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Detection of new hosts for white spot syndrome virus of shrimp using nested polymerase chain reaction

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Abstract

The presence of white spot syndrome virus (WSSV) of shrimp in various marine crustaceans was studied by using polymerase chain reaction (PCR). The incidence of the virus in non-cultured crustaceans from shrimp farms was also studied. The results indicate that wild-caught asymptomatic marine shrimp such as *Metapenaeus dobsoni*, *Parapenaeopsis stylifera*, *Solenocera indica* and *Squilla mantis* carry WSSV. This virus could be detected in apparently healthy marine crabs *Charybdis annulata*, *C. cruciata*, *Macrophthalmus sulcatus*, *Gelasimus marionis nitidus* and *Metopograpsus messor*. The virus could also be detected in asymptomatic *Macrobrachium rosenbergii* cultured inland far away from coast. Detection of carrier animals required two-step nested PCR.

Keywords: White spot syndrome virus; Polymerase chain reaction; Carriers; Crustaceans; Shrimp

1. Introduction

The rapid expansion and intensification of shrimp farming worldwide has been accompanied by the occurrence of diseases which threaten the development of the industry. White spot syndrome virus (WSSV) is the causative agent of shrimp viral

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disease, which presently overshadows all other disease agents as the leading cause of production losses in Asia (Flegel, 1997; Flegel et al., 1997) and is posing a major threat to the shrimp farming industry.

The disease was first noticed in the northern part of Asia in China and Japan and spread southwards (Inouye et al., 1994; Nakano et al., 1994; Takahashi et al., 1994; Chen, 1995; Wang et al., 1995; Wongteerasupaya et al., 1995; Karunasagar et al., 1997; Lo et al., 1999). In the USA, WSSV has been found ever since 1995 in wild shrimp, or in shrimp growing facilities in Texas and South Carolina (Lightner et al., 1997; Nunan and Lightner 1997; Nunan et al., 1998). A serious disease outbreak caused by WSSV was investigated in crayfish also (Wang et al., 1999). Presently, WSSV has been detected in Central American countries (Guatemala, Honduras, Nicaragua, Panama and Ecuador) and has devastated the shrimp industry there (Infofish, 1999).

WSSV is extremely virulent and targets various tissues of ectodermal and mesodermal origin in shrimp (Chang et al., 1996). The clinical signs of the syndrome include lethargy, anorexia, the presence of white spots on the cuticle and, often, a generalized reddish to pink discoloration (Durand et al., 1997). The mortality rate has been reported to be very high reaching 100% within 3–10 days of the onset of clinical signs (Inouye et al., 1994; Nakano et al., 1994; Chou et al., 1995; Wang et al., 1995; Manohar et al., 1996). WSSV is reported in many farms irrespective of the type of culture, species cultured, stocking densities, size/age of the shrimp (Panchayuthapani, 1997) and even in areas where the pond environment was apparently good (Karunasagar et al., 1997).

The disease has also been described in a wide range of wild crustaceans including crabs, lobsters and shrimp (penaeid and non-penaeid) by DNA based detection methods (Lo et al., 1996a,b; Peng et al., 1998; Wang et al., 1998; Otta et al., 1999). Information regarding the incidence of natural carriers and potential risk they pose is of vital importance to shrimp farmers, if they are to institute effective measures to prevent viral infections (Kanchanaphum et al., 1998). The pest species (crabs, prawns) are very likely asymptomatic carriers of WSSV in farms (Lo et al., 1996a). Certain organisms such as krill, *Acetes* sp., crabs, and other marine shrimp have been found to be carriers of WSSV and many other crustacean species could be suspected as WSSV carriers (Supamattaya et al., 1998). The presence of WSSV in broodstock captured from the sea has been reported (Lo et al., 1997; Itami et al., 1998; Otta et al., 1999) and if other marine crustaceans also serve as hosts of WSSV, these animals could be a source of infection for the broodstock. In this study, we have examined the presence of WSSV in different crustaceans in India using PCR and evaluated different PCR primers for detection of WSSV in various asymptomatic carrier species.

