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Experimental host range and histopathology of white spot syndrome virus (WSSV) infection in shrimp, prawns, crabs and lobsters from India

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Abstract

Experimental studies were conducted by injecting or feeding white spot syndrome virus (WSSV) derived from infected shrimp, *Penaeus monodon* (Fabricius), collected from the south-east coast of India, to five species of shrimp, two species of freshwater prawns, four species of crabs and three species of lobsters. All species examined were susceptible to the virus. Experimental infections in the shrimp had the same clinical symptoms and histopathological characteristics as in naturally infected *P. monodon*. A cumulative mortality of 100% was observed within 5–7 days in shrimp injected with WSSV and 7–9 days in shrimp fed with infected tissue. Two species of mud crab, *Scylla* sp., survived the infection for 30 days without any clinical symptoms. All three species of lobsters, *Panulirus* sp., and the freshwater prawn, *Macrobrachium rosenbergii* (De Man), survived the infection for 70 days without clinical symptoms. However, bioassay and histology using healthy *P. monodon* revealed that crabs, prawns and lobsters may act as asymptomatic carriers/reservoir hosts of WSSV. This is the first report to suggest the carrier/reservoir capacity of these hosts through histological and bioassay evidences. Ultrastructural details of the virus in experimentally infected shrimp, *P. vannamei*, (Boone), were also studied.

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Introduction

White spot syndrome virus infection, with its associated mortality, is emerging as one of the most challenging problems for the global shrimp industry, especially in Asia and south-east Asia (Inouye, Miwa, Oseko, Nakano, Kimura, Momoyama & Hiraoka 1994; Nakano, Koube, Umezewa, Momoyama, Hiraoka, Inouye & Oseko 1994; Takahashi, Itami, Kondo, Maeda, Fiji, Tomonaga, Supamattaya & Boonyaratpalin 1994; Chen 1995; Chou, Huang, Wang, Chiang & Lo 1995; Wang, Lo, Leu, Chou, Yeh, Chou, Tung, Chang, Su & Kou 1995; Wongteerasupaya, Vickers, Sriurairatana, Nash, Akarajamorn, Boonsaeng, Panyim, Tassanakajon, Withyachumnarnkul & Flegel 1995). The causative viral pathogen has been named differently by various workers (Huang, Song, Yu & Yang 1994; Takahashi *et al.* 1994; Wang *et al.* 1995; Wongteerasupaya *et al.* 1995; Lo, Ho, Peng, Chen, Hsu, Chiu, Chang, Liu, Su, Wang & Kou 1996). However, Lightner (1996) has regrouped all the members of these non-occluded viruses under the name white spot syndrome (WSS). Accordingly, the viral pathogen is called white spot syndrome virus (WSSV) in the present paper.

Although this disease was reported in India in early 1993, it was only in 1994 that adequate attention was paid to it as a consequence of its increased severity and large-scale mortality. In 1995, the causative organism of the epizootic was identified for the first time as SEMBV (Anonymous 1995). Thereafter, three reports were published on this viral epizootic from India (Manohar, Sundararaj, Selvaraj, Sheela, Chidambaram, Mohan &

Ravishankar 1996; Karunasagar, Otta & Karunasagar 1997; Rajendran, Vijayan, Alavandi, Rangaswamy, Srinivasagam & Rajababu 1998). The above reports were limited to information relating outbreaks of the disease in cultured shrimp, *P. monodon* and *P. indicus* (Edwards). However, the susceptibility of other species of shrimp and the role of other crustaceans in the epizootiology of Indian WSSV have not been investigated.

The susceptibility of five species of shrimp, two species of freshwater prawns, four species of crabs and three species of lobsters (all from India) to WSSV was investigated in the present study. The associated histological and morphological manifestation, the carrier/reservoir potential of experimental animals, and the ultrastructural details of the virus in experimentally infected shrimp were also analysed.

