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Experimental infection of *Aeromonas hydrophila* in pangasius

J Sarker[1], MAR Faruk*

Department of Aquaculture, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh, [1]Department of Aquaculture, Chittagong Veterinary and Animal Sciences University, Khulshi, Chittagong, Bangladesh

Abstract

Experimental infections of *Aeromonas hydrophila* in juvenile pangasius (*Pangasianodon hypophthalmus*) were studied. Five different challenge routes included intraperitoneal (IP) injection, intramuscular (IM) injection, oral administration, bath and agar implantation were used with different preparations of the bacteria to infect fish. The challenge experiments were continued for 15 days. A challenge dose of 4.6×10^6 colony forming unit (cfu) $fish^{-1}$ was used for IP and IM injection and oral administration method. Generally, IP route was found more effective for infecting and reproducing clinical signs in fish that caused 100% mortality at the end of challenge. IM injection, oral and bath administration routes were also found effective for infecting and reproducing the clinical signs in fish to some extent. Agar implantation with fresh colonies of bacteria also caused 100% mortality of challenged fish very quickly with no visible clinical signs in fish. The major clinical signs of challenged fish included reddening around eyes and mouth, bilateral exophthalmia, hemorrhage and ulceration at fin bases and fin erosion.

Key words: Experimental infection, *Aeromonas hydrophila,* pangasius

 ***Corresponding Author:** hasin96@yahoo.com

Introduction

Bacterial diseases are the most common infectious problem of commercial fish farms causing much mortality. Among the bacterial genera, *Aeromonas* spp. are one of the major bacterial pathogens which are widely distributed in aquatic environment. *A. hydrophila* and other motile aeromonads are the most common bacteria in freshwater habitats throughout the world and frequently cause disease among cultured fishes. *A. hydrophila* is particularly responsible for Motile Aeromonas Septicemia (MAS) disease of fish in aquaculture. The disease is characterized by swollen abdomen, red mouth, hemorrhage in external surface and surrounding the anus (Panigua *et al*., 1990; Alam, 2009). *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; Alam, 2009). Sabur (2006) isolated and identified five isolates of *Aeromonas* bacteria in polyculture environment of five carp species. *A. hydrophila* was also found to be associated with epizootic ulcerative syndrome affected stinging catfish, *Heteropneustes fossilis* (Mamnur Rashid *et al.,* 2008). Mamnur Rashid *et al.,* (2013) also isolated and identified *A. hydrophila* from silver carp and its culture environment from Mymensingh region. Nahar *et al.,* (2016) isolated and identified ten isolates of *A. hydrophila* from juvenile farmed pangasius (*P. hypophthalmus*) in a commercial fish farm of Bangladesh.

Experimental infection is done to know the pathogenicity of a pathogen of its susceptible host species. In a recent study, Zhang *et al.,* (1016) tested the effectiveness of the waterborne challenge model using four field isolates including *A. hydrophila* and *Aeromonas veronii*. However, establishing an effective experimental challenge model of *A. hydrophila* has been found difficult. There is lack of information about establishing effective experimental infection method of *A. hydrophila* infecting *P. hypophthalmus*. Therefore present study was carried out to develop an effective experimental infection model of *A. hydrophila* using different challenge routes in junenile pangasius.

Materials and Methods

Experimental fish

Healthy juvenile pangasius of average weight of 50 g were collected from a private fish farm in Mymensingh and stocked in 50 L rectangular glass aquariums at the laboratory of the Department of Aquaculture, Bangladesh Agricultural University (BAU), Mymensingh. Before starting the experiment, all the fish were acclimatized for 5 days providing adequate feed and better aeration by using air pump. After acclimatization the fish were used for experimental infection with laboratory stock of *A. hydrophila* bacteria. For challenge experiment, replicate groups of fish were placed in 15 rectangular 15-litre capacity well labeled glass aquariums. Each aquarium was aerated and one third of the water was replaced daily, dead fish were removed and debris was siphoned from the bottom of the aquarium.

Isolate of A. hydrophila and preparation of bacterial suspension

A. hydrophila isolate P1K was used for infecting fish. This isolate was obtained from the laboratory stock of Fish Disease Laboratory in the Department of Aquaculture, BAU. The isolate was previously isolated from the kidney of diseased pangasius from a local farm and identified as *A. hydrophila* by Nahar *et al*., (2016). It was Gram negative, motile, fermentative, oxidase positive, grown at 37ºC, resistant to O/129, TSI positive and hydrolyzed esculin. It was further confirmed using API20E microbiological kit. This isolate was found pathogenic on juvenile pangasius as determined by Nahar *et al.,* (2016). It was cultured on Triptic soya agar (TSA) and broth at 25ºC for 48 h prior to the experiment. An amount of 5 mg of fresh culture of *A. hydrophila* was carefully scraped and mixed with 1 ml sterile physiological saline (0.85% NaCl) and then the desired dilution was prepared by serial decimal dilution method. In the case of broth culture, an amount of 30 µl was taken and mixed with 2.7 ml sterile physiological saline and the desired dilution was prepared by decimal dilution method.

