Virus Research 270 (2019) 197666

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

VP19 is important for the envelope coating of white spot syndrome virus

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ARTICLE INFO

Keywords:
White spot syndrome virus
RNA interference
VP19
Envelope coating

ABSTRACT

VP19 is a major envelope protein of white spot syndrome virus (WSSV), an important pathogen of farmed shrimp. However, the exact function of VP19 in WSSV assembly and infection is unknown. To understand the function of VP19, the gene was knocked down by RNA interference. We found that the dsRNA specific for *vp19* gene dramatically reduced the replication of WSSV genomic DNA in infected animals. Further investigation by transmission electron microscopy showed that inhibition of VP19 prevented envelope coating of progeny virions, resulting in a high amount of immature virus particles without outer layer (envelope) in the host cells. This finding was further confirmed by SDS–PAGE analysis, which showed the loss of VP19 and other envelope proteins from the improperly assembled virions. These results suggest that VP19 is essential for WSSV envelope coating.

1. Introduction

White spot syndrome virus (WSSV), the only member of the family *Nimaviridae*, genus *Whispovirus*, is an enveloped double-stranded DNA virus (van Hulten et al., 2001a; Leu et al., 2009). The virus infects a wide range of crustaceans, including shrimp, crab, and crayfish (Wang et al., 1998), and has caused catastrophic damage (Lo et al., 1996a, 1996b; Flegel, 1997). So far, the complete genome of eight WSSV isolates have been sequenced (Yang et al., 2001; van Hulten et al., 2001a; Chen et al., 2002; Chai et al., 2013; Li et al., 2017; Kawato et al., 2018; Restrepo et al., 2018), and more than 58 structural proteins have been identified (Tsai et al., 2004; Xie et al., 2006; Liang et al., 2011).

Because the envelope of virus plays a key role in virus assembly and infection, envelope proteins of WSSV become the focus of research. The envelope of WSSV contains at least 35 proteins (Xie et al., 2006). Among them, VP28, VP26, VP24 and VP19 are four major envelope proteins, which can bind to each other and form a complex (Zhou et al., 2009). Of these four proteins, VP28 was reported to be involved in the attachment and penetration of WSSV into host cells (Yi et al., 2004). VP26 was identified as a linker protein between the envelope and nucleocapsid of virion by binding with nucleocapsid protein VP51 (Wan et al., 2008). VP24 was found to be a chitin-binding protein involved in WSSV infection (Li et al., 2016), which could bind with many proteins including VP28, VP26, VP19, VP33, VP38, VP52A (the counterpart of VP51A of WSSV-TW), VP53A and wsv010 (Xie et al., 2006; Jie et al., 2008; Lin et al., 2010; Liu et al., 2014; Huang et al., 2014; Li et al., 2015). The antiserum of VP19 was found to neutralize the virus and thus reduced the mortality rate of diseased shrimp (Mi et al., 2008). It was reported that the long dsRNA specific for *vp19* could induce both innate and target gene specific responses in *Litopenaeus vannamei* infected with WSSV, whereas siRNA failed to induce similar responses (Robalino et al., 2005). However, the exact function of VP19 in WSSV assembly and infection remains to be explored.

RNA interference (RNAi), a powerful experimental tool to study gene function and virus infection (Berkhout, 2004; Hu et al., 2005; Travella et al., 2006; Saurabh et al., 2014;), has been widely used in antiviral studies of WSSV (Ongvarrasopone et al., 2008; Attasart et al., 2009; Sudhakaran et al., 2011; Sanjuktha et al., 2012; Thammasorn et al., 2015; Alenton et al., 2016; Shu et al., 2016; Nilsen et al., 2017; Feng et al., 2017;). Here, to understand the function of VP19 during WSSV infection, we knocked down *vp19* gene by RNAi and investigated the possible outcome by transmission electronic microscopy (TEM). We demonstrate that VP19 is essential for WSSV envelope coating.

