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Assessment of the safety and efficacy of an attenuated live vaccine based on highly virulent genotype 2b porcine epidemic diarrhea virus in nursing piglets

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ABSTRACT

We have previously reported the generation of the attenuated KNU-141112-S DEL5/ORF3 virus by continuous propagation of highly virulent G2b porcine epidemic diarrhea virus (PEDV) in Vero cells. The present study aimed to assess the safety of S DEL5/ORF3 and to evaluate its effectiveness as a live vaccine for prime-booster vaccinations. Reversion to virulence experiments revealed that the S DEL5/ORF3 strain retains its attenuated phenotype and genetic stability after five successive passages in susceptible piglets. Pregnant sows were primed orally with an S DEL5/ORF3 live vaccine and boosted intramuscularly twice with a commercial killed vaccine at 2-week intervals prior to parturition. This sow vaccination regimen completely protected nursing piglets against virulent G2b challenge, as evidenced by the increase in survival rate from 0% to 100% and the significant reduction in diarrhea intensity, including the amount and duration of PEDV fecal shedding. In addition, despite a 2–3 day period of weight loss in piglets from vaccinated sows after challenge, their daily weight gain was recovered at 7 days post-challenge and became similar to that of unchallenged pigs from unvaccinated sows over the course of the experiment. Furthermore, strong antibody responses to PEDV were verified in the sera and colostrum of immunized sows with the prime-boost treatment and their offspring. Altogether, our data demonstrated that the attenuated S DEL5/ORF3 strain guarantees the safety to host animals with no reversion to virulence and is suitable as an effective primary live vaccine providing durable maternal lactogenic immunity for passive piglet protection.

1. Introduction

Porcine epidemic diarrhea (PED) is a highly epizootic and fetal swine disease that causes watery diarrhea, vomiting, severe dehydration, and significant mortality rates in newborn piglets (Lee, 2015, 2019; Saif et al., 2012). PED virus (PEDV), a member of the genus *Alphacoronavirus* within the family *Coronaviridae* of the order *Nidovirales*, is the causative agent of this disease (Gorbalenya et al., 2006; Lee, 2015). PEDV is a large, enveloped virus containing a single-stranded positive-sense RNA genome of approximately 28 kb in length with a 5′ cap and a 3′ polyadenylated tail (Kocherhans et al., 2001; Saif et al., 2012). The PEDV genome is composed of seven canonical genes, including the open reading frame (ORF) 3, flanked by 5′- and 3′- untranslated regions (UTRs) (Kocherhans et al., 2001). The first two large ORFs, ORF1a and ORF1b, encode replicase polyproteins (pp) 1a and 1ab, which are later proteolytically processed into 16 mature nonstructural proteins (nsp1–16). The remaining genes encode four canonical coronavirus structural proteins, including spike (S), membrane (M), envelope (E), and nucleocapsid (N), and a single accessory gene, ORF3 (Duarte et al., 1994; Lai et al., 2007; Lee, 2015). PEDV can be phylogenetically divided into two genotypes with two sub-genotypes: genogroup 1 (G1a and G1b; classical and recombinant, respectively, with low pathogenicity) and genogroup 2 (G2a and G2b; field epidemic and pandemic, respectively, with high pathogenicity) (Lee, 2015, 2019;

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Lee et al., 2010; Lee and Lee, 2014; Oh et al., 2014).

Although PED epizootics have been reported in Europe and Asia, the most serious outbreaks have mainly occurred in Asian swine-producing countries over the past two decades. However, in early 2013, PED first emerged in the United States (US) and rapidly spread nationwide and to North and South American countries, sustaining tremendous damage to pig health and the pork industry (Mole, 2013; Stevenson et al., 2013; Vlasova et al., 2014). Subsequently, highly virulent US prototype-like highly virulent G2b PEDV strains almost concurrently struck several Asian nations, including South Korea, Japan, and Taiwan, resulting in the recurrence of massive PED epidemics (Lee and Lee, 2014; Lin et al., 2014; Suzuki et al., 2015). Furthermore, the independent re-emergence of recombinant G1b or pandemic G2b PEDV in multiple countries in western and central Europe since 2014 has been reported (Boniotti et al., 2016; Dastjerdi et al., 2015; Grasland et al., 2015; Hanke et al., 2015; Mesquita et al., 2015; Steinrigl et al., 2015; Theuns et al., 2015). As a result of these pandemics, PEDV has gained international attention as a porcine coronavirus that represents a considerable financial threat to the global pork business.

PED has been considered one of the most economically serious swine diseases in South Korea since the first epizootic in 1992 (Kweon et al., 1993). Although all four su**b-**genotypes of PEDV have been reported in South Korea, the current dominant epidemic strains are mostly of the G2b su**b-**genotype, with frequent moderate-scale regional outbreaks occurring across the nation since the 2013–2014 PED re-emergence (Lee, 2015; Lee et al., 2014, 2015; Lee and Lee, 2014, 2017, 2018. Due to incomplete cross-protection between two genetic clusters, classical G1a PEDV vaccines have failed to defend against recent virulent strains, and thus new G2**b-**based vaccines have been developed and commercialized in South Korea. However, only inactivated/killed vaccines against G2b PEDV, which may be incapable of effectively controlling chronic PEDV enzootics, are commercially available in the domestic market. In our previous study, the double-dose immunization of pregnant sows with inactivated G2b vaccines conferred important passive protection to their litters (Baek et al., 2016). However, this study pointed out potential drawbacks of killed vaccines against PED in their effectiveness that does not fully mitigate mortality and morbidity rates of nursing piglets, probably owing to scant lactogenic immunity. Furthermore, since it is acknowledged that the vaccine administration route is one of the critical factors in stimulating optimum mucosal immunity in sows, the effects of parenteral vaccination with inactivated vaccines in PEDV-naïve sows on immune responses and preweaning mortality may be limited (Crawford et al., 2016; Langel et al., 2016; Schwartz et al., 2016). It has also been found that the oral administration of live attenuated vaccines can induce a sufficient lactogenic immune response against PEDV in naïve pigs and is thus most effective for PEDV-naïve sows (Chattha et al., 2015; Langel et al., 2016). Considering these issues, there is an urgent demand for the invention of a next-generation modified live virus (MLV) vaccine that can replicate in the gut and provide the stimulation required to induce protective mucosal immunity without leading to clinical disease.

Previously, we produced KNU-141112-S DEL5/ORF3 (S DEL5/ORF3), a genetically stable, fully attenuated PEDV G2b strain that retains its immunogenicity, using traditional cell culture adaptation procedures (Lee et al., 2017). In the present study, we assessed the phenotypic and genotypic stability of the attenuated S DEL5/ORF3 virus as a candidate vaccine strain using reversion to virulence experiments in highly susceptible neonatal piglets. In addition, the efficacy of S DEL5/ORF3 as an MLV vaccine was evaluated using a live prime-killed boost vaccination approach. Our results demonstrated that this vaccine provides safe and complete protection against challenge with highly virulent PEDV and can serve as a primary vaccine in prime-booster maternal vaccination strategies to prevent PEDV epidemics and endemics in herds.

