Veterinary Microbiology 234 (2019) 44–50

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Evaluation of novel inactivated vaccine for type C foot-and-mouth disease in cattle and pigs

Su-Hwa You, Hye-Eun Jo, Joo-Hyung Choi, Mi-Kyeong Ko, Sung Ho Shin, Min Ja Lee, Su-Mi Kim, Byounghan Kim, Jong-Hyeon Park*

Center for Foot-and-Mouth Disease Vaccine Research, Animal and Plant Quarantine Agency, 177 Hyeoksin 8-ro, Gimcheon, Gyeongsangbuk-do, Republic of Korea

ARTICLE INFO

Keywords:
FMD
Vaccine
Type C
Pig
Cattle
Protection

ABSTRACT

Foot-and-mouth disease virus (FMDV) is the cause of an economically devastating disease in major cloven-hoofed livestock. Although type C foot-and-mouth disease (FMD) has not occurred anywhere worldwide since 2004, the antigen bank should be preserved in preparation for an unexpected outbreak. We therefore conducted experiments to develop inactivated vaccines that are safer and exhibit improved characteristics over existing vaccines. Our previous study showed that the replacement of the capsid-encoding gene (P1) from the vaccine strain O1 Manisa could be rescued successfully from the vaccine strains. In addition, novel point mutation in the 3C region in the virus genome, for induction of properties with low pathogenesis to create a safe vaccine, and $3B_1B_2$ replacement, for differential diagnosis with the wild type virus, were performed. The modified FMD vaccine strain, C3 Resende-R, was shown to provide lower pathogenesis in young mice than the wild-type virus. To identify the immune responses after vaccination with 146S antigen (15 μg/mL/dose), we conducted a virus neutralization test using serum from pigs and cattle vaccinated with the inactivated vaccine. The neutralizing titers in the cattle were higher than those in the pigs and maintained mean antibody titers of around 1:100 until the end of the experiment. The vaccine showed protection capability of 16 PD_{50} against C3 Resende virus in the pigs. The replacement of the structural protein-coding gene for the new FMDV was a useful tool in the development of an effective vaccine candidate strain. This inactivated vaccine will be used for the establishment of a safe vaccine strain for the antigen bank.

1. Introduction

Foot-and-mouth disease (FMD) is an acute contagious disease affecting cloven-hoofed animals, such as cows, pigs, sheep, goats, and deer. This infection induces fever, lameness, and vesicles on the mouth, tongue, snout, teats, and feet (Alexandersen and Mowat, 2005). The FMD virus (FMDV) belongs to the *Aphthovirus* genus of the *Piconaviridae* family; it has a single-stranded, plus-sense RNA genome. The virus consists of seven serotypes: A, O, C, Asia1, and South African Territories 1, 2, and 3 (SAT1, SAT2, and SAT3). There is no cross-protection and immunity between the different serotypes (Grubman and Baxt, 2004). The capsid of the FMDV is composed of 60 copies of structural proteins VP1, VP2, VP3, and VP4. Eight proteins are nonstructural protein (NSPs, L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D) (Grubman and Baxt, 2004).

Inactivated vaccines should be developed in safe forms to effectively control the disease when FMD has occurred and to minimize side effects that may occur in domestic animals after vaccination (de Los Santos et al., 2018). There is a risk of leaking the live virus due to the necessary production of large amounts of pathogenic vaccine viruses. Therefore, if safe vaccines are to be produced using improved bio-techniques, this risk must also be reduced, and thus making vaccine strains into more attenuated viruses would be safer than the existing vaccines being used (Park, 2013). In addition, since the formation of granulomas at vaccination sites is problematic in the pig industry, reducing the volume of vaccines used, while maintaining the same effect as existing vaccines, would be another method of improving the safety of vaccines (Park et al., 2017).

Type C FMDVs are largely divided into EURO-SA, AFRICA, and ASIA topotypes (Sangula et al., 2011). C2, C3, C4, and C5 were identified as subtypes, with C3 as the most common type in South America, which is why this subtype was selected as a vaccine strain (Sanchez-Vazquez et al., 2019). However, topotypes are not particularly meaningful because type C FMD has not occurred recently. After the last outbreak in Brazil and Kenya in 2004 (Sangula et al., 2011), type C FMD has not occurred anywhere in the world. Thus, when live virus experiments are conducted, close attention should be paid to preventing virus release.

* Corresponding author.
E-mail address: parkjhvet@korea.kr (J.-H. Park).

https://doi.org/10.1016/j.vetmic.2019.05.016
Received 31 January 2019; Received in revised form 22 May 2019; Accepted 22 May 2019

Vaccines are still produced using type C in South America and are kept in the form of antigen banks in Europe and elsewhere. Since type C FMDV is, fortunately, not particularly variable (Sangula et al., 2011), large numbers of vaccine strains are not recommended—unlike type A—and the only vaccine strain internationally recommended for use by the World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD) at a low priority is C1 Noville, which originated in Switzerland in 1965. In the case of South America, strain C3 Indaial, which originated in Brazil in 1971, is currently used as a vaccine strain. In the case of South Korea, the antigen bank currently being created by an overseas vaccine company has been established using C1 Oberbayern, which originated in Germany in 1960, as a vaccine antigen.

For self-production of an FMD vaccine, South Korea has prepared and focused on the development of seven serotype vaccine strains since 2011. In our previous study, we synthesized various P1 genes—which we then used to replace the P1 genes in the O1 Manisa virus, a representative vaccine strain for FMD, with serotype specific antigenicity—to make an infectious clone and a new vaccine virus (Kim et al., 2015; Lee et al., 2017). In this study, we attempted to develop new vaccine strains with better functions and to make vaccines that were improved from existing vaccines. The residues138-150 of FMDV $3C^{pro}$ in the β-ribbon structure overlie the substrate binding cleft (Birtley et al., 2005). The residue C142 is one of a catalytic triad within the 3C. The C142 at the apex of this loop plays an important role in substrate binding (Nsamba et al., 2015). We checked the pathogenic characteristic by changing a single amino acid (C142) of the 3C site. Furthermore, we replaced $3B_1B_2$ with $3B_3B_3$ to enable differential diagnoses of vaccine strains and wild strains based on the 3B site. The $3B_1$ (CGPYAGPLERQKPLK) or $3B_2$ (PMERQKPLKVKAKA) peptide was used as the basis for the 3B ELISA to differentiate vaccinated from infected animals (Hohlich et al., 2003; Yang et al., 2015). Even if the FMD vaccine excluded the NSP antigen during antigen purification, multiple vaccination in animals resulted in the formation of the NSP ELISA antibody because of the mainly B-cell epitopes $3B_1$ and $3B_2$ (Hohlich et al., 2003). Therefore, we replaced $3B_1B_2$ with $3B_3B_3$ in the source virus to differentiate between infected and vaccinated animals.

