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Experimental pathogenesis of *Aeromonas hydrophila* bacteria in shing *Heteropneustes fossilis* (Bloch)

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Abstract
Pathogenicity of *Aeromonas hydrophila* bacteria was tested on the stinging catfish *Heteropneustes fossilis*. Before artificial infection the morphological, biochemical and physiological characters of *Aeromonas hydrophila* were studied. The infections were done by two different methods, *viz.*, intramuscular (IM) and intraperitoneal (IP) injection. In infection experiment, each group of 10 fish were injected either intramuscularly or intraperitoneally with one dose higher than the LD_{50} dose (9.6×10^7 CFU/fish). All the fish tested died within 1 to 9 days. Both in cases of intramuscular and intraperitoneal injection, external pathology were found. Haemorrhagic lesions were evident at the site of injection. The posterior end of the body surface was found to develop greyish-white lesion that was extended up to caudal fin. Hyperemic anal region and the fin bases were also observed. Total bacterial loads in liver, kidney and intestine were determined. *Aeromonas hydrophila* could be isolated from liver, kidney and intestine of the experimentally infected fish. In case of intramuscular injection the highest and the lowest bacterial load was found to be 2.4×10^7 CFU/g of liver and 2.1×10^2 CFU/g of kidney and in case of intraperitoneal injection they were found to be 3.6×10^6 CFU/g of kidney and 1.2×10^4 CFU/g of kidney respectively. It was concluded that *A. hydrophila* could cause serious disease condition to *Heteropneustes fossilis* and its pathogenesis in the fish was also very efficient.

Key words: Experimental pathogenesis, *Heteropneustes fossilis*, *Aeromonas hydrophila*

Introduction

Stinging catfish *Heteropneustes fossilis* is a hardy fish having much economic importance in Bangladesh. It can withstand wide environmental variation in relation to temperature and oxygen content. So it was supposed that the fish should be resistant to many fatal infections like epizootic ulcerative syndrome (EUS). However, only recently, the fish was found to be affected by EUS like lesion in an established farm in Mymensingh, Bangladesh. *Aeromonas hydrophila* could be isolated from those lesions (Hasan 2007). There are many evidences that *A. hydrophila* was associated with EUS in different fishes.

Laillier *et al.* (1980) stated that *A. hydrophila* might be the global bacteria associated with the fish diseases. *Aeromonas* spp. contributed to the pathogenesis of the

EUS disease (Lutwyche *et al.* 1995). Certain strains of *A. hydrophila* could induce EUS-like lesions were reported by Lio-Po *et al.* (1990). *Aeromonas* spp. contributes to the pathogenesis of MAS (Motile *Aeromonas* septicemia) disease (Roberts *et al.* 1990).

The present study was carried out to understand the pathogenesis of *A. hydrophila* in experimentally infected shing, *H. fossilis* and thereby to know the fate of the pathogen in the tissues of the fish. Thus the study might be helpful for the management and control of the aeromonads and other bacterial diseases in at least stinging catfish.

Materials and methods

Experimental site and duration of the study

The infection experiments were conducted at the wet laboratory of the Faculty of Fisheries. Duration of the experiment was from July to December 2006.

Experimental preparations

A recycle system was set with pipe fittings and electric motor in the wet laboratory. The recycle system consisted of 5 metallic drums each having 150 l capacity, twelve aquaria of fibre glass each having 40 l capacity and an over head tank. The water was supplied at first to the drums and then pumped up to the overhead tank. It was then drained to the aquaria by downward pipe ventilated to each aquarium. From the aquaria it was collected by a collecting tube which was passed through an ultra-violet tube light complex to sterilize the circulating water during the experimental period and then opened at the first drum. The circulation system was filled with both pond and supply water. Prior to the experiment the water was kept under circulation for 7 days. Glass wares (Petri dishes, test tubes, L-sticks, morter and pastle) were dry sterilized at 170°C for 1 hour by a dry sterilizer (Memmert). The tips were autoclaved at 121°C for 15 min and then dried at 70°C for overnight. Tryptic soya agar (TSA, Oxoid) plates were used for the culture of bacteria and TSA agar slants, for stocking of bacteria.

Fish stocking in the recycle system

The fish collected from the selected pond by seine net, were kept in the above mentioned 12 aquaria of the recycle system for 7 days for acclimatization. The aquaria were covered with synthetic fibre net to prevent fish escaping. Every day 50% of total water was changed. Aerators were set in the aquaria for continuous aeration during the whole experimental period.

Collection and characterization of Aeromonas hydrophila

An *Aeromonas hydrophila* isolate CK602 previously isolated, characterized and stocked by Sabur (2006) was collected, restocked in TSA slants and recharacterized before starting the experiment as follows.

