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Pathogenicity of *Vibrio parahaemolyticus* causing Acute Hepatopancreatic Necrosis Disease (AHPND) in shrimp (*Litopanaeus vannamei*) in Serang, Banten, Indonesia

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Abstract. *Saputra A, Maftuch, Andayani S, Yanuhar U. 2023. Pathogenicity of Vibrio parahaemolyticus causing Acute Hepatopancreatic Necrosis Disease (AHPND) in shrimp (*Litopanaeus vannamei*) in Serang, Banten, Indonesia. Biodiversitas 24: 2365-2373.* Vannamei shrimp target EMS (Early Mortality Syndrome)/AHPND (Acute Hepatopancreatic Necrosis Disease) cases in several Asian nations, including China, Vietnam, Malaysia, Thailand, India, and Indonesia. AHPND cases on shrimp farms have been reported in several Indonesian regions. The aim of this study was to evaluate the histopathological pathogenicity of *Vibrio parahaemolyticus* in different shrimp tissues exposed to different doses and durations of exposure; from Serang City/District, Banten, Indonesia. PCR amplification of *V. parahaemolyticus* was performed using Vp.flaE-79F (5′-GCA GCT GAT CAA AAC GTT GAG T-3′) and Vp.flaE-934R (5′-GATACCTTGTTACGACTT -3′) primers for molecular identification of bacteria isolated from shrimp exhibiting AHPND symptoms. *V. parahaemolyticus* positive target band at 897 bp. Bacterial isolates with green colonies on TCBS agar media were chosen for other test. Challenge tests with healthy shrimp were conducted using the *V. parahaemolyticus* strain. The challenge test on shrimp demonstrated the same clinical and pathological symptoms as the signs of infected shrimp in rivers, including swimming into the container's corner and changes in the carapace's pale color and empty midgut. The results showed that challenged shrimp displayed necrosis, atrophy, granulomas, cell sloughing, and had significantly considerable hemocyte infiltration that led to melanization. The highest percentage (63.33%) of damage was recorded in the treatment with a concentration of 10^8 CFU mL^{-1}. It was also observed that virulence of *V. parahaemolyticus* strain varied with different concentrations. At concentrations of 10^8 CFU mL^{-1} and 10^6 CFU mL^{-1}, virulence of *V. parahaemolyticus* strain caused 100% mortality in 73 hour post-infection (p.i). The lowest concentration limit for pathogenicity was 10^4 CFU mL^{-1} with a mortality of 48% at 73 p.i. The challenge test results showed that *V. parahaemolyticus* strain that causes AHPND was the main pathogen affecting vannamei shrimp in Serang, Banten, Indonesia.

Keywords: Acute Hepatopancreatic Necrosis Disease, hepatopancreas histology, pathogenicity, polymerase chain reaction, *Vibrio parahaemolyticus*

INTRODUCTION

The production of vannamei shrimp (*Litopenaeus vannamei*) accounts for approximately 75% of the total shrimp production in Indonesia (FAO 2016). The Asia-Pacific region is the world's leading producer of aquaculture products. However, shrimp farming has suffered economic losses due to high mortality rates. In recent years, pathogenic bacteria have been responsible for fatal diseases. Due to poor environmental or physiological conditions, stressed biota is more susceptible to contracting infectious illnesses. The spread of *Vibrio* spp. is very difficult to control in the environment if transmission occurs (Helmi et al. 2020). There is a relevant relationship between high production intensity like high doses of feeding with increased growth of vibrio bacteria and low dissolved oxygen (Amalisa et al. 2021; Riandi et al. 2021).

Numerous researches have revealed pathogenic bacteria, and bacteria of the family Vibrionaceae are regarded as one of the most significant causes of shrimp aquaculture deaths globally. Recently, a disease known as Early Mortality Syndrome (EMS) has been detected in shrimp *L. vannamei*. Acute Hepatopancreatic Necrotic Disease (AHPND), caused by *P. monodon* and *P. chinensis* showed significant (100%) mortality rate (FAO 2013; Kumar et al. 2014). EMS (Early Mortality Syndrome) and AHPND have been observed in vannamei shrimp in several Asian nations, including China, Vietnam, Malaysia, Thailand, and India (Trisniaty 2015). Vannamei shrimp have reportedly been affected by AHPND in China (2009), Indonesia and Vietnam (2010), Malaysia (2011), and Thailand (2012) (Soto-Rodriguez et al. 2015). *Enterocytozoon hepatopenaei* (EHP) and *Vibrio parahaemolyticus* accompanied with Acute Hepatopancreatic Necrosis Disease (VpAHPND) were identified in imported samples from several Asian nations, including Indonesia and Vietnam (Han et al. 2020). EMS/AHPND caused by *V. parahaemolyticus* was detected in the hepatopancreas and intestines of infected vannamei shrimp (Joshi et al. 2014; Kumar et al. 2014;

