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PCR detection of white spot syndrome virus (WSSV) from farmed Pacific white shrimp (*Litopenaeus vannamei*) in selected sites of the Philippines

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Abstract. Great losses caused by white spot syndrome virus (WSSV) in shrimp culture have been attributed to poor screening procedures in farms and the lack of sufficient access to specific pathogen free brood stock. Thus, early detection of the virus is considered the best option for shrimp farmers. The study, thus, assessed viral incidence in the Philippines and partially sequenced and characterized the Philippine WSSV isolate with regards to other isolates in GenBank. Developed primers for PCR can detect target genes from 0.4 pg of DNA extract from shrimp samples. PCR detection revealed that 6.67 % (1/15) of market samples from Zambales are infected with WSSV. Shrimp samples from a local shop and a public market in General Santos City showed 46.67% (7/15) and 20% (3/15) WSSV-positive samples respectively. Shrimp sources from Capiz and Batangas, however, showed negative detection for WSV. No significant difference in the number of infected samples from the sampling sites was found. Combined detections reveal that the Philippines has a low infection rate of 14.67%. The study has partially sequenced and characterized Philippine isolate. During the sampling period, most shrimps in General Santos City were WSSV-positive by PCR detection.
Key Words: WSV, Philippines, shrimp, *Litopenaeus vannamei.*

Introduction. Global production of sea foods from aquaculture has grown rapidly over the past four decades, contributing to 45% of the world's supply of fish (including crustaceans, mollusks, echinoderms and amphibians) for human consumption (Subasinghe et al 2009). The shrimp aquaculture industry has grown rapidly over several decades to become a major global industry that has contributed significantly to socio-economic development in many poor coastal communities (Walker & Mohan 2009). However, the ecological disturbances and changes in patterns of trade associated with the development of shrimp farming have unfortunately hindered further growth because of the emergence and spread of viral and bacterial disease.

At least four viruses have adversely affected the global penaeid shrimp farming industry since 1980's (Lightner 2003). In the Philippines, the presence of these viruses have been confirmed as studies on shrimp viruses started when Albaladejo et al (1998) reported the presence of yellowhead virus (YHV) in some cultivated *Penaeus monodon*. A year after, Belak et al (1999), reported the presence of infectious hypodermal and hematopoietic necrosis virus (IHHNV), with an overall prevalence of 53%, but not of white spot syndrome virus (WSSV) in wild spawners obtained from Palawan, Quezon, Capiz, and Negros Occidental. However, Tapay et al (2000) detected evidence of the high prevalence of WSSV in cultured *Penaeus monodon* in the Philippines. Detection of WSSV in *P. monodon* from local shrimp farms in Negros Island from 2000-2006 followed

(Tendencia & Usero 2007). Recent reports on shrimp done by de la Peña et al (2008) determined the prevalence of monodon baculovirus (MBV) in wild *P. monodon*. The latest reports are done by Tendencia et al (2010) exploring the effect of rainfall and atmospheric temperature on the prevalence of the WSSV in pond cultured *P. monodon*. Most of these data, however, are comprehensive only for the Visayas region of the Philippines, even if the presence of viruses is continuously confirmed by shrimp farmers in Luzon.

To date, viral disease problem is still in full effect largely because there has not been any significant advancement in effectively eradicating or minimizing the effects of these pathogenic shrimp viruses. The Philippines, in its effort to prevent the introduction of these viruses in the country, has imposed a ban on the importation of shrimps from other countries. However, this ban has been lifted to revive the ailing shrimp aquaculture industry that was once included in the largest producers of shrimp in the world (Aguiba 2007).

In May 2008, during 6th Philippine Shrimp Congress, three key factors have been identified in order to successfully revive the country's ailing shrimp industry. These include 1) the culture of specific-pathogen free (SPF) and specific-pathogen resistant (SPR) broodstock and "high health" fry ; 2) use of best management practices (BMPs) such as probiotics and biosecurity measures; and 3) marketing and compliance to food safety regulations (Mojica 2008). However, for these initiatives to be successful, especially the development of BMPs and SPF/SPR brood stocks and fries, it is imperative that recent information on the prevalence and detection of pathogens in the different regions of the country affecting the industry be documented. Utilization of currently available molecular biology and biotechnology-based applications like polymerase chain reaction (PCR) can provide such important information and applications. Thus, it is the aim of the study to detect and determine WSSV prevalence in the country through the selected sampling sites and partially sequence and characterize the Philippine WSSV isolate with regards to the WSSV isolates stored in Genbank. It is noteworthy to mention that this is the first report on WSSV detection in *L. vannamei* in the country.

