RESEARCH REPORT
SWINE HEALTH

Title: Evaluation of cross-protective immunity induced by porcine intestinal mesenchymal stromal cell-adapted PEDV – **NPB #14-282**

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Industry Summary:

Porcine epidemic diarrhea virus (PEDV) which was isolated in 2013 in the US is causing huge economic losses to Pork Industry. PEDV causes an acute enteric infection in pigs of all ages but infection is severe in suckling piglets causing up to 90-95% mortality in this age group. Therefore, there is immediate need to device control measures to control both these infections in pigs. A few commercial vaccines were granted conditional license for use in pigs in the US to control PEDV. However, their protective efficacy in field conditions is not known. In this proposal, we attempted to develop live attenuated vaccine by passaging virulent strain of PEDV in mesenchymal stromal (stem) cells (MSCs) isolated from duodenum of newborn piglets. We passaged KS 14-01 strain 40 times (KS-P40) in MSCs and examined the pathogenesis of passaged virus in neonatal pigs and protective efficacy of passaged virus against challenge with virulent field PEDV. Inoculation of KS-P40 in piglets caused mild diarrhea and significantly less virus shedding in the feces as compared to virulent PEDV-inoculated piglets. KS-P40 also induced the production of IgG and IgA antibodies and provided protection against clinical disease and reduced fecal virus shedding upon challenge with virulent field PEDV. Additional passages in MSCs may be required to obtain a complete attenuated live attenuated vaccine for PEDV.

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Keywords: porcine epidemic diarrhea virus (PEDV), mesenchymal stem cells, pathogenicity, cross-protection

Scientific Abstract
Since its first detection in April, 2013, porcine epidemic diarrhea virus (PEDV) is continuing to cause colossal economic losses to the US pork industry. PEDV causes an acute enteric infection in pigs of all ages but infection is severe in suckling piglets causing up to 90-95% mortality in this age group. PEDV replicates in enterocytes of small intestine and causes diarrhea, vomiting and dehydration. Recently, variants of original virulent PEDV were also isolated in the U.S., thus making vaccine development against this devastating disease even more challenging. In this study, we attempted to generate attenuate strain of PEDV by passaging in mesenchymal stromal (stem) cells (MSCs) isolated from the duodenum of newborn piglets. We passaged virulent PEDV Kansas 14-01 in MSCs 40 times (KS-P40). Next, we examined the pathogenesis of KS-P50 in 5-day-old piglets and examined the protective efficacy of KS-P40 against a virulent PEDV challenge. Compared to virulent PEDV, KS-P40 did not cause mortality in piglets and induced milder clinical signs and diarrhea and shed lower virus and for shorter duration in feces. KS-P40 also induced serum IgG, IgA and neutralizing antibodies and protected inoculated piglets against challenge virus-induced clinical disease. However, vaccinated challenged pigs shed virus in feces albeit at lower levels compared to non-vaccinated challenged pigs. These data indicate that additional passages in MSCs are needed to obtain a complete attenuate strain.

Introduction

Porcine epidemic diarrhea virus (PEDV) was first isolated in Belgium and the United Kingdom in 1978 and was later detected in other European countries, Canada and Asia (12). Most recently PEDV was detected in the United States in April, 2013 (13). PEDV belongs to the genus *Alphacoronavirus* of the family *Coronaviridae* and is single-stranded, positive sense RNA virus. PEDV causes highly contagious, acute intestinal infection resulting in diarrhea, vomiting and dehydration (9).

PEDV is prevalent in almost all swine-raising countries in Europe, and in China, Korea, and Japan. The seasonal outbreaks of PEDV in winter cause economic losses to pork producers. In Asia, live attenuated and killed vaccines are used with varying degree of protection. The efficacy of commercially available vaccines is limited in field conditions, and the protective immunity induced is insufficient due to the introduction of variant strains of PEDV (10). Recently, variants of original virulent PEDV were also isolated in the U.S. (15).

