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Pathogenicity test of *Vibrio parahaemolyticus* in Pacific white shrimp *Penaeus vannamei*

Widanarni[1)], Badar Kautsar [1)], Dinamella Wahjuningrum[1)], dan Muhamad Gustilatov[1*)]

[1] Department of Aquaculture, Faculty of Fisheries and Marine Science, IPB University, Indonesia 16680

*Corresponding author: mgustilatov@apps.ipb.ac.id

Raya Dramaga St. IPB Dramaga Campus, Bogor, Indonesia 16680

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ABSTRACT

One of the common diseases affecting the Pacific white shrimp (*Penaeus vannamei*) culture is vibriosis, caused by infection with *Vibrio* species, including *Vibrio parahaemolyticus*. Certain strains of *V. parahaemolyticus* that carry the PirA and PirB toxins are responsible for causing acute hepatopancreatic necrosis disease (AHPND). This study aimed to assess the pathogenicity of *V. parahaemolyticus* in *P. vannamei* using bacterial isolates from different sources. A challenge test was conducted using *P. vannamei* with an average weight of 0.9±0.1 g, exposed to bacterial concentrations of 10^4 CFU/mL, as determined by lethal concentration 50% (LC_{50}). The experiment followed a completely randomized design with three treatments and three replicates: a control group (K) of uninfected shrimp, shrimp infected with *V. parahaemolyticus* from Tasikmalaya, West Java (Vp-1), and shrimp infected with *V. parahaemolyticus* from Situbondo, East Java (Vp-2). Parameters observed included the confirmation of *V. parahaemolyticus* and AHPND via polymerase chain reaction (PCR), mortality rate, clinical symptoms, bacterial load, and immune response indicators, including total haemocyte count, phagocytic activity, respiratory burst, phenoloxidase activity, and histopathological examination of the hepatopancreas and intestines. Both Vp-1 and Vp-2 isolates were identified as *V. parahaemolyticus* AHPND strains, infecting shrimp with clinical signs such as pale hepatopancreas, empty intestines, and lethargic movement. Tissue damage, including extensive necrosis in the hepatopancreas and intestines, was observed, leading to mortality rates of 73.33-81.67% with an average time to death ranging from 24.28 to 65.44 hours postinfection.

Keywords: AHPND, histopathology, pathogenicity, shrimp, *V. parahaemolyticus*.

1. INTRODUCTION

The Pacific white shrimp (*Penaeus vannamei*) is a highly valuable aquaculture commodity in both Indonesia and globally. In 2022, global production of white shrimp reached 6.8 million tons, making it the most produced aquaculture species, followed by *Crassostrea* spp. and grass carp (FAO, 2022). Global production of white shrimp experienced significant growth between 2010 and 2020, increasing by 3.2 million tons (FAO, 2022). In Indonesia, shrimp production reached 881,599 tons (KKP, 2022), contributing approximately USD 2 billion to global exports in 2019 (FAO, 2022). The advantages of white shrimp include rapid growth, short production cycles, and high stocking densities.

However, increased shrimp production faces several challenges, one of which is disease outbreaks. One of the most common diseases in shrimp farming is vibriosis, caused by bacterial infections from *Vibrio* species, including *Vibrio parahaemolyticus* (Aguirre-Guzman et al., 2010). Infections by *V. parahaemolyticus* can cause abnormalities in shrimp, such as gill necrosis, lethargy, anorexia, and in acute conditions, mortality rates can reach 100% (Abdel-latif et al., 2022).

Certain strains of *V. parahaemolyticus* that produce PirA and PirB toxins can cause hepatopancreatic dysfunction, leading to Acute Hepatopancreatic Necrosis Disease (AHPND) (Han et al., 2015). These toxins damage the hepatopancreas tissue of shrimp, resulting in clinical symptoms that often lead to mass mortality in white shrimp. *V. parahaemolyticus* infections cause substantial economic losses in the shrimp aquaculture industry. The disease was first identified in China in 2009, referred to as Early Mortality Syndrome (EMS) due to rapid mortality during the first month of cultivation, and has since spread to several countries in Asia and the Americas (Hong et al., 2016; Zorriehzahra & Banaederakhshan, 2015). Between 2009 and 2018, AHPND caused by *V. parahaemolyticus* led to global economic losses amounting to USD 44 billion (Tang &

Bondad-Reantaso, 2019). Therefore, developing effective control and prevention strategies is critical to protecting shrimp populations from AHPND.

Pathogenicity tests on *V. parahaemolyticus* in white shrimp are essential for understanding how the bacteria cause infections in shrimp, thus aiding in the development of more effective prevention and treatment strategies. These tests involve exposing healthy shrimp to the test bacteria and monitoring infection symptoms, such as behavioral changes, morphological damage, tissue necrosis, and mortality (Saulnier et al., 2000).

Pathogenicity tests can help shrimp farmers identify and control infections in white shrimp, ultimately reducing the economic losses caused by *V. parahaemolyticus*. This study aims to evaluate the pathogenicity of *V. parahaemolyticus* from different bacterial isolate sources in white shrimp to compare the effects of isolate variation and its relationship to pathogenicity in causing disease in shrimp.

2. MATERIAL AND METHOD

2.1 Experimental Design

This study employed a completely randomized design with three treatments, each with three replicates. The treatments included: white shrimp uninfected by *Vibrio parahaemolyticus* (K) as the control group, white shrimp infected with *V. parahaemolyticus* isolated from Tasikmalaya, West Java (Vp-1), and white shrimp infected with *V. parahaemolyticus* isolated from Situbondo, East Java (Vp-2).

2.2 Bacterial Preparation

The bacteria used in this study were *Vibrio parahaemolyticus* isolated from Tasikmalaya, West Java, and Situbondo, East Java. *V. parahaemolyticus* was cultured on thiosulfate citrate bile salt sucrose (TCBS) agar, with rifampicin (50 µg/mL1) as an

antibiotic marker. Bacterial colonies grown on TCBS were then cultured in liquid sea water complete (SWC) medium and incubated in a water bath shaker at 29°C with a shaking speed of 130 rpm for 18-24 hours.

