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Veterinary Immunology and Immunopathology 174 (2016) 45–49

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

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

Efficacy of an inactivated genotype 2b porcine epidemic diarrhea virus vaccine in neonatal piglets

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ARTICLE INFO

Article history:
Received 7 March 2016
Received in revised form 15 April 2016
Accepted 22 April 2016

Keywords:
Porcine epidemic diarrhea virus
Field isolate
Inactivated vaccine
Protective efficacy

ABSTRACT

Massive outbreaks of porcine epidemic diarrhea virus (PEDV) recurred in South Korea in 2013–2014 and affected approximately 40% of the swine breeding herds across the country, incurring a tremendous financial impact on producers and consumers. Despite the nationwide use of commercially available attenuated and inactivated vaccines in South Korea, PEDV has continued to plague the domestic pork industry, raising concerns regarding their protective efficacies and the need for new vaccine development. In a previous study, we isolated and serially cultivated a Korean PEDV epidemic strain, KOR/KNU-141112/2014, in Vero cells. With the availability of a cell culture-propagated PEDV strain, we are able to explore vaccination and challenge studies on pigs. Therefore, the aim of the present study was to produce an inactivated PEDV vaccine using the KNU-141112 strain and evaluate its effectiveness in neonatal piglets. Pregnant sows were immunized intramuscularly with the inactivated adjuvanted monovalent vaccine at six and three weeks prior to farrowing. Six-day-old piglets born to vaccinated or unvaccinated sows were challenged with the homogeneous KNU-141112 virus. The administration of the inactivated vaccine to sows greatly increased the survival rate of piglets challenged with the virulent strain, from 0% to approximately 92% (22/24), and significantly reduced diarrhea severity including viral shedding in feces. In addition, litters from unvaccinated sows continued to lose body weight throughout the experiment, whereas litters from vaccinated sows started recovering their daily weight gain at 7 days after the challenge. Furthermore, strong neutralizing antibody responses to PEDV were verified in immunized sows and their offspring, but were absent in the unvaccinated controls. Altogether, our data demonstrated that durable lactogenic immunity was present in dams administrated with the inactivated vaccine and subsequently conferred critical passive immune protection to their own litters against virulent PEDV infection.

1. Introduction

Porcine epidemic diarrhea (PED) is a highly contagious and deadly swine disease that is characterized by watery diarrhea, vomiting, severe dehydration, and high mortality rates in neonatal piglets (Lee, 2015; Saif et al., 2012). Although this enteric disease was first recognized in England in 1971, it was not identified until 1978 when a coronavirus was described as the etiological agent of PED (Oldham, 1972; Pensaert and Debouck, 1978). PED virus (PEDV) is a member of the genus *Alphacoronavirus* within the family *Coronaviridae* of the order *Nidovirales* (Pensaert and Debouck, 1978; Lee, 2015). PEDV is a large, enveloped virus that contains a single-stranded positive-sense RNA genome of approximately 28 kb with a 5′ cap and a 3′ polyadenylated tail (Pensaert and Debouck, 1978; Saif et al., 2012). The PEDV genome is composed of a 5′ untranslated region (UTR), at least 7 open reading frames (ORF1a, ORF1b, and ORFs 2–6), and a 3′ UTR (Kocherhans et al., 2001). The two large ORF1a and ORF1b encode two replicase polyproteins (pp), 1a and 1ab, which are later proteolytically processed into mature

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http://dx.doi.org/10.1016/j.vetimm.2016.04.009
0165-2427/

non-structural proteins. The remaining ORFs in the 3′ terminal region code for four major structural proteins, namely, the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins (Duarte and Laude, 1994; Lai et al., 2007; Lee, 2015). Among these, the S glycoprotein has been considered an appropriate viral gene for sequencing in order to investigate genetic relatedness and molecular epidemiology of PEDV isolates (Chen et al., 2014; Gerber et al., 2014; Lee et al., 2010; Lee and Lee, 2014; Oh et al., 2014). On the basis of phylogenetic analysis of the S gene, PEDV can be genetically divided into 2 groups: genogroup 1 (G1; classical or recombinant and low-pathogenic) and genogroup 2 (G2; field epidemic or pandemic and high-pathogenic), each of which is composed of two subgroups, 1a and 1b, and 2a and 2b, respectively (Lee, 2015; Lee et al., 2010; Lee and Lee, 2014; Oh et al., 2014).

