Progress. Agric. 22(1 & 2): 105 – 114, 2011 ISSN 1017-8139

EXPERIMENTAL INFECTION OF INDIGENOUS CLIMBING PERCH *Anabas testudineus* WITH *Aeromonas hydrophila* BACTERIA

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ABSTRACT

The present study was conducted to know the pathogenicity and LD_{50} of *Aeromonas hydrophila* isolated from diseased climbing perch *Anabas testudineus* against apparently healthy homologous fish and the distribution of the bacteria in the organs of the experimentally infected fish. A total of 10 fish of average body weight of 18 g were used. For pathogenicity test, two different doses *viz.* 9.2×10^7 and 9.2×10^6 CFU/fish were injected intramuscularly. Pathogenicity of *A. hydrophila* was confirmed at water temperature of 28.53°C by the mortality of 40% to 100% of all tested fish within 4 to 9 days. The highest bacterial load was found to be 9.4×10^8 CFU/g in the intestine and the lowest bacterial load was found to be 2.8×10^3 CFU/g in the kidney of the tested fish. Four different serial concentrations, *vide* 9.2×10^7, 9.2×10^6, 9.2×10^5 and 9.2×10^4 CFU/fish of the bacteria were injected in each of four different groups of 10 fish. The calculated LD_{50} value at 27.3°C water temperature was 2×10^7 CFU/fish of 18 g of average body weight. In all the cases of intramuscular injection, external pathology was found. Reddish anal region and fin bases were observed. Injected *A. hydrophila* was re-isolated from liver, kidney and intestine of the challenged fish. It was understood that the isolate was a high virulent pathogen for *A. testudineus*.

Key Words: LD_{50}, *Aeromonas hydrophila, Anabas testudineus*, Pathogenicity

INTRODUCTION

A. hydrophila was frequently observed in various species of diseased farmed and wild freshwater fishes in different locations of Bangladesh (Rahman and Chowdhury, 1996; Sarker *et al*., 2000). It was recognized as a causative agent of ulcer type disease occurred in farmed fishes (Chowdhury, 1998). *A. hydrophila* had been frequently isolated from various lesions of epizootic ulcerative syndrome (EUS) of different fishes (Dooly *et al*., 1986; Torres *et al*., 1990; Roberts *et al*., 1990). Iqbal *et al.* (1998) detected *A. hydrophila, A. veronii sobria* and *A. jandaei* as pathogenic bacteria recovered from EUS affected mrigal. Mamnur Rashid *et al.* (2008) identified *A. hydrophila* from EUS affected shing *Heteropneustes fossilis*. Hasan *et al*. (2008) found histopathological changes in liver and kidney caused by this bacterium in the fish. Mostofa *et al.* (2008) studied experimental pathogenesis of *A. hydrophila* bacteria from the shing fish. Islam *et al.* (2008) studied histopathological changes in experimentally

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infected shing with the same bacteria. *A. hydrophila* is the causative agent of MAS (motile *Aeromonas* septicemia). Both farmed and wild fishes have been found to be affected by this disease. Sabur (2006) isolated and identified five species of *Aeromonas* bacteria in polyculture environment of five carp species namely *Labeo rohita, Cyprinus carpio, Cirrhinas cirrhosus, Catla catla* and *Hypophthalmichthys molitrix*. Lately the bacteria *A. hydrophila* was isolated from Thai pangus *Pangasianodon hypophthalmus* (Siddik, 2009) and from climbing perch *Anabas testudineus* (Sayed, 2010). In the present work, experimental infection was done to know the pathogenicity of *A. hydrophila* in *Anabas testudineus*. The virulence of the pathogen was estimated by experimental studies of the LD_{50} (median lethal dose) of *A. hydrophila* in the climbing perch.

