NON-CLOTTING HAEMOLYMPH OF WSSV-INFECTED SHRIMP: IS IT A FACTOR IN INFECTION PROCESSES?

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ABSTRACT

White spot syndrome virus is recognized as the most prominent pathogen of penaeid shrimp and has been affecting this shrimp farming industry around the world. The virus may reduce the shrimp's immune response and alter enzymatic and biochemical composition of tissues. Similar to other environmental stressed or other pathogen-infected shrimp, in late stages of WSSV infection, shrimp will fail to clot the haemolymph, so any minor injury will lead to increased haemolyph loss. A series of experiments to determine the effect of non-clotting haemolymph on WSSV infection were carried out in controlled facilities in Indonesia. The preliminary test showed that normal clotting time was 13.3 seconds while WSSV-injected shrimp mostly failed to clot their haemolymph 16 hours post infection (hpi). Some other clinical signs such as abnormal swimming, red discoloration, white spots and mortality were consistent with those observed by previous studies. Three shrimp species: banana shrimp (P. merguiensis) 9 g , white leg shrimp (P. vannamei) 7 g and the tiger shrimp (P. monodon) 16.5 g were water-borne-challenged with non-clotting, WSSV-infected haemolymph (NCH) from tiger shrimp donor in duplicate tanks each with 12 shrimp. The control were tiger shrimps fed with WSSV-infected tissue at the rate of 40% of bodyweight (BW) and other tiger shrimps were used as negative controls fed with commercial feed only. The study revealed that NCH dosages of 1.46%; 2.03%; and 2.06% (v/v) for each species were sufficient to infect and kill all shrimps in less than two days compared to eight days for the shrimps fed on infected tissue. The WSSV in non-clotted haemolymph eventuallyattaches into the living tissues of healthy shrimp. This mode of infection is likely more difficult to control by the ordinary fine mesh screening method.

KEYWORDS: **non-clotting-haemolymph, WSSV, infectious, *Penaeus monodon, P. merguiensis, P. vannamei***

INTRODUCTION

White spot syndrome virus (WSSV) has devastated the penaeid shrimp industries since the early 1990s in the main producing countries in Asia and Latin America (APHIS, 1999; Crockford, 2001; O.I.E, 2003). After its discovery in 1992, this highly contagious disease has now been found to infect wild penaeid shrimp and other wild crustaceans (APHIS, 1999; Lightner, 1999). This disease clinically infects the penaeid shrimp although it may infect a wider range of crustaceans (Chang *et al.*, 1998; Hossain *et al.*, 2004; Kanchanaphum *et al.*, 1998; Lo *et al.*, 1996; Rajendran *et al.*, 1999). Moreover, this disease has been experimentally and incidentally proven to cause mortality and severe infections among freshwater crustaceans (Corbel *et al.*, 2001; Jiravanichpaisal *et al.*, 2001; O.I.E, 2003). Furthermore, the present WSSV lineages are more virulent than their 96's ancestor, perhaps due

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to their 16 kb smaller genome (Marks *et al.*, 2005).

A white spot disease (WSD) outbreak in a shrimp farm is characterized by declining appetite and abnormal swimming on the water surface of affected shrimp (Corbel et al., 2001; Flegel, 1995). At later stages, some shrimps develop a red discoloration, die at the pond edges and at a more chronic phase, show white spots all over the body (Flegel, 1995). Histologically, shrimp will suffer massive apoptosis in all tissues including vital organs (Sahtout et al., 2001) and haemocytes (Hameed et al., in press). WSSV can be identified by PCR and in situ hybridization in the pleopods, gills, stomach, abdominal muscle, haemolymph, midgut, heart, pereiopods, lymphoid organs, integument, nervous tissue, hepatopancreas, testes, ovaries, spermatophores, and eye stalks (Lo et al., 1997). Clinically, WSSV-infected shrimp will be similar to environmentally-stressed animals by higher phenoloxydase (PO), lower prophenoloxidase (ProPO), lower total haemocyte counts (THC) (Sritunyalucksana et al., 1999), lower haemolymph protein level, lower clotting protein, and reduction in antioxidant enzymes (Mohankumar & Ramasamy, 2006b; Yoganandhan et al., 2003). WSSV-infected shrimp will also exhibit significantly reduced activity of membrane-bound phosphatases in many tissues that lead to an increase in the production of free radicals and decreasing mitochondrial enzyme activity in infected tissues (Mohankumar & Ramasamy, 2006a). Eventually the shrimps fail to coagulate their haemolymph which also occurs in TSV- infection (Song et al., 2003; Yoganandhan et al., 2003). At later stages of infection, viral particles can be found in any tissues of the shrimp (Rout et al., 2005) including in the haemocytes (Sahul Hameed et al., in press).

