Veterinary Microbiology 236 (2019) 108374

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Improved foot-and-mouth disease vaccine, O TWN-R, protects pigs against SEA topotype virus occurred in South Korea

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

[a] Animal and Plant Quarantine Agency, 177 Hyeoksin-8 ro, Gimcheon City, Gyeongsangbuk-do, 39660, Republic of Korea
[b] Veterinary College, Chungnam National University, Yuseonggu, Daejeon, 34134, Republic of Korea

ARTICLE INFO

Keywords:
FMD
Type O
Vaccine
Protection
Pig

ABSTRACT

Foot-and-mouth disease (FMD) is a highly contagious disease and causes economic damage at a national level. In particular, the type O FMD virus (FMDV) is a serotype that causes FMD outbreaks most frequently in the world. In recent years, Southeast Asia (SEA), Middle East-South Asia (ME-SA), and Cathay topotype-mediated FMD are prevalent in Asia, among which the SEA and ME-SA topotypes cause a majority of the outbreaks. The SEA topotype virus is more likely to infect both cattle and pigs simultaneously, thereby resulting in more severe damages; thus, it is necessary to study the protection ability of the candidate vaccines of this topotype after immunization. In this study, an experimental vaccine for pigs was produced using a vaccine strain that contains the structural protein of the O Taiwan97 strain, which was derived from the Cathay topotype, and its effect was evaluated. In the immunization test in pigs and cattle, the antibody titers were found to be elevated two weeks after immunization and very high titers of neutralizing antibodies were formed after four weeks. After the second inoculation, very high titers of neutralizing antibodies were produced in both species in the fourth week after immunization, and the antibodies maintained for up to six months and three months in cattle and pigs, respectively. No significant immunological difference in antibody production was observed in cattle and pigs. This study confirmed that complete protection from the challenge of the SEA topotype virus (O/Jincheon/SKR/2014), although the antibody titers against O/Jincheon/SKR/2014 strain were not that high, was achieved through immunization with the newly developed Cathay topotype vaccine in pigs.

1. Introduction

Foot-and-mouth disease (FMD) is a disease that rapidly spreads in major livestock—such as cattle, pigs, sheep, goats, and deer—and causes huge economic damages nationwide. There are seven serotypes of the FMD virus (FMDV)—namely O, A, Asia1, C, SAT1, SAT2, and SAT3. O serotype is the most prevalent worldwide (Mahapatra and Parida, 2018).

The O serotype is also the most frequently occurring serotype in Asia, among which the Southeast Asia (SEA) and Middle East-South Asia (ME-SA) topotypes occur frequently; the Cathay topotype occurs in Hong Kong, Southeast Asia, and China, but with a relatively low frequency (Di Nardo et al., 2014; Huang et al., 2001; Ko et al., 2019). Among the O Cathay topotype vaccine strains, the O Taiwan strain is the only vaccine strain that is recommended as a low priority by the OIE WRLFMD (www.wrlfmd.org). Internationally used FMD vaccine strains are O Manisa and O PanAsia-2 (which belong to the ME-SA topotype) as well as O Campos and O BFS (which belong to the EURO-SA topotype) (Galdo Novo et al., 2017; Mahapatra and Parida, 2018). In Asia, there have been a large number of SEA and ME-SA-mediated FMD outbreaks (Park et al., 2018), and studies have evaluated the ME-SA or SEA topotype vaccines against the SEA topotype (Park et al., 2014; Singanallur et al., 2017). However, only a few studies have evaluated the protection ability of the O Taiwan97 strain, which belongs to the Cathay topotype, against the SEA and ME-SA topotypes.

Thus, although it is desirable to use a vaccine of the same topotype as the circulating virus strain, it has already been demonstrated that O Campos of EURO-SA topotype was able to protect the SEA topotype (Galdo Novo et al., 2018).

This study aimed to investigate whether the vaccine strain that composed of the structural protein of O Taiwan97 virus—belonging to the O Cathay topotype—can induce immunogenicity in cattle and pigs against O/Jincheon/SKR/2014 of the SEA topotype and, thereby, determine whether the vaccine can provide protection from the SEA

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

https://doi.org/10.1016/j.vetmic.2019.07.026
Received 31 March 2019; Received in revised form 21 July 2019; Accepted 24 July 2019
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topotype; this topotype was responsible for the FMD outbreak in December 2014 in Korea and persisted for five months despite the enforcement of vaccination in livestock.