2.3 Shrimp Maintenance and Challenge Test

The white shrimp (*P. vannamei*) used in this study were sourced from the Specific Pathogen Free (SPF)-certified hatchery of PT Syaqua, Anyer, Banten Province, with an average body weight of 0.9±0.1 g. The shrimp were stocked at a density of 1 shrimp per liter in aquariums measuring 60 cm x 40 cm x 35 cm, containing 20 L of water. They were reared for seven days and fed commercial pellets containing 40% protein four times a day, at 07:00, 12:00, 17:00, and 22:00. The challenge test was conducted using *Vibrio parahaemolyticus* (10^4 CFU/mL; based on LC_{50} test) through an immersion method starting at the beginning of the maintenance period (Widanarni *et al*. 2024). Water quality parameters, including dissolved oxygen, pH, temperature, and salinity, were measured with values ranging from 5.2-6.4 mg/L, 7.58-7.75, 28.1-29.5 °C, and 30-32 g/L, respectively.

2.4 Observation Parameters

2.4.1 Confirmation of *V. parahaemolyticus* and AHPND

The characterization of *V. parahaemolyticus* was performed by culturing the isolates on TCBS and CHROMagar media. Colony color and morphology were observed and compared to reference characteristics for each medium. The confirmation of AHPND in *V. parahaemolyticus* was conducted using the PCR method prior to the challenge test. The PCR process included DNA extraction, amplification, and electrophoresis. Specific primers used for AHPND confirmation were AP4 (targeting *pirA* and *pirB* genes). The AP4 primer set for the first reaction (first-step PCR) was AP4-F1 (5’-ATG-AGT-AAC-

AAT-ATA-AAA-CAT-GAA-AC–3') and AP4-R1 (5'-ACG-ATT-TCG-ACG-TTC-CCC-AA–3'), while for the second reaction (nested PCR), AP4-F2 (5'-TTG-AGA-ATA-AGG-GAC-GTG-GG–3') and AP4-R2 (5'-GTT-AGT-CAT-GTG-AGC-ACC-TTC–3') were used. The amplicon size indicating *V. parahaemolyticus* AHPND strain was 230 bp (OIE 2019).

2.4.2 Mortality and Average Time of Death in White Shrimp

Shrimp mortality observations were made every 12 hours. Changes in the shrimp death rate were monitored to determine the peak and lowest mortality times. Mortality rate, one of the key parameters, indicates the percentage of dead shrimp from the initial population. Mortality rates were observed after the maintenance period following bacterial infection. Shrimp death numbers during the challenge test were used to assess the pathogenicity of the bacteria. The time of death was recorded every 12 hours, starting from the challenge test, and daily mortality was calculated using the formula from Nitimulyo et al. (2005).

$$\text{MTD} = \frac{\sum_{i=1}^{n} a_i \, b_i}{\sum_{i=1}^{n} b_i}$$

Notes:

MTD = Mean time to death

a = Time of death (hours)

b = Number of death shrimp (shrimp)

i = Summation index

n = Upper limit of summation

2.4.3 Total Bacterial Count

Bacterial population counts were conducted using the plate count method at the beginning, middle, and end of the maintenance period. Observations included the

abundance of *V. parahaemolyticus* in the rearing water and shrimp body. The media used were TCBS with rifampicin at 50 µg/mL and CHROMagar. Bacterial population calculation was performed using the formula from Madigan et al. (2003) as follows:

$$\text{Bacterial population (CF/mL)} = \sum \text{Colony} \times \frac{1}{\text{Dilution Factor}} \times \frac{1}{\text{Sample Volume (mL)}}$$

2.4.4. White Shrimp Immune Response

A total of 0.3 g of shrimp larvae in a mortar was added to 0.9 mL of anticoagulant (1:3 w/v) and then gently ground until the larvae's bodies were crushed. The hemolymph-anticoagulant mixture was pipetted and placed into a microtube and homogenized by gently swirling in a figure-eight motion. The hemolymph-anticoagulant mixture was then applied to a hemocytometer, and Total Hemocyte Count (THC) was directly counted under a microscope at 100× magnification (Tampangallo et al. 2012). The THC was calculated using the following formula:

$$\text{THC} = \frac{\Sigma \text{ Counted cells}}{\text{Grid volume}} \times \text{Dilution factor}$$

Hemolymph (0.1 mL) was placed in a microplate and evenly mixed with 25 µL *Staphylococcus aureus* suspension in PBS (10^7 cells/mL), incubated at room temperature for 20 minutes. A drop of the hemolymph-bacteria mixture (5–10 µL) was placed on a glass slide, smeared, and air-dried. The smear was then fixed with 100% methanol for 5 minutes and air-dried. It was stained by immersing the slide in Giemsa stain for 15 minutes, rinsed with running water, and air-dried with tissue. The number of cells undergoing phagocytosis out of 100 observed phagocytes was counted (Anderson and Siwicki 1993).

$$\text{AF (\%)} = \frac{\text{Number of Phagocytic Cells}}{\text{Number of Phagocytes}} \times 100$$

The respiratory burst activity of hemocytes was measured based on the reduction of nitroblue tetrazolium (NBT), indicating superoxide anion (O2-) production. A total of 250 µL of the hemolymph-anticoagulant mixture was incubated at room temperature for 30 minutes. It was then centrifuged at 3,500 rpm for 20 minutes, and the supernatant was discarded. Then, 100 µL of 0.3% NBT in Hank's Buffered Salt Solution (HBSS) was added and left at room temperature for 2 hours. The mixture was centrifuged again at 3,500 rpm for 10 minutes, the supernatant was discarded, and 100 µL of absolute methanol was added. The pellet was washed twice with 70% methanol. Finally, 120 µL of KOH (2M) and 140 µL of dimethyl sulfoxide (DMSO) were added to dissolve the pellet. The dissolved pellet was transferred to a microplate to measure the optical density (OD) using a microplate reader at 630 nm (Cheng et al. 2004).

