Prevalence, diversity and co-occurrence of the white spot syndrome virus, monodon baculovirus and Penaeus stylirostris densovirus in wild populations of Penaeus monodon in the Philippines

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ABSTRACT: The farming of the black tiger shrimp Penaeus monodon in the Philippines relies on wild broodstock. PCR was thus used to determine the prevalence of white spot syndrome virus (WSSV), monodon baculovirus (MBV) and Penaeus stylirostris densovirus (PstDV) in a total of 178 shrimp from 6 geographically disparate locations where broodstock are captured for use in hatcheries. PCR amplicons were also sequenced to identify phylogenetic relationships of the virus haplotypes detected. Shrimp from southeastern Luzon (Camarines Norte) had the highest prevalence of each of the 3 viruses and were frequently co-infected with 2 or more viruses. No viruses were detected in shrimp from northwestern Luzon (Pangasinan). MBV was most prevalent and PstDV strains displayed the most genetic diversity. WSSV was detected at 3 sites, and a VP28 gene sequence examined was invariant and consistent with strains found in many countries, including Thailand, China, Japan, Korea, Indonesia, Iran, Brazil and Mexico. WSSV open reading frame 94 gene sequence analysis identified location-specific repeat types. MBV sequences were dissimilar to haplotypes detected in India. PstDV sequences were diverse and included 2 lineages detected either in Australia or in the United States, Ecuador, Taiwan, China and Vietnam. The PCR data confirmed that WSSV, MBV and PstDV are endemic in P. monodon in the Philippines but that populations at some locations might remain free of infection.

KEY WORDS: White spot syndrome virus · WSSV · Penaeus stylirostris densovirus · PstDV · Monodon baculovirus · MBV · Penaeus monodon · Prevalence · Diversity · Philippines

INTRODUCTION

Several viruses are pathogenic for the black tiger shrimp Penaeus monodon. Diseases caused by viruses, including white spot syndrome virus (WSSV), monodon baculovirus (MBV) and P. stylirostris densovirus (PstDV), have resulted in serious economic impacts for farming of this species (Lightner 1996, Flegel 2006). Since the discovery of white spot disease (WSD) in Taiwan in 1992 (Chou et al. 1995), mass mortality resulting from WSD has devastated shrimp aquaculture industries worldwide. WSSV is classified in the genus Whispovirus of the family Nimaviridae and has a genome comprised of ~310 kb circular double-stranded DNA (Fauquet et al. 2005). MBV was first detected in P. monodon farmed in Taiwan, is classified in the genus Nucelopolyhedrovirus of the family Baculoviridae and has a genome comprised of long circular double-stranded DNA (Rohrmann 1986, Belcher & Young 1998). MBV replication occurs in
the nucleus of hepatopancreatic and midgut epithelial cells, and mortality due to disease primarily impacts larval life stages in hatcheries (Lightner & Redman 1981, Fegan et al. 1991, Lightner 1996, Bondad-Reantaso & McGladdery 2001). PstDV was first detected in juvenile pacific blue shrimp *P. stylirostris* being cultured in Hawaii in 1981 (Lightner et al. 1983), has subsequently been detected in most penaeid species farmed in different regions of the world (Lightner 1996, Tang et al. 2003), is classified in the genus *Brevidensovirus* of the family *Parvoviridae* (Bonami et al. 1990) and has a genome comprised of ~4.1 kb single-stranded DNA (Bonami et al. 1990, Mari et al. 1993). While PstDV infection can result in mortality, disease is more commonly associated with retarded growth and cuticle and rostrum deformities referred to as runt deformity syndrome (Primavera & Quintio 2000).

To assist the shrimp farming industry in the Philippines in understanding and managing the risks of utilizing wild-caught *P. monodon* broodstock, the prevalence and genetic makeup of WSSV, MBV and PstDV was assessed at 6 disparate geographical locations where broodstock are captured for use in hatcheries.

**MATERIALS AND METHODS**

**Shrimp**

A total of 178 wild female *Penaeus monodon* were trawled by fishermen at 6 locations in the northern, central and southern Philippines (Camarines Norte, Quezon, Leyte, Pangasinan, Cagayan, Zamboanga del Sur) representing the primary sources of broodstock used in hatcheries. Shrimp from each location were captured by local fishermen provided with a standard procedure for shrimp handling and storage. This procedure specified that shrimp be frozen immediately after collection and shipped frozen to the laboratory within 24 h. On arrival at the laboratory, shrimp were inspected and only those in visibly good condition were used. Information on the collection sites, dates and sample sizes is summarized in Table 1. WSSV, MBV and PstDV positive control material was obtained from the Fish Health Management and Quality Assurance Laboratory, Bureau of Fisheries and Aquatic Resources, Department of Agriculture (BFAR-DA). Tissue samples were dissected aseptically from each shrimp and refrozen at −20°C until processed.