In the US, PEDV is continuing to cause colossal economic losses to the US pork industry. Similar to PEDV infection in Asia and Europe, PEDV strains in US cause an acute enteric infection in pigs of all ages but infection is severe in suckling piglets causing up to 90-95% mortality in this age group (13). PEDV replicates in enterocytes of small intestine and causes diarrhea, vomiting and dehydration (9).

Recently, we succeeded in adapting PEDV to grow in porcine duodenal mesenchymal stromal cells (D-MSCs). Additionally, we were also able to isolate virulent PEDV in these cells from the intestinal contents/rectal swabs of piglets obtained from the Ohio pig farms. The growth of PEDV in D-MSCs is a significant step towards the development of vaccine for the control of PEDV in the pig population in the US. In this study we passaged virulent Kansas 14-01 strain 40 times in D-MSCs and examined the pathogenesis and protective efficacy of MSC-passaged virus in pigs. Our hypothesis was that D-MSC adapted PEDV will replicate in intestinal MSCs and *due to antigen presentation properties of MSCs, PEDV antigen will be presented to immune cells that will stimulate protective immunity and provide protection against virulent PEDV.*

Objective: To examine the pathogenesis of MSC-adapted PEDV in pigs.

Materials and Methods

Passage of PEDV in D-MSCs:

The KS 14-01 strain (obtained from Dr. Gourapura from our department) of PEDV was used to passage in D-MSCs. For passaging, the cells were infected with PEDV at an MOI of 0.1 and cultured in serum-free DMEM supplemented with 0.25% bovine serum albumin (infection medium) and 0.5 µg/ml tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (infection medium) for 36 hr and then infected cultures were frozen and thawed 2 times and cellular debris was removed by centrifugation and supernatants were frozen in -80

(Passage 1). We passaged PEDV 40 times in D-MSC and examined the pathogenesis of passaged virus in piglets. We have isolated virulent PEDV from the RT-PCR positive for PEDV intestinal contents/rectal swabs (Obtained from Dr. Zhang, Oho Department of Agriculture) of piglets collected from the Ohio farm. Virulent local PEDV isolates were used as a positive control for comparing the pathogenesis of KS-P40 in piglets and in challenge studies.

Experimental Design:

Groups (n=8 in each group) of 5-day-old pigs born to PEDV-negative sows were inoculated orally with 250 $TCID_{50}$ of KS-P40 or virulent PEDV (OH12503) (4). Pigs were examined daily for clinical disease and diarrhea. At days 1-7 after inoculation rectal swabs were collected for the detection of virus in the feces by titration in Duck intestinal epithelial cells (MK-DIEC) (5) as described (6). At day 21 after inoculation, all pigs in virulent PEDV group and 4 pigs in KS-P40- inoculated group were euthanized and serum and small intestinal contents were collected for the detection of virus-specific IgG and IgA by ELISA (6). At 21 days post inoculation, 4 pigs in KS-P40 group and 3 pigs in non-vaccinated (NV) group were challenged with $1x10^4$ $TCID_{50}$ of virulent PEDV OH1147. Pigs were monitored for clinical signs and rectal swabs were collected from 1-6 days post challenge (dpc) for the detection and quantification of challenge virus by titration in DIEC cells. In addition, small and large intestinal tissues will be collected for the examination of microscopic lesions.

Detection of PEDV in rectal swabs:

Serial tenfold dilutions of rectal swab were prepared in DMEM supplemented with 0.5 µg of TPCK trypsin/ml. DIEC cells cultured in 96-well tissue-culture plates were inoculated with the dilutions and were examined for cytopathic effects after 48 h incubation at 37°C and virus NP was detected by Immunofluorescence assay using anti-PEDV Mab (Madgene Labs). Virus titers were calculated by Reed and Muench method.

