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Immunogenicity and protective efficacy of a novel foot-and-mouth disease virus empty-capsid-like particle with improved acid stability

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ABSTRACT

Foot-and-mouth disease virus (FMDV) is the etiological agent of a highly contagious disease that affects cloven-hoofed animal species. The FMDV capsid is highly acid labile and viral particles lose their immunogenicity when they disassemble at mildly acidic pHs. The viral capsid of FMDV serotype O is more sensitive than those of other serotypes, making it more difficult to acquire enough empty-capsid-like particles in the acidic insect cell environment for research. In this study, novel FMDV mutants with increased acid resistance were isolated using BHK-21 cell cultured under low-pH conditions. Amino acid substitutions Q25R, K41E, and N85A in the VP1 capsid protein and K154Q in the VP3 capsid protein were detected in all six mutants. Based on these amino acid replacements, empty-capsid-like particles of FMDV serotype O, which were resistant to the acid-induced dissociation of the capsid into pentameric subunits, were produced in insect cells. We characterized the protective immunity induced by these acid-resistant empty capsid particles. Significant humoral and cellular immune responses were elicited in mice after immunization with the acid-resistant empty capsid particles. The acid-resistant empty-capsid-like particles also induced strong neutralizing antibodies in guinea pigs and protected all the guinea pigs from FMDV challenge. Our results suggest that these acid-resistant empty-capsid-like particles have potential utility as a vaccine against serotype O FMDV infection.

1. Introduction

Foot-and-mouth disease (FMD) is one of the most highly contagious viral diseases of cloven-hoofed animals, including important livestock species such as cattle, swine, sheep, and goats [1,2]. The disease is characterized by fever, lameness, and the appearance of vesicular lesions on the mouth, tongue, nose, feet, and teats, and it severely damages milk and meat production. The infection spreads rapidly, generating large-scale epidemics, and the high morbidity and associated trade restrictions cause significant economic losses [3]. The causative agent, FMD virus (FMDV), belongs to the genus *Aphthovirus* within the family *Picornaviridae* [4]. FMDV displays high genomic and antigenic variability, and includes seven serotypes (A, O, C, SAT 1–3, and Asia 1) and multiple subtypes within each serotype [5]. Vaccination with a chemically inactivated viral vaccine is currently the major measure used for FMD control in endemic countries. However, the production of the inactivated vaccine requires expensive high-containment manufacturing facilities and there is a risk of viral release during vaccine production or the improper inactivation of the virus, leading to vaccine-related outbreaks [6]. Therefore, more new-generation vaccines that circumvent these limitations are urgently required.

The FMDV genome consists of a positive-sense, single-stranded RNA molecule of about 8.5 kb in length. The RNA molecule is enclosed within an icosahedral capsid with a 30 nm diameter. The FMDV capsid is composed of 60 copies each of four structural proteins (VP1, VP2, VP3, and VP4) arranged into 12 pentameric subunits, which constitute the intermediates in capsid assembly and disassembly [7]. Empty capsid particles can also be produced during infection, which resemble the mature virus in their structure and immunogenicity [8,9]. The empty FMDV-virus-like particles can be synthesized with recombinant techniques and are a promising alternative subunit vaccine.

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The FMDV capsid is extremely labile to acidic pH, which causes the viral particles to lose their infectivity because they disassemble at pHs slightly below neutrality [10–12]. The dissociation of the FMDV virion into 12 pentameric subunits results in strongly reduced immunogenicity [9,13–16]. This instability makes it difficult to develop vaccines with long shelf-lives [17]. Therefore, understanding the molecular basis of capsid stability is not only relevant to our basic knowledge of the biology of viruses, but will also allow us to improve the stability of conventional vaccines based on inactivated virions or future vaccines based on empty capsids [17–22].

In recent years, some FMDV variants with enhanced acid resistance have been selected with acid treatment, and the point mutations in the viral capsid responsible for the increased resistance to acid inactivation have been identified. These correlated with an increased resistance to the acid-induced dissociation of FMDV into its pentameric subunits [18,23–25]. In previous studies, FMDV was incubated for ≤ 1 h in phosphate-buffered saline (PBS) after acid treatment and then neutralized, and the samples were used to infect BHK-21 cells cultured under normal conditions. In the present study, after acid treatment, the virus was used to infect BHK-21 cells that were then cultured at an acidic pH. Six FMDV variants with increased resistance to acid inactivation were isolated with an acid-induced inactivation assay. Four amino acid substitutions in the capsid proteins were detected in all six mutants. The empty FMDV capsid carrying these amino acid substitutions was then produced in a baculovirus expression system. The empty-capsid-like particles elicited significant humoral and cellular immune responses in mice. They also induced strong neutralizing antibodies in guinea pigs and protected all the vaccinated guinea pigs against FMDV serotype O challenge.

2. Materials and methods

2.1. Virus, cells, and animals

The FMDV O strain China/1/99 (GenBank: AF506822.2) was separated and persevered by the Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. Baby hamster kidney (BHK)-21 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific, Waltham,USA) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific, Waltham, USA) under standard culture conditions or at pH 6.0. The BHK-21 cells cultured at acidic pH were used to screen for acid-resistant FMDV variants. *Spodoptera frugiperda* (*Sf*9) insect cells were maintained at 27 °C in Grace medium (Thermo Scientific,Waltham,USA) supplemented with 2% FBS for the production of the control and recombinant baculoviruses. BALB/c mice and guinea pigs were provided by the Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Lanzhou, China). All the experimental protocols involving animals were approved by the Institutional Animal Use and Care Committee of Lanzhou Veterinary Research Institute and were performed according to the national guidelines for the use of animals in scientific research.

2.2. Acid-induced inactivation assay

A previously published protocol was followed [26], with minor modifications. Briefly, equal amounts (10 µl containing 10^7 50% tissue culture infective doses ($TCID_{50}$)/100 µl) of the viruses tested were mixed with 300 µl of PBS (pH 6.0) for 60 min at room temperature and the infectivity remaining in each sample was determined as the $TCID_{50}$ of the virus in BHK-21 cells.