2. Materials and methods

2.1. Gene cloning

We replaced $3B_1B_2$ with $3B_3B_3$ in the source virus to differentiate between infected and vaccinated animals based on 3B region of the virus genome. A plasmid that was already secured by removing the $3B_1B_2$ site and manipulating the site into $3B_3B_3$ as well as an infectious clone, where the 142nd residue of the 3C site was manipulated such that C was replaced by T(C142 T) to reduce the virulence of FMDV (You et al., 2019), were both used in this study. The FMDV P1 region was obtained through a polymerase chain reaction (PCR) using the PCR primers O TWN sense (5′-AGGTCCAGAAAAGGCTCAAGGGCGCCGGCA TAAGGGCGCCGGGCAATCCAGCCC-3′) and antisense (5′- AGCAGGTC AAAATTTAGAAGCTGTTTTGCGGGTGTTTTTGCGGGTGCCACAA-3′) (Ko et al., 2019). In the same manner as done in the method employed by Lee et al. (2017), the P1 region of O Taiwan97 virus was replaced to the P1 region in O1 Manisa genome through cloning, and an O TWN-R virus expressing the inserted P1 was obtained (Fig. 1A).

2.2. Construction and verification of the virus

The recombinant plasmid (pO-TWN-R) containing P1 of O Taiwan97 virus was reacted with the restriction enzyme *Spe*1 (NEB, USA) for 24 h at 37 °C in order to divide the gene into single piece; BHK T7-9 cells (cell line where T7 RNA polymerase is expressed) were transfected with the purified plasmid DNA using lipofectamine 2000 (Invitrogen, USA) and cultivated for 2–3 days. Besides, the chimeric FMDV with O Taiwan97 P1 were harvested. Thereafter, the secured viruses were multiplied through successive passages using ZZ-R (fetal goat tongue epithelium cell) 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 cells responsible for the production of FMDVs. Sixteen hours after virus infection, the viruses were inactivated by 0.003 N of binary ethylenimine for 24 h and 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 done in the previous study (Lee et al., 2017), the final inactivated antigen (FMD viral particles) was tested using transmission electron microscopy. For the lateral flow device (LFD), a rapid diagnosis kit for FMD antigen (Princeton Biotech, USA), which can detect SP and NSP of FMDV, was used and a positive/negative result was made on the basis of the presence or absence of band formation within 15 min. after the sample application.

