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Detection of white spot syndrome virus (WSSV) of shrimp by means of monoclonal antibodies (MAbs) specific to an envelope protein (28 kDa)

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ABSTRACT: The *vp*28 gene encoding an envelope protein (28 kDa) of white spot syndrome virus (WSSV) was amplified from WSSV-infected tiger shrimp that originated from Malaysia. Recombinant VP28 protein (r-28) was expressed in *Escherichia coli* and used as an antigen for preparation of monoclonal antibodies (MAbs). Three murine MAbs (6F6, 6H4 and 9C10) that were screened by r-28 antigen-based enzyme-linked immunosorbent assay (ELISA) were also able to recognize viral VP28 protein as well as r-28 on Western blot. Three non-overlapping epitopes of VP28 protein were determined using the MAbs in competitive ELISA; thus, an antigen-capture ELISA (Ac-ELISA) was developed by virtue of these MAbs. Ac-ELISA can differentiate WSSV-infected shrimp from uninfected shrimp and was further confirmed by a polymerase chain reaction (PCR) and Western blot. Approximately 400 pg of purified WSSV sample and 20 pg of r-28 could be detected by Ac-ELISA, which is comparable in sensitivity to PCR assay but more sensitive than Western blot in the detection of purified virus. Hemolymph and tissue homogenate samples collected from a shrimp farm in Malaysia during December 2000 and July 2001 were also detected by Ac-ELISA and PCR with corroborating results.

KEY WORDS: *Aeromonas salmonicida* · Furunculosis · Detection · Identification · Experimental infections · Polymerase chain reaction

INTRODUCTION

White spot syndrome (WSS) is an economically important disease of penaeid shrimp. Mortalities approaching 100% can be reached within 3 to 10 d after infection in shrimp populations. The first reported epizootic occurred in Taiwan in 1992 (Chou et al. 1995), followed by Japan in 1993 (Nakano et al. 1994), China in 1995 (Huang et al. 1995) and many other parts of the world. The causative agent of WSS is named white spot syndrome virus (WSSV) and can infect almost all commercially important species of penaeid shrimp (Lightner 1996). The virus has also been isolated from a wide range of wild crustaceans living in marine and fresh water, such as crayfish, crabs, lobsters, prawns and freshwater shrimp (Lo et al. 1996a,b, Peng et al. 1998, Wang et al. 1998, Otta et al. 1999, Wang et al. 1999), posing a potential threat to shrimp culture. Due to the devastation of the disease on shrimp culture worldwide, a great deal of effort has been made to develop tools for diagnosis of WSSV. A number of methods based on nucleic acid such as gene probes and polymerase chain reaction (PCR) have been successfully applied for detection of the disease (Chang et al. 1996, Kimura et al. 1996, Nunan & Lightner 1997, Lightner & Redman1998, Kasornchandra et al. 1998, Lourdes et al. 1999). However, the complexity of these approaches limits their practical application. Some immunological protocols using polyclonal antibodies to whole virus antigens have also been reported (Nadala et al. 1997, 2000, Hameed et al. 1998, Zhang

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et al. 2000, Shih et al. 2001), but their efficiency is affected by non-specific and false-positive reactions. In order to develop a specific tool for diagnosis of WSSV in this study, we prepared monoclonal antibodies (MAbs) specific to an envelope protein (VP28) of WSSV and developed a MAb-based antigen-capture enzyme-linked immunosorbent assay (Ac-ELISA) for detection of WSSV antigen from shrimp tissue homogenate and hemolymph.

MATERIALS AND METHODS

Virus isolation. The WSSV isolate used in the study was derived from a shrimp farm in Johor Bahru, Malaysia. Ten shrimp were pooled and homogenized using a mortar and pestle in the presence of liquid nitrogen. The homogenate was suspended in phosphate-buffered saline (PBS), pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma). The purification procedure was adapted from the method of Wang et al. (2000a) with slight modification. Briefly, the suspension was frozen and thawed 3 times followed by centrifugation at 3000 × *g* for 30 min. The supernatant fluid was pelleted at 30 000 × *g* for 30 min at 4°C. The pellet containing the virus was resuspended in PBS and then loaded onto the top of a 10 to 50% (w/w) discontinuous sucrose gradient solution in PBS. The gradients were centrifuged at 110 000 × *g* for 16 h at 4°C. The visible bands were drawn out with syringes and diluted 10 times with PBS. The virus was pelleted at 30 000 × *g* for 30 min at 4°C and the pellets were resuspended in PBS. The purified virus was negatively stained with uranyl acetate, pH 6.0, and observed for ultrastructure under a transmission electron microscope.