**DNA extraction**

Total genomic DNA was extracted from hepatopancreas tissue according to the Bioline®, DNA Isolation Kit protocol. DNA was quantified using a Shimadzu UV Biospec-Nano spectrophotometer and diluted to a concentration of 10 ng µl$^{-1}$ in PCR-grade purified water.

**PCR detection of WSSV, MBV and PstDV**

WSSV, MBV and PstDV were detected by PCR or nested PCR using published primer sequences and thermal cycling conditions (Table 2). Each 20 µl reaction contained Vivantis 1 × Taq polymerase buffer, 0.2 mM dNTPs, 0.5 µM forward and reverse primers, 1 U Taq DNA polymerase and 1–2 µl DNA. The sizes of amplified DNA products were confirmed using agarose gel electrophoresis and amplicons were purified and sequenced at First BASE Laboratories, Selangor, Malaysia.

**Amplicon sequence analyses**

DNA amplicons were sequenced in forward and reverse directions using primers employed in each PCR and sequence chromatograms were checked and edited using DNA Baser version 3.5 (Heracle Biosoft). ClustalW in MEGA 6 (Tamura et al. 2013) was used to align sequences and infer phylogenetic relationships according to the lowest Bayesian’s information criterion and Akaike’s information criterion scores. Bootstrap support was estimated with 1000 replicates. Phylogenograms were edited using Adobe® Illustrator CS5.

<table>
<thead>
<tr>
<th>Location</th>
<th>Shrimp number</th>
<th>Sample code</th>
<th>Collection date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daet, Camarines Norte</td>
<td>30</td>
<td>CAM 101–130</td>
<td>December 2014</td>
</tr>
<tr>
<td>Tagkawayan, Quezon</td>
<td>30</td>
<td>QUE 101–130</td>
<td>February 2015</td>
</tr>
<tr>
<td>Tacloban, Leyte</td>
<td>30</td>
<td>LEY 101–130</td>
<td>March 2015</td>
</tr>
<tr>
<td>Alaminos, Pangasinan</td>
<td>30</td>
<td>PAN 101–130</td>
<td>March 2015</td>
</tr>
<tr>
<td>Buguey, Cagayan</td>
<td>28</td>
<td>CAG 101–128</td>
<td>April 2015</td>
</tr>
<tr>
<td>Pagadian, Zamboanga del Sur</td>
<td>30</td>
<td>ZAM 101–130</td>
<td>May 2015</td>
</tr>
</tbody>
</table>
RESULTS

WSSV

A WSSV VP28 PCR primer pair (Syed Musthaq et al. 2006) was used to detect WSSV in hepatopancreas tissue sampled from groups of 28–30 wild *Penaeus monodon* (total = 178) collected from each of 6 disparate locations in the Philippines. WSSV DNA was amplified from 36 shrimp collected at 3 of the 6 locations: Camarines Norte (25/30), Zamboanga del Sur (7/30) and Leyte (4/30) (Table 3). For all 36 WSSV-positive shrimp, sequences determined for a 202 bp region of the VP28 gene were identical (GenBank accession numbers: KY273305 to KY273340).

The VP28 gene sequence detected was consistent with WSSV strains analyzed from Thailand (EF194079), China (AY249440), Japan (AY249443), Korea (AY324881), Indonesia (AY249441), India (DQ681069), Iran (AB855742), Brazil (HQ130032) and Mexico (FJ756456).

The open reading frame (ORF) 94 PCR designed to amplify across a variable-number tandem repeat (VNTR) region of the WSSV genome (Wongteerasupaya et al. 2003) generated amplicons for 27 of the 36 WSSV-positive shrimp. All shrimp yielded a single amplicon suggesting the presence of a single WSSV strain. Nucleotide sequences determined for the 27 amplicons (GenBank accession numbers KY273341 to KY273366) had 5 to 11 tandem copies of the 54 bp repeat and were designated ORF94-5 to ORF94-11, with repeat frequencies of ORF94-10 (n = 6), ORF94-11 (n = 6), ORF94-7 (n = 5), ORF94-6 (n = 4), ORF94-9 (n = 4), ORF94-5 (n = 1) and ORF94-8 (n = 1).

