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Distribution of *Vibrio parahaemolyticus* in pacific white shrimp *Litopenaeus vannamei* through immersion as a natural infection model

Distribusi *Vibrio parahaemolyticus* pada udang vaname *Litopenaeus vannamei* melalui perendaman sebagai model infeksi alami

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ABSTRACT

Vibrio parahaemolyticus is a pathogenic bacterium that indusces mortality is pacific white shrimp. This study aimed to analyze the distribution of *V. parahaeomyticus* in shrimp through immersion and evaluate, polymerase chain reaction, histopathology, and immune response. The study employed two treatments with four replicates each. Shrimp were infected through immersion with *V. parahaemolyticus* at a concentration of 10^7 CFU/mL for 30 minutes, followed by transferring the shrimp to a rearing container. Gill, hepatopancreas, gut, and hemolymph samples were collected at 6, 12, and 24 hours post-infection. Infected shrimp exhibited clinical symptoms such as a pale body and hepatopancreas, along with empty gut. Shrimp mortality reached 97.08%, while no mortality occurred in the control grouph. The abundance of *V. parahaemolyticus* in organs exceeded 105 CFU/mL. Histopathological examination revealed mild, moderate, and severe damage. PCR confirmation tests at 12 and 24 hours yielded positive results, with a DNA band at 230 bp. Immune responses, including total hemocytes, phagocytic activity, *phenoloxidase*, and *respiratory burst*, demonstrated significantly different results ($P<0.05$). It was concluded that the distribution of *V. parahaemolyticus* in the shrimp body, specifically in the gills, hepatopancreas, and gut, exceed 10^5 CFU/mL, as confirmed by a 230 bp DNA band in the PCR test. Tissue damage, manifested as lesions and necrosis, occurred in the gills, hepatopancreas, and gut. Additionally, the imun response of Vibrio-infected shrimp was found to be lower than that of uninfected shrimp (negative control).

Keywords: distribution, immersion, *Litopenaeus vannamei*, *Vibrio parahaemolyticus*

ABSTRAK

Vibrio parahaemolyticus adalah bakteri patogen yang menyebabkan kematian pada udang vaname. Penelitian ini bertujuan menganalisis distribusi *V. parahaemolyticus* pada udang melalui perendaman serta kerusakan yang ditimbulkan melalui pengamatan gejala klinis, angka lempeng total bakteri, polymerase chain reaction, histopatologi dan respons imun. Penelitian menggunakan dua perlakuan dan empat ulangan. Udang diinfeksi melalui perendaman menggunakan *V. parahaemolyticus* 10^7 CFU/mL selama 30 menit, kemudian udang dipindah pada wadah pemeliharaan. Pengambilan organ insang, hepatopankreas, usus dan hemolim dilakukan pada jam ke-6, 12, dan 24 pascainfeksi. Udang terinfeksi memiliki gejala klinis seperti tubuh dan hepatopankreas pucat, usus kosong. Mortalitas udang mencapai 97.08% dan tidak ada kematian pada udang kontrol. Kelimpahan *V. parahaemolyticus* pada organ lebih dari 10^5 CFU/mL. Pengamatan histopatologi menunjukkan kerusakan ringan, sedang, hingga parah. Uji konfirmasi PCR jam ke-12 dan ke-24 menunjukkan hasil positif pada 230 bp. Respons imun seperti total hemosit, aktivitas fagositik, *phenoloxidase*, *respiratory burst* menujukkan hasil yang berbeda nyata ($P<0.05$). Disimpulkan bahwa distribusi *V. parahaemolyticus* di dalam tubuh udang pada terdapat pada insang, hepatopankreas dan usus lebih dari 105 CFU/mL dan terkonfirmasi uji PCR pada pita DNA 230 bp. Kerusakan jaringan seperti lesi dan nekrosis terjadi pada insang, hepatopankreas, dan usus. Respons imun udang yang terinfeksi Vibrio lebih rendah dibandingkan udang yang tidak terinfeksi (kontrol negatif).

Kata kunci: distribusi, *Litopenaeus vannamei*, perendaman, *Vibrio parahaemolyticus*

INTRODUCTION

Litopenaeus vannamei (Pacific white shrimp) is the most popular species cultivated in Asia and Latin America (Kongchum *et al*., 2016). For the Crustacea group, the production of white shrimp ranked first, reaching 5.8 million tons in 2020 (FAO, 2022). With the increasing shrimp production, there are challenges in the cultivation process, especially regarding infectious pathogen attacks. Pathogen infections, especially bacteria, have been a constraint in white shrimp farming activities, as *Vibrio* can cause massive mortality, resulting in decreased production (Thitamadee *et al*., 2016). The emergence of diseases is a dynamic process involving interactions between the host, pathogen, and environment (Kenconojati *et al*., 2023; Valente & Wan, 2021).

