



Short communication

Chimeric vaccine strain of type O foot-and-mouth disease elicits a strong immune response in pigs against ME-SA and SEA topotypes

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ABSTRACT

Foot-and-mouth disease (FMD) is an acute infectious disease occurring in cloven-hoofed animals. There are many variations of the virus, making it difficult to protect against the various strains with one virus vaccine. The immunogenicity has generally been evaluated in pigs using neutralizing antibodies to determine the protection level against foot-and-mouth disease virus type O. Therefore, the vaccine from the chimeric vaccine strain of ME-SA (VP4, VP2, and VP3) and SEA (VP1) topotypes developed in this study is expected to be able to protect with high neutralizing antibody titers against most of the eight FMD viruses of the four different topotypes (ME-SA, SEA, Cathay, and EURO-SA) of type O in pigs. This is a new technique for powerful vaccine development, with multiple preventive roles against various epidemic FMD strains.

1. Introduction

Foot-and-mouth disease (FMD) occurs in domestic cloven-hoofed animals, such as pigs, cows, and goats, and it is highly infectious (Grubman and Baxt, 2004). There are seven serotypes of FMD (O, A, Asia1, C, SAT1, SAT2, and SAT3), which can be classified by the antigenicity of the structural proteins involved and their nucleotide sequences. The capsid of the FMD virus (FMDV) is composed of 60 copies of structural proteins VP1, VP2, VP3, and VP4. VP1 is known to be critical for preventing foot-and-mouth disease (Grubman and Baxt, 2004).

FMD spreads quickly and can be prevented from spreading by administering emergency vaccinations in the event of an outbreak. New vaccines are continuously developed, but it is difficult and labor- and time-intensive to develop a new vaccine each time a new virus appears (Mahapatra and Parida, 2018; Mahapatra et al., 2017; Paton et al., 2005). Thus, efforts have been made to develop vaccines with various improved functions that can respond to evolving versions of the virus (Caridi et al., 2017; Lee et al., 2017; Park et al., 2016).

Current problems with FMD vaccines include the instability of their antigens, the loss of antigens during the purification process, low immunogenicity, insufficient maintenance of the antibodies, and a narrow protection range of the antigens (Mahapatra et al., 2017; Park, 2013; Rudreshappa et al., 2012). For example, the vaccine from the SEA

topotype virus, O/SKR/2010, has shown a narrow antigenic spectrum for protection from various wild-type viruses (Park et al., 2018, 2014).

Therefore, to develop the upgraded vaccine platform, we replaced some nucleotide sequences of the O1 Manisa virus vaccine, which is a representative vaccine strain of foot-and-mouth disease, to produce a new FMD antigen composed of mixed amino acid sequences that can provide enhanced resistance to the general environment condition, be differentiated from wild-type strains, and have broad antigenicity for protection against various viruses. For this vaccine strain, VP1, the part of the vaccine antigen with the highest antigenicity, was combined with O/Andong/SKR/2010, which is the SEA topotype. In addition, to increase the stability and immunity of the virus, amino acids were replaced with VP1 N17D and VP2 H145Y, which were referred to in a previous study (Park et al., 2016), and for differentiation from wild-type strains, 3B₁ and 3B₂ were replaced with two 3B_{3s} (Fig. 1). An experimental vaccine was produced, and its protection effect was verified in mice. Pigs were also administered the vaccine, and their immune reactions to eight typical wild-type strains of type O, including ME-SA and SEA topotypes, were investigated for their life span.

