tions. Eventually, natural infection of GBNV on other crops should also be monitored and the virus isolates from different hosts should be further examined to assess biological and molecular diversity and ascertain their taxonomic status.

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Prevalence of white spot syndrome virus in wild crustaceans along the coast of India

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Crustacean samples comprising of shrimps, crabs and squilla were collected from various landing centres and local fish markets of Kolkata, Chennai, Thiruvananthapuram, Mangalore and Mumbai along the east coast and west coast of India. Nested polymerase chain reaction (PCR) was employed for screening the samples for white spot syndrome virus (WSSV). A total of 89 crustacean samples were examined, which comprised of 40 shrimps, 36 crabs and 13 squilla samples. The results indicate that the wild-caught marine shrimps such as *Penaeus monodon*, *Heterocarpus* sp., *Aristeus* sp., *Parapenaeopsis stylifera*, *Metapenaeus dobsoni*, *M. elegans* and *Squilla* sp. carry WSSV. This virus could be detected in apparently healthy crabs such as *Scylla serrata*, *Portunus sanguinolentus*, *P. pelagicus*, *Calappa lophos*, *Charybdis hoplites*, *C. lucifera*, *C. cruciata* and *Pseudograpsus intermedius*. The virus was also found to be present in the freshwater prawn *Macrobrachium rosenbergii*, collected from the fish market. Sensitivity of detection of WSSV by PCR improved greatly, with PCR primers yielding amplicons of smaller size. Marine shrimps such as *Heterocarpus* sp., *Aristeus* sp., *Metapenaeus elegans* and crabs such as *C. hoplites* are being reported as carriers of WSSV.

IN less than three decades, shrimp farming, which used to be a traditional practice has metamorphosed into a multi-billion dollar industry. The process of rapid expansion has brought along with it increased incidence of diseases caused by various infectious agents. Viral diseases in particular have become an important limiting factor for shrimp production in recent years, all over the world[1,2]. Among the various viral diseases, white spot syndrome virus (WSSV) presently overshadows all other disease agents as the leading cause of production losses[2,3] and has become the single most recognized biological threat to the shrimp culture industry.

White spot syndrome was first reported in 1992 in *Penaeus japonicus* cultured in northeast Taiwan[4]. Since then, the shrimp culture industry in various other countries like Japan, Thailand, China, Korea[5–9] and more recently Indonesia, India and North America[2,10–13] has

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been facing the menace of WSSV. The causative viral pathogen has been named differently by various workers[5–7,14–16], but all of them were found to produce disease with similar clinical signs and pathology. However, presently these viruses are collectively referred to as WSSV[17].

The principal clinical signs of the disease include the presence of white spots on the exoskeleton ranging from barely visible to 3 mm in diameter[4,5], lethargy, reduction in food consumption and often a generalized reddish-to-pink discoloration[2,10,17,18]. WSSV is highly virulent and can induce 100% mortality in infected shrimps within 3 to 5 days from the onset of clinical signs, targeting the organs of ectodermal and mesodermal origin[7]. WSSV has been reported in many farms irrespective of the type of culture, stocking densities and size or age of the shrimp[2,10].

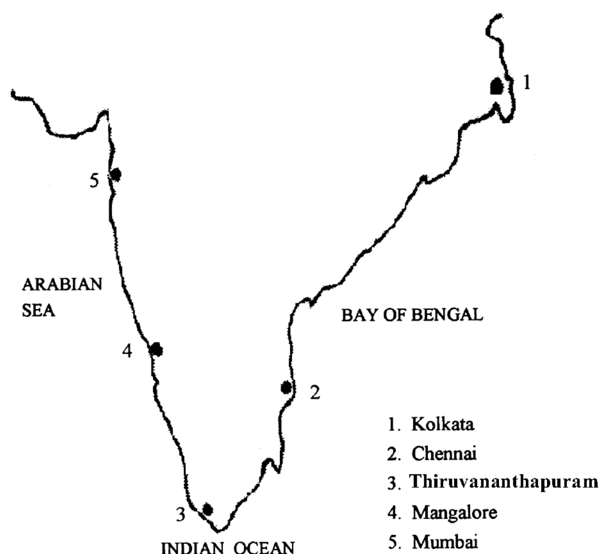

Figure 1. Map showing location of sampling sites.