Expression of recombinant VP28 envelope protein in *Escherichia coli*. The viral DNA was extracted from a purified WSSV sample using a DNAzol kit according to the manufacturer's instructions (Gibco BRL) and quantified by measuring optical absorbance at 260 nm using a visible (Vis) UV spectrophotometer. DNA purity was estimated by determining the ratio of 260/280 nm. The purified DNA was used to construct a bacterial artificial chromosome (BAC) library, essentially as described by Wang et al. (1995) with slight modifications. Briefly, 4 µg DNA was partially digested with *Hin*dIII and fractionated by pulse field gel electrophoresis (CHEF MAPPER™, Bio-Rad) on a 1% agarose gel. The DNA-size selection was employed at 6.0 V cm^{-1} with a 120° pulse angle, using a 1 s initial and 20 s final switch time for 14 h at 14°C. The fragments ranging from 10 to 50 kb were selected and cloned into pIndigoBAC-5 (Epicentre®). The BAC library was screened by Southern blot using a ^{32}P-deoxy-CTP-labeled oligo-nucleotide probe, which is the complement to 1 to 30 bases of *vp*28 (Genbank accession no.: AF380842). A clone positive for *vp*28 was used as a template for PCR, and the full length of *vp*28 was amplified using oligo-primers (forward: 5′-CGG GAT CCA TGG ATC TTT CTT TCA CTCT-3′, reverse: 5′-ACT CTG CTA CTT ACT CGG TCT CAG TGC CAG-3′). The PCR fragment was cloned into pQE30 expression vector (Qiagen) at *Bam*HI and *Sal*I sites. The cloned fragment was confirmed for *vp*28 using ABI Prism BigDye terminator cycle sequencing ready reaction kit (PerkinElmer) by an Applied Biosystems Automated DNA sequencer (PerkinElmer). VP28 was expressed as a fusion protein with a 6-histidine tag in *E. coli* JM105 strain according to the manufacturer's instructions (Qiagen). The recombinant VP28 (r-28)-histidine fusion protein was probed by anti-histidine MAb in Western blot and purified by affinity chromatography using Ni-NTA column performed according to the manufacturer's instructions (Qiagen).

Preparation of MAbs against r-28. Balb/c mice, weighing 18 to 20 g, were inoculated subcutaneously (sc) with 50 µg of purified r-28 in an equal volume of Freund's complete adjuvant and boosted (sc) 2 wk later with the same dose of r-28 mixed with an equal volume of incomplete Freund's adjuvant. The second boost was administered intraperitoneally (ip) with the same dose of antigen in PBS, pH 7.4, 2 wk after the previous boost. Four days later, mice were sacrificed and spleen cells were harvested. Spleen cells and X63Ag8.653 mouse myeloma cells (ATCC) were fused in the presence of PEG1500 according to conventional protocols. Hybridoma supernatant fluids from 96 well plates were screened by an indirect ELISA as mentioned below. The positive wells of cells were cloned 2 or 3 times by limiting dilution. The supernatant fluids were collected from cell culture and the ascites was harvested from hybridoma-inoculated mice that were primed with pristane. MAbs from the supernatants and ascites fluids were purified by protein A-coupled affinity column chromatography (Pierce) and quantified by a micro-bicinchonic acid (BCA) assay (Pierce) according to the manufacturer's instructions.

Indirect ELISA. An indirect ELISA was set up for screening of supernatant fluids from hybridoma cultures. Based on routine protocols, 96 well microtiter plates were coated overnight at 4°C with 100 ng $well^{-1}$ of r-28 antigen for which the coating concentration was optimized by a checkerboard titration. After blocking at 37°C for 1 h with 5% skim milk in PBS containing 0.1% Tween 20 (PBST), the wells were loaded with hybridoma supernatant fluid and incubated for 1 h at 37°C. The plates were washed 3 times with PBST and loaded with anti-mouse-horseradish peroxidase (HRP) conjugate (DAKO) at 1:2000 dilution. After 1 h incuba-

tion at 37°C and 3 washes with PBST, the plates were loaded with *o*-phenylenediamine dihydrochloride (OPD) substrate (Sigma) and the color reaction was measured at 490 nm on an ELISA reader (BioDot). The wells with high readings were cloned and further analyzed.