For the present study, the P1 gene of C3 Resende, which originated in Brazil in 1955, was inserted to replace the P1 gene of the O1 Manisa vaccine strain to make a vaccine strain for protection against type C. Although type C FMD outbreaks do not occur at present, the new vaccine is being made based on C3 Resende from South America, so the vaccine strain can be used for vaccines when type C FMD reoccurs.

2. Materials and methods

2.1. Preparation of infectious clone

The plasmid already secured by Ko et al. (2019) by removing the $3B_1B_2$ site and manipulating the site into $3B_3B_3$ was used, as well as an infectious clone where the 142 amino acid position in the 3C was manipulated such that C was replaced by T (C142 T) (Ko et al., 2019). C3 Resende was used to synthesize cDNA, and primer C3 sense (5'-AACA AAGGTCCAGAAAAGGCTCAAGGGAGCGGGACAGTCATCA3′) and primer C3 antisense (5'-TTTGAGCAGGTCAAAATTTAGAAGTGCACAG TTGTTTCTCGACG3′) were used for the polymerase chain reaction (PCR) to amplify P1 gene.

The PCR conditions were reactions with a mixed solution of 5x buffer (Finnzymes, 10 μl), 10 mM dNTPs (1 μl), Phusion enzyme (1 μl of 2 U/μl), and sterilized distilled water (35 μl) for 30 s at 98 ℃, followed by 10 s at 98℃, 30 s at 72 ℃, 2 min at 72 ℃, 25 cycles of 30 s at 72 ℃, and, finally, 10 min at 72 ℃. The PCR in which P1 was amplified was made to react under the ligation condition in the Gibson Assembly® Cloning Kit. The PCR to amplify only the genes used as vectors, in order to replace the P1 of FMDV C3 Resende at the infectious plasmid (pO-Manisa $3B_{333}$ 3Cmut : p3B3C), was carried out using template p3B3C (100 ng/μl, 1 μl), primer VF sense 5'-ACTTCTAAATTTTGACCTGC3′, and primer VR antisense 5'-CTTGAGCCTTTTCTGGAC3′. The PCR conditions were reactions with a mixed solution of 5X buffer (FINNZYMES, 10 μl), 10 mM dNTPs (1 μl), Phusion enzyme (2 U/μl, 1 μl), and sterilized distilled water (35 μl) for 30 s at 98 ℃, followed by 10 s at 98 ℃, 30 s at 65 ℃, 2 min at 72 ℃, 25 cycles of 30 s at 72℃, and, finally, 10 min at 72 ℃. The insertion of C3 Resende P1 into p3B3C was carried out with reactions of C3 Resende P1 PCR product (200 ng/μl, 1 μl), the PCR product in which P1 was removed from p3B3C (200 ng/μl, 1 μl), Gibson Assembly Master Mix (2X, 10 μl), and sterilized distilled water (8 μl) for 30 min at 56℃. Thereafter, the clone was transferred to the competent cell included in the Gibson Assembly® Cloning Kit. Finally, the base sequence of the clone was analyzed to determine whether the P1 in the plasmid of p3B3C had been replaced by the P1 of C3 Resende. As in the case of C1 Oberbayern-R (Om-C-Ob) that was secured in the previous study (Lee et al., 2017), infectious clones were produced by the same method.

2.2. Virus recovery and cell culture

To secure FMDVs from the infectious clone, experiments were conducted using the previous experimental method (Lee et al., 2017). In particular, the C1 Oberbayern-R (Om-C-Ob) virus rescued in our previous study was also used in this study.

The recombinant plasmid (pO-C3 Resende-R) into which P1 was inserted was induced to react with restriction enzyme *Spe*I (NEB, USA) for 24 h at 37 ˚C to make the genes into single pieces; BHKT7-9 cells (cell line where T7 RNA polymerase is expressed) were transfected with the purified DNA using lipofectamine 2000 (Invitrogen, USA) and cultivated for 2–3 days; and the FMDVs into which C3 Resende P1 was newly inserted were secured. Thereafter, the secured viruses were multiplied through successive subcultures using ZZ-R (fetal goat tongue epithelium) cells or BHK-21 (baby hamster kidney) cells.

To produce antigens for vaccine preparation, the viruses were multiplied using BHK21 or BHK21-suspension cells, which are the cells for production of FMDVs. Sixteen hours after infection, the viruses were inactivated by 0.003 N of binary ethylene immine for 24 h and inactivation of the virus was confirmed with non-cytotoxic effect after three serial passages in the ZZ-R and BHK-21 cells. The inactivated antigens were concentrated with polyethylene glycol 6000 (81260; Sigma Aldrich, USA).

The virus was layered on 15–45% sucrose-density gradients and centrifuged. After ultracentrifugation, the bottom of the centrifuge tube was punctured, and 1 ml fractions were collected. As in the previous study (Lee et al., 2017), the final inactivated antigen (FMD viral particles) was tested by transmission electron microscopy (TEM) (Fig. 2).

2.3. Nucleotide and amino acid similarities among the type C FMD strains

The available nucleotide and amino acid sequences of the P1 region (VP4, VP2, VP3, and VP1) were compared with those of the strains from South America and Europe, and their similarities were determined using the Clone Manager Professional 9 program (Scientific & Educational Software, USA). The viruses for similarity comparison of nucleotides and amino acid sequences were presented in the following order: GenBank accession; C3 Resende (AY593807, Brazil in 1955), C1 Noville (AY593804, Switzerland in 1965), C1-Oberbayern (AY593805, Germany in 1960), C1 Haute Loire FR/69 (L29061, France in 1969), C-S8c1 (AJ133357, Spain in 1970), C3 Indaial (M90376, Brazil in 1971), and C3 Argentina 85 (L29062, Argentina in 1984).

2.4. Preparation of experimental vaccine

The vaccine was prepared following method used in the previous study (Ko et al., 2019). The method was as follows; 15 μg (1-dose) of purified 146S antigen of C3 Resende-R and C1 Oberbayern-R was mixed with ISA206VG (Seppic, Paris, France) at a ratio of 1:1 (volume [v]/v),

and 10% aluminum hydroxide gel (Rehyragel® HPA; General Chemical, NJ, USA) and Saponin 0.5 μg were added to that mixture to prepare the vaccine in the form of water-in-oil-in-water mentioned in the previous study (Ko et al., 2019).

