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Haematological Alterations in *Penaeus monodon* Artificially Infected with White Spot Syndrome Virus (WSSV)

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Abstract

The present study examined the haematological alterations occurring in *Penaeus monodon* infected with white spot syndrome virus, a major havoc causing organism in aquaculture of Asian countries. White spot disease was developed by injecting white spot syndrome virus (WSSV) inoculum into the fourth abdominal segment of *P. monodon*. Morphological changes observed were very much similar to those developed during natural infections of WSSV. Clotting time, total haemocyte count, (THC), protein content, glucose and creatinine concentrations and albumin/globulin ratio in haemolymph of normal saline - injected and virus – infected *P. monodon* were monitored at 0, 24 and 48h after injection. In the WSSV injected shrimp, clotting ability was completely lost. A drastic THC reduction of 45% at 24h and 76% at 48h post infection was also observed. Twenty-five and 45% of reduction in haemolymph protein was noted at 24 and 48h, respectively. Haemolymph glucose level was reduced to 31% and creatinine to 12.6% of the original values by 48h post injection. Haemolymph globulin content increased and albumin content decreased significantly, reducing the albumin/globulin ratio. The ratio was reduced to 49% of its original value. The results of the present investigation indicate that WSSV infection induced significant alterations in the haematology of *P. monodon*.

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Introduction

White spot disease caused by white spot syndrome virus (WSSV) (assigned to the genus Whispovirus and family Nimaviridae) results in 80–100% mortality in shrimp within 3-7 days of viral injection (Vlak et al. 1999; Mayo 2002). Early detection of the virus has been recognized as the best control measure and the methods like polymerase chain reaction (PCR) assay and histology are used for this purpose (Karunasagar and Otta 1997). Bachere (2000) opined that practically no general criteria exist for specific evaluation of the health status of shrimp and other invertebrates. Though PCR has been used as the most viable method for the detection of WSSV, haematology is only occasionally used as a diagnostic option in penaeid shrimp pathology (Van De Braak 2002). Variables such as total plasma protein content, glucose concentration, alkaline phosphatase activity, clotting time, haemocyte count, prophenol oxidase activity, phagocytic index, release of reactive oxygen species (ROS) and antibacterial activity have been considered recently as potential health or disease markers in crustaceans (Rodriguez and Le Moullac 2000).

Biochemical indices may be helpful not only in understanding the exact mechanism involved in the pathology of WSSV and health status of the shrimp; but also in finding therapeutic alternatives in controlling and prevention of the disease. Though extensive studies have been carried out mainly focusing on the detection of WSSV, studies on the biochemical and haematological alterations are relatively scanty. Earlier, we have reported the biochemical changes occurring in the tissue defense system (Suseela et al. 2007a) and carbohydrate metabolism (Suseela et al. 2007b) in WSSV-infected *Penaeus monodon.* In the present study, we have attempted to assess the haematological changes occurring in WSSV-infected shrimp with respect to changes in clotting time, total haemocyte count, protein content, glucose and creatinine concentrations and albumin/globulin ratio in haemolymph of *P. monodon* injected with white spot syndrome virus.

Materials and Methods

Shrimp (*P. monodon)*, 18 ± 1 g body weight), were collected from grow-out ponds and maintained in 1000 L fibre glass tanks with air-lift biological filters at room temperature (27–30^{o}C) and salinity between 20

and 25 mg·ml^{-1} and dissolved oxygen level 6 mg·l^{-1}. The shrimp were kept in these tanks for 10 days for acclimatization before the experiments.

Shrimps were fed with artificial pelleted feed. To confirm absence of WSSV infection, 5 shrimp from a group of 30 were randomly selected and screened for WSSV by polymerase chain reaction (PCR) using the primer designed by Takahashi et al. (1996), which was sufficient to confirm with 95% confidence that the group of shrimp was free from WSSV, as described by Cameron (2002).