Finding early signs of mortality and abnormality has always been difficult in relatively large ponds. The farmers are usually aware of the problem at a very late stage and total mortalities most often occur 3 days after the initial discovery of some mortalities (Flegel, 1995). Some shrimps have red feces as a sign of eating rotten shrimp tissues. Healthy shrimp would prefer to eat recently dead shrimp (mostly appendages on cephalothorax) instead of the ration feed (Soto et al., 2001). These conditions are believed to instantly increase the total viral intake and trigger massive mortalities in the newly infected shrimps (Wu et al., 2001).

Studies of viral transmission among shrimps have been conducted by many authors via feeding infectious tissues (Wang et al., 1998; Soto et al., 2001), injection and immersion (Chou et al., 1998; Rajendran et al., 1999; Wu et al., 2001) and cohabitation with crabs (Rajendran et al., 1999). Studies of the effect of WSSV-transmission on shrimp were mostly carried out by oral route (Kanchanaphum et al., 1998), injections (Chang et al., 2003; Perez et al., 2005) or cohabitation. In the cohabitation experiment, the shrimp were positively infected by WSSV within 24 to 48 hours. In viral injected experiment, the mortality of shrimp at D-14 at low density reached 18% compared to 46% and 72% at higher densities. Removal of dead shrimp or physical separation may significantly reduce the cumulative mortalities (Rout et al., 2005). The study by Rout et al. (2005) concluded that transmission occurred horizontally via cannibalism and via waterborne route. It was found that the LD50s of two different WSSV injection preparations were estimated at 1:4.444 × 106 and 1:4.505 × 106 (Wu et al., 2001) while with immersion only 39% of the challenged shrimp died (Prior et al., 2003)

The cohabitation studies presumed that WWSV-naïve shrimp contracted the disease by digesting faecal materials or by exposure to free viral particles (Prior et al., 2003). As WSSV infects the haemocytes (Di Leonardo et al., 2005) and reduces the haemolymph clotting ability, small-free-infected haemocytes can easily be distributed in the water column and potentially infect shrimp or other crustaceans under cohabitation circumstances. There is little information on this possibility. These series of studies was carried out to determine the role of non-clotted-infected haemolymph as a possible viral transmission mechanism instead of cannibalism as previously known.

MATERIALS AND METHODS

Experimental Animals

WSSV-free penaeid shrimp were obtained from the Center of Brackishwater Aquaculture Development (CBAD) hatchery and controlled pond facilities in Jepara, Central Java, Indonesia. These experimental animals comprised batches of 24 banana shrimps (P. merguiensis) with 8 g mean bodyweight (BW), 24 white leg American shrimps (P. vannamei) 6 g BW, and 58 tiger shrimps (P. monodon), 15-18 g BW. All were confirmed to be WSSV-free by two step PCR

prior to the experiment. All shrimps were selected in physically good condition based on the criteria of rough skin and firm bodies in moult stage C. These shrimps were reared in recirculation tanks for at least three days to adapt before being challenged with the virus. Sterile sea water used in this experiment was kept at salinity of 31 ppt, pH 8.1 ± 0.6, temperature 28.0 ± 1.5°C and dissolved oxygen level at 5.2 + 1.7 ppm.

