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Development and application of monoclonal antibodies for the detection of white spot syndrome virus of penaeid shrimp

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ABSTRACT: Monoclonal antibodies (MAbs) were produced against white spot syndrome virus (WSSV) of penaeid shrimp. The virus isolate used for immunization was obtained from China in 1994 and was passaged in *Penaeus vannamei*. The 4 hybridomas selected for characterization all produced MAbs that reacted with the 28 kD structural protein by Western blot analysis. The MAbs tested in dot-immunoblot assays were capable of detecting the virus in hemolymph samples collected from moribund shrimp during an experimentally induced WSSV infection. Two of the MAbs were chosen for development of serological detection methods for WSSV. The 2 MAbs detected WSSV infections in fresh tissue impression smears using a fluorescent antibody for final detection. A rapid immunohistochemical method using the MAbs on Davidson's fixed tissue sections identified WSSV-infected cells and tissues in a pattern similar to that seen with digoxigenin-labeled WSSV-specific gene probes. A whole mount assay of pieces of fixed tissue without paraffin embedding and sectioning was also successfully used for detecting the virus. None of the MAbs reacted with hemolymph from specific pathogen-free shrimp or from shrimp infected with infectious hypodermal and hematopoietic necrosis virus, yellow head virus or Taura syndrome virus. In Western blot analysis, the 2 MAbs did not detect any serological differences among WSSV isolates from China, Thailand, India, Texas, South Carolina or Panama. Additionally, the MAbs did not detect a serological difference between WSSV isolated from penaeid shrimp and WSSV isolated from freshwater crayfish.

KEY WORDS: White spot syndrome virus · Monoclonal antibodies · Immunodetection

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INTRODUCTION

Epizootics due to white spot syndrome (WSS) were first reported in China in 1993, where the disease was responsible for catastrophic losses of cultured *Penaeus chinensis* (Huang et al. 1995). By 1995 WSS virus (WSSV) outbreaks in shrimp culture facilities had been reported in Japan, Korea, Thailand, Taiwan, Indonesia and India affecting farmed populations of *P. chinensis*, *P. monodon*, *P. japonicus* and *P. indicus* (Nakano et al. 1994, Chou et al. 1995, Winaro 1995, Wongteerasupaya et al. 1995, Heo et al. 1998, Mohan et al. 1998). Partly because of the rapid spread of the virus and its identification by numerous laboratories, the virus has been referred to by various other names including rod-shaped nuclear virus of *P. japonicus* (Inouye et al. 1994), systemic ectodermal and mesodermal baculovirus (Wongteerasupaya et al. 1995), hypodermal and hematopoietic necrosis baculovirus (Huang et al. 1995), white spot baculovirus (Wang et al. 1995), Chinese baculovirus (Nadala et al. 1997) and *P. monodon* non-occluded baculovirus (Chang et al. 1996). Very rapidly, WSSV spread to other regions of the world. The disease was reported in the USA in late 1995 in Texas and South Carolina in cultured stocks of *P. vannamei*, *P. stylirostris* and *P. setiferus* (Lightner et al. 1997). By 1998, WSSV had been introduced into Central and South America and it was reported in Mexico in 1999. The virus was recently reported in cultured

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shrimp in the Philippines (Magbanua et al. 2000). In the 6 years following the first reported epizootic, losses to shrimp farmers worldwide due to WSSV were thought to be in excess of several billion dollars (Flegel & Alday-Sanz 1998). Perhaps even more disturbing is the potential for this virus to infect decapod crustacean species other than penaeid shrimp.

Beginning in December 1995, the National Zoological Park in Washington, DC, USA noted significant mortalities in 2 species of freshwater crayfish (Richman et al. 1997). On histological analysis and *in situ* hybridization with WSSV-specific gene probes, it was determined that the animals were infected with an agent that appeared to be identical or very similar to WSSV. During this same time, Lo et al. (1996) identified WSSV, using DNA amplification techniques, in cultured and captured species of shrimps, crabs and other arthropods. WSSV is a major concern in all shrimp-growing regions of the world and the presence of the virus in wild populations of shrimp and other crustaceans has raised serious concerns about the impact of WSSV on the environment and native aquatic species.

Three methods are used routinely for the detection of WSSV: histological analysis of specimens fixed in Davidson's alcohol-formalin-acetic acid (AFA) solution; *in situ* hybridization on fixed tissues with WSSV-specific gene probes; and the PCR method using WSSV-specific oligonucleotide primers. Several investigators have reported on the production of polyclonal (PAbs) and monoclonal antibodies (MAbs) to the virus, but serological methods have not been put into routine use by diagnostic laboratories for the detection of WSSV in clinical specimens, in part because the antibodies are not commercially available (Sahul-Hameed et al. 1998, Zhan et al. 1999, Nadala & Loh 2000, van Hulten et al. 2000a). The current report details the production of MAbs to WSSV that react with the virus both in its native form and in a denatured state, thus lending themselves to several serological formats that can be designed for rapid testing of clinical specimens.

MATERIALS AND METHODS

WSSV purification. WSSV was purified from experimentally infected *Penaeus vannamei* tissue or hemolymph. Hemolymph from moribund shrimp was withdrawn from the ventral sinus using 10% sodium citrate to prevent coagulation. The tissue and hemolymph were kept frozen at –70°C until purification. For purification of the virus from tissue, epidermal tissue from the tails and cephalothoraces were combined with cuticular epithelium scraped from the inside of the carapace for homogenization in 3 volumes of Tris-NaCl buffer (TN; 20 mM Tris-HCl, pH 7.4, 400 mM NaCl). For purification from hemolymph, the frozen hemolymph was thawed slowly at room temperature and then frozen again for 5 min and thawed for 10 min to disrupt infected cells. The hemolymph was diluted 1:3 in TN buffer. From this point, the purification procedure for both preparations was identical. The samples were clarified by 2 low speed centrifugations (500 × *g* for 10 min followed by 5000 × *g* for 20 min) at 4°C. The supernatant fluids were then centrifuged at 112 400 × *g* at 4°C for 1 h and the resulting pellets were resuspended in TN buffer. The resuspended pellets were layered onto 10 ml gradients prepared with 15 to 45% Renografin-76 (Solvay Animal Health Inc., Mendota Heights, MN, USA) in TN buffer. After centrifugation at 153 200 × *g* at 4°C for 1.5 h, the visible virus band was collected by puncturing the tube with a 25 gauge needle and removing the band with a 1 ml syringe. The purified virus was washed with TN buffer and centrifuged at 153 200 × *g* at 4°C for 1.5 h, and the pellet was resuspended in TN buffer. The purified virus preparation was checked for the presence of enveloped virions and nucleocapsids by TEM using 2% phosphotungstic acid as the negative stain. Tissue from specific pathogen-free (SPF) (Wyban 1992) *P. vannamei*, which are free of all category 1, 2 and 3 pathogens as defined by the US Marine Shrimp Farming Consortium (Lotz et al. 1995), was taken through the purification protocol described for WSSV for use as a negative control in the Western blot analysis. One milliliter of the Renografin-76 gradient (from the same position as the WSSV band) was withdrawn and then washed and resuspended in TN buffer.

