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Research article

Effect of bacteriophage on histopathology and disease resistance of Whiteleg shrimp (*Litopenaeus vannamei*) infected by *Vibrio parahaemolyticus*

Le Hoang Bao Ngoc[1,3,4], Le Viet Dung[2], Tran Thi Lieu[1], Truong Thi Bich Van[1]*

[1] *Institute of Food and Biotechnology, Can Tho University, Can Tho 900000, Vietnam*
[2] *College of Agriculture, Can Tho University, Can Tho 900000, Vietnam*
[3] *Department of Biotechnology, An Giang University, An Giang 880000, Vietnam*
[4] *VietNam National University, Ho Chi Minh City 700000, Vietnam*

Abstract

Vibrio parahaemolyticus is the causative agent of shrimp diseases, the most serious of which is acute hepatopancreatic necrosis disease (AHPND). Bacteriophage is a virus of bacteria that can parasitize and destroy bacteria, so it is considered a potential alternative to antibiotics. The study was carried out to evaluate the treatment ability of bacteriophages on *Litopenaeus vannamei* after being infected by *V. parahaemolyticus* B4XOT2.2 isolated from the bottom mud of shrimp ponds infected with AHPND. The study used the histopathological survey method on shrimp at all three ages: postlarvae, adult shrimp at the ages of 30–45 days, and 55–60 days old, which were arranged into three treatments: healthy shrimp, diseased shrimp, and bacteriophage-treated shrimp. The results showed that: the concentration of *Vibrio* spp. in bacteriophage-treated treatment was decreased in all three groups (postlarvae, shrimp at the ages of 30–45 days, and 55–60 days old) from $3,7x10^3$ CFU/mL to $2,2x10^2$ CFU/mL after two days, from $4,6x10^4$ CFU/mL to $3,3x10^2$ CFU/mL after two days and from $4,6x10^4$ CFU/mL to $5,6x10^3$ CFU/mL after three days, respectively. The pathological signs and histological features of the infected shrimp samples were similar to those typical of acute hepatopancreatic necrosis disease and recovered in shrimp tissue after being treated with bacteriophages such as the hepatopancreas was also darker, the intestines gradually filled, no hematoma around the tubules were found, in the lumen of the tube reduced sloughing cells and blood cells, no melanization was observed.

Keywords: Bacteriophage, *Histopathology, Litopenaeus vannamei, Vibrio parahaemolyticus,* Whiteleg shrimp.

***Corresponding author:** Truong Thi Bich Van, Institute of Food and Biotechnology, Can Tho University, Can Tho 900000, Vietnam. Email: ttbvan@ctu.edu.vn

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INTRODUCTION

Whiteleg shrimp (*Litopenaeus vannamei*) is one of the main export products of Vietnam's seafood. Therefore, the production and farming area of *L. vannamei* have continuously increased in recent years. However, shrimp production also faces many challenges, as climate change and intensive farming with high densities complicate the growth of pathogenic bacteria. One of them is *Vibrio parahaemolyticus*, one of the main causative agents of Acute Hepatopancreatic Necrosis Disease (AHPND), with a mortality rate of up to 100%, causing serious damage to the global shrimp farming industry. The traditional and common method used in the treatment of bacterial infections is the use of antibiotics (Baticados and Paclibare, 1992). However, antibiotics are also used for prevention and growth promotion purposes (Holmstrom et al., 2003; Kowalska et al., 2020). This has led to the emergence and spread of antibiotic-resistant strains of bacteria in the culture system. Furthermore, the use of antibiotics in shrimp farming has created problems with antibiotic residues in food systems (Treiber and Beranek-Knauer, 2021). Therefore, in the trend towards safe agriculture, finding a biological therapy to inhibit the disease is an urgent issue in Vietnam in particular and the world in general. One of the biological methods that has been and is being studied with the hope of being able to replace antibiotics in the prevention of diseases caused by bacteria is bacteriophage therapy.

Bacteriophages were discovered by Twort in 1915 and d'Heralle in 1917 (Kutter and Sulakvelidze, 2005). The bacteriophage method was evaluated as a safe method with no side effects (Harada et al., 2018). Besides, according to (Culot et al., 2019), bacteriophages work very effectively in liquid conditions, so the application of bacteriophages in aquaculture in general and shrimp farming in particular as a biological control agent is very appropriate and can handle bacteria both in the aquatic environment and in aquatic animals. Because of that great potential, there have been many studies on the application of phages in inhibiting *V. parahaemolyticus* in the world, with good results (Jun et al., 2017; Wong et al., 2019; Yang et al., 2020; Thammatinna et al., 2020; Tan et al., 2021). However, besides the research of Phuong et al. (2022) about the application of bacteriophages capable of inhibiting *Salmonella*, currently, very few studies have been published in the country on the application of bacteriophages to inhibit *V. parahaemolyticus*, so the study was conducted to evaluate the ability to treat diseases in shrimp caused by *V. parahaemolyticus* by performing a pathological and histopathological survey.