Enzyme-linked immunosorbent assay for IgG and IgA detection:

PEDV specific (OH 1147) serum IgG and IgA antibodies were detected by ELISA as previously described (11). PEDV antigen for ELISA was prepared in DIEC cells. The viral antigen was concentrated and partially purified by ultracentrifugation on a 20% sucrose cushion. ELISA plates were coated with PEDV (5 µg/ml) diluted in a coating buffer (0.5 M carbonate bicarbonate buffer, pH 9.6). The coated plates were incubated overnight at room temperature (RT). After blocking the plates with blocking solution (5% skim milk in PBS) for 1 h at RT, serum samples dilutions 1:200, 1:800 and 1:3200 were added in the plates. IgG and IgA antibodies in test samples were then detected by adding HRP conjugated anti-porcine IgG or IgA antibodies and color was developed by adding 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. and the optical density (OD) was recorded at 450 nm.

Serum neutralization assay:

The serum neutralization test (SNT) was performed in DIEC as described previously (7). Twofold dilutions of serum from KS-P40 inoculated pigs were incubated with 50 $TCID_{50}$ OH 1147 virulent PEDV for 1 h at 37 C in a 96 well plate. After the incubation, virus and samples mixtures were transferred on to confluent monolayers of DIEC in 96 well plates.

After adsorption for 1 h, the inoculums were removed and fresh medium containing 0.5 µg/ml TPCK trypsin was added. The plates were incubated for 24 h and processed for immunocytochemistry. PEDV nucleoprotein (NP) was detected by using FITC-labeled mouse anti-NP Mab (Madgene labs).

Serum Neutralization (SN) titers were expressed as the reciprocals of the highest serum dilution causing 50% inhibition of PEDV.

Results:

Characteristics of pig duodenal mesenchymal stromal cells:

We isolated MSCs from the duodenum of piglets by collagenase digestion as described previously (8). Duodenal MSCs showed characteristic features of MSCs such as adherence to plastic surface, fibroblastic morphology, and expression of mesenchymal markers CD29, CD44 and CD90 on their surface (Fig. 1A and B).

Replication and passage of PEDV in D-MSC:

After confirming the purity of D-MSCs, next, we examined the replication of PEDV in MSCs. MSCs were infected with Kansas (KS) strain 14-01 of PEDV obtained from Dr. Gourapura from our department. MSCs were infected with the virus at MOI of 0.1.

After adsorption for 1 h, virus inoculum was removed and cells were cultured in infection medium. At 24 h post-infection (hpi), PEDV nucleoprotein (NP) was detected by using FITC-labeled anti-PEDV NP Mab (Fig. 2).

In PEDV infected MSCs CPE was visible around 20 hpi, CPE became more prominent in PEDV -infected MSCs at 24 hpi and extensive CPE characterized by syncytium formation and cellular detachment was observed at 36 hpi (Fig. 2). At 36 h, PEDV-infected MSC cultures were freeze-thawed two times and centrifuged to remove the cellular debris and supernatant was stored in -80 C labeled as passage 1. PEDV KS was passaged further 40 times in MSCs. KS-PEDV passsage 1 in MSCs had a titer of approximately $1x10^{4.5}$ $TCID_{50}$/ml in MSC, further passages of this virus in MSCs resulted in significant increase in the titer and passage 40 had a titer of $1x10^{6.5}$ $TCID_{50}$/ml.

Additionally, we successfully isolated PEDV (OH 12503 and OH 1147) in MSCs from the intestinal contents/rectal swabs of piglets (Fig. 2) from Ohio, obtained from our collaborator, Dr. Zhang at Ohio Department of Agriculture. We used local virulent PEDV strains as a positive control to compare the clinical signs and virus shedding in piglets and as a challenge virus to examine the protective efficacy of MSC-passaged KS-40 PEDV.