Virus isolates. WSSV was obtained from several geographic regions: China, Thailand, India, Panama, Texas and South Carolina. WSSV was also obtained from crayfish maintained at the National Zoo in Washington, DC. All isolates of WSSV were passaged by injection of infected tissue homogenates into SPF *Penaeus vannamei* according to the protocol of Durand et al. (1997). Other penaeid shrimp viruses, used to determine the specificity of the antibodies, were infectious hypodermal and hematopoietic necrosis virus (IHHNV), hepatopancreatic parvovirus (HPV), Taura syndrome virus (TSV) and yellow head virus (YHV). IHHNV, HPV and TSV were purified from infected shrimp tissue according to published procedures (Bonami et al. 1990 1995, 1997). Hemolymph, collected from the ventral sinus using 10% sodium citrate to prevent coagulation, was obtained from shrimp infected with TSV, IHHNV or YHV. Hemolymph collected from SPF *P. vannamei*, which were negative for all of the above viral pathogens, was used as the uninfected control in the dot-immunoblot assays. The disease status of all shrimp was confirmed by showing the presence or absence of characteristic lesions using routine histopathological techniques (Lightner 1996).

MAb production. MAbs were prepared according to the procedure described by Poulos et al. (1999), which was adapted from previously described protocols (Kohler & Milstein 1976, Kearney et al. 1979, Oi & Herzenberg 1980, Galfre & Milstein 1981). Briefly, BALB/cByJ mice (Jackson Laboratories, Bar Harbor, ME, USA) were injected intraperitoneally with purified WSSV emulsified in synthetic adjuvant (MPL+TDM; RIBI Immunochem Research, Inc., Hamilton, MT, USA). Sera from the mice were tested for antibody production after 2 booster immunizations. The final immunization was performed with WSSV structural proteins in the range of 15 to 35 kD, which were eluted from SDS-PAGE gel slices and emulsified in synthetic adjuvant. Spleen cells obtained from 1 mouse 2 d after the final immunization were fused with SP2/0-Ag-14 myeloma cells (American Type Culture Collection, Rockville, MD, USA) using polyethylene glycol (Curtin Matheson Scientific, Houston, TX, USA). A dot-immunoblot assay using purified WSSV was used to screen the hybridomas for the production of WSSV-specific antibodies. The immunoglobulin isotype of the MAbs was determined using an ELISA-based Mouse Hybridoma Subtyping Kit (Roche Molecular Biochemicals, Indianapolis, IN, USA). Selected hybridomas were subsequently cloned 3 or more times by limiting dilution. MAbs were concentrated 8 to 10 times from hybridoma supernatant fluids using Centriprep-30 centrifugal concentrators (Amicon, Inc., Beverly, MA, USA).

Dot-immunoblot assay. The dot-immunoblot assay was performed in 96 well MA-HA-N45 assay plates (Millipore, South San Francisco, CA, USA) using unconcentrated hybridoma supernatant fluids according to the protocol described in Poulos et al. (1999). Hemolymph or purified virus (1 µl) were dotted onto the membrane. The reactions were graded as described, such that a maximum +4 intensity was equivalent to the reaction generated using the mouse anti-WSSV polyclonal serum antibody at a dilution of 1:1000 against purified WSSV or WSSV-infected hemolymph. The negative control reaction, in which no colored spot was visible in the well, was performed using normal mouse serum in place of the primary antibody.

SDS-PAGE. WSSV structural proteins were separated using purified virus denatured by boiling for 3 min in Laemmli buffer (Laemmli 1970) with the addition of 10 M urea, followed by electrophoretic separation using a 12% resolving gel and a 4% stacking gel (Bonami et al. 1997). The gels either were stained with 0.1% Coomassie blue (Wilson 1983) or were transferred to nitrocellulose membranes for Western blot analysis. A prestained low mass molecular marker (BioRad Laboratories, Hercules, CA, USA) was run in all SDS-PAGE gels for reference. The proteins contained in the marker had apparent molecular weights of 107, 74, 49.3, 36.4, 28.5 and 20.9 kD. Another marker containing proteins with the following molecular weights was also run for the Coomassie-stained gels: 97, 66, 45, 31, 20.1 and 14.4 kD (Amersham Pharmacia Biotech, Piscataway, NJ, USA). For final immunization of the mice, the structural proteins in the range of 15 to 35 kD were eluted from unstained SDS-PAGE gels by overnight incubation of gel pieces in TN buffer at 4°C. The eluted proteins were then concentrated using Centriprep-10 centrifugal concentrators (Amicon, Inc.). A portion of the eluted and concentrated proteins was checked for the presence of the polypeptides by SDS-PAGE followed by Coomassie blue staining.

Western blot analysis. The structural proteins of purified WSSV separated by SDS-PAGE were electrotransferred to nitrocellulose membranes for 3 h at 300 mA constant current using a 0.5× Towbin transfer buffer (Towbin et al. 1979). The Western blot assay was performed according to the protocol described in Poulos et al. (1999) using a Mini-Protean II Multi Screen apparatus (BioRad Laboratories) and hybridoma supernatant fluids concentrated 8 to10 times as the primary antibody.