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https://doi.org/10.1016/j.virusres.2019.197666
Received 3 April 2019; Received in revised form 10 July 2019; Accepted 12 July 2019
Available online 12 July 2019

2. Materials and methods

2.1. Animals

Freshwater crayfish, *Procambarus clarkii*, can serve as an alternate proliferation host for WSSV. A larger amount of intact virions can be produced in *P. clarkii* than in the original penaeid shrimp host (Huang et al., 2001). *P. clarkii* were purchased from a local agricultural market in Xiamen City, China. Individuals with similar size and weight (20˜25 g) were selected for the experiment, and each individual was confirmed to be WSSV free by PCR with WSSV specific primers N4 and C4 (Supplementary Table.1) (Shi et al., 2014). To avoid individual death from sampling, abdominal leg muscle of the individuals were selected for genomic DNA /RNA extraction.

2.2. Virus preparation

WSSV virions were purified as previously described (Xie et al., 2005). The tissues of WSSV-infected crayfish excluding hepatopancreas were homogenized in TNE buffer [50 mM Tris − HCl, 400 mM NaCl, 5 mM ethylene diamine tetraacetic acid (EDTA), pH 8.5] containing protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, and 1 mM $Na_2S_2O_5$] and then centrifuged at 3,500 × g for 5 min. The supernatant was filtered through a nylon net (400 mesh) and centrifuged at 30,000 × g for 30 min at 4 °C. Then the supernatant was discarded, the upper loose layer (pink) of pellet was rinsed out carefully, and the lower compact layer (gray) was resuspended in 10 ml TM buffer (50 mM Tris − HCl, 10 mM $MgCl_2$, pH 7.5). The crude virus suspensions were centrifuged at 3,000 × *g* for 5 min, and the supernatant was centrifuged again at 30,000 × *g* for 20 min. After the supernatant and pink loose layer were removed, and the purified virus (white) were resuspended and kept in appropriate volume of TM buffer containing 0.05% sodium azide. The purified WSSV virions were quantified as described before (Zhou et al., 2007).

2.3. Synthesis of dsRNA

Long dsRNAs specific for WSSV genes (vp19-dsRNA: 343bp; vp28-dsRNA: 581bp) were synthesized in vitro by using T7 RiboMAX™ Express RNAi System (Promega), according to the manufacturer's instructions. The dsRNA specific for *EGFP* gene was synthesized and used as a control. All primers were listed in Supplementary Table. 1.

2.4. dsRNA injection and virus challenge

1×10^5 freshly purified virions were injected into each crayfish via the lateral area of the fourth abdominal segment immediately following the injection of 30 μg dsRNA. The second dsRNA injection (30 μg) was done 12 h post WSSV challenge. Sequence-specific dsRNAs corresponding to the gene of WSSV VP19 was used. The dsRNAs corresponding to the gene of WSSV VP28, a major envelope protein essential for WSSV infection (van Hulten et al., 2001b; Li et al., 2010; Qiu et al., 2012), was used as a specific dsRNA control. The dsRNA of EGFP was used as a nonspecific dsRNA control, and saline (0.9% NaCl) served as a mock-infected control.

2.5. Q-PCR analysis of WSSV genomic DNA

Genomic DNA was extracted using DNAzol reagent following the manufacturer's manual (MRC) at different time points (24, 48 and 72 hpi) after WSSV challenge. WSSV replication in crayfish was determined by q-PCR analysis with WSSV specific primers N4 and C4 (Supplementary table.1). 10-fold serial dilutions of internal standard plasmid were used to measure standard curve (Wang et al., 2008). Each sample was amplified and WSSV copies were determined according to standard curve.

The DNA was added in a 20 μl reaction which contained 10 μl 2 × SYBR green PCR mix buffer (ABI) and 0.4 μM of each primer. Q-PCR was carried out by denaturing at 95 °C for 2 min, followed by 40 cycles of 95 °C/10 s and 68 °C/40 s. For each experiment, five individuals were used in each group. The viral load was determined for each individual and the average viral load of each group was calculated. The experiment was performed for three times, and the three average datas were further analyzed by one-way ANOVA to calculate the mean and standard deviation.

