Detection of monodon baculovirus and whitespot syndrome virus in apparently healthy *Penaeus monodon* postlarvae from India by polymerase chain reaction

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Abstract

The simultaneous presence of monodon baculovirus (MBV) and whitespot syndrome virus (WSSV) in apparently healthy postlarvae of *Penaeus monodon* from different hatcheries in India was studied by nested polymerase chain reaction (PCR). MBV could be detected in 54% of the samples. However, only 15% of samples were positive by non-nested reaction. WSSV could be detected in 75% of samples, 19% being positive by non-nested reaction. The results show simultaneous presence of WSSV and MBV in many samples at various degrees of infection. Only 14% of the samples analysed were negative for both viruses.

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1. Introduction

Viral diseases are a major problem for shrimp aquaculture all over the world. Although several viral diseases have been reported in Asia (Flegel, 1997), widespread mortalities have been reported mainly due to monodon baculovirus (MBV) and whitespot syndrome virus...
(WSSV). MBV was reported to be the major cause of shrimp culture crash in Taiwan (Chen et al., 1989). Mass mortalities have also been reported from other Asian countries (Lightner et al., 1992; Natividad and Lightner, 1992).

Although, presently MBV is not considered a major threat to shrimp grow-out ponds, it can still cause serious mass mortalities in hatchery-reared larvae. Incidence of MBV has been reported to be 70–100% in various hatcheries and ponds in Thailand, The Philippines, Indonesia, Taiwan, Malaysia and Singapore (Brock et al., 1983; Lightner et al., 1983, 1985, 1992; Anderson, 1988; Johnson and Lightner, 1988; Nash et al., 1988; Chen et al., 1989; Baticados et al., 1991; Fegan et al., 1991). In a histopathological and electron microscopic study, Ramasamy et al. (1995) recorded 81% prevalence of MBV in Penaeus monodon larvae in a hatchery in India with infected postlarvae showing 90% mortality. Using direct microscopic observation, Karunasagar et al. (1998) noted an incidence of MBV ranging from 0% to 58% in different hatcheries in India.

At present, WSSV is considered the most serious problem for shrimp aquaculture in Asia. This virus affects all life stages of P. monodon and mortality rate can reach 100% within 3–10 days of the onset of clinical signs (Inouye et al., 1994; Nakano et al., 1994; Chou et al., 1995; Wang et al., 1995; Karunasagar et al., 1997). WSSV has been shown to affect a wide range of wild crustaceans including crabs, lobsters and shrimp, both penaeid and non-penaeid (Lo et al., 1996a,b; Kanchanaphum et al., 1998; Peng et al., 1998; Supamattaya et al., 1998; Wang et al., 1998; Otta et al., 1999; Hussain et al., 2001). Presence of WSSV in wild-captured broodstock of P. monodon has also been reported (Lo et al., 1997; Itami et al., 1998; Otta et al., 1999). Using in situ hybridisation, Lo et al. (1999) showed that WSSV can be present in oocytes, follicle cells and connective tissue cells in ovary and thus suggested that vertical transmission of the virus could occur. Presence of WSSV in both wild as well as hatchery reared postlarvae has been recorded (Lo et al., 1997; Tsai et al., 1999; Hao et al., 1999).

However, not much data are available on the simultaneous presence of WSSV and MBV in P. monodon postlarvae and the degree of infection. We report here the detection of WSSV and MBV in postlarvae of P. monodon from hatcheries along the coast of India using polymerase chain reaction (PCR).

2. Materials and methods

2.1. Samples

Samples were obtained from four hatcheries situated along the west coast of India. Random samples of P. monodon postlarvae (PL) aged 15–18 days (PL 15–18) from the different corners and center of the hatchery tanks were collected in a polythene pack.

A minimum of 200 larvae were collected from each tank in 2.5 L clean oxygenated hatchery water in polythene bag and transported to the laboratory for further analysis. Some seed samples (PL 15–18) for PCR analysis for MBV and WSSV were submitted by farmers from undisclosed hatcheries for stocking in their ponds.
2.2. Extraction of DNA

A subsample of 50 larvae was taken for DNA extraction. The larvae were transferred to a UV-sterilised plastic sachet and crushed well. To this 1 ml of guanidine hydrochloride buffer (10 mM Tris–HCl, pH 8.0, 0.1 M EDTA, pH 8.0, 6 M guanidine hydrochloride and 0.1 M sodium acetate) was added, mixed and allowed to react for 30 min. The homogenate was transferred to a 1.5-ml microfuge tube and centrifuged at 5000 rpm for 5 min. Five hundred microliters of supernatant was transferred to a fresh microfuge tube and 500 μl of cold ethanol was added. This was mixed few times by inverting and was subjected to centrifugation at 14,000 rpm for 20 min. The pellet obtained was washed once with 95% ethanol followed by one washing with 70% ethanol. The DNA pellet was dried in a vacuum drier and dissolved in 100 μl sterile distilled water.

