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Experimental infection of shing *Heteropneustes fossilis* with *Aeromonas hydrophila*

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Abstract

Experimental infections of shing *Heteropneustes fossilis* were conducted with *Aeromonas hydrophila* to test the pathogenicity of the bacteria in this fish and to determine the median lethal dose (LD_{50}) of the bacteria. Previously isolated and characterized *A. hydrophila* (CK602) was injected intraperitoneally to shing of 35 g body weight at two doses of 10^5 and 10^8 CFU/fish. All experimentally infected fish died within 15 days. In the next experiment, to calculate the LD_{50} of the pathogen, four different doses *viz.* 10^5, 10^6, 10^7 and 10^8 CFU/fish were injected to each group of 10 shing of average body weight 35 g. Mortality was found with the doses between 10^6 and 10^8 CFU/fish. LD_{50} was calculated to be 9.6×10^6 CFU/fish.

Keywords: LD_{50}, *Aeromonas hydrophila*, Shing

Introduction

Stinging catfish *Heteropneustes fossilis* (shing) is an important air breathing catfish in Bangladesh. It was believed that it was a resistant fish against many diseases like epizootic ulcerative syndrome (EUS). But now a days there are many evidences that *A. hydrophila* was associated with EUS in different fishes (Majumder *et al.,* 1999; Subasinghe *et al.,* 1990; Karunasagar *et al.,* 1995, Boonyaratpalin, 1987). Shing fish has been reported to be affected by bacterial diseases (Sahoo and Mukherjee, 1997), parasitic infestations (Sanaullah, 1976) and also by EUS in India (Sahoo *et al.,* 1998). Bacterial diseases have been found responsible for heavy mortalities of both wild and cultured fishes. Among them *Aeromonas* spp. are the major pathogens which are widely distributed in aquatic environment (Sabur, 2006). Recently shing was found to be affected by EUS like lesions in Mymensingh and *Aeromonas hydrophila* has been isolated from those lesions (Hasan, 2007). He mentioned that the EUS lesion of shing was a result of concurrent pathogenicity of both *Aphanomyces invadans* and *Aeromonas hydrophila*. He observed necrosis, haemorrhage, vaculation, atrophy and fatty deposition in liver together with fungal granuloma and in kidney, degeneration of renal tubule, missing of glomerulus, pyknosis and other lesions like liver together with similar fungal granuloma. Thus he proved that the above histopathological lesions were due to the above two pathogens. Chowdhury *et al.* (2002) proved that the presence of *Aeromonas hydrophila* potentiated the EUS lesions to be more severe.

The virulence of a pathogen can be estimated from experimental studies of the LD_{50} (median lethal dose) which is the amount of pathogen required to kill 50 percent of uniformly susceptible animals inoculated with the pathogen. Information on LD_{50} of a pathogen to a specific fish is very important for any type of research related to the interaction of that pathogen and the fish. So, the present study was conducted to test the pathogenicity of *A. hydrophila* and to work out LD_{50} of the pathogen in the shing fish.

Materials and Methods

The experiments were conducted at the wet laboratory of the Faculty of Fisheries, BAU. duration of the experiment was from July to December 2006.

A recirculatory system was set with pipe fittings and electric motor in the wet laboratory. The system consisted of 5 metallic drums each of 150 L capacity, twelve aquaria of fibre glass each of 40 L capacity and an over head tank of L capacity. 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. Glasswares (Petri dishes, test tubes, L-sticks, morter and pastle) were driest and sterilized at 170ºC for 1 hour by a dry sterilizer (Memmert). The plastic materials like tips were autoclaved at 121ºC 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 (TSA, Oxoid) agar slants for stoking of bacteria.

The fish were collected from the stock pond of the Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh, by seine net and kept in the above mentioned 12 aquaria of the recirculatory system for 7 days for acclimatization. The aquaria were covered with synthetic fibre net to prevent the fish from escaping. Everyday 50% of total water was changed and continuous aeration was maintained during the whole experimental period.