Viral propagation

WSSV was propagated from the fourth generation of local stock of severely infected tiger shrimp stored in a deep freezer (-80°C). The new viral solution was extracted by slightly modified previous procedures (Corbel et al., 2001; Namikoshi et al., 2004). Briefly 3 g of gill tissue was chill-homogenized in 10 mL Leibowitz medium and after centrifugation at 3,000 G for 10 min at 4°C, the supernatant was filtered through a 0.22 µm sterile membrane. The supernatant was then centrifuged at 10,000 G for 20 min at 4°C. The supernatant was then diluted 10-fold with phosphate buffered saline PBS (pH 7.4) for direct injection into the recipients at the rate of 0.05 mL per 10 g of shrimp.

All the five tiger shrimps receiving deep frozen WSSV-infected tissue homogenate and the ten shrimps at the subsequent injections showed a typical WSSV infection including inactivity, becoming moribund and rapid mortalities in less than 2 days.

Haemolymph collection

Donor tiger shrimps that received WSSV inoculation showed a declining appetite, moribund swimming, red discoloration and white spots on bodies. Non-clotting haemolymph (NCH) was obtained from inactive red discoloration or white spotted shrimp. Each 20 g shrimp may produce 1.5-2.5 mL of grey-colored NCH. After a two-minute clotting test, each individual NCH was stored in Eppendorf tubes, and then stored in a styrofoam box filled with ice flakes. A reconfirmation of non-clotting condition was performed on all haemolymph in tubes by gentle agitation. All NCH was mixed in one large sterile tube (50 mL) before it was divided into two smaller (20 mL) tubes. All tests and mixing were carried out under a biohazard hood.

Injection and negative control experiment

Two groups of twelve shrimps were injected with WSSV homogenate (0.05 mL/10 g) from two separate viral stocks at the dorsolateral third abdominal inter segment. Another 12 x 2 shrimps were injected similarly with PBS solution (Wu et al., 2002). Commercial food was given at 3% BW once per day but mostly was uneaten.

Eighteen negative control shrimps were reared in six separate stagnant water tanks fed with commercial feed (CP diet-3) 3% BW three times a day. Three shrimps were taken each day and returned to other tanks after 0.04 mL of their haemolymph was withdrawn. These shrimps were then fed with 5 pieces of frozen anchovies per shrimp at first day of recovery until their haemolymph was retaken on the fifth day.

Clotting time determination

Haemolymph was drawn from the base of fourth pereiopod. Soon after ejection from the needle and timing started, haemolymph was stirred with the needle tip on a glass surface. All needles and syringes were used only once. If the haemolymph-clott diameter reached approximately 3-4 mm in size, the clotting time measurement ended. All times were recorded with a digital stop watch.

NCH challenge and Controls

The NCH challenges were conducted in the isolated wet lab of CBAD Station, Jepara Central Java. All facilities and procedures were run with the highest biosecurity possible. All three species of shrimp (24 each) were subdivided (12 for each species) into six 20 L water containers filled with 10 L sea water (30 ppt, 28°C, 5.2 ppm DO) with gentle aeration. The NCH was dropped with a 5 mL syringes simultaneously into the containers: 1.5 mL for each P. merguiensis and P. vannamei and 4.5 mL for P. monodon. Fifteen minutes after NCH immersion, all water and the shrimps were removed into separate larger 300 L fiberglass tanks with stagnant water system (30 ppt, 27°C-28°C) with gentle aeration to get the 4.8-5.1 ppm DO. Commercial feed was given at the rate of 3% of biomass three times a day.

