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Article

Evaluation of pathogenicity of motile *Aeromonas* species in air-breathing catfish Magur (*Clarias batrachus*)

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Abstract: The present study was carried out to evaluate the comparative capability of producing infections and causing mortality of the experimental Magur (*Clarias batrachus*) with motile *Aeromonas* species. A total of 200 apparently healthy *C. batrachus* were acclimatized to the laboratory conditions for experimental study. Nine different groups (each group consisting of 20 fish) of healthy *C. batrachus* was injected with nine motile *Aeromonas* isolates (*A. hydrophila*-3, *A. sobria*-3 and *A. caviae*-3). Experimental *C. batrachus* were infected with motile *A. hydrophila*, *A. sobria* and *A. caviae* to groups 1-3, 4-6 and 7-9, respectively while group 10 was injected with sterile physiological saline (0.85% NaCl) and served as the control. The selected motile bacterial species via intramuscularly were injected at the rate of 4.5×10^5 cfu/fish for pathogenicity study on *C. batrachus* and monitored up to two weeks. The highest clinical infections were noticed 90% in group-3 whereas only 35% in group-8 within the experimental period. After two weeks of the experiment, the cumulative mortality rate was also found highest (60%) in group-3 but lowest (15%) in group-9 while no infection or mortality showed in group-10 (control group). The development of infection and mortality to the injected *C. batrachus* was associated more severely by *Aeromonas hydrophila* than *A. sobria* and *A. caviae* used in this study. However, the isolates motile *Aeromonas* species could serve as the primary cause of skin lesions as well as mortality in cultured *C. batrachus*.

Keywords: motile *Aeromonas* species; Magur (*Clarias batrachus*); pathogenicity; artificial infected

1. Introduction

Among the different air-breathing catfishes, Magur (*Clarias batrachus*) is very popular and highly valuable fish species in Bangladesh. It is not only recognized for its delicious taste and market value but it is also considered as a medicinal fish and traditionally remained a strike among the pregnant & lactating mothers, the elderly and children. It is prescribed prophylactically to the anemic & malnourished individuals as well as for the convalescent of the patients due to the nutritional superiority (Debnath, 2011). It is a very hardy fish that can survive for quite a few hours outside the water due to presence of accessory respiratory organs. *C. batrachus* was abundantly available in open water of Bangladesh but presently, it is threatened due to over exploitation and various ecological changes in its natural habitat. Although, the appropriate breeding, nursing and rearing technology of fry and fingerlings of *C. batrachus* had been developed by Bangladesh Fisheries Research Institute (BFRI) in few years ago but various diseases of this fish causes huge economic losses because of their high mortality under farming conditions.

Catfish like Shing (*H. fossilis*), Magur (*Clarias batrachus*) and Pangasius (*Pangasianodon hypophthalmus*) are teleosts having entire body surfaces, fins and barbells those covered with skin composed with non-keratinized stratified squamous epithelial cells (Zhao *et al*., 2008; Esteban, 2012; Monir *et al*. 2016). Actually fish skin plays an important role with the environment to maintain homeostatic conditions including protection from external environment and antimicrobial activity (Bordas, 1996; Esteban, 2012). It provides the first attachment site for a wide range of microorganisms in aquatic environment (Bordas, 1996; Esteban, 2012). As a result, the attachment of microorganisms in skin frequently cause lesions and rupture which make possible for the pathogens to invade and multiply into the body. Thus, skin lesions and rupture causes harmful effect on normal growth and reproduction of the fishes as well as mass mortality (Monir *et al.,* 2015).

Intensification of aquaculture has increased the various disease outbreaks in different cultured fish in the past few decades. The motile Aeromonad species by far the most common among the bacterial diseases of freshwater fish that has been associated with diffrent species of the *Aeromonas* such as *A. hydrophila*, *A. caviae*, *A. veronii*, *A. schuberti*, *A. salmonicida* and *A. sobria* etc. However, *A. hydrophila* were documented as causative agents for large scale mortality in fish (Wahli *et al*., 2005). Additionally, the pathogenesis of fish diseases or disease outbreak can be medium to high in fishes (Yardimci and Aydin, 2011; Kumar *et al*., 2016) but all types of disease results in economic losses. The acute form of the disease may result in fatal sepsis without any symptoms (Yardimci and Aydin, 2011) while chronic infections may show the symptoms like hemorrhagic septicaemia with ulceration, inflammation, and dermal lesions (Cipriano *et al*., 2001). Despite the knowledge of severity and symptoms of the Aeromonad septicaemia in other species, factors such as host-pathogen interaction, temperature requirement of the bacteria, course of pathogenesis, and immunity to pathogenesis may vary for fish species (Wu *et al*., 2007). So far a very few experimental works were conducted on bacterial diseases especially on pathogenicity of *Aeromonas* species in Magur (*C. batrachus*). Therefore, the present study was carried out to evaluate the comparative pathogenicity of motile *Aeromonas hydrophila*, *A. sobria* and *A. caviae* in air-breathing catfish Magur (*C. batrachus*) of Bangladesh.

