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Full Length Research Paper

Prokaryotic expression and immunodot-blot detection of Taura syndrome virus capsid proteins VP2 and VP3

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Capsid protein genes vp2 and vp3 of Taura syndrome virus (TSV) were cloned into pet-16b-1 and pGEX-4t-3 expression vector respectively, and transformed into *Escherichia coli* BL21 or DH5α for protein expression and purification. After induction with IPTG, recombinant VP2 (rVP2) and recombinant VP3 (rVP3) were produced in *E. coli*, purified by SDS-PAGE and used to immunize Balb/c mice for the production of polyclonal antisera: anti-rVP2 and anti-rVP3. Two antigenic peptides originating from TSV capsid protein VP2 and VP3 respectively were synthesized as antigen for the production of monoclonal antibodies (Mabs). Mab specific to VP2, VP3, anti-rVP2 and anti-rVP3 antisera all showed specific immunoreactivities to corresponding recombinant viral proteins by Western blot assay. The Mabs as well as the polyclonal antisera could detect VP2 and VP3 in homogenates from gills of TSV-infected shrimp by immunodot-blot. This is the first step towards our target of preparing TSV capsid proteins as oral vaccine and developing simple immuno-diagnostic test kits for TSV detection in *Penaeus vannamei* shrimp.

Key words: Taura syndrome virus (TSV), *Penaeus vannamei,* Mabs, VP2 and VP3 protein, Western blot.

INTRODUCTION

Taura syndrome virus (TSV) is one of the major viral pathogens causing high mortality in the cultured shrimps, *Penaeus vannamei* (Brock et al., 1995) and has caused serious economic losses in the shrimp farming industry (Lightner, 1996). It has a large geographical distribution in the Americas (Hasson et al., 1999) and spread rapidly across many countries through the regional and international transfer of broodstock and postlarva. TSV was first found in Taiwan of China in the Eastern Hemisphere at the end of the 20th century (Tu et al., 1999). To date, with the expanding of the shrimp farming industry, a serious trend in the incidence of Taura syndrome and large-scale incidence of death phenomenon had appeared in many areas such as Guangdong, Fujian, Zhejiang and other provinces of China with cumulative mortalities ranging from 0 to 90% (WHO, 2000).

The TSV, an unassigned species, is considered to be the member of the family Dicistroviridae (Mayo, 2002, 2005). TSV has been characterized to be a non-enveloped, 31 to 32 nm icosahedral, single stranded positive-sence RNA virus (Bonami et al., 1997). Its genome consists of 10,205 nucleotides with two open reading frames (ORF). ORF1 encodes a putative nonstructural protein of 24 KDa and ORF2 codes for three major capsid proteins of 55, 40 and 24 KDa called VP1, VP2 and VP3, respectively (Bonami et al., 1997; Mari et al., 2002). Currently, detections of TSV by PCR-based techniques have been widely used (Mari et al., 1998; Nunan et al., 1998). Quantitative real-time RT-PCR

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Abbreviations: TSV, Taura syndrome virus **PCR,** polymerase chain reaction; **RT-PCR,** reverse transcription polymerase chain reaction; **SDS-PAGE,** SDS polyacrylamide gel electrophoresis; **Mab,** monoclonal antibodies.

(Dhar et al., 2002; Mouillesseaux et al., 2003; Nunan et al., 2004; Tang et al., 2004) and other nucleic acid amplification methods have been developed with high specificity and high sensitivity for TSV detection (Kenneth et al., 1999; Teng et al., 2006, 2007; Kiatpathomchai et al., 2007). But these techniques are not practical for rapid detection because the detection system should be quick and easy to operate with high specificity and optimal sensitivity. In contrast, specific detection by immunoassay is an alternative choice for low cost screening of large numbers of samples, which has been the trend of clinic diagnostic in different parts of the world. Polyclonal antisera and monoclonal antibodies against TSV have been produced using purified viral antigens (Poulos et al., 1999). Polyclonal antibodies against recombinant proteins of VP1 and VP3 were developed (Chaivisuthangkura et al., 2006). Later, the monoclonal antibodies (Mabs) specific to the VP2 and VP3 capsid protein of TSV were developed (Chaivisuthangkura et al., 2010). In this study, we expressed and purified the recombinant fusion protein rVP2 and rVP3 from the local strain of TSV, and developed specific polyclonal antibodies and monoclonal antisera against rVP2 or rVP3 for sensitive immunodot-blot detection of infected shrimps.

