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.

Keywords
Aeromonas hydrophila, hemorrhagic disease, Pangasianodon hypophthalmus, salinity

1. INTRODUCTION
Striped catfish (Pangasianodon hypophthalmus) is one of the species that are widely cultured and have economic value in the Mekong Delta of Vietnam, 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 Delta region.

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 m³) 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 10³ CFU/mL). The density of bacteria was double-checked using the plate counting method.

2.4. Experimental setup and follow up

2.4.1. Determination of lethal dose of 50% (LD₅₀) 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 10²-10⁷ 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 (LD₅₀) was determined using the formula of Reed and Muench (1938): LD₅₀ = 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 LD₅₀ 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 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 LD₅₀ 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 28°C, 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 95°C 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 -20°C.
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; 1.5 mM MgCl₂; 200 µM dNTPs; 2.5 U Taq DNA polymerase; 0.4 µM forward (AeroFd) and 0.4 µM reverse primers (AeroRs) and 20 ng DNA samples. The thermal cycle was 95°C in 4 minutes, then 95°C for 30 seconds, 60°C for 45 seconds, 72°C for 30 seconds; repeat the cycle above 30 times; 72°C for 10 minutes and keep at 20°C.

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% (LD₅₀) 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 10⁷ 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 un-challenged treatment had no dead fish during the experiment. The LD₅₀ value of *A. hydrophila* to the fish was 6.4 x 10⁴ 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](image)

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

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 started after 16-hour injection with bacteria in treatments of salinities 0‰, 3‰, 5‰, and 8‰ with cumulative mortalities 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 third day (in treatment with salinity 11‰) and fourth day ((in treatment of salinity 14‰) after injection. Cumulative mortality of these treatments were 11.7% and 13.3%, respectively.
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

<table>
<thead>
<tr>
<th>Treatment salinity</th>
<th>Cumulative mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0‰</td>
<td>56.7±5.8(^D)</td>
</tr>
<tr>
<td>3‰</td>
<td>56.7±7.6(^D)</td>
</tr>
<tr>
<td>5‰</td>
<td>46.7±2.9(^C)</td>
</tr>
<tr>
<td>8‰</td>
<td>16.7±2.9(^B)</td>
</tr>
<tr>
<td>11‰</td>
<td>11.7±2.9(^A)</td>
</tr>
<tr>
<td>14‰</td>
<td>13.3±2.9(^A)</td>
</tr>
</tbody>
</table>

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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REFERENCES


