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Pathogenicity Test of *Vibrio parahaemolyticus* in Pacific White Shrimp *Penaeus vannamei*

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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* carrying the PirA and PirB toxins are responsible for acute hepatopancreatic necrosis disease (AHPND). This study aimed to assess the pathogenicity of *V. parahaemolyticus* in *P. vannamei* by 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 104 CFU mL^{-1}, 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). The parameters observed included 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 the 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, *Vibrio parahaemolyticus*.

I. INTRODUCTION

The Pacific white shrimp (*Penaeus vannamei*) is a highly valuable aquaculture commodity both in 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). The global production of white shrimp experienced significant growth between 2010 and 2020, increasing by 3.2 million tons (FAO, 2022). In Indonesia, shrimp production has 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 density.

However, increased shrimp production faces several challenges, one of which is disease outbreaks.

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One of the most common diseases in shrimp farming is vibriosis, which is caused by bacterial infections from Vibrio species, including *Vibrio parahaemolyticus* (Aguirre-Guzman *et al*., 2010). Infections caused by *V. parahaemolyticus* can cause abnormalities in shrimp, such as gill necrosis, lethargy, anorexia, and in acute cases, 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 the rapid mortality observed 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 protect 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 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 economic losses caused by *V. parahaemolyticus.* This study aimed to evaluate the pathogenicity of *V. parahaemolyticus* from different bacterial isolate sources in white shrimp to comparing the effects of isolate variation and its relationship to pathogenicity in causing disease.

II. MATERIALS AND METHODS

2.1 Experimental Design

This study employed a completely randomized design with three treatments, each with three replicates. The treatments were as follows: 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 mL^{-1}) 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 was 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 (104 CFU mL^{-1}; based on the 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^{-1}, 7.58-7.75, 28.1-29.5 °C, and 30-32 g L^{-1}, respectively.

2.4 Observation Parameters

2.4.1 Confirmation of V. parahaemolyticus and AHPND

V. parahaemolyticus was characterized by culturing the isolates on TCBS and CHROMagar. Colony color and morphology were observed and compared with the reference characteristics for each medium. AHPND in *V. parahaemolyticus* was confirmed using the PCR 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 was observed every 12 hours. Changes in mortality rate were monitored to determine the peak and lowest mortality times. The 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 the bacterial infection. Shrimp deaths numbers during the challenge test were used to assess bacterial pathogenicity. 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).

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

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

The bacterial population counts were determined using the plate count method at the beginning, middle, and end of the maintenance period. Observations included the abundance of *V. parahaemolyticus* in rearing water and shrimp boies. The media used were TCBS with rifampicin (50 µg mL^{-1}) 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 gente swirling in a figure-eight motion. The hemolymph-anticoagulant mixture was then applied to a hemocytometer, and the 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:

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

Hemolymph (0.1 mL) was placed in a microplate, evenly mixed with 25 µL of *Staphylococcus aureus* suspension in PBS (107 cells mL^{-1}), and 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. The sides were stained with Giemsa stain for 15 minutes, rinsed with running water, and air-dried. The number of cells undergoing phagocytosis out of the 100 observed phagocytes was counted (Anderson and Siwicki, 1993).

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

The respiratory burst activity of hemocytes was measured based on the reduction of nitroblue tetrazolium (NBT), indicating superoxide anion (O^{2-}) 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 then 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) at 630 nm using a microplate reader (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

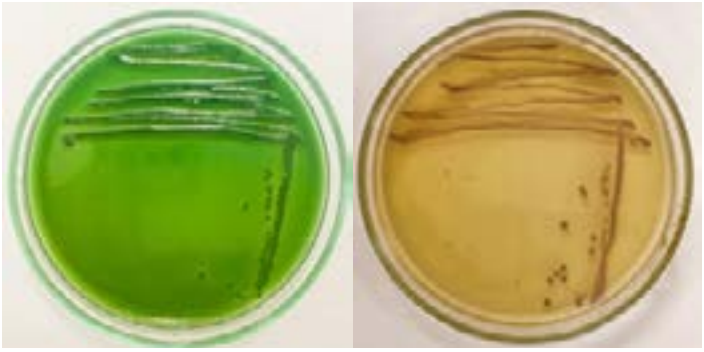


Figure 1. Colonies of *V. parahaemolyticus* on TCBS and CHROMagar media

was then centrifuged again at 3,500 rpm for 10 minutes at 4°C. After discarding the supernatant, 200 µL cacodylate buffer was added. A 100 µL cell suspension was incubated with 50 µL trypsin (1 mg mL^{-1} in cacodylate buffer) as an activator for 10 minutes at 25-26°C. Subsequently, 50 µL of L-DOPA (3 mg mL^{-1} 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 the OD was measured using a microplate reader at 490 nm (Liu and Chen 2004).

