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INVESTIGATION ON WHITE SPOT SYNDROME VIRUS (WSSV) IN *PENAEUS MONODON* BROOD, NAUPLII, POST LARVAE AND CULTURED SHRIMP IN COX'S BAZAR, BANGLADESH BY USING NESTED PCR TECHNIQUES

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ABSTRACT: Investigations were carried out between April 2014 to June 2016 for detecting white spot syndrome virus (WSV) in tiger shrimp, *Penaeus monodon* broods, nauplii, post larvae and cultured shrimp by using nested PCR techniques. The presence viral DNA was observed in shrimp culture farms in Cox's Bazar. Of 558 grow out shrimp samples tested, 239 (42.83%) were found to be positive for WSSV by PCR assay. Of 91 post larvae collected in adjacent river and canal, 41 (45.05%) were infected with WSSV and 70 juvenile shrimps collected, 45 (64%) of the samples were positive for WSSV infection. Selected shrimp hatcheries in the region also showed continual occurrence of WSSV infection and 149 nauplii were tested, 39(26.17%) were positive for WSSV by PCR assay. In hatcheries, 248 nos. *P. monodon* broods collected, 125(50.40%) were found positive for WSSB. The average WSSV infection in brood shrimps were increased as 32%, 34% and 69% for the years 2014, 2015 and 2016, respectively. *P. monodon* broods collected from the deep sea zone, WSSV prevalence was 57% in summer (May), falling to 0% during winter month (November). Many of the brooders and juveniles did not exhibit any external symptoms of WSSV infection, however, following PCR amplification with WSSV detection primers clear products were revealed, indicating the presence of latent infection. Thus, effective prevention and control measures are urgently needed to control the spread of the WSSV disease in the shrimp industry. Diagnostic PCR can be applied to screen for carrier brood stock and shrimp larvae used for shrimp culture.

KEYWORDS: *Penaeus monodon*, WSSV, PCR.

INTRODUCTION

The shrimp sector in Bangladesh has become important commodity for its potential to increase export earning of the country. The black tiger shrimp, *Peneaus monodon* is the major species contributing highly in shrimp production in Bangladesh coast added US$ 457 million to export earnings in 2016-17 with a production of about 40,000 MT shrimp (DoF, 2017). About 49 hatcheries now operated in Cox's Bazar to supply shrimp post-larvae to the shrimp farmers in western region and Cox Bazaar of the country.

White spot syndrome virus (WSSV), is a highly virulent rapidly replicating large, enveloped double-stranded DNA virus of shrimp, assigned by the ICTV to its own new genus *Whispovirus*, and family, Nimaviridae (Fauquet *et al.*, 2005), has been responsible

for a major loss in shrimp aquaculture, the production loss worth 4–6 billion USD in Asia from 1992 to 2012 (Lightner *et al*., 2012) and the mortality rate of shrimp due to WSSV is 100% within a few days (Vaseeharan *et al.*, 2003; Sanchez- Martinez *et al.*, 2007; Ayub *et al.*, 2008; Karim *et al.*, 2011). The virus is found to have the ability to infect and replicate in a wide host range of more than 98 species (Escobedo-Bonilla *et al*., 2008) and can also be vertically transmitted from infected shrimp brooders to post larvae (Lo *et al.*, 1997; Lo and Kou, 1998). WSSV DNA has been reported to be detected in shrimp farms soil, in surrounding canal water samples, and even in seawater environments (Natividad *et al*., 2008; Quang *et al.*, 2008) and reported to be infective for at least 40 days at 30°C in seawater under laboratory conditions (Momoyama *et al.*, 1998) and viable for at least 3-4 days in ponds (Nakano *et al.*, 1998). So, this kind of viral infections not only affects the shrimp but also the aquatic surrounding. Keeping in view of these conditions the objective of present work was designed to employ PCR to detect WSSV on wild broods of *P. monodon* shrimp **(**hatcheries and also broods those collected from deep sea origin), hatchery produced nauplii, post larvae (PL), and shrimp from grow out ponds and wild catches (adjacent river) in Cox's Bazar region of Bangladesh for developing consciousness and instruct farmers and business people about the WSSV infections in shrimps and make them take initiatives to reduce the risk of WSSV.

