

PEDV Research Updates 2013

Porcine Epidemic Diarrhea virus (PEDV) has caused significant challenges to the swine industry. The virus had not been previously identified in the United States prior to April of 2013. To assist producers and their veterinarians in the management, control and potential elimination of the virus, the National Pork Board funded key research projects to better understand PEDV. In order to provide timely information to producers from those projects, the objectives and initial updates will be periodically reported.

NOTE: The updates from the proposal represent interim information only and are not intended to be a final report. The final and formal reports will be provided at the end of the terms of the projects and then posted online at pork.org. The update information is intended to inform stakeholders of progress but are not intended to be the final outcome. For further information, please contact Dr. Lisa Becton at lbecton@pork.org.

#13-222: The Ohio State University

Propagation of PEDV in tissue culture and development of standardized reference samples for use in diagnostic testing

Objectives:

Objective 1. Development and validation of diagnostic testing for PEDV antigen and antibody detection

Objective 1.2. Development of standardized reference samples that can be utilized by Veterinary Diagnostic Labs (VDLs) for diagnostic test validation

Objective 1.3. Develop and validate antibody-based diagnostic tests for serologic monitoring and surveillance

Final Update: 12-18-13

Propagation of PEDV in tissue culture and development of standardized reference samples for use in diagnostic testing

Dec. 18, 2013 Final report

PI and Co-PI: Qihong Wang and Linda J. Saif

The Ohio State University

1. We have successfully passaged one Ohio PEDV strain PC22A in Vero cells. Currently PC22A strain replicates in Vero cells consistently and infectious virus titers reach 5-6 \log_{10} 50% tissue culture infectious dose (TCID₅₀) or plaque forming units (PFU) per mL. **The isolation of PEDV in cell culture is the first step toward the development of an attenuated vaccine. Our tissue cultured (TC) PEDV has adequate infectious titers to be developed as an inactivated booster vaccine to enhance lactogenic immunity in sows previously infected with PEDV to control endemic PED. It also can be used to evaluate disinfection/decontamination efficiency *in vitro* to promote the control of virus spread.** Our long term goal is to continue to passage this strain to develop a live attenuated PEDV vaccine for use in naïve PEDV sero-negative sows to protect their nursing piglets against epidemic PED.

2. **We have plaque-purified PC22A and generated a virus pool with high infectious titers (7.7 \log_{10} PFU/mL) in germfree pigs.** It can be used as challenge pool in future vaccine studies.

3. We have developed conventional reverse-transcription (RT)-PCR and real-time RT-PCR (RT-qPCR) for the detection of PEDV nucleic acids, TC PEDV-based immunofluorescent assay (IFA) and plaque reduction virus neutralization assay (VN) for the detection of isotype (IgG) and virus neutralizing antibodies, respectively, from pig serum samples. **Such assays are essential to: 1) screen fecal and serum samples from swine herds for PEDV prevalence; 2) detect antibodies to PEDV to determine pigs' PEDV infection status or to permit their export if PEDV sero-negative status is required; and 3) evaluate vaccines to induce protective immunity.**

4. We have investigated the pathogenesis of US PEDV in nursing germfree pigs. By using germfree animals, the precise role of a single pathogen in the disease process can be evaluated. We found that the germfree pig-passaged Ohio PEDV PC21A strain were free of other bacteria and enteric viruses. The virus pool can be used as challenge pool in the future vaccine studies. The infected pigs exhibited acute severe diarrhea/vomiting between 24-48 hpi, followed by dehydration and collapse. Pathologic lesions were limited to the small and large intestines, although mainly in the jejunum and ileum. Severe atrophic enteritis was identified microscopically. All infected pigs had viral RNA detected in feces and serum. Overall, our data suggest that the **US PEDV PC21A strain acutely infects the entire intestine, leading to severe atrophic enteritis, and is highly enteropathogenic.**

Are there steps that producers can take to incorporate this into daily management steps?