Although PED has been described in Europe and Asia, the most serious epizootics have occurred predominantly in Asian swine-producing countries over the past two decades. Despite a notorious reputation in Asia, PED was not globally well recognized until the disease struck the United States in early 2013. Since its incursion into the US, PEDV has rapidly spread nationwide and to neighboring countries, sustaining enormous damages in pig health and the pork industry (Mole, 2013; Stevenson et al., 2013; Vlasova et al., 2014). Soon thereafter, severe PED epidemics recurred in South Korea, Japan, and Taiwan, and US prototype-like G2b PEDV strains were responsible for recent outbreaks in these countries (Lee and Lee, 2014; Lin et al., 2014; Suzuki et al., 2015). More recently, PEDV re-emerged throughout western and central Europe (Boniotti et al., 2016; Hanke et al., 2015; Grasland et al., 2015; Mesquita et al., 2015; Steinrigl et al., 2015; Theuns et al., 2015). These re-emergent PEDV strains were phylogenetically similar to new low-pathogenic G1b variants identified first in China and later in the US, South Korea, and Japan (Lee et al., 2014b; Li et al., 2012; Suzuki et al., 2015; Wang et al., 2014). Therefore, PED is now considered an emerging and re-emerging viral disease of swine around the world, leading to significant financial concerns in the global pork business.

The first PED epizootic in South Korea was reported in 1992 (Kweon et al., 1993). Since then PED outbreaks have continually occurred, resulting in substantial economic losses to the domestic swine industry. Moreover, the recent 2013–2014 PED epidemics swept through the national herd and killed hundreds of thousands of piglets across mainland South Korea followed by Jeju Island (Lee et al., 2014a; Lee and Lee, 2014). Meanwhile, all four different genotypes of PEDV are present in South Korea, including vaccine strains (G1a), new variants (G1b), past epidemic strains (G2a), and current dominant epidemic strains (G2b) (Lee, 2015; Lee et al., 2010; Lee et al., 2014b; Lee and Lee, 2014). Although both modified live and inactivated/killed vaccines against PED are commercially available in South Korea, their efficacy in the field is still being debated. The low to moderate effectiveness of current PEDV vaccines may be attributed to antigenic, genetic, and phylogenetic differences between the major S glycoproteins of the vaccine and field epizootic strains (Kim et al., 2015; Lee, 2015; Lee et al., 2010; Lee and Lee, 2014; Lee et al., 2014a; Oh et al., 2014). Considering these issues, G2b epidemic or related strains prevalently circulating in the field should be employed for the development of next-generation vaccines to control PED. However, isolating PEDV in cell culture has proven fastidious, and even the isolated virus may be incapable of retaining infectivity upon further *in vitro* passage. This laboratory hurdle makes the production of efficacious vaccines difficult. Recently, a highly virulent Korean G2b strain KOR/KNU-141112/2014 was isolated in our laboratory and sequentially passaged in cell culture. In the present study, we developed a Korean field isolate-derived inactivated vaccine and assessed its efficacy on suckling piglets against homogeneous PEDV challenge.

2. Materials and methods

2.1. Cells and virus

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 solutions (100×; Invitrogen) and maintained at 37 °C in a humidified 5% CO_2 incubator. A Korean PEDV strain, KOR/KNU-141112/2014, was isolated and propagated in Vero cells in our lab as described previously (Lee et al., 2015). A viral stock at the 10th passage in cell culture (KNU-141112-P10) was prepared and used in this study. Briefly, confluent Vero cells grown in 100-mm diameter tissue culture dishes were washed with PBS and inoculated with 1 ml of 10-fold diluted PEDV KNU-141112 containing trypsin (USB, Cleveland, OH). After incubating at 37 °C for 1 h, 7 ml of virus growth medium [α-MEM supplemented with antibiotic-antimycotic solutions, 0.3% tryptose phosphate broth (TPB; Sigma, St. Louis, MO), 0.02% yeast extract (Difco, Detroit, MI), 10 mM HEPES (Invitrogen), and 5 μg/ml of trypsin] was added. The inoculated cells were maintained at 37 °C under 5% CO_2 and monitored daily for cytopathic effects (CPE). When 70% CPE appeared, inoculated cells were subjected to three rounds of freezing and thawing. The culture supernatants were then centrifuged for 10 min at 400*g* (Hanil Centrifuge FLETA5, Incheon, South Korea) and filtered through a 0.45-μm-pore-size filter (Millipore, Billerica, MA). The clarified supernatants were aliquoted and stored at −80 °C as the viral stock until use.