Materials and methods

Experimental animals

The penaeid shrimp, *Penaeus monodon* and *P. indicus*, were collected from a farm which stocked hatchery-reared postlarvae with no history of WSSV infection. *Penaeus semisulcatus* (De Hann) were collected from a hatchery and reared in the laboratory to 2–5 g in size. *Metapenaeus monoceros* (Fabricius) and *M. dobsoni* (Miers) were collected from the wild. Mudcrabs, *Scylla serrata* (Forskal) and *S. tranquebarica* (Fabricius), were collected from Pulikat lake, a brackishwater lake in Chennai, India, and other species of crabs, *Metapograpsus* sp. and *Sesarma* sp., were obtained from lagoons in Muttukkadu, Chennai. Freshwater prawns. *Macrobrachium rosenbergii*, were procured from a private hatchery, and all species of lobsters, *Panulirus homarus* (Linnaeus), *P. ornatus* (Fabricius) and *P. polyphagus* (Herbst), were collected from the Bay of Bengal off the Chennai coast. The animals used in the experiments are listed in Tables 1–4. All the experimental animals were observed for one week in a clean laboratory environment for clinical symptoms of white spot syndrome virus (WSSV) infection. Additionally, representative samples of these animals were subjected to histological studies to examine WSSV specific pathology (Lightner 1996).

Experimental and control animals were maintained in vertical, 100-L fibreglass tanks, which were kept in an isolated wet laboratory at an ambient temperature of 30–32 °C. Water salinity was maintained at 25–30$^{0}/_{00}$. The prawns were reared in fresh water. The number of animals kept in a single tank varied according to the size and species of the hosts. There were duplicated experimental tanks for all groups in addition to the non-exposed controls. The control groups were kept isolated from the experimental sets. Except for the days of administration of the infected tissues *per os*, dietary food for the shrimp and prawns consisted of commercial pellets fed *ad libitum*. Crabs and lobsters were fed with fresh clam meat *ad libitum*. All the experimental and control animals were examined four to five times a day to establish their health status. Samples of experimental animals were collected periodically for bioassay and pathological observation.

Experimental infection studies

White-spot-syndrome-infected *P. monodon* were collected from two farms at Nellore, Andhra Pradesh, on the south-east coast of India, during a widespread epizootic during the last quarter of 1996. The infected material which was collected was frozen and kept at –70 °C until use. Before checking the susceptibility of the experimental animals, the frozen material was used for testing the virulence using healthy *P. monodon* and was found to produce consistent histopathological alterations and mortality. The gills, stomach and epidermal layer collected from frozen diseased shrimp were used for challenge trials according to the methods followed by Takahashi *et al.* (1994), with slight modifications. The infected tissues were homogenized using a manual glass homogenizer (BOROSIL, India) in sterile marine phosphate buffered saline (PBS) (1 g of tissue material in 1 mL PBS) and centrifuged at 2000 r.p.m. for 10 min at 4 °C. The supernatant was filtered through a 0.22-μm membrane filter fitted to a syringe. This virus containing supernatant was diluted to one part of filtrate to 10 parts of PBS, and injected intramuscularly into healthy shrimp, prawns, crabs and lobsters [0.1% (v/w) body weight]. Sterile PBS was injected into the negative controls. In all injection treatments, a single injection was given to the experimental and control animals. In the case of *per os* infection, experimental animals were fed with diced infected material on the basis of 8–10% body weight. The feeding rates for the experimental animals are presented in Tables 1–4.

Table 1 Details of the experimental shrimp, mortality pattern, histopathology and bioassay

