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Characterization and application of monoclonal antibodies against white spot syndrome virus

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Abstract

Three hybridoma clones secreting monoclonal antibodies (MAbs) were produced from mouse myeloma and spleen cells immunized with white spot syndrome virus (WSSV) isolated and purified from *Penaeus monodon* (Fabricius), collected from north-eastern Taiwan. By sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), the protein profile of this isolate contained four major proteins with sizes of approximately 35 (VP35), 28 (VP28), 24 (VP24), and 19 kDa (VP19). Western blot analysis revealed that two MAbs (1D7 and 6E1) recognized epitopes on VP28 and one MAb (3E8) recognized an epitope on VP19. The MAb 6E1 isotyped to the IgG_1 class was used in both an indirect immunofluorescence assay (IFA) and in an immunochemical staining protocol for successful identification and localization of WSSV in infected shrimp tissues. Antigenic similarity of isolates from Indonesia and Malaysia to the Taiwan isolate was illustrated by IFA with MAb 6E1. A MAb (2F6) which bound specifically to two shrimp proteins, 75 and 72 kDa, and reacted to the healthy and non-target tissues of WSSV in infected shrimp, such as hepatopancreas, is also described here and shows the necessity for specific identification of antibodies.

Keywords: diagnosis, MAbs, *Penaeus monodon*, white spot syndrome virus, WSSV, IFA.

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Introduction

White-spot disease, caused by white spot syndrome virus (WSSV), is a relatively recent but widespread epizootic of cultured shrimp in Asia. This epizootic probably began in Taiwan in 1992 (Chen 1995) and subsequently spread to most shrimp growing countries in Asia (Flegel & Alday-Sanz 1998), and to the south-eastern United States (Nunan, Poulos & Lightner 1998). The disease has occurred naturally in cultured penaeid shrimp such as *Penaeus chinensis* (Zhan, Yu & Meng 1995), *P. japonicus* (Inouye, Miwa, Oseko, Nakano, Kumura, Momoyama & Hiraoka 1994), *P. monodon* (Chou, Huang, Wang, Chiang & Lo 1995) and has been produced experimentally in *P. vannamei*, *P. stylirostris* (Durand, Lightner, Nunan, Redman, Mrai & Bonami 1996) and *P. monodon* (Kanchanaphum, Wongteerasupaya, Sitidilokratana, Boonsaeng, Panyim, Tassanakajon, Withyachumnarnkul & Flegel 1998). Recently the virus was observed in wild *Metapenaeus ensis* in Taiwan (Wang, Tsai, Kou & Chen 1997) and in other crustaceans (Lo, Ho, Peng, Chen, Hsu, Chiu, Chang, Liu, Su, Wang & Kou 1996a).

Because of the devastation WSSV has caused in affected shrimp farms various detection methods have been developed to help monitor and control its spread. The most extensively used protocols are based on the polymerase chain reaction (PCR) technique (Kimura, Yamano, Nakano, Momoyama, Hiraoka & Inouye 1996; Lo, Leu, Ho, Chen, Peng, Chen & Chou 1996b). The DNA-based hybridization methods have also been reported (Durand *et al.* 1996; Chang, Tsai & Wang 1998). These techniques are highly sensitive and allow for detection of WSSV in asymptomatic animals.

Immunological diagnostic methods have also been exploited. A Western blot protocol for the specific and early detection of Chinese baculovirus (CBV) in experimentally infected shrimps as well as WSSV-infected primary shrimp cell cultures was developed using polyclonal antibodies prepared in rabbits (Nadala, Tapay, Cao & Loh 1997). A nitrocellulose-enzyme immunoblot (NC-EIB) method was used to detect the presence of systemic ectodermal and mesodermal baculovirus (SEMBV) in various tissues of infected *P. monodon*. Furthermore, polyclonal antiserum raised against a prominent viral protein (27 kDa) in mice was successfully used to detect SEMBV in shrimp by NC-EIB and competitive enzyme-linked immunosorbent assay (ELISA) techniques (Hameed, Anilkumar, Raj & Jayaraman 1998). With the development of hybridoma technology, a panel of monoclonal antibodies (MAbs) was produced and used in an indirect immunofluorescence assay (IFA) for rapid diagnosis of WSSV (Zhan, Wang, Fryer, Okubo, Fukuda, Yu & Meng 1999).