2.5. Pathogenesis in suckling mice

Seven-day-old ICR mice (Orient Co. Ltd, Republic of Korea) were used for this experiment. The animals were kept in the Animal and Plant Quarantine Agency (APQA) and were used with the approval of the APQA's Animal Care and Use Committee. Twenty-four mice were divided into two groups and the mice were administered C3-Resende-R or C3-Resende wild type virus by intraperitoneal (IP) injection with 0.1 ml of 1×10^5 $TCID_{50}$. The mice were observed for 7 days after the challenge.

2.6. Virus challenge after vaccination in adult C57BL/6 mice

Seven-week-old C57BL/6 female mice (KOSA BIO Co. Ltd, Republic of Korea) were used for this experiment (4 per group). The animals were kept in the APQA and were used with the approval of the APQA's Animal Care and Use Committee. The mice were inoculated by intramuscular (IM) injection with experimental vaccine (0, 1/10, 1/40, 1/160, and 1/640 doses for pigs and cattle use, 15 μg per dose) mixed with ISA206 and 10% aluminum hydroxide gel adjuvant. The mice were challenged by IP injection with 0.1 ml of C3 Resende at 100 LD_{50} (50% of a lethal dose); the actual dose was $10^{3.75}$ $TCID_{50}$. All mice were observed for 7 days after the challenge.

2.7. Test of the immunogenicity of the test vaccine in pigs and cattle

For the immunogenicity test, 20 FMDV antibody–negative farm pigs and five head of antibody-negative farm cattle aged at least 5 months were used. After the pigs and cattle were inoculated with the test vaccine, blood was collected at 0, 14, 28, 42, 56, and 70 days for pigs, and at 84, 112, and 140 days for cattle to measure the antibodies after the IM vaccination. Briefly, the neutralizing antibody titers in the serum were measured using the virus neutralization test (VNT) specified in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the World Organisation for Animal Health (OIE). Serum samples were collected from the animals after vaccination. The sera were heat inactivated at 56 °C for 30 min. Following 1 h incubation in serial diluted sera and virus suspension, LF-BK cells were added to the plate and incubated for a period of 2–3 days. The neutralizing antibody titers were calculated as the $\log_{10}$ of the reciprocal antibody dilution to neutralize 100 $TCID_{50}$ of virus.

2.8. Challenge test after vaccination in pigs

The experimental vaccines for the C3-Resende-R vaccine were prepared with 146S antigen at different inoculation amounts of 15 μg, 3.75 μg, and 0.9375 μg, while keeping other compositions unchanged. The animals were divided into Group 1: C3-Resende-R (1 dose: 15 μg), Group 2: C3-Resende-R (1/4 dose: 3.75 μg), Group 3: C3-Resende-R (1/16 dose: 0.9375 μg), and Group 4: C1-Oberbayern-R (1 dose: 15 μg) by assigning four animals to each group and two animals to a control group. Blood was collected at 0, 7, 14, 21, and 28 days after vaccination and the pigs in all groups were challenged with FMDV C3 Resende at a titer of 10^5 $TCID_{50}$ on their tongues at 28 days after vaccination. After the virus challenge, the pigs in individual groups were separately raised and then isolated when clinical symptoms of FMD appeared. Oral swabs were collected from 0 days post challenge (dpc) to 12 dpc, and the serum was collected at 0, 3, and 5 dpc. The blood samples were collected by venipuncture (anterior vena cava) and placed into Vacutainer serum tubes (BD Biosciences, USA). The oral swabs were collected using the BD™ Universal Viral Transport Kit (BD Biosciences, USA). The FMDV viral RNA was identified by extracting the viral RNA from oral swab samples and quantitative real-time reverse transcription PCR (RT-PCR). The MagNapure 96 system (Roche, Germany) was used for the extraction of the viral RNA, and the quantitative real-time RT-PCR was conducted using the same method as in the previous experiment (Lee et al., 2017). The clinical score was determined by the addition of points distributed as described below (Lee et al., 2017). Clinical observation was performed daily after the challenge. The clinical scores were calculated using the following criteria: (a) elevated body temperature of 40 °C (1 point), > 40.5 °C (2 points), or > 41 °C (3 points); (b) lameness (1 point); (c) hoof and foot vesicles (1–2 points per foot); and (d) snout, lips, and tongue vesicles (1 point for each affected area), for a maximum of 15 points. NSP ELISA Kit (Bionote Kit, Republic of Korea)—an ELISA kit for the detection of FMDV NSP antibodies in serum samples of pigs and bovines—was employed to detect NSP antibodies.

2.9. Virus detection in immunized and challenged pigs

Quantitative real-time RT-PCR was carried out on sera and swab samples of the experimental animals. Swab samples were gathered from the mouth and nose using cotton swabs. Total cellular RNA was extracted using the MagNA Pure 96 System (Roche, Germany) according to the manufacturer's protocol. Quantitative real-time RT-PCR was conducted using the one-step prime-script RT-PCR kit (Bioneer, Republic of Korea) according to the manufacturer's instructions.

Primers targeting the FMDV 3D region were sense 5'-GGAACYGGGTTTTAYAAACCTGTRAT3′ and antisense 5'-CCTCTCCTTTGCACGCCGTGGGA3′. The probe was 5'-CCCADCGCAGGTAAAGYGATCTGTA3′; its 5' end was labeled with 6-FAM, and the 3′ end was labeled with TAMRA. The CFX96 Touch™ Real-Time PCR Detection system (Bio-Rad, USA) was used for virus quantification.

2.10. Statistical analysis

The statistical relationships between the inoculated groups and negative control groups were determined. The *t*-tests and log rank test were conducted using GraphPad Prism (Ver 5.0; GraphPad Software, USA) and GraphPad Instant (Ver 3.05; GraphPad Software, USA).