Species	Mode of infection*	Day-wise mortality										Cumulative mortality (%)	Histopathology	Bioassay
		0	1	2	3	4	5	6	7	8	9			
Penaeus monodon (*n* = 40; 8–10 g)	IM	0	0	8	10	7	5	5				100	+	+
Control (*n* = 10; 10–12 g)	IM	0	0	0	0	0	0	0				0	–	–
P. monodon (*n* = 40; 6–8 g)	OF	0	0	0	5	8	7	5	6	4		100	+	+
Control (*n* = 10; 6–8 g)	OF	0	0	0	0	0	0	0	0			0	–	–
P. indicus (*n* = 40; 8–10 g)	IM	0	3	10	8	9	5					100	+	+
Control (*n* = 10; 8–10 g)	IM	0	0	0	0	0	0	0				0	–	–
P. indicus (*n* = 40; 8–10 g)	OF	0	0	0	4	7	5	8	5	6		100	+	+
Control (*n* = 10; 8–10 g)	OF	0	0	0	0	0	0	0	0	0		0	–	–
P. semisulcatus (*n* = 30; 2–5 g)	OF	0	0	4	3	9	3	4	2			100	+	+
Control (*n* = 10; 2–5 g)	OF	0	0	0	0	0	0	0	0			0	–	–
Metapenaeus monoceros (*n* = 40; 8–10 g)	IM	0	0	0	5	8	1	0	7	5		100	+	+
Control (*n* = 10; 8–10 g)	IM	0	0	0	0	0	0	0	0	0		0	–	–
M. monoceros (*n* = 30; 8–10 g)	OF	0	0	0	0	3	2	4	7	5	4	100	+	+
Control (*n* = 10; 8–10 g)	OF	0	0	0	0	0	0	0	0	0	0	0	–	–
M. dobsoni (*n* = 25; 2–4 g)	OF	0	0	1	4	1	5	7	2			100	+	+
Control (*n* = 10; 2–4 g)	OF	0	0	0	0	0	0	0	0			0	–	–

*Key: (IM) intramuscular injection; and (OF) oral feeding (single).

Cross-infection experiments

Challenge bioassay trials were carried out to study the dormant infection/carrier capacity of experimentally infected prawns, crabs and lobsters. A representative sample (two animals of each species) of the experimentally infected prawns, crabs and lobsters were sacrificed at weekly/fortnightly intervals. The gills and foregut of these animals were minced and fed twice a day to healthy *P. monodon* (10–15 g in size). Similarly, tissues from experimentally infected shrimp were also subjected to bioassay using healthy *P. monodon*.

Histopathology

The gills, foregut, epidermis, lymphoid organ, eye stalk and muscles from the control and experimental animals were used for histopathological studies. In the case of prawns, crabs and lobsters, two animals of each species were sacrificed after one week of infection and subjected to histological analysis. Samples of shrimp (two animals of each species) were taken after 2 days of infection. Tissues from these animals were fixed in Davidson's fixative, processed, embedded, sectioned (thickness = 5–6 μm), and stained with haematoxylin and eosin, according to the procedure outlined by Bell & Lightner (1988) and Lightner (1996). The tissues were examined for histomorphological changes.

Electronmicroscopy

Ultrastructural studies using WSSV collected from *P. monodon* in India were conducted by examining

Table 2 Details of experimental freshwater prawns, mortality pattern, histopathology and bioassay

Species	Mode of infection*	Day-wise mortality											Total time of experiment (days)	Cumulative mortality (%)	Histopathology	Bioassay
		0	1	2	3	4	5	6	7	8	9	10				
Macrobrachium rosenbergii (*n* = 20; 30–40 g)	IM	0	0	0	0	1	0	0	0	0	0	2	70	20	+	+
Control (*n* = 10; 30–40 g)	IM	0	0	0	0	0	0	0	0	0	0	0	70	0	–	–
M. rosenbergii (*n* = 20; 30–40 g)	OF	0	0	0	0	0	0	1	0	0	0	0	70	10	+	+
Control (*n* = 10; 30–40 g)	OF	0	0	0	0	0	0	0	0	0	0	0	70	0	–	–
M. idella (*n* = 20; 3–5 g)	OF	0	0	0	0	0	0	0	0	0	0	0	20	10	+	+
Control (*n* = 10; 3–5 g)	OF	0	0	0	0	0	0	0	0	0	0	0	20	0	–	–

*Key: (IM) intramuscular injection; and (OF) oral feeding (single).

