



Investigations on white spot disease outbreak in *Penaeus monodon* (Fabricius, 1798) in association with *Vibrio mimicus* infection in the Sunderbans, West Bengal, India

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ABSTRACT

The present study investigated the outbreak of white spot disease (WSD) in association with *Vibrio mimicus* infection, which occurred twice consecutively in a black tiger shrimp (*Penaeus monodon*) farm located in the Sunderbans, West Bengal, India. The farm stocked with post-larvae (PL) @ 16 PL m⁻², encountered disease outbreak on the 41st day of culture (DOC) followed by a second outbreak on the 54th DOC with 100% mortality each time. Shrimp samples were collected for molecular diagnosis as well as for microbiological investigations. White spot syndrome virus (WSSV) infection was confirmed by polymerase chain reaction (PCR). *V. mimicus* isolated from the outbreak, was characterised by morphological, physiological and biochemical characteristics. *V. mimicus* isolated was found to be pathogenic by challenge studies which caused 100% mortality in *P. monodon* juveniles. Microbial load was studied in the natural infection and also in challenge trials in relation to the day of advancing infection. LD₅₀ value for *V. mimicus*, isolated from the outbreak, was 10^{7.32} in *P. monodon* juveniles. Co-infection of *V. mimicus* with WSSV led to 100% mortality within seven days from the onset of clinical signs.

Keywords: Minimum inhibitory concentration, Vibriosis, *Vibrio mimicus*, White spot disease

Introduction

In the recent past, catastrophic outbreaks of white spot disease (WSD) in cultured penaeid shrimps wreaked havoc worldwide, especially in Asian countries. Cultured penaeid shrimp production decreased tremendously due to the outbreaks of WSD. WSD causing mass mortality was first reported in northern Taiwan during 1992 (Chou *et al.*, 1995; Escobedo-Bonilla *et al.*, 2008), followed by outbreaks in other countries. In India, it was first noticed in 1994 by Karunasagar *et al.* (1997). The etiological agent was identified as white spot syndrome virus (WSSV) belonging to a new viral genus *Whispovirus* under the family Nimaviridae (Mayo, 2002). The virus was found to be extremely virulent and cumulative mortality reached 80 to 100% within 3-7 days post-infection (Karunasagar *et al.*, 1997). Such an outbreak of WSD, in association with vibriosis was noticed consecutively twice in a shrimp farm located in the Sunderbans, West Bengal and the present study reports the result of an investigation carried out during the outbreak.

Materials and methods

Shrimp pond details and sample collection

Shrimp culture with zero water exchange was followed in the farm with water depth not less than eight feet. Initial chlorination was done @ 20 ppm. The water intake was done with a 20 mesh netting (20 slits inch⁻¹) fixed at the foot valve of the pump and 80 and 100 mesh netting at the delivery points. The pond, covering an area of 0.48 ha, was stocked with black tiger shrimp, *Penaeus monodon* post-larvae (PL) @ 16 nos. m⁻². Blind feeding was done twice a day with commercial pelleted feed up to 40th day of culture (DOC), followed by four times a day @ 10 to 3.5% of the body weight. The pond encountered disease outbreak on the 41st DOC followed by a second outbreak on the 54th DOC with 100% mortality each time. The average body weight (ABW) of the infected shrimp was recorded. About 10 shrimps, with signs of red colouration and white spots, were collected for five consecutive days from the first day of onset of clinical signs for PCR diagnosis as well as for microbiological investigations. Samples of pond water, sediment, shrimp muscle, haemolymph and hepatopancreas were collected aseptically for microbiological analyses.

Polymerase chain reaction (PCR)

For two-step PCR amplification, four DNA oligonucleotide primers were used (Kimura *et al.*, 1996). PCR was performed in a 25 μ l reaction mixture as reported by Ananda Raja *et al.* (2012a).