3. Results

3.1. Genetic variations among prototypes of type C FMDV

C3 Resende, which is a strain that circulated in South America, showed genetic similarity to European and other South American strains, to the extent that it was found to be little different from those strains. Although it shows a nucleotide difference of about 10% from both South American and European strains, it showed only a 5–7% difference in amino acids (Fig. 1A). C1 Oberbayern, which is a strain that occurred in Europe and was used as a vaccine strain reserved in South Korea as an antigen bank, showed at least a 95% genetic similarity to viruses that occurred in Europe, such as C Noville, C-S8c1 (Spain in 1970) and C Haute Loire FR (France in 1969), while showing relatively low genetic similarity to C3 Resende, C3 Indaial, and C3 Argentina85, strains which originated in South America, but the difference in amino acids was 5–8% (Fig. 1B). In the case of South American and European-type viruses among type C FMDVs, the largest number of amino acid changes could be observed in VP1 130–153 amino acid sites. Residues130, 152, and 204 in the South American types were identified as regional unique amino acid sequences.

3.2. Low pathogenicity and differentiation between the vaccine virus and wild type virus

The seven-day-old mice were inoculated with C3 Resende-R and C3 Resende wild type viruses at a titer of 1×10^5 $TCID_{50}$ into their

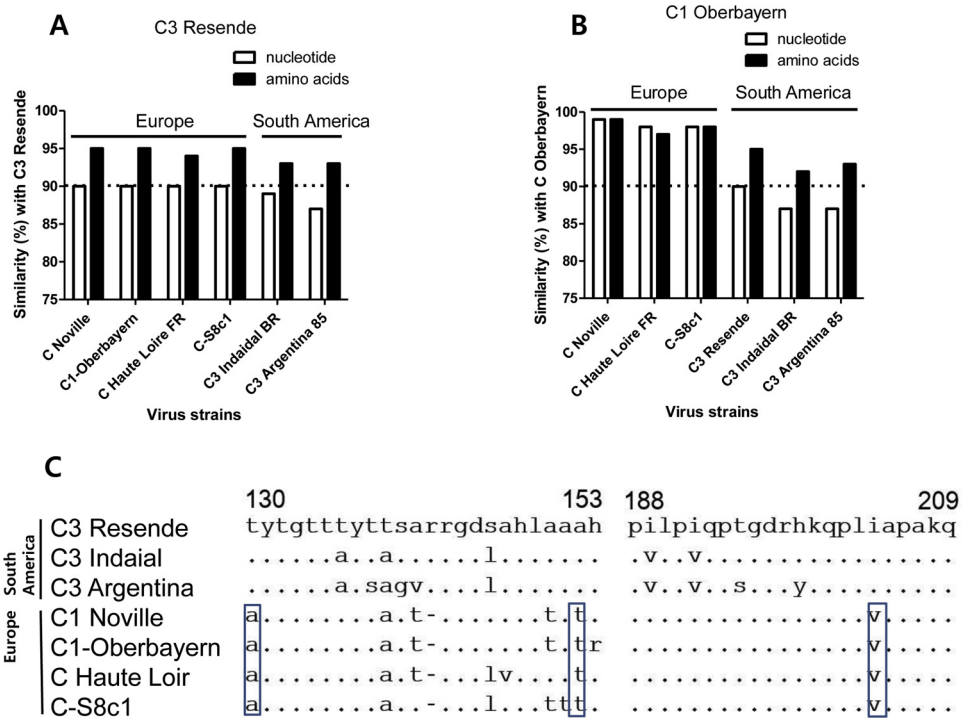

Fig. 1. Comparison of genetic variations of type C FMDV between strains from South America and Europe. A. Similarity (%) of nucleotide and amino acids based on P1 of C3 Resende; B. Similarity (%) of nucleotide and amino acids based on P1 of C1 Oberbayern; C. Comparison of VP1 variable antigenic sites (residues 130–153, and 188–209) between strains from South America and Europe. The boxes (residues 130, 152 and 204) represent unique sites in the viruses from South America. GenBank accessions : C3 Resende (AY593807), C1 Noville (AY593804), C1-Oberbayern (AY593805), C1 Haute Loire FR/69 (L29061), C-S8c1 (AJ133357), C3 Indaial (M90376), and C3 Argentina 85 (L29062).

abdominal cavities. All the mice in the group inoculated with the wild type virus died at 3 dpc. However, the group inoculated with C3 Resende-R virus showed a survival rate of 80% until 3 dpc and a survival rate of 20% at 7 dpc. Nevertheless, significant decreases in pathogenicity were identified ($p = 0.0082$) (Fig. 2B). In antigen rapid tests for FMDV diagnosis, no NSP band was identified (Fig. 2C). The inactivated viruses were examined through an electron microscope for a confirmation of formation of virus particle and we identified FMDVs of a size of about 25 nm diameter (Fig. 2D). The viral titers ($TCID_{50}$) of two viruses cultured in BHK-21 suspension cells were similar, which were 7.9×10^5 /ml in C3 Resende-R and 5.78×10^5/ml in C Oberbayern-R, respectively.

3.3. Protection in immunized mice after challenge

The protection capability of C3-Resende-R vaccine was tested through 7–8-week-old C57BL/6 mouse models. The immunogenicity of the vaccine was checked in the mouse model for prior estimation before applying the vaccine to pig models. In the case of C3 Resende-R vaccine (based on 1-dose for cattle and 1 for pigs), a 100% survival rate was identified at 1˜1/40 dose, while a 25% survival rate was identified at 1/640 dose. When the mouse models were challenged at 7 days after vaccination (0.1 mL/dose), 32 mouse PD_{50} (mPD_{50}) was identified. In the case of the C1 Oberbayern-R vaccine, a 100% survival rate was identified at 1˜1/160 dose, while a 50% survival rate was identified at