In aquaculture, if the balance between these three factors is maintained, diseases will not occur. Diseases emerge when the environment is suboptimal, and the balance is disrupted. Generally, the emergence of shrimp diseases is the result of complex interactions among three components in the aquaculture ecosystem: weak hosts (shrimp) due to various stressors, virulent pathogens, and suboptimal environmental quality, which can be abiotic and biotic. Abiotic factors include various interdependent factors that can affect living organisms, such as temperature, sunlight, soil, water, and chemicals. Biotic factors include live feed, plants, animals, and their interactions.

The survival and well-being of an organism depend on food and interactions with other living creatures. The initiation of diseases, particularly involving higher vertebrates, is a complex process that involves more than just the interaction between the host, pathogen, and environment. Host vulnerability, pathogen virulence, and environmental factors all must interact (Kenconojati *et al*., 2023). Another possible infection route is horizontal infection through water, feed, and carriers. One of the pathogenic bacteria infecting shrimp is *Vibrio* bacteria. *Vibrio i*s a normal microflora in shrimp or aquatic media and can act as a major pathogen when its density increases in water or as an opportunistic pathogen in secondary infections (Lee *et al*., 2015).

One of the *Vibrio* bacteria attacking white shrimp is *V. parahaemolyticus*. This bacterium is Gram-negative, halophilic, single-celled, rod-shaped, found in estuarine, marine, and coastal environments (Hasrimi *et al*., 2017). *Vibrio* bacteria can cause Vibriosis disease, resulting in mass mortality at all shrimp stages, from nauplius, zoea, mysis, and postlarvae in hatcheries to mature shrimp in grow-out ponds (Chandrakala & Priya, 2017). *V. parahaemolyticus* are the main pathogens causing acute hepatopancreatic necrosis disease (AHPND), which can lead to high mortality in cultured shrimp (Tran *et al*., 2013). This situation causes serious economic losses in shrimp farming (Shinn *et al*., 2018). Infected shrimp exhibit clinical symptoms such as decreased appetite, changes in body color, pale hepatopancreas, soft shell, and passive swimming (Zorriehzahra & Banaederakshan, 2015; Saputra *et al*., 2023). Disease outbreaks in shrimp farming occur naturally, possibly due to an imbalance between the environment, pathogens, and shrimp.

Further study is needed to understand how diseases affect cultured shrimp and cause mortality. This study used an immersion model as a transmission test. Previous studies that using immersion methods, such as (Agriandini *et al*., 2021), stated that *Mycobacterium fortoitum* adheres to and enters through the skin and gills, then spreads through the bloodstream to target organs, thereby increasing the number of leukocytes and respiratory burst (non-specific immune response). *V. parahaemolyticus* can cause Height Lethal Vibrio Disease (HLVD) and AHPND. HLVD is more aggressive than AHPND because it produces specific toxins (Yang *et al*., 2022).

Research has also been conducted on zebrafish using immersion and injection methods, where the infection method significantly affects the immune response, i.e., the infection method will activate innate response genes and induce different defense pathways (Guo *et al*., 2021). Transmission testing is one method to understand the pathogenesis of *V. parahaemolyticus* in shrimp. There are two transmission tests: horizontal transmission, which is the spread from one host to another through the environment, and vertical transmission, which is the spread from parent to offspring. A model of infection like that occurring in nature is the immersion method. Therefore, this study aims to analyze the distribution of *V. parahaemolyticus* on shrimp bodies through immersion and the resulting damage through clinical symptom observation, total bacterial plate count, polymerase chain reaction, histopathological analysis, and immune response.

MATERIAL AND METHOD

Time and place

This research was conducted from October 2022 to March 2023. The study was carried out at the Laboratory of Aquatic Organism Health, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University.

Shrimp test

The test shrimp used were Pacific white shrimp (*L. vannamei*) PL 8, then reared until reaching a size of 1.51 ± 00.04 g, obtained from the PT. Syaqua Indonesia hatchery in Serang Regency, Banten Province, Indonesia. The bacteria used were *V. parahaemolyticus* obtained from the collection of the Laboratory of Aquatic Organism Health, IPB University.

Preparation of shrimp containers and maintenance

The maintenance used containers sized 60×30×30 cm^3 totaling 32 units. Prior to use, white shrimp PL 8 were first reared with a protein-rich feed of 30-40%. Feeding was conducted five times a day at satiation at 06:00, 10:00, 14:00, 17:00, and 20:00 WIB. Shrimp were reared at a temperature of 28°C, pH 7.42, DO 4.3 mg/L, and salinity of 28 ppt.

Bacterial preparation

The *V. parahaemolyticus* obtained were subcultured on Thiosulfate Citrate Bile Sucrose (TCBS) selective media and incubated at 28°C for 24 hours. The resulting culture was then subcultured several times to obtain pure isolates. The selected isolates were then identified using polymerase chain reaction and cultured as stocks on sea water complete (SWC) media. Bacteria from the SWC media were taken in a volume of 100 µL, rinsed twice, serially diluted, and then 50 µL was plated on TCBS supplemented with rifampicin 50 µg/mL. Resistant test bacteria were then subjected to Koch's postulates by immersion method. After that, shrimp organs were taken for total plate count to obtain new isolates which could then be stored as stocks for further testing.