Western blotting. According to routine protocols, protein samples were placed in loading buffer and heated at 100°C for 5 min before loading onto 12% acrylamide resolving gel with a 4% stacking gel, and separating the proteins on a mini-gel electrophoresis apparatus (Bio-Rad) at 100 V for 2 h. A mini-electrotransfer system (Bio-Rad) was used to electroblot the protein to a nitrocellulose membrane at 80 V for 2 h. The membrane was blocked with 5% skim milk in PBST for 1 h before treating with antibody or antibody conjugate according to routine protocols for the immunoblot assay. The blots were developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Pierce).

Isotyping MAbs. The subclass and light chain type of individual MAbs were determined with a commercial mouse MAb isotyping kit (Pierce) according to the manufacturer's protocols. Briefly, 96 well microtiter plates were coated with 100 ng purified r-28 antigen followed by blocking with 5% skim milk-PBST, and then incubated with MAbs and finally with isotype-specific anti-mouse antibodies immunoglobulin (Ig) G1, IgG2a, IgG2b, IgG3, IgA, IgM, and kappa and lamda light chains. A 3,3′,5,5′-tetramethybenzidine (TMB) substrate was added to each well and optical density (OD) was measured at a dual wavelength of 450 and 562 nm on an ELISA Reader (BioDot).

Competitive ELISA. The topological relationship of antigenic epitopes that MAbs recognized were determined by a competitive binding between biotinylated MAb and non-biotinylated MAb (Ignjatovic et al. 1991). Three purified MAbs were individually labeled with biotin according to the manufacturer's instructions (Pierce). The concentration of antigen used for coating, and the dilution of labeled and unlabeled MAbs were determined in advance by checkerboard titration. The 96 well plates were coated with 20 ng well^{-1} of r-28 antigen and blocked with 3% BSA in PBST, followed by incubation at 37°C for 1 h with each competing MAb serially diluted 4 times and subsequently with each biotinylated MAb at 37°C for 1 h, and finally with streptavidin-HRP conjugate at 37°C for 1 h. The OD for each well was measured at 450 and 562 nm after incubation with TMB. Each MAb was used as a competing antibody and as a biotin-labeled antibody as well. Competing antibodies were considered to be directed against the same epitope of the protein if reciprocal competition for binding was greater than 50%, against overlapping epitopes if competition was between 25 and 50%, and against 2 different epitopes if competition was below 25%. Competition is calculated as follows: competition (%) = (1 – OD of unlabeled MAb / OD of biotinytated MAb) × 100%.

Ac-ELISA. By using MAbs that are directed to different epitopes, an AC-ELISA was developed. Optimal conditions for this assay were determined through checkerboard titration (data not shown). Two MAbs (100 ng well^{-1} of each) were used to coat 96 well plates by incubation at 4°C overnight. The plates were blocked with 3% BSA-PBST at 37°C for 1 h. Tissue homogenate, hemolymph samples or both were added to the plates and incubated at 37°C for 1 h. A third MAb, which was labeled with HRP according to the manufacturer's instructions (Pierce), was added to each well and incubated at 37°C for 1 h. The OPD substrate was added to each well and the OD was measured at 492 nm.

PCR. In order to determine whether the shrimp were infected with WSSV, a PCR approach was carried out using a pair of oligo-nucleotide primers (upstream primer: 5′-ATG GAA TTT GGC AAC CTA ACA-3′; downstream primer: 5′-TTA CTT CTT CTT GAT TTC GTC-3′) corresponding to the sequence of the *vp*26 gene from Genbank (accession no.: AF272980). The total DNA was extracted from either hemolymph or tissue homogenate using the DNAzol kit (Gibco BRL) and used as a template for the PCR assay. Thirty µl of reaction mixture was prepared in a thin-walled tube with 3 µl 10× *Taq* polymerase buffer (Qiagen), 0.3 µl of each primer (25 pmol µl^{-1}), 2 µl deoxynucleotide triphosphates (dNTPs) (10 mmol µl^{-1} each), 4 µl template DNA, 0.3 µl (5 unit µl^{-1}) *Taq* polymerase (Qiagen) and 20 µl sterile distilled water. The tubes were placed in a GeneAmp9600 (PE Applied Biosystems) for amplification. After a predenaturing step at 94°C for 10 min, a 50 cycle reaction was employed for the amplification. Each cycle consisted of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. A final extension step of 72°C for 10 min was applied. An aliquot of 10 µl product was subjected to electrophoresis on a 1.2% agars gel and photographed with a UV trans-illuminator. To confirm the positive results, bands of the expected size were gel purified and sequenced using ABI Prism BigDye terminator cycle sequencing ready reaction kit.