MATERIALS AND METHODS

Viral preparation

Natural TSV infected *Penaeus vannamei*, as verified by RT-PCR (Nunan et al., 1998), were obtained from a farm at Pudong New Area of Shanghai in China. Gills from infected shrimp were homogenized in 2X PBS (phosphate buffered saline, pH 7.2), centrifuged at 3000 x*g* for 30 min and aliquots of the supernatant were stored at –70°C.

Chemicals, enzymes and bacterial strains

Enzymes (Taq DNA polymerase, restriction enzymes of BamH I, Sal I, Nde I and T4 DNA ligase) and IPTG (dioxane free) were purchased from TaKaRa (Dalian, China). The protein marker, 2×SDS Loading Buffer, Universal DNA Purification Kit, TIANprep Mini Plasmid Kit, the *Escherichia coli* (*E. coli*) DH5α competent cells and BL21(DE3) competent cells were purchased from TIANGEN BIOTECH(BEIJING, China). Micro Protein PAGE Recovery Kit was purchased from Sangon Bioengineering CO, Ltd. (Shanghai, China). Anti-GST Antibody, Anti-His Antibody, AP AffiniPure Goat Anti-Mouse IgG (H+L) and NBT/BCIP color reagent kit were purchased from Beyotime Institute Biotechnology (Jiangsu, China). Other materials were purchased from China National Medicines Corporation Ltd. Female BALB/c mice of four-week-old were purchased from Shanghai Lab Animal Resources Center.

TSV RNA preparation

Gills from naturally TSV infected *L. vannamei* were homogenized in lysis buffer (50 mM Tris-HCl, pH 9, 100 mM EDTA, 50 mM NaCl, 2% SDS.) and RNA was extracted from homogenate using TRNzol-A$^+$ Reagent (Tiangen Biotech). Subsequently, the extracted RNAs were stored at -30°C with 75% alcohol.

Designing of VP2 and VP3 primers

According to the complete genome sequences of TSV in GeneBank (NC_003008), the major structural proteins of the virus VP2 and VP3 gene primers were designed with the addition of restriction enzyme restriction sites at 5` and 3` end. The primers were synthesized by Sangon Bioengineering CO, Ltd. (Shanghai, China).

Synthesizing of VP2 and VP3 peptide sequences

Based on the full amino acid sequences of VP2 and VP3, amino acids sequences with best immunogenicity and antigen-specific were selected using the Kolaskar and Tongaonkar method (Peptide VP2: RFTHGSISYKIIPKNKGDLYC; Peptide VP3: KLESDFESKAPVKFTPGNYTC) and synthesized as immunogens for the production of monoclonal antibodies against VP2 or VP3 of TSV.

Reverse transcription polymerase chain reaction (RT-PCR) and cloning of VP2 and VP3

TSV-infected shrimp RNA was utilized as the template for cDNA synthesis using Reverse Transcriptase M-MLV (RNase H$^-$) and Oligo(dT)$_{12\text{-}18}$ primers in a 10 µl reaction volume. Conditions for reverse transcription were as follows: 70°C for 10 min and then rapidly quenched on ice for 2 min or more , 42°C for 1 h, followed by 70°C for 15 min cooling on ice. Polymerase chain reaction (PCR) was used with this cDNA as template, Pfx polymerase (Invitrogen) and primers VP2-F (5'-CGCGGATCCTTAGACAATCCCCTGTTTAGAAG-3') and VP2-R (5'-GGAATTCCATATGGCTAACCCAGTTGAAATTGAT-3') containing added restriction sites (underlined) to amplify the VP2 gene, primers VP3-F (5'-CGCGGATCCGCGGCTGGTCTGGACTACTCCAGC-3') and VP3-R (5'-ACGCGTCGACGTCAAGCCAATTCGGTCCAA-3'), with added restriction sites (underlined), to amplify the VP3 gene. PCR amplification was performed as followed: 94°C for 5 min, 35 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 1 min and a final elongation step of 72°C for 10 min for the two primer pairs. Amplified cDNAs were run in 1% agarose gel, and purified with the Universal DNA Purification Kit (Tiangen Biotech). The fragments of VP2 and VP3 gene obtained were stored at –30°C.