Determining lethal concentration 50%

The determination of the lethal concentration 50% (LC_{50}) value followed the Reed and Muench method (1938). A total of 40 shrimp were used for each concentration, with 10 shrimp immersed in 1 L of seawater, resulting in the requirement of 6 L of seawater for each concentration, with LC50 concentrations of 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 CFU/mL + negative control. Observations were made by counting the number of dead and alive shrimp over a period of three days. The bacterial dose obtained in the LC_{50} test would be used for the challenge test. The LC_{50} value was calculated using the formula:

$$LC_{50} = \text{Log dose A} + \frac{50\% - B}{A - B}$$

Note:
A = Mortality >50%
B = Mortality <50%

Shrimp challenge test

The challenge test used 80 shrimp with four repetitions. Infection with *V. parahaemolyticus* was conducted by immersion for 30 minutes using the LC_{50} dose, which was 10^7 CFU/mL, and then transferred to a new aquarium. Organ sampling was performed at 6, 12, and 24 hours post-challenge. Observations included clinical symptoms, cumulative mortality, total bacterial plate count (gills, hepatopancreas, and gut), immune response (total hemocyte count, phagocytic activity, phenoloxidase activity, respiratory burst), organ histopathology (gills, hepatopancreas, and gut), and polymerase chain reaction (hepatopancreas). The shrimp were maintained at a temperature of 28°C, pH 7.42, dissolved oxygen level of 4.3 mg/L, and salinity of 28 ppt.

Observation parameters

Clinical symptoms

Clinical symptom observation was conducted post-challenge at 6, 12, and 24 hours. Clinical symptoms of shrimp affected by *V. parahaemolyticus* include decreased appetite, slow swimming, empty gut, pale coloration, and pale hepatopancreas (Zorriehzahra & Banaderakshan, 2015; Saputra *et al*., 2023).

Cumulative mortality

To determine the mortality of white shrimp post-infection with *V. parahaemolyticus* through immersion, cumulative mortality can reveal the pattern of shrimp mortality. The number of white shrimp deaths post-infection is calculated using the formula (Effendie, 1997) as follows:

$$\text{Cumulative mortality (\%)} = \frac{N_t}{N_0} \times 100$$

Note:
Nt = The number of shrimp at the end of the rearing period
No = The number of shrimp at the initial of the rearing period

Total bacterial plate count

Organ retrieval such as hepatopancreas, gills, and gut during post-immersion observation was conducted at 6, 12, and 24 hours post-reinfection. The bacterial count was performed using serial dilution and the pour plate method. Each target organ weighing 0.1 g was homogenized and dissolved in 1 mL of sterile PBS, then vortexed, and subjected to serial dilution. The entire dilution was spread on agar media in 50 µL aliquots and then incubated at 37°C for 24 hours. Colony counts were conducted (30-300) using the formula (Madigan *et al*., 2014) as follows:

$$\sum \text{bacteria colony (log cfu/g or log cfu/mL)} = \sum \text{colony} \times \frac{1}{\text{Spread volume (mL)}} \times \frac{1}{\text{Dilution factor}}$$

Histopathology

Histopathological observations were conducted at 6, 12, and 24 hours post-challenge, involving the examination of the hepatopancreas, gills, and gut. The histopathological method, according to (Short & Meyers, 2000), was stained using hematoxylin and eosin (HE) staining. Changes were observed by calculating the percentage of cell damage (degeneration and necrosis) in each organ from five fields of view. Degenerative cells are cell abnormalities resulting from minor injuries, whereas necrotic cells are conditions of cell injury resulting in early cell death and living tissue factors with three replications (Izwar *et al*., 2020), using the following formula:

$$P\ (\%) = \frac{\sum KS}{\sum TS} \times 100$$

Note:
P (%) = Percentage of normal cells or degenerated cells or necrotic cells
∑ KS = The total number of normal cells or necrotic cells in five fields of view
∑ TS = Total number of cells in five fields of view

Scoring the gills, hepatopancreas, and gut of shrimp refers to the study by Wolf *et al*. (2015) with a graded scoring system (Table 1).

Polymerase chain reaction

Sample preparation

The preparation was conducted at the Fish Health Laboratory, Bogor Agricultural University. Samples were rinsed using distilled water and dissected to extract the target organs using a dissecting set. The target organs taken for bacterial examination were the hepatopancreas at 12 and 24 hours post-challenge.

Extraction

Before the process began, the incubator machine was preheated to 60°C. 30 mg of the sample was placed into a 1.5 ml microtube. 200 µl of GT buffer and 20 µl of proteinase K were added, then ground using a micropestle. It was incubated at 60°C for 30 minutes (vortexed every five minutes), and the Elution Buffer was also incubated at the same temperature. 200 µl of GBT buffer was added and vortexed (mixed) for five minutes.

It was incubated for 20 minutes at 60°C (vortexed every five minutes), then centrifuged at 16.000 ×g for two minutes. 400 µl of the supernatant was transferred to a new 1.5 ml tube containing 200 µl of 96% absolute ethanol. 600 µl of the sample was transferred to the GS column with its 2 ml collection tube. Then it was centrifuged at 16.000 ×g for two minutes. The Collection Tube (bottom part) was discarded and the GS column was transferred to a new 2 mL Collection Tube.

