Recombinant Bivalent Vaccine against Foot-and-Mouth Disease Virus
Serotype O/A Infection in Guinea Pig

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Abstract In this study, two DNA fragments encoding amino acid (141–160)-(21–40)-(141–160) of the VP1 of FMDV (foot-and-mouth disease virus) serotype O and (138–160)-(21–40)-(138–160) of the serotype A FMDV were chemically synthesized. These two tandem-repeat fragments were ligated and transfected into prokaryotic expression vector pTrcHis A to construct pTH-O-A. The other vector called pTH-O-sciG-A was constructed similarly only that the two tandem-repeat DNA fragments were linked by the bovine-IgG heavy chain coding sequence. Guinea pigs immunized with the two bivalent vaccines pTH-O-A and pTH-O-sciG-A showed both specific antibody activity and T cell proliferation responses. FMDV challenge tests showed that 85% and 70% of guinea pigs vaccinated twice with 200 µg of the fusion protein of pTH-O-A were protected from FMDV serotype O and serotype A infection respectively. 70% and 57% of the guinea pigs immunized with the fusion protein of pTH-O-sciG-A were protected from FMDV serotype O and serotype A infection respectively.

Key words foot-and-mouth disease; FMDV serotype O; FMDV serotype A; VP1 protein; bivalent vaccine; guinea pig

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals such as cattle and pig. The disease causes explosive epidemics and heavy economic losses in the agriculture worldwide [1]. FMD virus (FMDV) shows a high genetic and antigenic variability, and has seven serotypes: O, A, C, Asial, SAT1, SAT2 and SAT3 [2]. The FMDV control is mainly implemented using chemically inactivated virus vaccines, which may contain residual living virus and pose a risk of virus release. In FMD-free countries, these types of vaccines are held only for emergency situations [3]. Another problem is that the vaccines for seven FMDV serotypes do not induce cross-protective immunity, so vaccination with one serotype does not confer protection against the infection of other FMDV serotypes [4]. However it is very difficult to anticipate which FMDV serotype will break out in one area before vaccination. Furthermore, multiple serotypes can circulate and one outbreak could be caused by more than one serotype. Many FMDV vaccination programs failed due to these reasons, especially in developing countries lacking sufficient technical/financial sources and fast and exact diagnosis on serotype [4,5]. To effectively protect the livestock from the FMDV infection, a multivalent vaccine is needed to vaccinate animals.

FMDV serotype O and A often breakout together in many countries, so we studied the recombinant bivalent vaccine against these two serotypes. We have previously developed a recombinant FMD vaccine that can express fusion protein gal-FMDV of ß-galactosidase and an immunogenic segment of FMDV VP1 protein containing amino acid (141–160)-(21–40)-(141–160). This fusion protein induced protective immune responses in guinea pig and swine. All immunized animals were efficiently protected against FMDV challenge [6,7]. Immunoglobulin (Ig) has been suggested to be an ideal carrier protein...
of recombinant vaccine [8,9]. Capon et al. [10] found that the CD4 molecule (the receptor for human immuno-deficiency virus type I, HIV-1) fused with IgG prolonged the half-life, and could bind and block the HIV-1 isolate. Bona et al. [11,12] reported that the microbial peptides fused to IgG could improve its immunogenicity. Our previous study reported that the FMDV peptide was fused to the IgG constant region to create the chimeric protein IgG-FMDV which can elicit a strong immune response in guinea pigs and fully protect the animals from FMDV infection [13,14].

In the current study, two bivalent vaccines pTH-O-A and pTH-O-sclG-A were created by fusing an immunogenic peptide consisting of B and T cell epitopes of VP1 gene of FMDV serotype O and A respectively. The results showed the two recombinant proteins not only elicited high antibody levels in vaccinated guinea pigs, but also effectively protected guinea pigs from the infection of FMDV serotype O and A.