Khimmakthong and Sukkarun 2017; Raja et al. 2017; Zheng et al. 2018; Kewcharoen and Srisapoome 2019; Lim et al. 2020; Haryani et al. 2022). *Vibrio parahaemolyticus* bacteria are halophilic and gram-negative, causing gastroenteritis (Paola et al. 1998). To induce hepatopancreas cell death (necrosis), *V. parahaemolyticus* attaches to the shrimp stomach organs by attaching with pili (Soonthornchai et al. 2015). Clinical symptoms in vannamei shrimp tissue infected with *V. parahaemolyticus* include epidermal degeneration and tissue necrosis (Joshi et al. 2014; Raja et al. 2017; Khimmakthong and Sukkarun 2017).

The impact of vibriosis-infected vannamei shrimp on aquaculture production, which has decreased, as measured by the percentage of survival rate at the start of maintenance, is between 20 and 30%. After infection, there is an increase in the feed conversion ratio (FCR), which ranges from 1.7 to 2.5 (Sriurairatana et al. 2014). *V. parahaemolyticus* becomes pathogenic if it is exposed to an environment that promotes the growth of these bacteria. This gram-negative bacterium thrives in environments with a lot of organic materials (Browdy et al. 2001). *Vibrio* spp. can kill shrimp at certain concentrations within 72 hours (Rokhmani 1994); and at 10^4 CFU mL^{-1} concentration, *V. parahaemolyticus* is found to be pathogenic (Abdul Manan and Kharisma 2012). Due to the high level of pathogenicity of VpAHPND, researchers focused their attention on identifying and isolating *V. parahaemolyticus*. *V. parahaemolyticus* causes many diseases when the shrimp's immune system is impaired or when the bacterial concentration is excessive (Soto-Rodriguez et al. 2015). The aim of this research was to assess the histopathological pathogenicity of *V. parahaemolyticus* in diverse shrimp tissues exposed to varying doses and exposure durations. This research contributes to the pathogenesis study of *V. parahaemolyticus* in vannamei shrimp.

MATERIALS AND METHODS

Statement of compliance with the ethics guideline

The experimental biota used in the present research were transported and tested following the recommendations of the Nationwide Advisory Committee for Laboratory Animal Studies (CCAC 2005).

Sampling and isolation of bacteria

Field samples were collected from the Kasemen Sub-district, Serang City (-6.025758, 106.159687) and Pontang Subdistrict, Serang District (-5.973887, 106.258592) of Banten Province, Indonesia (Figure 1). Infected prawns were placed in a plastic bag of pond water and kept in a styrofoam container with ice. The hepatopancreas (HP) was weighed and homogenized in 10 or 1.0 mL sterile 2.5% NaCl. Serial 10-fold dilutions of the supernatant with 2.5% NaCl were made; 0.1 mL of the solution was inoculated onto Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar to enumerate the *Vibrio* spp., and 0.1 mL was inoculated onto marine agar for the heterotrophic bacteria (Soto-Rodriguez et al. 2015). The pure culture of the desired colonies was grown on TCBS agar plates and incubated for 24 hours at 35 °C. Follow-up test for suspected *V. parahaemolyticus* bacteria with graded Tryptic Soy Agar (TSA) (0% up to 10% NaCl) (Pramono et al. 2015; Azis et al. 2022).

Histological analysis

The histopathological analysis was conducted as described by Joshi et al. (2014); Raja et al. (2017); Khimmakthong and Sukkarun (2017). The shrimp body was dissected from the head to the intestinal system, and the organs were then stored in Davidson's fixative. Hepatopancreas organs were also collected in this manner. Hematoxylin and eosin (HE) were used to dye the tissue sections.

