Foot-and-mouth disease (FMD) is a most contagious livestock disease and causes tremendous economic losses in cloven-hoofed animals. FMD outbreaks are devastating to the livestock industry because of their high rates of morbidity and rapid transmission across a broad range of susceptible animal species. FMD is characterized by fever, lameness, and vesicular lesions on the tongue, feet, snout, and teats [1]. The causative agent is FMD virus (FMDV), which is a non-enveloped, single-stranded, positive-sense RNA virus with a genome size of 8,400 nucleotides (nt). The virus belongs to the genus Aphthovirus within family Picornaviridae and is further classified into seven serotypes (O, A, Asia1, SAT1, SAT2, SAT3, and C).

Tremendous economic loss by FMD drives the requirement of an effective strategies to control FMDV and eventually to achieve FMD-free statue. Control and prevention of FMD outbreaks can be achieved by various control measures that can reduce a basic reproduction number of FMDV [2]. Control programs include (1) removal sources of infection (pre-emptive culling, slaughtering), (2) preventing contact between infected and susceptible animals (restriction of animal and instrument movement), and
(3) reduction in the proportion of susceptible animals (vaccination). Vaccination has been widely used to eradicate and control FMD due to its high effectiveness, especially when it is implemented together with other preventive tools [3].

In Korea, massive FMD outbreak in 2010 led to the decision on the use of nationwide vaccination with emergency monovalent (O) vaccine. Nevertheless, this disastrous outbreak resulted in an economic damage at US $3 billion, which include culling 150 thousands of culled cattle and 3 millions of pigs [4]. After outbreak ended in 2011, the government maintained the vaccination policy changing to trivalent (O, A, and Asia1) vaccine. However, the mandatory vaccination in all susceptible animals did not prevent the reoccurrence of FMD outbreak caused by serotype O in 2014/2015. This outbreak was characterized by nationwide spread of FMDV over 50% geographical provinces and most FMDV infection predominantly in pig farms (180 pig farms and five cattle farms), as officially reported by the World Organization for Animal Health (OIE; http://www.oie.int/wahis_2/public/wahid.php/Review-report/Review/viewsummary?reportid=16695). These facts raised a question on the efficacy of commercial FMDV vaccine in pigs. In addition, currently available research data on FMDV vaccine in pigs remains limited because a majority of FMDV research and FMD control programs have exclusively focused on use of vaccine in cattle. Therefore, the aim of this study was to evaluate the molecular characteristics of commercial FMDV vaccine and serological response in pigs.

A commercially available FMDV vaccine (Aftopor, Merial Animal Health Ltd., Pirbright, UK) was used in this study. The vaccine was labeled as a high-potency, trivalent, inactivated vaccine with an oil-based adjuvant. The antigen content of vaccine was labeled as more than 6 protective dose (PD50) of the O, A, and Asia 1 strains.

To identify and quantify each vaccine strains, reverse-transcription polymerase chain reaction and quantitative polymerase chain reaction (qPCR) were performed. Because oil-based, inactivated vaccines contain various surfactants and additives that could have a quenching effect on further processing, instability of the vaccine was induced for the phase separation by physical method. The vaccine was frozen at -70°C for 24 hours and then thawed to room temperature for 30 minutes. After the centrifugation at 3,000×g for 10 minutes, aqua and turbid phases were collected.

RNA of the FMDV vaccine was extracted using the Viral Gene-spin Virus DNA/RNA Extraction Kit (iNtRON Biotech).

