Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Available online at www.sciencedirect.com
ScienceDirect
Research in Veterinary Science 82 (2007) 134–140
RVS
www.elsevier.com/locate/rvsc

Oral efficacy of Vero cell attenuated porcine epidemic diarrhea virus DR13 strain

D.S. Song [a], J.S. Oh [a], B.K. Kang [a], J.S. Yang [b], H.J. Moon [b], H.S. Yoo [b], Y.S. Jang [c], B.K. Park [b,*]

[a] *Research Unit, Green Cross Veterinary Products, YongIn 227-5, Republic of Korea*
[b] *Department of Veterinary Microbiology Virology Lab and Infectious Disease Lab, The Xenotransplantation Research Center, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Republic of Korea*
[c] *Division of Biological Science and the Institute for Molecular Biology and Genetics, Chonbuk National University, Chonju 561-756, Republic of Korea*

Accepted 27 March 2006

Abstract

A Vero cell attenuated porcine epidemic diarrhea virus (PEDV) strain, DR13, was distinguished from wild-type PEDV using restriction enzyme fragment length polymorphism (RFLP). Cell attenuated DR13 was orally or intramuscularly (IM) administered to late-term pregnant sows, and mortality resulting from the highly virulent PEDV challenge was investigated in passively immunized suckling piglets of the two different groups. The mortality rate of the oral group (13%) was lower than that of the IM group (60%). In particular, the concentration of IgA against PEDV was higher in piglets of sows in the oral group, compared to the IM group. The attenuated DR13 virus remained safe, even after three backpassages in piglets. The findings of this study support the theory that the Vero cell attenuated DR13 virus may be applied as an oral vaccine for inducing specific immunity in late-term pregnant sows with a high margin of protection against PEDV infection.

Keywords: Porcine epidemic diarrhea virus; Oral inoculation; Vaccine candidate

1. Introduction

Porcine epidemic diarrhea virus (PEDV), a coronavirus, is an etiological enteropathogenic agent in swine (Debouck and Pensaert, 1980; Ducatelle et al., 1981; Wood, 1977). The virus replicates in differentiated enterocytes covering the villi of the small intestine, leading to villous atrophy and maladsorption (Debouck and Pensaert, 1980; Moon, 1978). PED occurs in most swine-raising countries in Europe, as well as China, Korea, and Japan. Thus, economic losses from the disease are serious every winter in these countries (Pensaert, 1999; Saif and Wesley, 1999). Infection is associated with >20% diarrheal cases in neonatal pigs in Korea (Kweon et al., 1999). Live vaccines for PED are used in Korea (KPEDV-9) and Japan (P-5V), due to the endemic nature of the disease (Kweon et al., 1999; Kadoi et al., 2002). The efficacy of commercially available vaccines is limited in field conditions, and the protective immunity induced is insufficient. This is directly attributed to the attenuated strain employed, and the intramuscular (IM) route. In a previous study, a serially Vero cell passaged PEDV, designated ''DR13'', with a restriction fragment length polymorphism (RFLP) pattern distinct from wild-type PEDVs was tested for pathogenicity in piglets and safety in pregnant sows after oral inoculation (Song et al., 2003).

In this investigation, we report the results of a field trial comparing oral versus IM inoculation of the DR13 virus in pregnant sows for protecting piglets against PEDV challenge.

* Corresponding author. Tel.: +82 2 885 0263.
E-mail address: parkx026@snu.ac.kr (B.K. Park).

0034-5288/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.rvsc.2006.03.007

2. Materials and methods

2.1. Cells and viruses

The continuous Vero cell line (ATCC, CCL-81) was maintained in α-minimum essential medium (α-MEM) supplemented with 5% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml).

Vero cell attenuated PEDV DR13 was propagated, as described previously (Hofmann and Wyler, 1988; Kweon et al., 1999). Briefly, prior to virus inoculation, the growth medium of confluent cells in 25 cm^2 flasks (Falcon, USA) was removed, and cells washed three times with phosphate-buffered saline (PBS, pH 7.4). Next, cells in each flask were inoculated with 1 ml virus. After adsorption at 37 °C for 1 h, cells were incubated in α-MEM supplemented with 0.02% yeast extract, 0.3% tryptose phosphate broth, and 2 μg trypsin.

Challenge PED virus was prepared from small intestines of suckling pigs inoculated orally with the field isolate, DR13, before cell attenuation. Intestines were homogenized to a 10% (v/v) suspension with phosphate-buffered saline (PBS; 0.1 M, pH 7.2). Suspensions were mixed by vortexing, and clarified by centrifugation for 10 min at 4800*g*. Supernatant fractions passed through a 0.2 μm syringe filter (Acrodisk, Gelman) were used for challenge experiments.