Colony forming unit (cfu ml^{-1}) were determined for the bacterial suspension, prepared according to the drop method described by Miles and Misra (1938) or drop count method using TSA plates. Briefly, the bacterial suspension was diluted 10 fold seven times either with fresh saline or TSA broth. Replicate drops (20 µl $drop^{-1}$) from each dilution were then placed onto a TSA plate that had been previously divided into six sections. The plates were allowed to dry before incubation at 25ºC for at least 24 h until colonies were visible and could be counted. The average number of colonies per drop was counted and cfu ml^{-1} determined for the bacterial suspension using following:

cfu ml^{-1} = Number colonies × 20 (volume added) × dilution factor × 50

Experimental infection

Five different challenge routes were used to administer the bacteria in fish which included intramuscular (IM) and intraperitoneal (IP) injection, oral administration, agar implantation with fresh colonies of bacteria and bath. The summary of experimental infection protocol is given in Table 1.

Injection method

The bacterial inoculates used in the IM and IP injection were prepared from bacteria cultured either in broth or on agar plates to examine it there was any effect on

mortality between the two preparations. Two groups of 20 fish were used for each challenge route. Fish were injected either IM (below the left dorsal fin of the animal) or IP with 0.1 ml of bacterial suspension contains 4.6×10^7 cfu ml-1. Two corresponding group of control fish were included for each challenge route and they were given 0.1 ml of sterile broth in place of the bacteria.

Table 1. Summary of experimental infection protocol

Challenge route	No. of experimentally challenged fish	No. of control fish	Challenge dose (cfu $fish^{-1}$)
IP agar	20	20	4.6×10^6
IP broth	20		4.6×10^6
IM agar	20	20	4.6×10^6
IM broth	20		4.6×10^6
Oral administration	20	20	4.6×10^6
Agar implantation	20	20	4.6×10^6
Bath	20	20	200 ml broth in 30 L water

Oral administration

For oral administration two groups of 10 fish were exposed to the bacteria by dropping 0.1 ml of 4.6×10^7 cfu ml^{-1} of bacterial suspension into the pharynx of the animal using 1 ml sterile syringe. Only broth culture was used for oral administration. This was washed with a further 0.1 ml of water.

Agar implantation

For agar implantation, bacteria were implanted under the skin of the group of the animals. A small cut was made left dorsal side with a sterile scalpel blade and few colonies of *A. hydrophila* from 24 h TSA culture were cut from agar and placed under the skin of the animal. The control groups of fish received agar only.

Bath administration

For bath administration, bacteria were cultured in 200 ml broth for 24 h and added to 30 litre water in an glass aquaria. Then fishes were released into it. Control fishes were released in water containing sterile broth. The experimentally infected fishes and control fishes were released in well labeled glass aquarium.

Follow-up of experimental infection

The experimentally infected fish were observed for 15 days. Continuous aeration was maintained and no feed was given to the experimental fish. One-third of the water was replaced daily and debris siphoned from the bottom of the tank. Infection was recorded by observation of red mouth and fins, lesion, clinical appearance and mortality. Moribund fishes were attended, observed and waited for their death. The numbers of dead fish were recorded. Immediately after death, they were removed from the aquaria.

Results

Five different challenge routes were used to determine the most effective infection model for *A. hydrophila*. During the experimental period the average water temperature was 28.56ºC. The percent cumulative mortality (PCM) of fish obtained at the end of the experimental challenge at 15 days post-challenge ranged from 70% to 100% for different route of challenge (Table 2). The PCM was more than 50% for all the challenged route.

Injection method challenges

Daily mortality, cumulative mortality, percent cumulative mortality of fish was recorded regularly for each challenge method to determine the efficacy of the method. IP route was found to be the most effective way of introducing infections in pangasius under laboratory condition. IP route resulted 100% mortality at a dose of 4.6×10^6 cfu $fish^{-1}$ both from bacteria grown on agar and broth medium (Figure 1). Mortality started

to occur slowly by IP injection method. In IP agar method mortality started to occur on day 4 but in case of IP broth method mortality started to occur on day 3. For both the IP broth and IP agar method PCM 50% occurred at day 9 of post infection and PCM 100% occurred at 14 day of post infection. IM route resulted 70% and 80% mortality after 15 days post infection at a dose of 4.6×10^6 cfu $fish^{-1}$ from bacteria grown on agar and broth of the experimental fishes (Fig. 1). Mortality started to occur quickly than IP injection method. For both the IM agar and IM broth routes mortality of fish started to occur on day 2. In case of IM agar route PCM 50 occurred on day 9 where as IM broth route resulted PCM 50% on day 10 (Figure 1).

