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NOTE

White spot syndrome virus (WSSV) in cultured *Penaeus monodon* in the Philippines

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ABSTRACT: The prevalence and geographic distribution of white spot syndrome virus (WSSV) infection among cultured penaeid shrimp in the Philippines was determined from January to May, 1999, using PCR (polymerase chain reaction) protocol and Western blot assays. A total of 71 samples consisting of 18 post-larvae (PL) and 53 juvenile/adult shrimp samples (56 to 150 days-of-culture, DOC) were screened for WSSV. Of the 71 samples tested, 51 (72%) were found positive for WSSV by PCR: 61% (31/51) after 1-step PCR and 39% (20/51) after 2-step, non-nested PCR. Of the PL and juvenile/adult shrimp samples tested, 50 and 79% were positive for WSSV, respectively. By Western blot, only 6 of the 51 (12%) PCR-positive samples tested positive for WSSV. Of the 20 samples negative for WSSV by PCR, all tested negative for WSSV by Western blot assay. This is the first report of the occurrence of WSSV in the Philippines.

KEY WORDS: White spot syndrome virus · Polymerase chain reaction · Western blot

In the last decade, disease outbreaks in cultured penaeid shrimp have wreaked havoc worldwide, especially in Asian countries, including the Philippines. Although the outbreaks in the Philippines have been attributed mainly to luminous vibriosis (Lavilla-Pitogo et al. 1990), the possible role of viruses could not be totally ruled out. After the reports of Natividad (1991) and Lightner et al. (1992), no studies on shrimp viruses were made in the Philippines until 1998, when Albaladejo et al. (1998) reported the presence of yellowhead virus (YHV) in some cultivated *Penaeus monodon*. In 1999, Belak and associates (Belak et al. 1999) reported the absence of white spot syndrome virus (WSSV) by polymerase chain reaction (PCR) assay in wild spawners obtained from Palawan, Quezon, Capiz and Negros Occidental. To date, there is a lack of information on the true status of shrimp viral diseases, such as WSSV, in the Philippines and this may consequently pose a great threat to the growth of the shrimp aquaculture industry. It is therefore imperative that screening and monitoring programs initiated by the Philippines' Bureau of Fisheries and Aquatic Resources, Department of Agriculture, be continued in order to determine the prevalence of some of the highly pathogenic shrimp viruses emerging in the farms and hatcheries in the Philippines.

Two important viral pathogens, YHV and WSSV, have been reported in Asia and elsewhere (Boonyaratpalin et al. 1993, Nakano et al. 1994, Chou et al. 1995, Wang et al. 1995, Wongteerasupaya et al. 1995, Durand et al. 1996, Lo et al. 1996a, Lu et al. 1997, Wang et al. 1997, Hameed et al. 1998), and several nucleic acid and/or immunologically based detection protocols have been developed for them (Kimura et al. 1996, Lo et al. 1996b, Nadala et al. 1997, Hameed et al. 1998, Kim et al. 1998, Tapay et al. 1999). For example, a combined SDS-PAGE/Western blot/nitrocellulose-enzyme immunoassay method for the early detection of both YHV and WSSV has been used to demonstrate the presence of specific viral proteins in the hemolymph of experimentally infected animals as early as 43 h post infection (Nadala et al. 1997). Also, a diagnostic probe for WSSV was reported by Durand et al. (1996) based

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on *Eco*RI-digested fragments of the WSSV genome that were cloned and labeled with DIG-11-dUTP. With respect to PCR assays, Kim et al. (1998) developed a PCR procedure for WSSV that is able to detect 1 pg of WSSV DNA after 30 cycles using a template comprising of total nucleic acid extracted either from diseased shrimp or from hatchery shrimp with no signs of viral infection. Lo et al. (1996b) also developed PCR primers and nucleic acid probes for diagnosis of WSSV. In addition, Tapay et al. (1999) developed a PCR protocol that can detect 4 different geographic isolates of WSSV. These PCR protocols can detect low levels of WSSV in the hemolymph of broodstocks and so do not require animal sacrifice.

In this paper, employing both the PCR and Western blot viral detection protocols, we report, for the first time, the occurrence of WSSV in the Philippines.