2. Results

A schematic of the produced recombinant virus genome is shown in Fig. 1. The VP1 sequence of the O1 Manisa virus was replaced with that

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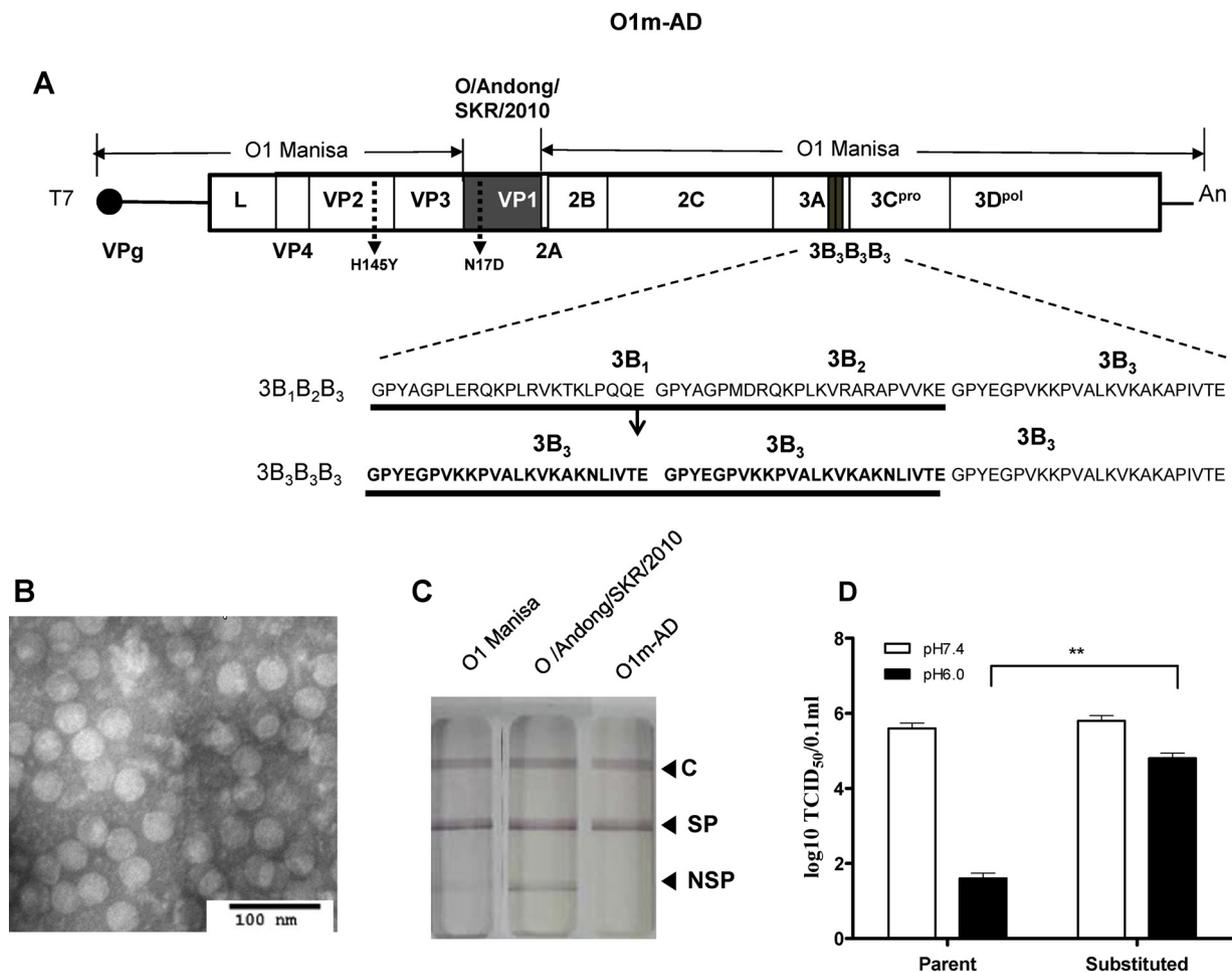


Fig. 1. Characteristics of chimeric foot-and-mouth disease virus with replaced VP1, VP2, 3B₁, and 3B₂.

