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WSSV proteins and DNA genome released by ultrasonic rupture can infect crayfish as effectively as intact virions

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ABSTRACT

Proteins and nucleic acids from ultrasonically ruptured white spot syndrome virus (WSSV) can infect crayfish and cause death as effectively as intact WSSV virions. In this study, ultrasound was used to rupture the virus and the resulting suspension was filtered through a 50 nm membrane. Analysis by PCR and SDS-PAGE showed that both viral genes (VP19, VP26, VP28 and DNA polymerase) and proteins (VP15, VP19, VP26 and VP28) were present in the filtered solution. Electron microscopy showed that there were no intact virions in the filtered solution. When crayfish were injected with the filtered solution or with intact WSSV, the mortality in each group was 100 %. The same result was seen when crayfish were challenged orally with the filtered solution and intact WSSV. The filtered solution of ultrasonically ruptured virus, which contains viral proteins and residual DNA genome, can thus infect the host as effectively as intact virions. When the solution of viral proteins and residual DNA genome was digested with DNase I and then injected into crayfish, the survival rate was 100 %. We also found that, although viral proteins (except VP15) in the solution of ruptured virus were destroyed by treatment with DNase I, DNase I did not destroy the structural proteins of intact virions. A remaining viral protein in the DNase I-treated solution protects the DNA genome from degradation and we concluded that this protein is VP15, which is a DNA-binding protein. Our study highlights the extreme danger in producing vaccines from proteins obtained by ultrasonic rupture of viruses sincethe viral DNA genome is difficult to degrade and, if present, will lead to viral infection.

1. Introduction

White spot syndrome virus (WSSV) is an important viral pathogen that causes major economic loss to cultured shrimp producers around the world. The virus is not only present in shrimp but also occurs in other freshwater and marine crustaceans, including crabs and crayfish (Kim et al., 1998). Intact WSSV is rod-shaped and somewhat elliptical, 260–350 nm in length and 110–130 nm in diameter, with a long, tail-like envelope extension. The naked viral nucleocapsid is approximately 80 × 350 nm and has 15 spiral and cylindrical helices composed of 14 globular capsomers along its long axis, with a 'ring' structure at one terminus (Huang et al., 2001). The virus contains double stranded DNA, with an estimated size of 305,107 bp, and has 181 open reading frames encoding 39 structural proteins (Yang et al., 2001). Structural proteins are particularly important in the characterization of viruses because these are the first molecules to interact with the host and therefore play critical roles in cell targeting as well as triggering host defenses (Tsai et al., 2004). VP26, VP24 and VP15 are located in the nucleocapsid, while VP28 and VP19 are located in the envelope. VP28 plays a key role in infection of the tiger shrimp, *Penaeus monodon*, by WSSV (van Hulten et al., 2001). The virions have a complicated protein profile on sodium dodecylsulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) and, so far, SDS-PAGE, coupled with western blotting and protein N-terminal sequencing, has identified only six structural proteins, VP35, VP28, VP26, VP24, VP19 and VP15 (van Hulten et al., 2000).

Since the discovery of WSSV, much effort has been devoted to preventing infection. For example, DNA vaccines (Badhul Haq et al., 2012) and recombinant proteins (Thomas et al., 2016) have both been used to protect shrimp from WSSV infection. DNA vaccines inject naked DNA into the host and this is then translated into immunogenic proteins by the host cells (Badhul et al., 2012). A WSSV recombinant protein vaccine has been constructed in vitro and expressed in *Escherichia coli* to provide purified immunogenic proteins, which were then used to immunize the host (Thomas et al., 2016). Because WSSV virions are relatively large, we speculated that it may be possible to obtain immunogenic proteins that could prevent WSSV infection directly from

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the virions. Our initial goal was to obtain immunogenic WSSV proteins directly from the virions and test their safety. We found, however, that viral proteins and residual DNA genome released by ultrasonic rupture of WSSV can infect the host just as effectively as intact virions.

2. Materials and methods

2.1. Crayfish

Healthy crayfish (*procambarus clarkii*) (approximately 20–25 g each) were purchased from a local seafood market in Hangzhou, China. The crayfish were reared at 25℃ in fresh water aerated using an air pump and fed with commercial pellet feed at 5 % of body weight per day. Walking legs from randomly selected individuals were used for PCR assays to confirm that the crayfish were WSSV-free before experimental challenge.

