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EFFECT OF STORAGE (-20°C) OF WHITE SPOT SYNDROME VIRUS (WSSV) INFECTED SHRIMP TISSUE ON PATHOGENICITY TO *PENAEUS MONODON* AND *MACROBRACHIUM ROSENBERGII*

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Abstract: The WSSV infected shrimp samples stored at -20°C for one year was found to be infectious to laboratory reared *Penaeus monodon*. Intramascular injection with 0.1 ml viral inoculum could produce 100% mortality by 48 hrs in experimental shrimps. All the infected moribund shrimp tested positive for WSSV by non-nested PCR. Freshwater prawn, *Macrobrachium rosenbergii* was found to be tolerant to WSSV in experimental condition with intramascular injection. *M. rosenbergii* carry WSSV for a long period without showing any clinical signs of infection. It was observed that the stomach tissue is not the favorable target for WSSV in M. rosenbergii.

Key words: *P. monodon, M. rossenbergii*, WSSV, PCR, Pathogenicity

INTRODUCTION

White spot syndrome (WSS), a shrimp disease caused by the white spot syndrome virus is responsible for mass mortalities of shrimp and thereby cause huge economic losses. The disease was first reported in *Penaeus japonicus* in northeastern Taiwan in 1992 (Chou *et al.* 1995). Since then, the disease has spread into virtually all shrimp growing countries in Asia (Inouye *et al.* 1994; Chou *et al.* 1995; Larkins, 1995; Wongteerasupaya *et al.* 1995; Lo *et al.* 1996a, b; Karunasagar *et al.* 1997, Park *et al.* 1998; Magbanua *et al.* 2000) and America (Nunan *et al.* 1998, Jory 2000, Yap 2001). The WSSV has brought the cultured penaeid shrimp industries in Asia and the Latin America to a critical condition. The losses caused by WSS has been estimated to be approximately US$ 1 billion in China in 1993, US$ 500 million in Thailand in 1996 (Wang *et al.* 1998) and about US$ 250-300 million in India in 1994-95 (Karunasagar *et al.* 1998). Due to this diseas, the loss was estimated to be US$ 5 million from only 21 farms in Bangladesh in 1994 (Larkins 1995).

The signs of the disease are the appearance of white spots on the inner surface of the carapace and shell, reddish-pink body coloration and rapid reduction in food consumption by the victim (Lo *et al.* 1996a, Durand *et al.* 1997, Karunasagar *et al.* 1997). The WSSV is extremely virulent and has a wide host

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range among penaeid shrimps and other crustaceans including non-penaeid shrimps, prawns, squilla, crabs, crayfishes, etc. (Lightner 1996; Lo *et al.* 1996a,b; Maeda *et al.*1998; Otta *et al.* 1999; Hossain *et al.* 2001 a,b). Most of the penaeid shrimps are susceptible to the WSSV infection (Takahashi *et al.* 1994, Lightner 1996, Wang *et al.* 2000). Among cultured shrimps, the cumulative mortality caused by this disease can reach as high as 100% within 3-10 days of the onset of clinical signs (Nakano *et al.* 1994; Chou *et al.* 1995; Sahul Hameed *et al.* 1998).

The WSSV has been found to be highly pathogenic to *P. monodon*, *P. indicus*, *P. japonicus* and other cultured penaeid shrimps (Sahul Hameed *et al.* 1998, Jory 2000). The information regarding the pathogenicity of WSSV in frozen shrimps is limited. The aim of the present study was to detect WSSV in shrimp stored at -20°C and to study the pathogenicity of the WSSV to *P. monodon* and freshwater prawn *Macrobrachium rosenbergii* using samples of shrimp naturally infected with WSSV, stored at -20°C.

MATERIAL AND METHODS

Collection of WSSV infected shrimp : A batch of about 40 cultured shrimp, *Penaeus monodon* (each weigh 20-25 g), naturally infected by white spot syndrome viruses were collected from a shrimp farm at Kundapur, India and kept them in a freezer at -20°C. Every two months, two frozen shrimps were taken out for DNA extraction.

Collection of test animals for infectivity study : A batch of adult shrimps, *P. monodon* (each weigh 25-30 g) were collected from a pond at katpadi, India and they were transported to the laboratory. All the shrimps were maintained in two aerated tubs each containing 120 litre of 20 ppt seawater at room temperature (27 - 28°C) for two weeks to acclimatize them prior to the experiment. The shrimps were fed on a dry commercial shrimp feed diet twice a day at 3% of the body weight. A batch of adult freshwater prawns, *Macrobrachium rosenbergii* (each weigh 25 - 30 g) were collected from a prawn farm located at Mysore (250 km east of Mangalore) and maintained in 120 litre tank at room temperature for 10 days and fed with artificial pelleted feed.