Pathogenesis of KS-P40 in commercial neonatal piglets:

We obtained 5 day-old confirmed PEDV negative OSU farm. Groups of piglets were inoculated orally with 250 $TCID_{50}$ of KS-P40 (n=8) or virulent field PEDV OH12503. Pigs in mock group (n-4) were inoculated with virus-free culture medium. Piglets inoculated with virulent PEDV started showing clinical signs and diarrhea from 2 dpi which continued up to 10 dpi and shed high titers of virus in rectal swabs starting from 1 dpi and continued to shed up to 7 dpi (last sampling point during acute phase of disease). One piglet in this group died on 8 dpi (Table 1). However, no virus in rectal swabs was detected at 21 dpi. All remaining piglets in this group were euthanized at 21 dpi. Piglets inoculated with KS-P40 also developed diarrhea at 2 dpi which continued for 4-5 days and piglets also shed virus in the rectal swabs starting from 1 dpi. The virus shedding in KS-P40-inoculated pigs were significantly lower than the virulent PEDV- inoculated piglets (Table 1). KS-P40 inoculated piglets also developed serum IgG, IgA and neutralizing antibodies against the challenge virus when examined at 21 dpi. Piglets in KS-P40 inoculated group had a mean virus neutralizing antibody titer of 192±74. Four piglets in this group were euthanized at 21 dpi and remaining 4 pigs were challenged with virulent field PEDV

OH1147. None of the piglet in mock-inoculated group developed clinical signs and diarrhea and no virus shedding was detected in rectal swabs.

Response of KS-P40-inoculated pigs to challenge with virulent PEDV:

At 21 DPI, KS-P40-inoculated pigs (n=4) and NV pigs (n-3) were challenged orally with $1x10^4$ $TCID_{50}$ of virulent OH 1147 PEDV. One NV non-challenged pig was also included to serve as a control. Pigs were monitored daily and rectal swabs were collected daily up to 6 dpc. NV pigs challenged with virulent developed diarrhea at 1 dpc which continued up to 6 dpc (last day of observation) and high titers of virus shedding were also observed in the rectal swabs of challenged pigs. Pigs in KS-P40-inoculated and challenged pigs did not develop diarrhea but virus shedding was detected starting from 2 dpc, however, virus shedding was significantly lower than the NV challenged pigs (Table 2).

Discussion:

In this study, we generated the partially attenuated strain of virulent PEDV (KS-P40) by serially passaging the virulent virus in D-MSC.KS-P40 had the following characteristics:

i) replicates to higher titers in D-MSCs

ii) did not cause mortality in neonatal pigs
iii) highly immunogenic and induced the production of virus-specific IgG, IgA and neutralizing antibodies
iv) protected inoculated piglets against the clinical disease caused by the virulent PEDV

Vero cell line is generally used for the isolation of PEDV from clinical samples, virus replication and vaccine production. More than 100 serial passages in vero cells are required to generate attenuated PEDV vaccine strains which are still not very effective in protecting pigs against PEDV outbreaks under field conditions (14). In this study, we have used cells from the homologous host to passage virulent PEDV and based on clinical and histopathological examination, KS-P40 is partially protective suggesting that a few additional passages may generate an attenuated strain with higher protective efficacy. MSCs are present in almost all organs of the body including intestines and possess the properties of self-renewal, differentiation, and immunoregulation (1). MSCs have also been shown to induce and regulate the proliferation and differentiation of epithelial cells including intestinal epithelial cells (3). More importantly, MSCs also possess antigen presentation potential (2) and due to this property these cells may better present the antigen to immune cells, thus, may induce the generation of protective immune response and may prove to be a better substrate for the production of live-attenuated and high tittered killed vaccines.

Summary: Inoculation of virulent PEDV passaged in MSCs in 5 day-old piglets did not cause mortality and virus shedding in feces was lower as compared to un-passaged virulent PEDV. Passaged virus induced IgG and IgA antibodies in the inoculated piglets and provided protection against the challenged virus induced clinical disease. However, virus shedding was observed albeit at a lower level as compared to non-vaccinated challenged pigs. Additional passages in MSCs will be required to obtain a complete attenuated strain of PEDV.