2.2. Virus inactivation

Before inactivation, KNU-141112-P10 virus was purified as described previously (Lee and Lee, 2013), and the purified virus ($10^{7.0}$ $TCID_{50}$/ml) was inactivated for use as a vaccine. Inactivation of PEDV with binary ethylenimine (BEI) was performed as described previously (Vanhee et al., 2009). Briefly, virus was inactivated by the addition of 0.1 M BEI to a final volume of 5% and incubating at 37 °C for 24 h. Excess BEI was then neutralized by incubation with sodium thiosulfate at 37 °C for 2 h. Inactivated virus was stored at −80 °C until use. Virus inactivation was verified by inoculation of Vero cells with the BEI-treated virus. The inoculated cells were analyzed for CPE, followed by immunofluorescent staining with a PEDV N protein-specific monoclonal antibody (Lee et al., 2015).

2.3. Experimental design

Swine vaccination and challenge experiments described here were performed at the Choongang Vaccine Laboratory Animal Facility under the guidelines established by its Institutional Animal Care and Use Committee. A total of 5 commercial crossbred sows (Great Yorkshire × Dutch Landrace) with the same parity and expected farrowing date were chosen at a conventional breeding farm with a high health status and no known prior PED outbreak or vaccination with PEDV. All animals were confirmed negative for PEDV, transmissible gastroenteritis virus (TGEV), porcine deltacoronavirus, and porcine rotaviruses by virus-specific PCRs on rectal swabs and determined to be free of antibodies to PEDV as well as to TGEV and porcine reproductive and respiratory syndrome virus. Pigs were randomly assigned to 2 experimental groups: vaccinated group 1 ($n = 3$; V1, V2, and V3) and unvaccinated group 2 ($n = 2$; C1 and C2). Three sows in group 1 were intramuscularly (IM) vaccinated twice at 6 and 3 weeks prior to farrowing with 2 ml of the experimental vaccine (1 ml of the inactivated virus in a 1 ml water-in-oil adjuvant) containing 10^7 $TCID_{50}$ of the BEI-inactivated PEDV KNU-141112 strain. The remaining two sows in group 2 were not vaccinated and served as controls. At 5 days post-farrowing,

8 suckling piglets per litter (a total of 40 newborn piglets) were randomly selected from each vaccinated or unvaccinated sow and were relocated to the experimental facility. Upon arrival, piglets from the same litter were housed together in 5 separate rooms: piglets (Gv1, Gv2, and Gv3, $n = 24$) from V1, V2, and V3 in rooms 1–3 and piglets (Gc1 and Gc2, $n = 16$) from C1 and C2 in rooms 4 and 5, respectively. Pigs were fed commercial milk replacer frequently (4–6 times daily) and had ad libitum access to water for the duration of the study (17 days). Following a 1-day acclimation period, all piglets (6-days old) from the vaccinated and unvaccinated groups were challenged orally with a 1 ml dose of $10^{2.7}$ $TCID_{50}$/ml of KNU-141112-P10 virus (Lee et al., 2015; Lee and Lee, 2015). Clinical signs of vomiting, diarrhea, and mortality were monitored daily throughout the experiment. Stool samples from all groups were collected prior to inoculation and daily with 16 inch, cotton-tipped swabs and subjected to RT-PCR using an *i*-TGE/PED Detection Kit (iNtRON Biotechnology, Seongnam, South Korea) and real-time RT-PCR to detect the presence of PEDV shedding as described previously (Lee et al., 2015). Clinical significance score (CSS) was determined with the following scoring criteria as a measure of diarrheal severity based on both visual examination and fecal PEDV shedding for 7 days post-challenge (dpc): 0, normal and no diarrhea (mean 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. Piglets were weighed daily until the end of the experiment. Blood samples were collected from sows prior to vaccination and at farrowing and also from 3 representative piglets per litter from each dam at 5 days after birth.

2.4. Virus neutralization

The presence of PEDV-specific neutralizing antibodies in serum samples collected from sows and representative suckling pigs from all groups was determined using a virus neutralization test in 96-well microtiter plates using PEDV isolate KNU-141112, as previously described (Lee and Lee, 2015) with minor modifications. Briefly, Vero cells were grown at 2×10^4/well in 96-well tissue culture plates for 1 day. The KNU-141112-P10 viral stock was diluted in serum-free α-MEM to make 200 TCID in a 50 μl volume. The diluted virus was then mixed with 50 μl of 2-fold serial dilutions of individual inactivated sera 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 5 times with PBS and maintained in virus growth medium 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 all of the duplicate wells.

