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DETECTION OF *INFECTIOUS MYONECROSIS VIRUS* (IMNV) IN *LITOPENAEUS VANNAMEI* USING NESTED REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (PCR) AT PT. CENTRAL PROTEINA PRIMA SIDOARJO EAST JAVA

Deteksi *Infectious Myonecrosis Virus* (IMNV) pada *Litopenaeus vannamei* dengan Metode *Nested Reverse Transcription-Polymerase Chain Reaction* (PCR) di PT. Central Proteina Prima Sidoarjo Jawa Timur

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ABSTRACT

Increased production and stocking density have elevated the risk of viral diseases, which can lead to significant economic losses. This study aimed to detect the presence of *Infectious Myonecrosis Virus* (IMNV) in vannamei shrimp using the *Nested Reverse Transcription-Polymerase Chain Reaction* (nRT-PCR) method. 54 vannamei shrimp samples from various regions in Indonesia were tested to detect the presence of IMNV. The test results showed 2 samples were detected positive for IMNV, namely samples from Central Kalimantan and East Java. The test results were confirmed and reinforced by other supporting factors that influence the spread or occurrence of IMNV disease infection in the sample's region of origin. This study highlights the importance of routine IMNV surveillance using sensitive molecular methods to control disease spread and protect the shrimp aquaculture industry in Indonesia.

Key words: *Infectious Myonecrosis Virus, Nested Reverse Transcriptase - Polymerase Chain Reaction, Litopenaeus vannamei*.

ABSTRAK

Peningkatan produksi dan kepadatan tebar telah meningkatkan risiko penyakit virus yang dapat menyebabkan kerugian ekonomi signifikan. Penelitian ini bertujuan untuk mendeteksi keberadaan IMNV pada udang vannamei menggunakan metode *Nested Reverse Transcription-Polymerase Chain Reaction* (nRT-PCR). 54 sampel udang vannamei dari berbagai wilayah di Indonesia dilakukan uji untuk mendeteksi keberadaaan IMNV. Hasil uji menunjukkan 2 sampel terdeteksi positif IMNV yaitu sampel Kalimantan Tengah dan Jawa Timur. Hasil pengujian dikonfirmasi dan diperkuat dengan faktor pendukung lainnya yang mempengaruhi

penyebaran atau terjadinya infeksi penyakit IMNV di wilayah asal sampel. Penelitian ini menyoroti pentingnya surveilans rutin IMNV menggunakan metode molekuler yang sensitif untuk mengendalikan penyebaran penyakit dan melindungi industri akuakultur udang di Indonesia.

Kata Kunci: *Infectious Myonecrosis Virus*, *Nested Reverse Transcriptase - Polymerase Chain Reaction*, *Litopenaeus vannamei*.

INTRODUCTION

Indonesia's shrimp production ranks fourth globally, making it an important commodity that can contribute to increasing state income (Koesharyani *et al.*, 2015). Shrimp cultivation in Indonesia is a fishery business that has been carried out for a long time. Vannamei shrimp (*Litopenaeus vannamei*) is one of the fishery commodities that offers promising business opportunities and potential to be managed and expanded (Kamil *et al.*, 2023). Shrimp production is estimated to reach 40 billion dollars in the global market (Baladrat *et al.*, 2022). Currently, aquaculture is one of the fastest growing sectors. The industrialization of the fisheries sector continues to grow along with the increasing public demand for these fishery products (Anggara *et al.*, 2024). Basically, increasing shrimp stocking density increases the possibility of disease spread. Shrimp become more aggressive and attack each other, which causes cannibalism and higher mortality (Baladrat *et al.*, 2022). The risk of infectious diseases is correlated with increased demand and production of fishery products. This risk can reduce the productivity of aquaculture products, which in turn results in a decrease in the quality of fishery products (Anggara *et al.*, 2024).