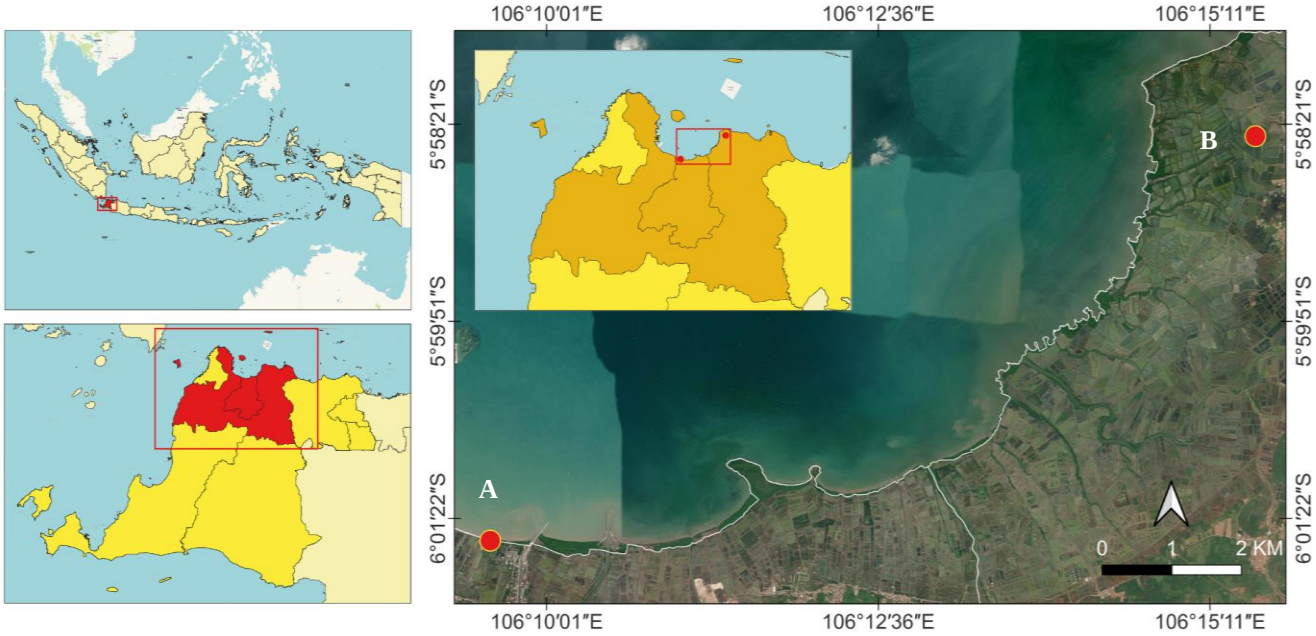

Figure 1. Sampling locations of *Litopenaeus vannamei* cases of AHPND in Kasemen Subdistrict (A), Serang City and Pontang Subdistrict (B), Serang District of Banten Province, Indonesia

Challenge test

The vannamei shrimp (*L. vannamei*), which had an average weight of 10 ± 0.5 g and were fed twice a day. The challenge test was performed according to Tran et al. (2013), Soto-Rodriguez et al. (2015) and Fu et al. (2018) with minor modifications. The challenge test was conducted in a 60 L container with in a density of 10 shrimp. Vannamei shrimp were inoculated by injection of *V. parahaemolyticus* for 72 hours at 0 (the control), 10^2, 10^4, 10^6, and 10^8 CFU mL^{-1} (Zheng et al. 2018). The treatment and control variables were performed in triplicates and in a completely randomized design. The experiment was conducted in a 12 hour dark/light photoperiod. Salinity of the water was 29 ppt, DO was kept at or above 5 ppm, pH 7, and the heater regulates the water's temperature at or below 29°C.

Observation and sampling

Commercial shrimp feed pellets (35% protein) were given to shrimp twice a day. Until all shrimp died (72 hours), and morphology and physiology were assessed every four hours. Moribund shrimp were cleaned with 70% ethanol. HP was removed for histopathological examination (Raja et al. 2017).

Bacterial reisolation

Within 24 hours of infection, HP samples were taken from moribund shrimp that displayed light HP signs (p.i). Bacterial streak from HP sample was plated on TCBS agar and incubated for 24 hours at 35°C. For further comparison with the tested isolates, GCs was plated on TCBS agar. GCs DNA was extracted and used for PCR testing (Soto-Rodriguez et al. 2015).

Molecular identification

Molecular identification was performed using the SensoQuest PCR. Bacterial genomic DNA was extracted with phenol-chloroform and precipitated with two volumes of ethanol in sodium acetate 0.3 M, pH 5.2; the pellet was washed twice with 70% ethanol, resuspended in sterile distilled water and stored at 4°C until use (Cordova et al. 2002). The isolates' GCs DNA was addressed to PCR amplification of *V. parahaemolyticus* using the primers Vp.flaE-79F (5′-GCA GCT GAT CAA AAC GTT GAG T-3′) and Vp.flaE-934R (5′-GATACCTTGTTACGACTT-3′) for bacterial identification. Extract samples were prepared for PCR as follows. Species *Vibrio* spp. from HP sample was grown at 35°C on TCBS for 24 h. After incubation, a single green colony was scraped from the plate surface and suspended in 200 µL of 1xTris-EDTA, pH 8.0. The suspension was then heated at 95°C for 10 min and centrifuged for 2 min to pellet cellular debris. PCR was carried out under the following conditions: initial denaturation step at 93°C for 15 min, followed by 35 cycles of denaturation at 92°C for 40 s, annealing at 57°C for 1 min, and extension at 72°C for 1.5 min. The process was completed with a final extension at 72°C for 7 min. PCR amplicons were electrophoresed in 1.5% agarose for 1 h at 100 V (Tarr et al. 2007).

Data analysis

The data obtained from the intestinal histology scoring was analyzed using the nonparametric method of the Kruskal Wallis Test to determine the differences treatment. The obtained data were analysed with SPSS 21 software and Microsoft Excel, while Koch's postulate experiment was subjected to a one-way ANOVA.