2. Materials and methods

2.1. Collection of samples

Wild captured crustaceans viz. *Penaeus monodon* (broodstock), *Metapenaeus dobsoni* and *Squilla mantis* were collected from fish landing centre at Mangalore, *Parape-*

naeopsis stylifera from fish landing centres at Karwar and *Solenocera indica* from fish landing centres at Mangalore and Malpe in Karnataka along the west coast of India. Wild captured crabs (details in Table 2) were collected from fish landing centre at Mangalore and from creeks at Kundapur and Katpadi regions. Non-cultured crustaceans viz. *P. indicus*, *Acetes* sp., *Macrobrachium rosenbergii* and crabs were collected from shrimp farms at Kundapur and Katpadi. Cultured fresh water prawn, *Macrob. rosenbergii* were collected from prawn farms at Mysore. After collection, shrimps and other crustaceans were wrapped in polythene bags and transported to the laboratory in ice box. Crustacean samples were collected from March 1999 to December 1999.

2.2. Extraction of viral DNA

WSSV DNA extraction was carried out as previously described by Yang et al. (1997) with some modification. Tissues of shrimp and other test animals (around 150 mg of gill, stomach, cuticle, etc. of juvenile/adult) were taken and homogenised individually with 1.5 ml TESP buffer (50 mM Tris–HCl, pH 8.5; 10 mM EDTA, 100 mM NaCl, 1 mM phenyl methyl sulfonyl flouride (PMSF)) in a pestle and mortar and then transferred to a 1.5-ml microcentrifuge tube. The sample was centrifuged at $1500 \times g$ for 10 min at 4°C in a refrigerated centrifuge (Remi, C 24, Remi sales, India) and the supernatant was transferred to another microcentrifuge tube and recentrifuged at $15{,}600 \times g$ for 30 min at 4°C. The pellet was suspended in 400 μl TESP buffer containing 4 μl Triton X100 and centrifuged first at $1500 \times g$ for 10 min and then at $16{,}300 \times g$ for 20 min at 4°C. Then the pellet was redissolved in 400 μl TESP buffer and centrifuged at $1500 \times g$ for 10 min and $16{,}300 \times g$ for 20 min at 4°C. The pellet was again dissolved in 400 μl TMP buffer (100 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM PMSF) and centrifuged at $1500 \times g$ for 10 min and $16{,}300 \times g$ for 20 min at 4°C. The pellet was finally dissolved in 25 μl of TESP buffer and used for PCR analysis.

2.3. Detection of WSSV in collected crustacean samples by WSSV diagnostic PCR

Diagnostic 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 and the one designated Lo 5–6 corresponded to 146 F4 and 146 R3 derived from the sequence described by Lo et al. (1996a). The primers named IK 1–2 (amplicon size, 486 bp) were based on sequence of WSSV 1461 bp Sal/I fragment described by Lo et al. (1996a) and internal to fragment amplified by primers Lo 5–6. The primers named IK 3–4 (amplicon size, 310 bp) were also based on the sequence of same fragment and internal to fragment amplified by IK 1–2. The DNA samples extracted from shrimp (*P. monodon*) naturally infected with WSSV was used as positive control. A 50μl of reaction mixture was prepared in sterile PCR tubes (PCR-03-C, Axygen, USA). The reaction mixture contained 38.40 μl of sterile distilled water, 5.0 μl of *Taq* polymerase assay buffer, 1.0 μl each of first and second primer (0.5 μg) from each set of primers, 1.0 μl dNTP (200 μM), 3 μl template DNA and 0.60 μl Taq DNA polymerase (2.25 U). For second-step PCR, 3.0 μl of the first-step reaction mixture was added to PCR cocktail. For positive

control 1.0 μl of WSSV DNA was used with PCR cocktail. The components were mixed thoroughly and the tubes arranged in the thermocycler (M.J. Research, USA) and 30 cycles programme employed for the amplification. Each cycle consisted of the conventional three-step reaction, i.e. denaturing of target DNA at 94°C for 1 min, annealing of primers at 55°C for 1 min and extension of primers at 72°C. The programme included an initial delay of 5 min at 94°C and final delay of 5 min at 72°C before and after 30 cycles, respectively. From each of the PCR reaction product, 20 μl was mixed with 5 μl gel loading buffer and subjected to electrophoresis in 0.8% agarose gels containing ethidium bromide at a concentration of 0.5 μg ml^{-1} except for the 310-bp product, which was resolved using 2.0% agarose gel. The gel was then observed on a UV transilluminator and photographed using Kodak electrophoresis documentation and analysis system (Pharmacia Biotech, USA).