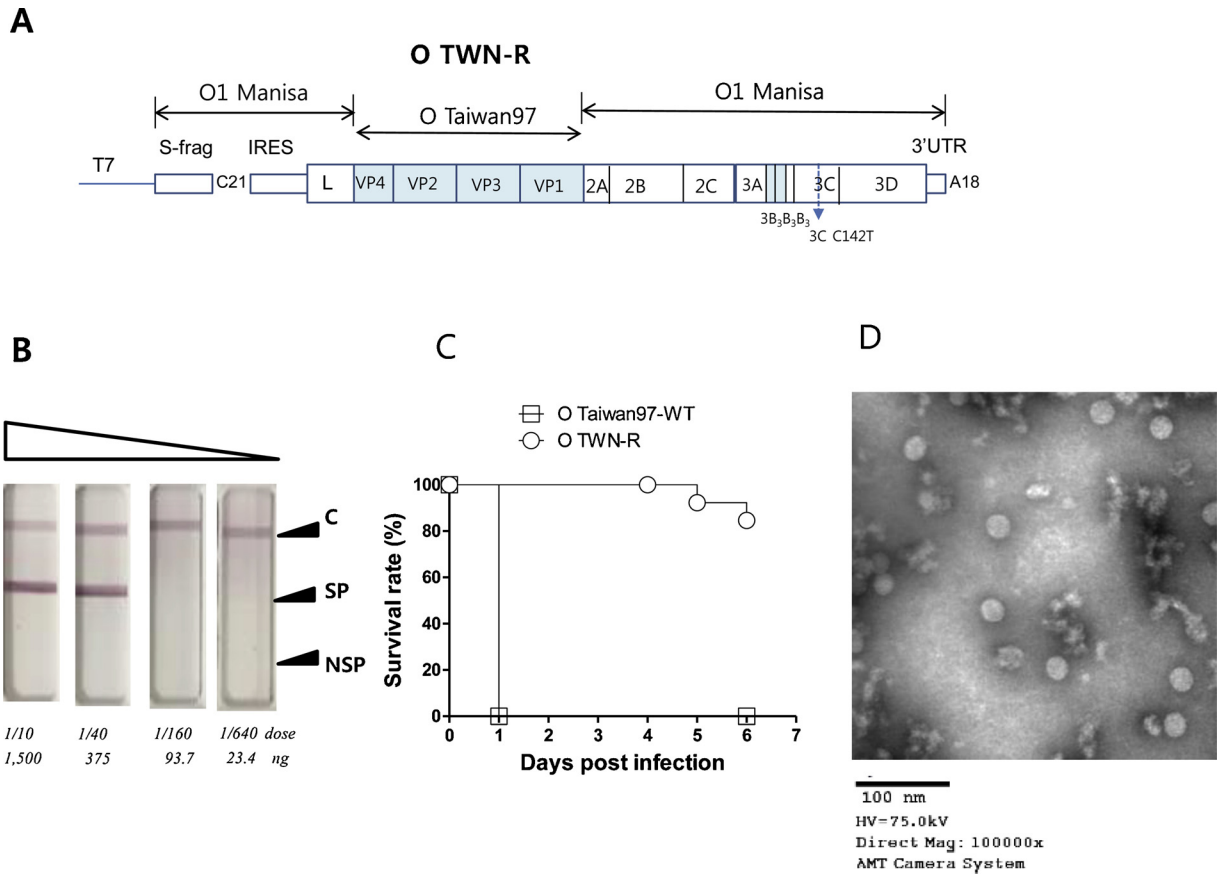

Fig. 1. Characterization of FMDV, O TWN-R.
A. Schematic diagram of type O TWN-R FMDV genome; $3B_1B_2$ was replaced using the method given by Ko et al. (2019), and mutated C142 T in the 3C region using the method given by You et al. (2019). B. No detection of the NSP antigen in the virus-cultured supernatant using FMDV antigen rapid kit (PBM, USA) for differentiation between vaccine and wild-type virus using the same method as that in (Ko et al., 2019). C: Control line, SP: FMDV structural protein line, NSP: FMDV non-structural protein line. C. Pathogenesis of O Taiwan97-wild type and O TWN-R virus in seven-day-old suckling mice. D. Electron microscopy of the FMDV vaccine strain, O TWN-R. The bar represents 100 nm.

2.3. Pathogenicity in seven-day-old mice

Seven-day-old ICR mice, supplied by the 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 Animal Care and Use Committee. A total of twenty-four ICR mice were divided into two groups (n = 12/group) and were administered O TWN-R or O Taiwan 97 wild-type virus by intraperitoneal (IP) injection with 0.1 ml of 1×10^5 $TCID_{50}$. All the mice were observed for seven days after the challenge.

2.4. Preparation of experimental vaccine

The vaccine was prepared according to the previous method (Ko et al., 2019). To briefly explain the method, 15 μg (1 dose) of purified 146S particles of inactivated O TWN-R virus for induction of strong immunity against heterologous virus and O TWN-R antigen was mixed with ISA206VG adjuvant (Seppic, Paris, France) in a ratio of 1:1 (volume [v]/v), and 10% aluminum hydroxide gel (Rehyragel® HPA; General Chemical, NJ, USA); in addition, saponin 0.5 μg was added to the mixture to prepare the vaccine in the form of water-in-oil-in-water.

