

# Enhanced production of Canine parvovirus VP2 protein using improved Baculovirus expression system and its immunogenicity

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## Research article

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# Abstract

**Background :** Canine parvovirus (CPV) is now recognized as a serious threat to dog industry worldwide. Vaccination remains the principal tool to control CPV infection. However, due to low yield, production of VP2 protein of CPV in baculovirus expression system remains challenging. The aim of this study was to increase the VP2 protein production by using a improved baculovirus expression system (Multibac) and evaluate the immunogenicity of the purified VP2 protein in mice.

**Results:** The results showed that CPV VP2 protein was successfully expressed in the improved baculovirus expression system efficiently. A high level of expression of the full length VP2 protein was achieved using our modified system. The recombinant virus carrying two copies of VP2 showed the highest expression level, with a productivity of 186 mg/L, which is about 1.4-1.6 fold that of the recombinant viruses carrying only one copy. The purified protein could react with Mouse anti-His tag monoclonal antibody and Rabbit anti-VP2 polyclonal antibody with good reactogenicity. The mice were then immunized with purified full length VP2 protein to evaluate its immunogenicity. After vaccination, VP2 protein could induce the mice produce high level of hemagglutination inhibition antibodies.

**Conclusions:** Full length CPV VP2 protein was successfully expressed at high level and purified efficiently. And it stimulated mice to produce high level of antibody. The full length VP2 expressed in this study could be used as a putative economic and efficient subunit vaccine against CPV infection.

## Background

The canine parvovirus disease is an acute and highly contagious infectious disease caused by canine parvovirus (CPV), which is manifested as hemorrhagic enteritis in dogs of all ages and fatal myocarditis in young puppies aged 2–3 week<sup>[1, 2]</sup>. CPV is of significant economical importance as it can cause large losses in breeding farms<sup>[3]</sup>. Vaccination is considered as the most effective method to prevent and control CPV infection. Current commercial vaccines against CPV are mainly inactivated and attenuated virus<sup>[4]</sup>. However, their application is limited by the risk of incomplete inactivation or reversion to virulence. In addition, large scale production of the virus in established cell lines is usually expensive and laborious<sup>[5]</sup>. To overcome these problems, attempts were made to develop alternative vaccines, such as subunit vaccines.

Canine parvovirus is a small, non-enveloped virus containing linear ssDNA (single-stranded DNA) genomes of approximately 5 kb, which encoding three structural proteins (VP1, VP2 and VP3) and two non-structural proteins (NS1 and NS2) <sup>[6, 7]</sup>. As the main component of the virion, VP2 proteins constitute about 90% of viral capsid and play an important role in the transmission and infection of CPV <sup>[8, 9]</sup>. In addition, VP2 protein contains several important B cell antigen epitopes in the N-domain and loop-domain, which could induce effective neutralizing antibody during the infection of CPV<sup>[10]</sup>. Therefore, the VP2 protein is generally considered as a potent protective antigen and a promising candidate protein for the CPV genetic engineering vaccine.

The baculovirus expression vector system (BEVS) is an excellent eukaryotic expression system with advantages of the expression of foreign proteins and the ability of post-translational modification, thus it has been widely used in the production of recombinant proteins and subunit vaccines<sup>[11, 12]</sup>. An improved baculovirus express system based on MultiBac system is constructed in our previous study, the recombinant baculovirus carry multiple expression cassettes could be produced rapidly and simply by using Bacmid-containing diminopimelate-

auxotrophic *Escherichia coli* infecting insect cells<sup>[13, 14]</sup>. In this study, the improved baculovirus expression system was used to produce VP2 protein by co-expression two copies of the vp2 gene, and the immunogenicity of the purified protein in mice was investigated.

## Methods

### Materials

*Spodoptera frugiperda* (Sf9) cell lines were grown and maintained at 27°C in SF900 medium (Invitrogen Corporation, USA). Plasmid pET28a-CPV-VP2 containing CPV vp2 gene (GenBank: MK518021.1) and Rabbit anti-VP2 polyclonal antibody were kindly provided by Dr. Qinghai Tang<sup>[15]</sup>. The improved baculovirus expression system including the vector pYBDM-IM (an IRES driven mcherry fragmemnt is insert into the sphI and kpnI sites of pFBDM), *Escherichia coli* AcMultiBacmid/rSW106/asd<sup>-</sup>/inv<sup>+</sup> were constructed as described previously<sup>[14]</sup>.

### Construction Of Recombinant Transfer Vectors

To facilitate expression of the VP2 genes in insect cells, full-length *vp2* gene was amplified by PCR from plasmid pET28a-CPV-VP2. The primers used for *vp2* gene amplification were VP2 Forward primer (5'-GGATCCCGGGATGAGTGATGG AGCAGT-3' containing one site of BamHI and SmaI each) and VP2 Reverse primer (5'-TCTAGAGTCTGACTTAATATAATTTCTAG-3' containing one site of XbaI and Sall each). The PCR product encoding VP2 ORF was treated with BamHI/XbaI and cloned into the multiple cloning sites (MCS) under the polyhedrin promoter, the resultant plasmid was designated pYBDM-IM-ph-VP2. Similarly, the amplified VP2 fragment was inserted into SmaI/XhoI restriction sites, downstream from the p10 promoter of the vector pYBDM-IM or pYBDM-IM-ph-VP2, to generate the plasmid of pYBDM-IM-p10-VP2 or pYBDM-IM-2VP2 respectively (Fig. 1). All constructs were confirmed by DNA sequencing.

