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Experimental transmission and tissue tropism of white spot syndrome virus (WSSV) in two species of lobsters, *Panulirus homarus* and *Panulirus ornatus*

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Abstract

The susceptibility of two species of lobsters, *Panulirus homarus* and *Panulirus ornatus* to white spot syndrome virus (WSSV) was tested by oral route and intramuscular injection. The results revealed that these lobsters were as highly susceptible as marine shrimp when the WSSV was administered intramuscularly. The WSSV caused 100% mortality in both *Panulirus homarus* and *Panulirus ornatus*, at 168 and 120 h, respectively, after intramuscular injection and failed to cause mortality when given orally. The presence of WSSV in moribund lobsters was confirmed by single-step and nested PCR, Western blot, histology, and bioassay test. It was found in eyestalk, gill, head muscle, tail muscle, hemolymph, appendages, and stomach. In lobsters with oral route infection, all tested organs except stomach and head muscle was negative for WSSV by nested PCR at 120 h post-inoculation. The stomach and head muscle was positive by nested PCR at 120 h p.i., but negative at 168 h p.i. Western blot analysis was negative in all the tested organs of both species of lobster at 120 h post-inoculation by oral route.

Keywords: *Panulirus*; White spot syndrome virus; Susceptibility; PCR; Western blot

1. Introduction

White spot syndrome is considered to be one of the most serious viral diseases of shrimp, and the causative organism has been identified as white spot syndrome virus (WSSV). This virus has caused the loss of several million dollars in shrimp culture industries in India and the loss continues even now (Anon., 1996). The WSSV has been isolated from *Penaeus monodon* and its morphology was studied (Takahashi et al., 1994; Wongteerasupaya et al., 1995; Lo et al., 1996; Sahul Hameed et al., 1998). WSSV has been found to be highly pathogenic to penaeid shrimp and has a wide host range that includes crabs, copepods and other arthropods. Forty-three species of arthropods have been reported as host or carriers of the WSSV either from culture facilities, the wild or experimental infection. Currently known hosts include the penaeid shrimp *P. monodon*, *P. indicus*, *P. japonicus*, *P. chinensis*, *P. penicillatus*, *P. semisulcatus*, *P. aztecus*, *P. vannamei*, *P. merguiensis*, *P. duorarum*, *P. stylirostris*, *Trachypenaeus curvirostris*, and *Metapenaeus ensis*, caridean shrimp *Exopalaemon orientalis* and *Macrobrachium rosenbergii* and crayfish, *Procambarus clarkii* (Cai et al., 1995; Chang et al., 1998; Lightner et al., 1998; Wang et al., 1998). WSSV has also been reported in crabs *Scylla serrata*, *Portunus pelagicus*, *Portunu sanguinolentus*, *Charybdis* sp., *Helice tridens*, lobsters *Panulirus* sp., planktonic copepods and pupae of an *Ephydridae* insect by PCR, in situ hybridization or immunological methods (Huang et al., 1995; Lo et al., 1996; Chang et al., 1998; Wang et al., 1998; Chen et al., 2000). Studies on WSSV pathogenicity for juveniles and adults of *M. idell*a, *M. lamerrae*, and *M. rosenbergii* indicated that *M. idella* and *M. lamerrae* have been found to be susceptible, whereas *M. rosenbergii* was resistant (Sahul Hameed et al., 2000). Experimental

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infection of WSSV has been demonstrated in freshwater crabs, 20 species of marine crabs and *Artemia* sp. (Sahul Hameed et al., 2001, 2002, 2003). The present work was carried out to examine the infectivity and pathogenicity of the WSSV to two species of lobsters, *Panulirus homarus* and *Panulirus ornatus*, by two routes namely intramuscular injection and oral route. We also examined the target organs of WSSV in these lobsters by PCR, Western blot, and histology to determine whether these target organs are the same as in marine shrimp and possibility of their acting as carriers or reservoir of WSSV to marine shrimp.