Materials and methods. A total of 71 samples from various geographic locations in the Philippines (Fig. 1) collected between January and May 1999 were analyzed for WSSV by Western blot and PCR assays. The samples, which consisted of 18 post-larvae (PL) and 53 juvenile/adult shrimp, were obtained from shrimp farms either with reported cases of mortality or solely for monitoring purposes. Samples were obtained from the following provinces: on Luzon — Bataan (4), Batangas (5), Bulacan (5), Camarines Norte (1), Mindoro Oriental (2), and Quezon (3); on Visayas — Cebu (17) and Negros Occidental (9); and on Mindanao — Agusan del Norte (11), Sarangani (9) and Zamboanga del Sur (5) (Table 1).

Fig. 1. Map of the Philippines showing the sources of the *Penaeus monodon* samples collected between January and May 1999 and used in this study. 1 = Bataan; 2 = Bulacan; 3 = Batangas; 4 = Quezon; 5 = Camarines Norte; 6 = Mindoro Oriental; 7 = Cebu; 8 = Negros Occidental; 9 = Agusan del Norte; 10 = Zamboanga del Sur; 11 = Sarangani

Ten-percent homogenates (in Tris-NaCl-EDTA buffer [TNE]) from whole animals (PL) or from gill tissues of adult shrimp (when hemolymph samples were unavailable) or hemolymph were used as sources of DNA. Prior to DNA extraction, the samples were collected and processed according to the following protocol: about 100 µl of either hemolymph or 10% gill homogenate in TNE (0.05 M Tris, 0.1 M NaCl, 0.001 M EDTA, pH 7.4) was collected in microfuge tubes containing 500 µl DNAZol® Reagent (GIBCO Life Technologies). The DNA was extracted according to the manufacturer's protocol.

For PCR amplification, 2 DNA oligonucleotide primers with the following sequences (Tapay et al. 1999) were used: 5′ GAA ACT ATT GAA AAG GCT TTC CCT C 3′ (forward primer) and 5′ GTT CCT TAT TTA CTA CTA CGG CAA 3′ (reverse primer). The primers were synthesized by the Biotechnology/Molecular Biology Instrumentation and Training Facility (University of Hawaii, Honolulu, USA). A single PCR reaction mixture containing 1× PCR buffer, 40 µM dNTP mix (Takara Chemicals), 3.0 U/100 µl DNA polymerase (ULTma Perkin Elmer), 0.2 µM Primer 1, 0.2 µM Primer 2, and template DNA (10 µl) was prepared for each run. $MgCl_2$ (1.75 mM) was separately added to each reaction tube after the temperature had reached 80°C. PCR runs were carried out using a Gene Cycler™ thermal cycler (Bio-Rad Laboratories) for 30 cycles according to the following protocol: denaturing temperature of 95°C for 30 s, annealing temperature of 60°C for 1 min, elongation temperature of 72°C for 30 s, and final extension of 72°C for 7 min. A negative control consisting of shrimp DNA or sterile 3× distilled water and a positive control consisting of WSSV DNA were used as templates and analyzed simultaneously.

For 2-step PCR, the final PCR reaction mixture from samples which tested negative for WSSV after the 1-step PCR protocol were used as DNA templates for a second round of amplification. Conditions for amplification were the same as above.

The PCR amplification products were separated electrophoretically in agarose gel (2% in Tris-Borate EDTA buffer, pH 7.6: Tris-base 121.1 g, H_3BO_3 55.6 g, Na-EDTA·$2H_2O$ 3.7 g, distilled water 1 l) containing ethidium bromide (EtBr, 0.25 µg ml^{-1}) at 100 V for about an hour. The EtBr-stained gel was viewed on a Chromatovue Transilluminator™ and documented using a Mitsubishi Gel Documentation System™. A positive PCR reaction for WSSV consisted of an amplicon band of 217 bp.