Fluorescent antibody detection of WSSV in fresh tissue impression smears. Experimentally infected SPF *Penaeus vannamei* were observed for signs of morbidity at which time the stomachs were harvested to prepare impression smears on positively charged microscope slides (SuperFrost Plus; Fisher Scientific, Pittsburgh, PA, USA). A sagittal section was made and the epithelial cells were transferred to the slide and then quickly spray-fixed using Surgipath cytology fixative (Surgipath Medical Industries, Richmond, IL, USA) and stored at room temperature until used for reaction with the antibodies. Just before antibody staining the smears were fixed by immersion in 100% methanol for 10 min. The smears were rehydrated by incubation in 3 changes of PBS for 2 min. Five hundred microliters of a blocking reagent consisting of PBS with 10% normal goat serum and 0.2% Hammersten casein (US Biochemical Corp., Cleveland, OH, USA) was applied to the smears for 15 min at room temperature. The MAbs (concentrated 8 to 10 times) or the mouse PAb (diluted 1:300) were then applied for 45 min at room temperature. After the smears were washed in 3 changes of PBS, a fluorescein-conjugated goat anti-mouse IgG secondary antibody (Rockland, Inc., Gilbertsville, PA, USA) was applied for 45 min at room temperature. After being washed 3 times in PBS, the smears were mounted with a coverslip using a fluorescent mounting medium (Dako Corp., Carpinteria, CA, USA) and observed under epifluorescence. Some of the impression smears were not reacted with antibody, but were stained with Diff-Quick stain (Baxter Diag-

nostics Inc., McGaw Park, IL, USA) to determine the integrity of the cells after treatment with the cytology fixative.

Immunohistochemistry on fixed sections. SPF *Penaeus vannamei* were experimentally infected with WSSV as described previously. Moribund animals were fixed with Davidson's AFA fixative for 24 to 48 h (Bell & Lightner 1988). The tissues were embedded in paraffin and 4 µm sections were placed onto Superfrost Plus positively charged microscope slides (Fisher Scientific, Pittsburgh, PA, USA). After being heated at 65°C to melt the paraffin, the sections were rehydrated through a series of washes in Hemo-De (a xylene substitute; Fisher Scientific), 95% alcohol, 80% alcohol, 50% alcohol and distilled water. The sections were incubated for 5 min in PBS and then blocked with PBS containing 10% normal goat serum and 2% Hammersten casein for 15 min at room temperature. The MAbs (concentrated 8 to 10 times) were then applied to the sections for 30 min at room temperature. After being washed in 3 changes of PBS, the sections were reacted with a goat anti-mouse IgG (H&L) $F(ab')_2$ antibody conjugated to alkaline phosphatase (Zymed, South San Francisco, CA, USA) and diluted 1:1000 in PBS for 30 min at room temperature. The reactions were then developed for 15 to 30 min using nitroblue tetrazolium and bromochloroindoyl phosphate in a pH 9.5 buffer. The sections were counterstained with Bismarck brown and dehydrated through a series of alcohol washes ending with Hemo-De. They were coverslipped with permanent mounting medium and examined under light microscopy for the presence of a blue-black precipitate.

Whole mount assay using pieces of Davidson's fixed tissue. Davidson's AFA fixed tissue from WSSV-infected shrimp were transferred to 70% alcohol after 24 to 48 h. The tissue was cut into 2 to 4 mm blocks using a razor blade and placed into a microfuge tube where all subsequent reactions were carried out either at room temperature or 37°C. The reagents, 250 to 500 µl, were added to the tube, the tissue was allowed to incubate and then the solution was removed with a pipet. The tissue was first rehydrated by immersion in 50% ethanol for 15 min and then washed 6 times with distilled water over a 10 min period. The tissue blocks were briefly homogenized with a plastic pestle and treated with a blocking reagent (PBS, 2% Hammersten casein, 10% normal goat serum) for 15 min at 37°C. The tissues were reacted with 10× concentrated MAb tissue culture supernatant fluid for 30 min at 37°C. The tissues were washed with several changes of PBS and the secondary antibody (goat anti-mouse IgG [H&L] $F[ab']_2$ conjugated to alkaline phosphatase and diluted 1:1000) was added for 30 min at 37°C. The tissue was again washed with PBS after which the detection reagent (nitroblue tetrazolium and bromochloroindoyl phosphate in pH 9.5 buffer) was added for 30 to 60 min at room temperature. The tissue was washed with distilled water and then counterstained with Bismarck brown and dehydrated through a series of alcohol washes ending with Hemo-De. The crushed tissue was placed onto a microscope slide and coverslipped with permanent mounting medium and examined under light microscopy for the presence of a blue-black precipitate within the nuclei of WSSV-infected cells.

RESULTS

MAb production

The techniques described were used to induce the production of antibodies to WSSV that would react with the virus in the native and denatured forms. Previous attempts to produce MAbs to WSSV using intact virions emulsified in synthetic adjuvant resulted only in antibodies that reacted with the virus in its native form. When viral proteins purified from denatured SDS-PAGE gels were used for the final booster immunization of the mice, antibodies were obtained that reacted with intact as well as denatured WSSV. Several hybridoma clones that produced MAbs that reacted with the virus in dot-immunoblots as well as in Western blots were chosen for subcloning and further characterization. Four clones were eventually chosen that had IgG isotypes and that reacted in the dot-immunoblot assay with intact, native virus and in the Western blot to the denatured viral proteins of WSSV (Table 1). Two of the clones, designated 8B9 and 9F1, were stable in culture and were used for development of detection formats.

Table 1. Characteristics of selected monoclonal antibodies (MAbs) to white spot syndrome virus (WSSV). Immunoglobulin isotypes were determined using an ELISA-based mouse subtyping kit. In the dot-immunoblot assay, hybridoma supernatant fluids were tested before concentration and the reactions were scored on a scale of increasing intensity (+1 to +4). In the Western blot assay, hybridoma supernatant fluids were tested after concentration

Hybridoma/MAb designation	Immunoglobulin isotype	Reactivity to WSSV in dot-immunoblot	Reactivity to WSSV in Western blot
3H12	IgG ($\gamma_1\kappa$)	+3	19, 28 kD
6B9	IgG ($\gamma_1\kappa$)	+3	28 kD
8B7	IgG ($\gamma_{2A}\kappa$)	+4	28 kD
9F1	IgG ($\gamma_{2A}\kappa$)	+4	28 kD

Dot-immunoblot analysis

Purified virus preparations were obtained for the various isolates of WSSV (China, Thailand, India, Panama, Texas, South Carolina and crayfish) and for IHHNV, HPV and TSV. In the dot-immunoblot assay, the 4 MAbs detected all of the WSSV isolates and did not react with the other purified viruses (Table 2). Likewise, the MAbs reacted with hemolymph obtained from WSSV-infected shrimp (China isolate), and they did not react with hemolymph from SPF (uninfected) shrimp or with hemolymph from shrimp infected with the other penaeid shrimp viruses, IHHNV, TSV or YHV (Table 3). The purified WSSV virus preparations from the Panama and Texas isolates reacted less intensely than the other WSSV isolates. By SDS-PAGE analysis there appeared to be less virus in these 2 preparations (data not shown). In addition, throughout the dot-immunoblot testing using unconcentrated supernatant fluids, MAbs 3H12 and 6B9 consistently reacted less intensely than MAbs 8B7 and 9F1.