The TC PC22A with high infectious titers can be used to evaluate the efficiency of disinfection/decontamination methods in vitro based on virus infectivity assays, thereby helping to control virus spread. Cleaning and disinfection of trucks and trailers and the farm premises are widely practiced in the swine industry and post-disinfection assessment is important. Currently RT-PCR is used to assess disinfection efficiency. Because many disinfectants may inactivate PEDV, but do not disrupt the viral RNA, RT-PCR usually underestimates the disinfection efficiency. This results in samples testing positive when in fact the virus has been inactivated, meaning pork producers must treat RT-PCR positive vehicles as a threat to the health of their herds. This situation compromises production efficiently by resulting in extra cleaning and disinfecting and equipment downtime. The TC PEDV and infectivity assays could be used to perform disinfection studies *in vitro* to provide efficient PEDV disinfection protocols for the pork industry.

What are the remaining gaps in knowledge for the project?

We need to know whether there are mutations in the genome of PEDV PC22A strain during passage in Vero cells. Also unknown is at which passage level the TC PC22A will become attenuated and can be used as a live vaccine candidate. Which gene is most related to virulence? Is there cross-reactivity among US PEDV strain PC22A, the European PEDV strain CV777, and TGEV? We also need to examine immunity to PEDV infections in different ages of pigs, and the mechanisms for why PEDV is so deadly in suckling pigs, but causes low mortality in older pigs.

Publications:

1. Kwonil Jung*, Wang, Q.H.*, Scheuer, K.A., Lu, Z., Zhang, Y., Saif, L.J.* Pathogenesis of a US strain of porcine epidemic diarrhea virus (PEDV) in gnotobiotic pigs. *Emerging Infectious Diseases*. (Submitted). (*corresponding authors)
2. Wang, T. Oka, S. M. Esseili, Sommer-Wagner, T. Meulia, Y. Zhang, L. J. Saif. Isolation and characterization of porcine epidemic diarrhea virus (PEDV) for development of serologic tests and vaccine. 2013 North American PRRS Symposium. Abstract #55. Chicago, IL. December 7-8, 2013.

References

1. **Chen, Q., G. Li, J. Stasko, J. T. Thomas, W. R. Stensland, A. E. Pillatzki, P. C. Gauger, K. J. Schwartz, D. Madson, K. J. Yoon, G. W. Stevenson, E. R. Burrough, K. M. Harmon, R. G. Main, and J. Zhang.** 2013. Isolation and Characterization of Porcine Epidemic Diarrhea Viruses Associated with the 2013 Disease Outbreak in US Swine. *Journal of clinical microbiology*.
2. **Song, D. S., J. S. Yang, J. S. Oh, J. H. Han, and B. K. Park.** 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.
3. **Zhang, X., M. Hasoksuz, D. Spiro, R. Halpin, S. Wang, S. Stollar, D. Janies, N. Hadya, Y. Tang, E. Ghedin, and L. Saif.** 2007. Complete genomic sequences, a key residue in the spike protein and deletions in nonstructural protein 3b of US strains of the virulent and attenuated coronaviruses, transmissible gastroenteritis virus and porcine respiratory coronavirus. *Virology* **358**:424-435.

Update: 11-27-13

PI and Co-PI: Qihong Wang and Linda J. Saif
The Ohio State University

We have successfully passaged the US PEDV isolate in Vero cells for 13 passages. Virus infectivity assays by observation of cytopathic effect (CPE) (titers in TCID₅₀/mL) and plaque assay [titers in plaque forming units (PFU)/mL] have been optimized. Several passages of tissue culture-adapted (TC) PEDV PC22A strains have been titrated using the two methods in parallel. We found that the two methods gave similar titers. Fig.1 showed the virus titers and ΔCt values of PEDV PC22A strain at passage levels (by P11) in Vero cells.

We have plaque-purified the virus which will be propagated and titrated soon. Immunofluorescent assay (IFA) for the detection of isotype antibodies (IgG) was performed successfully (Fig. 2). Two convalescent serum samples from pigs recovered from PED were titrated as 64 and 256, respectively. Viral neutralization test for the detection of neutralizing antibodies are under development. Such assays are essential to screen sera from swine herds for neutralizing antibodies to PEDV to determine their PEDV infection status or to permit their export if PEDV sero-negative status is required.