MATERIALS AND METHODS

Sample collection and shrimp preparation

All procedures were approved by the Animal Welfare Assessment (BQ2020-02/VNCPTCNSH). A bacterial source was isolated from the bottom sludge of diseased shrimp ponds in the U Minh Thuong district, Kien Giang province. *L. vannamei* sources provided from intensive farms. In this study, a total of 600 shrimps include 3 groups: postlarvae, adult shrimp at the ages of 30–45 days and 55–60 days old were used.

V. parahaemolyticus isolation

Sludge samples were diluted and inoculated on Thiosulfate Citrate Bile Salt Sucrose (TCBS) medium of Himedia (India). The plates were incubated at room temperature for 24 h. Select colonies specific to *V. parahaemolyticus* (large, blue) for domestication by the streak plate culture method.

Bacterial DNA was extracted through centrifugation at 12,000 rpm for 10 min. To break the bacterial cell wall, after 10 min at room temperature, 800 µL of lysis buffer was added to 800 µL of chloroform:isoamyl (24:1 v/v), mixed well, and incubated at 28 °C for 10 min. Centrifuge for 10 minutes at 12,000 rpm to collect clear liquid. The DNA was acquired and purified by running 700 µL of supernatant through 2.2 mL Eppendorf tubes, after which 700 µL of a 95% ethanol solution was added, and the mixture was mixed well. The mixture was then centrifuged at 12,000 rpm for 20 minutes to collect the precipitate. The precipitate was then washed with 500 µL of a 70% ethanol solution and centrifuged at 12,000 rpm for 10 min. Subsequently, the samples were vacuum-dried at 45 °C for 10 min. Afterward, DNA was dissolved in 100 µL of 0.1X TE solution and stored at 4 °C. Check DNA quality by electrophoresis on 1% agarose gel.

V. parahaemolyticus was identified by PCR using specific primers targeting on toxR gene (Kim et al., 1999) with the sequences as follows F: GTCTTCTGACGCAATCGTTG, R: ATACGAGTGGTTGCTGTCATG, and the expected length of the PCR product is 368 bp. PCR products were obtained, electrophoresis was carried out on a 1.5% agarose gel in 0.5X TBE buffer, and the results were read on a gel imager (Biorad). Strain positive for PCR with the toxR primer will continue to sequence the 16S rRNA gene to confirm the identification of *V. parahaemolyticus*

V. parahaemolyticus bacteriophage isolation

Isolation of bacteriophage was carried out according to the double agar overlay plaque assay (Kropinski et al., 2009). The 10 µL of crude bacteriophage mixture (20 mL sample + 5 mL TSB + 5 mL *V. parahaemolyticus* suspension incubated overnight at 30 °C. Add 1% chloroform and centrifuge at 12,000 rpm for 10 minutes at 4 °C, collect the supernatant) and 100 µL of isolated *V. parahaemolyticus* culture (allowed to stand for 10 min) were aliquoted into 5 ml of sterile soft nutrient medium (TSA 0.4% agar) (kept warm at 55 °C), were all mixed and spread on hard nutrient medium (TSA 1.7% agar). Incubate the plate at 35 °C for 24 h and observe the formation of a plaque. Using a sterile tip, collect an individual plaque, add it to 1 ml of sterile SM buffer, and store it at 4 °C.

Bacteria and bacteriophage inoculation

The experiment was designed according to the method of Jun et al. (2017) and Muthukrishnan et al. (2019) with some modifications as follows: Shrimps are cultured in seawater with a salinity of 15 ‰ for at least 72 h before the experiment, Shrimp culture water is disinfected with chlorine (concentration 30 ppm), aeration continuously to remove chlorine. Each experimental unit was arranged in a tank with a capacity of 30 L. Shrimp density was: postlarvae shrimp group: 50 shrimps/tank; 30-45 days old shrimp group: 30 shrimps/tank and 55-60 days old shrimp group: 20 shrimps/tank. Each type of shrimp is arranged in 3 tanks. Each treatment was performed with 3 replicates (Table 1). Spreading on the TCBS agar plate (Sanders, 2012) was used to measure the concentration of bacteria in water samples.

Table 1 Experimental design

Shrimp groups	Tank	Bacteria (*)	Bacteriophage (**)
Postlarvae	1 (Negative control): Healthy shrimps	No	No
	2 (Positive control): Infection	Yes	No
	3 Treat with bacteriophage	Yes	Yes
30-45 days old	1 (Negative control): Healthy shrimps	No	No
	2 (Positive control): Infection	Yes	No
	3 Treat with bacteriophage	Yes	Yes
55-60 days old	1 (Negative control): Healthy shrimps	No	No
	2 (Positive control): Infection	Yes	No
	3 Treat with bacteriophage	Yes	Yes

(*): Bacteria were inoculated into the shrimp culture water of tanks 2 and 3, respectively, to avoid shock and mass mortality: 5 mL of bacteria suspension with a density of 10^8/mL/day until reaching the final density in the shrimp culture tank postlarvae is 10^3 CFU/mL, in the 30-45 days and 55-60 days old shrimp tank is 10^5 CFU/mL. Recorded signs of disease development and histological characteristics of shrimp in tanks 1, 2, and 3 at the same time.