2. Materials and methods

2.1. Collection and maintenance of experimental animals

Two species of lobsters, *Panulirus homarus* and *Panulirus ornatus*, were collected from fish-landing centers at Chennai along the east coast of India. Taxonomic identification of these lobsters was carried out based on the manual published by FAO, Rome (Carpenter and Niem, 1998). The lobsters (125–150 g body weight) were transported to the laboratory and acclimated in separate 500 L aquaria (10 lobsters per aquarium, 30–34 ppt, 27–30 °C) for 5 days prior to the onset of experiment. They were fed minced fresh fish ad lib. Marine shrimp, *Penaeus monodon* (10–15 g body weight) were collected from grow-out ponds or the sea and maintained in a 500 L aquaria (30 shrimp per aquarium, 20–25 ppt, 27–30 °C) for 5 days prior to experiment. The shrimp were fed with commercial pellet feed. Saltwater was pumped from the adjacent sea and allowed to sediment, thus removing sand and other particulate matter before use for shrimp and lobster. Five per species were randomly selected and screened for WSSV using appendages by PCR with the primers designed by Sahul Hameed et al. (2003).

2.2. Preparation of viral inoculum

WSSV-infected shrimp *Penaeus monodon* with prominent white spots were collected from shrimp farms and hemolymph was drawn directly from the heart using sterile syringes. The hemolymph was centrifuged at 3000*g* for 20 min at 4 °C, after which the supernatant fluid was recentrifuged at 8000*g* for 30 min at 4 °C. The final supernatant fluid was then filtered through a 0.4 μm filter before storage at −20 °C, until used in infectivity experiments. Before storage, the total protein content was determined by the method of Lowry et al. (1951) and the presence of WSSV was determined by PCR as mentioned above (Sahul Hameed et al., 2003).

2.3. Infectivity experiments

The pathogenicity of WSSV was tested by intramuscular injection of the hemolymph filtrate described above and oral route by feeding of WSSV-infected shrimp meat.

For injection method, the lobsters (five/group/tank) maintained in tanks at room temperature with salinity 30–34 ppt were challenged by injecting hemolymph filtrate (300 μg of total protein/animal) into soft tissue at the base of walking legs. Marine shrimp (10/group/tank) were maintained in tanks at room temperature with salinity ranging between 20 and 25 ppt and inoculated intramuscularly at the third abdominal segment with hemolymph filtrate at the dose of 300 μg of total protein/animal. Control animals were inoculated with hemolymph collected from uninfected shrimp. Each trial was conducted in triplicate.

For oral infection, five lobsters (*Panulirus homarus* or *Panulirus ornatus*), or 10 *Penaeus monodon* were placed separately in a 100 L aquarium tanks and starved for 24 h. They were then fed with meat of WSSV-infected shrimp showing prominent white spots at the rate of 5% fresh body weight/day. The shrimp meat was divided into three portions and given at interval of 8 h for 3 days. After the final feeding, the lobsters were fed with minced fresh fish, and shrimp were fed with commercial pellet feed. In the control groups, the animals were fed with uninfected shrimp meat followed by normal diet as mentioned above. Each experiment was conducted in triplicate.

In all the experiments, the animals were examined twice per day for gross signs of infection, and the number of dead animals was recorded so that cumulative percentage mortality could be recorded.

2.4. Confirmation of WSSV infection

WSSV infection was confirmed by the PCR technique, Western blot analysis, bioassay or by histological examination of ectodermal and mesodermal organs for the presence of lesions characteristic of WSS (Lightner, 1996). Template DNA for PCR tests was prepared from experimental animals by extraction from eyestalks, gill, head muscle, tail muscle, hemolymph, appendages, and stomach following the method described by Lo et al. (1996). Hemolymph was drawn directly from the heart. The organs to be tested were removed and homogenized separately. Hemolymph or homogenized tissue samples were centrifuged at 3000*g* at 4 °C, after which the supernatant fluid was placed in another centrifuge tube together with an appropriate amount of digestion buffer 100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 50 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulfate, and 0.1 mg ml^{-1} proteinase K. After incubation at 65 °C for 2 h, the digest was deproteinized by successive phenol/chloroform/isoamyl alcohol extraction, recovered by ethanol precipitation, and dried; the dried DNA pellet was resuspended in TE buffer. Single-step and nested PCR were used to confirm the WSSV infection status. For single-step PCR, the primers designed by Tapay et al. (1999) to amplify a 211-bp sequence of WSSV-DNA were used. The reaction mixture contained 2 μl of template DNA, 1 μM of each primer, 200 μM of deoxynucleotide triphosphate, and 1.25 U of *Taq* DNA polymerase in PCR buffers supplied with a commercially available kit (Finnzymes, Espoo,

Finland). The PCR protocol comprised 35 cycles of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C with a final extension of 5 min at 72 °C. For nested PCR, the above-mentioned primers were used as external primers. Internal primers designed in our laboratory based on the sequence described by Tapay et al. (1999) to amplify 155-bp sequence of WSSV-DNA were used. The sequence of the internal primers is: 5′-GAG AGA TGC ATA ATT CTA GTA GAG G-3′ and 5′-ACG GCA AAA ACC AAA CAA TCA TCA-3′. The primary product was 211 bp and the nested PCR product was 155 bp. PCR products were analysed by electrophoresis in 1.2% agarose gels stained with ethidium bromide and visualized by ultraviolet transillumination.