Microbiological analyses

In order to understand the microbial dynamics during the outbreak, total *Vibrio* count (TVC) and total plate count (TPC) were done for water, sediment, shrimp muscle, haemolymph and hepatopancreas samples for five consecutive days from the day of the clinical signs first observed (Biswas *et al.*, 2012; Ananda Raja *et al.*, 2014). All the procedures were done aseptically under laminar air flow. Initial bacterial isolation was done from the haemolymph and hepatopancreatic tissue. Predominant colonies observed on 1.5% NaCl supplemented tryptone soya agar (TSA) plates were streak plated on to thiosulfate citrate bile salts sucrose (TCBS) agar plates. The predominant non-sucrose fermenting (green coloured) colonies grown on the TCBS plates were further screened and identified based on the phenotypic, physiological and biochemical characters according to Alsina and Blanch (1994).

Minimum inhibitory concentration (MIC)

The most dominant bacterial isolate (V18) was tested for the antimicrobial sensitivity and the MIC level was determined for 22 commercially available antimicrobial compounds using the HiComb strip (HiMedia). Single colony was inoculated to 1.5% NaCl supplemented tryptone soya broth (TSB) and incubated at 30°C for 3 h. Optical density (OD) value was recorded at 625 nm and the plate count was determined per ml of broth. The turbidity was compared between the bacterial suspension and McFarland 0.5 standard prepared by mixing 0.5 ml of 1.175% barium chloride and 99.5 ml of 0.36 N sulfuric acid (10^5 - 10^6 cells ml^{-1}). Any increase or decrease in turbidity to the standard was adjusted either by adding sterile normal saline (NS) or by further incubating the inoculum. Then, the bacterial suspension was inoculated on to Muller Hinton Agar (MHA) plates, using sterile non-toxic cotton swabs. The plates were dried for 10 min before placing the HiComb strips. Strips containing 22 antibacterial agents such as ampicillin, amoxycillin, amoxycylav, nalidixic acid, enrofloxacin, ciprofloxacin, norfloxacin, kanamycin, streptomycin, noemycin, gentamicin, vancomycin, chloramphenicol, erythromycin, tetracycline, oxytetracycline, chlortetracycline, metronidazole, furazolidone, sulphafurazole, co-trimoxazole and trimethoprim were placed on the MHA plates individually and incubated at 30°C for 18 h. After incubation, the diameter of the zone of inhibition

was measured and interpreted as per the zone diameter interpretative chart (HiMedia). The sensitivity of the isolates to the antibiotics was determined accordingly when the zone of inhibition equaled 8 mm.

Pathogenicity and LD₅₀

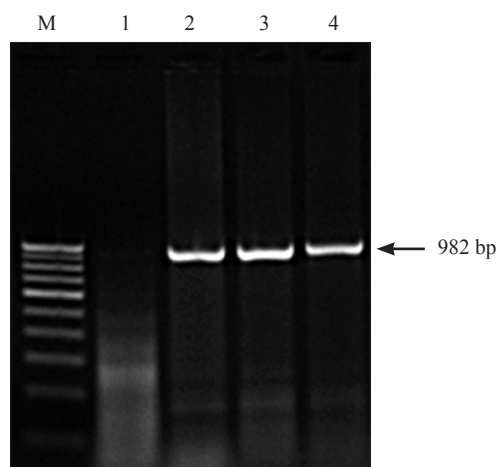
Hatchery produced PCR screened healthy juveniles of *P. monodon* were procured and maintained @ 10 shrimps per tank in 50 l glass aquaria with pre-treated water at 30 \pm 1°C with 7-8‰ salinity. The weight and length of each shrimp were recorded. A slant culture of the bacterial isolate (V18) stored at 4°C was sub-cultured, and an 18 h shake culture was centrifuged at 4800 g for 15 min. The cell pellets were washed twice using normal saline (NS). The shrimps were challenged by intramuscular injection of 10^4 to 10^8 colony forming units (cfu) in 50 μ l NS per shrimp in the ventral side of each shrimp, between II and III abdominal segments. The same quantity of NS alone was injected to the control groups. Clinical signs and mortality pattern were observed every 15 min during the first hour post-injection (hpi) followed by every 1 h until 6 hpi. The experiment was continued for seven days and the animals were monitored every 12 h (Joseph Selvin and Lipton, 2003). Parameters such as pH, temperature and salinity were recorded thrice a day for seven days. Tissue homogenate of moribund shrimp hepatopancreas was used for re-isolation of bacteria using Zobell marine agar (ZMA) plates to prove Koch's postulates. LD₅₀ value was calculated as per Reed and Muench (1938).

