

# Chimeric virus-like particles elicit protective immunity against serotype O foot-and-mouth disease virus in guinea pigs

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**Abstract** Foot-and-mouth disease (FMD) is an acute and highly contagious disease caused by foot-and-mouth disease virus (FMDV) that can affect cloven-hoofed animal species, leading to severe economic losses worldwide. Therefore, the development of a safe and effective new vaccine to prevent and control FMD is both urgent and necessary. In this study, we developed a chimeric virus-like particle (VLP) vaccine candidate for serotype O FMDV and evaluated its protective immunity in guinea pigs. Chimeric VLPs were formed by the antigenic structural protein VP1 from serotype O and segments of the viral capsid proteins (VP2, VP3, and VP4) from serotype A. The chimeric VLPs elicited significant humoral

and cellular immune responses with a higher level of anti-FMDV antibodies and cytokines than the control group. Furthermore, four of the five guinea pigs vaccinated with the chimeric VLPs were completely protected against challenge with 100 50% guinea pig infectious doses (GPID<sub>50</sub>) of the virulent FMDV strain O/MAY98. These data suggest that chimeric VLPs are potential candidates for the development of new vaccines against FMDV.

**Keywords** FMDV · Virus-like particles · *Baculovirus* expression system · Guinea pig challenge

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## Introduction

Foot-and-mouth disease virus (FMDV) is a positive-sense, single-stranded RNA virus that belongs to the *Aphthovirus* genus and *Picornaviridae* family (Knowles and Samuel 2003). The RNA genome of FMDV has approximately 8500 bases and is surrounded by four structural proteins that form an icosahedral capsid with a diameter of 30 nm. The icosahedral viral capsid consists of 60 copies each of the four structural proteins (VP1 [1D], VP2 [1B], VP3 [1C], and VP4 [1A]) (Grubman and Baxt 2004). During self-assembly, the capsid precursor P12A is cleaved by the 3C protease to produce 1AB (VP0), 1C (VP3), and 1D (VP1), and VP0 is cleaved during encapsidation of the genome to make VP4 and VP2. VP1, VP2, and VP3 are exposed on the virion surface, whereas VP4 is located internally (Jamal and Belsham 2013).

Although conventional inactivated vaccines play a vital role in current FMD control, their disadvantages have become apparent in recent years. For instance, the virus sometimes escapes from vaccine production facilities and has caused FMDV infections in vaccinated animals due to incomplete inactivation (Dong et al. 2015; Wang et al. 2002). Additionally, distinguishing carrier animals from uninfected vaccinated animals is difficult (Wang et al. 2002). Therefore, to avoid the deficiency of the conventional inactivated vaccine, there is an urgent need to develop novel vaccines for the prevention and control of FMDV, such as recombinant subunit vaccines, synthetic peptide vaccines, and virus-like particle (VLP) vaccines (Mohana Subramanian et al. 2012; Nanda et al. 2014; Shao et al. 2011; Wang et al. 2002). One of the most promising candidates is VLP vaccines, because these vaccine types have antigenic and immunogenic properties similar to natural FMDV particles but are noninfectious since they do not contain viral genes (Grubman and Baxt 2004).

FMDV is characterized by a wide variety of host species, an extensive distribution, and a high mutation rate. FMDV exists as seven serotypes (O, A, C, Asia 1, SAT 1, SAT 2, and SAT 3) as well as numerous variants and lineages described as topotypes (Gao et al. 2016; Klein 2009). There is no cross protection between the serotypes, and the serotype of a virus involved in an outbreak cannot be ascertained on the basis of clinical signs (Jamal and Belsham 2013). The seven FMDV serotypes are not distributed equally worldwide. Serotype O is the most widespread of the FMDV serotypes, especially in Asia, followed by serotypes A and Asia 1 (Brito et al. 2015). As a result, the development of a new vaccine against FMDV serotype O has become the key to the prevention and control of FMDV in recent years.

Insect cell-based expression systems are widely used for VLP production in the laboratory or on an industrial scale due to a number of advantages, such as the fast growth rates in animal product-free media, the capacity for large-scale cultivation, and the ability to post-translationally modify the

recombinant proteins similar to mammalian cells (Zeltins 2013). Although an insect cell-baculovirus expression system was reported for the production of FMDV serotype O VLPs (Bhat et al. 2013; Kumar et al. 2016; Li et al. 2016; Mohana Subramanian et al. 2012), related reports showed that the FMDV serotype O viral capsid was more acid-sensitive than the other types and was difficult to assemble in the low-pH insect cell (Li et al. 2016; Vlijmen et al. 1998). Therefore, in the present study, an FMDV serotype A-based chimeric VLP containing the major antigenic protein VP1 from serotype O instead of serotype A was generated using an insect cell-based expression system. Furthermore, the immunogenicity and protective efficacy of chimeric VLPs as a vaccine candidate against FMDV serotype O were evaluated in guinea pigs. The chimeric P1-2A protein (VP1 from serotype O and VP2, VP3, VP4, and 2A from serotype A) was cleaved by the 3C protease from serotype A and correctly self-assembled to form chimeric VLPs. An efficient immune response and protective efficacy similar to the inactivated FMDV vaccine were induced in guinea pigs by the chimeric VLPs.