Materials and Methods. Sample collection of *L. vannamei* was done in Batangas, Zambales, Capiz and General Santos (South Cotabato) from October 2009 and June 2010. During each collection, 2 shrimp samples of each kind were preserved in ethanol to be used for identification. Shrimp sample identification was done at the National Fisheries Resources Development Institute (Quezon City, Philippines) where the samples were identified according to morphological standards used in shrimps.

Fifteen shrimps with an average size of 110 mm, from each sampling site, were dissected and the extracted muscle tissues from the 1st abdominal segment were placed in 1.5 mL microcentrifuge tubes with ethanol and stored until DNA extraction. DNA was extracted using Modified CTAB Extraction Method (Santos et al 2010). The tissues were minced and placed in tubes containing cetyl trimethyl ammonium bromide (CTAB) Extraction buffer (600 μL 2% CTAB pH 8.5, 30 μL of 1% Proteinase K) and finally incubated overnight in a water bath at 55 °C with occasional shaking. After incubation, 600 μL of chloroform: isoamyl (3:1) solution was added to each of the sample, shaken by hand for about 3 min and then centrifuged for 5 min. at 8,000 rpm. The upper aqueous supernatant was then transferred in new, marked 1.5 mL tubes, avoiding inclusion of the organic phase. Described steps of clean-up were done twice. DNA precipitation was carried out by mixing 50 μL of 3M sodium acetate (NaOAc) and 900 μL 95 % ethanol to the tubes containing the supernatant. These were then hand shaken for 3 min. and placed overnight in a –20 °C freezer. After precipitation, the tubes were spun in a microcentrifuge at 12,000 rpm for 10 min and then the aqueous phase was carefully pipetted out by leaving the DNA pellet at the bottom of the tube. The pellet was rinsed by adding 500 μL 70% ethanol and spun for 12,000 rpm for 5 min before the removal of ethanol. This rinsing step was done twice. Air drying was carried out right after. DNA pellets were then rehydrated in 300 μL of 1X TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). Resulting stock DNA extracts were stored in microcentrifuge tubes at -20 °C.

Each PCR reaction contained 1 unit Taq polymerase, 0.6 μM each of designed WVF (5' GTA CGG CAA TAC TGG AGG AGG 3')and WVR primers (5' GGA GAT GTG TAA GAT GGA CAA GG 3') or published primers (Tapay et al 1999), 0.2 μM dNTP solution mix, and 1x buffer with 1.5 mM $MgCl_2$. Reactions were cycled in the program: initial denaturation temperature at 95 °C for 5 minutes, 35 cycles of denaturation temperature at 95 °C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72 °C for 30 seconds, and Final DNA extension at 72 °C for 5 minutes and followed by 4 °C storage. PCR products were then run on a 1% agarose gel and viewed under UV light.

Results and Discussion. Two frozen shrimp samples with gross signs of WSSV were subjected to PCR detection using LMT primers published by Tapay et al (1999). The primer set was successful only in detecting WSSV in one of the samples (Figure 1), though success and efficiency in the amplification of Philippine shrimp WSSV isolates have been confirmed by Natividad et al (2006). These primers were designed from a cloned fragment of the WSSV genome which may have a low copy number DNA in the samples.

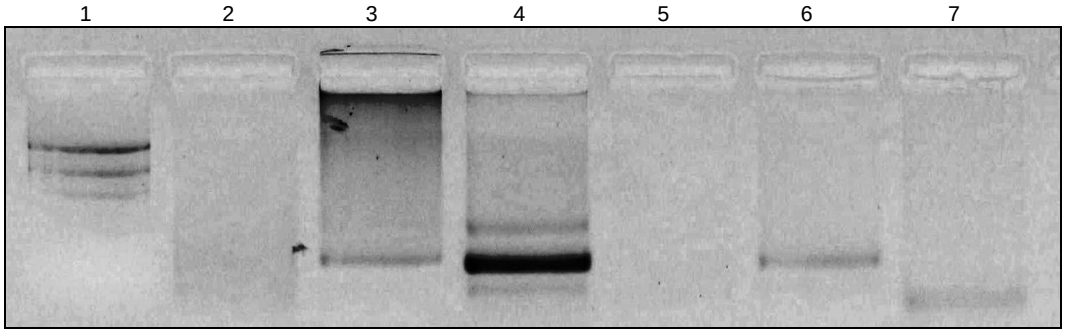

Figure 1. PCR detection of 2 shrimp samples from the Philippines using designed (WVF) and published primers (LMT). Lane 1: low mass DNA ladder, lane 2: negative control for WVF primers, lane 3: sample 1, lane 4: sample 2, lane 5: negative control for LMT primers, lane 6: sample 1, Lane 7: sample 7.