Some of the most significant diseases listed in the World Organization for Animal Health report are caused by viruses. One of the listed diseases is *Infectious myonecrosis* (IMN). This disease is caused by the *Infectious Myonecrosis Virus* (IMNV) and is also included in the Quarantine Fish Pests and Diseases. This shows that in a relatively short time, it can become an epidemic and have a negative impact on the economy and social aspects of the country. According to Tang *et al.*, (2019), vannamei shrimp is one of the most common penaeid shrimp species infected by IMNV. This disease is important because mortality in infected populations can reach 40-70%. In Indonesia, the first case of myonecrosis occurred in Situbondo in 2006, with reports of clinical symptoms similar to the myonecrosis outbreak that occurred in Brazil. Shrimp are characterized by the presence of focal to extensive white necrotic areas in the striated muscle (skeleton), especially in the distal abdominal segment and uropods, which can become necrotic and red in some individual shrimp in the acute phase. The main target tissues of IMNV include striated muscle (skeleton), connective tissue, hemocytes, and parenchymal cells found in lymphoid organs (WOAH, 2023). Several factors that influence the incidence of IMNV are poor water quality, stocking density, shrimp stress, and the impact of climate change (Ramadhan *et al.*, 2024).

IMNV is a double-stranded Ribonucleic acid (RNA) virus. To detect this virus, molecular methods such as *Nested Reverse Transcription - Polymerase Chain Reaction* (nRT - PCR) can be used. Nested PCR is a method used to increase the sensitivity and specificity of target DNA detection. This procedure involves two stages of PCR amplification, where the product of the first PCR reaction is used as a template for the second PCR reaction. This method is a molecular technique recommended by WOAH for the diagnosis of pathogens found in fishery products but still with high sensitivity and specificity (WOAH, 2023). This study aims to detect shrimp infected with IMNV using *Nested Reverse Transcription - Polymerase Chain Reaction* (nRT - PCR). So it can be used as an early diagnosis of IMNV infection. It is important to know the

early detection method so that it can suppress infection and the spread of IMNV in shrimp farming.

RESEARCH METHODS

Sampling

This research was conducted at PT. Central Proteina Prima, Sidoarjo Regency, East Java, from September 21, 2024 to December 13, 2024. The vannamei shrimp samples used in this study were obtained from East Java, West Java, Bali, West Nusa Tenggara, and Kalimantan. Furthermore, the samples were extracted for PCR testing.

Tools and Materials

The tools used include tweezers, microtube racks, micropipettes, grinders, vortexes, centrifuges, water baths, ovens, beaker glasses, measuring cups, agarose gel molds, electrophoresis, UV transilluminators, memo cards, and cooler racks. While the materials used include microtubes (1.5 ml & 0.5 ml), microtips (200 µl & 1000 µl), 0.2 ml PCR tubes, distilled water, RNA extraction, 70-96% ethyl alcohol, isopropanol, DEPC ddH_2O, IMNV premix, IQzyme, RT enzyme, agarose, TBE 1x, hydra green, and loading dye.

DNA Extraction

DNA extraction was performed using the RNA Extraction kit (GeneReach Biotechnology), according to the manufacturer's instructions. To ensure the accuracy of the extraction procedure, positive and negative controls were also included during testing.

PCR Testing and DNA Amplification

The testing and sample amplification process followed the instructions of the IQ2000TM IMNV (nested) kit. The Nested Reverse Transcription-Polymerase Chain Reaction (nRT-PCR) method for IMNV uses two sets of PCR primers that produce a first step amplicon product of 255 bp and a nested amplicon of 510 bp. The reaction volume for first step PCR amplification is 10 µl and for nested step 25 µl. The cycle conditions during amplification for the first step are 15 cycles of 94oC/20 seconds; 62 oC/20 seconds; 72oC/30 seconds and for nested 30 cycles of 94oC/20 seconds; 62 oC/20 seconds; 72oC/30 seconds. After amplification, PCR products were dyed with loading dye and separated by electrophoresis on a 1.5% (w/v) agarose gel using 1x TBE buffer (Zaujat *et al.*, 2016). The agarose gel was prepared by adding HydraGreen dye. Furthermore, the DNA bands were visualized using a UV transluminator.