2.3. Polymerase chain reaction

For detection of MBV, PCR primers described by Belcher and Young (1998) were used. For WSSV, primers described from our laboratory previously (Hossain et al., 2001) were employed. PCR was carried out in 50 μl volume, the reaction mixture consisting of 1 × PCR buffer, 10 pmol of each primer, 200 μM of dNTPs, 2.25 units of Taq DNA polymerase and 3 μl of DNA extract. The PCR programme for MBV (both I step and II step) was as described by Belcher and Young (1998) and for WSSV (both I step and II step) as described by Hossain et al. (2001).

The amplified products were electrophoresed in 1.5% agarose gel incorporated with ethidium bromide at a concentration of 0.5 μg/ml in 1 × Tris–acetate–EDTA (TAE) buffer (4.84 g Tris base, 1.14 ml glacial acetic acid, 2.0 ml 0.5 M EDTA, pH 8.0, made up to 1 L) (Sambrook et al., 1989) and the gel analysed using Gel-Doc System (Herolab, Germany).

3. Results and discussion

The PCR primers used in this study for MBV amplified a 533-bp fragment in the non-nested reaction and a 361-bp fragment in the nested reaction (Fig. 1). The WSSV primers amplified a 486-bp fragment in non-nested reaction and a 310-bp fragment in the nested reaction (Fig. 2). Results in Table 1 show that out of 116 samples tested from four different hatcheries, 68 (59%) were positive for MBV and 85 (73%) were positive for WSSV. Of these, 19 (16%) were positive for MBV by non-nested reaction and 24 (21%) were positive for WSSV by non-nested reaction. Out of 31 larval samples of unknown source studied, 12 were positive for MBV, 3 in non-nested reaction and 9 in nested reaction. Of the 31 larval samples, 25 were positive for WSSV, 4 in non-nested reaction and 21 in nested reaction.

Although mortality due to MBV in hatcheries in India has been reported (Ramasamy et al., 1995), there is very little data on the incidence of this virus in apparently healthy larvae. Using direct microscopic observation, Karunasagar et al. (1998) reported an incidence ranging from 0% to 58% in different hatcheries in India. In this study, PCR has been used to detect MBV and this technique is expected to be much more sensitive compared to direct microscopic observation. This study demonstrates that there is a gradation of infection in
various hatcheries. Positivity in non-nested PCR would indicate a higher load of pathogen. Nested PCR has been observed to increase the sensitivity of detection of WSSV by $10^3$ to $10^4$ fold (Lo et al., 1996a) and it is possible to expect a similar situation in the case of MBV. As shown in Table 1, 22/147 (15%) samples were positive for MBV by non-nested reaction indicating a high virus load while 58/147 (40%) were positive only by the nested reaction indicating low viral load.

Most earlier reports on the incidence of MBV studied in Asia has been based on histological data. Hao et al. (1999) noted less than 40% prevalence in wild shrimp seeds in Vietnam. MBV has been reported to occur frequently in *P. monodon* broodstock in Asia. Liao et al. (1992) noted that the prevalence of MBV in female broodstock in Taiwan was only 33% in 1987, but it was 100% in 1989. Natividad and Lightner (1992) reported 85–100% prevalence of MBV in *P. monodon* postlarvae in the Philippines. The results of this study show that MBV is widely present in hatcheries in India. Presence of MBV need not necessarily result in disease and mortalities as this virus is well tolerated by *P. monodon* in light to moderate infections (Lightner, 1988). It has been suggested that the transmission of MBV occurs through oral route in water contaminated with MBV from fecal matter of broodstock (Chen et al., 1992). It has also been proposed that MBV infection of eggs/larvae can be avoided by washing the fertilised eggs in clean filtered seawater (Chen et al., 1992). The differences observed in the incidence in different hatcheries may be due to variations in the incidence of MBV in broodstock or due to hygienic practices in the hatchery.

