SHORT REPORT
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Production of Polyclonal Antibodies against Recombinant VP26 Structural Protein of White Spot Syndrome Virus (WSSV)

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ABSTRACT: A portion of the VP26 gene (VP26F109) encoding a structural protein of white spot syndrome virus was cloned into an expression vector and transformed into *E. coli*. The objective was to produce a truncated VP26 structural protein lacking the N-terminal transmembrane region. After induction, the recombinant protein rVP26F109 was produced, purified by SDS-PAGE and used to immunize Swiss mice for polyclonal antibody production. The mouse anti VP26 antiserum demonstrated specific immunoreactivity to viral antigen in white spot syndrome virus (WSSV) infected *Penaeus monodon*, as verified by immunohistochemistry and western blot. This constitutes the first step in producing monoclonal antibodies against rVP26F109 that can be combined with anti-VP28 monoclonal antibodies to enhance the sensitivity in WSSV immunological assays.

KEYWORDS: immunohistochemistry, polyclonal antibody, VP26, Western blot, WSSV.

INTRODUCTION

White spot syndrome virus (WSSV) is one of the most virulent pathogens that causes major losses in shrimp farming. Genome based diagnostic methods, such as *in situ* hybridization[1], PCR[2] and real time PCR[3] have been developed for WSSV detection. Immunological based diagnostic methods using polyclonal antibodies[4] and monoclonal antibodies specific to the VP28 envelope protein have also been developed.[5-8] However, in most cases the detection limit of the immunodiagnostic methods is inferior to that of PCR. This study aimed to clone a structural protein gene, VP26 in order to produce antigen and then antibody. The antibody against VP 26 was expected to be used in combination with an antibody specific to VP28 in order to improve the sensitivity of various immunoassays for WSSV.

MATERIALS AND METHODS

Viral Preparation

Natural white spot syndrome virus (WSSV) infected *P. monodon* was obtained from a farm at Nakhon Srithamarat Province, Thailand. Gills from the infected shrimp were homogenized in 2X PBS (phosphate buffered saline, pH 7.2), then centrifuged 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, 100 mM EDTA, 50 mM NaCl, 2% SDS; Timothy Flegel personal communication). DNA from 200 µl of the the homogenate was prepared using a High pure viral nucleic acid kit from Roche Molecular Biochemicals as described in the product manual.

Cloning and Expression of Truncated VP26

Primers, VP26F109 (5'- CG GGA TCC CGT GTT GGA AGA AGC GTC GTC-3'; 109 nucleotides downstream of the ATG start site) and VP26RPST (5'T GCA CTG CAG TTA CTT CTT GAT TTC G-3') with added restriction sites (underlined) were used to amplify a truncated VP26 gene by polymerase chain reaction (PCR) using *pfx* polymerase (GIBGO BRL). The PCR product 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 VP26F109

E. coli with pQE30-VP26F109 plasmid was cultured in LB broth to the 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 4,000 g 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 with a 15% gel. After staining with Coomassie brilliant blue, the recombinant protein bands of rVP26F109 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) at 70 V for 6 h. The protein solution was dialysed to eliminate SDS and salt before determining the protein content by Bradford protein assay.[9] The protein solution was divided into small aliquots and stored at −70°C.

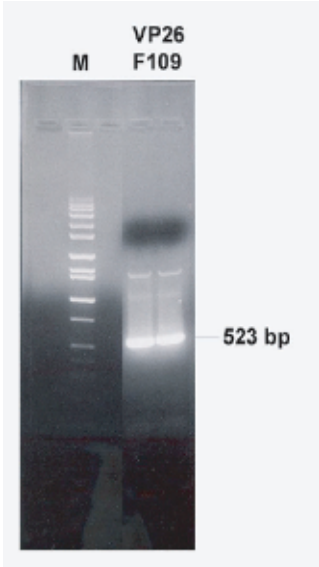

Fig 1. Ethidium bromide stained gel of VP26F109 PCR product. M=DNA marker.

Polyclonal Antibody Production

Three Swiss mice were injected intra-peritoneally with purified rVP26F109 (0.05 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 two weekly intervals. One week after the fourth injection, mouse antisera were collected and tested against *E. coli* lysate, purified rVP26F109 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.

Western Blot Analysis

Lysate of *E. coli* M15 (pREP4) with pQE30 or with pQE30-VP26F109, purified rVP26F109 and gill extract from WSSV infected *P. monodon* were separated by 15% SDS-PAGE according to method described by Laemmli.[10] Samples were electrophoresed for 6 h at 30 V and gels were stained using Coomassie brilliant blue. For western blot analysis , samples resolved by SDS-PAGE were electroblotted onto nitrocellulose membranes using a Transblot apparatus (BioRad) then incubated for 4 h with mouse anti rVP26F109 antiserum at dilution of 1:3000 in 5% Blotto (5% nonfat dry milk, 0.1% Triton X-100 in PBS). 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 in 5% Blotto for 4 h. The membranes were then washed extensively as before and incubated for 5 min in a substrate mixture containing 0.006% hydrogen peroxide, 0.03% diaminobenzidine (DAB), and 0.05% cobalt chloride in PBS and washed extensively in distilled water. Immunoreactive protein appeared as dark gray bands. The membrane was also reprobed with W29 monoclonal antibody (specific to the VP28 enveloped protein) obtained from previous work[8] for comparison with the immunoreactivity from the antiserum.

