ScienceAsia 30 (2004): **359-363**

Monoclonal antibodies against a truncated viral envelope protein (VP28) can detect white spot syndrome virus (WSSV) infections in shrimp

Parin Chaivisuthangkura,[a] Jarasporn Tangkhabuanbutra,[a] Siwaporn Longyant,[a] Weerawan Sithigorngul,[a] Sombat Rukpratanporn,[b] Piamsak Menasveta[b] and Paisarn Sithigorngul[a,*]

[a] Department of Biology, Srinakharinwirot University, Bangkok 10110, Thailand.
[b] Marine Biotechnology Research Unit at Chulalongkorn University, BIOTEC, Bangkok 10330, Thailand.

* Corresponding author, E-mail: paisarn@swu.ac.th

Received 12 May 2004
Accepted 27 Aug 2004

ABSTRACT: A portion of the VP28 gene (VP28F118) of white spot syndrome virus was cloned into an expression vector and transformed into *E. coli*. The objective was to produce a truncated VP28 envelope protein lacking the N-terminal transmembrane region. After induction, the recombinant VP28F118 protein (rVP28F118) was produced, purified by SDS-PAGE and used to immunize Swiss mice for monoclonal antibody production. Seven hybridoma clones were obtained. The monoclonal antibodies bound well to VP28 protein and could be used to detect WSSV by immunohistochemistry and haemolymph dot blot. Both methods detected WSSV in experimentally infected *Penaeus monodon* at 12 hr. These monoclonal antibodies can be used to develop simple, low cost immuno-based test kits for WSSV detection at high specificity and sensitivity.

KEYWORDS: dot-blot, white spot syndrome virus (WSSV), immunohistochemistry, monoclonal antibody, *Penaeus monodon*.

INTRODUCTION

White spot syndrome (WSS) is one of the most serious diseases in penaeid shrimp farming world wide. It is caused by white spot syndrome virus (WSSV). Mortality rates can reach 100% within 3-10 days after outbreaks begin. The first report was from Taiwan in 1992.[1] Later the disease was reported from various parts of the world.[2] WSSV infects not only all the economically important shrimp species, but also various marine and freshwater crustaceans including crayfish, crabs, and freshwater palaemonid shrimp.[3-5]

Recently, immunologically based diagnostic methods have been developed for shrimp viruses. A polyclonal antibody against purified WSSV was generated, but it cross reacted with various shrimp proteins.[6] Monoclonal antibodies raised against purified WSSV were also produced. These antibodies were capable of detecting WSSV infection by dot blot, Western blot, immunohistochemistry and immunofluorescence, but could not distinguish among various WSSV isolates from China, Thailand, India, Texas, South Carolina and Panama.[7]

WSSV contains five major structural proteins, VP28 and VP19 are located in the envelope and VP26, VP24 and VP15 are located in the nucleocapsid.[8] Recombinant VP28 protein has been expressed in *Escherichia coli* and used as an antigen for production of monoclonal antibodies. These monoclonal antibodies were used for development of antigen-capture ELISA which gave a high sensitivity comparable to that of single-step PCR assay.[9]

In the present study, a truncated version of VP28 protein was expressed in *E. coli* and used for production of monoclonal antibodies. The antibodies had high specificity for WSSV in Western blot, immunohistochemical and dot blot assays.

MATERIALS AND METHODS

Antigen Preparation

Preparation of WSSV Stock.

Naturally WSSV infected *P. monodon* were obtained from a farm at Nakhon Srithamarat Province, Thailand. Gills from infected shrimp were homogenated in 2X PBS (Phosphate buffered saline, pH 7.2), then centifuged at 3,000 g for 30 min. Aliquots of the supernatant were collected and stored at -70°C.

WSSV DNA Preparation

Gills from naturally WSSV infected *P. monodon* were homogenized in lysis buffer (50 mM Tris-HCl pH 9.0, 100 mM EDTA, 50 mM NaCl, 2% SDS; Flegel personal

communication). DNA from 200 µl of the homogenate was prepared using a High pure viral nucleic acid kit from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.) as described in the product manual.