2.4.5 Clinical Sympyoms and Histopathology of Shrimp

Clinical symptoms were observed during the pathogenicity test. Shrimps 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 procedures included fixation, dehydration, clearing, embedding, sectioning, and staining. The prepared slides were examined under a microscope at 40 × 100 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 the high accuracy of the results.

2.5 Statistical Analysis

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

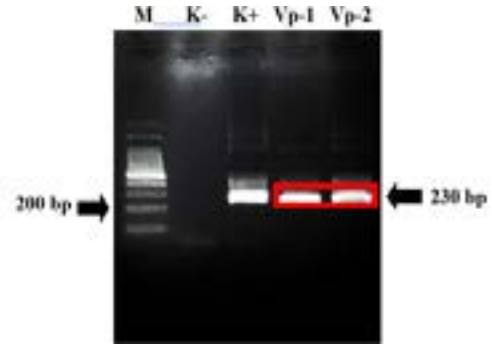


Figure 2. Visualization of V. parahaemolyticus bacteria PCR using primer AP4

Note: M: marker; K-: Negative control; K+: Positive control; Vp-1: Treatment bacteria Vp-1; Vp-2: Treatment bacteria Vp-2

III. RESULT

3.1. V. parahaemolyticus and AHPND Confirmation

The colony color of *V. parahaemolyticus* grown on selective media for *Vibrio* sp., namely thiosulfate citrate bile salt sucrose (TCBS) and CHROMagar, was consistent with that of *V. parahaemolyticus* (Figure 1). On TCBS medium, the bacterial colonies appeared green, whereas on CHROMagar medium, 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, are presented in Figure 2. The PCR visualization results indicated that both isolates tested positive for AHPND, with an amplicon size of 230 bp. This was 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),

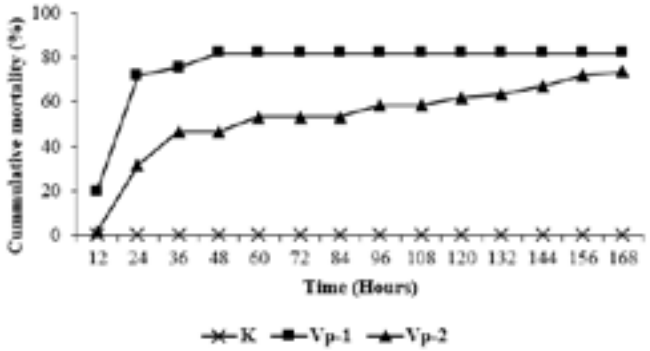


Figure 3. Cumulative mortality of white shrimp infected with *V. parahaemolyticus*

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

Treatmment	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)

no mortality occurred during the maintenance period. In the Vp-1 treatment, most mortality occurred between the 12th and 48th hour, with no further mortality observed from the 48th to the 168th hour. In the Vp-2 treatment group, 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 Mean Time to Death (MTD) showed different results among treatments K, Vp-1, and Vp-2 (Table 1). In treatment K, no shrimp died during the maintenance period, resulting in 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. The 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 populations of *V. parahaemolyticus* on TCBS and CHROMagar media were not significant different 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^{-1} and CHROMagar (4.50±0.09 Log CFU mL^{-1}). The bacterial population in the water continued to decrease until reaching H7. The ANOVA results for treatment Vp-1 regarding the bacterial population in the water on TCBS and CHROMagar indicated no significant differences. The highest bacterial population for Vp-1 on the shrimp body was at H1 on TCBS media (4.29±0.50 Log CFU g^{-1}) and CHROMagar (4.01±0.53 Log CFU g^{-1}). 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

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^{-1})	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^{-1})	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)

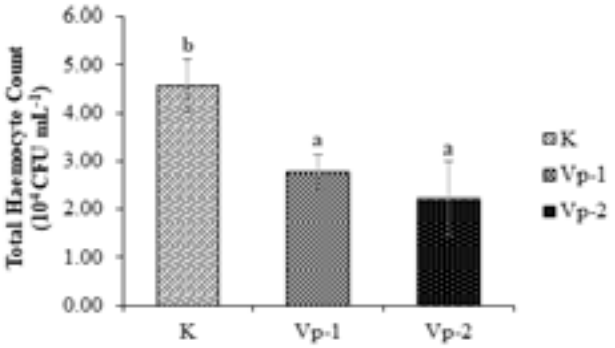


Figure 4. Total hemocyte count of white shrimp infected with *V. parahaemolyticus*

Note: Different letters above the bars indicate significantly different results (Duncan P<0.05).

