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The rescue and evaluation of FLAG and HIS epitope-tagged Asia 1 type foot-and-mouth disease viruses

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ABSTRACT

The VP1 G–H loop of the foot-and-mouth disease virus (FMDV) contains the primary antigenic site, as well as an Arg-Gly-Asp (RGD) binding motif for the αv-integrin family of cell surface receptors. We anticipated that introducing a foreign epitope tag sequence downstream of the RGD motif would be tolerated by the viral capsid and would not destroy the antigenic site of FMDV. In this study, we have designed, generated, and characterized two recombinant FMDVs with a FLAG tag or histidine (HIS) inserted in the VP1 G–H loop downstream of the RGD motif +9 position. The tagged viruses were genetically stable and exhibited similar growth properties with their parental virus. What is more, the recombinant viruses rFMDV-FLAG and rFMDV-HIS showed neutralization sensitivity to FMDV type Asia1-specific mAbs, as well as to polyclonal antibodies. Additionally, the r_1 values of the recombinant viruses were similar to that of the parental virus, indicating that the insertion of FLAG or HIS tag sequences downstream of the RGD motif +9 position do not eradicate the antigenic site of FMDV and do not affect its antigenicity. These results indicated that the G–H loop of Asia1 FMDV is able to effectively display the foreign epitopes, making this a potential approach for novel FMDV vaccines development.

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1. Introduction

Foot-and-mouth disease virus (FMDV) is the etiological agent of foot-and-mouth disease (FMD), FMD is one of the most economically devastating viral disease in cattle and other cloven-hoofed animals. The disease is distributed worldwide, and it has a greatly negative economic impact on livestock health and production, as well as on international trade. This virus includes seven distinct serotypes (e.g., O, A, C, Asia 1, and SAT1–3), and in each serotype there are multiple antigenic variants and subtypes. Thus, a vaccine against one serotype will provide poor cross-protection against the other serotypes (Grubman and Baxt, 2004).

FMDV belongs to the Aphthovirus genus, Picornaviridae family. The genome of FMDV encodes a single polyprotein, the polyprotein is proteolytically processed to generate the intermediate precursors and related mature proteins that are required for viral replication and assembly. The four structural proteins (VP1, VP2, VP3, and VP4) were produced via cleavage of the N-terminal P1-2A region of the polyprotein (Mason et al., 2003). Specifically, a highly conserved Arg-Gly-Asp (RGD) motif is included in the prominent surface-exposed loop of the VP1 capsid protein, also known as the G–H loop (Acharya et al., 1989; Baxt et al., 1989; Logan et al., 1993). RGD motif has been shown to be a recognition domain for the αv-integrin family of cell surface receptors (Baxt and Becker, 1990; Brown et al., 1999; Burman et al., 2006; Fox et al., 1989). The RGD site is highly adapted for binding to integrins, composing stable complexes that are dependent on two conserved residues at positions RGD +1 and RGD +4 (Dicara et al., 2008). This loop is a major viral antigenic site, which is conformationally flexible and mediates cell attachment by binding integrin receptors (Acharya et al., 1989; Aggarwal and Barnett, 2002; Barnett et al., 1989; Baxt et al., 1989; Broekhuijsen et al., 1987; Brown, 1988; Crowther et al., 1993a,b; Kitson et al., 1990; Mateu et al., 1990; Thomas et al., 1988).

As reported, inserting a foreign epitope-tagged sequence downstream and upstream of the RGD motif was tolerated by the viral capsid (Lawrence et al., 2013; Seago et al., 2012; Wang et al., 2012). The insertion of foreign epitope tags in FMDV offers a feasible alternative to current methods of vaccine manufacturing, as it is a potential tool for FMDV research that may lead to the development of FMD vaccine conjugates (Seago et al., 2012). It can also be

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used to generate a positively marked vaccine to support DIVA (differentiating infected from vaccinated animals) diagnostic testing, which is crucial in outbreak control and sero-surveillance (Prentoe and Bukh, 2011; Wegelt et al., 2011). Seago et al. produced serotype O recombinant FMDV by insertion of exogenous HA or Flag tags into the VP1 GH loop downstream of RGD +8, and the rescued viruses were viable, stable and can infect cells via integrins receptors (Seago et al., 2012). Wang et al. generated recombinant Asia 1 FMDV with insertions of type O FMDV neutralizing epitopes at the upstream of RGD +6, and the insertion downstream of this position produced no viable viruses (Wang et al., 2012).

In this study, we designed, generated, and characterized two recombinant Asia 1 FMDVs with a FLAG or histidine (HIS) tags inserted in the VP1 G–H loop downstream of the RGD motif at the RGD +9. It is shown that the tagged viruses are genetically stable and exhibit growth properties that are similar to those of their parental virus, without affecting the antigenicity of the viruses.

2. Materials and methods

2.1. Cells lines and viruses

Baby hamster kidney (BHK) cells, strain 21, (maintained in modified Eagle's medium) (MEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (Delta Bioproducts, Atascadero, USA) were used for transfection. Based on the reverse-genetics system used in our laboratory (Lian et al., 2015), we constructed an infectious cDNA of O/CHA/99, named pO-FMDV. The P1 coding sequence of Asia 1was chosen as the site for exchange with the corresponding region of the pO-FMDV, and the recombinant virus was named pRe-Asia1-FMDV.

