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Occurrence of white spot syndrome virus and *Vibrio parahaemolyticus* in brackishwater shrimp culture systems of Sundarban, West Bengal, India

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ABSTRACT

White spot disease (WSD) and acute hepatopancreatic necrosis disease (AHPND) are two important and highly fatal diseases of cultured shrimps. The occurrence of white spot syndrome virus (WSSV) was found to be very high in brackishwater aquaculture systems of Sundarban area in West Bengal, with 40.6% of the samples being positive of which 18.75% being positive in the first step PCR. The prevalence of WSSV in traditional and semi-intensive culture systems was found as 59.3 and 27%, respectively. On the other hand, presence of *Vibrio parahaemolyticus* was detected in 53.1, 55.5 and 75.9% of the shrimp, water and soil samples, respectively. All the isolates of *V. parahaemolyticus* contained *toxR* gene as detected by PCR. However, none of the isolates was realised as AHPND causing isolate by PCR. Moreover, *tdh* gene, which causes gastroenteritis in human beings, was also not detected in any of the isolates. The present study indicated that WSSV is the major cause of crop failure in the brackishwater shrimp culture systems of Sundarban, India. Although, *V. parahaemolyticus* is highly prevalent in brackishwater shrimp culture systems of Sundarban, strains causing gastroenteritis or AHPND were not detected during the present study.

Keywords: Acute hepatopancreatic necrosis disease, AHPND, Aquaculture, Gastroenteritis, Shrimp, *Vibrio parahaemolyticus,* White spot syndrome virus, WSSV

Introduction

White spot syndrome virus (WSSV) is a devastating pathogen of penaeid shrimps causing white spot disease (WSD), which is a major cause of crop failure in India as well as other shrimp producing countries. In India, the gross national loss due to this disease was 48,717 t of shrimps, with a value of ₹10,220 million during 2006-08 (Kalaimani *et al.*, 2013). Since the first outbreak of this disease in Taiwan during 1992, WSD played havoc in shrimp farms of different shrimp growing countries across the world. The *Whispovirus*, a double stranded DNA virus under family Nimaviridae, is recognised as the causative agent of WSD (OIE, 2009). In India, almost all the areas practicing brackishwater shrimp culture had occurrence of this disease.

Different species of *Vibrio* are extensively distributed in brackishwater aquaculture environment. *Vibrio parahaemolyticus* is an important pathogen, both for human beings and shrimps. This pathogen causes vibriosis, which affects both hatchery production and grow-out culture of shrimps, leading to heavy economic losses (Saulnier *et al.*, 2000). Of late, some strains of this pathogen have been designated as the causative agent of acute hepatopancreatic necrosis disease (AHPND), which has been observed to be a devastating malady in cultured shrimps, very often, leading to 100% mortality and total crop loss (Tran *et al.*, 2013). This disease caused mayhem in different shrimp growing nations of south-east Asia and Mexico since 2009 with a high degree of financial loss to the farmers. *V. parahaemolyticus* strains bearing 69 kb pVPA3-1 plasmid are responsible for this disease (Han *et al.*, 2015). On the other hand, in case of human beings, *V. parahaemolyticus* causes gastroenteritis and is transmitted usually through consumption of raw and improperly cooked seafood (DePaola *et al.*, 1990). The pandemic strain of *V. parahaemolyticus* O3:K6 is known to cause acute gastroenteritis in human beings (Matsumoto *et al.*, 2000). Two genes *tdh* and *trh*, that encode thermostable direct haemolysin (TDH) and TDH-related haemolysin (TRH), are considered as responsible for this gastroenteritis related virulence (Shirai *et al.*, 1990).