Using designed primer sets, an approximately 150 bp DNA fragment was amplified from the two samples (Figure 1). Serial dilution reveals that the designed primers, PCR mix and conditions have a detection limit of 0.4 pg. This protocol proves to be more sensitive than that of Tapay et al (1999) which has a detection limit of 10 pg.

Pairwise sequence alignment of the 2 field samples shows 100% identity with each other (Figure 2).

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CLUSTAL 2.1 multiple sequence alignment


MUS1_WSV        ---------AGGCTGGTCACATACATTGGGTAGTAAACACTGGGTACAGATCAGGGAACA 51
PV42_WSV        CATCTCATTAGGCTGGTCACATACATTGGGTAGTAAACACTGGGTACAGATCAGGGAACA 60
                         **************************************************

MUS1_WSV        TTTGCTATCACCAGTTTCTCCCTCCACCATCTTAAAGAGTTTAACGGGCGGTCTGAATCT 111
PV42_WSV        TTTGCTATCACCAGTTTCTCCCTCCACCATCTTAAAGAGTTTAACGGGCGGTCTGAATCT 120
                ************************************************************

MUS1_WSV        ATTATCCACCACAGTAAATTT- 132
PV42_WSV        ATTATCCACCACAGTAAATTTT 142
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Figure 2. Nucleotide alignment of the partial sequences of WSV from Philippine sampling sites. An asterisk indicates conserved nucleotide composition.

Global alignment using Blastn indicates that the target DNA template is conserved among other isolates in Genbank with 100% identity (Figure 3). This reveals that, based on the DNA fragments amplified, the sequences of Philippine isolates are very similar to the published sequences of China, Taiwan and Thailand isolates (Natividad et al 2006). Thus, this designed primer set was used in all subsequent WSSV detections.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
AF099142.1	White spot syndrome virus small subunit of ribonucleotide reductase gene, partial cds; l	263	263	100%	2e-67	100%	G
AF332093.1	White spot syndrome virus, complete genome	263	263	100%	2e-67	100%	
AF369029.2	White spot syndrome virus, complete genome	263	263	100%	2e-67	100%	
AF536176.1	Shrimp white spot syndrome virus wsv168 gene, complete cds	263	263	100%	2e-67	100%	G
AF440570.1	Shrimp white spot syndrome virus, complete genome	263	263	100%	2e-67	100%	

Figure 3. Sequences producing significant alignments in GenBank (blastn).

PCR detection for Luzon revealed Batangas samples from a specific pond to be negative for WSSV, despite the presence of a disease outbreak during the sampling period in nearby ponds. According to the pond operator and owner, the non-infection of the pond may be caused by getting water from a source different from the ones that got infected. Meanwhile, one (1/15 or 6.67%) market sample from Zambales returned a positive result (Figure 4).

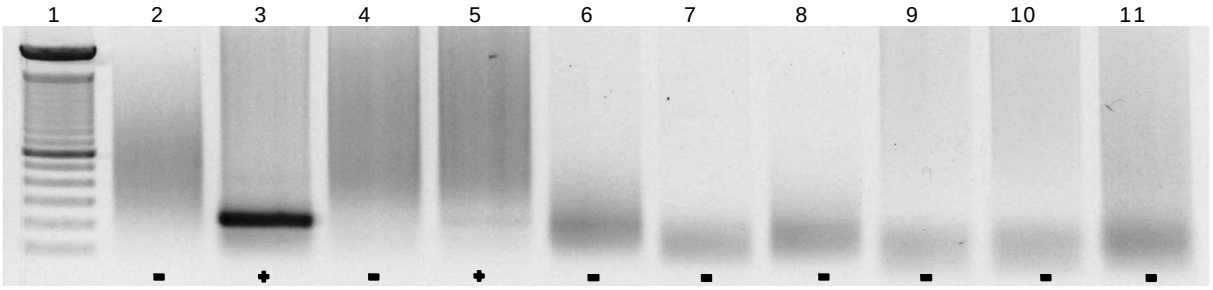

Figure 4. WSSV detection for Zambales samples, lane 1: ladder, lane 2: negative control, lane 3: positive control, lane 4 to 11: samples 1 to 8.

A very low viral load in the sample may have resulted in a very faint positive band in the PCR detection. On the other hand, all Capiz market samples are found to be negative for WSSV, showing a different scenario in shrimp farms in Negros, where WSSV infections are observed annually (Tendencia et al 2010). Samples from a farm and a shop in General Santos City were found to be positive for WSSV. For instance, fresh samples bought from a local shop in the city were found positive, 7/15 or 46.67% (Figure 5). Meanwhile, 3/15 (20%) pond samples from same sampling region were also found positive for WSSV. This is the second report for high prevalence of WSSV in the area (Leung & Engle 2006).