Immunohistochemistry

Cephalothoraces from WSSV infected *P. monodon* 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 mouse anti-rVP26F109 antiserum at 1:1000 dilution and GAM-HRP at 1:1000 dilution in 10% calf serum in PBS for 5 h at 37 °C for each step. After extensive washing with PBS, peroxidase activity was revealed by incubation with 0.03% DAB, 0.006 % hydrogen peroxide in PBS for 5 min. Preparations were counterstained with haematoxylin and eosinY, dehydrated in a graded ethanol series, cleared in xylene and mounted in Permount.[11] Positive reactions were visualized as brown coloration against the pink and purple colors of haematoxylin and eosin. A nearby section was also treated with W29 monoclonal antibody in the same fashion for comparison.

Results and Discussion

The truncated VP26 gene, VP26F109, of WSSV structural protein could be amplified as a 523 bp PCR product (Fig 1.). This was cloned, expressed in *E. coli*, and visualized by Coomassie blue staining as a band

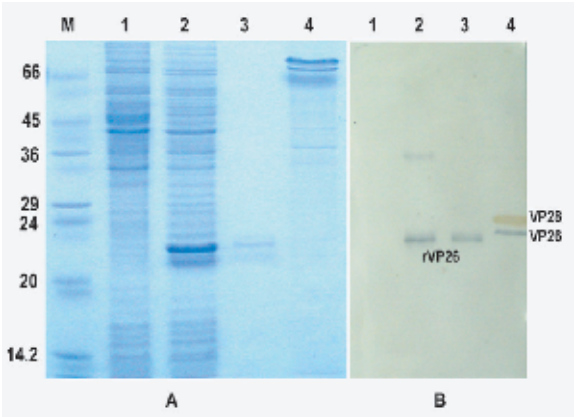

Fig 2. SDS-PAGE and Western blot analyses. (A) Coomassie blue stained gel, (B) Western blot using polyclonal antibody against rVP26F109 (lane 1-4) and reprobed with W29 monoclonal antibody specific to VP28 (lane 4, upper band). M = protein marker, 1 = lysate of *E. coli* strain M15 (pREP4) containing pQE30, 2= lysate of *E. coli* strain M15 (pREP4) containing pQE30-VP26F109, 3 = purified rVP26F109 protein, 4 = gill homogenate of *P. monodon* infected with WSSV.

with molecular mass of ~23 kDa that was slightly smaller than natural VP26 (Fig 2A lane 2). After this band was cut and eluted, high purity rVP26F109 was obtained (Fig 2A lane 3) and adjusted to 1 mg/ml protein before storage in small aliquots. The truncated VP26 structural protein was in the form of a recombinant fusion protein with a 6-histidine tag at the N-terminus.

After immunization of Swiss mice with rVP26F109 protein, antisera obtained from 3 mice displayed very strong immunoreactivities and specificities on western blot to purified rVP26F109 protein and lysate of *E. coli* containing VP26F109-pQE30 (Fig 2B lane 2 and 3) but not to the lysate of *E. coli* containing only pQE30 (Fig 2B lane 1). Immunoreactivities against VP26 (Fig 2B lane 4, lower band) and VP28 (Fig 2B lane 4, upper band) were observed in gill homogenates from *P. monodon* infected with WSSV. By immunohistochemistry, the immunoreactivity of anti-VP26 antibody occurred in a similar pattern to that of W29 monoclonal antibody specific to VP28 of WSSV (Fig 3). Therefore, this result confirmed that the rVP26 protein obtained from transformed *E. coli* shared similar epitopes with natural VP26 of WSSV.

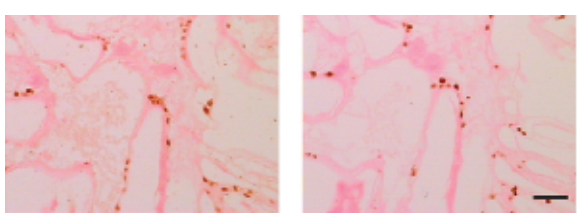

Fig 3. Immunohistochemistry of gill tissues from WSSV infected *P. monodon* using polyclonal antibody against rVP26F109 (A) and W29 monoclonal antibody specific to VP28 (B). Bar = 50 μm.

In western blot analysis, the content of VP26 structural protein in WSSV appeared to be slightly less than that of VP28. However, the tissue immunoreactivity demonstrated by the antibody against VP26 was comparable to that of the monoclonal antibody specific to VP28, as revealed by immunohistochemistry (Fig 3). Therefore, the antibody against VP26 could be used well for localization of WSSV infection in tissues similarly to antibodies specific for VP28. Hydrophobicity analysis of the VP26 protein demonstrated that there was a strong hydrophobic region at the N terminus suggesting that it is a membranous protein. Immunogold electron microscopy revealed that VP26 is an envelope protein.[12] Therefore, the combination of antibodies against VP26 and VP28 should be able to enhance the sensitivity for detection of WSSV. Recently, recombinant VP28 and VP19 were applied as injected [13] or oral [14] protectants against WSSV. The recombinant VP26 may be potentially applied in the same fashion for additional efficacy in protection against WSSV.

Acknowledgements

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