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SIMULTANEOUS PATHOGEN DETECTION OF SHRIMP VIRUSES ON CULTURED TIGER SHRIMPS (*Penaeus monodon*) IN INDONESIA

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ABSTRACT

The multiple-pathogen infection causes severe economic impact to shrimp industry in Indonesia and worldwide due to mass mortality and multiple abnormalities of the survived infected shrimps. However, multiple-pathogen detection tools in shrimp diseases have not yet widely used. The purpose in this study was to develop and applied simultaneous detection system using multiplex polymerase chain reaction (PCR) assay from natural infections caused by white spot syndrome virus (WSSV), infectious hypodermal and haematopoietic necrosis virus (IHHNV) and monodon baculovirus (MBV) in Black tiger shrimp culture. To analyze multiple-pathogen infections in the shrimp, the study designed and used three pairs of specific primers targeting DNA virus from the shrimp diseases. All amplifications used a specific master mix for multiplex PCR assay and standardized extracted nucleic acid from the samples. This mPCR assay successfully amplified the DNA of three viruses in a single tube-run by multiplex PCR for each virus. Based on the results, the study confirms that multiple-pathogen infection contributes the highest mass mortality rather than from single infection by either WSSV, IHHNV or MBV. This study also confirms that the mPCR assay is a faster, cheaper, and efficient method to detect and subsequently prevent the spreading of multi-pathogen shrimp diseases.

KEYWORDS: IHHNV*; *MBV*; *multiplex PCR*; *Penaeus monodon*; *WSSV

INTRODUCTION

The Ministry of Fisheries and Marine Affairs has set a target of increasing shrimp production up to 250% by 2024 (MMAF's 2020). In order to achieve this optimistic target, various shrimp aquaculture systems and technologies must be improved by following good aquaculture practices including shrimp disease prevention and mitigation. Currently, shrimp disease outbreaks increase with an alarming rate primarily due to unsustainable shrimp farming practices. More than twenty shrimp diseases are caused by viruses, bacteria, fungi and protozoa. The diseases caused by viruses were the most common impediments faced in shrimp farming industry (Lee *et al*., 2022; Yu *et al*., 2022). Between 2019 and 2021, Office *International* des Epizooties (OIE) released a list containing important viral diseases infecting shrimp farms in Asia and Indo-Pacific in which white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), penaeus monodon-type baculovirus (MBV), hepatopancreatic parvovirus (HPV) and yellowhead diseases genotype-1 (YHV) were included.

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Other diseases, such as Taura syndrome (TSV), and baculovirus penaeid (BP), are of more concern in American countries (Lightner & Redman, 1998).

In Indonesia, black tiger shrimp (*Penaeus monodon*) farming business started in 1990 and increased rapidly but then slowed down due to diseases outbreaks (Flegel, 2006; Taukhid *et al*., 2008; Amelia *et al*., 2021). As a result, a new shrimp species, pacific white shrimp (*Litopenaeus vannamei*), was introduced to substitute *P. monodon* in an effort to revive and increase shrimp production in Indonesia. The introduction of pacific white shrimp has led to massive development of shrimp farming throughout Indonesia complicated by the increasing outbreaks of diseases caused by viral infections, especially WSSV, IHHNV and MBV (Taukhid *et al*., 2008).

The economic loses of shrimp culture in Indonesia due to disease outbreaks still occurs in both *P. monodon* and *L. vannamei* farming despite good aquaculture practices have been implemented rigorously such as brood stock system, certified seeds and improved pond management (Amelia *et al.,* 2021). Mass mortality accompanied by multiple abnormalities in affected shrimp farms is strongly linked to the possibility of multiple-pathogen infections (Feijó *et al*., 2013; Koesharyani *et al*., 2012). Polymerase chain reaction (PCR) assay is commonly used to detect viral infections in shrimp diseases. In general, the detection is usually carried out with a single specific primary target (Lightner & Redman, 1998). However, detecting multiple-pathogen infection requires longer time and needs a different approach. Therefore, this study developed a technique of single-tube run of a multiplex-PCR (mPCR) diagnostic technique for WSSV, MBV and IHHNV. Using this technique, it is possible to simultaneously detect natural multiple-pathogens of viral infections from a shrimp farm. This finding provides a rapid yet efficient technique to detect and prevent the spreading of viral diseases in a shrimp farming area by using the PCR assay.

