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Prevalence Analysis of Acute Hepatopancreatic Necrosis Disease (AHPND) in Vannamei Shrimp (*Litopenaeus vannamei*) by PCR Method in Bengkalis District

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ABSTRACT

Vannamei shrimp is one of the shrimps that are being cultivated in Indonesia because it has superior quality compared to other types of shrimp. Cultivation activities cannot be separated from disease attacks that attack cultured commodities. One of the diseases that attack vannamei shrimp is Acute Hepatopancreatic Necrosis Disease (AHPND). The disease is caused by Vibrio parahaemolyticus bacterial infection (VpAHPND) which produces toxins and causes death in shrimp with mortality reaching 100%. Therefore, it is necessary to conduct research or early detection in the aquaculture area of Bengkalis Regency using the PCR (Polymerase Chain Reaction) method. The method used is the survey method. This research was conducted from March to April 2023. The results of this study are the prevalence of AHPND (Acute Hepatopancreatic Necrosis Disease) in vannamei shrimp (*Litopenaeus vannamei*) in the Bengkalis Regency Aquaculture area is 0% with the category of infection never. AHPND examination by PCR method using AP4 primer measuring 230 bp showed that vannamei shrimp samples from Bengkalis District, Bantan District, and Bukit Batu District of Bengkalis Regency were negative for AHPND. The morphological condition of vannamei shrimp from the three research locations did not show any clinical symptoms of shrimp infected with AHPND.

Keywords: AHPND, PCR, Vannamei Shrimp

1. INTRODUCTION

Vannamei shrimp (*Litopenaeus vannamei*) known as white shrimp is one of the shrimp that is being cultivated in Indonesia. Vannamei shrimp in 2019 has a large contribution to the country with an average export volume reaching 85% (KKP, 2019). Many people cultivate Vannamei shrimp because it has superior qualities compared to other types of shrimp, namely being able to live in low water quality, tolerant of high density, have a good appetite, can grow well with low protein feed, and are resistant to disease (Irfandy et al., 2016).

The superiority of this shrimp creates a lot of local and world market demand that makes farmers rampant Vannamei shrimp cultivation in several regions in Indonesia. One of the areas that has the potential to develop Vannamei shrimp farming is Bengkalis Regency, where this district is directly adjacent to the East Coast of Sumatra. According to Sari (2021), Bengkalis Regency, which has an area of 1,300 ha and has utilized as much as 469.66 has the potential to develop aquaculture

Aquaculture activities cannot be separated from the attack of diseases that attack aquaculture commodities. One of the diseases that attack vannamei shrimp is Acute Hepatopancreatic Necrosis Disease (AHPND). This disease first appeared in China in 2009, known as Covert Mortality Syndrome (CMS). In 2010, the disease increasingly spread to various farms in China. Then in 2011, EMS/AHPNS was detected in and infected ponds in Vietnam and Malaysia, the disease was also confirmed in Thailand in 2012 (FAO, 2013). Based on the estimated total decline in shrimp production from 2011 to 2014, the total economic loss from AHPND disease was estimated at USD 0.49 billion (Kua et al.,

2016).

AHPND disease is caused by infection with *Vibrio parahaemolyticus* bacteria (Vp AHPND) that produce toxins and cause death in shrimp with mortality reaching 100%. Shrimp mortality due to AHPND occurs at the age of less than 40 days after stocking in the pond. The main causes of AHPND cases occur mostly due to high stocking density, high salinity, poor water quality, dry season, incomplete pond preparation, post larva stress during transport and acclimatization, low dissolved oxygen, and poor feed quality and management (Muslimah, 2022).

The presence of AHPND in Vannamei shrimp will have a very detrimental impact on the production of shrimp farmers. One of the measures to prevent the spread of AHPND disease in ponds is the distribution of AHPND disease-free shrimp and the application of strict biosecurity. Therefore, it is necessary to conduct research or early detection in the pond area of Bengkalis Regency using the Polymerase Chain Reaction (PCR) method. By researching the analysis of the prevalence of AHPND in Bengkalis Regency, this research aims to find out how much AHPND attacks vannamei shrimp farming. In addition, early efforts can also be made to prevent AHPND disease attacks

2. RESEARCH METHODS

Time and Place of Research

This study was conducted from March to April 2023. Vannamei shrimp samples were taken from 3 sub-districts in Bengkalis Regency, Riau Province, namely Bantan District, Bengkalis District, and Bukit Batu District. Sample analysis was conducted at the Pekanbaru Fish Quarantine, Quality Control, and Fishery Product Safety Station (SKIPM) Laboratory.