Cloning and Expression of Truncated VP28

Two primers, VP28F118 (5'-CGGGATCCCACACA GACAATATCGA-3';118 nucleotides downstream of the ATG start site) and VP28R (5'-TGCACTGCAGTTACT CGGTCTCAGTGCCAG-3'), with added restriction sites (underlined) were used to amplify a truncated VP28 gene by polymerase chain reaction (PCR) using *Pfx* polymerase (GIBCO BRL). The PCR product VP28F118 was cloned into the pQE30 expression vector at the *Bam*HI and *Pst*I sites and transformed into *E. coli* strain M15(pREP4). The integrity of the open reading frame of the recombinant plasmid was verified by DNA sequencing.

Preparation of Recombinant VP28F118

E. coli with VP28F118-pQE30 plasmid was cultured in LB broth to exponential phase and expression of the recombinant proteins was induced with 1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) for 4 h. After centrifugation at 4,000g for 20 min, the bacterial pellet was dissolved in 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 8, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated until a clear lysate was obtained. The lysate was separated by SDS-PAGE on a 15% gel. After staining with Coomassie brilliant blue, the recombinant protein bands of rVP28F118 were cut out and destained until the gels were clear. They were collected in dialysis bags and the protein was eluted with a transblot apparatus (Biorad Corp, Hercules, CA, U.S.A.) at 70 volts for 6 h. The protein solution was dialysed to eliminate SDS and salt before determining the protein content by Bradford protein assay.[10] The solution was divided into small aliquots and stored at –70°C.

Monoclonal Antibody Production

Immunization: Three swiss mice were injected intra-peritoneally with purified rVP28F118 (0.1 mg/ mouse) mixed with complete Freund's adjuvant in a 1:1 ratio. Mice were subsequently injected three more times with the protein mixed with incomplete Freund's adjuvant at bi-weekly intervals. One week after the fourth injection, mouse antisera were collected and tested against *E. coli* lysate, purified rVP28F118 and gill extract from WSSV infected *P. monodon* by Western blot. They were also tested against head tissues from WSSV infected *P. monodon* by immunohistochemistry. The best performing mouse was later boosted 3 days before hybridoma production.

Hybridoma Production: A cell fusion procedure was adapted from the method developed by Kohler & Milstein[11], with modifications described by Mosmann et al.[12] A P3X myeloma cell line was used as the fusion partner. Fusion products from 1 mouse were plated on 30 microculture plates (96 wells/plate). After identification of positive cultures by screening methods described below, cells were cloned by the limiting dilution method.

Screening Methods

Dot-Blotting: Purified rVP28F118 (0.01 mg/ml) was spotted on a nitrocellulose membrane (1 µl/spot), subsequently baked at 60°C for 10 min and then incubated with hybridoma conditioned media from each culture well (1:20 dilution in 5% Blotto: 5% nonfat drymilk, 0.1% Triton-X-100 in PBS) for 8 h. After extensive washing in 0.5% Blotto, the membrane was incubated in horseradish peroxidase conjugated goat anti-mouse IgG heavy and light chain specific antibody (GAM-HRP; BioRad) at 1:1000 dilution for 8 h. The membrane was then washed extensively as before and incubated in a substrate mixture containing 0.006% hydrogen peroxide, 0.03% diaminobenzidine (DAB),0.05% cobalt chloride in PBS. Hybridoma clones that displayed immunoreactivity were confirmed for viral specificity by Western blot and immunohistochemistry before cloning and cryopreservation for further investigation.

SDS-PAGE and Western Blot Analysis: Lysate of *E. coli* M15(pREP4) with and without VP28F118-pQE30, purified rVP28F118 and haemolymph from WSSV infected *P. monodon* were separated by 15% SDS-PAGE according to the method described by Laemmli.[13] Samples were electrophoresed for 6 h at 30 V and gels were stained using Coomassie brilliant blue. For Western blotting, samples resolved by SDS-PAGE were electroblotted onto nitrocellulose membranes using a transblot apparatus, then incubated with each monoclonal antibody and processed as described in dot blotting assay above.