2.6. sqRT-PCR analysis for dsRNA knock-down efficiency

For each experiment, five individuals were used in each group. The total RNA was extracted from the five individuals at different time points (24, 36, 48 and 60 hpi), using RNeasy Mini Kit (Qiagen, Germany), and pooled together. Genomic DNA contamination was eliminated by DNase I treatment. Two microgram of total RNA was used for first-strand cDNA synthesis with MonsterScript™ 1st-Strand cDNA Synthesis Kit (Epicentre, America). *vp19* and *vp28* genes were analyzed by PCR with gene specific primers (Supplementary table. 1), and crayfish β-actin gene was used as a control for normalization. Equal amounts of PCR products were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining. The specific bands of PCR products were analyzed by Image-Pro Plus 6.0 system. RT-PCR was performed for three times independently.

2.7. Antibodies and Western blotting

The three recombination proteins rVP28, rVP19 and rVP664 were purified by our lab as decribed in our previous studies (Xie et al., 2006; Zhou et al., 2009; Xiong and Yang, 2007). Polyclonal antibodies against specific WSSV structural proteins (VP28, VP19 and VP664) were generated in mice by using the expressed recombinant proteins in *Escherichia coli* as described previously (Li et al., 2005). VP664 is the major nucleocapsid protein of white spot syndrome virus (Leu et al., 2005), and its antibodies are used to identify the nucleocapsid in this study. The antibodies were purified by protein A-Sepharose. Pre-immune serum (IgG) was used as a negative control.

For Western blotting analysis, purified virions were lysed in SDS-loading buffer and boiled for 10 min. The proteins were separated by SDS-PAGE (12% gel) and transferred to a polyvinylidene fluoride membrane (PVDF membrane, Amersham) by semi-dry transfer (0.5 mA cm2 for 1.5 h). The membrane was immersed in blocking buffer (1% BSA, 20 mM Tris/HCl (pH 7.2), 150 mM NaCl, 0.05% Tween 20) at room temperature for 1 h, and incubated with specific antibodies (1:1000) for 1 h. Subsequently, alkaline phosphatase-conjugated goat antimouse IgG (Promega) was added at a dilution of 1:7500 and signals were detected by using a substrate solution containing 4-chloro-1-naphthol and X-phosphate (Promega).

2.8. Transmission electron microscopy (TEM) and immunoelectron microscopy (IEM) analysis of crayfish mid-gut

The mid-gut of WSSV-infected crayfish was collected at 48 hpi after injection of vp19 dsRNAs. One half of the mid-gut tissue was used for electron microscopy analysis, and the other half was used for IEM. The mid-gut tissue of WSSV-infected crayfish without dsRNA injection at 48 hpi was used as a control. The experiment was performed three times with three different individuals.

Samples were fixed in 0.5% glutaraldehyde and 4% paraformaldehyde for 4 h. After stepwise dehydration in ethanol, the specimens were embedded in Epon resin, polymerized at 60 °C for 24 h, and sectioned. The thin-sections were blocked with 1% bovine serum albumin in PBS for 30 min, and probed with antibodies (1: 250) specific for VP19, VP28 and VP664. After 3 times wash with PBS, the sections were incubated with colloidal gold-conjugated goat anti-mouse immunoglobulin G