## Materials and methods

### Cells, viruses, and animals

*Spodoptera frugiperda* (Sf9) insect cells (Invitrogen, USA) were cultured using Sf-900™ II SFM (Invitrogen, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Gibco, USA) at 27 °C. Baby hamster kidney (BHK-21) cells (stored in our lab) were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA) supplemented with 10% heat-inactivated FBS (Gibco, USA), 100 µg/mL of streptomycin, and 100 IU/mL of penicillin at 37 °C in 5% CO<sub>2</sub>. The FMDV vaccine strain O/BY/CHA/2010 (GenBank No. JN998085) was propagated in BHK-21 cells. The viruses used in this study were only maintained and made available at Lanzhou Veterinary Research Institute (LVRI). Healthy male and female guinea pigs weighing 250 to 350 g with no FMDV-specific antibodies were obtained from the Experimental Animal Center of LVRI and provided pathogen-free food and water.

### Construction and generation of recombinant baculovirus

Gene fragments encoding chimeric P12A (2259 bp; VP2, VP3, VP4, and 2A gene sequences from the vaccine strain AF/72; VP1 gene sequence from strain O/BY/CHA/2010, GenBank No. KY523524) and 3C (639 bp; gene sequence from the vaccine strain AF/72, GenBank No. KY523525) were synthesized with introduced restriction enzyme sites using the PCR-based accurate synthesis method (PAS) (Zoonbio, Nanjing, China). The synthesized gene product

encoding chimeric P12A was digested with *EcoRI* and *HindIII* and cloned into the pFast-Bac Dual vector (Invitrogen, USA) under the control of the polyhedron promoter. Similarly, the 3C fragment was digested with *NcoI* and *KpnI* and cloned into the pFast-Bac Dual vector (Invitrogen, USA) under the control of the P10 promoter. The resulting transfer plasmids were named pFBDual-P12A3C(O-VP1) and were confirmed by double digestion and sequencing. The recombinant baculovirus rBac-P12A3C(O-VP1) was subsequently generated using the Bac-to-Bac® baculovirus expression system (Invitrogen, USA). Briefly, the transfer plasmid pFBDual-P12A3C(O-VP1) was transformed into DH10Bac™ *Escherichia coli* to generate the recombinant bacmid rBacmid-P12A3C(O-VP1). Positive recombinant bacmids were obtained by blue-white screening and confirmed by PCR amplification using the universal primers M13-F/R. Subsequently, the positive bacmids were transfected into Sf9 insect cells using the Cellfectin® II Reagent (Invitrogen, USA) to produce recombinant baculoviruses, which were titrated and stored. The chimeric P1-2A and 3C genes present in the recombinant baculovirus stock produced in Sf9 cells were amplified using the specific primers P12A-F/R (P12A-F: 5'-ATGAGTGGTGCCCCACCGACCGACT-3' and P12A-R: 5'-TCACTCGTGGTGTGGTTCCGGGGTCG-3) and 3C-F/R (3C-F: 5'-ATGGGCGCCGGGCAATCCAG-3' and 3C-R: 5'-TCACCCAGGGTTGGACTCCACGTCT-3'), respectively.

### Expression of the chimeric FMDV VLPs in Sf9 cells

The Sf9 insect cell monolayer was grown in a 175-cm<sup>2</sup> culture flask (Corning, USA) at a density of  $2 \times 10^6$  cells/mL, infected with the recombinant baculovirus at a multiplicity of infection (MOI) of 5 and incubated at 27 °C. At 72 h post-infection when the cytopathic effect (CPE) was almost 100%, the infected cells were lysed with lysis buffer [phosphate-buffered saline (PBS) with 0.1% Triton X-100, pH 7.4], and the proteins were harvested for western blotting. The proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane. The transferred membrane was blocked with 5% skimmed milk at 37 °C for 1 h. The proteins on the transferred membrane were reacted with rabbit anti-FMDV serotype O polyclonal sera (Diagnostic Products Center, LVRI, Lanzhou, China) at a 1:1000 dilution as the primary antibody and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (Sigma, USA) at a 1:5000 dilution as the secondary antibody. After washing three times with TBS-Tween (50 mM Tris, 150 mM NaCl and 0.05% Tween 20, pH 7.6), the protein bands were visualized using an ECL chemiluminescent substrate reagent kit (Thermo Scientific, USA). Cell lysates from normal Sf9 cells and whole virus inactivated antigen from FMDV serotype O (Diagnostic

Products Center, LVRI, Lanzhou, China) were used as the controls.

The antigenicity of the proteins was confirmed by an indirect sandwich enzyme-linked immunosorbent assay (IS-ELISA). Briefly, a 96-well ELISA plate (Corning, USA) was coated with rabbit anti-FMDV serotype O polyclonal sera (Diagnostic Products Center, LVRI, Lanzhou, China) at a 1:1000 dilution at 37 °C for 1 h and then blocked with 1% bovine serum albumin (BSA). The plates were incubated with cell lysates from recombinant baculovirus rBac-P12A3C(OVP1)-infected Sf9 cells (72 h after infection) at 37 °C for 1 h. The whole virus inactivated antigen from FMDV serotype O (Diagnostic Products Center, LVRI, Lanzhou, China), and the cell lysates and supernatants from normal Sf9 cells were used as the controls. After washing three times with PBS Tween-20 Buffer (PBST), the plate was incubated with guinea pig anti-FMDV serotype O polyclonal sera (Diagnostic Products Center, LVRI, Lanzhou, China) at 37 °C for 1 h and then HRP-labeled rabbit anti-guinea pig IgG (Diagnostic Products Center, LVRI, Lanzhou, China) at 37 °C for 1 h, followed by the tetramethylbenzidine (TMB) substrate. The absorbance was determined at 450 nm.