For Western blot assays, gill tissue homogenates from adult shrimp or whole animal homogenates for PL were used for analysis according to the method of

Table 1. Detection of WSSV in adult and post-larvae (PL) of *Penaeus monodon* from shrimp hatcheries and grow-out farms in the Philippines. na: not applicable

Location	No. of samples collected	No. of samples WSSV-positive Western blot[a]	PCR[b] 1-step	PCR[b] 2-step[c]	WSSV[d]-negative
Agusan del Norte[e]	11[f,g]	0	1	8	2
Bataan	4[g]	1	1	2	1
Batangas	5[g]	0	5	na	0
Bulacan[e]	5[h]	0	3[a]	0	2
Camarines Norte	1[i]	0	1	na	0
Cebu[e]	17[j]	4	7[k]	3[l]	7
Negros Occidental[e]	9[g]	0	5	2	2
Mindoro Oriental[e]	2[g]	1	1[a]	1	0
Quezon	3[i]	0	1	1	1
Sarangani Province	9[g]	0	6[a]	2	1
Zamboanga del Sur[e]	5[g]	0	0	1	4
Total	71	6	31 + 20 = 51		20

[a]Gills of adult shrimp
[b]Hemolymph of adult shrimp or whole animals for PL, except when otherwise specified
[c]The PCR product from the first step was used as a DNA template for the 2-step PCR. Same PCR reagents and conditions were followed for both 1-step and 2-step PCR
[d]By either PCR or Western blot analysis
[e]With field cases of mortality
[f]Fry (PL) were obtained from a Cebu hatchery which tested positive for WSSV
[g]Adult shrimp
[h]3 adult and 2 PL
[i]PL
[j]5 adult and 12 PL
[k]2 hemolymph and 5 PL
[l]2 hemolymph and 1 PL

Nadala et al. (1997). Briefly, PL or fry samples and gill tissues were pooled separately and processed by grinding in TNE buffer (10% final concentration) using a sterile mortar and pestle. The resulting homogenate was centrifuged at 5000 rpm (1779 × *g*; Force 14 Microcentrifuge, Denver Instrument Co.) for 30 min at 4°C. The supernatant was collected and further clarified by centrifugation at 3000 rpm (641 × *g*) for 30 min. The final supernatant was used for SDS-PAGE according to the method of Laemmli (1970). Briefly, the crude sample preparation was mixed with an equal volume of 2× loading buffer (2.5 ml 0.5 mM Tris-HCl, pH 6.8; 4.0 ml 10% sodium dodecyl sulfate; 2.0 ml glycerol; 1.0 ml β-mercaptoethanol; and 0.5 ml de-ionized distilled water) heated at 95°C for 5 min and centrifuged briefly in a microcentrifuge. Fifteen microliters of each of the test samples was loaded into the wells of a 12.0% sodium dodecyl sulfate-polyacrylamide gel slab and electrophoresed at 200 V for 30 to 45 min. The electrophoresed gels were blotted onto nitrocellulose membranes (Schleicher and Schuell, 0.1 µm pore size) and used in an immunoassay according to the method of Nadala et al. (1997). The sensitivity and specificity of the hyperimmune polyclonal antibody (IgG) used in this study have been described by Nadala et al. (1997).

Results and discussion. All the samples obtained from farms with field cases (16/16) tested positive for WSSV by PCR (Table 2). A field case refers to a shrimp population reported by farms experiencing mortalities due to unknown causes. Gross manifestations ranged from lack of appetite to reddish discoloration, external fouling and mortality. No macroscopic 'white spots' were visible at the time of sampling. Sixty-two percent of the samples obtained for monitoring purposes tested positive for WSSV (Table 1), although at the time of sampling, the shrimp appeared apparently healthy. It is noteworthy that at least 1 farm in Agusan del Norte

Table 2. Incidence of WSSV in shrimp samples obtained for monitoring purposes and field cases. A field case refers to a shrimp population reported by farms experiencing mortalities due to unknown causes. Gross manifestations ranged from lack of appetite to reddish discoloration, external fouling and mortality. Samples of apparently healthy animals for monitoring purposes were randomly taken from farms/hatcheries in major shrimp-producing provinces of the Philippines

	No. of samples	WSSV-positive
Field case	16	16
Monitoring	55	35

which tested positive for WSSV obtained their PL from a hatchery which also tested positive for WSSV.

Of the total samples (71) tested, 51 (72%) were found positive for WSSV by PCR assay (Table 2). Thirty-one samples (61%) were positive after a 1-step PCR (Figs. 2 & 3) while the remaining 20 samples (39%) were positive only after 2-step PCR. Twenty samples (28%) were negative for WSSV even after 2-step PCR.