Research Methods

The method used in this research is the survey method. The survey method is used to obtain or collect information and data from a large population with a relatively small sample. The sampling method used purposive sampling method.

Research Procedures

Water Quality Measurement

Water quality measurements in Vannamei shrimp ponds were conducted in situ. The measured water quality parameters include pH, temperature, salinity, dissolved oxygen, and ammonia.

Sample Handling

The samples used in this study were vannamei shrimp with an age range of 33-36 days. Shrimp samples were taken using anco as many as 5 shrimp per pond and measured its length, then put into plastic samples and stored in an icebox.

Preparation of Bacterial Enrichment Media

The media used for isolation and enrichment of bacteria is TSB (Trypticase Soy Broth) 3%. The method of making 3% TSB media is weighing 6 g of TSB media and 3 grams of NaCl then dissolved in 191 ml of distilled water in an erlenmeyer and then covered with aluminium foil. Furthermore, it was heated on a hotplate using a stirrer until homogeneous, then the media was sterilized in an autoclave at 121ºC and 2 atm pressure for 15 minutes. The media was cooled at room temperature until warm and then poured into a 5 ml tube.

Bacterial Enrichment

Vannamei shrimp samples were cultured by taking the shrimp hepatopancreas using scissors and tweezers and then put into 3% TSB media. Furthermore, the suspension was stored in an incubator at room temperature (25-28 ºC) and isolated for 18-24 hours before the DNA extraction process. TSB media contains casein and soy peptone which provide amino acids and other nitrogenous substances that make it a nutritious medium (Setiadji et al., 2017). It is often used for the enrichment of various microorganisms, one of which is Vibrio bacteria which is the causative agent of AHPND disease.

DNA Extraction

DNA extraction was carried out using the precipitation method (SKIPM Pekanbaru 2022). The isolated 3% TSB medium was taken as much as 1 ml and put into a 1.5 ml microtube. Then incubated at 95 ºC for 10 minutes and centrifuged at 8000 rpm for 5 minutes then the supernatant was discarded. The pellet formed was added 600 µl CTAB solution and incubated at 75 ºC for 5 minutes then let stand at room temperature. Next, 700µl

chloroform was added, vortexed for 20 seconds, and centrifuged at 12,000 rpm for 5 minutes. The supernatant formed was transferred into a new microtube as much as 200 µl then added 100 µl CTAB solution and 900 µl NFW (Nuclease Free Water). Vortex for 20 seconds, incubated at 75 ºC for 5 minutes, and allowed to stand at room temperature, then centrifuged at 12,000 rpm for 10 minutes then the supernatant was discarded. Pellets were dissolved with 150 µl dissolve solution and incubated at 75 ºC for 5 minutes, allowed to stand at room temperature, and then centrifuged at 12,000 rpm for 5 minutes. Then the supernatant (clear layer) was transferred as much as 200 µl into a new microtube measuring 1,5 ml, added 300 µl of 95% Ethanol and vortexed for 20 seconds, and then centrifuged at 12,000 rpm for 5 minutes. The supernatant was discarded and the pellet was dried, then 100 µl NFW was added. The sample extraction results were stored at -20 ºC until they were amplified.

Amplification

Amplification aims to multiply the target DNA that will be analyzed by agarose gel electrophoresis. After DNA extraction, the next step is the preparation of reagents for the First PCR Master mix as much as n + 1 with a total of 25 µl reagents in 1 reaction, consisting of 1 µl F1 primer, 1 µl R1 primer, 12.5 µl Go Taq Green, and 6.5 µl NFW then vortexed until homogeneous and spindown for 5 seconds. Next, the First PCR master mix was distributed into a 0.2 ml tube with as much as 21 µl to each tube and added 4 µl each of the DNA template, control (+), and control (-) according to the tube code then vortexed until homogeneous and spindown for 5 seconds. Microtubes were inserted into the thermal cycler and run First PCR with the following temperature settings: 1) 94ºC 2 minutes, lyse face, 2) 94ºC 30 seconds, denaturation; 55ºC 30 seconds annealing phase, and 72ºC 90 seconds extension, repeated for 30 cycles; and 3) 72ºC 2 minutes, until the cycle ends.