Morphological characters such as shape, size, Gram character, flagellation and motility were observed 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. 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%.

Experimental infections

For preparation of bacterial suspension *A. hydrophila* were freshly cultured onto TSA plate, incubated at 25°C for 48 hours and then 52 mg of the bacterial colonies were mixed with 4 ml of sterile physiological saline (0.85% NaCl in distilled water). For the experimental infection method 1 ml sterile plastic (disposable) syringe was used. Each of the 10 experimental fish was injected with 0.2 ml bacterial suspension either intramuscularly or intraperitoneally. One dose higher than the calculated LD_{50} (Mostafa 2007) was planned to be injected. Accordingly the suspension was made and the desired dilution was prepared and counted to be 9.6×10^7 CFU/ml.

An allocate of 0.2 ml of the above bacterial suspension was injected at the base of the dorsal fin of each fish after disinfection with 70% alcohol cotton. For intraperitoneal infection, bacterial suspension was injected in the peritoneal cavity at a dose of 0.2 ml of each selected fish. Injections were given with utmost care to avoid puncture of internal organs. At the injection period care also was taken so that the inoculum (bacterial suspension) would not come out after pushing back of the syringe.

All the injected fish were then transferred to the aquaria and observed for 15 days of the experimental period. No feed was supplied during this time. The injected fish were observed daily several times for any abnormal clinical appearances and were recorded properly. Water temperature was also recorded daily. Moribund fish were attended, observed and waited for their death. Freshly dead fish were collected immediately, transferred to the laboratory and used for bacterial isolation.

Isolation of bacteria from liver, intestine and kidney

Intestine, liver and kidney of each freshly dead fish were dissected out aseptically and placed in sterilized separate plastic petri dishes. After weighing, sample of each of the above organ was homogenized and suspended in sterile physiological saline (1 part of sample: 9 parts of PS) to obtain a stock solution. Two consecutive decimal dilutions, 10^{-1} and 10^{-2}, from the stock solution were made for each organ. The dilutions (stock, 10^{-1} and 10^{-2}) of each organ were spreaded onto duplicate TSA plates. All such plates were incubated at 25°C for 48 hours. Appeared colonies were counted and their numbers were used to interpret the pathogenesis of the pathogen in the organs of the experimentally infected fish, expressed by bacterial loads.

The bacterial load was calculated by using the following formula worked out by Mamnur Rashid *et al.* (1994).

$$\text{Bacterial CFU/g of fish organ} = \text{No. of colonies counted in the plate} \times 10^n \times 100$$

where, n is the dilution factor

Results and discussion

Morphological, biochemical and physiological characters of the *Aeromonas hydrophila* isolate (CK 602) have been shown in Table 1. They were found to possess same characteristics like those tested by Popoff (1984) and Sabur (2006).

Table 1. Characters of *Aeromonas hydrophila* isolates in comparison to other studies

Characters	Characterization by Popoff (1984)	Characterization by Sabur (2006)	Present results
Gram stain	-[1]	-	-
Shape	Rod	Rod	Rod
Motility	+[2]	+	+
Oxidase	+	+	+
Catalase	+	+	+
OF test	F[3]	F	F
Acid and gas production from Glucose	+	+	+
Acid production from			
Lactose	+	+	+
Sucrose	+	+	+
Maltose	+	+	+
Manitol	+	+	+
Insitol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Methyl-red test	-	-	-
Voges-Proskaur	+	+	+
Indole	+	+	+
H_2S production	+	+	+
Arginine decomposition	+	+	+
Lysine decarboxilation	-	-	-
Ornithine decarboxilation	-	-	-
Citrate utilization	+	+	+
Growth in : 4°C	-	-	-
: 5°C	-	-	-
: 37°C	+	+	+
: 40°C	+	+	+
: 0% NaCl	+	+	+
: 1% NaCl	+	+	+
: 2% NaCl	+	+	+
: 3% NaCl	+	+	+
: 4% NaCl	-	-	-

[1] Negative, [2] Positive , [3] Fermentative

The results of the pathogenesis of *A. hydrophila* in the liver, intestine and kidney *i.e.* their fate after experimental infection with different doses and by two different methods, *viz.*, intramuscular injection and intraperitoneal injection are shown in Table 2 and 3.