2. Materials and methods

2.1. Cells and viruses

Vero cells (ATCC CCL-81) were cultured in alpha-minimum essential medium (α-MEM; Invitrogen, Carlsbad, CA) with 5% fetal bovine serum (FBS; Invitrogen) and antibiotic-antimycotic solution (100×; Invitrogen) and maintained at 37 °C in a humidified 5% CO_2 incubator. The highly virulent Korean PEDV G2b strain KOR/KNU-141112/2014 was isolated and propagated in Vero cells as described previously (Lee et al., 2015). Viral stock from fifth passage cell culture (KNU-141112-P5) was prepared and used as the challenge virus in this study. The fully attenuated S DEL5/ORF3 strain was cultivated in Vero cells as described previously (Lee et al., 2017) and prepared as the live vaccine stock (T317PEDL01; $10^{5.0}$ $TCID_{50}$/ml) according to instructions from ChoongAng Vaccine Laboratories (CAVAC; Daejeon, South Korea).

2.2. Animal infection experiments and clinical examinations

The *in vivo* swine studies described here were performed at the ChoongAng Vaccine Laboratory Animal Facility under the guidelines established by its Institutional Animal Care and Use Committee. All animals were obtained from a conventional breeding farm with a good health record and either vaccination against PEDV or no known prior PED outbreak and were also tested to confirm that they were not infected with any porcine enteric viruses.

Conventional five-passage reversion to virulence tests were performed using a total of 24 suckling piglets (3 days old at the start of the experiment) from commercial crossbred sows (Great Yorkshire × Dutch Landrace). The experimental protocol consisting of passaging the virus in the piglets in accordance with the World Organization for Animal Health (OIE)'s recommendations, as described in the Manual of Diagnostic Test and Vaccines for Terrestrial Animals (OIE, 2012), with minor modifications. Briefly, four animals were assigned to each reversion to virulence experiment, except for the final passage, which included eight piglets. The animals were fed commercial milk replacer 3–4 times daily and had *ad libitum* access to water for the duration of the study. Following a 2-day acclimation period, a group of four piglets (now 5 days old) initially received an oral 1-ml dose of $10^{6.5}$ $TCID_{50}$/ml S DEL5/ORF3 (Lee et al., 2017). Piglets were observed for 5 days post-inoculation (dpi), with daily recording of clinical signs of vomiting, diarrhea, and mortality. Stool samples were collected prior to inoculation and daily afterward using 16-inch cotton-tipped swabs and subjected to RT-PCR using an *i*-TGE/PED Detection Kit (iNtRON Biotechnology, Seongnam, South Korea) and to real-time RT-PCR to detect the presence of PEDV shedding, as described previously (Lee et al., 2015, 2017). All piglets were euthanized at 5 dpi for post-mortem examination, except for four animals from the 5th passage, which were euthanized at 21 dpi. Fecal and small intestine (SI) tissue samples (collected at 3–5 dpi and during the necropsy, respectively) were used to prepare a 10% w/v homogenate suspension for subsequent inoculation as described previously (Lee et al., 2015, 2017; Lee and Lee, 2018). Unless otherwise indicated, 1-milliliter doses of fecal and SI suspension with the maximum titer from all four euthanized piglets were used to orally inoculate a new group of piglets. This procedure was repeated 5 times in total (i.e., 5 back passages).

Swine vaccination and challenge experiments were conducted with a total of 6 commercial crossbred sows (Great Yorkshire × Dutch Landrace) with the same parity and expected farrowing date. Pigs were divided randomly into three experimental groups: vaccinated group 1 (n = 3; S1, S2, and S3), challenge control group 2 (n = 2; S4 and S5), and strict negative control group 3 (n = 1; S6). A live prime-killed/killed boost (L/K/K) multiple-dose PED vaccination schedule at 2-week intervals starting prior to farrowing was applied. The three sows in group 1 were orally administered once with 2 ml of a live vaccine and were then intramuscularly (IM) boosted twice with a commercial

inactivated G2b PEDV vaccine (ISU46065IA13 strain, PED-X®, CAVAC) at 6, 4, and 2 weeks pre-partum, respectively. The remaining sows in groups 2 and 3 were unvaccinated and served as controls. Stool samples were collected at days 0, 3, 5, 7, and 14 after oral primary immunization with the live vaccine and analyzed with real-time RT-PCR to detect viral shedding and used as an inoculum for the challenge experiment using PEDV-naïve piglets. All sows were monitored daily for clinical changes and adverse effects following vaccination. To mimic the field conditions for nursing piglets, all sows were allowed to farrow naturally and nurse their piglets freely for the duration of the study. After removing the atrophic and weak newborn piglets from each litter at farrowing, 10 healthy piglets per sow were allocated to each group. Ten 3-day-old suckling piglets per litter (a total of 50 newborn piglets) in groups 1 and 2 were challenged orally with a 1-ml dose of $10^{2.7}$ $TCID_{50}$/ml KNU-141112-P5 virus, which was equivalent to a 100 median pig diarrhea dose (PDD_{50}) (Lee and Lee, 2015; Liu et al., 2015). The sham-inoculated piglets from group 3 were administered with cell culture media as a placebo. Animals were monitored daily for clinical signs of vomiting, diarrhea, and mortality throughout the study. Fecal specimens from all groups were collected and examined for PEDV shedding as described above. A clinical significance score (CSS) for diarrheal severity was determined using the following scoring criteria based on a visual examination for 21 days post-challenge (dpc): 0 = normal and no diarrhea (mean cycle threshold [Ct] values of > 45); 1 = mild and fluidic feces; 2 = moderate watery diarrhea; 3 = severe watery and projectile diarrhea (mean Ct values of < 20); 4 = death. Blood samples were collected from sows before (during each vaccination), at, and after farrowing (up to 4 weeks) and also from 5 representative piglets per litter from each dam at 0, 7, 14, and 21 dpc. Colostrum was collected on the day of farrowing, and the piglets were weighed daily until the end of the experiment. Piglets that died after challenge during the study were necropsied upon their death, while the surviving pigs were euthanized at 21 dpc for post-mortem examination, except for six piglets from each of groups 1 and 3, which were kept alive for an additional four weeks and weighed daily to measure their average postweaning daily weight gain.