(**): After the density of bacteria reached final density, the dead shrimp showed signs of disease, then proceeded to the phage treatment with a dose of 2 mL with a density of 2×10^6 PFU/mL in tank 3. Recording signs of disease development and histopathological characteristics of shrimp in tanks 1, 2, and 3 at the same time.

Histological assessment

Three shrimps in each experimental tank were collected for histological assessment. Tank shrimp sample 1: No bacterial strain was collected after 2 days of stocking. Tank shrimp sample 2 consisted of only bacterial strain (infectious) and was collected when shrimp showed obvious disease symptoms and reached the density of pathogenic bacteria. Tank shrimp sample 3 consisted of treatment with phages after the shrimp showed obvious disease symptoms and was collected after 4 days of phage inoculation. Shrimp samples, after removing the hepatopancreas and intestines, were fixed in Davidson's AFA solution (at the ratio of 1 part muscle to 10 parts Davidson's solution) for about 48 h, then washed under gently running water for at least 12 h. The sample was further processed through dehydration steps with increasing alcohol concentrations from alcohol 50°, 60°, 70°, 80°, 90°, absolute alcohol, and n-Butanol; The sample was then clarified with xylene, then impregnated in paraffin and melted beeswax in an oven at 60 °C. The specimen was mass-cast and, after at least 24 h, sliced, glued onto a laminar, and stained with hematoxylin and eosin (H&E) dyes. The specimen was observed and photographed with a characteristic specimen under a Primo Star Carl Zeiss microscope in Germany with 10X and 40X objectives.

RESULTS

V. parahaemolyticus isolation

From 4 collected sludge samples, *V. parahaemolyticus* bacteria was isolated on TCBS medium, selecting round, regular, and blue colonies with a diameter of 2 mm or more after 24 h of incubation. Ten strains that are likely to be *V. parahaemolyticus* were established. However, in order to accurately conclude that these are *V. parahaemolyticus* strains, the strains continue to be cultured, screened, extracted DNA, and performed toxR-specific primer PCR. The

results showed that 4 samples showed positive results. The line appeared at position 368 bp, in which the control sample well was a positive control for *V. parahaemolyticus*. This showed that in 10 suspected bacterial strains, there were 3 strains identified as *V. parahaemolyticus*: B3XD2, B4XOT2.2, and B4XOT2.4 (Figure 1).

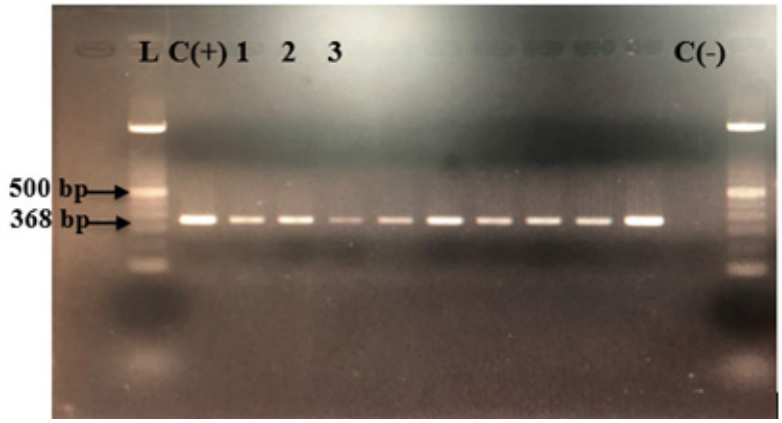

Figure 1 Results of amplification of toxR gene fragment in isolated bacterial strains. L: GeneRulerTM 100 bp DNA ladder (Thermo Scientific, USA); C(+): Positive control ATCC *V. parahaemolyticus* 17802; 1: strain B3XD2; 2: strain B4XOT2.2; 3: strain B4XOT2.3; C(-): negative control CTX2.

To confirm the identification results, the bacterial strain B4XOT2.2 (The morphology and color of colonies on TCBS medium did not change during storage) was sequenced with the 16S RNA gene and compared with the data on GenBank. The result is that the nucleotide sequence of strain B4XOT2.2 has high homology (96%) with the sequence of *V. parahaemolyticus* strain 2016030305-1, code CP034298. From the above results, the B4XOT2.2 strain was identified as *V. parahaemolyticus* and was used for the screening bacteriophage and subsequent bacterial infection experiments.