Table 3 Details of experimental crabs, mortality pattern, histopathology and bioassay

Species	Mode of infection*	Day-wise mortality 0	1	2	3	4	5	6	7	8	9	10	Total time of experiment (days)	Cumulative mortality (%)	Histopathology	Bioassay
Scylla serrata (*n* = 10; 80–110 g)	IM	0	0	0	1	0	0	0	0	0	0	0	30	30	+	+
Control (*n* = 5; 80–110 g)	IM	0	0	0	0	0	0	0	0	0	0	0	30	0	–	–
S. serrata (*n* = 10; 80–110 g)	OF	0	0	0	0	0	0	0	0	0	0	0	30	10	+	+
Control (*n* = 5; 80–110 g)	OF	0	0	0	0	0	0	0	0	0	0	0	30	0	–	–
S. serrata (*n* = 10; 80–110 g)	OFD	0	0	0	1	0	0	0	0	0	1	0	30	20	+	+
Control (*n* = 5; 80–110 g)	OFD	0	0	0	0	0	0	0	0	0	0	0	30	0	–	–
S. tranquebarica (*n* = 20; 100–110 g)	IM	0	0	0	0	1	0	0	0	0	0	1	30	20	+	+
Control (*n* = 5; 100–110 g)	IM	0	0	0	0	0	0	0	0	0	0	0	30	0	–	–
S. tranquebarica (*n* = 20; 100–110 g)	OFD	0	0	0	0	0	0	0	0	0	0	0	30	10	+	+
Control (*n* = 5; 100–110 g)	OFD	0	0	0	0	0	0	0	0	0	0	0	30	0	–	–
Metapograpsus sp. (*n* = 20; 2–5 g)	OF	0	0	0	0	0	6	0	2	0	2	1	15	100	+	+
Control (*n* = 10; 2–5 g)	OF	0	0	0	0	0	0	1	0	0	1	0	15	20	–	–
Sesarma sp. (*n* = 20; 4–10 g)	OF	0	0	1	0	0	0	5	0	3	1	0	15	100	+	+
Control (*n* = 5; 4–10 g)	OF	0	0	0	0	1	0	0	0	0	0	0	15	20	–	–

*Key: (IM) intramuscular injection; (OF) oral feeding (single); and (OFD) oral feeding (daily for one week).

the foreguts of experimentally infected *P. vannamei*. Tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer with 0.6 M sucrose, post-fixed in 1% osmium tetroxide, stained *en bloc* in 1% uranyl acetate and embedded in Spurr's epoxy resin (Spurr 1969). Ultrathin sections were cut on a Reichert–Jung Ultracut-E ultratome and stained with lead citrate solution (Reynolds 1963). The sections were examined with a JEOL-JEM 100SX transmission electron microscope. This ultrastructural study was carried out at the Institute of Marine Sciences, Gulf Coast Research Laboratory, Ocean Springs, MS, USA.

Results

The details of the experimental animals, the mortality pattern, histopathology and bioassay are listed in Tables 1–4. All the shrimp, prawn, crab and lobster species used in the experiment were found to be susceptible to WSSV. All the experimentally infected shrimp species exhibited marked white spots or patches on the carapace, and showed a similar mortality pattern and clinical symptoms as naturally infected animals(Wongteerasupaya *et al.* 1995; Wang, Tang, Kou & Chen 1997). The mortality pattern of experimentally infected shrimp varied with the method of disease induction. Mortality was observed within 2–3 days, when the pathogen was administered intramuscularly, and cumulative mortality reached 100% in 5–7 days. In the case of oral infection, mortality was observed at 2–4 days and cumulative mortality reached 100% in 7–9 days. Bioassay using fresh tissues of all the experimentally infected shrimp species produced the characteristic clinical symptoms of WSSV in *P. monodon*.

Macrobrachium rosenbergii, which were administered the infection intramuscularly, recorded a cumulative mortality of 20% during 70 days of

Table 4 Details of experimental lobsters, mortality pattern, histopathology and bioassay