Statistical analyses

The data were analysed with independent samples t-test, one way ANOVA and Duncan's multiple range test (DMRT) using SPSS for Windows v.17.0 programme (SPSS Inc. 2007). Results were expressed as mean \pm standard error (SE).

Results and discussion

Average body weight (ABW) of the infected shrimps was 6.44 \pm 0.15 and 8.12 \pm 0.10 g at 41st and 54th DOC, respectively. The major clinical signs such as lethargy, loss of balance, preening and no response to stimulus, reduced feeding, presence of white spots initially on the carapace which later progressed to the whole body, reddish discolouration, dark necrotic spots and finally death (Wongteerasupaya *et al.*, 1995, Tan *et al.*, 2001; Wu *et al.*, 2001) were observed during the outbreak. PCR analyses showed that the shrimp samples were I and II step positive for WSSV (Fig. 1, 2). Lo *et al.* (1998) reported that the WSSV infected shrimps with white spots lesions might survive indefinitely under non-stressful conditions. In addition, Ananda Raja *et al.* (2012b) reported that the

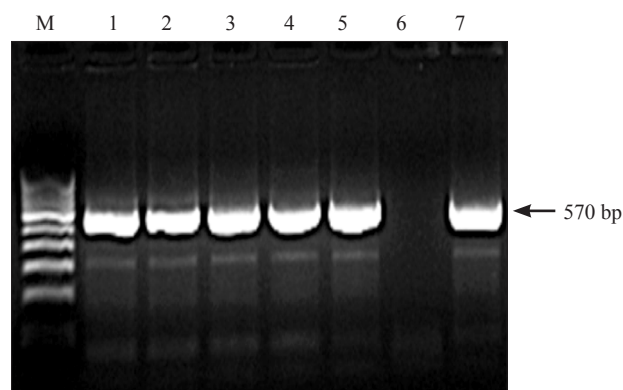


M:100 bp Marker; Lane 1: Negative control; Lane 2: Positive control; Lane 3 & 4: Shrimp positive

Fig. 1. Gel picture of 1st step PCR for WSSV

animals were apparently healthy with no clinical signs and lesions though they harbour I step PCR positive viral load. But in the present study, the animals were I step PCR positive in association with *Vibrio* infection which led to the stress and disease outbreak.

The microbial load in pond water, sediment, muscle, haemolymph and hepatopancreas were found increasing significantly as days advanced and significant increase in gross lesions was noticed (Table 1, 2). Significant difference ($p < 0.01$) was observed in the TVC and TPC of the water, sediment, muscle, haemolymph and hepatopancreas (Table 3), but, from the LD₅₀ trials, only TVC was statistically significant ($p < 0.05$). Based on morphological, physiological and biochemical



M:100 bp Marker; Lane 1 to 5: Shrimp positive; Lane 6: Negative control; Lane 7: Positive control

Fig. 2. Gel picture of 2nd step PCR for WSSV

characteristics (Alsina and Blanch, 1994), it was confirmed that all the shrimp were infected with a single type of bacterium, *Vibrio mimicus* (V18). In addition, all the 50 shrimps collected for five consecutive days showed characteristic lesions of WSD and this was confirmed by PCR. Therefore, the co-infection of *V. mimicus* and WSSV in the pond was ascertained. Four species of bacteria viz., *Vibrio alginolyticus*, *V. parahaemolyticus*, *V. anguillarum* and *Pseudomonas aeruginosa* were so far reported, in association with WSD in shrimps (Jayasree *et al.*, 2000, Joseph Selvin and Lipton, 2003; Liu *et al.*, 2004). Results of the present study showed that *V. mimicus* infection can also cause severe mortality in association with WSD. In addition, it was observed that the isolate was highly susceptible to enrofloxacin, ciprofloxacin, norfloxacin, vancomycin, chloramphenicol and trimethoprim and susceptible to amoxycillin, nalidixic acid, kanamycin,