In the present paper, the production and characterization of another panel of MAbs against WSSV is described. After analysis by Western blot, a MAb (MAb 6E1) identified to be specific for a VP28 envelope protein was used to develop a specific IFA and an immunochemical staining protocol for WSSV detection in tissue sections. In addition, we assessed its usefulness for investigating the antigenic relationships of three WSSV isolates from Asia, using IFA.

Materials and methods

Virus purification

Samples of moribund *P. monodon* were collected from shrimp farms in north-eastern Taiwan. The samples were examined by gross anatomy and PCR for confirmation of the disease using a protocol described previously (Wang *et al.* 1997). To purify the virus, gill and head soft tissues were harvested from infected shrimps. The tissues were suspended in TNE buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.4) at 10% w/v and homogenized manually in an ice bath. Virus purification was carried out as described by Takahashi, Itami, Maeda, Suzuki, Kasornchandra, Supamattaya, Khongpradit, Boonyaratpalin, Kondo, Kawai, Kusuda, Hirono & Aoki (1996), with modification. Tissue debris was pelleted at 3000 *g* for 10 min. The supernatant was filtered through a 0.45-μm membrane. The filtrate was ultracentrifuged at 100 000 *g* for 1 h at 4° (Hitachi 85H2, RPS40T rotor, Tokyo, Japan). The pellet was then soaked in a small volume of TNE buffer at 4° overnight. The pellet was gently resuspended and the suspension was layered onto the top of a discontinuous gradient of 10 and 50% (w/v) sucrose and then ultracentrifuged at 100 000 *g* for 1 h at 4°. A single virus band was observed at the interface between the 10 and 50% sucrose solutions (Fig. 1) and harvested with a Pasteur pipette. The virions were washed with TNE buffer by ultracentrifugation at 100 000 *g* for 1 h at 4°. The virus preparation was assayed for protein concentration by Bio-Rad protein assay kit and used for mouse immunizations, ELISA, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), and Western blot.

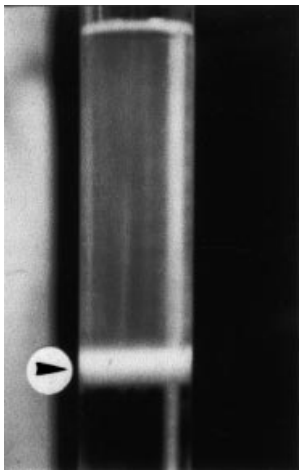

Figure 1 The band of concentrated WSSV virions formed in the sucrose discontinuous density gradient. Arrowhead indicates the virus band.

Immunization

Balb/c strain mice were immunized with the virus preparation (25 μg mouse^{-1}) emulsified in Freund's complete adjuvant. Starting 3 weeks after the first immunization, booster injections of antigen in incomplete adjuvant were given twice at 2-week intervals. The mice were killed 3 days after the last injection.

Fusion

Spleen cells from immunized mice were fused with P3-X63-Ag8U1 myeloma cells in 50% (w/v)

polyethylene glycol 1500 (Merck, NJ, USA). Fused cells were resuspended in RPMI1640 medium (Flow Laboratories Inc., VA, USA) supplemented with 20% foetal bovine serum (FBS) and hypoxanthine–aminopterine–thymidine (HAT)(GIBCO-BRL, NY, USA) and plated into 96-well culture plates. After 2 weeks of selection in HAT medium, hybridomas were screened for antibody production by ELISA. The cells from wells showing positive reactions were cloned twice by limiting dilution. The MAb preparations used in the following studies were unpurified cell culture supernatants from these hybridomas.