(A) Schematic depiction of the chimeric viruses. (B) Images of O1m-AD virus particles via electron microscopy. (C) Identification of recombinant FMDV differentiation through non-structural proteins using a rapid diagnosis kit (PBM, USA). (D) Increased acid resistance via the VP1 N17D and VP2 H145Y substitutions in the virus. ** $P < 0.01$.

of O/Andong/SKR/2010, and the amino acids were replaced with VP1 N17D and VP2 H145Y to provide resistance to an acidic condition. Furthermore, both 3B₁ and 3B₂ were replaced with two 3B₃s to enable differentiation from wild-type strains, given the deficiency of 3B₁ and 3B₂ (Fig. 1A). The produced recombinant antigen was observed with a transmission electron microscope (Fig. 1B). Differentiation from wild-type strains was confirmed with a FMD virus rapid test kit (Princeton BioMeditech Corporation, USA), as 3B was not detected in the NSP band of the recombinant virus replaced with 3B₃B₃B₃ (Fig. 1C). In addition, it was confirmed that the recombinant virus had greater resistance than the parental virus in an acidic environment with a pH of 6.0, which is an inactivation condition for FMDV (Fig. 1D).

To evaluate protection in mice, experimental vaccines were produced with 1500 ng – 46.75 ng of antigen per mouse, and the mice ($n = 4$ /group) were given experimental vaccines. The mice were challenged with O Vietnam 2013 (ME-SA topotype) 7 days after vaccination, and all the mice immunized with a dose of antigen at 46.75 ng (1/320 dose) or higher survived, resulting in a survival rate of 38.1 mouse PD₅₀ (1/10 dose of pig = 1 dose [0.1 mL] for mice) (Fig. 2A and B). To compare the protective effect between the commercial vaccine (trivalent vaccine-O1 Manisa, A22, and Asia1 Shamir [Merial], > 6PD₅₀, 0.2 mL/mouse) and the experimental vaccine, the mice were vaccinated (1/10 dose of pig, 0.1 mL/mouse) and challenged with the O Vietnam 2013 virus at 21 days after vaccination. All the non-vaccinated mice died at 4 days post-infection, and the group given either the commercial vaccine or the experimental vaccine showed 100% protection until 7 days post-

infection (Fig. 2C and D). When viremia was measured after virus infection, viral RNA was detected 3 days post-infection only in the controls that were not vaccinated (Fig. 2E).

To evaluate the immunogenicity of the vaccine in pigs ($n = 20$ /group), the pigs were vaccinated with the commercial vaccine or the test vaccine (15 µg of 146S antigen/1 mL/dose), and a virus neutralization test (VNT) and structural protein enzyme-linked immunosorbent assay (SP-ELISA) were performed to detect the antibody against FMDV. The VNT after vaccination for the test vaccine showed higher levels of antibodies than for the commercial vaccine, and the antibody titers increased further after boosting (Figs. 3A and B). Testing the protection against other foot-and-mouth disease type O viruses revealed that a high level of neutralizing antibodies, above 1:100, was formed in the viruses of the Cathay (Fig. 3D) and EURO-SA (Fig. 3E) topotypes as well as in the ME-SA (Fig. 3B) and SEA (Fig. 3C) topotypes.

3. Discussion

FMD type O has consistently been found in East and Southeast Asia (Bachanek-Bankowska et al., 2018; Qiu et al., 2018; Zhu et al., 2018). Concerns about this type are increasing. In South Korea, the introduction of the O/SEA/Mya98 virus during 2010–2016 and the O/ME-SA/Ind2001d virus in 2017 was unexpected seven years ago. FMD virus strains are quickly changing, meaning that new vaccines that can be used in emergencies are always necessary. However, it takes significant amounts of time and money to develop vaccine strains against