After the First PCR is complete, the microtube is removed from the thermal cycler, then the First PCR amplification results are used as a template for Nested PCR. Nested PCR master mix was prepared in a 0,2 ml tube according to the number of previous templates, then prepared a mixture of Nested PCR master mix reagents consisting of 1 µl F2 primer, 1 µl R2 primer, 12.5 µl Go Taq Green, and 6.5 µl NFW Master mix Nested PCR that has been prepared is vortexed until homogeneous and dispindown for 5 seconds. Then the Nested PCR Master mix was distributed as much as 21 µl to each tube and added 4 µl each of the First PCR results according to the previous sample code. Microtubes were inserted into the thermal cycler and run Nested PCR with the following temperature settings: 1) 94^{o}C 2 minutes; 2) 94^{o}C 20 seconds, 55^{o} C 20 seconds, 72^{o}C 20 seconds, repeated for 30 cycles; and 3) 72^{o}C 2 minutes, until the end of the cycle.

After the Nested PCR amplification ends, the tube is removed and the thermal cycler is turned off. The PCR product is ready for electrophoresis.

Electrophoresis

The first electrophoresis process is making 1.5% agarose. Agarose was weighed as much as 0.75 g then put into an erlenmeyer and added 50 ml TAE Buffer. Then heated in a microwave at a temperature of 100-150 ºC for ±3 minutes, when it boils the agarose is removed from the microwave and then cooled to room temperature, added 5 µl sybr safe and shaken in a circle so that it is evenly distributed, then poured into the casting gel that has been fitted with a comb. The hardened agarose gel was put into the electrophoresis machine and filled with 500 ml TAE Buffer 1x until the agarose gel was submerged. The amplicons were inserted into the wells sequentially, negative control, sample, positive control, and marker each as much as 5 µl then electrified 50 volts for 50 minutes. In the process of electrophoresis, negatively charged DNA will move to the positive pole.

DNA Visualization

DNA observation was carried out by documenting samples that had been PCR. The gel electrophoresis results were lifted and placed on a UV transluminator, then a picture was taken with a Gel documentation system. After the UV transluminator is switched on, the DNA bands will glow. Bands or DNA bands that appear on the sample are compared with the bands on the positive control (Fahmi et al., 2022). The sample infected with AHPND measures 230 bp and is aligned with the DNA band from the positive control.

Prevalence Test

The main parameter observed was the prevalence of AHPND infecting Vannamei shrimp, and the supporting parameters were clinical symptoms and water quality before examination. The prevalence value was used to determine the number of shrimp infected with AHPND. Samples of Vannamei shrimp positive for AHPND were calculated using a formula to determine the prevalence value. Prevalence can be calculated using the formula according to Cameron (2002).

$$\text{Prevalence (\%)} = \frac{\sum \text{infected shrimp}}{\sum \text{shrimp samples}} \times 100$$

Data Analysis

Data obtained from the examination results were presented in tables and figures, then analyzed descriptively to explain the prevalence value of AHPND infecting vannamei shrimp according to Williams & Williams (1996) based on relevant references.

3. RESULT AND DISCUSSION

Description of Research Location

Vannamei shrimp samples were taken at three different locations. The first, vannamei shrimp sample came from the Berkat Yakin Business Group, Kelubuk Village, Bengkalis District. The second, vannamei shrimp sample came from Anak Kempas Village, Bantan Sub-district. The third, vannamei shrimp sample came from the Ketutu Joint Business Group, Batang Duku Village, Bukit Batu Sub-district.

Water Quality Parameters

Water quality is one of the supporting factors that closely affects the production of aquaculture. Good water quality will produce superior cultivation products. The water quality data at the research location can be seen in Table 1.

Table 1. Water quality data at the research site

Parameters	Quality Standard SNI 8037.1: 2014	Water Quality Parameter Values		
		Workshop	Bantan	Bukit Batu
Temperature (ºC)	28-33	30	28	30
pH	7,5 - 8,5	7,5	7,7	8
Salinity (ppt)	30-33	22	19	25
DO (ppm)	>4	3,9	5	4,7
Ammonia (ppm)	<0,1	0,5	1,5	0,34

Based on the value of water quality Table 1 shows different results from each research location, this is due to differences in the source water used to fill the ponds and the treatment given at each research location. The source water used in ponds in the Bengkalis and Bukit Batu sub-districts comes from the Bengkalis Strait, while the source water used in ponds in the Bantan sub-district comes from the Liong River. Good water quality for shrimp growth is a parameter that is in optimal environmental conditions based on water quality standards.