Prophenoloxidase (PO) activity in hemocytes was measured based on the formation of dopachrome produced by L-dihydroxyphenylalanine (L-DOPA). A total of 1 mL of the hemolymph-anticoagulant mixture was centrifuged at 3,500 rpm for 10 minutes at 4°C. The supernatant was discarded, and the pellet was slowly resuspended with 1 mL of cacodylate-citrate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.10 M trisodium citrate, pH 7). The mixture was centrifuged again at 3,500 rpm for 10 minutes at 4°C. After discarding the supernatant, 200 µL of cacodylate buffer was added. A 100 µL cell suspension was incubated with 50 µL trypsin (1 mg/mL in cacodylate buffer) as an activator for 10 minutes at 25-26°C. Then, 50 µL L-DOPA (3 mg/mL in cacodylate buffer) was added and left for 5 minutes before adding 800 µL of cacodylate buffer. A 200 µL suspension was transferred to a microplate, and OD was measured using a microplate reader at 490 nm (Liu and Chen 2004).

2.4.5 Gejala Klinis dan Pemeriksaan Histopatologi Udang Vaname

Clinical symptom observations were conducted throughout the pathogenicity test. Shrimp were observed macroscopically post-challenge, noting the condition of the body, organs, and behavioral changes (Saulnier et al., 2000).

Histopathological preparations were made by dissecting shrimp to collect the hepatopancreas and intestines, which were preserved in Davidson's solution. The histopathological procedure included fixation, dehydration, clearing, embedding, sectioning, and staining. The prepared slides were examined under a microscope at 40x100 magnification to observe the integrity or damage of the cells in each treatment. According to Izwar et al. (2018), tissue examination was conducted by observing five different fields of view to ensure high accuracy in the results.

2.5 Statistical Analysis

The research parameter data was tabulated using Microsoft Excel 2019 software. The tabulated data was then analyzed using the analysis of variance (ANOVA) method with a 95% confidence interval, and if significant results were obtained, the analysis would be continued using Duncan's test with IBM SPSS version 26.0 software.

3 RESULT

3.1 *V. parahaemolyticus* and AHPND Confirmation

The colony color of *V. parahaemolyticus* bacteria grown on selective media for *Vibrio* sp., namely thiosulfate citrate bile salt sucrose (TCBS) and CHROMagar, showed colors consistent with *V. parahaemolyticus* (Figure 1). On TCBS media, the bacterial colonies appeared green, while on CHROMagar media, the colonies appeared purple. The colony colors on both media corresponded to the characteristics of *V. parahaemolyticus*. Furthermore, the PCR test results using the AP4 primer, a specific primer for detecting

AHPND, showed visualizations as presented in Figure 2. The PCR visualization results indicated that both isolates tested positive for AHPND with an amplicon size of 230 bp. This is evidenced by the alignment of the positive control amplicon with both samples, as well as above the 200 bp marker.

3.2 White Shrimp Mortality Pattern

The observation results of white shrimp mortality every 12 hours are presented as a cumulative mortality percentage graph (Figure 3). In the control group (K), no mortality occurred during the maintenance period. In the Vp-1 treatment, most mortality occurred between the 12th and 48th hours, with no further mortality observed from the 48th to the 168th hour. In the Vp-2 treatment, mortality began at the 12th hour and continued until the end of the maintenance period at the 168th hour.

3.3 Mortality and Mean Time to Death (MTD)

The observations of mortality and MTD (Mean Time to Death) showed different results between treatments K, Vp-1, and Vp-2 (Table 1). In treatment K, no shrimp died during the maintenance period, resulting in a mortality and MTD value of 0. The highest mortality and MTD were found in treatment Vp-1 at 81.67±15.28%, with an MTD of 24.28 hours. Meanwhile, treatment Vp-2 had a mortality rate of 73.33±20.21% with an MTD of 65.44 hours. ANOVA results for the three treatments indicated that treatment K was significantly different ($P<0.05$) from treatments Vp-1 and Vp-2.

3.4 Bacterial Population

The population of *V. parahaemolyticus* on TCBS and CHROMagar media showed no significant difference from one another, except for treatment K (Table 2). In treatment K, no bacteria grew until the end of the maintenance period on both media. Treatment Vp-1 had the highest bacterial population in the water at H0 on TCBS media (4.66±0.07

Log CFU/mL and CHROMagar (4.50±0.09 Log CFU/mL). The bacterial population in the water continued to decrease daily until H7. The ANOVA results for treatment Vp-1 regarding the bacterial population in the water on TCBS and CHROMagar indicated no significant difference. The highest bacterial population for Vp-1 on the shrimp body was at H1 on TCBS media (4.29±0.50 Log CFU/g) and CHROMagar (4.01±0.53 Log CFU/g). The ANOVA results for treatment Vp-1 regarding the bacterial population on the body on TCBS and CHROMagar showed no significant difference. Treatment Vp-2 had the highest bacterial population in the water at H0 on TCBS media (4.95±0.44 Log CFU/mL) and CHROMagar (4.74±0.35 Log CFU/mL). The bacterial population for Vp-2 in the water continued to decrease until H7. The ANOVA results for treatment Vp-1 regarding the bacterial population in the water on TCBS and CHROMagar indicated no significant difference. The highest bacterial population for Vp-2 on the body was at H1 on TCBS media (4.35±1.05a Log CFU/g) and CHROMagar (4.08±0.92 Log CFU/g), and it continued to decrease until H7. The ANOVA results for treatment Vp-1 regarding the bacterial population on the body on TCBS and CHROMagar showed no significant difference.

3.5 White Shrimp Immune Response

The results of the total haemocyte count (THC) measurements for all treatments are presented in Figure 4. The THC of white shrimp in treatment K had the highest value ($4.58\pm0.55 \times 10^4$ CFU/mL), while the lowest value was found in treatment Vp-2 ($2.22\pm0.76 \times 10^4$ CFU/mL). The ANOVA results for the three treatments showed that treatment K was significantly different ($P<0.05$) from treatments Vp-1 and Vp-2.

The data for the measurements of phagocytic activity (AF) for each treatment can be seen in Figure 5. AF in treatment Vp-1 had the highest value (74.67±4.04%), while

the lowest value was observed in treatment K (64±3.00%). The ANOVA results for the three treatments showed that treatment K was significantly different ($P<0.05$) from treatments Vp-1 and Vp-2.