Table 2. Summary of experimental infection of *Aeromonas hydrophila* in *Pangasianodon hypophthalmus*

Challenge route	Days post infection	PCM
IP agar	15	100
IP broth	15	100
IM agar	15	70
IM broth	15	80
Oral administration	15	80
Agar implantation	15	100
Bath	15	70

Oral administration, bath and agar implantation challenges

The PCM obtained by oral administration route was 75% at the end of the experiment with challenge with 4.6×10^6 cfu $fish^{-1}$ (Fig. 2). Mortality of fish started to occur later in this method than IP and IM method. Mortality started to occur on day 4 and fish showed clinical signs lately. PCM 50% occurred on day 11 through this method. Bath administration method was found less effective than IP, IM and oral administration challenge. This challenge route resulted low level of mortality of fish and there was no major clinical signs in the infected fish. This challenge route resulted 70% PCM mortality of the experimental fishes at the end of the experiment (Figure 2). Mortality started to occur on day 2. The highest mortality in this method 70% occurred on day 15. In this method clinical signs were found lately among fishes.

Agar implantation method was a newly applied method which resulted 100% mortality of the experimental fishes where few colonies of bacteria were inserted into the fish. A small injury was created before inserting bacteria. Fish became stressed for that injury. Death occurred very quickly by this method. The highest PCM 100% occurred on day 7 (Figure 2). In this method mortality of fishes occurred so quickly that there were no major clinical signs in the infected fishes.

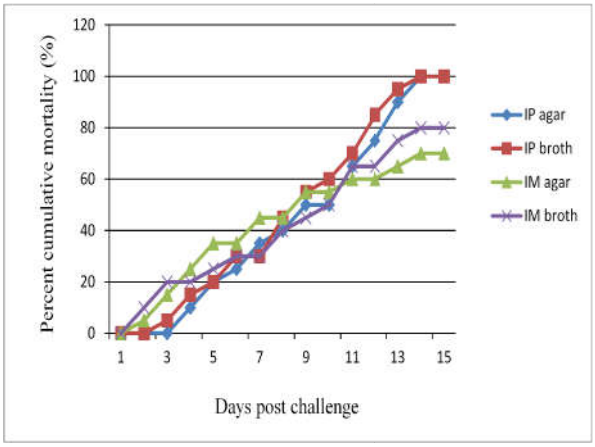

Figure 1. Percent cumulative mortality (PCM) of fish after 15 days post- challenge for IP and IM injection routes

Clinical and Gross Pathology of experimentally infected fish

All pangasius fish were apparently healthy, bright and of good appearance before experimental infection. After the challenge test they lost their normal appearance and abnormal movement were also observed. Tip of the mouth, anal region and fin bases developed red colour. Bilateral exophthalmia, reddening around eyes and mouth were the most

common clinical signs in infected fishes especially in fishes challenged with injection methods (Fig. 3). The common clinical signs of oral and bath challenged fish included fin loss and hemorrhagic ulceration at the fin bases (Figure 3). In moribund condition of all experimentally infected fish, abnormal movement and loss of balance were observed. Some fish were gulping and showed irritated movement. The posterior end of the body surface was found to develop grayish- white lesion that was extended up to caudal fin.

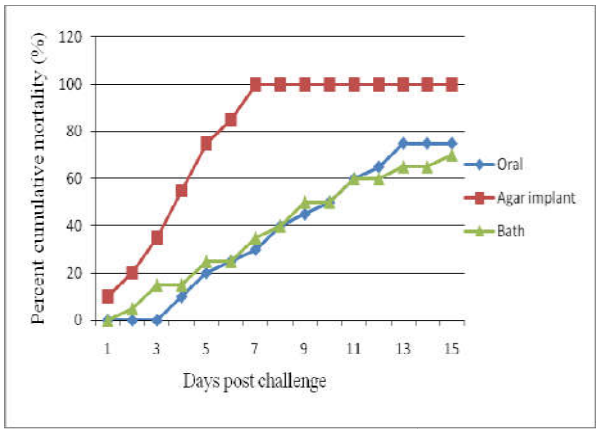

Figure 2. Percent cumulative mortality (PCM) of fish after 15 days post- challenge for Oral administration, bath and agar implantation methods