Sensitivity of Ac-ELISA, PCR and Western blot. The purified r-28 and the purified virus mentioned above were serially diluted and used as antigen for detection in Ac-ELISA and Western blot. Total DNA extracted from the purified virus preparation was also serially diluted to be used as a template for PCR detection. The protein concentration of the purified virus was measured by a micro-BCA assay (Pierce) and the total

DNA was quantified by measuring optical absorbance at 260 nm with a UV-Vis spectrophotometer. A pool of the 3 MAbs was used as the primary antibody in the Western blot.

Shrimp samples. Six batches of live adult tiger shrimp, 2 to 20 g in weight, were purchased from a shrimp farm in Johor Bahru, Malaysia, during December 2000 and July 2001. Apparently, the majority of the shrimp were grossly normal without white spot signs. Hemolymph samples were collected in tubes containing 10% sodium citrate. Tissue samples were prepared by homogenizing the whole shrimp body with a mortar and pestle. PMSF was added to each sample, which was stored at –80°C before use.

RESULTS

Identification of WSSV isolate

The morphology of the purified viral isolate from Malaysian shrimp was observed under a transmission electron microscope. As shown in Fig. 1, typical rod-shaped virions and nucleocapsids were observed. The size ranges of enveloped virions and nucleocapsids were 280–350 × 80–150 nm and 300–380 × 70–130 nm, respectively. The *vp28* and *vp*26 genes amplified from the virus isolate were found to have 100% homology to those reported in Genbank (accession nos: AF380842, AF272980).

Expression of VP28 in *Escherichia coli*

Total DNA was extracted from purified WSSV and quantified to be 7.5 µg µl^{-1}. The viral DNA was partially digested by *Hin*dIII and subsequently cloned into the BAC vector. Three clones from the BAC library were screened to be *vp*28-positive in Southern blots. One positive clone was used as a template in PCR for amplifying the *vp28* gene. Amplified *vp*28 was then cloned and r-28 was expressed in *E. coli* in the presence of isoprophyl-β-D-thiogalactoside (IPTG). The expressed r-28 was visualized by Coomassie blue staining on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as shown in Fig. 2a and confirmed to beVP28 by probing with anti-histidine MAb in a Western blot as shown in Fig. 2b. The purified r-28 was quantified by BCA assay and estimated to be 0.4 µg µl^{-1}.

Production and characterization of MAbs

Supernatant fluids from hybridoma cultures were screened by r-28-based ELISA at 8 to 15 d after fusion. Three clones, 6F6, 6H4 and 9C10, that showed high OD readings in ELISA were obtained. The MAbs were identified by Western blot using the purified virus as well as r-28 antigens. The viral VP28 and the r-28 could both be recognized by all 3 MAbs as shown in Fig. 3. It is noted that the 3 MAbs did not react with VP24, VP26 or any other viral proteins, or normal shrimp tissues (data not shown) in Western blot. The MAbs in supernatant fluids of cell cultures were titrated by r-28-based ELISA, and the titers of 6F6, 6H4 and 9C10 were $1{:}4^9$, $1{:}4^4$ and $1{:}4^8$, respectively. The isotype of each MAb was determined to be to IgG1 with a kappa chain. The reciprocal competition between every 2 MAbs was assayed in competitive ELISA. The results showed that no competition greater than 25% was detected between any 2 MAbs at each concentration (Fig. 4), suggesting that the 3 MAbs may be directed to different epitopes that do not overlap one another in topology.