2.3. Quantitative real-time RT-PCR

Viral RNA was extracted from fecal samples prepared as described above using an *i*-TGE/PED Detection Kit according to the manufacturer's protocol. PEDV S gene-based quantitative real-time RT-PCR was performed using a One Step SYBR PrimeScript RT-PCR Kit (TaKaRa, Otsu, Japan) and primers (forward primer 5′-ACGTCCCTTT ACTTTCAATTCACA-3′, reverse primer 5′-TATACTTGGTACACACATCC AGAGTCA-3′) and a probe (5′-FAM-TGAGTTGATTACTGGCACGCCTA AACCAC-BHQ1-3′) described elsewhere (Kim et al., 2007; Lee et al., 2017; Sagong and Lee, 2011). The reaction was performed using a Thermal Cycler Dice Real Time System (TaKaRa) according to the manufacturer's protocol under the following conditions: 1 cycle of 45 °C for 30 min, 1 cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The results were analyzed using the system software as described previously (Lee et al., 2017; Sagong and Lee, 2011). A PEDV isolate with a known infectivity titer was 10-fold serially diluted to generate a standard curve for each PCR plate. The virus concentrations (genomic copies/ml) in the samples were calculated based on this standard curve. The mean Ct values were calculated based on PCR positive samples, and the mean virus titers were calculated based on all pigs within the group.

2.4. Nucleotide sequence analysis

The structural protein gene sequences of PEDV isolates from each reversion to virulence experiment were determined using the traditional Sanger method. Four overlapping cDNA fragments spanning the entire structural protein-coding region (S-ORF3-E-M-N) of each isolate were amplified by RT-PCR as previously described (Lee et al., 2017; Lee and Lee, 2018). The individual cDNA amplicons were gel-purified, cloned using the pGEM-T Easy Vector System (Promega, Madison, WI), and sequenced in both directions using two commercial vector-specific T7 and SP6 primers and gene-specific primers. In addition, the complete genome of PEDV collected from the final back passage experiment was also sequenced. Ten overlapping cDNA fragments spanning the whole genome were RT-PCR-amplified as described previously (Lee and Lee, 2014; Lee et al., 2015, 2017; Lee and Lee, 2018) and each PCR product was sequenced as described above. The 5′ and 3′ ends of the genome of the isolate were determined by the rapid amplification of cDNA ends (RACE) as described previously (Lee and Lee, 2013).

2.5. Histopathology and immunohistochemistry of the small intestines

Intestinal tissue and other major organs were grossly examined during a necropsy. Small intestinal tissue specimens (< 3 mm thick) collected from each piglet were fixed with 10% formalin for 24 h at room temperature (RT) and embedded in paraffin according to standard laboratory procedures. The formalin-fixed paraffin-embedded tissue samples were cut in 5–8-μm thick sections on a microtome (Leica, Wetzlar, Germany), floated in a 40 °C water bath containing distilled water, and transferred to glass slides. The tissue was then deparaffinized in xylene for 5 min and washed in decreasing concentrations of ethanol (100%, 95%, 90%, 80%, and 70%) for 3 min each. The deparaffinized intestinal tissue sections were stained with hematoxylin and eosin (H&E; Sigma, St. Louis, MO) to observe histopathological changes or were analyzed with immunohistochemistry (IHC) for the detection of PEDV antigens using a monoclonal antibody specific to PEDV N protein (CAVAC) as described previously (Lee et al., 2015, 2017). Villous height and crypt depth were also measured throughout the H&E-stained jejunal sections and the mean ratio of jejunal villous height to crypt depth (VH:CD) was calculated as described previously (Jung et al., 2006).

2.6. Virus neutralization

The presence of PEDV-specific neutralizing antibodies in serum and colostrum samples collected from the pigs in all groups was determined using serum neutralization (SN) tests in 96-well microtiter plates with PEDV isolate KNU-141112 as previously described (Oh et al., 2014), with minor modifications. Briefly, Vero cells (2×10^4/well) were grown in 96-well tissue culture plates for 24 h. KNU-141112-P5 virus stock was diluted in serum-free α-MEM to produce 200 $TCID_{50}$ in a 50-μl volume. The diluted virus was then mixed with 50 μl of 2-fold serial dilutions of each inactivated serum sample in 96-well plates and incubated at 37 °C for 1 h. The mixture was inoculated into Vero cells and incubated at 37 °C for 1 h. After removing the mixture, the cells were thoroughly rinsed five times with PBS and maintained in virus growth medium (Lee et al., 2017; Lee and Lee, 2018) at 37 °C in a 5% CO_2 incubator for 2 days. The neutralization titer was calculated as the reciprocal of the highest dilution of serum that inhibited virus-specific CPE in duplicate wells.

2.7. Enzyme-linked immunosorbent assays

Recombinant PEDV S1 protein was obtained from PK-rS1-Ig cells (Oh et al., 2014) and purified as described previously (Oh et al., 2014). Anti-PEDV immunoglobulin (Ig) A antibodies in serum and colostrum were detected using an *in-house* PEDV G2b S1-based indirect enzyme-linked immunosorbent assays (ELISA) as described previously (Gerber et al., 2014; Gerber and Opriessnig, 2015), with minor modifications. Briefly, microtiter plates (Nunc, Naperville, IL) were coated with 1.6 ng per well of the S1 antigen diluted in coating buffer (50 mM bicarbonate buffer, pH 9.6) and incubated overnight at 4 °C. After three washes with PBS containing 0.05% Tween 20 (PBST), the plates were blocked with

5% powdered skim milk (BD Biosciences, Belford, MA) in PBST for 2 h at 37 °C and then incubated with serum or colostrum diluted 1:100 in PBST containing 10% goat serum (Vector Laboratories, Burlingame, CA) for 1 h at 37 °C. After washing, a 1:20000 diluted peroxidase-conjugated goat anti-porcine IgG (Santa Cruz Biotechnology, Santa Cruz, CA) or IgA (Abcam, Cambridge, UK) was added and incubated at 37 °C for 1 h. The peroxidase reaction was visualized using tetramethylbenzidine-hydrogen peroxide as the substrate (R&D Systems, Minneapolis, MN) for 20 min at RT in the dark and was stopped by adding 2 N sulfuric acid as the stop solution (R&D Systems) to each well. Optical densities (ODs) were measured at 450 nm using a SPARK 10 M multimode microplate reader (TECAN, Männedorf, Switzerland). Positive, negative, and blank (sterile water) samples included on each plate, and all clinical and control samples were tested in duplicate.

2.8. Statistical analysis

All values are expressed as the means ± standard deviation of the mean (SDM). Statistical significance was evaluated using Student's *t*-tests with GraphPad Prism 5.0 (GraphPad Prism Inc., San Diego, CA). *P*-values of less than 0.05 were considered statistically significant.

