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Effect of different salinities on the susceptibility of striped catfish (*Pangasianodon hypophthalmus*) to *Aeromonas hydrophila* bacteria causing hemorrhagic disease

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ABSTRACT

This study was conducted to determine the effect of different salinities on striped catfish (Pangasianodon hypophthalmus) susceptibility to hemorrhagic disease causing by Aeromonas hydrophila bacteria. The experiment was set up in plastic tanks at various salinities (0, 3, 5, 8, 11 and 14‰). Experimental fish (about 25 ± 0.8g/fish) were challenged with A. hydrophila bacteria by intramuscular injection. After infection, the mortality and disease signs were recorded for 14 days. At the same time, bacteria re-isolation and re-identification were carried out with infected fish samples that displayed signs of lethargy, disorientation, hemorrhagic in anal, peritoneal sinus and spleen. Recorded cumulative mortality after 14 days was 86.7% at salinity 0‰ and 8.3% and 11.7% at salinities 11 and 14‰, respectively. At higher salinity, striped catfish is less susceptible to A. hydrophila bacteria than at lower salinities.

1. INTRODUCTION

Striped catfish (*Pangasianodon hypophthalmus*) is one of the species that are widely cultured and have economic value in the Mekong Delta of Viet Nam, which has contributed to creating jobs and bringing economic benefits for the country.

One major factor affecting the sustainable development of striped catfish culture in the Delta which has been reported so far as the annual cycle of bacterial pathogen infections and hemorrhagic septicemia caused by *A. hydrophila* is being one of the most common types of diseases in cultured striped catfish (Hoang *et al*., 2019).

On the other hand, climate change has an impact on the ecological environment in general and aquaculture in particular, including catfish farming. Excessive saline intrusion will partly affect the living environment and fish's ability to grow. An increase of 20-45 cm in sea water level which is projected to rise between 2030 and 2090 (Khang *et al*., 2008) will facilitate saltwater intrusion deeply into freshwater fish farming areas in the Mekong Deltaregion.

According to Phuc *et al*. (2014), high salinity levels (at 14 g/L and above) had a clear effect on Tra catfish growth performance and created stressful conditions. However, study on the effects of salinity on the susceptibility of striped catfish to common diseases, such as hemorrhagic disease, is limited. In this report, the effect of different salinities on the susceptibility of striped catfish to *A. hydrophila* bacteria causing hemorrhagic disease is presented.

2. MATERIALS AND METHODS

2.1. Experimental systems

The experiment was conducted at the laboratory of the College of Aquaculture and Fisheries, Can Tho

University. Plastic tanks (60 L) were disinfected with chlorine 200 ppm, dried and filled with water to 2/3 of the volume with continuous aeration.

Salinity acclimation: Salinities in the experimental tanks were being increased 2‰ per day to the experimental salinities. After 7-day acclimation with required salinities, there was no dead fish recorded in all treatments.

2.2. Experimental fish

Healthy striped catfish fingerlings (25 ± 0.8 g/fish) were used for the experiment, fish were acclimated in composite tanks (1 m^3) for 1 week and prior to being distributed to experimental tanks, 10 fish were randomly checked for parasites (on gill and internal organs) in and bacteria (*Edwardsiella ictaluri* and *A. hydrophila*) to ensure the fish are free from these pathogens.

2.3. Bacterial preparation for challenge

A. hydrophila strain A.1 from the collection of Can Tho University (Hoang *et al*., 2019) was used for injection. The bacterial strain was recovered from glycerol stock preserved at -80°C by culturing on Tryptone Soya Agar (TSA, Merck) and incubated at 28°C for 24 hours. Pure bacterial strain was re-cultured in 100 mL of Tryptone Soya Broth (TSB, Merck) at 28°C for 24 hours. Bacterial cells were harvested by centrifugation at 7,500 rpm for 15 minutes and washed 2 times with 0.9% NaCl solution after discarded TSB. Bacteria density was determined by using a spectrophotometer at a wavelength of 610 nm with OD = 1.2 (density equivalent to 10^9 CFU/mL). The density of bacteria was double-checked using the plate counting method.

2.4. Experimental set up and fowllow up

2.4.1. Determination of lethal dose of 50% (LD_{50}) of A. hydrophyla on fish

Experimental fish were allocated into the plastic tanks (10 fish/tanks) with 6 treatments (TM) in triplicate: (TM1) control treatment inject with 0.9% NaCl solution; (TM2-6) bacterial injection treatments with a density of 10^2-10^7 CFU/fish, respectively. Fish were monitored for pathological signs for 14 days after bacterial infection. Lethargy fish were collected to observe and record pathological signs, isolate and re-identify bacteria from kidney by polymerase chain reaction method.