400 µL of W1 buffer was added to the GS Column and centrifuged at 16.000 ×g for 30 seconds. The liquid in the collection tube was discarded and the GS column was placed back

Table 1. Histopathology scoring and degree of damage to gill, hepatopancreas, and intestinal tissues.

Score	Necrotic	Degree of damage
1	Necrotic <25% Total fields of view	Normal
2	Necrotic 25-<50% Total fields of view	Mild damage
3	Necrotic 50-<75% Total fields of view	Moderate damage
4	Necrotic >75% Total fields of view	Severe damage

into the 2 ml Collection Tube. 600 µl of Wash Buffer was added and centrifuged at 16,000 ×g for 30 seconds. The liquid in the collection tube was discarded and the GS column was placed back into its collection tube. It was centrifuged at 16.000 ×g for three minutes to dry the column matrix (GS Column). The dried GS column was transferred to a new 1.5 ml tube. 100 µl of pre-heated Elution buffer (heated to 60°C) was added to the middle of the column matrix. It was left to sit for five minutes. Then it was centrifuged at 16.000 µl ×g for 30 seconds. Ready for amplification.

Amplyfication

The specific primers used for AHPND confirmation were AP4 (pirA and pirB) (Table 3), 1 µL of the DNA target fragment was amplified in 9 µL of Master Mix reagent (5 µL 2× MyTaq HS Red Mix, 0.8 µL Primer Reverse, 0.8 µL Primer Forward, and 2.4 µL Nuclease Free Water). The PCR reaction was conducted on a peqSTAR XS Thermocycler (The peqSTAR, Germany). Testing was performed using the nested PCR method. The specific PCR gene primers for AP4 (pirA and pirB) were as follows: pir AB Step 1 AP4F1-5'-ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC-3' AP4R1-5'-ACG-ATT-TCG-ACG-TTC-CCC-A, pir AB Step 2 AP4F2-5'-TTG-AGA-ATA-CGG-GAC-GTG-GG-3', AP4R2-5'-GTT-AGT-CAT-GTG-AGC-ACC-TTC-3'.

Immune response

Total hemocyte count

Observation of total hemocyte count (THC) followed the method by Huynh *et al*. (2018), which involved extracting 0.1 mL of shrimp hemolymph from the ventral sinus using a one-drop syringe (1 mL) pre-filled with anticoagulant fluid in a 1:2 ratio. Subsequently, the hemolymph was pipetted onto a hemocytometer and covered with a cover glass. The total number of hemocytes was observed and counted under a microscope at 100× magnification. The calculation of THC was based on the following formula:

$$\text{THC} = \frac{\text{Observed number cells}}{\text{Observed squares}} \times \frac{1}{\text{Hemacytometer volume}} \times \text{dilution factor}$$

Phagocytic activity

The phagocytic activity (PA) was observed by mixing 100 µl of shrimp hemolymph with 25 µl of *Staphylococcus aureus* suspension (10^7 CFU/mL) and then incubating for 20 minutes. A smear slide was prepared and dried. It was then fixed using methanol and left for five minutes before being dried again. After staining with Giemsa stain, it was observed under a microscope at 400 times magnification (Anderson & Siwicki, 1993).

Phenoloxidase activity

The phenoloxidase activity (PO) was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA). The method followed the procedure described by Liu and Chen (2004), which involved mixing 1 mL of hemolymph and anticoagulant and centrifuging at 1500 rpm for 10 minutes. The resulting supernatant was discarded, and the pellet was gently resuspended in 1 mL of cacodylate citrate buffer solution (0.01 M sodium cacodylate, 0.45 M sodium chloride, and 0.10 M Trisodium citrate, pH 7). This solution was then centrifuged again at 1500 rpm for 10 minutes.

The supernatant was discarded, and the pellet was resuspended in 200 µL cacodylate-citrate buffer. The suspension was then incubated with 50 µL trypsin (1 mg/mL cacodylate buffer) as an activator for 10 minutes at a temperature of 25 to 26°C. After five minutes, 50 µL LDOPA (3 mg/L cacodylate buffer) was added to the pellet suspension, followed by the addition of 800 µL cacodylate buffer. The contents of the standard solution were 100 µL cell suspension, 50 µL cacodylate buffer (to replace trypsin), and 50 µL L-DOPA. The optical density (OD) was measured using a spectrophotometer at a wavelength of 490 nm. The PO activity was expressed as dopachrome formation in 100 µL of hemolymph.

Respiratory burst activity

The respiratory burst (RB) activity was measured based on the reduction of nitroblue tetrazolium (NBT) to formazan as an indicator of superoxide anion production. Hemolymph and anticoagulant were mixed in a volume of 300 µL and incubated for 30 minutes at room temperature. Subsequently, the mixture was centrifuged at 3000 rpm for 20 minutes. The supernatant was discarded, and 100 µL of NBT (0.3%) was added, followed by a two-hour incubation at room temperature.