ELISA

The 96-well EIA plates (Nunc, Denmark) were coated overnight at 4° with 100 μL well^{-1} of the virus preparation diluted to 1 μg mL^{-1} in carbonate buffer (50 mM, pH 9.6). The plates were then washed three times in phosphate buffered saline (PBS) with 0.1% Tween 20 (PBS-T) followed by blocking with 3% bovine serum albumin (BSA) in PBS-T, 100 μL mL^{-1}, for 1 h at room temperature. All subsequent incubations were performed at 37°. The plates were drained and incubated for 1 h with 100 μL well^{-1} of the hybridoma supernatants to be examined. Serum from the mouse that was used for the fusion served as the positive control, and the conditional media pooled from myeloma cell culture, P3-X63-Ag8U1, was used as the negative control. After washing the plates three times in PBS-T, 100 μL well^{-1} of peroxidase-conjugated goat anti-mouse Ig (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA), 1/2000 in PBS-T, was added and the plates were incubated for a further 1 h. Finally, following washing, 100 μL well^{-1} of H_2O_2–OPD substrate (3.5 mM o-phenylenediamine and 0.012% hydrogen peroxide in 35 mM citrate/150 mM phosphate, pH 5.0) was added. The optical density was read at 492 nm using an ELISA reader (DigiScan, ASYHITECH, Austria) after 30 min incubation. In initial screening, a hybridoma supernatant was regarded as ELISA-positive when its absorbance value was above 1.0, which was 20 times higher than that of the negative control wells.

Monodon-type baculovirus (MBV), purified from shrimp hepatopancreas as described previously (Chang, Wang, Lo, Kou & Chen 1992), was also coated (0.1 μg well^{-1}) in EIA plates (Nunc) as the control antigen to determine the specificity of the MAbs produced. In this second screening, a hybridoma supernatant was regarded as WSSV-specific when its absorbance value was above 0.5 in the WSSV-coated well, provided that the absorbance of the corresponding well coated with control antigen, MBV, did not exceed 10% of the value of the WSSV antigen.

SDS–PAGE

The structural proteins of the virus were analysed by 12.5% SDS–PAGE according to the method of Laemmli (1970). Samples were electrophoresed for 90 min at 100 V and the gels stained using a silver stain kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Molecular weight standards were coelectrophoresed.

Western blotting

The unstained, electrophoresed gel was blotted onto a nitrocellulose membrane in blotting buffer (3.03 g Tris base, 14.4 g glycine, and 200 mL methanol L^{-1}) at 100 V for 1 h. The membrane was then rinsed in TBS buffer (20 mM Tris-HCl, 137 mM NaCl, pH 7.5), soaked in 5% skim milk (in TBS) for 1 h, and rinsed again in TBS for 5 min. The membrane was treated with hybridoma culture fluids as the primary antibody for 1 h, rinsed three times with TBS for 5 min, and then treated with 1:4000 dilution of the secondary antibody (peroxidase-conjugated goat anti-mouse Ig, KPL) for 1 h. The membrane was again rinsed three times with TBS for 5 min and then treated with the 3,3′,5,5′-tetramethylbenzidine (TMB) membrane peroxidase substrate (KPL) until the bluish purple colour developed. The membrane was soaking in distilled water to stop the reaction and air-dried. All of the incubations were carried out at room temperature.

Isotype determination

Monoclonal antibody class and subclass were determined by using the MAb-based mouse Ig isotyping kit (PharMingen International, San Diego, CA, USA) according to the protocol recommended by the manufacturer.