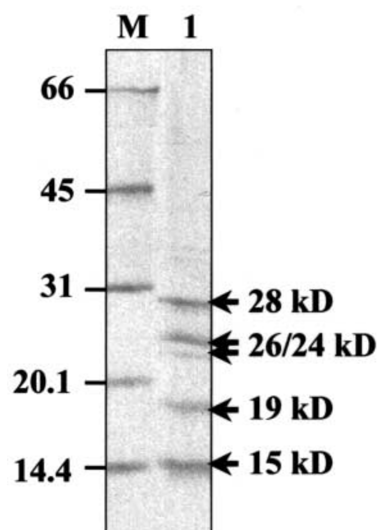

Fig. 1. SDS-PAGE gel of white spot syndrome virus (WSSV) China isolate. Lane M contains the molecular weight marker; lane 1 contains purified WSSV. Size of WSSV proteins indicated on right side of photo. Gel stained with Coomassie blue

SDS-PAGE and Western blot analysis

The isolate used for mouse immunizations was originally from China, and the Coomassie-stained SDS-PAGE gel of this virus isolate (Fig. 1) had 5 of the structural proteins in the range of 15 to 28 kD that have been previously reported (van Hulten et al. 2000b, Wang et al. 2000a). After separation by SDS-PAGE and transfer to nitocellulose, the purified WSSV China isolate was reacted with the MAbs in a Western blot assay. All 4 MAbs reacted with this isolate in the Western blot assay (Fig. 2). MAbs 6B9, 8B7 and 9F1 all reacted with a single band approximately 28 kD in size; however, the reaction with MAb 6B9 was very weak. MAb 3H12 reacted with the 28 kD band but also showed a second reaction with a band corresponding to a molecular weight of 19 kD. The pooled PAb from the immunized mice reacted with 3 bands at 74, 28 and 19 kD (Fig. 2). Nadala et al. (1998) has reported a putative WSSV protein band at 75 kD. This high molecular weight protein was not evident in the Coomassie-stained gel (Fig. 1), but in the Western blot, a band at 74 kD was detected with the mouse PAb. The 3 strongly reacting MAbs were then tested in Western blot assays using WSSV isolates from various geographic regions. Initially the isolates from Thailand, India and South Carolina were tested (Fig. 3). With each of these isolates, the reaction pat-

Table 2. Specificity of the anti-WSSV MAbs against purified virus preparations as determined by dot-immunoblot assay using unconcentrated hybridoma supernatant fluids. The reactions were scored on a scale of increasing intensity (+1 to +4). NR: no reaction. HPV: hepatopancreatic parvovirus; IHHNV: infectious hypodermal and hematopoietic necrosis virus; TSV: Taura syndrome virus

Purified virus	MAb 3H12	MAb 6B9	MAb 8B7	MAb 9F1
China WSSV	+3	+3	+4	+4
Thailand WSSV	+3	+3	+4	+4
India WSSV	+3	+3	+4	+4
Panama WSSV	+2	+2	+3	+3
Texas WSSV	+2	+2	+3	+3
S. Carolina WSSV	+3	+3	+4	+4
Crayfish WSSV	+3	+3	+3	+3
IHHNV	NR	NR	NR	NR
HPV	NR	NR	NR	NR
TSV	NR	NR	NR	NR

Table 3. Specificity of the anti-WSSV MAbs against virus infected or specific pathogen-free (SPF) hemolymph as determined by dot-immunoblot assay using unconcentrated hybridoma supernatant fluids. The reactions were scored on a scale of increasing intensity (+1 to +4). YHV: yellow head virus

Hemolymph	MAb 3H12	MAb 6B9	MAb 8B7	MAb 9F1
WSSV (China)	+3	+3	+4	+4
IHHNV	NR	NR	NR	NR
TSV	NR	NR	NR	NR
YHV	NR	NR	NR	NR
SPF	NR	NR	NR	NR

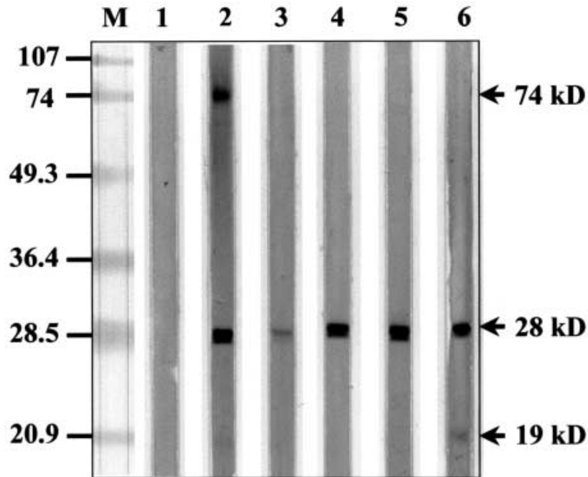

Fig. 2. Western blot of WSSV, China isolate, reacted with antibodies. Lane M contains the prestained molecular weight marker; lane 1 reacted with secondary antibody only; lane 2 reacted with mouse polyclonal antibody to WSSV; lane 3 reacted with monoclonal antibody (MAb) 6B9; lane 4 reacted with MAb 8B7; lane 5 reacted with MAb 9F1; lane 6 reacted with MAb 3H12. Size of WSSV proteins indicated on right side of photo

tern was similar to that seen with the China isolate; i.e., the 3H12 MAb detected 2 bands (at 28 and 19 kD) whereas the 8B7 and 9F1 MAbs detected only a band at 28 kD. Subsequently, SPF tissue and WSSV isolates from Texas, Panama and crayfish were tested (Fig. 4). The uninfected SPF tissue that had been 'purified' using the WSSV purification protocol was unreactive with any of the MAbs. The isolates from China and India were re-run in this Western blot and, although the reactions were weak, the 28 and 19 kD bands were again detected with MAb 3H12 whereas only the 28 kD band was detected with MAbs 8B7 and 9F1. The