2.3. Screening the acid-resistant FMDV variants

BHK-21 cells were propagated in DMEM supplemented with 10% heat-inactivated FBS (Thermo Scientific, Waltham, USA) at pH 6.0 and infected with acid-induced FMDV at a multiplicity of infection (MOI) of 1. BHK-21 cells cultured under standard culture conditions were infected with FMDV as the negative control. The supernatant was harvested when most of the cells showed a cytopathic effect (CPE) and was used for the next round of acid-induced inactivation and the screening assay. Three parallel tests were performed and the assay was repeated 10 times to isolate the acid-resistant variants. The acid-resistant variants were subjected to three rounds of plaque assay to isolate individual viral plaques.

2.4. Viral RNA extraction, cDNA synthesis, and DNA sequencing

Viral RNA was extracted from the supernatants of the infected cell cultures using RNeasy Mini Kit spin columns (Qiagen, Dusseldorf, Germany). The nucleotide sequence of the capsid-encoding region of FMDV was amplified with reverse transcription–PCR (RT–PCR). The following forward and reverse synthetic oligonucleotide primers were used: forward primer, 5′-GGCGGCGGGCAA TCCAGCCCGGCGAC-3′; and reverse primer, 5′-CAAAAGCTGTTTCA CAGGCGCCACA-3′. DNA sequencing of RT- PCR products were performed by GENEWIZ. Inc (Suzhou, China). The nucleotide substitutions were analyzed by DNAstar software. The structural model of a pentameric subunit of the FMDV capsid was built using the Python (https://www.python.org/) and the structures were visualized using PyMOL (DeLano Scientific) (http://www.pymol.org/).

2.5. Construction and screening of recombinant baculoviruses

The region of the FMDV genome encoding the polyprotein and 3C viral protease was amplified and cloned into the multiple cloning site in the pFastBacDual vector under the control of the polyhedrin promoter to generate pFastBacDual–P12A3C. Five selected nucleotide substitutions from the acid-resistant FMDV variants were introduced into pFastBacDual–P12A3C. All mutagenesis was conducted with the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, USA), according to the manufacturer's protocol. The recombinant plasmid constructed, pFastBacDual–mP12A3C, was sequenced to confirm that the correct nucleotide substitutions had been introduced and the absence of other changes.

The recombinant baculoviruses rBac–P12A3C, rBac–mP12A3C, and the negative control rBac–NC were then generated according to the Baculovirus Expression System Manual (Thermo Scientific, Waltham,USA). Briefly, competent DH10Bac™ *Escherichia coli* cells (Thermo Scientific,Waltham,USA) were transformed with the plasmid pFastBacDual–P12A3C, pFastBacDual–mP12A3C, or the empty vector pFastBacDual. Positive colonies were identified with blue–white screening, confirmed with PCR amplification using M13 primers, and separately used to transfect *Sf*9 insect cells with a liposome-mediated method. The supernatants were collected after culture for 72 h. The viruses were purified with plaque assays and amplified by infecting Sf9 cells at a multiplicity of infection (MOI) of 5 pfu/cell. High-titer viral stocks were obtained with several rounds of infection in *Sf*9 insect cells. The viral genomes were extracted and amplified with the appropriate combinations of primers to confirm that the target genes were correctly inserted into the recombinant baculoviruses.

2.6. Expression and identification of recombinant proteins

*Sf*9 cells in six-well plates were infected with the recombinant baculovirus rBac–mP12A3C, rBac–P12A3C, or rBac–NC at an MOI of 5. The recombinant proteins were detected with either an indirect immunofluorescence assay (IFA) or western blotting. For the indirect IFA, the cells were stained with a polyclonal rabbit antibody directed against FMDV (1:250 dilution), and then with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG secondary antibody (Sigma, St. Louis, MO, USA; 1:200 dilution), and analyzed under a fluorescence microscope. For western blotting, the cell lysates were resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes, probed with a polyclonal rabbit antibody directed against FMDV (diluted 1:1000), and then with a horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG secondary antibody (Sigma, St. Louis, MO, USA; 1:10000 dilution). The bands were visualized with a chemiluminescence kit (Thermo Scientific, Waltham, USA).

2.7. Antigenicity of the recombinant proteins

The recombinant proteins harvested from the cells infected with the recombinant baculovirus rBac–mP12A3C, rBac–P12A3C, or rBac–NC were tested with an antigen-capture sandwich enzyme-linked immunosorbent assay (ELISA), performed as described by the World Organization for Animal Health, Office International des Epizooties standard for FMDV serotyping. Briefly, the plates were coated with a rabbit anti-FMDV serotype O antibody diluted 1:1000 in carbonate/bicarbonate buffer (pH 9.6) and incubated at 4 °C overnight. On the following day, serial two-fold dilutions of the recombinant proteins in PBS buffer were added to the wells. The plates were incubated with a guinea pig anti-FMDV serotype O antibody (1:1000 dilution), followed by an HRP-conjugated rabbit anti-guinea pig IgG secondary antibody (Sigma, St. Louis, MO, USA) diluted 1:10,000. A substrate solution containing orthophenylene diamine/hydrogen peroxide was added and the plates were allowed to stand for 15 min. The reaction was stopped by the addition of 1 M H_2SO_4. The optical density (OD) of each well was determined at 490 nm.

2.8. Observation of FMDV empty-capsid-like particles

The cell lysate containing rBac–mP12A3C was observed with immunoelectron microscopy. The cell lysate was fixed on a copper grid. After incubation with a polyclonal rabbit antibody directed against FMDV (1:200 dilution), the copper grid was treated with a gold-conjugated goat anti-rabbit IgG secondary antibody (Sigma, St. Louis, MO, USA) diluted 1:200. The diameter of the colloidal gold particles was 10 nm. The copper grid was then negatively stained with 1% uranyl acetate solution and observed under a transmission electron microscope.

2.9. Acid stability assay

The cell lysates were incubated with PBS at pH 7.2 (control) or pH 6.0 (acid treated) at room temperature for 30 min, and the proportions of virions and pentamers in each sample were determined by ultracentrifuging the samples in a 15%–60% sucrose density gradient at 4 °C in an SW40 rotor (Beckman Instruments) at 35,000 rpm for 2.5 h. The gradient was fractionated into 0.5 ml aliquots and analyzed with dot blotting using a rabbit anti-FMDV polyclonal antibody and chemiluminescent detection.