Immunofluorescence assay

The cephalothorax of naturally diseased shrimp was treated in Davidson's fixative for 48 h. The specimens were then processed routinely for

histology. Paraffin embedded sections 6–7 μm in thickness were rehydrated through graded alcohol (3 min each in 100, 80, 40, and 20% ethanol) to water. The slides were fixed in cold acetone for 20 min and rinsed with PBS. They were then incubated in blocking solution (5% skim milk/PBS) for 1 h at room temperature. The slides were then covered with hybridoma culture fluids as primary antibody. After incubation for 1 h at 37° in a humidified chamber, the slides were rinsed three times for 5 min with PBS. The secondary antibody, fluorescein isothiocyanate-conjugated goat anti-mouse Ig (1:200 in 1% skim milk/PBS)(KPL), was applied at 37° for 1 h. The preparations were rinsed again with PBS, mounted in glycerol and examined using fluorescence microscopy.

To compare the antigenicity of geographic isolates of WSSV, the cephalothorax of infected shrimps collected from Indonesia and Malaysia were also assayed with IFA.

Immunochemical staining

Deparaffinized sections were fixed and rehydrated followed the procedures described above. The endogenous peroxidase activity of tested tissues was blocked by immersion of the slides into an extra blocking solution (0.3% H_2O_2/100% methanol) for 30 min. After rinsing in distilled water for 5 min and soaking in PBS for 10 min, the slides were incubated with a serum block solution (10% normal goat serum/PBS) for 20 min to minimize the background reaction. The slides were then covered with hybridoma culture fluids as the first antibody and incubated for 1 h at 37°. A secondary antibody, peroxidase-conjugated goat anti-mouse Ig (1:200 in PBS)(KPL), was applied onto these slides. After 60-min incubation at 37°, the slides were washed and treated with a TrueBlue peroxidase substrate (KPL) containing TMB and H_2O_2 for 10 min. They were rinsed in distilled water for 1 min, and counter-stained with 1% of aqueous eosin for 1 min. The slides were rinsed again with distilled water, mounted in glycerol, and examined under the light microscope to identify the positive nuclei stained blue to purple.

Results

Two trials were conducted to obtain hybridoma clones secreting MAbs against WSSV. Fourteen days after fusion, approximately 90% of the 576 seeded wells contained hybridomas. In the initial ELISA screening assay, approximately 30% of the hybridomas gave strong positive reactions with purified WSSV. Some of the strongest ELISA-positive hybridomas were selected for subcloning by limiting dilution. Subcultivation and subsequent screening by ELISA yielded 20 positive hybridoma clones and four representative clones are described in Table 1. Specificity of these MAbs was tested with a second agent, MBV. Indirect ELISA using MBV as antigen showed that none of these four MAbs reacted with MBV (Table 1).

Table 1 Detection of hybridomas secreting monoclonal antibodies and their specificity by ELISA, immunoglobulin class or subclass, and their epitopes

MAb	Class or subclass	ELISA (OD_{492}) to WSSV*	MBV	Western blot†
1D7	$IgG_{1,\kappa}$	2.52 ± 0.21	<0.03	VP28
2F6	$IgM,_{\kappa}$	1.88 ± 0.29	<0.05	RS
3E8	$IgM,_{\kappa}$	1.87 ± 0.32	<0.04	VP19
6E1	$IgG_{1,\kappa}$	2.98 ± 0.15	<0.05	VP28

* Mean ± SD of three individual tests.
† Western blot: VP28 = epitope on the envelope structural protein of WSSV with a molecular mass of 28 kDa; VP19 = epitope on the envelope structural protein of WSSV with a molecular mass of 19 kDa; RS = reaction with shrimp-origin proteins with molecular masses of 75 and 72 kDa.

Isotype determination showed that MAb 1D7 and 6E1 were IgG_1 with a κ light chain. The other two MAbs, 2F6 and 3E8, were IgM with a κ light chain (Table 1).

After separation by SDS–PAGE, more than 20 bands were observed in the WSSV extract. Six major proteins were identified with an apparent molecular mass of 75, 72, 35, 28 (VP28), 24 (VP24) and 19 kDa (VP19), respectively (Fig. 2A). The 75 and 72 kDa bands are shrimp proteins copurified with the virions (van Hulten, Westenberg, Goodall & Vlak 2000).