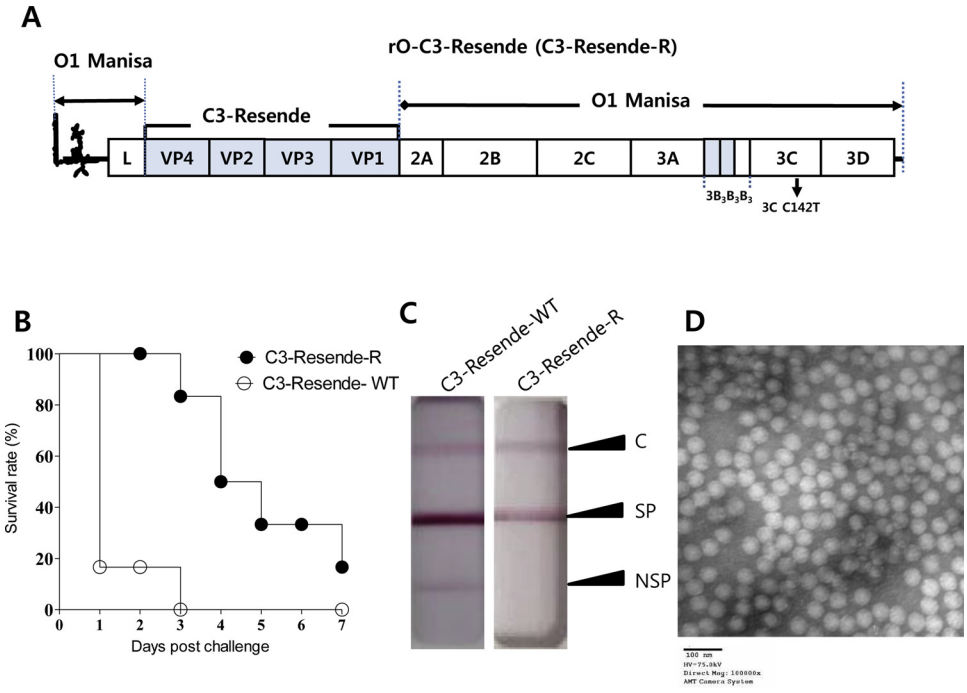

Fig. 2. Characterization of type C FMDV and C3-Resende-R. A. Schematic diagram of type C FMDV genome, C3-Resende-R, the $3B_1B_2$ were replaced by the method by Ko et al. (2019), and mutated C142 T in 3C region; B. Pathogenesis of C3-Resende-R and wild type virus in 7-day-old suckling mice, $p = 0.0082$; C. No detection of NSP antigen in virus cultured supernatant using FMDV antigen rapid kit (Princeton Bio-Meditech Corporation, USA) for differentiation between the vaccine and the wild-type virus (Ko et al., 2018 referred to the same method), C: Control line, SP: FMDV structural protein line, NSP: FMDV non-structural protein line; D. Electron microscopy of FMDV vaccine stain, C3-Resende-R. The bar represents 100 nm.

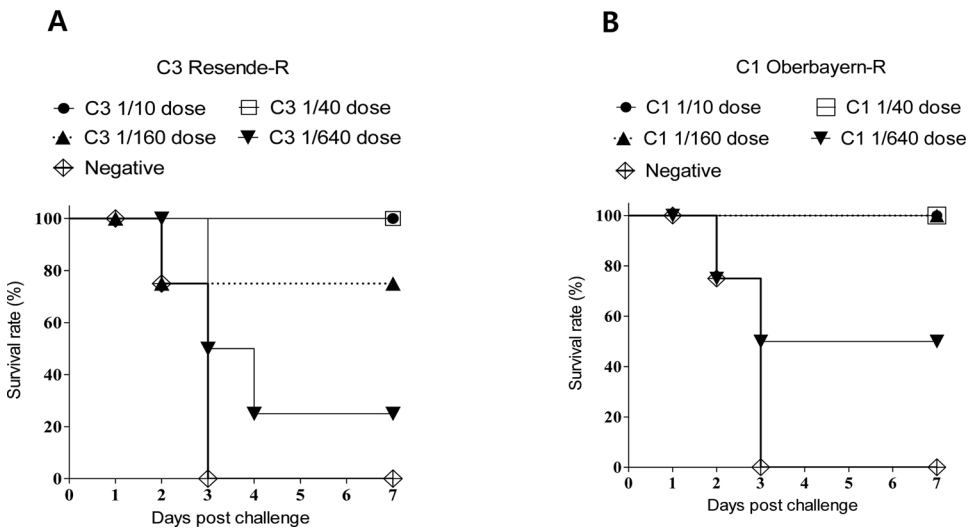

Fig. 3. Survival rate in adult mice (C57BL/6) challenged with C3-Resende wild-type virus at 7 days after vaccination with a serially diluted vaccine for antigen doses in mice. A. C3-Resende-R; B. C1-Oberbayern-R (Om-C-Ob). The vaccine contains sucrose gradient purified-146S inactivated antigen (C3-Resende-R or Om-C-Ob), ISA206, and 10% Gel. The vaccinated mice were monitored for 7 days after the C3-Resende virus challenge.

1/640 dose. The result was identified as 64 mPD_{50} (Fig. 3).

3.4. Evaluation of the immunogenicity of the candidate vaccine (C3-Resende-R) in cattle and pigs

After C3-Resende-R vaccination, the pigs were tested until 70 days post-vaccination (dpv), and it was found that virus-neutralizing antibodies were produced two weeks after vaccination. After the second vaccination at 28 dpv, the antibody titer was slightly decreased at 42 dpv, but it was determined that the neutralizing antibody was maintained at a level of at least 1:100, which is the level of protective antibodies, until 70 dpv (Fig. 4A). After the vaccine of the C3-Resende-R vaccine candidate strain was produced, the vaccine was inoculated into farm cattle (cattle $n = 5$/group) and rapid increases in the neutralizing antibody were identified at 14 days after vaccination (Fig. 4B). A serum virus-neutralizing antibody level of at least 1:100 was identified until 70 dpv, but gradually decreased after 84 dpv. After the second vaccination at 28 dpv, an antibody level of 1:100 on average was maintained, even at 140 dpv.

3.5. Virus challenge inoculation in immunized pigs

C3-resende-R vaccine made with different amounts of antigen—1, 1/4, and 1/16 doses—were inoculated into pigs. One individual (#1-3) in the C3-Resende-R 1-dose group showed vesicle formation at the inoculation site on its foot at 6 dpc and low virus titers of about 10^2 were identified in the swabs collected from some other animals (Fig. 5). After the inoculation, no viremia was measured in the saliva samples from two pigs, and the individuals showed no clinical symptoms. In the 1/4-dose group, a low clinical index was assigned to individual #3-8 because a low level of virus titer was identified, and a vesicle formed on the foot at 5 dpc. A low virus titer was also identified in the blood (Table 1). In the case of individuals #1-9 and #3-3 in the 1/4-dose group, although no clinical symptoms appeared, discharge of viruses at low concentrations could be seen in the saliva. In the 1/16-dose group, individual #5-9 died at 4 dpc, but no clinical symptoms were observed at the time of death. Discharge of viruses at low concentrations could be identified in the saliva of the remaining individuals, #5-3 and #5-8. In the case of the C1 Oberbayern-R 1-dose group, used as a comparison group, virus titers of 10^{4-5} were temporarily identified in individual #6-4 at 3 dpv and in individual #3 at 5 dpv, and individuals #3, #6-3, and #6-4 were shown to be NSP antibody positive (Table 1).