The inoculum was prepared as per the method of Van Hulten et al. (2000) with slight modification. The WSSV-infected *P. monodon* with prominent white spots were collected from shrimp farms at Kannamali, Cochin. Said WSSV infection was confirmed by PCR (Takahashi et al. 1996). The heads of the infected shrimps were homogenized in sterile buffer (1: 2) followed by centrifugation (3000 x g) for 20 min at 4°C. The supernatant fluid was filtered through a 0.45µm filter. The protein content of the filtrate was determined (9mg·ml^{-1}) and the presence of WSSV was confirmed by PCR (Takahashi et al. 1996). The filtrate comprised the viral inoculum and was stored at -40°C.

Thirty shrimps were maintained in 200 l fiberglass tanks at room temperature (27–30°C) with salinity ranging between 20 and 25 mg·ml^{-1}. They were injected with 100µl of thawed viral inoculum intramuscularly in the fourth abdominal segment as described by Van Hulten et al. (2000) with modification (Suseela et al. 2007a). The prawns were sacrificed at 24h and at the moribund stage (48h). Haemolymph was drawn directly from the cephalothorax before sacrifice using a specially designed, pointed and tapered sterile thin glass pipette pre-rinsed with anticoagulant (10mM Tris-HCl, 250mM sucrose, 100mM trisodium citrate pH 7.6) at 0, 24 and 48h post injection. Mortality reached 100% in 72–84h and hence animals could not be sampled live after 48h. The WSSV infection was confirmed at 48h by PCR (Takahashi et al. 1996) (Fig 1). Another set of 30 shrimp, maintained in similar conditions, were treated as control and were injected with normal physiological saline. Haemolymph samples were collected similarly at 0, 24 h and 48 h from control shrimps.

Collected haemolymph from control and infected shrimp was stored in eppendorf tubes with 100µl anticoagulant at 4°C. Fixed haemolymph was diluted 2, 4, 8, 16, and 32 times with ice-cold phosphate buffer saline (PBS, 20 mM, pH 7·2) and total haemocyte counts (THC, cells·ml^{-1}) were done using a Burker haemocytometer (Le Moullac et al. 1997) in the

dilutions with a light microscope. The THC was measured for both infected and control shrimp. Haemolymph clotting time was measured according to Jussila et al. (2001).

Total haemolymph protein was determined spectrophotometrically based on the method of Lowry et al. (1951) using bovine serum albumin as a standard. The albumin and globulin content of the haemolymph was estimated by the method of Varley et al. (1980). Haemolymph glucose was estimated by the method of Sasaki et al. (1972). Creatinine in haemolymph was estimated by the method of Slot (1965). Results were compared using Duncan's Multiple Range test and differences were considered significant at $p< 0.05$.

Results

At 24h after intramuscular injection of viral inoculum, *P. monodon* developed clinical signs of white spot disease. The WSSV infection was confirmed by PCR (Fig. 1). The shrimp stopped feeding, became lethargic and showed a tendency to move towards the edges of tanks, near the surface. Signs included appearance of white circular inclusions or spots in the cuticle, with more pronounced spots occurring on the sides of the cephalothorax. A red discolouration also was seen all over the body. When clear white spots were developed, they varied in size from minute spots to large white plates. In a few cases, shrimp did not develop any white spots. However there was 100% mortality in 72-84h. In the normal saline-injected control shrimp, no morphological changes were observed and survival was

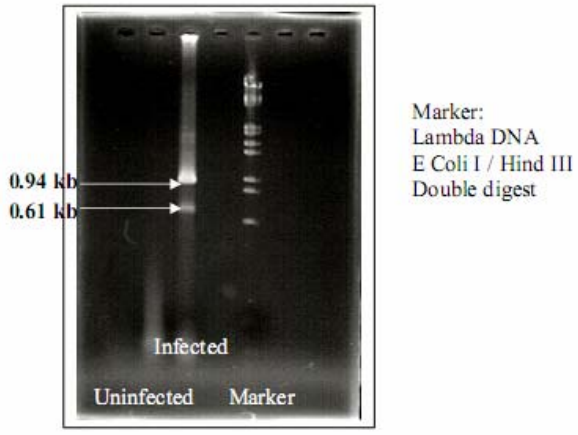

Figure 1. PCR Electrophoretic pattern of control and experimental *P.monodon* for confirmation of WSSV infection

100%. They were very active, showed no abnormality and behaved the same way as uninfected shrimps.