2.10. Quantification of the empty-capsid-like particles

Sf9 cells infected with the recombinant baculovirus rBac–P12A3C or rBac–mP12A3C were harvested from a T-75 tissue culture flasks and resuspended with 1 ml PBS. After ultrasonic decomposition, the supernatant was collected after centrifuging at 16,000 rpm for 30 min at 4 °C. The empty FMDV capsid dissociates into specific viral capsid degradation products (12S). Therefore, the empty-capsid-like particles in supernatant were quantified with a specific double-antibody sandwich ELISA (named M3 ELISA), using a single-domain antibody fragment (M3ggsVI-4Q6E) that binds specifically to the 12S particles, according to a previously published procedure [16,27]. Briefly, a 96-well ELISA plate was coated with 0.5 mg/l M3ggsVI-4Q6E. The supernatant of cell lysates were diluted 25-fold and separated into two samples. One sample was heated at 56 °C for 1 h to convert the empty-capsid-like particles into 12S particles, and the other sample was incubated at 4 °C. The plate was washed and 50 μl of each cell lysates were added. All the reagents were serially diluted two-fold. The plate was incubated at 37 for 1 h, washed, and then successively incubated with biotinylated M3ggsVI-4Q6E at a weight-ratio of protein to biotin of 1, 0.5 mg/l peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), and stained with 3,3′,5,5′ tetramethylbenzidine. After the reaction was stopped by the addition of 0.5 M H_2SO_4 (50 μl per well), the absorbance at 450 nm was measured with an ELISA plate reader.

2.11. Immunogenicity of FMDV empty-capsid-like particles in mice

Thirty-six female BALB/c mice (aged 6 weeks) were randomly divided into three groups, with 12 mice in each group. Group A was intramuscularly vaccinated with 100 μl of solution containing 5 μg of empty-capsid-like particles with ISA-206 adjuvant (Seppic, Paris, France). Group B was inoculated with100 μl of a commercial inactivated FMDV vaccine containing ISA-206 adjuvant (China Agricultural Vet. Bio. Science and Technology Co., Lanzhou, China) as the positive control, and group C was vaccinated with rBac–NC as the negative control. All the mice were boosted with the same dose 14 days after the primary immunization (dpi). Blood samples for serum preparation were collected before and 14 and 28 days after the primary immunization. At 28 days after the primary immunization, the mice were killed and their splenocytes harvested with Mouse 1 × Lymphocyte Separation Medium (Dakewe Biotech Company, Shenzhen, China) for the detection of cytokines.

2.12. Antibody and cytokine tests

Anti-FMDV antibodies were detected with a liquid-phase blocking (LPB)-ELISA, as previously described [28]. Splenocytes were isolated from the mouse spleens and cultured in DMEM supplemented with 10% heat-inactivated FBS. The levels of mouse cytokines interferon γ (IFN-γ) and interleukin 4 (IL-4) in the splenocytes at 28 dpi were measured with commercially available ELISPOT kits (Dakewe Biotech Company, Shenzhen, China), according to the manufacturer's instructions, as described in our previous report [29].

2.13. Challenge study of the protective response in guinea pigs

Twelve healthy guinea pigs, weighing 300–400 g, were randomly divided into three groups, with four guinea pigs in each group. Group A was immunized with an intramuscular injection of 100 μl of solution containing 5 μg of empty-capsid-like particles and ISA-206 adjuvant. Group B was immunized with 100 μl of a commercial inactivated FMDV vaccine containing ISA-206 adjuvant (China Agricultural Vet. Bio. Science and Technology Co.) as the positive control, and group C was vaccinated with rBac–NC as the negative control. Blood samples for serum preparation were collected before and 25 days after the primary immunization. At 28 days dpi, all the guinea pigs were challenged with 100 GID_{50} (50% guinea pig infective dose) of FMDV strain China/1/99 by intradermal injection in the left rear foot. All the guinea pigs were monitored for lesions on the footpads for 10 consecutive days post challenge (dpc) and were then euthanized on day 11 (or before in cases of severe signs of disease). No lesions on footpads other than the inoculated footpad indicated the guinea pig was ‘protected’, whereas the appearance of a secondary lesion on any footpad other than the inoculated footpad was scored as ‘unprotected’.

2.14. Quantification of neutralizing antibodies in guinea pigs

The neutralizing antibody titers in individual sera were measured with a virus neutralization test (VNT). The serum from each guinea pig was heat-inactivated at 56 °C for 30 min and the neutralizing anti-FMDV antibodies in it were measured with a VNT in a 96-cell tissue culture plate, according to the method described in the OIE Manual (OIE, 2015). The neutralizing antibody titers were calculated with the Reed–Münch method and expressed as log10 of the reciprocal of the final serum dilution that neutralized 100 $TCID_{50}$ of homologous FMDV in 50% of the wells.

3. Results

3.1. Isolation of serotype O FMDV variants with increased resistance to acid inactivation

A viral suspension (10 µl) containing about 10^7 $TCID_{50}$/100 µl of FMDV strain China/1/99 was incubated for 60 min in PBS at pH 6.0, and then used to infect BHK-21 cells, which were subsequently cultured at pH 6.0. The progeny viruses were subjected to subsequent rounds of treatment for a total of 10 passages. The viruses were purified by plaque assay and six acid-resistant variants (AR1 to AR6) and six negative control strains (N1 to N6) were isolated. The purified viruses were evaluated for acid resistance at pH 6.0. The mutants analyzed showed reduced acid sensitivity compared with that of the control strains and the parental strain China/1/99 (Fig. 1). No significant reduction in the titers of the mutants was observed after incubation in PBS at pH 6.0, whereas the titer of the parental virus decreased more than 10^6-fold after incubation in PBS at pH 6.0 (Fig. 1). These results demonstrate that the acid-selected serotype O FMDV variants had increased resistance to acid-induced inactivation.

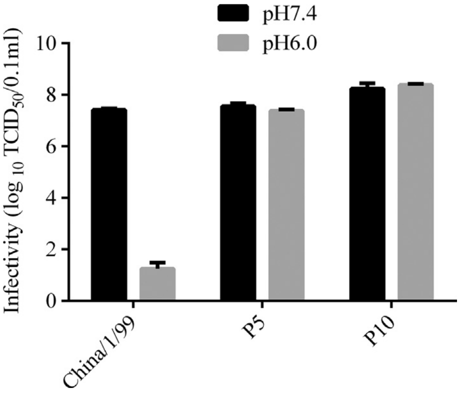

Fig. 1. Resistance to acid-induced inactivation of FMDV mutants selected with a pH 6.0 treatment. Viral samples P5, P10, and the parental strain China/1/99 were treated with acidic buffer (pH 6.0) or neutral buffer (pH 7.4) as the control. The samples were plated on BHK-21 monolayers and titered with $TCID_{50}$ assays.