2.4. Confirmation of PCR by probe hybridisation

To confirm that the PCR products obtained were from WSSV, probe hybridisation was used. The 310-bp fragment obtained using a known virus extract was purified using

Table 1
Incidence of WSSV in wild captured shrimp and mantis shrimp

Species	No. trawl catch examined	Primers used for PCR and no. of samples positive			
		One-step		Two-step	
Penaeus monodon (brooders)	2	Lo 1–2	0	Lo 5–6	0
	1	IK 3–4	0		
		IK 1–2	0	IK 3–4	1
	1	Lo 1–2	0	Lo 5–6	0
		IK 3–4	0		
		Lo 5–6	0	IK 3–4	1
		IK 1–2	0	IK 3–4	1
Metapenaeus dobsoni	3	Lo 1–2	0	Lo 5–6	0
	6	Lo 5–6	0	IK 1–2	5
		IK 3–4	1		
	3	IK 3–4	1		
		IK 1–2	0	IK 3–4	1
Parapenaeopsis stylifera	7	Lo 5–6	0	IK 1–2	6
		IK 3–4	1		
Solenocera indica	6, Gill	IK 1–2	2	IK 3–4	6
	Gill	IK 3–4	6		
	Stomach	IK 3–4	5		
	19	IK 3–4	11		
		IK 1–2	0	IK 3–4	8
	7	IK 3–4	0		
		IK 1–2	0	IK 3–4	7
Squilla mantis (mantis shrimp)	5	Lo 1–2	0	Lo 5–6	1
	5	IK 3–4	2		
		IK 1–2	0	IK 3–4	2

concert PCR purification system (Gibco BRL, USA) and labelled with Biotin 14 dATP (Gibco BRL) using Nick Translation kit (GIBCO BRL). The labeled product was purified using concert PCR purification system. This labeled product was used as probe in hybridisation assays. PCR products obtained from wild crustaceans were resolved in 2.0% agarose gel, transferred to nylon membrane (Nitran NY 12N, Schleicher and Schuell, Germany) by capillary transfer (Dyson, 1994). Hybridisation was performed as described by Rashtchian and Mackey (1992). The probe was detected using streptavidin–alkaline phosphatase conjugate (Bangalore Genei, Bangalore) and chromogenic substrate following the manufacturer's protocol.

3. Results

Results in Table 1 show that WSSV could be detected in all species of wild shrimp and mantis shrimp examined. Out of the four *P. monodon* broodstock studied, two

