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Original Paper

THE WHITE SPOT SYNDROME VIRUS (WSSV) LOAD IN *Dendronereis* spp.

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ABSTRACT

The white spot syndrome virus (WSSV), the causative agent of White Spot Syndrome Disease (WSSD), is a major shrimp pathogen in Indonesia. Dendronereis spp. is a ubiquitous Polychaetes and natural food of shrimp raised in brackishwater pond in Indonesia. The objective of this research is to determine the occurrence of WSSV and the viral load in Dendronereis spp. obtained from the shrimp pond. Dendronereis spp. was obtained with PVC (10 cm in diameter) from a traditional shrimp pond in Semarang vicinity. As a comparison, healthy looking Penaeus monodon was also obtained from the same pond. The occurrence of WSSV in Dendronereis spp. was determined with 1-step and nested PCR using primer for WSSV major envelope protein, VP 28. The viral load was counted with 1-step Real Time PCR. The WSSV was detected in Dendronereis spp. with 1-step and nested PCR. The point prevalence of WSSV infection in Dendronereis spp. is 90 %. The viral load ranged from 0 to 1.9 x 10^4 copy of DNA/µg total DNA. The viral load in Dendronereis is comparable with that of naturally infected and at carrier state P.monodon from the same pond. This is the first report of WSSV load in naturally infected Dendronereis spp.

Key words: *WSSV; Dendronereis spp.; viral load*

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INTRODUCTION

Shrimp farming is an important aquaculture industry in Indonesia. For the last two decades, this industry has become source of export earnings for Indonesia that lead Indonesia to be one of top shrimp producing countries in the world (Lem, 2006; FAO, 2010). White spot syndrome disease (WSSD) is a major shrimp disease that hampered cultured shrimp production in Indonesia. It was reported for the first time in Java island in 1994 and has caused serious impact on the shrimp culture (Sunarto *et al.,* 2004), since then WSSD has become endemic in Indonesia. In the pond, the onset of the diseases usually occurs during the second month of culture (PL are 42-50). Once the disease occurs, it can cause high mortality in one week.

The causative agent is the white spot syndrome virus (WSSV), a large DNA virus belong to genus Whispovirus and family Nimaviridae (Lo *et al*., 2012). WSSV is very pathogenic and highly virulent on penaeid shrimp, which the most affected hosts of this virus (Hameed *et al.,* 2005; Flegel, 2006). Moreover, it has a broad host range in addition to penaeid shrimp. WSSV DNA detected by 1-step as well as nested PCR in various benthic invertebarate carriers and vectors such as crabs (Supamattaya *et al.,* 1998; Kanchanaphum *et al*., 1998; Chen *et al.,* 2000; Meng *et al.,* 2009; Liu, *et al.,* 2011), rotifers (Yan *et al*., 2004), polychaetes (Vijayan *et al.,* 2005), copepods (Zhang *et al.,* 2008), marine microalgae (Liu, *et al.,* 2007) plankton (Esparza-Leal *et al.,* 2009), mollusk (Chang *et al.,* 2011).

Qualitatively, WSSV load has been classified heavy infection when WSSV DNA was detected with 1-step PCR and light infection when it was detected with nested PCR (OIE, 2006). Nowadays, Real-Time PCR was has been developed to quantitatively

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determined the number of viral copy in the host (Schmitt and Anderson, 2005).

Polychaetes is a common benthic fauna in the shrimp pond. Among Nereidids polychaetes ubiquitous in shrimp pond in Indonesia is *Denronereis* spp.. *Dendronereis* spp. is potential to be infected by WSSV because it lives in the sediment, and detritofeeder. WSSV infects tissues of ectodermal and messodermal origin especially gills, the fore and hind-gut, hemopoietic tissue, antennal gland and lymphoid gland (Chang *et al.*, 1996, Chen *et al.*, 2000, Rahman *et al.*, 2008, Escobedo-Bonilla 2007). In the previous study we were able to detect the presence of WSSV in *Dendronereis* spp. from shrimp pond in Semarang vicinity with one step and nested PCR (Desrina *et al*., 2011). This research is to determine the WSSV load in *Dendronereis* spp. with natural infection with1-step, nested and 1-step RT-PCR.

MATERIAL AND METHODS

Dendronereis spp.