After incubation, the solution was centrifuged at 3000 rpm for 10 minutes, the supernatant was discarded, and 100 µL of absolute methanol was added. It was then centrifuged again at the same speed and duration. The resulting pellet was washed with 70% methanol. Then, 120 µL

of 2M potassium hydroxide (KOH) and 140 µL of dimethyl sulfoxide (DMSO) were added, and the sample was transferred to a microplate well. The respiratory burst value was measured using a microplate reader at a wavelength of 630 nm. KOH or NBT per 10 µL of hemolymph was used as a blank (Cheng *et al*., 2004).

Data analysis

The data obtained, including clinical symptoms, cumulative mortality, total bacterial plate count, total hemocyte count, phagocytic activity, phenoloxidase activity, and respiratory burst activity, were analyzed quantitatively using SPSS 26 software. If the results of the Analysis of Variance (ANOVA) indicate a significant difference (P<0.05), post-hoc testing using Duncan's test with a confidence interval of 95% was conducted. Histopathological tissue data were analyzed using the Kruskal-Wallis test, and if P<0.05, further analysis was conducted using the Mann-Whitney test.

RESULT AND DISCUSSION

Result

Clinical symptoms

The observation of clinical symptoms post-infection (Figure 1) showed that the shrimp's body color became pale, the hepatopancreas appeared pale, the gut looked empty, the shell softened, and swimming behavior became passive. No clinical symptoms were found in the shrimp in the negative control group.

Cumulative mortality

The results of cumulative mortality observations of white shrimp post-infection are presented in (Figure 2). The cumulative mortality values indicate that mortality occurred in shrimp immersed in *V. parahaemolyticus*, with 27.08% at the 6th hour and 58.75 ± 4.99% at the 12th hour, increasing to 97.08% at the 24th hour post-infection. In contrast, there was no mortality in the negative control group until the end of the observation period.

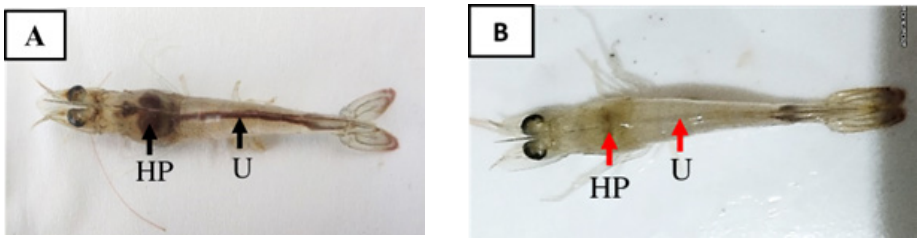

Figure 1. Clinical symptoms of post infection white shrimp: (A) Negative control and (B) Treatment-pale body color, empty gut, pale hepatopancreas.
Note: HP: Hepatopancreas and U: Gut.

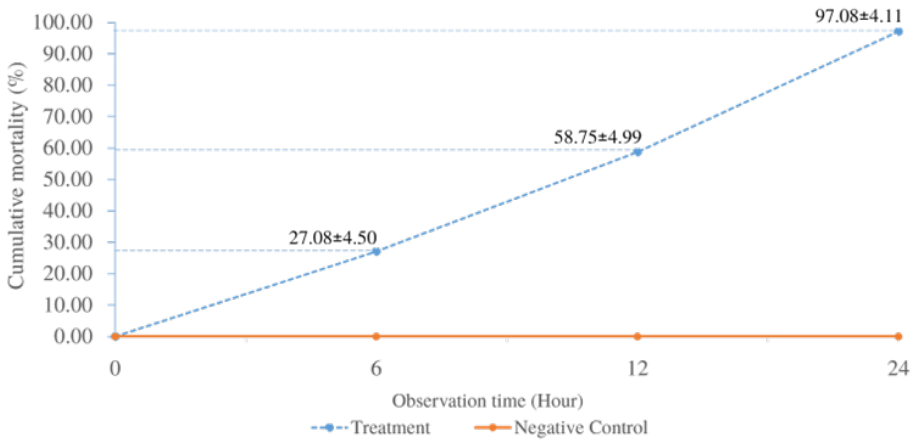

Figure 2. Cumulative mortality of white shrimp at 6, 12, and 24 hours post-infection with *V. parahaemolyticus.* Cumulative mortality values exhibited significanly different result between treatments and the negative control (duncan p<0.05).

Total plate count

The observations of total *V. parahaemolyticus* Rf^R and total Vibrio count in the gill, hepatopancreas, and gut organs began to appear at the 6th hour post-infection. *V. parahaemolyticus* Rf^R levels in the gills peaked at the 12th hour with $7.0\times10^5\pm0.53$. The hepatopancreas showed the highest levels at the 24th hour with $2.0\times10^6\pm0.03$, showing significant differences at each observation time except in the gut, which had the highest levels at the 12th hour with $2.3\times10^6\pm0.34$. In contrast, the total Vibrio count in the gill organs did not vary significantly between observation times, with the highest value recorded at the 6th hour, at $3.8\times10^6\pm0.28$. The hepatopancreas showed significant variations at each observation time, peaking at the 6th hour with $9.7\times10^6\pm0.04$. In the gut, there were no significant differences, with the highest value observed at the 12th hour, at $6.0\times10^6\pm0.24$, as shown in Table 2.

