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ISOLATION OF *Aeromonas hydrophila* FROM EUS AFFECTED SHING *Heteropneustes fossilis* OF A FISH FARM IN MYMENSINGH

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ABSTRACT

Aeromonas hydrophila bacteria was isolated from the suspected EUS-affected shing fish, *Heteropneustes fossilis* (Bloch). The disease investigations were primarily based on clinical signs and subsequently confirmed by the isolation of bacterial pathogen *Aeromonas hydrophila* from lesion of liver and kidney. The *A. hydrophila* isolates were identified by a series of morphological, physiological and biochemical tests. The total bacterial load in liver, intestine and kidney were 1.67×10^4 to 6.46×10^8 CFU/g, 1.71×10^3 to 1.18×10^9 CFU/g and 1.47×10^4 to 3.70×10^8 CFU/g respectively.

Key words : Shing, EUS, *Aeromonas hydrophila*, Load

INTRODUCTION

Epizootic ulcerative syndrome (EUS) is a very harmful disease of fishes. This disease has been causing a large scale mortality of freshwater fish species since 1988 in Bangladesh and over 120 freshwater fish species are susceptible to EUS (Barua, 1994). Shing fish has been reported to be affected by some metazoan parasitic diseases (Sanaullah, 1976) and bacterial diseases (Sahoo and Mukherjee, 1997). In India, *Gyrodactylus neonephrotus* was detected from the skin of *H. fossilis* by Singh and Agarwal (1994). In Bangladesh *Aeromonas* bacteria were found to be associated with EUS of different fishes. *Aeromonas hydrophila* bacteria were found in EUS affected carps and silver barb (Majumder *et al.*, 1999). Chowdhury *et al.* (1995) reported *Aeromonas* sp. as the dominant bacteria in the kidney of EUS affected different culture fishes. Rahman and Chowdhury (1996) also found *Aeromonas* as dominant bacteria from the lesions and kidney of EUS affected carps. In Bangladesh the shing fish was supposed to be an EUS resistant one, until 2006. Recently, in last September 2006, in "Agro 3 fish farm" situated at Boilor, Trishal, Mymensingh, the fish became severely affected by ulcer type disease, suspected to be EUS and mass mortality of the fish occurred. The present study was undertaken to work on the bacteria associated with the ulcer type disease in *H. fossilis* fish to characterize and identify the bacteria associated with the disease.

MATERIALS AND METHODS

Study area and duration

A fish farm namely "Agro-3 fish farm" was selected for the experiment, which was situated at Boilor, Trishal, Mymensingh. Duration of the study was 5 months starting from September 2006.

Collection of sample

A total number of 20 *Heteropneustes fossilis* (Shing) fish were collected from one of the selected ponds in which the fish were suffering from suspected EUS like lesions with severe mortality. Such diseased fish were caught by seine net during sampling. No dead fish were collected. After netting, the fish were taken in a plastic bucket with pond water and immediately transferred to the fish disease laboratory, Bangladesh Agricultural University, for the study.

Preparation of bacterial plate and stock bottles

Tryptic soya agar (TSA, Oxoid) was mixed to prepare bacterial culture medium at a rate of 40 g/L of distilled water in conical flask. The mixture was heated on hot plate and then autoclaved at 121^{o}C for 15 minutes. After autoclaving it was placed in clean chamber waited up to cooling to 60^{o}C and then poured to sterile petri dishes at an amount of 30 ml. After complete cooling and solidification, all the TSA plates were turned upside down. Half of each sterile stock bottle was poured with the above sterile TSA medium and kept in a slant position till the medium became cool and solidify. To test any growth of *Aeromonas* like bacteria, *Aeromonas* isolation medium was prepared. An amount of 28.15 g of *Aeromonas* selective supplement medium was mixed with 500 ml distilled water and boiled to dissolve completely. The contents of the commercially available Ampicillin vial for this purpose (Ampicillin SR 136 E - 2.5 mg) was rehydrated with a mixture of 1 ml sterile distilled water and 1 ml absolute alcohol which was then shaked to dissolve completely. It was then added aseptically to above prepared 500 ml sterile *Aeromonas* isolation medium at 59-55^{o}C. *Aeromonas* selective supplement medium base plates were then prepared as the above method of preparing TSA plates and were kept in incubator at 22^{o}C for 24 hours to examine contamination. All the plates and stock bottles were kept at 4^{o}C for future use.