For histological observations gill tissue was cut and preserved in Davidson's fixative for 48 h and then transferred to 70% alcohol for subsequent histological preparation (Bell and Lightner, 1988). Sections of 4–5 μm in thickness were stained with haematoxylin and eosin.

Western blot analysis was done to detect the WSSV in infected shrimp by the method of Talbot et al. (1984). After separation on SDS–PAGE the normal and infected samples were transferred to nitrocellulose paper (NCP) at 300 mA for 2 h at 4 °C. After transfer, the NCP was blocked for 1 h with 3% skimmed milk in PBS. The NCP was incubated in primary antiserum raised against VP28 protein of WSSV (Yoganandhan et al., 2004) at a dilution of 1/10,000 in PBS containing 0.2% BSA for 3 h. Subsequently, the membrane was then incubated in ALP-conjugated goat anti-rabbit IgG (Sigma) for 1 h and VP28 was detected with a substrate solution of 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma) in substrate buffer.

Bioassay tests were carried out in *Penaeus monodon* by injecting the hemolymph collected from experimentally WSSV-injected lobsters and shrimp.

3. Results and discussion

The cumulative percent mortalities for lobsters (*Panulirus homarus* and *Panulirus ornatus*) and shrimp (*Penaeus monodon*) are presented in Table 1. The results showed that the lobsters were found to be susceptible to WSSV, like shrimp. The WSSV caused 100% mortality in *Panulirus homarus* and *Panulirus ornatus* at 168 and 120 h, respectively, by intramuscular injection, but failed to cause mortality in these two species of lobsters when the virus was administered orally in the experimental period of 50 days. The WSSV caused 100% mortality in *Penaeus monodon* at 48 h after intramuscular injection and at 120 h by oral infection. No mortalities were observed in all the control groups during the period of experimental study.

The gross signs of disease observed were lethargy and lack of appetite, together with dark coloration on the dorsal side, reduced swimming activity, and lack of movement in case of lobsters and shrimp. The white spots, typical symptom of white spot syndrome, were not observed in lobster. Orally infected lobsters appeared normal and no mortality was observed.

Lobsters (*Panulirus homarus* and *Panulirus ornatus*) challenged with intramuscular injection of WSSV were positive of all tested organs by one-step PCR (Fig. 1). Western blot analysis using antiserum to r-VP28 with WSSV-injected lobsters gave positive in all tested organs (Fig. 2). In lobsters with oral infection, all tested organs except stomach and head muscle was negative by nested PCR at 120 h post-inoculation (Table 2). The stomach and head muscle was positive by nested PCR at 120 h p.i., but negative at 168 h p.i. (Table 2). Western blot analysis was negative in all the tested organs of both species of lobster at 120 h post-inoculation by oral route (Table 2). Histological observation in gill tissue of moribund lobsters injected with WSSV revealed degenerated cells characterized by hypertrophied nuclei with basophilic intranuclear inclusion bodies in tissues of ectodermal and mesodermal origin (Fig. 3). Hemolymph collected from moribund lobsters injected with WSSV caused 100% mortality in *Penaeus monodon* in 48 h.

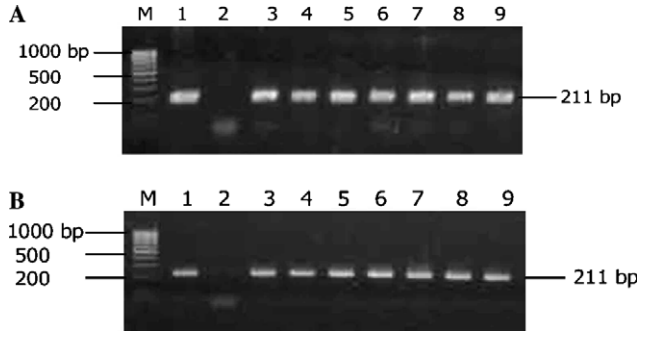

Fig. 1. Detection of WSSV-DNA in the experimentally WSSV-infected lobsters (A, *Panulirus homarus*; B, *Panulirus ornatus*). Lane M, marker; lane 1, positive control; lane 2, normal lobsters; lane 3, eyestalk; lane 4, gills; lane 5, head muscle; lane 6, tail muscle; lane 7, hemolymph; lane 8, appendages; lane 9, stomach.