Table 1. Percentage of gross lesions observed from the day 1 to 5 of the disease outbreak

Days after initial sign	White spot**	Reddish discoloration**	Dark necrotic spots**	No lesion**
1	14.72±1.43 ^a	0.85±0.42 ^a	4.75±1.12 ^a	79.68±1.40 ^a
2	21.43±0.84 ^b	1.04±0.64 ^a	8.76±1.68 ^{ab}	68.77±2.28 ^b
3	26.51±0.46 ^b	3.46±0.45 ^b	10.89±1.14 ^b	59.14±0.64 ^c
4	37.25±0.90 ^c	5.09±0.83 ^{bc}	17.35±1.14 ^c	40.31±1.07 ^d
5	52.75±3.13 ^d	6.78±0.18 ^c	25.98±2.35 ^d	14.49±5.63 ^e

** $p < 0.01$, Values bearing different superscripts in a column differ significantly

Table 2. Total plate count (TPC) and total *Vibrio* count (TVC) in WSSV and *Vibrio mimicus* infected shrimp monoculture ponds for five consecutive days

Water		Sediment		Muscle		Haemolymph		Hepatopancreas	
TPC (10 ⁶ cfu ml ⁻¹)**	TVC (10 ⁵ cfu ml ⁻¹)*	TPC (10 ⁶ cfu ml ⁻¹)**	TVC (10 ⁵ cfu ml ⁻¹)**	TPC (10 ⁶ cfu ml ⁻¹)**	TVC (10 ⁵ cfu ml ⁻¹)**	TPC (10 ⁶ cfu ml ⁻¹)**	TVC (10 ⁵ cfu ml ⁻¹)*	TPC (10 ⁶ cfu ml ⁻¹)**	TVC (10 ⁵ cfu ml ⁻¹)*
0.49±0.02 ^a	0.16±0.01 ^a	28.84±1.14 ^a	13.58±0.3 ^a	0.21±0.01 ^a	0.003±0.00 ^a	3.63±0.22 ^a	2.17±0.05 ^a	62.25±1.3 ^a	19.98±0.6 ^a
1.03±0.003 ^b	0.19±0.01 ^a	31.0±1.2 ^a	19.38±0.4 ^b	0.29±0.01 ^a	0.004±0.00 ^a	5.00±0.16 ^b	3.50±0.02 ^b	86.75±4.8 ^b	18.83±3.4 ^a
1.44±0.07 ^c	0.24±0.01 ^b	51.0±1.8 ^b	22.23±1.1 ^c	1.54±0.05 ^b	0.008±0.00 ^b	5.43±0.14 ^b	4.52±0.04 ^c	133.25±4.8 ^c	25.10±1.2 ^{ab}
2.07±0.07 ^d	0.28±0.02 ^c	87.13±2.4 ^c	27.6±0.7 ^d	2.08±0.08 ^c	0.01±0.00 ^c	7.34±0.18 ^c	5.83±0.05 ^d	187.25±7.3 ^d	28.15±1.2 ^b
2.62±0.07 ^e	0.30±0.003 ^c	117.13±10.1 ^d	29.55±0.3 ^d	2.38±0.01 ^d	0.01±0.00 ^c	8.94±0.02 ^d	6.97±0.02 ^e	222.5±6.5 ^e	28.83±0.9 ^b

* $p < 0.05$; ** $p < 0.01$, Values bearing different superscripts in a column differ significantly

Table 3. Total plate count (TPC) and total *Vibrio* count (TVC) in shrimp monoculture ponds infected with WSSV and *Vibrio mimicus*, and in the LD₅₀ experiment (*V. mimicus*) tanks