WSSV DNA extraction: The WSSV DNAs were extracted from infected shrimps and the experimental shrimps (*P. monodon* and *M. rosenbergii)* using rapid extraction method previously described by Hossain *et al.* (2001 b). Around 150 mg of shrimp tissue including gill, pleopod, cuticle and stomach was homigenised 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) in a

disposable UV sterilized polythene sachet and then transferred to a microfuge tube. After adding 4 ml Trition x 100, the sample was incubated for 30 min at 45°C and then centrifuged at 1,500 × g for 10 min at 4°C in a refrigerated centrifuge (Remi C 24, Remi Instruments, India). The supernatant was transferred to another microfuge tube and recentrifuged at 16,300 × g for 30 min at 4°C. The pellet was suspended in 400 µl TESP buffer and centrifuged again at 16,300 × g for 30 min at 4°C. The pellet was finally resuspended in 25 µl of TESP for PCR.

Polymerase chain teaction (PCR) amplification of WSSV DNA: The primer pairs Lo 1-2, Lo 5-6, IK 1-2 and IK 3-4 were used to amplify the WSSV DNA. The primers designated Lo 1-2 (amplicon size 1447 bp) corresponded to primers 146 F1 and 146 R1 and Lo 5-6 (775 bp) corresponded to 146 F4 and 146 R3 (Lo et al, 1996a,b). The primers named IK 1-2 (486 bp), internal to fragment amplified by Lo 5-6 and primers named IK 3-4(316 bp), internal to fragment amplified by IK 1-2 were based on sequence data from Gene bank accession No. U50923. The DNA extracted from *P. monodon* naturally infected with WSSV served as positive control and DNA from juvenile shrimp negative for WSSV by nested PCR was used as negative control. PCR reaction was carried out in sterile PCR tubes (PCR-03-C, Axygen, USA). A 50.0 µl of reaction mixture that consisted 5.0 µl of Taq polymerase assay buffer (2 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 50 mgml-1 nuclease free BSA), 1.0 ml (0.5 mg) of each primer, 1.0 µl (200 µM) dNTP mix, 3.0 µl of template DNA and 0.60 µl (2.25 units) Taq DNA polymerase. For nested PCR, 5.0 µl of the I-step PCR product was added to 45.0 µl of PCR cocktail containing the internal primer (IK 3-4). The components were mixed gently and PCR was performed in a PTC 100 thermocycler (MJ Reserach Inc, USA) for 30 eycles, each cycle consisting of a denaturation of target DNA at 94°C for 1 min, annealing of primers at 50°C for 1 min and extension of primers at 72°C for 2 min. The programme included an initial delays and final of 5 min each at 94°C and 72°C, respectively.

Agarose gel electrophoresis: A 20 µl PCR product was subjected to electrophoresis in agarose gels containing 0.5 µg/ml ethidium bromide. For more than 700 bp product, 1% agarose gel was used, for 486 and 316 bp, 1.5% agarose gel was used. The gel was then observed on a UV transilluminator and photographed using Gel doc (Pharmacia Biotech, USA).

Preparation of viral inoculum: The *P. monodon* stored for 1 year and for 2 months at -20°C were used to prepare viral inoculum. The Gill tissues from the shrimps were homogenized in 1% NaCl. After centrifugation at 1000 × g for 10 min, the supernatant fluid was filtered (0.2 mm filter) and used as inoculum.

Infection via intramuscular injection: Eight *P. monodon* (Weighing 25-30 g each) maintained separately in well aerated 12 litre injected intramuscularly with the WSSV preparation from the shrimps stored at -20°C for 1 yr. A similar experiment was performed with the six *M. rosenbergii* maintained as described before. However, in this case, WSSV preparation was made from the shrimps stored at -20°C for 2 months. The experimental prawns were observed for mortality. The PCR was performed to detect the WSSV in the different tissues viz. gills, pleopods, cuticles and stomachs.