V. parahaemolyticus bacteriophage isolation

From 15 shrimp samples, isolated 5 bacteriophages, they have displayed an ability to infect *V. parahaemolyticus.* However, only 1 bacteriophage has the ability to create clear plaque on the double-layer agar plate with *V. parahaemolyticus* B4XOT2.2. To evaluate the effectiveness of the isolated bacteriophages, the study was continued on shrimp models as follow-up experiments (Figure 2).

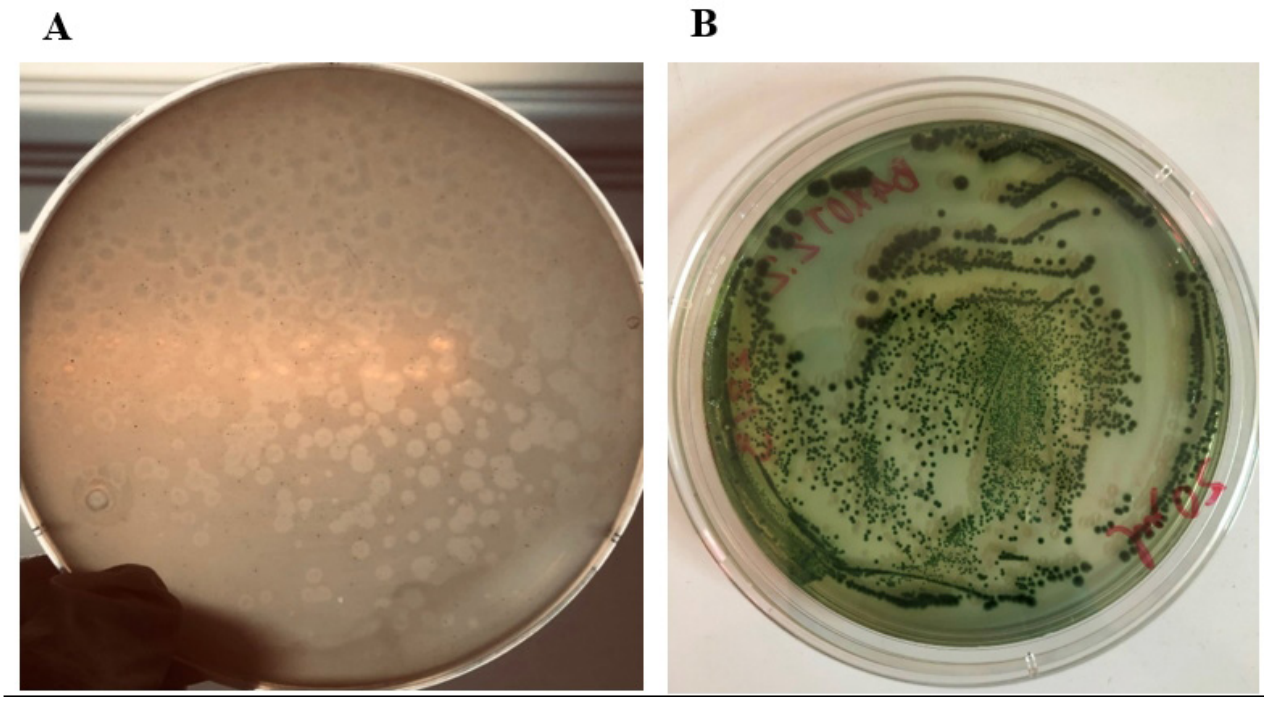

Figure 2 (A): Plaque morphology of bacteriophage on the double-layer agar plate *V. parahaemolyticus* B4XOT2.2 + Phage C2. (B): The colony morphology of *V. parahaemolyticus* B4XOT2.2 after treatment by Phage C2.

Pathological signs of shrimp after infection with *V. parahaemolyticus* and after bacteriophage treatment

In healthy shrimp tanks (tank 1) of 3 groups, the concentration of *Vibrio* spp. always fluctuates in the range of 10^2 CFU/mL because the shrimp intestine always has a certain amount of *Vibrio* spp. (Holt et al., 2021), but the density does not reach the pathogenic threshold as well and *Vibrio* spp. may not be the causative strain. This is proved by shrimp in healthy tanks without any signs of disease, still active, dark liver and pancreas, and full intestines throughout the experiment. Meanwhile, in tank 2 and tank 3 of 3 groups, bacterial concentration gradually increased after specific inoculation days as follows: Postlarvae shrimp group reached 3.7×10^3 CFU/mL after 2 days of inoculation, 30-45 days old shrimp group reached 4.6×10^4 CFU/mL after 9 days of inoculation and 55-60 days old shrimp group reached 8.2×10^4 CFU/mL after 10 days of inoculation. At these concentrations, shrimp began to show clear signs of disease, and shrimp started to die, so the bacteriophages inoculation was carried out in tank 3 of all 3 groups. The results were as follows: the bacterial density in tank 3 of 3 groups decreased sharply; for the postlarvae, shrimp group was 2.2×10^2 after 2 days; 30-45 days old shrimp group was 3.3×10^2 after 2 days; and 55-60 days old shrimp group was 5.6×10^3 after 3 days.