Data Analysis

Data analysis in this study used a qualitative approach through direct interpretation of PCR product visualization. DNA amplification results were evaluated based on the band pattern formed on the electrophoresis gel after the electrophoresis process was carried out. The presence and size of DNA bands were analyzed to determine the presence of target sequences.

RESULT

Nested PCR amplification involves two different primer sets, the first primer is used for the first PCR amplification to produce a DNA product. This product is then used as a template for a second PCR amplification with a second primer, which amplifies a more specific DNA region. Interpretation of the electrophoresis results produces a DNA product at 255 bp or 510 bp. Figures 1 and 2 show the results of IMNV detection. A total of 54 vannamei shrimp samples were tested, 23 of which were from East Java, 4 from Bali, 4 from Central Java, 15 from West Nusa Tenggara, and 4 from Central Kalimantan. Of the 54 samples, 2 samples were detected positive for IMNV, namely sample no. 31 from Central Kalimantan (Palangkaraya) and sample no. 54 (Tr) from East Java (Probolinggo).

A	B	C	D	E	F	G	H	I	J
No	Tgl Uji	Jenis Sampel	Wilayah	Kode	DOC	Organ Target	Pemeriksaan Laboratorium	Positive	Negative
1	17-Nov-24	*Litopenaeus vannamei*	Banyuwangi	1012	53	Insang dan Pleopod	Nested PCR (IMNV)	-	V
2	17-Nov-24	*Litopenaeus vannamei*	Banyuwangi	1013	53	Insang dan Pleopod	Nested PCR (IMNV)	-	V
3	17-Nov-24	*Litopenaeus vannamei*	Banyuwangi	1014	53	Insang dan Pleopod	Nested PCR (IMNV)	-	V
4	17-Nov-24	*Litopenaeus vannamei*	Banyuwangi	1015	53	Insang dan Pleopod	Nested PCR (IMNV)	-	V
5	17-Nov-24	*Litopenaeus vannamei*	Banyuwangi	1016	53	Insang dan Pleopod	Nested PCR (IMNV)	-	V
6	17-Nov-24	*Litopenaeus vannamei*	Banyuwangi	1017	28	Insang dan Pleopod	Nested PCR (IMNV)	-	V
7	17-Nov-24	*Litopenaeus vannamei*	Banyuwangi	1018	28	Insang dan Pleopod	Nested PCR (IMNV)	-	V
8	17-Nov-24	*Litopenaeus vannamei*	Banyuwangi	1019	28	Insang dan Pleopod	Nested PCR (IMNV)	-	V
9	17-Nov-24	*Litopenaeus vannamei*	Banyuwangi	1020	28	Insang dan Pleopod	Nested PCR (IMNV)	-	V
10	17-Nov-24	*Litopenaeus vannamei*	Probolinggo	1021	36	Insang dan Pleopod	Nested PCR (IMNV)	-	V
11	17-Nov-24	*Litopenaeus vannamei*	Probolinggo	1022	36	Insang dan Pleopod	Nested PCR (IMNV)	-	V
12	17-Nov-24	*Litopenaeus vannamei*	Probolinggo	1023	36	Insang dan Pleopod	Nested PCR (IMNV)	-	V
13	17-Nov-24	*Litopenaeus vannamei*	Bali	1024	41	Insang dan Pleopod	Nested PCR (IMNV)	-	V
14	17-Nov-24	*Litopenaeus vannamei*	Bali	1025	41	Insang dan Pleopod	Nested PCR (IMNV)	-	V
15	17-Nov-24	*Litopenaeus vannamei*	Bali	1026	41	Insang dan Pleopod	Nested PCR (IMNV)	-	V
16	17-Nov-24	*Litopenaeus vannamei*	Bali	1027	41	Insang dan Pleopod	Nested PCR (IMNV)	-	V
17	17-Nov-24	*Litopenaeus vannamei*	Jember	1028	56	Insang dan Pleopod	Nested PCR (IMNV)	-	V
18	17-Nov-24	*Litopenaeus vannamei*	Tulungagung	1029	48	Insang dan Pleopod	Nested PCR (IMNV)	-	V
19	17-Nov-24	*Litopenaeus vannamei*	Tulungagung	1030	48	Insang dan Pleopod	Nested PCR (IMNV)	-	V
20	17-Nov-24	*Litopenaeus vannamei*	Purworejo	1031	56	Insang dan Pleopod	Nested PCR (IMNV)	-	V
21	17-Nov-24	*Litopenaeus vannamei*	Purworejo	1032	56	Insang dan Pleopod	Nested PCR (IMNV)	-	V