RESULTS AND DISCUSSION

Infected shrimp symptoms and pathogen identification

The result showed that shrimp with AHPND symptoms had a low appetite, pale carapace, lysed empty midgut contents, and HP becomes atrophic and looked whitish (Figure 2.A-B). Different conditions in healthy shrimp showed morphology carapace with dark pigmentation, with full midgut (Figure 2.C). A similar condition was reported by Soto-Rodriguez et al. (2015) that shrimp infected with AHPND showed slightly dilated chromatophores, lethargy, erratic swimming, and empty intestine. The results of PCR analysis revealed that *V. parahaemolyticus* showed a positive band of target 897 bp, detected in shrimp that displayed positive clinical signs (Figure 3). A similar result was also reported by Tarr et al. (2007), where the DNA band was 897 bp in size.

Shrimp farmer suffered losses due to the AHPND disease, which struck several shrimp farmers in Serang (Kasemen and Pontang Districts), Banten. Previously, the bacteria *V. parahaemolyticus* was identified at an 897 bp DNA band using the primer pairs 79F and 934R. For *V. parahaemolyticus* PCR testing, various primers have been used by many researchers. Nunan et al. (2014); Dabu et al. (2017) and Khimmakthong and Sukkarun (2017) claimed to use primer 89F/R under 470 bp DNA band to identify *V. parahaemolyticus*.

Isolates from shrimp ponds affected by AHPND

The isolates of hepatopancreas target organ (HP) on TCBS media were associated with clinical symptoms that characterize infection with AHPND disease caused by *V. parahaemolyticus* in shrimp. Tran et al. (2013) and Soto-Rodriguez et al. (2015) reported that the causative agent of AHPND is *V. parahaemolyticus* in Asia and Mexico. On TCBS agar media, the samples showed two different colony colors, namely yellow colonies (YCs) and green colonies (GCs) (Figure 4.A). A pure culture of green colonies was maintained on TCBS agar (Figure 4.B).

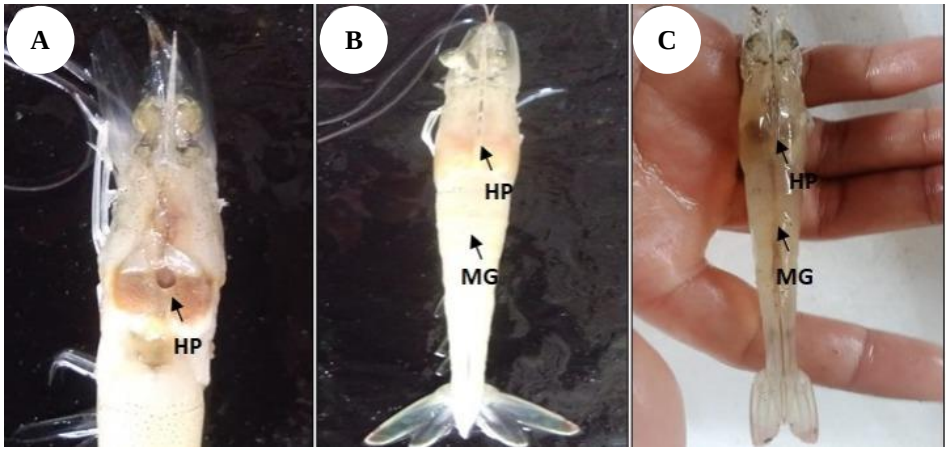

Figure 2. A. Signs of hepatopancreas (HP) (arrow) of vannamei shrimp infected with AHPND, B. Empty midgut (MG) (arrow), and pale HP (arrow) of AHPND-infected vannamei. C. Midguts (MG) (arrows), and HP (arrows) of normal vannamei