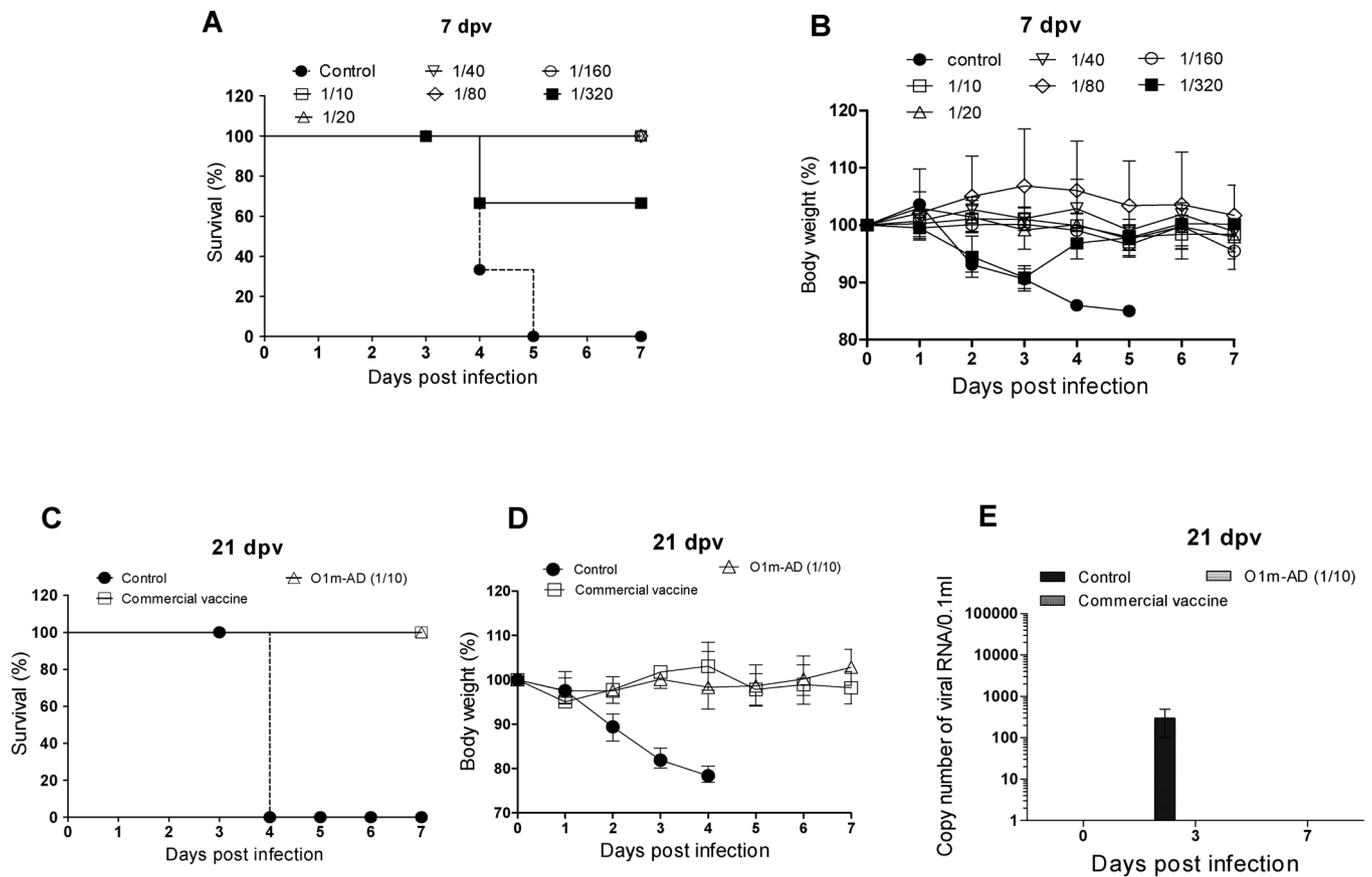


Fig. 2. Survival in the immunized C57BL/6 mice challenged with foot-and-mouth disease virus at 7 and 21 days after vaccination.