2.2. Efficacy of Vero cell attenuated PEDV DR13

Five commercial farms were used for the PEDV DR13 virus field trials. Three farms had no outbreaks or vaccination of PED, while the other two farms had a history of PED. A mixed breed of pigs was employed in this study (Yorkshire × Landrace × Duroc). All sows from farms A, B, and C, located in the Kyungsang and Chuchung provinces, were serologically negative for PED virus neutralizing antibodies. A number of pregnant sows in the three PED-free commercial farms (500 sows in Farm A; 300 in B; 200 in C) were inoculated orally (O group) or intramuscularly (IM group) with 1 ml DR13 (passage level 100) at a titer of $10^{6.0}$ $TCID_{50}$/0.1 ml, 4 weeks prior to farrowing. A second inoculation with an equivalent titer of virus was administered after 2 weeks. Sows that were not vaccinated with the virus comprised the control group. Paired serum samples before and after inoculation were collected at two-week intervals. Paired sera (2 and 4 weeks before farrowing, and at farrowing) and colostrum at delivery were tested for the presence of antibodies against PEDV using ELISA, as reported previously (Kweon et al., 1999). Piglets of all groups were housed with their mothers with no artificial supply of colostrum or milk. Thirty 3-day-old piglets were selected randomly from farrowing sows in vaccinated (O and IM) and control groups for challenge exposure with virulent PEDV. Piglets from all groups were challenged orally with 5 ml wild-type PEDV (the virus titer was not determined). Before challenge, sera from piglets were collected for ELISA and serum neutralization (SN) analysis of antibodies against PEDV. Nursing 3-day-old pigs were removed from the sow 1 h prior to challenge with 5 ml virulent PEDV (which previously caused 100% mortality in 3-day old pigs) (Song et al., 2005). Piglets from each group were housed in a 1.3 m × 1.3 m (1.69 m^2) pen of an environmentally controlled building, and fed substitute milk. Clinical signs of diarrhea and mortality in challenged piglets were observed for 10 days, and antibody response against PEDV was examined during the experimental period. The duration of PEDV shedding after challenge with wild-type PEDV was monitored using RT-PCR. Twenty-four pregnant sows from the two other farms (D and E) were inoculated orally with 1 ml virus (passage level 100) containing $10^{6.0}$ $TCID_{50}$/0.1 ml at 2 and 4 weeks prior to farrowing. The 24 colostrum samples were collected at delivery from each farrowing sow. Moreover, two colostral samples were collected from control sows of each farm within 24 h after parturition. Serum samples were obtained from the cranial vena cava, and colostrum samples obtained manually from 2 or 3 glands and pooled after IM injection of 1 IU oxytocin. Whey was obtained from colostral samples for use in a SN test. Eight 3-day-old piglets were selected randomly from farrowing sows of farms D and E, and challenged using a similar method. Non-vaccinated sows provided the source of control pigs in farms A, B, and C. Control piglets (Yorkshire × Landrace × Duroc) of farms D and E (seronegative against PEDV, 1: $\leqslant$ 2 in SN test), which aged at 3days, were provided by YANGSUNG Laboratory Animal Inc (YongIn, Korea).

All animal experiments complied with the current laws of Korea. Animal care and treatment were conducted in accordance with guidelines established by the Seoul National University Institutional Animal Care and Use Committee.

2.3. ELISA

Antigen was prepared, as described previously, with minor modifications (Kweon et al., 1999; Oh et al., 2005). Briefly, dilutions of antigen and secondary antibody were adjusted to an optical density (OD) of around 0.1 (A_{405}), using negative porcine sera. Each well in a 96-well microplate (Costar) was coated with 0.1 μg protein in 50 mM carbonate buffer (pH 9.6) at 5 °C overnight, followed by blocking with 5% rabbit serum and 3% gelatin at 37 °C. The 1/100 diluted sample porcine sera, colostrum and negative porcine sera (used for adjusting the antigen concentration) in PBS with 0.05% Tween 20 (PBST) were reacted at 37 °C for 1 h, and washed 5 times with PBST. The collection of positive and negative porcine sera was described previously (Oh et al., 2005). The reacted plate was re-washed under the same conditions, and incubated with 10,000-fold (IgG) and 2000-fold (IgA) diluted horseradish peroxidase (HRP)-labeled anti-porcine IgG or IgA (KPL) for 1 h at 37 °C. The plate was developed in ABTS

substrate (KPL) at room temperature for 20 min. The reaction was terminated with 2 M H_2SO_4 before OD measurement at 405 nm. All samples were tested at the same time at the same plate in triplicate for minimizing intraassay variation.