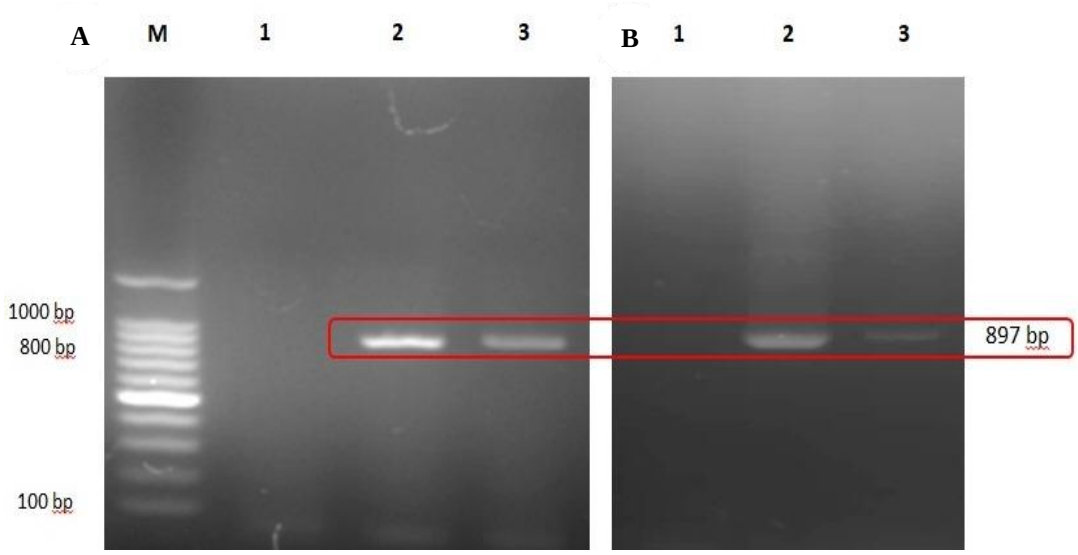

Figure 3. A. The results of nested PCR analysis: (M) marker, 1. negative control, 2. positive control, 3a. shrimp with clinical symptoms of AHPND in ponds in Pontang District, B. 1. negative control, 2. positive control, shrimp with clinical symptoms of AHPND in ponds Kasemen District

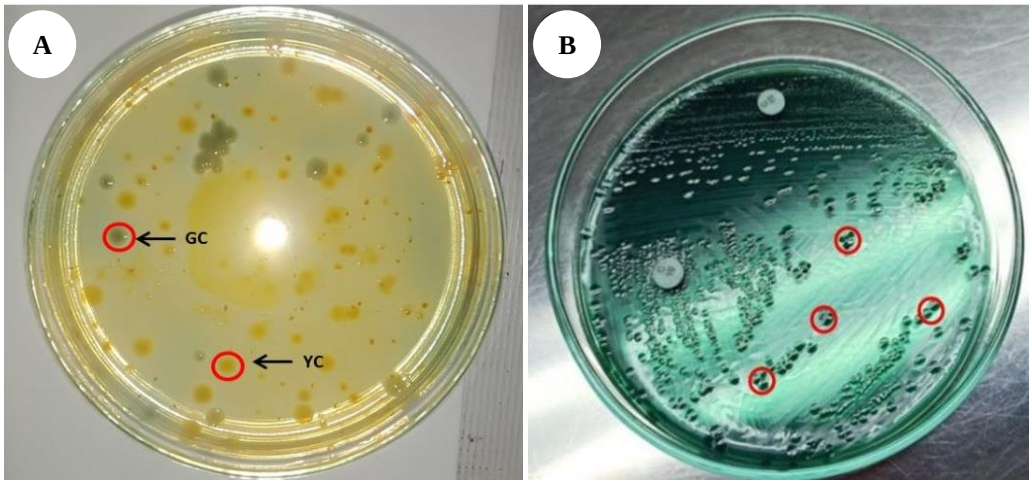

Figure 4. A. Samples with two different colony colors, yellow colonies (YCs) (arrow) and green colonies (GCs) (arrow) on TCBS agar. B. Green colonies on TCBS agar

Reisolation of *V. parahaemolyticus* from challenge test

In the challenge test, shrimp in the four experimental groups with *V. parahaemolyticus* showed low appetite, then stop eating after an hour. Pratiwi et al. (2022) reported that the results of challenge test of shrimp with *V. parahaemolyticus* showed changes in morphology and the shrimp began to passively swim. Additionally, at 10^8 CFU mL^{-1} concentration, the first death in shrimp occurred at 1 p.i (Figure 5). Before death, infected shrimp from the treatment group had the same clinical symptoms as infected shrimp from ponds. Shrimp from the control group showed no distinguishable changes in their HP, while shrimp in the four experimental groups challenged with *V. parahaemolyticus* displayed empty midgut and blanching changes. At bacterial concentrations of 10^8 and 10^6 CFU mL^{-1}, the mean lethal times (LD_{50}) were 4 hours, and 8 hours p.i, respectively (Figure 5). Bacterial concentration of 10^4 and 10^2 CFU mL^{-1} resulted in mortality rates of 48% and 27% at 73 hours p.i. At 10^8 and 10^6 CFU mL^{-1}, the mortality was 100% in 16 and 28 hours, respectively. After 73 hours post-injury, mortality rate in the control group was 7%. Shrimp exhibited clinical sign of AHPND when challenged with *V. parahaemolyticus.* The outcomes of this challenging test revealed that the isolated strain of *V. parahaemolyticus* displayed virulence comparable to that of the strain described by Dong et al. (2017) and Fu et al. (2018) before when a concentration of 10^6 CFU mL^{-1} resulted in 100% death at 20 to 24 hours p.i. Soto-Rodriguez et al. (2015) revealed that at a concentration of 10^6 CFU mL^{-1}, death in various strains of *V. parahaemolyticus* reached 100% at 16 to 25 hours p.i. Different virulent conditions were reported by Dabu et al. (2017), whereas *V. parahaemolyticus* demonstrated a death rate of 40% to 50% in the challenge test with a concentration of 10^6 CFU mL^{-1} for up to 120 hours p.i. Nunan et al. (2014) reported that *V. parahaemolyticus* at a concentration of 10^6 CFU mL^{-1} caused 96-hour-long shrimp mortality. Based on a challenge test of 12 strains of VpAHPND identified by shrimp from various experimental groups, it was revealed that 10 strains caused 100% mortality of 10 strains on day 4 p.i (Feng et al. 2017).