**Materials and Methods**

**Plasmid and virus**

The expression vector pTrcHis A used in this study is from Invitrogen Company. The FMDV serotype O is strain HongKong/1997. The serotype A is strain A5. The chemically inactivated vaccine O and A were purchased from Lanzhou Veterinary Institute of the Chinese Academy of Agricultural Sciences.

**Construction of the expression plasmids of pTH-O-A and pTH-O-sclG-A**

For construction of plasmid pTH-O-A, the DNA fragment encoding amino acid 141–160 and 21–40 of VP1 gene of serotype O, and amino acid 138–160 and 21–40 of VP1 gene of serotype A were chemically synthesized. These two fragments were joined to construct a tandem-repeat gene with PstI site (141–160)-(21–40)-(141–160)-PstI(138–160)-(21–40)-(138–160), which was then inserted into the vector pTrcHis A between BamHI and HindIII sites (Fig. 1).

For construction of plasmid pTH-O-sclG-A, bovine IgG constant region (sclG) gene was generated by RT-PCR amplification. The total mRNAs from cattle blood were isolated using mRNA purification kit. Bovine IgG gene specific primers used for sclG gene amplification were: sense primer 5’-CGCGGATCCCCCCCT-GAGCTCCCCC-3’ and antisense primer 5’-ACC-GTGCGT-GTTGACTAT-3’. The tandem-repeat gene (141–160)-(21–40)-(141–160) of FMDV serotype O and (138–160)-(21–40)-(138–160) of FMDV serotype A were chemically synthesized, and ligated to the N-terminus and C-terminus of sclG gene respectively. The fusion gene was inserted into the expression vector pTrcHis A between BamHI and HindIII sites, which was named pTH-O-sclG-A (Fig. 2).

![Fig. 1](https://academic.oup.com/abbs/article-abstract/36/9/589/87)
Expression of recombinant protein

*E. coli* strain TG1 was transformed with the vector pTH-O-A and pTH-O-scIgG-A respectively and grown overnight at 37 °C with shaking. Then the culture was diluted 20 fold with fresh LB medium containing 50 µg/ml ampicillin, incubated for 2.5 h at 37 °C, and then induced by 50 µg/ml IPTG and extended for another 5 h.

Western blot analysis

The IPTG induced expression profile of pTH-O-A and pTH-O-scIgG-A in *E. coli* TG1 were obtained by SDS-PAGE. The recombinant proteins were electro-transferred from the SDS-PAGE gel to nitrocellulose membrane (Gelman). The blots were blocked with 10% skim milk and 0.02% Tween-20 in phosphate-buffered saline (PBS), incubated with guinea pig anti-FMDV serotype O and serotype A serum respectively at 37 °C for 2 h, washed 3 times with PBS, and then incubated with a 1:3000 dilution of guinea pig antiserum IgG rabbit-peroxidase conjugate at 37 °C for 1.5 h for staining. Diaminobenzidine (Sigma) and 0.003% H2O2 (V/V) were used as chromogen and substrate, respectively. The color developing reaction was stopped with distilled water.