Histopathology

Histopathological observations of target organs such as the gills, hepatopancreas, and gut in both the negative control (A) and treatment groups (B) are presented in (Figure 3). The images reveal visual changes and differences between the control and treated organs. Post-infection with *V. parahaemolyticus* shows damage to the gills, including necrosis and rupturing of the gill lamellae, lesions in the hepatopancreatic tubules, and damage to the intestinal epithelium with cell wall degradation. The histopathological scoring results indicating cell damage are shown in (Table 3).

Polymerase chain reaction

The electrophoresis results of the confirmation using polymerase chain reaction (PCR) for AHPND testing post-infection are presented in (Figure 4). The PCR results show that the hepatopancreas organ at 12 and 24 hours post-infection displays a DNA band of 230 bp, aligning with the positive control. This PCR result confirms that the bacteria used during the challenge test stage were *V. parahaemolyticus* with the AHPND strain.

Table 2. Results of total bacterial plate counts in gill, hepatopancreas, and gut organs at 6, 12, and 24 hours post-infection.

Organ	Hour	*V. parahaemolyticus* Rf^R (CFU/mL)	*Total Vibrio Count* (CFU/mL)
Gill	6	$2.0\times10^5\pm0.46$	$3.8\times10^6\pm0.28$
	12	$7.0\times10^5\pm0.53$	$2.7\times10^6\pm0.12$
	24	$1.3\times10^4\pm0.48$	$2.0\times10^6\pm0.31$
Hepatopancreas	6	$3.9\times10^5\pm0.45$	$9.7\times10^6\pm0.04$
	12	$7.4\times10^5\pm0.11$	$4.0\times10^6\pm0.03$
	24	$2.0\times10^6\pm0.03$	$2.1\times10^6\pm0.13$
Gut	6	$1.0\times10^5\pm0.06$	$2.0\times10^6\pm0.25$
	12	$2.3\times10^6\pm0.34$	$6.0\times10^6\pm0.24$
	24	$2.0\times10^6\pm0.20$	$5.0\times10^6\pm0.23$

Note: Superscript letters in the same organ and column indicate statistically significantly different results. (Duncan $P<0.05$).

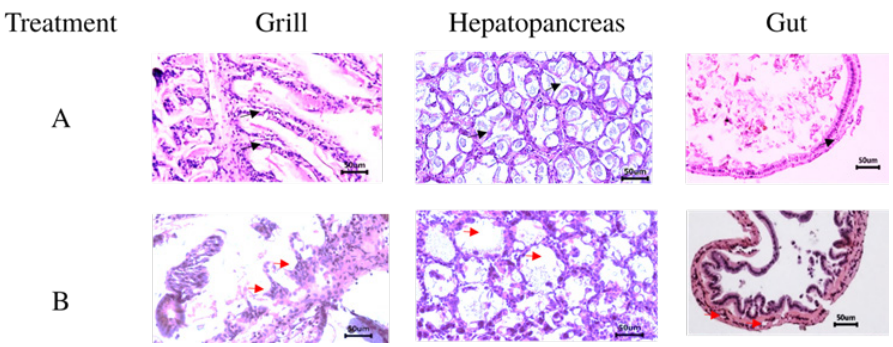

Figure 3. Histopathological observations on gill, hepatopancreas, and gut organs post-infection.

Immune response

The observations for total hemocyte count, phagocytic activity, phenoloxidase activity, and respiratory burst in white shrimp are presented in (Table 4). The total hemocyte count observed at 6, 12, and 24 hours post-infection were 5.81 ± 0.03c, 5.76 ± 0.10bc, and 5.53 ± 0.07ab, respectively. Phagocytic activity values were 82.36 ± 8.48b, 75.69 ± 14.74b, and 64.71 ± 16.96b. The phenoloxidase activity results were 0.18 ± 0.01b, 0.17 ± 0.01b, and 0.15 ± 0.01b. Meanwhile, respiratory burst observations yielded results of 0.10 ± 0.01b, 0.10 ± 0.01b, and 0.09 ± 0.01b. The results indicate significant differences (P<0.05) between the negative control and the treatment groups.

Table 3. Total score in gill, hepatopancreas and gut organs post-infection.

Organ	Hour	Percentage of necrotic (%)	Score	Degree of damage
Gill	6	26.67 ± 8.65^{a}	2	Mild damage
	12	32.67 ± 6.24^{a}	2	Mild damage
	24	49.00 ± 12.57^{a}	2	Mild damage
Hepatopancreas	6	65.67 ± 9.74^{a}	3	Moderate damage
	12	87.67 ± 3.68^{b}	4	Severe damage
	24	88.67 ± 2.87^{b}	4	Severe damage
Gut	6	47.67 ± 6.24^{ab}	2	Mild damage
	12	67.00 ± 25.66^{b}	3	Severe damage
	24	70.67 ± 13.91^{b}	3	Severe damage

Note: Superscript letters in the same organ and column indicate statistically significantly different results. (*Mann Whitney*) P<0.05).