### Table 1. Primer and probe sets used in this study

| Name                                      | Orientation | Sequence (5’-3’)                                             | Size (bp) |
|-------------------------------------------|-------------|---------------------------------------------------------------|-----------|
| **Primers for identification of inactivated FMD antigen** |             |                                                              |           |
| O seq F                                   | Forward     | GACAGATTTGTGAAAGATGACACCA                                     | 434       |
| O seq R                                   | Reverse     | TTCATGC GGTTAAACACAGTGCA                                      | 404       |
| A seq F                                   | Forward     | GCAGGCCACCTACACTCTTC                                            |           |
| A seq R                                   | Reverse     | TGTGTGTCTCTGTCTGCGAC                                           |           |
| Asia 1 seq F                              | Forward     | ACTTCTGTCTGCGACGCTTACT                                         | 439       |
| Asia 1 seq R                              | Reverse     | CAAAACCTGCCCTTCTCAGGTC                                         |           |
| **Primers for quantification of inactivated FMD antigen** |             |                                                              |           |
| SA-IR-219-246F                            | Forward     | CACYTYAAGRTGACAYTGRACTGATGAC                                   | 97        |
| SA-IR-315-293R                            | Reverse     | CAGATYCCRAGTGWCICITGTTA                                       |           |
| SAmuti2P-IR-292-269R                      | Forward     | FAM - CCTGGGCTACCTGAGCGCATCC - TAMRA                          | 108       |
| O quanti                                  | Forward     | CACCAACCCACAGCTTACC                                            |           |
| O quanti R                                | Reverse     | CCATACCTGGCTGTCCCAGTTG                                        |           |
| O quanti Probe                            | Forward     | FAM - CGA CCTGGCTGTCCCAGGTCAC - TAMRA                         | 127       |
| A quanti                                  | Forward     | CACAGCTCCTGTGCTTCTCA                                           |           |
| A quanti R                                | Reverse     | TTGGCACAACACCTGCCACTG                                          |           |
| A quanti Probe                            | Forward     | FAM - CTACTGCCCAGGCTGAC - TAMRA                               |           |
| Asia 1 quanti F                           | Forward     | GCAGTWAAGGSCYGAGGACATYAC                                       | 75        |
| Asia 1 quanti R                           | Reverse     | GCARAGGCTCGAGGACGATG                                           |           |
| Asia 1 quanti                             | Forward     | FAM - AGCTTTTGATC CGCATGAAGCSYGGCG - TAMRA                     |           |

FMD, foot-and-mouth disease.
nology Inc., Seongnam, Korea) according to the manufacturer’s instruction. cDNA was synthesized with the SuPrimeScript RT Premix (GenetBio, Daejeon, Korea) using step-specific reverse primers (Table 1).

Amplification efficiency ($E$) of the untreated vaccine, aqua and turbid phases were calculated to estimate gene recovery. cDNA was five-fold diluted with distilled water ($5^0$ to $5^{-5}$). Diluted cDNA was mixed with pan-serotypic primers and probes targeting FMDV internal ribosomal entry site [5] in order to perform qPCR using the iQ Supermix (Bio-Rad, Hercules, CA, USA) in a Bio-Rad Chromo Real-Time PCR system (Bio-Rad). Each experiment was conducted in quadruplicate to calculate the amplification curves and efficiencies ($E=10^{1/slope}$ -1) using slopes of the regression lines [6,7]. The most efficiently amplified phases were used for identification and quantification of antigen content in vaccines.

After cDNA of each strain (O, A, Asia 1) was synthesized using serotype-specific reverse primers, conventional polymerase chain reaction (PCR) was performed using serotype-specific primers (Table 1) targeting the partial VP1 gene using the HS Prime Taq DNA polymerase (GenetBio). PCR products were electrophoresed on a 1.5% agarose gel and each target band was extracted by Dokdo-Prep Gel Extraction Spin-type Kit (ELPIS BIOTECH Inc., Daejeon, Korea). PCR products were sequenced on both strands at the commercial sequencing facility (Macrogen Inc., Seoul, Korea).

Based on the published FMDV nucleotide sequences, primers and probes targeting the VP1 gene were designed. With the strain-specific primer and probe sets, qPCR was conducted to quantify the amount of FMDV antigens in vaccine. Specifically, because of the epidemiologic importance of serotype O in South Korea, the amplicon was inserted into a pGEM-T vector (Promega Corp., Madison, WI, USA) as an internal amplification control. The vector was then transformed into a chemically competent *Escherichia coli* DH5α cell (iNtRON Biotechnology Inc.). With a 10-fold serial dilution of plasmid DNA ($10^0$ to $10^{-5}$), standard curve analysis was performed, and the nucleotide copy number was calculated.