The Western blot analysis illustrated the binding ability of MAbs 1D7 and 6E1 to VP28 (Fig. 2B, lane 1), and MAb 3E8 to VP19 (Fig. 2B, lane 2). One representative (MAb 2F6) with binding ability to both the 75 and 72 kDa proteins is shown in Fig. 2B, lane 3. The MAb 6E1 was subsequently applied in IFA and immunochemical staining to detect WSSV in diseased shrimp.

Using MAb 6E1 in IFA, nuclei exhibiting positive reactions for WSSV were located in gill epithelial cells (Fig. 3A) and in the stomach epidermis (Fig. 3B). Circulating haemocyte

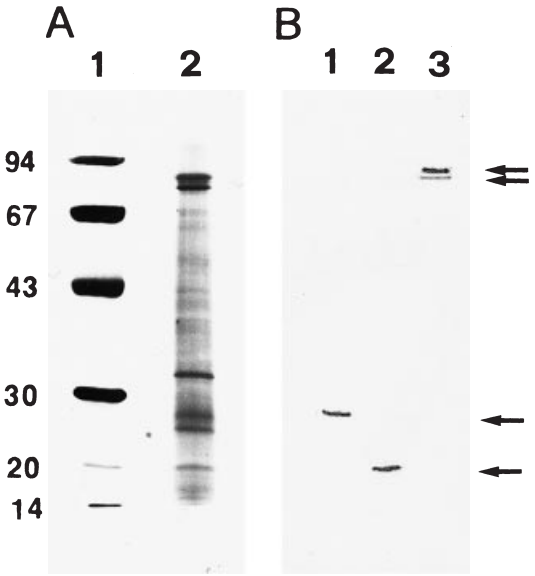

Figure 2 Western blot and SDS–PAGE of purified WSSV Taiwan isolate. (A) 12.5% of silver-stained gel; lane 1: Bio-Rad low molecular weight marker; lane 2: purified Taiwan isolate. (B) Western blot of Taiwan isolate using MAbs; lane 1: MAb 6E1 binding to viral envelope protein VP28; lane 2: MAb 3E8 binding to another viral envelope protein VP19; lane 3: MAb 2F6 binding to shrimp-origin proteins, 75 and 72 kDa (arrows).

aggregates (Fig. 3C) or haemocytes fixed amongst the striated skeletal muscles also showed positive reactions (Fig. 3D). Background fluorescence was only observed in healthy shrimp tissue, such as gill (Fig. 3E). In contrast, MAb 2F6 specific to shrimp proteins (75 and 72 kDa) reacted to healthy shrimp hepatopancreas (Fig. 3F).

In addition, MAb 6E1 also detected WSSV in various tissues collected from Indonesian and Malaysian shrimp, although the strength of positive reactions were different compared with the Taiwan isolate (Table 2). Strong fluorescence was observed in stomach epidermis of a sample from Indonesia, but a comparatively weak reaction was shown in gill epithelium of the Malaysian samples.

Immunochemical staining, using MAb 6E1, clearly distinguished infected and uninfected cells in the various tissues tested, including gill epithelium and lymphoid organ. Infected cells with clearly hypertrophied nuclei gave positive signals when stained by MAb 6E1 (Fig. 4A,B). Although the positive signal in immunochemical staining was primarily nuclear, a positive signal was also evident in the cytoplasm. The normal nuclei in healthy cephalothoracic epidermis showed no reactions (Fig. 4C).