2.4. Serum neutralization (SN) test

The SN test was performed using a previously published method, with some modifications (Kusanagi et al., 1992). The hyperimmune PEDV reference serum was prepared from an antibody free and PEDV-free 2-week-old pig inoculated with 1 ml of PEDV, KPEDV-9 strain (105.5 TCID50/0.1 ml) and then the serum was collected at 4 weeks post-inoculation. Negative reference serum was also obtained from a mock-infected pig of the same age. Briefly, swine sera were inactivated at 56 °C for 30 min, and stored at −20 °C until use. After twofold dilution, serum was mixed with PEDV (200 $TCID_{50}$/0.1 ML) at an equal volume, and incubated for 1 h at 37 °C. Subsequently, 0.1 ML of each virus–serum mixture was transferred to Vero cell monolayers of a 96-well tissue culture plate washed twice with PBS. After adsorption for 1 h at 37 °C, inocula were discarded, and washed twice with PBS. Next, maintenance medium containing trypsin (2 μg/Ml) was added to each well, and the plate incubated for 5 days at 37 °C. SN titers were expressed as reciprocals of the highest serum dilution, resulting in cytopathic effect (CPE) inhibition.

2.5. Detection of PEDV shedding after challenge

Fecal samples from all groups were collected every day with cotton swabs (3 cotton tipped sticks, 15 cm long per pig) upto 10 days post-challenge. PEDV was identified by the presence of the S gene in RT-PCR experiments (Kim et al., 2001; Song et al., 2006). The following primers were employed for amplification of PEDV: 5′-TTCTGAGTCACGAACAGCCA-3′ (forward), and 5′-CATATGCAGCCTGCTCTGAA-3′ (reverse). The size of the amplified product was 651 bp. For the positive control, we utilized cell-attenuated PEDV (KPED-9 strain) provided by the Virology Lab, National Veterinary Research and Quarantine Service of Korea. Reverse primer was used for the synthesis of complementary DNA. Three-step procedures were performed in a thermal cycler (Perkin–Elmer, Applied Biosystems, Inc., Foster City, CA). Samples were amplified using a program that consisted of: incubation at 94 °C for 5 min; followed by five cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; and 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 30 s, adding 1 s every cycle. At the completion of cycling, samples were kept at 72 °C for 7 min and then cooled. Specificity of PCR was evaluated using several causative agents of diarrhea, TGEV, porcine rotavirus, and bovine viral diarrhea virus.

2.6. Passage of DR13 (passage level 100) in colostrum-deprived 3-day-old pigs

DR13 in piglets was passaged as reported previously, with some modifications (Welter, 1998). The RFLP marker stability was assessed in vivo at passage level 100 of DR13. Colostrum-deprived 3-day-old pigs were employed in three consecutive passages (3, 3 and 2 pigs in order), allowing one pig as a control for each passage. At 3 days PI, a pig was sacrificed, and duodenum, jejunum and ileum were collected for gross histopathology and RT-PCR detection of virus (Kim et al., 2001). To prepare an inoculum for the next passage, a 10% (w/v) tissue suspension of small intestine was additionally prepared. The 5 ml suspension was fed orally to the next pig passage. The *Hin*dIII and *Xho*II-specific restriction pattern of the RFLP marker of shedding virus was analyzed, as described above.

2.7. Statistical analysis of immune response results

Results were expressed as means ± SD of at least two independent experiments. All data of immune response (SN test) were converted into log2 and used in statistical analysis. A one-way ANOVA using SAS version 8.0 software was employed for multiple comparisons, and a value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Antibody response of Vero cell attenuated PEDV DR13 in pregnant sows

IgG-specific ELISA results revealed no significant differences between the OD values of O- and IM-inoculated groups from farms A and B. However, a difference in OD values between vaccinated and control groups was evident. Interestingly, in IgA-specific ELISA, significant OD differences were observed between the sera of O- and IM-inoculated groups at delivery and colostrums (Figs. 1A and B) ($P < 0.05$). In colostrums samples from farm A and B, a difference in OD values between O and IM group was evident (O group: 0.515 ± 0.13, IM group: 0.226 ± 0.07). Moreover, OD values of all groups increased considerably after a second vaccination (Fig. 1B). An IgG-specific ELISA comparison of the OD values obtained from serum with those of colostrum of corresponding sows at delivery disclosed slightly higher values in colostrum, regardless of the inoculation route. Interestingly, in IgA-specific ELISA, the values of the O group were significantly increased in colostrum, compared to sera. In contrast, no major differences were observed between the two sample types (colostrum and sera) in the IM group. Furthermore, the antibody responses of sows in the SN test were distinct between samples of O and IM groups in the B farm (Figs. 2A and 2B).