(A) Survival rate of 7 dpv groups after the challenge with 100 LD₅₀ of O Vietnam 2013 (ME-SA toptotype). (B) Change in body weight of 7 dpv groups after the challenge. (C) Survival rate of 21 dpv groups after the challenge. The FMD trivalent vaccine (O1 Manisa, A22, and Asia1 Shamir, Merial) was used as a commercial vaccine control. (D) Change in body weight of 21 dpv groups after the challenge. (E) Detection of viremia at 0, 3, and 7 days post-infection in 21 dpv groups. The vaccine dose in this study was referred to a dose for cattle or pigs (15 µg/mL = one dose).

numerous epidemic FMD viruses.

The technique of replacing the target genes using the O1 Manisa vaccine strain has been successful in previous studies (Kim et al., 2015; Lee et al., 2017). However, O1 Manisa was not a good match with FMDVs of the SEA toptotype, which have recently occurred in Asian countries (Park et al., 2018). The vaccine strains originating from the EURO-SA and Cathay toptotypes were not very well matched with the FMDVs of the SEA toptotype (Park et al., 2018). Vaccine strains from the SEA toptotype have shown a narrow antigenic spectrum against other toptypes (unpublished data).

In this study, VP1, which is an important structural protein for the production of a protective antibody, was replaced with amino acids that provide antigen stability, which means that the vaccine developed in this study has more resistance to general environmental conditions and can be differentiated from wild-type strains, as shown in previous reports (Oem et al., 2007; Yang et al., 2015). To test the effect of the vaccine, an experimental vaccine was produced, and its protection capacity was tested after using it to immunize mice. The vaccine proved to have a protective effect even with low levels of doses against the ME-SA toptotype (O Vietnam 2013). Furthermore, when the effect of the vaccine was tested in pigs, it showed higher immunogenicity than a commercial vaccine. The vaccine showed a protection level of neutralizing antibody titers of approximately 1:100, indicating almost protection (Black et al., 1984) against eight different viruses in four toptypes of type O FMD through 70 days post-vaccination. In a previous study, the animals with neutralizing antibody titers of > 1:32–1:45 were all protected against virus challenges (McCullough et al., 1992; Zheng et al., 2015).

This indicates that the vaccine is applicable to a wide variety of recent pandemic type O FMDVs. Future research should be conducted to prove the effects of the vaccine via inoculation tests for various viruses.

4. Materials and methods

4.1. Cells and viruses

The BHK 21 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Paisley, UK). The BHK 21 suspension cells were maintained in ProVero-1 (Lonza, Belgium) supplemented with Dextran sulfate sodium salt and pluronic F-68. The viruses selected for this study: O Manisa, O1 Manisa/Turkey/69 (Genbank No. AY593823), and O/Andong/SKR/2010 (GenBank No. KC503937), were rescued from the infectious cDNA clones (pO Manisa-FG) of pO Manisa.

4.2. Site-directed mutagenesis and subcloning

For this study, O1 Manisa/Turkey/69 (GenBank No. AY593823) was replaced with the VP1 part of O/Andong/SKR/2010 (GenBank No. KC503937) and rescued from the infectious cDNA clones (pO Manisa-FG) of pO Manisa. A previously reported procedure was followed to substitute the VP1 of O/Andong/SKR/2010 (Kim et al., 2015; Lee et al., 2017) through polymerase chain reaction (PCR) using Phusion® High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Vantaa, Finland). The VP1 region were amplified using the oligonucleotide primers (5'-