MATERIALS AND METHODS

2.1. Sample collections

All shrimp (*Penaeus monodon*) and wild crab (*Scylla* spp.) specimens were collected during outbreak with an unknown diseases with mass mortality cases in organic shrimp farm at Sidoarjo and Bangil, East Java, Indonesia in 2009. The shrimp samples were collected with exhibited symptom of shrimp virus infection such as white spot on the carapace, highly disparate shrimp growth, damaged of hepatopancreas organ and blackened gills (Figure 1). The water samples were collected from the ponds and then directly analyzed.

Figure 1. Condition of organic pond of *P. monodon* farm with mass mortality case at Sidoarjo, East Java (A): organic traditional pond; (B) shrimp samples were exhibited symptom of variable sizes; (C) shrimp with blackened gills.

2.2. Total nucleic acid (DNA) purification

Total DNA was extracted from gill of the specimens using DNeasy Blood & Tissue Kit (Qiagen, Germany) following the manufacturer's protocols. The extracted DNA was stored at –20 ºC until used. Briefly, 25 mg of homogenized shrimp organ of every singles specimen was added to buffer ATL, proteinase-K and buffer AL and Ethanol. Put the suspension into the spin column, centrifuged and discard flow through. washing two times with buffer AW1 and AW2 respectively and added onto spin column, centrifuged and discard flow through, repeated this protocol twice. Change the new collection tube and elute the nucleic acid with buffer AE onto the spin column, centrifuged in rotor bucket for last purification and collected the DNA. Total extracted DNA was quantified using NanoDrop (Thermo Scientific, CA, USA) with absorbance 260 nm and ratio of optical density (OD_{260}/OD_{280}) measurements, respectively.

2.3. Oligonucleotide primers

Using free software from (https://primer3.org/), specific oligonucleotide primers for WSSV and MBV were created using data from GenBank with Accession Nos. U50923.1 and NC 024692.1. respectively (https://www.ncbi.nlm.nih.gov/genbank/). The paired sequences used WS-F: 5'–ATG-GTA-CAT-TTT-CCG-GGC-G–3' (forward) and WS-R: 5'–AGA-TAG-CGA-AAC-

AAC-ATC-CAA-C–3' (reverse) for WSSV, and MBV-F: 5'–CAT-ATC-GGC-CGA-ATA-GTG-GTC–3' (forward) and MBV-R: 5'–TGG-CAT-GCA-CTC-CCT-GAG-AT–3' (reverse) for MBV. IHHNV, primer sequence was followed by Tang *et al*. (2007) with accession No. AF218266. The size of the DNA amplification were 113 base pair (bp) for WSSV, 526 bp for MBV and 389 bp for IHHNV.

2.4. Multiplex polymerase chain reaction assay (mPCR)

All specimens were analyzed in parallel by single PCR using WSSV, MBV and IHHNV primers. Positive samples were continued to multiplex PCR assay (mPCR). Firstly, three primer sets WSSV, IHHNV or MBV were mixed in 1 tube in the ratio of 1:1:1 (0.4 µM of each final concentration) as the primer cocktail solution. Reaction assay were performed in 25 µl. consist of 12.5 µl of 2× QIAGEN® Multiplex PCR master mix (containing: HotStarTaq® DNA Polymerase, Multiplex PCR Buffer*, dNTP Mix), 2.5 µl of Q-Solution, 5 x, 3 µl of primer cocktail, and 6 µl of nucleic acid-free water. Twenty-four µl of reaction solution was transferred to a new PCR tube and added 1 µl of 50 ng/µl extracted DNA sample into the tube as a template. Amplification of these mixture was done by PTC-200 thermal cycler (MJ Research, NH, USA) and setup to the following program: first step by 15 min at 95°C; second step by 40 cycles of 95°C for 30 secs, 58°C for 60 secs, and 72°C for 90 secs, followed by a final step for 5 min at 72°C and final incubation of unlimited time of 4 °C. The amplified products were analyzed by electrophoresis in 1.5 % agarose gels stained with ethidium bromide 50 - 1500 low range ladder was used as molecular weight marker. Electrophoresis was carried out at 100 V in a Mupid Mini gel electrophoresis unit for 30 min and viewed under UV-transilluminator (Bio-Rad Laboratories, CA, USA).

