Concurrent infection with WSSV and MBV in Tiger prawn, *Penaeus monodon* (Fabricius) in West Bengal and their detection using PCR and DNA Dot-blot hybridization technique

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Prevalence status of white spot disease (WSD), caused by white spot syndrome virus (WSSV) and Monodon baculovirus (MBV), was studied in different prawn farms in North and South-24 paragana districts of West Bengal, specifically in the areas previously affected by WSD. In total 283 samples of giant tiger prawn *Penaeus monodon*, Indian white prawn *P. indicus* and giant freshwater prawn *Macrobrachium rosenbergii* from 12 different farms were screened and presence of MBV occlusion-bodies (Obs) were detected. Of samples screened, 30 samples showed multiple spherical Obs typical of MBV infection. Of 80 samples screened using PCR, 17 (21.25%) were positive for MBV and 51 samples (63.75%) were positive for WSSV, and amplified DNA of 674 bp and 643 bp, respectively was visualized on 1% agarose gel electrophoresis. Significantly all samples showing positive reaction with MBV were also positive for WSSV in PCR, indicating co-infection of prawns with both MBV and WSSV. However, none of the *M. rosenbergii* samples were positive of MBV-Obs but 5 samples were positive for WSSV in PCR. Both MBV and WSSV amplified DNA were labeled with alkaline phosphatase using Alkphos DNA labeling reagent kit and samples were screened using DNA dot-blot and slot-blot hybridization. All samples showing positive PCR reaction were also found positive in DNA hybridization.

**Keywords**: DNA probe, MBV, PCR, Tiger prawn, white spot disease, WSSV

**IPC Code**: Int.Cl.7 C12N15/10,G01N33/53

**Introduction**

White spot syndrome associated baculovirus (WSBV), the causative agent of white spot disease (WSD) in prawns, and *Penaeus monodon*-type baculovirus (MBV), also designated as *P. monodon* singly enveloped nuclear polyhedrosis virus (PmSNPV)¹, have been responsible for high mortalities and severe damage to shrimp culture in India²-⁵ as well as in different East Asian countries⁶-⁷. Since the first report of MBV in Taiwanese *P. monodon*⁸ and WSSV in cultured shrimp from Taiwan⁹ and China¹⁰-¹¹ in 1992 and 1993, respectively, both MBV and WSSV have been reported in several penaeid prawns from different geographical regions⁶,¹²,¹³. WSBV is a rod shaped, enveloped, non-occluded virus¹¹,¹⁵ and belongs to the Nudibaculovirus subfamily of the Baculoviridae, whereas MBV belongs to Baculovirus group in Baculoviridae family. MBV and WSSV are wide spread in both wild and cultured stocks of penaeid prawns and can be transmitted from brood stock to their offspring³,¹⁶, indicating that their detection and removal from culture system is an area of serious concern. The shrimp postlarvae (PL) act as asymptomatic, latent carriers of the virus and stocking of WSSV-infected PL has been reported a risk factor for WSD and MBV in culture ponds. In India, occurrence of MBV³-⁴ and WSSV²,¹⁷,¹⁸ from 1994-1999, leading to severe economic losses and virtual collapse of *P. monodon* farming in many regions in maritime states of India. As a result, farmers are led to adopt alternate farming system, like extensive or modified extensive culture instead of semi-intensive culture and replacement of *P. monodon* with giant freshwater prawn *Macrobrachium rosenbergii*, in order to minimize the loss. Subsequent reports have also indicated the occurrence of WSSV in many farms, specifically along south-east coast of India¹⁹,²⁰.

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In our previous studies, we reported the usefulness of PCR in detection of WSSV in prawn samples\cite{17,21}, suitability of various tissues\cite{22} and utility of WSSV-DNA probe hybridization in diagnosis\cite{17,23}. In continuation to our previous work, we have investigated the present status of WSSV and MBV prevalence in different prawn farms in West Bengal and evaluated the utility of PCR and specific DNA probes in detection of WSSV and MBV in prawn samples.