2.2. Ultrasonic rupture of virions

A muscle sample (5 g) from moribund WSSV-positive crayfish was placed in a beaker together with 200 mL TNE Buffer (50 mM Tris; 400 mM NaCl; 5 mM EDTA, pH7.5) containing 1 mM serine protease inhibitor (phenylmethylsulphonylfluoride, Solarbio, China) (Zhu et al., 2009). The mixture was homogenized at 3000 rpm for 15 min using a high speed disperser and the homogenate was then centrifuged at 6000 *g* for 10 min using a flop-mounted ultrahigh speed freezing centrifuge. The supernatant was removed and centrifuged three more times under the same conditions. The virus was present in the supernatant which basically removes muscle tissue. The final supernatant was passed through a 300-mesh cell sieve, centrifuged at 6000 *g* for 30 min and then passed through a 450 nm filter membrane to provide a crude extract. Centrifugation at 30,000 *g* for 30 min then gave a white precipitate of WSSV virions. The precipitated virions were dissolved in 10 mL PBS. The dissolved viral particles were ruptured using an FS-200 T ultrasonic homogenizer (Shengxi, Shanghai, China). The solution of ultrasonically ruptured virions was centrifuged at 30,000 *g* for 30 min, and the supernatant is the ultimate rupture virions.

2.3. Filter membrane

We need to filter the above mentioned virus ultrasonic rupture fluid to ensure that the final liquid does not contain intact virus particles. We placed a 50 nm membrane(whatman, UK) in a filter extruder(PhD Technology LLC, USA). The ultimate rupture virions was passed filter membrane to provide a solution containing viral proteins and residual DNA genome of WSSV, which we termed WSSV + U.

2.4. Oral challenge

WSSV + U (1 mL) or a solution of purified WSSV (1 mL) was mixed with ordinary aquatic feed (6 g). After the WSSV + U or purified WSSV solution was evenly mixed with the feed, the feed was coated with propolis and mixed evenly using a glass rod, as previously described (Zhu et al., 2009). The feed was allowed to dry for 10 min and then fed to the crayfish. A negative control group received ordinary feed. Ten individuals in each group were raised separately and mortality was recorded every 12 h.

2.5. Treatment with DNase I

WSSV + U and a solution of WSSV were treated separately with DNase I (40 U/mL, Tiangen Company, Beijing, China) for 30 min at 37°C.Specially, The part of WSSV + U used for the PCR analysis should purified to remove proteins by Protease K and then treated with DNase I.

2.6. PCR analysis for WSSV

The DNA of WSSV was purified using a DNA purification kit (TaKaRa, Tokyo, Japan), which was used as the DNA template. The DNA of WSSV derived from the sample solution that requires PCR detection. The samples were tested using four primer sets: VP19, (5′-GAGGAACAGAAGAGCGGACC-3′ and 5′-ATGGCCACCACGACTAA CAC-3′); VP26, (5′-CAATAACAGTTGGCCG-3′ and 5′-TGGAATTTGGCA ACCTAACA-3′); VP28, (5′-ATGGATCTTTCTTTCACTCTTTC-3′ and 5′-CTCGGTCTCAGTGCCAGAGT-3′); DNA polymerase (5′-AGTGGGTG GAACAATGTAGC-3′ and 5′-TCTACAGATTGCTCCTTCTC-3′). Sections of VP19, VP26, VP28 and the DNA polymerase gene were amplified to determine whether the important genes of WSSV are still present in WSSV + U. The VP28 gene, in particular, has previously been was used to screen for WSSV-positive animals (Zhu et al., 2009). PCR was performed with the VP19, VP26, VP28 and DNA polymerase primer pairs using the following protocol: 5 min at 94 ℃,35 cycles at 94 ℃ for 15 s,45–65℃(depending on the annealing temperature of the primer) for 30 s and 72℃ for 30 s, followed by elongation at 72 ℃ for 5 min. The PCR products were analyzed by electrophoresis on 1 % agarose gels, stained with Golden View (Aidlab, Shanghai, China) and visualized using an ultraviolet transilluminator.

2.7. Electron microscopy and SDS-PAGE

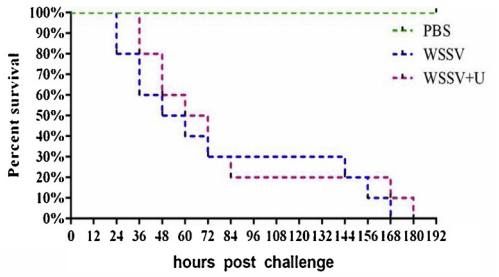
WSSV virions and WSSV + U particles were negatively stained with 2 % sodium phosphotungstate (pH 7.0) on collodioncarbon coated grids [9] and observed using a JEOL 1010 transmission electron microscope (JEOL, Japan), operating at 70 kV. WSSV virions and WSSV + U were also digested with DNase I. WSSV virions and WSSV + U, before and after digestion by DNase I, were analyzed by SDS-PAGE, as previously described (Laemmli, 1970). The gels were stained with Coomassie brilliant blue R-250 (0.1 % solution in 1 % acetic acid and 40 % methanol). A premixed protein molecular weight marker (15–170 kDa, Thermo Fisher, USA) was co-electrophoresed to determine the molecular weights of the WSSV proteins.