The *Dendronereis* spp. was obtained from a traditional shrimp pond located in Semarang vicinity. The main cultivant raised is *Penaeus monodon* monoculture or polyculture with tilapia (*Tilapia nilotica*) and milkfish (*Chanos chanos*) with shrimp density was 2 shrimp/ m^2. The pond has experienced reoccurring of WSSV infection based on farmers report and clinical signs such as lethargic, low appetite, white discoloration on the body and carapace and low number of shrimp mortality that occurred over extended period of time, usually started about one months after stocking. Three months prior to sampling, the pond experienced mass mortality and has been fallow since. The sediment was obtained using a PVC (diameter 10 cm and high 40 cm) and sieved thorough a series of sieve shaker (mesh: 2 mm,0,6 dan 0,3 mm). *Dendronereis* spp. was easily recognized by its bright red color and the gills. The *Dendronereis* spp. retained was cleaned with sterile PBS (pH 7.4) and stored in the deep freezer (-80 ^{o}C) for PCR analysis. Eight worms were randomly picked to be used in this study. Along with the worm, we also obtained three *P.monodon* (average weight 25 g/piece) that survived and remained in the pond, treated and stored as done on Dendronereis spp.

Template DNA preparation for PCR analysis

A piece of the head section (first 15-20 segments, 25 mg) of frozen *Dendronereis* spp. was cut with sterile scalpel and placed in a sterile microcentrifuge tube. DNA extraction and purification was done using DNeasy Blood Tissue Kit (Qiagen) according to the instruction manual. In the case of *P.monodon*, the gills was used as the source of DNA and processed as for *Dendronereis* spp..

1-step and Nested-PCR

PCR was performed with 1 µl of DNA template using Taq Polymerase, the same amount of the first-step product was used as template for nested-PCR.. WSSV DNA obtained from known infected shrimp served as positive control of the PCR process. The DNA was amplified using VP 28 (Marks *et al.*,, 2003) and VP28 nested primer. The PCR was done with Gene Amp PCR System 9700 (Applied Biosystem) for 30 cycles for 1-step PCR and 25 cycles for nested PCR. Primer sequence is VP28-F1 CACAACACTGTGACCAAG (Forward) and VP28-R1 TTTACTCGGTCTCAGTGCCAG (Reverse) produce amplimer 529 bp. The sequence of the second primer used in nested- PCR is VP28-F1 nested CATTCCTGTGACTGCTGAGG (Forward) and VP28-R1nested CCACACACAAAGGTGCCAAC (Reverse) produce amplimer 383 bp. Both primer used the same annealing temperature 50 ^{o}C (50 sec). WSSV DNA obtained from known infected shrimp served as positive control of the PCR process. The result was visualized using UV illuminator Gel Doc XR System (Biorad).

Real Time PCR to quantify viral load

WSSV load was quantify by ABI PRISM 7300 Real Time-PCR system (Applied Biossystem) using TAQMAN master mix following instruction of manufacturer. WSSV DNA copy was quantified by analysing the cycle threshold value (Ct) using Step One software v2.1 (Applied Biosystem). ROX dye was used to monitored background fluorescence. Standard quantification was based on a series of WSSV-

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recombinant plasmid dilution with known copy number.

RESULTS AND DISCUSSION

WSSV was detected in *Dendronereis* spp. with 1 step and nested PCR (**Fig. 1**).

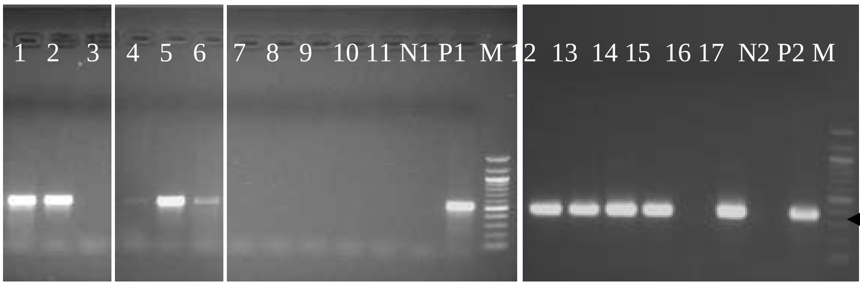

Fig. 1. 1-step and nested- PCR of *Dendronereis* spp. and *P. monodon* from shrimp pond. Lane 1-11 are result of 1-step PCR and lane 12-17are result of nested PCR. Lane 1- 3: *P. monodon* , Lane 4-11: *Dendronereis* spp., , Lane 12-16 *Dendronereis* spp.., lane 17 *P. monodon*. P1: Positive control for the 1-step PCR, P2: Positive control for nested- PCR, N1= Negative Control for 1-step PCR, P2= Positive control for nested- PCR. M= Marker (100 bp DNA ladder).