Results in Table 1 show that WSSV is also present in 75% of apparently healthy larvae. Although WSSV is considered a highly virulent virus, the presence of this virus could be
detected in apparently healthy postlarvae by PCR (Lo et al., 1996a; Otta et al., 1999; Magbanua et al., 2000). In this study, WSSV could be detected in 19% of the PL tested by non-nested reaction and in 55% by nested PCR. WSSV has been reported to be present in wild shrimp broodstock from Taiwan, Japan and India (Lo et al., 1997; Itami et al., 1998; Otta et al., 1999). WSSV has been detected in oocytes, follicle cells and connective tissue cells in ovary and it has been suggested that there can be vertical transmission of this virus (Lo et al., 1997). Thus, its presence in a majority of postlarval samples is to be expected. Lo et al. (1997) and Tsai et al. (1999) noted that larvae obtained from broodstock that are PCR positive for WSSV could be PCR positive by nested reaction or PCR negative. Their studies also showed that positivity at the egg to PL stage was low, but that it increased

Table 1
Incidence of WSSV and MBV in apparently healthy larvae of *P. monodon*

<table>
<thead>
<tr>
<th>Hatchery</th>
<th>No. of samples</th>
<th>No. positive for MBV</th>
<th>No. positive for WSSV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Non-nested</td>
<td>Nested</td>
</tr>
<tr>
<td>A</td>
<td>30</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>B</td>
<td>31</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>C</td>
<td>25</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>D</td>
<td>30</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>Unknown source</td>
<td>31</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>147</td>
<td>22</td>
<td>58</td>
</tr>
</tbody>
</table>
from PL 1 to PL 20 and they proposed that this could be due to the inability of the virus to replicate in early larval stages. In this study, however, we have examined PL 15–PL 18 and that might explain the high positive rate (19%) even by the non-nested reaction.

Results in Table 2 show the simultaneous presence of WSSV and MBV in PL. In 5 samples (3.4%), both WSSV and MBV were detected by non-nested reaction, while in 15 samples (10.2%), WSSV was detected by non-nested reaction and MBV by nested reaction. While 11.6% of sample showed MBV by non-nested reaction and WSSV by nested reaction, 18% samples showed the presence of both WSSV and MBV by nested PCR. The data presented in Table 2 were subjected to Pearson correlation test. The test indicated no significant correlation ($P=0.29$) between WSSV and MBV severity. Results in Table 3 indicate number of samples that showed presence of single virus detected by either nested or non-nested PCR. Of the 147 samples, 21 (14.2%) were negative for both viruses. Forty-six samples that were positive for WSSV were negative for MBV. Of these, 8 were positive for WSSV by non-nested reaction. Sixteen samples that were positive for MBV by nested reaction were negative for WSSV.

There are not much data on the simultaneous presence of WSSV and MBV in *P. monodon* postlarvae intended for stocking. An earlier study of Hao et al. (1999) showed the simultaneous presence of MBV (detected by the microscopic method) and WSSV in shrimp seeds in Vietnam, but they did not indicate the viral status of individual samples or degrees of infection (nested vs. non-nested PCR). This study provides data on both MBV and WSSV by PCR. Knowledge on the presence of viruses and viral load in postlarvae intended for stocking in ponds will greatly help the farmer to understand the risk associated with

<table>
<thead>
<tr>
<th>Hatchery</th>
<th>Number of samples showing positive reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WSSV-N/MBV-N</td>
</tr>
<tr>
<td>A</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
</tr>
<tr>
<td>D</td>
<td>7</td>
</tr>
<tr>
<td>Unknown source</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
</tr>
</tbody>
</table>

N = nested PCR, NN = non-nested PCR.

<table>
<thead>
<tr>
<th>Hatchery</th>
<th>WSSV-non-nested/MBV-negative</th>
<th>WSSV-nested/MBV-negative</th>
<th>WSSV-negative/MBV-non-nested</th>
<th>WSSV-negative/MBV-nested</th>
<th>WSSV-negative/MBV-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>3</td>
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<tr>
<td>B</td>
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<td>3</td>
</tr>
<tr>
<td>C</td>
<td>2</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>D</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Unknown source</td>
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<td>11</td>
<td>0</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>38</td>
<td>0</td>
<td>16</td>
<td>21</td>
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</table>
stocking infected larvae and appropriate management measures for their culture. It would be interesting to study the relationship between crop success and simultaneous presence of two viruses.

Acknowledgements

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