Assay of FMDV-specific antibody

Two weeks after boost, the blood of the immunized guinea pigs was taken and allowed to clot at 37 °C for 1 h. The clots were allowed to contract overnight at 4 °C, and the serum was aspirated, clarified by centrifugation and stored at −20 °C. A liquid-phase blocking ELISA (LP-ELISA) was used to detect FMDV-specific antibody in guinea pigs as previously described [15,16]. Briefly, the 96-well flat-bottom plates were coated with 100 µl of 1:1000 dilution of rabbit antiserum in carbonate/bicarbonate (pH 9.6) overnight at room temperature. The serum was 2-fold serially diluted (i.e. 1/32, 1/64, 1/128, 1/256, 1/512) and incubated with 50 µl of a constant dose of pure FMDV antigen overnight at 4 °C. Both serum and FMDV antigen were diluted with PBS containing 0.05% Tween-20 and phenol red indicator (PBST). 50 µl of the serum/antigen mixture were then added to the 96-well plates and incubated at 37 °C for 1 h on a rotary shaker. The plates was washed and then incubated for 1 h at 37 °C with 1:1000 dilution of homologous guinea pig antiserum diluted with PBST containing 10% normal bovine serum and 5% normal rabbit serum. After washing, 50 µl of 1:1000 dilution of rabbit anti-guinea pig IgG conjugated to horseradish peroxidase was added to each well. The plates were incubated at 37 °C for 1 h. After washing, 50 µl of O-phenylenediamine (OPD) containing 0.05% H2O2 (30 % W/V) was added to each well. After 15 min, the reaction was stopped with 1.25 M H2SO4. Plates were read by spectrophotometer at 492 nm. The degree blocking or inhibition for each serum dilution was then determined using Formula (1):

\[
\text{Inhibition(%) = } \frac{\text{Max}(A_\infty) - \text{Test}(A_m)}{\text{Max}(A_\infty)} \times 100\% 
\]
Serum titer was calculated as the dilution where 50% of the Max($A_{492}$) was inhibited, using the method of Kärber [15].

**Assay of T cell proliferation**

To determine whether the two fusion proteins could stimulate T cell proliferation at the existence of purified FMDV serotype O and serotype A antigens respectively, the T cells were isolated from the spleens of immunized guinea pigs and resuspended in the RPMI 1640 medium containing 10% fetal bovine serum, $5 \times 10^{-2}$ M $\beta$-mercaptoethanol, 50 μg/ml penicillin and 50 μg/ml streptomycin to $4 \times 10^6$ cells/ml. An aliquot of 100 μl the T cell suspension per well was added to the 96-well roll-bottomed plate. A total of 50 μl of the diluted pure FMDV serotype O and serotype A antigens were added to their respective wells in triplicate. The plates were incubated at 37 °C for 72 h and extended with 1 μCi/25 μl of [3H]thymidine for 18 h. Then the cells were harvested and subjected to liquid scintillation counting (Beckman LS6500) for measurement.

**Animal vaccination with the fusion proteins**

The fusion proteins were prepared from the inclusion bodies of *E. coli*. They were then dissolved in urea, purified as described by Strebel and emulsified with the adjuvant Montanide ISA 206 1:1 (SEPPIC). The serotype O and A antibody free guinea pigs were initially injected intramuscularly with 200 μg of the fusion proteins and boosted 3 week later with an equal amount of the fusion proteins.

**Viral challenge assays in guinea pigs**

FMDV challenge assays in immunized animals were performed as described previously [6]. Briefly, 3 week after second immunization, each guinea pig was injected intradermally in each of the rear feet with 0.2 ml of viral solution containing 100 guinea pig infectious dose (100 ID$_{50}$) FMDV serotype O or serotype A respectively. After challenge assay, all animals were examined daily for clinical symptoms of FMD such as an increase in body temperature (above 41 °C) and the appearance of vesicles on the mouth or hooves. Observation was terminated after 14-day challenge.

**Results**

The expression profile by SDS-PAGE and Western blot analysis of the fusion proteins pTH-O-A and pTH-O-sclgG-A

The fusion protein pTH-O-A with 18 kD in size and the fusion protein pTH-O-sclgG-A with 48 kD in size were highly expressed in the form of inclusion body. After induced with 0.2 mM IPTG for 5 h, the proteins on the SDS-PAGE gel were electro-transferred to nitrocellulose membrane, reacted with standard guinea pig anti-FMDV serotype O serum and serotype A serum, and then with peroxidase conjugated rabbit anti-guinea pig antibodies. The results proved that the two fusion proteins had the immunogenicity of both FMDV serotype O and serotype A (Fig. 3).