To investigate the amino acid substitutions responsible for the increased resistance to acid inactivation, the complete capsid-encoding regions of the acid-resistant variants (AR1 to AR6) and the negative control strains (N1 to N6) were sequenced. Nucleotide and amino acid replacements were found in the acid-resistant variants and are shown in Table 1. All the mutants showed common amino acid (nucleotide) substitutions: VP3 K154Q (A1369C) and VP1 Q25R (A1643G), VP1 K41E (A1690G), and VP1 N85A (A1822G and A1823C). The location of the mutations on the 3-dimensional structure of the pentameric subunit in the capsid was identified (Fig. 2). The result showed that replacement VP3 K154Q was located remarkably close to the interfaces between the pentameric subunits, into which the capsid is known to dissociate upon acidification. While replacements VP1 K41E and VP1 N85A were located at the outer surface of the capsid, not far from the capsidpore (5-fold symmetry axis), and replacement VP1 Q25R was located at the internal surface of the capsid, not far from the interpentamer interfaces.

3.2. Expression and characterization of FMDV empty-capsid-like particles

After the infection of *Sf*9 cells with recombinant baculoviruses encoding the P1-2A and 3C expression cassette, where P1 (derived from FMDV China/1/99) was either the wild-type sequence or carried the mutations K154Q in VP3 and Q25R, K41E, and N85A in VP1. The infected cells were stained with a polyclonal rabbit antibody directed against FMDV and analyzed with an IFA. Obvious green fluorescence was observed in the *Sf*9 cells infected with rBac–mP12A3C or rBac–P12A3C, whereas only very weak background fluorescence was observed in the mock-infected control cells (Fig. 3A). The infected cells were pelleted and lysed, and their extracts were analyzed with western blotting at 3 dpi, at which point most of the cells showed CPEs. Bands of 27 kDa, corresponding to VP3/VP1, and of 36 kDa, corresponding to VP0, were

Table 1
Mutations found in the capsid-encoding regions of FMDV mutants with increased resistance to acid-induced inactivation, selected with 10 serial acid treatments.

Genomic region	Mutations found in viral population[a]					
	AR1	AR2	AR3	AR4	AR5	AR6
VP4						
VP2						
VP3	A1369C	A1369C	A1369C	A1369C	A1369C	A1369C
	K154Q	**K154Q**	**K154Q**	**K154Q**	**K154Q**	**K154Q**
VP1	A1643G	A1643G	A1643G	A1643G	A1643G	A1643G
	Q25R	**Q25R**	**Q25R**	**Q25R**	**Q25R**	**Q25R**
	A1690G	A1690G	A1690G	A1690G	A1690G	A1690G
	K41E	**K41E**	**K41E**	**K41E**	**K41E**	**K41E**
	A1822G	A1822G	A1822G	A1822G	A1822G	A1822G
	A1823C	A1823C	A1823C	A1823C	A1823C	A1823C
	N85A	**N85A**	**N85A**	**N85A**	**N85A**	**N85A**

[a] Nucleotide positions are as in the China/1/99 genome and the resultant amino acid substitutions are indicated. Nonsynonymous amino acid replacements are indicated in boldface.

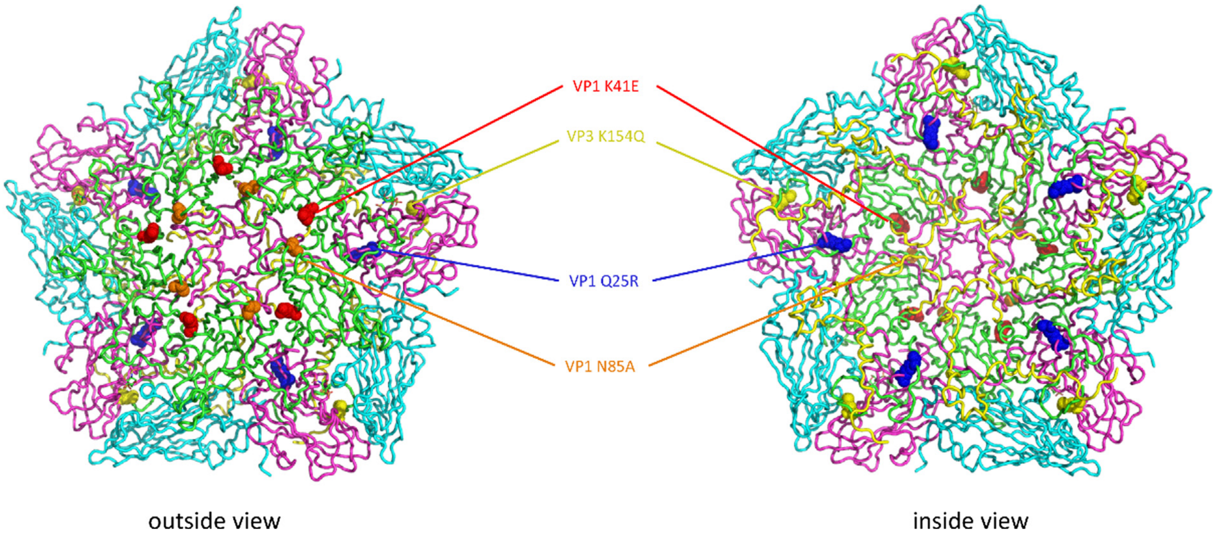

Fig. 2. Outside and inside schematic views of a pentameric subunit in the capsid. VP1 is colored green, VP2 cyan, VP3 magenta, and VP4 yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