Count	Water	Sediment	Muscle	Haemolymph	Hepatopancreas
Pond					
TPC (10 ⁶ cfu ml ⁻¹)**	1.53±0.38 ^a	63.02±17.1 ^b	1.3±0.45 ^a	6.20±0.90 ^a	138.40±29.96 ^c
TVC (10 ⁵ cfu ml ⁻¹)**	0.23±0.03 ^a	22.47±2.87 ^b	0.007±0.002 ^a	4.62±0.85 ^a	24.18±2.06 ^b
LD ₅₀ trial					
TPC (10 ⁶ cfu ml ⁻¹)	236.94±191.77	-	13.37±4.03	5.93±0.97	420.1±171.10
TVC (10 ⁵ cfu ml ⁻¹)*	2.76±1.78 ^a	-	9.84±2.22 ^a	4.57±0.84 ^a	404.93±237.95 ^b

*p<0.05; **p<0.01, Values bearing different superscripts in a row differ significantly

streptomycin, neomycin, gentamicin, erythromycin, tetracycline, oxytetracycline, metronidazole, furazolidone, sulphafurazole and co-trimoxazole. The isolate was resistant to β -lactam antibiotics, such as ampicillin and amoxycyclav (Table 4). No unusual resistance was observed with 22 antimicrobials, as observed by Manjusha *et al.* (2005).

The total length and weight of juvenile shrimp used for the challenge trials with the *V. mimicus* isolate (V18) ranged between 83.3±1.32 and 85.1±1.08 mm and 5.20±0.60 and 5.40±0.10 g, respectively. Parameters such as pH, temperature and salinity recorded during the experiment were: 7.9±0.02, 30.5±0.31°C and 7.5±0.02 g l⁻¹, respectively. All shrimps died at 6 to 24 hpi with a high dose of 10⁸ cfu per shrimp. But, no mortality was observed within 7 days post-infection (dpi) at 10⁵ cfu per shrimp, though *Vibrio* infection was noticed on 5 dpi. Based on this observation, the LD₅₀ value was

Table 4. Minimum inhibitory concentration (MIC) of V18 to different antimicrobial compounds

Antibiotics group	Antibiotics	MIC (μ g)
β -Lactam antibiotics	Ampicillin	>256
	Amoxycillin	<0.01
	Amoxycyclav	>240
Fluoroquinolones/Quinolones	Nalidixic acid	0.05
	Enrofloxacin	<0.001
	Ciprofloxacin	<0.001
	Norfloxacin	<0.001
Aminoglycosides	Kanamycin	3
	Streptomycin	7.5
	Noemycin	1
	Gentamicin	5
Glycopeptides	Vancomycin	<0.001
Macrolides	Chloramphenicol	<0.001
	Erythromycin	0.01
Tetracyclines	Tetracycline	0.25
	Oxytetracycline	0.5
	Chlortetracycline	0.5
Nitroimidazole	Metronidazole	40
Nitrofurans	Furazolidone	5
Sulphanomides	Sulphafurazole	0.01
	Co-Trimoxazole	0.05
	Trimethoprim	<0.001

found to be 10^{7.32} cfu per shrimp. Signs of infection among the challenged animals included lethargy, lack of food intake, empty midgut, abnormal swimming behaviour, brown to black spots on the shell, pink or brown gills, reddish discolouration, murky whitish muscle, flexure of the abdominal musculature, folded tail base and dark necrotic areas in the tail fan (NIO, 1998). As per the earlier reports, lower stocking density, good management practices and stress free environment, even in presence of WSSV infection could lead to successful grow-out culture (Ananda Raja *et al.*, 2012b). Present study revealed that co-infection of *V. mimicus* with WSSV can lead to 100% mortality within seven days from the onset of clinical signs. Moreover it was confirmed that the *V. mimicus* isolate was also pathogenic which could cause 100% mortality in *P. monodon* juveniles.

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