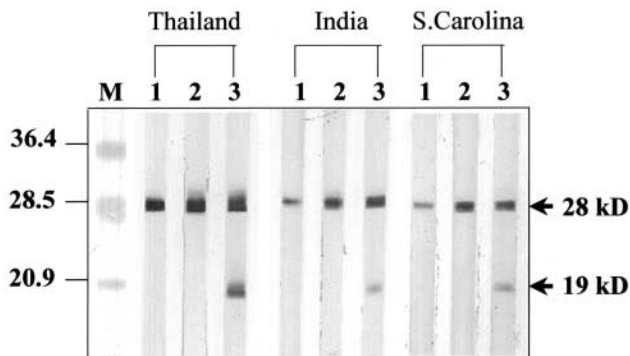

Fig. 3. Western blot of WSSV purified from different isolates and reacted with MAbs 9F1, 8B7 and 3H12. Lane M contains the prestained molecular weight marker; lanes 1 reacted with MAb 9F1; lanes 2 reacted with MAb 8B7; lanes 3 reacted with MAb 3H12. Names above lanes indicate source of WSSV isolate. Size of WSSV proteins indicated on right side of photo

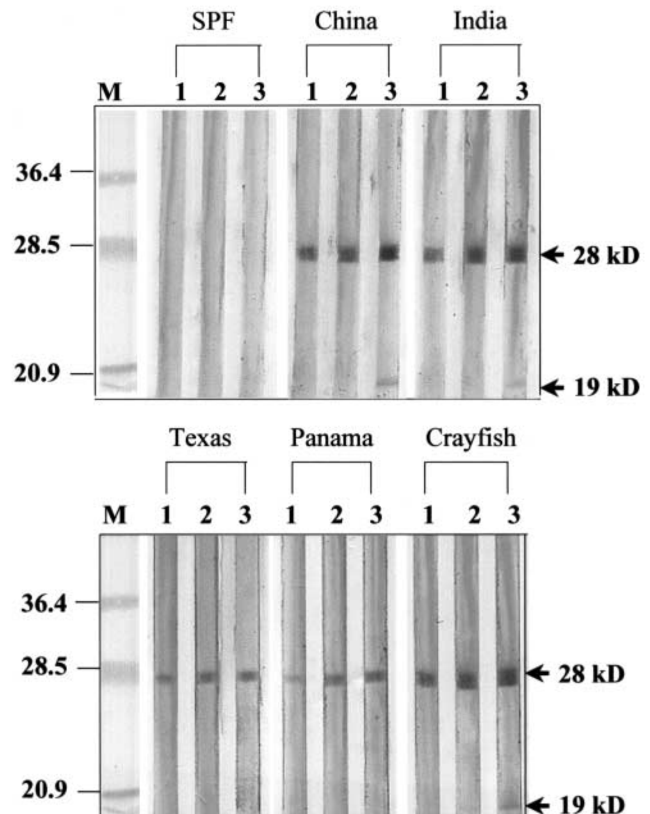

Fig. 4. Western blot of WSSV purified from different isolates and reacted with MAbs 9F1, 8B7 and 3H12. Lanes M contain the prestained molecular weight marker; lanes 1 reacted with MAb 9F1; lanes 2 reacted with MAb 8B7; lanes 3 reacted with MAb 3H12. Names above lanes indicate source of WSSV isolate. SPF (specific pathogen-free) indicates uninfected shrimp tissue 'purified' using virus purification protocol. Size of WSSV proteins indicated on right side of photo

WSSV isolated from crayfish also had the same reaction pattern in the Western blot. Interestingly, with the isolates from Texas and Panama, only the 28 kD band was detected with all 3 MAbs; MAb 3H12 did not detect the 19 kD band in these 2 purified isolates.

Comparison of different antibody assay formats using MAbs 8B7 and 9F1

The 2 MAbs that were most stable in culture were 8B7 and 9F1. These 2 MAbs were chosen for examination of different assay formats to determine their usefulness for detecting WSSV in clinical specimens. Before the fluorescent antibody test was done, impressions of fresh stomach tissue, harvested from moribund WSSV-infected animals, were stained with a general cytological stain (Diff-Quick) to determine the integrity of the cells after fixation with the spray-on fixative. As

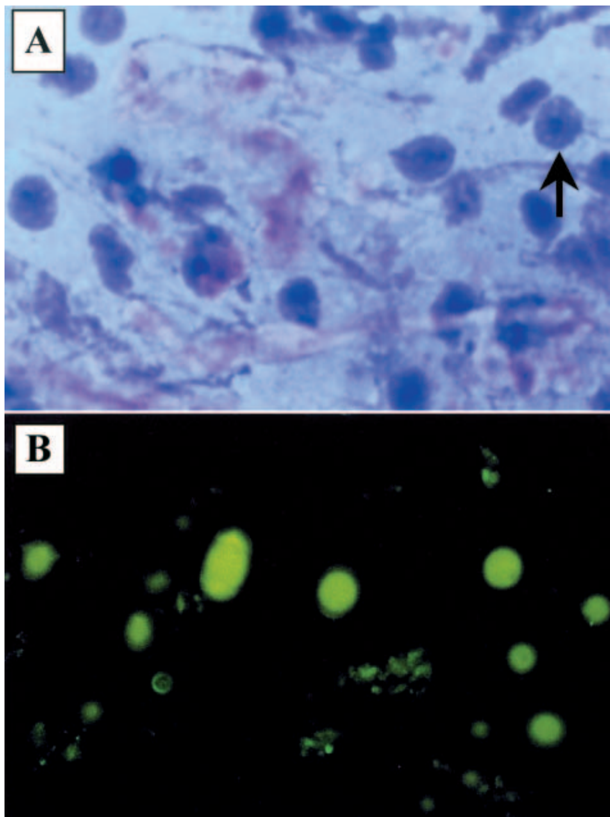

Fig. 5. Fluorescent antibody staining of fresh impression smears prepared from dissected stomach of WSSV-infected *Penaeus vannamei*. (A). Smear reacted with Diff-Quick cytological stain. Arrow indicates WSSV-infected nucleus with nucleolus visible. Magnification is 481×. (B) Smear reacted with MAb 9F1. Green reactions are fluorescing WSSV-infected nuclei. Magnification is 281×