A positive control experiment was carried out by feeding 12 x 2 tiger shrimps with

Table 1. Summary of NCH experimental design

Prawns		Biomass (g)	NCH Vol. (mL)	NCH-prawn ratio (v/v)1) (%)	NCH-water ratio (v/v) in 300 L tanks
P. merguiensis	(24)	184	1.5	146.00%	5 ppm
P. vannamei	(24)	132	1.5	203.00%	5 ppm
P. monodon	(24)	390	4.5	206.00%	15 ppm

[1)] healthy shrimp are assumed to have specific gravity 1.12

grounded whole infected shrimp tissue. The shrimps were starved for two ration feeding times before fed with the tissues at the rate of 20% of body weight (BW) per feeding, for two feeding times. The next feeding resumed with the commercial feed (CP diet 3) at the rate 3% BW three times a day.

The negative control was arranged similar to the positive control but fed with commercial feed only. Limited amount of new water was added into each tank daily (approximately 7%-10%). All waste and waste water from infected and non-infected tanks were collected in several 40 L water buckets, disinfected with calcium hypochlorite and drained into isolated septic tanks for further treatment.

Tools and facilities for all experiments were separated and treated with disinfectant (500 ppm Sodium hypochlorite) before and after use.

Observation of clinical signs

Three clinical factors were monitored during the NCH experiment: red discoloration (ReD), abnormal swimming (Abn), white spot development (WSp) and cumulative mortality (CM). The NCH immersion data were collected within 48 hours due to quick reactions while the feeding and control experiment were monitored for 8 days.

All clinical sign data were calculated in relative value (%) to the current survivor at time of observation. Mortality data (CM) was arranged in cumulative total death at a designated time.

Data analysis

Log natural transformation was conducted for normal distribution of all data of NCH –challenged study before further analysis. One way ANOVA analyses were applied for observation data by using statistical software SPSS version 12. Data were compared by the least significant difference (LSD) analysis and presented by mean ± standard error. A significant level of 0.05 was set for all statistical analysis.

RESULTS

Clotting time of normal shrimp

All of the normal shrimps showed a similar clotting time. Statistically there was no significant effect between time in captivity (9 days) with the clotting time (one way ANOVA, F= 0.16, d.f = 2, 29 and P >0.1). The mean haemolymph clotting time under these conditions was 13.3 seconds (Figure 1.)

Clotting time of WSSV-injected prawns and the controls

After injection by WSSV homogenate, shrimps showed a longer clotting time compared to PBS injection and the negative control treatments (Figure 2A and 2B). All treatments had a significant effect for clotting time (one way ANOVA, F= 59.06 d.f= 2,35 and P<0.001).

In the first two hours post-injection, the clotting time patterns were similar (Figure 2 A.). At 8 hpi and 16 hpi shrimps received WSSV injection (WS inj) had significantly longer and non clotting haemolymph. Overall, injections had significantly longer mean clotting time than the other two controls (Figure 2B). Mean clotting time for WS-inj. was 98.42 ± 17.45 sec compared to PBS-inj. (13.33 ± 1.62) sec and negative control (9.92 ± 0.72) sec with no significant difference between the last two.

Non Clotting Haemolymph (NCH) challenges

The NCH collected from heavily infected animal used in immersion tests showed that

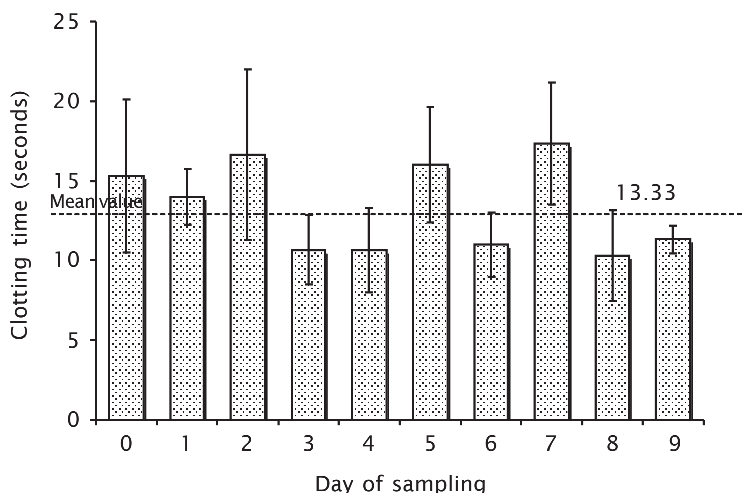

Figure 1. Daily haemolymph mean clotting time ± standard error of three shrimp per sampling reared in five separate stagnant-water tanks. Haemolymph collection was repeated after five days