At 24h post injection, haemolymph lost its clotting ability, while haemolymph of control shrimp clotted within 160 to 170 sec. THC declined significantly by 45% at 24h and 76% at 48h post-viral injection (Table 1). No significant variation occurred in the THC in saline injected samples. Glucose content decreased rapidly ($p< 0.05$) 33% of its original value at 24h of infection (Table 1). Protein content of haemolymph decreased significantly ($p< 0.05$) by 25% at 24 h and 45% at 48 h (Table 1).Thereafter the rate of decrease was less. Creatinine also showed a significant decrease ($p< 0.05$) to about 35% of the original value at 24 h and about 12% at 48 h post injection (Table 1). The globulin content increased and albumin content decreased significantly ($p< 0.05$) in the haemolymph, thus reducing the albumin/globulin ratio (Table 1). The ratio was reduced to 49% of its original value at 48h. The control group of shrimps, did not exhibit any significant variations in protein, glucose and creatinine content. The same trend was observed in the control shrimps with regard to albumin/globulin ratio also.

Table 1. Variation in THC, heamolymph protein, glucose, creatinine and albumin/globulin ratio in haemolymph of *Penaeus monodon* during WSSV infection

	0h		24h		48h	
	Control	WSSV Infected	Control	WSSV Infected	Control	WSSV Infected
THC cells·ml^{-1}	50±2x10^{6a}	50±2x10^{6a}	50±2x10^{6a}	28±2x10^{6b}	50±2x10^{6a}	10±2x10^{6c}
Protein mg·ml^{-1}	83±1^{a}	83±1^{a}	82±1^{a}	62±1^{b}	81±1^{a}	46±1^{c}
Glucose mg·dl^{-1}	30±1^{a}	30±1^{a}	29±1^{a}	10±1^{b}	29±1	8±1^{c}
Creatinine mg·dl^{-1}	12±1^{a}	12±1^{a}	11±1^{a}	4±0.5^{b}	11±1	1±0.05^{c}
Albumin/ Globulin Ratio	0.55 ±0.05^{a}	0.55 ±0.05^{a}	0.52 ±0.05^{a}	0.35 ±0.03^{b}	0.52 ±0.05^{a}	0.25 ±0.01^{c}

Discussion

White spot syndrome virus (WSSV) has been causing havoc by producing devastating epidemics in Asia since 1988 (Primavera 1997). The

exact pathophysiology and biochemical alterations occurring in WSSV infection are still not clear. Compared to studies in vertebrates and other crustaceans, studies on economically important *P. monodon* in relation to its haematology in pathogenic condition are relatively scanty. The focus of the present study is to determine the alteration occurring in the haematology of WSSV-infected *P. monodon.*

The clinical signs and mortality occurred, and further detection of WSSV by PCR in the injected animals confirmed that injection of viral extract at the administered dosage could induce disease in the organism. This present observation concurs with previous reports of Sen et al. (1999) and Van Hulten et al. (2000), which have shown 80–100% mortality in *P. monodon* injected with purified WSSV.

In the present experiment, at 24h post injection, haemolymph lost its clotting ability, while haemolymph of control shrimp clotted within 160 to 170 sec**.** This is in accordance with an earlier reported study (Lightner and Lewis 1975). Clotting in crustaceans results from the action of a clot-initiating factor secreted by the haemocytes, which is a transglutaminase enzyme. This enzyme acts on the fibrinogen (coagulogen) present free in the haemolymph to produce the clot. In most infections, the haemocytes and hence the clot initiating factor decline much more rapidly than haemolymph proteins. As a result of the disappearance of the clot- initiating factor through the disappearance of the haemocytes, the clotting increases as observed in the present study.

