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Full Length Research Paper

Characterization of a tailless white spot syndrome virus from diseased *Penaeus vannamei* and *Procambarus clarkii* in China

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In this study, a virus similar to the causative agent of white spot syndrome virus (WSSV) but without tail-like extension was identified and characterized from diseased *Penaeus vannamei* and moribund *Procambarus clarkia.* Contrary to previous reports, white spots were not observed on the carapace of the diseased *P. vannamei* but with ulceration on the carapace and red tail. All samples were analyzed for WSSV and *Sporoplasma eriocheiris* using polymerase chain reaction (PCR) methods. Samples were negative for *S. eriocheiris* but positive for WSSV. Following the World Organization for Animal Health (OIE) standard protocol, the result of nest-PCR showed a characteristic band of 1447 bp, suggesting that the pathogen of *P. vannamei* and *P. clarkii* was similar to the causative agent of WSSV. Interestingly, transmission electron microscopy of sectioned tissues and negatively stained samples revealed an elliptical shaped virus-like particle but without tail-like extension, which was different from previous reported cases of WSSV. The major envelope proteins, VP19, VP26 and VP28 were cloned and sequenced. Results show that the present isolates had over 97% DNA and 100% amino acid sequence similarity to the known WSSV. These results suggest that this tail less virus may be a different strain of the WSSV virus exhibiting a different disease sign but equally virulent. Results of this study broaden our understanding of WSSV symptoms and diversity.

Key words: White spot syndrome virus (WSSV), morphology, crustacean.

INTRODUCTION

As the only representative of a new virus group (family Nimaviridae; genus Whispovirus), white spot syndrome virus (WSSV) is the most pathogenic among the penaeid shrimp viruses (Vlak et al., 2006). The virus exists in an extremely wide range of potential hosts, including shrimps and other crustaceans such as crabs and crayfish (Wang et al., 1998), and has caused catastrophic appendages (Durand et al., 1997; Wang et al., 1995). The sizes of the WSSV genome reported for three isolates were contradictory (GenBank accession nos: AF332093, AF369029 and AF440570). It has a double-stranded circular DNA with size approximately 300 kb based on complete genome analysis (van Hulten et al., 2001a; Yang, 2001). WSSV consist of at least 5 predominant proteins with estimated sizes of 28, 26, 24, 19 and 15 kDa. Among the viral structural proteins, envelope proteins often play an important role in virus entry and assembly (Chazal and Gerlier, 2003). VP28 as an envelope protein was previously reported to be involved in the systemic infection of shrimp. More than 40 WSSV proteins had been identified, now, the envelope protein of WSSV has become the focus of numerous studies (Seok et al., 2004; Tang and Hew, 2007; Tsai et

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Abbreviation: WSSV, White spot syndrome virus.

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al., 2004a; van Hulten et al., 2001b; Wu et al., 2005; Xu et al., 2009).

Although, several researches have been done in characterizing the virus, information of the host genes involved in WSSV pathogenesis is limited. To identify the interactions between WSSV and their hosts, a variety of approaches have been used, including mRNA differential display technique (Astrofsky et al., 2002), differential hybridization (He et al., 2005), cDNA microarrays (Dhar et al., 2003; Wang et al., 2006), suppression subtractive hybridization (SSH) (Pan et al., 2005) and expressed sequence tags (Rojtinnakorn et al., 2002). *Penaeus vannamei* and *Procambarus clarkii* are valuable aquatic animals and they remain vulnerable to outbreaks of various viral diseases, especially WSSV. In this study, the diseased shrimps and crayfishes were sampled from the different ponds where epidemic disease occurred. The pathogens of WSSV and spiroplasma were detected with transmission electron microscopy and molecular biology methods, which were primary pathogens of *P. vannamei* and *P. clarkii* in recent years (Nunan et al., 2005). Furthermore, the major viral structure protein genes of WSSV from these crustaceans were cloned and analyzed.

MATERIALS AND METHODS

Shrimp and crayfish sampling

Samples were collected from aquaculture farms that had experienced white spot syndrome or *Spiroplasma eriocheiris* infection in recent years. High shrimp and crayfish mortality were observed in the region at the time the samples were collected. Twenty-four diseased *P. vannamei* with ulceration on the carapace and red tail were collected from Gaoyou; while twenty-four moribund *P. clarkii* were taken from Baoying and Yancheng (Jiangsu Province, China). A portion of the samples (four shrimps and four crayfishes) were processed for negative staining and electron microscopy; and the remaining samples were stored at -20°C for further study.