the infection was as severe as that of the injection treatment. The NCH challenge, compared with infected tissue feeding and the negative control, has a significant effect for most of the bioindicators measured.

The first clinical symptom observed, red discoloration (ReD), occurred on P. merguiensis did not get any color changes until 36 hours post exposure (hpe), which is statistically significant (F=3.76 d.f = 4,49 and P<0.01) but most of the P. vannamei had shown this sign of infection 12 hpe. Meanwhile *P. monodon* immersed in NCH increased in ReD from 36 hpe to 96 hpe. Increasing ReD was also occurred in *P. monodon* fed with infected tissue from 48 hpe. The negative control showed little ReD (Figure 3 A).

No white spots on the epicuticle were detected in either P. merguiensis or P. vannamei exposed to NCH (Figure 3 B). However the P.

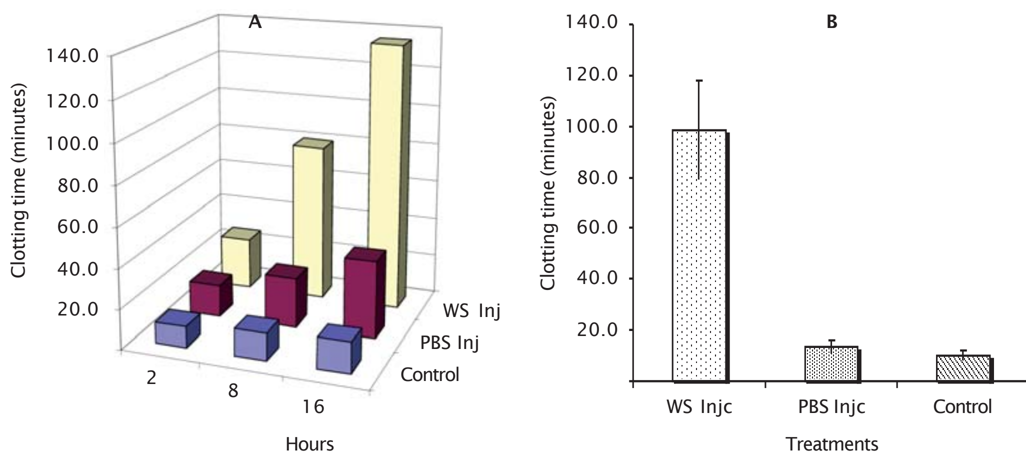

Figure 2. Clotting time on WSSV injected shrimps (WSinj) compared to those injected with PBS (PBS inj) and negative control (Control) in 2, 8 and 16 hpi (A) and differences among mean clotting time ± standard error of each treatment (P< 0.05) (B)