### Immunofluorescence assay

Sf9 cells were cultured on sterile cell slides placed in 24-well plates and infected at an MOI of 5. At 0, 12, 24, and 36 h post-infection, the cells were fixed using cold methanol/acetone (1:1) at –20 °C for 15 min, washed with PBS and blocked with 1% BSA at 37 °C for 30 min. Subsequently, the cells were incubated with rabbit anti-FMDV serotype O polyclonal sera (Diagnostic Products Center, LVRI, Lanzhou, China) at a 1:1000 dilution at 37 °C for 1 h. After washing three times with PBS, the cells were incubated with Dylight™ 488-conjugated goat anti-rabbit IgG (Cayman, USA) at a 1:1000 dilution at 37 °C for 1 h and then counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma, USA). Finally, the cell staining was visualized using a fluorescence microscope (Olympus, Japan).

### Transmission electron microscopy

Lysates from recombinant baculovirus rBac-P12A3C(OVP1)-infected Sf9 cells were purified by sucrose density gradient centrifugation as previously reported (Li et al. 2016). Then, 10 mL of the purified fractions were placed onto a copper grid (300 mesh, Pelco, CA, USA) coated with a formvar-carbon film at room temperature for 3 min. The grid was washed with PBS, stained with 1% ammonium phosphotungstic acid, and examined under a transmission electron microscope (TEM).

## Guinea pig immunization

Healthy male and female guinea pigs weighing 250 to 350 g with no FMDV-specific antibodies were randomly divided into three groups of five animals each. The experimental group was immunized by intramuscular injection of 0.5 mL of crude extracted VLPs containing approximately 10 µg of chimeric VLPs with the ISA-206 adjuvant (China Agricultural Vet. Bio. Science and Technology Co., Lanzhou, China) at an adjuvant/antigen ratio of 1:1 on days 0 and 21. Additionally, two groups of guinea pigs were immunized with 0.5 mL of the commercial inactivated FMDV serotype O vaccine containing approximately 5 µg of the 146S antigen (China Agricultural Vet. Bio. Science and Technology Co., Lanzhou, China) and PBS as a positive and negative control, respectively. Serum samples were collected by heart punctures on 0, 7, 14, 21, and 28 days post-vaccination (dpv) to perform the antibody and cytokine analyses.

## Indirect enzyme-linked immunosorbent assay

Serum samples were examined to detect FMDV-specific IgG antibodies using an indirect ELISA method. First, 96-well flat-bottomed plates (Corning, USA) were coated with whole virus inactivated antigen from FMDV serotype O (Diagnostic Products Center, LVRI, Lanzhou, China) in 0.1 M bicarbonate buffer (pH 9.6) overnight at 4 °C. After washing three times with PBST, the plates were blocked with 5% skim milk at 37 °C for 1 h and then incubated with duplicate serum samples (1:16 dilution) at 37 °C for 1 h. Then, HRP-labeled rabbit anti-guinea pig IgG (Diagnostic Products Center, LVRI, Lanzhou, China) was added and incubated at 37 °C for 1 h, followed by the *o*-phenylenediamine dihydrochloride (OPD) substrate. The absorbance was determined at 492 nm.

## Virus neutralizing antibody test

The FMDV-specific neutralizing antibody titers from 28 dpv serum samples were determined using virus neutralizing antibody test (VNT) with BHK-21 cells according to the OIE protocol (OIE 2009). Briefly, 50 µL of twofold serial serum dilutions starting at 1:2 was co-incubated with equal volumes of viral stock containing 100 TCID<sub>50</sub> (50% tissue culture infective doses) of FMDV O/BY/CHA/2010 in 96-well plates (Corning, USA) at 37 °C for 1 h. Then, cells were added to the mixture as indicators of residual infectivity. The plates were incubated at 37 °C for 72 h, and the cells were fixed and stained with 10% methanol and 0.05% methylene blue solution (prepared with formaldehyde solution). The neutralizing antibody titers were evaluated as the reciprocal log<sub>10</sub> of the highest dilution that neutralized 100 TCID<sub>50</sub> of FMDV in 50% of the wells.

## Cytokine analysis

Serum IFN-γ, IL-2, IL-4, and IL-6 levels were compared among the experimental groups using the Rat Cytokine Antibody Array CYT-1 (Ray Biotech, Norcross, GA, USA) at 28 dpv. The experiments were performed by the RayBiotech Technical Service Department (Guangzhou, China) following the manufacturer's instructions.

## Lymphocyte proliferation assay

The FMDV-specific lymphocyte proliferative responses of all experimental groups were measured at 28 dpv. At 28 dpv before challenge, guinea pig lymphocytes from each immunization and control group were separated from the blood using an animal lymphocyte separation solution kit (Solarbio, China). The cells were re-suspended at a density of  $5 \times 10^6$  cells/mL with RPMI 1640 medium (Gibco, USA) containing 10% heat-inactivated FBS (Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA) and then seeded into 96-well plates with 50 µL per well. Each sample was stimulated with 50 µL of inactivated FMDV antigen (10 µg/mL) in quadruplicate. Concanavalin A (ConA, 5 µg/mL), unstimulated wells, and complete RPMI 1640 medium were used as the positive control, negative control, and blank, respectively. After incubation for 72 h at 37 °C in 5% CO<sub>2</sub>, the proliferative responses were detected using the MTT Lymphocyte Proliferation Assay Kit (Solarbio, China). The results were expressed as a stimulation index (SI, ratio of stimulated sample/unstimulated sample at OD490 nm).