Table 2. PCR primer sequences and amplicon lengths

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’−3’)</th>
<th>Product size (bp)</th>
<th>PCR annealing temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSSV VP26F</td>
<td>ATGGAAATTGTGGCAACCTAACAACACCTG</td>
<td>304</td>
<td>52</td>
<td>Dieu et al. (2004)</td>
</tr>
<tr>
<td>WSSV VP26R</td>
<td>GGCGGTGTGACGAGGATGAGACTG</td>
<td>245</td>
<td>45</td>
<td>Syed Musthaq et al. (2006)</td>
</tr>
<tr>
<td>WSSV VP28F</td>
<td>GAAAAACACAGACAAATATCG</td>
<td>245</td>
<td>45</td>
<td>Syed Musthaq et al. (2006)</td>
</tr>
<tr>
<td>WSSV VP28R</td>
<td>CTTCCCTCAAAGGTGAGATTCC</td>
<td>245</td>
<td>45</td>
<td>Syed Musthaq et al. (2006)</td>
</tr>
<tr>
<td>WSSV ORF94R</td>
<td>AGCCAGGTGTGACGACGAC</td>
<td>533</td>
<td>45</td>
<td>Belcher &amp; Young (1998)</td>
</tr>
<tr>
<td>MBV1.4F</td>
<td>CGATTCCATATCGCCGGAATA</td>
<td>314</td>
<td>50</td>
<td>Belcher &amp; Young (1998)</td>
</tr>
<tr>
<td>MBV1.4R</td>
<td>TGGCGATGCACCTCCTGAGAT</td>
<td>314</td>
<td>50</td>
<td>Belcher &amp; Young (1998)</td>
</tr>
<tr>
<td>MBV1.4NF</td>
<td>TCCAATCCTCGCTGCGGATCTA</td>
<td>648</td>
<td>45</td>
<td>Rai et al. (2009)</td>
</tr>
<tr>
<td>MBV1.4NR</td>
<td>CGCTAAATGGGGGACCAAAATTC</td>
<td>648</td>
<td>45</td>
<td>Rai et al. (2009)</td>
</tr>
<tr>
<td>IHNV648F</td>
<td>GAAGCGTGTTGGATTTTT</td>
<td>309</td>
<td>55</td>
<td>Tang et al. (2007)</td>
</tr>
<tr>
<td>IHNV648R</td>
<td>AGCGTAGGACTTGGCGGATTA</td>
<td>309</td>
<td>55</td>
<td>Tang et al. (2007)</td>
</tr>
</tbody>
</table>

Table 3. Numbers of wild *Penaeus monodon* captured at the 6 locations in the Philippines in which white spot syndrome virus (WSSV), monodon baculovirus (MBV) and/or *Penaeus stylirostris* densovirus (PstDV) were detected by PCR

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Camarines Norte (n)</th>
<th>Quezon (n)</th>
<th>Leyte (n)</th>
<th>Pangasinan (n)</th>
<th>Cagayan (n)</th>
<th>Zamboanga del Sur (n)</th>
<th>Total (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1</td>
<td>15</td>
<td>17</td>
<td>30</td>
<td>21</td>
<td>14</td>
<td>98</td>
</tr>
<tr>
<td>MBV only</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>PstDV only</td>
<td>1</td>
<td>9</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>WSSV and MBV</td>
<td>11</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td>19</td>
</tr>
<tr>
<td>MBV and PstDV</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>WSSV and PstDV</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>WSSV, MBV and PstDV</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>WSSV total</td>
<td>25</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>36</td>
</tr>
<tr>
<td>MBV total</td>
<td>18</td>
<td>13</td>
<td>9</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>44</td>
</tr>
<tr>
<td>PstDV total</td>
<td>14</td>
<td>8</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>13</td>
<td>47</td>
</tr>
</tbody>
</table>
addition, VNTR types detected at each of the 3 locations were unique: Camarines Norte, ORF94-7, -10 and -11; Leyte, ORF94-5 and -8; Zamboanga del Sur, ORF94-6 and -9. A cytosine thymine (C/T) polymorphism occurred at position 48 in each 54 bp repeat, with T being dominant in strains with ≥10 repeats and C dominant in strains with <10 repeats.