Optical density (OD) values of colostrum samples from pregnant sows (farm D and E) orally inoculated with

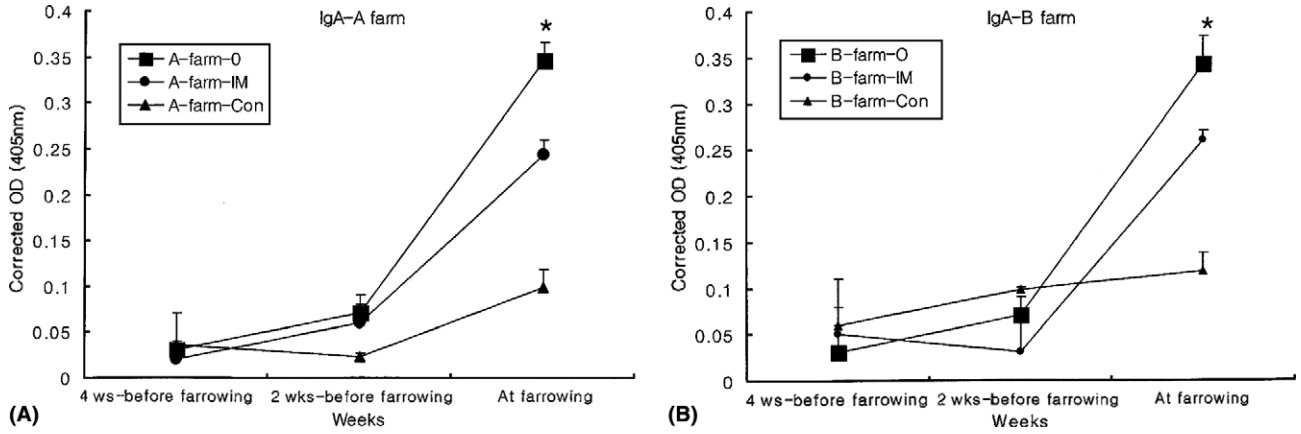

Fig. 1. Immune responses of serum samples from pregnant sows inoculated with Vero cell attenuated PEDV DR13, using IgA-specific ELISA (1A; A farm, 1B; B farm). $^{*}P < 0.05$.

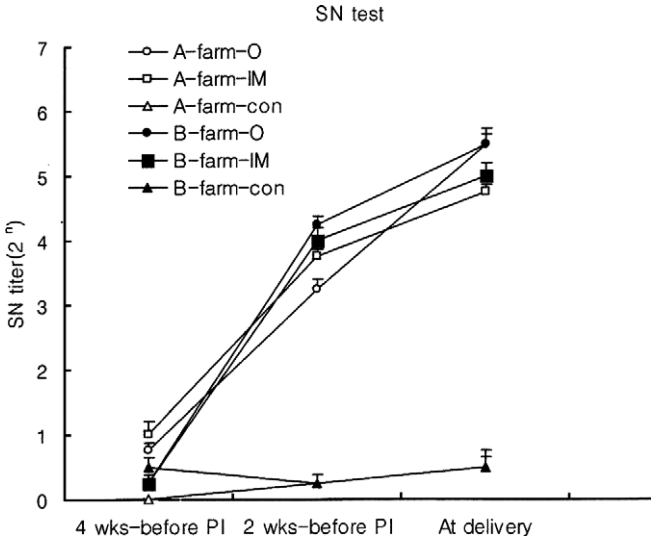

Fig. 2A. Antibody responses from serum samples observed with the SN test against the porcine epidemic diarrhea virus in pregnant sows. Titers are presented as means ± SD.

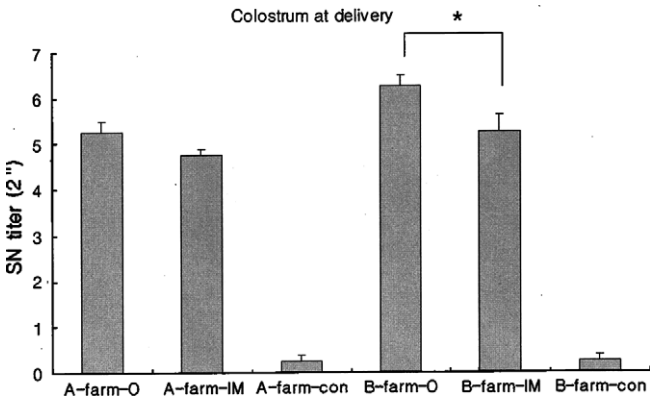

Fig. 2B. Antibody responses from colostrums observed with the SN test against the porcine epidemic diarrhea virus in pregnant sows. Titers are presented as means ± SD ($^{*}P < 0.05$).

cell-adapted PEDV DR13 ranged from 0.409 to 0.733 (0.53 ± 0.11 from 24 colostrum samples), while those of the control group were from 0.129 to 0.155 (0.151 ± 0.02 from 4 colostrum samples).