Ten 8-week-old pigs which were NSP enzyme-linked immunosorbent assay (ELISA) negative for FMDV were used to evaluate the immune response of commercially available FMDV vaccine under the field conditions. According to the manufacturer’s instructions, 2.0 mL of vaccine was administered intramuscularly and booster vaccination was performed after two weeks. Blood samples were collected at 0-, 2-, 4-, and 6-week post-vaccination (wpv). ELISA was carried out on the samples to detect antibodies against structural proteins of serotype O (PrioCHECK FMDV type O ELISA, Prionics, Lelystad, Netherlands) and nonstructural proteins (VDPro FMDV NSP AB ELISA, Median Diagnostics Inc., Chuncheon, Korea) according to manufacturer’s instructions. Positive inhibition (PI) values above 50% as calculated by ELISA were considered serological positives.

Physical method (freezing, thawing, and centrifugation) successfully separated vaccine into two phases (aqua and turbid phase). To select the suitable phase for the molecular analysis, amplification efficiency of two phases and vaccine stored in 4°C were compared. As expected, inactivated FMDV vaccine in recommended storage temperature (4°C) was not suitable to successfully amplify FMDV-specific genetic materials ($E$, 58.05%). The results indicated that the efficiency of aqua phase was higher than that of turbid phase, implying that aqua phase was appropriate for molecular analysis. Coefficient of determination ($r^2$) and $E$ of the amplification curves were summarized in Table 2.

The partial VP1 gene was successfully amplified from the selected phases of oil-based vaccine, and these results revealed the presence of three different serotypes (O, A, and Asia 1) in vaccine. Sequencing analysis indicated that the partial nucleotide sequence of VP1 showed 99.4% identity with O1 Manisa strain (accession no. AY593823) for serotype O, 98.0% identity with A Malaysia 97 strain (accession no. KJ933864) for serotype A, and 100% identity with Asia 1 Shamir strain (accession no. JF739177) for serotype Asia 1. Mutations in serotype O resulted in one nonsynonymous and one synonymous substitution (Fig. 1). For A Malaysia 97 strain, seven nucleotide mutations were identified, and resulted in four non-synonymous mutations and three synonymous mutations.

Quantification using qPCR results showed that quantification cycle (Cq) values of three different serotypes in the inactivated FMDV vaccine were as following: serotype O, 18.38±0.06; serotype A, 20.86±0.2; and serotype Asia 1, 18.25±0.05. These
amplification efficiencies were 97.51% for serotype O, 91.53% for serotype A and 91.25% for serotype Asia 1. Based on standard curve analysis of serotype O antigen, nucleotide copy number of antigen in aqua phase was estimated as 9.63±0.01 (log_{10}).

To evaluate antibody response against structural protein of serotype O after vaccination, blocking ELISA was conducted. The mean PI value and antibody positive rate for structural protein of serotype O were as follows: 21.24±3.72% and 0% at 0 wpv, 49.54±8.73% and 40% at 2 wpv, 58.37±6.43% and 60% at 4 wpv, and 62.92±6.05% and 90% at 6 wpv. Both PI value and antibody positive rate gradually increased and reached an acceptable level (over 80% seroprevalence) for herd immunity at 6 wpv during the experiment period (Fig. 2) [8,9].

The results of the NSP ELISA tests were negative in samples from all experimental animals over the entire study period (data not shown).