Clinical observation

Before collection of diseases fish, their movement and swimming were observed and recorded. Collected fish were examined to observe any external lesion, injury or any other abnormalities and were recorded properly. Magnifying glass and careful eye observations were applied for this study.

Bacteriological procedure

Any external lesions were touched with a sterile inoculating loop and streaked onto the TSA plates. The plates were incubated at 25°C for 36-48 hours. Individual colonies were then separated from the plates on the basis of colour, shape and size and were further cultured onto to the TSA plates again to obtain pure culture of each type of colonies. Individual isolates were streaked onto the agar slant, incubated at 25°C for 24 hours and after the growth of the bacteria the TSA slant bottles were preserved at 4°C as stocks. Similar streakings were also done onto *Aeromonas* isolation medium plates from each lesion.

Bacterial isolation from liver, kidney and intestine

Body cavities of the collected fish were opened aseptically by the help of sterilized scissors. Portions of intestine, liver and kidney samples were weighed and taken in separate sterile petri dishes. Each of the samples was mixed with sterile physiological saline (0.85% NaCl = PS) at the ratio of 1 part of sample**:** 9 parts of the PS and homogenized by mortar and pestle. The homogenized solution was designated as stock solution. To prepare decimal dilution 100 µl of the stock solutions were transferred into 0.9 ml sterile physiological saline to make a serial decimal dilution. Similar 0.1 ml allocates were transferred into each 0.9 ml PS to prepare two more next serial decimal dilutions. A 100 µl of each dilution was inoculated onto duplicate TSA plates and inoculated at 25^{o}C for 48 hours for colony appearances. An allocate of 100 µl from the stock solutions of each organ was also spreaded onto *Aeromonas* isolation medium plates.

Colony counting

Incubated plates were observed for colony appearance. The colonies from TSA plates were counted with the help of digital colony counter. Total bacterial load of each organ was calculated using the following formula worked out by Mamnur Rashid *et al.* (1994).

Total bacterial load = Number of colonies in the plate × 10^{n} × 100
Where, n = dilution number

Identification of Aeromonas like bacteria

All grown colonies of *Aeromonas* like bacteria from the streaked and spreaded plates of *Aeromonas* isolation medium were subcultured onto TSA plates and then undergone morphological characterization such as shape, size, Gram character, flagellation and motility using fresh 24-hour cultures. Biochemical characters such as, oxidase, catalase, oxidative-fermentative (OF), acid and gas production from sugars (glucose, lactose, maltose, sucrose and manitol), methyl-red, Voges-Proskauer (VP), indole and H_2S production, decarboxylase and citrate utilization were studied to confirm their generic and specific natures. Physiological characters were checked by observing the growth of each isolate at temperatures of 4°C, 5°C, 37°C and 40°C and in different concentrations of NaCl such as 0%, 1%, 2%, 3% and 4% to confirm the characteristics of *Aeromonas* bacteria.

RESULTS

Clinical pathology

External ulcerative lesions were found on the body surface of naturally infected shing fish (*H. fossilis*) from the "Agro 3 fish farm" which were characterized by deep red ulcers surrounded by whitish margins. Some fish were showed to loss their fins and in some cases the myotomes was observed by naked eyes. Reddish fin bases were also observed. Affected fish showed pale body colour, sluggish movement and lethargic swimming (Fig. 1).

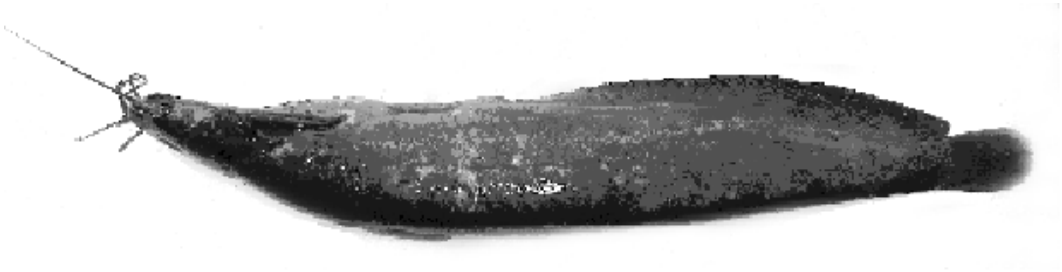

Fig.1. A moribund fish collected from the Agro-3 fish farm showing pale body colour and a deep red ulcer surrounded by whitish margin.