MATERIALS AND METHODS

Experimental fish and set up

Apparently healthy indigenous climbing pearch *Anabas testudineus* (koi) were collected from different fish markets of Mymensingh stocked in cemented cistern and acclimatized for 15 days providing adequate feed and better aeration by circulating water. A Celsius thermometer was set with an aquarium for temperature recording. The infection experiments were conducted at the wet laboratory and fish disease laboratory of the Department of Aquaculture, Bangladesh Agricultural University, Mymensingh. Duration of the experiment was 6 months from July to December 2010. In the Wet laboratory, a recycle system was set with having 150 l capacity drums, filled with coconut straw for filtering of bacteria. Twelve aquaria of fiber glass each having 40 l capacity, an over head tank and an ultraviolet tube light complex chamber for disinfection had the access to an electric pump. The recycle system was filled with both pond and supply water. Prior to the experiment the water was kept under circulation for 7 days.

Characterization of A. hydrophila

A. hydrophila, isolated from diseased indigenous climbing pearch *Anabas testudineus* (koi) (Sayed, 2010) were sub-cultured and morphological, biochemical as well as physiological characters were verified.

Pathogenicity experiment

For the pathogenicity experiment, intramuscular injection method was used to know the efficacy of the method in initiation of infection and pathogenicity of the pathogen. One ml insulin syringes (sterile and disposable) were used for the injection. For the intramuscular (IM) injection, a total of 10 fish were injected intramuscularly with 0.1 ml of each of two sired bacterial doses (9.2×10^7 and 9.2×10^6 CFU/fish) just below the anterior part of the dorsal fin after disinfecting with 70% alcohol cotton. The base of the dorsal fin was selected as the most suitable place of injection because it contains the target tissue 'deep muscle'. The above two groups of 10 fish were realised in two separate aquaria and were observed upto 15 days of the experimental period for any abnormal clinical appearances and were recorded properly. Water recirculation and aeration were given continuously

during the study period and no feed was supplied. Water temperature was 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. Intestine, liver, and kidney of each dead fish were dissected out aseptically, homogenized with 1:10 volume of sterile physiological saline (0.87% NaCl = PS), 100 µl of each organ was spreaded onto TSA plats, incubated and the appeared colonies were counted to express the fate of the bacteria in the organ of the experimentally infected fish with the following formula: Bacterial CFU/g of fish organ = No. of colonies counted in a plate × 10^n ×100 Where, n is the dilution factor.

Median lethal dose (LD50) experiment

An amount of 10 mg of fresh culture of the bacteria was carefully scraped and mixed with 1 ml PS and desired dilutions were prepared by serial decimal dilution method. In a preliminary test the above stock dilution (10 mg in 1 ml) was calculated to contain around 10^7 CFU/ml. Four serial dilutions having an estimated concentration of 10^7, 10^6, 10^5 and 10^4 CFU/ml were used for the (LD_{50}) experiment. From each of the above 4 dilutions, 0.1 ml bacterial suspension was injected intramuscularly to each of previously stocked and acclimatized 10 fish making a group. Each group was then released in one aquarium properly labeled to understand the dose. The injected fish were observed up to 15 days. No feed was given to the experimental fish and water temperature was recorded twice daily. Immediately after death, each fish was transferred to laboratory, kidney was dissected out, touched with a sterilized loop and streaked onto TSA plates. The plates were incubated at 25°C for 48 hours for *A. hydrophila* colony appearance. From the mortality record, LD_{50} value was worked out according to the following formula:

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

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

Results of morphological, biochemical and physiological characters of *A. hydrophila* compared with the characters shown by Popoff *et al.* (1984) are shown in Table 1.

Pathogenicity of A. hydrophila

During the experimental period of pathogenicity test the average water temperature was 28.53°C. Intramuscular method resulted in 100% mortality at a dose of 9.2 × 10^7 CFU/fish and 40% mortality at a dose of 9.2 × 10^6 CFU/fish of the experimental fishes. Kidney

streaking from all dead fish gave rise to the growth of *A. hydrophila* and thus the isolates were proved to be pathogenic. No fish died from the control group of the experimental fish. Results of pathogenicity tests are shown in Table 2. *Anabas testudineus* was proved to be sensitive to *A. hydrophila* as shown by their mortality upto 100%, at a dose of 9.2×10^7 CFU/fish and 40%, at a dose of 9.2×10^6 CFU/fish. Post infection days of mortality were observed to be from 2 to 5 days and 4 to 9 days respectively.