3. Results

3.1. KNU-141112-S DEL5/ORF3 was phenotypically and genotypically stable during reversion to virulence experiments

To assess its attenuated phenotype and genetic stability, S DEL5/ORF3 was tested in a conventional five-passage reversion to virulence experiment During the acclimation period, all piglets were active and exhibited normal fecal consistency with no clinical symptoms, while no PEDV genetic material was detected in the fecal samples from any piglet. None of the piglets used in the five S DEL5/ORF3 passages developed clinical signs typical of PED, remaining asymptomatic and alive throughout the 5 or 21 days of observation. All piglets inoculated with the stock virus (first-passage group [P1]) were positive for PEDV by 1 or 2 dpi as determined by RT-PCR, and they continuously shed low amounts of PEDV in feces with mean titer ranges of $10^{3.14}$–$10^{3.52}$ genomic copies/ml from 2 dpi until they were euthanized (Fig. 1). Similar levels of PEDV fecal shedding were also recorded for the piglets in all four subsequent passage groups (P2–P5), and the kinetics for the mean titer values in groups P2–P5 were comparable to those from the piglets in P1. Interestingly, the quantities of virus in the feces from four piglets in the fifth-passage group (P5), which were euthanized at 21 dpi, were lower at 7 dpi, with a mean titer of $10^{0.83}$ genomic copies/ml; thereafter, their feces were negative for the presence of PEDV until the termination of the study (Fig. 1). Thus, fecal shedding of the S DEL5/ORF3 virus occurs for up to a week after challenge in neonatal piglets.

PEDV RNA titers were measured in the intestinal tissue samples of piglets collected during a necropsy at 5 dpi for each passage (Fig. 1B). Compared to the dose of the S DEL5/ORF3 oral inoculum, all of the inoculated piglets contained approximately 1-log lower levels of PEDV, with mean titer ranges of $10^{4.52}$–$10^{5.13}$ genomic copies/ml. There were no significant differences in the intestinal PEDV quantities between the five groups. Under macroscopic examination, it was observed that entire animals in all passage groups (P1–P5) had no gross lesions in their gastrointestinal tracts and a normal bowel wall thickness (Fig. 2A–E). Although mild histopathology was evident in some cases, the microscopic lesions in the small intestine of the inoculated pigs appeared normal (Fig. 2F–J). There were no significant differences in the mean (± SDM) jejunal VH:CD ratios between the inoculated nursing piglets in the different passage groups: 5.3 ± 0.5 in P1, 5.8 ± 0.4 in P2, 6.1 ± 0.5 in P3, 5.8 ± 0.6 in P4, and 5.5 ± 0.9 in P5. These values were slightly lower than the normal VH:CD ratios (7.6 ± 0.2) in uninoculated negative control pigs but were significantly higher than the VH:CD ratios (1.2 ± 0.8) in the positive control pigs inoculated with the parental KNU-141112-P5 strain. Under IHC analysis, no virus antigen was found in the small intestine of any animal in the first two passages (P1 and P2; Figs. 2K–O). On the other hand, PEDV antigen-positive cells were detected in some small intestinal villi from the inoculated pigs in P3–P5, with a similar range of 0–3 per jejunal villus within all three groups. Altogether, the results demonstrated that attenuated S DEL5-ORF3 did not revert to a virulent phenotype after five continuous back passages in susceptible piglets.

To identify the genomic changes that might have occurred during sequential back passages in highly susceptible piglets, the structural protein gene sequences of the PEDV inoculums for each reversion to virulence experiment (P2–P5) were determined and compared to parental attenuated S DEL5/ORF3. No mutations arose in the S-ORF3-E-M-N protein-coding region in any of the isolates from the passages, while the distinguishing deletion (DEL) signature present in S and ORF3 within the S DEL5/ORF3 strain (Lee et al., 2017) remained completely unchanged during the reversion to virulence experiment. The full-length genome sequence of PEDV collected from the final (i.e., 5th)

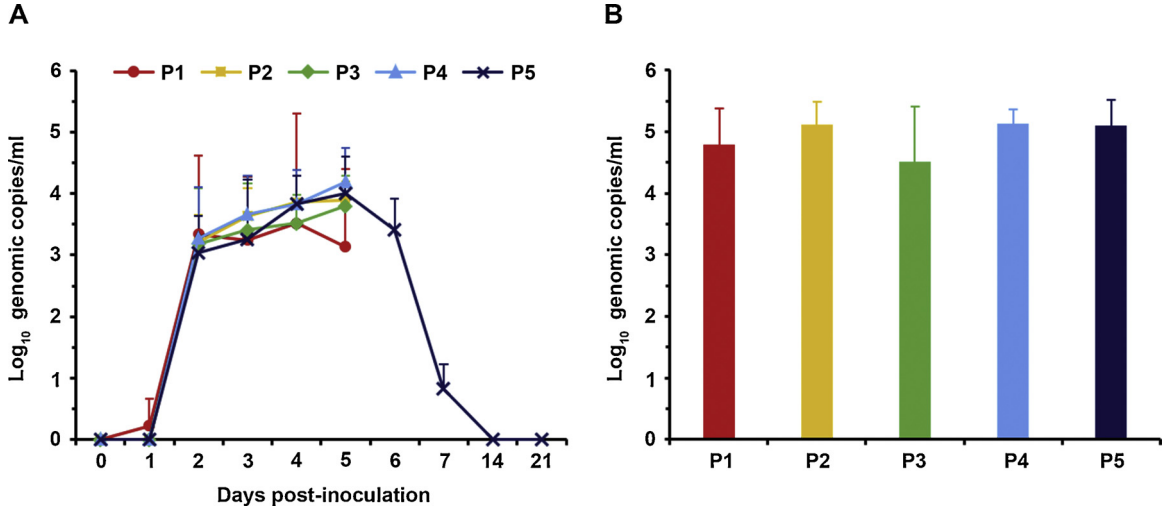

Fig. 1. PEDV detection in fecal and intestinal samples from piglets in the five back passage groups (P1–P5). PEDV RNA titers in rectal swabs collected at the indicated time points (A) and jejunal tissue collected at 5 dpi for each back passage (B) were determined using quantitative real-time RT-PCR analysis. The virus titer (log_{10} genomic copies/ml) values represent the mean virus titer from all pigs in each reversion to virulence test and the error bars represent the mean ± SDM. Statistical significance was assessed using Student's *t*-tests. There were no significant differences between the groups.