In our study, the total haemolymph counts (including granular, semi granular and hyaline cells) in uninfected shrimp were around 50.0×10^6 cells·ml^{-1} and this is comparable to the value 50.9×10^6 cells·ml^{-1} reported by Sritunyalucksana and Soderhall (2000) for healthy *Penaeus monodon.* The THC drop in infected shrimp could be due to a number of factors. Earlier, Van de Braak (2002) has reported that haemocytes migrated to sites of infection, resulting in low THC. Prince (1997) noted lower levels of serum proteins and lower numbers of circulating haemocytes in the haemolymph of infected lobsters. In the present study, THC dropped to 76% of original value in 48h of infection. Stress and infection can reduce THC significantly. The reduction in THC in the present study during WSSV infection might be due to apoptosis of haemocytes, as a mechanism to tide over the viral infection.

Glucose, the major sugar in the circulating haemolymph in crustace has been reported to be 0.34 mg·ml^{-1} (Verri et al. 2001) in *P. monodon*. This corresponded well with the concentration of glucose (0.30 mg·ml^{-1})

obtained in our study for uninfected *P. monodon.* . It is interesting to note that in our study, shrimp survived, even when the glucose level declined to one third of its original level. Our findings correlate with investigation done by Quzon et al. (2000) who reported that crustaceans lack an efficient mechanism of glucose homeostasis and that they tolerate large variations in its haemolymph glucose concentration. The present observation of a reduction in haemolymph glucose level to 33% of the original value at 24h after injecting viral inoculum might be the result of an attempt of an organism to tide over stress and diseased state.

There was a significant decrease in plasma protein content (from 83 to 46 $mg \cdot ml^{-1}$) as a result of viral infection (Table 1). Sritunyalucksana and Soderhall (2000) reported a mean haemolymph plasma protein content of 79.9 $mg \cdot ml^{-1}$ and this corresponded with 83 $mg \cdot ml^{-1}$ of protein reported by us in healthy shrimp. Shrimp have high ability to use protein as a source of energy because they use gluconeogenesis to produce carbohydrates (Rosas et al. 2001). Since it was observed that shrimp stopped feeding after injection, energy from feed would not be available. Thus the low protein value in haemolymph might be the result of utilizing protein as an energy source during energy crisis. The energy requirements of shrimp during non-feeding periods were met by catabolizing nitrogenous substances such as proteins (Oliveira et al. 1997). Whole animal analysis of copepods revealed a significant decline in total proteins, lipids and carbohydrates during fasting (Claybrook 1983). Floreto et al. (2000) reported a decline in total protein values in haemolymph of crustaceans during starvation, stress and infection. The decrease in haemolymph protein content might be due to an energy crisis and other stress factors arising from viral infection.

Creatinine, a major nitrogenous waste in the circulatory system originates from the urea cycle or from the break down of creatine phosphate (Cheng and Cheng 1998). In the present investigation, creatinine showed a significant decrease ($p< 0.05$) to about 35% of the original value at 24h and about 12% at 48h post injection. During infection, the feeding rate was low and hence metabolic processes should have been reduced, resulting in less nitrogenous waste in the form of creatinine in the haemolymph. According to Takabataka et al. (1988) low levels of creatinine might result from decreased hepatic production because of severe hepatic damage or due to inadequate dietary protein. Our observations confirmed the findings of Takabataka et al. (1988) indicating severe hepatic damage due to viral infection. It is inferred that during viral infection, there could be hepatopancreatic damage especially of interstitial cells, resulting in low levels of creatinine.

In the present study, there was a significant reduction in the albumin/globulin ratio observed in WSSV-infected *P. monodon*. The albumin/globulin ratio in plasma is an indicator of health status (Chanutin et al. 1938). The low albumin/globulin ratio noted might be due to overproduction of γ globulin or due to low production of albumin. Significant decrease in albumin/globulin ratio is an indication of severe damage to the hepatopancreatic interstitial cells.

Conclusion

In conclusion, the results of the present investigation indicate that WSSV infection induced significant alterations in the haematology of *Penaeus monodon*. Our earlier observations indicated that the antioxidant defence system and energy status were operating at a lower rate in WSSV infected *P. monodon.* However further studies on finding permissible therapeutic alternatives possessing antioxidant, antiviral and cytoprotective properties to prevent WSSV infection have to be carried out in comparison with viral infection of vertebrates and other higher animals.

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