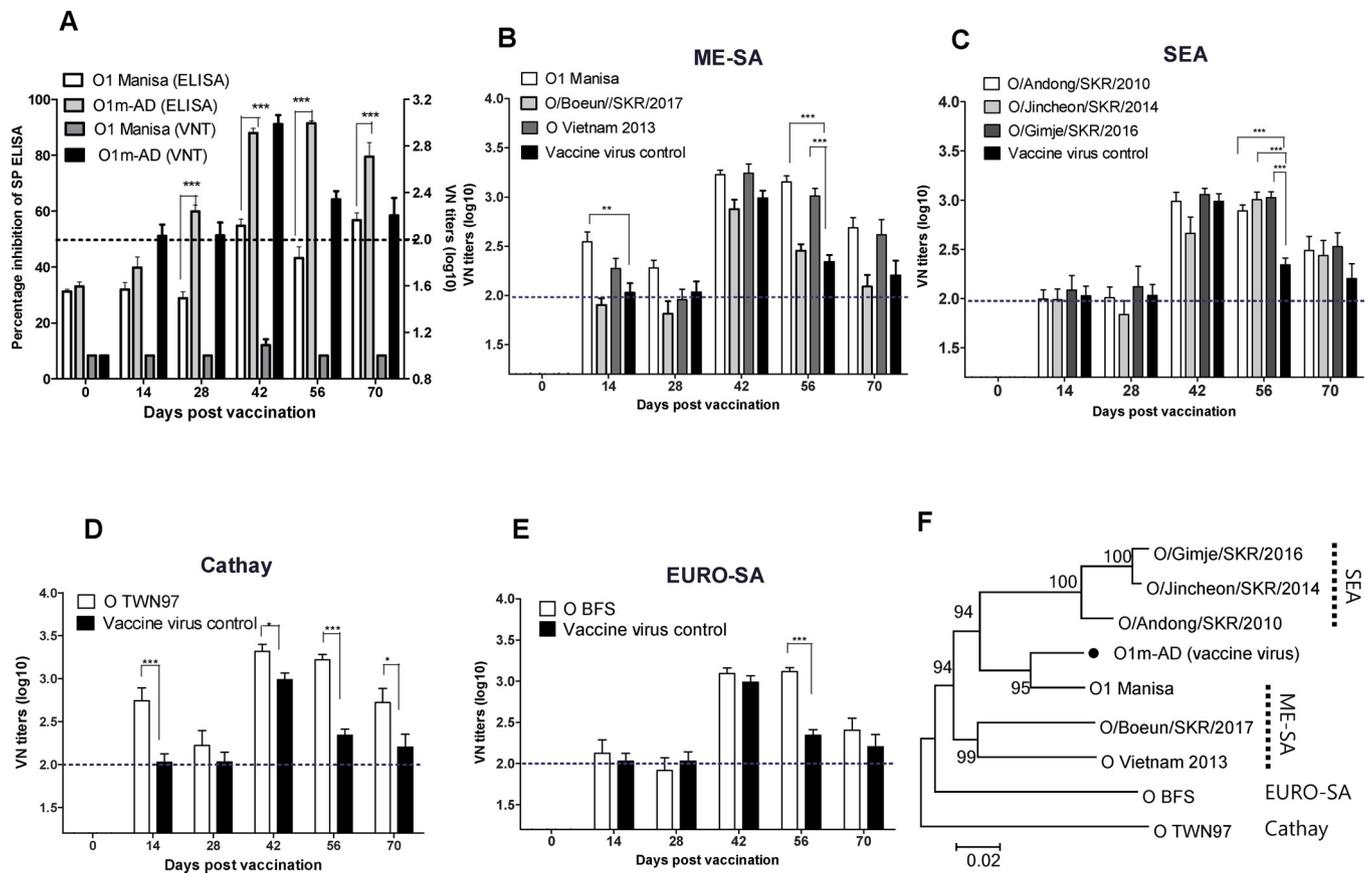


Fig. 3. Immune responses against four different topotypes measured by a virus neutralization test in immunized pigs and the genetic relationships among tested viruses.