The results of measurements taken at the research site obtained temperature values in the range of 28-30 ºC, with the lowest temperature found in Bantan District. The low temperature in Bantan District is thought to be due to measurements taken in the afternoon, but the temperature values obtained in the three research locations are by the optimum quality standards for shrimp growth according to SNI 8037.1: 2014. In Suryana et al. (2023) positive samples came from ponds with a temperature value of 32.8-35.8ºC, this indicates a temperature that is too high and does not comply with quality standards. According to Muslimah (2022), the main causes of AHPND cases appear mostly due to high stocking density, high salinity, poor water quality, and dry season.

The pH value obtained from the research location ranges from 7.5-8. This shows that the pH values of the three research locations have met the quality standards of water quality according to SNI 8037.1: 2014 (7,5-8,5). Daily pH changes that are too significant can cause stress in cultured shrimp. Too many pH values can be stabilized by liming to reduce fluctuations in daily pH values.

The salinity value is in the range of 19-25 ppt, this indicates that the value is still following the water quality standards according to Soemardjati & Suriawan (2007), where vannamei shrimp can grow well and optimally in the range of salt levels of 15-25 ppt. Other opinions also explain that Vannamei shrimp can live in a wide salinity range between 0.5-45

ppt and have good resistance to changing environmental conditions (KKP, 2019).

The DO value obtained from the 3 research locations is 3.9-5 ppm with the lowest value found in Bengkalis District, which is 3.9 ppm. Based on SNI 8037.1: 2014, the value of DO in Bengkalis District is less appropriate because it is below the standard quality of water quality. This is due to the higher level of shrimp stocking so the distribution of oxygen in the pond becomes uneven. According to Wahyuni et al. (2022), the cause of the relatively high oxygen demand for vannamei shrimp due to an increase in shrimp biomass, so more aerators are needed that provide oxygen for shrimp respiration.

The ammonia value at 3 research locations is between 0.34-1.5 ppm based on SNI 8037.1: 2014 ammonia value in the three research locations is not appropriate because it has exceeded the standard quality of water quality is <0.1 ppm. Generally, the increase in ammonia levels in aquaculture ponds is caused by residual feed and shrimp metabolism in the form of shrimp feces so there is a buildup of organic matter at the bottom of the pond. Therefore, feeding must be limited with the intention of according to the needs of shrimp feed at its age. According to Wulandari (2015), if organic matter reaches a value of 17.73 ppm it will produce ammonia (NH_3) at the maximum limit (0.1 ppm) where the value is the maximum limit of ammonia value for vannamei shrimp farming.

Vannamei Shrimp Morphological Measurements

The results of morphological measurements on 35-day-old vannamei shrimp samples in Bengkalis District can be seen in Table 2.

Table 2. Measurement results of shrimp samples in Bengkalis Sub-district

Code Sample	Measurement		
	Length (cm)	Weight (g)	Body Condition
BE1	8,0	3,25	Normal
BE2	9,5	5,18	Normal
BE3	8,5	3,60	Normal
BE4	8,5	3,58	Normal
BE5	8,5	3,21	Normal

Description: BE: Bengkalis Sub-district; 1-5: Sample number

Based on Table 2, the results of measuring the length of vannamei shrimp samples with the highest value in sample BE2 is 9.5 cm and the lowest value in sample BE1 is 8 cm. While the results of weight measurements obtained the highest value in sample BE2 is 5.18 g and the lowest value in sample BE5 is 3.21 g. Furthermore, the results of morphological measurements on vannamei shrimp samples aged 33 days in Bantan District can be seen in Table 3.

Table 3. Measurement results of shrimp samples in Bantan sub-district

Code Sample	Measurement		
	Length (cm)	Weight (g)	Body Condition
BA1	7,0	1,78	Normal
BA2	6,5	2,24	Normal
BA3	6,5	1,19	Normal
BA4	6,5	1,63	Normal
BA5	6,0	1,28	Normal

Description: BA: Bantan District; 1-5: Sample number

Based on the measurement results of shrimp samples in Table 3, the length of the highest value in sample BA1 is 7 cm and the lowest value in sample BA5 is 6 cm. The results of measuring the weight of vannamei shrimp samples obtained the highest value in sample BA2 is 2.24 g and the lowest value in sample BA3 is 1.19 g. The results of morphological measurements on 5 samples of vannamei shrimp aged 36 days in District Bukit Batu can be seen in Table 4.