MATERIALS AND METHOD

Detecting White Spot Syndrome Virus (WSSV):

Investigations were carried out between April 2014 to June 2016 for detecting white spot syndrome virus (WSV) in shrimp brood stocks, nauplii, post larvae and cultured shrimp by using nested PCR techniques. Wild brood samples were collected from two shrimp hatcheries and also directly from shrimp brood harvesting industrial trawler. A tip of pleopod (20-30 mg) was collected from live brood shrimp and was taken into Eppendorf tube containing 70% alcohol. Ship's brood samples were brought directly to laboratory of Marine Fisheries and Technology Station, BFRI, Cox's Bazar in November 2015 (winter season) and May 2016 (summer season) those were collected from St. Martin (Depth 46-52.2 m) and Elephant Point (Depth 46.6-48 m) of Bay of Bengal. The shrimp nauplii (1000 nos.), post larvae (150-200 nos.) were collected from hatcheries and was put into poly bag in live condition. Immediately after collection, the samples were transported to the laboratory for analysis. Samples of cultured shrimp were collected from four selected shrimp farms located at different geographical locations at four Upazillas *viz*. Cox's Bazar Sadar, Teknaf, Moheshkhali, and Chakaria of Cox's Bazar District and wild samples were collected from adjacent river/canals. Physico-chemical parameters of water *viz.* water depth (cm), transparency (cm), temperature (^{0}C), pH, dissolved oxygen (DO ppm), ammonia (ppm) and alkalinity (ppm) were recorded at fortnight interval from each location to assess the environmental health of shrimp farming. Dissolved oxygen (DO) and temperature was measured using DO meter (Lutron PDO-519), water salinity by using a hand Salinity refractometer (Model RHS-10), water pH by pH meter (model PH5011), alkalinity and ammonia by Test kit (HNNA). Monitoring of shrimp hatchery was also done whether shrimp hatchery contributes to degradation of coastal environment through its effluent discharge. This study was

involved with only one hatchery for three month from February-May, 2016 as other hatcheries was not cooperated to perform this kind of investigations. Every three-week interval samples were collected from inlet and outlet of a hatchery.

DNA extraction and PCR test:

DNA was extracted as per the IQ 2000 WSSV detection and prevention system protocol. Samples were homogenized with lysis buffer provided in the IQ 2000 WSSV detection system kit. After 5 minutes of incubation at 100^0C and a brief cool down in ice flakes, samples were centrifuged at 1500x g for 10 min. After collecting the liquid phage, DNA was precipitated in absolute ethanol. Finally, the pellet was dissolved in sterile distilled water, and used as a PCR template. Then PCR was performed using the method described in the protocol of the IQ 2000 WSSV detection and prevention system (Chang *et al.*, 1996; Chou *et al*., 1995; Lightner, 1996; Lo *et al.,* 1997; Peng *et al.*, 1996; Wang *et al.*, 1995; Wongteerasupaya *et al.*,1995).

We have used IQ 2000TM WSSV Detection and Prevention System Kit (GeneReach Biotechnology Crop., Taiwan validates and certified by OIE (Reg. No.: 20080304)).

Reaction condition:

First PCR reaction profile: 94°C30 seconds; 62°C30 seconds; 72°C30 seconds, repeat 5 cycles, then 94°C15 seconds; 62°C15 seconds; 72°C20 seconds, repeat 15 cycles, then add 72°C30 seconds; 20°C30 seconds at the end of the final cycle.

Nested PCR reaction profile: 94°C20 seconds; 62°C20 seconds; 72°C30 seconds, repeat 25 cycles, add 72°C30 seconds; 20°C30 seconds at the end of the final cycle.

RESULTS AND DISCUSSION

The results of WSSV screening indicated widespread occurrence of WSSV infection in shrimp hatcheries and grow-out ponds along the south east coast of Bangladesh at Cox's Bazar. The average WSSV infection in brood shrimp were 32%, 34 % and 69% for the years 2014, 2015 and 2016, respectively, and three years data revealed an increasing trend i.e. prevalence of WSSV increasing from 2014 than 2016 (Fig. 1).