See Figures 1 and 2 on next page.

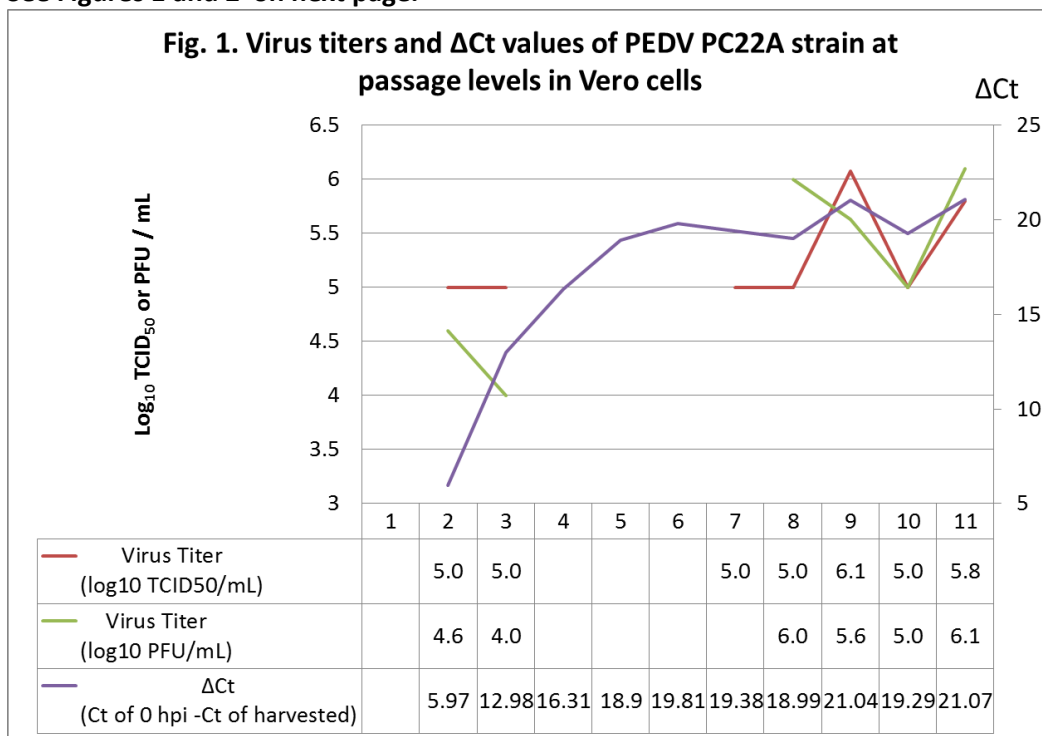
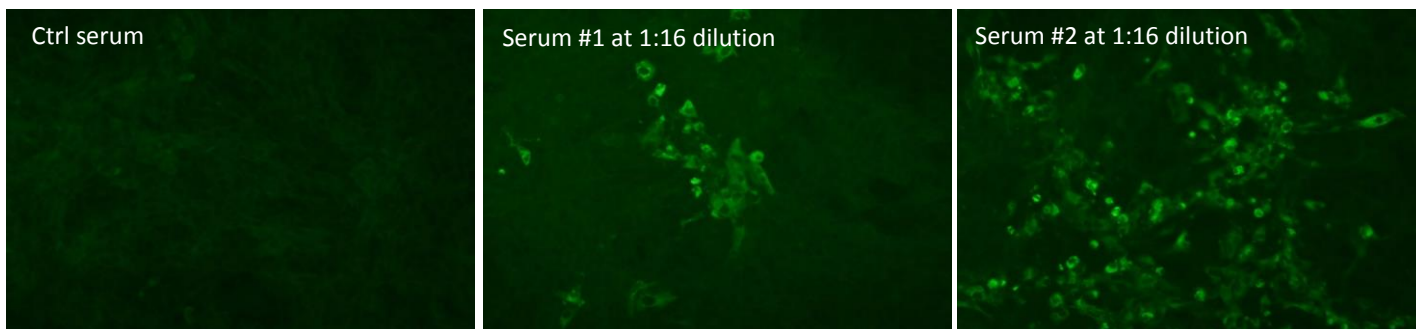


Fig. 2. IFA for the detection of IgG antibodies anti-PEDV from pig convalescent serum samples



Update: 11-13-13

No new report this week. The project is progressing as stated.