Characteristics of Aeromonas like bacteria

Streaked and spreaded plates with *Aeromonas* isolation medium showed growth of *Aeromonas* bacteria confirming their association with the ulcerative lesions of shing fish. The isolates rose to yellowish opaque colonies on TSA agar. These colonies were smooth, raised and round in shape. The results of their comparative, morphological, biochemical and physiological tests with those of Popoff (1984) and Sabur (2006) are shown in Table 1.

Table 1. Characters of *Aeromonas hydrophila* isolates in comparison to those shown by Popoff (1984) and Sabur (2006)

Characters		Popoff (1984)	Sabur (2006)	Present isolates
Gram stain		-	-	-
Motility		+	+	+
Shape		Rod	Rod	Rod
Oxidase		+	+	+
Catalase		+	+	+
Acid and gas production from sugar	Glucose	+	+	+
	Sucrose	+	+	+
	Maltose	+	+	+
Acid production	Lactose	+	+	+
	Manitol	+	+	+
Voges-Proskaur		+	+	+
H_2S production		+	+	+
Arginine hydrolysis		+	+	+
Esculin hydrolysis		+	+	+
Growth at	4°C	-	-	-
	37°C	+	+	+
	40°C	-	-	-
Growth in NaCl Solution	0%	+	+	+
	1%	+	+	+
	2%	+	+	+
	3.5%	-	-	-
	4%	-	-	-

The bacteria were Gram negative, motile, fermentative, oxidase positive and catalase positive rods. They produced acid and gas from dextrose, maltose and sucrose. In lactose and manitol, the bacteria produced only acid. The bacteria gave positive results in Voges-Proskauer, H_2S, arginine hydrolysis and esculine hydrolysis. Incase of physiological study the growth of the isolates were observed at 37°C but no growth was observed at 4°C and 40°C. Also the growths of isolates were observed in 0%, 1% and 2% NaCl solution but no growth was observed in 3.5% and 4% NaCl.

Bacterial load in liver, intestine and kidney

Results of the bacterial loads in the liver, intestine and kidney of the naturally EUS affected shing are shown in Table 2. Numbers of the viable cells of *A. hydrophila* were found to be 1.67 x 10^4 to 6.46 x 10^8 CFU/g in liver, 1.71 x 10^3 to 1.18 x 10^9 CFU/g in intestine and 1.47 x 10^4 to 3.70 x 10^8 CFU/g in kidney.

Table 2. Bacterial load in liver, intestine and kidney of shing fish sampled from Agro 3 fish farm during September 2006

Sl. No. of investigated fish	Liver (CFU/g)	Intestine (CFU/g)	Kidney (CFU/g)
F_1	1.67×10^4	3.20×10^6	1.47×10^4
F_2	1.51×10^5	3.78×10^5	5.51×10^5
F_3	9.45×10^4	1.18×10^9	9.83×10^7
F_4	6.33×10^5	1.01×10^5	3.20×10^5
F_5	2.00×10^6	1.71×10^3	1.10×10^7
F_6	2.09×10^6	4.91×10^4	5.06×10^7
F_7	4.18×10^8	2.88×10^5	9.02×10^6
F_8	7.37×10^7	7.04×10^7	6.05×10^6
F_9	2.20×10^6	1.30×10^6	3.30×10^5
F_{10}	3.26×10^6	9.94×10^4	2.00×10^5
F_{11}	1.84×10^7	6.63×10^6	4.00×10^5
F_{12}	2.38×10^6	5.22×10^3	3.70×10^8
F_{13}	6.46×10^8	5.25×10^8	3.17×10^6
F_{14}	4.38×10^8	3.56×10^8	3.50×10^6
F_{15}	2.70×10^7	3.23×10^8	5.10×10^7

DISCUSSION

In the present study, round ulcers on body surface with haemorrhage and skin erosion were found from EUS like diseased catfish *H. fossilis* in a fish farm from Mymensingh. Shing fish was not found to develop such ulcerative lesions before this work in Bangladesh. Infected shing fish lost their normal appearances and formed pale body colour. Reddened fin bases were also observed. Only Sahoo *et al.* (1998) found an outbreak of ulcer disease in Indian shing fish having similar clinical symptoms.