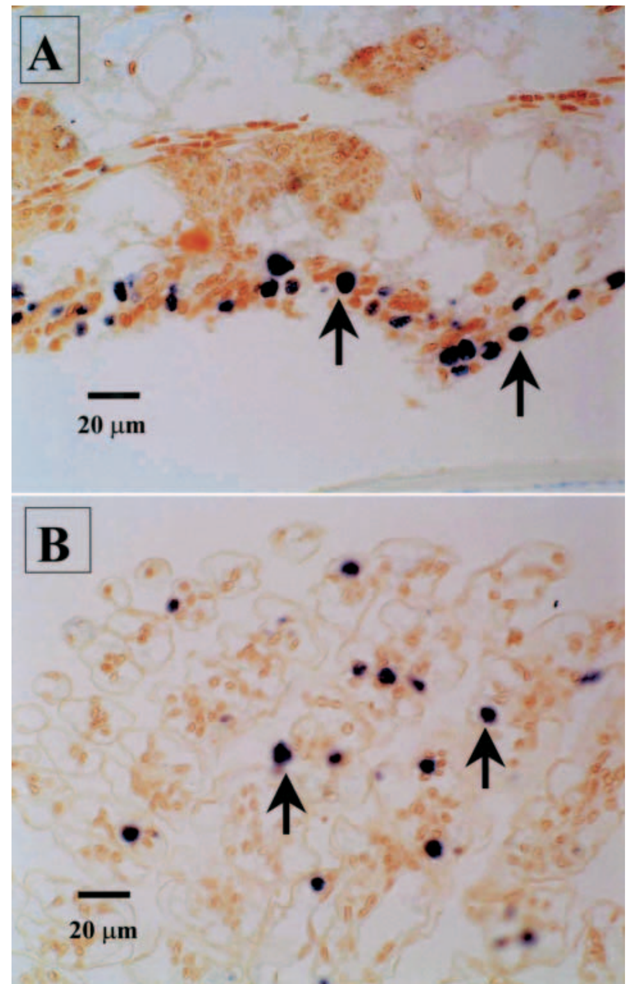

Fig. 6. Immunohistochemical analysis using Davidson's alcohol-formalin-acetic acid (AFA) fixed tissue sections from shrimp infected with WSSV. (A) Reaction of MAb 8B7 with WSSV-infected periopod tissue. (B) Reaction of MAb 9F1 with WSSV-infected gill tissue. Arrows indicate reactive cells

seen in Fig. 5A, there were intact cells on the slide, indicating that the impression smears would be useable for this test. The fluorescent antibody test was then performed using impressions of fresh stomach tissues, harvested from moribund WSSV-infected animals. In Fig. 5B, MAb 9F1 is shown to react with infected cells from WSSV-infected shrimp. MAb 8B7 also reacted strongly in the fluorescent antibody test, whereas the control slide, which did not receive anti-WSSV MAb, had no fluorescent reaction (data not shown). The assay method was rapid, requiring less than 3 h from the time the tissue impressions were immersed in methanol until they were observed under the microscope.

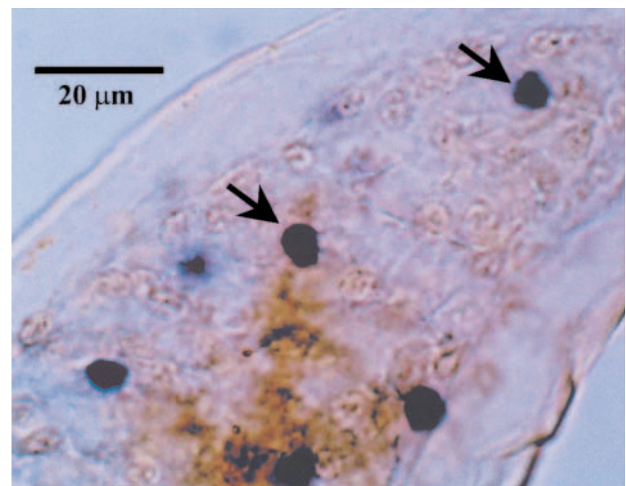

Fig. 7. Whole mount assay of Davidson's AFA fixed tissue blocks from shrimp infected with WSSV. Reaction of MAb 8B7 with gill filaments. Arrows indicate reactive cells

Two other assay formats were tested that used tissues that had been fixed in Davidson's AFA. The first was an immunohistochemical format that was performed on 4 µm thick tissue sections, analogous to what is used for *in situ* hybridization with gene probes. Again, both MAbs reacted with the WSSV-infected tissue sections (Fig. 6) but not with tissue sections from SPF shrimp (data not shown). The pattern of reactivity using the MAbs was similar, if not identical, to that seen using specific digoxigenin-labeled gene probes. Only areas showing lesions typical of acute WSSV infection were marked by the deposition of the MAb and subsequently developed with the chromogenic substrate. The reactions were easily visualized by light microscopy. Once the tissues were sectioned onto microscope slides the immunohistochemical method was rapid, requiring less than 4 h to complete. Fixation, paraffin embedding and sectioning of the tissues, however, required an additional 48 h before the reaction with MAb.

The third assay format that was tested also used Davidson's AFA fixed tissues, but eliminated the need for paraffin embedding or sectioning. This method is called the 'whole mount' assay because intact pieces of tissue are used instead of sectioned material. For this method, only MAb 8B7 was tested and the results showed clear deposition of the blue-black precipitate in the area of WSSV lesions (Fig. 7). No reaction was seen in the negative control from which the primary antibody (MAb 8B7) was omitted (data not shown). The time required to complete this test was 3 h and there was no need for paraffin embedding or sectioning.

DISCUSSION

The purpose of this research was to develop MAbs to WSSV and to test them against various isolates of the virus and in different assay formats. A strategy was chosen for the immunization of the mice to obtain MAbs that would react with both intact virions and denatured viral proteins. The rationale for obtaining such MAbs was that they could then be used for studies using whole virus particles and could also be used for detection of the virus in various types of clinical specimens. The hybridomas were screened initially using a dot-immunoblot assay that incorporated intact virions, either purified or in hemolymph. This method and others similar to it have been described previously (Poulos et al. 1999, Nadala & Loh 2000). While it is an excellent screening tool, the dot-immunoblot assay as described here is not highly sensitive when used with clinical specimens. The MAbs chosen for the dot-immunoblot method were then screened by Western blot to select MAbs that could also detect viral proteins after they had been denatured. Although 4 MAbs were chosen for further testing, only 2 (8B7 and 9F1) proved highly stable in culture.

The Western blots illustrated 2 important points. First, the 28 kD viral protein appeared to be the most common antigen recognized by the panel of MAbs obtained using the immunization protocol described here. According to van Hulten et al. (2000a,b) and others (Nadala et al. 1998), this protein is an envelope protein. Interestingly, MAb 3H12 also reacted with the protein at 19 kD, which appears to be a second envelope protein (Nadala et al. 1998, van Hulten et al. 2000a,b). Whether MAb 3H12 recognized an epitope common to both proteins or was not truly 'monoclonal' was not determined before the hybridoma stopped producing antibody. Regardless, neither the PAb or any of the 4 MAbs examined by Western blot reacted with the protein bands that van Hulten et al. (2000a) describe as being the nucleoproteins of WSSV (i.e., the 24 or 26 kD proteins) nor did they react with the 15 kD protein, which may also be a nucleoprotein (J. M. Vlak pers. comm.). The PAb also reacted with a protein at 74 kD, which is probably identical to the 75 kD protein described by Nadala et al. (1998) but which has not been confirmed in other publications as being derived from the virus. Production of MAbs to the 74 kD protein was likely precluded in this study by the antigen preparation that used for the final booster immunization of the mice; i.e., only proteins less than 35 kD in size were eluted from SDS-PAGE gels for final immunization of the mice. Hence, it is not surprising that none of the 4 MAbs reacted with a 74 kD protein in purified WSSV preparations or in the 'purified' SPF preparation. Although the PAb reacted with a band at 74 kD in the purified WSSV preparation, there remains the possibility that the 74 kD protein is a host-derived protein made in response to WSSV infection, which is co-purified with the WSSV by the purification procedure described here.