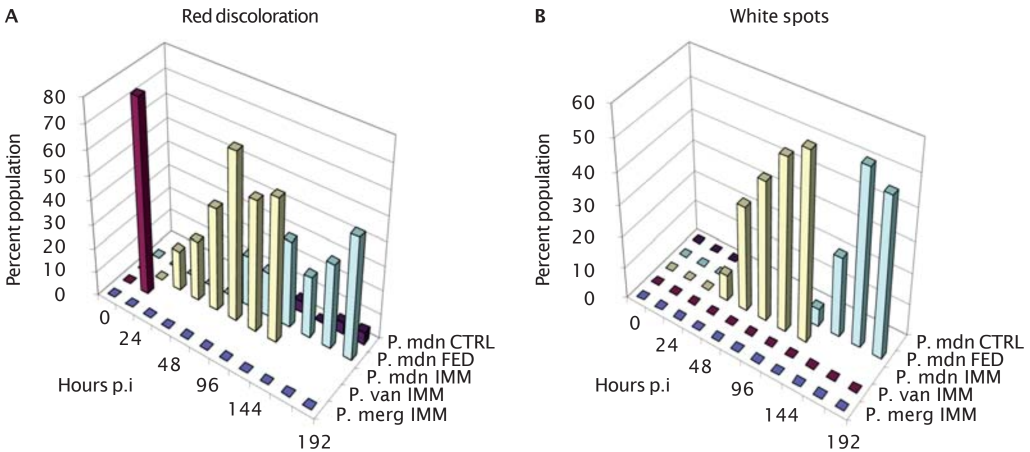

Figure 3. Percentage of surviving prawn populations challenged with different WSSV : *P. merguiensis* immersion (P merg IMM), P. vannamei immersion (P van IMM), *P. monodon* immersion (P mdn IMM), *P. monodon* fed on infected tissue (P mdn FED), *P. monodon* negative control (P mdn CTRL) based on two clinical signs : Red discoloration (A) and White spots (B)

monodon immersed in NCH showed rapid development of white spots (1.5 days) compared to shrimp fed with infectious tissue (4 days) and none in the negative control (F = 3.427 d.f = 4,49 and P<0.05).

Signs of cute infection (laying on side, moribund swimming, and curling pereiopods) were detected in all infection-challenged treatments and statistically significant (F = 4.584, d.f = 4, 49 and P<0.005). In P. merguiensis and *P. vannamei*, the abnormality was first observed at 6 hpi and reached its peak (100%) at 18 hpi. While for P. vannamei the peak (75%) was reached quicker at 12 hpi. In P. monodon, the infection of NCH progressed more slowly than the previous white prawns; it took 24 hpi for the first sign of abnormalities and progressed until its peak at 120 hpi (5 days). In the fed-challenged experiment the earliest sign of abnormalities was at 48 hpi and slowly increased until it reached the peak at 192 hpi (8 days).

NCH caused dramatic mortalities amongst tested shrimp (F = 6.93, d.f = 4,49 and P< 0.001). P. merguiensis and P. vannamei all died within 18 hours (presented at 24 hpi- on Figure 4B) while P. monodon all died at 120 hpi. P. monodon fed infected tissue reached 100% mortality in 192 hpi. The negative control shrimps showed no significant mortalities (9.09% or 2 out of 22 shrimp) in 8 days (Figure 4B).

Discussion

The injection of WSSV homogenate in donor tiger shrimps had general clinical signs such as red discoloration, lost appetite and moribund swimming (abnormalities), white spots and rapid mortalities. The normal clotting time of 15 shrimps reared in fiber glass tanks to represent handling stress was 13.3 seconds.

Immersion with NCH at approximately 1.46% and 2.03% (v/v) for the P. merguiensis and P. vannamei and 2.06% for P. monodon may kill both shrimps in less than 2 days which was similar to injection in viral propagation steps in this study. The immersion at the first 15 minutes was equal to 150 ppm to 450 ppm NCH in the 10 L of clean seawater and only 5 and 15 ppm onward. On the other hand, feeding infected tissue killed some of the shrimp after 6 days and totally killed the shrimps in 9 days (not accounted in 8 days-analysis).