All individuals in the control group showed the clinical symptom of blisters appearing on the feet, such that a clinical score of 4–5 points was assigned. In addition, discharges of viruses were identified in the saliva from 2 dpc. Low levels of viruses were also measured in the blood at 3 dpc and 5 dpc (Fig. 5 and Table 1).

4. Discussion

Type C FMD has not occurred since 2004. Type C is largely divided into three topotypes: EURO-SA, AFRICA, and ASIA. Historically, type C was first recorded when it occurred in Germany in 1926 (C/GER/c.26; CGC). Its vaccines are still produced in South America using C3 Indaial as a vaccine strain, and the vaccines have been inoculated in Argentina, Bolivia, Brazil, and Paraguay (Sanchez-Vazquez et al., 2019). However, even the international organizations Food and Agriculture Organization (FAO) and World Organisation for Animal Health (OIE) have reviewed

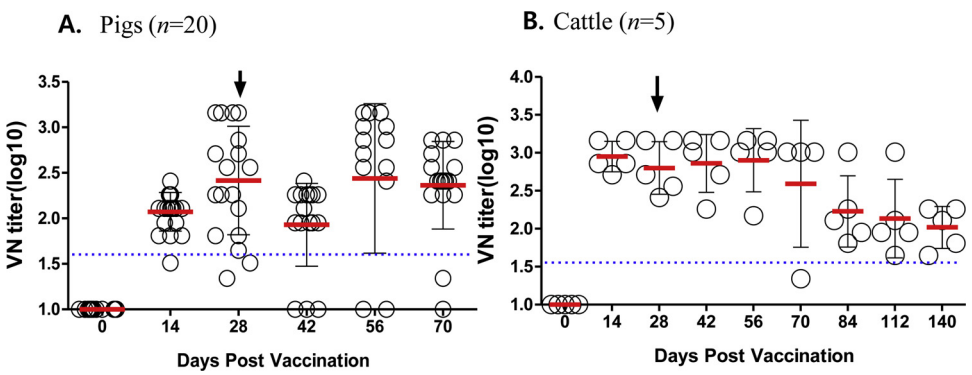

Fig. 4. Virus-neutralizing antibody titers in pigs and cattle vaccinated with experimental FMD vaccine with ISA 206 oil adjuvant. A. pigs ($n = 20$); B. cattle ($n = 5$). The dotted lines show 1.65 log virus neutralizing (VN) titers (1:45), which would be protected.

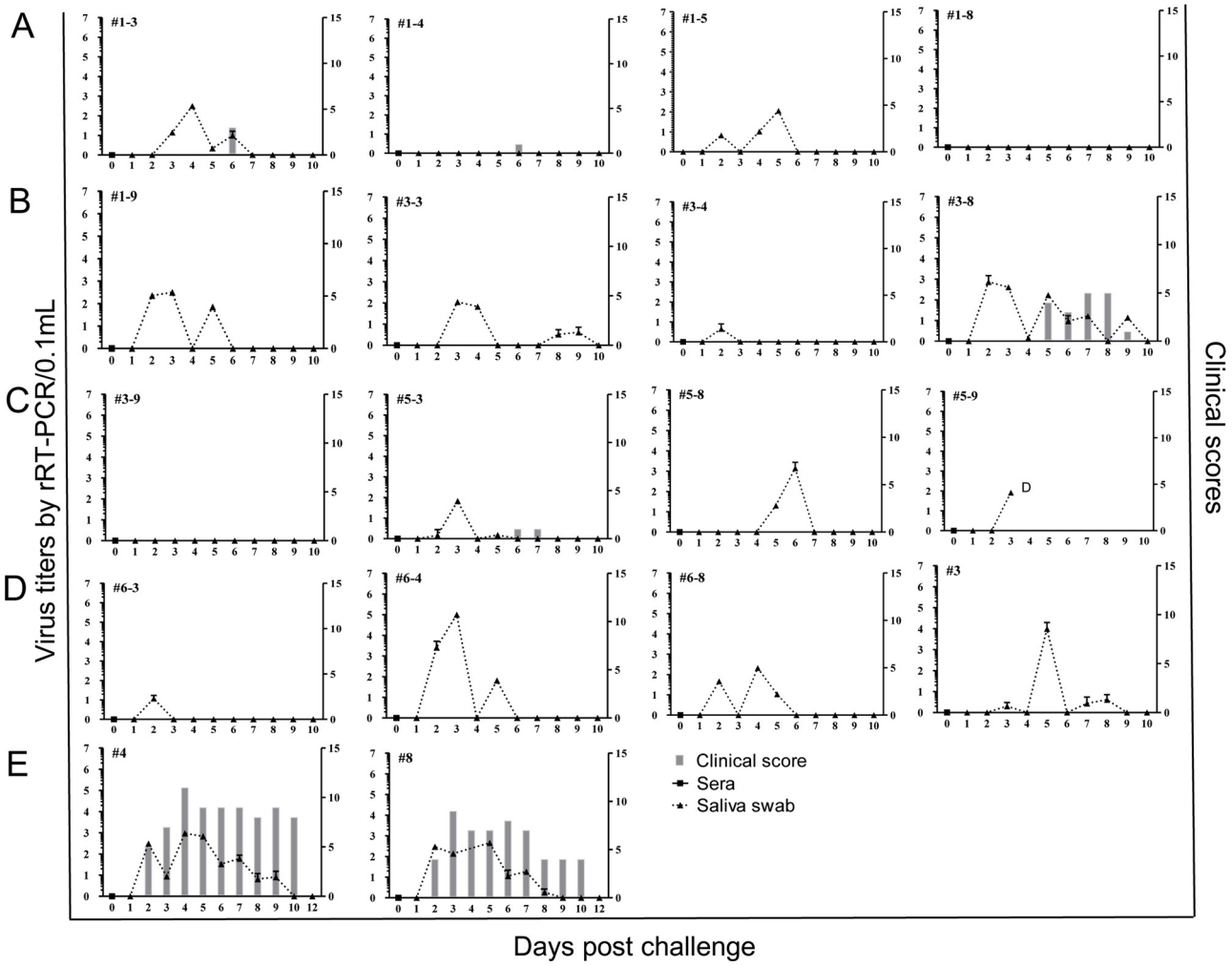

Fig. 5. Clinical scores and virus shedding in the C3-Resende-R-immunized pigs after C3-Resende virus challenge. A. C3 Resende-R 1-dose (15 μg), animal ID #1-3, 1–4, 1–5, and 1–8; B. C3 Resende-R 1/4-dose (3.75 μg), animal ID #1-9, 3-3, 3–8, 3–4; C. C3 Resende-R 1/16-dose (0.93 μg), animal ID #3-9, 5-3, 5–8, 5–9; D. C1-Oberbayern-R 1-dose vaccination (15 μg), animal ID # 3, 6–8, 6-3, 6-4; E. Negative control, animal ID # 4 and 8. Animal ID #5-9 died at 4 dpc.

