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Detection of White Spot Syndrome Virus (WSSV) in Wild Captured Shrimp and in Non-cultured Crustaceans from Shrimp Ponds in Bangladesh by Polymerase Chain Reaction

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ABSTRACT—The presence of white spot syndrome virus (WSSV) in wild *Penaeus monodon*, other wild shrimps, and in non-cultured crustaceans from shrimp ponds/ghers in Bangladesh was studied by polymerase chain reaction (PCR). Wild shrimps viz. *P. mondon*, *P. semisulcatus*, *P. indicus*, *Metapenaeus monoceros*, *M. brevicornis* and *Palaemon styliferus* were positive for WSSV. The non-cultured shrimps *M. monoceros*, *M. brevicornis*, freshwater prawn *Macrobrachium rosenbergii*, crabs *Scylla serrata* and *Pseudograpsus intermedius* from shrimp ponds/ghers were WSSV positive. This is the first study on the detection of WSSV in Bangladesh by PCR and first report of *P. styliferus*, *M. monoceros*, *M. brevicornis* and *P. intermedius* as WSSV carriers.

Key words: white spot syndrome virus, WSSV, wild crustacean, potential carrier, PCR, Bangladesh

White spot syndrome virus (WSSV) is the causative agent of a severe shrimp viral disease. A number of commercially important shrimp species such as *Penaeus monodon, P. indicus, P. japonicus, P. chinensis, P. merguiensis, P. stylirostris* and *P. vannamei* are susceptible to WSSV[1]. Certain crustaceans like mud crabs, pest crabs, pest prawns, copepods, insects, wild crabs, wild lobsters, wild shrimps and wild mantis shrimps and fresh water prawn *Macrobrachium rosenbergii* harbour this virus but are asymptomatic and have been reported as reservoirs of WSSV[2–6]. Since 1992, white spot syndrome has caused high mortalities and consequent serious damage to the shrimp culture industry in Japan, China, Taiwan, Thailand, Korea, Indonesia, India and North America[7–12]. Recently WSSV positive animals have been found in both wild and farmed shrimp populations in most of the Central and South American countries[13]. In Bangladesh, White spot syndrome first appeared in Cox's Bazar in 1994. Economic losses caused by shrimp crop failures from 21 farms (244 ha intensive pond area) in 1994 in Bangladesh were estimated at 891 metric tonnes of shrimp and valued at US$ 5.0 million[14]. Since 1994, the disease has been regularly occurring and consequently there is no intensive or semi intensive shrimp culture in Bangladesh. In this study, we have examined the presence of WSSV in different wild cultured shrimps and non-cultured crustaceans from shrimp ponds and ghers (low lying paddy fields used for shrimp culture) in Bangladesh using polymerase chain reaction (PCR).

During May 2000, wild captured shrimps viz. *P. monodon, P. semisulcatus, P. indicus, Metapenaeus monoceros, M. brevicornis* and *Palaemon styliferus* were collected from Khulna (South-Western Bangladesh) and wild captured shrimp *P. monodon* from Cox's Bazar (South-Eastern Bangladesh). The non cultured shrimps/prawns *M. monoceros, M. brevicornis, P. styliferus, Macrobrachium rosenbergii*; crabs *Scylla serrata* (mud crab) and *Pseudograpsus intermedius* (pest crab) were collected from shrimp ponds and ghers at Khulna; non cultured *P. styliferus* and *P. indicus* were collected from shrimp ponds at Cox's Bazar. Immediately after collection of shrimp/crabs, a portion of gill tissue from each animal was removed aseptically and transferred to different tubes containing 70% alcohol. The samples were brought to the laboratory at Mangalore, India for PCR analysis.

The following modified protocol of Yang *et al*[15] was used for extraction of WSSV DNA. Around 150 mg of gill tissue of shrimps or crabs were homogenized 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 fluoride (PMSF) in a disposable polythene pouch and then transferred to a microfuge tube. Four μl Triton × 100 was added to the sample and incubated for 30 min at 45°C. The sample was then centrifuged at 1,500 × g for 10 min at 4°C in a refrigerated centrifuge (Remi C-24, Remi Instruments, India) and the supernatant was transferred to another microfuge tube and recentrifuged at 16,300 × g for 30 min at 4°C. The supernatant was discarded and the pellet resuspended in 25 μl of TESP to be used for PCR.

The primers Lo 1–2 corresponded to primers 146 F1 and 146 R1 and Lo 5–6 corresponded to 146 F4 and 146 R3 described by Lo *et al*.[3,16]. The primers IK 1–2 (nucleotide binding site 464 to 484 and 1016 to 1035) were based on sequence of WSSV 1461 bp *Sal* I fragment described by Lo *et al*.[16] and would amplify 486 bp fragment. The primers IK 3–4 (nucleotide binding site 628 to 648 and 922 to 943 of the *Sal* I fragment), ampli-

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fied a 316 bp fragment internal to the fragment amplified by IK 1–2. The DNA samples extracted from *P. monodon* showing clinical signs of WSSV were used as positive control. PCR was performed and results recorded as described earlier[5].