FMDV antibody responses of guinea pigs vaccinated with the fusion protein pTH-O-A and pTH-O-sclgG-A

To determine if the fusion protein of pTH-O-A or pTH-
O-scIgG-A could induce a specific antibody response against both serotype O and A of FMDV, the guinea pigs were inoculated with 200 µg of the fusion protein and boosted 3 weeks later with an equal amount of the fusion protein. As a comparison, we also inoculated guinea pigs once with commercial chemically inactivated vaccines O and A respectively. ELISA experiment revealed that the guinea pigs inoculated with fusion proteins were able to elicit specific antibody response for either serotype O or serotype A of FMDV. However, the antibody titers of guinea pigs vaccinated by the fusion proteins were lower than the commercial inactivated virus vaccines. The results are shown in Table 1.

**Assay of T cell proliferation**

Spleen cells from guinea pigs immunized with pTH-O-A and pTH-O-scIgG-A exhibited apparent specific proliferative response to inactivated FMDV. After stimulated with different dilutions of inactivated FMDV, there wasn’t apparent difference between the SI (stimulation index) of FMDV serotypes O and A, but the SI induced by the fusion protein was lower than that induced by inactivated virus vaccines (Fig. 4).

**Protection of immunized guinea pigs against viral challenge**

We tested whether the fusion proteins of pTH-O-A or pTH-O-scIgG-A can protect guinea pigs against FMDV infection. For this purpose, all immunized guinea pigs were challenged by direct injection with 100 ID₅₀ serotype O or serotype A FMDV. The animals were then observed daily for clinical signs of FMD. Table 2 indicated that 85% (6/7) and 70% (5/7) of the guinea pigs vaccinated twice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of guinea pigs</th>
<th>Dosage of inoculation</th>
<th>Specific antibody titer a,b, inoculated 21 d later</th>
<th>Specific antibody titer, inoculated 42 d later</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>6</td>
<td>0</td>
<td>0 (to serotype O)</td>
<td>0 (to serotype O)</td>
</tr>
<tr>
<td>Inactivated vaccine serotype O</td>
<td>6</td>
<td>4×10³ ID₅₀</td>
<td>2.1 (to serotype O)</td>
<td>4.55 (to serotype O)</td>
</tr>
<tr>
<td>Inactivated vaccine serotype A</td>
<td>6</td>
<td>4×10³ ID₅₀</td>
<td>1.9 (to serotype A)</td>
<td>4.21 (to serotype A)</td>
</tr>
<tr>
<td>pTH-O-A</td>
<td>6</td>
<td>200 µg×2</td>
<td>2.1 (to serotype O)</td>
<td>4.1 (to serotype O)</td>
</tr>
<tr>
<td>pTH-O-scIgG-A</td>
<td>6</td>
<td>200 µg×2</td>
<td>1.8 (to serotype A)</td>
<td>3.9 (to serotype A)</td>
</tr>
</tbody>
</table>

Fig. 4 Proliferation of spleen cells of guinea pigs vaccinated with pTH-O-A (A) and pTH-O-scIgG-A (B) fusion proteins

Results are expressed as the stimulation indexes, ratio of count per min (cpm) with or without antigen. SI: cpm of culture with FMD viral protein stimulation/mean cpm of culture without FMD viral protein stimulation. SI larger than 1.5 is considered a positive response.

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*the specific antibody titers were the mean value of the six guinea pigs; the titer was expressed as the log₁₀ reciprocal of the dilution, where 50% of the Max(Aₜₜₚₚ) reading was inhibited.*
with 200 µg of the fusion protein of pTH-O-A were protected from FMDV serotype O and serotype A infection respectively. 70% (5/7) and 57% (4/7) of the guinea pigs immunized with the fusion protein of pTH-O-scIgG-A were protected from FMDV serotype O and serotype A infection respectively. In contrast, in the negative control group all the guinea pigs presented characteristic signs of FMD within 3 day after challenge, including vesicles on the feet and tongue and high body temperature.