Expression of rVP2 and rVP3

The PCR product of VP2 gene was cloned into BamH I(F) and Nde I(R) sites of the pET-16b expression vector, and the PCR product of VP3 gene was cloned into BamH I and Sal I sites of the pGEX-4t-3 expression vector, and then transformed into *E. coli* strain BL21 (DE3) and DH5α, respectively. Colony PCR method was used for the screening of the positive recombinant clone. And the recombinant expression vectors were named as pET-16b-vp2 and pGEX-4t-3-vp3, respectively.

E. coli with each recombinant plasmid was cultured on Luria-Bertani (LB) both to exponential phase, and expression of the recombinant proteins was induced with 1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) for 4 h at 37°C. After centrifugation at 4000 ×*g* for 20 min for each recombinant, the bacterial pellet were washed in PBS (pH 8) and sonicated until a clear lysate was obtained. The lysate was separated by 10% SDS-PAGE. After staining with Coomassie brilliant blue R-250, the recombinant protein bands were cut out destained and eluted using the Micro Protein PAGE Recovery Kit (Sangon). The two proteins (GST-VP3 and His-VP2) elutions were adjusted to 4 mg/ml and stored at -70°C in small aliquots.

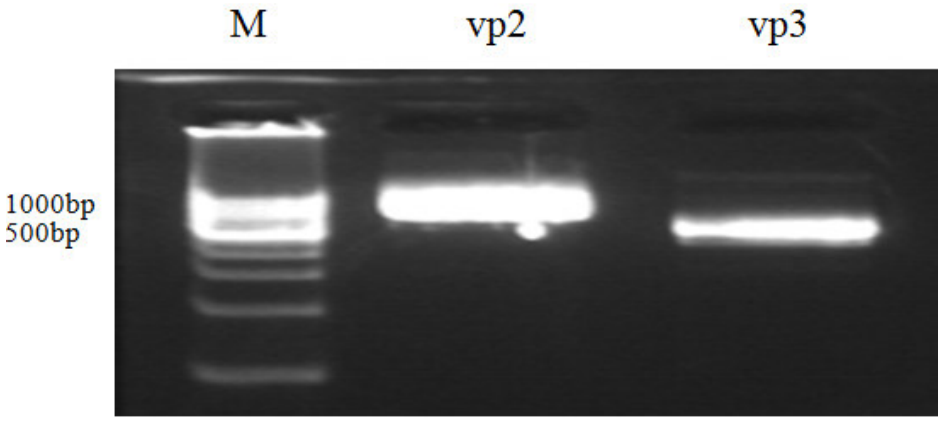

Figure 1. The 1% agarose gel electrophoresis of VP2 and VP3 gene fragment amplified from TSV genome by RT-PCR.

Preparation of polyclonal antibody

For each recombinant protein, 2 Balb/c mice were injected intra-peritoneally with 0.4 mg protein per mouse mixed with complete Freund's adjuvant in a 1:1 ratio. Mice were subsequently injected at 2-weekly intervals with the protein mixed with incomplete Freund's adjuvant, then mice were injected 2 more times in caudal vein at 1-weekly interval with the protein. One week after the 4th injection, mouse antisera were collected and tested against *E. coli* lysate and purified recombinant proteins by Western blot. The antibodies were also evaluated by immunodot blot using crude preparation of TSV.

Production of monoclonal antibodies

The immunization protocol was performed as described earlier. And the two kinds of immunogen were polypeptide VP2 and polypeptide VP3. Three days after the third booster, mice antisera were collected and tested against the polypeptide of VP2 and VP3 by ELISA. The final booster was given intravenously 3 days before fusion.