Update: 10-29-13

Biweekly report for NPB Project #13-222: Propagation of PEDV in tissue culture and development of standardized reference samples for use in diagnostic testing

PI and Co-PI: Qihong Wang and Linda J. Saif

The Ohio State University

We have passaged the US PEDV isolate in Vero cells for 10 passages. Viral RNA titers increased steadily up to 5-6 log₁₀-fold in the presence of 10 µg/mL trypsin (Invitrogen) after the 4th passage by quantitative real time RT-PCR. The 7th and 8th passages of virus were titrated as 5 log₁₀ TCID₅₀/mL by observing cytopathic effects. We have successfully conducted a plaque assay once. Figure 1 shows the PEDV particles (arrow heads) on the cell surface and in a vesicle (arrow) containing coronavirus particles inside the infected Vero cell by transmission electron microscopy.

Next, we will plaque-purify the virus and to develop a viral neutralization test for the detection of neutralizing antibodies. Such assays are essential to screen sera from swine herds for neutralizing antibodies to PEDV to determine their PEDV infection status or to permit their export if PEDV sero-negative status is required.

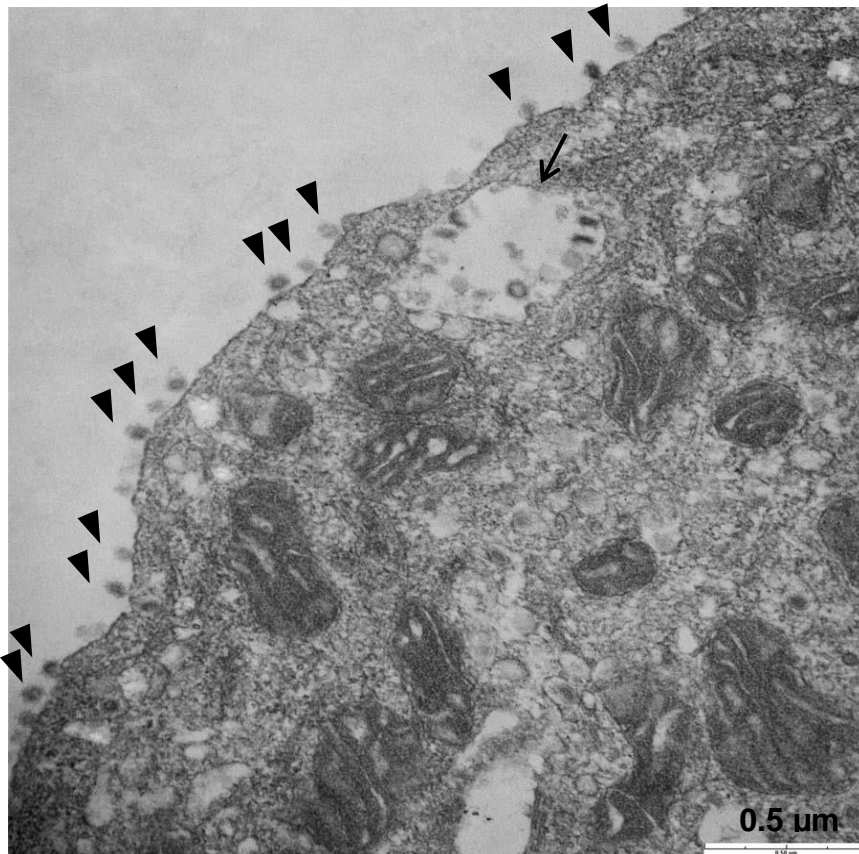


Fig. 1. Transmission electron micrograph of a PEDV-infected Vero cell.