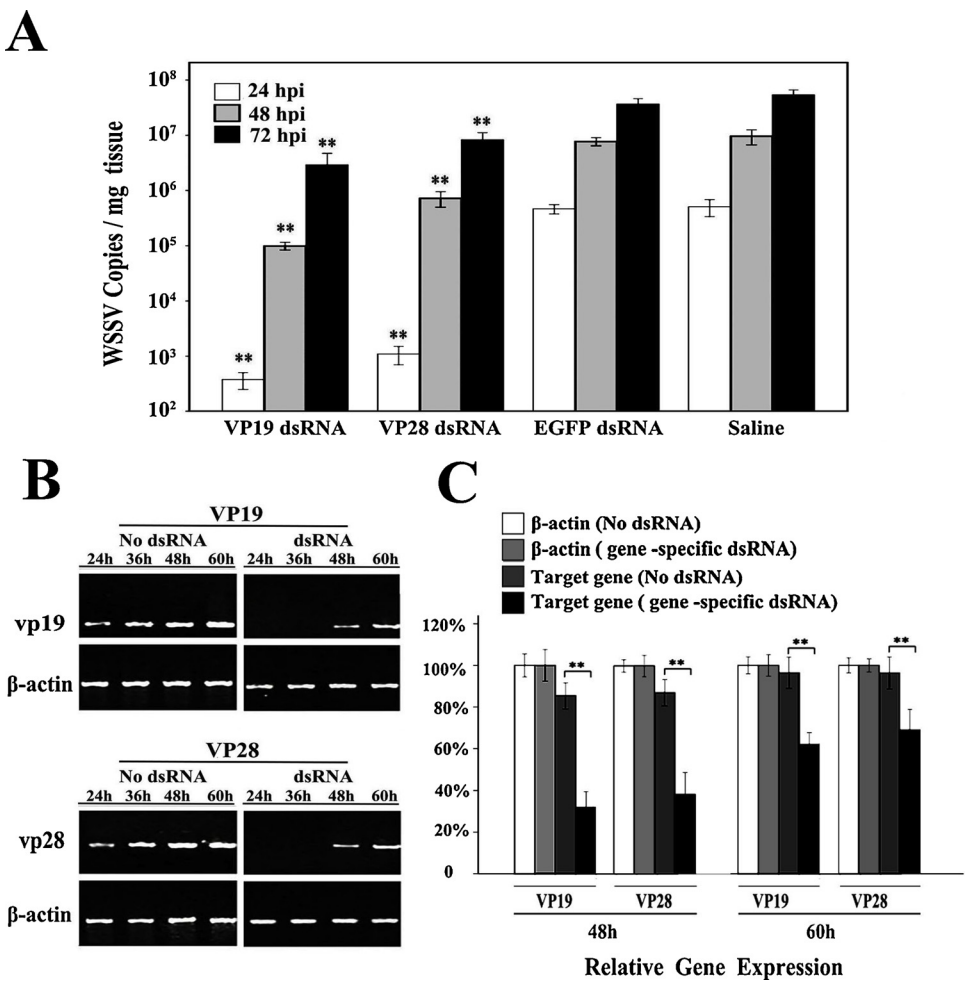

Fig. 1. Sequence-specific dsRNAs inhibition in crayfish.

(A) Detection of WSSV replication by q-PCR. DNA was extracted from the muscle of the abdominal leg of each crayfish, and the viral copies was examined at different time intervals (24, 48 and 72hpi) after treatment with virus and VP19 dsRNA. VP28 dsRNA, EGFP dsRNA, and saline (0.9% NaCl) were used to replace VP19 dsRNA as the controls. In each group, five individual crayfish were analyzed and the mean viral load was calculated. Data represent Mean ± SD of three independent experiments. The statistical differences between specific dsRNA treated and saline treated groups were analyzed by ANOVA (** $P < 0.01$).

(B) Determination of dsRNA knock-down efficiency by sqRT-PCR.

The pooled total RNA was extracted from the muscle of the abdominal leg of five individuals after virus and sequence-specific dsRNA challenge at different time points (24, 36, 48 and 60 hpi). sqRT-PCR was performed to determine the knock-down efficiency of sequence-specific dsRNA of *vp19* and *vp28*. The transcription of neither gene was observed at 24 hpi and 36 hpi, whereas a low level of transcription was detected at 48 hpi, compared with no dsRNA control.

(C) The relative expression level of vp19 or vp28 mRNA after treatment of the sequence-specific dsRNA.