## Challenge experiment

Twenty-eight days after the first immunization, all guinea pigs were challenged intradermally in the left rear leg with 0.2 mL of 100 GPID<sub>50</sub> (50% guinea pig infectious doses) of FMDV O/BY/CHA/2010. Clinical examinations of the guinea pigs were performed daily for 7 days post-challenge. Protection against FMDV was defined as previously reported (Wang et al. 2011). Briefly, guinea pigs showing FMD-compatible lesions only at the original injection site were judged to be protected, whereas guinea pigs showing any FMD clinical signs in the other three feet were judged to be unprotected.

## Statistical analysis

Statistical significance among the different experimental groups was determined by a two-tailed *t* test. Differences were considered significant when the *P* value was less than 0.01.

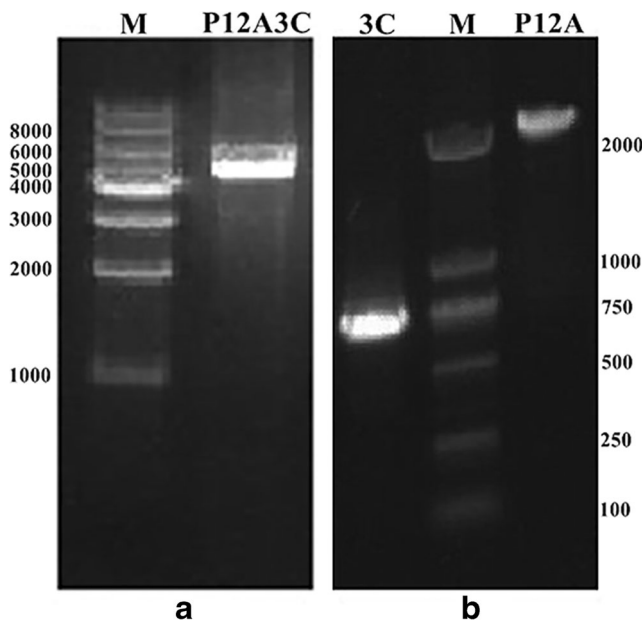
## Results

### Confirmation of the presence of chimeric P12A and 3C by PCR amplification

To verify the presence of the P12A3C gene in the recombinant bacmid, PCR amplification was performed using the universal primers M13-F/R. A 5506-bp fragment corresponding to the size of the P12A3C gene was amplified, as shown in Fig. 1a. Furthermore, the P12A and 3C genes present in the recombinant baculovirus stock produced by the Sf9 cells were amplified using the primers P1-2A-F/R and 3C-F/R, respectively. The sizes of the PCR-amplified products were 2283 and 663 bp, which were consistent with the target genes (Fig. 1b). These results indicated that the P12A and 3C genes were correctly inserted after transposition.

### Analysis of the expression of the chimeric VLPs in Sf9 cells

The recombinant baculovirus was expanded by serial passage in Sf9 cells to produce P3 stock viruses with titers up to  $10^{7.5}$  plaque-forming units (pfu)/mL. Cells and supernatants were harvested and analyzed 48 h post-infection as indicated by the cytopathic effects. The immunofluorescence assay (IFA) results showed that Sf9 cells infected with recombinant baculovirus at 12, 24, and 36 h reacted with the rabbit anti-FMDV serotype O sera (Fig. 2). Four bands approximately 24, 33, 47, and 82 kDa in size corresponding to the sizes of



**Fig. 1** Identification of recombinant bacmids and baculovirus by PCR amplification. **a** Lane M DNA marker, lane P12A3C PCR results for recombinant bacmids with the universal primers M13-F/R. **b** Lane 3C PCR results for recombinant baculovirus with the specific primers 3C-F/R, lane P12A PCR results for recombinant baculovirus with the specific primers P12A-F/R

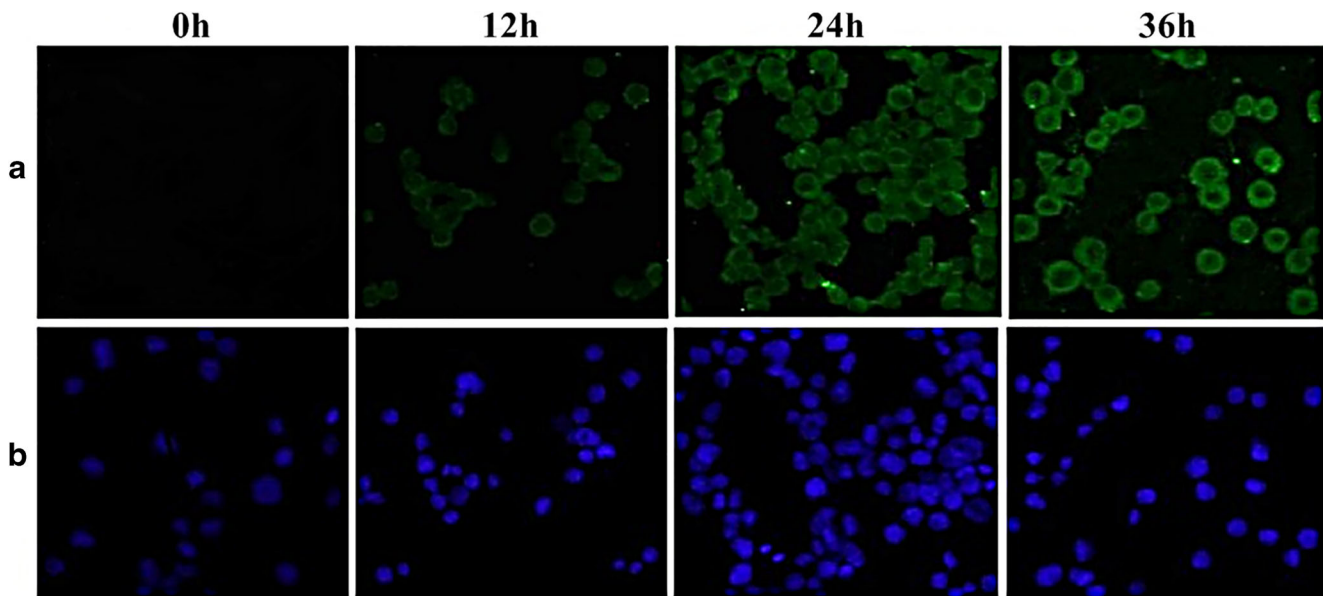
VP1/VP3, VP0, VP31, and chimeric P12A were detected by western blotting (Fig. 3). These results indicated that the chimeric P12A polyproteins were expressed correctly in the Sf9 cells and efficiently cleaved by the 3C protease to yield the viral proteins required for chimeric VLP formation. Additionally, the expressed chimeric VLPs were tested in an IS-ELISA using rabbit and guinea pig anti-FMDV serotype O polyclonal sera. The average  $OD_{450}$  values of the lysates and supernatants from the three chimeric VLP batches were 2.499 and 0.846, respectively, whereas the average  $OD_{450}$  values for the positive control (whole virus inactivated antigen from FMDV serotype O) and two negative controls (lysates and supernatants from normal Sf9 cells) were 2.867, 0.548, and 0.024, respectively. These results indicated that the chimeric VLPs had antigenicity.