### Generation Of Recombinant Bacmids

Individual recombinant plasmids were transformed into *E. coli* AcMultiBacmid/rSW106/asd<sup>-</sup>/inv<sup>+</sup> competent cells, allowing the transposition of CPV VP2 gene to Bacmid between the mini-Tn7 element on the transfer vector and the mini-att Tn7 target site on the Bacmid to generate the recombinant bacmids.

The obtained recombinant bacmids were characterized by white–blue and PCR screening using vp2 forward primer and M13 reverse primer to confirm the insertion of the VP2 gene.

## Production Of Recombinant Baculoviruses

*E.coli* AcMultiBacmid/rSW106/asd-/inv + cells with recombinant VP2 genes were grown in LB broth supplemented with 0.5 mM DAP, 10 µg/ml tetracyclines, 7 µg/ml gentamicin, 25 µg/ml spectinomycin and 50 µg/ml kanamycin at 30 °C. The overnight culture were collected by centrifugation (3000 × g) and resuspended in distilled ultrapure water for three times. The pellet was resuspended in SF900 medium and adjusted to different densities (10<sup>5</sup>-10<sup>8</sup> cells/mL). Sf9 cells at 10<sup>5</sup>/ml were incubated overnight in 24-well plates (70–80% confluent single layer). After removing the medium, 500µL bacterial cells at different concentrations were added to each well to infect sf9 cells. After culturing at 27 °C for 4–5 h, each well was washed three times. 500µL fresh SF900 medium was then added and incubated for 3–5 days. When the mCherry fluorescence was observed using reverted fluorescence microscopy (507 nm excitation), indicating that sf9 cells were infected successfully. The supernatant containing recombinant baculovirus was harvested and infected again with sf9 cells. Titters of the baculovirus were determined by a plaque assay.

## Production And Purification Of Cpv Vp2 Protein

2 × 10<sup>8</sup> of sf9 insect cells were grown in 100 mL volumes in 500 ml baffled glass flasks and incubated in a rotary shaker at 80 rpm and 28 °C. The initial cell density was 2.0 × 10<sup>5</sup> cells/mL. The cultures were infected with the recombinant baculoviruses, Ac-IM-ph-VP2, Ac-IM-p10-VP2 and Ac-IM-2VP2 at a multiplicity of infection (MOI) of 5. At 96 hours post infection (h.p.i.), the cells were harvested by centrifugation (1000 × g, 10 min, 4 °C) and lysed by sonication. The lysed Sf9 cells were centrifuged at 12,000 × g for 10 min, the supernatant was purified using Ni-NTA agarose according to the manufacturer's instructions. The concentration of VP2 protein was measured with the His Tag ELISA Detection Kit (GenScript).

## Western blot analysis of recombinant CPV VP2 protein

The expression of CPV VP2 proteins was determined by Western blot assay. The supernatants of samples, which were purified as described above, were separated by 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes and then blocked with 10% skimmed milk for 2 h. After five washes with PBST (PBS plus 0.05% Tween-20) for 5 min each time, the membranes were incubated overnight with mouse anti-His monoclonal antibody (1: 5000 dilution) and rabbit anti-VP2 polyclonal antibody (1: 200 dilution) at 4 °C, respectively. After five washes with PBST for 5 min each time, the membranes were incubated with HRP-conjugated goat anti mouse IgG antibody (1: 2000 dilution) and HRP-conjugated

goat anti rabbit IgG antibody (1: 2000 dilution), respectively. After five washes with PBST for 5 min each time, detection was performed with DAB (3, 3'-diaminobenzidine) solution (Boshide, Wuhan, China).

## Animals Experiment

The purified VP2 proteins were adjusted at a final concentration of 1 mg/mL. All animal protocols were performed in accordance with the guidelines of the ethical committee of Nanyang Normal University. The mice were purchased from Wuhan Biological products Research Institute Co., Ltd. Fifteen BALB/c mice (6-week-old, female) were randomized into three groups (n = 5). Mice in group A were intramuscularly injected with 100 $\mu$ L of purified VP2 protein mixed with Freund's adjuvant. Mice in group B was injected with 100 $\mu$ L commercial live-attenuated vaccine as a positive control. Mice in group C were injected with 100 $\mu$ L/mouse of PBS as a negative control. Mice from all groups were injected 2 times at 2-week intervals (Days 0 and 14). Blood samples were collected from the forelimb vein at 0, 7, 14, 21 and 28 days post-vaccination (dpv).

## Hemagglutination Inhibition (hi) Test

Serum samples from mice were inactivated at 56 °C for 30 min, then serially diluted two-fold (25 $\mu$ L of serum) in V-96-well plates. Subsequently 25 $\mu$ L 4 hemagglutination units of CPV were added. The mixture were incubated at 37 °C for 1 h after which 50 $\mu$ L 0.8% pig erythrocytes were added. Hemagglutination inhibition antibody titers were expressed as the reciprocal of the highest serum dilution that completely inhibited hemagglutination.

## Statistical analysis

The experimental data were analyzed using a one-way analysis of variance (ANOVA), combined with Tukey's post hoc test.  $P < 0.05$  was considered statistically significant.