Histogram graphs show relative values of each group at 48 hpi and 60 hpi measured by sqRT-PCR. β-actin was used as an internal control for normalization. The data showed that the relative expression levels of *vp19* and *vp28* gene were significantly decreased after sequence-specific dsRNA treatment. The experiments were performed in triplicate. ** indicate the significant difference at $P < 0.01$.

(Sigma) for 1 h. Pre-immune serum was used as a negative control. The samples were observed using a transmission electron microscope (JEM-1230, JEOL).

2.9. TEM and SDS-PAGE analysis of purified virions

Virions were purified from WSSV-infected crayfish with VP19 dsRNAs treatment at 48 hpi, and quantified by q-PCR. One half of the purified virions were used for TEM analysis, and the other half were used for SDS-PAGE analysis. The virions purified from WSSV infected crayfish without VP19 dsRNA treatment was used as the control. Three different individuals were analyzed for each group.

For TEM analysis, the virions purified from different treatment groups (in TNE) was mounted onto formvar-coated, carbon-stabilized copper grids (200 meshs) and negatively stained with 2% sodium phosphotungstate (PTA, pH 7.0), respectively. And, the similar amount of purified virions were lysed in SDS-loading buffer and boiled for 10 min. The proteins were separated by SDS-PAGE (12% gel).

3. Results and discussion

VP19 is one of the major envelope proteins of WSSV, but the specific role of VP19 in virus infection is still unknown. In this study, RNA interference was used to study the role of VP19 in WSSV infection. According to a previous study, long VP19 dsRNA can induce not only innate immune responses but also target gene specific responses in *L. vannamei* infected with WSSV, whereas siRNA fails to induce a similar response (Robalino et al., 2005). In this study, to understand the specific function of VP19 during WSSV infection, *vp19* gene was knocked down by injection of long VP19 dsRNA and WSSV into crayfish. To make sure that the injected virus could overwhelm innate immunity and successfully infect each individual crayfish, 1×10^5 freshly purified virions, a dose that could generate effectively WSSV proliferation (Huang et al., 2001), was injected into each crayfish. In our previous experiment, 10^3 virions purified with our method could generate a productive WSSV infection in the challenged crayfish (Xie et al., 2005). In our experiment, the injection of WSSV virions at the dose of 1×10^5 could cause crayfish (20 individuals) to become moribund at 36 hpi and die within 4 days. In addition, to ensure the sequence-specific antiviral effect, we conducted two high-dose dsRNA (30 ug) injections after the virus challeng. One injection was done immediately after the virus challenge and the other was done 12 h later.

Compared with the groups injected with EGFP dsRNA or saline alone, injection of both VP19 and VP28 dsRNA significantly inhibited the replication of WSSV ($P < 0.01$) (Fig.1A). This result further confirmed that only sequence specific dsRNA can trigger an effective RNAi response upon a high dose of WSSV infection (Robalino et al., 2005; Claudio et al., 2011; César et al., 2015). Knockdown efficiency of *vp19* and *vp28* genes were evaluated by sqRT-PCR. β-actin was used as internal control. As shown in Fig. 1B, transcription of neither genes were observed at 24 hpi and 36 hpi, whereas a low level of transcription was detected at 48 hpi. The relative expression levels of both genes were decreased significantly at 48 and 60 hpi ($P < 0.01$) (Fig. 1C), indicating that *vp19* and *vp28* genes were effectively knocked down by

