff, RP Subasinghe and JR Arthur (eds). Diseases in Asian Aquaculture I Fish Health Section, Asian Fisheries Society, Manila, 1992, 341-436.
30. Roberts RJ, Wootten R, Macrae I, Millar S, Struthers W. Ulcerative disease survey, Bangladesh: Final report to the Government of Bangladesh and Overseas Development Administration, Institute of Aquaculture, University of Sterling, Scotland, 1989, 104.
31. Santos Y, Romalde JL, Bandin I, Magarinos B, Nunez S, Barja JL, *et al*. Usefulness of API-20E system for the identification of bacterial fish pathogens. Aquaculture. 1993; 116:111-120.
32. Sarker MGA, Faruk MAR, Uddin MN, Sarker MA, Chowdhury MBR. Bacteriological studies on farmed catfish Thai-pangas, *Pangasius sutchi* Progressive Agriculture 1999; 10(1-2):209-212.
33. Sindermann CJ. Pollution associated diseases and abnormalities of fish and shell-fish a review Fishery Bulletin 1979; 76(4):717-749.
34. Thune RL, Stanley LA, Cooper RK. Pathogenesis of gram negative bacterial infections in warm water fish. In: M Faisal and FM Hetrich (eds). Annual Review of Fish Diseases. Pergamon Press, New York, 1993, 37-68.
35. Tonguthai K, Chinabut S, Somsiri T, Chanratchakool P, Kanchanakhan S. Diagnostic Procedures for Finfish Diseases. Aquatic Animal Health Research Institute, Bangkok, Thailand, 1999, 23.
36. Toranzo AE, Combarro P, Lemos ML, Barja JL. Plasmid coding for transferable drug resistance in bacteria isolated from cultured rainbow trout, Applied Environmental Microbiology 1984; 48(4):872- 877.
37. Torres JL, Tajima K, Shariff M. Numerical taxonomy and virulence screening of *Aeromonas* spp. isolated from healthy and epizootic 58 ulcerative syndrome positive fishes, Asian Fisheries Science 1993; 6(1):11-22.