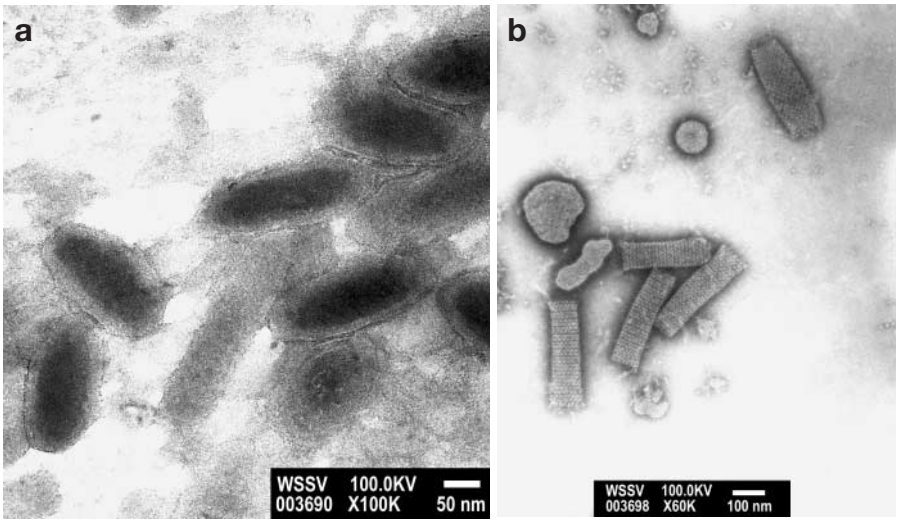

Fig. 1. Transmission electron microscopic photographs of purified white spot syndrome virus (WSSV) virions negatively stained by uranyl acetate, pH 6.0. (a) Intact virions; (b) naked virions

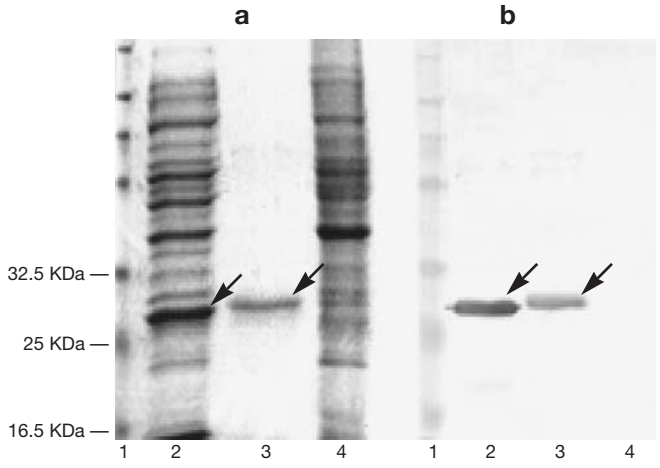

Fig. 2. Analysis of the expressed and purified recombinant VP28 (r-28) by (a) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12%) and (b) Western blot using anti-histidine monoclonal antibody. Lane 1: pre-stained protein standards; Lane 2: r-28 in crude cell lysate; Lane 3: purified r-28; Lane 4: non-induced control. Arrows indicate position of VP28

Ac-ELISA

The concentration of each MAb used for the assay was optimized by checkerboard titrations. One hundred nanograms per well of 6F6 and 6H4 was used to coat the 96 well plates. The 9C10-HRP conjugate, diluted 1:1000, was used for detection to capture the VP28 antigen. With these optimized conditions, samples of hemolymph and tissue homogenates obtained from PCR-negative and PCR-positive shrimp were tested, and the criterion for positive reaction was determined to be OD of sample / OD of negative control (S/N) ≥ 2.0 (data not shown). Ten-fold serial dilutions of r-28 and purified virus samples were assayed to determine the detection limit for both sample types. The purified r-28 and the purified virus were quantified by BCA assay and the protein concentrations were estimated to be 0.4 µg µl^{-1} and 8 µg µl^{-1}, respectively. The titers of the purified r-28 and the purified virus preparation in the Ac-ELISA are approximately 1:10^6 (20 pg) and 1:10^6 (400 pg), respectively.

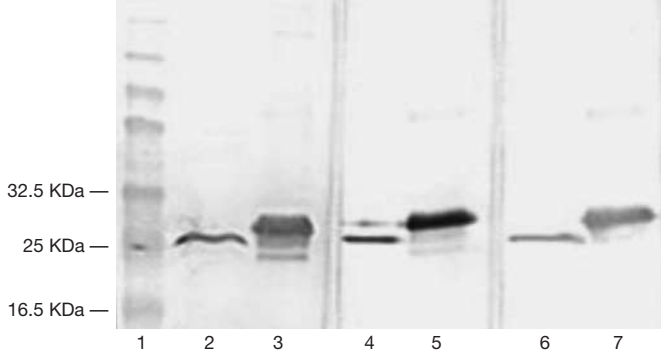

Fig. 3. Analysis of reactivity of monoclonal antibodies (MAbs) on Western blot. Lane 1: pre-stained protein standards; Lanes 2, 4 &6: purified WSSV as antigen; Lanes 3, 5 & 7: purified r-28 as antigen; Lanes 2 & 3: recognized by MAb 6F6; Lanes 4 & 5: recognized by MAb 9C10; Lanes 6 & 7: recognized by MAb 6H4