Table 1. Characters of *A. hydrophila* isolates in comparison to those of Popoff *et al.* (1984)

Characters	Characterization by Popoff *et al.* (1984)	Present results	Characters	Characterization by Popoff *et al.* (1984)	Present results
Gram stain	–[1]	–	Esculin hydrolysis	ND	+
Shape	Rod	Rod	Methyl-red test	–	–
Motility	+[2]	+	Voges-Proskaur	+	+
Sensitivity to 0129	ND[3]	-	Indole	+	+
Oxidase	+	+	H_2S production	+	+
Catalase	+		Arginine decomposition	+	+
OF test	F[4]	F	Lysine decarboxilation	-	-
Acid and gas production from glucose	+	+	Ornithine decarboxilation	-	-
Acid production from			Citrate utilization	+	+
Lactose	+	+	Growth at: 4°C	-	-
Sucrose	+	+	5°C	+	+
Maltose	+	+	37°C	+	+
Manitol	+	-	40°C	-	-
Insitol	-	-	Salt tolerances (NaCl): ≤3%	+	+
Sorbitol	-	-	4%	-	-

[1]: Negative, [2]: Positive, [3]: Not Done, [4]: Fermentative

Table 2. Results of pathogenicity test of *Aeromonas hydrophila* in experimental fish by intramuscular injection method (five fish were infected with each dose of the bacteria)

Injected with	Dose (CFU/fis)	Average weight of fish (g)	No. of fish died	Mortality (%)	Post infection days of mortality
A .testudineus	9.2×10^7		5	100	2-5
	9.2×10^6	18	2	40	4-9
Control (PS)	0.1 ml	18	0	0	0

The present study was carried out to understand the pathogenicity of *A. hydrophila* and median lethal dose (LD_{50}) in experimentally infected climbing perch *Anabas testudineus*. The average body weight of the experimental fish was 18g. During the experimental period of pathogenicity test and LD_{50} the average water temperature was 28.53°C and 27.3°C respectively. Akhlaghi and Vafaie (2002) isolated pathogenic *A. hydrophila* from diseased frog-eyed fish (*Carassius* sp.) at 20°C. Mostafa (2007) calculated LD_{50} of *A. hydrophila* in *Heteropneustes fossilis* at 28°C. In the present pathogenicity test, the bacterial isolates were proved to be highly invasive. Pathogenicity of *A. hydrophila* was measured intramuscularly at 28.53°C with two different doses of 9.2×10^7 CFU/fish and 9.2×10^6 CFU/fish and showed mortality to 100% and 40% within 2 to 5 days and 4 to 9 days in *Anabas testudineus* of 18g.

Experimental infection by *A. hydrophila* of the fish showed that the fish were seriously affected which caused mortality. Thus it was proved that *A. hydrophila* was pathogenic to *A. testudineus*, causing 100% mortality by a suspension of the bacterial cells of 9.2×10^7 CFU/fish and 40% mortality by 9.2×10^6 CFU/fish. Sarker (2009) conducted experimental infections in carps (rui, catla, and mrigal), perch, and catfishes (shing and magur). The author found that by intramuscular injection method 100% of *Labeo rohita* died at a dose of 6.7×10^6 CFU/fish and 80%, at a dose of 6.7×10^5 CFU/fish. *Cirrhinus cirrhosus* showed their mortality as 100% at a dose of 6.7×10^6 CFU/fish and 60%, at a dose of 6.7×10^5 CFU/fish. It caused 100% mortality in *Heteropneustes fossilis* at a dose of 6.7×10^6 CFU/fish and 80%, at a dose of 6.7×10^5 CFU/fish. Same type of experiment was conducted by Angka (1990). The author found that *A. hydrophila,* injected intraperitonealy, was pathogenic to *Clarias batrachus* fingerlings, causing 93% mortality in fish infected with 10^7 CFU/ml. At lower dosage mortalities were significantly lower. Islam (2006) found 85% mortality of shing by 6.4×10^7 CFU/fish of *A. hydrophila.* Mostafa *et al.* (2008) found 100% mortality of *Heteropneustes fossilis* with 9.6×10^7 CFU/fish of *A. hydrophila.* Sabur (2006) observed that *A. hydrophila* was found to be pathogenic for both indigenous (rui, *Labeo rohita*, catla, *Catla catla* and mrigal, *Cirrhinus cirrhosus*) and exotic carps (silver carp, *Hypophthalmichthys molitrix* and common carp, *Cyprinus carpio*). The author observed that intramuscular method was found to be the most effective method that resulted 80 to 100% mortality at a dose of 2×10^6 CFU/fish and 60 to 80% mortality at a dose of 2×10^5 CFU/fish for three indigenous and two exotic carp species within 2 to 12 days. Yambot (1998) performed experimental infection of Nile tilapia *Oreochromis niloticus* with *A. hydrophila* by immersion method at a dose of 1.5×10^6 CFU/fish and 100% mortalities were observed within 96h.