Update: 10-16-13

The project is in process and the next report will be available October 23rd, 2013.

Update: 10-3-13

We have passaged the US PEDV isolate in Vero cells for 6 passages. Viral RNA titers increased up to 5-6 log₁₀-fold in the presence of 10 μg/mL trypsin (Invitrogen) for the 5th and 6th passages by quantitative real time RT-PCR (Table 1).

Table 1. US PEDV PC22A strain grown in Vero cells at the presence of Trypsin tested by RT-qPCR

Pass. #	Trypsin	0 hpi (Ct)*	2 dpi lysates** (Ct)	4 dpi lysates** (Ct)	Δ Ct	Fold increase of RNA ($2^{\Delta Ct}$)	Clear cytopathic effect (CPE)
1st	with	NT#	NT	13.54	N/A§	N/A	Yes
	w/o	NT	NT	24.43	N/A	N/A	No
2nd	with	18.95	12.98	NT	5.97	63	Yes
	w/o	20.00	17.28	NT	2.72	7	Yes/No
3rd	with	25.01	NT	12.03	12.98	8,079	Yes
	w/o	24.98	NT	23.93	1.05	2	No
4th	with	28.79	NT	12.48	16.31	81,245	Yes
5th	with	29.72	NT	10.82	18.90	489,178	Yes
6th	with	31.05	NT	11.24	19.80	919,188	Yes

* Culture supernatant was sampled at 0 hour-post-inoculation (hpi).

** Cell cultures were frozen at 2 or 4 days post-inoculation (dpi) and thawed once for RNA extraction.

NT: not tested.

§ N/A: not available.

Update: 9-21-13

1. The germfree pig-passaged US PEDV pool was tested negative for other enteric viruses by RT-PCR or PCR. The tested viruses include porcine rotavirus groups A, B and C, sapoviruses (GIII, GV, GVI, GVII, GVIII, GX, GXII, and GXIII), norovirus and St-Valerien-like virus, bocavirus, kobuvirus, and astrovirus. The last three viruses were tested by Veterinary Diagnostic lab of UMN. The virus pool can be used as a challenge virus in a vaccine study.
2. We have passaged the US PEDV isolate in Vero cells for 4 passages and still have high virus titers (Table 1).

Table 1. One US PEDV strain grown in one Vero-BI at the presence of Trypsin tested by RT-qPCR

Pass. #	Trypsin (10 µg/mL)	0 hpi (Ct)*	2 dpi lysates** (Ct)	3 dpi lysates** (Ct)	4 dpi lysates** (Ct)	Δ Ct	Fold increase of RNA ($2^{\Delta Ct}$)	Cytopathic effect
1st	with	NT#	NT	NT	13.54	N/A§	N/A	Yes
	w/o	NT	NT	NT	24.43	N/A	N/A	No
2nd	with	18.95	12.98	NT	NT	5.97	63	Yes
	w/o	20.00	17.28	NT	NT	2.72	7	Yes/No
3rd	with	25.01	NT	NT	12.03	12.98	8079	Yes
	w/o	24.98	NT	NT	23.93	1.05	2	No
4th	with	28.79	NT	NT	12.48	16.31	81245	Yes

* Culture supernatant was sampled at 0 hour-post-inoculation (hpi).

** Cell cultures were frozen at 2-4 days post-inoculation (dpi) and thawed once and used for RNA extraction.

NT: not tested.

§ N/A: not available.

Update 9-4-13:

The project attempted to propagate virus from several PEDV-positive field fecal samples and germfree pig. Virus was passaged in different cell types and the Ohio sourced strain could survive for three passages. The project will continue to passage this PEDV strain to ensure PEDV titers do not decrease during passaging and to attenuate the wild-type PEDV virulence. Then the virus will be titrated and purified and PEDV growth curve will be analyzed. The ability to grow and propagate the virus in cells is critical in order to develop further diagnostic tests and potential vaccines.