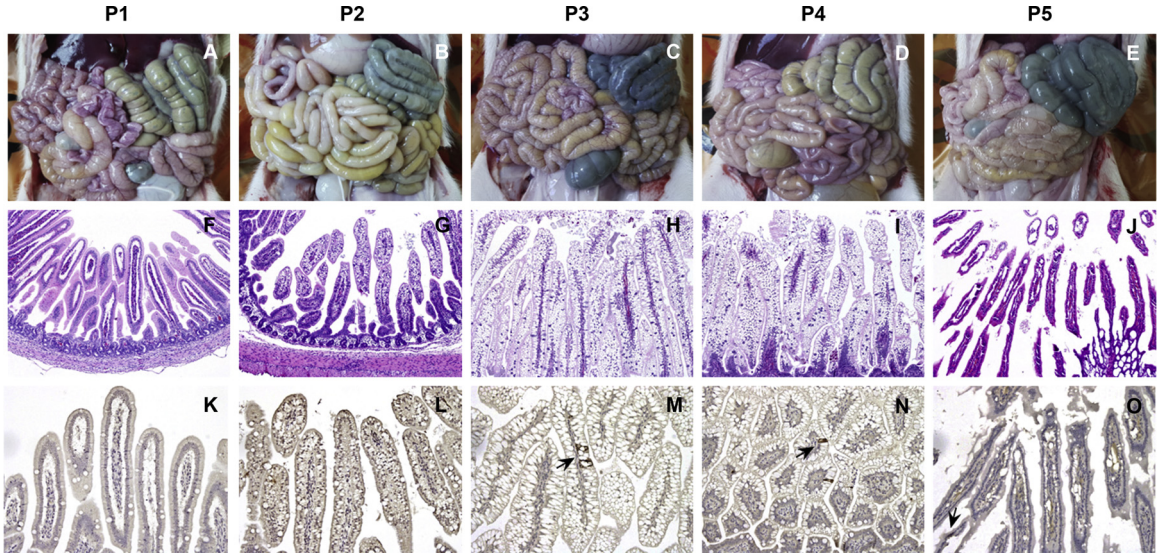

Fig. 2. Macroscopic and microscopic small intestine lesions in piglets from the five back passage groups (P1–P5). (A–E) The small intestine from representative piglets in each reversion to virulence test was examined for gross lesions. (F–J) Hematoxylin and eosin-stained tissue sections of the jejunum from representative piglets in each reversion to virulence test (100× magnification). (K–O) Detection of PEDV antigens using IHC analysis of jejunum tissue sections from representative piglets in each reversion to virulence test (200× magnification). Immunostaining of PEDV antigen appears as brown staining (arrows) and was detected in the epithelial cells of the jejunum in representative pigs from passage groups P3–P5 (panels M–O).

Table 1
Summary of nucleotide and amino acid differences between the parental KNU-141112-S DEL5-ORF3 (P1) and 5th *in vivo*-passaged (P6) viruses.

Gene	Position		S DEL5/ORF3	
	Nucleotide	Amino acid	P1	P6
ORF1ab				
nsp3	3847	1283	**A**TT (I)	**C**TT (L)
	3860	1287	TG**C** (C)	TT**C** (F)
S	1108	370	**T**TT (F)	**C**TT (L)

The bold letters indicate mutated nucleotides based on the KNU-141112-S DEL5-ORF3 virus.

passage of the experiment was also determined. Compared to the S DEL5/ORF3 genome, the virus isolate (P6) had three amino acid (aa) substitutions that include 2-aa (Ile to Leu and Cys to Phe) and 1-aa (Phe to Leu) changes at positions 1283 and 1287 of nsp3 and position 370 of S, respectively. Additional nsp3 sequencing revealed that I1283 L and C1287 F mutations were independently acquired during P4 and P3, respectively. Details of the mutations of the 5th *in vivo*-passaged virus are summarized in Table 1. Our sequence analysis thus proves that S DEL5/ORF3 was genetically stable during serial passages in piglets.

3.2. Live attenuated KNU-141112-S DEL5/ORF3 vaccine provided complete protection against PEDV challenge

To evaluate the protective effect of the S DEL5/ORF3 live vaccine, the present study employed an oral prime-parenteral booster scheme. None of the sows in the vaccinated and unvaccinated groups experienced any clinical signs associated with PEDV or any adverse reactions to the vaccines, such as fever, hypersensitivity, or abortion/stillbirth. No PEDV genetic material was detected using RT-PCR in fecal samples from any of the sows over the duration of the study. On the other hand, rectal swab samples from all orally primed sows, which were negative for PEDV RNA, were collected for 2 weeks, pooled, and subsequently used to inoculate three PEDV-naïve 2-day-old piglets for 5 days. No PED-related clinical symptoms were observed in any of these piglets. Furthermore, no piglet exhibited detectable PEDV shedding in their feces throughout the experimental period or a viral load in their small intestine at the end of the study (data not shown). In addition, no significant differences in reproductive performance were observed between the vaccinated and unvaccinated sows at farrowing.

Ten piglets per litter were assigned to each sow for nursing. During the pre-challenge period, all of the piglets were active, exhibited no clinical symptoms, and had normal fecal consistency, with the fecal samples from all piglets found to be negative following PEDV-specific RT-PCR. After challenge, PEDV-exposed piglets from unvaccinated sows (group 2) experienced clinical signs, including lethargy and diarrheic feces, within 1 dpc (mean CSS of > 2.5) and experienced severe watery diarrhea with vomiting thereafter (Fig. 3A). However, piglets from vaccinated sows (group 1) had mild diarrhea (mean CSS of < 1.0) beginning at 1 or 2 dpc and remained clinically normal after 7 or 8 dpc; they were thus comparable to the unchallenged piglets from the group 3 sow. These results revealed that litters from vaccinated sows developed notably lesser and milder diarrhea after challenge compared to those from unvaccinated sows. PEDV-associated mortality occurred in 5 of the 20 piglets (25%) from unvaccinated sows at 2 dpc, and all of the remaining animals had died by 4 dpc, thus representing a 100% mortality rate for the unvaccinated challenge control group 2 (Fig. 3B). In contrast, there was a 100% survival rate among the litters from the vaccinated sows (group 1) and the negative control sow (group 3) over the course of the experiment. After challenge, fecal shedding was detected in all piglets from the unvaccinated sows (group 2) by 1 or 2 dpc, and they continuously shed high amounts of PEDV in their feces until death (Fig. 3C). In contrast, a majority of piglets (26/30) from the vaccinated sows (group 1) had detectable amounts of PEDV RNA in feces by 3–7 dpc with a peak mean titer of $10^{1.27}$ genomic copies/ml at 5 dpc and continued shedding until 14 dpc. As with all of the unchallenged piglets, viral shedding was not detected in the rectal swabs of two challenged piglets from vaccinated sows over the entire experimental period. All dead piglets from the unvaccinated sows displayed archetypal PED-like gross and histopathologic lesions including dilated and fluid filled intestines with thin transparent walls as a result of villous atrophy, whereas all surviving piglets from vaccinated sows appeared normal both macroscopically and microscopically (data not shown). Therefore, it can be concluded that the oral live prime-parenteral killed/killed booster maternal vaccination regimen provided complete piglet protection against challenge by virulent PEDV