The presence of WSSV has been described in a wide range of captured and cultured crustaceans and other arthropods, including wild crabs (*Calappa lophos*, *Portunus sanguinolentus*, *Charybdis* sp., *Helice tridens*), wild lobsters (*Panulirus* sp.), palamonidae pest shrimp, (*Exopalaemon orientalis*), copepods, pupae of Ephydridae insects, cray fish (*Orconectes punctimanus* and *Procambus clarkii*), pest crabs (*Sesarma pictum*), mud crabs (*Scylla serrata*), krill (*Acetes* sp.) and many other marine crustaceans[16,19–23]. Presence of WSSV in broodstock captured from sea has also been reported[22,24]. Wild crustaceans serving as hosts for WSSV may act as natural reservoirs of the virus in the marine environment. Information regarding the presence of carriers in natural environment is of vital importance to the shrimp farmers and hatchery operators. It is also important to have data on the distribution of WSSV in different locations along both east coast and west coast of India.

Against this background, various crustaceans collected from different fish-landing centres and fish markets were examined for the presence of WSSV.

Wild-captured crustaceans, shrimps, crabs and squilla were collected from various fish-landing centres and

Table 1. Location, source and type of sample collected for analysis

Location	Market/landing centre	Shrimp	Crab	Squilla
Kolkata	Sealdah fish market	4	3	–
	Gariahat fish market	10	3	–
	Khidirpore fish market	3	–	–
	Esplanade fish market	–	2	–
	Park circus fish market	3	–	–
	Digha fish-landing centre	–	2	3
	Shankarpur fish-landing centre	–	3	3
Chennai	Chennai fish-landing centre	–	4	–
	Palavakkam fish market	3	–	–
Thiruvananthapuram	Vizhinjam fish-landing centre	8	–	–
	Palayam fish market	5	1	–
Mangalore	Central fish market	2	3	–
	Kankanady fish market	–	2	–
	Ullal fish market		2	
	Mulky fish market	–	2	–
	Mangalore fish-landing centre	2	–	3
	Malpe fish-landing centre	–	1	2
Mumbai	Colaba fish market	–	2	–
	Crawford fish market		2	
	Sasoon dock-landing centre	–	2	2
	Bhau ka dhakka landing centre	–	2	–
Total		40	36	13

local fish markets of five different sampling locations, viz. Kolkata and Chennai on the east coast and Thiruvananthapuram, Mangalore and Mumbai on the west coast of India (Figure 1, Table 1). The samples were dipped in 40% formalin overnight and then packed in sealed plastic containers containing 10% formalin and brought to the laboratory. The period of sample collection was from March to April 2000.

The samples were first deformalinized by keeping them in water for 12–15 h. Then WSSV nucleocapsid isolation was carried out as described by Yang *et al.*[25], with some modification. Tissues of shrimps, crabs and squilla (approx. 150 mg of gill and cuticle) were taken and homogenized with 1.5 ml TESP buffer (50 mM Tris-HCl, pH 8.5; 10 mM EDTA; 100 mM NaCl; 1 mM phenylmethylsulphonyl flouride (PMSF)) in a pestle and mortar and then transferred to a 1.5 ml microcentrifuge tube, centrifuged at 1500 *g* for 10 min at 4°C (Remi C-24, India). The supernatant was recentrifuged at 15,600 *g* for 30 min at 4°C. The pellet was suspended in 400 µl of TESP buffer containing 1% Triton × 100, centrifuged at 1500 *g* for 10 min and again at 16,300 *g* for 20 min at 4°C. The pellet, resuspended in equal volume of TESP, was centrifuged at 1,500 *g* for 10 min and 16,300 *g* for 20 min at 4°C. The pellet thus obtained was suspended in 400 µl TMP buffer (100 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM PMSF) and centrifuged at 1500 *g* for 10 min and 16,300 *g* for 20 min at 4°C. The pellet was finally dissolved in 25 µl of TESP buffer and used for further analysis.