The Sundarban delta is the largest single block of tidal halophytic mangrove forest in the world, spreading over southern part of Bangladesh and West Bengal State of India. Indian part of Sundarban, which is around 4110 km^2 spreading over two districts (North and South 24 Parganas), is of utmost importance for brackishwater aquaculture, due to abundance of desired saline water in different

riverine systems. Like other shrimp growing places in India, Sundarban area also faces the problem of WSD very often. However till date, there is no confirmed report on occurrence of AHPND in India. However, in the wake of many outbreaks of AHPND in various shrimp growing nations in the recent past, it is also very much required to screen shrimp samples for *V. parahaemolyticus*. Till date, no attempt was made to assess the occurrence of AHPND causing *V. parahaemolyticus* in brackishwater aquaculture system of this area. In this context, the present study was undertaken to assess the occurrence of WSSV and *V. parahaemolyticus* in brackishwater aquaculture systems of Sundarban area of West Bengal.

Materials and methods

Sampling location

The shrimp, pond water and pond soil samples were collected from different shrimp farms of Sundarban area of West Bengal, covering two districts, namely North 24 Parganas and South 24 Parganas during January, 2014 to June, 2016. The sampling area lies between latitude 21.539 N to 22.544 N and longitude 88.012 E to 88.921 E.

Isolation and identification of V. parahaemolyticus

A total of 138 samples comprising 64, 45 and 29 nos. of shrimp, pond water and pond bottom soil samples, were screened for the presence of *V. parahaemolyticus*. For isolation of the organisms from shrimp, approximately 50 µl of haemolymph was aseptically collected, using a 2 ml syringe and inoculated into 10 ml of alkaline peptone water (APW). In case of pond soil samples, 10 g of respective samples were inoculated into 90 ml of APW. It was incubated at 30°C for 18 h and was streaked on to tryptone soy agar supplemented with triphenyltetrazolium chloride (TSAT agar) (Kourany, 1983). The plates were incubated at 37°C for 24 h. Smooth, bright red colonies were presumptively selected as *V. parahaemolyticus* colonies. They were reconfirmed by streaking on thiosulphate citrate bile salt sucrose (TCBS) agar containing 3% NaCl to select sucrose non-fermenting green colonies. The identification of the cultures was carried out by Gram-staining and different biochemical tests including catalase, cytochrome oxidase, triple sugar iron, glucose oxidation-fermentation tests, arginine dehydrolase, lysine and ornithine decarboxylase and O/129 susceptibility tests. Sugar fermentation tests for sugars including arabinose, lactose, mannitol, salicin and inositol were carried out following standard procedures (USFDA, 2001).

Screening of shrimp samples for WSSV

The shrimp samples (n = 64), which were screened for *V. parahaemolyticus*, were also screened for the presence of WSSV. Crude template DNA was isolated from gill and pleopods of the shrimp samples by boiling in lysis buffer (0.025 N NaOH and 0.0125% sodium dodecyl sulfate) as described by Kiatpathomchai *et al.* (2001). Two step nested PCR was carried out using the primers 146F1 (5′-ACTACTAACTTCAGCCTATCTAG-3′)/146R1(5′-TAATGCGGGTGTAATGTTCTTACGA-3′) for first step PCR and 146F2 (5′-GTAACTGCCCCTTCCATCTCCA-3′)/146R2 (5′-TACGGCAGCTGCTGCACCTTGT-3′) for nested PCR (Lo *et al.*, 1996). PCR protocol mentioned by Lo *et al.* (1996) was followed with slight modifications, *i.e.,* number of cycles was limited to 30 in both the steps and the annealing temperature in the second step PCR was set as 65ºC instead of 55ºC.

Preparation of crude bacterial DNA from V. parahaemolyticus isolates for PCR template

Preparation of crude bacterial lysate was carried out as per the method of Chakraborty *et al.* (2008), with slight modifications. The overnight grown culture in tryptic soy broth containing 2% NaCl was centrifuged at 9000 g for 5 min. The bacterial pellet was washed with 0.85% sterile normal saline and resuspended in 200 µl sterile distilled water. The suspension was heated at 98°C for 10 min and the lysate was centrifuged at 4000 g for 10 min and 5 µl of the supernatant was used as PCR template.