Polymerase chain reaction (PCR)

DNA extraction

WSSV DNA samples for PCR test were prepared from diseased shrimp and crayfish by extraction from muscle tissues following the modified method (Miller et al., 1988; Tsai et al., 2002). Briefly, muscle tissues to be extracted were dissected and dispersed in a Potter-Elvehjem tissue grinder at 8000 rpm with 10 × TN buffer (50 mM Tris-HCL, (pH 7.6), 0.4 M NaCl) on ice, then centrifuged at 8000 g for 6 min at 4°C, after that the supernatant was placed in other centrifuge tubes together with an appropriate amount of DS buffer (0.1 mg/ml proteinase K, 50 mM KCl, 10 mM Tris-HCl, (pH 8.3), 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween-20). After incubation at 99°C for 10 min, it was ice bathed for 5 min immediately, with 13000 g for 4 min at 4°C, supernate was the WSSV DNA samples and it was stored at -20°C. The DNA for *S. eriocheiris*, another important novel pathogen on shrimp and crayfish, was prepared following the chelex-100 method (Ding et al., 2007).

DNA amplification

Using the World Organization for Animal Health (OIE) WSSV protocol, nested PCR with the outlier primer (146F1 and 146R1) and the inner primer (146F2 and 146R2) were employed (Table 1). For the first-step of PCR reaction, 1 µl DNA template solution was added to a 0.2 ml PCR tube containing 100 µl of reaction mixture including 10 mM Tris/HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 µM of dNTP, 100 pmol of each outer primer and 2 units of thermal stable DNA polymerase. The PCR reaction condition was 94°C for 4 min, 55°C for 1 min and 72°C for 2 min, followed by 39 cycles for 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, and a final 5 min extension at 72°C. For the second-step of the PCR reaction, 10 µl of the first-step PCR reaction product was added to 90 µl of PCR cocktail of the same composition as earlier mentioned, except when it contained the inner primers. The same PCR conditions as earlier mentioned were used.

To detect the *S. eriocheiris*, the method described by Ding was employed (Ding et al., 2007). In the PCR reaction, the primers F28 and R5 were used (Table 1).

Following PCR, the amplified products were analyzed by electrophoresis in 1.5% agarose gel stained with ethidium bromide, visualized and recorded by ultraviolet transillumination.

Transmission electron microscopy

Negative staining and ultrathin sectioning for transmission electron microscope (TEM) were carried out in this study. As for negative staining, sodium phosphotungstate (2%) was employed. Drops of the tissue supernatant (from diseased shrimp and crayfish) were fixed with 2.5% glutaraldehyde and placed on formvar-coated copper grids for 1 min. The samples were immediately covered with the stain for 30 to 40 s, and then withdrawn by filter paper. The grid was air-dried before examination under Hitachi H-7600 TEM.

When preparing the ultrathin section, the gill tissues, nervous tissues and cardiac muscular tissues from sampled shrimp and crayfish were pre-fixed in 2.5% glutaraldehyde dissolved in 0.1 M phosphate buffer (pH 7.2) and transferred to 1% osmium tetroxide made with the same buffer, then the samples were serially dehydrated with acetone and embedded in Epon 812. Ultrathin sections with thickness of 50 to 80 nm were made by Reichet–Jung ultramicrotome and double-stained with uranyl acetate and lead citrate. The sections were observed and photographed by Hitachi H-7600 TEM.

Sequence analysis

VP19, VP26 and VP28 were amplified using prepared PCR mixture (30 µl). The mixture contained 0.2 µl DNA polymerase, 2 µl primer mixture (Table 1), 3 µl 10× STR buffer (including dNTP and Mg^{2+}, Promega), 5 µl DNA template and 19.8 µl sterile water. The amplification cycle protocol was as follows: 5 min at 94°C, then 25 cycles of 1 min denaturation at 94°C, 1.5 min of annealing at 53°C and 1.5 min extension at 72°C, followed by a final extension of 10 min at 72°C. Agarose gel electrophoresis of PCR products was done using a 1.5% agarose gel in the presence of a molecular size ladder standard and followed by UV visualization after ethidium bromide staining. PCR products were cleaned with gel-purified PCR DNA kit (TaKaRa) and sequenced in Shanghai Sangon Biological Engineering Technology and Services Co., Ltd using an Applied Biosystems 3700 capillary DNA Genetic Analyzer. Homology searches in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), prediction of transmembrane helices in proteins through TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and functional features in 3-D analysis by SWISS-MODEL

Table 1. Sequences of the primers used in this study.