2. Materials and Methods

2.1. Experimental fish and set up

A total of 200 healthy Magur (*C. batrachus*) weighting 80-90 g were collected from local commercial fish farms of Mymensingh district located in 24°38′3′′N 90°16′4′′E of Bangladesh during this experiment. Prior to the artificial infection by selected motile *Aeromonas* spp., the collected fish were kept in aquariums to acclimatize in laboratory conditions from 28 to 30°C for at least 7 days providing adequate feed and better aeration by circulating water. The pathogenicity test was conducted at the Fish Disease and Health Management Laboratory of Bangladesh Fisheries Research Institute, Mymensingh.

2.2. Collection of motile *Aeromonas* species

Motile *Aeromonas hydrophila, A. sobria* and *A. caviae* were isolated from infected Shing (*Heteropneustes fossilis*) showing severe disease symptom of erosions at the bases of fins and tail, hemorrhages and skin lesions on body surface (Monir *et al*., 2015; Monir *et al*., 2016).

2.3. Bacterial culture and preparation of suspension

The **c**ollected stocks of *A. hydrophila*, *A. sobria* and *A. caviae* were grown on Typtic Soya Agar (TSA) at 30 °C for 24 hours and identity confirmed using different biochemical characteristics prior to the experiment. The bacterial suspensions were prepared with 0.85% NaCl (physiological saline) that resulted in a concentration of 4.5 to 5.6 x 10^5 cfu/ml.

2.4. Experimental infection

For the purpose of this study, intramuscular injection method was used for the experimental infection to know the efficacy of the selected motile *Aeromonas* spp. in initiating the infection as well as observe mortality. After acclimatization for 7 days, 80-90 g, apparently healthy Magur (*C. batrachus*) were randomly assigned to 10 groups as 20 fish per group. For the intramuscular (IM) injection, one ml insulin syringes (sterile and disposable) were used to inject intramuscularly with 0.1 ml of pre-selected (Ahmed, 2009) bacterial dose (4.5×10^5 cfu/fish) as follows: groups 1 to 3 - *A. hydrophila*, groups 4 to 6 - *A. sobria*, while groups 7 to 9 were infected with *A. caviae*. A negative control group-10 of 20 fish were injected with sterile physiological saline as above Monir *et al*., 2015). Prior to the experiment, one day before, feeding was stopped. During experiment fish were fed with commercial feed once in a day. Water was exchanged thrice in a week and residual feed was removed every two days by siphoning. The temperature, pH and dissolved oxygen, ammonia concentration of the water was kept at acceptable limit during the experiment (Sofiq *et al*., 2013). The injected fishes were then observed clinical signs, symptoms and mortalities daily up to 14 days.

2.5. Re-isolation of challenged pathogens

Re-isolation of inoculated bacteria were carried out by collecting samples from skin lesions, kidney, liver of moribund and freshly dead or sacrificed experimental infected Magur (*C. batrachus*) fish and grown on TSA plates to check the presence and absence of bacterium. Positive bacterial culture was confirmed by the morphological and biochemical characteristics of the re-isolated bacteria were identical with those of the isolates used in the experimental infection.

2.6. Statistical analysis

The data collected for rates of developing infection and mortality were subjected to descriptive statistics and expressed in percentages.

3. Results

3.1. Clinical and gross pathology of experimental Magur (*C. batrachus*)

It was observed that after one day post-infection of intramuscularly injected all Magur (*C. batrachus*) groups expressed abnormal movement, loss of balance and constant rubbing of body with the aquaria glass except control groups. By day 4 post-infection, most of the fishes were noticed to develop hemorrhages at the base and tips of the fins. The liver, spleen and kidney of the infected freshly dead fish were observed to be hemorrhage, enlarged, unsmooth, and turned slide blackish. After day 6 post-infection, severally diffused hemorrhage was observed on fin bases, edge of head and body surface in groups 1, 2, 3 (Figure 1). In some fishes, hyperaemic patches of the fins were also observed in these groups. The hemorrhagic ulcerative lesions, body, fins and tail erosions were noticed in groups 4, 5, 6, 7, 8, 9 (Figure 2) after day 6 post-infection. But corrosion of the barbells and severe hemorrhagic ulcerative lesions on the caudal area were also observed in most of the fishes especially in groups 7, 8, 9 (Figure 3). However, bacteria showed clinical signs in natural infection were found to be more or less similar in the injected experimental fish. But, no clinical signs and mortality were observed in the control group 10.