3.2. Antibody responses and mortality in 3-day-old piglets

The mortality of piglets after challenge with wild-type PEDV was 13% in the O-inoculated group (3/23), compared to 100% in the control group (14/14). In contrast, piglet mortality in the IM-inoculated group was 60% (9/15).

Antibody responses of 3-day-old piglets before challenge are presented in Figs. 3 and 4A. Piglets delivered from O-inoculated sows displayed a higher concentration of IgA and SN titer than those from IM-delivered sows ($P < 0.05$). However, no significant differences in the IgG level were observed between piglets from the O- and IM-inoculated groups.

3.3. Virus shedding in piglets after challenge exposure

There were no significant differences in virus shedding between the O- and IM-inoculated groups. Virus shedding in the Ogroup lasted for 3–4.3 days after challenge. In the IM group, shedding lasted for 4–5.3 days after challenge. However, the endpoint of virus shedding in the control group was not detected, due to mortality 3–6 days post-challenge.

3.4. Virus shedding through pig passage of PEDV DR13 in colostrum-deprived 3-day-old pigs

At the first passage, virus shedding lasted for 6 days, similar to data shown in Table 1. From the second passage, the duration of virus shedding was decreased to 4 days, and only lasted for 3 days in the third passage (Table 1). The

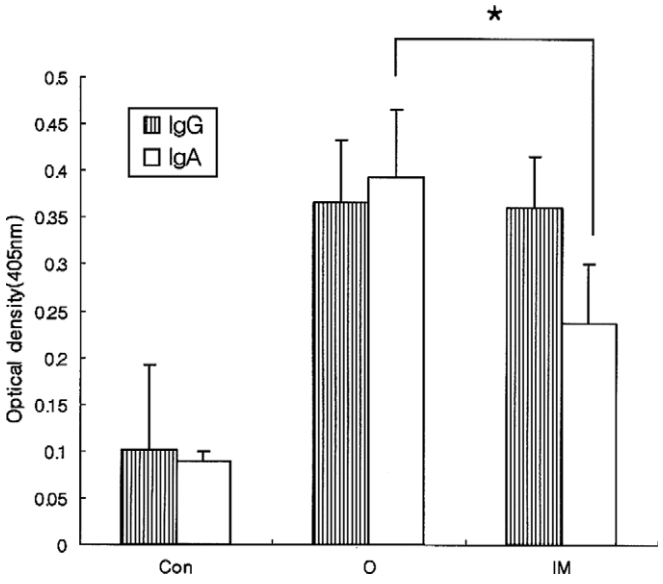

Fig. 3. Antibody responses from serum samples against porcine epidemic diarrhea virus in 3-day-old piglets using IgG and IgA-specific ELISA before challenge exposure ($^{*}P < 0.05$).

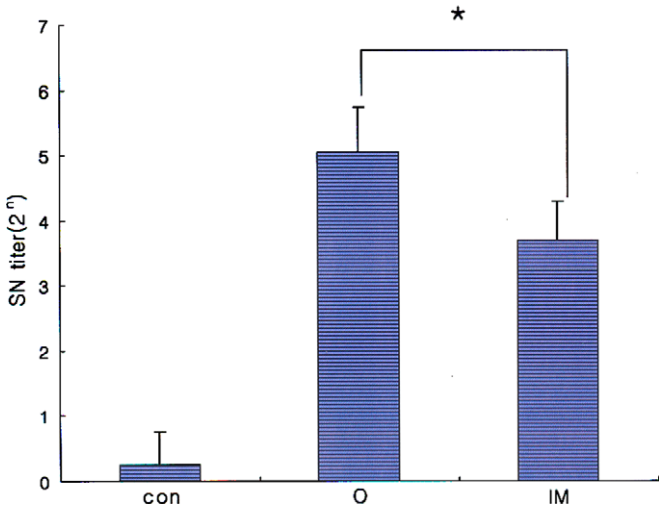

Fig. 4. Antibody responses from serum samples against porcine epidemic diarrhea virus in 3-day-old piglets using the SN test before challenge exposure ($^{*}P < 0.05$).

RFLP marker of the DR13 inoculum was maintained during passage, as identified by restriction enzyme patterns (*Hin*dIII and *Xho*II). In three consecutive passages, inoculated pigs remained clinically and histopathologically normal with no villous atrophy. Moreover, no reversion of virulence was observed during passage.