## Results

### Identification Of The Recombinant Bacteria Containing Positive Bacmids

After transferring the recombinant donor plasmids into competent *E. coli* AcMultiBacmid/rSW106/asd-/inv + cells, recombinant bacmids were obtained and the correct insertion of the target genes was confirmed by PCR analysis. Recombinants were amplified with one gene-specific primer and one M13 primer. If transposition has occurred, the recombinant baculovirus could amplify a 2380 bp band (including the size of vp2 gene 1777 bp and primers 603 bp) or 4157 bp band (including the size of two copies of vp2 gene 3554 bp and primers 603 bp) whereas non-recombinant baculovirus could not amplify any band, suggesting that the vp2 gene had been successfully recombined into a bacmid shuttle vector (Fig. 2).

# Production of recombinant baculovirus

The recombinant bacteria carrying positive bacmids was used to infect Sf9 cells to produce recombinant baculovirus as described previously [10]. The Sf9 cells were infected with the recombinant baculovirus at an MOI of 0.1 for virus amplification. When examined by fluorescence microscopy, it was found that obvious red fluorescence in virus-infected sf9 cells (Fig. 3). The presence of the *vp2* gene in recombinant viruses was confirmed by direct PCR of the viral genomic DNA. As expected, PCR amplification of the recombinant viral genomic DNA revealed a specific band of 1.77 kb. Non-specific amplification was not observed in the negative control sample (Fig. 4). These results indicated that *vp2* gene was inserted into recombinant baculovirus.

## Expression of recombinant VP2 protein in insect cells

$2 \times 10^8$  of Sf9 cells (100 mL cell culture) were infected respectively with the recombinant baculoviruses Ac-IM-ph-VP2, Ac-IM-p10-VP2 and Ac-IM-2VP2 at an MOI of 5 for protein expression. At 96hpi, the expression of VP2 protein in different virus-infected insect cells was determined by SDS-PAGE, and then the expression level of VP2 protein was quantified with the His Tag ELISA Detection Kit. As shown in Fig. 5A, the recombinant VP2 band had a size of 56 kDa, which correlates to its expected size. In addition, VP2 expression is considerable higher in Sf9 cells infected with Ac-IM-2VP2 (0.186 mg/mL cell suspension or 186 mg/L) when compared to Ac-IM-ph-VP2 (0.139 mg/mL, or 139 mg/L) and Ac-IM-p10-VP2 (0.117 mg/mL or 117 mg/L), respectively (Fig. 5B). A high expression level of the full length VP2 protein was achieved when using our modified system. And the recombinant virus with two copies of VP2 showed the highest level, which is about 1.4–1.6 fold that of the virus with one copy. Therefore, Ac-IM-2VP2 was used as seed virus to infect sf9 cells in the following experiments.

## Purification And Characterization Of Recombinant Vp2 Protein

Based on its C-terminal His-tag, the recombinant VP2 protein was purified by Ni<sup>2+</sup>- affinity chromatography. The SDS-PAGE results showed that a specific band at 65 kDa was observed in the elution buffer (Fig. 6A), and the purity of recombinant VP2 protein was 90.5%. Western blotting analysis further confirmed that the purified protein is VP2 protein by reacting with mouse anti-His monoclonal antibodies (Fig. 6B) or rabbit anti-*vp2* polyclonal antibodies (Fig. 6C).

## Serum Antibody Against Recombinant Vp2 Protein In Mice

Serum samples collected from mice were subjected to the HI test to determine the antibody titer against CPV. As shown in Fig. 7, all PBS buffer-inoculated mice were negative for HI antibody throughout the study. In group A mice that were vaccinated with VP2 protein, HI antibody was positive at 7 dpv and

steadily increased thereafter, and then HI antibody reached to the highest (1: 2<sup>8.4</sup>) at 28 dpv. These results indicated that the VP2 protein could induce specific antibody response as expected.

## Discussion

VP2 protein of Canine parvovirus (CPV) is the major determinant of eliciting specific neutralizing antibodies and has been used as recombinant protein-based subunit vaccine in previous studies<sup>[6, 16–18]</sup>. In addition, subunit vaccines comprise selected pathogen specific antigens are a safe alternative to traditional whole organism vaccines<sup>[19]</sup>. In this study, we expressed the full length VP2 capsid protein as a first step in the development of CPV subunit vaccines. An improved baculovirus expression system was used to increase the expression of VP2 protein. Further, the immunogenicity of purified VP2 proteins in mice were evaluated. The results showed that the VP2 protein was expressed in Sf9 cells at very high level and the purified recombinant VP2 protein could induce strong immune responses in mice.

Previous studies reported that VP2 protein can be produced in E.coli and insect expression system<sup>[17–18, 20–23]</sup>. Although E.coli expression system has been used widely for recombinant proteins production in laboratory and industrial scale due to its simplicity and economy, however, the solubility of target proteins is low<sup>[24–26]</sup>. Particularly, VP2 protein has high molecular weight and weak hydrophilicity, which make it prone to aggregate easily in inclusion bodies. Previous reports demonstrated that SUMO tag or molecular chaperone Tf16 could promote the solubility of VP2 proteins, however, the procedure is complicated and inconveniently<sup>[27, 28]</sup>. Therefore, baculovirus - insect cell expression system (BEVS) was used to produce VP2 protein in this study.