### Observation of the chimeric VLPs

Chimeric VLPs were purified from the culture supernatants using sucrose density gradient centrifugation. Fractions were observed by TEM at sucrose densities between 15 and 45%. TEM analysis of the fractions revealed that the negatively stained sample contained intact spherical VLPs ( $25 \pm 5$  nm, Fig. 4) that were morphologically similar to empty particles from FMDV-infected cells.

### Humoral immune responses elicited by chimeric VLPs in guinea pigs

To evaluate the humoral immune responses induced by the chimeric VLPs, FMDV serotype O-specific IgG for each guinea pig was assessed by I-ELISA. The guinea pigs in the VLP and commercial vaccine groups exhibited detectable IgG antibody levels at 14 dpv (Fig. 5). The IgG titers were significantly increased at 21 dpv in both the VLP and commercial vaccine groups, and the titers further increased after the boost immunization (28 dpv) (Fig. 5). Importantly, the VLP and commercial vaccine groups showed significantly enhanced IgG titers in contrast to the PBS group ( $P < 0.01$ , Fig. 4), whereas the difference in the IgG titers between the VLP and commercial vaccine groups was not significant ( $P > 0.01$ , Fig. 5). Next, the anti-viral serum neutralizing antibody titers of the immunized guinea pigs on the day of challenge (28 dpv) were assessed by VNT (Fig. 6). The antibody responses of the guinea pigs in the VLP and commercial vaccine groups showed higher FMDV-neutralizing activity than the antibody responses of the PBS group ( $P < 0.01$ ), indicating that the VLPs could induce serum neutralizing antibodies to FMDV. These results indicated that the VLPs could effectively induce humoral antibody responses comparable to the commercial vaccine.



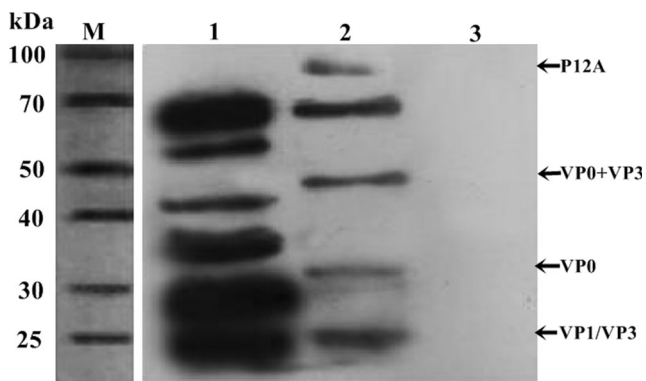
**Fig. 2** Immunofluorescence assay of chimeric polyprotein expression in Sf9 cells at 0, 12, 24, and 36 h post-infection with recombinant baculovirus. **a** Sf9 cells infected with recombinant baculovirus were

incubated with rabbit anti-FMDV serotype O polyclonal sera and stained with Dylight™ 488-conjugated goat anti-rabbit IgG (*green*). **b** Sf9 cells nucleoli were stained with 6-diamidino-2-phenylindole (DAPI) (*blue*)

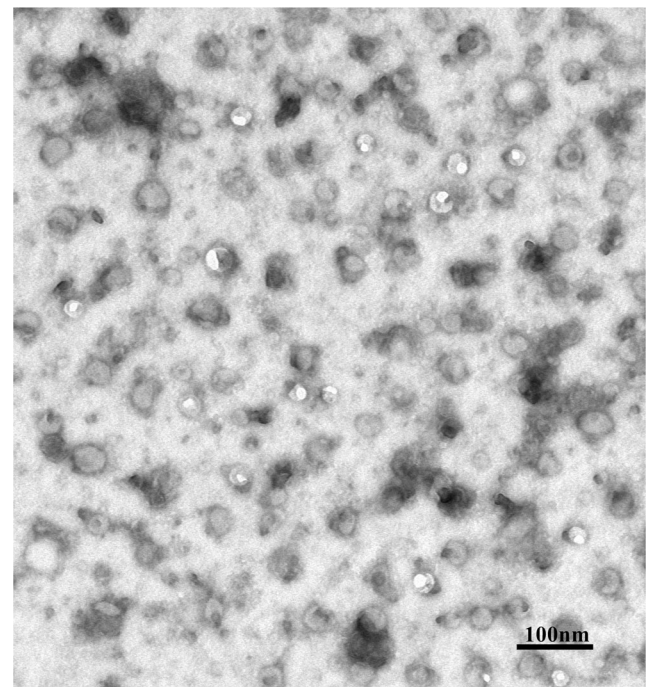
### Cell-mediated immune response promoted by chimeric VLPs in guinea pigs