Clinical and gross pathology

In moribund condition of each group of intramuscularly injected fish, abnormal movement and loss of balance were observed. Clinical external pathologies were also evident. The posterior end of the body surface was found to develop grayish-white lesion that was extended up to caudal fin. Anal region and the fin bases developed red color. After dissection of the freshly dead fish, the liver was observed to be swollen, unsmooth, and uneven and turned blackish. Ahmed *et al*. (2006) reported scale loss, rough skin

haemorrhagic lesions and reddish spots in naturally infected exotic carp *Barbodes gonionotus*. Akter *et al*. (2006) observed red sports subcutaneus lesions and rough skin in small indigenous fishes as clinical features of natural disease. Ahmed *et al*. (2007) found that naturally infected Thai Koi, *Anabas testudineus* showed scale loss, dermal lesion, ulcer and loss of caudal fin. Mamnur Rashid *et al*. (2008) observed pale body colour and fin loss in EUS affected stinging catfish *Heteropneustes fossilis*. In an experimental pathogenesis of *Aeromonas hydrophila* in shing Mostofa *et al*. (2008) experienced haemorrhagic lesions at the injection site, hyperemic anal region and fin bases and grayish white lesion on the caudal area of the experimental fish.

Fate of A. hydrophila bacteria in the tissues of experimental fish

A. hydrophila could be isolated from liver, kidney and intestine of experimentally infected fish. The results are shown in Table 3. In case of intramuscular injection, the highest bacterial load was found to be 9.4×10^8 CFU/g in the intestine and the lowest, 2.8×10^3 CFU/g in the kidney.

Table 3. Fate of *Aeromonas hydrophila* in liver, kidney and intestine of experimentally infected *Anabas testudineus* injected by intramuscular injection at a dose of 9.2×10^7 CFU/fish and 9.2×10^6 CFU/fish

Species of fish	Fish No.	Bacterial colony count		
		Liver	Intestine	Kidney
Anabas testudineus	F_1	6.3×10^4	4.9×10^5	3.6×10^4
	F_2	3.2×10^5	2.8×10^6	3.3×10^5
	F_3	5.6×10^7	3.7×10^6	2.8×10^4
	F_4	2.9×10^6	**9.4×10^8**	4.9×10^5
	F_5	2.3×10^4	3.8×10^5	**2.8×10^3**