The values for the respiratory burst (RB) in each treatment showed different numbers (Figure 6). RB in treatment K had the highest value (1.08±0.12), while the lowest value was found in treatment Vp-2 (0.63±0.06). The ANOVA results for the three treatments indicated that treatments K and Vp-1 were significantly different ($P<0.05$) from treatment Vp-2.

The results of the phenoloxidase activity measurements can be seen in Figure 7. PO in treatment K had the highest value (0.13±0.01), while the lowest value was found in treatment Vp-1 (0.08±0.02). The ANOVA results for the three treatments showed that treatment K was significantly different ($P<0.05$) from treatments Vp-1 and Vp-2.

3.6 White Shrimp Clinical Symptoms

Clinical symptoms caused by *V. parahaemolyticus* infection during rearing can be seen in Figure 8. The movement of white shrimp in treatments Vp-1 and Vp-2 at 12 hours after the challenge test started showed weakness, swimming in a tilted or unbalanced manner. The shrimp were also seen at the bottom of the aquarium with minimal movement. Macroscopic observations of the bodies in both treatments showed that the shrimp appeared pale, with the hepatopancreas appearing whitish and the intestines appearing empty. Meanwhile, in the control treatment (K), there were no changes during the treatment. Macroscopic observations of the control treatment showed that the shrimp's body appeared bluish-clear, with the hepatopancreas reddish in color, and the intestines appeared full.

3.7 White Shrimp Histopathology

Histopathological observations of the hepatopancreas showed differences between the control treatment (K) and treatments Vp-1 and Vp-2 (Figure 9). The control treatment showed many normal tubules and normal cells. In treatment Vp-1, there was noticeable necrosis of B cells and numerous vacuoles. Necrotic tubules were commonly found, appearing shattered and shapeless. Treatment Vp-2 also exhibited similar damage, including significant necrosis and vacuoles, with many damaged tubules observed. The tissue in the control treatment appeared more stained compared to treatments Vp-1 and Vp-2.

Histopathological observations of the intestines revealed differences between the control treatment (K) and treatments Vp-1 and Vp-2 (Figure 3). The tissue in infected shrimp (Vp-1 and Vp-2) exhibited massive damage or necrosis of the intestinal wall, with numerous B cell necroses also present in both treatments. Inflammation or inflammatory cells were found at several points. Meanwhile, in the uninfected treatment (K), the intestinal wall tissue was intact with a regular lumen. Necrosis and tissue inflammation were not found in this treatment.

4 DISCUSION

The role of a microbe in attacking a host to cause a disease is called pathogenesis (Amrullah 2014). Certain strains of *V. parahaemolyticus* are the cause of acute hepatopancreatic necrosis disease (AHPND). Testing was conducted by observing the pathogenicity level of the bacteria against white shrimp. Characterization tests for *V. parahaemolyticus* were performed on TCBS and CHROMagar media. TCBS media is specific for *Vibrio* sp., while CHROMagar is specific for *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, and *V. alginolyticus*. Both media contain specific compositions to

298 inhibit the growth of non-target bacteria. Bacteria grown on TCBS media show green-
299 colored colonies. *Vibrio* sp. that cannot ferment sucrose in TCBS media, such as *V.*
300 *parahaemolyticus* and *V. vulnificus*, will produce green colonies, while those that can
301 ferment sucrose, like *V. cholerae* and *V. alginolyticus*, have yellow colonies (Lee et al.
302 2020). Meanwhile, on CHROMagar, the bacterial colonies appear purple. According to
303 Lee et al. (2020), *V. vulnificus* and *V. cholerae* have blue colonies, while *V. alginolyticus*
304 appears yellowish-white, and *V. parahaemolyticus* has purple colonies. A confirmation
305 test for AHPND was then conducted using the Polymerase Chain Reaction (PCR)
306 method.

307 The AHPND testing was performed with two PCR steps: first-step PCR and
308 nested PCR. According to Hoang et al. (2021), nested PCR is performed to obtain high-
309 accuracy amplicons that match the target. Positive and negative controls were used as
310 comparisons for sample results. The main stages included DNA extraction, amplification,
311 and electrophoresis, followed by visualization of the PCR results. The visualization of the
312 PCR electrophoresis results showed that the bacteria Vp-1 and Vp-2 tested positive for
313 AHPND with DNA bands measuring 230 bp. This aligns with Dangtip et al. (2015),
314 which states that *V. parahaemolyticus* is considered positive for AHPND if it has a DNA
315 band measuring 1269 bp in first-step PCR and 230 bp in nested PCR.

316 Shrimp infected by *V. parahaemolyticus*, the cause of AHPND, will experience
317 early mortality. The disease previously known as Early Mortality Syndrome typically
318 causes deaths in shrimp farms during the early months of rearing, before DOC-35
319 (Rodriguez et al. 2019). The mortality pattern shows the cumulative mortality percentage
320 over time (Figure 3). The highest percentage of mortality in treatments Vp-1 and Vp-2
321 occurs from hour 12 to hour 24. A drastic increase in mortality at hour 24 occurs in

treatment Vp-1, where the mortality percentage exceeds 50% of the population. Treatment Vp-1 shows the highest mortality percentage (81.67±15.28b) with the fastest average time to death (ATD), while mortality in treatment Vp-2 has a lower percentage and a slower ATD compared to Vp-1. According to Hong et al. (2016), vannamei shrimp infected with AHPND have mortality rates ranging from 40% to 100%.

The bacterial population in the water in the challenge treatments (Vp-1 and Vp-2) has the highest density on day 0 and continues to decline until the end of the observation. According to Valente and Wan (2021), bacteria that do not find a host will die because they do not receive nutrients to survive. This shows that the bacterial density in the water continues to decrease due to the declining or even absent population of vannamei shrimp as hosts. The bacterial population on the shrimp bodies also decreases daily. This can be linked to the decreasing number of bacteria in the water, leading to fewer bacteria entering the shrimp's body.