To assess the serum cytokine levels in the immunized guinea pigs, IFN- $\gamma$ , IL-2, IL-4, and IL-6 levels were determined using a cytokine antibody array. Significant IFN- $\gamma$  and IL-6 levels were detected in the VLP and commercial vaccine groups compared to the PBS group ( $P < 0.01$ , Fig. 7a, d). However, no significant differences in the IL-2 and IL-4 levels were detected between the VLP and PBS groups ( $P > 0.01$ , Fig. 7b, c). Furthermore, T lymphocyte proliferative responses were evaluated using the MTT lymphocyte proliferation assay. The VLP groups produced significantly higher T

lymphocyte proliferation levels following stimulation by inactivated FMDV antigen as a specific stimulant compared with the PBS group ( $P < 0.01$ , Fig. 8), whereas no significantly higher levels were observed compared with the commercial vaccine group ( $P > 0.01$ , Fig. 8). These results suggested that

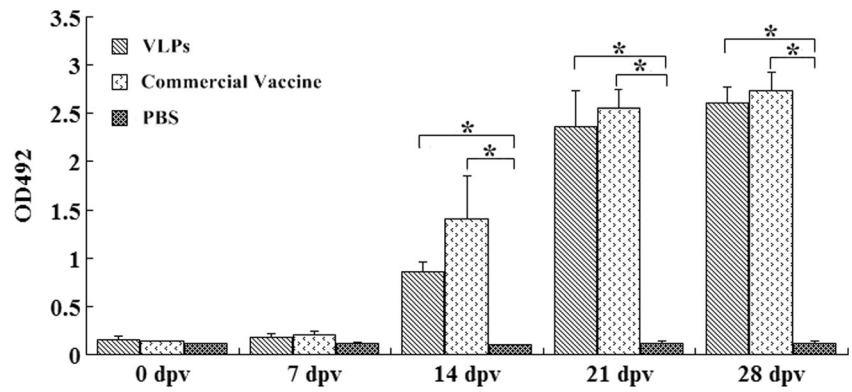


**Fig. 3** Western blotting analysis of chimeric polyprotein expression in Sf9 cells probed with rabbit anti-FMDV serotype O polyclonal sera. *M* Protein molecular weight marker, *lane 1* inactivated antigen of FMDV serotype O, *lane 2* cell lysates from Sf9 cells infected with the recombinant baculovirus rBac-P12A3C(O-VP1), *lane 3* cell lysates from normal Sf9 cells



**Fig. 4** Transmission electron microscope (TEM) image of negatively stained chimeric VLPs. These VLPs are approximately  $25 \pm 5$  nm in diameter and are morphologically similar to the empty capsids of FMDV

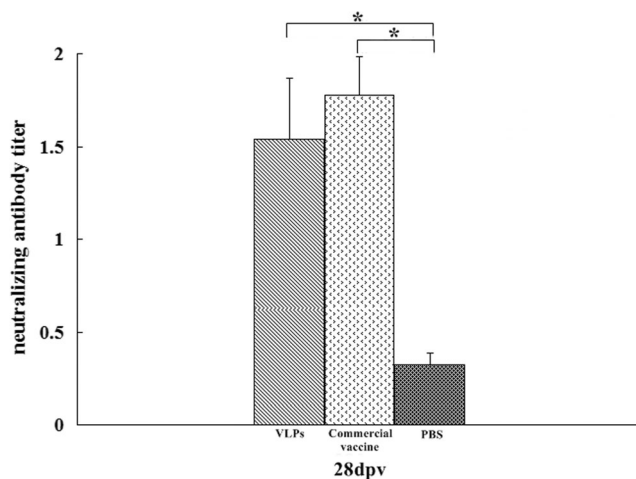
**Fig. 5** The IgG titers of guinea pigs after primary and booster immunizations analyzed by indirect ELISA. Sera were sampled at 0, 7, 14, 21, and 28 dpv (days post-vaccination) and tested for IgG at a 1:32 dilution. Bars represent the arithmetic means of the antibody titers in each group  $\pm$  standard errors. Significant values ( $P < 0.01$ ) are indicated by an asterisk



the chimeric VLPs could elicit an effective cellular immune response against FMDV.

### Protective effect of chimeric VLPs in guinea pigs after challenge

As shown in Table 1, all five guinea pigs in the PBS group developed severe lesions on both rear feet after challenge with FMDV on 28 dpv, whereas no clinical symptoms were observed in the commercial vaccine group. One of the guinea pigs in the VLP group developed mild FMD lesions on the uninoculated feet, whereas the remaining three guinea pigs did not show clinical symptoms. Therefore, the protective effect of the chimeric VLPs (80%) was similar to the protective effect of the commercial FMDV vaccine (100%). These results indicated that the chimeric VLPs were able to elicit a protective immune response in guinea pigs.