The lethal dose of 50% experimental fish (LD_{50}) was determined using the formula of Reed and Muench (1938): LD_{50} = 10a-pd (pd = (L% -50/L% -H%)); where, a is the exponent at which lowest mortality rate but over 50%; H% is the highest mortality rate but less than 50%; L% is the lowest mortality rate but over 50%).

The LD_{50} value was used to induce infection in an experiment to evaluate the effect of different salinities on the susceptibility of striped catfish to *A. hydrophila* bacteria causing hemorrhagic disease.

2.4.2. Determine the effect of different salinities on the susceptibility of striped catfish to A. hydrophila

Striped catfish were randomly distributed into to plastic tanks (60L in volume) with a density of 20 fish/tank, then salinity was increased by 2‰ per tank per day until the experimental salinity were reached. There were 6 treatments with 3 replications, including 1 control treatment at 0‰ and 5 treatments at salinity of 3‰, 5‰, 8‰, 11‰ and 14‰. After being acclimated to required salinities, fish were infected with *A. hydrophila* bacteria with LD_{50} and monitored for 14 days.

Recorded data after injection includes (1) behavior of experimental fish and daily mortality; (2) examination, observation and record disease signs; (3) bacterial re-isolation on TSA medium by randomly selecting one diseased fish/tank and confirmed by PCR method.

2.4.3. Re-isolation and identification of challenged A. hydrophila strain

Lethargic fish were collected from experimental tanks and recorded signs of pathology both outside and inside the body, then 70% ethanol solution was used to disinfect the outside of the fish body, the fish were dissected with a scalpel and a pair of sterile scissors. Kidney from specimen was inoculated on TSA plate, incubated at 28°C for 24 hours. The colonies growing on TSA medium were recorded in color, shape and size. Recovered strains were subjected to PCR for detection of *A. hydrophila*.

PCR detection for *A. hydrophila*

Bacterial DNA was extracted following the method of Bartie et al. (2006). Bacteria were cultured for 24 hours in 5 mL of TSB medium at 28ºC, then 1.5 mL of bacterial suspension was added to a centrifuge tube containing 100 µL TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0). The mixture was heated at 95ºC for 15 minutes, cooled on ice and centrifuged for 2 minutes at 14,000 rpm to separate the DNA from the solution and kept at -20ºC.

PCR detection was performed according to the procedure of Panagala et al. (2007). The total reaction volume of 25 µL, including: 1X 10X buffer solution; 1.5mM $MgCl_2$; 200 µM dNTPs; 2,5 U Taq DNA polymerase; 0.4 µM forward (AeroFd) and 0.4 µM reverse primers (AeroRs) and 20ng DNA samples. The thermal cycle was 95ºC in 4 minutes, then 95ºC for 30 seconds, 60ºC for 45 seconds, 72ºC for 30 seconds; repeat the cycle above 30 times; 72ºC for 10 minutes and keep at 20ºC.

Gel electrophoresis was carried out by loading 10 µL of PCR product and run on gel 1.0% agarose (Promega, USA) in TAE 0.5X buffer solution (10 mM Tris, 5 mM acetate, 0.1 mM EDTA). DNA fragments were recorded by UV reading table. The 100 bp DNA ladder (Promega) was used to determine the size of the DNA fragments. The amplified product of *A. hydrophila* is 209 bp.

2.5. Data analysis

Data on cumulative mortality between treatments were statistically analysis using Student's t-test (at significance level $P<0.05$) using SPSS 16 software.

3. RESULTS AND DISCUSSION

3.1. Lethal dose 50% (LD_{50}) of experimental fish

After 24 hours of challenge, infected fish appeared typical pathological signs of disease caused by *A. hydrophila* (Figures 2&3). After 14 days of infection, the cumulative mortality in the treatment of 10^7 CFU/fish was 100%. Cumulative mortalities of fish which were injected with bacterial concentrations of 103, 104, 105 and 106 CFU/fish were 40%, 50%, 60% and 70%, respectively. The unchallenged treatment had no dead fish during the experiment. The LD_{50} value of *A. hydrophila* to the fish was 6.4×10^4 CFU/fish.

3.2. Effect of salinities on the susceptibility of catfish to *A. hydrophila* infection

Pathological signs: Infected fish displayed lethargic swimming on the water surface. External signs showed hemorrhages in the gills, fins, anus and fin roots (Figure 1). The internal organs (liver, kidney and spleen) also showed hemorrhages and pale color (Figure 2).