Species	Mode of infection*	Day-wise mortality 0	1	2	3	4	5	6	7	8	9	10	Total time of experiment (days)	Cumulative mortality (%)	Histopathology	Bioassay
Panulirus homarus (*n* = 6; 100–150 g)	IM	0	0	0	0	0	0	1	0	0	0	0	70	33.2	+	+
Control (*n* = 3; 100–150 g)	IM	0	0	0	0	0	0	0	0	0	0	0	70	0	–	–
P. ornatus (*n* = 6; 80–110 g)	OFD	0	0	0	0	0	0	0	0	0	1	0	70	16.6	+	+
Control (*n* = 3; 80–110 g)	OFD	0	0	0	0	0	0	0	0	0	0	0	70	0	–	–
P. polyphagus (*n* = 6; 80–100 g)	OFD	0	0	0	0	1	0	0	0	0	0	0	70	16.6	+	+
Control (*n* = 3; 80–100 g)	OFD	0	0	0	0	0	0	0	0	0	0	0	70	0	–	–

*Key: (IM) intramuscular injection; and (OFD) oral feeding (daily for one week).

the experimental period. In the feeding experiment, the cumulative mortality was found to be 10%. The surviving animals did not show any manifestation of the disease except for the tiny spots on the carapace under microscopic examination. In the case of *M. idella* (Hilgendorf), cumulative mortality reached 10% in the experimental period of 20 days. Both the species showed tiny spots on the carapace upon examination under a microscope. The surviving prawns exhibited normal activity and feeding. A challenge bioassay in *P. monodon* using experimentally infected prawn tissue produced WSSV infection.

Out of the 10 mud crabs injected with filtrate, the cumulative mortality was found to be 30% in 30 days of experiment. *Scylla serrata* which were orally infected showed 10% and 20% mortality in 30 days. The remaining animals in the experiments showed no signs of the disease at termination of the experiment after 30 days. Experiments with *S. tranquebarica* also showed similar results, and in both cases, none of the experimental crabs showed any white spots on the carapace. The gills and stomach tissues of experimentally infected crabs exhibited a remarkable degree of infectivity in the cross-infection trials using *P. monodon*, and the shrimp showed all the clinical symptoms and associated mortality at 2–3 days post-infection. Other species of crabs, i.e. *Metapograpsus* sp. and *Sesarma* sp., were also found to be susceptible to WSSV. These crabs were infected through the oral route and all these experimental animals died within 15 days. Bioassays using the fresh carcasses of the experimental animals were found to be positive.

All three species of lobsters used in the present experiments were found susceptible to WSSV. *Panulirus homarus*, which received an intramuscular injection with the filtrate, showed a cumulative mortality of 33.2% after 70 days. In the case of *P. ornatus* and *P. polyphagus*, which were infected by the oral route, 16.6% showed cumulative mortality within 70 days. The remaining animals appeared healthy at the termination of the experiment. White spots could not be detected on the carapace of any of these animals. Bioassay using gills and foregut tissues of experimental animals gave positive results in 2–4 days.

In all species of shrimp, marked histomorphological changes characteristic of WSSV have been observed in infected tissues of ectodermal and mesodermal origin. Furthermore, all the experimentally infected animals showed characteristic pathological manifestations similar to those in naturally infected *P. monodon* (Figs 1–8).

The histological manifestations in the gills and foregut tissues of experimentally infected prawns, crabs and lobsters revealed a marked similarity to that of WSSV infection in shrimp. Gill lamellae showed acute degeneration, marked hypertrophy of nuclei with chromatin margination and karyorrhexis along with intranuclear inclusions (Figs 1, 3, 5 & 7). Haemocytes found in the gill lamellae and other tissues also showed marked hypertrophy. The cuticular ectodermal layer of gut showed a large number of prominent, deeply stained, variably sized eosinophilic to basophilic inclusions characteristic of WSSV (Figs 2, 4, 6 & 8). However, the inclusions found in the gut wall of mud crabs were more prominent, deeply basophilic, large and greater in number than those in lobsters and prawns (Fig. 6).

Ultrastructural studies of the stomach wall of experimentally infected *P. vannamei* showed infected nuclei enclosing rod-shaped virions 210–320 nm long and 80–100 nm wide. The nucleocapsid was 182–250 nm long and 60–80 nm wide. Virions were observed in crystalline arrays or scattered inside the nuclei (Figs 9–11).