Table 1
Immune response and virus detection in the immunized pigs after C3-Resende challenge.

Group	Animal no.	VN titer (log)					Viremia		NSP antibody detection	Protection
		0dpv	7dpv	14dpv	21dpv	28dpv	3dpc	5dpc		
C3 Resende-R 1-dose (15 μg)	1-3	< 1.20	1.34	1.20	< 1.20	1.34	Neg	Neg	Neg	Yes
	1-4	< 1.20	1.51	2.11	1.81	1.65	Neg	Neg	Neg	Yes
	1-5	< 1.20	1.65	1.81	1.65	1.34	Neg	Neg	Neg	Yes
	1-8	< 1.20	1.51	1.34	1.51	1.65	Neg	Neg	Neg	Yes
C3 Resende-R 1/4-dose (3.75 μg)	1-9	< 1.20	1.34	1.20	1.20	1.20	Neg	Neg	Neg	Yes
	3-3	< 1.20	1.20	1.34	1.34	1.95	Neg	Neg	Neg	Yes
	3-8	< 1.20	1.34	< 1.20	< 1.20	< 1.20	Neg	Pos	Neg	Yes
	3-4	< 1.20	1.95	1.51	2.11	1.51	Neg	Neg	Neg	No
C3 Resende-R 1/16-dose (0.93 μg)	3-9	< 1.20	1.20	1.51	1.34	1.95	Neg	Neg	Neg	Yes
	5-3	< 1.20	1.20	< 1.20	< 1.20	1.20	Neg	Neg	Neg	Yes
	5-8	< 1.20	1.34	1.81	1.34	1.95	Neg	Neg	Neg	Yes
	5-9	< 1.20	< 1.20	< 1.20	1.34	< 1.20	Neg	n.a.	n.a.	No
C1-Oberbayern-R 1-dose vaccination (15 μg)	3	< 1.20	1.51	1.81	1.51	1.65	Neg	Neg	Neg	Yes
	6-8	< 1.20	1.51	< 1.20	< 1.20	< 1.20	Neg	Neg	Pos	Yes
	6-3	< 1.20	1.81	1.20	1.34	1.65	Neg	Neg	Neg	Yes
	6-4	< 1.20	1.20	1.51	1.20	1.65	Neg	Neg	Neg	Yes
Negative control	4	< 1.20	< 1.20	< 1.20	< 1.20	< 1.20	Pos	Pos	Pos	No
	8	< 1.20	< 1.20	< 1.20	< 1.20	< 1.20	Pos	Neg	Pos	No

n.a ; not available.

the global suspension of type C vaccine experiments, vaccination and virus challenge to reduce a risk of type C outbreak.

C3 Resende and C3 Indaial have been used as vaccine strains in South America, but only C3 Indaial has been used recently (Sanchez-Vazquez et al., 2019), and the reserve vaccine strain recommended by the OIE for global antigen banks is C Noville, which originated in Europe (www.WRLFMD.org).

Countries such as South Korea cannot be free from the threat of FMD outbreak caused by the virus occurring in surrounding countries. South Korea relies on overseas vaccine companies for vaccine import, but with the large-scale FMD outbreaks in 2010 (Park et al., 2013), studies to develop local FMD vaccines for all serotypes, even type C, in South Korea have been conducted since 2011. Although type C FMD has not occurred recently anywhere in the world, FMD serotypes that have not occurred should be kept in the form of antigen banks to be prepared for infection by viruses such as type C, SAT1, SAT2, and SAT3.

Since type C FMDV does not show large genetic variations (Sangula et al., 2011), and there have been few studies of it recently, analysis of the genetic variation of the virus is difficult. The last outbreaks of type C FMD were in Kenya, Africa, and in the Amazon region in Brazil, South America, and the virus has not re-occurred since 2004. Europe-originated C1-Oberbayern is highly homologous to the European viruses but has relatively low similarity with the South American viruses, and C3-Resende has similar antigenic similarities to those of Europe and South America. In addition, C1-Oberbayern has low antigenic similarity with C3-Resende, so that it is possible to maintain a complementary relationship in the use of vaccines. In Asian regions, type C FMD occurred in 1982–1996, mainly in Nepal, Bhutan, and India, but was eradicated. FMD also spread in Europe and South American regions, but, through monitoring of regions such as the Amazon, it has been proven that type C FMD is not re-occurring (Sanchez-Vazquez et al., 2019).

In this study, we prepared vaccine strains to make safe FMD vaccines by developing them in the form of viruses with lower pathogenicity, rather than the highly pathogenic field viruses that have been prepared in the past for the outbreak of type C FMD, even though it has been almost completely eradicated. In previous study, the residue C142 was one of catalytic triads within the 3C. The C142 at the apex of this loop was found to play an important role in substrate binding (Birtley et al., 2005; Nsamba et al., 2015). Moreover, the protease activity of the intermediated level by modified 3C with C142 T of four sequences (C142 T, C142 L, C142S, and C142A) resulted in the efficient expression of empty FMDV capsid (Porta et al., 2013). The mutation was the most productive approach leading to almost complete processing of P1-2A. We believe that the mutation in 3C influenced the activity of $3C^{pro}$ determining the pathogenic property of the virus.

The experimental FMD vaccine sufficiently protected the pigs against the homologous challenge and the virus was not discharged, unlike in the control group. Therefore, the potential of the vaccine for type C was confirmed. As for the range of protection of this vaccine, its similarity to South American and European strains in virus structural proteins and amino acids was higher than topotypes within other serotypes. If animals are not protected against type C FMD, even when the C3 Resende-R vaccine strain has been used, supplementary use of the C1 Oberbayern-R strain, with its antigenicity which is significantly different from the C3 Resende-R vaccine strain, should enable complementary protection.

Type C has mainly occurred in cattle in Asia, South America, and Africa. In the case of cattle, immunogenicity of the vaccine is very high, as shown in the results of our experiments, and very high antibody levels were maintained after 2 weeks following only a one-time of vaccination. Therefore, the importance of the vaccine can be said to be higher for pigs, which are known to have lower immunogenicity than cattle, than for cattle with excellent immunogenicity.