In the case of intramuscular injection, the highest bacterial load were found to be 9.4×10^8 CFU/g in the intestine and the lowest bacterial load was found to be 2.8×10^3 CFU/g in the kidney. Sarkar (2009) found that in case of intramuscular injection, the highest bacterial load in carps were 4.9×10^9 CFU/g in the liver of catla, 7.7×10^8 CFU/g in the intestine of rui and 5.8×10^8 CFU/g in the intestine of mrigal and the lowest bacterial load was found to be 2.7×10^4 CFU/g in the kidney of catla, 3.0×10^4 CFU/g in the kidney of rui, 5.6×10^3 CFU/g in the kidney of mrigal. In the present experiment, the highest and lowest bacterial load in perch (koi) was found to be 6.4×10^7 CFU/g in the intestine and 1.6×10^2 CFU/g in the kidney. The highest bacterial load in catfishes were found to be 5.5×10^8 CFU/g in the liver of shing and 5.6×10^7 CFU/g in the intestine of magur and the lowest bacterial load was found to be 2.2×10^2 CFU/g in the kidney of shing, and 2.4×10^3 CFU/g in the liver of magur. Mamnur Rashid *et al.* (2008) observed the highest and the lowest loads of *A. hydrophila* in liver, intestine and kidney to be 6.46×10^8 CFU/g, 1.18×10^9 CFU/g and 3.70×10^8 CFU/g and 1.67×10^4 CFU/g, 1.71×10^3 CFU/g and 1.47×10^4 CFU/g in the natural EUS affected shing *Heteropneustes fossilis* respectively. Mostofa *et al.* (2008) conducted infection experiment of shing *Heteropneustes fossilis* with 10^5 and 10^8 CFU/fish of *A. hydrophila* and found the highest bacterial load in the kidney, intestine and liver of the experimentally infected fish to be 1.3×10^7 CFU/g, 3.5×10^6 CFU/g and $2.42 \times$

10^7 CFU/g and the lowest bacterial load to be 2.1 × 10^2 CFU/g, 9.0 × 10^3 CFU/g and 2.0 × 10^4 CFU/g respectively. Roshid (2009) performed experimental infection in pangus *Pangasianodon hypophthalmus* with *A. hydrophila* at different doses of 5.07 × 10^5 CFU/fish (intramuscular injection), 4.1 × 10^5 CFU/fish (intraperitoneal injection) and 2.7 × 10^5 CFU/fish (oral intubation) and the highest bacterial load was found to be 2.8 × 10^7 CFU/g, 2.9 × 10^6 CFU/g and 7.7 × 10^6 CFU/g in the liver, 4.3 × 10^6 CFU/g, 2.1 × 10^6 CFU/g and 4.7 × 10^6 CFU/g in the intestine and 2.8 × 10^6 CFU/g, 3.3 × 10^6 CFU/g and 4.4× 10^6 CFU/g in the kidney and the lowest bacterial load was found to be 3.7 × 10^4 CFU/g, 2.9 × 10^4 CFU/g and 2.5 × 10^4 CFU/g in the liver, 5.0 × 10^3 CFU/g, 2.3 × 10^4 CFU/g and 2.3× 10^4 CFU/g in the intestine and 2.9 × 10^2 CFU/g, 2.5 × 10^4 CFU/g and 2.4× 10^5 CFU/g in the kidney respectively. In the LD_{50} experiment and in the pathogenicity test the average water temperatures were 27.3°C and 28.53°C respectively, which were favorable for the infection experiments. Sarker *et al.* (2000) conducted water borne infection method to infect *Puntius gonionotus* with *A. hydrophila* isolates at 30°C. All the isolates were found to have the highest pathogenicity for fish at 25°C. Akhlaghi and Vafaie (2002) used previously isolated pathogenic *A. hydrophila* for its pathogenicity tests to frog-eyed, red rukin and moor fish (all *Carassius* spp.). Experiments were conducted at temperatures of 20°C and 28°C; the highest pathogenicity was showed at 28°C and the lowest pathogenicity at 20°C. Sarker (2009) conducted experimental infections in carps, perch, and catfishes where average temperature for the pathogenicity test and LD_{50} were 29°C and 30°C respectively.

Median lethal dose (LD_{50})

Results of LD_{50} test are presented in Table 4. All the fish died with 9.2 × 10^7 CFU *A. hydrophila* bacteria/fish within 2 days. With the dose of 9.2 × 10^6 CFU/fish, 5 fish died out of 10. Among them three fish died at the day of injection, one fish died at 2nd day, one fish died at 7th day of injection. In the case of 9.2 × 10^5 CFU/fish, 2 fish died out of 10. Among them one fish died at 3rd day and another fish died at 10th day. In case of 9.2 × 10^4 CFU/fish, kidney streaking and incubation from each dead fish gave rise to the appearance of pure colonies of *A. hydrophila*.