Primer	Sequence (5'-3')
146F1	ACTACTAACTTCAGCCTATCTAG
146R1	TAATGCGGGTGTAATGTTCTTACGA
146F2	GTAACTGCCCCTTCCATCTCCA
146R2	TACGGCAGCTGCTGCACCTTGT
F28	CGCAGACGGTTTAGCAAGTTTGGG
R5	AGCACCGAACTTAGTCCGACAC
VP19-1	ATGGCCACCACGACTAACACTC
VP19-2	TTACTGCCTCCTCTTGGGGTAAG
VP26-1	TTGGCAACCTAACAAACCTGG
VP26-2	TACTTCTTCTTGATTTCGTCC
VP28-1	GGAATAGACCCTGGCTTACTG
VP28-2	ACCAGGAAAAGACCAATAGGC

(http://swissmodel.expasy.org/) (Arnold et al., 2006) were carried out in this study.

RESULTS

Polymerase chain reaction (PCR)

The PCR test results show that all the samples (shrimps and crayfishes) were solely infected by WSSV. The results of electrophoresis of the amplified DNA for WSSV (the second-step of nested PCR) and *S. eriocheiris* are shown in Figure 1A and B, respectively. The nested-PCR results showed a special band of about 1447 bp, indicating a positive WSSV infection (Figure 1A). On the contrary, PCR test did not show the characteristic (271 bp), indicating negative results for *S. eriocheiris* (Figure 1B).

Electron microscopy

The results of negatively stained intact WSSV virions for shrimp and crayfish's tissues supernatant under TEM examination revealed that the virions were elliptical and without tail-like extension (Figure 2A, B and C). In this experiment, tail-like extension was not found in all the virions. Different sectional WSSV were observed in the epidermic cells of gills (Figure 3), in hemocytes and blood sinus of connective tissues of cardiac muscular tissues or nervous tissues (Figure 4).

Sequence analysis

Envelope structural proteins play a critical role in viral infection and are considered to be the first molecules to interact with the hosts (Tsai et al., 2004b; Yi et al., 2004). Homologous contrast showed that the sequences of VP19, VP26 and VP28 in the study possess over 97% DNA and 100% amino acid sequence similarity to WSSV in the NCBI database. Prediction of transmembrane helices results suggested that all of the three structure proteins had the transmembrane region and they should belong to the transmembrane proteins, thus the position of VP26 was predicted on the outer membrane. Functional features in 3-D analysis are shown in Figure 5. Both VP26 and VP28 adopted β-barrel architecture. Additionally, there was a two-turn α-helix hanging outside the β-barrel structure in VP26. Similar to VP26, one α-helix hung outside the β-barrel in VP28, and the pore of the β-barrel was highly hydrophobic in nature. However, VP19 was not found in the SWISS-MODEL workspace.

DISCUSSION

With the rapid growth and development of *P. vannamei* and *P. clarkii* in aquaculture industry, some of the most significant pathogens such as WSSV (Natividad et al., 2008) and *S. eriocheiris* (Wang et al., 2005) were detected and reported. The two pathogens could induce the high mortalities in aquaculture. Infection of *P. vannamei* by WSSV could result to up to 100% mortality during 3 to 7 days (Lightner, 1996); while infection of *S. eriocheiris* caused tremor disease in crabs and *P. clarkii* had up to 100% mortality for about two weeks (Liang et al., 2009). Many researches showed that crayfish *P. vannamei* was the model animal as the host for WSSV (Huang et al., 2001; Jiravanichpaisal et al., 2001, 2006; Shi et al., 2000). Herein, the diseased shrimps from wild with canker on the carapace and red tail symptoms were collected. Due to the fact that all the aquaculture farms in this study had a history of infection by WSSV and/or *S. eriocheiris*, the both pathogens might induce the high mortalities in aquaculture probably. So, WSSV and *S. eriocheiris* were detected in this study. From the PCR test results, all the samples (shrimps and crayfishes) collected were infected only by WSSV. Interestingly, unlike the earlier reports, the samples collected had no white spots in the carapace which was one of the significant characters in white spot syndrome. Indeed, the WSSV had still induced high mortalities and caused heavy losses to aquiculture in Jiangsu, China.