2.8. Analysis of infection and survival

Seven groups of at least ten crayfish were randomly transferred into separate tanks containing freshwater. Each group received a different injection (100 μL) into the soft tissue between the second and third dorsal segments as follows: PBS (control), WSSV, WSSV + U, WSSV + U digested with DNase I (WSSV + U + DNase I),purified WSSV solution digested with DNase I (WSSV + DNase I), WSSV + U incubation at 37 ℃ for 30 min (WSSV + U + incubation)or purified WSSV solution incubation at 37 ℃ for 30 min (WSSV + incubation). The number of live crayfish in each group was counted every 12 h. Dead crayfish were removed immediately and dirty water was replaced with clean freshwater every day. To ensure the statistical accuracy of the survival analysis, each group contained at least ten individuals. The PCR method used for analysis of WSSV has been previously described (Zhu et al., 2009).

2.9. Analysis of WSSV replication

For analysis of WSSV copy numbers, at least three crayfish (as a technical repeat) were collected from the control and WSSV challenge groups 48 h after injection. WSSV copy numbers were determined as previously described (Baozhen et al., 2017; Wang and Zhu, 2016). Briefly, DNA was extracted from a mixture of hemocytes collected from three randomly selected crayfish in each group, and TaqMan real-time quantitative PCR was performed using WSSV specific primers (5′-TTG GTTTCATG CCCGAGATT-3′ and 5′-CCTTGGTCAGCCCCTTGA-3′) and TaqMan fluorogenic probe (5′-FAM-TGCTGCCGTCTCCAA-TAMRA-3′).



groups	PBS	WSSV	WSSV+U
mortality	0	100%	100%

Fig. 1. Survival of crayfish after oral challenge. Crayfish were fed ordinary food mixed with PBS (PBS), ordinary feed mixed with WSSV virions (WSSV) or ordinary feed mixed with ultrasonically ruptured virus (WSSV + U). The table shows statistical mortality rates.

2.10. Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD). Data from three independent experiments were analyzed by one-way analysis of variance to calculate the means and standard deviations of the triplicate assays. Statistical differences were estimated using one-way ANOVA followed by least-significant differences (LSD) and Duncan's multiple range test. The differences between the different treatments were analyzed by multiple *t*-test method. All statistics were measured using SPSS software version 19 (IBM, USA). A probability level of 0.01 was used to indicate statistical significance ($P < 0.01$). All graphs were made using Microsoft GraphPad 5.0.

3. Results

3.1. Survival after oral infection

The mortality rate of crayfish fed both purified WSSV and WSSV + U was 100 % (Fig. 1), whereas the negative control group (fed PBS) survived. The viral proteins and genome in WSSV + U are thus intact and retain sufficient biological activity to cause crayfish death comparable to purified WSSV.

3.2. PCR analysis of ultrasonically ruptured WSSV

PCR analysis confirmed the presence of important WSSV genes (VP19, VP26, VP28 and DNA polymerase) in WSSV + U (Fig. 2A). These viral genes were also found to be present in WSSV + U following treatment with DNase I (Fig. 2B). The bands attributable to VP19, VP26 and VP28, as well as the DNA polymerase nucleic acid fragment, all disappeared when the proteins were removed from the WSSV + U using a DNA purification kit before treatment with DNase I (Fig. 2C). These results show that the four viral genes, VP19, VP26, VP28 and DNA polymerase, as well as WSSV proteins, are present in WSSV + U and that the viral proteins may protect viral DNA from degradation by DNase.