Table 4. Measurement results of shrimp samples in Bukit Batu Sub-district

Code Sample	Measurement		
	Length (cm)	Weight (g)	Body Condition
BB1	9,5	5,10	Normal
BB2	8,0	3,55	Normal
BB3	9,0	4,86	Normal
BB4	8,0	3,02	Normal
BB5	7,5	3,33	Normal

Description: BB: Bukit Batu sub-district; 1-5: Sample number

Based on the measurement results in Table 4, Vannamei shrimp body length is obtained with the highest value in sample BB1

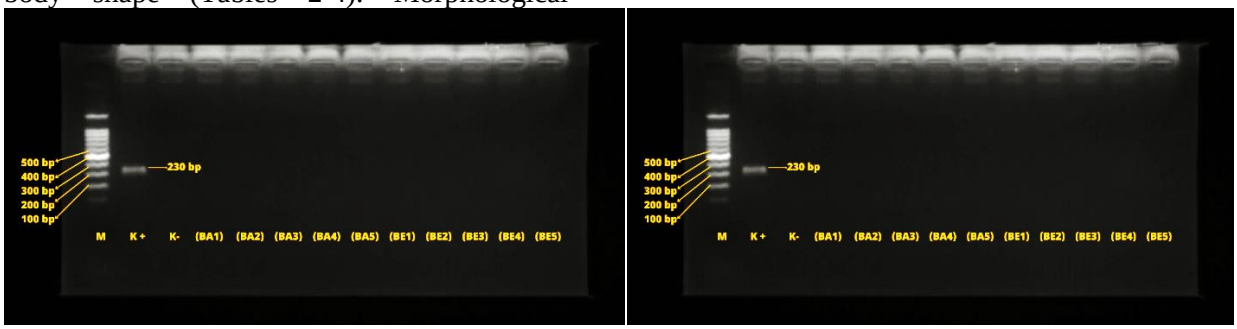
which is 9.5 cm, and the lowest value in sample BB5 which is 7.5 cm. The measurement of the weight of Vannamei shrimp samples obtained the highest value in sample BB1 which is 5.10 g and the lowest value in sample BB4 which is 3.02 g. Measurement of the length and weight of vannamei shrimp samples in each research location showed different results, this is due to differences in the age of vannamei shrimp in each research location.

According to research by Suryana et al. (2023), shrimp that were positive for AHPND disease weighed between 0.2-2.5 g and showed that the shrimp samples did not develop according to the shrimp at their age. While the shrimp examined weighed 1.19-5.18 grams, this indicates that the shrimp samples examined have growth following the age of the shrimp. AHPND disease generally attacks Vannamei shrimp at the age of 30-35 days after stocking post-larvae in the rearing pond (Han et al., 2015), this is also supported by the opinion of Muslimah (2022) that shrimp mortality due to AHPND occurs at the age of less than 40 days after stocking in ponds.

The results of morphological observations based on vannamei shrimp at each study site were all samples of vannamei shrimp brownish white, agile movements, brown hepatopancreas and not pale, stomach and intestines full of food, and straight and normal body shape (Tables 2-4). Morphological symptoms of vannamei shrimp infected with AHPND are lethargic shrimp, delayed growth rate, spiral swimming, pale hepatopancreas, empty intestines, and stomach, pale yellowish body color (Muslimah 2022), hepatopancreas necrosis, atrophy, and pale appearance (Suryana et al., 2023). How to diagnose the possibility of AHPND disease among other things, when siphoning shrimp die in large numbers, the pattern of death that occurs continuously, weakened larval movement and spiral movement, sudden death in larval and post-larval stadia up to >30%. (Muslimah, 2022).

Examination of AHPND by PCR Method

The examination of AHPND was carried out molecularly using the PCR method with AP4 primers. The analysis of the results is declared positive if the sample DNA band size has the same base length as the positive control of 230 bp, following the Office International des Epizooties standard (OIE, 2021). Furthermore, the sample is declared negative if there is no DNA band. The results of the examination of AHPND disease with the PCR method showed negative results which can be seen in Figure 1. Based on the results of DNA visualisation there is no extension in the DNA band of Vannamei shrimp samples measuring 230 bp or parallel to the positive control.