**Materials and Methods**

**Collection of Prawn Samples and Analysis**

Postlarvae (PLs), juveniles and sub-adults of prawns, *P. monodon*, *P. indicus* and *M. rosenbergii*, were collected from 12 different prawn culture farms located in North and South 24-Paragana districts, West Bengal (Table 1), where outbreaks of WSD was reported earlier. A total of 283 samples were screened and a representative random sample of 10 infected shrimps from each site was kept for microbiological analysis, PCR and DNA hybridization screening. At farm site, tissue samples (hepatopancreas, gills) from suspected or moribund shrimp were inoculated onto Zobell marine broth 2216 (ZMB, HiMedia, Mumbai) and stored at 4°C for further analysis. For histopathological examination, PCR and DNA hybridization screening. At farm site, tissue samples (hepatopancreas, gills) from suspected or moribund shrimp were inoculated onto Zobell marine broth 2216 (ZMB, HiMedia, Mumbai) for microbiological analysis. The hepatopancreas was removed from prawns and squash were made on clean glass slides. After air drying, the squash were fixed with few drops of methanol for 5 min and brought to the laboratory. For histopathological examination, gills, hepatopancreas and gut from prawns were fixed in cold Davidson’s fixative overnight prior to transfer to 50% ethanol for subsequent histopathological preparation and analysis\cite{24}. For electron microscopy, gills samples (3-4 mm size) were fixed in cold 3% glutaraldehyde (Electron Microscopy Grade, Sigma, USA) in 0.1 M phosphate buffer (PB, NaH2PO4.2H2O, 3.2 g; Na2HPO4.2H2O, 27.6 g; distilled water up to 1000 mL), kept in small glass vials, brought to the laboratory and stored at 4°C for further analysis.

**Microbiological Analysis of Samples**

Hepatopancreas squash prepared on glass slides were stained using hematoxyline and eisin stain (H&E) and presence of multiple spherical intranuclear, eosinophilic occlusion bodies (Obs) were examined using standard protocol\cite{25}. For microbiological analysis, samples inoculated onto ZMB were further streaked onto Zobell’s Marine 2216 E agar (ZMA), Aeromonas isolation agar (AIA) and Thiosulphate-citrete-bilesalt-sucrose (TCBS) agar (HiMedia, Mumbai). The pure cultures of bacteria obtained on ZMA, AIA and TCBS were tested for biochemical reaction, and bacteria were identified using the standard microbiological method\cite{6} and following Bergey’s Manual of Determinative Bacteriology\cite{26}.

**Extraction of DNA**

DNA extraction was carried out from both gills\cite{27} and hepatopancreas tissue samples\cite{28} for its use as template in PCR for detection of WSSV and MBV. Briefly, the hepatopancreas samples stored at –70°C were trichurated to make a fine suspension (50% w/v) using TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Treatment with sodium dodecyl sulphate (SDS) or protease K was avoided and DNA was directly extracted using phenol:chloroform (1:1) equilibrated with TE. The aqueous phase was collected and again extracted with equal volume of chloroform. DNA was precipitated using 3 M sodium acetate (to final 0.3 mM concentration) and 2 volume of chilled ethanol. After centrifugation at 12000 g × 5 min, the DNA was resuspended in 100 µL of TE. Similarly, eyestalk tissue homogenates (50% w/v) were mixed with TE containing 0.5% (w/v) SDS, 100 mM NaCl and Proteinase K (1 mg/mL) and incubated in water bath at 55°C for 1 h. After phenol-choloform (1:1) extraction, the DNA was precipitated using 3 M sodium acetate, pH 4.0 (to a final concentration of 0.3 M) and two volume of chilled absolute ethanol (Merck, Germany) and solubilized in 100 µL of distilled water. DNA were extracted from selected prawns obtained from different farms and used as template in PCR.