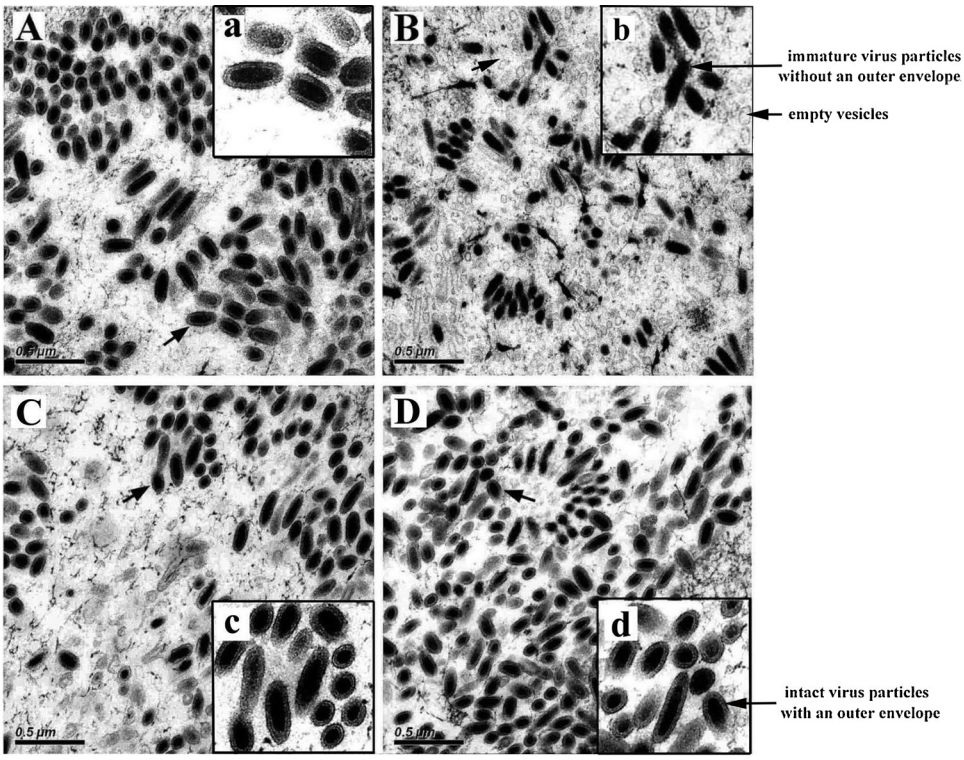

Fig. 2. TEM analysis of the midgut tissues of infected crayfish.
In each treated group, 1×10^5 freshly purified virions was injected into each crayfish immediately following the injection of saline (A), sequence-specific dsRNA corresponding to vp19 (B), vp28(C) or the unrelated dsRNA corresponding to gfp (D). The second dsRNA injection was done 12 h post WSSV challenge. Then, 48 h post WSSV challenge, midgut was collected to produce ultrathin section. Different from any other group (a, c, d), VP19 dsRNA group exhibited a high percentage of immature virus particles and empty vesicles. Bar, 0.5 μm. The small panel in each image shows the same field indicated by arrow (magnification = 2).