Some virus particles were observed in the infected *P. vannamei* and *P. clarkii* tissues by negative stained and ultrasectioned samples. Similar to other studies, the virus showed rod-shaped to elliptical, and the length was approximately 300 nm (Figure 2A, B and C). The most typical trait was the absence of tail-like extension on the end of the virus, which was not observed and described in detail before. From our TEM results, this virus can infect gills' epithelium and proliferate in the nucleus. These data further suggest that the WSSV multiplication was still going on when we sampled the diseased shrimp and crayfish from the aquaculture farms.

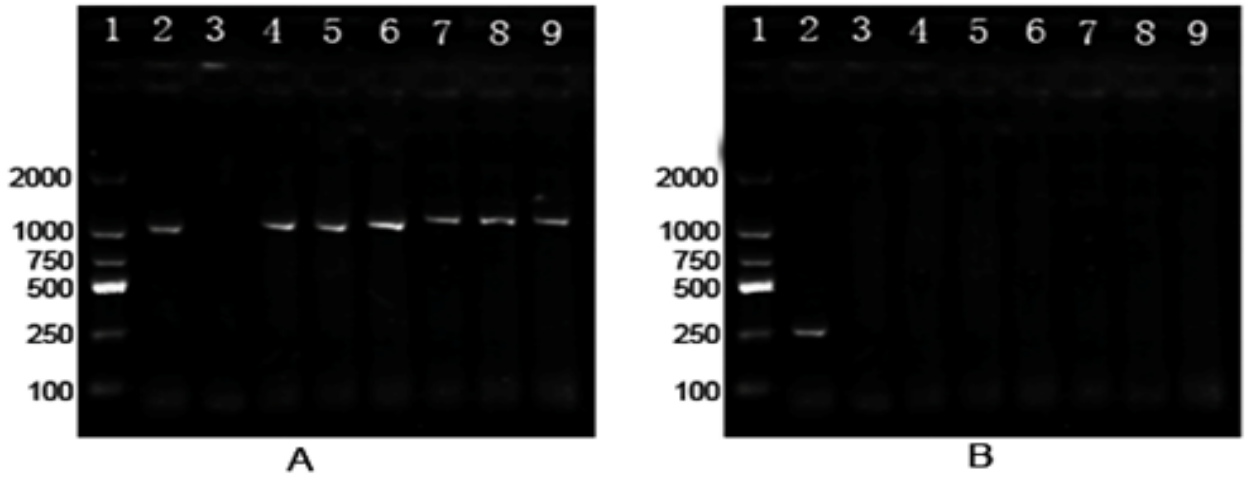

Figure 1. Agarose gel electrophoresis profile of PCR-amplified for WSSV (the second-step of nested PCR results) (A) and spiroplasma (B) were shown. In A and B, the lanes were marked as fellows: lane 1, molecular size marker (2000 bp); lane 2, positive control; lane 3, negative control; lanes 4 to 6, infected shrimp samples; lanes 7 to 9, infected crayfish samples. A special band about of 1447 bp (A) or 271 bp (B) showed the positive results.