2.2. Construction of full-length clones of epitope-tagged viruses

Using a reverse genetic system, we constructed two infectious clones containing FLAG (DYKDDDDK) and HIS (HHHHHH) epitope tags. The P1 cDNA of Asia 1 was amplified with sense (P1-F) and antisense (P1-R) primers using AdvanTaq DNA Polymerase (Clontech, Mountain View, USA). P1-F contained an AflII restriction endonuclease site, and P1-R contained a ClaI restriction endonuclease site.

The PCR amplification product was ligated to the pMD20-T vector at 16 °C for 16 h. Then, the FLAG- or HIS-tag sequences were inserted into the subclones by performing consecutive rounds of PCR amplification using primers (FLAG: 9FLAGF/9FLAGR and HIS: 9HISF/9HISR, respectively), yielding plasmids 9FLAG-T and 9HIS-T, respectively (Table 1). After PCR amplifications of tagged VP1 in the 9FLAG-T and 9HIS-T plasmids, the products were purified using the Wizard SV Gel and PCR Clean-Up System Kit (Promega, Shanghai, China), the purified products and pO-FMDV plasmid were digested with the restriction endonucleases *Afl*II and *Cla*I (New England Biolabs, Ipswich, Massachusetts, USA), followed by ligation, and the rightly constructed plasmids were designated as prFMDV-FLAG and prFMDV-HIS, respectively.

2.3. Transfection and recovery of infectious recombinant viruses

The purified plasmids prFMDV-FLAG and prFMDV-HIS were prepared using QIAGEN Plasmid Midi Kits (Qiagen, Hilden, Germany) based on the manufacturer's protocol. Using Lipofectamine^{TM} 2000 (Invitrogen, Carlsbad, CA, USA), the plasmids were transfected directly into sub-confluent BHK-21 cells. After 4 h of incubation at 37 °C, the supernatants were discarded and supplemented with MEM medium supplemented with 10% FBS, and the cells were further cultured at 37 °C until cytopathic effects (CPE) appeared. The cell monolayers generally showed CPE at 48 to 60 h post-transfection. The rescued virus were designated as rFMDV-9HIS or rFMDV-9FLAG, and used to infect BHK-21 cells for the further generations.

2.4. Immunofluorescence assays (IFA)

BHK-21 cells were cultured in a six-well plate, infected with rFMDV-FLAG or rFMDV-HIS at a multiplicity of infection (MOI) of 0.1; mock-infected and rFMDV-infected cells were included as negative and positive controls, respectively. At 6 h post-infection (hpi), the cells were washed with phosphate-buffered saline (PBS) for three times and fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 and subsequently, blocked with blocking buffer (PBS, 10% bovine serum albumin). After washing three times with 0.5% PBST (PBS containing 0.5%Tween), the cells were incubated at 37 °C for 1 h with primary antibody (mouse anti-FLAG mAb or mouse anti-HIS mAb). After washing with PBST for three times, the cells were incubated with secondary FITC-conjugated goat anti-mouse IgG antibody for 1 h. Finally, the fluorescence was observed under an Olympus BX40 fluorescence microscopy (Li et al., 2012). In addition, the 50% tissue culture infective dose ($TCID_{50}$) was also determined on BHK-21 cells.

2.5. Antigenic integrity identification of VP1 of recombinant virus and tag expression analysis by an indirect ELISA

An indirect ELISA assay was performed to determine whether the native structure of VP1 was affected by the insertion of the 9FLAG or 9HIS. The specific monoclonal antibody 7312 targeting the G–H loop of VP1 was used and ELISA was performed according to the standard methods (Yang et al., 2011). Briefly, microplates were coated with the recombinant virus at a concentration of 1:100 diluted in carbonated-bicarbonate buffer (pH 9.6) overnight at 4 °C, with the wild-type virus as a positive control and serum as a negative control. The 7312 monoclonal antibody was diluted 1:2000 in PBST, 50 ul were added to each well, and incubated at 37 °C for 1 h. Before and after incubation of HRP-conjugated goat anti the mouse antibody(Sigma–Aldrich, St. Louis, USA), the plates were washed three times with PBST as described above, then, the plates were incubated at 37 °C for 10 min with OPD(*o*-phenylenediamine) containing H_2O_2 (1%), and the reaction was stopped with 0.2 M sulfuric acid. The optical density value was determined at 490 nm. The same indirect ELISA assay was performed for detecting the expression of FLAG and HIS tags, ELISA plates were coated with rFMDV-FLAG or rFMDV-HIS recombinant virus, primary mouse monoclonal anti-

Table 1
Primers used for the construction of modified full-length clones of tagged FMDV.

Primer	Sequence(5′ → 3′)	Nucleotide Position
P1-F	TTTTCC*CTTAAG*GGACAAGAACATGCTGTGTTTGCCTGTG	1541–1574
P1-R	ACTCACATCGATGTCAAAGTGAAACCTCC	5100–5122
9HIS-F	CACCACCACAACCGGCTGCCCACTTCCTTCA	3755–3785
9HIS-R	GTGGTGGTGGCTCACTCTGCGTGCGAGGGCGG	3723–3754
9FLAG-F	GATGATGACAAGAACCGGCTGCCCACTTCCTTCA	3758–3791
9FLAG-R	GTCCTTATAGTCGCTCACTCTGCGTGCGAGGGCG	3724–3757

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