3.3. SDS-PAGE profiles of intact and ultrasonically ruptured WSSV

SDS-PAGE confirmed the presence of all four important WSSV proteins, VP15, VP19, VP26 and VP28, in WSSV + U (Fig. 3A, lane 2). Surprisingly, VP19, VP26 and VP28 protein disappeared following treatment with DNase I, although VP15 proteins remained in the solution (Fig. 3A, lane 4). The four protein bands were all present in WSSV virions treated with DNase I (Fig. 3A, lane 3) and, to exclude the influence of incubation treatment in the DNase I digestion process, the experiment was repeated with WSSV + U incubation at 37 °C for 30 min. Treatment at 37 °C for 30 min did not affect the presence of viral proteins (Fig. 3B, lanes 3 and 4), and SDS-PAGE (Fig. 3B, lanes 1 and 2) confirmed that all the important WSSV proteins remained in the solution of ruptured WSSV. Similarly, VP15 protein band was still present in WSSV + U following treatment with DNase I (Fig. 3A, lanes 4). Another important finding is that treatment with DNase I did not destroy the proteins of intact virions but did destroy viral proteins in WSSV + U.

3.4. Electron microscopy of intact and ultrasonically ruptured WSSV

Electron microscopy of purified WSSV showed relatively intact virions (Fig. 4A). Electron microscopy of WSSV + U, on the other hand, showed no intact virions (Fig. 4B), confirming that WSSV + U contains only viral proteins and viral DNA.

3.5. Measurement of WSSV copy number in challenged crayfish

To investigate the infectivity of ruptured virus, WSSV copy numbers were measured 48 h after challenge with intact WSSV and virus that had been ultrasonicated for different periods of time. The viral copy number of the intact WSSV group (3.00E + 05) was lower than that of ruptured virus following ultrasonication for4 h (9.04E + 05, P = 0.00273), 8 h (1.88E + 06, P = 5.122E-05), 12 h (2.79E + 06, P = 0.002) or 15 h (8.33E + 05, P = 0.00755) (Fig. 5). Ultrasonication of WSSV for only 1 h did not significantly increase the viral copy number.

3.6. Survival of challenged crayfish

The mortality rate of challenged crayfish was used to determine the infectivity of WSSV following different treatments. No deaths occurred in the control (PBS) group during the 192 h experimental period. Following injection of WSSV that had been ultrasonicated for 1 h, 4 h, 8 h, 12 h and 15 h, however, the mortality rate of the crayfish was 100 % 168 h after challenge (Fig. 6). There were no deaths among crayfish in the WSSV + U + DNase I group (Fig. 7). The death of crayfish in the WSSV group began at 72 h and 100 % mortality was reached 168 h after challenge. In the WSSV + U group, the death of crayfish began at 72 h and 100 % mortality was reached 180 h after challenge (Fig. 7). These data suggest that ultrasonically ruptured WSSV can infect and kill crayfish just as effectively as purified WSSV. In the WSSV + DNase I group, the death of crayfish began at 72 h and all had died by 192 h after challenge. Moreover, Incubation ruptured virus at 37 °C for 30 min did not alter the mortality rate of the crayfish, which reached 100 %, as in the WSSV group (Fig. 7). These data show that DNase I was unable to digest the DNA genome of WSSV when viral proteins were present in the solution.

3.7. Detection of WSSV in challenged crayfish using PCR

PCR showed that, after injection with WSSV + U, both dead and live crayfish were infected with WSSV. Crayfish injected with WSSV that had been both ultrasonically ruptured and digested with DNase I, on the other hand, did not show any sign of infection, as determined by PCR (Fig. 8). These results show that WSSV + U can infect crayfish just as well as intact WSSV, but that further digestion with DNase I destroys the ability of WSSV + U to infect crayfish.

4. Discussion

The initial purpose of our study was to obtain immunogenic viral proteins and test their safety. We first tested safety using a feeding challenge and found that WSSV + U was 100 % fatal to crayfish. We then tried to explain why a solution of ruptured WSSV can still infect crayfish. Ultrasonication and filtration did not prevent the virus from infecting crayfish but virus that had been both ruptured and subjected