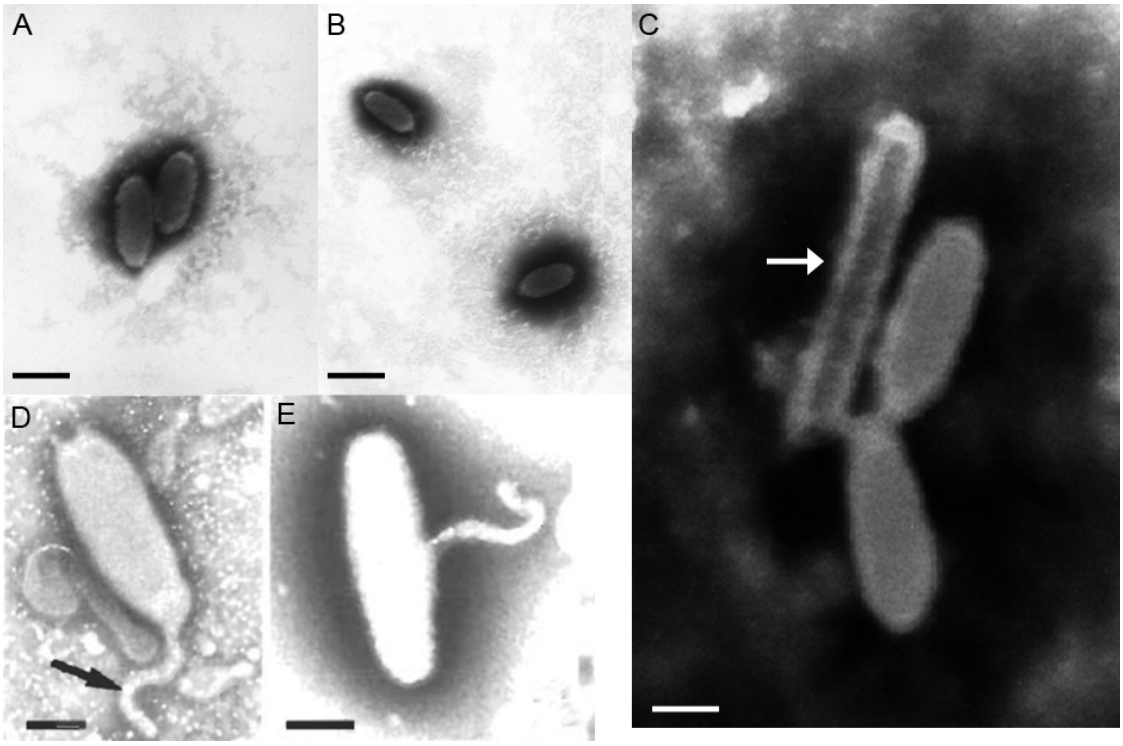

Figure 2. A and B: Electron micrograph of negatively stained *P. vannamei*'s tissues supernatant showing intact WSSV virions without tail-like extension. Scale bar = 300 nm. C: Negatively stained samples of *P. clarkii*'s tissues supernate showing WSSV virions and nucleocapsids (the white arrow indicates nucleocapsid). Scale bar = 100 nm. D and E: Negative stain for WSSV shows an overwhelming evidence of tail-like extension. Scale bar = 100 nm (Durand et al., 1997).

Furthermore, the WSSV can also transmit to the nerves tissues and cardiac muscular tissues by hemocytes and circulation.

Many major envelope proteins of WSSV were identified in some studies (Liang et al., 2005; Liu et al., 2009a, b; Zhou et al., 2009). In light of the interesting phenomenon

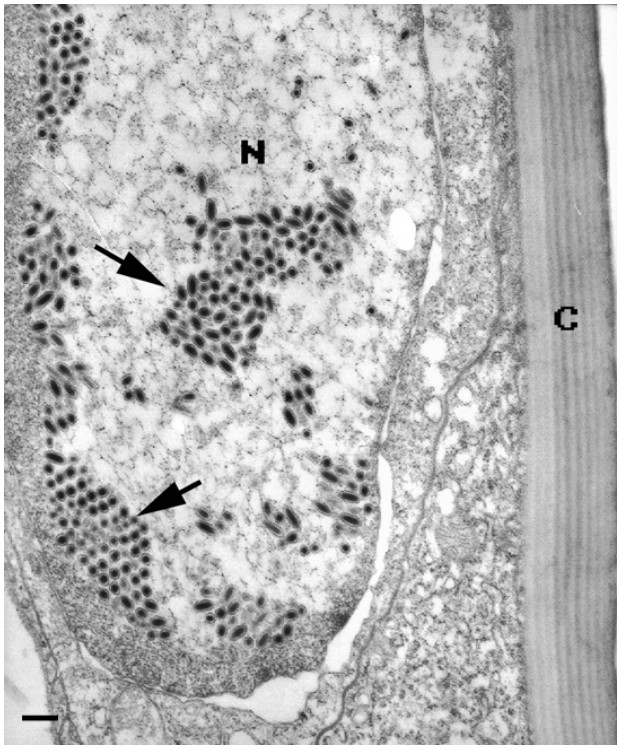

Figure 3. Different sectional WSSV (black arrow) in ultrathin sections of *P. vannamei*'s gills tissues. C: Corneous layer, N: cell nucleus. Scale bar = 500 nm.