Table 4. Formulated data from the mortalities in the experimental infection of *Anabas testudineus* with *Aeromonas hydrophila* by intramuscular injection for the calculation of LD_{50}

Pathogen dilution	Mortality ratio	Mortalities	Survivors	Accumulated values			
				Total dead	Total survived	Mortality	
						Ratio	Percent
9.2× 10^7	10/10	10	0	10	0	10/10	100
9.2 × 10^6	5/10	5	5	15	5	15/20	75
9.2× 10^5	2/10	2	8	17	13	17/30	56.66
9.2× 10^4	0/10	0	10	17	23	17/40	42.5

The value of LD_{50} of *A. hydrophila* was found to be 2 × 10^7 CFU/fish by intramuscular injection where average body weight was 18 g at an average temperature of 27.3°C calculated from the mortality report of Table 4.

Calculation of LD_{50} for koi is given below:

$$\text{Proportionate distance (PD)} = \frac{5 - 2}{10 - 2} = 0.375$$

Dilution factor (DF) = 7
PD×DF = 0.375 × log7 = 0.312
Log LD_{50} = 0.312 +7 = 7.312
$LD_{50} = 10^{6.584} = 2 \times 10^7$

The calculated value of LD_{50} by intramuscular injection of *A. hydrophila* at an average water temperature of 27.3°C was 2×10^7 CFU/fish of 18 g of average body weight. Sarker (2009) conducted experimental infections in carps (rui, catla, and mrigal), climbing perch (*Anabas testudineus*) and catfishes (shing and magur) and calculated value of LD_{50} by intramuscular injection of *A. hydrophila* at an average water temperature of 29°C was 1.8×10^6 CFU/fish for *Catla catla* of 25.7g, 3.3×10^6 CFU/fish for *Labeo rohita* of 35.2g, 1.6×10^6 CFU/fish for *Cirrhinus cirrhosus* of 30.5g, 2.5×10^6 CFU/fish for *Heteropneustes fossilis* of 20.4g, 2.9×10^6 CFU/fish for *Clarias batrachus* of 25.6g and 3.8×10^6 CFU/fish for *Anabas testudineus*. Shen *et al*. (2001) isolated *A. hydrophila* from liver and kidney of rice field eel *Monopterus albus* and determined LD_{50} of the three isolates of the bacteria as 2.84×10^6 CFU/fish, 6.12×10^6 CFU/fish and 2.13×10^6 CFU/fish. Islam (2006) determined the LD_{50} value to be 6.4×10^6 CFU/ fish of *A. hydrophila* against *Heteropneustes fossilis* of 35g average body weight at an average water temperature of 27°C by intramuscular injection method. Mostafa (2007) calculated the value of LD_{50} of *A. hydrophila* to be 9.6×10^6 CFU/fish by intraperitoneal injection against *Heteropneustes fossilis* of 35g average body weight at an average water temperature of 28°C.

The study proved that *A. hydrophila,* though oppurtunistic, was a serious pathogen for koi. It was also proved that the pathogenesis of the pathogen was very active at least in liver, kidney and intestine of the experimental fish, investigated. As a ubiquitous species, *A. hydrophila* are available in water, fish body, and other aquatic animals and even in their feed. From the above discussion it is clear that the pathogen might be an important disease causing agent of fishes in Bangladesh aquaculture. Generally *A. hydrophila* are found to cause disease in fishes associated with fungus, *Aphanomyces invadans* to produce EUS (Hasan, 2007). As a bacterial pathogen, it is causing severe losses of fish by decreasing fish production and ultimately hampering the national economy. It has been isolated from lesions of almost all infectious diseases. So, proper preventive as well as curative measures should be taken for the reduction of the disease conditions caused.

CONCLUSIONS

It was confirmed that *A. hydrophila* bacteria was found to be a serious pathogen for the climbing perch as was also found for carps, catfishes, eels and snakeheads. Pathogenesis

of *A. hydrophila* in the liver, kidney and intestine was very active. Further researches are necessary to prepare antibody against this bacteria, to serotype all Bangladesh isolates, to prepare whole vaccines and purified vaccines and to try vaccination in susceptible fishes to save our fish folks against this pathogen.

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