Discussion

Previous characterizations of the structural proteins of WSSV and other related viruses show that they consist of a complex protein profile, and that at least three dominant polypeptides, 27.5, 23.5 and 19 kDa, are present in the WSSV virion (Nadala, Tapay & Loh 1998; Nadala & Loh 1998; Hameed *et al.* 1998). Three dominant polypeptides with similar sizes (25, 23 and 19 kDa) have also been detected in six geographic isolates of WSSV from China, India, Thailand, as well as three locations in the USA (Wang, Poulos & Lightner 2000). Furthermore, van Hulten *et al.* (2000) observed four major proteins in the same size range with approximate masses of 19 (VP19), 24 (VP24), 26 (VP26) and 28 kDa (VP28), respectively. According to these authors two proteins, VP24 and VP26, are the major components of the WSSV nucleocapsid. Two other major virion proteins, VP19 and VP28, are most likely constituents of the virion envelope. A complex protein composition of the Taiwan isolate is indicated by SDS–PAGE analysis in the present study. Four major and several less prominent protein bands were observed. The sizes of the three major virion proteins, i.e. VP19, VP24 and VP28, were the same as those described by van Hulten *et al.* (2000) (Fig. 2A). In our study VP26 was not detected, but a 35-kDa major band was observed. This band has only so far been identified in a Taiwanese isolate.

Three other major protein bands in the range of 67–78 kDa have been detected in shrimp haemolymph and copurified with the virions (van Hulten *et al.* 2000). Two proteins within this size range, 75 and 72 kDa, were found in this study (Fig. 2A). As these two proteins were usually copurified with the viral preparation, MAbs specific to them were selected by ELISA using a WSSV preparation as the antigen. It is, therefore, most important to ensure specificity of antibodies (polyclonal or monoclonal) produced against the viral preparation prior to detection of specific WSSV proteins. The use of identified MAbs in immunodiagnostic protocols will significantly improve their specificity.

The IFA and an immunochemical staining method, using MAb 6E1, clearly localized WSSV in infected shrimp sections. Strong labelling was limited to infected nuclei and was stronger when there was hypertrophy of the nucleus in the immunochemical staining assay (Fig. 4). Infection