For the postlarvae group, in tank 1, shrimps: Light gray body, glossy shell, dark yellow or dark brown hepatopancreas (Figure 3A). Swim in a swarm against the current of the water. Quick response to sudden impacts. After the infecting bacteria reached the pathogenic concentrations, shrimp began to slow down, stop eating, lethargic, and sluggish. Because shrimp show signs of not eating, slow growth leads to the difference in size between the same

group. Tanks 2 and 3 have a lot of food, while healthy ones (tank 1) are all gone. Infected tanks recorded an increase in bottom dead shrimp, which had soft shells and molting. When pale hepatopancreas is observed, the bowel is empty (Figure 3B). In tank 3, after 48 h of inoculating bacteriophages, the shrimp began to eat again, and the shrimp intestines were darker. After 6 days of treatment with bacteriophages, the shrimp ate well, and the color of the hepatopancreas and intestines was stable. After 8 days of treatment, the color of the hepatopancreas and intestines gradually returned to the original, and the shrimp intestines were full (Figure 3C).

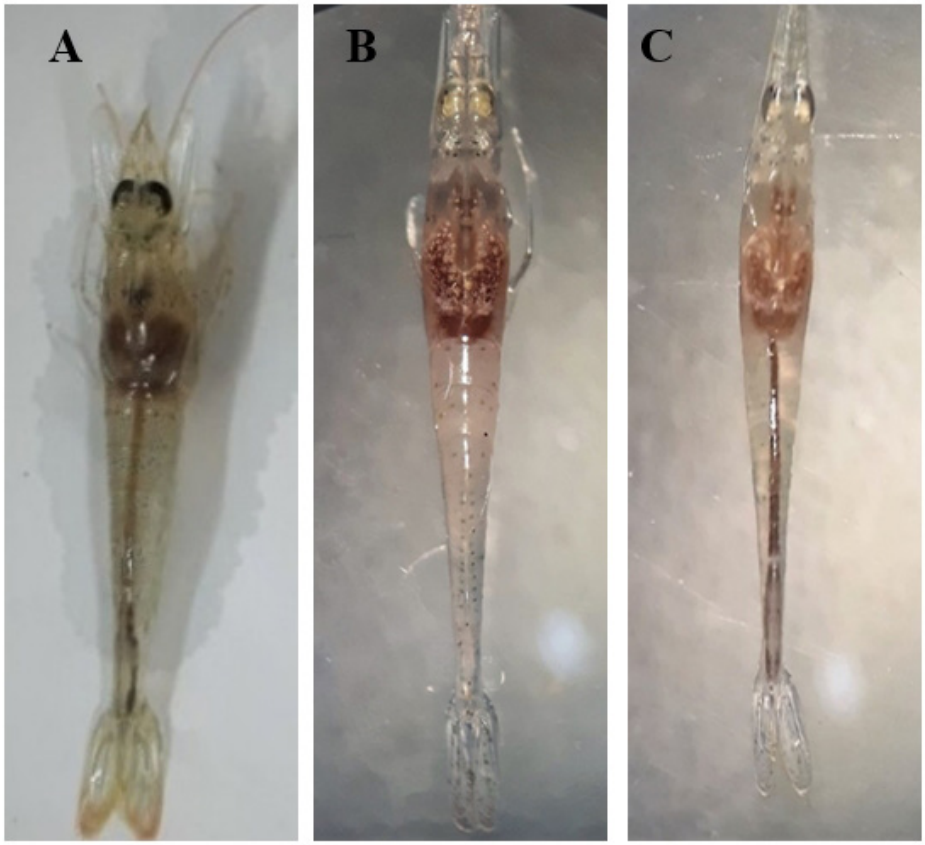

Figure 3 External signs of healthy postlarvae (A), diseased shrimp (B), and phage-treated shrimp (C).

For the 30-45 days-old group, shrimp have bright color, quick response, no external damage, healthy hepatopancreas, and full intestine (Figures 4A and B). After the bacteria reached the pathogenic threshold, the shrimp began to slow down, stop eating, lethargy, and drool. The amount of food recorded between the inoculated and healthy shrimp tank (control) was significantly different, while the healthy tank was exhausted. Infected tanks also recorded an increase in bottom dead shrimp with soft shells. When observed under the stereo microscope, the hepatopancreas is pale, and the intestines are empty. (Figures 4C and D). After 24 h of inoculating bacteriophages, it was observed that the shrimp started to work quickly again, less sluggish. When observed under the microscope, the hepatopancreas gradually recovered, became darker, and the intestines were full because the shrimp began to eat again (Figures 4E and F).