Quick Take

- PEDV strains need to be cultivated to help create testing capabilities.

Update: 8-31-13

1. **Screened for PEDV-positive field pig fecal samples that were negative for other porcine enteric viruses**, such as Transmissible Gastroenteritis Virus (TGEV), Porcine Respiratory Coronavirus (PRCV), rotavirus group A (Rota-A), rotavirus group C (Rota-C). These field samples will be the first choice for cell culture adaptation and making virus pools in germfree pigs.

Representative PEDV-positive samples were confirmed by sequence analysis.

First, a conventional reverse transcription (RT)-PCR was designed based on the N gene sequences of new US PEDV strains. Forward primer PEDN219 (5'-GCATTCTACTACCTCGGAACA) and reverse primer PEDN557 (5'-CTCCACGACCCTGGTTATTT) were designed to amplify a 338 nt-fragment. Samples from field pigs (commercially-raised pigs) were received from different Midwestern farms and were analyzed for different viruses – PEDV, TGEV, PRCV, Rota-A, and Rota-C using conventional RT-PCR [1-3]. Out of the field diarrheic samples tested, it was determined that 19 were positive for PEDV, 9 were positive for Rota-A, 12 were positive for Rota-C, and no samples were positive for either PRCV or TGEV. When considering mixed infections (co-infections), 7 samples were positive for both PEDV and Rota-C, while 3 samples were positive for all three – PEDV, Rota-A, and Rota-C. This co-infection data is summarized in Figure 1.

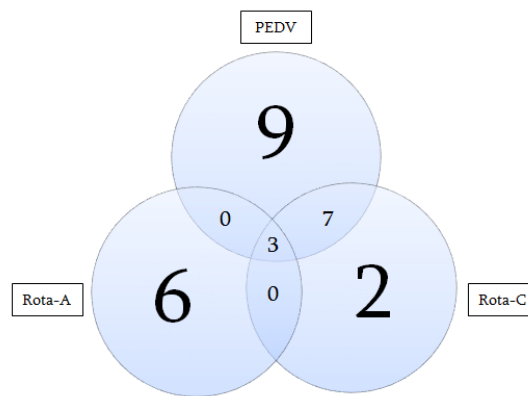


Figure 1. Porcine field sample viral co-infection

2. **Developed a sensitive and specific real-time reverse transcription PCR (RT-qPCR) for the titration of PEDV RNA.** A new TaqMan RT-qPCR assay was developed, specifically for sensitive and selective PEDV detection. Forward primer PEDNFnew (5'-CGCAAAGACTGAACCCACTAAC), reverse primer PEDNR (5'-TTGCCTCTGTTGTTACTTGGAGAT), and probe PEDprobe (FAM-TGYACCAAYACCACGACTCCTGC-BHQ3) were designed based on the partial N gene sequences of new US PEDV strains and previous publication [4].

The RT-PCR products of primer pair PEDN219/557 was used to generate a standard curve for the calculation of template RNA titer. The detection limit is 10 genomic equivalents (GE) per reaction. It has been used to detect PEDV from field and germfree pig fecal samples, cell culture supernatants and cell lysates, and from field and germ-free pig serum samples.

3. **Tested different culture supplemental enzymes and concentrations to propagate US PEDV strains in cell culture.** This was first performed with two PEDV-positive field fecal samples (PC21A and PC15) in Primary Porcine Kidney (PPK) cells and Vero-81 (ATCC #CCL-81) cells. The enzymes tested include trypsin (Invitrogen) at 0, 2.5, 10 and porcine pancreatin (Sigma) at 37.5 µg/mL. There was no significant difference in PEDV replication between all conditions based on viral RNA titers by real time RT-qPCR. Then we tested one PEDV-positive field fecal sample (PC21A) in Vero-81 cells with different enzymes, including trypsin (Invitrogen) at 100 µg/mL,

porcine pancreatin at 75 µg/mL and 100 µg/mL, and L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) at 2, 4, 10 and 100 µg/mL. There was no significant difference in PEDV replication between all conditions based on viral RNA titers by real time RT-qPCR. Based on cell monolayer integrity we decided to use 10 µg/mL trypsin (Invitrogen) for the following trials.