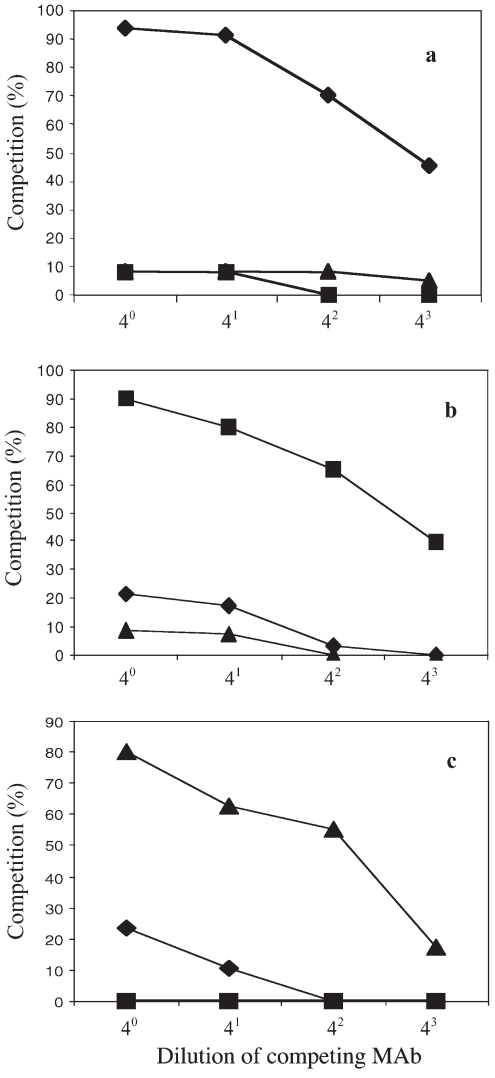

Fig. 4. Competitive interaction of biotinylated MAbs with unlabeled MAbs in competitive enzyme-linked immunosorbent assay (ELISA). (a) Competition with biotinylated 6F6; (b) competition with biotinylated 6H4; (c) competition with biotinylated 9C10. diamond: unlabeled 6F6; triangle: unlabeled 6H4; square: unlabeled 9C10

Comparison of AC-ELISA, PCR and Western blot

PCR and Western blots were carried out to verify the Ac-ELISA detection. The results are summarized in

Table 1. Sensitivity of polymerase chain reaction (PCR), Western blot (WB) and antigen-capture enzyme-linked immunosorbent assay (Ac-ELISA) in detection of white spot syndrome virus (WSSV)

Sample	Detection limit		
	PCR	WB	ELISA
Purified WSSV r-28	300 pg DNA	375 ng protein 12 ng	400 pg protein 20 pg

Table 2. Comparison of PCR, Ac-ELISA and WB in detecting WSSV samples collected from a shrimp farm in Malaysia

Sample batch	No. sample	No. positive in PCR	No. positive in ELISA	No. positive in WB
1	10	10	10	7
2	11	11	11	10
3	20	3	3	1
4	30	2	2	0
5	33	4	4	0
6	40	2	2	1

Tables 1 & 2. When the purified virus and the DNA extracted from the purified virus preparation were serially diluted 10-fold and tested in Ac-ELISA and PCR, respectively, approximately 300 pg of total DNA ($1:10^5$ dilution) could be detected by PCR (Fig. 5) and 400 pg of purified virus ($1:10^6$ dilution) could be detected by Ac-ELISA. The minimum detectable amount of purified virus by the Ac-ELISA (i.e., 400 pg) was used for the extraction of viral DNA. The DNA extracted from this 400 pg purified virus was also determined to be the minimum amount of template for PCR detection. DNA templates extracted from the purified virus less than 400 pg were not detectable by PCR (data not shown). Therefore, Ac-ELISA is comparably sensitive to PCR in terms of detection of virus amount. Nevertheless, Western blot is less sensitive than Ac-ELISA; the minimum amount of purified virus that could be detected in Western blot was 375 ng (figure not shown). When r-28 was tested, 20 pg r-28 could be detected by Ac-ELISA as compared with 12 ng that was detected by Western blot (figure not shown). As for field samples, 19 samples that were detected positive in Western blot were also positive in both PCR and Ac-ELISA. All 30 samples that were positive in PCR were also positive in Ac-ELISA.