Screening for toxR and tdh gene

Detection of *toxR* gene was carried out using the primer set toxRF (5`- GTCTTCTGACGCAATCGTTG-3`) and toxRR (5`-ATACGAGTGGTTGCTGTCATG-3`) (Kim *et al.*, 1999). PCR amplification was carried out in a reaction volume of 25 µl consisting of 5 µl of template (lysate supernatant), 2.5 µl of Dream *Taq* buffer containing 20 mm $MgCl_2$, 10 pmol of each primer, 1 U Dream *Taq* DNA polymerase (Thermo Fisher Scientific) and 200 µm of each dNTPs (ThermoFisher Scientific). PCR cycling condition at 63°C annealing temperature as mentioned in Kim *et al.* (1999) was followed, but the number of cycles was restricted to 30.

The primer pair L-tdh (5′-GTAAAGGTCTCTG ACTTTTGGAC-3′) and R-tdh (5′-TGGAATAGAAC CTTCATCTTCACC-3′) was used to screen for *tdh* gene (Bej *et al.*, 1999). Amplification was carried out in a 25 µl reaction volume containing 5 µl of template, 2.5 mm $MgCl_2$, 20 pmol of each primer, 200 µm of each dNTPs and 1U Dream *Taq* DNA polymerase. Amplification was performed with an initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C (denaturation) for 1 min, 58°C for 1 min (primer annealing) and 72°C for 1 min (primer extension). Final extension was carried out at 72°C for 5 min.

Screening for AHPND producing strain by AP3 primer

AP3 primer pair (Forward 5′-ATGAGTAACA ATATAAAACATGAAAC-3′/Reverse 5′-GTGGTAATA GATTGTACAGAA-3′), which yields 336 bp amplicon from AHPND producing *V. parahaemolyticus*, was used (Sirikharin *et al.*, 2014). All the *V. parahaemolyticus* isolates were screened. PCR was carried out in a 25 µl reaction volume and at 53°C annealing temperature (Sirikharin *et al.,* 2014).

PCR products were resolved by electrophoresis on 1.5% agarose gel containing 0.3 µg ml^{-1} ethidium bromide at 7 V cm^{-1} for 1 h in 0.5 x TBE buffer and were photographed under an UV gel documentation system (Syngene, GBOX, UK).

Results and discussion

Presence of WSSV in shrimp samples

Of the 64 shrimp samples screened, presence of white spot virus was detected in 26 samples. Out of those, 12 samples were positive in the first step PCR itself with the evidence of a 1447 bp amplified product and rest of the samples were positive only in the second step as indicated by the presence of a 941 bp amplified product (Fig. 1 and 2). All the samples, which were positive in the first step PCR, were associated with crop failure (Table 1). However, one *Penaeus monodon* sample, which was positive only in nested PCR, was also associated with crop failure. The occurrence of WSSV was found higher in traditional culture (59.3%) compared to semi-intensive culture (27%). Some of the WSSV positive samples did not exhibit typical white spot on the exoskeleton (Tables 1 and 2).

Occurrence of V. parahaemolyticus

Following isolation and biochemical confirmation, out of 138 samples, the presence of *V. parahaemolyticus*

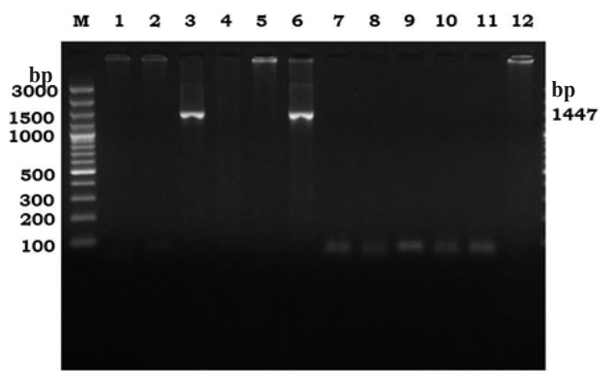

Fig. 1. WSSV specific PCR 1st step; Lane M: GeneRuler™ 100 bp plus DNA ladder (Thermo scientific), Lane 3, 6: Samples positive in 1st step PCR (1447 bp product), Lane 1, 2, 4, 5, 7-12: Samples negative in 1st step PCR