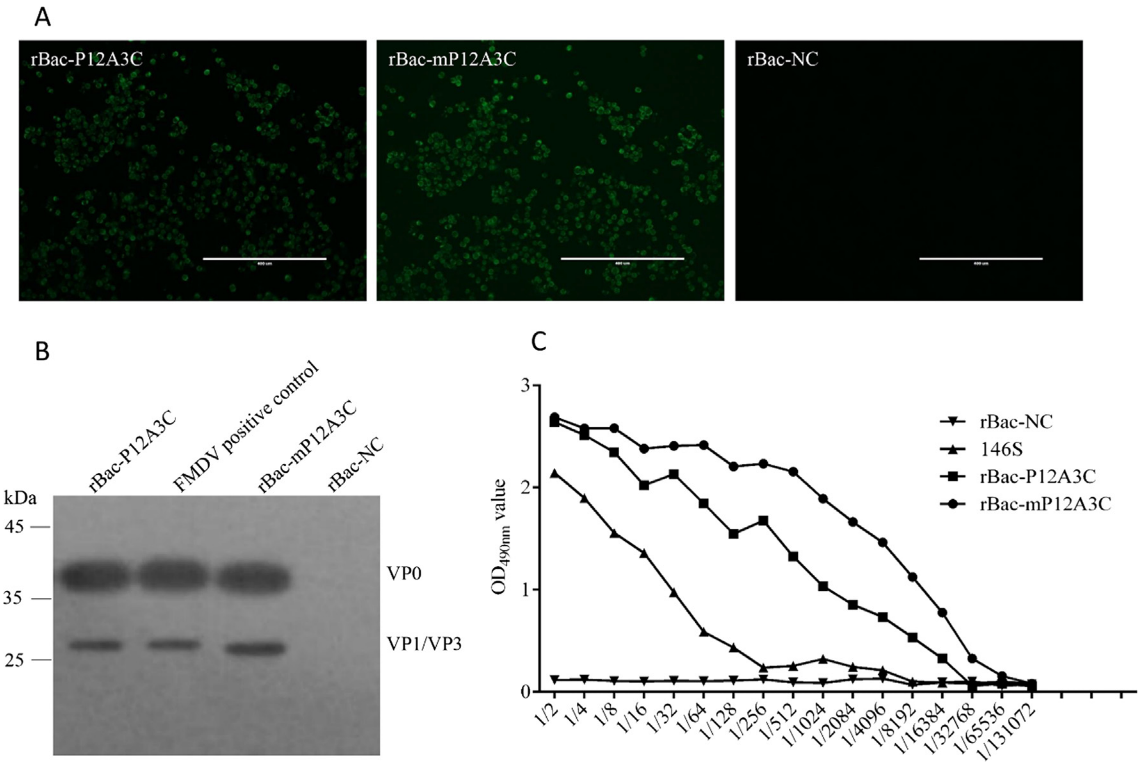

Fig. 3. Expression, identification, and antigenicity of the recombinant proteins. (A) Immunofluorescence assays of rBac–P12A3C-, rBac–mP12A3C-, and rBac–NC-infected *Sf*9 cells with a polyclonal rabbit antibody directed against FMDV. (B) Immunoblot of rBac–P12A3C-, rBac–mP12A3C-, and rBac–NC-infected *Sf*9 cells probed with a polyclonal rabbit antibody directed against FMDV. Inactivated FMDV was used as the positive control. (C) Antigenicity of rBac–P12A3C-, rBac–mP12A3C-, and rBac–NC-infected *Sf*9 cells tested with an antigen-capture sandwich ELISA, with inactivated 146S as the positive control.

confirmed on western blots (Fig. 3B) probed with a polyclonal rabbit antibody directed against FMDV, whereas no specific band was detected in the mock-infected cell lysates. These results demonstrate that the recombinant baculoviruses efficiently expressed the target proteins in *Sf*9 cells.

The extracts of infected *Sf*9 cells were analyzed with antigen-capture ELISA to determine their antigenicity. The results of the antigen-capture sandwich ELISA indicated that a large number of recombinant proteins were expressed when *Sf*9 cells were infected with the recombinant baculovirus rBac–mP12A3C. The OD of the harvested cell lysates infected with rBacP12A3C decreased with dilution, in agreement with the variation in the concentration of FMDV antigen in the positive control. No FMDV antigen was detected in the mock-infected cells (Fig. 3C).

To confirm the nature of the empty-capsid-like particles, extracts obtained from the infected cells were analyzed with immunelectron microscopy, using a polyclonal rabbit antibody directed against FMDV and a colloidal-gold-particle-conjugated secondary antibody. The prepared capsid-like particles had an orthohexagonal morphology and a diameter of approximately 30 nm (Fig. 4A). The extracts obtained from the infected cells were purified by ultracentrifugation in a sucrose gradient. The purified empty-capsid-like particles were then incubated at pH 6.0 for 60 min, subjected to ultracentrifugation, and analyzed with dot blotting using a rabbit anti-FMDV polyclonal antibody to determine the amounts of empty-capsid-like particles remaining after treatment. The results (Fig. 4C) showed that the empty-capsid-like particles from rBac–mP12A3C were substantially more resistant than the rBac–P12A3C particles to the acid-induced dissociation of the capsid into pentameric subunits.

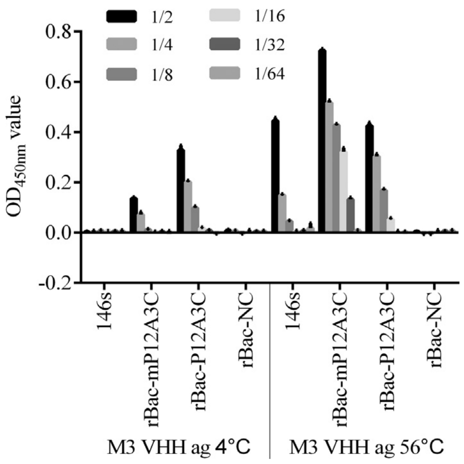

Fig. 5. Quantification of empty-capsid-like particles. Extracts of rBac–P12A3C- and rBac–mP12A3C-infected *Sf*9 cells were separated into two samples. One sample was heated at 56 °C for 1 h to convert the empty-capsid-like particles into 12S particles, and the other sample was incubated at 4 °C. All the samples were analyzed with a specific double-antibody sandwich ELISA using the single-domain antibody M3ggsVI-4Q6E. The increase in the concentration of 12S particles after heating at 56 °C represented the empty-capsid-like particle concentration present in the sample before heating at 56 °C.