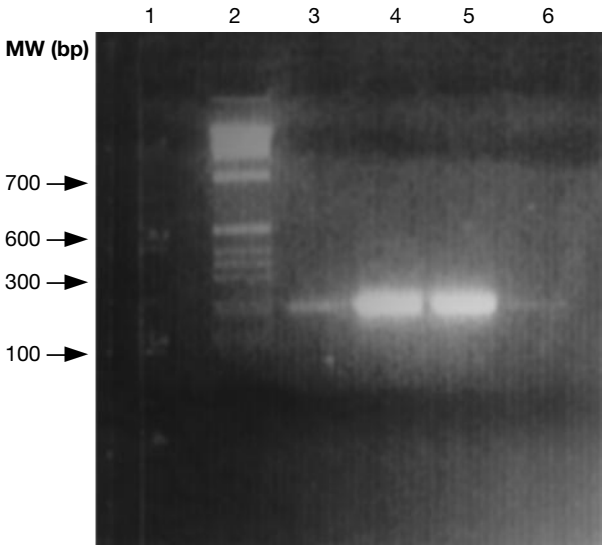

Fig. 2. Electrophoresis gel of PCR products from representative samples with medium to heavy WSSV infections: lane 1, distillled water; lane 2, 1 kb DNA ladder; lane 3, WSSV-positive control; lanes 4 and 5, WSSV-infected ('heavy') samples; and lane 6, WSSV 'light' infection

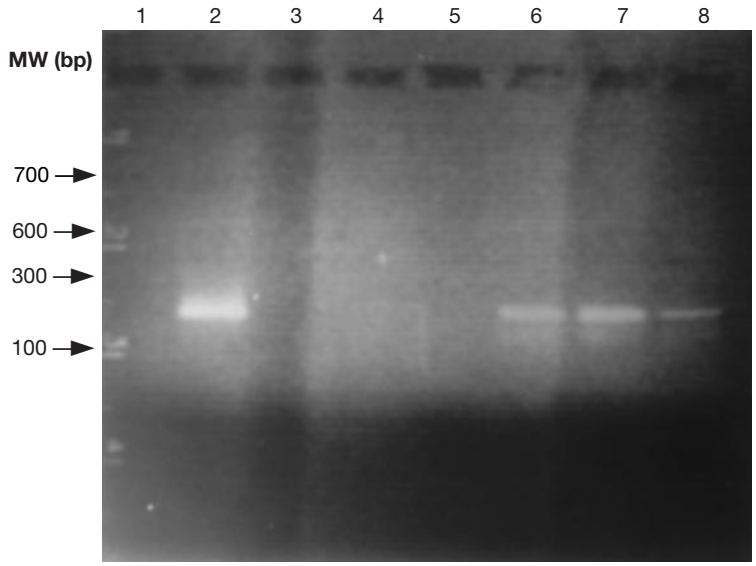

Fig. 3. Electrophoresis gel of PCR products from representative samples with 'light' infection: lane 1, distilled water; lane 2, WSSV-positive control; lanes 3 to 5, PCR products of WSSV-infected samples after a 1-step amplification; and lanes 6 to 8, PCR products of the same samples in lanes 3 to 5 after a 2-step amplification

Only 6 samples (12%) of the 51 WSSV PCR-positive samples were found positive for WSSV by Western blot assay (Table 1). Fig. 4 shows a representative gel with a band which approximates the prominent non-glycosylated viral envelope component (27.5 kDa) of the WSSV control. The 2 other protein bands (23.5 and 19 kDa), representing the capsid and envelope components of WSSV, respectively (Nadala et al. 1998), were not observed. It is noteworthy that 2 samples from Cebu which tested positive for WSSV by Western blot assay were found positive by PCR only after a 2-step amplification. The 2 assay samples came from the same animal, although gill tissues were used for the Western blot assay and hemolymph was used for the PCR. All samples that tested negative by PCR assay also tested negative by Western blot assay.