Clinical and Gross Pathology of experimentally infected fish

All pangasius fish were apparently healthy, bright and of good appearance before experimental infection. After the challenge test they lost their normal appearance and abnormal movement were also observed. Tip of the mouth, anal region and fin bases developed red colour. Bilateral exophthalmia, reddening around eyes and mouth were the most common clinical signs in infected fishes especially in fishes challenged with injection methods (Figure 3). The common clinical signs of oral and bath challenged fish included fin loss and hemorrhagic ulceration at the fin bases (Figure 3). In moribund condition of all experimentally infected fish, abnormal movement and loss of balance were observed. Some fish were gulping and showed irritated movement. The posterior end of the body surface was found to develop grayish-white lesion that was extended up to caudal fin.

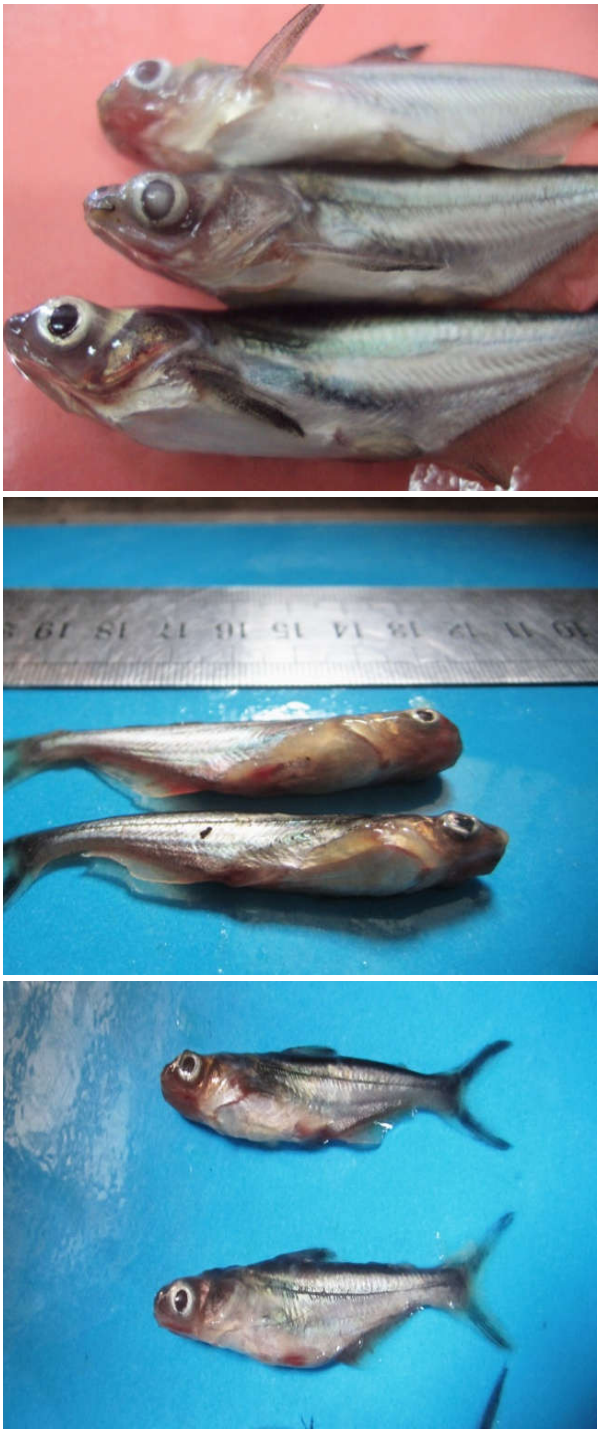

Figure 3. Clinical signs and pathology of experimentally infected pangasius with *A. hydrophila*. (a) and (b): Bilateral exophthalmia developed by IP challenge; (c) and (d): Reddening around eyes, mouth and head resulted by IM challenge; (e) and (f): Hemorrhagic ulceration at fin bases occurred by oral and bath administration challenge.

Discussion

The experimental challenges of *A. hydrophila* presented here clearly demonstrated that the IP challenge route appeared to be the most effective way of establishing infections in pangasius fry under laboratory condition. IP method showed 100% mortality at a dose of 4.6×10^{6} cfu $fish^{-1}$ with peak mortalities occurring on 8 to 13 days. It was found that bacteria cultured in either broth or on agar did not show any major difference in their virulence when injected IP. Clinical signs like exophthalmia, hemorrhage and reddening around eyes and mouth were evident in infected fishes when injected IP.