**Polymerase Chain Reaction**

PCR was carried out for detection of MBV\cite{29} and WSSV\cite{27} using the standard protocols. The sequence of primers used in WSSV-PCR were Forward F1: 5'-GAC AGA GAT ATG CAC GCC AA –3', Reverse R1: 5'-ACC AGT GTT TCG TCA TGG AG –3, and for MBV-PCR Forward: p35-5'-ACT TAT GTG TCA GAC AAC AAA TAT TAC AAA –3', Reverse : 5'-GGC GGC TCT GGT GCT GCA AAC TCT TTT ATT TTG AA –3'. The reaction conditions for WSSV-PCR were 100 ng of extracted DNA, 1.0 µM of each primer, 200 µM deoxynucleotide triphosphate (dNTPs), 1.0 U of Taq DNA polymerase in 10X buffer containing 15 mM MgCl2 supplied with the PCR kit (Bangalore Genei, Bangalore). The total volume was made up to 50 µL with sterile distilled
water. The mixtures were incubated at 95°C for 5 min followed by 30 cycles of amplification in an automated thermal cycler (Applied Biosystems, Gene Amp 2400, USA) programmed for 0.5 min at 95°C, 1 min at 58°C, and 1 min at 72°C. For MBV-PCR, all reaction components were same except that MgCl₂ was used at 0.25 mM final concentration and amplification was carried out for 35 cycles (95°C for 1 min, 45°C for 1 min and 72°C for 2 min). Final extension of 5 min at 72°C was given after which the 20 µL PCR products were mixed with 5 µL of DNA sample buffer (Bangalore Genei, Bangalore) and electrophoresed in 1.0% agarose gel containing ethidium bromide (Sigma-Aldrich, USA) at a concentration of 0.5 µg mL⁻¹. The DNA bands were visualized under ultraviolet transilluminator (Gel Doc 2000, Bio-rad Laboratories, USA).

Preparation of Labeled Probes and DNA Dot-blot Hybridization

The PCR amplified WSSV (643 bp) and MBV (674 bp) DNA fragments were purified from template DNA and dNTPs using QIA quick PCR purification kit (Qiagen Inc, USA). The purified DNA was labeled with alkaline phosphatase enzyme using Alkphos direct labeling reagents (Amersham Pharmacia Biotech UK Limited, England) following manufacturer’s instructions. The labeled probes were kept in ice for 30 min by which time the sample preparation was carried out. Crude lysates (500 µL) of gills and hepatopancreas samples were boiled in hot water bath for 5-10 min and snap cooled on ice. To each tube 100 µL of 0.4 N NaOH were added and after 10 min 100 µL of 2 M ammonium acetate were added. The samples were either spotted (10 µL) onto Hybond-plus membrane (Amersham Pharmacia Biotech) directly using micropipette or using a 48 well slot-blot vacuum filtration manifold apparatus (Biorad laboratories, Hercules, CA, USA). The membranes were air dried and kept separately in hybridization buffer containing WSSV and MBV labeled probe. Hybridization was carried out at 42°C overnight in a hybridization incubator (Bibby Stuart Scientific, UK) with constant rotation (8 rev/min). Stringency washes were given to the membranes using primary and secondary wash buffers, prepared following manufacturer’s protocol, then treated with signal detection buffer containing luminol and the signal was trapped on X-ray film (Kodak). Development of clear black spots on the film was taken as positive reaction.

Transmission Electron Microscopy

Gill samples of WSD infected prawns kept in 3% glutaraldehyde were given several rinses with PB. Small tissue sections (1-3 mm²) were selected and post-fixed in 1% osmium tetroxide (OsO₄, Johnson Metty Chemicals, London) prepared in PB (2% OsO₄ in distilled water mixed with equal volume of 0.2 M PB) for 1 h. The tissues were dehydrated in a series of ethanol (50, 70, 95 & 100%) with 15 min washing in each, followed by 15 min washing in two changes of propylene oxide (PO). In the mean time, Epon-Araldite embedding medium was prepared [Epon resin812, 25 mL; Araldite resin502, 20 mL; Dodecenyl sucinic anhydride (DDSA), 60 mL; 2,4,6-Tri-dimethyl aminomethyl phenol (DMP), 3 mL, Sigma Aldrich, USA]. The tissues were then treated with PO: Epon-Araldite (2:1) mix for 1 h, followed by PO:Epon-Araldite (1:2) mix for overnight. Blocks in Epon-Araldite embedding medium was prepared in beam capsules by heating at 60°C for 24-48 h in a hot air oven. Ultrathin sections were cut using Ultramicrotome (LKB Broma, 8800) and glass block knife. The sections were stained with 2% uranyl acetate (BDH, England) and 0.5% lead citrate (Sigma). Presence of viral bodies was viewed under transmission electron microscope (Jeol Gem 100X, Japan).