(A) Antibody responses by SP ELISA for type O or VN titers for O1m-AD in pigs ($n = 20$ /group) at 0, 14, 28, 42, 56, and 70 days post-vaccination for the O1m-AD experimental vaccine and O1 Manisa commercial vaccine (trivalent vaccine containing O1 Manisa, A22, and Asia1 Shamir). The booster was injected at 28 dpv. The dotted line represents the cut-off value for a positive reaction. (B) The VNTs for O1 Manisa, O/Boeun/SKR/2017, and O Vietnam 2013 for ME-SA (Middle Eastern-South Asian) topotypes were performed. The results were compared with VNT by a virus vaccine (O1m-AD). The dotted line represents 1:100 of neutralizing antibody titers. (C) O/Andong/SKR/2010, O/Jincheon/SKR/2014, and O/Gimje/SKR/2016 for SEA (South Eastern Asian) topotypes were used for VNTs. The results were compared with VNT by a virus vaccine (O1m-AD). (D) O Taiwan 97 for the Cathay topotype was used for a VNT. The result was compared with VNT by a virus vaccine (O1m-AD). (E) O BFS for EURO-SA topotype was used for a VNT. The result was compared with VNT by a vaccine virus (O1m-AD). (F) Phylogenetic tree of surface protein-coding sequences (VP2, VP3, and VP1) of the viruses, which have antigenic determinants for the neutralizing antibody. O1 Manisa (AY593823.1), O/Boeun/SKR/2017(MG972599.1), O Vietnam 2013 (KJ608371), O/Andong/SKR/2010 (KC503937), O/Jincheon/SKR/2014 (KX162590), O/Gimje/SKR/2016(KY086465), O Taiwan 97(KR149697.1), and O BFS (EU448370.1).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

ACCACCTCGACAGCGGAGT - 3' and 5' - CTGCTTTACAGATGCCACT - 3') and inserted to pO Manisa according to the referred instructions (Kim et al., 2015; Lee et al., 2017). The replaced plasmid was used as a PCR template for the amino acid substitutions of VP1 N17D (using 5'-TGTTCTCGCGCCGTAATCCTCA-3' oligomer) and VP2 H145Y (using 5'-TCCTTACCAGTTCATCAACCTC-3' oligomer). For the replacement to two 3B3s from 3B1 and 3B2 in 3B region, we were used full genome cDNA vector deleted both 3B1 and 3B2, and we were inserted the 3B3 dimer genes amplified with specific primers (5'- GGACCTTAC GAGGGACCGGTGAAG-3' and 5'- TTCAGTGACAATCGGGGCTTTG CT-3') using synthetic 3B3 dimer genes as the PCR template (Fig. 1). The constructed recombinant plasmids were sequenced to confirm the amino acid substitutions. The virus was rescued by transfection into BHK-T7-9 cells.

4.3. Rescue of mutant viruses

The recombinant plasmids were linearized by treatment with a restriction enzyme *SpeI*(NEB, Beverly, USA) and the linearized plasmids were transfected in BHK-T7-9 cells using lipofectamine 2000 (Invitrogen, Carlsbad, USA) to recover the chimeric viruses,

respectively. Following transfection and incubation for 48 h at 37 °C, viruses were harvested in three freeze-thaw cycles and the chimeric viruses were used to infect fresh ZZ-R cells. For the differentiation from wild-type FMDV and recombinant viruses, structural protein and non-structural protein were checked using an FMDV rapid kit (Princeton BioMeditech Corporation, USA).

4.4. Acid-induced inactivation assays

To determine the stability of FMDV, a virus comprising equal amounts of infectious virus (10 μ l of the virus containing 10⁶ 50% tissue culture infectious doses (TCID₅₀)) was incubated for 30 min in PBS solution (50 mM NaH₂PO₄ and 140 mM NaCl) with a pH of 6.0 or 7.4 at room temperature. The mixed solution was neutralized with the addition of 120 μ l of 1 M Tris solution (pH of 7.6). The remaining infectious titers in each solution were determined using BHK21 cells grown in 96-well tissue culture plates according to standard procedures, and they were expressed as TCID₅₀.