PCR for WSSV was carried out using the following primer sets: The primers designated Lo 1–2 corresponded to primers 146 F1 and 146 R1, originally described by Lo *et al.*[16] and yielded an amplicon of 1447 bp. The primers named IK 1–2 (yielding an amplicon of 486 bp) were based on the sequence of a cloned WSSV *Sal*I 1461 bp DNA fragment[26] and internal to the fragment amplified by primers Lo 5–6 which corresponded to the primers 146F4 and 146R3 derived from the sequence described by Lo *et al.*[26]. The primers named IK 3–4 (yielding an

Table 2. Details of first step and nested PCR analysis of various crustacean samples collected

Location	Type	Species	Total no. tested	Positive in 1st step PCR			Positive in 2nd step PCR IK 1–2 to IK3–4	Negative after 2nd step PCR
				LO 1–2	IK 1–2	IK3–4		
Kolkata	Shrimp	*Penaeus monodon*	8	0	0	3	1	4
		Macrobrachium rosenbergii	4	0	0	3	–	1
		Metapenaeus dobsoni	8	0	0	0	5	3
	Crab	*Scylla serrata*	3	0	0	0	3	0
		Portunus sanguinolentus	2	0	0	0	2	0
		Pseudograpsus intermedius	2	0	0	2	–	0
		Matuta planipes	6	0	0	0	4	2
	Squilla	*Squilla* sp.	6	0	0	6	–	0
Chennai	Shrimp	*Penaeus monodon*	3	0	1	1	1	0
	Crab	*Portunus sanguinolentus*	2	0	0	0	1	1
		Charybdis lucifera	2	0	0	1	–	1
Thiruvananthapuram	Shrimp	*Penaeus monodon*	3	0	0	1	2	0
		Heterocarpus sp.	4	0	0	3	1	0
		Aristeus sp.	2	0	0	1	1	0
		Parapenaeopsis stylifera	2	0	0	1	1	0
		Metapenaeus elegans	2	0	0	1	1	0
	Crab	*Scylla serrata*	1	0	0	1	–	0
Mangalore	Shrimp	*Metapenaeus monoceros*	4	0	0	4	–	
	Crab	*Portunus pelagicus*	3	0	0	1	2	0
		Charybdis hoplites	2	0	0	2	–	0
		Charybdis cruciata	1	0	0	0	1	0
		Portunus sanguinolentus	1	0	0	1	–	0
		Scylla serrata	2	0	0	0	2	0
		Calappa lophos	1	0	0	1	–	0
	Squilla	*Squilla* sp.	5	0	0	4	1	0
Mumbai	Crab	*Charybdis lucifera*	6	0	0	5	1	0
		Portunus pelagicus	1	0	0	1	–	0
		Scylla serrata	1	0	0	0	1	0
	Squilla	*Squilla* sp.	2	0	0	0	2	0
Total			89	0	1	43	33	12

amplicon of 316 bp) were also based on the sequence of the same fragment and internal to the fragment amplified by IK 1–2. A 50 µl of reaction mixture was prepared in sterile PCR tubes (PCR – 05-C, Axygen, USA) with the following components: 38.40 µl of sterile distilled water, 5 µl of Taq polymerase assay buffer, 1.0 µl (0.5 µg) each of primer 1 and primer 2 from each set of primers (Lo 1–2, IK 1–2 and IK 3–4), 1.0 µl (200 mM) dNTP mix, 3.0 µl template DNA and 0.6 µl of Taq polymerase (2.25 U).

For second step PCR, 5.0 µl of the first step reaction mixture was added to the PCR cocktail. Positive control (WSSV DNA from naturally-infected *Penaeus monodon*) and negative control (shrimp DNA from uninfected animals and distilled water) were used in each batch of reactions. The amplification was performed in a thermocycler PTC-100 (MJ Research Inc., USA) for a 30-cycle programme, each cycle consisting of three steps, i.e. denaturation of DNA at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension of primers at 72°C for 2 min with an initial delay at 94°C for 5 min and final delay of 5 min at 72°C. The amplified products were electrophoresed in 1.2% agarose gel containing ethidium bromide at 0.5 µg/ml and observed using Kodak electrophoresis documentation and analysis system (Hoefer, USA).

For probe hybridization, Biotin-14-dATP (GIBCO BRL, USA) labelled probe was prepared by nick translation kit (GIBCO BRL, USA) using another primer pair IK 5–6 which amplified 210 bp internal to the 316 bp fragment amplified by primer IK 3–4 (obtained from virus extract of a known positive sample). This was purified using CONCERT PCR purification system (GIBCO BRL, USA). PCR products obtained were resolved in 1.2% agarose gel and transferred to nylon membrane (Nitran NY 12N, Schleicher and Schuell, Germany) by capillary transfer[27]. Hybridization was performed as described by Rashtchian and Mackey[28] and detected using streptavidin–alkaline–phosphatase conjugate (Bangalore Genei, Bangalore) and chromogenic substrate (Bangalore Genei, Bangalore) following the manufacturer's protocol.