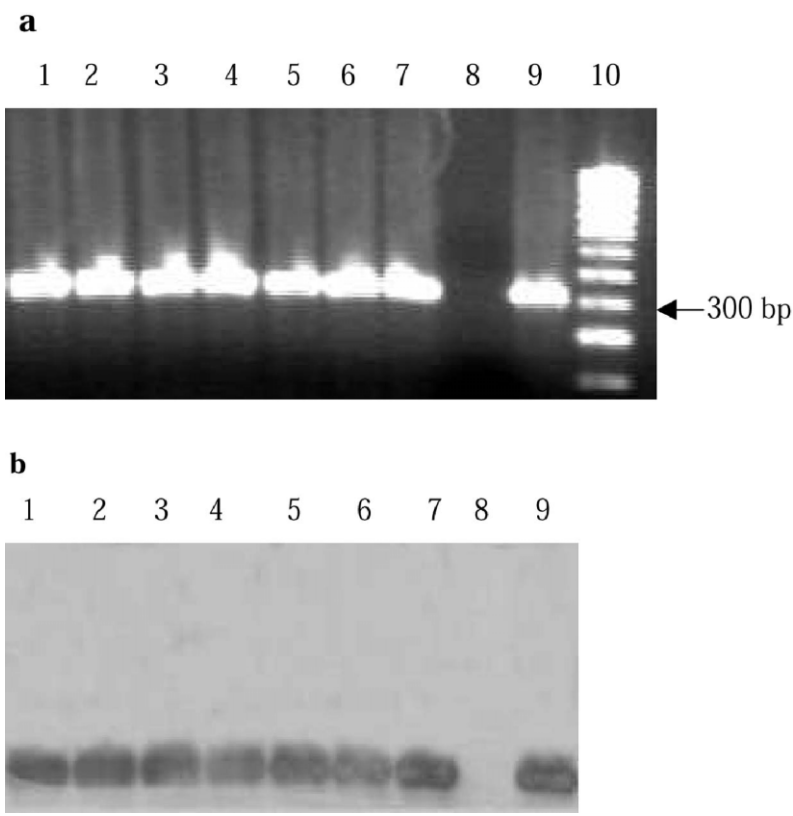

Fig. 1. Detection of WSSV from wild captured crustaceans by nested P1CR and confirmation by Southern hybridisation (a) agarose gel stained with ethidium bromide and (b) Southern blot hybridisation with 14-dATP biotin probe. Lanes (1) *Penaeus monodon* (brooder), (2) *Metapenaeus dobsoni*, (3) *Solenocera indica*, (4) *Squilla mantis*, (5) *Charybdis cruciata*, (6) *C. annulata*, (7) *Metopograpsus messor*, (8) negative control, (9) positive control and (10) mol. wt. marker (100 bp ladder).

individuals were positive by nested PCR. Interestingly, one brooder, in which different primer pairs were used, nested PCR was negative with one set (Lo 1–2 and Lo 5–6), but positive with two other sets (Lo 5–6 and IK 3–4; IK 1–2 and IK 3–4). Out of 12 samples of *M. dobsoni* tested, nine were positive, two by first step using primer pair IK 3–4 and seven by nested PCR (IK 1–2 and IK 3–4). All of the seven samples of *Par. stylifera* were positive, one by first step and six by nested PCR. All the 32 samples of *S. indica* were positive for WSSV. Primer pairs used greatly influenced the results. In one batch of six samples, two samples of gill tissue were positive in first step when the primer pair IK 1–2 was used, but all six were positive by nested PCR (Fig. 1a). Results of hybridisation analysis (Fig. 1b) confirm that the PCR products are derived from WSSV and do not represent contamination. However, when primer pair IK 3–4 was used, all samples of gills were positive by one-step PCR. The stomach of 5/6 samples showed WSSV by one-step PCR with primer IK 3–4. In another batch of 19 samples, 11 were positive by one-step PCR using IK 3–4 and eight by nested reaction. Another batch of seven samples was determined to be negative in one-step reaction but positive by nested reaction. Two batches of *Squ. mantis* were studied. In one batch, 1/5 samples was positive by nested reaction. In another batch of five samples, two were positive by first step and two more by nested reaction.

Among various marine crab species studied, WSSV could be detected in *Charybdis cruciata*, *C. annulata*, *Macrophthalmus sulcatus*, *Gelasimus marionis nitidus* and *Metopograpsus messor* (Table 2). Out of 14 samples of *C. cruciata*, seven were positive

Table 2
Incidence of WSSV in wild captured crabs

Species	No. of specimens	Primers used for PCR and no. of samples positive			
		One-step		Two-step	
C. cruciata	8	Lo 1–2	0	Lo 5–6	0
	17	IK 3–4	7		
		IK 1–2	0	IK 3–4	3
C. annulata	5	IK 3–4	0		
		IK 1–2	0	IK 3–4	2
Macrophthalmus sulcatus	1, Gill	Lo 1–2	0	Lo 5–6	1
		Lo 5–6	0	IK 1–2	1
	Cuticle	Lo 1–2	0	Lo 5–6	1
		Lo 5–6	0	IK 1–2	1
	Stomach	Lo 1–2	0	Lo 5–6	0
		Lo 5–6	0	IK 1–2	0
Gelasimus marionis nitidus	1	IK 3–4	1		
Metopograpsus messor	4	IK 3–4	0		
		IK 1–2	0	IK 3–4	2
C. callinassa	1	Lo 1–2	0	Lo 5–6	0
C. lucifera	16	Lo 1–2	0	Lo 5–6	0
Doclea gracilipes	1	Lo 1–2	0	Lo 5–6	0
Matuta planipes	3	Lo 1–2	0	Lo 5–6	0
Neptunus pelagicus	5	Lo 1–2	0	Lo 5–6	0
N. sanguinolentus	6	Lo 1–2	0	Lo 5–6	0
Scylla serrata	15	Lo 1–2	0	Lo 5–6	0