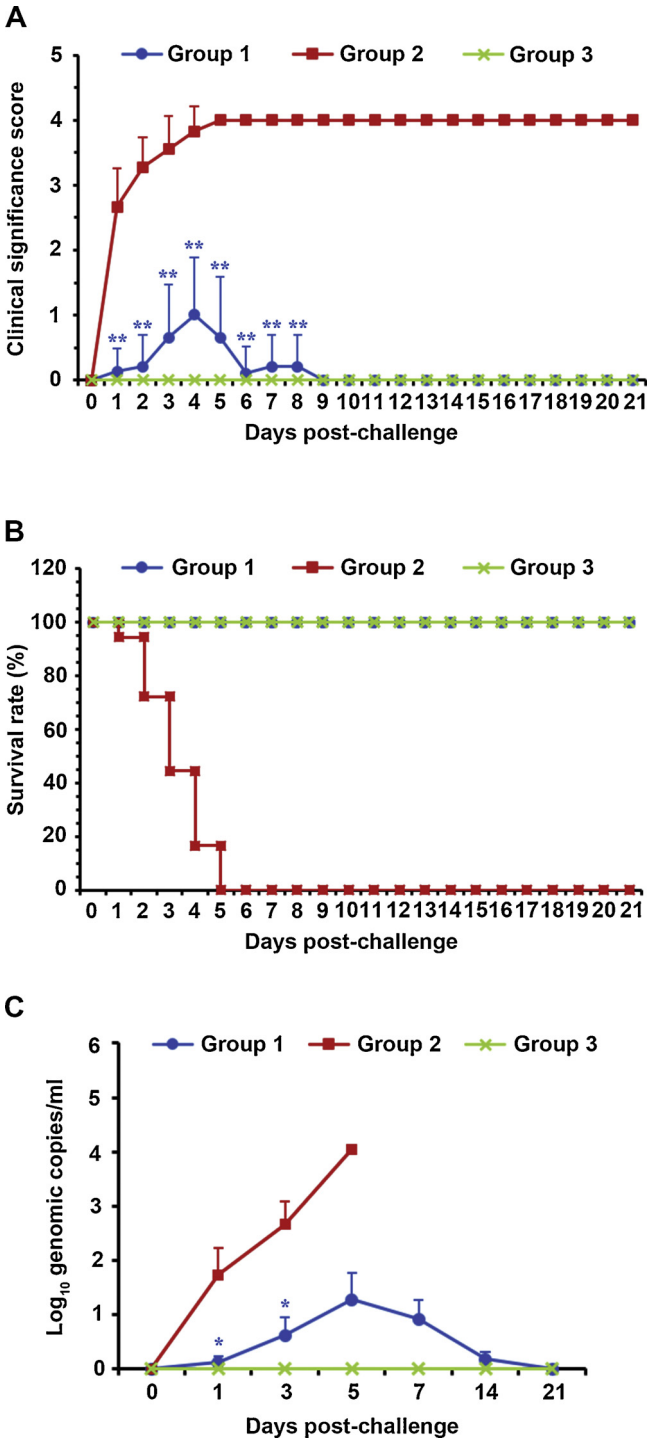

Fig. 3. Clinical significance scores, survival rates, and virus shedding in piglets from three experimental groups. Pregnant sows were primed orally with a live S DEL5/ORF3 vaccine and boosted twice with a commercial G2b PEDV vaccine at 2-week intervals pre-farrowing and their nursing piglets were challenged with virulent G2b PEDV at 3 days of age. (A) Clinical significance scores were measured as described in the Materials and Methods section. (B) Survival rate of piglets from vaccinated (group 1), challenge-control (group 2), and negative-control (group 3) sows through 21 dpc. (C) PEDV titers in rectal swab samples at each time point were determined using quantitative real-time RT-PCR analysis. The virus titer (log_{10} genomic copies/ml) values represent the mean virus titer from all pigs and the error bars represent the mean ± SDM. *P* values were calculated by comparing the data from the vaccinated and unvaccinated sow groups after challenge using Student's *t*-tests. *, $P = 0.001$ to 0.05; **, $P < 0.001$.

The average daily weight gain (ADG) per litter from the vaccinated and unvaccinated sows was calculated at different time during the experimental period (Fig. 4). With a 100% mortality rate by 4 dpi, the challenged piglets from unvaccinated sows (group 2) experienced significantly reduced ADG from 1 dpc until their death (data not shown). The overall ADG in the challenged piglets from vaccinated sows (group 1) was compared to that in the unchallenged piglets from the unvaccinated sow (group 3). Following virus challenge, the ADG of litters from vaccinated sows began to decrease at 3 dpc, with the loss of body weight continuing until 5 or 6 dpc. However, starting at 7 dpc, the animals in group 1 exhibited a gradual increase in their body weight and showed consistently enhanced weight gain during the remaining experimental period, which were comparable to the unchallenged pigs from the unvaccinated sow (group 3) (Fig. 4A). There were no significant differences in ADG between the two groups for the pre-weaning (0–21 dpc) and post-weaning periods (21–49 dpc) (Fig. 4B). The mean ADG for the entire experimental duration (0–49 dpc) was also similar for the challenged piglets from the vaccinated sows and unchallenged piglets from the negative control sow. Taken together, these results indicate that sow vaccination prevents detrimental effects on the growth performance of piglets resulting from PEDV infection.

3.3. Prime-boost maternal vaccinations elicited a sufficient antibody response

Measuring the quantities of neutralizing antibodies against PEDV in serum and colostrum is necessary to monitor immunity levels following sow immunization. Thus, individual samples were collected from pre- and post-partum sows and from neonatal piglets pre- and post-challenge and tested for the presence of anti-PEDV antibodies using SN assays and ELISA. As shown in Fig. 5A, all sows developed anti-PEDV neutralizing antibodies after the initial live prime vaccination, with titers ranging from 128 to 256 and a geometric mean of 213.3. At farrowing after the killed/killed vaccine boosters, the neutralizing antibody titers in the sera increased, ranging from 64 to 512 with a geometric mean of 362.7, while the titers in colostrum were all measured at 1024. All three sows maintained high levels of neutralizing antibody titer (512) for 4 weeks post-farrowing. Furthermore, representative nursing piglets representative from each litter within group 1 had neutralizing antibody levels ranging from 128 to 512 with a geometric mean of 307.2 prior to challenge, which were comparable to the levels of their own dam, and retained these antibodies with a slight reduction at 3 weeks post-challenge, indicating the adequate protective lactogenic immunity had been acquired from the vaccinated sows. These results were further verified by ELISA to determine anti-PEDV IgA antibody levels (Fig. 5B). The kinetics for the presence of antibodies in the sera and colostrum from vaccinated sows and their offspring were comparable to those of the neutralizing antibody levels in the corresponding samples. In contrast, neither anti-PEDV neutralizing nor IgA antibodies were detected in the serum and colostrum samples collected from the unvaccinated sows and their nursing piglets (groups 2 and 3). Taken together, our data demonstrate that prime/booster vaccination efficiently elicits potent antibody responses in sows, which are then transferred to their litters via lactation to provide full protection against PEDV.