RESULTS AND DISCUSSION

Unknown mass mortality was reported in an organic shrimp farm in Sidoarjo, East Java, Indonesia in February 2009 during the rainy season. When shrimp samples are discovered with serious signs including blackened gills, white patches on the carapace, various sized of shrimp, and damage to the hepatopancreas. Multiplex PCR assay revealed positive results for WSSH, IHHNV, and MBV. The worst conditions for shrimp farming were also due to the poor water quality in the organic ponds.

The shrimp farming area's ponds are approximately 30,000 m^2, with soil pond construction and a two-month harvesting time using a traditional culture system with algae as a supplement (Figure 1 A). The stocking density was 35.000 shrimp of 30 days' post larval stage (PL.30) per 10,000 m^2 and was provided by a brood stock company in Gresik, East Java, Indonesia. A problem occurred when rain floods made it unable to replace the water in the pond, which required to be done every two weeks. Lower water quality in the pond become 3‰ of salinity, 3.8 mg/l of oxygen density and 32.4°C of water temperature worsened the shrimp condition and reached 80% mortality. Shrimp farming needed water salinity level ranging from 16 – 32‰. (Maicá *et al*., 2014). This condition deteriorates shrimp health, and the virus easily infects the shrimp, resulting in mass mortality and significant economic loss. Furthermore, higher temperature and lower oxygen concentration in the ponds will stress the shrimp and facilitate virus infection in *P. monodon* shrimp farming (Gunalan *et al*., 2010).

The quantities and purity of the isolated DNA from the shrimp in this investigation ranged from 43.6 to 145.4 ng/l and 1.94 to 2.03 (Table 1). Therefore, in order to yield a valid test with 50 ng/L for the mPCR per reaction, extraction techniques provide the requirements. In a single PCR run, the WSSV primer showed positive results in all samples from a total of 15 shrimp collected from the ponds. Ten samples of the total samples were used in the mPCR test. WSSV, IHHNV, and MBV primers were useful for detecting from 10/10 (100%), 5/10 (50%) and 6/10 (60%), respectively, based on mPCR data (Table 2).

The application of mPCR produced three type of multiple-pathogen infection pattern, which were clearly visible from electrophoresis of PCR product (Figure 2). Thus, two samples were infected by a combination of WSSV, IHHNV, and MBV which detected from target bands at 113, 389 and 526 bp of amplified product. Three samples were found to have WSSV and IHHNV infections (113 bp and 389 bp), while four samples were found to have WSSV and MBV infections (113 and 526 bp). A single infection of WSSV infected only one sample. Our study is the first report of a natural multiple-pathogens infection of WSSV, IHHNV and MBV in Black tiger shrimp (*P. monodon*) which was confirmed using single tube-run multiplex PCR method.

All shrimp with WSSV positive results displayed severe symptoms of red body staining, clearly white spots on the carapace, irregular shrimp size, blackened gills and hepatopancreas rupture (Figure 1 B-C, Figure 3 and Table 3). A typical symptom of WSSV infection is the presence of red body staining and white spots on carapace. WSSV was discovered for the first time in Japan as a result of mass mortality of *P. japonicus* shrimp (Nakano *et al*., 1994). Furthermore, blackened gill was observed in WSSV infec-

tion (Koesharyani *et al.,* 2001). WSSV is the most lethal virus with a very high mortality prevalence rate that causes significant losses and still present in Indonesia (Flegel & Fegan, 2002; Koesharyani *et al.,* 2001). The occurence of WSSV in crustaceans in general is currently being carried out (Xu *et al.*, 2020).