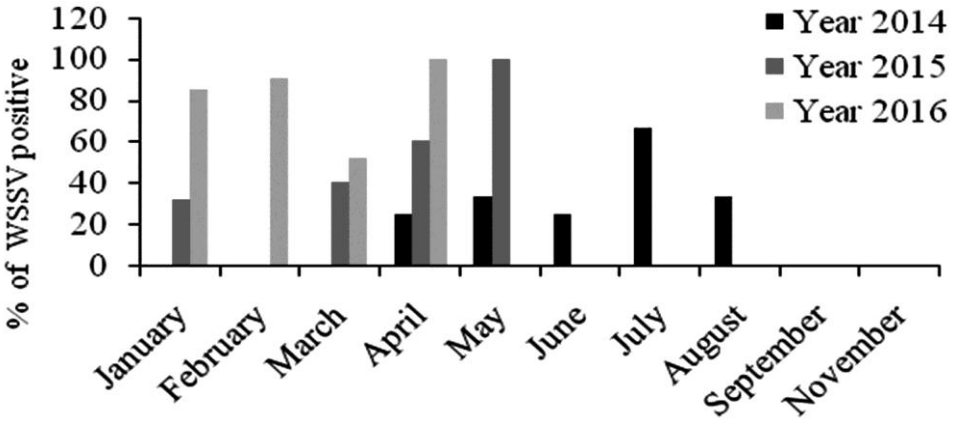

Fig. 1. Three years monthly status of WSSV positive shrimp broods.

The highest WSSV prevalence in brood shrimps was found 66.67% in the month of July, 2014 and lowest WSSV prevalence was found 0% in the month of September, 2014 which coincide with the findings of Iqbal *et al.* (2011) where broods sample showed prevalence zero in the month September but as high as 90% in May/June. In 2015, highest WSSV prevalence 100% was observed in the month of April and lowest was found 0% in the month of February and November where 12 brood samples were collected from deep sea zone area by Sea Resource Ltd. Bangladesh. In 2016, highest (100%) WSSV prevalence found in the month of April and it was lowest (52%) in March. Debnath *et al.* (2014) also reported higher 57 (63.3%) WSSV positive in the shallow zone than deep zone 21 (23.3%) of 90 brood samples in the Bay of Bengal.

On the other hand, shrimp sample those were collected from grow out farms located at Teknaf, Chakaria and their adjacent river showed 100% prevalence in 2014 but 80 to 60% prevalence prevailed in 2015 and 2016, respectively (Fig. 2). Shrimp farms at Moheshkhali showed 100% WSSV prevalence in 2016 which was 40% in 2015. Cox's Bazar sadar shrimp farm samples showed 40, 60 and 80% prevalence in 2014, 2015 and 2016, respectively (Fig. 2).

In case of shrimp nauplii, the highest WSSV prevalence was 66.67% in July and lowest was 0% in September, 2014 (Fig. 3), whereas 100% WSSV positive nauplii samples were observed in May 2015 and 2016. In 2015, highest WSSV prevalence in nauplii was found in the month of May and lowest was found 0% and 25% in February 2015 and 2016, respectively.

On the other hand, in the case Post Larvae (PL), 100% WSSV positive PL was observed during April and May in 2015 and 2016 but it was only 20% in April 2014. A lower prevalence of 25% and 71.42% was observed in April and March of 2015 and 2016, respectively (Fig. 4).

The finding of Debnath *et al.* (2014) revealed the prevalence of WSSV was found to be highest during the months of March and June, peaked at 93.3%, and lowest 33.3% in the month of April, although shrimp broods collected from the deep zone, showed highest WSSV prevalence of 33.3% in June, falling to 6.7% in April. In the present study, 24 brood samples were collected from deep sea zone where 12 samples those collected in November 2015 were WSSV negative but those collected in May 2016, seven (58%) were positive. Among the infected brooders from deep sea zone, 3 were males which may indicated May and June months are more susceptible for WSSV prevalence.

An assessment of physical, chemical and nutrient parameters of water in shrimp hatcheries recorded were found to be at optimal range (Table 1). Among the nutrient parameters, Ammonia-N (mg/l) was observed in hatchery outlet water undetectable range to 0.0 mg/l (0.03-00 mg/l). Both Nitrate-N and Nitrite-N were below the detectable levels in hatchery outlet and inlet water. Phosphate P (mg/l) was high in case of hatchery outlet that had high phosphate (0.91-0.09 mg/l) than inlet (0.12-0.07 mg/l). Biological oxygen demand (BOD) in inlet (1.91-0.4 ppm) was low compared to outlet water (2.371.41 ppm). Chloride was higher in inlet (17010-16180 mg/l) than outlet (12330-2802 mg/l). EC in inlet water was high (56,550-54,350 µs/cm) compared to outlet (36,150-7,460 µs/cm). TDS in inlet water was high (37,888-36,414 mg/l) compared to outlet water (24,220-4,958.2 mg/l).