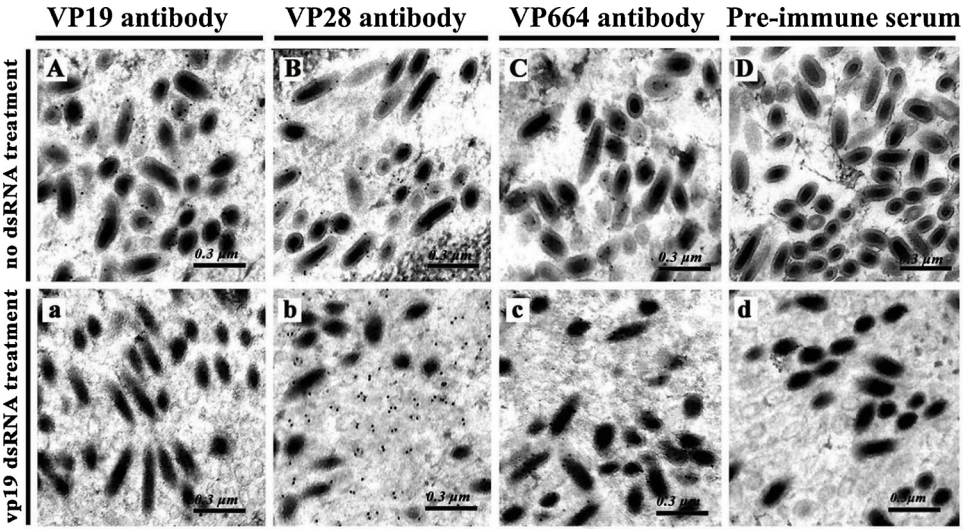

Fig. 3. IEM anlaysis of the midgut tissues of infected crayfish.
In VP19 dsRNA group, 1×10^5 freshly purified virions was injected into each crayfish immediately following the injection of VP19 dsRNA. The second dsRNA injection was done 12 h post WSSV challenge. In no-dsRNA group, saline was used instead of VP19 dsRNA. Ultrathin section then incubated with different primary antibody respectively, followed by gold-labeled secondary antibodies. Primary antibody included: anti-VP19 serum (A, a), anti-VP28 serum (B, b), anti-VP664 serum (C, c), and pre-immune serum was used as control (D, d). In no-dsRNA group, VP19 and VP28 distributed on the envelope of virions, while VP664 located in the nucleocapsid (A, B, C). Knockdown of *vp19* gene resulted in loss of both VP19 (a) and VP28 (b) from the virons, while the distribution of VP664 remained unaffected (c). Moreover, the empty vesicles in the host cells contained considerable amount of VP28 (b). No gold particles were observed in the samples probed with pre-immune serum (D, d). Bar, 0.3 μm.

sequence-specific dsRNA.

To further investigate wether the inhibition of VP19 and VP28 expression affects progeny virion production, midguts of the infected crayfish, the most prominent site of WSSV vaccines uptake and processing (Bright Singh et al., 2013), were collected at 48 hpi and analyzed by TEM. Under the electron microscope, numerous intact virus particles consisting of an electron-dense inner core (nucleocapsid) and an outer layer (envelope) were found in EGFP dsRNA group, VP28 dsRNA group and saline group (Fig. 2A, C, D). In contrast, most virions were improperly assembled in VP19 dsRNA group. There were large amounts of immature virus particles without envelopes and empty vesicles in host cells (Fig. 2B, b). The present of these structures were confirmed by subsequent IEM with antibodies specific for VP28, VP19 and VP664. The specificity of the antibodies was validated by western blot (Supplementary Fig. 1). IEM analysis showed that in no dsRNA group, VP19 and VP28 distributed on the envelope of virions, while VP664 located in the nucleocapsid (Fig. 3A–C). Knockdown of *vp19* gene resulted in loss of both VP19 (Fig. 3a) and VP28 (Fig. 3b) from the virons, while the distribution of VP664 remained unaffected (Fig. 3c). Moreover, the empty vesicles in the host cells contained considerable amount of VP28 (Fig. 3b). No gold particles were observed in the samples probed with pre-immune serum (Fig. 3D, d). Large amounts of VP28 were present on the surface of empty vesicles indicating that these vesicles may be envelope fractions that fail to coated onto nucleocapsids (Fig. 3b).

