



Note

Occurrence of pathogenic shrimp viruses in selected wild crab species of Sunderban, India

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ABSTRACT

Diseases in brackishwater aquaculture systems are the major hindrance to the growth of shrimp industry in India, with white spot viral disease being the most devastating one causing crop failure leading to heavy economic losses. In this study, wild crab species *viz.*, *Uca* spp. and *Sesarma* spp., which are frequent dwellers surrounding the shrimp culture ponds, were screened for the presence of white spot syndrome virus (WSSV), infectious hypodermal and haematopoietic necrosis virus (IHHNV) and hepatopancreatic parvovirus (HPV). Out of total 66 samples screened, presence of WSSV and IHHNV was detected in 18.2 and 1.5% of the samples, respectively. Results of the present study indicate that wild crab species found in and around shrimp ponds could act as carriers of shrimp pathogenic viruses and their effective control is essential for prevention of shrimp viral diseases.

Keywords: *Sesarma* spp., Shrimp viruses, *Uca* spp., Wild crabs

During 2013-14, India earned ₹3,02,132.6 million through export of fish and fishery products and the contribution of frozen shrimps alone was 64.11% (₹193683 million) on value basis (MPEDA, 2015). In spite of the tremendous growth of shrimp industry in India, both in terms of domestic and export market, very often diseases of varied etiology cause heavy damage to the industry leading to severe economic losses (Flegel *et al.*, 2008). Among different shrimp pathogens, the white spot syndrome virus (WSSV) is considered as the most important one, which causes heavy crop failure all over the world (Rosenberry, 2001). The main source of WSSV infection in shrimp is the infected broodstock. However presently, the use of specific pathogen free (SPF) broodstock helps to reduce the chances of vertical transmission of WSSV from broodstock. But, the contaminated environment, including water bodies remains a potential source of this viral infection. Different living vectors in and around aquaculture ponds are also responsible for transmission of WSSV to the cultured shrimps (Vijayan *et al.*, 2005). Infectious hypodermal and haematopoietic necrosis virus (IHHNV) and hepatopancreatic parvovirus (HPV) also cause economic losses to the farmers, although the mortality by these diseases is less than that of WSSV (Fauce *et al.*, 2007; Xia *et al.*, 2015). Different species of crabs very often act as carriers of WSSV transmission to cultured shrimps (Meng *et al.*, 2009). The wild crab *Uca* spp. (fiddler crabs) and *Sesarma* spp. (marsh crabs) are common dwellers around

shrimp culture ponds. WSSV has been experimentally transmitted from these crabs to *Penaeus monodon* (Kanchanaphum *et al.*, 1998). These crab species are the common inhabitants of intertidal mangrove zones of Asia-pacific region (FAO, 2015). Sunderban is considered as the largest mangrove forest of the world and this region is very important as far as brackishwater aquaculture is concerned. In India, till date, no work has been reported on the study of occurrence of different shrimp pathogenic viruses in these species of crabs. Keeping these in view, the present study was undertaken to examine the occurrence of WSSV, IHHNV and HPV in *Uca* spp. and *Sesarma* spp. in Sunderban area of West Bengal.

Crab samples belonging to *Uca* spp. and *Sesarma* spp. were collected from different shrimp culture areas of Sunderban, of South 24 Parganas District, West Bengal, India. A total of 66 crab samples (23 samples of *Uca* spp. and 43 samples of *Sesarma* spp.) were collected during the period July, 2014 to August, 2015 from different shrimp growing areas of Sunderban. All the samples were collected in individual sterile polypropylene bottles. On arrival to laboratory, the samples were stored at -40°C till processing.

For detection of WSSV, two methods were followed *viz.*, single tube WSSV detection kit (Genei Merck Bioscience, India) and protocol of Lo *et al.* (1996) for comparison of results. Portions of heart, muscle and gill were collected separately from each crab sample. A total of