22	17-Nov-24	*Litopenaeus vannamei*	Purworejo	1033	56	Insang dan Pleopod	Nested PCR (IMNV)	-	V
23	17-Nov-24	*Litopenaeus vannamei*	Purworejo	1034	56	Insang dan Pleopod	Nested PCR (IMNV)	-	V
24	17-Nov-24	*Litopenaeus vannamei*	Probolinggo	1035	40	Insang dan Pleopod	Nested PCR (IMNV)	-	V
25	17-Nov-24	*Litopenaeus vannamei*	Probolinggo	1036	40	Insang dan Pleopod	Nested PCR (IMNV)	-	V
26	17-Nov-24	*Litopenaeus vannamei*	Madura	1037	63	Insang dan Pleopod	Nested PCR (IMNV)	-	V
27	17-Nov-24	*Litopenaeus vannamei*	Madura	1038	63	Insang dan Pleopod	Nested PCR (IMNV)	-	V
28	17-Nov-24	*Litopenaeus vannamei*	Madura	1039	63	Insang dan Pleopod	Nested PCR (IMNV)	-	V
29	17-Nov-24	*Litopenaeus vannamei*	Madura	1040	63	Insang dan Pleopod	Nested PCR (IMNV)	-	V
30	17-Nov-24	*Litopenaeus vannamei*	Madura	1041	63	Insang dan Pleopod	Nested PCR (IMNV)	-	V
31	17-Nov-24	*Litopenaeus vannamei*	Palangkaraya	1042	66	Insang dan Pleopod	Nested PCR (IMNV)	*	-
32	17-Nov-24	*Litopenaeus vannamei*	Palangkaraya	1043	66	Insang dan Pleopod	Nested PCR (IMNV)	-	V
33	17-Nov-24	*Litopenaeus vannamei*	Palangkaraya	1044	66	Insang dan Pleopod	Nested PCR (IMNV)	-	V
34	17-Nov-24	*Litopenaeus vannamei*	Palangkaraya	1045	66	Insang dan Pleopod	Nested PCR (IMNV)	-	V
35	17-Nov-24	*Litopenaeus vannamei*	Nusa Tenggara Barat	1046	57	Insang dan Pleopod	Nested PCR (IMNV)	-	V
36	17-Nov-24	*Litopenaeus vannamei*	Nusa Tenggara Barat	1047	57	Insang dan Pleopod	Nested PCR (IMNV)	-	V
37	17-Nov-24	*Litopenaeus vannamei*	Nusa Tenggara Barat	1048	57	Insang dan Pleopod	Nested PCR (IMNV)	-	V
38	17-Nov-24	*Litopenaeus vannamei*	Nusa Tenggara Barat	1049	57	Insang dan Pleopod	Nested PCR (IMNV)	-	V
39	17-Nov-24	*Litopenaeus vannamei*	Nusa Tenggara Barat	1050	57	Insang dan Pleopod	Nested PCR (IMNV)	-	V
40	17-Nov-24	*Litopenaeus vannamei*	Nusa Tenggara Barat	1051	57	Insang dan Pleopod	Nested PCR (IMNV)	-	V
41	17-Nov-24	*Litopenaeus vannamei*	Nusa Tenggara Barat	1052	57	Insang dan Pleopod	Nested PCR (IMNV)	-	V
42	17-Nov-24	*Litopenaeus vannamei*	Nusa Tenggara Barat	1053	57	Insang dan Pleopod	Nested PCR (IMNV)	-	V
43	17-Nov-24	*Litopenaeus vannamei*	Nusa Tenggara Barat	1054	57	Insang dan Pleopod	Nested PCR (IMNV)	-	V
44	17-Nov-24	*Litopenaeus vannamei*	Nusa Tenggara Barat	1055	57	Insang dan Pleopod	Nested PCR (IMNV)	-	V
45	17-Nov-24	*Litopenaeus vannamei*	Nusa Tenggara Barat	1056	57	Insang dan Pleopod	Nested PCR (IMNV)	-	V
46	17-Nov-24	*Litopenaeus vannamei*	Nusa Tenggara Barat	1057	49	Insang dan Pleopod	Nested PCR (IMNV)	-	V
47	17-Nov-24	*Litopenaeus vannamei*	Nusa Tenggara Barat	1058	49	Insang dan Pleopod	Nested PCR (IMNV)	-	V
48	17-Nov-24	*Litopenaeus vannamei*	Nusa Tenggara Barat	1059	49	Insang dan Pleopod	Nested PCR (IMNV)	-	V
49	17-Nov-24	*Litopenaeus vannamei*	Nusa Tenggara Barat	1060	49	Insang dan Pleopod	Nested PCR (IMNV)	-	V
50	17-Nov-24	*Litopenaeus vannamei*	Tegal	1061	46	Insang dan Pleopod	Nested PCR (IMNV)	-	V
51	17-Nov-24	*Litopenaeus vannamei*	Tegal	1062	46	Insang dan Pleopod	Nested PCR (IMNV)	-	V
52	17-Nov-24	*Litopenaeus vannamei*	Tegal	1063	46	Insang dan Pleopod	Nested PCR (IMNV)	-	V
53	17-Nov-24	*Litopenaeus vannamei*	Tegal	1064	46	Insang dan Pleopod	Nested PCR (IMNV)	-	V
54	17-Nov-24	*Litopenaeus vannamei*	Probolinggo	1065	54	Insang dan Pleopod	Nested PCR (IMNV)	**	-