4. Discussion

Examination of the immunoprophylactic effect in pregnant sows after oral inoculation disclosed decreased mortality of piglets from vaccinated animals, indicating that cell-adapted DR13 induces immunity status. Previously, protective immunity was induced by an attenuated strain of PEDV inoculated intramuscularly (Kweon et al., 1999). PEDV DR13 was isolated through serial passage in Vero cell cultures, and differentiated from the wild-type strainusing RT-PCR RFLP with *Hin*dIII and *Xho*II enzymes (Song et al., 2003). Moreover, attenuation and safety of PEDV DR13 (passage level 100) after oral inoculation have been reported (Song et al., 2003). Since lactogenic immunity protects suckling pigs from PEDV infection, induction of mucosal immune responses in lactating pigs may be an effective way of protection. Efficacy studies on similar enteric diseases, such as bovine coronavirus, revealed that the modified live oral vaccine significantly protected calves from highly virulent challenges with newborn calf diarrheal coronavirus isolates (Welter, 1998). Highly attenuated PEDV (orally inoculated) conferred partial protection against challenge with the virulent virus in conventional pigs. This protection is related to the inoculation dose, and is enhanced with increasing concentrations (De Arriba et al., 2002).

In this study, we compare the efficacy of cell-adapted DR13 after inoculation via oral (O) and intramuscular (IM) routes, using the SN test and ELISA. Discrepancies between data obtained with ELISA and SN tests under our experimental conditions are possibly due to the addition of trypsin required for PEDV propagation, which degrades antibodies (Bae et al., 2003). The correlation of SN titer and ELISA was described previously (Oh

Table 1
RT-PCR detection of virus shedding in serial passages of colostrum-deprived 3-day-old pigs

Level of pig passage	Piglet No.	Days after oral inoculation							
		0	1	2	3	4	5	6	7
1	1-A	–	+/+[a]	+/+	+/+	+/+	+/+	+/+	–
	1-B	–	+/+	+/+	+/+[b]				
	Control	–	–	–	–	–	–	–	–
2	2-A	–	+/+	+/+	+/+	+/+	–	–	–
	2-B	–	+/+	+/+	+/+[b]				
	Control	–	–	–	–	–	–	–	–
3	3-A	–	+/+	+/+	+/+	–	–	–	–
	Control	–	–	–	–	–	–	–	–

[a] PEDV detection in feces using RT-PCR/RFLP patterns (*Hin*dIII and*Xho*II) of the virus.
[b] Pigs 1B and 2B were sacrificed at day 3 after oral inoculation, and small intestines collected for preparing the next oral inoculum.

et al., 2005). Briefly, the maximum agreement was obtained from the serum samples of 1: $\geqslant$ 32 of SN titer, however, the least was from the samples of 1:4 of SN titer. The ELISA has been shown to detect IgG, including maternal antibodies at a very low level. In contrast, the SN test detects both IgM and IgG antibodies. The SN test can detect maternal antibodies once the titers drop to level (1:<16) that are more difficult to interpret because of possible nonspecific reactions that can interfere with the test. The protective effects observed with high titers of IgA and SN from maternal colostrum are consistent with the findings of other reports (Ward et al., 1996). High titers of passive serum antibodies in colostrumfed conventional piglets are associated with reduced mortality, following inoculation of virulent viruses. The differences in mortality between the O and IM inoculated groups may be due to the IgA content in colostrum. In fact, the IgA concentration before challenge exposure in piglets delivered from the O-inoculated group was higher than that in the IM-inoculated group. The results indicate that IgA plays a more central role in preventing PED than IgG. Moreover, there is a strong correlation between the IgA concentration detected in blood and gut associated lymphoid tissue (De Arriba et al., 2002). Similarly, Yuan et al. (1996) reported that in pigs orally inoculated with rotavirus, the IgA antibody secretion response in blood was analogous to that in the gut.

In piglets, the extent of protection against PEDV is based on the presence of specific IgA antibodies in the milk of immune sows (De Arriba et al., 1995). After antigenic sensitization in the gut, IgA immunocytes migrate to the mammary gland, where they localize and secrete IgA antibodies into colostrum and milk. This ''gut mammary'' immunologic axis is an important concept in designing optimal vaccines to provide effective lactogenic immunity (Saif et al., 1972). Sows that are immune after infection with PEDV protect the suckling pigs through lactogenic immunity (Pensaert et al., 1994). Virus specific antigen stimulation of lymphocytes occurs during intestinal infection in the sow. The stimulated cells subsequently circulate in the body, mature to plasma cells, and migrate to the gut and settle in other organs, such as mammary gland. Secretory IgA production occurs at these sites. Lactogenic IgA-producing cells are thus stimulated in the intestine but produce IgA in the mammary gland itself. Pigs that regularly suckle the immune mother are constantly provided with milk-bound IgA antibodies in the lumen. This passive immunity is related to the presence of specific virus neutralizing antibodies in the gastrointestinal tract of suckling pigs. IgG accounts for more than 60% of the Ig in colostrum. However, IgA is more effective to neutralize the orally infected pathogens than IgG and IgM because IgA is more resistant to proteolytic degradation in the intestinal tract and has more virus neutralizing ability than IgG or IgM (Offit and Clark, 1985). Accordingly, we performed IgA-specific ELISA to elucidate the relationship between protection and IgA content in colostrum. Notably, variable piglet immunity is observed, according to the degree of colostrum uptake, litter size, antibody uptake, and quality of colostrum (Song et al., 2003). To determine the specific roles of IgA and IgG in preventing PED after vaccination, the changes in antibody concentrations in milk during lactation require investigation.