Following a previously published procedure, we used the M3 ELISA to indirectly quantify the empty-capsid-like particles and 12S particles in the samples before and after heating at 56 °C. The increase in the concentration of 12S particles after heating at 56 °C represented the concentration of the empty-capsid-like particles present in the sample before heating at 56 °C. This allowed the fraction of empty-capsid-like particles to be calculated. The cell lysates were diluted 25-fold before they were quantified. The OD of rBac–mP12A3C increased almost seven-fold after heating at 56 °C, whereas that of rBac–P12A3C increased only slightly (Fig. 5). The absorbance data were then evaluated with an Excel® spreadsheet template (Microsoft Corporation, Redmond, WA, USA). A four-parameter logistic curve was fitted to the absorbance and FMDV antigen concentrations of standards with a nonlinear least squares method, using the Excel® solver tool. The 12S concentrations in the unknown samples were then determined by interpolation. The difference in the 12S concentration, measured with both assays, was deemed to be the final concentration of empty-capsid-like particles, whereas the 12S concentration in the heated sample was deemed to be the total antigen concentration. The final concentrations of empty-capsid-like particles in the cell lysates are shown in

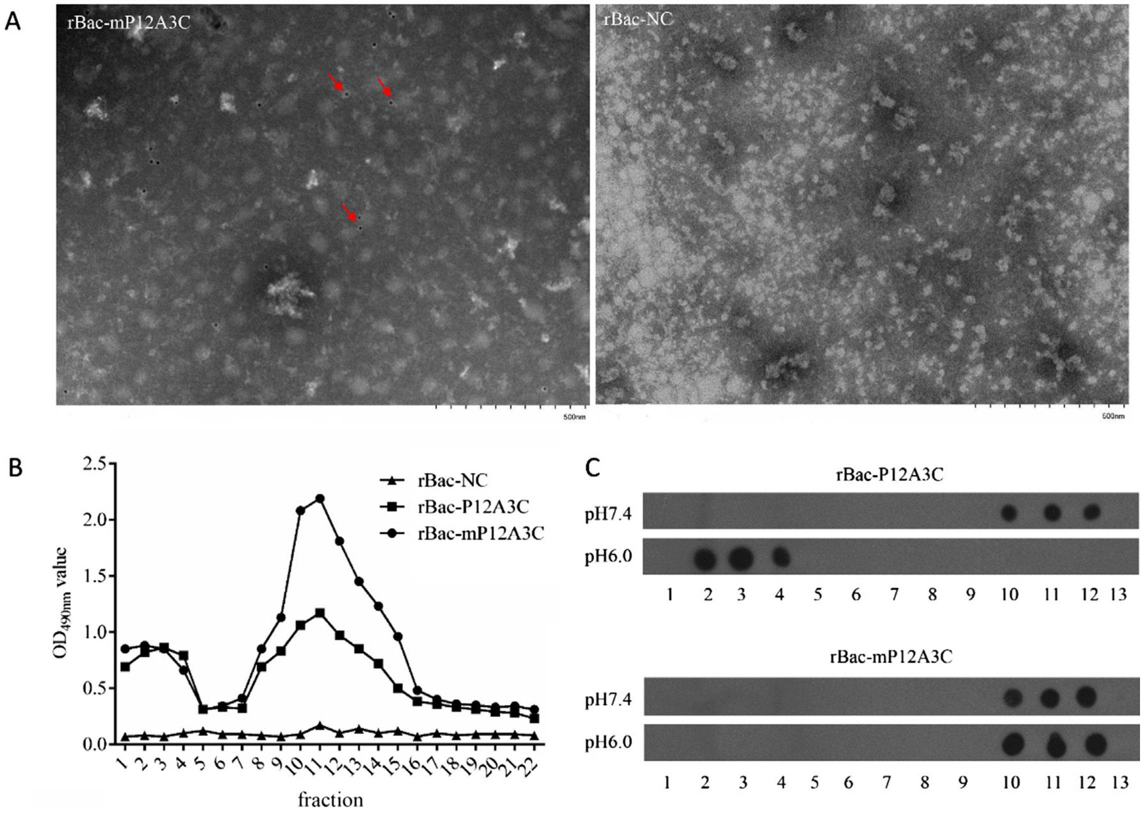

Fig. 4. Characterization of the empty-capsid-like particles. (A) Extracts of rBac–mP12A3C-infected *Sf*9 cells were analyzed with immunoelectron microscopy using a polyclonal rabbit antibody directed against FMDV and a colloidal-gold-particle-conjugated secondary antibody. (B) Extracts of rBac–P12A3C- and rBac–mP12A3C-infected *Sf*9 cells were centrifuged on a 15%–60% (wt/vol) sucrose gradient. Samples from each fraction were assayed with an antigen-capture sandwich ELISA. (C) Analysis of the integrity of the empty-capsid-like particles after acid treatment. Purified empty-capsid-like particles from extracts of rBac–P12A3C- and rBac–mP12A3C-infected *Sf*9 cells were incubated with PBS at pH 7.4 (control) or 6.0 (acid treatment) for 60 min at room temperature. The proportions of empty-capsid-like particles and pentamers in each sample were determined by ultracentrifugation of the samples in a 15%–60% sucrose density gradient and analysis with dot blotting using a polyclonal rabbit antibody directed against FMDV and chemiluminescent detection.

Table 2
Concentrations of antigens.

Antigen samples	Concentration (μg/ml)		
	M3 VHH ag 56 °C [a]	M3 VHH ag 4 °C [b]	empty-capsid-like particles [c]
rBac-mP12A3C	206.5	28.8	177.7
rBac-P12A3C	80.8	54.6	26.2
rBac-NC	0	0	0

[a] Samples were heated at 56 °C for 1 h to convert empty-capsid-like particles into 12S particles, which were detected with an M3 ELISA.

[b] Samples were incubated at 4 °C for 1 h and detected with an M3 ELISA.

[c] Difference in M3-treated antigen and M3-intact antigen was taken as the final concentration of empty-capsid-like particles.

Table 2. The concentration of empty capsid particles of rBac–mP12A3C after heating was 177.7 μg/ml, almost seven-fold higher than the concentration of rBac–P12A3C (26.2 μg/ml).

3.3. Empty-capsid-like particles elicit high titers of FMDV-specific antibodies and cellular immune responses in naïve BALB/c mice

To evaluate the humoral responses induced by the acid-resistant empty-capsid-like particles, BALB/c mice were immunized twice, at a 14-day interval, with 5 μg of acid-resistant empty-capsid-like particles combined with adjuvant. On day 14 after the primary immunization, anti-FMDV antibodies were detected with an LPB-ELISA in all the groups of mice. On day 14 after the booster, the mice immunized with either the acid-resistant empty-capsid-like particles or the inactivated vaccine showed high levels of anti-FMDV antibodies compared with the rBac–NC-treated control mice (Fig. 6A). The titers of anti-FMDV antibodies induced by the acid-resistant empty-capsid-like particles were higher than those induced by the inactivated vaccine when determined with an LPB-ELISA. These results indicate that the acid-resistant empty-capsid-like particles are immunogenic and induce a significant humoral response in mice.