The results of measuring total hemocyte count (THC) after the challenge test showed that the control treatment (K) had the highest value. Hemocytes play an important role in crustaceans as they help eliminate or compete with foreign particles entering the crustacean body, such as disease-causing pathogens (Hauton 2012). According to Sahoo et al. (2007), hemocytes can act as a cellular defense system in white shrimp, playing a role in phagocytosis, encapsulation, and nodulation. The number of hemocytes in the shrimp body can influence the infection level of *V. parahaemolyticus*. The more hemocytes present, the lower the infection level. This can affect the survival rate of vannamei shrimp after the challenge test. Treatment Vp-2 had the lowest THC value; according to Smith et al. (2003), low THC values indicate a defense mechanism occurring during pathogen infection. The decrease in THC is due to hemocytes migrating to the

infected tissues, thus affecting immune response performance at that site (Hamsah et al. 2019).

Phagocytic activity showed a significant difference ($P<0.05$) between treatment K and treatments Vp-1 and Vp-2. Phagocytic activity plays a role in the immune response mechanism of vannamei shrimp against pathogen infections and physiological processes such as tissue repair (Liu et al. 2020). Hemocytes are the cells involved in the phagocytosis process to combat various pathogens, including *V. parahaemolyticus*, to protect their hosts. The highest phagocytic activity value is found in treatment Vp-1, followed by Vp-2, while K has the lowest phagocytic value. According to Braak (2002), there are three types of hemocyte cells: hyaline, granular, and semi-granular. Hyaline cells play a role in phagocytic activity, while semi-granular cells function as temporary phagocytic cells. An increase in THC value can enhance phagocytic activity (Johansson et al. 2000).

Respiratory burst (RB) is part of the shrimp's immune response system against pathogen infections. White shrimp infected by pathogens like viruses or bacteria will respond through hemocytes with increased reactive oxygen production. This process is marked by the reduction of nitroblue tetrazolium (NBT) by hemocytes. RB is a continuation of phagocytosis, and the two processes are interconnected because the digestive enzymes of RB are bactericidal agents that are released from the phagolysosome, resulting in free radical release from the phagolysosome (Risjani et al. 2021). The RB results in the study showed that treatment K had the highest value, while Vp-2 had the lowest value. The assumption that treatment Vp-1 has a lower value than Vp-2 is due to the lower mortality rate in Vp-1. This is likely because sampling was conducted simultaneously at hour 24 across all treatments. It is assumed that treatment

Vp-1 reached its peak mortality before hour 24, so at the time of sampling, it was already in the recovery phase. According to Wang et al. (2012), the post-disease attack recovery process in vannamei shrimp can enhance RB values.

Phenoloxidase (PO) in shrimp plays a role in biological functions such as defense against pathogens, wound healing, and body color regulation. The mechanism of PO begins with phenol oxidase, forming quinone that generates dark brown pigments to inactivate and prevent pathogens. This process is called melanization (Amparyup et al. 2013). The measurement of PO in the challenged treatments (Vp-1 and Vp-2) showed lower values compared to the control treatment without challenge (K). According to Costa et al. (2009), a decrease in the immunity level or defense of shrimp will lower PO activity, while shrimp with good immunity will have high PO values.

Shrimp infected with AHPND exhibit lethargic movement, anorexia, slow growth, empty digestive tracts, and pale to white hepatopancreas (Kumar et al. 2021). The clinical symptoms exhibited in the infected treatments (Vp-1 and Vp-2) have similar characteristics. The shrimp's body appears pale, the carapace softens, and slowed movement is observed, with their bodies appearing unbalanced or tilted at the bottom. Internal organ attacks on the hepatopancreas show a pale white color, and the intestines are empty. Meanwhile, in the uninfected treatment (K), no symptoms are observed. The shrimp's body is bluish-clear, with active movement, reddish-brown hepatopancreas, and filled intestines. According to Nunan et al. (2014), healthy vannamei shrimp have a bright clear body color, are actively moving, and have reddish-brown hepatopancreas, with full intestines displaying an unbroken black line.

Histopathology is performed to observe pathological changes at the microscopic level in shrimp tissues. The pathogen's ability to infect a host can affect tissues at the

microscopic level. Tissues can suffer acute damage due to pathogen attacks, such as viruses or bacteria. *V. parahaemolyticus*, which causes AHPND, attacks the hepatopancreas and intestines with acute damage levels (Suryana et al. 2023). The hepatopancreas tissue in shrimp infected with AHPND shows high levels

5 CONCLUSION

Isolates Vp-1 and Vp-2 were identified as *V. parahaemolyticus* strains causing AHPND that infect Pacific white shrimp, presenting clinical symptoms such as pale hepatopancreas, empty intestines, and reduced activity. Tissue damage was evident with significant necrosis in the hepatopancreas and intestinal organs, resulting in mortality rates ranging from 73.33% to 81.67%, with an average time to death of 24.28 to 65.44 hours.

CONFLICT OF INTEREST

We declare that there are no conflicts of interest regarding financial, personal, or other relationships with individuals or organizations related to the material discussed in the manuscript.

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Table 1 Mortality and mean time of death (MTD) of white shrimp infected with *V. parahaemolyticus*

Treatment	Mortality (%)	MTD (Hours)
K	0[a]	0
Vp-1	81,67±15,28[b]	24,28
Vp-2	73,33±20,21[b]	65,44

Note: Different letters in the same column indicate significantly different results (Duncan P<0.05)

Table 2 Population of *V. parahaemolyticus* in the water and the bodies of white shrimp infected with *V. parahaemolyticus*

Sources	Time	K		Vp-1		Vp-2	
		TCBS	CHROM	TCBS	CHROM	TCBS	CHROM
Water (Log CFU/mL)	H0	0	0	4,66±0,07[a]	4,50±0,09[a]	4,95±0,44[a]	4,74±0,35[a]
	H1	0	0	4,44±0,04[a]	4,32±0,03[a]	4,63±0,52[a]	4,35±0,29[a]
	H2	0	0	4,09±0,53[a]	3,85±0,56[a]	4,09±0,47[a]	3,92±0,45[a]
	H4	0	0	3,19±1,02[a]	2,83±0,67[a]	3,69±0,09[a]	3,48±0,09[a]
	H7	0	0	2,73±0,51[a]	2,50±0,35[a]	2,66±0,32[a]	2,60±0,30[a]
Body (Log CFU/g)	H1	-	-	4,29±0,50[a]	4,01±0,53[a]	4,35±1,05[a]	4,08±0,92[a]
	H2	-	-	2,30±1,02[a]	2,30±0,48[a]	4,17±1,02[a]	3,70±0,67[a]
	H3	-	-	-	-	3,54±0,59[a]	3,29±0,49[a]
	H4	-	-	-	-	3,13±0,72[a]	2,89±0,53[a]
	H7	-	-	-	-	2,95±0,57[a]	2,53±0,40[a]