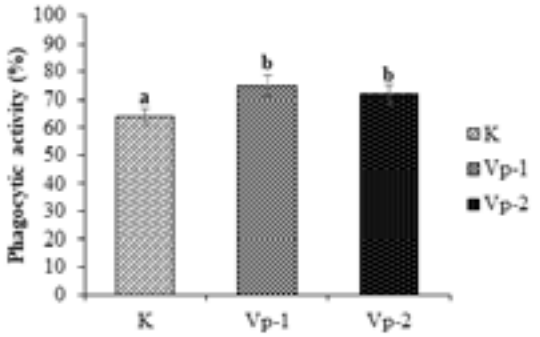
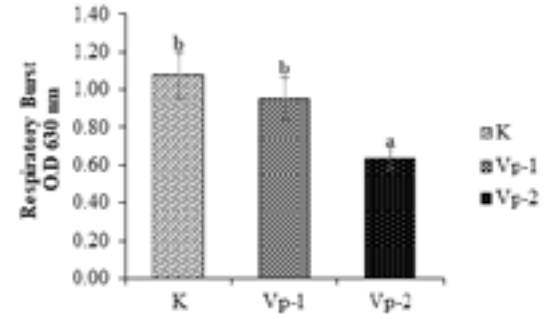


Figure 5. Phagocytic activity of white shrimp infected with *V. parahaemolyticus*

Note: Different letters above the bars indicate significantly different results (Duncan P<0.05).



Gambar 6. Respiratory burst of white shrimp infected with *V. parahaemolyticus*

Note: Different letters above the bars indicate significantly different results (Duncan P<0.05).

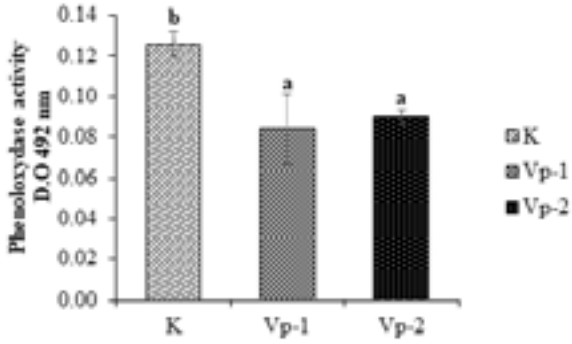


Figure 7. Phenoloxidase activity of white shrimp infected with *V. parahaemolyticus*

Note: Different letters above the bars indicate significantly different results (Duncan P<0.05).

in the water at H0 on TCBS media (4.95±0.44 Log CFU mL^{-1}) and CHROMagar (4.74±0.35 Log CFU mL^{-1}). 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±1.05 Log CFU g^{-1}) and CHROMagar (4.08±0.92 Log CFU g^{-1}), 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 differences.

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±0.55 × 104 CFU mL^{-1}), while the lowest value was found in treatment Vp-2 (2.22±0.76 × 104 CFU mL^{-1}). 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