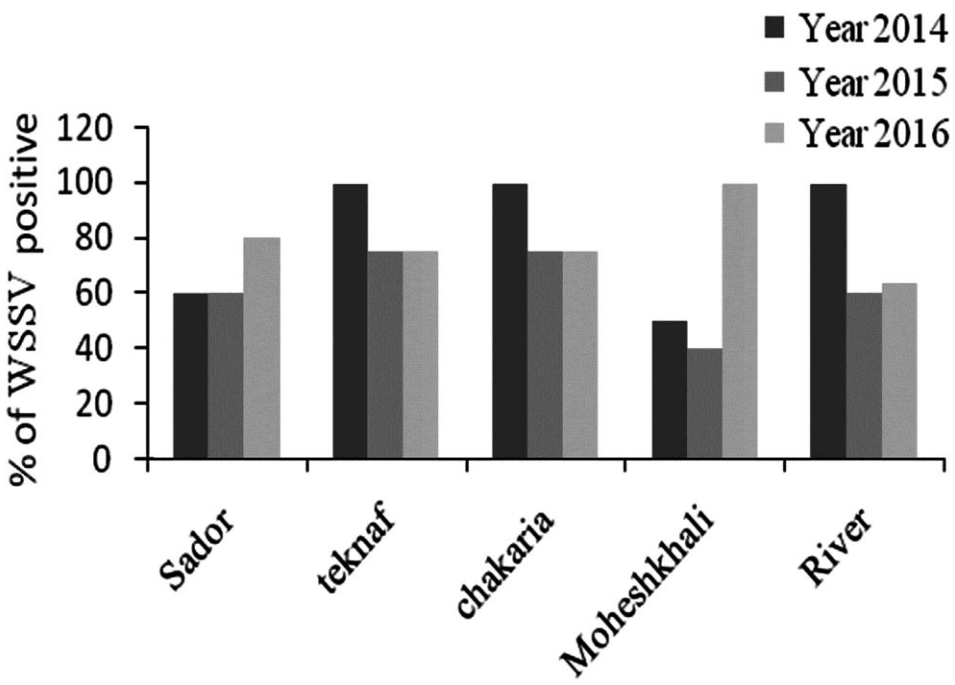

Fig. 2. Three years prevalence status of WSSV positive from five sources.

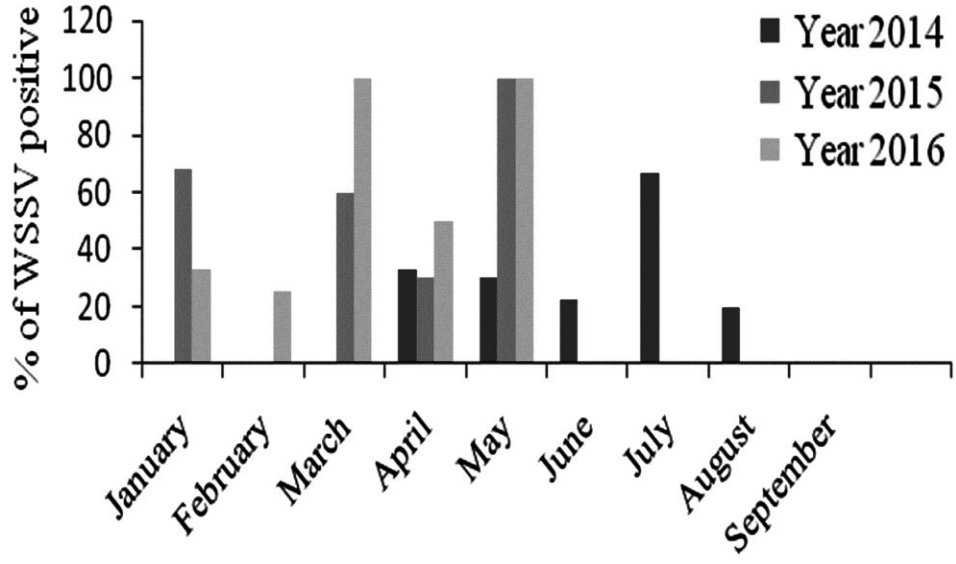

Fig. 3. Three years monthly status of WSSV positive shrimp nuplii.