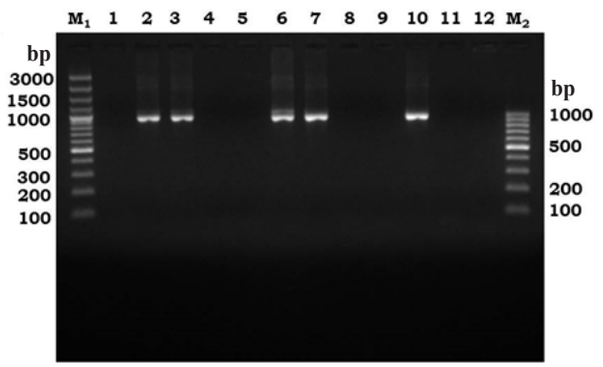

Fig. 2. WSSV specific PCR 2nd step; Lane M: GeneRuler™ 100 bp plus DNA ladder, Lane 2, 3, 6, 7, 10: Samples positive in 2nd step PCR (941 bp product); Lane 1, 4, 5, 8, 9, 11, 12: Samples negative in 2nd step PCR, Lane M2: GeneRuler ™ 100 bp DNA ladder

was detected in 34 shrimp (53.1%), 25 water (55.5%) and 22 soil (75.9%) samples. A total of 212 isolates were recovered from those positive samples. The isolates were confirmed by different biochemical tests as mentioned in USFDA (2001).

Screening for toxR and tdh genes

The presence of *toxR* gene was detected in all the isolates recovered from brackishwater aquaculture systems of Sundarban area, as evidenced by the presence of a 367 bp band in the PCR products, using the primer pair toxRF and toxRR (Fig. 3). However, none of the isolates was found positive to *tdh* gene.

Screening for AHPND producing V. parahaemolyticus strain

All the isolates were found negative to AHPND producing strain as evidenced by the absence of 336 bp amplicon while using AP3 primer pair.

Vibrio parahaemolyticus, a halophilic species of *Vibrio*, is widely distributed in brackishwater aquaculture systems and can survive for a long period in this environment (Gopal *et al.*, 2005). In cultured shrimp, some specific strains of this organism bearing a 69 kb plasmid pVA3-1 cause the deadly disease AHPND, which is also known as early mortality syndrome (EMS). This particular plasmid contains Photorhabdus insect-related (Pir) toxin-like genes (Han *et al.*, 2015) and it encodes a binary toxin PirAB (vp) (Lai *et al*., 2015). This disease caused a substantial mortality and crop loss both in *P. monodon* and *P. vannamei* in South-east Asian countries and Mexico. The mortality generally occurs possibly due to damage of hepatopancreas, leading to secondary bacterial infection. Recently, the rumour of AHPND outbreak in shrimp in India was in news (Tallaksen, 2013). Sundarban

Table 1. Presence of WSSV in normal and diseased farms:

	1st step +ve	2nd step +ve (1st step -ve)	White spot visible on exoskeleton of the sample
Shrimp samples associated with apparently diseased pond with crop failure	12	1	8
Shrimp samples associated with apparently normal pond	0	13	0
Total	12	14	8

Table 2. Presence of WSSV in traditional (bheri) and semi-intensive shrimp culture:

	Traditional culture (bheri)	Semi-intensive culture (ponds)	Total
Samples	27	37	64
WSSV 1st step +ve	7	5	12
WSSV 2nd step +ve (1st step -ve)	9	5	14
Total WWSV +ve samples	16 (59.3%)	10 (27%)	26 (40.6%)