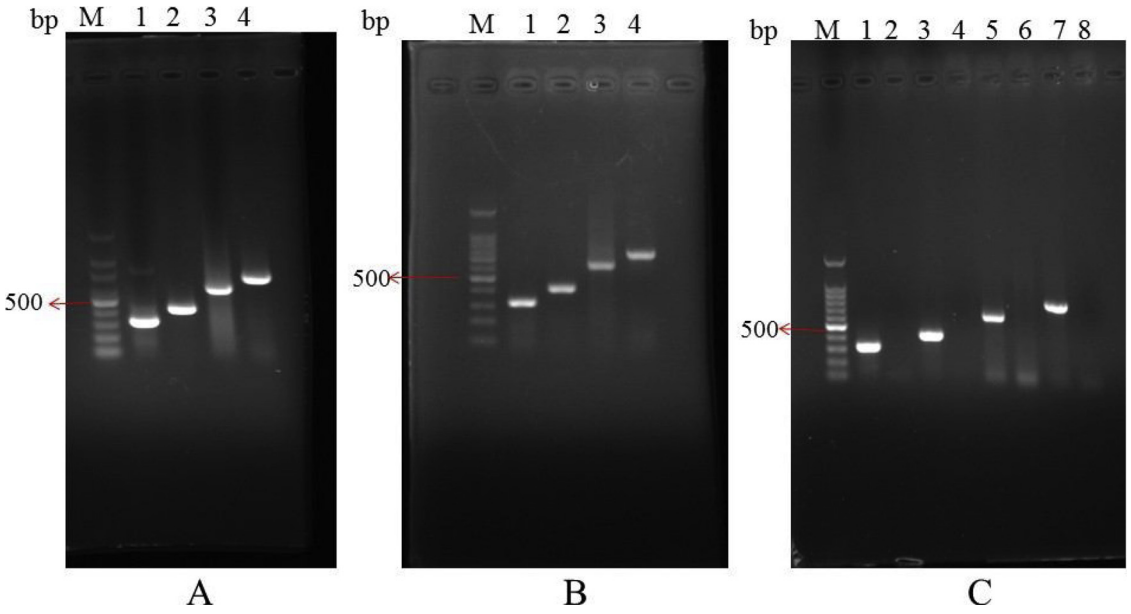


Fig. 2. Nucleic acid electrophoresis profiles of ultrasonically ruptured WSSV. (A) A solution of ultrasonically ruptured WSSV (WSSV + U) was purified and then used as a DNA template and amplified by PCR. Lane1, VP19 (318 bp); Lane 2, VP26 (420 bp); Lane 3, VP28 (612 bp); Lane 4, DNA polymerase (756 bp); Lane M, DNA marker (100 bp DNA ladder). (B) A solution of WSSV + U was digested with DNase I, purified(No protease K), and then used as a DNA template and amplified by PCR. Lane1, VP19 (318 bp); Lane 2, VP26 (420 bp); Lane 3, VP28 (612 bp); Lane 4, DNA polymerase (756 bp). (C) Four DNA primers were used as the destination band to confirm that DNA is protected by proteins. Lanes 1 and 2, VP19 (318 bp); Lanes 3 and 4, VP26 (420 bp); Lanes 5 and 6, VP28 (612 bp); Lanes 7 and 8, DNA polymerase (756 bp). Lanes 1, 3, 5 and 7, WSSV + U was digested with DNase I. Lanes 2, 4, 6 and 8, WSSV + U was purified to remove proteins(by protease K) and then digested with DNase I.