So far, Bac-to-Bac system is a common utilized BEVS for the production of heterologous proteins because it has many advantages, including proper post-translational modifications and protein processing<sup>[29]</sup>. Bac-to-Bac system enables protein expression controlled by its highly active promoters of polyhedrin (polh) or p10. We report here that employment of the improved system permits generated recombinant baculovirus containing single or double vp2 gene insertions. After compared the VP2 protein yield after infection with Ac-IM-p10-VP2 and Ac-IM-ph-VP2, we found largely similar VP2 protein yield upon polh- and p10- promoter driven expression. In addition, simultaneous expression from both promoters results in slightly increased CPV VP2 production (Fig. 5B). The enhancement of VP2 expression was 1.4 and 1.6-fold than polh- and p10-promoter driven expression, respectively. These findings are consistent with previous studies which reported similar conclusion upon single and combined polh and p10 promoter driven expression of HPV57 L1 protein<sup>[30]</sup>. Therefore, our study demonstrates that simultaneous expression from both p10 and polh promoters can be an effective way used to increase the expression level of heterologous proteins. In this study, the productivity is up to 186 mg/L, which means a very high level of VP2 protein expressed in insect cells using the improved system. And it is very useful to produce a novel vaccine economically.

Currently, HI is considered as the standard method for detection of CPV antibodies. To evaluate the immunogenicity of an experimental vaccine based on the purified VP2 protein, HI antibodies were tested in the immunized mice in the present study. The results appear as shown in Fig. 7, HI antibody titers increased rapidly after the second immunization. It is remarkable that the maximum titers of HI antibody in VP2 protein immunity group were 1: 2<sup>8.4</sup> at 28 dpv. It has been demonstrated in an earlier report that HI antibodies of > 1:80 can protect dogs from CPV infection<sup>[31]</sup>. Therefore, our results clearly demonstrated that the VP2 protein expressed in this system could induce enough immune responses to prevent CPV infection.

## Conclusion

In conclusion, our study describes the improvement in gene expression level and the high immunogenicity of CPV VP2 protein produced in insect cell lines using the improved system. These results suggest that, following further studies, CPV VP2 protein might be a safe, convenient and effective vaccine for preventing diseases associated with CPV. The use of our improved baculovirus system to produce viral proteins is low-cost and attractive.

## Abbreviations

BEVS: Baculovirus Expression Vector System; CPV: Canine parvovirus; DAB: 3,3'-diaminobenzidine; DAP: 2,6-diaminopimelic; dpv: days post-vaccination; ELISA: Enzyme linked immunosorbent assay; HI: Hemagglutination inhibition; hpi: hours post infection; HPV: Human papilloma virus; MOI: multiplicity of infection; ORF: Open reading frames; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ssDNA: single-stranded DNA

## Declarations

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This procedure was approved by the Northwest Agriculture & Forestry University (Belongs to Chinese Association for Laboratory Animal Sciences, CALAS), All animals were handled strictly in accordance with the good animal practice in order to minimize animal sufferings during blood sampling. Finally, the mice were euthanized by the physical method of cervical spine fracture.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests. The work is an original paper and is not under consideration in other journals.

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### Authors' contributions

YLG directed the project and participated in the coordination and management of the study. YLG and LYK designed the study. HXM analyzed the data. CD and CYY performed the experiments. BA, PA and BV guided the protein purification and drafted the manuscript. All authors read and approved the final manuscript.

### Acknowledgments

Not Applicable.

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## Figures

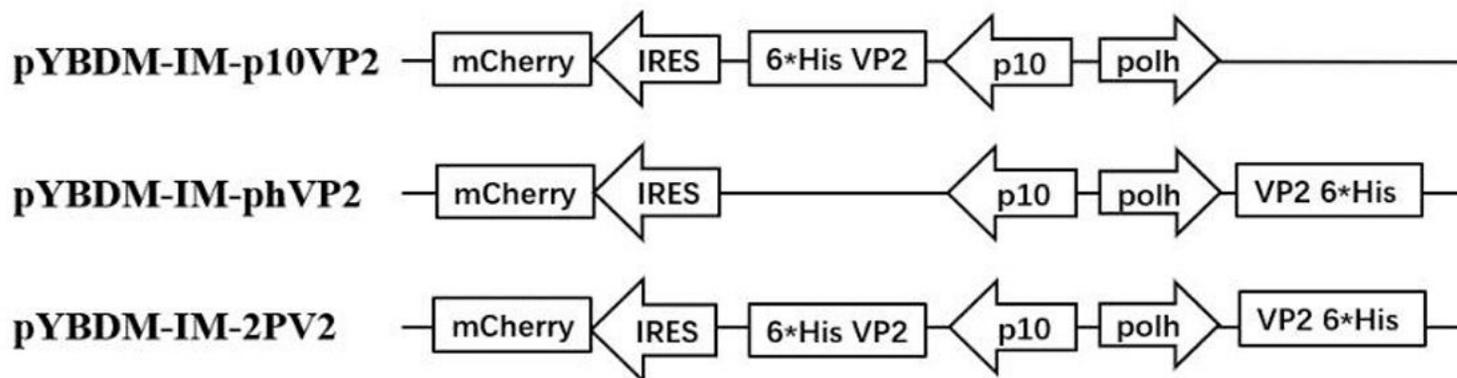
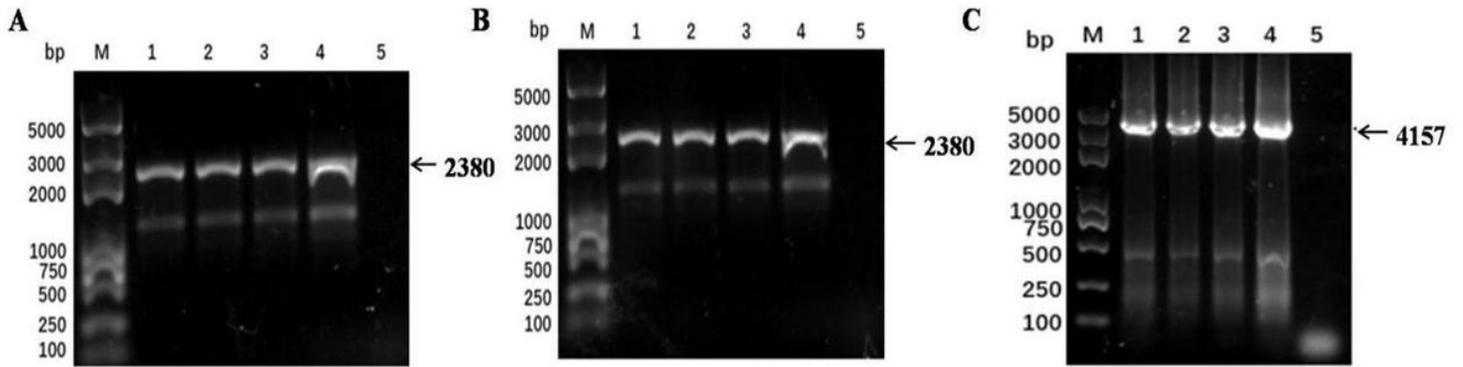