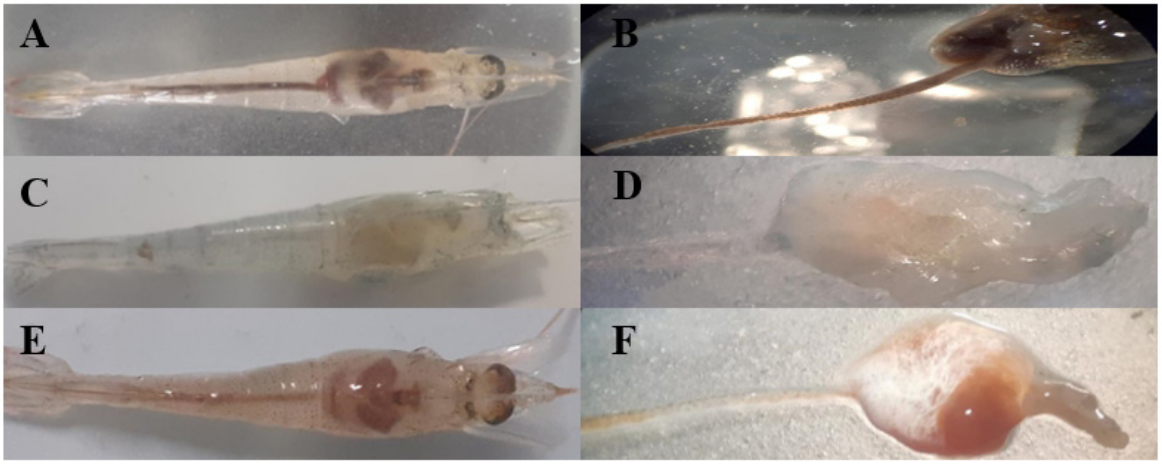

Figure 4 External signs and hepatopancreas of 30-45 days old shrimp (A, C, E: external signs of healthy shrimp, diseased shrimp, shrimp treated respectively; B, D, F: hepatopancreas of healthy shrimp, diseased shrimp, shrimp treated respectively).

For the 55-60 days old group, healthy shrimps are agile, eat quickly and run out of food according to portions. Shrimp have bright color, quick response, no external damage, dark hepatopancreas, and full intestine (Figures 5A and B). Bottom dead shrimp, anorexia, pale hepatopancreas, empty intestines or intermittent food storage, and soft shell phenomenon. There was a significant difference in the amount of feed recorded between the inoculated tank and the healthy shrimp (control) tanks. With the same number of shrimp, and the same amount of feed (5% of body weight), the results show that the tank 2 and 3 is still a lot while the healthy one is gone. Infected tanks recorded an increase in bottom dead shrimp, which had soft shells and molting. When the hepatopancreas is pale in color, the intestines are empty (Figures 5C and D). In tank 3, after 48 h of inoculating bacteriophages, shrimp began to eat again, less sluggish, when external morphological surgery observed that hepatopancreas gradually recovered, darker, intestines were full of blood (Figures 5E and F). The shrimp was eaten again. After 8 days, shrimp started working normally again.

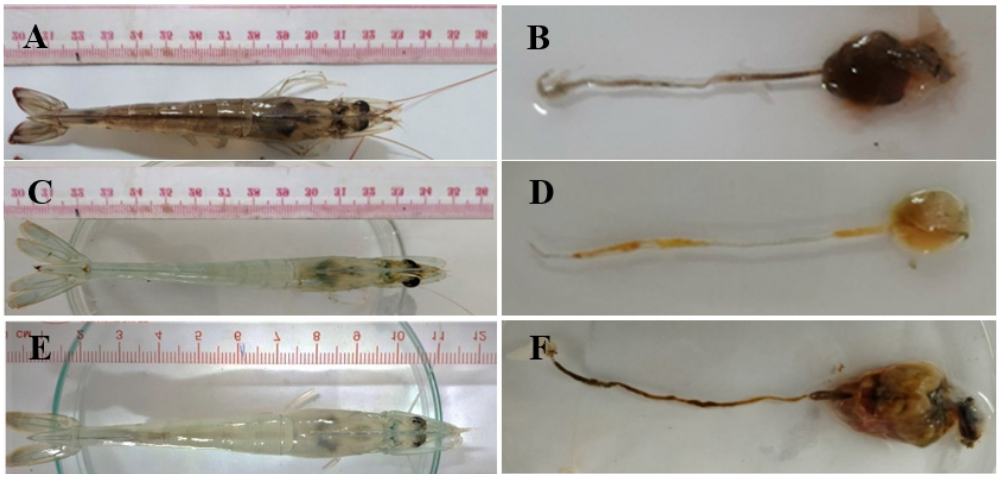

Figure 5 External signs and hepatopancreas of shrimp 55-60 days old: (A, C, E: external signs of healthy shrimp, diseased shrimp, shrimp treated respectively; B, D, F: hepatopancreas of healthy shrimp, diseased shrimp, shrimp treated respectively).