Figure 1. Hemorrhages in fins bases, edge of head and skin lesions in Magur (*C. batrachus*) experimentally-infected with *Aeromonas* species.

Figure 2. Hemorrhagic ulcerative lesions on body in Magur (*C. batrachus*) experimentally-infected with *Aeromonas* species.

Figure 3. Corrosion skin lesions on caudal region in Magur (*C. batrachus*) experimentally-infected with *Aeromonas* species.

3.2. Effect of the selected *Aeromonas* species to develop infections in experimental Magur (*C. batrachus*)

After 14 days of the experiment, the highest clinical infection in the challenged fishes showed up to 90% in groups-3 but lowest 35% in group-8. Additionally, seventy percent of the challenged fish in group-4 had developed infection and only 40% in groups-9. However, the highest average infection was found 82% among the groups-1, 2 and 3 where *Aeromonas hydrophila* was used but the lowest average infection was noticed 42% in group-7, 8 and 9 during the experimental period where *Aeromonas caviae* was used (Table 1).

Table 1. Cumulative progression of infection in experimental Magur (*C. batarchus*) infected with *Aeromonas* species.

Group	Infected bacteria	Number of healthy Magur used per group	Development of infections at days after injection														Percentage
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	
1		20	0	0	3	7	9	13	15	16	16	16	16	16	16	16	80
2	*A.*	20	0	0	3	8	10	14	14	15	15	15	15	15	15	15	75
3	*hydrophila*	20	0	0	4	9	12	15	16	18	18	18	18	18	18	18	90
		Average	**0**	**0**	**3**	**8**	**10**	**14**	**15**	**16**	**16**	**16**	**16**	**16**	**16**	**16**	**82**
4		20	0	0	2	5	7	9	12	14	14	14	14	14	14	14	70
5	*A. sobria*	20	0	0	1	3	5	8	10	11	11	11	11	11	11	11	55
6		20	0	0	1	3	3	6	8	12	12	12	12	12	12	12	60
		Average	**0**	**0**	**1**	**4**	**5**	**7**	**10**	**12**	**12**	**12**	**12**	**12**	**12**	**12**	**62**
7		20	0	0	0	2	3	5	6	9	10	10	10	10	10	10	50
8	*A. caviae*	20	0	0	0	0	2	4	5	7	7	7	7	7	7	7	35
9		20	0	0	0	1	3	4	4	6	8	8	8	8	8	8	40
		Average	**0**	**0**	**0**	**1**	**2**	**4**	**5**	**7**	**8**	**8**	**8**	**8**	**8**	**8**	**42**
10	Control	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 2. Cumulative progression of mortality rate in experimental Magur (*C. batrachus*) infected with *Aeromonas* species.

Group	Infected bacteria	Number of healthy Magur used per group	Mortality rate at days after injection														Percentage
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	
1		20	0	0	0	2	4	6	7	8	9	9	9	9	9	9	45
2	*A. hydrophila*	20	0	0	0	1	3	5	6	7	7	7	7	7	7	7	35
3		20	0	0	2	3	4	7	8	10	12	12	12	12	12	12	60
		Average	**0**	**0**	**1**	**2**	**3**	**4**	**6**	**7**	**8**	**9**	**9**	**9**	**9**	**9**	**47**
4		20	0	0	0	1	3	5	6	7	7	7	7	7	7	7	35
5	*A. sobria*	20	0	0	0	1	2	4	4	5	6	6	6	6	6	6	30
6		20	0	0	0	0	2	3	3	4	4	4	4	4	4	4	20
		Average	**0**	**0**	**0**	**1**	**2**	**4**	**4**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**28**
7		20	0	0	0	0	1	2	2	3	3	4	4	4	4	4	20
8	*A. caviae*	20	0	0	0	0	1	2	2	2	4	4	4	4	4	4	20
9		20	0	0	0	0	0	1	1	2	2	3	3	3	3	3	15
		Average	**0**	**0**	**0**	**0**	**1**	**2**	**2**	**2**	**3**	**3**	**3**	**3**	**3**	**3**	**18**
10	Control	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

3.3. Effect of the selected *Aeromonas* species on mortality of experimental Magur (*C. batrachus*)

At the end of the experiment (after 14 days), a total of 2 severely infected fish were died in group-3 after 3 days of post infection whereas no fish was died in other groups. The cumulative mortality rate was recorded highest (60%) in group-3 but the lowest (15%) in group-9. However, the highest average mortality rate was found 47% among the groups-1, 2 and 3 where *Aeromonas hydrophila* was used but the lowest average mortality rate was noticed 18% in group-7, 8 and 9 during the experimental period where *Aeromonas caviae* was used (Table 2).