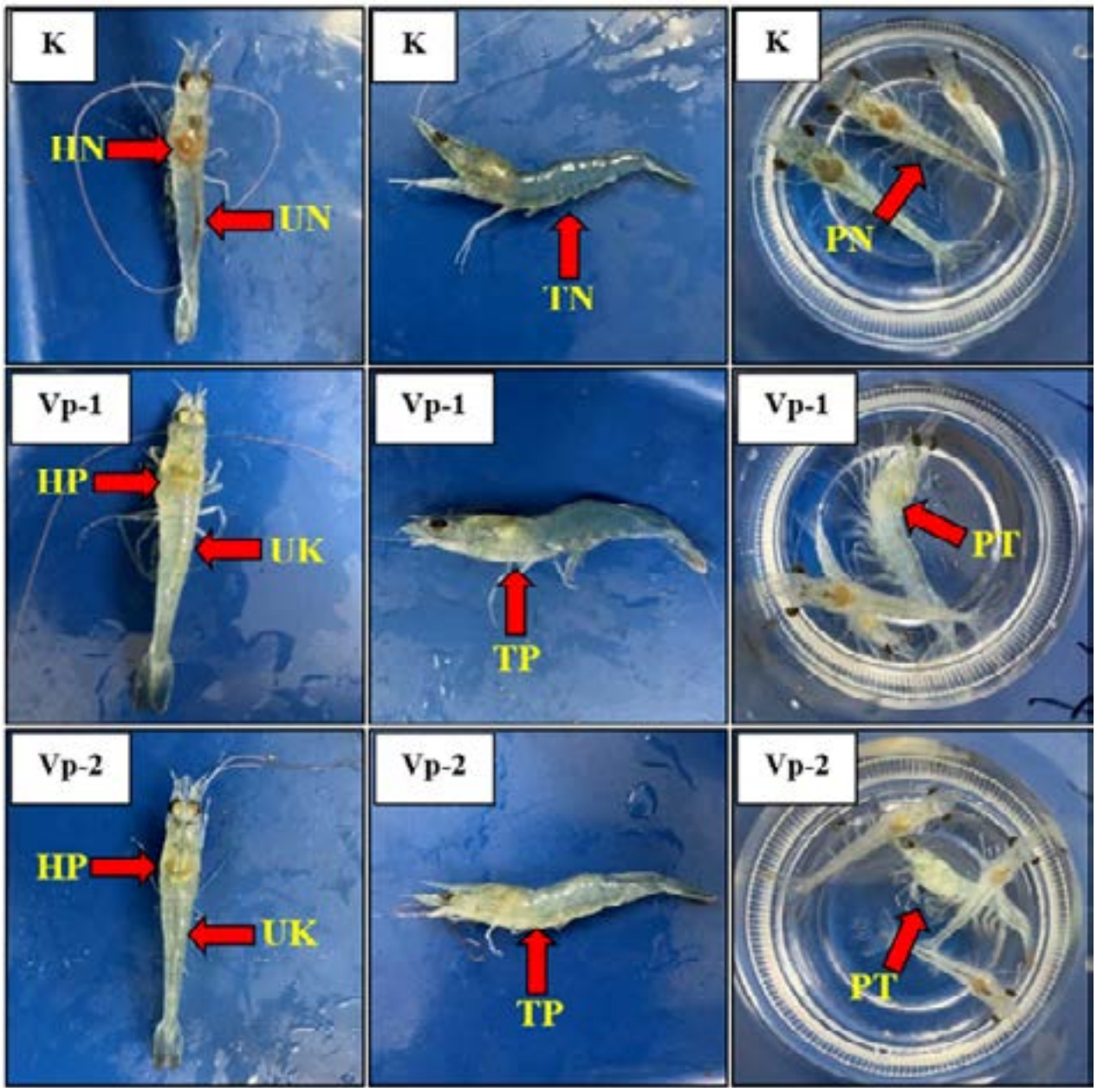


Figure 8 Clinical symptoms of white shrimp infected with *V. parahaemolyticus*
Note: TN: normal body; HN: normal hepatopancreas; UN: normal intestine; PN: normal movement; TP: pale body; HP: pale hepatopancreas; UK: empty intestine; PT: abnormal movement. K: Vannamei shrimp not infected with *V. parahaemolyticus*; Vp-1: Vannamei shrimp infected with *V. parahaemolyticus* from Tasikmalaya; Vp-2: Vannamei shrimp infected with *V. parahaemolyticus* from Situbondo.