RESULTS AND DISCUSSION

The WSSV was delected in all the samples stored at -20°C till 14 months and examined at 2 months intervals (Table 1). Except the 2 samples at the 6th month and at the 10th month), all the samples tested positive for the WSSV by non-nested reaction with all the primer pairs used (Lo 1-2, Lo 5-6, IK 1-2 and IK 3-4). The primers IK 3-4 were not used if the samples were positive by other primers. One sample at 6th month batch was not positive by primers Lo 1-2 and Lo 5-6, but positive by both IK 1-2 and IK 3-4 in no-nested reaction. Another sample at 10th month batch was not positive by only primer pair Lo 1-2, but was positive by other primer pairs. The PCR results of the WSSV for representative sample using different primer sets are shown in Fig. 1.

Table 1. Detection of WSSV in the infected *Penaeus monodon* tissues stored at - 20°C

Period of shrimp storing (months)	Smple No.	PCR result by non-nested reaction with diff. primer sets			
		Lo 1-2	Lo 5-6	IK 1-2	IK 3-4
Initial	1	+	+	+	NT
	2	+	+	+	NT
2	1	+	+	+	NT
	2	+	+	+	NT
4	1	+	+	+	NT
	2	+	+	+	NT
6	1	+	+	+	NT
	2	+	+	+	NT
8	1	+	+	+	NT
	2	+	+	+	NT
10	1	+	+	+	NT
	2	+	+	+	NT
12	1	+	+	+	NT
	2	+	+	+	NT
14	1	+	+	+	NT
	2	+	+	+	NT

NT = not tested; + = positive response; - = negative response.

The Pathogenicity was tested by after intramuscular injection of 0.1 ml filtrate (inoculum) of the WSSV infected one year stored shrimp samples to 8 healthy *P. monodon*. All the 8 shrimps died between 25 hrs to 48 hrs. All the infected shrimps exhibited pathological signs of white spot disease, with pink to reddish body colaboration. White spots appeared on the carapace after 24 hrs of injection. No pathological signs, lethargy or mortality were seen in 2 saline injected control shrimps. The gill tissues from all the moribund or recently desad shrimps tested postivie by non-nested PCR reaction with all the primer pairs (Lo 1-2, Lo 5-6 and IK 1-2).

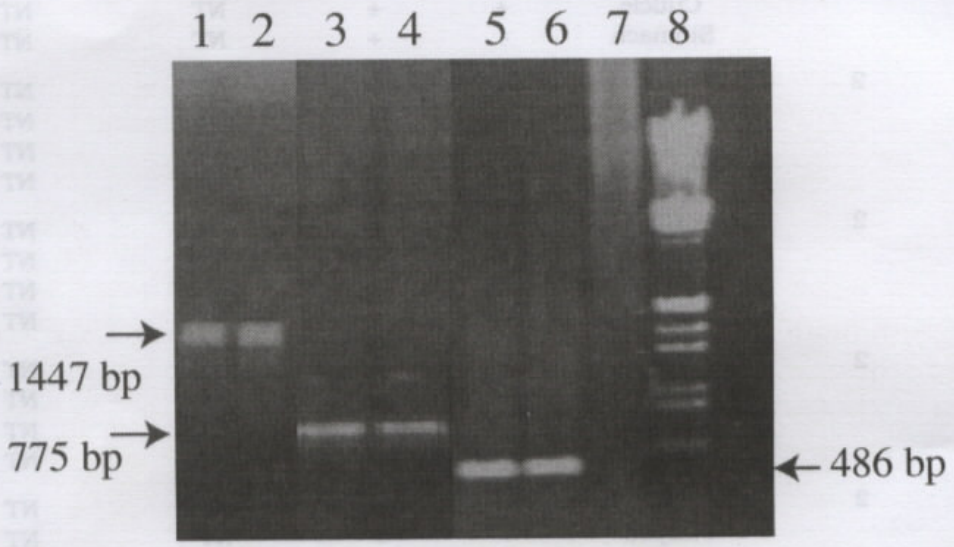

Fig. 1. Agarose gel electrophoresis of non-nested PCR of *P. monodon*. Lane 1, 3 & 5 - *P. monodon* amplified by primer pair Lo 1-2 (1447 bp), Lo 5-6 (775 bp) and IK 1-2 (486 bp), respectively. Lane 2, 4 & 6 - positive control, Lane 7 - Negative control, Lane 8 - Molecular wt marker (x DNA/Eco RI Hind III Double Digest).