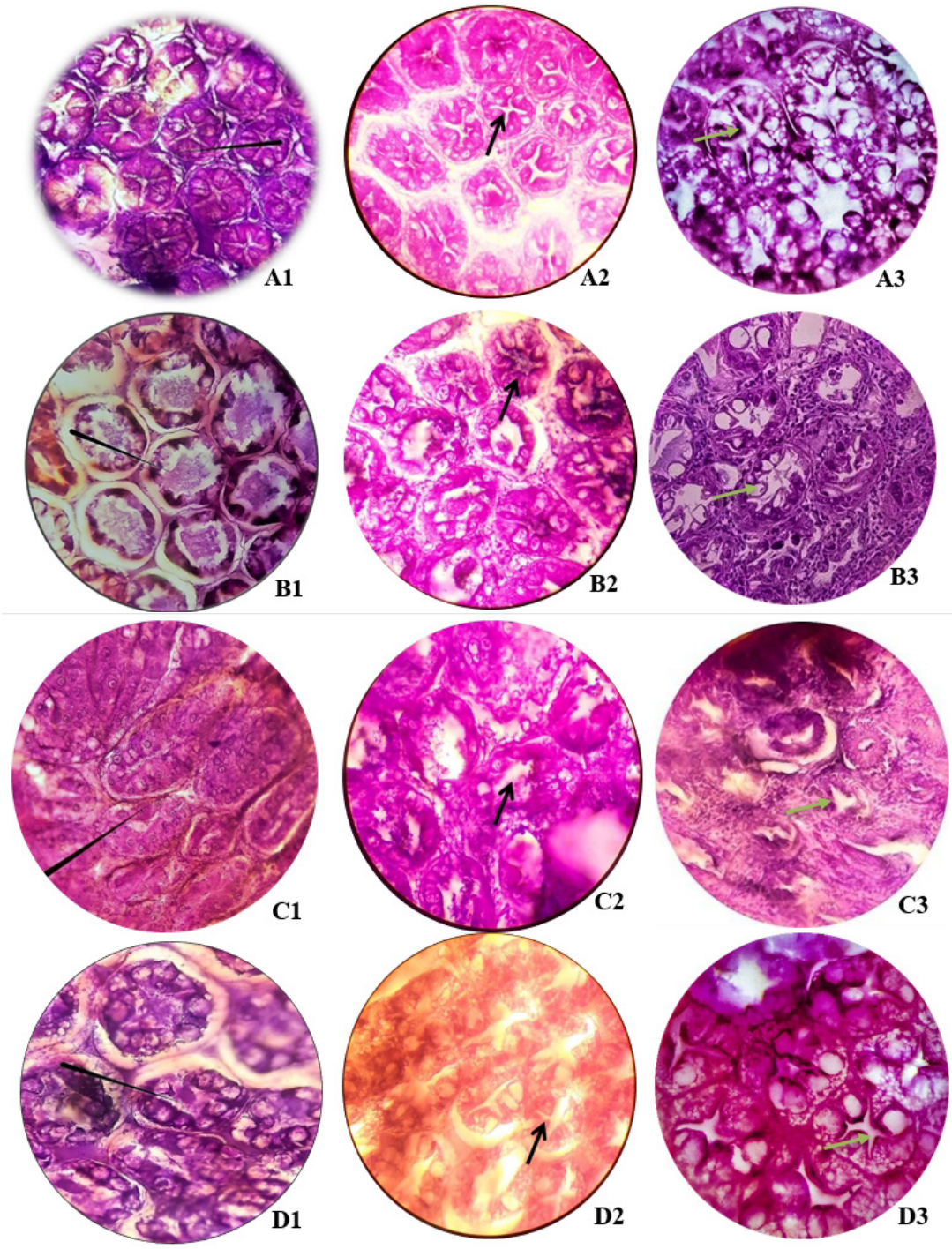

Figure 6 Shrimp's hepatopancreas tissue was taken under the microscope at 40X objective. (A) Shrimp hepatopancreatic tissue was not susceptible to bacteria (-) (A1: postlarvae; A2: shrimp 30-45 days old; A3: shrimp 55-60 days old). (B) Infected hepatopancreas tissue, showing signs of necrosis type 1 (B1: postlarvae; B2: shrimp 30-45 days old; B3: shrimp 55-60 days old). (C) Infected hepatopancreas tissue, showing signs of necrosis type 2 (C1: postlarvae; C2: shrimp 30-45 days old; C3: shrimp 55-60 days old). (D) Hepatopancreas tissue in the treatment trial (D1: postlarvae; D2: shrimp 30-45 days old; D3: shrimp 55-60 days old).