Immunohistochemistry: Cephalothoraces from *P. monodon* injected with WSSV were cut and fixed in Davidson's fixative solution for 24 h before processing for paraffin sectioning. Serial sections (8 µm thickness) were prepared and processed for indirect immunoperoxidase staining using various MAbs and GAM-HRP diluted to 1:1000 with 10% calf serum in PBS. Peroxidase activity was revealed by incubation with 0.03% DAB and 0.006% hydrogen peroxide in PBS. Preparations were counter-stained with haematoxylin and eosin Y (H&E), dehydrated in graded ethanol series, cleared in xylene and mounted in Permount.[14] Positive reactions were visualized as brown

coloration against the pink and purple colors of H&E.

Characterization of Monoclonal Antibodies

Classes and subclasses of mouse immunoglobulins produced by the hybridomas were determined by sandwich ELISA using Zymed's Mouse MonoAb ID kit (HRP).

Determination of WSSV infection in *P. monodon*

P. monodon (15-20 g) were experimentally injected with filtered haemolymph (0.45 μm) from WSSV infected *P. monodon* at 1:50 dilution. Haemolymph from 8 shrimps was collected individually at 12, 18, 24 and 36 h after injection, and the heads of these shrimps were fixed in Davidson's fixative and processed for immunohistochemistry using the monoclonal antibody. Haemolymph from individual shrimp was spotted onto a nitrocellulose membrane (1 μl/spot) and processed for dot blotting using the monoclonal antibody. Pooled haemolymph (10 μl from each individual) at each period of time after injection was tested by PCR using VP28F (5'-CGGGATCCATGGATGGGATCTTTCTTTCACTC TTTCG-3') and VP28R (see above) as primers to yield a PCR product of 633 bp.

Results and Discussion

Early attempts to generate antibodies against purified WSSV proved to be difficult because the virus was always damaged during purification. Therefore, expressed recombinant protein was utilized as an antigen. In order to obtain a high protein yield in *E. coli*, a truncated version of the protein lacking the N-terminal hydrophobic region was used, as previously described by You et al.[15]

The truncated VP28 gene, VP28F118, of the WSSV envelope protein could be amplified as a 516 bp PCR product. (Fig 1). This was cloned and expressed in *E. coli*, and recombinant protein was visualized by Coomassie blue staining. After purification by SDS-PAGE, high purity rVP28F118 was obtained (Fig. 2) and adjusted to 1 mg/ml protein for immunization. The truncated VP28 envelope protein was in the form of a recombinant fusion protein with a 6-histidine tag at the N-terminus. This recombinant fusion protein was used to generate monoclonal antibodies without removing the 6-histidine tag.

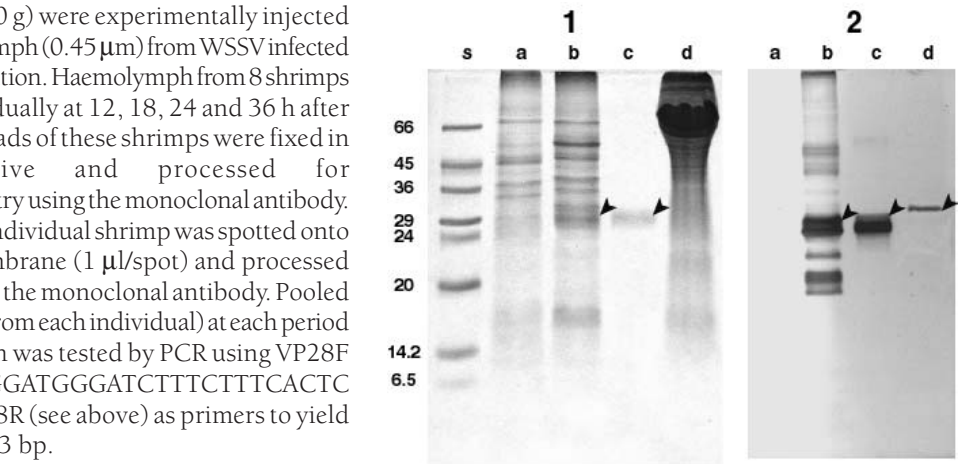

Fig 1. PCR product of VP28F118.