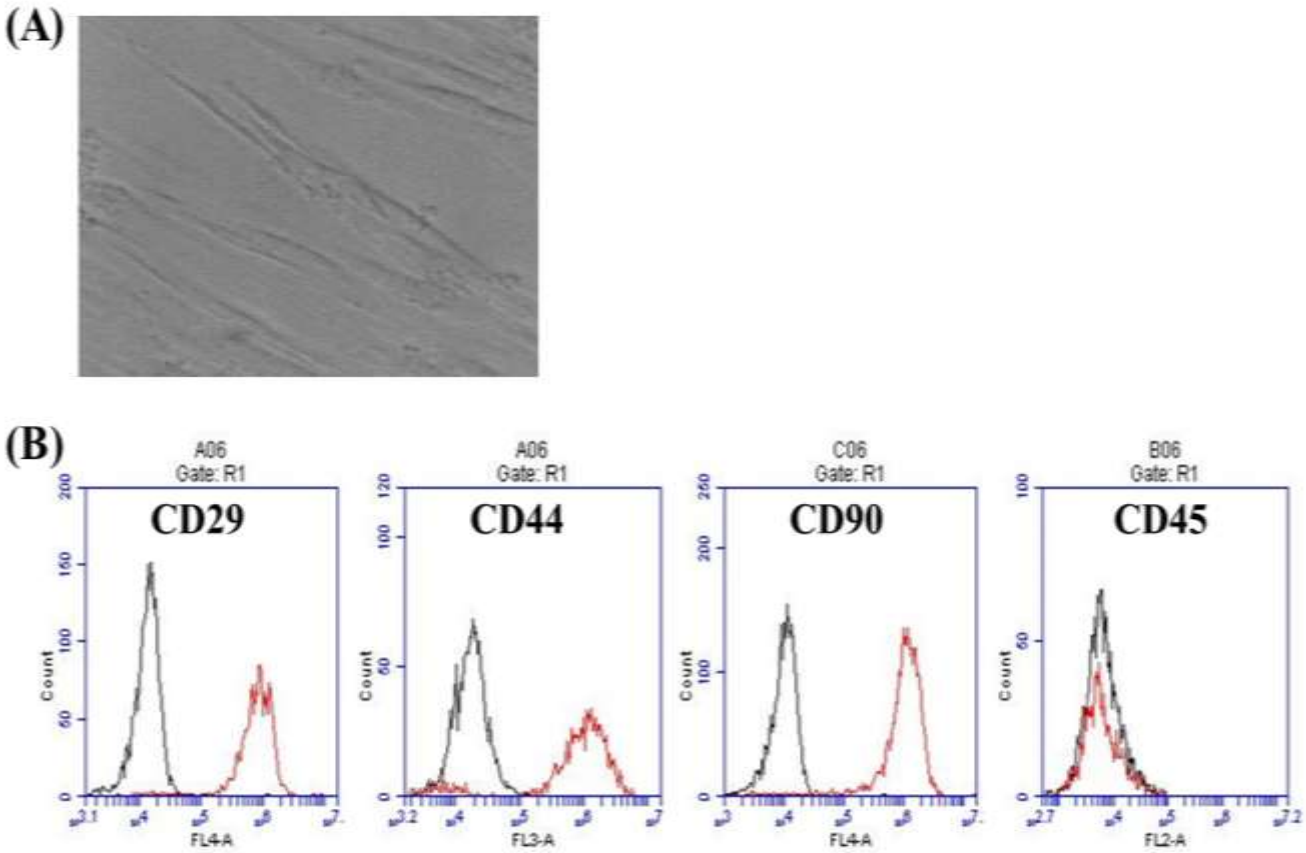

Fig. 1: Characteristics of duodenum derived mesenchymal stromal cells. Mesenchymal stromal cells were isolated from the duodenum of newborn piglets by collagenase digestion. Cells are spindle shaped **(A)** and express mesenchymal markers **(B)**.