The second point illustrated by the Western blots was that the MAbs 8B7 and 9F1 did not discriminate between WSSV isolates from various sources. The difference in reaction of MAb 3H12 to the Texas and Panama isolates was most likely due to a low virus concentration and not to a difference in the viral protein epitopes that were recognized by the MAb. However, since the hybridoma was unstable, it is not possible to re-evaluate the results. Thus, at the antigen level, the 2 MAbs 8B7 and 9F1 reacted with the same protein band regardless of whether the isolate was from penaeid shrimp or freshwater crayfish, or from widely separated geographic origins. Although they cannot therefore be used to determine differences among various WSSV isolates, which is known to occur based on

molecular evidence (Nadala et al. 1998, Wang et al. 2000b), the MAbs described here can prove useful as a tool for detecting WSSV regardless of its origin. This is important when considering the use of MAbs to develop rapid assay formats for detection of the virus that can be used worldwide.

The development of simple, sensitive and rapid assay formats for detection of shrimp viruses has been an elusive goal. Although the use of gene probes and DNA amplification techniques has provided valuable new testing formats, in general the technology is expensive, highly technical, not rapid and not amenable to field conditions. The use of antibodies for detection of shrimp viruses is an alternative that has been slow to progress primarily because of the difficulties associated with their production, due to limited amounts of purified antigen for immunization and testing, and because of the necessity for detecting the viral agent rather than the antibody produced by the host in response to the agent. The results described here indicate that these problems can be circumvented if certain characteristics are chosen for the antibodies and for the testing formats.

Four antibody testing formats were used in this study: dot-immunoblot, fluorescent antibody, immunohistochemistry and whole mount. The dot-immunoblot test as described here has not proved sensitive enough for clinical shrimp specimens using the direct detection method (Poulos et al. 1999) but it was extremely useful for the selection and characterization of the MAbs. Two of the methods, the fluorescent antibody test and immunohistochemistry, are widely used in human medicine. The fourth method, the whole mount assay, was an adaptation based on the need for a test that can be used under conditions that are highly limiting. In the fluorescent antibody test, the procedure for preparing the sample was relatively easy and required no expensive equipment or intense training. The major requirement was that the sample be obtained and dissected pond-side from moribund animals. The performance of the test itself was also relatively simple and very rapid. The primary drawbacks to this test were the requirement for a fluorescent microscope and the need for experience and training to distinguish fluorescing WSSV-infected cells. This method requires an investment both in the microscope and in adequately training the personnel who read the test. Another minor drawback is the limited life of the final specimen since fluorescence fades relatively quickly.

The immunohistochemical test format was chosen for study initially because of the large number of specimens received at this laboratory that are fixed in Davidson's AFA. This method of sample preservation is widely used in the shrimp culture industry and is relatively easy to perform correctly. The immunohistochemical test with MAbs was easy and rapid to perform provided that the end user has access to equipment or a laboratory designed for histological analysis. If histological services are accessible to the farmer, then the antibody method described here is far more specific than standard histological techniques and much more rapid than *in situ* hybridization with gene probes. In addition, it is easier to perform than the gene probe assay because there is no need to heat the sections to denature the viral DNA in the tissues. Reading the reactions requires only the use of a light microscope and minimal training in the determination of a positive reaction. The major limiting feature of this technique is that it requires the use of a highly trained technician to prepare the fixed tissues for testing.

The whole mount assay was used in an attempt to circumvent some of the limitations of the latter 2 procedures. The technique uses fixed tissues but does not require anything more than a scalpel or razor blade for cutting the tissue into small pieces. Thereafter, all the reactions were performed in a small test tube, using the same reagents that were used in the immunohistochemical test. Because of the elimination of paraffin embedding and sectioning, this procedure was much more rapid as well and required no special training to perform. As for the immunohistochemical test, reading the reactions in the whole mount assay technique required only the use of a light microscope and some training in the determination of a positive reaction. Of the various formats tested, this method proved to be the easiest to perform, was relatively rapid and required the least amount of special equipment and training.

In summary, the MAbs described here were highly specific for the detection of WSSV, reacted with several different isolates of the virus and were highly versatile in their ability to be adapted to various test formats that may prove useful to the shrimp culture industry.

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LITERATURE CITED

Bell TA, Lightner DV (1988) A handbook of normal shrimp histology. Special publication No. 1. World Aquaculture Society, Baton Rouge

Bonami JR, Trumper B, Mari J, Brehelin M, Lightner DV (1990) Purification and characterization of the infectious hypodermal and hematopoietic necrosis virus of penaeid shrimps. J Gen Virol 71:2657–2664

Bonami JR, Mari J, Poulos BT, Lightner DV (1995) Character-

ization of hepatopancreatic parvo-like virus, a second unusual parvovirus pathogenic for penaeid shrimps. J Gen Virol 76:813–817

Bonami JR, Hasson KW, Mari J, Poulos BT, Lightner DV (1997) Taura syndrome of marine penaeid shrimp: characterization of the viral agent. J Gen Virol 78:313–319

Chang PS, Lo CF, Wang YC, Kou GH (1996) Identification of white spot syndrome associated baculovirus (WSBV) target organs in the shrimp *Penaeus monodon* by *in situ* hybridization. Dis Aquat Org 27:131–139

Chou HY, Huang CY, Wang CH, Chiang HC, Lo CF (1995) Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Dis Aquat Org 23:165–173

Durand S, Lightner DV, Redman RM, Bonami JR (1997) Ultrastructure and morphogenesis of white spot syndrome baculovirus (WSSV). Dis Aquat Org 29:205–211

Flegel TW, Alday-Sanz V (1998) The crisis in Asian shrimp aquaculture: current status and future needs. J Appl Ichthyol 14:269–273