4. Discussion

Because suckling neonates are the population most susceptible to PEDV infection, maternal vaccination is an integral strategy for passive lactogenic protection to prevent and eradicate PED epidemic or endemic outbreaks. In Asian pig-producing countries, including South Korea, G1a PEDV-based vaccines have been extensively used to control PED for decades. However, the advent of a new-genotype PEDV that led to the 2013–2014 pandemic has raised concerns regarding the efficacy of existing vaccines and the calls for a new effective vaccine against

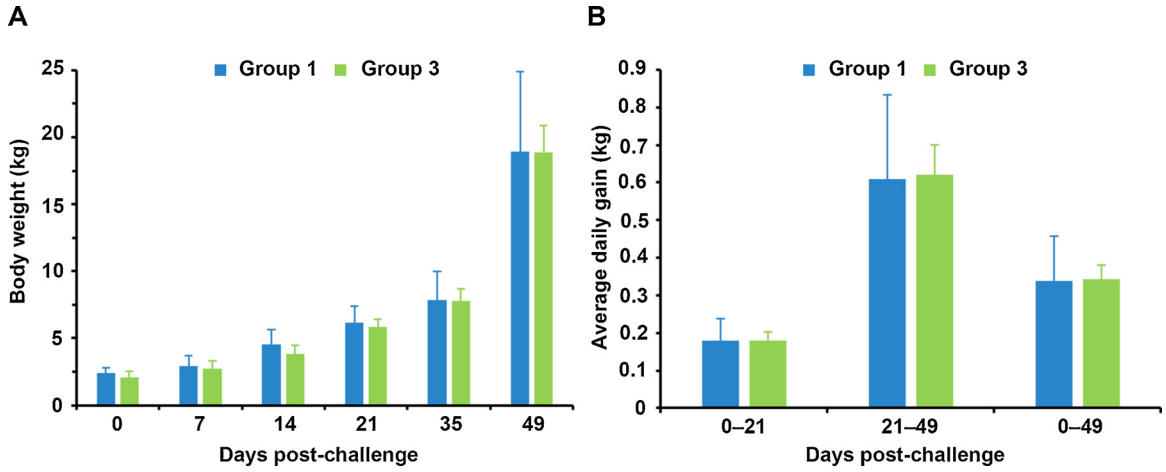

Fig. 4. Mean body weight and average daily weight gain for piglets from the vaccinated (group 1) and negative-control (group 3) sows after challenge through 49 dpc. Error bars represent the mean ± SDM. Statistical significance was assessed using Student's *t*-tests. There were no significant differences between the two groups.

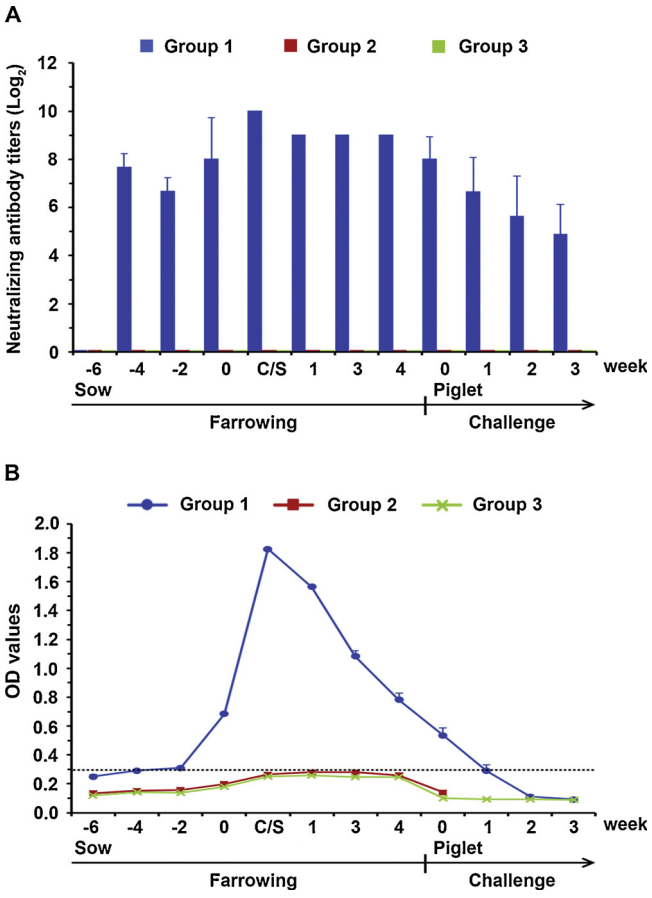

Fig. 5. PEDV-specific antibody responses in serum and colostrum samples of sows and their corresponding litters. Pregnant sows were primed orally with a live vaccine at 6 weeks prior to farrowing and boosted twice with a commercial killed vaccine at 4 and 2 weeks pre-farrowing and their nursing piglets were challenged with virulent G2b PEDV at 3 days of age. The samples were collected at the indicated time points and were tested with virus neutralization assays using KNU-141112 (A) and an in house IgA PEDV ELISA (B). C/S denotes colostrum samples collected at farrowing. Neutralizing antibody titers for individual samples were presented as a $\log_2$ scale. Values are representative of the mean from three independent experiments in duplicate and the error bars denote the mean ± SDM.

emergent epidemic strains, especially given that classical G1a vaccines are not fully protective against contemporary virulent G2b strains in the field. In 2015, the first licensed G2b PEDV vaccine was introduced to the domestic pig market, and currently three inactivated G2b vaccines are commercially available in South Korea for the immunization of sows against PEDV. Despite the certain benefits of these vaccines in preventing acute outbreaks, they often fail in endemic statuses and naïve herds. One reason for the variable vaccine efficacy observed under field conditions may be that parenteral (IM) inactivated vaccines induce a weaker and less persistent lactogenic immunity compared to oral live virus vaccines. Due to the impermeable placenta barrier in swine, piglets are born immunologically immature or agammaglobulinic and are hence readily vulnerable to a wide range of infections. Newborn piglets rely absolutely on colostrum and milk antibodies for maternal lactogenic immunity (Saif and Jackwood, 1990). Multiple enteric pathogens, including PEDV, require live viruses to migrate to the intestine and replicate to activate the gut-associated mucosal system in order to efficiently stimulate a local immune response that is passively transferred to suckling piglets via colostrum and milk (lactogenic immunity) (Langel et al., 2016). While natural infection or oral immunization with live virus can generate sufficient mucosal or lactogenic immunity, intramuscular administration is known to induce a systemic immune response that provides little lactogenic immunity to piglets (Chattha et al., 2015; Langel et al., 2016). Given these points in the design of PEDV vaccine, the development of next-generation live vaccines that can be applied orally is necessary for effective PED control in the swine industry. Recently, we successfully achieved the development of the cell culture-attenuated G2b strain S DEL5/ORF3, which is genetically stable and homologous to global G2b field strains, and immunogenically comparable to the parental virus (Lee et al., 2017). In this study, we sought to assess the safety and efficacy of the S DEL5/ORF3 strain as an MLV vaccine candidate under experimental conditions.