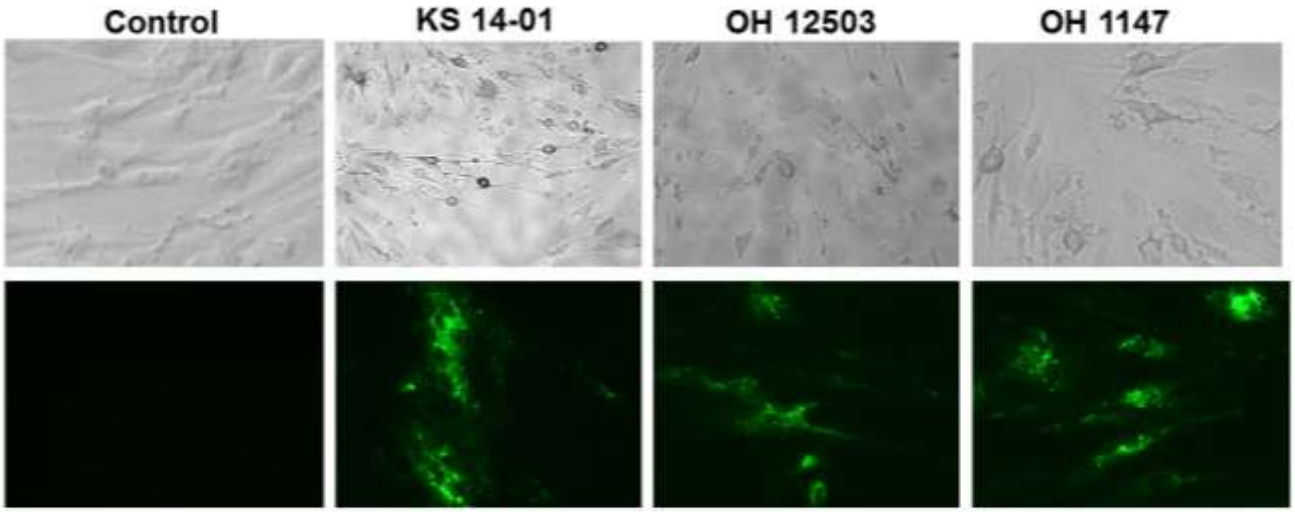

Fig. 2: Replication of PEDV. PEDV were isolated from the intestinal contents/rectal swabs of infected piglets in D-MSCs. PEDV KS-14-01 and field strains of PEDV grow well in D-MSCs when cells are infected in presence of 0.5 μg/ml TPCK-treated trypsin.