Cytokine assays were performed to investigate the ability of the acid-resistant empty-capsid-like particles to elicit a specific cellular immune response. The splenocytes from individual mice were removed 14 days after the final immunization and stimulated with inactivated FMDV (146S). The concentrations of IFN-γ and IL-4 secreted into the supernatants of the stimulated spleen cells were then measured. The concentrations of IFN-γ and IL-4 from mice treated with the acid-resistant empty-capsid-like particles or the inactivated vaccine were significantly higher than those of the control group ($P < 0.0001$) (Fig. 6B and C).

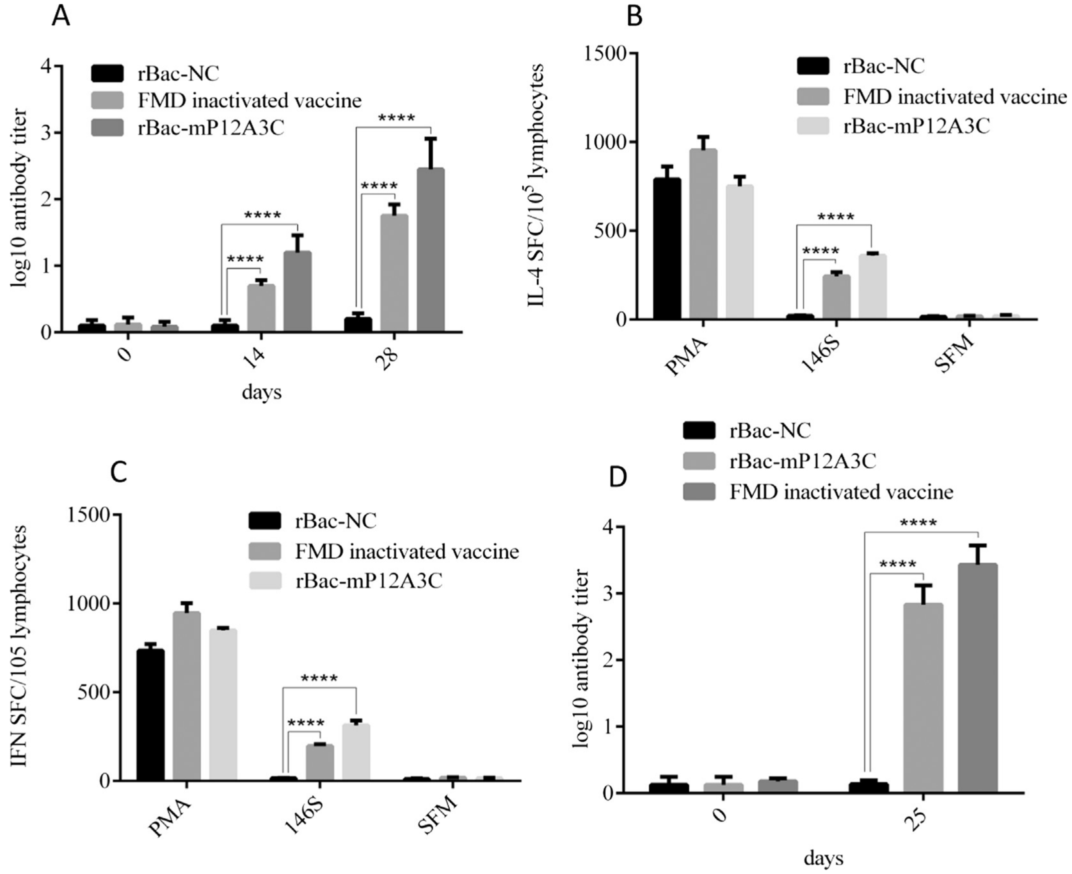

Fig. 6. Immunogenicity of FMDV empty-capsid-like particles in BALB/c mice and guinea pigs. (A) BALB/c mice were immunized twice with the empty-capsid-like particles at a 14-day interval. A commercial inactivated FMDV vaccine was used as the positive control and an extract of rBac–NC-infected *Sf*9 cells as the negative control. LPB-ELISA-detected antibodies against serotype O FMDV were measured with an LPB-ELISA in mouse serum samples on days 0, 14 and 28 dpi. The LPB-ELISA antibody titers were calculated and expressed as log10 of the reciprocal of the highest serum dilution. (B) Splenocytes were isolated from mouse spleens at 28 dpi and the levels of mouse cytokine IL-4 in the splenocytes was measured with an ELISPOT assay. The splenocytes were stimulated with the following materials: PMA+Ionomyin (positive stimulus), FMDV 146S and serum free medium (SFM, negative stimulus). (C) Levels of mouse cytokine IFN-γ in the splenocytes at 28 dpi were measured with an ELISPOT assay (n = 3 per group). (D) Guinea pigs were immunized with the empty-capsid-like particles. A commercial inactivated FMDV vaccine was used as the positive control and an extract of rBac–NC-infected *Sf*9 cells as the negative control. The neutralizing antibody titers in sera collected at 25 dpi were tested with micro VNT, calculated with the Reed–Münch method, and expressed as log10 of the reciprocal of the final serum dilution that neutralized 100 $TCID_{50}$ of homologous FMDV in 50% of wells. Data are means ± SEM. P values < 0.05 are considered significant. $^{*}0.01 < P \leq 0.05$; $^{**}0.001 < P \leq 0.01$; $^{***}0.0001 < P \leq 0.001$; $^{****}P \leq 0.0001$.

3.4. Protective effects in guinea pigs challenged with FMDV

Sera collected on 0 and 25 dpi were tested with a micro VNT. As shown in Fig. 6D, the mean FMDV-specific viral neutralizing titers in all three groups were low before vaccination, at 0.13–0.18 $\log_{10}$. The conventional vaccine group showed the highest mean titer of 3.43 $\log_{10}$ on 25 dpi, followed by 2.83 $\log_{10}$ in the rBac–mP12A3C-treated group. Although the titer induced by the conventional inactivated vaccine was higher than that induced by rBac–mP12A3C, the VNT of rBac–mP12A3C was not significantly different from that of the conventional inactivated vaccine (P greater than 0.05).