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

For the immunogenicity test, twenty FMDV antibody-negative three month-old-pigs and five head of antibody-negative cattle, aged at least 6 months, were used. After the cattle were inoculated with the test vaccine, blood was collected at 0, 14, 28, 56, 84, 112, 140, and 168 days after the vaccination to measure the presence of FMDV antibodies. For pigs, blood was collected at 0, 14, 28, 42, 56, and 70 days after the vaccination. Briefly, the virus-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 Organization for Animal Health (Liang et al., 2014). Serum samples were collected from the animals after vaccination and virus challenge. The sera were heat-inactivated at 56 °C for 30 min. Following 1 h incubation at 37 °C in serial diluted sera and virus suspension, LF-BK cells were added to the microplate and incubated for a period of 2–3 days. The neutralizing antibody titers were calculated as the log10 of the reciprocal antibody dilution to neutralize 100 $TCID_{50}$ of the virus. The virus used in VNT is O TWN-R

2.6. Challenge test after vaccination in pigs

The test vaccines for the O TWN-R vaccine were prepared with 146S antigen at 15 μg by assigning four animals to each group and three 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 intradermally challenged with FMDV O/Jincheon/SKR/2014 at a titer of 10^5 $TCID_{50}$ in the bulb of the heel of the foot at 28 days after vaccination. After the virus challenge, the pigs in individual groups were separately raised; when clinical symptoms of FMD appeared, they were isolated. The oral swabs and serum were collected in the period from 0 days post-challenge (dpc) to 10 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). Further, the FMDV viral RNA was identified by extracting the viral RNA from oral swab samples and quantitative real-time 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 that used in (Lee et al., 2017). The clinical score was determined by the addition of points distributed as described below (Lee et al., 2017). The 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) lethargy (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. The NSP ELISA Kit (Bionote Kit, Republic of Korea)—an ELISA kit for the detection of FMDV non-structural protein (NSP) antibodies in serum samples of pigs—was utilized to detect NSP antibodies.

2.7. Virus detection in immunized and challenged pigs

Real-time RT-PCR was performed on sera and swab samples from the experimental animals. Swab samples were collected from the mouth and nose using cotton swabs. The total cellular RNA was extracted using the MagNa pure 96 system (Roche, Germany), by following the manufacturer's protocol. Further, the real-time RT-PCR was conducted using the one-step prime-script RT-PCR kit (Bioneer, Republic of Korea), by following the manufacturer's instructions.

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

2.8. Antibody test after immunization with the vaccine

Two methods were used for the antibody test, where type-O SP-ELISA test (Prionics, Switzerland, the specificity of ELISA is > 99%) was performed according to the manufacturer's instructions, and the virus neutralization test for O/Jincheon/SKR/2014 or O TWN-R virus was conducted according to the OIE manual for the FMD neutralization test (Ko et al., 2019).

2.9. Statistical analysis

The statistical relationships between the inoculated groups and the control group were determined. The *t*-tests and the 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. Characteristics of the FMD vaccine virus

For the FMD virus (Fig. 1A), which was constructed to express P1 of the O Taiwan97 virus as the structural protein-expressing antigen, 0.357 μg of the antigen—corresponding to approximately 1/40 dose of the purified antigen—could be detected using a simple antigen kit (Fig. 1B). All suckling mice infected with the wild-type O Taiwan97 virus died within a day, whereas the survival rate of the suckling mice infected with the O TWN-R virus was much higher, at 80%, even after six days (Fig. 1C). Further, the O TWN-R virus that expresses the Taiwan97 surface antigen was observed by electron microscopy and 25 nm FMDV particles were identified (Fig. 1D).

3.2. Immunoreactivity in cattle and pigs after vaccination

Immunoreactivity in cattle increased two weeks after immunization with the vaccine, and after four weeks, the neutralizing antibody titers were determined to be 1:100, on average. After the second inoculation, a homogenous immunity was formed, and after four weeks, the neutralizing antibody titers were determined to be very high at 1:1000 (Fig. 2A and B). Pigs showed similar results; in particular, after four weeks, the neutralizing antibody titers were determined to be very high at 1:1000. After the second inoculation, the neutralizing antibody titers were maintained at high levels, between 1:100 and 1:1000. No significant difference was observed between individual pigs (Fig. 2C and