The role of free virions or viral-loaded haemocytes in infecting living or apparently healthy shrimps is still poorly understood. By feeding infected tissues, shrimps will show clinical symptoms within 5 to 18 days (Wang et al., 1998). In this experiment, the clinical symptoms occur within 5 to 7 days. Variation in time may be due to different feeding practices, viral strains and the concentration of viral loads and handling stress. However, both of these

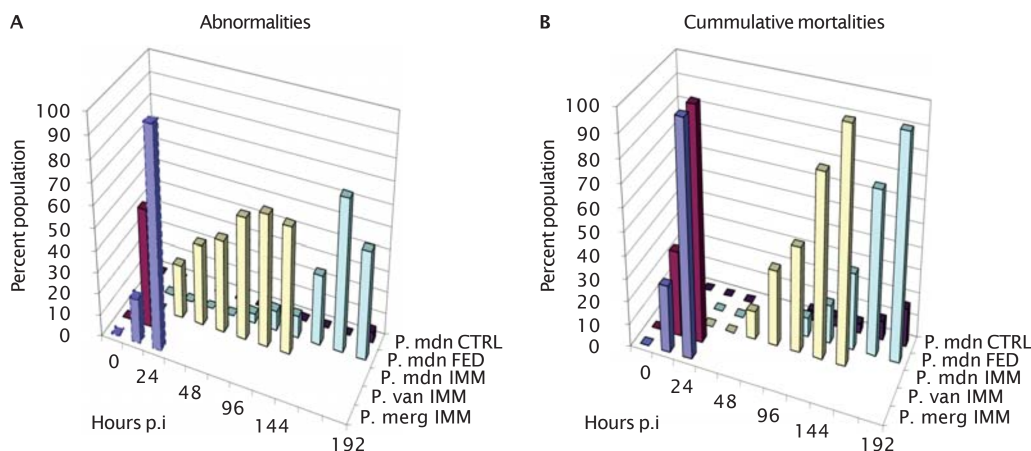

Figure 4. Percentage of surviving shrimp challenged with different WSSV : P. merguiensis immersion (P merg IMM), *P. vannamei* immersion (P van IMM), *P. monodon* immersion (P mdn IMM), *P. monodon* fed on infected tissue (P mdn FED) with *P. monodon* as negative control (P mdn CTRL) based on abnormalities (A) and cumulative mortalities (B)

explanations indicate that infection via ingestion of infected tissue did not induce disease as hypothesized for a WSD outbreak (3-4 days). During ingestion WSSV has to pass physical and chemical barriers before reaching the susceptible epithelial cells. Moreover the cuticle layers still protect any epithelial cells on the foregut while some protease, amylases and tripsin enzymes may form chemical barriers. Accordingly it is possible that the amount of WSSV may be significantly reduced (van de Braak, 2002). The foregut and hindgut constitute a very effective barrier against viruses essentially because of their structure: an inner layer of cuticle, then a digestive epithelium surrounded by a basal membrane tightly applied at the base of epithelial cells. The midgut possesses involve only the epithelial layer and the basal membrane (Escobedo-Bonilla et al., 2005). So at a very low level per os challenge on P. japonicus, the WSSV-positive nuclei could only be found in epithelial digestive cells (Okumura et al., 2004; Prior et al., 2003; Rajendran et al., 1999).

The clot formation is actually a physical mechanism to prevent the loss of body fluid and dissemination of foreign materials into haemocoel after an injury or infection (Di Leonardo et al., 2005). Biologically, this mechanism was carried out by crosslink or protein polymerization of coagulogen (a clotting protein with a molecular mass of 340-400 kDa) (Soto et al., 2001), into clottable proteins (CP); this process is triggered by innate catalyses of a calcium dependent transglutaminase (TG)(Aono & Mori, 1996; Martin et al., 1991; Okumura et al., 2004; Perazzolo et al., 2005) which is abundantly available in hyaline cells (Aono & Mori, 1996; Perazzolo et al., 2005; Yeh et al., 1998). A series of studies on western rock lobster (Panulirus cygnus) revealed that after exercise or handling stress, total haemocyte counts (THC) will increase by increasing number of semi granulocyte cells but a decline in the number of hyalinocyte and granulocyte cells. These physically-stressed lobster showed a significant declining clotting time (Montano-Perez et al., 1999). But contrary to the above test, under WSSV infection, THC reduced drastically since most haemocytes (including the hyalinocytes) migrated to the infected tissues (Martin et al., 1991). Hyalinocytes, also known as explosive corpuscles dehisce, release their products to catalyze the coagulogen so loss of clotting is likely to be dependent on the decline of hyalinocytes (Chen et al., 2005).