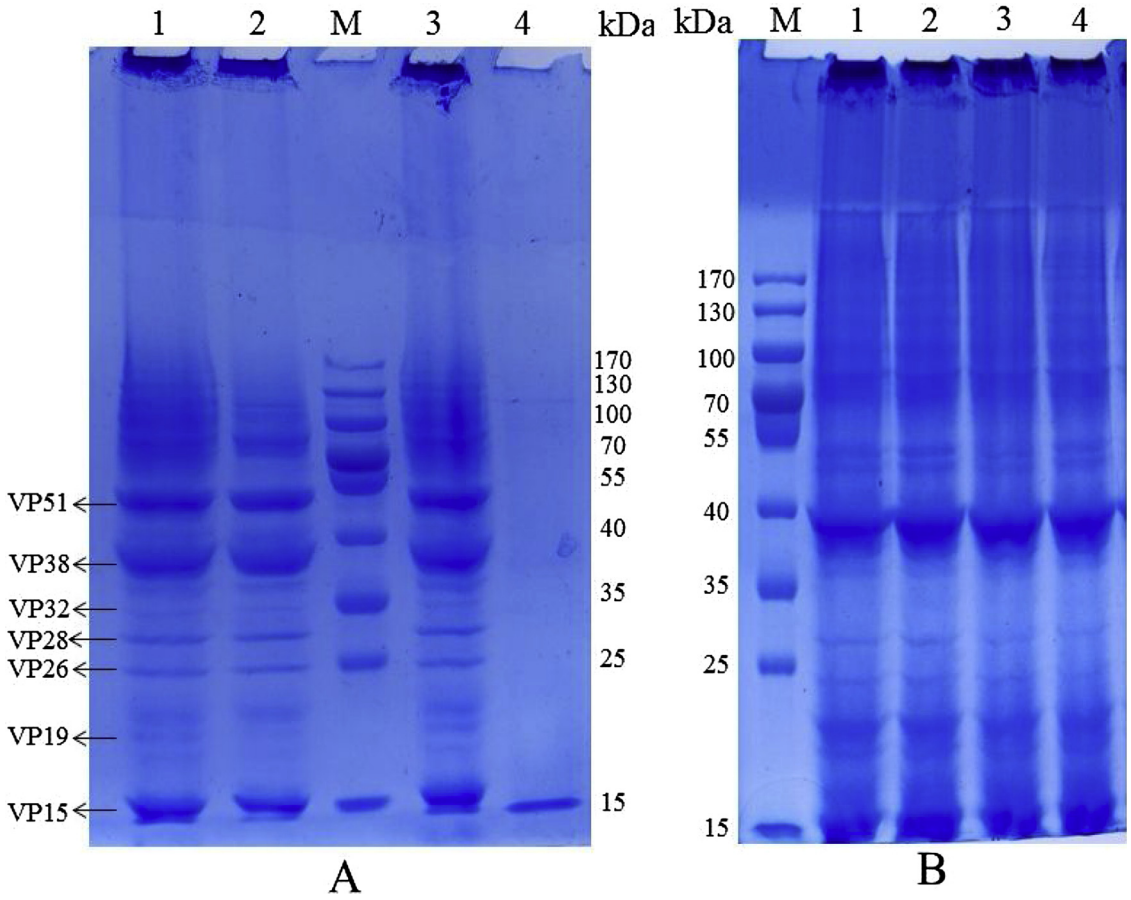


Fig. 3. SDS-PAGE profiles of WSSV and ultrasonically ruptured WSSV. (A) Lane 1, WSSV; Lane 2, ultrasonically ruptured WSSV (WSSV + U); Lane 3, WSSV digested with DNase I; Lane 4, WSSV + U digested with DNase I; Lane M, protein molecular weight marker (kDa). (B) SDS-PAGE profile confirming effect of incubation to 37 ℃ for 30 min on WSSV + U. Lanes 1 and 2, WSSV + U; Lanes 3 and 4, WSSV + U incubation to 37 ℃ for 30 min; Lane M, protein molecular marker (kDa).

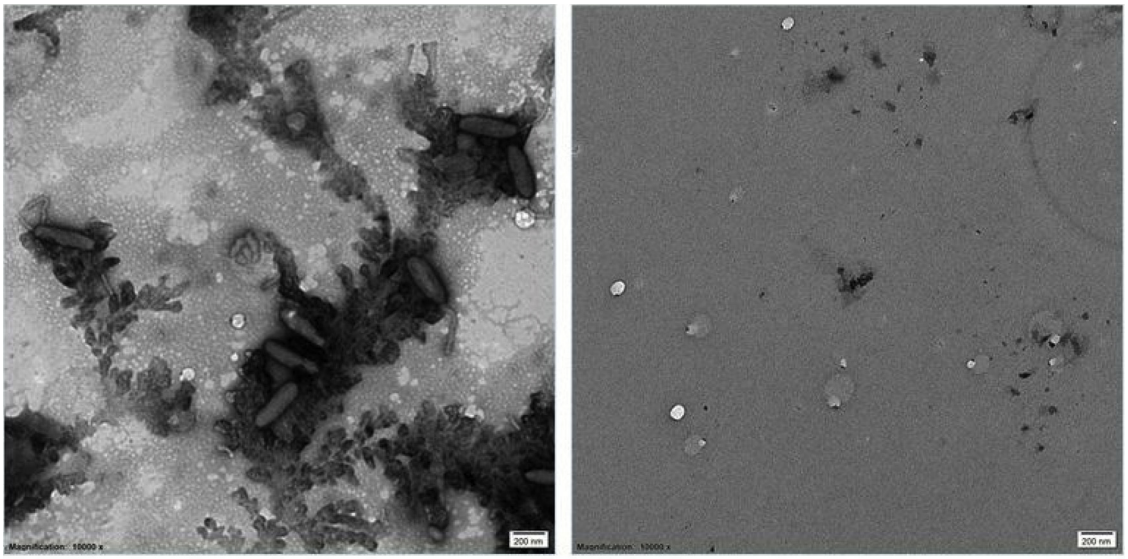


Fig. 4. Electron micrographs of WSSV and ultrasonically ruptured WSSV. (A) Electron micrograph of negatively stained intact WSSV virions (10,000×); Scale bar = 200 nm. (B) Electron micrograph of ultrasonically ruptured WSSV (10,000×); Scale bar = 200 nm.