For preparation of bacterial suspension, the inocula from CK602 isolate of *Aeromonas hydrophila* were sub cultured onto TSA plate and incubated at 25ºC for 48 hours. An amount of 52 mg of such cultured bacterial colonies were mixed with 4 ml of sterile physiological saline (0.85% NaCl in distilled water = PS). This mixture was designated as stock solution. Nine serial dilutions were made from this one as follows. From each earlier suspension 0.1 ml was added to 0.9 ml sterile PS. The 10^{-7} to10^{-9} dilutions were used for colony count to find out the bacterial CFU in the dilutions used for experimental infection using the formula according to Mamnur Rashid *et al.* (1994)..

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

The stock solution and dilution 10^{-3} were used for pathogenicity test. From each suspension, 0.2 ml portion were injected intraperitoneally to each of 10 fish. Each of another group of 10 fish were injected with 0.2 ml PS which was designated as negative control group. Each two groups of bacterial inoculated fish and the control group were then released in 3 separate aquaria labeled properly. The injected fish were observed up to 15 days. No feed was given to the experimental fish. Temperature was recorded daily. Each moribund fish was attended, waited for its death and transferred to the laboratory immediately after death. Each dead fish was dissected out aseptically, kidney was touched with a sterilized loop and the loop was streaked onto TSA agar plate. The plates were incubated at 25°C for 48 hours to test *A. hydrophila* colony appearance. Each death case was recorded properly and a data of mortality was prepared.

The stock solution and next three serial dilutions (10^{-1}, 10^{-2} and 10^{-3}) were used for this experiment. From each of the above four dilutions 0.2 ml bacterial suspension were injected intraperitoneally to each of 10 fish of a group. A negative control group of 10 fish were maintained as above. Each of the above five groups of experimental fish were transferred to 5 separate aquaria. The experiment was continued for 15 days with continuous observation. Moribund fish were attended as above and freshly dead fish was dissected out aseptically in the laboratory. Kidney was touched with a sterilized loop and the loop was streaked onto TSA agar plate. The plates were incubated at 25°C for 48 hours to test *A. hydrophila* colony appearance. Each death case was recorded properly and a data of mortality was prepared. LD_{50} was calculated using the data from the pathogen-dilution and mortality record of intraperitoneally infected shing fish according to the following formulae worked out by Mamnur Rashid *et al.* (1996).

$$\text{Proportionate} = \frac{\%\text{mortality at dilution next above 50\%} - 50\%}{\text{distance (PD) \% mortality at dilution next above 50\%} - \%\text{mortality at dilution next above 50\%}}$$

Dilution factor (DF) = Negative Log of lower dilutions (next above 50% mortality)..... (i)

PD × DF..... (ii)

Log LD_{50} titer = (i) + (ii)

LD_{50} titer = $10^{[(i)+(ii)]}$

Results and Discussion

In case of pathogenicity test, the plate count of colony appearances of the 10^{-7} to 10^{-9} dilutions, the bacterial CFU/ml of the decimal dilutions were found to be 6.5×10^8 CFU/ml in stock solution. So, the inoculated dose (0.2 ml) from the stock solution and the 10^{-3} dilution were calculated to be 1.3×10^8 and 1.3×10^5 CFU/fish. From the above two test groups all the 10 inoculated fish (100%) died within 2 to 7 days from the first group (inoculated with 1.3×10^8 CFU/fish) and 4 fish (40%) died within 5-7 days from the second group (Table 1). No fish died from the negative control group. Alam *et al.* (1999) used six selected isolates of *Edwardsiella tarda* bacteria for injecting Thai pangas (*Pangasius sutchi*) intramuscularly to study the pathogenicity of the isolates. The doses ranged from 1.18×10^3 to 4.81×10^4 CFU/fish. All the tested isolates were proved to be pathogenic. Mortality rate was 33% to 100% within 6 to 10 days. Pal *et al.* (1997) used five isolates of *Pseudomonas fluorescens* in order to detect their pathogenicity to silver barb (*Puntius gonionotus*) at doses of $2\text{-}6 \times 10^6$ CFU/ml. They found 40-100% mortalitiy. Miyashita (1984) performed pathogenicity test of *Edwardsiella tarda* to tilapia by intramuscular injection at doses of 10^7 and 10^6 CFU/fish. At the dose of 10^7, 40% of the tested fish died within 1-3 days and at the dose of 10^6, 40% fish died within 4-6 days. Sabur (2006) observed the pathogenicity of *Aeromonas hydrophila* to five carps, *viz., Labeo rohita, Catla catla, Cirrhina cirrhosus, Hypophthalmichthys molitrix and Cyprinus carpio* by intramuscular and intraperitoneal injections at doses of $2 \times 10^{5\text{-}6}$ CFU/fish and by immersion method at doses of $2.2 \times 10^{7\text{-}8}$ CFU/fish. Mortality rates were from 40% to 100%. The present experiment proved that *Aeromonas hydrophila* was also pathogenic for *Heteropneustes fossilis.*