Note: (-): No shrimp died. Different letters in the same row indicate significantly different results (Duncan P<0.05)

Figure 1 Colonies of *V. parahaemolyticus* on TCBS and CHROMagar media

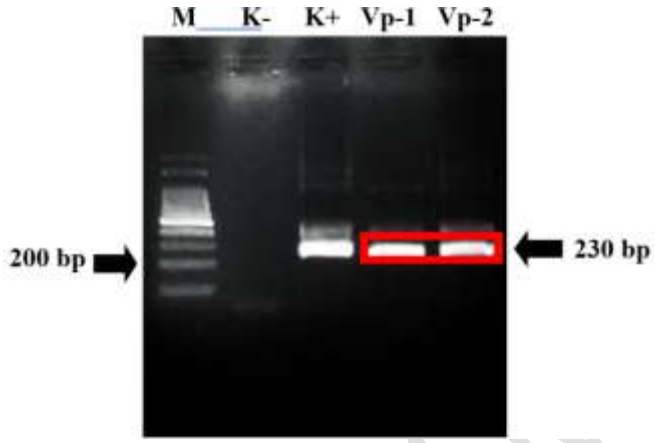


Figure 2 Visualization of *V. parahaemolyticus* bacteria PCR using primer AP4
Note: M: marker; K-: Negative control; K+: Positive control; Vp-1: Treatment bacteria Vp-1; Vp-2: Treatment bacteria Vp-2

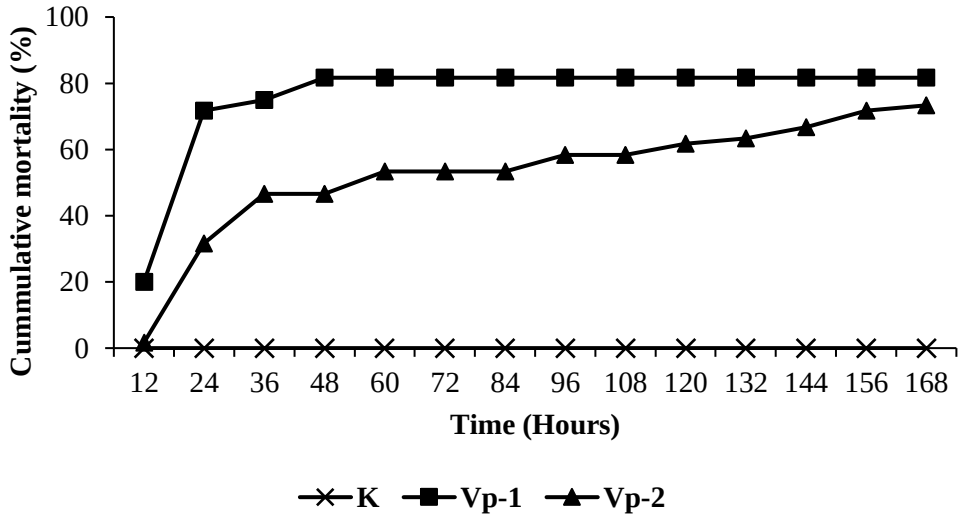


Figure 3 Cumulative mortality of white shrimp infected with *V. parahaemolyticus*

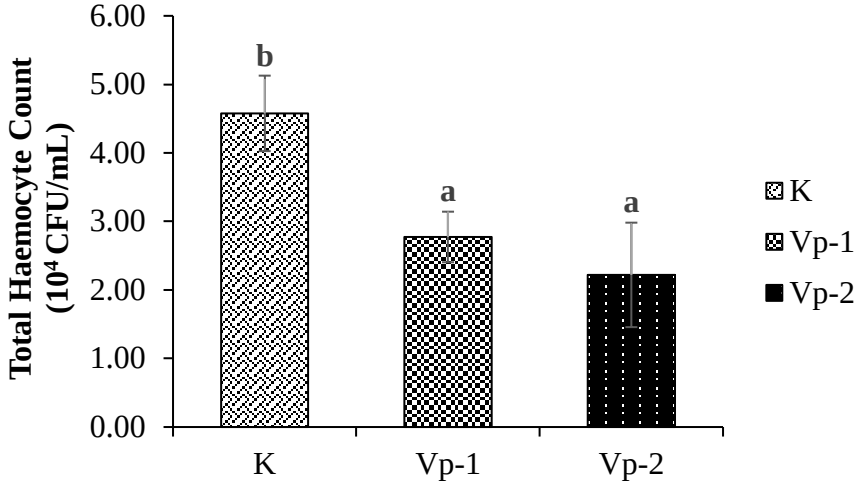


Figure 3 Total hemocyte count of white shrimp infected with *V. parahaemolyticus*
Note: Different letters above the bars indicate significantly different results (Duncan P<0.05)