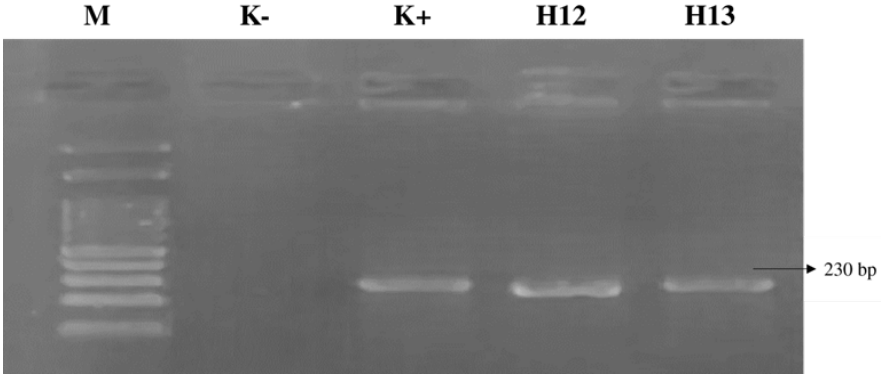

Figure 4. Confirmation of AHPND in white shrimp postinfection with *V. parahaemolyticus* M= Marker; K-= Negative Control; K+= Positive Control; H12= 12th hour hepatopancreas; H24= 24th hour hepatopancreas.

Table 4. Observation of post-infection immune response.

Parameter	**Treatment**	**Observation time (hour)**		
		6	**12**	**24**
Total Hemocyte Counts (THC (×10^{3} cell/mm^{-3})	Negative	5.42 ± 0.27^{a}	5.38 ± 0.15^{a}	5.28 ± 0.12^{a}
	Treatment	5.81 ± 0.03^{c}	5.76 ± 0.10^{bc}	5.53 ± 0.07^{ab}
Phagocytic Activity (AF) (%)	Negative	29.80 ± 8.39^{a}	26.99 ± 6.58^{a}	28.90 ± 4.58^{a}
	Treatment	82.36 ± 8.48^{b}	75.69 ± 14.74^{b}	64.71 ± 16.96^{b}
Phenoloxidase (PO) ƛ 490 nm (100µL)	Negative	0.12 ± 0.00^{a}	0.12 ± 0.01^{a}	0.12 ± 0.00^{a}
	Treatment	0.18 ± 0.01^{b}	0.17 ± 0.01^{b}	0.15 ± 0.01^{b}
Respiratory Burst (RB) (ƛ 630 nm (10 µL))	Negative	0.08 ± 0.01^{a}	0.07 ± 0.01^{a}	0.07 ± 0.01^{a}
	Treatment	0.10 ± 0.01^{b}	0.10 ± 0.01^{b}	0.09 ± 0.01^{b}

Note: Superscript letters in the same parameter and column indicate statistically significantly different results. (Duncan P<0.05).

Discussion

The spread of diseases in shrimp can occur horizontally and vertically. Horizontal disease transmission occurs due to contact from one shrimp to another, while vertical transmission occurs from parent to offspring (Arbon *et al*., 2022). Disease transmission can occur through immersion, oral, and cohabitation (OIE, 2019). Additionally, invasion or transmission can occur through four ways: direct contact, digestive tract, phoresis (requiring intermediary hosts), and penetrating the skin surface (Hardi, 2015).

Direct contact can occur between healthy shrimp and shrimp infected with bacteria. In the digestive tract, bacterial invasion (entry, attachment, colonization) occurs after ingestion with food. Furthermore, phoresis involves transmission from one host to another through other animals. Subsequently, penetrating the skin surface involves the bacteria attacking the carapace, which then adheres to it. Moreover, *V. parahaemolyticus* is an opportunistic pathogen whose survival depends on the host, and therefore, the spread of this bacterium can occur through direct contact with water contaminated with bacteria, especially *V. parahaemolyticus*.

This study used the immersion method as a model for naturally infecting white shrimp with *V. parahaemolyticus*. The immersion method can directly explain how *V. parahaemolyticus* adheres, enters, proliferates, and spreads virulence within the host's body. Shrimp infected with *V. parahaemolyticus* experience changes in body color, decreased appetite, pale hepatopancreas, soft shells, and passive swimming (Zorriehzahra & Banaederakshan, 2015; Saputra *et al*., 2023). Subsequently, in the final phase of infection, shrimp will sink and lead to death (Hong *et al*., 2015).

White shrimp immersed in *V. parahaemolyticus* result in shrimp mortality observed from cumulative mortality showing initial mortality occurring between the sixth and twenty-fourth hours. The high concentration of *V. parahaemolyticus* during immersion influences the time of death and the number of shrimps observed. The bacterial concentration is suspected to affect shrimp mortality rates based on the observed number of shrimp deaths. Additionally, shrimp size can affect mortality rates; smaller shrimp tend to die faster compared to larger ones (Soto-Rodriguez *et al*., 2022).