Table 2 shows that a total of 77 crustacean samples were positive for WSSV out of 89 samples examined. Among these, 44 were positive by first-step PCR and 33 by second-step (nested) PCR. From Figure 2 *b*, it can be seen that the percentage positivity in the first step was more between squilla and shrimp samples, whereas the crab samples showed higher percentage positivity in the second step. The squilla samples showed 100% positivity, with 76.92% positive at the first step and 23.07% positive at the second step. The crab samples showed an overall positivity of 88.88% with 41.66% at the first step and 47.22% at the second step. The overall percentage positivity in shrimp samples was least among all the sample types and it showed 80% positivity with 47.55% and 37.07% at first and second step respectively. When all the samples are pooled together, the positivity was quite high at 86.52%.

The results also indicate that there was not much difference in percentage positivity when the market samples and landing centre samples were compared (Figure 2 *c*). In Thiruvananthapuram, Mangalore and Mumbai both the market and landing centre samples showed 100% positivity when both first step and second step results were taken together, whereas in Kolkata a higher positivity

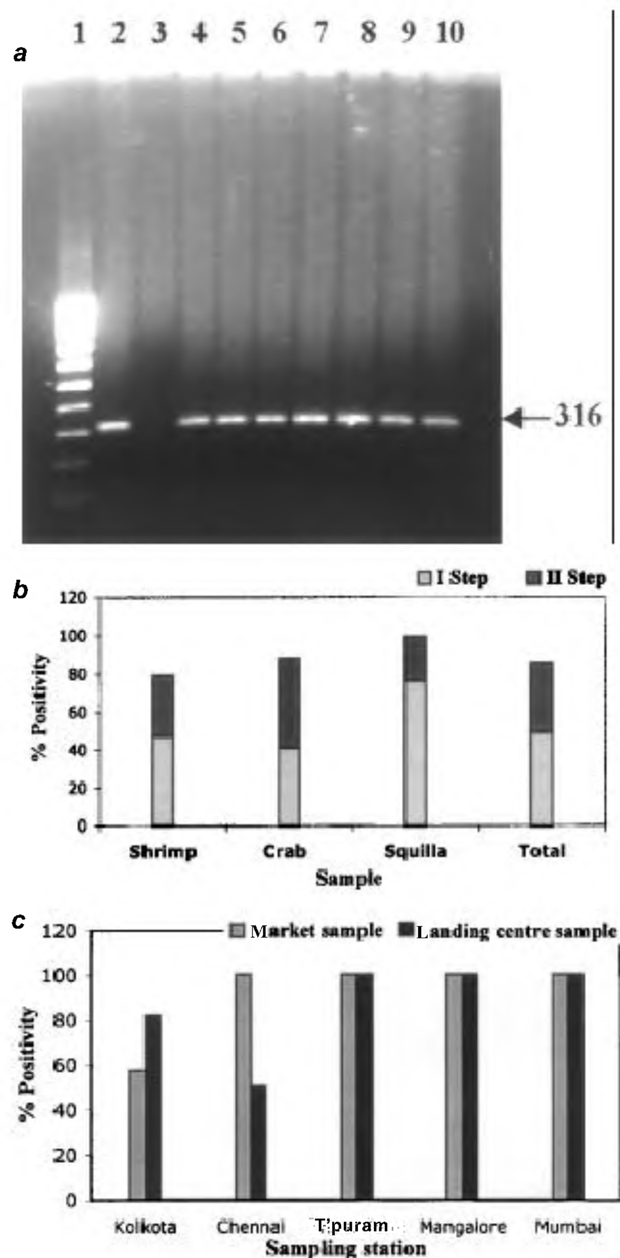

Figure 2. ***a***, Detection of WSSV from wild captured crustaceans in east coast and west coast of India by nested PCR. Lane 1, DNA molecular weight marker (100 bp ladder); Lane 2, Positive control; Lane 3, negative control; Lane 4, *Heterocarpus* sp.; Lane 5, *Aristeus* sp.; Lane 6, *Metapenaeus elegans*; Lane 7, *Squilla* sp.; Lane 8, *Charybdis hoplites*; Lane 9, *M. planipes*; and Lane 10, *Calappa lophos*; ***b***, Percentage positivity of WSSV in various samples by first-step and second-step PCR; ***c***, Percentage positivity of WSSV in market and landing centre samples at various sampling stations.

was observed in samples from the landing centre (81.81%) compared to market samples (57.14%). Samples from Chennai showed a higher positivity in market (100%) compared to landing centre (50%).