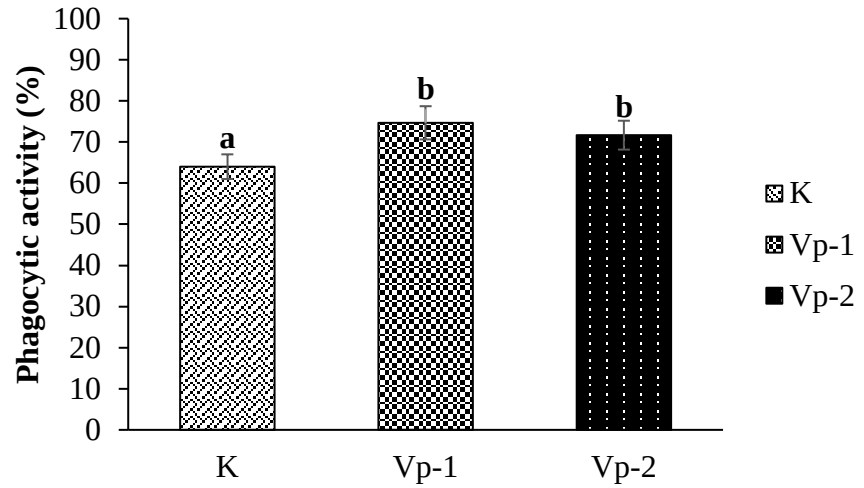
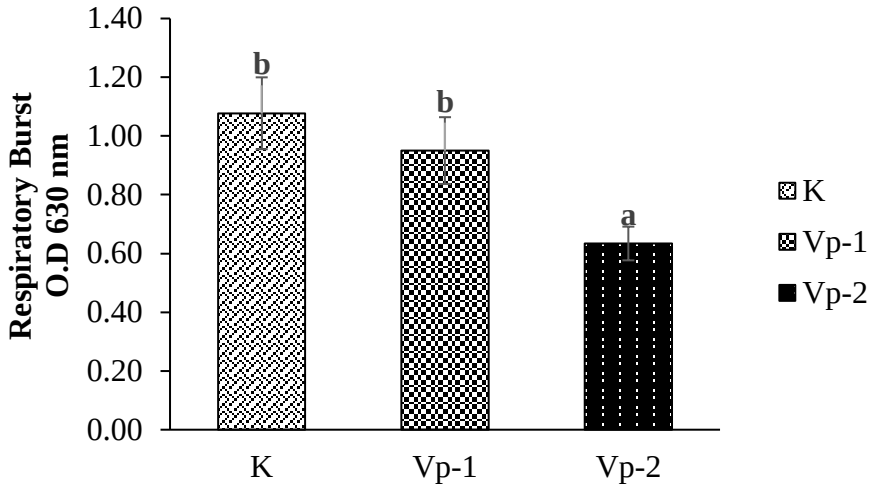


Figure 4 Phagocytic activity of white shrimp infected with *V. parahaemolyticus*
Note: Different letters above the bars indicate significantly different results (Duncan P<0.05).



Gambar 5 Respiratory burst of white shrimp infected with *V. parahaemolyticus*
Note: Different letters above the bars indicate significantly different results (Duncan P<0.05).

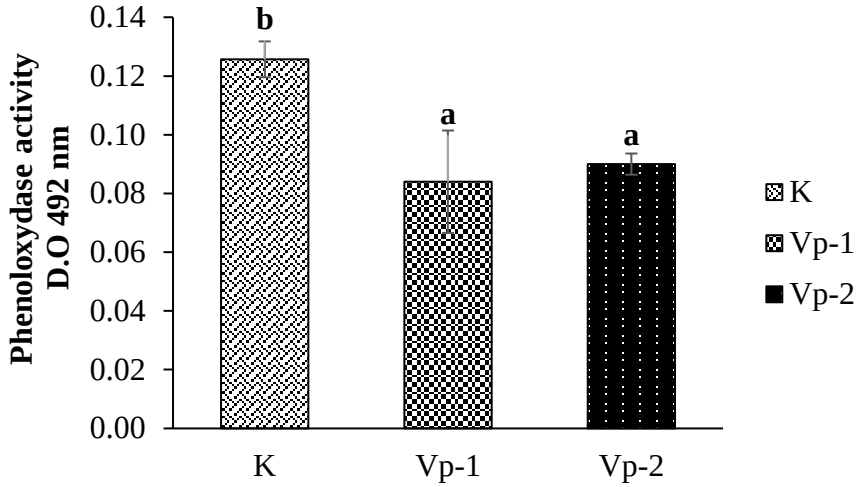


Figure 7 Phenoloxidase activity of white shrimp infected with *V. parahaemolyticus*
Note: Different letters above the bars indicate significantly different results (Duncan P<0.05).