The duration of virus shedding after virulent challenge is an indication of the protective level (Yuan et al., 1998; Ward et al., 1996). Three of the farms selected for the investigation were PEDV-free and the piglets from those farms did not shed PEDV before challenge. Therefore, the virus shed after challenge exposure was possibly the same as inoculum with no RFLP marker. Earlier studies report that complete protection from PEDV infection prevents virus shedding after challenge exposure (De Arriba et al., 2002). High titers of serum antibodies in piglets are related to reduced severity and duration of diarrhea, and shorter period of virus shedding (Ward et al., 1996). Accordingly, virus shedding in piglets was assessed after challenge, using RT-PCR. No significant differences in duration of virus shedding were observed between O and IM groups. PEDV shedding may be variable, depending on the sensitivity of the detection tool. Moreover, shedding may be influenced by the viral strain employed. In the case of cell attenuated DR13, virus shedding lasted for 8 days, and that in sows for 3 days (Song et al., 2005). A comparison of the duration of virus shedding between vaccinated and control groups was not performed due to the death of control piglets after 3–6 days post-challenge. These results indicate that passive immunity by PEDV DR13 does not prevent virus shedding after challenge. Additionally, a study on reversion to virulence was conducted through serial pig passage. Cell-adapted DR13 PEDV remained safe, even after three passages in colostrum-deprived pigs. Accordingly, we propose that cell-adapted DR13 may be applied as a vaccine candidate under commercial farm conditions, although there is rising concern over the possibility of confounding factors introduced by genetic variation or housing variables (Hoblet et al., 1986). In conclusion, cell-adapted PEDV DR13 prevents mortality after challenge, and induces higher IgA concentrations in colostrum. This elevated IgA concentration may explain the reduced mortality in the O-inoculated group, compared to IM-inoculated and control groups. Further experiments, including lymphoproliferative response and antibody-secreting cells in lymphoid tissue of pigs after challenge, are required to improve PEDV immunity in pigs.

Acknowledgments

This work was supported by the Korea Research Foundation Grant (KRF-2002-070-C00069), and the Brain Korea 21 Project of the Ministry of Education & Human Resources Development, Republic of Korea.

References

Bae, J.L., Lee, J.G., Kang, T., Jang, H.S., Jang, Y.S., Yang, M.S., 2003. Induction of antigen specific systemic and mucosal immune responses by feeding animals transgenic plants expressing antigen. Vaccine 21, 4052–4058.

De Arriba, M.L., Carvajal, A., Lanza, I., Rubio, P., Carmens, P., 1995. Development of an ELISA for the detection of antibody isotypes against porcine epidemic diarrhea virus (PEDV) in sow's milk. In Immunology of viral infections. Proc. Congr. Eur. Soc. Vet. Virol. 3, 222–225.

De Arriba, M.L., Carvajal, A., Pozo, J., Rubio, P., 2002. Mucosal and systemic isotype-specific antibody responses and protection in conventional pigs exposed to virulent or attenuated porcine epidemic diarrhea virus. Vet. Immunol. Immunopathol. 85, 85–97.

Debouck, P., Pensaert, M., 1980. Experimental infection of pigs with a new porcine enteric coronavirus CV777. Am. J. Vet. Res. 41, 219–223.

Ducatelle, R., Coussement, W., Pensaert, M., Debouck, P., Hoorens, J., 1981. In vivo morphogenesis of a new porcine enteric coronavirus, CV777. Arch. Virol. 68, 35–44.

Hoblet, K.H., Saif, L.J., Kohler, E.M., Theil, K.W., Bech-Nielsen, S., Stitzlein, G.A., 1986. Efficacy of an orally administrated modified-live porcine origin rotavirus vaccine against postweaning diarrhea in pigs. Am. J. Vet. Res. 47, 1697–1703.

Hofmann, M., Wyler, R., 1988. Propagation of the virus of porcine epidemic diarrhea in cell culture. J. Clin. Microbiol. 26, 2235–2239.

Kadoi, K., Sugioka, H., Satoh, T., Kadoi, B.K., 2002. The propagation of a porcine epidemic diarrhea virus in swine cell lines. Microbiologica 25, 285–290.