PCR products were confirmed by dot bolt hybridization. The 316 bp PCR product was purified using Concert PCR purification kit (Gibco BRL, USA) and labelled with biotin 14-dATP using Nick Translation kit (Gibco BRL, USA). The labelled product was purified again using Concert PCR purification kit (Gibco) and used as a probe in hybridization assays. PCR products obtained from wild molluscs, water, sediment and feed were initially visualised using UV transilluminator before performing hybridization. The products were boiled for 10 min in a water bath and snap-cooled on ice. Two ml of each of the samples was spotted on to a nylon membrane (Nitran, NY 12 N, Schleicher and Schuell, Germany) and blotting was done as detailed by Dyson[17]. The DNA was fixed to the membrane using a UV crosslinker (UVC 500, Hoefer, USA) and hybridization assay performed as described by Rashtchian and Mackey[18]. The hybridized probe was detected using streptavidin alkaline phosphatase conjugate (Bangalore Genei, Bangalore) and chromogenic substrate (Bangalore Genei, Bangalore) as per manufacturer's instructions.

As shown in Table 1, 21 out of 43 samples of wild captured shrimp were positive for WSSV by nested PCR. Only 5 of these were positive by non-nested PCR with primer pair yielding 316 bp fragment. However, none of the samples were positive by non-nested PCR with primer pair yielding amplicons of higher size. Interestingly only samples of *P. monodon* were positive by non-nested PCR. Out of 6 *P. monodon* samples examined from Cox's Bazar, 2 were positive by non nested reaction and 5 by nested reaction. From Khulna area, 3/12 *P. monodon* samples were positive by non- nested reaction and 7/12 by nested reaction. None of the other species of wild captured shrimp were positive by non-nested PCR but 2/5 samples of *P. semisulcatus*, 1/4 samples of *P. indicus*, 3/9 samples of *M. monoceros*,

Table 1. Detection of WSSV in wild captured shrimps by PCR

Sampling area	Species	No. examined	No. positive by non-nested PCR		No.positive by nested PCR
			Lo1-2, Lo5-6 IK 1-2	IK 3-4	IK 1-2→IK 3-4
Cox's Bazar	*Penaeus monodon*	6	0	2	5
Khulna	*P. monodon*	12	0	3	7
	P. semisulcatus	5	0	0	2
	P. indicus	4	0	0	1
	Metapenaeus monoceros	9	0	0	3
	M. brevicornis	4	0	0	2
	Palaemon styliferus	3	0	0	1
	Total	**43**	**0**	**5**	**21**

Table 2. Detection of WSSV in non-cultured crustaceans from shrimp ponds/ ghers by PCR

Sampling area	Species	Source pond/gher	No. examined	No. positive by non-nested PCR		No. positive by nested PCR
				Lo1-2, Lo5-6 IK 1-2	IK3-4	IK 1-2→IK 3-4
Khulna	*M. monoceros*	Pond	4	0	1	4
		Gher	7	0	0	5
	M. brevicornis	Pond	2	0	1	2
		Gher	3	0	0	2
	P. styliferus	Pond	2	0	0	2
		Gher	4	0	0	2
	Macrobrachium rosenbergii	Gher	9	0	0	7
	Scylla serrata	Pond	3	0	2	3
		Gher	6	0	0	4
	Pseudograpsus intermedius	Pond	5	0	3	5
		Gher	4	0	1	2
Cox's Bazar	*P. styliferus*	Pond	2	0	0	1
	P. indicus	Pond	3	0	0	2
	Total		**54**	**0**	**8**	**41**

2/4 samples of *M. brevicornis* and 1/3 samples of *P. styliferus* were positive by nested PCR.

All species of non-cultured crustaceans from shrimp ponds tested were positive for WSSV (Table 2). None of the samples were positive by non-nested PCR when primers yielding amplicons of higher size were used (1.4 kb to 486 bp). Only 8/54 samples were positive by non-nested PCR yielding product of 316 bp while 41/54 were positive by nested PCR. *M. rosenbergii, P. styliferus* and *P. indicus* were positive only in nested PCR while some samples of *M. monoceros, M. brevicornis, S. serrata* and *P. intermedius* were positive also by non-nested PCR. From Khulna area samples were drawn from both ponds and ghers and among these 100% of pond samples were positive for WSSV by nested PCR while some samples in each species from ghers were negative.

PCR is a highly sensitive technique for detection of WSSV and using this technique the virus can be detected even when it is present in very low numbers in asymptomatic or carrier animals[3–5]. Results in Table 1 show that 49% of wild caught shrimp were positive for WSSV when nested PCR was used while only 12% were positive when non-nested PCR was used. The sensitivity of PCR varies depending on the size of amplicons and generally primers yielding smaller amplicons will be able to detect lower levels of target organisms[5]. The results of this study support this. Nested PCR increases the sensitivity of detection by about 10^3–10^4 times[3]. The observation that most samples were positive in nested PCR suggests that the viral load in the animals is very low. The animals tested did not show any clinical signs of WSSV and therefore detection of virus by nested PCR suggests that the animals are asymptomatic carriers.

Earlier studies have shown that wild shrimp such as *P. japonicus, P. semisulcatus, P. penicillatus*[3], *Metapenaeus dobsoni, Parapenaeopsis stylifera, Solenocera indica* and *Squilla mantis*[2] are carriers of WSSV. In this study, we are showing for the first time that *Metapenaeus monoceros, M. brevicornis, Pseudograpsus intermedius* and *Palaemon styliferus* are carriers of WSSV. Presence of WSSV in 49% of wild caught shrimp from Bangladesh show that the virus is widely prevalent in the marine environment in Bangladesh. The results should be of value to shrimp farmers and aquaculture managers in Bangladesh.

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