The pathological signs from experimental fish in this experiment were similar to other reports on hemorrhagic disease caused by *A. hydrophila* bacteria in other fish species such as crucian carp *Carassius carassius* (Miyazaki & Kaige, 1985) rice eel *Monopterus albus* (Oanh & Hien, 2012) and clown knife fish *Chitala chitala* (Thy et al., 2014).

Figure 1. External pathological signs of infected fish

(A) un-injected fish (control), (B) hemorrhages on the fin, (C) hemorrhages on the skin and anus

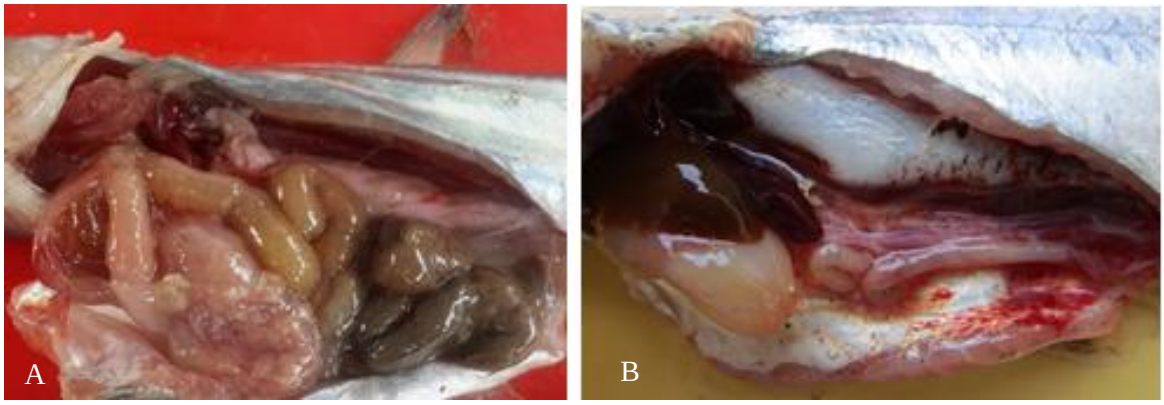

Figure 2. Internal pathological signs of infected fish

(A) un-injected fish (control), (B) injected fish

Re-isolation and identification of bacteria from infected fish: The bacteria were isolated from fish showing lethargic swimming signs, external and internal hemorrhages. Bacteria grew on TSA medium after 24 hours at 28°C, giving a round, slightly convex, smooth, light yellow cream colony with a size of 2-3 mm (Figure 3A). Typical colonies are pure, Gram negative (Figure 3B) and rod-shaped and positive result with PCR analysis using primer pair to detect the specific aerolysis gene of *A. hydrophila* (Figure 3C).

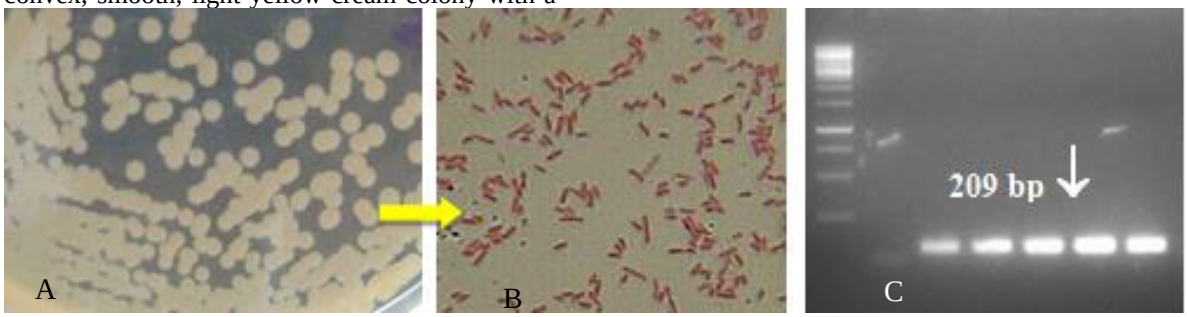

Figure 3. Re-isolated *A. hydrophila* bacteria from infected fish

(A) Bacteria on TSA medium (after 24 hours), (B) Gram-negative, rod-shaped (100X) bacteria, (C) A. hydrophila specific genes in re-isolated bacteria from kidneys of infected fish

The time of disease occurrence: After 14 days of experiment, all bacterial infected treatments had mortality and showed pathological signs of hemorrhagic disease. The timing of disease occurrence and mortality were different among treatments (Figure 4 and Table 1).

Mortality strated after 16-hour injection with bacteria in treatments of salinities 0‰, 3‰, 5‰, and 8‰ with cumulative mortalites were 56.7%, 56.7%, 46.7% and 16.7%, respectively.