The isolated bacteria *V. parahaemolyticus* had a virulence that resulted in mortality starting at 1-hour p.i and reaching the first 100% death at 16 hours p.i at a concentration of 10^8 CFU mL^{-1}. The cause of EMS/AHPND in shrimp has been recognized as toxin-producing *V. parahaemolyticus* (Maralit et al. 2018). The *V. parahaemolyticus* strain, which secretes PirA/B in the hepatopancreas, is the cause of AHPND (Lai et al. 2015; Foo et al. 2017). Han et al. (2015) and Tinh et al. (2021) discovered that the protein toxins pirA (336 bp) and pirB (1317 bp), which encode for proteins 13 and 50 kDa, respectively, were produced by AHPND as a result of *V. parahaemolyticus* infection. While Peña-Navarro et al. (2020) used the primers gene sequences for pirA (284 bp) and pirB (392 bp) to identify pirA and pirB toxins. The 69-kb plasmid contains protein-coding gene of AHPNS strains pirA and pirB (Theethakaew et al. 2017). Sirikharin et al. (2015) and Lazarte et al. (2021) reported two toxin proteins of *V. parahaemolyticus* strain, encoding for 15-16 kDa (pirA) and 50-55 kDa (pirB) proteins, respectively, homologous to the insect toxin. The toxin that VpAHPND employs to eliminate shrimp affects its stomach epithelium, which leads to increased propagation of the toxin protein and the bacteria into the hepatopancreas and the sloughing off of the epithelial cells lining the tubules of the organ (Maralit et al. 2018). Bile acids positively control the virulence of PirABVp toxin that causes AHPND pathogenicity (Kumar et al. 2020). PirBVp can be adequate to damage cells, according to some research, whereas binary PirABvp toxin is necessary to exert toxic effects (Lai et al. 2015). According to Noble et al. (2021), the expression of pirA and pirB toxin in *V. parahaemolyticus* using qPCR revealed no discernible change in pirA or pirB toxin gene expression. The VpAHPND injection challenge test (with pirA and pirB) revealed the presence of responsive genes, including those involved in important metabolic processes such as carbohydrate, lipid, and amino acid metabolism, as well as the strong activation of genes involved in cell growth and anti-apoptotic responses (Zheng et al. 2018). The in vivo tests show that PirBVp is more harmful than PirAVp confronted with Artemia, PirAVp and PirBVp toxins operate synergistically in parallel, where the two toxins create a complex (Kumar et al. 2019). AHPND's pathophysiology involves the VpAHPND toxin receptor, which mediates the entry of toxins into hemocytes like LvAPN1, and shows that rPirAVp and rPirBVp toxins are directly bound by rLvAPN1 (Dong et al. 2019). LvAPN1 inhibition prevents the toxin from severely damaging hemocytes and maintains total haemoglobin levels (THC) (Luangtrakul et al. 2021). When shrimp were exposed to pure strains of *V. parahaemolyticus*, they displayed clinical signs of infection, such as halting to eat. The actions of swimming to the container's corner and alterations in the carapace's pale color and empty gut. After 1 hour of p.i, first death occurred, and after 16 hours of p.i (10^8 CFU mL^{-1}) the mortality rate was 100% . The distinct marks on infected shrimp from the pond were due to the clinical signs shown in shrimp tested against the pure strain of *V. parahaemolyticus* identified.

AHPND histopathology from challenge test

It was observed that when shrimp exposed to pure strain of *V. parahaemolyticus* they displayed histological signs of AHPND infection. Treated shrimp HP shows different conditions in the test groups compared to the control. Based on the general histology of shrimp infected with *V. Parahaemolyticus* (treatment), necrosis, sloughing of cells and tubules, granulomas, and atrophy and inflammation of the midgut, acute illness were detected from 4 to 24 hours p.i (Figure 6a-h).

The histopathology of acute circumstances in shrimp hepatopancreas was characterized in a challenge test investigation of the pure strain of *V. parahaemolyticus.* Attacks by acute disease manifest as necrosis and atrophy. Hemocyte infiltration between the tubules was seen along with a granuloma. Numerous sloughing cells were seen in the tubule lumen, and hemocytes that produced melanization were infiltrated quite heavily all around the

tubules. Tran et al. (2013) reported *V. parahaemolyticus* challenge test displayed lesions, peeling and necrotic HP tubules, and hemocyte infiltration between tubules. The same ailment has also been reported by Nunan et al. (2014) and Han et al. (2017).