: stellate structure of postlarvae, shrimp 30-45 days old, shrimp 55-60 days old respectively in stage A,B,C,D

Histopathology

The results of histological analysis on shrimp samples at all 3 different ages: from postlarvae to adult shrimp, the specific histological features are shown in Figure 6.

In all age groups studied, for shrimp not susceptible to *V. parahaemolyticus*, the normal shrimp hepatopancreas with lumens had a pronounced stellate structure without cell shedding (Figures 6A1, A2, and A3). In the first stage, when shrimp are newly infected with *V. parahaemolyticus*, the hepatopancreas when observed under the microscope, will show signs of necrosis 1: the star-shaped structure in the lumen of the tube gradually disappears due to the peeling of the cells. Some cells with enlarged nuclei and darker dye-stained cytoplasm have an infiltrate of intraluminal hematopoietic cells (Figures 6B1, B2, and B3). When the number of bacteria in the shrimp hepatopancreas increased and reached the pathogenic threshold, the necrosis of the cells increased. Observation under the microscope will show signs of necrosis 2. The structure of the hepatopancreatic tubule has completely disappeared, making it impossible to distinguish the cell types. Besides, there is the appearance of countless blood cells surrounding the necrotic mass (Figures 6C1, C2, and C3). The characteristics of hepatopancreatic tissue, when infected with *V. parahaemolyticus*, were similar to those described by Lightner et al., 2012; Joshi et al., 2014; and Aguilar-Rendón et al., 2020, which describe in detail the characteristics of histopathological features of acute hepatopancreatic necrosis disease. After treatment with bacteriophages, the recorded results initially show the influence of the phage on shrimp infected by *V. parahaemolyticus*: the shrimp hepatopancreatic tissue structure gradually recovered, some tissues regained their stellate structure, and the number of sloughed cells and blood cells in the tubule lumen decreased significantly (Figures 6D1, D2, and D3). The results are also similar to the study by Jun et al. 2017, which documented signs of recovery in shrimp injured by acute hepatopancreatic necrosis disease after bacteriophage treatment.

DISCUSSION

From the above results, it can be seen that in all 3 groups with different ages, namely postlarvae, 30-45 days old shrimp, and 55-60 days old shrimp, in the healthy shrimp treatment (control shrimp), shrimp remained healthy, active, ate quickly and ran out of food according to the ration. Shrimp were brightly colored, had a quick response, no external lesions, dark hepatopancreas, and full intestines. This shows that when there is no impact of infectious bacteria, shrimps develop normally, so the pathological signs of shrimp are caused by the infection of pathogenic bacteria. Meanwhile, in the treatment of diseased and treated shrimp, during the infection period, similar pathological signs appeared as described by Lightner et al., 2012 and Tran et al., 2013, such as anorexia, empty intestine, pale hepatopancreas, and soft shell phenomenon. After shrimp were infected and 50% of shrimp died in the infected and treated shrimp tanks, bacteriophage was introduced into the treated shrimp tanks. It was observed that shrimp began to eat again after 24 h of phage inoculation; they were less sluggish. When observed under the microscope, the hepatopancreas

gradually recovered, became darker, and the intestines were full because the shrimp had eaten again. The hepatopancreas tissue samples of shrimp strain *V. parahaemolyticus* showed signs of peeling and falling into the lumen of the hepatic duct and the phenomenon of melanization of black blood cells around the necrotic area. After bacteriophage treatment, the stellate structure gradually recovered, no hematoma around the tubules was found, reducing of sloughing cells and blood cells in the lumen of the tube, and no melanization was observed. Based on the obtained results, it can be concluded that bacteriophages are promising as an alternative biological solution to antibiotics in the treatment of diseases caused by *Vibrio* spp. cause.

CONCLUSIONS

V. parahaemolyticus is one of the dangerous bacteria groups for shrimp; indeed, this has been verified through bacterial infection experiments; Shrimp showed obvious disease symptoms, and especially shrimp mortality increased gradually over time. However, the research results also showed a positive effect of phages on the treatment of shrimp diseases. The first is the direct effect of phages on bacterial concentration. The rapid decrease in bacterial concentration helped shrimp to survive through pathological and histopathological signs. Histopathological investigations recorded signs of recovery in shrimp with typical lesions caused by acute hepatopancreatic necrosis disease after being treated with bacteriophages. Furthermore, pathological signs of shrimp after infection with *V. parahaemolyticus* and after bacteriophage treatment showed the potential phage application in preventing diseases caused by the bacteria *V. parahaemolyticus* in shrimp.

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AUTHOR CONTRIBUTIONS

Le Hoang Bảo Ngoc, Tran Thi Lieu; Investigation, methodology, formal analysis, manuscript preparation, editing, and finalization
Truong Thi Bich Van; Conceptualization and design of the experiment, investigation, supervision, editing, and finalization
Le Viet Dung; Conceptualization and design of the experiment, investigation, editing, and finalization

CONFLICT OF INTEREST

We have no conflict of interest.

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