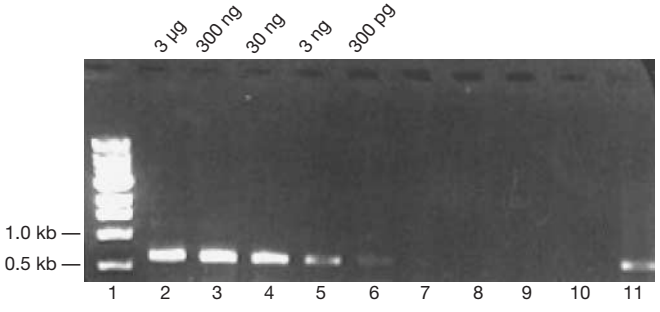

Fig. 5. Detection of purified WSSV sample by polymerase chain reaction (PCR). Lane 1: 1.0 kb DNA ladder; Lanes 2 to 10: 10-fold serial dilution of purified total DNA template; Lane 11: positive control: *vp*26 fragment

DISCUSSION

In this study, we isolated WSSV from shrimp and found its size to be within the range of WSSV reported by others (Wang et al. 2000a). The sequences of *vp*28 and *vp*26 were 100% homologous to those in Genbank, indicating that the isolate was indeed WSSV.

The WSSV virion consists of at least 5 structural proteins with approximate sizes of 28 kDa (VP28), 26 kDa (VP26), 24 kDa (VP24), 19 kDa (VP19) and 15 kDa (VP15). VP28 and VP19 are associated with the virion envelope while the others constitute the nucleocapsid (van Hulten et al. 2000b, 2001). Although WSSV has a wide geographic distribution and host range, previous studies have shown that viral isolates from different locations share high homology in nucleotide sequence (Hameed et al. 1998, van Hulten et al. 2000a, Wang et al. 2000a, b). Wang et al. (2000a) reported that 6 geographic isolates from shrimp and crayfish of China, India, Thailand and the USA had limited differences among them. The protein profiles of different WSSV isolates on SDS-PAGE have shown that VP28 is a predominant protein (Hameed et al. 1998, van Hulten et al. 2000a, Wang et al. 2000a,b), which could be recognized by WSSV antisera. These findings suggest that VP28 is a suitable target antigen for immunological detection.

In this study, we raised MAbs against r-28 that can also recognize the viral VP28 antigen. Although VP26 and VP24 share about 40% homology with VP28 (van Hulten et al. 2000a), MAbs that were raised against VP28 did not cross-react with VP26 or VP24 in Western blot, suggesting that the MAbs are VP28 specific. Three non-overlapping antigenic epitopes were elucidated by reciprocal competitions in a competitive ELISA. The difference in epitope recognition among MAbs enables the development of an Ac-ELISA. By coating 2 MAbs to capture WSSV antigen that was subsequently recognized by the third MAb-HRP conjugate, the Ac-ELISA developed in this study exhibited remarkable sensitivity in detection of WSSV from both shrimp hemolymph and tissue homogenates.

High sensitivity and specificity of PCR assays have been well documented in WSSV detection. We set up a PCR assay in which the *vp*26 rather than the *vp*28 gene was amplified. It was considerably easier to amplify *vp*26 than *vp*28 in our studies (data not shown) for reasons that are not clear. With a hot start and 50 cycle reaction, the distinguishing bands of predicted size could be amplified and were further confirmed by sequencing to be the *vp*26 gene.

A total of 144 samples were tested by PCR and Western blot, and used to evaluate Ac-ELISA. The criterion used for Ac-ELISA was thus determined by comparison with the results of PCR detection. Ac-ELISA exhibits comparable sensitivity to PCR but much higher sensitivity than Western blot in detection both of purified virus sample and of field samples. Therefore, Ac-ELISA is sufficiently sensitive and can be employed as an alternative for PCR in field detection. Additionally, both hemolymph and tissue homogenate samples can be applied in AC-ELISA for detection. In practical terms, it is easier to test hemolymph directly without special treatment, facilitating in particular large scale detection.

WSS was first observed in Malaysia in 1994, and over 80% of the shrimp farms were affected by 1996 (Wang YG et al. 1999). In this study, a follow-up surveillance of WSSV in a shrimp farm off Singapore-Johor strait showed a high prevalence of WSSV during December 2000 and July 2001, although only a few shrimp exhibited white spots on the carapace. The prevalence of WSSV in the shrimp farm seems to correlate with the death of shrimp in this farm because the mortalities during December 2000 and January 2001 (Batches 1 and 2, Table 2) were also higher than those of April to July 2001 (Batches 3 to 6, Table 2). More data are still required using the Ac-ELISA to elucidate the epidemiology of WSSV and further develop strategies for control of the problem in these areas.