Figure 3 *b* shows that the percentage positivity of shrimps after first-step PCR was highest in Mangalore followed by Chennai, Thiruvananthapuram and Kolkata. Crab samples had highest percentage positivity in Thiruvananthapuram and least in Kolkata (Figure 3 *c*) after first-step PCR. The squilla samples could be collected only from three sampling stations and after first-step PCR, the positivity was highest in Kolkata and least in Mumbai (Figure 3 *d*).

When second-step PCR results were compared (Figure 3 *b–d*) it was seen that except Kolkata (80%) all the other sampling stations showed 100% positivity of WSSV in shrimps, whereas crab samples collected from Thiruvananthapuram, Mangalore and Mumbai showed 100% positivity, and the Chennai samples showed the least value (50%). However, among squilla samples collected from Kolkata, Mangalore and Mumbai, there was 100% positivity.

Species-wise analysis of the results shows that a number of species of shrimps, crabs and squilla are found to be positive for WSSV. While some of them are already reported to be carriers of WSSV, few shrimp species such as *Heterocarpus* sp., *Aristeus* sp., *Metapenaeus elegans* and crab species such as *Charybdis hoplites* are found to be positive for the first time (Table 2).

The results of this study also clearly illustrate the influence of PCR amplicon size on sensitivity of detection of WSSV. As shown in Table 1, none of the samples were positive by primer LO 1–2 which yields amplicon of 1447 bp. Only one sample of shrimp was positive by one-step PCR with primer pair IK 1–2 which yields amplicon of 486 bp. However, 43 samples were positive by one-step PCR with primer pair IK 3–4 which yields amplicon of 310 bp.

This study shows that WSSV is widely distributed in wild crustaceans collected from both the east coast and west coast of India. The percentage positivity was quite high and the virus is prevalent among the wild crustaceans irrespective of place of collection of samples, either commercial markets or fish-landing centres. WSSV has been found to be present in wild brood stock of *P. monodon* in Taiwan[24], Japan[29] and India[22]. Wild populations of *P. monodon*, *P. japonicus*, *P. semisulcatus*, *P. penicillatus*[16], *Metapenaeus dobsoni*, *Parapenaeposis stylifera* and *Solenocera indica*[23] were found to be carriers of WSSV. Several species of wild crabs such as *Portunus pelagicus*, *P. sanguinolentus*, *Charybdis feriatus*, *Macrophthalmus sulcatus*, *Gelasimus marionis nitidus*