WSSV was detected in 7 out of 8 *Dendronereis* spp. tested with various degree of infection. Three out of 8 worm tested were positive with 1-step PCR (lane 4, 5 and 6) and 4 others were positive with nested PCR. 2 out of 3 of the shrimp tested were positive with 1 step PCR. Result of Real time PCR confirmed the findings of conventional PCR, indicating that WSSV load in the *Dendronereis* spp. that shows positive response with 1-step PCR is quite high and comparable with that in the naturally infected shrimp taken from the same pond. In contrast, those that only gave positive signal with nested PCR, the WSSV DNA is undetectable with RT PCR (**Table 1**).

Table 1. PCR analysis of WSSV infection in *Dendronereis*spp. detected with 1-step, nested and Real Time-PCR

Specimen	One step PCR	Nested -PCR	RT-PCR (WSSV copy / µg total DNA
Dendronereis spp.	N	P	ND
Dendronereis spp.	N	P	NT
Dendronereis spp.	P	NA	1.9×10^4
Dendronereis spp.	P	NA	1.2×10^2
Dendronereis spp.	P	NA	1.5×10^2
Dendronereis spp.	N	P	ND
Dendronereis spp.	N	P	NT
Dendronereis spp.	N	N	NT
P. monodon	P	NA	3.0×10^4
P. monodon	P	NA	1.57×10^5
P. monodon	N	P	NT

ND= Not detected, NA= Not applicable because specimen was positive with 1-step PCR; NT= Not tested, N= Negative, P= Positive

DISCUSSION

OIE (2009) recommended 1 step and nested PCR to detect WSSV infection in shrimp, because this method is highly specific and accurate. When tested shrimp give positive signal with 1-step PCR, it considers having heavy infection. On the other hand, since nested PCR can detect DNA in a much lower copy

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number than 1-step PCR, specimen that gave positive signal with nested PCR is considered to have light infection (OIE 2006) and considered at carrier state (de la Pena *et al.,* 2007) . WSSV has been detected with 1-step and nested PCR in wild broodstock of *P. monodon* (Withyachumnarnkul *et al.,* 2003, Shahadat Hossain *et al.,* 2004, de la Pena *et al.,* 2007), broodstock and post larvae of fleshy shrimp *Fenneropenaeus chinensis* (Jang *et al.,* 2009), as well as cultured shrimp (Shahadat Hossain *et al.,* 2004). Unlike conventional PCR, RT-PCR can detect the viral DNA concentrartion in the tissue. Viral load in infected shrimp varied according to intensity of infection. Moribound penaeid shrimp ranged 2.0 10^4 to 9.0 · 10^{10} WSSV copies g–1 of total DNA (Durand and Lightner 2002) and slightly higher in shrimp with acute infection (Durand *et al.,* 2003).

The WSSV load in the *Dendronereis* spp. and shrimp observed in this study is quite high, because it was detected with 1-step PCR in some specimen. Moreover, result of RT-PCR this study within the range reported by Jang et (2009), but lower than reported on the experimentally infected shrimp (Durand and Lightner, 2002, Durand *et al*., 2003). The degree of infection is varied among the *Dendronereis* spp. examined and in some specimen it is comparable with that of the naturally infected shrimp from the same pond that look healthy with no clinical sign of WSSV. It can be implied that the WSSV load in *Dendronereis* spp. is comparable with that in *P.monodon* at the carrier state. It has been suggested that polychaetes is a mechanical vector of WSSV and the virus is accumulated in the digestive tract by artificial infection (Vijayan *et al.,* 2005). The part of *Dendronereis spp.* that we used included the front gut. However, whether WSSV merely accumulated or replicate in this organ need further study with immunohistochemistry or probe specific for WSSV.

CONCLUSSION

In conclusion, WSSV DNA load in infected *Dendronereis* spp obtained from shrimp pond is varied, and in some specimens is comparable with that in the infected shrimp.

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