30 mg of sample (heart, muscle and gill pooled together) were used for extraction of DNA. The tissue was blended in 300 µl of DNA extraction buffer (provided with the kit) followed by heating at 95°C for 10 min. Then the vials containing blended tissue were centrifuged at 10,000 g for 10 min and 2 µl from the supernatant was used as PCR template. Another set of samples, consisting of similar type of tissues (heart, muscle and gill pooled together), were processed for WSSV nested PCR (Lo *et al.*, 1996) and IHNV specific PCR. Tissue processing was done as per the method of Kiatpathomchai *et al.* (2001) with slight modification. Briefly, 30 mg of tissue was homogenised in a 1.5 ml microcentrifuge tube with 300 µl of buffer containing 0.025 N NaOH and 0.0125% sodium dodecyl sulfate, using a sterile micropestle, incubated in a boiling water bath for 10 min and was placed immediately on ice. The suspension was centrifuged at 10,000 g for 10 min and 2 µl from the supernatant was used as template for PCR reaction. For preparation of template DNA for detection of hepatopancreatic parvovirus (HPV), 20 mg of crab tomalley was used. Isolation of DNA template was carried out as per the protocol of Manjanaik *et al.* (2005).

PCR for WSSV using single tube detection kit was carried out in a 30 µl reaction volume as per manufacturer's instructions. In PCR assay, three different annealing temperatures (70, 58 and 55°C for 5, 18 and 25 cycles, respectively) were used, as mentioned in the instruction manual.

WSSV nested PCR was carried out as per the OIE protocol of Lo *et al.* (1996) with a slight modification using primer pairs 146F1 (5'-ACTACTA ACTTCAGCCTATCTAG-3')/146R1 (5'-TAATGCGGG TGTAATGTTCTTACGA-3') for first step PCR and 146F2 (5'-GTAAGTCCCTTCCATCTCCA-3')/146R2 (5'-TACGGCAGC TGCTGCACCTTGT-3') for nested PCR. In the first step, PCR was run in a 25 µl reaction volume containing 10 pmol of each primer (146F1 and 146R1), 1 U Dream *Taq* DNA polymerase (ThermoFisher Scientific, USA), 200 µM of each dNTP, 2 µl of template and 2.5 µl of 10x Dream *Taq* Buffer with 20 mM MgCl₂ (final concentration 1x Dream *Taq* buffer with 2 mM MgCl₂). Dream *Taq* buffer also contained KCl and (NH₄)₂SO₄ at a ratio for optimised performance of Dream *Taq* DNA polymerase as mentioned in the product information. The first step PCR was carried out in a thermocycler (Mastecycler, Eppendorf, Germany) as per Thakur *et al.* (2002), but the number of total cycles was limited to 30. In the second step PCR, 2 µl of product of first step PCR was used as template. The reaction mixture of second step PCR was similar to that of first step PCR with the primer pair (146F2/146R2) being different. The PCR cycling condition remained

same as that of first step PCR, except for the annealing temperature used (65°C instead of 55°C). PCR assay for IHNV was carried out using the primers 392F (5'-GGGCGAACCAGAATCACTTA-3') and 392R (5'-ATCCGAGGAATCTGATGTG-3') (Tang *et al.*, 2000; OIE, 2015). The PCR reaction mixture (25 µl) used was similar to that of WSSV first step PCR mentioned previously with the exception of primer pair 392F/392R. The amplification was carried out at 55°C annealing temperature, using the cycling protocol as mentioned by Tang *et al.* (2000). Detection of HPV was done by nested PCR (Manjanaik *et al.*, 2005). The primers H441F (5'-GCATTACAAGAGCCAAGCAG-3') and H441R (5'-ACACTCAGC CTCTACCTTGT-3') were used for first step PCR (Phromjai *et al.*, 2002; Manjanaik *et al.*, 2005; OIE, 2007). In second step PCR, 2 µl of product of first step PCR was amplified using the forward primer 5'-ATAGAACGCATAGAAAACGCT-3' and reverse primer 5'-GGTGGCGCTGGAATGAATC GCTG-3' as per Manjanaik *et al.* (2005). First step PCR was done following the protocol as optimised by Phromjai *et al.* (2002). The cycling condition as per Manjanaik *et al.* (2005) was used for second step PCR. The expected sizes of the amplified products were 441 and 265 bp for first and second step PCR, respectively. In each case, electrophoresis of the PCR products along with molecular weight markers was done on 1.5% agarose gel containing 0.3 µg ml⁻¹ ethidium bromide in 0.5 x TBE buffer at 6 V per cm for 1 h 15 min and was observed and photographed in a gel documentation system (GBOX, Syngene, UK).