Figure 1. Nested PCR sample data

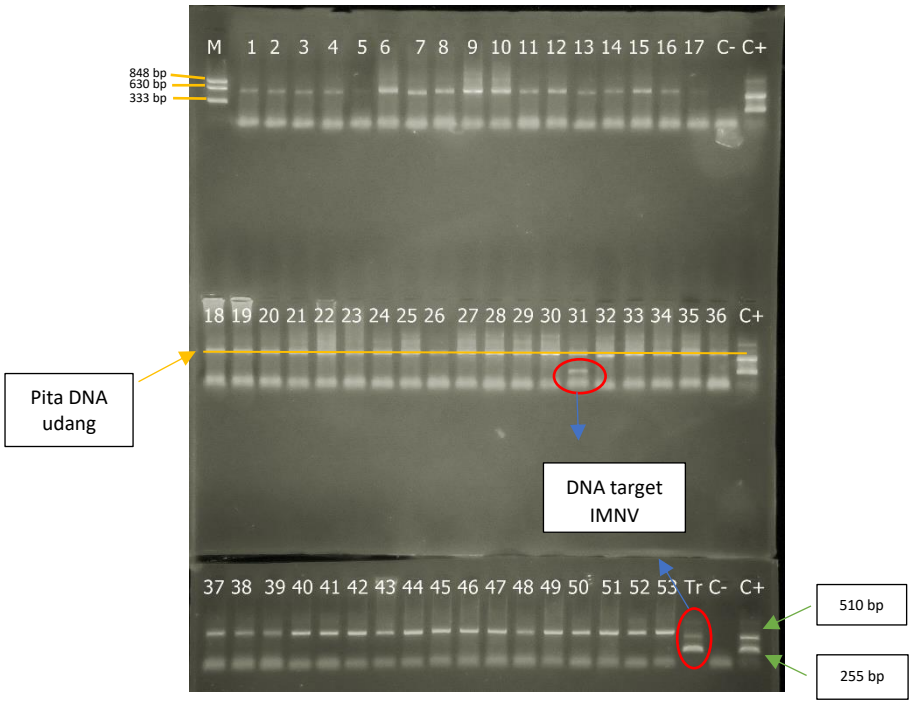

Figure 2. PCR Test Results of Vannamei Shrimp. M: DNA Marker; C+: positive control; C-: negative control; (1-Tr): vannamei shrimp sample

