

Membrane Binding Activity with Virus in White Spot Syndrome Virus-Infected *Fenneropenaeus chinensis*

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Abstract: Problem Statement: White Spot Syndrome Virus (WSSV) is one of the most disastrous pathogens in shrimp culture, having caused high mortality in many cultured shrimp species. It is well known that the most important step of viral infection is cell attachment. **Approach:** Therefore determining the binding activity of shrimp cells to WSSV is an important to evaluate anti-infection ability and understanding the procedure of a viral infection. So this study aims to analyze binding activity of cell membrane with WSSV in shrimp of *Fenneropenaeus chinensis* (*F. chinensis*). WSSV was used to stimulate *F. chinensis* and binding activity in gills, muscle and hepatopancreas with virus within the procession of WSSV was measured. **Results:** The results showed that binding activity in gills with WSSV within infection stage (0-96 h) had no significant change. The highest binding activity was attained after infection 24-48 h in muscle. The binding activity in hepatopancreas with WSSV was lower during infection stage (0-96 h). **Conclusion/Recommendation:** The higher binding activity of WSSV to gills and muscle indicated that gills and muscle are the importance target tissue for WSSV infection. This suggested that receptor of WSSV existed in gills and muscle.

Key words: White spot syndrome virus, *Fenneropenaeus chinensis*, binding activity, Infection

INTRODUCTION

White Spot Syndrome Virus (WSSV) is one of the most disastrous pathogens in shrimp culture, having caused high mortality in many cultured shrimp species^[1]. White spot syndrome virus-positive cells occur initially in the stomach, gill, lymphoid organ, cuticular epidermis and hepatopancreas and spread rapidly to other organs during a WSSV infection^[2,3]. The immune corrected factor and enzyme activity infected by WSSV were evaluated^[4,5].

It is well known that the most important step of viral infection is cell attachment. Therefore determining the binding activity of shrimp cells to WSSV is an important to evaluate anti-infection ability and understanding the procedure of a viral infection. Many previous study mainly focused on physiological response of prawns such as total protein content, haemocyte count, phenoloxidase (PO) activity, phagocytic index, release of reactive oxygen intermediates and antibacterial activity, superoxide dismutase (SOD), alkaline phosphatase (ALP), acid phosphatase (ACP) activities^[6,7]. However, cell membrane binding activity with WSSV could be

another criterion to be considered when selecting breed. In shrimp, no data is available on binding activity in relation to WSSV. The present study aims to analyze binding activity of cell membrane with WSSV in shrimp of *Fenneropenaeus chinensis*.

MATERIALS AND METHODS

Shrimp and challenge experiments: Experimental Chinese shrimp (*Penaeus chinensis*), body length 12.32 ± 1.23 cm ($n = 60$) and body weight 16.33 ± 4.40 g were obtained from a shrimp farm and held in tanks for 10 d prior to challenge experiments. WSSV was injected into the second abdominal segment. Muscle tissue, gills, hepatopancreatic tissue and hemolymph of 6 individual shrimp from both the control group (injected 0.9% NaCl) and the challenge group were collected at various times (from 0-72 h) after challenge. Haemolymph was withdrawn from the ventral sinus of the first abdominal segment into an equal volume of anticoagulant (27 mmol mL^{-1} sodium citrate, 336 mmol mL^{-1} NaCl, 115 mmol mL^{-1} glucose, 9 mmol mL^{-1} EDTA, pH 7.0), while muscle tissue and gills were harvested by dissection.

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Virus preparation: Tissues (1 g) from *P. chinensis* confirmed for WSSV infection by DNA dot-blot assay were homogenized in 5 mL TE buffer (20 mM Tris-HCl and 400 mM NaCl, pH 7.4). After centrifugation at 2000×g, the supernatant was diluted to 1:100 with 0.9% NaCl and filtered (0.22 mm filter). This crude WSSV extract solution was stored at -70°C as the challenge inoculum.

DNA purification: Shrimp gill or muscle tissue (100 mg) was placed in a 1 mL Eppendorf tube with 300 µL TE buffer (10 M Tris-HCL, 1 M EDTA, pH 8.0) and homogenized using a sterile stick. After boiling for 10 min, the homogenates were clarified by centrifugation at 130 g for 10 min at 4°C. Nucleic acids were flocculated by adding 500 µL 100% ethanol and pelleted by centrifugation at 10,000 g for 10 min at 4°C. The pellets were then washed with 500 µL 70% ethanol and re-pelleted by 10 min centrifugation at 10,000 g at 4°C. Then pellets were dissolved in 80 µL of TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) and kept at -20°C.