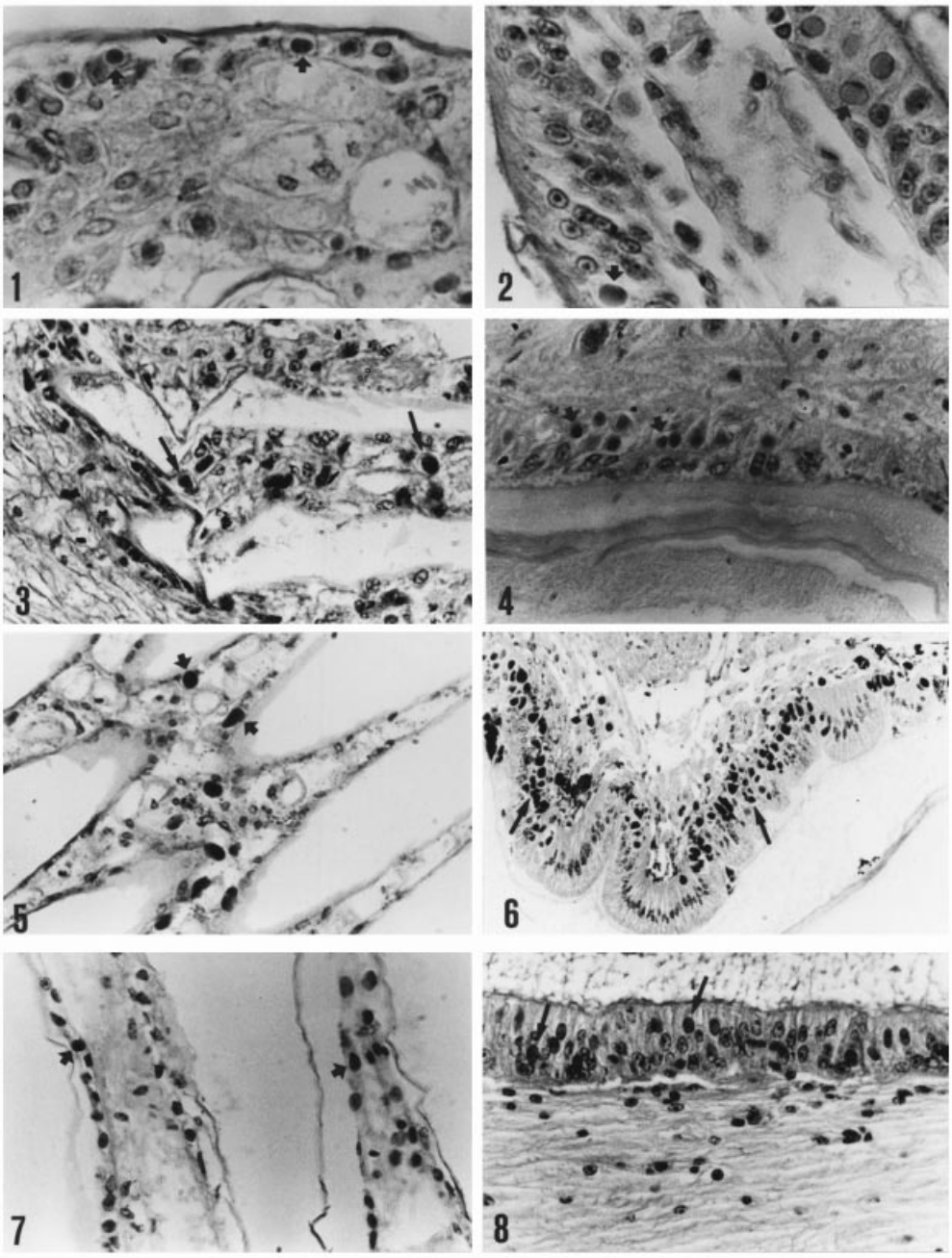
Discussion

A list of susceptible species of WSSV has been presented by Flegel (1996) and Lo *et al.* (1996). The present study demonstrates the susceptibility of five species of shrimp, two species of freshwater prawns, four species of crabs and three species of lobsters to Indian WSSV. All the experimentally infected shrimp showed marked histopathological changes in ectodermal and mesodermal tissues, with prominent eosinophilic to basophilic intranuclear inclusions similar to those reported earlier for WSSV in naturally infected shrimp (Wongteerasupaya *et al.* 1995). The mortality pattern of experimentally infected shrimp was found to vary with respect to the mode of infection. The early onset of mortality in the injected shrimp might be partly attributed to the high concentration of pathogen in the filtrates and possible stress as a result of handling.