DISCUSSION

Based on the WOAH report (2023), shrimp in the acute phase showed focal to extensive white necrotic areas on the striated muscles (skeleton), especially on the distal abdominal segments and tail fans, which could become necrotic and red in some individual shrimp. This is also in accordance with what was stated by Prasad *et al.,* (2016) that the muscles and appendix can show a reddish color, making them look like cooked shrimp. During the preparation, clinical symptoms were observed on the shrimp body. From the positive samples, there were no specific clinical symptoms of IMNV such as focal white necrotic areas or redness. The gills and pleopods of shrimp were chosen as the test targets because their structural integrity was maintained. The samples tested macroscopically showed healthy conditions, with no signs of decomposition or visible lesions. The selection of the gill organ as the test target, because the gills are organs that are susceptible to infection and can provide accurate information about the infection status of shrimp, while pleopods are highly recommended for screening juveniles and adult shrimp. Although the clinical symptoms of the shrimp did not show any IMNV infection, PCR detection results revealed the presence of the pathogen in several individuals. This finding indicates that shrimp can act as carriers/latent, which have the potential to spread the infection without showing obvious signs of disease. Jha *et al.* (2020), stated that shrimp that survive IMNV infection can be lifelong carriers of the virus. Although some carriers may not show obvious signs of disease, it is still possible that they can still shed the virus and infect other shrimp.

IMNV disease was tested using IQ2000 with the nRT-PCR method. The nested method can detect at least 100 copies of IMNV RNA in the first step and 10 copies of IMNV PCR RNA in the second step (WOAH, 2023). The nested PCR method is very useful in early detection of the disease, because it is able to identify shrimp infected with IMNV even though they do not show obvious clinical signs. Currently, nested RT-PCR is used and recommended for the detection and surveillance of IMNV from infected shrimp tissue (Kokkattunivarthil *et al.,* 2018). The results obtained in this study showed that only 2 out of 54 samples were detected positive, the other samples showed negative results. However, this negative test result does not mean that the sample is not infected, but is possible because the sensitivity of the primers used in this study showed a low detection limit. According to Lin *et al.,* (2022), explained that another factor that affects the detection limit is that a highly specific and efficient primer design is very important for accurate amplification. And inhibitors in the sample can interfere with the amplification process and reduce sensitivity. Based on the IQ2000TM IMNV (nested) kit manual instructions, there are different detection limits for each type of sample. The detection limit for amplifying 5 copies/µl of IMNV DNA plasmid is 10 copies/reaction. Meanwhile, for in vitro transcribed RNA samples (20 copies), <PL 12 (10 tails), PL 12 - PL 20 (5 tails), pleopods (1-2 pieces or 20 mg), and muscles (20 mg) the detection limit is 20 copies/reaction.

The results of the gel documentation visualization are in accordance with the references used (GeneReach, 2022), this directly provides validation that the PCR reagents and reaction conditions are working properly. Factors that cause reaction failure in a PCR test can also be eliminated. These factors include: 1) Contamination of the sample or target DNA. PCR is a very sensitive technique, which means that even the slightest contamination can affect the results. Contamination can be seen by the appearance of a DNA band in the negative control. 2) The absence of a shrimp DNA band as an internal control. If the target virus DNA band does not appear in the positive control, it indicates that the positive primary condition has been degraded or damaged. Positive samples are marked by the presence of a DNA band in the sample according to the IMNV positive control (Maharani & Sumsanto, 2024). In Figure 2, it is observed that sample number 31 from Central Kalimantan (Palangkaraya) was detected positive 1 (*). This means that only fragments at 255 bp were detected. While sample number

54 (Tr) from East Java (Probolinggo) was detected positive 2 (**). This means that nested PCR successfully amplified two specific target DNA fragments from the sample with a high level of IMNV infection. The interpretation of these results was then recorded and reported for confirmation. These results are in accordance with research conducted by Naim (2012), which created a map of the distribution or geographical distribution of IMNV in Indonesia. The red color is the province infected with IMNV (Figure 3).