The clotting mechanism relies on clotting proteins but under WSSV infection, protein profiles are drastically altered. Four new proteins and three intensely expressed high molecular weight proteins in haemolymph of infected shrimps were previously identified (Jussila et al., 2001). Furthermore WSSV infection creates higher glucose level and lower fatty acid in the haemolymph (van de Braak, 2002). The fail-

ure of clotting formation under WSSV infection must involve one or all of the above factors.

The non-clotting haemolymph may contain free living virions (FLV) (Smith et al., 1995). In the haemolymph, these free viruses have been observed as enveloped virions and nucleocapsids associated with envelope debris (Rameshthangam & Ramasamy, 2005). In contrast to the histology-based study by Yoganandhan et al. (2003) which stated that circulating haemocytes were not the target of WSSV infection, the NCH was found to contain the viral-loaded infected haemocytes (IH) especially at the granular and semi granular cells (Corbel et al., 2001; Okumura et al., 2005; van de Braak, 2002). These infectious materials are freely available in the water column for a certain time (Wang et al., 2002a). NCH probably released into the water from open cuts and wounds during cannibalism. In previous cohabitation studies, no information of any viral forms (FLV or IH) that had infected the healthy hosts (Wang et al., 2002b) and mostly using the virions homogenates from infected tissues in immersion studies.

The release mechanisms of FLV from unwounded hosts (Wang et al., 2002a) are still unknown and neither are the entrance mechanism into internal body system. The most likely exit is the hindgut via excretes and the entrance is the gill which is a relatively open organ. Gills, with their semi-permeable cuticle and mucosa layers, are highly vascularised. With its complex surfaces, the gill may trap some foreign particles including infected haemocytes.

The NCH volume (1.5 mL) required to kill 100% of P. merguiensis and P. vannamei was withdrawn from shrimps at the size of 15 grams. This NCH amount is analogous with two to three dead white shrimps or three dead tiger shrimps. In dying-shrimp-removal experiment (Anonymous) the shrimps took longer time to be infected in both removal and separation of dead shrimps, but after the current study, it is possible that separation of dead shrimps was actually preventing NCH leakage from an open cut during cannibalism. NCH is not exclusive to WSSV infection, it may also found in other pathogen's symptoms or shrimp under environmental and pollution stresses. This study has proven that NCH can be used as alternative way of WSSV survival strategy.

CONCLUSION

The cohabitation study on WSSV pathogenesis is likely more important for understanding the ultimate threat of this virus against cultivated and wild crustaceans. Infection from non-clotting haemolymph is similar to the induction of disease by injection in that it rapidly creates systemic infection. The feeding-infection route should take longer time. Quick and safe removal of dying shrimps from the pond edge before cannibalism is a critical point to prevent an outbreak. This study reveals the danger of NCH but the main question of the actual infective agents (FV or IH) and how to reduce the infectivity of these waterborne viruses are still unanswered. The next study should put more emphases on negative NCH traces after using well-filtered and aged water before and after being used in shrimp cultivation.

ACKNOWLEDGMENT

The authors would like to thank the ACIAR for funding travel and materials for this study under project FIS 2000/061. The help from staff of CBAD Jepara, Indonesia (Arif, Astuti, Evi, and Retno) with the provision of WSSV-infected specimens and the PCR testing, and Zaenal for providing space, tanks and field equipment, is much appreciated. Special thanks also given to Sardi and Siska who assisted us during preparation and sample collection, Mr. Darmawan and Mr. Kadek Ariawan who kindly provided WSSV-free shrimps used in this experiment. At last, the author would like to express his gratitude to Associate Professor Jesmond Sammut for reviewing the manuscript and the BRKP Indonesia for accepting this paper.

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