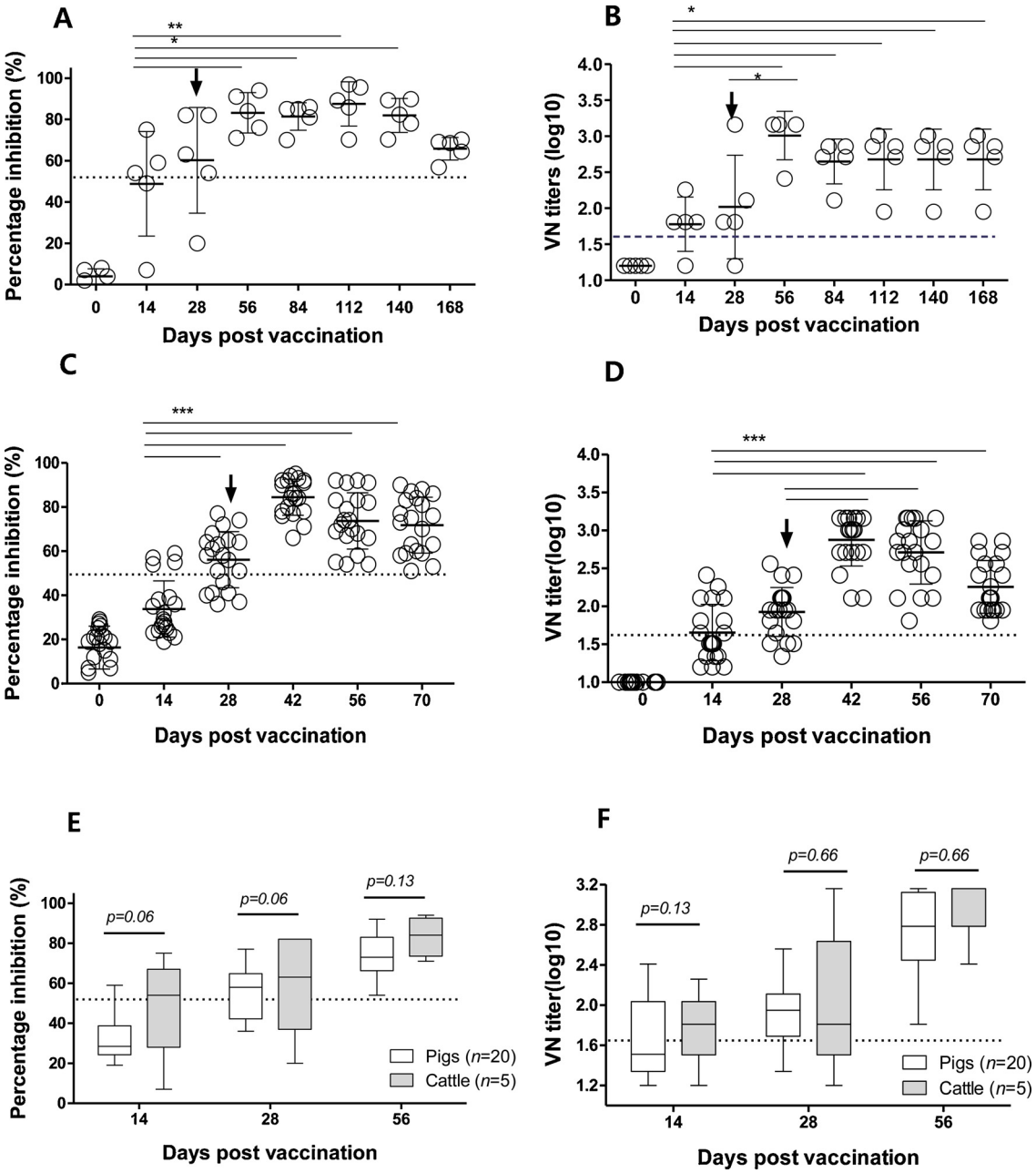

Fig. 2. Antibodies detected by virus neutralization test and ELISA in pigs and cattle vaccinated with the experimental FMD vaccine using an ISA 206 oil adjuvant. A. SP-ELISA in cattle (n = 5), B. VN titers in cattle (n = 5), C. SP-ELISA in pigs (n = 20), D. VN titers in pigs (n = 20), E. Comparison of SP-ELISA PI value in pigs and cattle. F. Comparison of VN titers in pigs and cattle. The dotted lines in SP-ELISA show 50% inhibition (PI), which would be positive in a test. The dotted lines in the VN test represent 1.65 log virus-neutralizing antibody (VN) titers (1:45). The virus used in VNT is O TWN-R. The arrows represent the second vaccination time. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

D).