Table 1
Cumulative percent mortality of lobsters and shrimp at different time intervals after intramuscular injection of WSSV, and results of PCR and Western blot analysis

Species	Hours to 100% mortality by injection	PCR test						
		E	G	H	T	HL	A	S
Panulirus homarus	168	+/w	+/w	+/w	+/w	+/w	+/w	+/w
Panulirus ornatus	120	+/w	+/w	+/w	+/w	+/w	+/w	+/w
Penaeus monodon	48	+/w	+/w	+/w	+/w	+/w	+/w	+/w

E, eyestalk; G, gill; H, head muscle; T, tail tissue; HL, hemolymph; A, appendages; S, stomach; +, WSSV PCR positive; w, Western blot WSSV positive.

Fig. 2. Detection of WSSV by Western blot analysis using r-VP28 antiserum in different organs of WSSV-infected lobsters (A, *Panulirus homarus*; B, *Panulirus ornatus*). Lane M, marker; lane 1, positive control; lane 2, normal lobsters; lane 3, eyestalk; lane 4, gills; lane 5, head muscle; lane 6, tail muscle; lane 7, appendages.

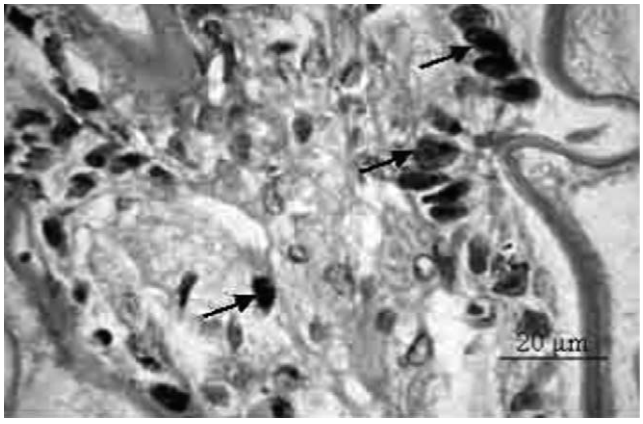

Fig. 3. Photomicrograph of WSSV-infected gill tissue of lobster, *Panulirus homarus*. Note hypertrophied nuclei in the cells (arrow) (H&E). 40× magnification.

In *Penaeus monodon*, all tested organs from either infection routes were positive by one-step PCR, Western blot, and histology. The susceptibility of *M. idella*, *M. lamerrae*, and *M. rosenbergii* to WSSV has been tested by immersion challenge, oral route, and intramuscular injection, and the results indicate the susceptibility of *M. idella* and *M. lamerrae* to this virus but not to *M. rosenbergii* (Sahul Hameed et al., 2000). This virus failed to produce mortality with any of the methods of infection applied in *M. rosenbergii*.

WSSV has a wide host range and is a highly pathogenic virus that is transmitted to cultured shrimp via contaminated water and ingestion of WSSV-infected animals (Supamattaya and Boonyaratpalin, 1996). Transmission is also possible through cohabitation of infected species with uninfected stocks in both the shrimp farming environment and the wild (Flegel, 1997; Flegel and Alday-Sanz, 1998). The mortality data, together with PCR tests, Western blot analysis, histological observations, and bioassay test presented here, confirmed that two species of lobsters were found to be susceptible to WSSV when it was administered intramuscularly but failed to cause mortality by oral route. The histological observations in WSSV-injected lobsters revealed typical lesions (hypertrophied nuclei with intranuclear inclusion bodies) as described by various workers in shrimp (Wongteerasupaya et al., 1995; Lightner, 1996; Wang et al., 1997; Yoganandhan et al., 2003) in cells of ectodermal and mesodermal organs such as heart tissue, head tissue, eyestalk, gills, tail muscle, and appendages. In addition, the presence of WSSV in these organs was confirmed by single-step and nested PCR, and Western blot analysis suggests a systemic infection in these lobsters, like marine shrimp. This virus failed to cause mortality by oral route and was negative by all diagnostic methods employed in all the organs except stomach and head muscle. These organs were positive by nested PCR at 120 h p.i. and became WSSV-negative at 168 h p.i, showed the resistance of orally infected lobsters to WSSV. Snieszko and Taylor (1947) were unable to infect American lobsters with *Gaffkya homari* introduced with the food, but succeeded in transmitting gaffkemia disease to healthy lobsters by injection of bacteria. It was later found that gaffkemia is transmitted only through ruptures in the integument and not through the consumption of infected food (Stewart and Rabin, 1970). This may also be the case of WSSV infection in these lobsters. Different organs of orally infected lobsters were screened for WSSV by nested PCR and Western blot analyses and the results showed that all the organs tested were found to be negative for WSSV. This indicates that the lobsters were resistant to WSSV by oral route, but not by injection. This might be the reason for not reporting any natural WSSV infection in lobsters. Ingestion of WSSV-