2.5. Statistical analysis

The nonparametric Mann-Whitney *U* test was used for all statistical analyses, and *P*-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Efficacy of an inactivated KNU-141112 vaccine

Three sows were vaccinated twice with the inactivated vaccine at 3-week intervals prior to farrowing, while 2 control animals were not vaccinated. Sows in vaccinated and unvaccinated groups experienced no signs of clinical infection with PEDV during gestation. No PEDV genetic material was detected in fecal samples from any sow by RT-PCR over the duration of the study.

Eight piglets per litter from each sow were delivered to the animal facility at 5 days following farrowing and allowed to acclimate

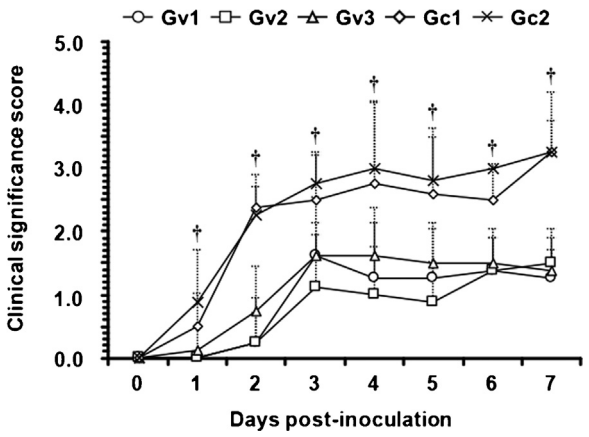

Fig. 1. Clinical significance scores in piglets from vaccinated (Gv) and unvaccinated (Gc) sows during the first 7 dpc. CSS was measured as described in Section 2. Error bars represent standard deviations. *P* values were calculated by comparing Gv and Gc groups using the Mann-Whitney *U* test. *, $P = 0.001$ to 0.05; †, $P < 0.001$.

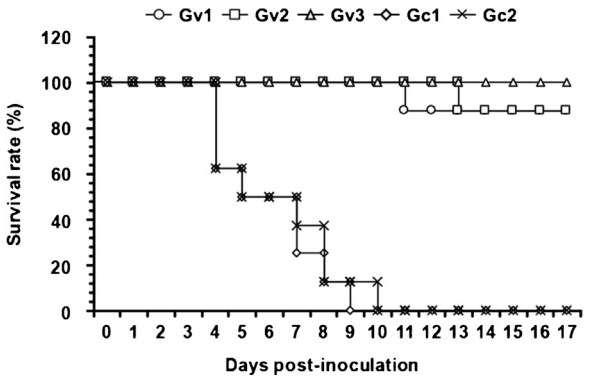

Fig. 2. Survival rate of piglets from vaccinated (Gv) and unvaccinated (Gc) sows after challenge through 17 dpc.

for 24 h. Challenge-exposure was performed orally to the 6-day-old piglets with 1 ml of the virulent KNU-141112 virus. Clinical signs were recorded daily and fecal swabs were collected prior to and after challenge for the duration of the study. During the acclimation period, all piglets were active and had no clinical symptoms with normal fecal consistency, and the fecal samples from all 40 piglets were negative by PEDV-specific RT-PCR. PEDV-challenged piglets from unvaccinated sows exhibited clinical signs including lethargy and diarrheic feces by 1 dpc (mean CSS of 0.7) and experienced severe watery diarrhea with vomiting thereafter (mean CSS of > 2.5), whereas piglets from vaccinated sows had mild diarrhea beginning at 2 dpc (mean CSS of < 1.5) (Fig. 1). These results revealed that litters from vaccinated sows exhibited significantly less diarrhea compared to those from unvaccinated sows. PEDV-associated mortality occurred in 6 of 16 piglets (37.5%) from unvaccinated sows at 4 dpc, and all of the remaining animals died by 9 dpc, indicating 100% mortality in the unvaccinated control group (Fig. 2). In contrast, nearly all litters from vaccinated sows remained alive throughout the study, with the exception of 2 piglets that died at 11 and 13 dpc, respectively.