Kim, S.Y., Song, D.S., Park, B.K., 2001. Differential detection of transmissible gastroenteritis virus and porcine epidemic diarrhea virus by duplex RT-PCR. J. Vet. Diagn. Invest. 13, 516–520.

Kusanagi, K., Kuwahara, H., Katoh, T., Nunoya, T., Ishikawa, Y., Samejima, T., Tajima, M., 1992. Isolation and serial propagation of porcine epidemic diarrhea virus infection in cell cultures and partial characterization of the isolate. J. Vet. Med. Sci. 54, 303–318.

Kweon, C.H., Kwon, B.J., Lee, J.G., Kwon, G.O., Kang, Y.B., 1999. Derivation of attenuated porcine epidemic diarrhea virus (PEDV) as vaccine candidate. Vaccine 17, 2546–2553.

Moon, H.W., 1978. Mechanism in the pathogenesis of diarrhea: a review. J. Am. Vet. Med. Assoc. 172, 443–448.

Offit, P.A., Clark, H.F., 1985. Protection against rotavirus induced gastroenteritis in a murine model by passively acquired gastrointestinal but not circulating antibodies. J. Virol. 54, 58–64.

Oh, J.S., Song, D.S., Yang, J.S., Song, J.Y., Moon, H.J., Kim, T.Y., Park, B.K., 2005. Comparison of an enzyme-linked immunosorbent assay with serum neutralization test for serodiagnosis of porcine epidemic diarrhea virus infection. J. Vet. Sci. 6 (4), 349–352.

Pensaert, M.B., 1999. Porcine epidemic diarrhea. In: Straw, B.E., D'Allaire, S., Mengeling, W.L., Taylor, D.J. (Eds.), Disease of Swine, Eighth ed. Iowa State University Press, Ames, IA, pp. 179–185.

Pensaert, M.B., Callebaut, P., Cox, E., 1994. Enteric coronaviruses of animals. In: Kapikian, A.Z. (Ed.), Viral Infections of the Gastrointestinal Tract, second ed. Marcel Dekker, New York, pp. 627–696.

Saif, L.J., Bohl, E.H., Gupta, R.K., 1972. Isolation of porcine immunoglobulins and determination of the immunoglobulin classes of transmissible gastroenteritis viral antibodies. Infect. Immun. 6, 600–609.

Saif, L.J., Wesley, R.D., 1999. Transmissible gastroenteritis and porcine respiratory coronavirus. In: Straw, B.E., D'Allaire, S., Mengeling, W.L., Taylor, D.J. (Eds.), Disease of Swine, Eighth ed. Iowa State University Press, Ames, IA, pp. 295–323.

Song, D.S., Yang, J.S., Oh, J.S., Han, J.H., Park, B.K., 2003. Differentiation of a Vero cell adapted porcine epidemic diarrhea virus from Korean field strains by restriction fragment length polymorphism analysis of ORF 3. Vaccine 21, 1833–1842.

Song, D.S., Oh, J.S., Kang, B.K., Yang, J.S., Song, J.Y., Moon, H.J., Kim, T.Y., Yoo, H.S., Jang, Y.S., Park, B.K., 2005. Fecal shedding of a highly cell adapted porcine epidemic diarrhea virus after oral inoculation in pigs. J. Swine Health Prod. 13 (5), 269–272.

Song, D.S., Kang, B.K., Lee, S.S., Yang, J.S., Moon, H.J., Oh, J.S., Ha, G.W., Jang, Y.S., Park, B.K., 2006. Use of an internal control in a quantitative RT-PCR assay for quantitation of porcine epidemic diarrhea virus shedding in pigs. J. Virol. Meth. 133 (1), 27–33.

Ward, L.A., Yuan, L., Rosen, B.I., To, T.L., Saif, L.J., 1996. Development of mucosal and systemic lymphoproliferative response and protective immunity to human group A rotavirus in a gnotobiotic pig model. Clin. Diag. Lab. Immunol. 3, 342–350.

Welter, M.W., 1998. Adaptation and serial passage of bovine coronavirus in an established diploid swine testicular cell line and subsequent development of a modified live vaccine. Adv. Exp. Med. Biol. 91, 707–711.

Wood, E.N., 1977. An apparent new syndrome of porcine epidemic diarrhea. Vet. Rec. 100, 243–244.

Yuan, L., Ward, L.A., Rosen, B.I., To, T.L., Saif, L.J., 1996. Systemic and intestinal antibody secreting cell responses and correlations of protective immunity to human rotavirus in a gnotobiotic pig model of disease. J. Virol. 70, 3075–3083.

Yuan, L., Kang, S.Y., Ward, L.A., To, A.W., Saif, L.J., 1998. Antibody-secreting cell response and protective immunity assessed in gnotobiotic pigs inoculated orally or intramuscularly with inactivated human rotavirus. J. Virol. 72, 330–338.