The results of this nationwide screening indicate widespread occurrence of WSSV infection in the Philippines, both in hatcheries (50% of the PL samples tested) and in grow-out ponds (79% of the juvenile/adult shrimp samples tested). This occurred despite the ban on importation of all morphogenetic stages of *Penaeus* species (except marketable sizes of 25 to 50 g) into the country set by the Department of Agriculture in 1993 through Fisheries Administrative Order 189, Series of 1993. This ban, however, did not prevent the entry of fry through the 'back door' in the south, thereby evading quarantine regulations.

There is currently a fry evaluation procedure being implemented in the Philippines. However, this does not include testing for WSSV. Thus, it is not surprising that PL being stocked in some farms in the country would test positive for WSSV. This was documented in PL samples from some of the hatcheries which were included for monitoring purposes.

Still another possible source of the virus would be the spawners, although a recent study by Belak et al. (1999) revealed that spawners from Palawan, Quezon, Capiz and Negros Occidental provinces tested negative for WSSV by PCR. Their data, however, were limited to 4 sampling sites. Hence, the presence of WSSV in spawners cannot be totally ruled out.

Our most recent samples of wild shrimp from unfiltered sea water pumped into ponds during the cultivation period have tested positive for WSSV by PCR (data not shown). This data strongly suggests that the virus may have been introduced from wild populations of shrimp.

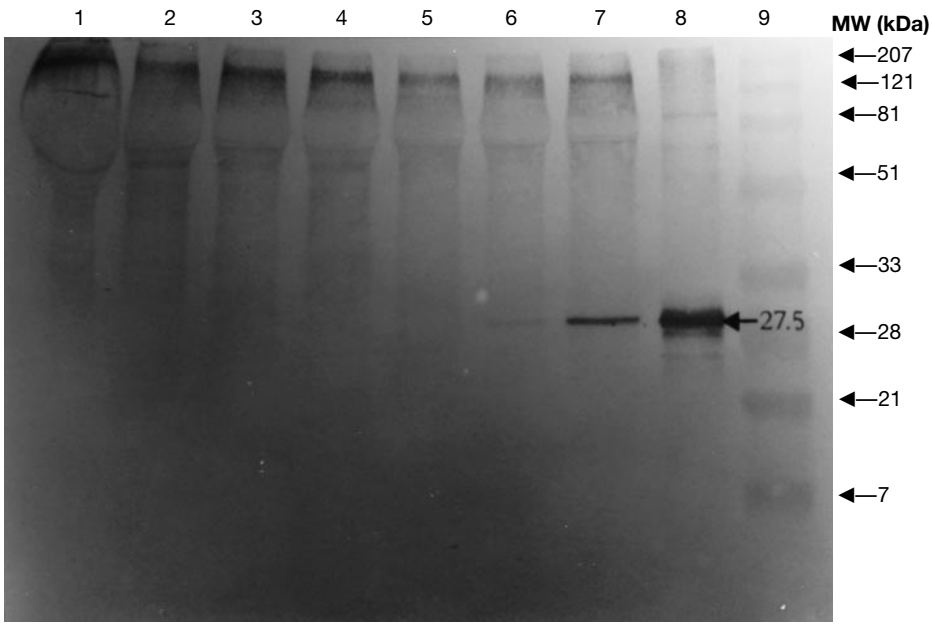

Fig. 4. Western blot analysis of WSSV proteins: lanes 1 to 5, WSSV-negative samples; lanes 6 and 7, WSSV-positive samples; lane 8, WSSV-positive control; and lane 9, prestained broad range molecular weight marker (Bio-Rad Laboratories)

It is noteworthy that, despite the high prevalence of WSSV in the country, no mass mortalities were reported; only loss of appetite, stunted growth and heterogeneous weight sizes (data not shown) were reported from ponds with 'light-grade' infections (e.g. Agusan del Norte samples). This observation may suggest that the infections were at an early stage so that no gross signs of the disease (e.g. 'white spots') were visible. It is also possible that a less virulent, variant strain of the virus was being detected or that the affected shrimp had developed some form of resistance to the virus so that outbreaks of mortality did not occur.

The PCR protocol used in the study was very sensitive. It was capable of distinguishing between light and heavy infections and was more sensitive than the antibody (Ab)-based Western blot assay used. However, despite its lower sensitivity, the Western blot assay proved useful in validating the PCR-positive results obtained from the 'heavily' infected samples (e.g. some samples from Cebu, Bataan and Mindoro Oriental).

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