In addition, the average daily weight gain (ADG) per litter from vaccinated or unvaccinated sows was calculated at the indicated time points (Fig. 3). Following virus challenge, litters from vaccinated sows began to decline in ADG at 2 dpc and continued to lose body weight until 6 dpc. However, starting at 7 dpc, those animals exhibited a gradual increase in body weight gain and consistently had an ADG of 100–300 g during the remaining experimental period. In contrast, after 1 dpc, litters from unvaccinated

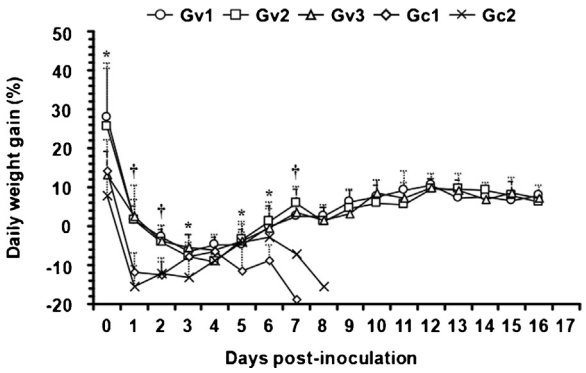

Fig. 3. Average daily weight gain rate of piglets from vaccinated (Gv) and unvaccinated (Gc) sows after challenge through 17 dpc. Due to no piglets surviving after 9 dpc in the Gc group, daily weight gain could not be determined. Error bars represent standard deviations. *P* values were calculated by comparing Gv and Gc groups using the Mann-Whitney *U* test. *, $P = 0.001$ to 0.05; †, $P < 0.001$.

Table 1
PEDV-specific virus neutralizing (VN) antibody titers of the sera of sows and their corresponding litters.

Sow[a]	VN	Piglet[b]	VN
V1	64	Gv1-1	64
		Gv1-2	>256
		Gv1-3	>256
V2	>256	Gv2-1	128
		Gv2-2	64
		Gv2-3	>256
V3	128	Gv3-1	128
		Gv3-2	128
		Gv3-3	>256
C1	<4	Gc1-1	<4
		Gc1-2	<4
		Gc1-3	<4
C2	<4	Gc2-1	<4
		Gc2-2	<4
		Gc2-3	<4

[a] Serum samples of sows collected before vaccination were not included, since they had VN antibody titers less than 1:4.
[b] Serum samples were collected from 3 representative piglets per litter at −1 dpc.

sows exhibited significantly reduced ADGs, over 10% less compared to those from vaccinated sows, and no piglets demonstrated an increase in ADG over the course of the study. Taken together, these data indicate that diarrheal severity by PEDV infection negatively affects the growth performance of piglets.

3.2. Antibody response of an inactivated KNU-141112 vaccine

Measuring quantities of neutralizing antibodies against PEDV in serum and colostrum is necessary to monitor the immunity level following sow immunization. Thus, individual samples were collected from sows before vaccination and at farrowing and from neonatal piglets before challenge and tested for their neutralizing activity against KNU-141112. As shown in Table 1, the vaccinated sow sera and colostra were highly effective in inhibiting KNU-141112 infection, with mean neutralizing antibody titers greater than 1:128 and 1:256, respectively. Furthermore, piglets representative of each litter had neutralizing antibody levels comparable to those of their own dam, indicating lactogenic immunity passively acquired from vaccinated sows. In contrast, none of the sera collected from unvaccinated sows and their offspring showed neutralizing activity against PEDV. Taken together, our data indicate that the inactivated PEDV vaccine elicits potent antibody responses in sows, which are then transferred to their litters via lactation to induce protection against the disease.

4. Discussion

Vaccination is a fundamental strategy for controlling and eradicating PED during epidemic or endemic outbreaks. As a first step toward developing an effectual vaccine for prevention of the disease, we must obtain a PEDV isolate that can grow productively in cell culture. In South Korea, three PEDV strains, SM98-1, DR-13, and Chinju99, were initially isolated almost two decades ago. Genetic and phylogenetic analyses revealed that SM98-1 and DR-13 are classified into the classical G1 genogroup, whereas Chinju99 belongs to the field epidemic G2 genogroup. Since only G1 strains (SM98-1 and DR-13) can be serially propagated *in vitro*, they have been employed as seeds for live attenuated or inactivated vaccines. Despite the nationwide implementation of vaccination against PED, the disease has continually affected pig farms and become endemic in South Korea, fueling doubt as to the effectiveness of domestic vaccines for PED control in the field. We have been monitoring the genetic diversity of PEDV circulating in the field and found that antigenic and genetic variations have occurred between vaccine and field strains, exhibiting a more than 10% amino acid difference in the S protein (Lee et al., 2010; Lee and Lee, 2014; Lee, 2015; Oh et al., 2014). These findings appear to contribute to the incomplete efficacy of current vaccines in South Korea, suggesting that the isolation of field PEDV is imperative for next-generation vaccine development. We recently accomplished the successful isolation and propagation of a Korean virulent PEDV strain in Vero cells that is genotypically almost identical to the global field strains causing the recent severe PED epidemics in Asia and the Americas (Lee et al., 2015). In this study, we sought to develop an inactivated vaccine using the Korean virulent G2b strain and investigated the effectiveness of this vaccine under experimental conditions