Figure 1

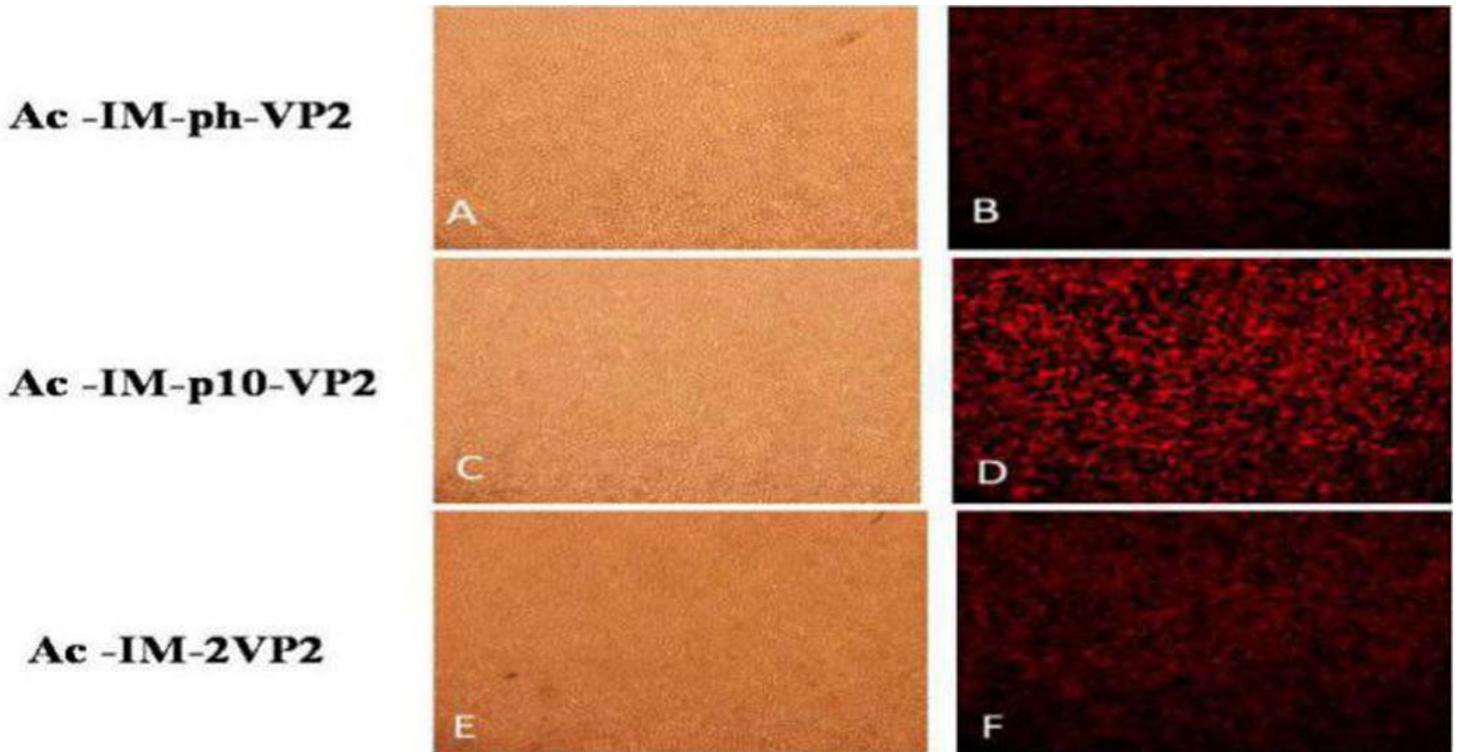
Schematic diagram of different transfer vectors containing CPV VP2 gene. polh: polyhedrin promoter; p10: p10 promoter; IRES: internal ribosome entry site derived from *Rhopalosiphum padi* virus; mCherry:

cDNA of red fluorescent protein derived from mushroom coral.



**Figure 2**

PCR analysis of the recombinant bacmids, using forward primers of vp2 gene and M13 reverse primers. Lane M: Trans2K Plus DNA Marker; (A) 1-3: PCR product of AcMultiBac-IM-p10-VP2; (A) 4: PCR product of plasmid pYBDM-IM-p10-VP2; (B) 1-3: PCR product of AcMultiBac-IM-ph-VP2; (B) 4: PCR product of plasmid pYBDM-IM-ph-VP2; (C) 1-3: PCR product of AcMultiBac-IM-2VP2; (C) 4: PCR product of plasmid pYBDM-IM-2VP2; (A-C) 5: PCR product of AcMultiBacmid/rSW106/asd-/inv+.