**MBV**

A nested PCR designed to amplify a 314-bp MBV genome region (Belcher & Young 1998) detected MBV DNA in 52 out of 178 hepatopancreas DNA samples examined at variable prevalence depending on shrimp capture location: Camarines Norte, 83.3%; Quezon, 43.4%; Leyte, 30.0%; Zamboanga del Sur, 10.0%; Cagayan, 6.7%; Pangasinan, 0% (Table 3).

For the 52 MBV PCR amplicons, sequence analysis of the 272 bp region internal to the nested PCR primers (GenBank accession numbers KY274526 to KY274571) identified variations at 12 nucleotide positions. Sequence comparisons identified 8 haplotypes (Fig. 1), but only a small number (up to 3) were detected at each site. Haplotype M-PH 2 was detected most commonly (n = 36) among MBV-positive shrimp from the 5 capture locations where MBV was detected, followed by M-PH 1 (n = 4) found only at Camarines Norte. Distinct haplotypes (M-PH 3, 4, 5, 6, 7 and 8) were detected in 6 shrimp from variable locations.

MBV haplotypes inferred using a maximum likelihood tree constructed using the Tamura-3 model with gamma distribution (T92+G) demarcated 2 lineages, highlighting divergence between MBV strains in the Philippines and India, although with low bootstrap support (61%), and indicated that the MBV strains detected at the different locations were closely related (Fig. 2).

**PstDV**

A PCR test designed to amplify a 309 bp region of the PstDV genome (Tang et al. 2007) generated amplicons for 49 of the 178 shrimp tested (Table 3). Detection prevalences varied markedly in shrimp captured at different sites (Camarines Norte, 50.0%; Zamboanga del Sur, 50.0%; Quezon, 23.3%; Cagayan, 23.3%; Leyte, 16.7%; Pangasinan, 0%).

![Fig. 1. White spot syndrome virus (WSSV), monodon baculovirus (MBV) and Penaeus stylirostris densovirus (PstDV) haplotype distribution in wild Penaeus monodon from 6 locations in the Philippines. Numbers outside the graphs are frequency of haplotypes at a given site; W-PH: Philippine WSSV haplotype; M-PH: Philippine MBV haplotype; I-PH: Philippine PstDV haplotype.](image-url)
Sequence comparisons of the 265 bp region internal to the PCR primers for the 49 PstDV-positive shrimp (GenBank accession numbers KY273367 to KY273413) identified nucleotide variations at 54 positions. The sequences grouped into 11 haplotypes (Fig. 1) with different haplotype numbers detected at different shrimp capture locations: Camarines Norte, n = 5; Cagayan, n = 3; Quezon and Leyte, n = 4; Zamboanga del Sur, n = 1. The I-PH 2 haplotype (n = 26) occurred most frequently across the 5 capture locations. Distinct PstDV haplotypes (I-PH 3, 5, 6, 9 and 11) were identified in 6 shrimp from different sites. Only 1 PstDV haplotype was identified in shrimp from Zamboanga del Sur.

Relationships identified among the PstDV haplotypes are shown using a maximum likelihood tree constructed using the Hasegawa-Kishino-Yano model with gamma distribution (HKY+G) (Fig. 3). Haplotypes were separated with low to high bootstrap support. PstDV haplotypes in the Philippines were distributed in the maximum likelihood tree, with several haplotypes (I-PH 3, 10 and 11) clustering with Lineage I and most strains from Australia (KM593909 to KM593913) and I-PH 4 and 6 clustering with Lineage II strains from India (EU552487), Vietnam (KC513422, KF031144), China (KP733858), Taiwan (AY355307) and Thailand (AY102034). I-PH 1, 2, 5, 7, 8 and 9 clustered with Lineage III strains. I-PH 1, the most frequently detected PstDV haplotype, was identical to strains from the USA (AF273215), Ecuador (AY362548), Taiwan (AY355306 to AY355308), China (KP733859 to KP733863), Vietnam (JX840067) and Australia (KM272862) (Fig. 3).