Table 3
Incidence of WSSV in non-cultured crustaceans from shrimp farms

Group	Species	No. of batches examined	Primers used for PCR and no. of samples positive			
			One-step		Two-step	
White Shrimp	*P. indicus*	2	Lo 1–2	0	Lo 5–6	2
			Lo 5–6	0	IK 1–2	2
Pest shrimp/ prawn	*Acetes* sp.	2	Lo 1–2	0	Lo 5–6	0
		3	IK 1–2	0	IK 3–4	3
	Macrobrachium rosenbergii	2	Lo 1–2	0	Lo 5–6	0
Mud crab	*Scy. serrata*	2	Lo 1–2	0	Lo 5–6	0
Pest crab	*Sesarma oceanica*	1	Lo 1–2	0	Lo 5–6	0
	Pseudograpsus intermedius	2	Lo 1–2	0	Lo 5–6	0

by one-step PCR and three by nested reaction. In the case of *C. annulata*, only two of three samples were positive by nested reaction. In one sample of *M. sulcatus*, gill and cuticle were positive by nested PCR while stomach was negative even by nested reaction. One sample of *G. nitidus* was positive in first-step reaction using primers IK 3–4. Both samples of *Meto. messor* were positive by nested PCR. All PCR products were confirmed by hybridisation assay and only representative data is presented in Fig. 1b.

WSSV could be detected in samples of wild *P. indicus* and *Acetes* sp. found in shrimp farms (Table 3). In both the species, WSSV could be detected only by nested PCR. Interestingly, WSSV could not be detected in few samples of wild *Macrob. rosenbergii*, *Scylla serrata*, *Sesarma oceanica*, *Pseudograpsus intermedius* found in shrimp farms.

Interestingly, few samples of cultured *Macrob. rosenbergii* obtained from an inland area (250 km from coast) were positive for WSSV by both non-nested and nested PCR (Table 4). Two gill samples were positive in first-step PCR using primer pair IK 3–4. Wherever reactions were obtained in first step, it was with this primer pair. None of the animals showed any signs of WSSV infection and were apparently healthy.

Table 4
Incidence of WSSV in *Macrob. rosenbergii* cultured in inland area

No. of samples	Tissues used for PCR	Primers used for PCR and no. of samples positive			
		One-step		Two-step	
3	Gill	IK 3–4	2		
2	Gill	Lo 5–6	0	IK 1–2	1
		IK 1–2	0	IK 3–4	2
	Cuticle	IK 3–4	2		
	Stomach	IK 3–4	2		
		Lo 5–6	0	IK 1–2	0