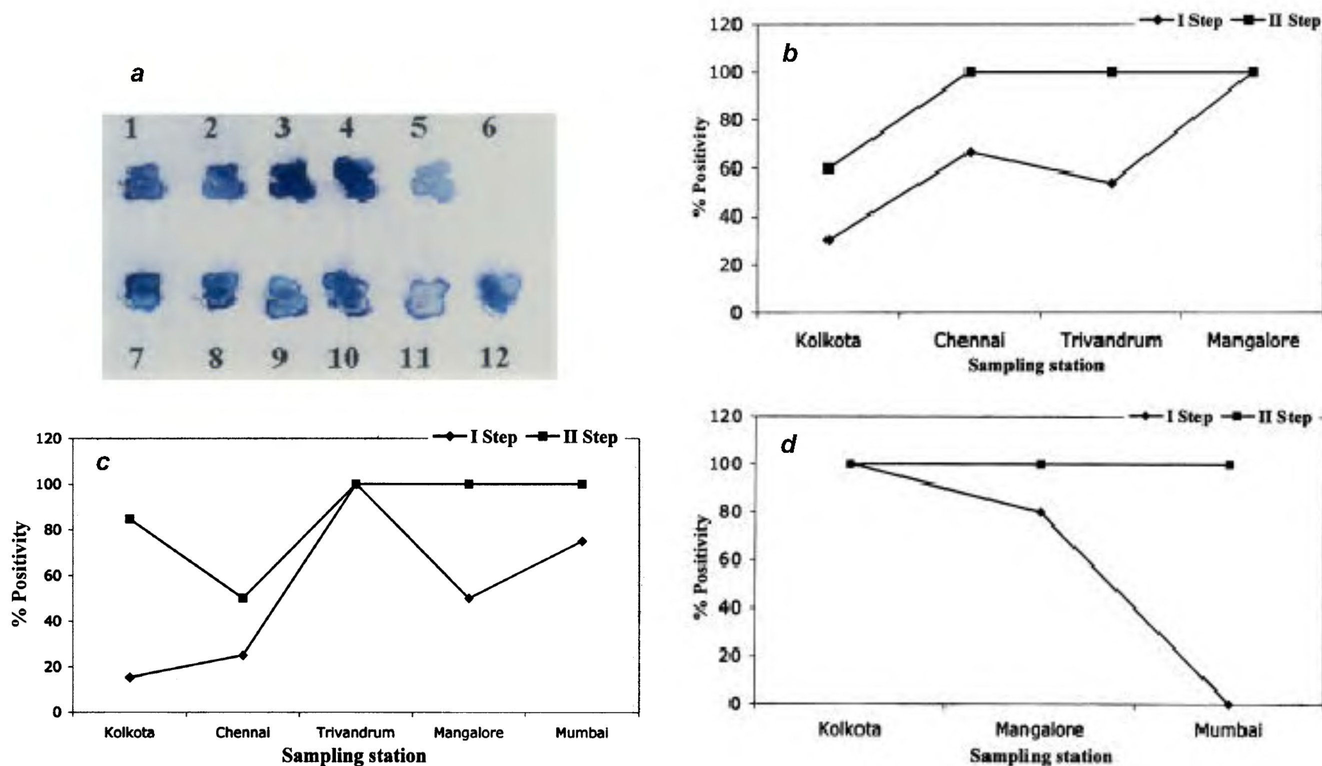

Figure 3. ***a***, Confirmation of WSSV PCR products of wild captured crustaceans from east coast and west coast of India by dot blot hybridization with 14-dATP biotin probe. Lane 1, *Heterocarpus* sp.; Lane 2, *Aristeus* sp.; Lane 3, *Metapenaeus elegans*; Lane 4, *Squilla* sp.; Lane 5, *P. sanguinolentus;* Lane 6, Negative control; Lane 7, *M. planipes*; Lane 8, *P. pelagicus*; Lane 9, *Charybdis hoplites*; Lane 10, *C. cruciata*; Lane 11, *Calappa lophos*; and Lane 12, Positive control. Percentage positivity of WSSV in (***b***) Shrimp, (***c***) Crab, and (***d***) Squilla by first-step and second-step PCR among various sampling stations.

and *Metapograpsus messor*[23] were also reported to harbour this virus. In this study, we have found that several additional wild caught shrimps and crabs are hosts for WSSV. These include shrimps such as *Heterocarpus* sp., *Aristeus* sp., *M. elegans* and crabs such as *C. hoplites* (Figure 3 *a*). It can also be observed that the prevalence is more in the west coast where Thiruvananthapuram, Mangalore and Mumbai showed 100% positivity after second-step PCR than the east coast samples where Kolkata and Chennai showed 80% and 71% positivity respectively. Reasons for higher positivity in the west coast are not clear. In fact there is not much of aquaculture activity in these areas. It raises the question whether wild animals are a source of virus for cultured shirmp or vice versa.

Among the various samples, squilla showed highest percentage positivity followed by crabs and shrimps. Hossain *et al.*[23] reported that *Squilla mantis* is one of the carriers of WSSV. None of the animals examined had white spots on their carapace or other parts of the exoskeleton. These results suggest that though samples were positive by one step PCR, they did not show signs of disease. It is possible that these animals are tolerant to the virus.

This study shows that the wild crustaceans could act as reservoirs of WSSV. They could be a source of virus to aquaculture systems. Further, the results suggest that sensitivity of detection can be improved by choosing primers that yield smaller amplicons.

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Occurrence of concurrent infections with multiple viruses in *Penaeus monodon* from culture ponds of north coastal Andhra Pradesh

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A histopathological study of *Penaeus monodon* showing external symptoms of white spot disease, collected from culture ponds at Mulakuddu and Rambilli villages (Visakhapatnam District) during May and July 2001, provided evidence for the occurrence of multiple viral infections. Each diseased shrimp carried concurrent infections with four different viruses, identified on the basis of nuclear changes, resulting in the formation of characteristic inclusion/occlusion bodies, as WSSV, YHV, MBV and IHHNV. The identification of the various viruses needs confirmation through molecular diagnostic methods. This constitutes a report recording concurrent infections with multiple viruses in a single shrimp.

VIRAL diseases have been seriously affecting the sustainability and economic success of the shrimp industry. To date over 22 different viruses are known to infect

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