Rangdale (1995) performed a comparative study using a number of different *Flavobacterium psychrophilum* isolates, in which 0.8g rainbow trout were injected IP with 10^{6} cell ml^{-1}. Resultant mortality ranged from between 17 and 74% with the different isolates tested. Madsen and Dalsgaard (1999) reported a successful IP challenge model using isolates with different elastin-degrading profiles and different sero-types.

The IM challenge method also showed better result to develop infections in pangasius for both the bacteria grow on agar or broth. In this challenge model difference in virulence of *A. hydrophila* was observed depending on that the bacteria cultured in either broth or on agar. IM agar method resulted 70% mortality whereas IM broth resulted 80% mortality of the challenged fish. Ostland *et al*. (1997) found 100% mortality in groups of rainbow trout injected IM with *F. psychrophilum* at 1.45×10^{6} cfu $fish^{-1}$. In the present study IM challenge route also reproduced some clinical signs of *Aeromonas* infection in challenged fish like hemorrhage and reddening around mouth and eyes. Mostafa *et al.* (2008) experimentally infected *H. fossilis* with *A. hydrophila* by IM and IP injection which resulted 100% mortality at a dose of 9.6×10^{7} cfu $fish^{-1}$ within 1 to 9 days. Iqbal *et al*. (1998) investigated the pathogenicity of *Aeromonas* species isolated from fishes with EUS through IM injection and immersion method in goldfish at 20ºC ($2\times10^{6.8}$ to 6×10^{8} cfu $fish^{-1}$)

and 25ºC ($2.6\times10^{4.5}$ to 1.2×10^{8} cfu $fish^{-1}$) and it showed that infection rates at 25ºC in most cases were higher than those at 20ºC. In the present study the average temperature was recorded as 28.56ºC during the experimental period.

Oral administration of *A. hydrophila* appeared to reproduce the symptoms in challenged fishes like hemorrhagic ulceration at fin bases and fin erosion. The PCM obtained by this route was 75% with 4.6×10^{6} cfu $fish^{-1}$. Mortalities started occur lately in this model. It has been shown by Sera and Ishida (1972) that few bacteria are able to survive the adverse conditions of the stomach where p^{H} values of less than 3 are encountered, as well as the presence of digestive enzymes. The present study suggested that *A. hydrophila* may cause infection when enter by oral or gastrointestinal route though the probable route of entry may be the gills or through the skin.

Low levels of mortality were observed when fish were challenged by bath administration. The PCM obtained here was 70% when fish were released in water where bacterial suspension was added. This method was less effective than IP and IM injection and oral administration route. The reason why the bath challenge was not effective as IP or IM injection may be that the laboratory conditions under which the challenge was performed were too clean and fish were not sufficiently stressed to succumb to the disease. Another reason for the low mortality in bath challenge may be that the fish secrete of mucus in the bath, which neutralises some of the bacteria, and some of the bacteria stick to the fish. Clinical signs observed in this method included reddening around mouth and eye and abnormal movement of fish. Rangdale (1995) and Madsen and Dalsgaard (1999) attempted to develop a bath challenge model, whereby groups of rainbow trout fry were dipped into bacterial suspensions. Using 10^{5}cfu ml^{-1}, Rangdale (1995), obtained PCM of 30% and 38% after 14 days post-challenge when fish were dipped into the bacterial suspension for 5 h and 10 h, respectively, while Madsen and Dalsgaard (1999) found PCM between of 27 and 31% when 10^{7}cfu ml^{-1} was applied for only 0.5 h.

In the present study quick mortality of pangasius occurred when fish were challenged by agar implantation. PCM obtained by this route was 100% within 7 days post challenge. The reason of quick mortality of fish may be the small injury that was created before agar implantation. This injury stressed the fish which may be caused quick mortality. No clinical signs were observed in dead fishes challenged by this route as because of too quick mortality.

The presence of clinical signs of the challenged fish was quite similar with that of naturally infected diseased pangasius as observed by several authors (Faruk *et al*., 2012; Nahar *et al.,* 2016). Major clinical signs like anal protrusion, hemorrhage, red eyes and mouth, ulcer on the body surface, grayish cotton wool patches on skin, eroded tail and fins in naturally diseased pangasius were observed.

Conclusion

This experiment established that infecting *P. hypophthalmus* with *A. hydrophila* through different routes can serve as a novel infection model that allows for the future study of host–pathogen interactions. The work presented here suggests that *P. hypophthalmus* represents a permissive host for *A. hydrophila* infections and can be used as a simple host model to assess the virulence of *A. hydrophila.*

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