PCR amplification: A primer set for WSSV was designed (W1: 5'-TATTGTCTCTCCTGACGTAC-3, W2: 5'-CACATTCTTCACGAGTCTAC-3') based on the WSSV genomic sequence which generated a 300-bp PCR product. PCR reactions were carried out in a 20 µL reaction mixture containing 2.5 µL 10×PCR buffer, 1.5 µL 25 mM MgCL₂, 0.5 µL 10 mM of each of four nucleotides, 2 µL of 10 mM of the two primers, 0.5 µL Tag DNA polymerase (Promega, France), 13.5 µL diethyl pyrocarbonate (DEPC)-treated water and 1 µL of the template DNA. PCR conditions were as follows: 95°C for 5 min followed by 30 cycles of 95°C for 40 s, 55°C for 40 s and 72°C for 1 min with a final extension for 2 min at 72°C. PCR reaction products (5 µL) were mixed with 1 µL of gel buffer and run on 1% agarose gels, followed by UV examination in Genefinder™.

SDS-PAGE analysis: The procedure for discontinuous, denaturing gel electrophoresis was carried out in tris-glycine buffer according to the method of Laemmli (1970). The gels were stained with Coomassie Brilliant Blue R-250 (CBB) and included a premixed molecular weight marker with proteins ranging from 14.4-97.4 kDa.

Extraction of membrane proteins in gills, muscle and hepatopancreas: At various stages post injection challenge (i.e., 0, 12, 24, 48 and 72 h), gills, muscle and hepatopancreas of shrimp (n = 6) were minced in 250 mM sucrose, 10 mM Hepes, 1 mM EDTA, 0.1 mM PMSF, pH 7.2 homogenized and centrifuged at 600 and

8,000 g for 10 min respectively. The supernatants were centrifuged at 100,000 g for 20 min in order to sediment the membrane proteins. Then the sediment was re-suspended in PBS. All subsequent steps were carried out at 0-4°C. The protein concentration was estimated by measuring the Optical Densities (OD) at 280 nm. The purity of the membrane proteins was evaluated by negative-staining transmission electron microscopy.

Virus production, purification and DIG-labeled: The virus used in this study was isolated from infected *Penaeus monodon* shrimps from China. Infected tissue was homogenized in Penaeid Physiological Buffer (PPB). The homogenates were centrifuged at 7000 rpm min⁻¹ (RP65T-856, Hitachi) for 20 min at 4°C, the supernatant was recentrifuged at 25000 rpm min⁻¹ (RP65T-856, Hitachi) for 60 min at 4°C and sediment was re-suspended in PPB (contain 35% sucrose). After centrifugation at 7000 rpm min⁻¹ for 10 min, the supernatant was over layered onto the top of a 35%-62% (W/W) (RPS65T-704, Hitachi) continuous sucrose gradient and centrifuged at 36000 rpm min⁻¹ for 3 h at 4°C. After centrifugation, viral band was visualized by top illumination and was removed using a pipette. The viral fraction was diluted in PPB and centrifugation at 25000 rpm min⁻¹ for 1h at 4°C. The pellet was then re-suspended in PBS. Then about 20 times membrane protein was mixed with Digoxigenin (DIG) (20 mg dissolved in 1 mL DMSO) and redundant DIG was removed through Sephadex G-50 column.

Binding assay of extraction membrane protein with WSSV by ELISA: A 10 µg mL⁻¹ solution of extraction membrane protein of gills, muscle and hepatopancreas was prepared. The polystyrene 96-well plate was covered with 100 µL extract membrane protein solution (diluted 1:15 with PBS) as the final volume in each well and incubated overnight at 4°C. Antigen solution was removed and the wells were washed three times with PBS. To each well was added 250 µL MPBS (5% Non-fat milk + PBS) and the plates were incubated for 2 h at room temperature. After washing the wells with PBS twice, 100 µL DIG-labeled WSSV was added diluted with MPBST (Tween 20, 0.1% (w/v)) to each well for 2 h at room temperature. Then the wells were washed with PBST and PBS five times respectively. Anti-DIG-Fab-HRP diluted in MPBS (1:3000) was added at room temperature for 1 h. The wells were washed with PBST and then PBS for five times. The substrate of 75 µL OPD was added to each well and incubated at 37°C until color appeared. The reaction was stopped by the addition of 50 µL of 1 M H₂SO₄ to each well. The

absorbance at 492 nm was read with 850 nm as reference. BSA ($10 \mu\text{g mL}^{-1}$) was as a control.