Table 2
Detection of WSSV by using PCR and Western blot to analyse WSSV in different organs of survived lobsters (*Panulirus homarus* and *Panulirus ornatus*) at different time intervals (h) after oral challenge

h	*Panulirus homarus*							*Panulirus ornatus*						
	E	G	H	T	A	S	I	E	G	H	T	A	S	I
24	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+
36	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+
72	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+	+/w+
96	−/−	−/−	+/w+	−/−	−/−	+/w+	−/−	−/−	−/−	+/w+	−/−	−/−	+/w+	−/−
120	−/−	−/−	++/w−	−/−	−/−	++/w−	−/−	−/−	−/−	++/w−	−/−	−/−	++/w−	−/−
144	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
168	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−

E, eyestalk; G, gill; H, head muscle; T, tail tissue; A, appendages; S, stomach; I, intestine; +, WSSV PCR positive; ++, WSSV nested PCR positive; w+, Western blot WSSV positive; w−, Western blot WSSV negative; −, PCR and Western blot negative.

infected tissue by shrimp is probably one of the principal infection routes of this virus, in both natural and farm environments. Failure of infection by oral route in lobsters and absence of WSSV in vital organs raises doubts about the possibility of these lobsters acting as reservoir or carriers of WSSV to shrimp in marine environment.

The WSSV caused 100% mortality in marine shrimp by either infection routes giving positive results by PCR and Western blot in all the organs tested (hemolymph, head tissue, eyestalk, gills, tail muscle, stomach, and appendages). The presence of WSSV in these vital organs was found to be responsible for failure of vital functions such as clotting of hemolymph, defense mechanism, exchange of respiratory gas, and excretion in WSSV-infected shrimp (Yoganandhan et al., 2003). The high mortality rate and distribution of this virus in all the vital organs of marine shrimps infected by either route indicates that it is more susceptible to WSSV than lobster. The vital organs of orally infected lobsters was negative by nested PCR and Western blot analysis. This indicates the failure of WSSV to infect lobster by oral route. Pathogenicity experiments carried out on *M. rosenbergii* indicate that the WSSV failed to produce mortality by the methods of immersion, injection, and oral routes (Sahul Hameed et al., 2000). The exact mechanism of tolerance to WSSV is not known in *M. rosenbergii* and also in orally infected lobsters, but resistance in some invertebrates includes the production of bactericidins, lysins, and agglutinins. These factors in certain invertebrates following exposure to foreign protein may account in part for increased resistance to certain pathogens (Bang, 1967; Mckay and Jenkin, 1969). This might be the reason for the resistance of orally infected lobsters to WSSV. Factor and Beekman (1990) have reported the fixed phagocytes in digestive system of American lobster, *Homarus americanus*, which are responsible for removing two types of non-biogenic, foreign particles (carbon particles and latex beads) that were injected into the blood. These cells play an important role in the cell-mediated immunity of lobsters. This might be true in the case of orally WSSV-infected lobsters. *Penaeid* shrimp lack fixed phagocytes in hemal spaces of digestive system (Martin et al., 1993) and this might be the reason for successful WSSV infection through oral route in shrimp. Based on the results of the present study and previous reports, responses of host to WSSV can be divided into three types. Type 1 hosts which includes marine shrimp are an acute infection with high mortality and highly susceptible to WSSV by oral route and intramuscular injection Type 2 hosts which includes lobsters are partially susceptible and resistant to WSSV by oral route. Type 3 hosts like *M. rosenbergii* and some marine crabs are resistant to WSSV by any method of infection. Further work needs to be carried out to determine the mechanism of resistance in the lobsters.

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