The percentage of damage caused by each treatment was different. The average level of damage is presented in (Table 1). The results of statistical analysis showed that there was a significant difference in the damage percentage between control and treatment of *V. parahaemolyticus* challenge test ($p<0.05$).

The highest percentage of damage in infection with the administration of a concentration of 10^8 CFU mL^{-1}, while the lowest percentage (26.67%) of damage was in the treatment with a concentration of 10^2 CFU mL^{-1} . The control treatment showed little damage. This shows that the higher the concentration of *V. parahaemolyticus* infection, the higher the percentage of hepatopancreas damage and mortality rate. Soto-Rodriguez et al. (2015) reported that VpAHPND strain's pathogenicity varied with exposure time and concentration. As reported by Sani et al. (2020) when shrimp challenged with VpAHPND at DOC 80, mortality reached 90% with HP level of necrosis (lesions). According to present research, these circumstances demonstrate that *V. parahaemolyticus* concentration and exposure period play a significant influence in defining the virulence conditions. Pathogenicity testing of the isolate *V. parahaemolyticus* revealed acute exfoliation of HP tubular epithelial cells. The results of a *V. parahaemolyticus* challenge test at various concentrations revealed HP tubular epithelial sloughing, HP necrotic sloughing, HP tubules surrounded by hemocytic infiltrate, cell sloughing was observed in the HP tubular lumen with significant bacterial colonization, and hepatopancreas showed collapsing tubular epithelium. The results of this research show that a specific criterion for the attack of the VpAHPND toxin invade the epithelium of the shrimp HP tubular cells. The virulence of the *V. parahaemolyticus* strain must also be taken into account, along with several other crucial elements.

Table 1. Average level of damage to the intestine of vannamei shrimp

Treatments	Average level of damage	Damage percentage (%)	Damage rates
control	0.27 ± 0.12	6.67	Light
10^2 CFU mL^{-1}	2.00 ± 0.20	26.67	Medium
10^4 CFU mL^{-1}	2.67 ± 0.12	56.67	Heavy
10^6 CFU mL^{-1}	3.00 ± 0.35	60.00	Heavy
10^8 CFU mL^{-1}	3.00 ± 0.20	63.33	Heavy

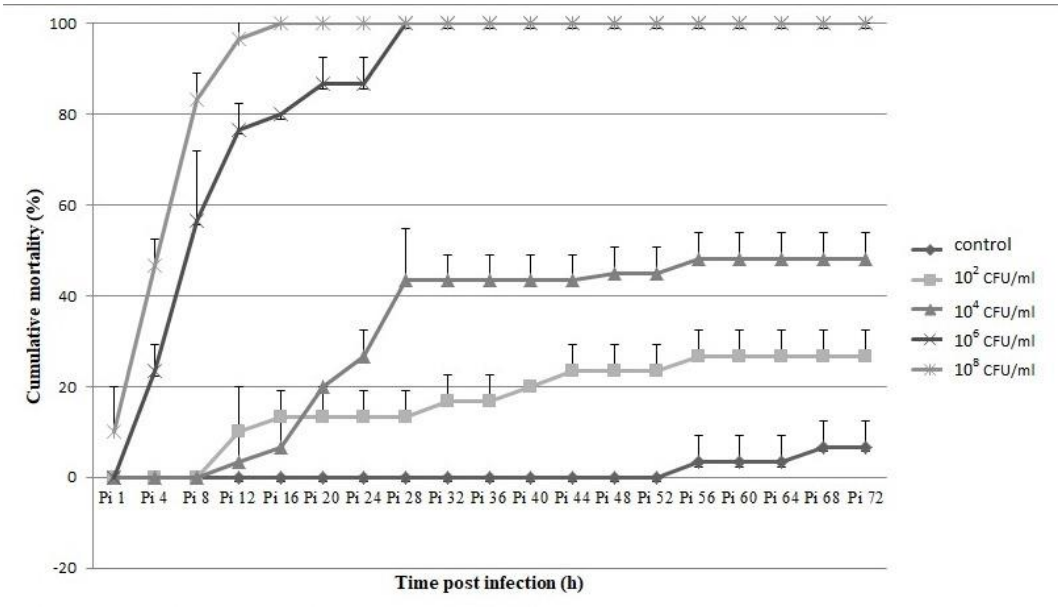

Figure 5. Cumulative mortality of vannamei shrimp challenged with *Vibrio parahaemolyticus*