In the present study, *Aeromonas hydrophila* was isolated from EUS-affected shing. Same type of experiment was conducted by Sahoo and Mukherjee (1997). They isolated three gram negative bacterial pathogens: *A. hydrophila, Edwardsiella tarda* and *Haemophilus piscicum* from the diseased catfish *H. fossilis* and *Clarias batrachus*. Roberts *et al.* (1989) carried out a survey of the ulcerative disease in Bangladesh where *A. hydrophila* and *A. sobria* were found to be the dominant bacteria in the EUS lesions and ulcers. Pal and Pradhan (1990) carried out a study on the bacterial involvement in ulcerative condition of air-breathing catfish in India and isolated four types of bacteria, two of which were fluorescent pseudomonads, one aeromonad (*A. hydrophila*) and one coccus (*Micrococcus varians*) from skin lesions of the fish. Chowdhury *et al.* (2002) observed the severity of the ulcerative disease in different fishes with concurrent infection of *Aphanomyces invadans* and *Aeromonas* bacteria. But in case of experimental infection with only *Aphanomyces*, they found less severe infection. In other part of this study (Hasan, 2007) *Aphanomyces* granuloma were found in the tissues of these ulcer diseased shing fish. This study showed the concurrent availability of *Aeromonas hydrophila* in the same lesions, which proved that the severe loss of shing fish in the investigated fish farm was due to the cumulative pathogenicity of the fungal and the bacterial pathogens. This result coincides with the findings of Chowdhury *et al.* (2002).

In the present study, bacterial load found in liver, intestine and kidney were 1.67×10^4 to 6.4×10^8CFU/g, 1.71×10^3 to 1.18×10^9 CFU/g and 1.47×10^4 to 3.70×10^8 CFU/g respectively. Rahman and Chowdhury (1996) isolated bacteria from kidney of carp fishes. In their case total load of bacteria in the kidney of different sampled fishes were found to be 2.6×10^5 to 1.7×10^6 CFU/g. Bastawrows and Mohammed (1999) isolated *A. hydrophila* from *Oreochromis niloticus* and *Clarias lazera* where average bacterial counts were 3.4×10^4 CFU/g and 4.1×10^3 CFU/g respectively. Al-Harbi and Uddin (2003) recorded total bacterial load in intestine of tilapia ranging from $8.2 \pm 1.6 \times 10^5$ to $9.9 \pm 1.5 \times 10^7$ CFU/g and $3.4 \pm 1.8 \times 10^6$ to $5.8 \pm 0.45 \times 10^7$ CFU/g. Rahman and Chowdhury (1997) investigated total bacterial load in the kidney of *Channa punctatus, C. striatus, Mastacembelus pancalus* and *H. fossilis* which varied from 2.7×10^5 to 4.7×10^5 CFU/g. In the present study, the bacterial loads in all the organs tested were found to be higher than those of the other investigators. This might be attributed to the weakness of the shing fish due to EUS and attack of the *Aeromonas* bacteria as a secondary invader as well as their primary infection.

It is established that the *Aeromonas hydrophila* bacteria is an ubiquitous pathogen in Bangladesh waters (Sabur, 2006). So this higher load of *Aeromonas* in EUS affected shing fish supports the above the ubiquitousness of the bacteria at least in Mymensingh region. In the light of the present findings, it is suggested that the fish culturists of Bangladesh may take much care of their culture fishes at least the shing, to save from fungal as well as bacterial infections specially from the mixed infections of *Aphanomyces* and *Aeromonas* by improving their culture techniques and culture environment.

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