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LITERATURE CITED

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, Chuang 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

Hameed ASS, Anilkumar M, Raj MLS 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

Huang J, Song X, Yu J, Yang C (1995) Baculoviral hypodermal and hematopoietic necrosis-study on the pathogen and pathology of the explosive epidemic disease of shrimp. Mar Fish Res 16:51–58

Ignjatovic J, McWaters PG (1991) Monoclonal antibodies to three structural proteins of avian infectious bronchitis virus: characterization of epitopes and antigenic differentiation of Australian strains. J Gen Virol 72:2915–2922

Kasornchandra J, Boonyaratpalin S, Itami T (1998) Detection of white-spot syndrome in cultured penaied shrimp in Asia: microscopic observation and polymerase chain reaction. Aquaculture 164:243–251

Kimura T, Yamano K, Nakano H, Momoyama K, Hiraoka M, Inouye K (1996) Detection of penaeid rodshaped DNA virus (PRDV) by PCR. Fish Pathol 31:93–98

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

Lightner DV, Redman RM (1998) Shrimp diseases and current diagnostic methods. Aquaculture 164:201–220

Lo CF, Leu Jh, Ho CH, Chen CH and 8 others (1996a) Detection of baculovirus associated with white spot syndrome (WSSV) in penaeid shrimp using polymerase chain reaction. Dis Aquat Org 25:133–141

Lo CF, Ho CH, Peng SE, Chen CH and 7 others (1996b) White spot syndrome baculovirus (WSSV) detected in cultured and captured shrimp, crabs and other arthopods. Dis Aquat Org 27:215–225

Lourdes M, Tapay E, Cesar B, Nadala EC Jr, Philip CL (1999) A polymerase chain reaction protocol for the detection of various geographical isolates of white spot virus. J Virol Methods 82:39–43

Nadala ECB Jr, 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, Lourdes MT, Cao SR, Loh PC Jr (1997) Detection of yellowhead virus and Chinese baculovirus in penaeid shrimp by western blot technique. J Virol Methods 69: 39–44

Nakano H, Koube H, Umezawa S, Momoyama K and 3 others (1994) Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: epizootiological survey and infection trials. Fish Pathol 29:135–139

Nunan LN, Lightner DV (1997) Development of a nonradioactive gene probe by PCR for detection of white spot syndrome virus (WSSV). J Virol Methods 63:193–201

Otta SK, Shubha G, Joseph B, Chakraborty A, Karunasagar I (1999) Polymerase chain reaction (PCR) detection of white spot syndrome virus (WSSV) in cultured and wild crustaceans in India. Dis Aquat Org 38:67–70

Peng SE, Lo CF, Ho CH, Chang CF, Kou GH (1998) Detection of white spot baculovirus (WSBV) in giant freshwater prawn, *Macrobrachium rosenbergii*, using polymerase chain reaction. Aquaculture 164:253–262

Shih HH, Wang CS, Tan LF, Chen SN (2001) Characterization and application of monoclonal antibodies against white spot syndrome virus. J Fish Dis 24:143–145

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

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

van Hulten MCW, Witteveldt J, Snippe M, Vlak JM (2001)

White spot syndrome virus envelope protein VP28 is involved in the systemic infection of shrimp. Virology 285: 228–233

Wang GL, Holsten TE, Song WY, Wang HP, Ronald PC (1995) Construction of a rice bacterial artificial chromosome library and identification of clones linked to the *Xa-21* disease resistance locus. Plant J 7:525–533

Wang Q, White BL, Redman RM (1999) Per os challenge of *Litopenaeus vannamei* post larvae *Farfantapenaeus duorarum* juveniles with six geographical isolates of white spot syndrome virus. Aquaculture 170:179–194

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

Wang YC, Lo CF, Chang PS, Kou GH (1998) Experimental infection of white spot baculovirus in some cultured and wild decapods in Taiwan. Aquaculture 164:221–231

Wang YG, Hassan MD, Shariff M, Zamri SM, Chen X (1999) Histophology and cytopathology of white spot syndrome virus (WSSV) in cultured *Penaeus monodon* from peninsular Malaysia with emphasis on pathogenesis and the mechanism of white spot formation. Dis Aquat Org 22: 1–11

Zhang AB, Xu LM, Xu X (2000) Detection of prawn white spot bacilliform virus by immunoassay with recombinant antigen. J Virol Methods 92:193–197

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