Table 1. Results of the pathogenicity test of the *Aeromonas hydrophila* CK602 isolate to 10 shing *Heteropneustes fossilis* injected intraperitoneally

Average fish body weight	Injected dose[1] CFU/fish	Mortality[2] (%)	Post injection day of mortality[3]
35 g	1.3×10^5	40	5-7
	1.3×10^8	100	2-7

[1] Injected amount was 0.2 ml/fish
[2] The mortality occurred at 28°C of the experimental temperature
[3] Observation was continued for 15 post injection days

In the LD_{50} experiment the 4 different doses were calculated to be 3.2×10^8 CFU/fish in the stock solution, 3.2×10^7 CFU/fish in the dilution 10^{-1}, 3.2×10^6 CFU/fish in the dilution 10^{-2} and 3.2×10^5 CFU/fish in the dilution 10^{-3}. All the 10 fish injected with stock solution, 3.2×10^8 CFU/fish, died within 1-5 days. From the dose of 3.2×10^7 CFU/fish, 6 fish died within 1 to 7 post infection days. In case of 3.2×10^6 CFU/fish, 3 fish died within 4 to 9 post infection days. No mortality was observed in case of the dose of 3.2×10^5 CFU/fish within 15 days of the experimental period as well as in the negative control group. Incubation of kidney streaked plates of each dead fish gave rise to the appearance of pure colonies of *A. hydrophila*.

Formulating the mortality report of the experimental fish in the Table 2, the LD_{50} was calculated to be 9.6×10^6 CFU/fish for the shing fish of 35 g average body weight at 28°C. Shen *et al.* (2001) determined LD_{50} of three isolates of *A. hydrophila* against rice field eel, *Monopterus albus* to be 2.84×10^6 CFU/fish, 6.12×10^6 CFU/ fish and 2.13×10^6 CFU/fish. Though these LD_{50} values were from different fish species, the similar findings in the present study implied the similar pathogenicity of *A. hydrophila*.

Table 2. Formulated data from the mortalities in the experimental infection of shing with *Aeromonas hydrophila* by intraperitoneal injection for the calculation of LD_{50} according to Mamnur Rashid *et al.* (1996)

Pathogen dilution	Mortality ratio	Morta-lities	Survi- vors	Accumulated values			
				Total dead	Total survived	Mortality	
						Ratio	Percent
3.2×10^8 = stock solution	10/10	10	0	19	0	19/19	100
$3.2 \times 10^7 = 10^{-1}$	6/10	6	4	9	4	6/9	60
$3.2 \times 10^6 = 10^{-2}$	3/10	3	7	3	7	3/3	27.27
$3.2 \times 10^5 = 10^{-3}$	0/10	0	10	0	11	0/0	0

From these results, LD_{50} value was worked out according to the following formulae worked out by Mamnur Rashid *et al.* (1996).

$$\text{Proportionate} = \frac{\text{\%mortality at dilution next above 50\% - 50\%}}{\text{distance (PD) \% mortality at dilution next above 50\% - \%mortality at dilution next above 50\%}}$$