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

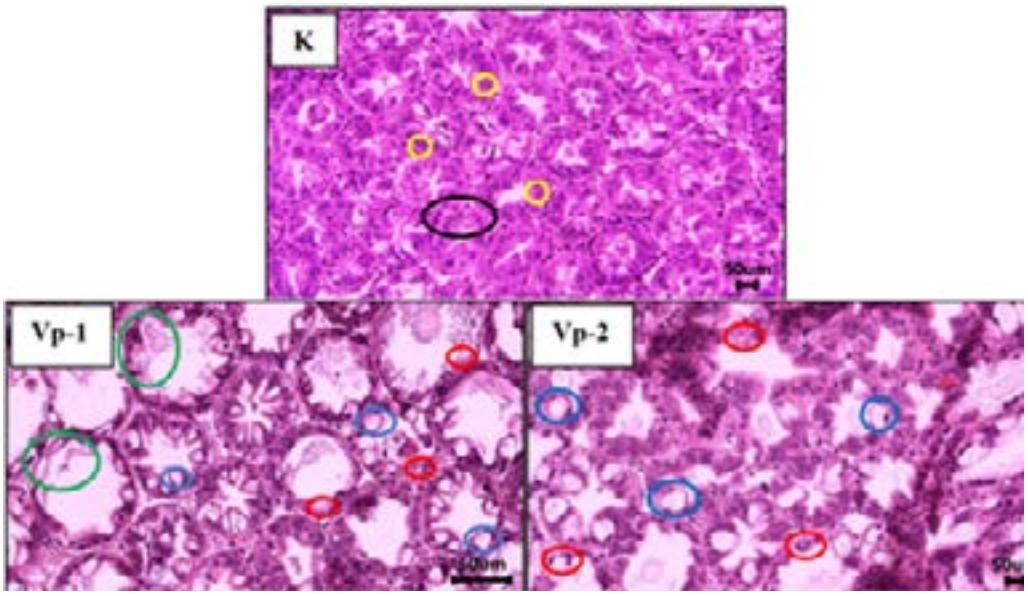


Figure 9. Histopathology of the hepatopancreas of white shrimp infected with V. *parahaemolyticus*. Normal tubule (**O**), normal cell (O), necrosis cell (O), vacuola (O), necrosis tubule (O).

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.

IV. DISCUSION

The role of a microbe in attacking a host to cause a disease is known as pathogenesis (Amrullah, 2014). Certain strains of *V. parahaemolyticus* can cause acute hepatopancreatic necrosis disease (AHPND). Testing was conducted by observing the pathogenicity level of the bacteria against white shrimp. Characterization of *V. parahaemolyticus* was 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 inhibit the growth of non-target bacteria. Bacteria grown on TCBS media showed green colonies. *Vibrio* sp. that cannot ferment sucrose in TCBS media, such as *V. parahaemolyticus* and *V. vulnificus*, will produce green colonies, while those that can ferment sucrose, like *V. cholerae* and *V. alginolyticus*, have yellow colonies (Lee *et al*., 2020). On CHROMagar, the bacterial colonies appeared purple in color. According to Lee et al. (2020), *V. vulnificus* and *V. cholerae* have blue colonies, while *V. alginolyticus* appears yellowish-white, and *V. parahaemolyticus* has purple colonies. A confirmation test for AHPND was conducted using the Polymerase Chain Reaction (PCR) method.

The AHPND testing was performed using two PCR steps: a first-step PCR and nested PCR. According to Hoang *et al*. (2021), nested PCR is performed to obtain highly accurate amplicons that match the target. Positive and negative controls were used to compare sample results. The main stages included DNA extraction, amplification, and electrophoresis, followed by visualization of the PCR results. Visualization of the PCR electrophoresis results showed that the bacteria Vp-1 and Vp-2 tested positive for AHPND with DNA bands measuring 230 bp. This aligns with the results of Dangtip *et al*. (2015), which stated that *V. parahaemolyticus* is considered positive for AHPND if it has a DNA band measuring 1269 bp in first-step PCR and 230 bp in nested PCR.

Shrimp infection by *V. parahaemolyticus*, which is the cause of AHPND, leads to early mortality. The disease, previously known as Early Mortality Syndrome, typically causes death in shrimp farms