3.3. Determination of protection ability in pigs through a virus challenge

Four weeks after O TWN-R vaccination, pigs were challenged with O/Jincheon/SKR/2014 virus. Similar VN titers against O TWN-R were obtained from the immunized pig group for the challenge as those for the immunity test (Fig. 2). Homogenous immunity against vaccine virus was formed only after two-three weeks of immunization, and the VN titers were determined to be in the ratio of 1:100 after three weeks (Fig. 3A). The immune response against O/Jincheon/SKR/2014 virus was formed only after four weeks of immunization, and the VN titers were very low (Fig. 3B).

When virus challenged in the fourth week after immunization, the neutralizing antibody titers were 1:160 on average, which were elevated to a higher level within seven days after the challenge (Fig. 3A). In the control group, the neutralizing antibody titers were elevated within four days, and the neutralizing antibody titers reached a similar level as that in the immunized group within seven days of the challenge.

However, the neutralizing antibody titers against the O/Jincheon/SKR/2014 virus, which was the challenge virus, were much lower compared to those against the vaccine virus. When challenged with the O/Jincheon/SKR/2014 virus, high clinical index scores of clinical symptoms began to appear after 2–3 days in the control group, peaking on the fifth day (Fig. 4). No clinical symptoms were observed in the

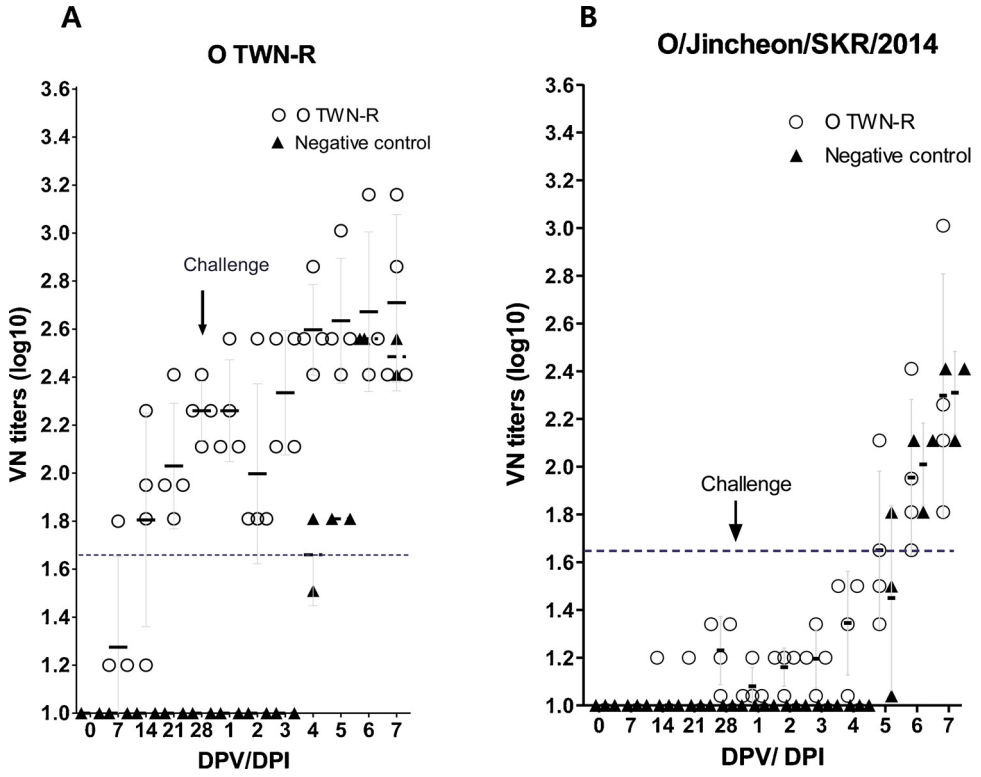

Fig. 3. Virus neutralizing antibody (VN) titers in the O TWN-R vaccine-immunized pigs after the O/Jincheon/SKR/2014 virus challenge.
A. VN titers against O TWN-R; B. VN titers against O/Jincheon/SKR/2014. The O/Jincheon/SKR/2014 virus challenge was attempted at 28 days post-vaccination

vaccinated group, although two animals (#3-4 and #4-1) exhibited low levels of virus shedding for a short period time.