**Co-occurrence of shrimp viruses**

The PCR data provided an opportunity to examine the prevalence at which 2 or more virus types were present in the hepatopancreas of the *P. monodon* examined (Table 3). Virus co-occurrence levels across the 5 capture locations were: WSSV + MBV, 7.3%; WSSV + PstDV, 6.7%; MBV + PstDV, 5.1%; WSSV + MBV + PstDV, 3.4%.
DISCUSSION

WSSV, MBV and PstDV were detected by PCR in wild Penaeus monodon captured from 5 of 6 locations in northern, central and southern regions of the Philippines, suggesting that they are endemic and widespread across the country. None of the 3 viruses were detected among any of the 30 shrimp captured at the Pangasian location in a northwestern region of the Philippines. While these data suggest the these viruses might not occur or occur at low prevalence in P. monodon endemic to this region, the frozen shrimp sent for analysis were only assessed visually for signs of inadequate storage; no DNA quality checks or PCR tests for endogenous shrimp genes were performed to assess its integrity. Therefore, it cannot be discounted that the DNA extracted from this group of shrimp had degraded beyond a point where it could be amplified effectively by the PCR tests.

MBV was detected most commonly, WSSV least commonly and sequences determined for PstDV strains were most diverse. Of the 5 out of 6 capture locations in the Philippines at which viruses were detected, all 3 viruses were most prevalent (50 to 83%) among the 30 shrimp captured at Camarines Norte, with many containing various combinations of 2 or all 3 viruses. These findings are consistent with other studies in which wild P. monodon have been found to carry multiple viruses (Manivannan et al. 2002, Chayaburakul et al. 2004, Umesha et al. 2006, Prakash et al. 2007, Tan et al. 2009). The prevalence of WSSV (13 to 80% depending on location) was higher than found in a dry and wet season comparison of wild P. monodon sourced from 7 locations (Capiz, Negros Occidental, Bohol, Quezon, Palawan, Misamis Occidental and Surigao del Sur) in the Philippines during 2005 (de la Peña et al. 2007). In that study, WSSV was detected at all locations except Bohol during the dry season (2 to 25% prevalence range) but only at the Palawan location during the wet season.

MBV prevalence levels (10 to 83% depending on location) detected in the groups of P. monodon captured at 5 locations were higher than those detected among P. monodon sampled in 2005 from the Philippines (de la Peña et al. 2008) using a PCR test employing the MBV1.4NF:1.4NR primer pair known to generate data consistent with the microscopic diagnosis of MBV infection in P. monodon postlarvae in hatcheries (Natividad et al. 2006). The widespread detection of MBV was also consistent with data from this study that showed MBV to be present at all evaluated locations except Palawan during the dry sea-
locations in the Philippines were genetically diverse and thus distributed across different clades. Most PstDV (I-PH 3, 5, 6, 9 and 11) sequences clustered within Lineage III, similar to PstDV types detected in shrimp from the United States, Ecuador, Taiwan, China and Vietnam. The most common PstDV haplotype in the Philippines, I-PH-1, was found to be identical to strains from the United States, Ecuador, Taiwan, China and Vietnam, suggesting the spread of this haplotype across Asia and the Americas. The possibility that PstDV-infected *P. monodon* from the Philippines might be the origin of PstDV strains now present in the Western Hemisphere (Mexico, Gulf of California, Hawaii, Ecuador, Panama, Colombia) was previously raised based on inference from data on introductions of *P. monodon* broodstock into the Americas (Lightner 1999) and on phylogenetic data (Tang et al. 2003). Moreover, PstDV strains from the Philippines clustering within Lineage I (I-PH 3, 10 and 11) together with Australian PstDV strains suggests that these haplotypes may be derived from a lineage that evolved in Indo-West Pacific *P. monodon*, as suggested previously (Jaroenram et al. 2015).

Overall, the data reported here are consistent with previous reports of WSSV, MBV and PstDV occurring in wild populations of *P. monodon* inhabiting islands throughout the Philippines. They also provide the first information on PstDV prevalence in different populations and on the prevalence of co-infections with these viruses. All shrimp studied appeared healthy and displayed no overt signs of disease, and there is no anecdotal evidence for the *P. monodon* populations from which they were captured being threatened due to disease. This leads to questions around what virus–host interactions and adaptations have evolved in *P. monodon* to accommodate such viral infections and often co-infection by several viruses (Fegan et al. 1991, Flegel et al. 2004). Hypotheses to explain how shrimp survive such assaults include an active viral accommodation concept, in which some physiological mechanism is utilized to tolerate virus infection in an active, but innocuous persistent state that hinders it from progressing to cause disease or from stimulating host-defense responses leading to cell destruction (Flegel & Pasharawipas 1998, Flegel 2007). Populations of wild *P. monodon* in the Philippines might thus provide a useful resource for validating or challenging such hypotheses.

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