**Figure 3**

Fluorescence microscopy of Sf9 cells infected with Ac-IM-ph-VP2, Ac-IM-p10-VP2 and Ac-IM-2VP2. The visible light image (A, C, E) and fluorescence image (B, D, F) were captured at 120 hours post-infection.

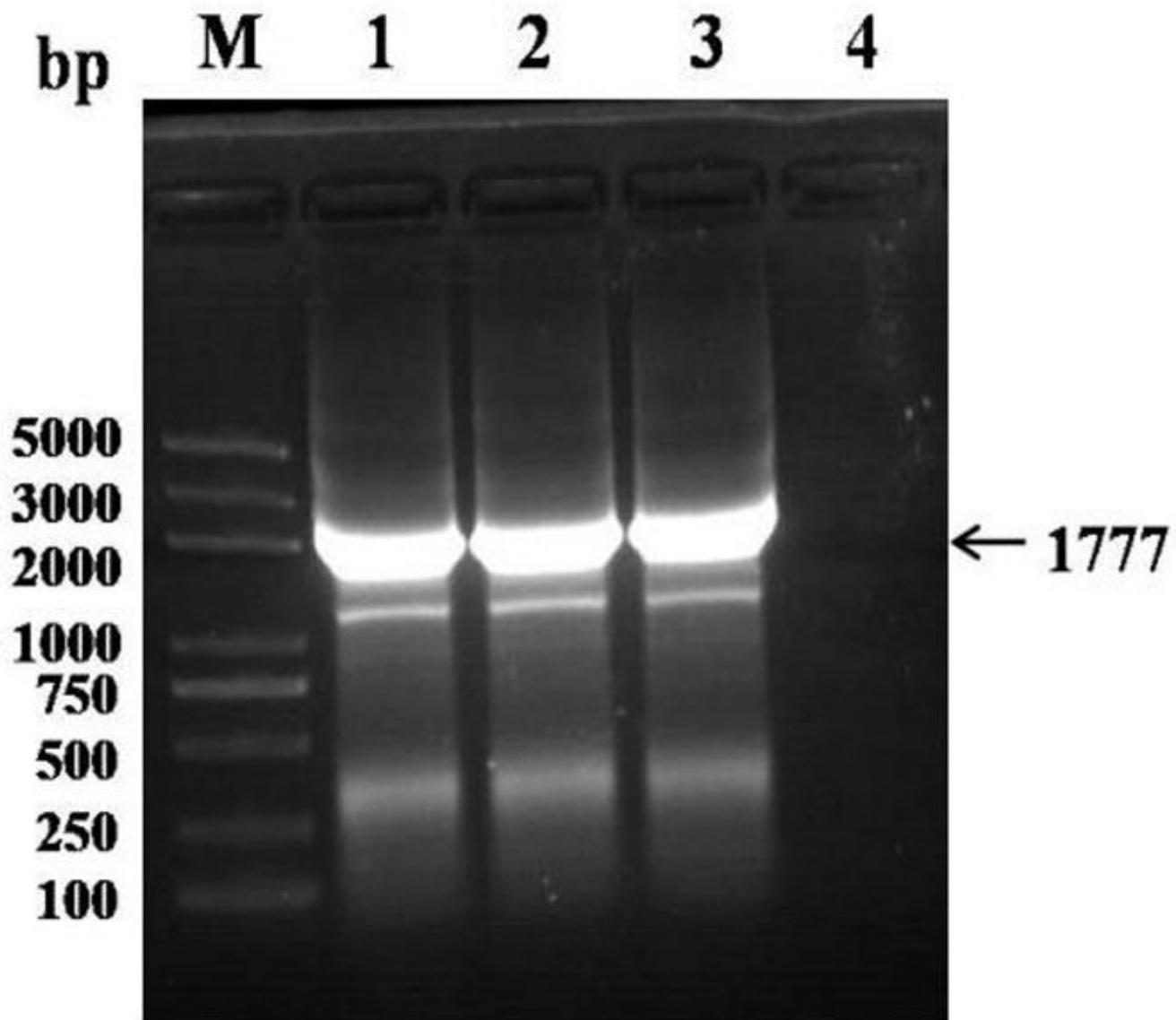
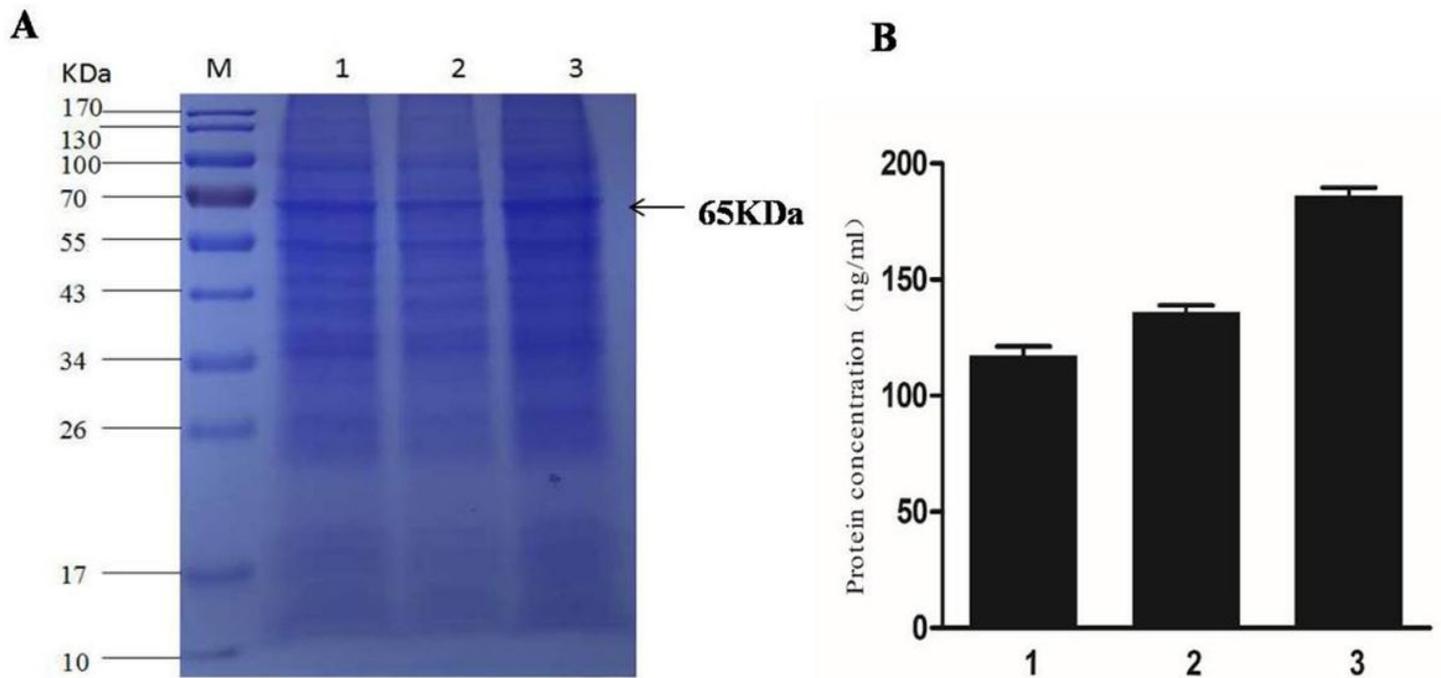