Galfre G, Milstein C (1981) Preparation of monoclonal antibodies: strategies and procedures. Methods Enzymol 73B:3–46

Heo MS, Ju JW, Jung CR (1998) Viral disease of the cultured *Penaeus chinensis* and *Penaeus japonicus*. J Korean Soc Microbiol 33:381–389

Huang J, Song XL, Yu J, Yang CH (1995) Baculoviral hypodermal and hematopoietic necrosis, study on the pathogen and pathology of the shrimp explosive epidemic disease of shrimp. Mar Fish Res 16:1–10

Inouye K, Miwa S, Oseko N, Nakano H, Kimura T, Momoyama K, Hiraoka M (1994) Mass mortalities of cultured Kuruma shrimp *Penaeus japonicus* in Japan in 1993: electron microscope evidence of the causative virus. Fish Pathol 29:149–158

Kearney JF, Radbruch A, Liesegang B, Rajewsky K (1979) A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. J Immunol 123:1548–1550

Kohler G, Milstein C (1976) Fusion between immunoglobulin-secreting and nonsecreting myeloma cell lines. Eur J Immunol 6:511–519

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lightner DV (1996) A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp. World Aquaculture Society, Baton Rouge

Lightner DV, Redman RM, Poulos BT, Nunan LM, Mari JL, Hasson KW (1997) Risk of spread of penaeid shrimp viruses in the Americas by the international movement of live and frozen shrimp. Rev Sci Tech Off Int Epiz 16: 146–160

Lo CF, Ho CH, Peng SE, Chen CH, Hsu HC, Chiu YL, Chang CF, Liu KF, Su MS, Wang CH, Kou GH (1996) White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. Dis Aquat Org 27:215–225

Lotz JM, Browdy CL, Carr WH, Frelier PF, Lightner DV (1995) USMFP suggested procedures and guidelines for assuring the specific pathogen status of shrimp broodstock and seed. In: Browdy CL, Hopkins JS (eds) Swimming through troubled waters. Proceedings of the Special Session on Shrimp Farming. The World Aquaculture Society, Baton Rouge, p 66–75

Magbanua FO, Natividad KT, Migo VP, Alfafara CG, de la Pena FO, Miranda RO, Albaladejo JD, Nadala ECB, Loh PC (2000) White spot syndrome virus (WSSV) in cultured *Penaeus monodon* in the Philippines. Dis Aquat Org 42: 77–82

Mohan CV, Shankar KM, Kulkarni S, Sudha PM (1998) Histopathology of cultured shrimp showing gross signs of yellow head syndrome and white spot syndrome during 1994 Indian epizootics. Dis Aquat Org 34:9–12

Nadala ECB, Loh PC (2000) Dot-blot nitrocellulose enzyme immunoassays for the detection of white-spot virus and yellow-head virus of penaeid shrimp. J Virol Methods 84:175–179

Nadala ECB, Tapay LM, Cao S, Loh PC (1997) Detection of yellowhead virus and Chinese baculovirus in penaeid shrimp by the Western blot technique. J Virol Methods 69:39–44

Nadala ECB, Tapay LM, Loh PC (1998) Characterization of a non-occluded baculovirus-like agent pathogenic to penaeid shrimp. Dis Aquat Org 33:221–229

Nakano H, Koube H, Umezawa S, Momoyama K, Hiroaka M, Inouye K, Oseko N (1994) Mass mortalities of cultured Kuruma shrimp, *Penaeus japonicus*, in Japan in 1993; epizootiological survey and infection trials. Fish Pathol 29: 135–139

Oi VT, Herzenberg LA (1980) Immunoglobulin-producing hybrid cell lines. In: Mishell BB, Shiigi SM (eds) Selected methods in cellular immunology. WH Freeman, San Francisco, p 351–372

Poulos BT, Kibler R, Bradley-Dunlop D, Mohney LL, Lightner DV (1999) Production and use of antibodies for the detection of Taura syndrome virus in penaeid shrimp. Dis Aquat Org 37:99–106

Richman LK, Montali RJ, Nichols DK, Lightner DV (1997) A newly recognized fatal baculovirus infection in freshwater crayfish. Proc Am Assoc Zoo Vet 262–264

Sahul-Hameed AS, Anilkumar M, Stephen-Raj ML, Jayaraman K (1998) Studies on the pathogenicity of systemic ectodermal and mesodermal baculovirus and its detection in shrimp by immunological methods. Aquaculture 160: 31–45

Towbin HT, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354

van Hulten MCW, Westenberg M, Goodall SD, Vlak JM (2000a) Identification of two major virion protein genes of white spot syndrome virus of shrimp. Virology 266:227–236

van Hulten MCW, Goldbach RW, Vlak JM (2000b) Three functionally diverged major structural proteins of white spot syndrome virus evolved by gene duplication. J Gen Virol 81:2525–2529

Wang CH, Lo CF, Leu JH, Chou CH, Yeh PY, Chou HY, TungMC, Chang CF, Su MS, Kou GH (1995) Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. Dis Aquat Org 23:239–242

Wang Q, Poulos BT, Lightner DV (2000a) Protein analysis of geographic isolates of shrimp white spot syndrome virus. Arch Virol 145:263–274

Wang Q, Nunan LM, Lightner DV (2000b) Identification of genomic variations among geographic isolates of white spot syndrome virus using restriction analysis and southern blot hybridization. Dis Aquat Org 43:175–181

Wilson CM (1983) Staining of proteins on gels: comparison of dyes and procedures. Methods Enzymol 91:236–247

Winaro B (1995) Shrimp aquaculture in Indonesia. In: Browdy CL, Hopkins JS (eds) Swimming through troubled water. Proceedings of the Special Session on Shrimp Farming.

World Aquaculture Society, Baton Rouge, p 24–28
Wongteerasupaya C, Vickers JE, Sriurairatana S, Nash GL, Akarajamorn A, Boonsaeng V, Panyim S, Tassanakajon A, Withyachumnarnkul B, Flegel TW (1995) A nonoccluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn, *Penaeus monodon*. Dis Aquat Org 21:69–77
Wyban JA (1992) Selective breeding of specific pathogen-free (SPF) shrimp for high health and increased growth. In: Fulks W, Main KL (eds) Diseases of cultured penaeid shrimp in Asia and the United States. The Oceanic Institute, Honolulu, p 257–268
Zhan WB, Wang YH, Fryer JL, Okubo K, Fukuda H, Yu KK, Meng QX (1999) Production of monoclonal antibodies (MAbs) against white spot syndrome virus (WSSV). J Aquat Anim Health 11:17–22

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