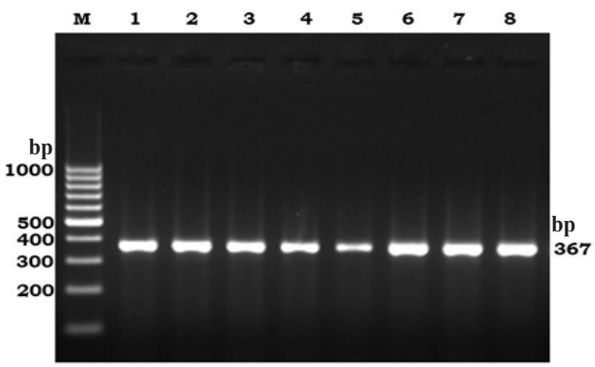

Fig. 3. *tox*R gene specific PCR: Lane M: GeneRuler™ 100 bp DNA ladder; Lane 1-8: toxR gene specific PCR product from *V. parahaemolyticus* isolates

areas of both India and Bangladesh are very important for brackishwater aquaculture production, particularly shrimp farming, due to availability of sufficient natural saline water (Primavera, 1994; Chakraborti *et al.*, 2002). In West Bengal, crop loss in shrimp culture very often takes place due to WSD (Mishra and Shekhar, 2006; Anandaraja *et al.*, 2012; Dutta *et al.*, 2013). Over a long period, this disease remained the major problem in shrimp aquaculture, both in semi-intensive and traditional culture systems. The present study also reiterated the fact that WSD is the major cause of crop failure in the West Bengal part of Sundarban area, as the crop failure was associated with all the samples, which were detected positive for WSSV in the first step PCR. However, in case of one farm in Kakdwip area, although the sample was found positive to WSSV only in the second step PCR, crop failure was noticed, which may be due to poor water quality, as high levels of total ammonia nitrogen (TAN) was recorded in the farm (unpublished data). Some of the WSSV positive samples did not exhibit typical white spots in the exoskeleton (Table 2). This indicates that mere absence of white spots on the exoskeleton does not rule out WSSV infection in shrimp. It was also observed that the occurrence of WSSV was more in traditional culture (bheri) compared to semi-intensive culture systems (Table 2). This may be due to lack of proper biosecurity measures and use of seeds which are not certified as specific pathogen free (SPF), in traditional systems in the bheries. *Vibrio parahaemolyticus* strains, which produce gastroenteritis in human beings, cause β-haemolysis on human or horse blood agar and this reaction is known as Kanagawa phenomenon. The *tdh* gene, which encodes TDH (thermostable direct haemolysin) toxin, is responsible for this phenomenon (Abbott *et al.*, 1989; Honda and Idaha, 1993). In this study, all the strains of this bacterium were free from *tdh* gene which indicates that the probability of causing gastroenteritis by these isolates in human beings is low. Previous studies also showed that the *tdh* gene is very common in clinical isolates of *V. parahaemolyticus*, but is rarely detected in the environmental isolates (Shirai *et al.*, 1990; DePaola *et al.*, 2000). Thus, the present study showed that *V. parahaemolyticus* is highly prevalent in brackishwater aquaculture environment of Sundarban. But, presence of neither AHPND causing nor *tdh* gene positive *V. parahaemolyticus* was detected in brackishwater aquaculture systems of this area. However, regular screening of shrimp samples for AHPND causing strains is highly recommended, as this disease is very much prevalent in various shrimp producing countries of Asia. More detailed studies are required on this aspect involving histopathology of hepatopancreas, muscle and other major organs of the apparently diseased shrimps.

It is also essential to create proper awareness among farmers on farm-level biosecurity measures for prevention of diseases and farmers also need to be sensitised to be more vigilant about AHPND like symptoms.

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