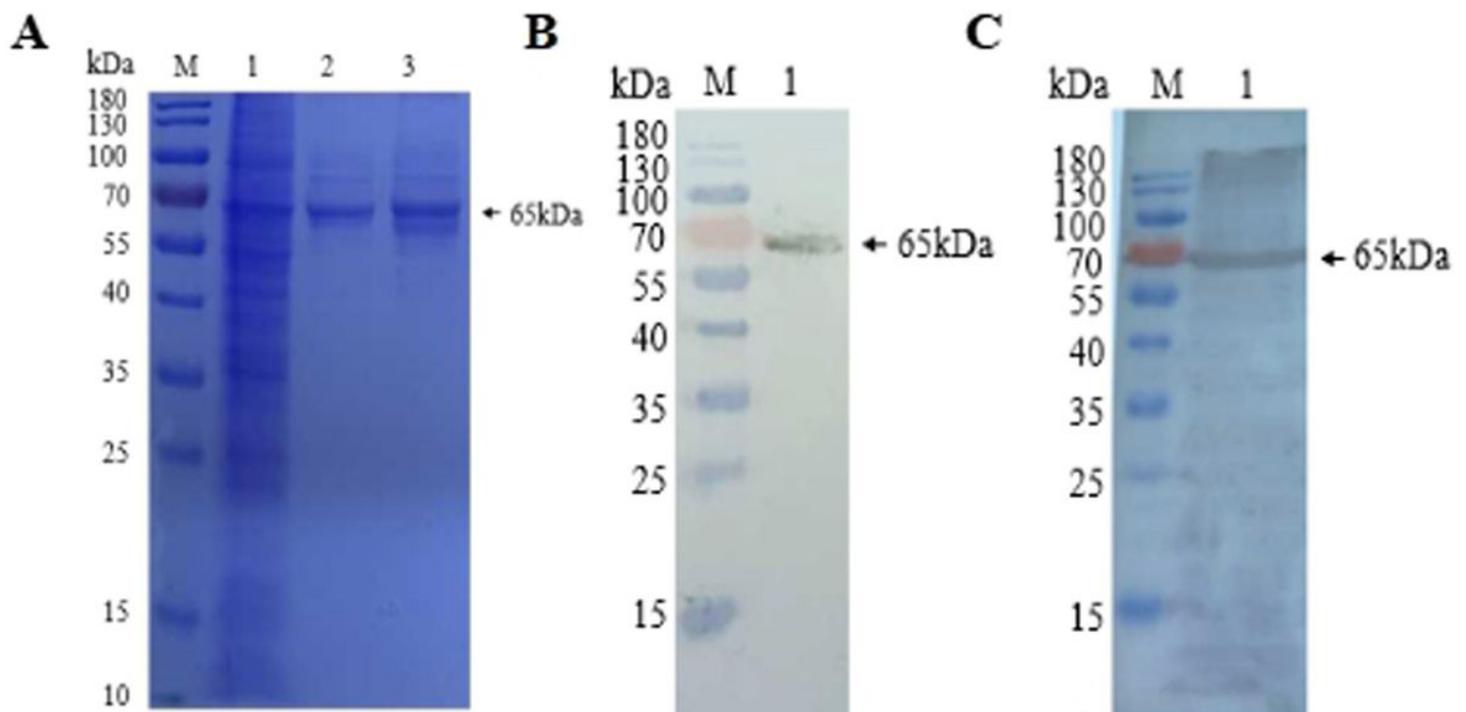
Figure 4

Identification of the baculoviral genomic DNA samples by PCR. Lane M: DL2000 Marker; Lane 1: genomic DNA of the recombinant virus Ac-IM-ph-VP2; Lane 2: genomic DNA of the recombinant virus Ac-IM-p10-VP2; Lane 3: genomic DNA of the recombinant virus Ac-IM-2VP2; Lane 4: negative control, genomic DNA of un-infected Sf9 cells.