RESULTS

Detection of shrimp infection: Following injection with WSSV, the first shrimp mortality was recorded at 24 h post-injection (p.i.). The presence of viral genomic DNA was determined by PCR in living shrimp sampled after injection. The expected band (i.e., at 300 bp) for WSSV infection (i.e., a positive result) was observed with the viral inoculum and with WSSV-injected shrimp at 12 h in gills and muscle (Fig. 1 and 2). The band was never obtained with the control shrimp.

Extraction cell membrane and SDS-PAGE analysis: Extraction membrane protein was negative stained and observed by electron micrograph. Membrane proteins in gills showed typical vacuolar. Membrane proteins in muscle were homogenous and larger membrane proteins were showed in hepatopancreas. Microscopy analysis indicated higher purity of extraction membrane proteins in gills, muscle and hepatopancreas (Fig. 3).

SDS-PAGE analysis of cell membrane in gills, muscle and hepatopancreas: SDS-PAGE of the membrane protein stained with coomassie blue revealed the presence of several proteins in gills, muscle and hepatohesis with a wide range of molecular masses.

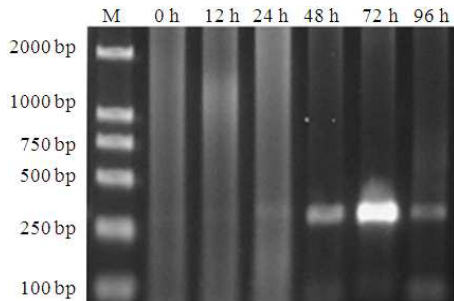


Fig. 1: Detection of WSSV genome by PCR in gills

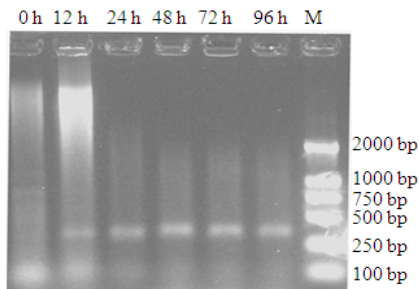


Fig. 2: Detection of WSSV genome by PCR in muscle. Protein bands in gills with apparent molecular mass of 171 kD (band 1), 157 kD (band 2), 145 kD (band 3), 139 kD (band 4), 122 kD (band 5), 101 kD (band 6), 86 kD (band 7), 78 kD (band 8), 65 kD (band 9), 60 kD (band 10), 50 kD (band 11), 42 kD (band 12), 39 kD (band 13), 37 kD (band 14), 33 kD (band 15), 31 kD (band 16) were present (Fig. 4). The main protein bands in muscle were 165 kD (band 1), 157 kD (band 2), 152 kD (band 3), 139 kD (band 4), 101 kD (band 5), 93 kD (band 6), 86 kD (band 7), 78 kD (band 8), 66 kD (band 9), 58 kD (band 10), 51 kD (band 11), 48 kD (band 12), 44 kD (band 13), 39 kD (band 14), 36 kD (band 15), 34 kD (band 16), 32 kD (band 17), 31 kD (band 18), 29 kD (band 19) (Fig. 4). Protein bands of 170 kD (band 1), 152 kD (band 2), 139 kD (band 3), 120 kD (band 4), 88 kD (band 5), 65.5 kD (band 6), 58 kD (band 7), 45 kD (band 8), 35 kD (band 9), 30 kD (band 10), 29.3 kD (band 11) were present in hepatopancreas (Fig. 4).