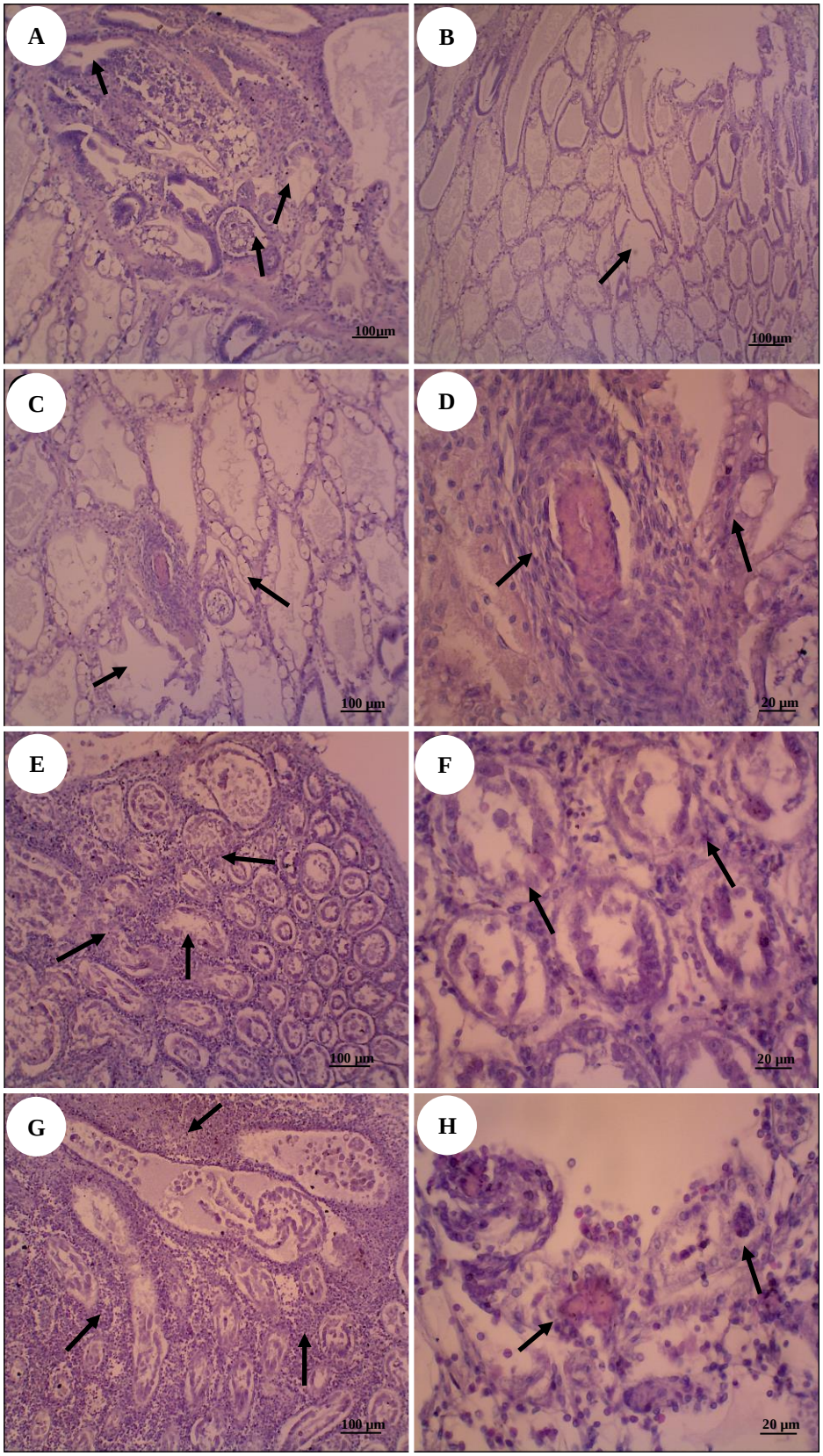

Figure 6. Hepatopancreas (HP) images of shrimp due to infection with *V. parahaemolyticus* 4 to 24 hours after infection: A. Tubules undergo necrosis (arrows) at 4 hours p.i, B. Tubules atrophy (arrows) at 4 hours p.i, C. Presence of granulomas (arrows) at 4 hours p.i, D. Hemocytee infiltration between tubules (arrows) at 4 hours p.i, E. showing necrosis (arrows) in tubules at 24 hours p.i, F. The lumen of the tubules observed many sloughing cells (arrows) at 24 hours p.i, G. Around the tubules hemocytic infiltration occurred (arrows) at 24 hours p.i, H. melanization (arrows) at 24 hours p.i. The hepatopancreas (HP) of shrimp due to infection with *V. parahaemolyticus* 4 to 24 hours after infection

In conclusion, our findings show that the VpAHPND strain found in Serang causes infection in shrimp. The results of this study, such as mortality and necrosis in HP, had an impact on which. Future research aims to broaden the research design to include Banten Province, Indonesia as well as Serang Serang City/District, allowing detailed monitoring of bacterial disease outbreaks caused by shrimp culture in Banten Province during field tests.

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