**Fig. 6** Serum neutralizing antibody titers of immunized guinea pigs on 28 dpv (days post-vaccination) before challenge. Bars represent the arithmetic means of the neutralizing antibody titers in each group  $\pm$  standard errors. Significant values ( $P < 0.01$ ) are indicated by an asterisk

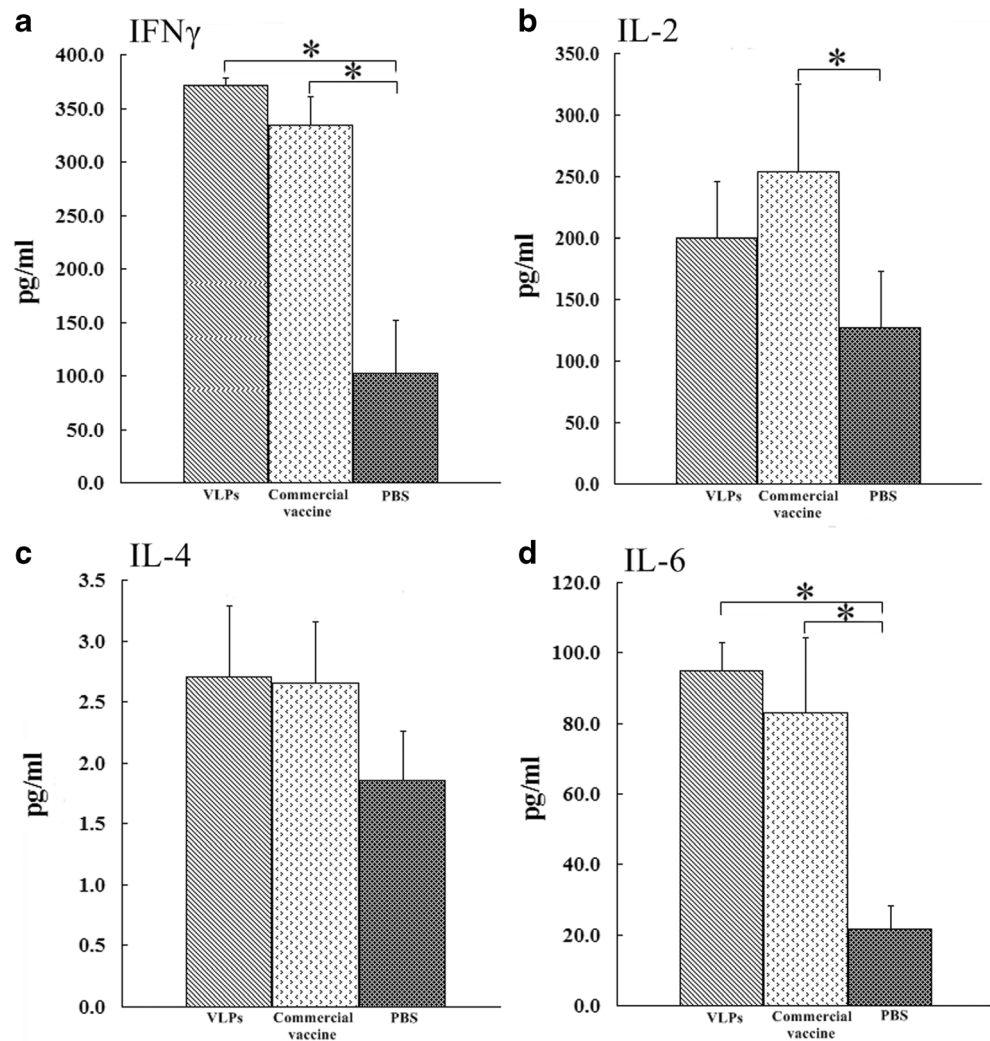
### Discussion

FMDV serotype O is regarded as the most important and extensive type distributed worldwide (Jamal and Belsham 2013). In particular, serotype O was responsible for outbreaks in Southeast Asia (SEA), including China, South Korea, Thailand, Japan, and Vietnam (Park et al. 2013; Valdazo-Gonzalez et al. 2013). Hence, there is an urgent need to prevent and control serotype O in these regions. Additionally, an efficient vaccine produced by a strain with wide antigenic coverage for serotype O is the most economical and effective measure. The FMDV strain O/BY/CHA/2010 used in this study belongs to the MYA-98 lineage within the SEA toptotype, which has been prevalent in southeast China, and confers excellent protection as a vaccine candidate strain against current epidemic strains. Therefore, the chimeric VLPs produced in this study were constructed based on the major protective antigen gene VP1 of FMDV strain O/BY/CHA/2010.

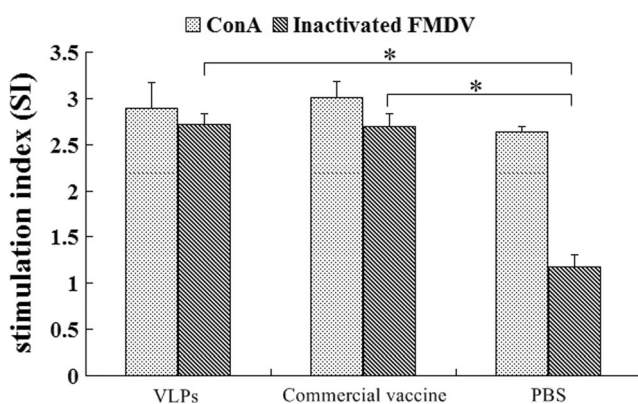
To date, many studies have shown that FMDV VLPs can be successfully expressed using insect cell-based expression systems and induce effective protective immunity similar to the inactivated vaccine (Bhat et al. 2013; Cao et al. 2009; Kumar et al. 2016; Mohana Subramanian et al. 2012; Porta et al. 2013). Moreover, some novel chimeric VLPs composed of antigenic VP1 from serotype O and segments of capsid proteins from FMDV or other species have been developed to overcome the sensitivity of FMDV serotype O to the low pH in the expression cells (Dong et al. 2015; Li et al. 2016). Hence, the objective of this study was to produce a chimeric VLP containing antigenic VP1 from strain O/BY/CHA/2010 using viral capsid protein segments from serotype A strain AF/72 as the vector. Then, chimeric VLPs were tested for their ability to elicit an immune response in guinea pigs to evaluate their protective efficacy as a vaccine.