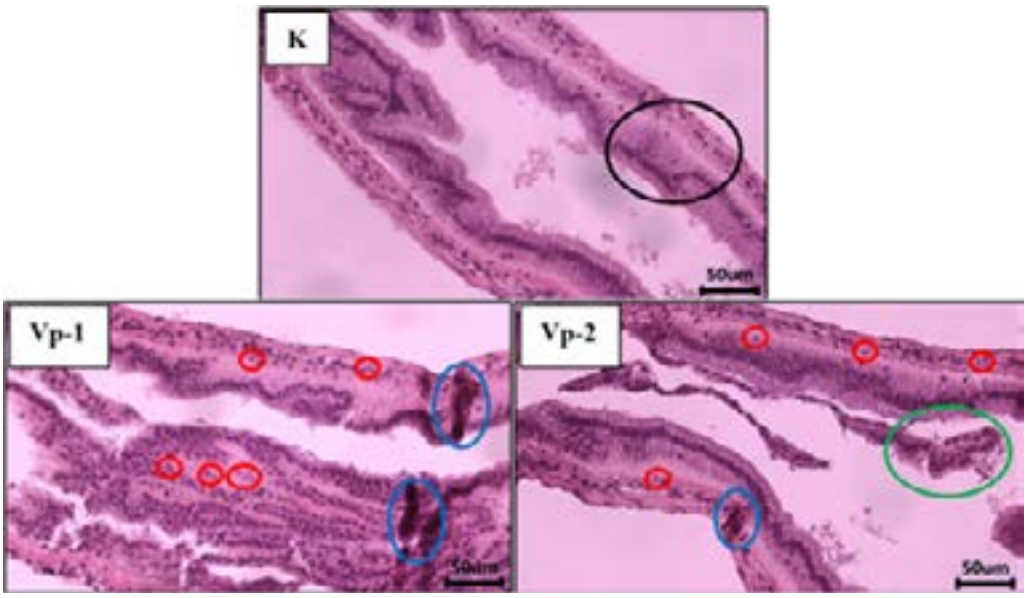


Figure 10 Histopathology of the intestine of white shrimp infected with *V. parahaemolyticus*. Normal intestine wall (o), inflammation (o), intestinal wall necrosis (o), Cell necrosis (o).

during the early months of rearing, before DOC-35 (Rodriguez *et al*., 2019). The mortality pattern shows the cumulative mortality percentage over time (Figure 3). The highest percentage of mortality in treatments Vp-1 and Vp-2 occured from hour 12 to hour 24. A drastic increase in mortality at 24 hour occured in treatment Vp-1, where the mortality percentage exceeded 50% of the population. Treatment Vp-1 showed the highest mortality percentage (81.67 ± 15.28^{b}) with the fastest average time to death (ATD), while mortality in treatment Vp-2 had a lower percentage and 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) had the highest density on day 0 and continued to decline until the end of the observation period. According to Valente and Wan (2021), bacteria that do not find a host die because they do not receive nutrients to survive. This shows that the bacterial density in the water continues to decrease because of the declining or even absent population of vannamei shrimp as hosts. The bacterial population on the shrimp bodies also decreased daily. This can be linked to the decreasing number of bacteria in the water, leading to fewer bacteria entering the shrimp bodies.

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 defence 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 higher the number of 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, and according to Smith *et al*. (2003), low THC values indicate a defense mechanism that occurs 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 plays a role in the immune response mechanism of vannamei shrimp against pathogenic 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 was found in treatment Vp-1, followed by treatment Vp-2, while K had the lowest phagocytic activity. 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 immune response system against pathogen infections. White shrimp infected by pathogens, such as viruses or bacteria, 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 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 treatment 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; therefore, 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 PO in the challenged treatments (Vp-1 and Vp-2) was lower than that in the control treatment without challenge (K). According to Costa *et al*. (2009), a decrease in the immunity level or defense of shrimp lowers PO activity, whereas shrimp with good immunity will have high PO values.

Shrimp infected with AHPND exhibited 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) had similar characteristics. The shrimp body appeared pale, the carapace softened, and slowed movement was 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 were observed. The shrimp 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 a reddish-brown hepatopancreas, with full intestines displaying an unbroken black line.

Histopathology was performed to observe pathological changes at the microscopic level in shrimp tissues. The ability of pathogen 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 was high.

V. CONCLUSION

Isolates Vp-1 and Vp-2 were identified as *V. parahaemolyticus* strains causing AHPND that infect Pacific white shrimp, presenting with 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 certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

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REFERENCES

Abdel-Latif, H. M., E. Yilmaz, M. A. Dawood, E. Ringø, E. Ahmadifar, & S. Yilmaz. 2022. Shrimp vibriosis and possible control measures using probiotics, postbiotics, prebiotics, and synbiotics: A review. Aquaculture. 551: 1-23.

Aguirre-Guzmán, G., J. G. Sánchez-Martínez, R. Pérez-Castañeda, A. Palacios-Monzón, T. Trujillo-Rodríguez, & N. I. dela Cruz-Hernández. 2010. Pathogenicity and infection route of *Vibrio parahaemolyticus* in American white shrimp,

Litopenaeus vannamei. J. World Aquac. Soc. 41(3): 464-470.

Amparyup, P., Charoensapsri, W., & Tassanakajon, A. 2013. Prophenoloxidase system and its role in shrimp immune responses against major pathogens. Fish. Shellfish. Immunol. 34(4): 990-1001.