Table 1. Total nucleic acid concentration and purity of shrimp samples for detection by multiplex Polymerase Chain Reaction

No	Species	Organ	Nucleic Acid Concentration (ng)	Purity Optical Density (260/280)
1	*P. monodon*	Gill	58.6	1.97
2	*P. monodon*	Gill	137.1	1.98
3	*P. monodon*	Gill	93.1	1.99
4	*P. monodon*	Gill	120.8	1.99
5	*P. monodon*	Gill	87.	2.03
6	*P. monodon*	Gill	131.8	1,96
7	*P. monodon*	Gill	130.0	1.98
8	*P. monodon*	Gill	43.6	1.94
9	*P. monodon*	Gill	145.4	1.98
10	*P. monodon*	Gill	104.8	1.95

Table 2. Results of Polymerase chain reaction detection from *P monodon* samples using different primers IHHNV, MBV, and WSSV

No	Species	Organ	Type of Virus		
			WSSV	IHHNV	MBV
1	***P. monodon***	**Gill**	**Post.**	**Post.**	**Post.**
2	*P. monodon*	Gill	Post.	Negt.	Post.
3	*P. monodon*	Gill	Post.	Post.	Negt.
4	*P. monodon*	Gill	Post.	Post.	Negt.
5	***P. monodon***	**Gill**	**Post.**	**Post.**	**Post.**
6	*P. monodon*	Gill	Post.	Negt.	Negt.
7	*P. monodon*	Gill	Post.	Post.	Negt.
8	*P. monodon*	Gill	Post.	Negt.	Post.
9	*P. monodon*	Gill	Post.	Negt.	Post.
10	*P. monodon*	Gill	Post.	Negt.	Post.
Insidence of Diseases (%)			100	50	60

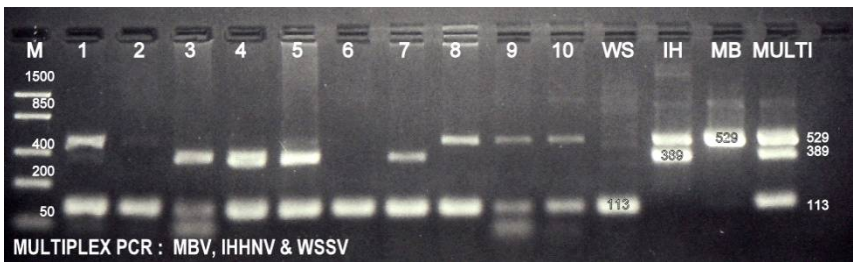

Figure 2. The multiple-pathogen infection pattern in *P. monodo*n showed from electrophoresed mPCR using primer cocktails: M. marker 50-1500 bp; 1. MBV, IHHNV & WSSV; 2. MBV &WSSV; 3. IHHNV & WSSV; 4. IHHNV & WSSV; 5. IHHNV & WSSV; 6. WSSV; 7. IHHNV & WSSV; 8. MBV & WSSV; 9. MBV & WSSV; 10. MBV & WSSV; WS. positive control for WSSV; IH. positive control for IHHNV; MB. positive control for MBV; Multi. mixed positive control for MBV, IHHNV & WSSV.

Figure 3. *P. monodon* samples collected from mass mortality case showed clinical sign of white spot in the carapace (A), red staining to blackened gills (B), hepatopancreas ruptured (C), and irregular size of shrimp (D).