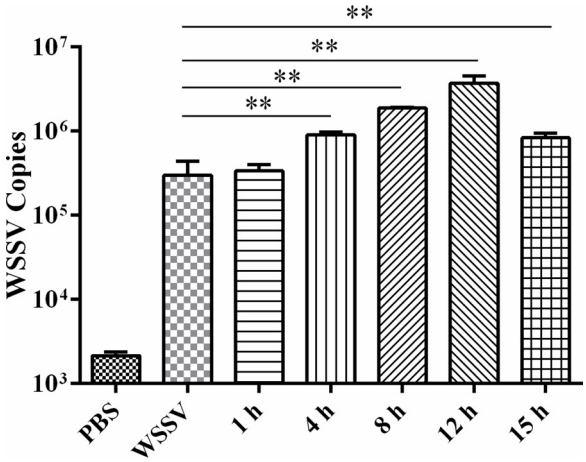
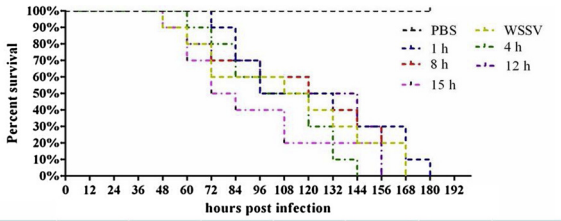
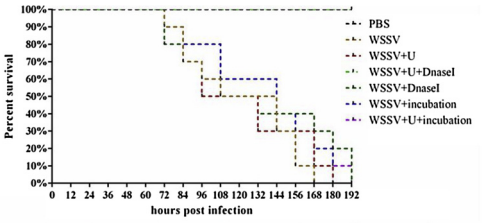


Fig. 5. WSSV copy numbers(The amount of WSSV in the DNA of the crayfish blood sample), detected 48 h post challenge, in crayfish injected with PBS, WSSV and ultrasonically ruptured WSSV (WSSV + U). The times shown for WSSV + U represent the duration of ultrasonication. Each experiment was repeated three times and the figure shows mean values, with standard deviations as error bars. Asterisks indicate the significance of differences between groups (*P < 0.05, **P < 0.01), analyzed by two way ANOVA and multiple *t*-test method.



groups	PBS	WSSV	1 h	4 h	8 h	12 h	15 h
mortality	0	100%	100%	100%	100%	100%	100%

Fig. 6. Survival of crayfish injected with PBS, WSSV and ultrasonically ruptured WSSV (WSSV + U). The times shown for WSSV + U represent the duration of ultrasonication. Each group contained at least 10 individuals. The table shows statistical mortality rates.



groups	PBS	WSSV	WSSV+U	WSSV+U+ DnaseI	WSSV+ DnaseI	WSSV+ incubation	WSSV+U+ incubation
mortality	0	100%	100%	0	100%	100%	100%

Fig. 7. Survival of crayfish injected with PBS (control), WSSV, ultrasonically ruptured WSSV (WSSV + U), ultrasonically ruptured WSSV digested with DNase I, (WSSV + U + DNase I),WSSV digested with DNase I (WSSV + DNase I), WSSV + U incubation at 37°C for 30 min (WSSV + U + incubation), and WSSV incubation at 37°C for 30 min (WSSV + incubation). Each group contained at least 10 individuals. The table shows statistical mortality rates.

to DNase I digestion was not infectious, although the specific mechanisms underlying this difference were unclear. Further experiments were carried out to test our hypothesis that the viral proteins and DNA of WSSV + U allow the ruptured virus to infect crayfish just as effectively as intact virions. We found that, no matter how long the ultrasound treatment lasted, solutions of ruptured WSSV all caused 100 % mortality in crayfish. The copy number of WSSV in the infected crayfish at 48 h increased with increasing ultrasound time, and did not decrease until the duration of ultrasound treatment exceeded 15 h. Only ultrasound treatment for 1 h gave WSSV copy numbers that were lower than those of the intact WSSV control group. We thus chose an ultrasound time of 1 h for further experiments.

Ultrasonically ruptured WSSV was analyzed using PCR and SDS-PAGE. PCR showed that the important genes, VP19, VP26, VP28 and DNA polymerase, of WSSV were present in the solution of ruptured WSSV and that these persisted even after digestion of viral DNA with DNase I. Since DNA that is bound to protein is known to be protected from degradation by DNase I (Galas and Schmitz, 1978), the WSSV genome is most likely bound to specific viral proteins, which protect it from digestion by DNase I. There have been other reports of proteins