4. Discussion

Type O FMDV is widely prevalent in Asia, Africa, and South America, and it is the virus serotype that causes FMD outbreaks most frequently among the seven serotypes (Mahapatra and Parida, 2018). Because the SEA topotype viruses have been circulating in East Asia for a long time, more attention is currently paid to this topotype (Park et al., 2018; Valdazo-Gonzalez et al., 2013; Zheng et al., 2012). In particular, simultaneous infections of this topotype in cattle and pigs have been identified in livestock farms; thus, the SEA topotype has been identified as a rather risky topotype in Asia, including Korea and Japan

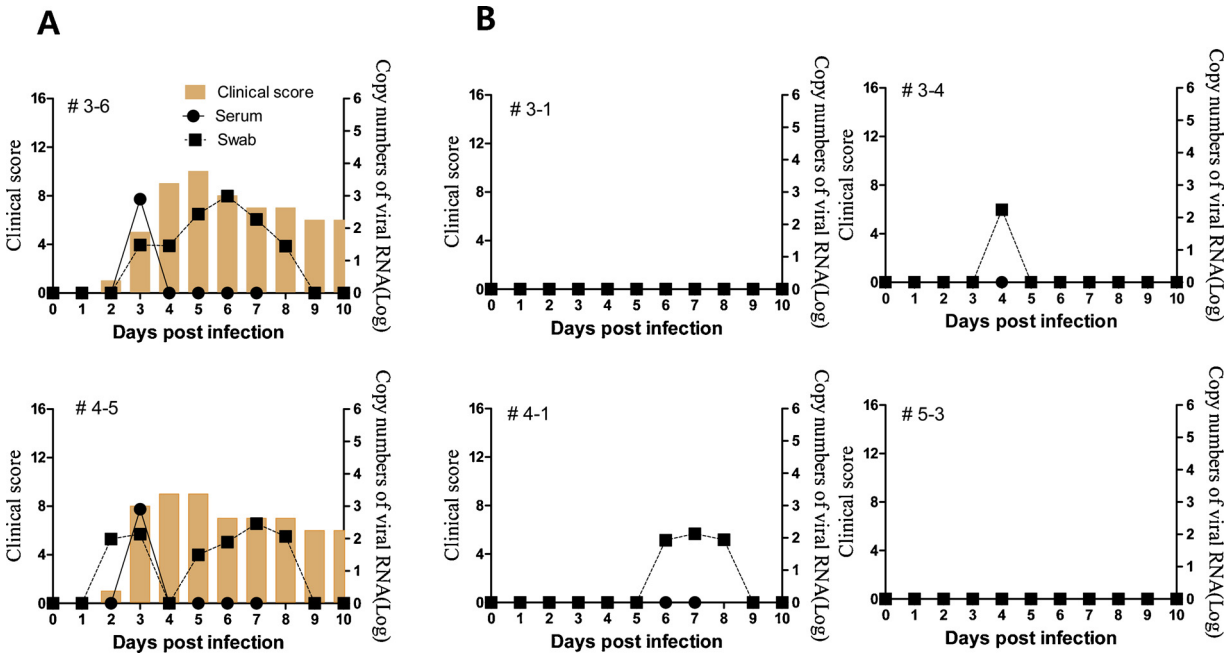

Fig. 4. Clinical scores and virus shedding in the O TWN-R vaccine-immunized pigs after the O/Jincheon/SKR/2014 virus challenge.
A. Negative control group (n = 2, #3-6, #4-5); B. Vaccinated group (n = 4, #3-1, #3-4, #4-1, #5-3). The O/Jincheon/SKR/2014 virus challenge was attempted at 28 days post-vaccination. The dotted lines in the VN test represent 1.65 log virus-neutralizing antibody (VN) titers (1:45).