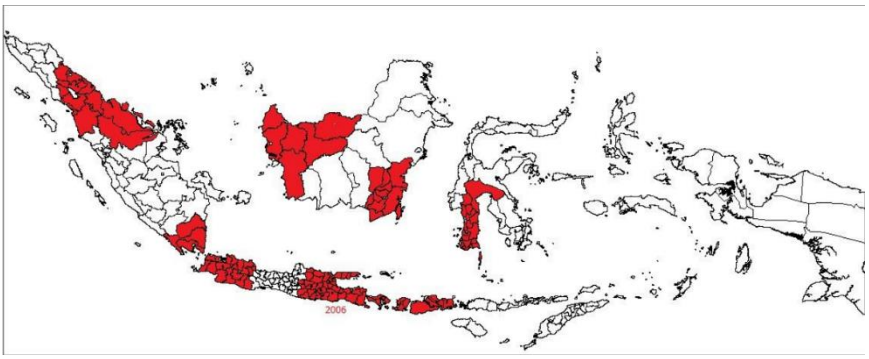

Figure 3. Distribution of provinces infected with IMNV
Source: Naim (2012)

IMNV infection has spread to East Java, South Kalimantan, Bali, West Nusa Tenggara, Lampung, West Java, Riau, North Sumatra, West Kalimantan, and South Sulawesi (Naim, 2012). Rekasana *et al.*, (2013) added that IMNV has spread to aquaculture areas in South Kalimantan and East Java, namely Probolinggo and Banyuwangi Regencies. This is in accordance with the area of origin of the samples tested for IMNV in this study. This event can be called endemic, which means a condition where a disease appears regularly in a certain area or community group with a fixed number within controllable limits. The risk of IMNV transmission globally is increasing due to the frequent cross-border trade in farmed shrimp (Wan *et al.*, 2023). However, the occurrence of this disease can be supported by other factors. One of these factors is high stocking density. This is in accordance with what was conveyed in the study by Baladrat *et al.*, (2022) that increasing shrimp stocking density can worsen water quality and reduce the Total Hemocyte Count (THC), which is an indicator of the shrimp's immune response. Higher stocking densities accelerate the development of IMNV. According to Sarah *et al.*, (2017), another factor that contributes to the high prevalence of IMNV is the influence of climate change. This climate change can be seen through extreme daily weather changes, which cause fluctuations in water temperatures. The samples were taken on November 17, 2024, when the weather conditions in Indonesia changed due to the shift from the dry season to the rainy season. This is in accordance with the results of the Prasetyaningtyas (2024) report on the analysis of atmospheric dynamics in the second decade of November 2024. This condition needs to be confirmed with the results of water quality testing. This is because water quality factors support the spread of IMNV disease. According to Cong & Thao (2023), water quality is an important factor that influences the spread and severity of IMNV in shrimp farming. Poor water quality is a significant factor influencing the incidence of IMNV.

CONCLUSION

This study successfully detected Infectious Myonecrosis Virus (IMNV) in vannamei shrimp using the nested PCR method, which showed high sensitivity in identifying pathogens even in samples without clinical symptoms. Of the 54 samples tested, two were positive for IMNV, indicating the presence of the virus in Central Kalimantan and East Java in accordance

with reports of the spread of IMNV in Indonesia. Factors such as primer sensitivity, sample conditions, and the cultivation environment, including water quality and stocking density, affect the results of IMNV detection and spread. Therefore, continuous surveillance, early detection with sensitive methods, and good cultivation practices are essential to prevent the spread of IMNV and maintain shrimp health.

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