Out of a total of 66 samples, 11 samples (6 samples of *Uca* spp. and 5 samples of *Sesarma* spp.) were positive for WSSV by single tube detection kit (Genei, Merck Bioscience, India). The infection level in all the positive samples were found to be of 'low' category, as evidenced by the presence of one band of 204 bp with the exception in a sample of *Sesarma* spp., in which the WSSV level was found to be moderate (presence of two bands of 525 and 204 bp). Only one sample of *Sesarma* spp. was found positive to WSSV in the first step of WSSV specific nested PCR using primers 146F1/146 R1, as evidenced by 1447 bp amplified product. However, in the second step PCR, 12 samples including 6 samples of *Uca* spp. and 6 samples of *Sesarma* spp. were found positive for WSSV showing 941 bp amplified product. All the 11 samples, which were found positive by single tube WSSV detection kit, were also detected as positive in the WSSV specific two-step PCR. The sample, which was detected positive in first step, was found moderately infected in single tube WSSV detection kit. Out of a total of 66 samples, one sample of *Sesarma* spp. procured from Kakdwip was found positive to IHNV, as evidenced by the presence of 392 bp band in PCR using 392F / 392 R primers. However,

none of the crab samples screened was found positive to HPV in both first step and second step PCR (Table 1).

In this study, presence of WSSV was detected in 12 out of 66 samples tested (18.2%). Both *Uca* spp. and *Sesarma* spp. are found common in brackishwater aquaculture areas of different regions (Kanchanaphum *et al.*, 1998). Though WSSV viral load was found 'low'

The current study showed that the wild crab species (*Uca* spp. and *Sesarma* spp.) can carry both WSSV and IHNV. The presence of HPV was not detected in any of the crab samples tested. But both IHNV and HPV share common ecological niche and host species. The occurrence of WSSV and IHNV indicates the risk of presence of HPV, too. Thus, it can be stated that the wild crabs occurring in the brackishwater aquaculture

Table 1. Occurrence of WSSV, IHNV and HPV in wild crabs (*Uca* spp. and *Sesarma* spp.)

Crab samples	No. of samples	WSSV (detected by single tube detection kit)	WSSV (detected by nested PCR, Lo <i>et al.</i> , 1996)		IHNV	HPV
			1 st step	2 nd step		
<i>Uca</i> spp.	23	6*	0	6	0	0
<i>Sesarma</i> spp.	43	5**	1	6	1	0
Total	66	11 [#]	1	12	1	0

* All six positive samples were of 'low' category (Only one band of 204 bp)

** One positive sample was of moderate category (two bands of 525 and 204 bp) and other four were of 'low' category (Only one band of 204 bp)

[#] Ten positive samples were of 'low' category and one sample was of 'moderate' category

in most of the positive samples, and only one sample was positive in first step PCR, there is a potential risk of transmission of those viruses to the cultured shrimp. The samples, which were detected positive by single tube WSSV detection kit (Genei, Merck Bioscience, India), were also detected positive in nested PCR (Lo *et al.*, 1996). However one sample, which was found negative by single tube WSSV detection kit, was detected positive in second step nested PCR. This may be due to the fact that the two-step nested PCR is more sensitive than the single tube detection kit, which uses one sense and three anti-sense primers. However, advantage of single tube detection PCR is that it saves time. In addition to WSSV, one sample of crab was also found to harbour IHNV as detected by PCR using 392F/392R primers. However, none of the crab samples was found positive to HPV (Table 1).

White spot disease (WSD) caused by WSSV is one of the major limiting factors for the growth of shrimp industry, causing crop loss leading to heavy economic loss. The first dreaded outbreak of WSSV was reported in 1994 in black tiger shrimp, affecting vast areas from Visakhapatnam in Andhra Pradesh to Sirkali in Tamilnadu (Shankar and Mohan, 1998). With the passage of time, WSD affected various farms engaged in brackishwater aquaculture activities in the country and still this disease remains a challenge to the shrimp farmers globally, including India. Although, IHNV and HPV generally do not cause heavy mortality in commonly cultured shrimp species in India, both diseases cause stunted and slow growth in shrimp leading to production loss (Rai *et al.*, 2009). However, IHNV causes severe infection in pacific blue shrimp (*P. stylirostris*) leading to heavy mortality (Bell and Lightner, 1984).

sites may act as a potential source of different shrimp viruses, including WSSV, and their effective control is essential for prevention of viral diseases in brackishwater aquaculture systems.

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