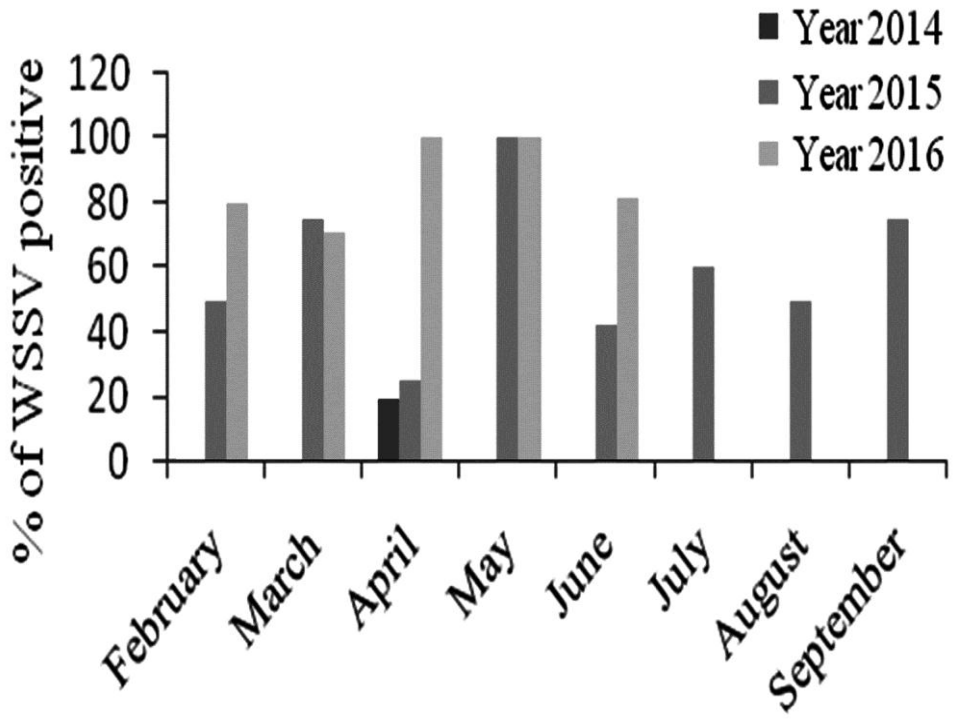

Fig. 4. Three years monthly status of WSSV-positive PL.

The losses of shrimp crop due to diseases in grow out ponds under study were not able to measure though we found the symptoms of bacterial diseases and the presence viral DNA in the ponds without severe mortality. So, simple detection of virus using PCR methods does not indicate its viable nature and ability to infection. A certain concentration of viral load is required to cause disease in individual. Mazumder *et al.* (2015) reported by stocking PCR negative PL where six farms failed in different days of culture due to WSS outbreaks though 39 (86.67%) farms successfully completed the production cycle out of 45 farms at Khulna region. In Cox's Bazar area semi-intensive farmers only stocked PCR tested PL. In studied area, cent percent farmers stocked with hatchery and natural PL without PCR test. Hence, screening for WSSV alone may not guarantee an outbreak of the disease in grow-out ponds unless the sources of further infection such as entrance of contaminated water, presence of weeds and invertebrate animals particularly mollusks and crustaceans were eliminated which is attributed with expression on horizontal contamination from the canals and neighboring farms and/or contaminated from already remaining other crustaceans including crabs (Kanchanaphum *et al.*, 1998; Karim *et al.*, 2011). So, there is no way to cure the WSSV affected farm without taking preventive measure along with improving management practices such as bleaching, liming, controlling water quality, maintaining proper stocking with PCR tested

Table 1. Physico-chemical and nutrient parameters of water in shrimp hatcheries at Cox's Bazar.

Parameter	Hatchery water	
	Inlet	Outlet
Temperature	30.73±2.59 (33.7-28.9)	29.33±1.04 (30.5-28.5)
pH	8.3±0.1 (8.4-8.2)	8.37±0.11 (8.5-8.3)
Salinity	30.25±4.79 (36-25)	14.5±11.35 (24-30)
Alkalinity	137±4.69 (142-133)	245±164.01 (434-140)
Ammonia	-*	0.01±0.02 (0.03-00)
DO	5.7±1.34 (6.78-4.51)	5.17±0.95 (6.22-4.37)
Transparency	-	-
Chloride	16595±586.9 (17,010-16,180)	7566.1±6737 (12,330-2,802)
Nitrate,	-	-
Nitrite	-	-
Phosphate	0.063±0.060 (0.12-0.07)	0.44±0.42 (0.91-0.09)
Iron	0.49±0.28 (0.52-0.45)	0.21±0.07 (0.15-00)
BOD	1.35±0.83 (1.91-0.4)	1.91±0.48 (2.37-1.41)
EC(µs/cm)	55450±1555 (56,550-54,350)	21805±20286 (36,150-7,460)
TDS	37151±1042.2 (37,888-36,414)	14589±13620 (24,220-49,582)

-* not in detectable range. In parenthesis the ranges.

WSSV free PL and also as part of the better management practices (BMPs), ploughing, tilling and sun-drying of shrimp culture ponds are advocated for prevention and control of this disease (Corsin *et al*., 2005).

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