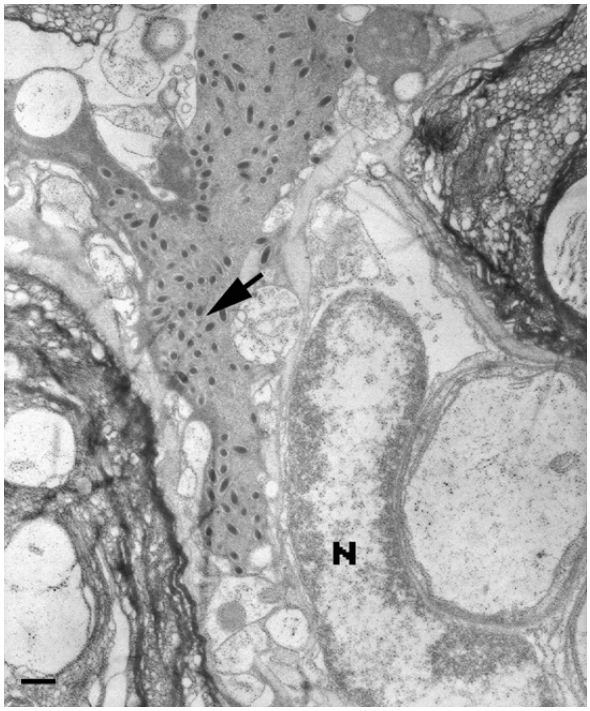

Figure 4. Different sectional WSSV (black arrow) in blood sinus of *P. vannamei*'s nervous tissues. N: cell nucleus. Scale bar = 500 nm.

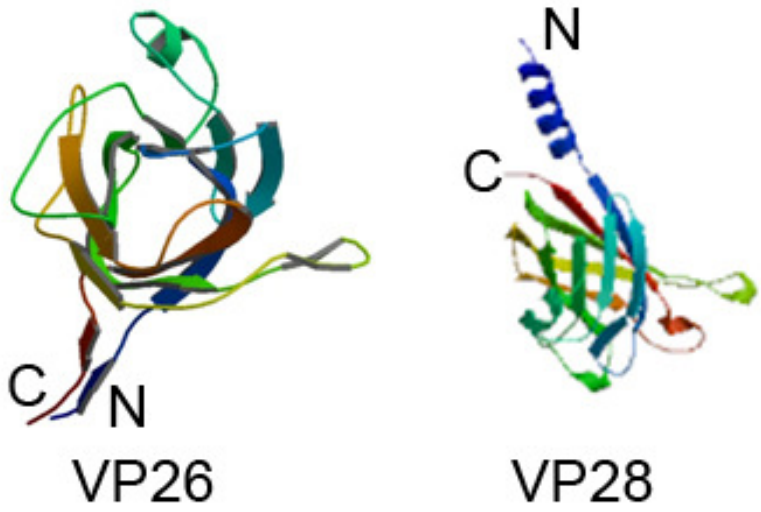

Figure 5. Based on the amino acid sequences, functional features like VP26 and VP28 in 3-D analysis were employed by SWISS-MODEL workspace.

of without tail-like extension, we tried to find some relationships between the external morphology and the major envelope proteins. In order to determine whether the three envelope proteins (VP19, VP26 and VP28) had differences with the other reported WSSV, we obtained and analyzed their nucleic acid sequences. From the homology comparison, no significant difference was found in the nucleotide and amino acid sequences. These results suggest that the virus observed should belong to WSSV.

Both VP26 and VP28 had the transmembrane regions at N-terminal by prediction of transmembrane helices. We speculated that they should be located on the outer surface of the virus. The functional features of VP19, VP26 and VP28 in 3-D analysis by SWISS-MODEL revealed that VP26 and VP28 were found with hits on the template identification in SWISS-MODEL, however, VP19 had no hits. Though VP26 and VP28 were not highly homologous to any of the viral envelope fusion proteins, they retained the overall β-barrel as well as trimmer architecture similar to the structure of other viral envelope fusion proteins (Tang et al., 2007). Figure 5 shows that the predicted N-terminal transmembrane region of VP26 and VP28 may anchor on the viral envelope membrane and these structures would make the core β-barrel protrude outside the envelope. We speculated that WSSV could fuse with the host cell membrane or infect the host receptor more effectively. Crystal structure of major envelope proteins VP26 and VP28 from WSSV had been studied (Tang et al., 2007), and their results also supported that VP26 was located on the outer surface instead of the nucleocapsid.

Based on our studies and relative literatures, we conclude that this virus without tail-like extension may be a different external form of WSSV; although, with lack of tail-like extension, it still has high virulence to crustacean. In addition, the report expands our knowledge on WSSV morphologic diversity.

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