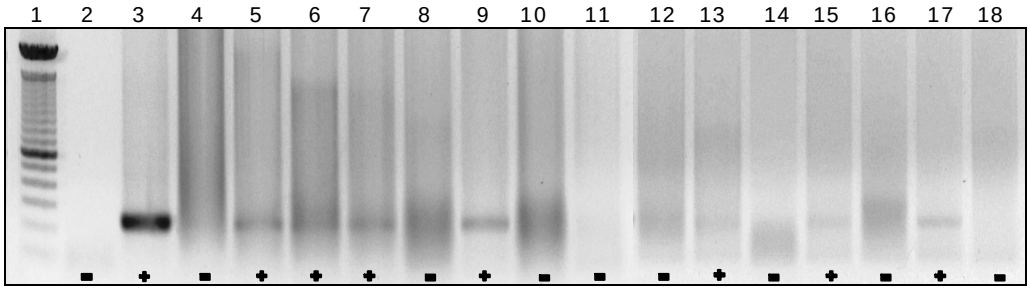

Figure 5. WSSV detection for shop samples from General Santos City, lane 1: ladder, lane 2: negative control, lane 3: positive control, lane 4 to 18: samples 1 to 15.

Figure 6 shows the summary of WSV incidence in each of the sampling sites. One sample t-test (SPSS Software Version 13) at 95% confidence interval reveals that there is no significant difference in the number of WSSV positive samples in the sampling sites. However, most of the WSSV infections were observed in the Mindanao sampling sites. Taking into account the results of WSSV detection from all the sampling sites, the Philippines has a low overall WSSV infection of 14.67% compared to the first report of WSSV outbreak in 2000 where an overall prevalence of 71.83% (51/71) was observed.

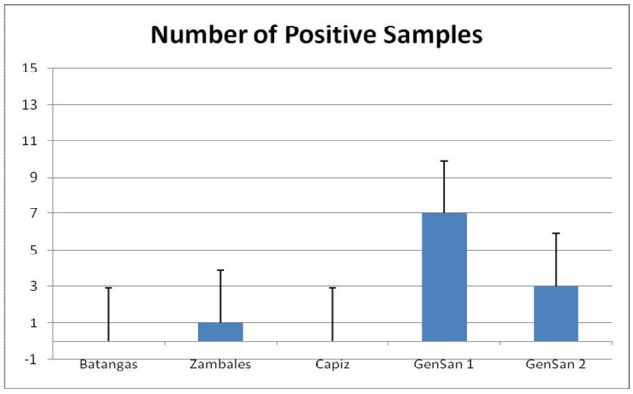

Figure 6. Distribution of WSV positive samples based on PCR detection.

In 1999, the Philippine shrimp, whether cultured or wild, is still WSSV free. However, frozen shrimp imported to the Philippines were found positive (Belak et al 1999). This may be the cause of the first WSSV report in 2000 (Tapay et al 2000) although strong evidence is lacking as viral dynamics of WSSV in the environment is not completely understood. Reports in the following years showed 20.75% average annual WSSV prevalence in shrimp farms in Negros Island (Tendencia & Usero 2007). This is supported by Leung & Engle (2006) listing Negros, Cebu and Mindanao, around the cities of Davao and General Santos as severely affected high density *P. monodon* farming areas. The current study confirms that General Santos City is still affected by WSSV, though not as severe as the previous report. It is apparent that the virus persists in localized regions suggesting that certain factors allow for the persistence of the virus in the environment over the years. However, general practices somehow make the virus manageable to a certain extent that large mortalities and infection in other areas, which have never been infected, are prevented. Subsequently, it is alarming that WSSV infection was detected in *L. vannamei*, which are supposed to be SPF (shrimp pathogen free) as these were imported from other countries. These findings have implications in the current best management practices of the shrimp aquaculture industry. The decree on the "lifting of the ban for the importation of *L. vannamei"* in the country might need revisiting.

Conclusions. The study has partially sequenced and characterized Philippine WSSV isolate. It concludes that the most WSSV- infected shrimps were found in General Santos City during the sampling period. On the other hand, no infections were found in Batangas and Capiz. No significant difference in the number of infections was found in the sampling sites. The study suggests that natural infections of WSSV are manageable in Philippine shrimp farms as shown by the low overall detection. It is alarming though, that some regions of the country tested positively, since *L. vannamei* shrimps are supposedly clear of any pathogen (SPF) as they enter the Philippine territory.

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