Fig 2. SDS-PAGE and Western blot analysis of (a) lysate from *E. coli* M15 (pREP4) without plasmid pQE30, (b) lysate from *E. coli* M15 (pREP4) with VP28F118-pQE30 plasmid (c) purified rVP28F118 protein (d) gill homogenate from WSSV infected *P. monodon*. (s) = molecular weight standards. (1) Gel stained with Coomassie brilliant blue (2) Western blot using W29-1A monoclonal antibody.

After immunization in Swiss mice, anti-rVP28F118 antisera obtained from 3 mice displayed very high immunoreactivities and specificities by immunohistochemistry and Western blot assays. Therefore, one mouse was used for hybridoma production.

Seven hybridoma clones producing antibodies specific to WSSV were obtained. Four were IgG1 and three were IgG2a. The yield of the monoclonal antibodies in this study was higher than those previously reported [i.e, only 3-4 clones were obtained by Poulos et al[7], Anil et al[16], and Lui et al[9]]. All MAbs were very specific and did not show any cross reactivity to either uninfected shrimp tissues and haemolymph or shrimp tissues and haemolymph infected with other viruses such as YHV, HPV and MBV as determined by immunohistochemistry and Western blot analysis (Table 1, Fig. 2). However, antibodies W28-8H and W29-1A showed strong binding to WSSV in haemolymph of infected shrimp and very intense immunoreactivity on dot blotting. Therefore, these monoclonal antibodies were used for determination of WSSV infection in other experiments.

These MAbs revealed WSSV infection in various

Table 1. Characterization of monoclonal antibodies specific to rVP28F118 protein of WSSV.

MAb	Subclass	dot-blot (gill extract)	Western blot	Immuno-histochemistry
W1-7D	IgG_1	+	+	+
W2-4G	IgG_{2a}	+	+	+
W6-5H	IgG_1	+	+	+
W13-8E	IgG_{2a}	+	+	+
W14-8A	IgG_1	+	+	+
W14-9A	IgG_{2a}	+	+	+
W15-7E	IgG_1	+	+	+
W28-8H	IgG_1	+	+	+
W29-1A*	IgG_1	+	+	+

* representative clone used for illustration.

tissues including muscle, heart, gills, lymphoid organ, subcuticular epithelium and interstitial cells of the hepatopancreas. WSSV immunoreactivity could be observed even in cells that did not show Cowdry type A nuclei, a histopathological characteristic of WSSV infection (Fig. 3). Therefore, they can be used to detect early stages of WSSV infection, before histopathological characteristics can be observed.

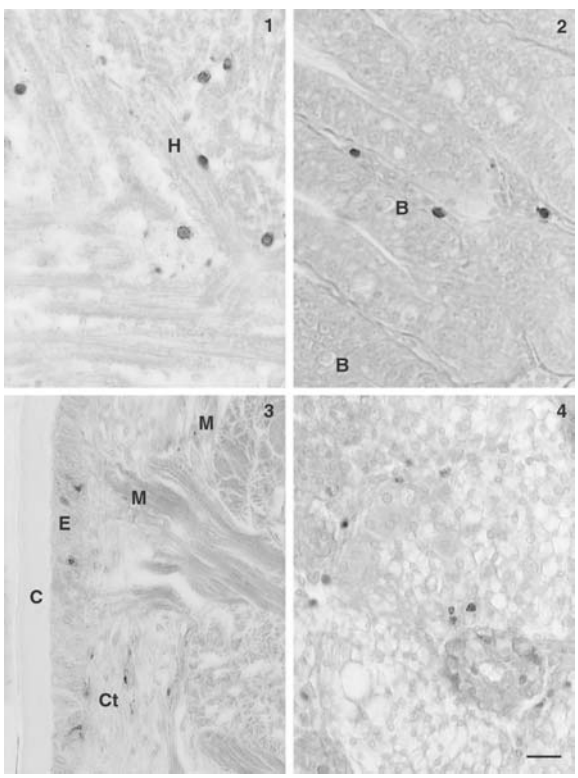

Fig 3. Immunohistochemistry using monoclonal antibody W29-1A with various tissues from *P. monodon* at 24 h post injection. (1) Cardiac muscle (H) (2) interstitial cells of the hepatopancreas (3) sub-cuticular tissues including epithelium (E), connective tissue (Ct) and muscle (M) (4) lymphoid organ. WSSV immunoreactivity was observed in infected cells with and without Cowdry Type A nuclei, but not in uninfected cells or HPV infected cells (B). C = cuticle. Bar = 25 μm.