4. Discussion

In the present study, intramuscular injected all Magur (*C. batrachus*) groups expressed abnormal movement, loss of balance and constant rubbing of body with the aquaria glass and develop hemorrhages at the base and tips of the fins. The internal organs such as liver, spleen and kidney of the infected freshly dead fish were observed to be hemorrhage, enlarged, unsmooth, and turned slide blackish. The intramuscular inoculation of motile *Aeromonas* spp. produced similar signs and symptoms, reported during the disease progression in other fish species (Rashid *et al*., 2008; Kumar *et al*., 2016). Khalil and Mansour (1997) reported that clinical signs including weakness, slower movement, swimming closer to the surface, fin hemorrhages and red patches at the gut region were observed in the challenge fish with *Aeromonas* spp. Yarmidici and Aydin (2011) noticed body rubbing against tank walls in Nile tilapia infected with *A. hydrophila* isolate after 8 hours of infection. However, similar observations were also documented by Borty *et al*. (2016); Monir *et al.* (2015).

The average highest percentage (82%) of infection in challenge fish were found among the groups-1, 2 and 3 indicates that *A. hydrophila* species used to infect the groups might be more pathogenic than the other used *Aeromonas* species in this experiment. Paniagua (1990), Janda (2010); Daood (2012) were observed that *A. hydrophila* is more pathogenic and the most frequently isolated species from naturally-infected fish than other *Aeromonas* species. The average percentage of infection in *A sobria* groups (28%) was higher than the *A. caviae* groups (15%) that suggests *A sobria* was more pathogenic compared to *A. caviae* in this experiment. However, these findings are in agreement with those reported by Daood (2012) and Monir *et al*. (2015).

Pathogenicity of *A. hydrophila* to Magur (*C. batrachus*) by intramuscular injection was ranged from 35-60% mortality among the groups-1, 2 and 3. This mortality rate was higher than that recorded in the groups of *A. sobria*-injected (20-35%) and *A. salmonicida*-injected (15-20%) groups. This result indicates that the *A. hydrophila* strains used in this experiment were more pathogenic than the other Aeromonas species. Kumer *et al*. (2016) reported that the LD_{50} value of 1.74×10^5 cfu per 100 g of body weight was standardized for *A. hydrophila* in Golden Mahseer. Sarkar and Rashid (2002) noticed that 100% and 60-80% mortality in Shing (*H. fossilis)* and Magur (*C. batrachus*), Carps and Thai koi (*Anabas testudineus*) injected with $6.7x10^7$ and $6.7x10^6$ cfu/ml of *A. hydrophila,* successively. Mostafa *et al.* (2008) carried out an experimental infection of Shing (*H. fossilis*) with *A. hydrophila* by two different methods viz. intraperitoneal and intramuscular injection at a dose of 9.6×10^7 cfu/fish that caused in 100% mortality of the tested fish within 1-9 days. Additionally, Monir *et al*. (2015) found 100% mortality by 14 days of injection when Shing (*H. fossilis*) was challenged with $6.7 \text{ x } 10^5$ cfu/fish of *A. hydrophila* isolate, successively. However, the infection and mortality rates were found variation in this study than other studies might be due to different species of fish used in experiment, immunity of the fish, various strains of *Aeromonas* species, environmental factors, used different doses of the infective pathogens, route of administration as well as experimental duration.

5. Conclusions

Motile *Aeromonas* species used in this study those were capable to develop infections as well as cause mortality in challenge Magur (*C. batrachus*). In the experiment, challenge through motile *Aeromonas* species will serve as a baseline data for further studies on Magur (*C. batrachus*). Also, the knowledge of various symptoms during pathogenesis help to understand the course of pathogenesis and overall immune response of fishes which can be very beneficial to control and prevent the disease outbreaks in farmed fishes. Further researches are necessary to prepare antibody against these bacteria strains to prepare vaccines and to try vaccination in susceptible to save catfishes against these pathogenic bacteria.

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Conflict of interest
None to declare.

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