4.5. Virus purification for vaccine antigens

To purify virus particle antigens, infected BHK21 suspension cells were harvested by being frozen first and then thawed when a complete cytopathic effect (CPE) was observed. Cell debris was removed after the inactivation of the virus with binary ethylenimine (BEI). The inactivated virus in the clarified culture was subsequently precipitated with 7.5% PEG 6000 and 2.3% NaCl overnight at 4 °C, and the pellet was resuspended in TN buffer. The resuspended pellet was subsequently purified by centrifugation through a 15–45% sucrose gradient in TN buffer at 30,500 rpm for 4 h in an SW41 rotor at 4 °C. The 146S antigen quantity was determined after spectrophotometer analysis at 259 nm. Purified antigens were separately adsorbed on carbon-coated copper grids, negatively stained with 2% uranyl acetate, and then imaged with a transmission electron microscope at 125 kV.

4.6. Vaccination and virus challenge

Animal experiments were performed in strict accordance with the recommendations of the guide for the care of laboratory animals of the Animal and Plant Quarantine Agency. To verify protection in vaccinated mice, 10 experimental groups of seven-week-old C57BL/6 mice (female, 4 per group) were inoculated by intraperitoneal injection with a commercial vaccine (trivalent vaccine O1 Manisa, A22, and Asia1 Shamir, Merial, and antigen (0, 1/10, 1/20, 1/40, 1/80, 1/160, and 1/320 dose groups of a dose [15 µg/dose] of the vaccination in pigs) mixed with ISA 206 and 10% aluminum hydroxide gel adjuvant.

After 7 (antigen 0–1/320 dose groups) or 21 (commercial vaccine and antigen 1/10 dose groups) days post-vaccination (dpv), all the mice were subjected to the O Vietnam 2013 virus following intraperitoneal injection with 1×10^4 TCID₅₀ for 100 LD₅₀ and observed for 7 days. After a virus challenge inoculation, the levels of the serum virus detected for the serum samples were collected at 0, 3, and 7 days using the real-time RT-PCR method.

4.7. Immunization in pigs with an experimental vaccine

The pigs (n = 20) were inoculated with 15 µg/ml antigen with mixed ISA 206, saponin, and aluminum hydroxide gel adjuvant. Sera were collected 10 weeks (0, 7, 14, 28, 42, 56, 70 dpv) after vaccination.

4.8. ELISA for the detection of structural protein antibodies

Structural proteins in sera using PrioCheck FMDV type O (Prionics, Switzerland) were detected. When the samples had a percent inhibition (PI) value of $\geq 50\%$, the animals were regarded as having immune responses. Animals showing PI values of $\geq 50\%$ were considered to have demonstrated immune responses.

4.9. Virus neutralization test

Serum samples were collected from pigs after vaccination and were heat-inactivated at 56 °C for 30 min. Following the incubation of the test serum with FMDV 100TCID₅₀ for 1 h, LFBK cells were added to the plate and incubated for 3 days. The CPE was checked to determine the titers, which were calculated as the log₁₀ of the reciprocal antibody dilution to neutralize 100 TCID₅₀ of the virus. FMDVs O1m-AD, O1 Manisa/Turkey/69 (O1 Manisa), O/Andong/SKR/2010, O/Jincheon/SKR/2014, O/Gimje/SKR/2016, O Vietnam 2013, O/Taiwan/97 (O TWN97), O/Boeun/SKR/2017, and O BFS 1860 (O BFS) were used for VNT. Data are presented as the mean \pm standard deviation (SD). Differences were considered statistically significant at $p < 0.05$. An analysis of the genetic similarity of a phylogenetic tree was conducted using BioEdit for Window 95/98/NT2K/XP/7 and MEGA software (ver. 6.0) with the neighbor-joining method. The percentage of replicates from the tree in which the associated taxa were

clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

4.10. Ethics statement

Animal experiments were performed in strict accordance with the recommendations of the guide for the care and use of laboratory animals of the Animal and Plant Quarantine Agency (APQA). All animal procedures were approved by the Institutional Animal Care and Use Committee of the APQA of Republic of Korea (approval no. 2017-357). All efforts were made to minimize animal suffering.

4.11. Statistical analysis

Data are presented as means \pm SD. Differences were considered statistically significant at $p < 0.05$.

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