Results and Discussion

Cultured prawns are menaced with a variety of infectious viral diseases. WSSV disease has presumably overshadowed all others as the leading cause of shrimp production loss in Asia¹². Because of the devastation that WSSV has caused in affected prawn farms, various detection methods have been developed to monitor and control its spread. The most extensively used protocols are histopathology and PCR. However, the DNA based hybridization methods have been reported to be highly sensitive and allow the detection of virus in low concentrations and even asymptomatic carrier animals¹⁹,³⁰, indicating their suitability in diagnosis.

Prawn samples were collected from 12 different prawn farms along coastal belt of West Bengal, specifically in the areas previously affected with WSD. In most cases, prawns at various days of culture in modified-extensive system were affected but occurrence was less in extensive or natural system. The common features of disease observed in different farms were appearance of dead or moribund prawns in the feed-check-tray at the margins of the ponds and the gut of the animals looked empty.
Affected prawns showed reddish colouration of body and appendages with presence of white spots on carapace overhead or spreading over body cuticle as characteristic feature of WSSV disease\textsuperscript{15,31,32}. In some samples of *M. rosenbergii* collected from culture ponds, prominent white spots were also visible on carapace and cuticle but not in samples collected from extensive system at Kakdwip, Namkhana and Bakhali. The significant feature of disease noted in many farms, was the delayed mortality continuing up to 3 weeks after the first symptoms of disease was noticed. In the previous reports, however, high mortality in prawns of various days of culture was reported with mass mortality within 2-4 days of the onset and the disease affected a number of farms in the region\textsuperscript{2,5,31}. This indicates a decrease in virulence of WSSV and adoption of prawns to culture environment to withstand WSD to some extent; although, a detailed study is warranted in this regard. Though most farmers did not report occurrence of MBV infection, as the gross signs are not typical to observe, some prawn samples in WSD affected ponds were also examined for MBV both by squash staining method and PCR.

Microbiological analysis revealed the presence of *Vibrio* spp. in hepatopancreas and gills in most of the samples, and *V. alginolyticus*, *V. parahaemolyticus* and *Aeromonas* spp. were isolated. Occurrence of MBV with bacterial septicemia causing mass mortality of cultivated shrimp had been reported earlier in the south-east coast of India\textsuperscript{33}, where more than 10\textsuperscript{6} bacteria per mL were recorded in haemolymph and these were predominantly *V. alginolyticus* and *V. harveyi*. Isolation of *Vibrio* spp. in various organs of WSSV infected shrimp have also been reported\textsuperscript{15,34}. MBV infected larvae harboured 10 times more bacteria than uninfected larvae\textsuperscript{3}, reflecting the role of secondary invaders in the mortality of shrimp larvae. However, rinsing broodstocks, eggs and nauplii with clean sea water containing iodophore could significantly reduce bacterial load and thereby MBV infection in prawns\textsuperscript{35}.

It was reported that MBV infects digestive epithelium of hepatopancreas and anterior mid-gut in prawns and can be diagnosed by histological detection of eosinophilic intranuclear multiple spherical Obs in hypertrophied nuclei of infected cells\textsuperscript{6}. In the present study, 14.46% *P. monodon* (Fig. 1) and 9.33% *P. indicus* (Fig. 2) were found positive for MBV-Obs in squash preparations of hepatopancreas (Table 1). Significantly, all these samples were also found
positive for WSD in PCR, indicating co-infection of prawns with both MBV and WSSV. However, all tested *M. rosenbergii* were found negative for MBV-Obs. The results correlate with the histopathological findings of hepatopancreas, where necrosis of hepatopancreatic tubules (Fig. 3) with the presence of multiple spherical Obs could be observed. The Obs were eosinophilic, multiple, spherical, 3-4 μm in diameter, often crossed and reported as characteristic of MBV\(^{25}\), which suggest that histological and impression methods to be of similar sensitivity. Obs were not noticed in any hepatopancreas squash of adult prawns collected from extensive or natural sources. This finding was typical as in our previous surveillance in various farms in coastal Andhra Pradesh, typical MBV-Obs were not observed in hepatopancreas squash of sub-adult and adult prawns, although most of the samples were positive for WSD in PCR\(^3\). On the other hand, others\(^4\) have noted high prevalence of MBV infection both in *P. monodon* (37%) and *P. indicus* (33%), but most of the prawns were not affected by WSD. Occurrence of MBV in prawn PLs with high mortality but less in juveniles, sub-adults and adult prawns have been reported\(^3,36,37\).