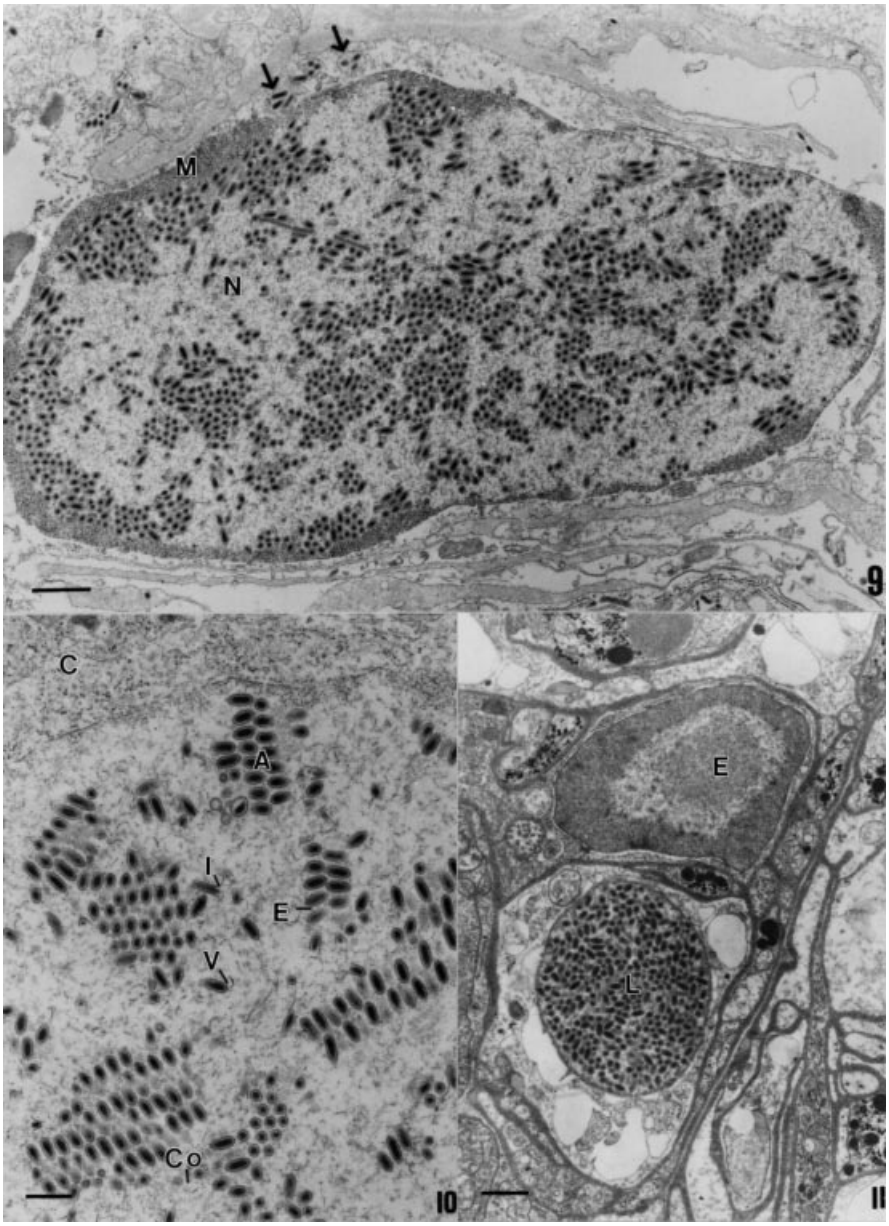
Although Flegel (1996) listed the freshwater prawn as a species resistant to WSSV, polymerase chain reaction (PCR) analysis of cultured *M.*

rosenbergii in Taiwan confirmed infection with WSSV (Kou, Ho, Chen, Hsu & Lo 1997). The present study confirmed the presence of WSSV in experimentally infected *M. rosenbergii* by histological and bioassay methods, possibly for the first time. The present study also revealed the tolerance of prawns to WSSV infection and the presence of microscopic spots on the carapace as a result of the infection.