FMD is a highly contagious, viral diseases of cloven-footed mammals with significant economic impact. Vaccination has been one of a most frequently used tool to control and prevent FMD outbreak because extensive use of vaccine efficiently eradicated FMD from Europe and some of South America. However, the extensive vaccination could not prevent the continuous FMD outbreaks in some Asian countries, especially China and South Korea, where pig is one of the major industrial animals. In addition, field situation in Korea showed...
that vaccination induced significantly poor antibody response in pigs than in cattle [10]. This circumstance has raise a ques-
tion whether commercial FMD vaccine is really effective to
to control and prevent FMD outbreak in pigs. In this study, in
order to exclude vaccine formulation-related factors for vac-
cine failure [3], we conducted the identification and quantifi-
cation of vaccine strains in post-manufactured commercial vaccine and then evaluated immune response in the field.

Selection of vaccine strain plays a critical role in successful
protection against FMDV [11]. It is therefore necessary to ver-
ify the presence of selected vaccine strains in post-manufac-
tured vaccines because of possibility of unintentionally con-
tamination of different strains and/or deliberately alteration
of vaccine strains. In addition, it is possible that a deletion
may occur at a coding gene of critical antigen-binding site in
the viral capsid during the production process [12]. Despite
the inactivation of FMDV antigen with binary ethyleneimine
that strongly reduce the amount of intact viral genome, we
successfully amplified partial VP1 gene of three different se-
rotypes in oil emulsified vaccine through phase separation
caused by freezing-thawing method. The result indicates that
one vaccine strains in commercial FMDV vaccine shared 100%
nucleotide identity to sequences deposited in GenBank, but
nucleotide mutations of vaccine strain of serotype O and Asia
1 resulted in some nonsynonymous substitutions in VP1 pro-
tein, which contains several antigenic determinants and par-
ticipates in viral pathogenesis. Although the effect of these
mutations on efficacy of FMDV vaccine was not mentioned
in this study, continuous quality control is needed to produce
a consistent level of immune responses.

The potency is defined as the concentration of the immu-
nologically active component, and normally represented as
50% cattle PD50. For emergency vaccine, more than 6 PD50 is
recommended to induce the rapid protective immunity against
a wide range of FMDV strains. However, because a potency
test method has been standardized in cattle, the protective
dose does not guarantee the efficacy of FMDV vaccine against
field strains of FMDV in pigs. In this study, we approached
the quantification of antigen payload using serotype-specific
quantitative PCR. The results showed low Cq values of three
different serotypes, which implied relatively high amounts of
antigen payload. However, we could not convert Cq values of
qPCR into infectious unit nor amount of the antigen. Instead,
absolute quantification using standard curve method could
determine viral copy number of serotype O antigen in inacti-
vated vaccine. Previously many researchers have attempted
to measure and quantify an immunogenic component, whole
virus particle (146S), in inactivated FMD vaccines using mono-
clonal antibodies [13] and chromatography [14]. Although
the integrity of the 146S particle was not evaluated in this study,
our approach is a rapid and convenient method to measure
the quantity of each antigen in multivalent FMD vaccine. Im-
proved qPCR-based techniques for antigen quantification
would be needed to determine antigen payload objectively.

It has been showed that high potency vaccine for emer-
gency use could clinically protect pigs against homologous
FMDV strain and reduce virus excretion and transmission
[15]. However, in the experimental studies on heterologous
challenge, O Manisa-based emergency vaccine partially pro-
tect clinical manifestations, and in a case, the virus excretions
were not decreased significantly in pigs [16,17]. Moreover,
our data showed that commercial oil-based vaccine induced
delayed immune response in the field, suggesting a possible
immunity gap in vaccinated pigs. These facts strongly sup-
ported a question whether current FMDV vaccine is still ef-
efective to control FMD outbreak. In this study, we identified
poor immune response against FMD in the field when pigs
were administrated twice with emergency vaccine that con-
tained high antigen payload of same serotype with similar
-genetic identity and with formulation as double-water emul-
sion (data not shown). Therefore, there is an urgent need for
an FMD vaccine to elicit a suitable immune response in pigs
as well as a standard protocol to evaluate FMD vaccine-in-
duced protective immunity in pigs.

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