In experimentally infected *P. monodon* examined at different time periods, immunohistochemistry could detect very light infection in 7 out of 8 shrimps at 12 h post injection, mostly in the cells of lymphoid organ, heart, sub-cuticular tissue and interstitial cells of hepatopancreas (Table 2). This was superior to *in situ* hybridization that required 16 h post infection.[17] WSSV infection was revealed in various tissues of all shrimps from 18 h post-injection onwards (Table 2). Immunohistochemistry results corresponded to those from dot blots (Fig.4). However, PCR detection also gave intensely positive PCR products from 12 h post-injection onwards (Fig.5).

Table 2. WSSV infection in *P. monodon* at different times after injection, determined by one step PCR, dot blot and immunohistochemistry.

Times after WSSV injection (Hr.)	PCR*	Dot-blot	Immuno-histochemistry
0	-	-(8)	- (8)
12	++	±(7/8)	+ (7/8)
18	++	+ (8/8)	++ (8/8)
24	++	++ (8/8)	++ (8/8)
36	++	++ (8/8)	++ (8/8)

\- No immunoreactivity observed.
± Light and variable detection.
\+ Clear detection.
++ Strong detection.
* All samples at each period were pooled.

Further development of other types of immuno-based assays, such as sandwich ELISA and a convenient strip test kit for WSSV detection using these monoclonal antibodies, is underway.

Acknowledgement

Funding for this research has been provided by the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand (W.S.)

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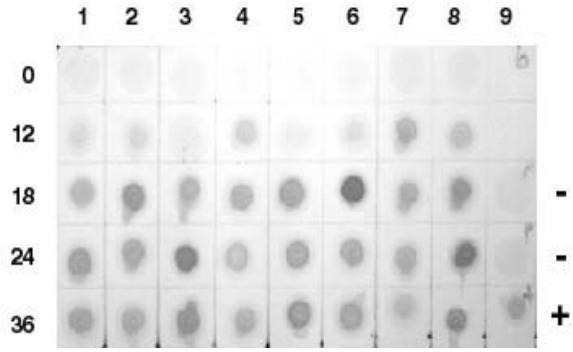

Fig 4. Dot blot of haemolymph from individual *P. monodon* (1-8) after injection with diluted infected haemolymph for 12, 18, 24 and 36 h. (0) = haemolymph from shrimp before injection. (–) = negative control, uninfected haemolymph and (+) = positive control, WSSV- infected haemolymph (9).

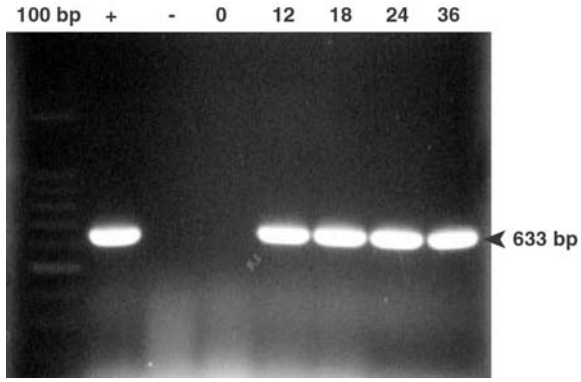

Fig 5. One step PCR of pooled haemolymph of *P. monodon* after injection with diluted WSSV-infected haemolymph for 12, 18, 24 and 36 h. (0) = haemolymph from shrimp before injection. (+) = positive control, haemolymph from infected shrimp and (-) = negative control, distilled water. 100 bp = DNA markers.

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