Lo *et al.* (1996) recorded natural WSSV infec-



Figures 1–8 Photomicrographs of tissues from shrimp, prawn, crab and lobster experimentally infected with white spot syndrome virus showing degenerated hypertrophied nuclei and viral inclusions (arrows): (1) *Penaeus monodon* gill tissue (H & E, × 1000); (2) *P. monodon* gut (H & E, × 1000); (3) *Macrobrachium rosenbergii* gill tissue (H & E, × 400); (4) *M. rosenbergii* gut (H & E, × 400); (5) *Scylla serrata* gill tissue (H & E, × 400); (6) *S. tranquebarica* gut (H & E, × 200); (7) *Panulirus ornatus* gill tissue (H & E, × 400); and (8) *P. ornatus* gut (H & E, × 400).



Figures 9–11 Transmission electron micrographs of the stomach epithelium of *Penaeus vannamei* experimentally infected with white spot syndrome virus from *P. monodon*: (9) A hypertrophied nucleus (N) with marginated chromatin (M) contains virus particles in various stages of development. Note the membrane-bound virus particles in the cytoplasm (arrow) (bar = 1 μm). (10) Viral assembly in a nucleus. Various stages of virion development include membranous envelope precursors (E), capsid originators (Co), immature virus particles (I), mature virions with a nipple-like projection (V) and virions in arrays (A); (C) cytoplasm (bar = 500 nm). (11) Nuclei with early and late stages of viral infection: (E) a nucleus with an area of viroplasm developing in the centre, surrounded by marginated chromatin; and (L) a nucleus filled with mature virions as the only distinguishable feature (bar = 1 μm).

tions in *S. serrata* by PCR analysis. However, the present study indicates that WSSV can be experimentally transmitted to mud crabs. Furthermore, the present investigation also provided evidence of infection in crabs by histological and bioassay methods. Since the crabs survived the infection for 30 days (at the termination of experiment) without exhibiting clinical symptoms, these animals may act as an asymptomatic carrier/reservoir host rather than an indicator species of infection. Although the other species of crabs used in the present experiment, i.e. *Metapograpsus* sp. and *Sesarma* sp., are

not of any importance to aquaculture, the susceptibility of these species suggests that they may be important in the spread of WSSV since they are abundant around shrimp farms in Asia and South-east Asia. Rajendran *et al.* (1998) reported the outbreak of WSSV in confined shrimp grow-outs where farms were rain-fed. One of the main sources of disease transmission in these confined systems might be carrier hosts such as crabs.

The present investigation demonstrated that lobsters could play a major role as a reservoir host of WSSV. Transmission of the disease to cultured shrimp is unlikely since lobsters have a marine habitat. However, there appears to be a possibility of infection of wild shrimp broodstock by lobsters carrying WSSV. The fact that lobsters can be infected experimentally with WSSV and can survive for a long period without any ill effect indicates that infected material released into the sea through birds, unscreened sea ranching, and untreated shrimp waste from farms and processing plants can act as a source of infection to lobsters.

According to information obtained from various hatchery operators, the prevalence of WSSV infection in wild broodstock of *P. monodon* and *P. indicus* collected off the south-east coast of India is high. This clearly suggests that a wild reservoir of WSSV exists in this geographic region. The present study indicates the wide range of hosts available for the Indian WSSV and the need for extreme precautions to prevent the dissemination of the disease.

In the present study, the heavily mineralized crustaceans, lobsters and crabs were positive for WSSV for long periods of time, but did not exhibit the characteristic clinical symptoms such as white spots or patches on the carapace. Perhaps, the name 'white spot syndrome' is not descriptive for all hosts of this virus. The defence mechanisms of prawns, crabs and lobsters may be different from those of shrimp. This is an area which warrants further investigation.

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