(a) (b)

Figure 1. **DNA visualisation of AHPND PCR results in shrimp from (a) Bantan Sub-district and Bengkalis Sub-district (b) Bukit Batu Sub-district**

Description: M : *Marker* DNA 100 bp; K+ : Positive Control; K- : Negative Control; BA : Bantan District; BE : Bengkalis Sub-district; BB : Bukit Batu sub-district; 1-5: Sample number

The results showed that there were no samples of Vannamei shrimp that were positive for AHPND. This is supported by water quality parameters that are still following the optimal quality standards for vannamei shrimp farming. In the research of Nainggolan et al. (2020), the results of the examination of all samples from Vannamei shrimp ponds using AP4 primers and OIE standards in 2018 showed negative results for AHPND infection. Research by Suryana et al. (2023) showed that samples from Socah District tested positive for AHPND with an amplicon size of 230 bp while samples from Kwanyar District and Sepuluh District showed

negative results. Samples that tested positive showed vannamei shrimp at the age should weighing ±8.5 g, suspected that the shrimp were sick because of slow growth. The appearance of AHPND disease is thought to be due to temperature and pH values that are not by water quality standards. According to Sarina (2018), clinical symptoms that are easily recognized when shrimp are infected with AHPND are the hepatopancreas of shrimp will be black, sometimes red, and generally yellow and shrunken.

Prevalence of AHPND Disease Attack

Prevalence is the total number of disease cases in a certain period calculated using the formula according to Cameron (2002), then identified with prevalence criteria according to Williams & Williams (1996). The number of vannamei shrimp samples examined was 5 per pond with negative AHPND test results in all samples. The prevalence value of the three locations was 0%, indicating the category of never infection. The negative AHPND test results and prevalence values are supported by the morphology of vannamei shrimp samples taken from 3 research sites look normal and do not show any infection or symptoms of AHPND disease.

In 2016, AHPND still exists in Malaysia but at a lower prevalence. The cause of AHPND, which is an infection of *V.parahaemolyticus*, can occur due to the low application of biosecurity and Good Aquaculture Practices (GAPs) from farmers, as well as hot weather that can cause fluctuations in temperature and salinity in ponds (Suryana et al., 2023).

The relatively low prevalence value of 0%, where no Vannamei shrimp samples were found with AHPND disease, is a success for vannamei shrimp farmers in the Bengkalis Regency. Careful preparation and planning are required. According to Poerwosoediro (2002), the success of shrimp farming is generally determined by management factors. The factors that support the success of Vannamei shrimp farming in Bengkalis Regency include the selection of disease-free fry, cleanliness in the form of land sterilization, daily and weekly water quality control, quality feed, treatments such as drugs and chemicals by the dosage and trained human resources (HR).

The success of pond shrimp farming is usually determined by management factors. There are other factors such as inappropriate site selection, untrained labor, and physicochemical and biological characteristics of water such as water quality and microalgae that have not been thoroughly considered. Important variables that need to be considered in shrimp farming are dissolved macro and microelements, dissolved oxygen, salinity, temperature, water color, pH, and dissolved toxic compounds. Furthermore, it is necessary to pay attention to the quality and quantity of feed and stocking density. Feed is very important in intensive cultivation. The provision of quality and appropriate feed contributes greatly to the success of production. The use of chemicals that are inappropriate both in type and dose is also a cause of failure of shrimp farming. The use of pesticides to control pond pests is recommended to use effective materials, that do not last long in pond water and soil, do not accumulate in living organisms, and are safe for the aquatic environment.

4. CONCLUSIONS

Based on the results of the study it can be concluded that the prevalence of Acute Hepatopancreatic Necrosis Disease (AHND) in vannamei shrimp is 0% with the category of infection never. AHPND examination by PCR method using AP4 primer measuring 230 bp showed that vannamei shrimp samples from Bengkalis District, Bantan District, and Bukit Batu District of Bengkalis Regency were negative for AHPND. The morphological condition of vannamei shrimp from the three research sites did not show any clinical symptoms of AHPND-infected shrimp. All vannamei shrimp samples were brownish white, not pale and straight (normal) body shape, brown hepatopancreas not shrunken, and intestines and stomach filled with feed. Based on the research conducted, it is recommended that the owners of shrimp farms in Bengkalis Regency conduct an AHPND examination using the PCR method.

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