However, in countries where pigs are raised on a large scale near cattle, such as South Korea, vaccines effective in pigs should be prepared. In the present study, when immunity tests were conducted in cattle and pigs, the animals were found to maintain antibodies at the protection level after the second inoculation, but some pigs did not induce any antibodies. As severe pathogenicity was identified after the challenge in the control group of pigs, the usability of the vaccine in pigs is could be considered to be sufficient, and protection effects could be identified in most animals.

In conclusion, the development of vaccine strains using C3 Resende-R induced the formation of sufficient antibodies in cattle and pigs and successful protection against FMDV challenges in pigs. In addition, C1 Oberbayern-R also protected animals against C3 Resende virus challenges, and it was found that sufficient cross-protection can be achieved between heterologous viruses.

Funding

This study was generously supported by Animal and Plant Quarantine Agency, Gimcheon, Gyeongsangbuk-do, Republic of Korea.

Acknowledgements

We wish to thank the staff of the Center for FMD Vaccine Research and Mr. Jung-Won Park for electron microscopy at the Animal and Plant Quarantine Agency.

References

Alexandersen, S., Mowat, N., 2005. Foot-and-mouth disease: host range and pathogenesis. Curr. Top. Microbiol. Immunol. 288, 9–42.
Birtley, J.R., Knox, S.R., Jaulent, A.M., Brick, P., Leatherbarrow, R.J., Curry, S., 2005. Crystal structure of foot-and-mouth disease virus 3C protease. New insights into catalytic mechanism and cleavage specificity. J. Biol. Chem. 280, 11520–11527.
de Los Santos, T., Diaz-San Segundo, F., Rodriguez, L.L., 2018. The need for improved vaccines against foot-and-mouth disease. Curr. Opin. Virol. 29, 16–25.
Grubman, M.J., Baxt, B., 2004. Foot-and-mouth disease. Clin. Microbiol. Rev. 17, 465–493.
Hohlich, B.J., Wiesmuller, K.H., Schlapp, T., Haas, B., Pfaff, E., Saalmuller, A., 2003. Identification of foot-and-mouth disease virus-specific linear B-cell epitopes to differentiate between infected and vaccinated cattle. J. Virol. 77, 8633–8639.
Kim, R.H., Chu, J.Q., Park, J.N., Lee, S.Y., Lee, Y.J., Ko, M.K., Hwang, J.H., Lee, K.N., Kim, S.M., Tark, D., Ko, Y.J., Lee, H.S., Seo, M.G., Park, M.E., Kim, B., Park, J.H., 2015. Antigenic properties and virulence of foot-and-mouth disease virus rescued from full-length cDNA clone of serotype O, typical vaccine strain. Clin. Exp. Vaccine Res. 4, 114–118.
Ko, M.K., Choi, J.H., You, S.H., Shin, S.H., Jo, H.D., Lee, M.J., Kim, S.M., Kim, B.H., Park, J.H., 2019. Chimeric vaccine strain of type O foot-and-mouth disease elicits a strong immune response in pigs against ME-SA and SEA topotypes. Vet. Microbiol. 229, 124–129.
Lee, S.Y., Lee, Y.J., Kim, R.H., Park, J.N., Park, M.E., Ko, M.K., Choi, J.H., Chu, J.Q., Lee, K.N., Kim, S.M., Tark, D., Lee, H.S., Ko, Y.J., Seo, M.G., Park, J.W., Kim, B., Lee, M.H., Lee, J.S., Park, J.H., 2017. Rapid engineering of foot-and-mouth disease vaccine and challenge viruses. J. Virol. 91.
Nsamba, P., de Beer, T.A., Chitray, M., Scott, K., Vosloo, W., Maree, F.F., 2015. Determination of common genetic variants within the non-structural proteins of foot-and-mouth disease viruses isolated in sub-Saharan Africa. Vet. Microbiol. 177, 106–122.
Park, J.H., 2013. Requirements for improved vaccines against foot-and-mouth disease epidemics. Clin. Exp. Vaccine Res. 2, 8–18.
Park, J.H., Lee, K.N., Ko, Y.J., Kim, S.M., Lee, H.S., Shin, Y.K., Sohn, H.J., Park, J.Y., Yeh, J.Y., Lee, Y.H., Kim, M.J., Joo, Y.S., Yoon, H., Yoon, S.S., Cho, I.S., Kim, B., 2013. Control of foot-and-mouth disease during 2010–2011 epidemic, South Korea. Emerging Infect. Dis. 19, 655–659.
Park, M.E., You, S.H., Lee, S.Y., Lee, K.N., Ko, M.K., Choi, J.H., Kim, B., Lee, J.S., Park, J.H., 2017. Immune responses in pigs and cattle vaccinated with half-volume foot-and-mouth disease vaccine. J. Vet. Sci. 18, 323–331.
Porta, C., Xu, X., Loureiro, S., Paramasivam, S., Ren, J., Al-Khalil, T., Burman, A., Jackson, T., Belsham, G.J., Curry, S., Lomonossoff, G.P., Parida, S., Paton, D., Li, Y., Wilsden, G., Ferris, N., Owens, R., Kotecha, A., Fry, E., Stuart, D.I., Charleston, B., Jones, I.M., 2013. Efficient production of foot-and-mouth disease virus empty capsids in insect cells following down regulation of 3C protease activity. J. Virol. Methods 187, 406–412.
Sanchez-Vazquez, M.J., Buzanovsky, L.P., Dos Santos, A.G., Allende, R.M., Cosivi, O., Rivera, A.M., 2019. Investigating the temporal and spatial distribution of foot-and-mouth disease virus serotype C in the Region of South America, 1968–2016. Transbound. Emerg. Dis. 66, 653–661.
Sangula, A.K., Siegismund, H.R., Belsham, G.J., Balinda, S.N., Masembe, C., Muwanika, V.B., 2011. Low diversity of foot-and-mouth disease serotype C virus in Kenya: evidence for probable vaccine strain re-introductions in the field. Epidemiol. Infect. 139, 189–196.
Yang, M., Parida, S., Salo, T., Hole, K., Velazquez-Salinas, L., Clavijo, A., 2015. Development of a competitive enzyme-linked immunosorbent assay for detection of antibodies against the 3B protein of foot-and-mouth disease virus. Clin. Vaccine Immunol.: CVI 22, 389–397.