To further verify this observation, similar amount of virions purified

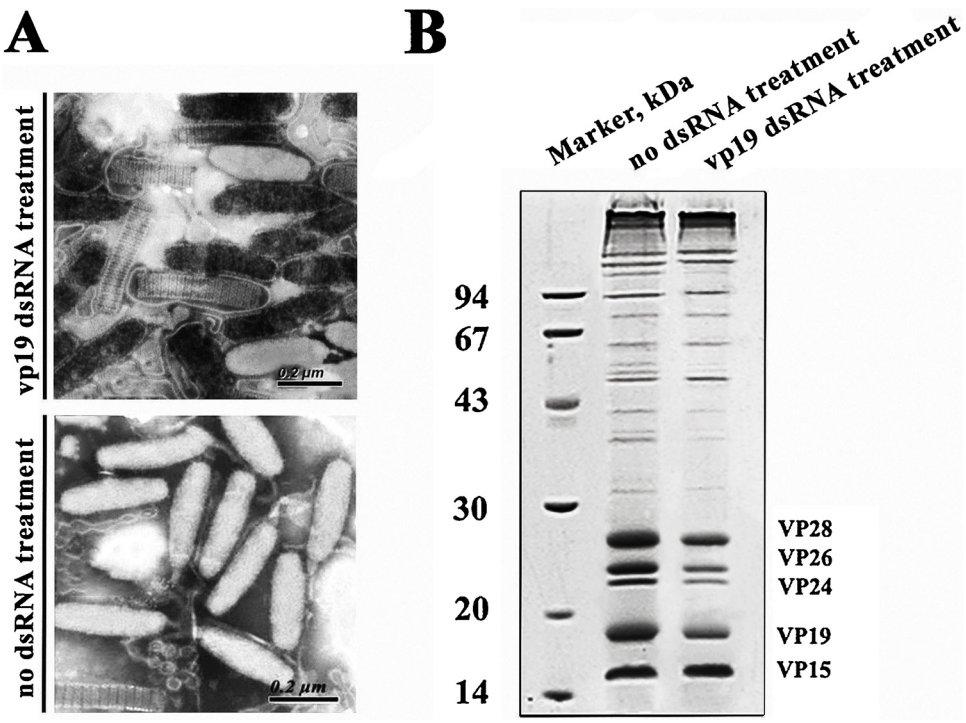

Fig. 4. TEM and SDS-PAGE analysis of purified progeny virions.
(A) TEM analysis of the purified virus from VP19 dsRNA group and no dsRNA group. Most particles are intact with tight rod-shape in no dsRNA group. However, most particles from VP19 dsRNA group were immature virus particles without an outer envelope. Bar, 0.2 μm.
(B) SDS-PAGE analysis of the purified virus from VP19 dsRNA group and no dsRNA group. In VP19 dsRNA group, the quantity of VP19 and the other envelope proteins (including major envelope protein VP24, VP26 and VP28) decreased significantly. The content of VP15, a major nucleocapsid protein (Xie et al., 2006), was no significantly difference.

(determined by q-PCR) from the VP19 dsRNA and no-dsRNA groups were subjected to TEM and SDS-PAGE analysis. Under the electron microscope, virions purified from no-dsRNA group were mostly intact in tight rod-shape. In contrast, the large majority of virus particles purified from VP19 dsRNA group were in the form of immature viral particles, which had no outer membrane coating on their surfaces (Fig. 4A). SDS-PAGE analysis showed these abnormal virus particles contained similar amount of VP15 (a major nucleocapsid protein, Xie et al., 2006), while they contained much less amount of envelope proteins including VP28, VP26, VP24, and VP19 (Fig. 4B). The loss of envelope proteins also suggests that when VP19 is knocked down, the membrane of the virus cannot be normally assembled onto the nucleocapsid. This result was consistent with our IEM analysis (Fig. 3b). Therefore, VP19 might play an important role in the envelopment of WSSV.

Furthermore, many empty vesicles contained major WSSV envelope protein VP28 were formed in the cells of *vp19*-knockdown animals, where there were also many naked nucleocapsids (Figs. 2 and 3). These results imply that during progeny virion formation, the nucleocapsid and envelope of WSSV are first assembled separately, and then packed together, which supported an earlier hypothesis that the development of the WSSV nucleocapsid is not dependent on its association with viral envelope (Durand et al., 1997).

4. Conclusion

In this research, we explored the role of VP19 in WSSV infection using RNA interference. By analyzing the replication of viral genome and production of progeny virions, we demonstrate that VP19 may play an important role in the envelopment of WSSV. These findings extend our knowledge on the mechanism of WSSV assembly.

Acknowledgments

This work was supported by Fujian province “2011 collaborative innovation center” special fund (fujian education science [2015] No.75), Discipline team construction fund of wuyi university (wu yuan zong [2007] No. 66), and the National Natural Science Foundation of China [grant number 31672675].

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197666.

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