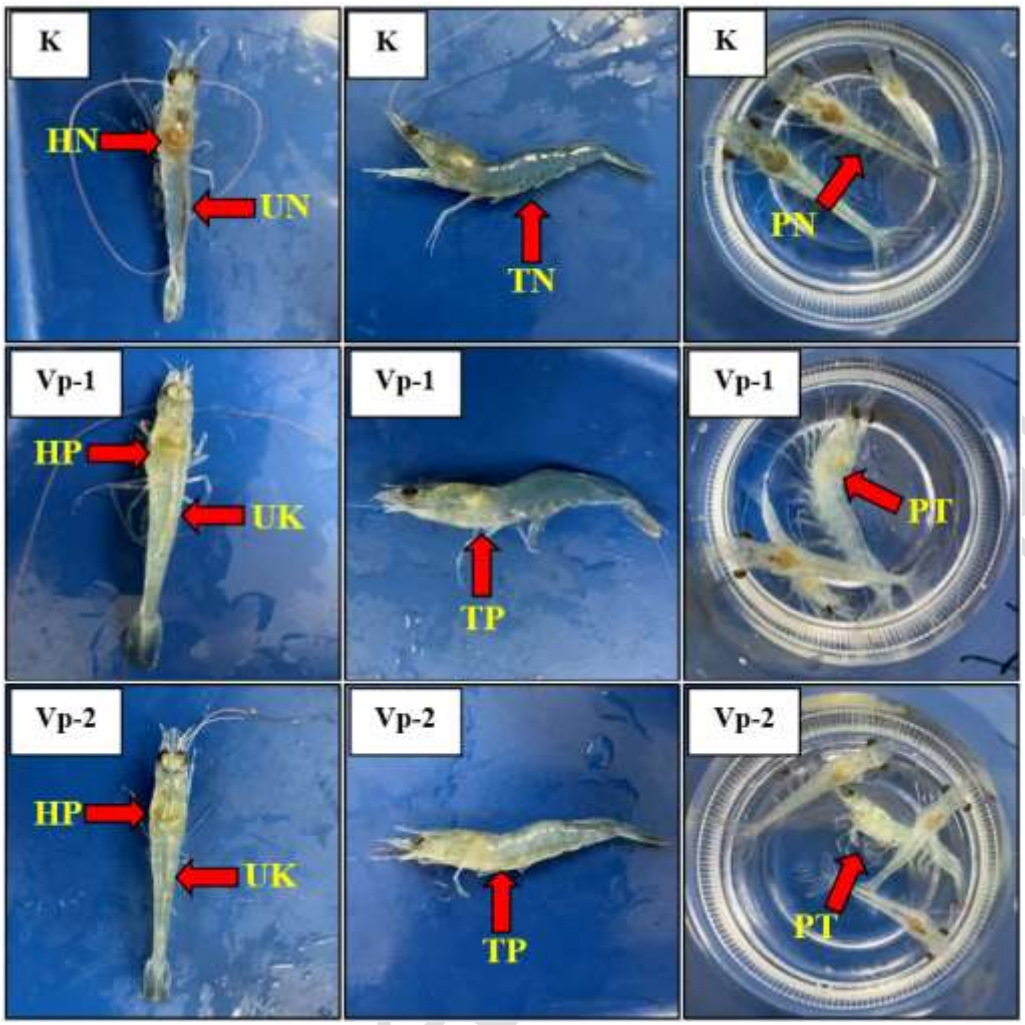


Figure 8 Clinical symptoms of white shrimp infected with *V. parahaemolyticus*

Note: TN: normal body; HN: normal hepatopancreas; UN: normal intestine; PN: normal movement; TP: pale body; HP: pale hepatopancreas; UK: empty intestine; PT: abnormal movement. K: Vannamei shrimp not infected with V. parahaemolyticus; Vp-1: Vannamei shrimp infected with V. parahaemolyticus from Tasikmalaya; Vp-2: Vannamei shrimp infected with V. parahaemolyticus from Situbondo.

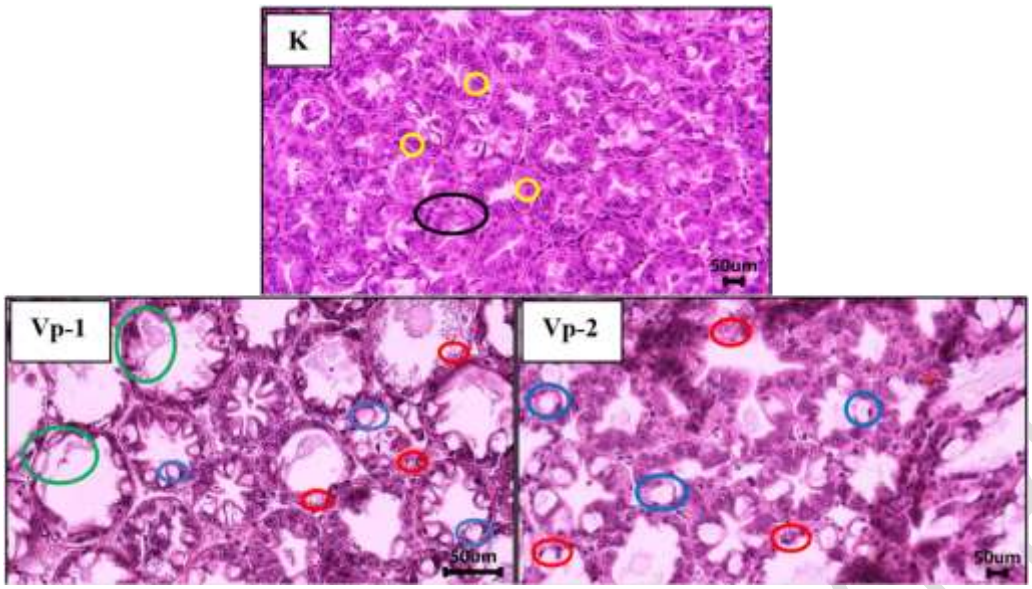


Figure 9: Histopathology of the hepatopancreas of white shrimp infected with *V. parahaemolyticus*

Note: Normal tubule (O), normal cell (O), necrosis cell (O), vacuola (O), necrosis tubule (O).

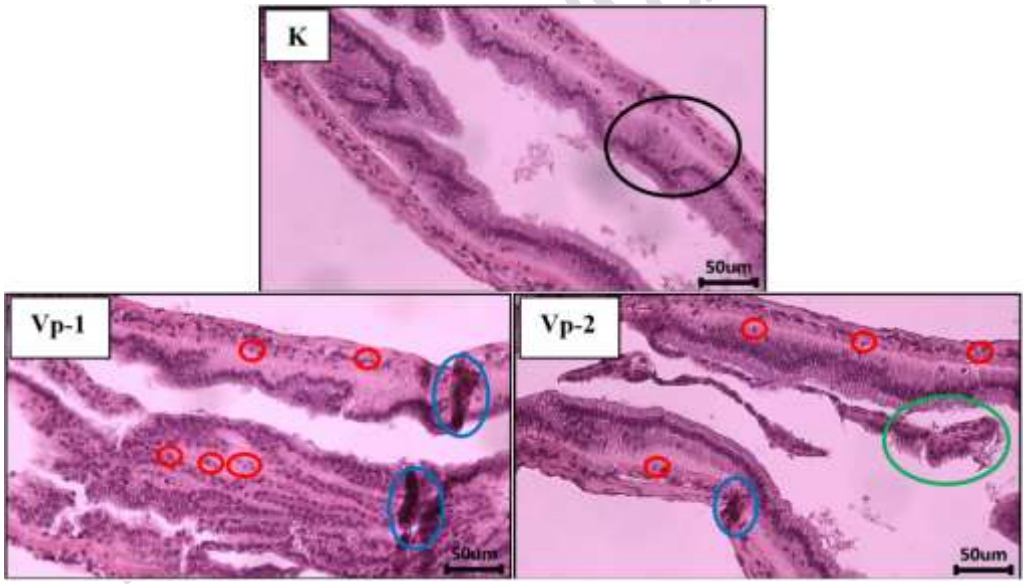


Figure 10 Histopathology of the intestine of white shrimp infected with *V. parahaemolyticus*

Note: Normal intestine wall (O), inflammation (O), intestinal wall necrosis (O), Cell necrosis (O).