The splenic cells of BALB/c mice and the oncocyte were fused and screened in HAT culture medium. About three hybridoma cells were positive for polypeptide VP2 and two hybridoma cells were positive for polypeptide VP3 in the second round of screening by ELISA. And both hybridoma clones were selected and cloned to establish cell lines. The Mabs against VP2 and Mabs against VP3 were analyzed by both Western blotting and immunodot blotting.

Western blot analysis

Lysates of *E. coli* BL21 with pET-16b plasmid, *E. coli* DH5α with pGEX-4t-3 plasmid, *E. coli* with VP2- pET plasmid and *E. coli* with VP3-pGEX plasmid were separated by 10% SDS-PAGE. Samples were electrophoresed at 80 V for 20 min followed by 1 h at a constant 120 V and gels were stained with Coomassie brilliant blue. For Western blot analysis, samples resolved by SDS-PAGE were electroblotted onto nitrocellulose membranes and then incubated for 2 h in blocking solution (5% skim milk solution) at 37℃, followed by washing with PBS. Immunodetection was achieved by mouse anti-rVP2 or anti-rVP3 antiserum, Mabs against VP2 or VP3 as primary antibody at a 1:500 dilution for 4 h at 37℃. And the positive control was operated by using mouse anti-GST antibody (monoclonal antibody against GST epitope) and anti-His antibody (monoclonal antibody against His epitope) (Beyotime) as primary antibody at a 1:2500 dilution. After washing with 0.01 M PBS, the alkaline phosphatase conjugated goat anti-mouse IgG were added as secondary antibody at a 1:1000 dilution for 2 h. The membranes were washed three times with PBS after each incubation. The antigen-antibody complexes were detected with NBT/BCIP alkaline phosphatase color reagent kit (Beyotime) for coloration away from light for 15 to 30 min at room temperature.

Immunodot blot analysis

The antisera and Mabs obtained earlier were detected by immunodot blot. Firstly, homogenates from gills of TSV-infected *P. vannamei* were spotted onto nitrocellulose (NC) membranes and air-dried (5 µ/spot), then blocked with 5% bovine albumin for 1 h at 37℃, and washed in PBS with 0.05% Tween-20 (PBST). The NC membranes were incubated in mouse anti-rVP2 or anti-rVP3 antiserum, Mabs against VP2 or VP3 as primary antibody at a 1:500 dilution for 1 h at 37℃, and washed in PBS with 0.05% Tween-20 (PBST). Finally, the alkaline phosphatase conjugated goat anti-mouse IgG (1:500) were added for 1 h at 37℃, and rinsed with PBST. The alkaline phosphatase reaction was developed in a substrate solution of NBT-BCIP for about 15 min. The Mabs of VP2 or VP3 were performed as the same protocol. Homogenates from gills of TSV-uninfected *P. vannamei* was used as negative control.

RESULTS

Cloning of the VP2 and VP3 gene

To produce rVP2 and rVP3 in *E. coli*, RT-PCR was performed with the template of the total RNAs extracted from the infected shrimps, and specific bands were shown by 1% agarose gel electrophoresis, a 1000-bp PCR product of the VP2 gene and a 580-bp PCR product of the VP3 gene were obtained (Figure 1) which matched the experimental design of VP2 and VP3 gene size.