Anderson, D. P., & A. K. Siwicki. 1993. Basic haematology and serology for fish health programs. Paper Presented in Second Symposium on Diseases in Asia Aquaculture "Aquatic Animal Health and The Environmental". Phuket, Thailand. 25-29th October 1993.

Amrullah. 2014. Immunoproteksi vaksin toksid bakteri Streptococcus agalactiae pada ikan nila (*Oreochromis niloticus*) [Disertasi]. Bogor (ID): Sekolah Pasca Sarjana, Institut Pertanian Bogor.

Braak, K. V. D. 2002. Haemocytic defence in black tiger shrimp (*Paneus monodon*) [Disertasi]. Wageningen (NL): Wageningen Institute of Animal Science.

Cheng, W., C. H. Liu, S. T. Yeh, & J. C. Chen. 2004. The immune stimulatory effect of sodium alginate on the white shrimp Litopenaeus vannamei and its resistance against *Vibrio alginolyticus*. Fish Shellfish Immunol. 17: 41-51.

Costa, A. M., C. C. Buglione, F. L. Bezerra, P. C. C. Martins, & M. A. Barracco. 2009. Immune assessment of farm-reared Penaeus vannamei shrimp naturally infected by IMNV in NE Brazil. Aquaculture. 291: 141-146.

Dangtip, S., R. Sirikharin, P. Sanguanrut, S. Thitamadee, K. Sritunyalucksana, S. Taengchaiyaphum, R. Mavichak, & T. W. Flegel. 2015. AP4 method for two-tube nested PCR detection of AHPND isolates of *Vibrio parahaemolyticus*. Aquaculture Reports. 2(1): 158-162.

Effendi, M. I. 1997. Biologi Perikanan. Bogor (ID): Yayasan Pustaka Nusantara.

[FAO] Food and Agriculture Organization of the United Nations. 2022. The State of World Fisheries and Aquaculture 2022 [Internet]. [Accessed 2023 Aug 6]; http://www.fao.org/fishery/statistics.

Hamsah, H., W. Widanarni, A. Alimuddin, M. Yuhana, M. Z. Junior, & D. Hidayatullah. 2019. Immune response and resistance of Pacific white shrimp larvae administered probiotic, prebiotic, and synbiotic through the bioencapsulation of *Artemia* sp. Aquac. Int. 27(2): 1-14.

Han, J. E., Tang, K. F., Tran, L. H., & Lightner, D. V. 2015. Photorhabdus insect-related (Pir) toxin-like genes in a plasmid of *Vibrio parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. Dis. Aquat. Org. 113(1): 33-40.

Hauton, C. 2012. The scope of the crustacean immune system for disease control. J. Invert. Pathol. 110(2): 251-260.

Hoang, T. D. M., H. L. Tien, H. H. M. C. Hoang, K. H. N. Phuoc, H. Q. Pham, T. L. Tran, & H. T. Van. 2021. A novel PCR method for simultaneously detecting acute hepatopancreatic necrosis disease (AHPND) and mutant-AHPND in shrimp. Aquaculture. 534: 1-7.

Hong, X., L. Lu, & D. Xu. 2016. Progress in research on acute hepatopancreatic necrosis disease (AHPND). Aquac. Int. 24: 577-593.

Izwar, A. 2018. Isolasi, identifikasi, dan uji patogenisitas bakteri penyebab penyakit pada ikan kakap putih Lates calcarifer [Tesis]. Bogor (ID): Institut Pertanian Bogor.

Johansson, M. W., P. Keyser, K. Sritunyalucksana, & K. Soderhall. 2000. Crustacean haemocytes and haematopoiesis. Aquaculture. 191: 45-52.

[KKP] Kementerian Kelautan dan Perikanan. 2022. Kelautan dan Perikanan Dalam Angka Tahun 2022. Jakarta (ID): Kementerian Kelautan dan Perikanan.

Kumar, V., S. Roy, B. Behera, P. Bossier, & B. K. Das. 2021. Acute hepatopancreatic necrosis disease (AHPND): virulence, pathogenesis and mitigation strategies in shrimp aquaculture. Toxins. 13(8): 1-28.

Lee, J. M., R. N. Azizah, & K. S. Kim. 2020. Comparative evaluation of three agar media-based methods for presumptive identification of seafood-originated *Vibrio parahaemolyticus* strains. Food Control. 116: 1-6.

Liu, C. H., & J. C. Chen. 2004. Effect of ammonia on the immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to Vibrio alginolyticus. Fish Shellfish Immunol. 16: 321-324.