Similarly, high prevalence of MBV (98% PLs and 5.7% in brood-stock) has also been reported in many hatcheries in Taiwan and Thailand\(^25\). In contrast, the present investigation recorded only 10.6% samples to be infected with MBV and all these samples were also positive for WSSV. Similarly a total of 41.16% *P. monodon*, 37.33% *P. indicus* and 4.08% *M. rosenbergii* (37.10% of total samples screened) were found positive for WSD (Table 1). This indicates a drastic fall in prevalence and virulence of MBV and WSSV in prawn juveniles, sub-adults and adults as compared to previous years.

PCR has been used for specific detection (643 bp amplified DNA) of RV-PJ and SEMBV viruses responsible for WSD in Japan\(^27\). Using the same set of primers in the present investigation, of 80 samples, 51 samples (63.75%) were found positive for WSSV in PCR and DNA band of 643 bp could be visualized on 1% agarose gel (Fig. 4). In MBV-PCR, of 80 samples screened, 17 samples (21.25%) were found positive and amplicon of 674 bp could be visualized on 1% agarose gel (Fig. 5). Significantly all those samples found positive for MBV in PCR were also found positive for WSSV, indicating concurrent infection of

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<th>Species affected</th>
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<th>No. infected with WSD(^a)</th>
<th>No. infected with MBV(^b)</th>
<th>Percentage prevalence</th>
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\(^a\) WSD in prawns as detected by gross signs of body discoloration with presence of minute white spots on carapace, cuticle and empty gut

\(^b\) MBV infection in prawns as detected by presence of single or multiple spherical occlusion bodies in hepatopancreas squash preparation

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**Table 1**—Prevalence of MBV and WSSV in cultured *P. monodon*, *P. indicus* and *M. rosenbergii* in different farms located in North and South-24 Paragana, West Bengal
Fig. 3—Histopathological analysis of hepatopancreas from WSSV infected prawn showing cellular degeneration of hepatopancreatic globules (arrow) with presence of MBV-Obs. See some healthy globules unaffected by MBV infection (H&E stain, ×400).

Fig. 4—Screening of prawn samples for detection of WSSV using PCR. Specific amplification of 643 bp was visualized on 1% agarose gel after staining with ethidium bromide (Lane 2, 3, 5, 8, 9, 11 & 12) and in negative cases no bands were observed. M & Lane 1 indicate position of DNA molecular weight marker pBR322 DNA-Hinf I digest (1631 bp-75 bp).

Fig. 5—Showing PCR amplification of MBV in hepatopancreas extract of WSD infected prawn samples. Amplification of 674 bp DNA (arrow) could be visualized on 1% agarose gel electrophoresis. Lane 1 & 14 indicate position of DNA molecular weight marker-pBR 322 Hinfl digest (1631 bp-75 bp).

prawns with both the viruses. This finding was important as occurrence of MBV in *P. monodon* and concurrent infection of prawns with both MBV and WSSV had not been previously reported in West Bengal, although occurrence of WSD had been recorded. However, prevalence of both MBV and WSSV in *P. monodon* hatcheries located along south-east and south-west coast in India have been reported, where MBV and WSSV were detected in 54 and 75% of the samples, respectively. Similar prevalence of MBV and WSSV in other parts has also been reported.

The occurrence and distribution of WSSV among cultured and captured penaeid shrimps and crustaceans in the south-east coast of India was studied from November 1999 to April 2002 using PCR. Of 419 samples of captured crustaceans, viz. *P. monodon* brooders, *P. indicus* juveniles, *Metapenaeus* spp., crab *Scylla serrata* and *Squilla mantis*, 23% of them were found positive for WSSV in PCR. PCR has also been found suitable for detection of MBV, and WSSV in *P. monodon* and *M. rosenbergii* larvae, post-larvae, juveniles and adults. It has been reported that WSBV from infected *P. monodon* and *M. rosenbergii* were closely related. Other researchers, using 2 oligonucleotide primers (102F1 & 102F2) specific for WSSV, observed that closely
related strains of putative baculovirus were involved in white spot disease of cultured penaeid prawns occurring in six Asian countries.