Expression of proteins and specificity of antisera

After IPTG induction, the supernatants and the

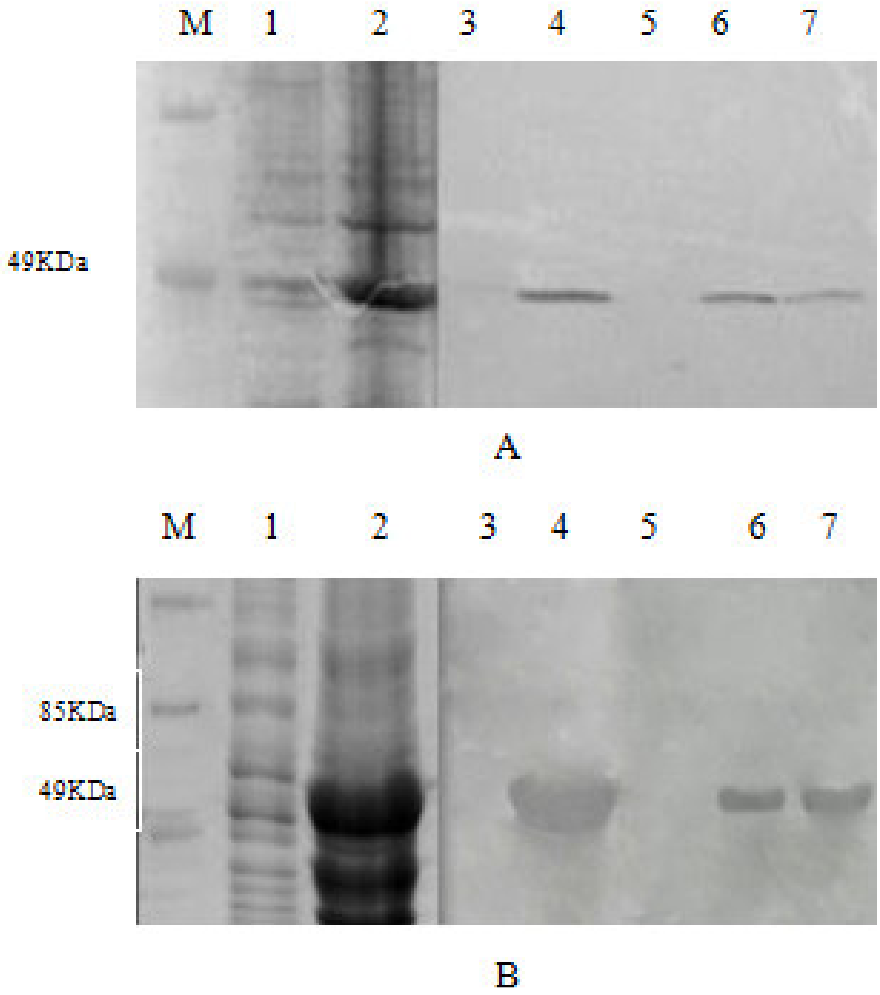

Figure 2. SDS-PAGE and Western blot analysis of the rVP2 (A) and rVP3 (B) expressed in *E.coli*. Lysate of uninduecd bacterial (lane 1) and lysate of induecd bacterial (lane 2) were electrophoresed and stained with Coomassie brilliant blue. Lysate of uninduced bacterial (lanes 3 and 5) and lysate of induecd bacterial (lanes 4, 6 and 7) were electrophoresed and transferred to a nitrocellulose membrane and incubated with Mab against VP2 (A, lane 4), Mab against VP3 (B, lane 4), anti-rVP2 antiserum (A, lane 7), anti-rVP3 antiserum (B, lane 7), anti-His antibody (A, lane 6), anti-GST antibody (B, lane 6).

precipitations of the bacteria of DH5α and BL21 were collected after sonication. According to the results of the 10% SDS-PAGE electrophoresis, the expression of the recombinant fusion protein His-VP2 and the recombinant fusion protein GST-VP3 were induced by IPTG. The recombinant protein was visualized as two bands with a molecular mass of 45 and 50 KDa by Coomassie blue staining of the gels (Figure 2A, lane 2; 2B, lane 2). Theoretically, the protein molecular weight of VP2 and VP3 capsid are 40 and 21.6 KDa. In addition, the expression of pET-16b vector starts from the T7 promoter to the end of the terminator, so the fusion peptide molecular weight is about 5 KDa, and the GST tag of pGEX-4t-3 can be expressed about in 26 KDa of the fusion peptide. Our results are consistent with expectations (the recombinant fusion protein His-VP2: 5 KDa + 40 KDa = 45 KDa, the recombinant fusion protein GST-VP3: 26KDa + 21.6KDa = 47.6KDa).