Discussion

In earlier work, it was demonstrated that the major epitopes of VP 1 exist in the region 141–160, 21–40 and 200–213 and the synthetic peptides of these regions can elicit virus-neutralizing antibodies in experimental animals [17–19]. The induced immuno-response was lower than that induced by inactivated virus and required approximately a 1000-fold higher dosage than the conventional vaccines [7,20]. The reasons are: (1) the molecules are so small that they are easily degraded \textit{in vivo} [21]; (2) the peptides have only the linear epitope without a conformational epitope like intact viruses [22]; (3) the peptides are not effectively presented by antigen-presenting cells (APCs) [23]. Many methods have been applied to solve these problems, such as increasing the copies of epitope, joining the epitope with a carrier protein to enhance the immunogenicity of the epitopes, joining the epitopes with targeted proteins or T cell epitope with B cell epitope to enhance the immune response of polypeptide vaccine in animals [24,25].

The recombinant peptide vaccines have many advantages as alternatives to traditional vaccines. The recombinant peptides are synthesized in bacteria, therefore, there is no involvement of infectious FMDV in vaccine production, and the peptides are more stable at room temperature than chemical inactivated virus vaccines. Further, there is no possibility of induction of antibody reactivity with non-structural proteins of FMDV, so it is easy to distinguish the vaccinated animals by peptide vaccine with the animals infected by FMDV. Finally, peptide vaccines can be designed to induce protection against multiple virus serotypes [26–28].

Multivalent peptide vaccines against many kinds of viruses have been constructed, but the results are usually disappointing because the protective rate against each serotype is low or the protective rate against one serotype is high, while the others are rather low. It is a great challenge to construct a polypeptide bivalent vaccine which can effectively protect animal from the infection of two FMDV serotypes [29–31]. In our research, for improving the effectiveness of FMDV polypeptide vaccine, two copies of B cell epitope and one copy of T cell epitope of FMDV serotype O and A were used. We constructed two plasmids consisting of two tandem-repeat sequences of amino acid (138–160)-(21–40)-(138–160) for serotype A and amino acid (141–160)-(21–40)-(141–160) for serotype O. One bivalent protein of pTH-O-A consists of only the epitopes and short spacers. The spacers will help to sustain the independence of each epitope to minimize the probability of the epitope inclusion. The epitope 138–160 aa and 140–160 aa of the VP1 includes the RGD site of FMDV. The antibodies of the polypeptides containing this epitope will effectively block the RGD site of FMDV, preventing FMDV from entering host cells. This may explain that the epitope peptide containing the RGD site is more effective than that containing no RGD site [6].

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose of second vaccination</th>
<th>Dose of first vaccination</th>
<th>Number protection (FMDV serotype)</th>
<th>Protection rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control 1</td>
<td>0</td>
<td>0</td>
<td>0/7 (O)</td>
<td>0</td>
</tr>
<tr>
<td>Negative control 2</td>
<td>0</td>
<td>0</td>
<td>0/7 (A)</td>
<td>0</td>
</tr>
<tr>
<td>Inactivated virus vaccine serotype O</td>
<td>4×10^7 ID₅₀/guinea pig</td>
<td>4×10^7 ID₅₀/guinea pig</td>
<td>0/7 (O)</td>
<td>100</td>
</tr>
<tr>
<td>Inactivated virus vaccine serotype A</td>
<td>4×10^7 ID₅₀/guinea pig</td>
<td>4×10^7 ID₅₀/guinea pig</td>
<td>6/7 (A)</td>
<td>85</td>
</tr>
<tr>
<td>pTH-O-A (group 1)</td>
<td>200 µg/guinea pig</td>
<td>200 µg/guinea pig</td>
<td>6/7 (O)</td>
<td>85</td>
</tr>
<tr>
<td>pTH-O-A (group 2)</td>
<td>200 µg/guinea pig</td>
<td>200 µg/guinea pig</td>
<td>5/7 (A)</td>
<td>70</td>
</tr>
<tr>
<td>pTH-O-scIgG-A (group 1)</td>
<td>200 µg/guinea pig</td>
<td>200 µg/guinea pig</td>
<td>5/7 (O)</td>
<td>70</td>
</tr>
<tr>
<td>pTH-O-scIgG-A (group 2)</td>
<td>200 µg/guinea pig</td>
<td>200 µg/guinea pig</td>
<td>4/7 (A)</td>
<td>57</td>
</tr>
</tbody>
</table>