At present, gene synthesis is emerging as a valuable tool to support recombinant protein expression. Gene synthesis can prevent point mutations in target genes caused by PCR amplification and allows optimization of codon usage to the recombinant host system, thereby promoting the effective operation

**Fig. 7** Serum levels of the cytokines **a** IFN- $\gamma$ , **b** IL-2, **c** IL-4, and **d** IL-6 in the blood of immunized guinea pigs on 28 dpv (days post-vaccination) before challenge. The cytokines were detected using the Rat Cytokine Antibody Array according to the manufacturer's protocols. Bars represent the arithmetic means of the cytokine titers in each group  $\pm$  standard errors. Significant values ( $P < 0.01$ ) are indicated by an asterisk



of the cellular translational machinery (Sequeira et al. 2016). To efficiently express chimeric P12A and 3C in Sf9 cells, the



**Fig. 8** Mean lymphocyte stimulation index (SI) of guinea pigs immunized with chimeric VLPs, the commercial vaccine, and PBS on 28 dpv (days post-vaccination) before challenge. Bars represent the arithmetic means of the SI value in each group  $\pm$  standard errors. Significant values ( $P < 0.01$ ) are indicated by an asterisk

coding genes were analyzed according to the codon usage frequency and preference of the insect cells and synthesized with introduced restriction enzyme sites using the PAS. The sequence analysis showed that the chimeric P12A and 3C coding regions had the same codon usage bias as the insect cells and could be used directly without further optimization (data not shown).

In this study, the synthesized gene fragments encoding chimeric P12A and 3C were cloned into the pFast-Bac Dual Vector (Invitrogen, USA) under control of the polyhedron and P10 promoters, respectively. The precursor proteins were expressed successfully by recombinant baculovirus in the Sf9 cells, were processed correctly by the 3C protease, and self-assembled into VLPs or intermediates as observed by electron microscopy and the appearance of the VP1, VP3, and VP0 bands in the western blotting analysis. These results indicated that the VP1 proteins from the chimeric capsid precursor protein were correctly cleaved by a 3C protease derived from a different serotype. The result is consistent with a previous study (Li et al. 2016).



**Table 1** Protection in guinea pigs after challenge with 100 GPID<sub>50</sub> FMDV serotype O

Group	Number of guinea pigs without clinical symptoms /challenged	Rates of protection
Commercial vaccine	5/5	100
Chimeric VLPs	4/5	80
PBS	0/5	0

Protective immunity induced by the chimeric VLPs was assessed in guinea pigs. FMDV-specific antibodies and neutralizing antibodies were induced in immunized guinea pigs at significantly higher levels than in un-immunized animals ( $P < 0.01$ ); however, their levels were slightly lower than the levels generated by the commercial vaccine. This result could have occurred because the guinea pigs were vaccinated with crudely extracted proteins containing chimeric VLPs, resulting in a lower quantity in the complete chimeric VLPs than in the commercial vaccine. This reason was also discussed in a related previous study (Cao et al. 2009). To date, FMDV VLP assembly has been limited by lower expression levels, which has restricted the development of VLPs as an effective vaccine. A key reason for this limitation is that the structural protein precursor P1-2A reduces expression significantly when the P12A and 3C coding sequences are cloned simultaneously into a dual promoter vector, resulting in incomplete precursor cleavage and predominantly pentameric assemblies rather than complete capsids, particularly in insect cells (Porta et al. 2013). Hence, further VLP vaccine studies are needed to enhance the protein expression levels in insect cells.

Additionally, chimeric VLP immunization induced cellular immune responses in the guinea pigs as evidenced by the cytokine levels (Fig. 7). High IFN- $\gamma$  and IL-6 levels were detected in the VLP-immunized group compared with those in the PBS group ( $P < 0.01$ , Fig. 7). In particular, the IFN- $\gamma$ , IL-4, and IL-6 levels in the VLP-immunized group were higher than the levels in the commercial vaccine group, indicating that the chimeric VLPs could elicit efficient T cell responses in guinea pigs. In contrast, the IL-4 levels induced by the VLPs and the commercial vaccine showed no significant differences compared with the PBS group ( $P > 0.01$ , Fig. 7), possibly because the IL-4 concentration was significantly enhanced 2 weeks after the second immunization (Zhao et al. 2013).

In summary, our study showed that chimeric FMDV VLPs elicited efficient humoral and cellular immune responses in guinea pigs and significantly protected the guinea pigs against FMDV challenge. These results indicate that chimeric VLPs may serve as an effective FMDV vaccine candidate.

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#### Compliance with ethical standards

**Competing interests** The authors declare that they have no competing interests.

**Ethics statement** All guinea pig experiments were performed in a bio-safety level 3 laboratory at Lanzhou Veterinary Research Institute (LVRI), Chinese Academy of Agricultural Sciences (CAAS). All of the animal protocols were approved by the Institutional Animal Use and Care Committee of the CAAS. All guinea pigs used in the present study were humanely bred during the experiment and euthanized at the end of the experiment.

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