For Western blot analysis, the samples resolved by SDS-PAGE were electroblotted onto a nitrocellulose membrane. The results of Western blot analysis showed that the rVP2 antiserum and rVP3 antiserum displayed relatively strong immunoreactivity and specificity against rVP2 (45 KDa) and rVP3 (50 KDa) (Figure 2A, lane 7; 2B, lane 7). Anti-His Mabs and anti-GST Mabs were used as positive control (Figure 2A, lane 6); significant bands were shown by using Mab specific to VP2 and Mab specific VP3 as primary antibodies (Figure 2A, lane 4; 2B, lane 4). The special bands presented on the membrane are on the same position with the stained

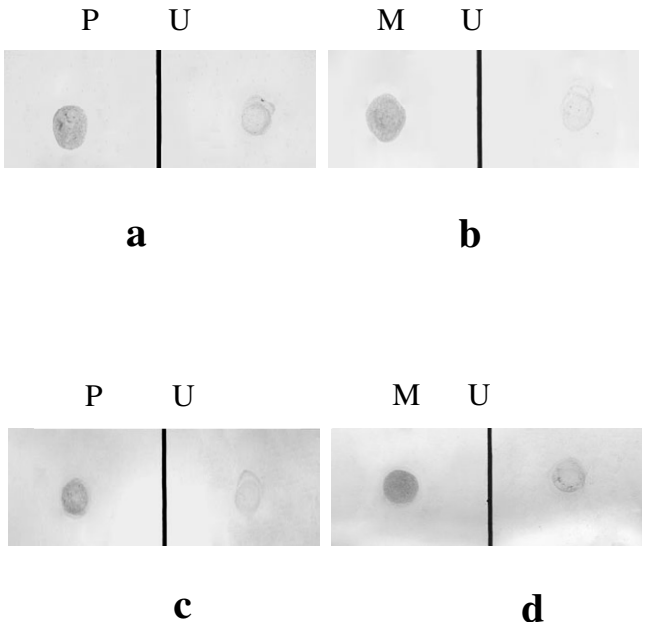

Figure 3. Immunodot blot analysis with the rVP2 antisera (a), rVP3 antisera (c), Mab specific to VP2 (b) and Mab specific to VP3 (d). Homogenates of gills from naturally TSV-infected *P. vannnamei* (aP, bM, cP and dM) or homogenates of gills from naturally uninfected *P. vannamei* (U). The samples were spotted onto nitrocellulose membrances (5 µl/spot) and then processed for dot blotting.

bands on the gels (Figure 2). Western blot analysis using Mab specific to VP2 and the antiserum specific to rVP2 revealed a single immunoreactive band for rVP2, likewise, only one band was presented on the nitrocellulose filter by Mab specific to VP3 and the antiserum specific to rVP3, which was similar to the results demonstrated in the previous study (Chaivisuthangkura et al., 2010).

Detection of TSV in shrimp infected naturally

Using the anti-rVP2 and anti-rVP3 antisera, Mabs against VP2 or VP3 for immunodot-blot, specific reactions were observed in homogenates of TSV-uninfected *P. vannamei* and crude preparation of TSV (Figure 3). The rVP2 antiserum as well as the Mab against VP2 reacted specifically with the homogenates of gills from naturally TSV-infected *P. vannnamei* (Figure 3a). Similarly, the rVP3 antiserum and Mab against VP3 both could bind to the homogenates of gills from naturally infected *P. vannnamei* (Figure 3c). These results confirm that the recombinant proteins demonstrated immunoreactivity similar to natural proteins of TSV.

Confirmation of multi-copy transformants

Recombinant plasmids were identified by restriction analysis of purified plasmid DNA and verified by DNA sequencing.

DISCUSSION

In this study, polyclonal antisera specific to the VP2 capsid protein or VP3 capsid protein of TSV were generated using the recombinant protein His-VP2 or GST-VP3 as an immunogen. Mab specific to VP2 and VP3 were produced by immunization with mice with synthetic peptides specific to VP2 or VP3 protein of TSV. The Mabs demonstrated a clear background in the dot blot assay, which indicated that they did not cross-react with uninfected shrimp tissues and had higher specificity than the polyclonal antisera specific to r VP2 or rVP3. Similarly, the Mabs recognized a single band corresponding to rVP2 or rVP3 in our Western blot analysis.