Furthermore, the total bacterial count in the hepatopancreas was found to be 10^9 CFU/g, while in the gills, the bacterial density reached 10^5 CFU/mL, and in the hepatopancreas, it reached 10^6 CFU/mL, whereas in the gut, it reached 10^6 CFU/mL. The presence of bacteria in shrimp organs can lead to mortality. According to Sirikhairin *et al*. (2014), if the bacterial density in the hepatopancreas exceeds 10^4 CFU/mL, it can cause lesions and hepatopancreas dysfunction. Moreover, in the gut, according to Soto-Rodriguez *et al*. (2015), bacterial density reached 10^7 CFU/mL, which was lower compared to the findings of this study.

However, in the study by Yang *et al*. (2022), it was stated that a bacterial density of 10^5 CFU/mL could cause mortality of up to 50%. Bacteria abundance is found in each target organ: gills, hepatopancreas, and gut. If we look at Table 1, the total *V. parahaemolyticus* and total vibrio count have varying values. Bacteria enter through the gills and then enter internal organs such as the hepatopancreas and gut. Bacteria will colonize the digestive tract until an outbreak occurs, leading to shrimp mortality. The number of bacteria at each observation hour increases, especially in the hepatopancreas.

According to Kumar *et al*. (2020), bacteria enter orally, then move to the gut, where they release toxins PirA and PirB. Subsequently, bacteria will colonize and replicate, releasing toxins PirA and PirB, which enter the lumen, triggering increased immune responses and RHO pathway activity. RHO signaling pathway activity causes epithelial cell damage in the stomach, allowing gap formation between cells, facilitating the migration of PirA and PirB and *V. parahaemolyticus* into the hepatopancreas intracellularly. From the histopathological observations, the hepatopancreas experienced severe damage compared to the gills and gut.

Shrimp infected with *V. parahaemoyticus* will experience lesions in the hepatopancreas due to continuous bacterial exposure, causing hepatopancreas dysfunction in the acute stage (Dhar *et al*., 2019; Aguilar-Rendon *et al*., 2020; Soto-Rodriguez *et al*., 2018). Hemocytes found in shrimp hemolymph play a crucial role in shrimp's defense system against pathogenic infections (Kulkarni *et al*., 2021; Liu *et al*., 2020). Immune response parameters such as total hemocyte count, phagocytic activity, phenoloxidase, and respiratory burst can be used to monitor the health of shrimp after immersion in test bacteria. Total hemocyte count is one of the parameters that can be used as an indicator to assess stress in

crustaceans (Celi *et al*., 2015; Arifin *et al*., 2014; Adiyana *et al*., 2014). In this study, there was a significant difference between the treatment and negative control, where the treatment challenged with *V. parahaemolyticus* was higher compared to the negative control.

This indicates a decrease in the immune system of shrimp due to the attack of test bacteria, causing stress in shrimp. Stress conditions in shrimp can be hazardous to their health as they become more vulnerable to pathogen attacks. According to (Laith *et al*., 2021), the high number of hemocytes in the hemolymph causes the body's inability to respond, which can reduce the body's immune system. Phagocytic activity describes a non-specific defense system that shrimp undergoes during bacterial infection in the body. The results of phagocytic activity in the study showed a significant difference between the treatment and negative control.

It is suspected that *V. parahaemolyticus* infection reduces phagocytosis and bactericidal activity in shrimp, as shown in previous studies (Lin *et al*., 2017; De Los Santos *et al*., 2022). Phenoloxidase activity plays a crucial role in generating protective immune responses against bacteria, including those caused by *Vibrio* (Oktaviana *et al*., 2014; Zubaidah *et al*., 2015; Kuo *et al*., 2021). Hemocyte numbers vary significantly in response to bacterial infection, the environment, and host life cycles (Zubaidah *et al*., 2015; Kuo *et al*., 2021). Prophenoloxidase plays a significant role in eliciting protective immune responses in crustaceans.

This study showed a, indicating the presence of PO activity against test bacteria. Peroxinectin is a protein associated with the proPO system, where peroxinectin's biological activity is generated simultaneously with proPO system activity (Phan *et al*., 2022). Respiratory burst activity is rapidly reactive oxygen species, including hydrogen peroxide, peroxide anions, and hydroxyl radicals, responsible for killing and minimizing foreign particles, including microbes (Alonzo *et al*., 2017). During respiratory burst activity, reactive oxygen species (ROS) are released as a defense mechanism against bacterial infections.

CONCLUSION

The distribution of *V. parahaemolyticus* in shrimp bodies is found in the gills, hepatopancreas, and gut with a quantity exceeding 10^5 CFU/mL and confirmed by PCR testing showing a DNA band at 230 bp. Clinical symptoms include pale body, pale hepatopancreas, empty gut, soft shell, and passive swimming. Tissue damage such as lesions and necrosis also occurs in the gills, hepatopancreas, and gut. Observations indicate that the immune response of Vibrio-infected shrimp is lower compared to uninfected shrimp (negative control).

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