Dilution factor (DF) = Negative Log of lower dilutions (next above 50% mortality)..... (i)

PD × DF..... (ii)

Log LD_{50} titer = (i) + (ii)

LD_{50} titer = $10^{[(i)+(ii)]}$

Yambot (1998) performed experimental infection of Nile tilapia, *Oreochromis niloticus* with *A. hydrophila* by immersion and mortalities were observed within 96 hrs. His results showed that LC_{50} was achieved at a dose of 1.5×10^6 at 96 h, LC_{100} at 10^8 doses and no mortality at 10^3 dose and control group. Though the injection method was different from the present study, the findings for LC_{50} were similar to the present study. Kumagai *et al.* (2006) observed experimental infection of atypical *A. salmonicida*. They confirmed that an isolate was highly pathogenic not only to marbled sole, *Pleuronectes yokohamae* but also to Japanese flounder *Paralichthys olivaceus* and spotted halibut *Verasper variegatus*, with LD_{50} of less than 10^2 CFU/fish (1.4 - 6.8 g body weight) by intramuscular injection. Itano *et al.* (2006) performed challenge test with *Nocardia seriolae* in yellow tail, *Seriola quinqueradiata*. The LD_{50} values for the infection trials were found to be 1.9×10^2 ml^{-1} (intraperitoneal injection), 1.5×10^4 ml^{-1} (immersion), 4.3×10^6 ml^{-1} (intradermal injection) and 1.7×10^7 ml^{-1} (oral administration).

Yldz and Aydn (2006) isolated *Arcobacter cryaerophilus* from naturally infected rainbow trout (*Oncorhynchus mykiss*) and its pathogenicity was tested by intramuscular injection into 40 healthy one year old rainbow trout at 16°C. The lethal dosage of 50% end point (LD_{50}) for *A. cryaerophilus* was calculated to be 2.25×10^4 ml^{-1} of viable cells. Shen *et al.* (2005) isolated two strains of *Streptococcus iniae* from the kidney of the infected red drum, *Sciaenops ocellatus*. The LD_{50} of these two isolates to red drum was 4.8×10^8 CFU/fish and 1.9×10^7 CFU/fish and in tilapia, 2.8×10^8 CFU/fish and 8.3×10^7 CFU/fish. Here, LD_{50} values of *S. iniae* was higher might be due to its low virulency to red drum fish.

Mekuchi *et al.* (1995) determined LD_{50} of *E. tarda* against *Paralichthys olivaceus* to be 7.1×10^1 CFU/fish by intramuscular injection, 1.7×10^2 CFU/fish by intraperitoneal injection, 3.6×10^6 CFU/ml by immersion and 1.3×10^6 CFU/fish by oral administration. These results showed that the Japanese flounder had high susceptibility to *E. tarda*. Mamnur Rashid *et al.* (1996) conducted an experimental infection of Japanese flounder *P. olivaceus* with *E. tarda* to determine LD_{50} of the bacteria. The calculated values of the LD_{50} were 4.8×10^4 CFU/fish in intraperitoneal method, 2.2×10^6 CFU/fish in oral intubation method and 1.7×10^7 CFU/ml in immersion method. In intraperitoneal method, the LD_{50} value was found to be lower because infection occurred here quickly than oral intubation and immersion methods. LD_{50} value of *E. tarda* for Japanese flounder was sometimes, lower than the LD_{50} value of *A. hydrophila* for shing fish might be due to the virulence of *E. tarda* over *A. hydrophila*.

From this study it was concluded that *A. hydrophila* is a virulent bacterium which causes 50% mortality to shing in a substantially low dose of 9.6×10^6 CFU/fish. The LD_{50} dose will be helpful for further study with this pathogen in shing to understand the pathogenesis in the different organs of fish. Further studies are necessary with the higher dose than this LD_{50} dose to observe the fate of the pathogen in the organs of the fish as well as to study the experimental histopathology of the fish with the pathogen.

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