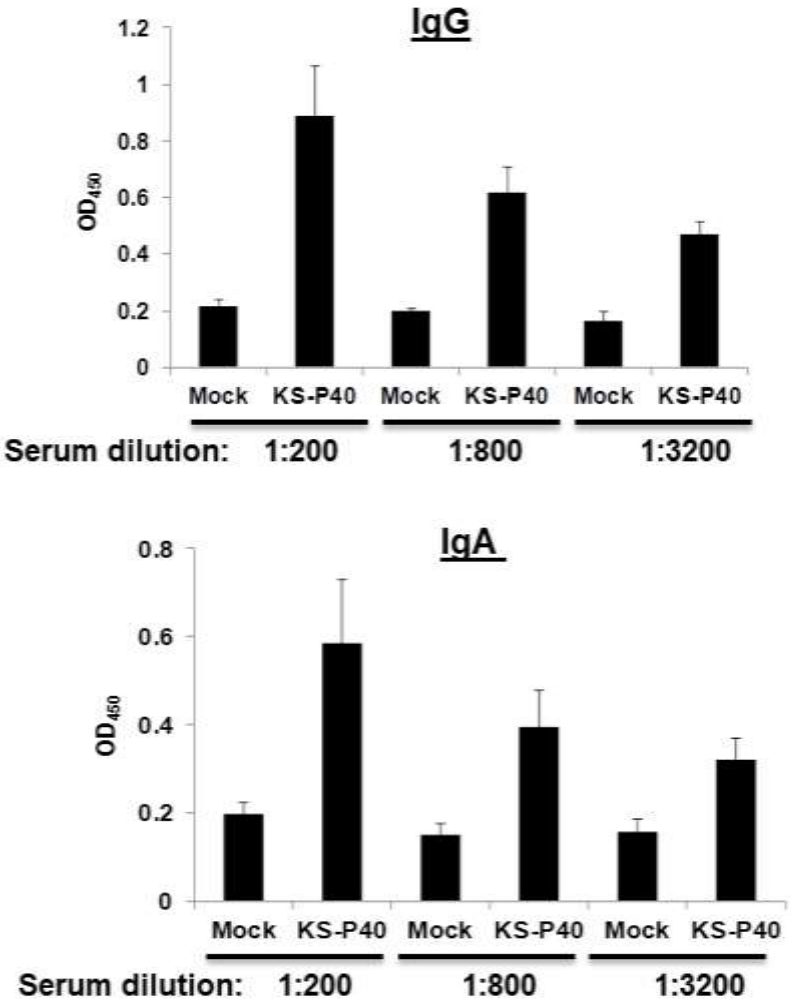

Fig. 3. D-MSC passaged KS-P40 induce the production of IgG and IgA antibodies. Five-day-old piglets were inoculated with KS-P40. At 21 days after inoculation, PEDV-specific IgG and IgA antibodies were detected in serum by ELISA. Each bar represents the mean ± SD OD values of 4 pigs.

Table 1. Clinical signs and fecal virus shedding of 5-day-old commercial piglets inoculated with virulent PEDV or KS-P40.

Observations	Pigs inoculated with virulent PEDV (OH12503) (n=8)	Pigs inoculated with KS-P40 (n=8)
Morbidity (%)	**100**	**100**
Mortality (%)	**12.5**	**0**
Onset of diarrhea (dpi)	**2**	**2**
Duration of diarrhea (day)	**8**	**4-5**
Onset of viral shedding (dpi)	**1**	**1**
Highest viral shedding titer (log_{10} $TCID_{50}$/ml)	**6**	**3.5**

Five-day-old piglets were inoculated with 250 $TCID_{50}$ of KS-P40 (n-8) or virulent field PEDV OH12503. Piglets were monitored for clinical signs and rectal swabs were collected from the piglets up to 7 dpi and at 21 dpi. Virus titers in rectal swabs were determined by titration in DIEC cells.
dpi - days post inoculation

Table 2. Clinical signs and fecal virus shedding in KS-P40 vaccinated piglets challenged with virulent PEDV

	Diarrhea rate	Onset of diarrhea (dpc)	Duration of diarrhea (days)	Onset of viral shedding (dpc)	Highest viral shedding titer (log_{10} $TCID_{50}$/ml)
Non-Vacc Challenged (n=3)	**100**	**1**	**6**	**1**	**5.5**
KS-P40 Vacc and Challenged (n=4)	**0**	**0**	**0**	**2**	**2.5**
Control NonVacc and non-Challenged (n=1)	**0**	-	-	-	-

Five-day-old piglets were inoculated with KS-P40. At 21 days after inoculation, pigs were challenged with $1x10^4$ $TCID_{50}$ of virulent OH 1147 PEDV. Pigs were monitored daily and rectal swabs were collected daily up to 6 dpc.
dpc-days post challenge

References:

1. **da Silva Meirelles, L., P. C. Chagastelles, and N. B. Nardi.** 2006. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci **119:**2204-13.
2. **Francois, M., R. Romieu-Mourez, S. Stock-Martineau, M. N. Boivin, J. L. Bramson, and J. Galipeau.** 2009. Mesenchymal stromal cells cross-present soluble exogenous antigens as part of their antigen-presenting cell properties. Blood **114:**2632-8.
3. **Gregoire, C., C. Lechanteur, A. Briquet, E. Baudoux, F. Baron, E. Louis, and Y. Beguin.** 2017. Review article: mesenchymal stromal cell therapy for inflammatory bowel diseases. Aliment Pharmacol Ther **45:**205-221.
4. **Jung, K., Q. Wang, K. A. Scheuer, Z. Lu, Y. Zhang, and L. J. Saif.** 2014. Pathology of US porcine epidemic diarrhea virus strain PC21A in gnotobiotic pigs. Emerg Infect Dis **20:**662-5.
5. **Khatri, M.** 2015. Porcine epidemic diarrhea virus replication in duck intestinal cell line. Emerg Infect Dis **21:**549-50.
6. **Khatri, M., V. Dwivedi, S. Krakowka, C. Manickam, A. Ali, L. Wang, Z. Qin, G. J. Renukaradhya, and C. W. Lee.** 2010. Swine influenza H1N1 virus induces acute inflammatory immune responses in pig lungs: a potential animal model for human H1N1 influenza virus. J Virol **84:**11210-8.
7. **Kusanagi, K., H. Kuwahara, T. Katoh, T. Nunoya, Y. Ishikawa, T. Samejima, and M. Tajima.** 1992. Isolation and serial propagation of porcine epidemic diarrhea virus in cell cultures and partial characterization of the isolate. J Vet Med Sci **54:**313-8.
8. **Lanzoni, G., F. Alviano, C. Marchionni, L. Bonsi, R. Costa, L. Foroni, G. Roda, A. Belluzzi, A. Caponi, F. Ricci, P. Luigi Tazzari, P. Pagliaro, R. Rizzo, F. Lanza, O. Roberto Baricordi, G. Pasquinelli, E. Roda, and G. Paolo Bagnara.** 2009. Isolation of stem cell populations with trophic and immunoregulatory functions from human intestinal tissues: potential for cell therapy in inflammatory bowel disease. Cytotherapy **11:**1020-31.
9. **Madson, D. M., D. R. Magstadt, P. H. Arruda, H. Hoang, D. Sun, L. P. Bower, M. Bhandari, E. R. Burrough, P. C. Gauger, A. E. Pillatzki, G. W. Stevenson, B. L. Wilberts, J. Brodie, K. M. Harmon, C. Wang, R. G. Main, J. Zhang, and K. J. Yoon.** 2014. Pathogenesis of porcine epidemic diarrhea virus isolate (US/Iowa/18984/2013) in 3-week-old weaned pigs. Vet Microbiol.
10. **Pan, Y., X. Tian, W. Li, Q. Zhou, D. Wang, Y. Bi, F. Chen, and Y. Song.** 2012. Isolation and characterization of a variant porcine epidemic diarrhea virus in China. Virol J **9:**195.
11. **Park, S., K. Sestak, D. C. Hodgins, D. I. Shoup, L. A. Ward, D. J. Jackwood, and L. J. Saif.** 1998. Immune response of sows vaccinated with attenuated transmissible gastroenteritis virus (TGEV) and recombinant TGEV spike protein vaccines and protection of their suckling pigs against virulent TGEV challenge exposure. Am J Vet Res **59:**1002-8.
12. **Song, D., and B. Park.** 2012. Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccines. Virus Genes **44:**167-75.
13. **Stevenson, G. W., H. Hoang, K. J. Schwartz, E. R. Burrough, D. Sun, D. Madson, V. L. Cooper, A. Pillatzki, P. Gauger, B. J. Schmitt, L. G. Koster, M. L. Killian, and K. J. Yoon.** 2013. Emergence of Porcine epidemic diarrhea virus in the United States: clinical signs, lesions, and viral genomic sequences. J Vet Diagn Invest **25:**649-54.
14. **Sun, D., X. Wang, S. Wei, J. Chen, and L. Feng.** 2015. Epidemiology and vaccine of porcine epidemic diarrhea virus in China: a mini-review. J Vet Med Sci **78:**355-63.
15. **Wang, L., B. Byrum, and Y. Zhang.** 2014. New variant of porcine epidemic diarrhea virus, United States, 2014. Emerg Infect Dis **20:**917-9.