Table 2. Fate of *Aeromonas hydrophila* in liver, kidney and intestine of stinging catfish, *Heteropneustes fossilis* artificially infected by intramuscular injection at a dose of 1.9×10^7 CFU/fish

Fish No.	Bacterial colony count		
	Liver	Intestine	Kidney
F_1	2.4×10^7	1.5×10^7	1.3×10^7
F_2	2.1×10^5	1.5×10^6	4.1×10^4
F_3	3.0×10^4	4.8×10^5	4.1×10^5
F_4	1.3×10^6	1.7×10^6	3.8×10^6
F_5	1.4×10^5	3.5×10^6	2.7×10^5
F_6	6.5×10^5	3.1×10^5	4.7×10^4
F_7	6.2×10^4	1.0×10^5	1.3×10^5
F_8	3.9×10^5	1.1×10^5	4.2×10^4
F_9	6.2×10^4	9.0×10^3	2.1×10^2
F_{10}	6.0×10^4	5.1×10^5	3.1×10^3

Table 3. Fate of *Aeromonas hydrophila* in liver, kidney and intestine of stinging catfish, *Heteropneustes fossilis* artificially infected by intraperitoneal injection at a dose of 1.9×10^7 CFU/fish

Fish No.	Bacterial colony count		
	Liver	Intestine	Kidney
F_1	2.1×10^5	2.2×10^5	1.4×10^5
F_2	3.9×10^5	5.0×10^4	3.6×10^6
F_3	2.0×10^4	1.3×10^4	1.2×10^4
F_4	1.9×10^6	2.9×10^6	3.7×10^5
F_5	2.0×10^5	3.7×10^5	3.6×10^6
F_6	5.2×10^5	2.5×10^4	6.6×10^4
F_7	1.3×10^5	1.1×10^6	3.3×10^4
F_8	1.5×10^6	2.9×10^5	1.9×10^5
F_9	2.7×10^6	2.8×10^5	4.5×10^5
F_{10}	3.2×10^4	3.0×10^5	2.1×10^5

In the present study, *A. hydrophila* could be isolated from liver, kidney and intestine of experimentally infected shing by the homologous bacteria. Highest bacterial load was found to be 2.42×10^7 CFU/g of liver and the lowest, 2.1×10^2 CFU/g of kidney of *H. fossilis*. The range was 2.0×10^4 to 2.42×10^7 CFU/g of liver. In case of intestine, 3.5×10^6 CFU/g and 9.0×10^3 CFU/g was respectively the highest and lowest bacterial count. The bacterial load was found to be 1.3×10^7 to 2.1×10^2 CFU/g of kidney. Rahman and Chowdhury (1996) found total bacterial load in kidneys of carps to be 2.6×10^5 to

1.7×10^6 CFU/g. Iqbal *et al.* (1996) found total bacterial load to be 5.4×10^3 to 4.7×10^7CFU/g in slime and undetectable to 1.7×10^4 CFU/g in kidney of *Cirrhinus mrigala*. In the experimental infection of the selected *A. hydrophila* isolate (CK602) done intramuscularly and intraperitoneally to shing fish at a dose of $1.92x10^7$ CFU/fish, mortalities were 100% within 1-9 days. Alam *et al.* (1999) used 1.18×10^3 to 4.81×10^4 CFU/fish of *Edwardsiella tarda* bacteria for infecting Thai pangas (*Pangasius sutchi*) and 33% to 100% mortalities were achieved within 6 to 10 days. Pal *et al.* (1997) found 40-100% mortalities of silver barb (*Puntius gonionotus*) with a dose of $2\text{-}6\times10^6$ CFU/ml of *Pseudomonas fluorescens* to detect their pathogenicity. Miyashita (1984) found 40% death of tilapia by *Edwardsiella tarda* at doses of $10^{6\text{-}7}$ CFU/fish within 1-6 days. In the present study external clinical pathologies were observed in the moribund *H. fossilis* experimentally infected by *A. hydrophila*. Haemorrhagic lesions at the site of injection were observed. Wanna (2000) found petechial haemorrhages at the injected area and congestion around the anus of *Clarias batrachus* intraperitoneally with *A. hydrophila*. Hasan (2007) isolated *A. hydrophila* from naturally EUS affected shing and found the load to be 1.67×10^4 to 6.46×10^8 CFU/g in liver, 1.71×10^3 to 1.18×10^9 CFU/g in intestine and 1.47×10^4 to 3.70×10^8 CFU/g in kidney. Mamnur Rashid *et al.* (1996) found 100% mortality from an experimental infection of Japanese flounder *Paralichthys olivaceus* with *Edwardsiella tarda* by the dose of 10^5 CFU/fish in intra-peritoneal injection, 10^8 CFU/fish in oral intubation and 10^9 CFU/fish in immersion method respectively.

Through experimental infection it was proved that *A. hydrophila* was seriously pathogenic to shing fish which may cause heavy loss of its commercial production as also evidenced from the first observation by Hasan (2007). The bacteria *A. hydrophila* could be reisolated from the kidney, liver and intestine of the experimentally infected shing fish which proved that the pathogenesis of the pathogen in the organs of the shing fish is very efficient. Further studies are necessary to understand the histopathology of shing fish by *A. hydrophila* bacteria in natural condition as well as in experimental infection.

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