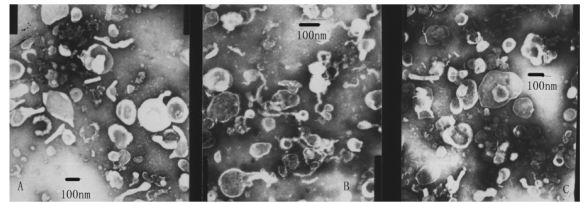
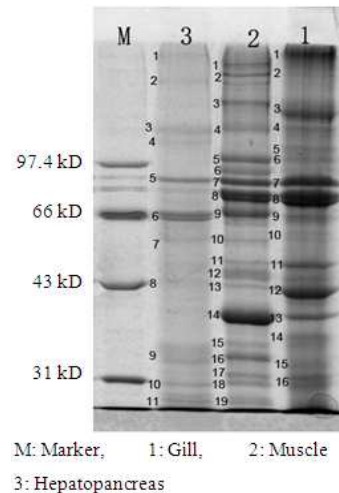


Fig. 3: Ultra-structure of membrane fragments from gills, muscle and hepatohesis. Outer membranes were isolated in materials and methods. The membrane were concentrated by ultracentrifugation. (A): Gills; (B): Muscle; (C): Hepatohesis, The bar represents 100 nm



M: Marker, 1: Gill, 2: Muscle, 3: Hepatopancreas

Fig. 4: SDS-PAGE analysis of membrane protein in gills, muscle and hepatopancreas of *P. chinensis*

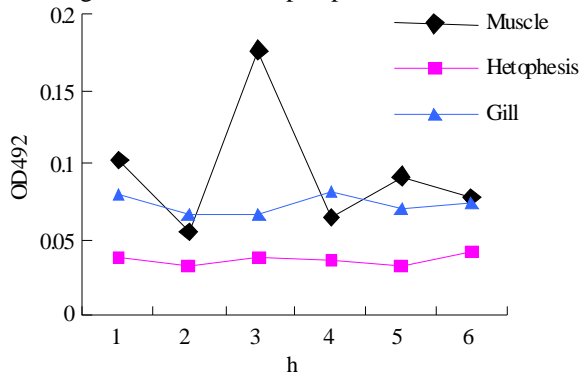


Fig. 5: The binding activity of gill, muscle and hepatopancreas with Dig-labeled WSSV (1, 2, 3, 4, 5, 6 in X axis indicated infection hours of 0, 12, 24, 48, 72 and 96 h)

Binding activity of cell membrane in different tissues with WSSV: The ability of WSSV adhere to cell membrane of gills, muscle and hepatopancreas was evaluated. Cell membrane from gills, muscle showed a higher level of adhesion activity than cell membrane from hepatopancreas. There was no significant change of WSSV binding activity with cell membrane in gills during the stage of WSSV infection (0-96 h). The highest binding activity was attained in cell membrane of muscle after 24 h infection. WSSV binding to cell membrane of hepatopancreas was kept at low level during whole infection stage (0-96 h) (Fig. 5).

DISCUSSION

WSSV is the most devastating viral pathogen of cultured Penaeid shrimp (*Penaeus* spp.) worldwide^[8,9]. The virus has a wide host range among crustaceans and infects all commercially important species of Penaeid shrimp^[8].

Receptor binding is only the first step in the infection process. The virus, or the virus genome alone, then has to enter the cell, a process that requires translocation of the genome or a sub-viral particle across the membrane into the cytoplasm and in some cases, into the nucleus. Unlike plant viruses, most animal and bacterial viruses attach to specific cellular receptors that, in part determine host range and tissue tropism. Some viruses recognize very specific molecules (e.g., adhesion molecule) whereas other viruses recognize widely distributed chemical groups. The tissue distribution of the receptor will in part determine the tropism of the virus and hence, the symptoms of the infection. Similarly, species

differences between receptor molecules can limit host range.

An interesting observation was that WSSV bound well to gills and muscle. This also indicates that WSSV adhesion may be in specificity tissue. The binding to gills and muscle appeared to be specific since the binding of WSSV to BSA was significantly less (data not shown). The higher binding activity of WSSV to gills and muscle indicated that gills and muscle are the importance target tissue for WSSV infection.

This study indicates that adhesion does indeed correlate to some extent with the presence of receptor. The present data make it clear that adhesion depend on specific tissue. It seems reasonable to deduced that binding activity was corrected with receptor. In view of this study, it is tempting to speculate receptor is of saturated. This research was initiated to test a working hypothesis that the adhesion of WSSV to shrimp cell was the result of the specific interaction between the virus adhesion and a receptor on the gills and muscle surface.

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