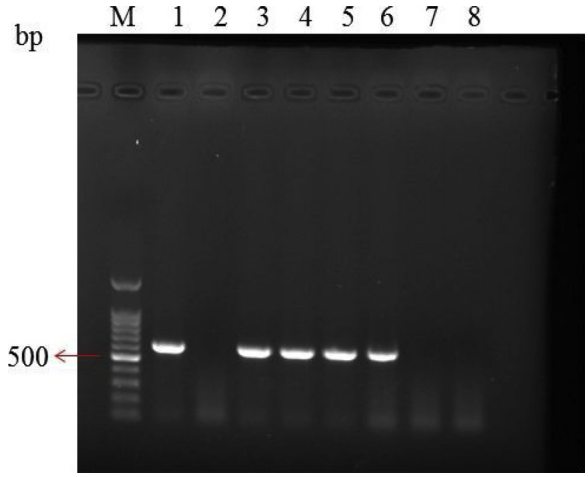


Fig. 8. PCR detection of WSSV in challenged crayfish. Total DNA was extracted from walking legs of surviving and dead crayfish. Lane M, DNA marker (100 bp DNA ladder); Lane 1, positive control; Lane 2, negative control; Lanes 3 and 4, crayfish that died after injection of ultrasonically ruptured WSSV (WSSV + U); Lanes 5 and 6, crayfish that survived after injection of WSSV + U; Lanes 7 and 8, crayfish that survived after injection of WSSV + U digested with DNase I.

protecting nucleic acids from degradation by DNases (Williams et al., 1981). To test this hypothesis, we removed the proteins using a DNA purification kit and then treated the solution with DNase I. The viral nucleic acid bands for VP19, VP26, VP28 and DNA polymerase were not present in WSSV + U following purification and digestion with DNase I. This confirms that the proteins protect the viral nucleic acids from digestion by DNase I and that the nucleic acids are digested by DNase I after removal of the proteins. SDS-PAGE showed that some immunogenic proteins, such as VP15, VP19, VP26 and VP28, are present in WSSV + U but that only VP15 persist after digestion with DNase I. The influence of incubation treatment in the DNase I digestion process on viral proteins was successfully excluded. DNase I was thus unable to digest the viral proteins of intact WSSV but could digest viral proteins (except VP15) in ruptured virus. Since previous studies have shown that DNA binding proteins protect viral DNA from degradation by DNase (Greetham et al., 2015) and VP15 is a DNA binding protein that is located in the nucleocapsid of WSSV (Escobedo-Bonilla et al., 2008), we conclude that VP15 may bind viral DNA and protect it from digestion by DNase I.

Previous studies have shown that intact WSSV virions are 210–380 nm in length and 70–167 nm in maximum diameter (Escobedo-Bonilla et al., 2008). Since WSSV + U had been filtered through a 50 nm membrane to ensure that the solution did not contain any intact virions, our results show that viral proteins and residual DNA genome can infect a host just as effectively as intact virions. This is likely because WSSV is a relatively large virus with a genome made up of DNA, which is more stable than RNA. We also found that DNase I can destroy a viral protein in WSSV + U that may protect viral DNA from degradation by DNase I. We concluded that this protein is VP15. After treatment with DNase I, the solution of ultrasonically ruptured WSSV, which contained only VP15 protein and viral DNA, was unable to infect crayfish. We also found that the proteins of ruptured, but not intact, WSSV could be digested by DNase I.

Our study highlights the dangers of producing immunogenic proteins by ultrasonic rupture of DNA viruses with an intact membrane protein structure and then using these to immunize animals. Because viral DNA is protected by DNA binding proteins, it is more difficult to digest with DNase than free DNA, leaving hidden dangers for infection and pathogenic diseases. The most effective way to inactivate WSSV is to use binary ethylenimine, which destroys viral nucleic acids but not viral proteins (Zhu et al., 2009).

Ethical statement

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang A & F University (Hangzhou, China). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All experiments in this study did not involve any mammals, only invertebrate crayfish was used in experiment. This article does not contain any studies with human participants performed by any of the authors

Authors' contributions

FZ and YL conceived and designed research. YL and YX conducted experiments. YL and FZ analyzed data. FZ and YL wrote the manuscript. All authors read and approved the manuscript.

CRediT authorship contribution statement

Yongyong Lai: Investigation, Data curation, Writing - original draft, Validation, Software. **Fei Zhu:** Investigation. **Yinglei Xu:** Conceptualization, Data curation, Writing - review & editing, Methodology.

Declaration of Competing Interest

There are no patents, products in development or marketed products to declare. This does not alter our adherence to all the gene policies on sharing data and materials. The authors declare that they have no competing interests. Yongyong Lai declares that he has no conflict of interest. Fei Zhu declares that he has no conflict of interest. Yinglei Xu declares that she has no conflict of interest.

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