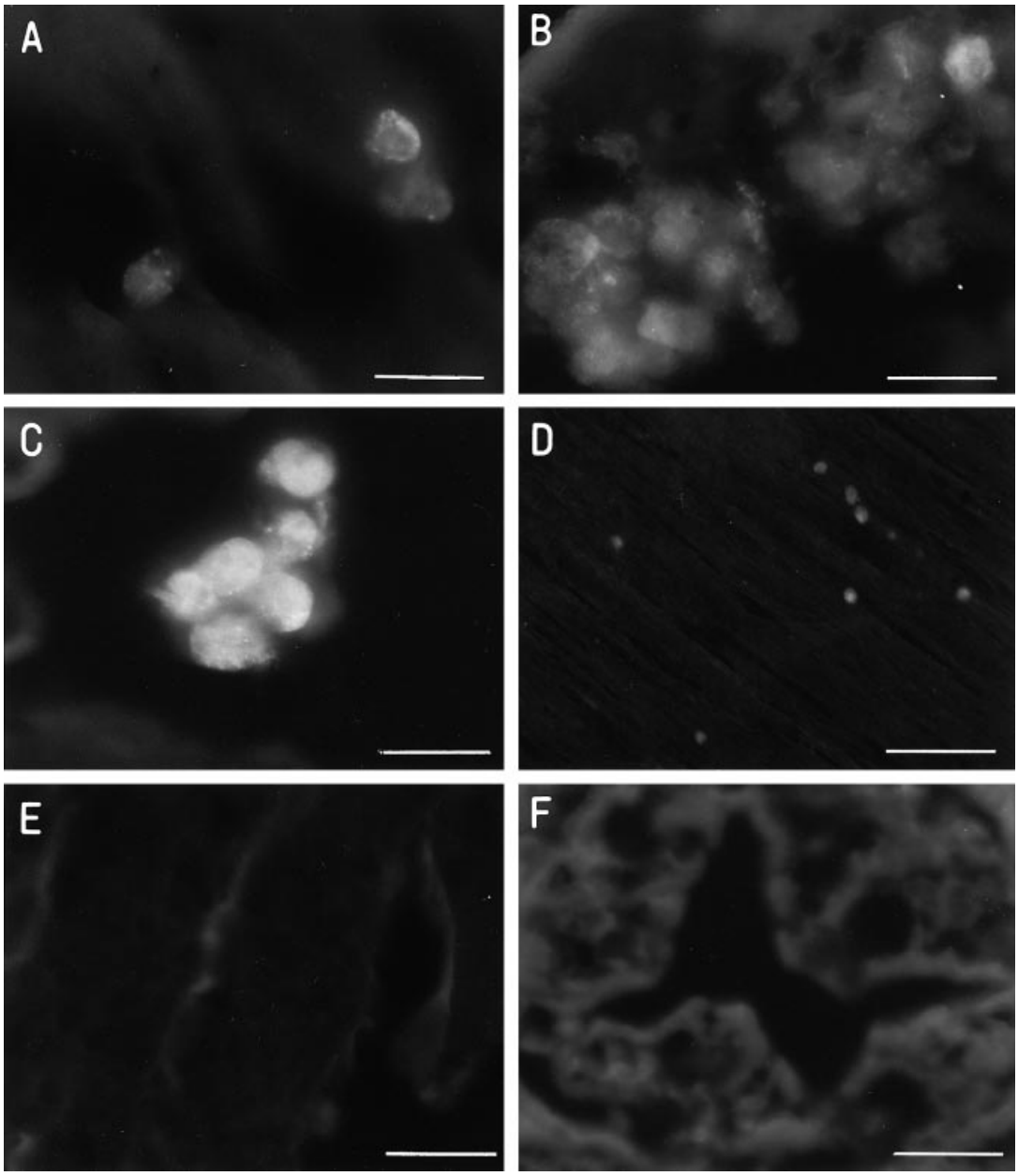

Figure 3 Immunofluorescence assays. Paraffin wax sections of the cephalothorax were prepared from WSSV-infected and uninfected shrimps. Anti-WSSV MAb (6E1) shows positive reactions with the gill epithelial cells (A), the epidermis of the stomach (B), circulating haemocyte aggregates (C), and fixed haemocytes amongst the striated muscle fibres (D) of infected shrimp, but negative reaction with the gill tissue of healthy shrimp (E). Another MAb (2F6) specific to shrimp-origin proteins reacted uniformly with healthy uninfected hepatopancreatocytes (F) [bars = 25 μm in (A, B, C, E, F), 100 μm in (D)].

was detected in gills, epidermis, haemocytes, lymphoid organ and the stomach. The target tissues for WSSV detected by MAb 6E1 are the same as those reported by Chen (1995) and Inouye *et al.* (1994). No positive reaction was found in healthy shrimp tissue, such as gills (Fig. 3E), or uninfected epidermis cells under the cephalothoracic cuticle (Fig. 4C).

A major envelope protein of WSSV and related viruses, VP28, has been detected in various geographic isolates including CBV from China (Nadala *et al.* 1997; Nadala & Loh 1998), white-spot virus

Table 2 Detection of WSSV-infections in diseased shrimp from three geographic locations by indirect IFA using MAb 6E1

	Tissue examined		
WSSV isolate	Gill epithelium	Stomach epidermis	Haemocytes
Taiwan	++	+++	+++
Indonesia	++	+++	++
Malaysia	+	ND	++

+++ = strong fluorescence; ++ = moderate fluorescence; + = weak fluorescence.
ND: Not done.