When evaluating the phenotypic stability of S DEL5/ORF3 by passaging the virus in highly susceptible 5 day-old piglets, no reversion to virulence was observed. Consistent with our previous research (Lee et al., 2017), the present study reproduced similar amounts and durations of PEDV fecal shedding, with the onset by 2 dpi, a peak at 4 dpi, and absence after 7 dpi. These kinetics were analogous among all animals and comparable for each passage. Most piglets from each passage had normal histopathological lesions but only mildly affected intestinal villi, with mean jejunal VH:CD ranges of 5.3–6.1, which are slightly below normal values of > 6 (Jung et al., 2015). PEDV antigen-positive cells were evident in piglets from later passages with a maximum of three cells per jejunal villus. Our experimental data indicated that the S

DEL5/ORF3 virus replicated in the gut of inoculated piglets, conserving its virologically and clinically attenuated phenotype during the five back passages. Accordingly, except for 3-aa changes (I1281 F and C1285 F in nsp3 and F375 L in S), no substitutions were found at the genome level after sequential back passages *in vivo*, demonstrating the genetic stability of S DEL5/ORF3. Furthermore, the genetic modifications, including a 5-aa DEL at each **N-** and C-terminus of S and a 70-aa DEL at the **N**-terminus of ORF3, that may have contributed to the attenuation to S DEL5/ORF3 were completely unaffected. The conservation of the 5-aa (KVHVQ) DEL of the S DEL5/ORF3 virus also supports a recent study that demonstrated that two intracellular sorting signals (endocytosis and endoplasmic reticulum-retention) in the cytoplasmic tail of the S protein are associated with PEDV virulence (Hou et al., 2019). It is thus likely that the discontinuous large DELs of S DEL5/ORF3 are highly secure and hinder reversion to the native sequences of the virulent parental virus.

Piglets from unvaccinated control sows exhibited much higher mean CSS, as determined by diarrhea severity, than those from vaccinated sows. When differences in CSS between the groups were assessed at 2 dpc, the challenged piglets from the vaccinated (group 1) and unvaccinated (group 2) sows exhibited mean CSS values of < 1.0 and > 3.0, respectively. As a result, all piglets in group 2 died within 4 dpc, whereas no piglets in group 1 died over the experimental period. The onset of PEDV fecal shedding in animals from the vaccinated sows was recorded at different times (3–7 dpc), and its termination varied among piglets, lasting up to 2 weeks after challenge. A recent study revealed that giving sows twice inactivated killed G2b vaccines (K/K vaccination) enhanced the survival rate of piglets against G2b challenge from 0% to 93% and relieved the severity and morbidity of diarrhea, including the fecal shedding of PEDV (Baek et al., 2016). Considering these results, the maternal L/K/K vaccinations applied in the present study were more efficacious than K/K treatment in terms of absolute improvement in survival rate and the significant mitigation of diarrheal severity, including the quantity and duration of viral shedding in stool. Although our L/K/K vaccination of sows still did not completely prevent morbidity in piglets after challenge, the remarkably shorter duration of PEDV fecal shedding and substantially declined titers would alleviate the environmental burden in the farrowing room by effectively eliminating the direct transmission source for the virus. Furthermore, neither adverse effects nor PEDV genetic and infectious materials in feces were found in any pregnant sows orally primed with S DEL5/ORF3. These data suggest that PEDV fecal transmission after sow immunization with the oral live vaccine is not possible under experimental conditions. None of the challenged piglets from vaccinated sows experienced body weight loss, and their ADGs returned to standard after a week post-challenge, indicating the favorable effects of vaccination on piglet growth rates. We were also able to confirm sufficient levels of anti-PEDV neutralizing and IgA antibodies in the sera of orally primed sows, which were boosted by the K/K vaccination, colostrum at farrowing, and the sera of sows at and after farrowing, as well as representative offspring sera, indicating that nursing piglets were protected by the transfer of maternal immunity via colostrum and milk from immune dams. These results further support the notion that colostrum and milk IgA and PEDV neutralizing antibody titers are correlated for protective immunity against PEDV; thus, the optimal vaccine regimens tend to promote high levels of both antibodies in the lactogenic secretions of vaccinated sows (Lee, 2015, 2019; Langel et al., 2016; Song et al., 2016).

Several studies on enteric virus vaccines have reported that, following the effectual priming of the gut by natural infection or oral attenuated vaccines, parenteral inactivated booster vaccines can augment and sustain mucosal or lactogenic immunity (Jafari et al., 2014; John et al., 2014; Langel et al., 2016). As a part of enzootic PEDV control in South Korea, a multiple-dose vaccination program with live (prime)-killed (boost)-killed (boost) vaccines at 2-week or 3-week intervals prefarrowing has been commonly recommended for pregnant sows for decades (Lee, 2015). Given the lessons from this traditional approach and the unavailability of an effective G2b-based MLV vaccine, the use of classical G1a MLV vaccines or feedback for prime-boost maternal vaccination strategies has been generally considered able to halt continuous devastation in the field since the overwhelming PED epidemic in 2013–2014. However, PEDV vaccination strategies based on historical G1a strains may create beneficial immune environments for the survival of epidemic G2b strains, which would continuously evolve to escape the host immune system under relieved immune pressures. In particular, aggressive feedback (i.e., intentional exposure) practices have since been adopted recklessly and involuntarily in a majority of domestic pig farms. Although feedback can artificially trigger rapid lactogenic immunity and hopefully shorten the outbreak period in PEDV-naïve herds, a number of potential negative consequences exist, including the widespread dissemination of other viral pathogens, insufficient gut stimulation due to heterogeneous and low titers of infectious PEDV in autogenous materials, the increased risk of recombination and viral diversity, and unexpected transmission between facilities and farms followed by an endemic establishment (Jung et al., 2006; Lee, 2015; Park et al., 2009; Saif et al., 2012). Therefore, it is anticipated that the proposed S DEL5/ORF3-based MLV vaccine will be able to replace the feedback approach used nationwide.

In conclusion, this is the first report to describe the safety and efficacy of an MLV vaccine based on a virulent G2b PEDV strain, and it is proven that this vaccine offers a greater preventive effect than do inactivated viral vaccines. The present work demonstrated that the fully attenuated phenotype of the S DEL5/ORF3 strain is not converted after five consecutive back passages in newborn piglets. Vaccination-challenge experiments revealed that sow immunization with an oral prime-parenteral boost L/K/K regimen provides protective lactogenic immunity for piglets, thus averting mortality completely, greatly reducing morbidity and fecal shedding, and sustainably maintaining piglet growth performance following exposure to virulent PEDV. One disadvantage of oral administration, however, is that it is impossible to ensure that individual pigs are vaccinated with the appropriate dose; this issue needs to be overcome in order to efficiently employ live vaccines for oral priming purposes under various field conditions. Because the introduction of a new, safe, and effective MLV vaccine to the market is expected to be useful for combating PED, particularly in seropositive and PEDV-naïve herds, customized implementation of vaccines such as the application of prime-boost maternal vaccination schemes should be established based on herd circumstances to prevent primary outbreaks, restrict the occurrence of re-infection or recurrent epidemics, and halt potential endemics. More importantly, stringent biosecurity/disinfection procedures and optimal farm and husbandry management practices must be combined to maximize vaccination as the most promising and effective approach to ensuring passive lactogenic immunity for the prevention and control of PED epizootics and enzootics.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgments

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