Table 2: The protection effect of the fusion proteins against FMDV serotype A and O infection in guinea pigs.
other bivalent protein vaccine pTH-O-scIgG-A is constructed by fusing the tandem-repeat sequence with the cattle IgG single heavy chain constant region, generating a chimeric protein. In the present study, we chose a bovine IgG heavy chain constant region as the epitope carrier for the following reasons: first, the previous studies indicated that the IgG molecule could prolong the half-life of peptides, therefore, linking the FMDV epitope peptide to scIgG would allow the chimeric protein to circulate in vivo for a prolonged period \[32\]; second, the IgG molecule could enhance the immunogenicity of short peptides; third, the IgG molecule could target fusion antigen peptide on the surface of APCs, greatly enhancing the efficiency of antigen-presenting; finally, since the bovine IgG was a self-molecule, using it as a cattle FMD vaccine would minimized unwanted side effects [33].

Valid vaccine efficacy tests must follow a well-defined protocol with appropriate control groups, vaccination method, route of inoculation and critical clinical scoring, and the most important factor is the virulence of the challenge virus. The challenge virus must be collected from the same species as the challenged animals and be fully pathogenic to the challenged animals. We followed closely the recommendations of “OIE Manual of Standards”. In this experiment, guinea pigs were used to test the efficacy of the fusion protein against FMDV serotype O and A, so the stock virus was passaged four generations in guinea pigs to restore its pathogenicity until the secondary lesion vesicles appeared on the feet within 18 hours after inoculation. The experiment results showed the recommendations of “OIE Manual of Standards”. In this experiment, guinea pigs were used to test the efficacy of the fusion protein against FMDV serotype O and A, so the stock virus was passaged four generations in guinea pigs to restore its pathogenicity until the secondary lesion vesicles appeared on the feet within 18 hours after inoculation. The experiment results showed the chimera proteins of pTH-O-A and pTH-O-scIgG-A were highly expressed in E. coli with the immunogenicity of FMDV serotype O and A proved by Western blot analysis, and elicited high level FMDV specific antibodies in guinea pigs. Finally, the pTH-O-A fusion protein protected 85% of the vaccinated guinea pigs from the challenge of serotype O FMDV, 70% of the vaccinated guinea pigs from the infection of serotype A FMDV. The pTH-O-scIgG-A fusion protein protected 70% vaccinated guinea pigs from the challenge of serotype O FMDV and 57% from the infection of serotype A FMDV. However, the protection rate of the fusion protein is lower than that of chemically inactivated virus. The reasons may be as follows: first, the chemically inactivated virus contains discontinuous epitope; second, the chemically inactivated virus contains many B cell and T cell epitopes of different capsid protein (VP1, VP2, VP3, and VP4), however, the fusion protein contains only two epitopes of VP1; third, the targeting effect of bovine IgG was not exhibited exactly because guinea pigs, not cattle, were used as experimental animals.

To overcome these drawbacks, further investigations are required to completely refold the fusion protein from inclusion body to its native structure, improve the purity of the target protein, and optimize the dosage of immunization and evaluate the protective effectiveness in cattle.

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References
13. Li GJ, Yan WY, Xu QX, Sheng ZT, Zheng ZX. Study on the DNA vaccine against foot-and-mouth disease virus using the heavy chain constant region of...


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