4. Discussion

Though it has been now accepted that WSSV has a broad host range, reports on the presence of this virus in wild crustaceans are limited. Presence of this virus in wild broodstock of *P. monodon* has been reported from Taiwan (Lo et al., 1997), Japan (Itami et al., 1998) and India (Otta et al., 1999). The presence of the virus in wild crustaceans such as wild -caught shrimp (*P. japonicus*, *P. semisulcatus* and *P. penicillatus*) and crabs (*C. feriatus*, *Portunus pelagicus* and *P. sanguinolentus*) was reported by Lo et al. (1996a). Otta et al. (1999) noted that wild-caught marine crabs such as *C. cruciata* and *Matuta planipes* carried WSSV. In this study, we show for the first time that several additional wild-caught shrimps and crabs are hosts for WSSV. These include shrimps such as *Metapenaeus dobsoni*, *Parapenaeopsis stylifera*, *S. indica* and *Squ. mantis* (Table 1). In the early part of the study, we used the primer pairs Lo 1–2 (which gave amplicon size of 1447 bp) and Lo 5–6 (amplicon size, 775 bp). Later we used primer pair IK 1–2 (amplicon size, 486 bp) and IK 3–4 (amplicon size, 310 bp). When the virus load is low, there are better chances of getting positive results with small amplicons. This is evident from results in Table 1. Of the four samples of broodstock tested, two were negative by nested PCR with Lo 1–2 and Lo 5–6. The two samples tested with smaller amplicons obtainable by primers IK 1–2 and IK 3–4 were positive. One sample of *P. monodon* broodstock was negative with nested PCR using Lo 1–2 and Lo 5–6, but was positive when Lo 5–6 and IK 3–4 were used. Therefore, the 50% incidence observed with broodstock may not be truly representative of the incidence of WSSV in broodstock in India and the incidence could be even higher. In a batch of six samples of *S. indica*, only two of the gill samples were positive with primer IK 3–4. Those that were negative by non–nested reaction with IK 1–2, tested positive when nested reaction was performed using IK 3–4. When all samples are considered together, highest number of positive non-nested reaction was with IK 3–4.

Results in Table 2 confirm the results of Otta et al. (1999) that wild -caught marine crabs could be hosts for WSSV. In this study, several additional species are being shown to carry WSSV for the first time. These include *C. annulata*, *Macrop. sulcatus*, *Gelasimus marionis nitidus* and *Meto. messor*. In these crab samples, PCR positivity by non-nested reaction was noticed only with primer IK 3–4. The results of this study provide further evidence for the wide spread prevalence of WSSV in several wild crustaceans. It is interesting to note that wherever negative reactions were obtained in a particular crustacean species, the primer pairs Lo 1–2 and Lo 5–6 were used. It is possible that these species carry low viral load and therefore might yield positive reactions in nested PCR for smaller amplicons. The results of this study emphasise the need to use primers giving smaller amplicons while screening for carrier states of WSSV. Presence of WSSV in non-cultured crustaceans from shrimp farms (Table 3) suggest that *Penaeus indicus* and *Acetes* sp. can also act as source of WSSV in shrimp culture environment. Earlier, we (Otta et al., unpublished) and Kasornchandra et al. (1998) had observed histological evidence of WSSV infection in *P. indicus* and PCR results confirm that *P. indicus* could be hosts of WSSV. However, the animals examined in this study did not show any signs of disease and PCR was positive only in nested reaction indicating low viral load. Lo et al. (1996a) noted that non-cultured

arthropods such as copepods, pest crab *Helice tridens*, pest Palaemonidae prawns/shrimp ponds affected by WSSV showed presence of the virus. Experimental transmission of WSSV to *Acetes* sp. has been reported by Supamattaya et al. (1998) and our results suggest that *Acetes* sp. may be naturally infected in the shrimp pond environment.

Another interesting observation in this study is the presence of WSSV in *Macrob. rosenbergii* cultured in an inland area, over 250 km from the coast. The animals did not show any clinical signs of WSSV. Presence of WSSV in cultured *Macrob. rosenbergii* has been reported by Peng et al. (1998) and Rajendran et al. (1999). However, in these studies, the animals were cultured in coastal areas where *P. monodon* is also cultured. The detection of WSSV in *Macrob. rosenbergii* cultured in inland area where there is no *P. monodon* cultures in the vicinity can perhaps be explained by the contamination of larvae since *Macrob. rosenbergii* hatcheries are located in coastal areas and these hatcheries draw sea water which may be contaminated with WSSV. It is possible that virus is persisting in the animal without causing any disease. Sahul Hameed et al. (2000) reported that *Macrob. rosenbergii* is highly tolerant of WSSV. In this study, *Macrob. rosenbergii* was subjected to stress in the laboratory such as low oxygen stress, exposure to ammonia but the animals did not develop any signs of WSSV suggesting tolerance of this species to WSSV. Such tolerant species may contribute to the spread of the virus to distant destinations.

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