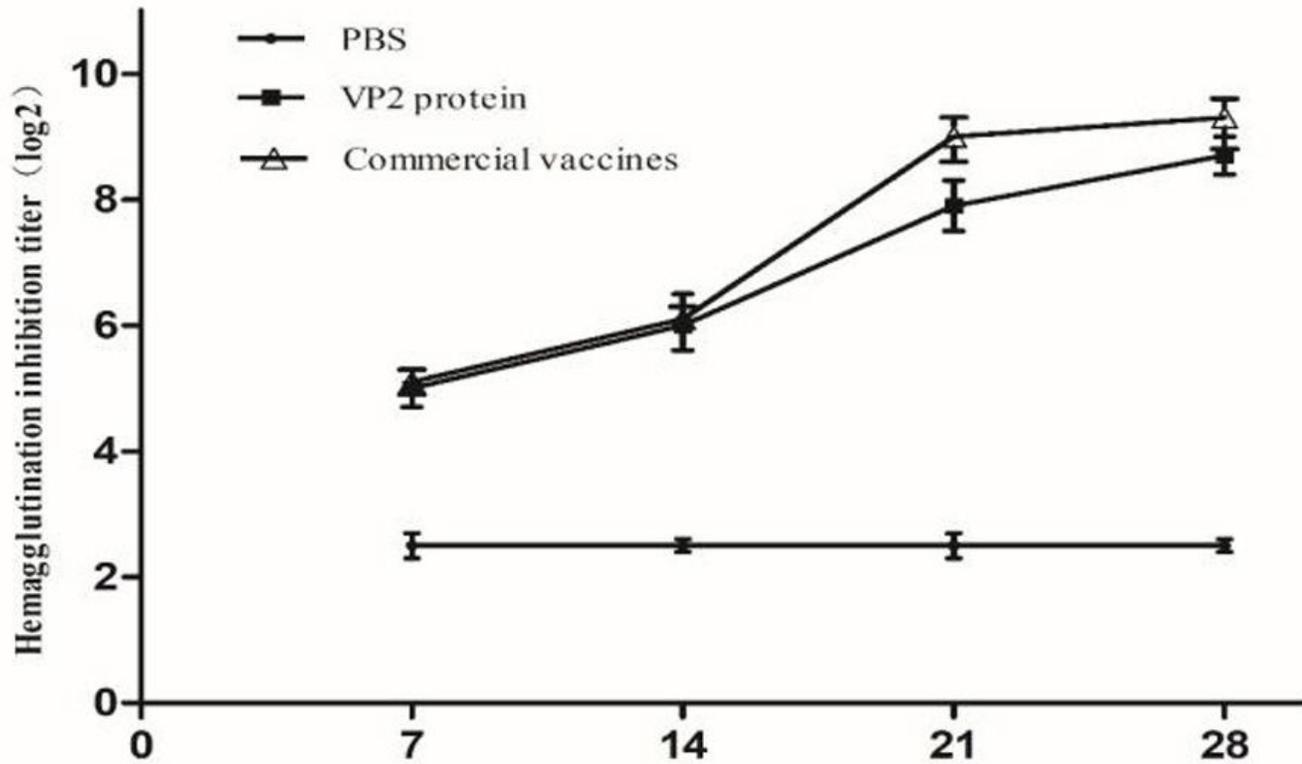
**Figure 5**

Expression of recombinant VP2 protein in insect cells. (A) SDS-PAGE analysis of VP2 expression in Sf9 cells at 96 h p.i. (B) The expression level of CPV VP2 protein in different virus-infected cells. 1: recombinant baculovirus Ac-IM-p10-VP2; 2: recombinant baculovirus Ac-IM-ph-VP2; 3: recombinant baculovirus Ac-IM-2VP2.



**Figure 6**

Purification and characterization of recombinant VP2 protein. (A) The SDS-PAGE analysis of purified recombinant VP2 protein. Lane M: PageRuler™ Prestained Protein Ladder, 10 to 180 kDa; Lane 1: ultrasound supernatant of the recombinant baculovirus Ac-IM-2VP2 infected sf9 cells; Lane 2: 200 mM Imidazole eluent; Lane 3: 300 mM Imidazole eluent. (B) Western blotting analysis of purified VP2 protein with mouse anti-His monoclonal antibodies. Lane 1: purified VP2 protein; (C) Western blotting analysis of purified VP2 protein with rabbit anti-vp2 polyclonal antibodies. Lane 1: purified VP2 protein.



**Figure 7**

Kinetics of hemagglutination inhibiting (HI) antibody in mice vaccinated with the VP2 protein at various times post-vaccination. Results represent mean values of each group sera samples  $\pm$  SD from three independent experiments. The statistical significance of antibody titer differences between different groups were analyzed by one-way ANOVA statistical analysis and significant difference is expressed as  $P < 0.05$  (\*).