As shown in Table 2, out of 6 *M. rosenbergii* injected intramusculary by the viral isolates of the WSSV (stored for 2 months at -20°C), 2 survived till two to three days. Form them the WSSV was detected from the gills, pleoponds and cuticle tissues by non-nested PCR with primers Lo 5-6 and all the tissues such as gills, pleopods, cuticles and stomachs were positive by non-nested PCR with primer pair IK 1-2. One of the prawn survived till 18 days and its gills, pleoponds and cuticle tissues were positvie by non-nested PCR with IK 1-2 and the stomach tissue by only IK 3-4. Another prawn survived till 52 days and only gill tissue was positive by non-tested PCR with 1K 3-4 and other tissues were positive by nested PCR (IK 1-2 and IK 3-4). The 5th and 6th prawns survived up to 74 and 127 days and the tissues, except stomach, were WSSV positve only by nested PCR (IK 1-2 and IK 3-4).

Table 2. Experimental pathogenicity of the WSSV to *M. rosenbergii* after intramascular injection of the WSSV innoculum

Sample No.	The prawn survival of after injection (in days)	Tissue examined for WSSV	PCR result for WSSV: Non-nested PCR		Nested PCR	
			Lo 5-6	IK 1-2	IK 3-4	IK 1-2 → IK 3-4
MR1	2	Gill	+	+	NT	NT
		Pleopod	+	+	NT	NT
		Cuticle	+	+	NT	NT
		Stomach	-	+	NT	NT
MR1	2	Gill	+	+	NT	NT
		Pleopod	+	+	NT	NT
		Cuticle	+	+	NT	NT
		Stomach	-	+	NT	NT
MR1	2	Gill	+	+	NT	NT
		Pleopod	+	+	NT	NT
		Cuticle	+	+	NT	NT
		Stomach	-	+	NT	NT
MR1	2	Gill	+	+	NT	NT
		Pleopod	+	+	NT	NT
		Cuticle	+	+	NT	NT
		Stomach	-	+	NT	NT
MR1	2	Gill	+	+	NT	NT
		Pleopod	+	+	NT	NT
		Cuticle	+	+	NT	NT
		Stomach	-	+	NT	NT
MR1	2	Gill	+	+	NT	NT
		Pleopod	+	+	NT	NT
		Cuticle	+	+	NT	NT
		Stomach	-	+	NT	NT
MR1	2	Gill	+	+	NT	NT
		Pleopod	+	+	NT	NT
		Cuticle	+	+	NT	NT
		Stomach	-	+	NT	NT
MR1	2	Gill	+	+	NT	NT
		Pleopod	+	+	NT	NT
		Cuticle	+	+	NT	NT
		Stomach	-	+	NT	NT

NT = not tested.

As shown in Table 1, the WSSV was dected in all the stored (-20°C) shrimp samples tested at every 2 months interval till 14 months. The pathogenicity tests on *P. monodon* showed pathological signs of white spots and pink-reddish coloration and died after variable time periods. Wongeerasupaya *et al.* (1995) observed first signs of pathology after 6 hrs of injection with WSSV. The development of reddish or pink-reddish color ation of moribund shrimp due to

WSSV infection have also been reported by Nakano *et al.* (1994); Durand *et al.* (1997); Sahul Hameed *et al.* (1998). Sahul Hameed *et al.* (1998) observed the clinecal signs of WSSV after 24 hrs post infection. They also observed 100% mortality by 48 hrs for *P. monodon* and 72 hrs for *P. indicus* of shrimp injected (i.m.) by WSSV. Nunan *et al.* (1998) reported that WSSV in the frozen imported products are infectious to shrimp. From this result it can be concluded that WSSV in frozen shrimp (– 20°C) could be infectious to *P. mondon* even after 1 year.

The fact that tha treated *M. rosenbergii* survived for a long period (up to 127 days) without showing any clinical signs of white spots. But, the virus was detected by nested PCR which suggests that the prawns were tolerant to the WSSV and they were symptomatic carriers. The WSSV was detected from gills, pleopods, and cuticle tissus but not detectd from the stomach tissue of the prawn. This suggests that the stomach was not the favorite target for WSSV in *Macrobrachium* sp. Peng *et al.* (1998) showed that *M. rosenbergii* is susceptible to WSSV. According to them infection by WSSV may vary in larval, post larval, juvenile and adult stages of *M. rosenbergii* and larvae showed stronger positive signals than the post larvae. On the other hand, Flegel (1996) listed the freshwater prawn as a species resistant to WSSV. Rajendran *et al.* (1999) and Sahul hameed *et al.* (2000) also reported that *M. rosenbergii* is highly tolerant to WSSV. In this study, *M. rosenbergii*, which survived for more than 50 days received feed and oxygen stress, by the prawns did not develop any signs of WSSV suggesting tolerance of this species to WSSV. Such tolerance species may contribute to the spread of the virus to distant destinations.

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