Since the polyclonal antisera and monoclonal antibodies (Mabs) against TSV had been produced using purified viral antigens in 1999 (Poulos et al., 1999), more efforts have been made on designing immune-detection method for TSV by researchers in different regions. For example, the Mab specific to VP1 (Designated 1A1) (Mari et al., 2002; Robles-Sikisaka et al., 2002) was developed and used for Western blot, dot blot and immunohistochemical assays (Robles-Sikisaka et al., 2002; Erickson et al., 2002, 2005). But the 1A1 Mabs

cannot react with isolates of TSV from different sources like Mexico, Nicaragua and Belize. It was demonstrated that the 1A1 Mab showed less sensitive than the ISH (*in situ* hybridization) method (Poulos et al., 2008). In 2006, polyclonal antisera against rVP1 and rVP3 were developed (Chaivisuthangkura et al., 2006) for immunoassays. Later, Mabs against capsid proteins of TSV, including VP1, VP2 and VP3 (Chaivisuthangkura et al., 2006, 2010; Longyant et al., 2008) were produced and developed with high specificity and easy for TSV detection. Consistent with these studies, Mab specific to the recombinant GST-VP3 or VP2 capsid protein of TSV demonstrated specificity and did not cross-react with uninfected shrimp tissues or other shrimp viruses. Since VP2 Mabs had shown higher sensitive than the VP3 Mabs (Longyant et al., 2008, Chaivisuthangkura et al., 2010), we believe that combination of Mabs against VP2 and VP3 will be a better choice in designing immunoassays.

The two capsid proteins VP1 and VP2 sequence comparison among 20 TSV isolates collected from America, Taiwan, Mexico and Nicaragua showed that the differences of VP1 gene is 0 to 2.4% and VP2 gene is 0 to 3.5% (Robles-sikisaka et al., 2002). By amino acid sequence, comparison of the structural proteins of TSV, VP2 (CP1) and VP3 (CP3) showed closer similarity, while VP1 (CP2) demonstrated the least similarity (Mari et al., 2002). Amino acid sequence comparison of VP2 from two Thai TSV isolates revealed a 1.5% difference with isolates obtained from Hawaii and South America (Srisuvan et al., 2005). At least, four genotypic variants (Americas group, the South-East Asia group, the Belize group and the Venezuelan group) have been isolated and identified based on the sequence of the capsid protein VP1 (Chang et al., 2004; Erickson et al., 2002, 2005; Nielsen et al., 2005; Tang and Lightner, 2005). Therefore, it is likely that the antiserum specific to VP2 in combination with the antiserum specific to VP3 in this study may be useful for the detection of most TSV isolates from various geographical regions. And using the combination of the two kinds of Mabs can increase the sensitivity of TSV detection and confirm TSV infection in *L. vannamei* for dot blotting and immunohistochemistry (Chaivisuthangkura et al., 2010). The anti-rVP2 and anti-rVP3 antiserum in this study presented a single immunoreactive band on the nitrocellulose membrane for rVP2 and rVP3 which demonstrated high specificity to the fusion protein. Likewise, Mabs against VP2 or VP3 did bind to the recombinant proteins and both recognized a single band. Furthermore, based on the result of immunodot blot analysis, both the polyclonal and monoclonal antibodies did bind strongly to the tissues of TSV-infected shrimp and had high sensitivity. Therefore, the combination of the two antisera as well as the two Mabs obtained would be expected to increase the sensitivity of TSV detection.

In summary, the Mabs against VP2 or VP3 peptide and polyantisera specific to these recombinant capsid proteins could be used for specific detection of TSV infection in shrimps by immunodot blotting. The stated results would provide premise basis for the development of immuno-assay kit for TSV antigen detection. For further study, we will be interested in testing the possibility of using the recombinant capsid proteins as oral vaccine against TSV infection besides the optimization of our immune-dot blot analysis.

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