Sows were allocated to 2 groups with or without 2 IM administrations of the inactivated vaccine at 3-week intervals before farrowing. Suckling piglets born to all sow groups were challenged with a homogeneous virulent PEDV strain. The disease intensity, characterized by mortality and morbidity, was compared daily between litters from vaccinated and unvaccinated sows throughout the study. Piglets from the unvaccinated control sows showed much higher mean CSS as determined by diarrhea severity, including fecal shedding of PEDV, than those from vaccinated sows. When differences in CSS between the groups were assessed in the first 7 dpc, the Gv and Gc piglets exhibited CSS values of 0.0–1.5 and 0.7–3.3, respectively. As a result, all Gc piglets died within 9 dpc, whereas no Gv piglets died over the same experimental period (though mortality occurred later in 2 piglets). Moreover, the Gc piglets experienced serious body weight loss, likely due to severe watery diarrhea and vomiting until death. A recent study revealed that commercially available inactivated vaccines based on classical G1 strains enhanced the survival rate of piglets challenged with a field isolate from 18.2% to 80%, but failed to relieve the morbidity rate of diarrhea including virus-shedding feces (Lee, 2015). Considering those data, the G2b strain-derived inactivated vaccine developed in this study was more efficacious than current domestic vaccines in terms of reductions in mortality rate and diarrheal severity. Although the administration of our vaccine to sows did not completely prevent morbidity in piglets upon challenge, a decrease in fecal shedding of PEDV would alleviate the environmental burden in the farrowing room by partially eliminating the source for direct transmission of virus to other animals. Moreover, we were able to confirm sufficient quantities of neutralizing antibodies in the sera and colostra of vaccinated sows, as well as representative offspring sera, indicating that neonatal piglets were protected

by a transfer of maternal immunity via colostrum and milk from immune dams. These results further support the notion that the optimal vaccine regimen is associated with retaining high levels of PEDV-specific neutralizing antibodies in the serum and colostrum of vaccinated sows, although protection against the enteric disease generally depends on the presence of secretory IgA antibodies in the intestinal mucosa (Lee, 2015; Park and Lee, 2009; Park et al., 2011).

In conclusion, the present work demonstrated that sow immunization with the new inactivated vaccine provides protective lactogenic immunity to piglets, thereby reducing mortality, morbidity, and fecal shedding through challenge-exposure with virulent PEDV. Further vaccination-challenge studies will be needed to evaluate the inactivated vaccine described in this study in 1- or 2-day-old piglets under field conditions, and this aspect is currently under investigation. Further, for improving morbidity and mortality to confer complete protection, we are now employing the Korean G2b field epidemic isolate in efforts to develop a modified live attenuated vaccine (MLV), which often proves to supply more preventive benefits than inactivated viral vaccines. Since the advent of new, effective, and safe PEDV vaccines on the market is expected in the near future to combat PED, the customized implementation of vaccines (*e.g.* the use of MLV or inactivated vaccines alone, or the application of inactivated vaccines as a booster following MLV or feedback) should be established on the basis of field circumstances to block a primary outbreak, to limit the occurrence of re-infection or secondary epidemics, or to discontinue endemic infection. More importantly, we should bear in mind that full biosecurity and husbandry management must be accompanied in the field to maximize vaccine effectiveness for prevention and control of PED.

5. Conclusions

In this study, sow vaccination with the G2b epidemic strain-derived inactivated vaccine protected piglets against virulent PEDV challenge. The new inactivated vaccine mediated strong neutralization antibody responses in vaccinated sows and offspring.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This research was supported by Bio-industry Technology Development Program through the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (iPET) funded by the Ministry of Agriculture, Food and Rural Affairs (315021-04).

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