4. **Passaged different field samples in different cell lines.** Four PEDV-positive field fecal samples from three states (KS/PC10, OH/PC15, OH/PC21A and OK/PC23) were TGEV-, PRCV-, rota A- and rota C-negative. They were passaged in 4 Vero cell lines: Vero-E6 (also known as Vero C1008, ATCC #CRL-1586), Vero-76 (ATCC #CRL-1587), Vero-81, and Vero-BI (a gift from a biological company) and PPK cells supplemented with 10 µg/mL trypsin and 37.5 µg/mL pancreatin for 2-3 passages (Table 1). There is no significant PEDV replication in all conditions based on viral RNA titers by real time RT-qPCR. We tested and found that the monoclonal antibody against Korean PEDV strains also worked for US PEDV strains using the PC21A-infected pig intestinal tissue (see section 5). We plan to perform immunofluorescence assay to detect PEDV antigens from the inoculated cells. We will continue to passage US PEDV strains to 10 passages as we proposed in the grant.
5. **Propagated an US PEDV strain, OH/PC21A, in gnotobiotic (Gn) or germfree pigs.** The obtained sterile PEDV pools can be used in downstream studies, including sample characterization, cell culture adaptation trials, molecular characterization of US PEDV strains, pathogenesis studies and future vaccine studies. The US PEDV strain PC21A was detected from the intestinal contents of one diarrheic 1-3-day-old piglet from a farm in Shelby county of Ohio in June, 2013. The partial N gene region was amplified and sequenced. The strain-specific 296 nt was genetically 100% identity to the two US PEDV strains USA/Colorado/2013 (GenBank # KF272920) and 13-019349 (GenBank # KF267450). The original intestinal contents were tested negative for rotavirus group A and C, TGEV, and PRCV.

Near-term Gn pigs were delivered aseptically by hysterectomy. Six 10 to 35-day-old pigs were randomly assigned to two groups: PEDV infection ($n = 5$; pigs # 1-5) and Mock ($n = 1$; pig # 6). This trial is summarized in Table 2. Pigs # 1-3 and 5 were inoculated with the PC21 strain orally ($6.8-9.0 \log_{10}$ GE) and/or intranasally ($6.3-8.3 \log_{10}$ GE), while pig # 4 was exposed to inoculated-pig # 3 in the same isolator. After PEDV inoculation, we monitored clinical signs daily. Pig # 4 was monitored for relatively longer-term clinical signs and virus shedding for approximately 120 hours after it showed clinical signs. Other infected or negative pigs were kept for shorter-term studies to create virus pools and were euthanized for histopathological examination at an acute to mid-stage (from onset of clinical signs to 24 hours thereafter), a mid-stage (from 24 to 48 hours after clinical signs appear), and a later-stage (longer than 48 hours after clinical signs appear) of PEDV infection. The Institutional Animal Care and Use Committee (IACUC) of the Ohio State University approved all protocols related to the animal experiments in this study. All animals used in this study were also handled in accordance with the guidelines of the IACUC of the Ohio State University.

All the infected Gn pigs exhibited acute, severe watery diarrhea, accompanied with acute vomiting and gut pain as suggested by abdominal crouch position on the isolator floor. Regardless of the different inoculum doses ($7-9 \log_{10}$ GE) and passage numbers (1-4) among the inocula, the 5 infected pigs had diarrhea and vomiting between 24 and 48 hours post inoculation (HPI) (Table 1). An exposed pig (pig # 4) was observed for a longer term (120 hours after onset of clinical signs). It also showed dehydration as evident by loss of body weight and lethargy, but still had enough appetite to finish the whole milk fed for the observing period. However, on day 5 after onset of clinical signs, the pig suddenly collapsed and suffered from disorientation and lethargy. The negative control pig did not have diarrhea or vomiting throughout the experiment.