In the present investigation, the samples were screened for WSSV using dot blot hybridization, whereas MBV samples were screened using slot blot hybridization. Clear deep black spots were visible on X-ray film, indicating most samples tested to be positive for WSSV (Fig. 6). In slot blot, similar reaction was also observed with MBV samples and deep black lanes were observed in positive cases (Fig. 7). All samples which were positive in PCR were also found positive by DNA hybridization. Similar observations have also been observed by others using DIG labeled 1447 bp DNA specific for WSBV. The PCR product hybridized with DNA extracted from WSBV infected shrimp but not with control samples, indicating the specificity of the 1447 bp PCR. Using DNA probe against white spot virus PmNOBII in Thailand, cross-reaction of the probe has been reported with WSSV infected specimens from different locations.

Other workers have also used DNA probe in dot blot and in situ hybridization for detection of WSSV. WSSV-digoxygenin (DIG) labeled DNA probes L46 and B15 have been used to test the hemolymph collected from moribund and dead animals. In situ hybridization with WSSV probe L46, sub-cuticular cells, gills, connective tissue, heart muscle cells in the haemal sinuses of hepatopancreas gave strong hybridization. Using a recombinant plasmid Pms146 probe labeled with DIG-dUTP, WSBV-DNA has been detected in the paraffin embedded, fixed tissue sections. Hossain et al. used nested PCR amplified 310 bp DNA labeled with biotin as probe in hybridization assay to assess similar products from wild crustaceans after PCR. Whereas other researchers have used DIG and biotin as label in WSSV-DNA probe. In the present investigation, we used alkaline phosphatase enzyme for DNA labeling and the results were found to be more accurate with strong and persistent colour reaction than HRP labeling as observed in our previous trials, indicating suitability of AP-DNA label in hybridization. Further, dot blot hybridization was found to be easier in application, did not require manifold vacuum and had equal sensitivity as slot blot; hence, preferred for sample screening application.

TEM examination of gill tissues revealed numerous rod shaped, non-occluded, cylindrical to ellipsoid in

Fig. 6—Detection of WSSV in gill extract of prawn using dot-blot hybridization technique. PCR amplified 643 bp DNA was labelled with alkaline phosphatase and used in hybridization. After development of X-ray film, clear black spots were visualized in positive samples and in negative case no spots were visible. Lane 1A indicates known positive and Lane 1F indicates known negative sample.

Fig. 7—Screening of hepatopancreas samples for MBV using slot-blot DNA hybridization. Samples after blotting on Hybond membrane were kept in hybridization buffer containing MBV-674 bp labeled probe. After signal detection, clear black bands were developed in MBV positive samples and in negative samples no spots or bands were formed Lane 1A indicate known positive and Lane 1E indicate known negative sample.
shape, enveloped virions accumulated in hypertrophied nuclei of gill tissue and some in the cytoplasm (Fig. 8). There was much variation in the size of virions and the mean size of the virion was in the range of 125x310 ± 20 nm. Some empty capsids and circular envelopes were also observed in the hypertrophied nuclei (Figs 8 B,C). Similar TEM observations of WSSV have also been made by others\textsuperscript{11,31,32}. Some workers\textsuperscript{15} observed rod shaped viruses in the nuclei of lymphoid organ and the size of complete virion was 83x275 nm and the size of nucleocapsid was 54x216 nm, whereas others\textsuperscript{48} have noted the virus measurement 298 ±21 x 107 ± 8 nm in \textit{P. monodon} and 248 ± 12 x 104 ± 8 nm in \textit{P. japonicus}, indicating size variability in different species. The presence of immature viruses with empty capsids, capsid originators, nucleocapsids and circular envelops was also observed. Similar observations were also made by others in WSSV infections\textsuperscript{44,48}.

Early detection of virus in larvae, PLs, juveniles and brood-stock has been an important factor in disease management and prevention of disease spread. Further, selection of virus free brood-stock is also equally important for the control of disease in hatchery and its eradication in subsequent culture. In this regard, PCR and DNA hybridization techniques can be useful in screening of samples for the presence of both MBV and WSSV, which could benefit the shrimp farmers. The advantages using these techniques include detection of virus in latent stage and in carrier animals, which are considered as an important source of virus transmission. The present study showed the prevalence of both MBV and WSSV in prawn farms in West Bengal and utility of both PCR and DNA dot-blot hybridization in detection of these viruses, which can be employed in diagnosis and disease management programmes.

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