(WSV) from Indonesia and the USA (Nadala & Loh 1998), SEMBV from India (Hameed *et al.* 1998), and WSSV from Thailand (van Hulten *et al.* 2000), India, China and the USA (Wang *et al.* 2000). In this study, it was also detected in a Taiwanese isolate and isolates from Indonesia and Malaysia. This result confirms and expands the observation that seven different geographical WSSV isolates from clinical samples (collected from China, India, two from Thailand and three from the USA) were genetically closely related based on dot hybridization data and the restriction fragment length polymorphism (RFLP) analysis of PCR products (Lo, Hsu, Tsai, Ho, Peng, Kou & Lightner 1999). Nadala & Loh (1998) showed that three isolates of WSV from the USA, Indonesia and China were antigenically identical by Western blot using polyclonal antiserum. The present study highlights the antigenic relatedness of WSSV isolates from Taiwan, Indonesia and Malaysia, using a MAb recognizing an epitope on a single viral structural protein, VP28.

Immunological, as well as nucleic acid hybridization detection assays often suffer from poor specificity, especially when tissue extracts or biological fluids are used. This is mainly because of either cross-reactions with non-target proteins (ELISA, NC-EIA, capture-ELISA) or nucleic acids (hybridizations) or simply non-specific molecular interactions (Nadala *et al.* 1997). With the development of hybridoma technology, a method has become available for producing more specific immunological reagents. The MAbs generated in this study have strong binding activity for WSSV but not for MBV, another virulent virus of penaeid shrimp. One MAb (6E1) described, locates WSSV successfully in infected target tissues by IFA and immunochemical staining. Furthermore, it has for the first time enabled assessment of the degree of antigenic similarity between WSSV isolates based on a single epitope located on the viral envelope protein VP28. These results strongly suggest that MAb 6E1 is useful for diagnosis of WSSV infections and gives useful information for the classification of WSSV isolates and related viruses.

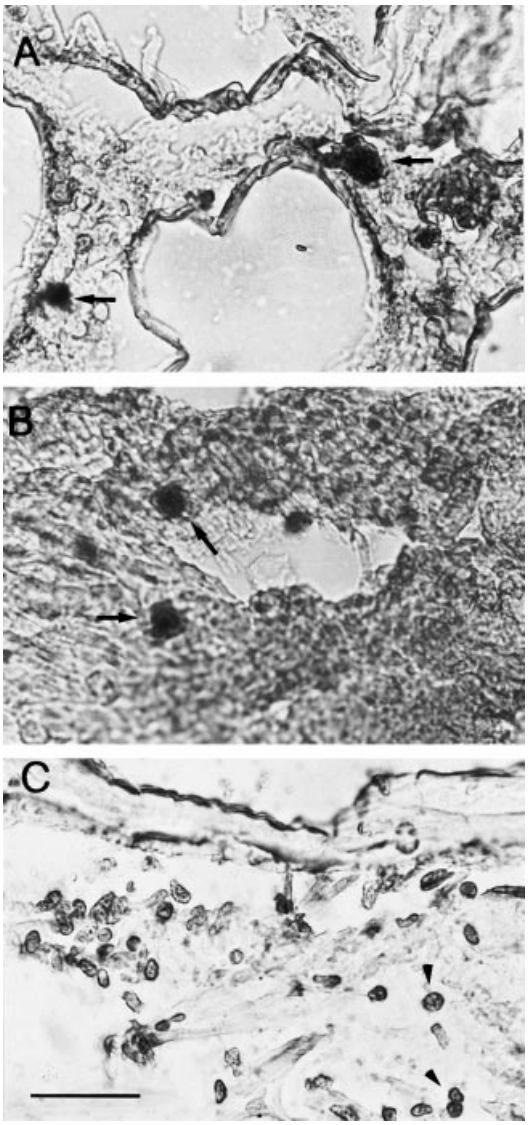

Figure 4 Detection of WSSV-infected nuclei in *P. monodon* by immunochemical staining with MAb 6E1. Hypertrophied nuclei in the infected gill (A) and lymphoid organ (B) show dark-staining positive signals (arrows). The normal nuclei in healthy cephalothoracic epidermis (C) show no reaction (arrowheads) (bar = 25 μm).

Acknowledgments

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