Analysis of virus shedding patterns and histopathology studies are now underway. Although the original PC21A was negative for rota A and C and TGEV, other porcine enteric viruses can cause similar or exacerbate clinical signs. Therefore, porcine rotavirus group B, circovirus, bocavirus, kobuvirus, astrovirus, and caliciviruses (sapovirus, norovirus and St-Valerien-like virus) are going to be tested by RT-PCR or PCR and immune electron microscopy (IEM).

6. **Failed to propagate the 2nd US PEDV strain, OK/PC23, in Gn pigs.** Pig#5 (31-day-old) was first inoculated with PC23 through oral and intranasal routes at a high dose of 8 log₁₀ GE, but it did not show any clinical signs or viral fecal shedding by 4 dpi. So, at 35-day-old it was re-inoculated with the 3rd passage of PC21A at a high dose (9 log₁₀ GE/pig). It had typical PEDV clinical signs at 26-30 hpi after re-inoculation with PC21A strain. We suspected that the PEDV in PC23 had lost infectivity during sample transportation and storage.

References

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3. Kim L, Chang KO, Sestak K, Parwani A, Saif LJ (2000) Development of a reverse transcription-nested polymerase chain reaction assay for differential diagnosis of transmissible gastroenteritis virus and porcine respiratory coronavirus from feces and nasal swabs of infected pigs. *J Vet Diagn Invest* 12:385-388
4. Kim SH, Kim IJ, Pyo HM, Tark DS, Song JY, Hyun BH (2007) Multiplex real-time RT-PCR for the simultaneous detection and quantification of transmissible gastroenteritis virus and porcine epidemic diarrhea virus. *J Virol Methods* 146:172-177

Table 1. Summary of cell culture trials for PEDV-positive samples in different types of cells supplemented with trypsin or pancreatin.

Field sample	Passages	Cell types	Supplement*
OH/PC21A	2	Primary pig kidney	T, Pan
	2	Vero-81	T, Pan
	2	Vero-BI	T, Pan
	2	Vero-E6	T, Pan
OH/PC15	3	Primary pig kidney	T, Pan
	3	Vero-81	T, Pan
	2	Vero-BI	T, Pan
	2	Vero-E6	T, Pan
KS/PC10	2	Primary pig kidney	T, Pan
	2	Vero-81	T, Pan
	2	Vero-BI	T, Pan
	2	Vero-E6	T, Pan
OK/PC23	2	Primary pig kidney	T, Pan
	2	Vero-81	T, Pan

	2	Vero-BI	T, Pan
	2	Vero-E6	T, Pan
PE22 (1st passage of PC21A in germfree pig)	1	Vero-81	T, Pan
	1	Vero-BI	T, Pan
	1	Vero-E6	T, Pan

*T: Trypsin concentration was 10 µg/ml.

Pan: Pancreatin concentration was 37.5 µg/ml.

Table 2. Summary of the germfree pig experiment.

Pig #	Pig age when inoculated (day)	Oral/intranasal Inoculum titer (log ₁₀ GE/pig)	Onset of clinical signs (hour-post-inoculation, HPI)	HPI when euthanized (acute-, mid-, or later-stages of infection)	Viral RNA titer (log ₁₀ GE/ml) of small/large intestinal contents when euthanized	PC21A number
1	10	7.7/7.3	25-26	30 (acute to mid)	11.9/11.6	1
2	18	6.8/6.3	44-46	72 (mid)	11.5/12.1	2
3	24	8.8/8.3	44-46	46 (acute to mid)	11.8/11.4	3
Infected 4	24 (age when exposed to pig#3, in the same isolator as #3, considered as natural transmission)	N/A (Exposed to pig#3 inside the same isolator)	2-4 hours after pig#3 showed clinical signs	120 hours after onset of clinical signs (later)	11.5/11.5	3
5	35	9.0/NA (only oral)	26-30	67 (mid)	12.4/11.6	4
Mock 6 (26 day-old when euthanized)	N/A	N/A	N/A	N/A	N/A	N/A