Table 3. The condition of the samples collected from the organic ponds of shrimp farm

No.	Species	Source of samples	Note
1	*P. monodon*	Sekardangan Urban Village, Sidoarjo, East Java	Shrimp age: 2 months, soil pond construction, traditional system. 80%, mortality occurred in February (rainy season) with symptoms of white spots
2	*P. monodon*		
3	*P. monodon*		
4	*P. monodon*		
5	*P. monodon*		
6	*P. monodon*		
7	*P. monodon*	Kedung Peluk Urban Village Candi district-Sidoarjo, East Java	Shrimp age : 2.5 months with length of 12.5-18 cm, traditional system, all shrimp with red stained symptom
8	*P. monodon*		
9	*P. monodon*	Kalang Anyar village Bangil district, Pasuruan, East Java	Shrimp age: 2 months, soil pond construction, traditional system, shrimp with symptoms of white spots
10	*P. monodon*	Segorotambak village Sedati district-Sidoarjo, East Java	Shrimp age: 3 months, traditional system + (CP Marine fed)

In addition, two wild crabs with no symptoms were found to have WSSV infection in this organic ponds (data not shown). This finding was supported by Maeda *et al*. (1998; 2000), who reported that wild crabs can be carriers and vectors of WSSV, and this can infect most decapod groups and crustaceans family, including sea and brackish shrimp, freshwater shrimp, crabs and lobsters. According to Xu *et al.* (2020), WSSV was found and well persisted in wild sea-crustacean and Rotifer (Yan *et al.*, 2004) and in wild mud crabs (Norizan *et al.*, 2019). This additional evidence that wild crab can act as a carrier and spread the virus more quickly.

The irregular size of shrimp was a common symptom for IHHNV infection according to Office International des Epizooties (2010), which that explained the virus causing runt-deformity syndrome. The IHHNV infection has a significant economic impact worldwide. This virus was susceptible and caused more than 90% of mortality in *P. stylirostris,* as well as in infected *P. monodon* and *L. vannamei* by causing rostrum deformities and different size of shrimp growth, but less mortality (Bell & Lightner, 1984; Tang *et al.,* 2007; Office International des Epizooties, 2019).

Shrimp with hepatopancreas ruptured and MBV positive mPCR are strongly associated. In 1977, this virus was first discovered in post-larvae stage of *P. monodon* in Taiwan and adult shrimp in Mexico (Lightner & Redman, 1981; Lightner *et al*., 1983; Yan *et al*., 2009). MBV was introduced in Indonesia in 1998 from PL. 30-50 of farmed shrimp (Nash *et al.,* 1998). Thus, it is typically present in hepatopancreatic tubules and duct epithelium (Lightner *et al.,* 1983; Surachetpong *et al.,* 2005; Rajendran *et al.,* 2012).

The PCR assay is a standard technique for detecting the presence of a pathogen infection, particularly for viruses. Furthermore, when compared to conventional PCR testing, this developed mPCR assay was to carry out the specificity of target and efficiency of cost and time consuming of assay by simultaneously amplification in single tube using primer cocktails. Moreover, because of this developed mPCR methods was non-nested PCR, the risk of cross-contamination between samples was minimized. This method was developed and widely used in identifying mixed infection of WSSV and TSV in *L. vannamei* (Tsai *et al.,* 2002), *Macrobrachium rosenbergii*-nodavirus and extra small virus in giant prawn *Macrobrachium rosenbergii* (Tripathy *et al.,* 2006). In addition, this approach has been used to identify multiplex bacteria of the *Aeromonas* genus (Chu & Lu, 2005).

The single tube-run multiplex PCR was designed to detect several pathogens of infection in an outbreak incident at shrimp farm and could be useful in the routine testing of viral disease screening at shrimp larvae farms or broodstock center. Furthermore, this mPCR need to develop for RNA shrimp viruses such as IMNV, TSV and YHV which are the most problematic in the shrimp farming, especially *L. vannamei*.

CONCLUSION

This mPCR assay can detect the presence of multiple-pathogen infections simultaneously in single tube-run with a specified target, requiring less time and cost efficiency. This method can be applied to identify and screen shrimp before they are introduced into the ponds, as well as routine monitoring and epidemiological studies of shrimp diseases. In the future, this assay for RNA shrimp viruses needs to be developed to prevent virus infection and prevent shrimp farmer from financial losses.

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