(Mahapatra and Parida, 2018; Mahapatra et al., 2017; Park et al., 2018; Wada et al., 2017). In particular, in Korea, the FMD outbreak pattern has become more serious, as revealed by three FMD outbreaks for one year in 2010 alone, which led to a policy change in terms of disease prevention to the implementation of a nationwide vaccination policy (Park et al., 2013).

Nevertheless, the problem that the vaccine matching results are not available quickly and accurately when new FMD viruses are introduced has been continuously highlighted. In order to deal with this problem, numerous efforts have been made to achieve faster results, such as determination of vaccine-matching rates using vaccinnated sera obtained from cattle and pigs (Galdo Novo et al., 2017) and application of various methods, including *in vivo* and *in vitro* (Lavoria et al., 2012; Lee et al., 2016); however, research on effective methods for improvement remains underway (Park et al., 2018). Newly developed local vaccine strains could not be frequently used due to their limited protection range. It suggests a need for the development of broadly protective vaccine strains (Mahapatra and Parida, 2018; Paton et al., 2005). International vaccine strains—such as O Manisa, O Camops, O BFS, O PanAsia-2, and O Taiwan97—are known to be recommended as wide-range vaccine strains (www.wrlfmd.org). Of these, O Manisa, O Camops (O BFS), and O PanAsia-2 have been evaluated for their ability to protect against most SEA topotype strains, and it has been found that they can be used as vaccine strains (Galdo Novo et al., 2018; Mahapatra and Parida, 2018; Mahapatra et al., 2017; Park et al., 2018). On the contrary, almost no study has been conducted on the O Taiwan97 vaccine strain, although there were a few evaluations of commercial vaccines for their ability to protect against O Taiwan97 of the Cathay topotype (Chen et al., 2007, 2008; Galdo Novo et al., 2017). Therefore, in this study, a vaccine strain equivalent to the O Taiwan97 virus was developed and tested through a post-immunization challenge test in pigs using the SEA topotype virus strain that afflicted Korea in 2014. This challenge test proved the protection ability of the newly developed vaccine, although the antibody titers against the SEA Mya-98 virus strain were not that high. Similar to the vaccine strains already developed in our laboratory (Ko et al., 2019; You et al., 2019), the O TWN-R strain developed in the present study has numerous advantages in that it was improved as a vaccine strain so that it could be distinguished from the wild-type strain by replacing $3B_1B_2$ with $3B_3B_3$ in the 3B region in the source virus in consideration of the potential problem of external leaking of this vaccine virus strain and the fact that it has low virulence. 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. It was evaluated that C142 T gave influence to the activity of $3C^{pro}$ determining a pathogenic property of virus (You et al., 2019). The *in vitro* vaccine-matching results revealed that the matching rate of the O Taiwan97 vaccine with the SEA topotype was not so high (Mahapatra and Parida, 2018; Park et al., 2018). In the previous study, the pigs protected against challenge with O Taiwan97 after vaccination with O Manisa, despite the relatively low r1 value (Eblé et al., 2006). Although antibodies are considered to be the most important element in the protective response against the heterologous virus, cell-mediated responses including production of cytokines are also induced by FMD vaccination. Cross-reactive lymphocyte proliferation after vaccination is caused by recognition of conserved epitopes within or even between serotypes (Eblé et al., 2006). Also, in this study, we had the results of complete protection with low antibody production against the heterologous virus in the pigs like the previous study. Nevertheless, the present study revealed that the immunogenicity of the O TWN-R strain was sufficiently high to stimulate higher levels of antibody formation in pigs than the levels of antibody titers in cattle and also induced homogenized immunoreactivity in pigs. These advantages indicated that a high protection ability of a vaccine strain can be achieved by increasing antibody titers even if the matching rate is relatively low, which is a unique feature of this vaccine as compared to other vaccine strains.

In conclusion, this study showed that the O TWN-R vaccine strain completely protected the SEA topotype virus challenge in pigs, and its immunogenicity was considered satisfactory in cattle and pigs based on the initial antibody induction levels and antibody maintenance. Overall, this vaccine strain was evaluated as a vaccine that was capable of providing sufficient protection against the SEA topotype virus.

Funding

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

Acknowledgments

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

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