In treatments of salinity 11‰ and 14‰, high mortality occurred at 2 days after challenge, then reduced and stabilized from the third3 days (in treatment with salinity 11‰) and fourth day ((in treatment of salinity 14‰) after injection. Cumulative mortality of these treatments were11.7% and 13.3%, respectively.

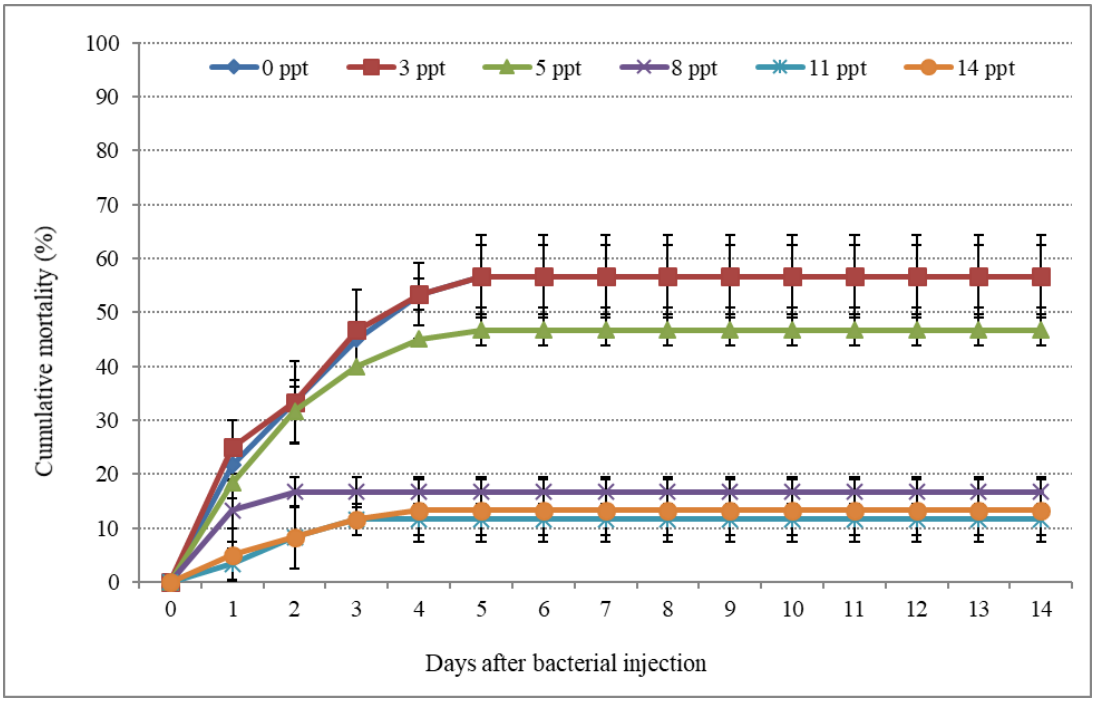

Figure 4. Cumulative mortality (%) of experimental fish after 14-day challenge

Cumulative mortality: Cumulative mortalities in the treatments of salinities 0‰ and 3‰ were similar (56.7±5.8% and 56.7±7.6%, respectively) and were higher than salinities 5‰, 8‰, 11 ‰ and 14‰ (ranging from 11.7±5.8% to 46.7±2.9%). In treatments of salinity 11‰ and 14‰, high mortality occurred at 2 days after challenge, then reduced and stabilized from the third days (in treatment with of salinity 11‰) and fourth day ((in treatment of salinity 14‰) after injection. Cumulative mortalities of these treatments were 11.7% and 13.3%, respectively. Therefore, salinity affects the susceptibility of striped catfish to *A. hydrophila* bacteria causing hemorrhagic disease was decreased with increasing salinity of water.

Table 1. Cumulative mortality (%) of experimental fish after 14 days of challenge

Treatment salinity	Cumulative mortality (%)
0‰	56.7 ± 5.8^{D}
3‰	56.7 ± 7.6^{D}
5‰	46.7 ± 2.9^{C}
8‰	16.7 ± 2.9^{B}
11‰	11.7 ± 2.9^{A}
14‰	13.3 ± 2.9^{A}

^{A}, ^{B}, ^{C} and ^{D}: data in the same column have different characters showing a statistically significant difference (P <0.05).

4. CONCLUSION

Salinity has an influence on susceptibility of striped catfish (about 25 ± 0.8g/fish) to *A. hydrophila* bacteria causing hemorrhagic disease. At higher salinity, striped catfish is less susceptible to *A. hydrophila* bacteria than that at lower salinities. It is necessary to study the effect of salinity in combination with other environmental indicators such as pH and temperature on the susceptibility of catfish to *A. hydrophila*.

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