When the immunized guinea pigs were challenged with FMDV type O on 28 dpi, the unvaccinated (rBac–NC) guinea pigs developed lesions on all uninoculated feet 2 dpc. Only one of the four guinea pigs immunized with the conventional vaccine showed clinical symptoms at 3 dpc. None of the four rBac–mP12A3C-immunized guinea pigs and all four of the rBac–NC-immunized animals developed severe vesicles. These protection data indicate that the rBac–mP12A3C-treated guinea pigs showed 100% protection, whereas those treated with the conventional vaccine group showed 75% protection and the rBac–NC-treated animals showed 0% protection (Table 3).

3.5. Discussion and conclusion

Unlike enteroviruses, which release RNA by receptor-mediated uncoating [30], FMDV appears to release its RNA genome by the dissociation of the viral capsid into pentamers at mildly acidic pHs [10–12]. Its acid-labile capsid is a key characteristic of FMDV RNA uncoating, but its instability increases the difficulty of developing a vaccine because FMDV loses its immunogenicity when it disassembles in culture environments below pH 6.8 [9,13–16]. In recent years, several FMDV variants with enhanced acid resistance have been isolated and a number of amino acid substitutions have been identified as responsible for this phenotype [18,23–25]. Based on the mutants rescued with reverse genetics technology, an improved inactivated vaccine with increased capsid stability was developed as a novel candidate FMDV vaccine [21].

The acid-labile capsid is a characteristic of FMDV, although capsid metastability appears to differ across the different FMDV serotypes [8,31]. Serotypes Asia 1 and A are relatively stable, whereas serotypes O and SAT are more sensitive to acidic pHs [20]. Therefore, inactivated FMDV serotype O vaccines require a 4–5-fold greater antigenic mass than the corresponding serotype A vaccines [32,33], and the empty-capsid-like particle vaccine based on genetic engineering technology may also require a greater antigenic mass. The insect cell/baculovirus expression vector system is one of the preferred platforms for the production of virus-like particles (VLPs), especially VLPs composed of multiple viral proteins [34–37]. In previous studies, we developed empty-capsid-like vaccines against serotypes Asia I and A using the baculovirus expression system [28,38,39], but we were unable to induce similar protection against serotype O with the same strategy. One reason may be that the viral capsid of FMDV serotype O is more acid sensitive than those of other serotypes, making it more difficult to acquire enough empty-capsid-like particles in the low-pH environment of insect cells. In the present study, novel FMDV mutants with increased acid resistance were isolated and empty capsid particles of FMDV serotype O with amino acid replacements VP3 K154Q, VP1 Q25R, VP1 K41E, and VP1 N85A were produced in insect cells. Unlike the previous strategy for screening for acid-resistant mutations, the viruses were not neutralized after acid treatment and were used to infect cultured BHK-21 under acidic conditions rather than under standard culture conditions. In our system, the BHK-21 cells can stably proliferate and be passaged at pH = 6. The cell morphology and proliferation rate are not different from cells cultured under neutral pH conditions. Thus, the whole screening procedure was performed in a low-pH environment. The quantity of empty-capsid-like particles of the acid-resistant virus obtained was much higher than that obtained from the parental virus. We also incubated the empty-capsid-like particles at pH 6.0 for 60 min to confirm their acid stability. The empty-capsid-like particles from the acid-resistant mutants were more resistant than the parental strain to the acid-induced dissociation of the capsid into pentameric subunits. Previously published article has showed that inclusion of mutated viral 3C protease in frame with the polypeptide (P1-2A), enhanced the yield of structural proteins [40]. We would like to introduce these mutants into our expression cassetteto attempt whether it can further enhance the yield of structural proteins in our system.

We then characterized the protective immunity induced by the acid-resistant empty capsid particles produced with baculovirus. After the immunization of mice with the acid-resistant empty capsid particles, the anti-FMDV titer, determined with an LPB-ELISA, was used to evaluate the humoral response. The acid-resistant empty capsid particles induced systemic immunity in the vaccinated mice, with LPB-ELISA titers higher than those induced by an inactivated vaccine. The role of cell-mediated immunity in the protection of animals against FMDV infection is unclear [41,42]. Previous studies have demonstrated the partial protection of animals vaccinated with Ad5–FMD vectors without detectable levels of neutralizing antibodies, suggesting that cell-mediated immunity may be responsible for this phenotype [43,44]. To evaluate the specific cellular immune responses induced by the acid-resistant empty-capsid-like particles, the levels of cytokines IFN-γ and IL-4 were determined with ELISPOT. The concentrations of IFN-γ and IL-4 were higher in the animals treated with the acid-resistant empty-capsid-like particles than in those treated with the inactivated vaccine. These results indicate that the acid-resistant

Table 3
Protection of and symptom severity in guinea pigs after challenge with FMDV[a].

Serial number of guinea pigs	Group A (rBac-mP12A3C)	Group B(FMD inactivate vaccine)	Group C (rBac-NC)
Protection			
1	Protective	Protective	Non-protective
2	Protective	Protective	Non-protective
3	Protective	Non-protective	Non-protective
4	Protective	Protective	Non-protective
Severity of symptoms	None	None	Severe
1	None	None	Severe
2	None	Mild	Severe
3	None	None	Severe
4	100 (4/4)	75 (3/4)	0(0/4)
Rate of protection (%)[b]			

[a] Observations were made 24 h after challenge. Appearance of secondary lesions was taken as ‘infected’.

[b] Ratio of the number of protected animals to the total number of challenged animals.

empty-capsid-like particles are immunogenic and induce significant humoral and cellular immune responses in mice. Because there is no excellent murine model system susceptible to FMDV O strain China/1/99 in our lab, guinea pigs were used to investigate whether the acid-resistant empty-capsid-like particles protected animals from homologous FMDV attack. After challenge with a virulent homologous virus, all four vaccinated guinea pig were protected, a rate similar to that observed in guinea pigs treated with the inactivated viral vaccine. The results of a VNT for rBac–mP12A3C did not differ significantly from the VNT results for the conventional inactivated vaccine. Therefore, the protection from challenge was consistent with the virus-neutralizing antibody titers. These results are evidence that these acid-resistant empty-capsid-like particles offer a promising strategy for the development of a vaccine against FMDV serotype O. Further investigations would be required to evaluate the vaccine efficiency of the in the target species. These studies would further confirm the potential of using this recombinant vaccine to control FMD in endemic areas.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

YX, HL, XQ, BY, SZ and XY performed experiments. ZL, YX, YM and HY analyzed data. ZL and YX conceived and designed experiments and wrote the manuscript. All the authors read and critically reviewed the manuscript.

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