Liu, S., Zheng, S. C., Li, Y. L., Li, J., & Liu, H. P. 2020. Hemocyte-mediated phagocytosis in crustaceans. Front. Immunol. 11: 268.

Madigan, M. T., J. Martinko, & J. B. Parker. 2003. The Biology of Microorganisms. New Jersey (US): Prentice Hall.

Muharrama, A. R. W., W. Widanarni, A. Alimuddin, & M. Yuhana. 2021. Gene expression and immune response of Pacific white shrimp given Bacillus NP5 probiotic and honey prebiotic and *Vibrio parahaemolyticus* infection. J. Appl. Aquac. 34(3): 1-17.

Nazaruddin, D. Aliza, S. Aisyah, Zainuddin, & Syafrizal. 2014. Gambaran histopatologis hepatopankreas udang windu (*Penaeus monodon*) akibat infeksi virus hepatopancreatica parvovirus (HPV). J. Kedokteran Hewan. 8(1): 27-29.

Nitimulyo, K. H., A. Isnansetyo, Triyanto, M. Murdjani, & L. Shoclchah. 2005. Efektivitas vaksin polivalen untuk pengendalian vibriosis pada kerapu tikus (*Cromileptes altivelis*). J. Perikanan. 7(2): 95-100.

Nunan, L., D. Lighner, C. Pantoja, & S. Gomez-Jimenez. 2014. Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. Dis. Aquatic Organ. 111(1): 81-86.

[OIE] Office International des Epizooties. 2019. Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.2.1. OIDE: France.

Risjani, Y., N. Mutmainnah, P. Manurung, S. N. Wulan, & Yunianta. 2021. Exopolysaccharide from Porphyridium cruentum (purpureum) is not toxic and stimulates immune response against vibriosis: the assessment using zebrafish and white shrimp *Litopenaeus vannamei.* Mar. Drugs. 19(133): 1-17.

Rodriguez, S., O. R. Lozano, P. Gonzales, M. D. A. Bolan, & R. Anguilar. 2019. Characterization and growth conditions of Vibrio parahaemolyticus strains with different virulence degrees that cause acute hepatopancreatic necrosis disease in *Litopenaeus vannamei.* J. World Aquac. Soc. 50(5): 1002-1015.

Sahoo, P. K., B. R. Pillai, J. Mohanty, J. Kumari, S. Mohanty, & B. K. Mishra. 2007. In vivo humoral and cellular reaction, and fate of injected bacteria Aeromonas hydrophila in freshwater prawn *Macrobacterium rosenbergii*. Fish Shellfish Immunol. 23: 327-340.

Saulnier, D., Haffner, P., Goarant, C., Levy, P., & Ansquer, D. 2000. Experimental infection models for shrimp vibriosis studies: a review. Aquaculture, 191(1-3): 133-144.

Smith, V. J., J. H. Brown, & C. Hauton. 2003. Immunostimulation in crustaceans: does it really protect against infection?. Fish Shellfish Immunol. 15(1): 71.

Takeuchi. 2003. Role of COX inhibition in pathogenesis of NSAID-induced small intestinal damage. Kyoto (JP): Kyoto Pharmaceutical University.

Tang, K. F. J., & Bondad-Reantaso, M. G. 2019. Impacts of acute hepatopancreatic necrosis disease on commercial shrimp aquaculture. Rev. Sci. Tech. 38: 477-490.

Tran, L., Nunan, L., Redman, R. M., Mohney, L. L., Pantoja, C. R., Fitzsimmons, K., & Lightner, D. V. 2013. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis. Aquat. Org. 105(1): 45-55.

Valente, C. D. S., & Wan, A. H. 2021. Vibrio and major commercially important vibriosis diseases in decapod crustaceans. J. Invertebr Pathol. 181: 107527.

Widanarni, W., Gustilatov, M., Ekasari, J., Julyantoro, P. G. S., Waturangi, D. E., & Sukenda, S. 2024. Unveiling the positive impact of biofloc culture on *Vibrio parahaemolyticus* infection of Pacific white shrimp by reducing quorum sensing and virulence gene expression and enhancing immunity. J. Fish. Dis. 47(6), e13932.

Zorriehzahra, M. J., & Banaederakhshan, R. J. A. A. V. S. 2015. Early mortality syndrome (EMS) as new emerging threat in shrimp industry. Adv. Anim. Vet. Sci. 3(2S): 64-72.