RESEARCH REPORT
SWINE HEALTH

Title: Propagation of PEDv in tissue culture and development of standardized reference samples for use in diagnostic testing **- NPB #13-222**

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Industry Summary:
The highly contagious and deadly porcine epidemic diarrhea (PED) virus (PEDv) was first discovered in the US in April-May 2013. Since then the virus has spread rapidly nationwide causing high mortality among nursing piglets and significant economic losses. Currently there are no efficacious preventive measures or therapeutic tools to control PEDv: therefore, development of an effective PEDv vaccine for swine in the US should be a high priority. Since most viruses accumulate mutations and become attenuated (reduced pathogenicity or virulence) after serial passage in cell culture, the isolation of PEDv in cell culture is the first step toward the development of an attenuated vaccine, to study the basic biology of PEDv, and to develop *in vitro* PEDv immunoassays, inactivation assays and screen for PEDv antivirals. Our labs have started to work on PEDv since June, 2013. What we have achieved on PEDV research is listed below:

1. We have successfully propagated one US PEDv strain PC22A in Vero cells to high titers. **The isolation of PEDv in cell culture is the first step toward the development of an attenuated vaccine.**
2. We have plaque-purified PC22A and generated a virus pool with high infectious titers in germfree pigs. **This plaque-purified and germfree pig-passaged PC22A pool can be used as a challenge pool in future vaccine studies.**
3. We have developed reverse-transcription (RT)-PCR and immunofluorescent assays (IFA) for the detection of PEDv nucleic acids and antigens, respectively. IFA and virus neutralization assay (VN) for the detection of isotype (IgA and IgG) and virus neutralizing (protective) antibodies, respectively, were also developed. **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 whether a PEDv vaccine induces protective immunity.**
4. We have investigated how US PEDv causes disease (pathogenesis) in nursing germfree pigs. We found that the **US PEDv PC21A strain acutely infected the entire intestine, leading to severe atrophic enteritis, and is highly enteropathogenic.**

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Keywords: porcine epidemic diarrhea (PED), PED virus (PEDv), cell culture, pathogenesis, reverse transcription (RT)-PCR

Scientific Abstract:

The highly contagious and deadly porcine epidemic diarrhea virus (PEDv) first appeared in the US in April 2013. Since then the virus has spread rapidly nationwide causing high mortality among nursing piglets and significant economic losses. Currently there are no efficacious preventive measures or therapeutic tools to control PEDv: therefore, development of an effective PEDv vaccine for swine in the US should be a high priority. Since most viruses accumulate mutations and become attenuated (reduced pathogenicity) after serial passage in cell culture, the isolation of PEDv in cell culture is the first step toward the development of an attenuated vaccine and to study the basic biology of PEDv and to develop *in vitro* PEDv immunoassays, inactivation assays and screen for PEDv antivirals. Our labs have started to work on PEDv since June, 2013. What we have achieved on PEDV research is listed below:

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 ($TCID_{50}$) 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 seronegative sows to protect their nursing piglets against epidemic PED.
2. We have plaque-purified PC22A at a low cell culture level (passage level 3) using plaque assays, confirmed that it retained high virulence similar to the wild-type PEDV PC21A strain, and generated a virus pool with high infectious titers (7.7 $\log_{10}$ PFU/mL) in germfree pigs. **It can be used as a challenge pool in future vaccine studies.**
3. We have developed conventional reverse-transcription (RT)-PCR and real-time RT-PCR (RT-qPCR) and immunofluorescent assays (IFA) for the detection of PEDv nucleic acids and antigens, respectively. TC PEDv-based immunofluorescent assay (IFA) and plaque reduction virus neutralization assay (VN) have been developed for the detection of isotype (IgA and 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 whether a vaccine induces protective immunity.**
4. We have investigated the pathogenesis of US PEDv in nursing germfree pigs. By using gnotobiotic (Gn) 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.**

Introduction:

The highly contagious and deadly porcine epidemic diarrhea virus (PEDv) first appeared in the US in April 2013. Since then the virus has spread rapidly nationwide causing high mortality among nursing piglets and significant economic losses. Currently there are no efficacious preventive measures or therapeutic tools to control PEDv: therefore, development of an effective PEDv vaccine for swine in the US should be a high priority. Since most viruses accumulate mutations and become attenuated (reduced pathogenicity) after serial passage in cell culture, the isolation of PEDv in cell culture is the first step toward the development of an attenuated vaccine and to study the basic biology of PEDv and to develop *in vitro* PEDv immunoassays, inactivation assays and screen for PEDv antivirals.

Objectives:

Obj. 1. Development and validation of diagnostic testing for PEDv antigen and antibody detection

Obj 1.1 Use multiple sources of Vero cells and primary pig kidney cells with various concentrations of porcine trypsin or pancreatin, and intestinal content preparations (ICP) from germfree piglets to propagate PEDv.

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

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

Materials & Methods:

Clinical pig fecal and intestinal samples. Samples from field pigs (commercially-raised pigs) were received from different Midwestern farms and were analyzed for different viruses – PEDv, Transmissible Gastroenteritis Virus (TGEV), Porcine Respiratory Coronavirus (PRCV), rotavirus group A (Rota-A), rotavirus group C (Rota-C) using conventional or real-time RT-PCR (1, 2, 4, 5). Representative PEDv-positive samples were confirmed by sequence analysis using primer set PEDN219/557 targeting the N gene sequences of new US PEDv strains (4). **These field samples tested positive for PEDv but negative for other enteric viruses were used for cell culture adaptation and making virus pools in germfree pigs.**

Cell lines. Several different Vero cell lines (Vero-81, ATCC No. CCL-81; Vero 76, ATCC No. CRL-1587; Vero-E6, ATCC No. CRL-1586; and Vero-BI (originally provided to Dr. Linda J. Saif by Dr. Louis Harris at Boehringer-Ingelheim in 1990) and primary porcine kidney cells isolated from mock Gn pigs were used for PEDv isolation trials. All vero cells were grown in Dulbecco Modified Eagle Medium (DMEM, Life Technologies,) supplemented with antibiotics (100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of Fungizone®) (Life Technologies), and 5% heat inactivated fetal bovine serum (Hyclone).

Preparation of PEDv inoculum for Vero cells. PEDv-positive intestinal or fecal samples were diluted 10-fold in Dulbecco's Phosphate buffered Saline without Mg^{2+} and Ca^{2+} [PBS (-), Sigma), and were vortexed briefly followed by centrifugation at 10,000 × g for 3 min at 4°C. The supernatants were filtered through 0.22 µm-pore size, and then used immediately for inoculation of Vero cells.

Virus isolation and propagation. One or two-day-old, semi-confluent Vero cell and porcine primary kidney cell monolayers were used for virus inoculation. Before inoculation, cells were washed with PBS (-) twice. Then inoculum was added. After incubating at 37°C for 30~60 min, PEDv growth medium [DMEM supplemented with antibiotics (100 units/mL of penicillin and 100 µg/mL of streptomycin, Life

Technologies), 0.3% tryptose phosphate broth (TPB, Sigma), and 10 µg/ml of trypsin (Life Technologies)] was added and the flasks/plates were incubated up to 7 days.

To propagate a virus pool of TC PEDv PC22A strain in gnotobiotic (Gn) pigs. One plaque-purified clone (P3-1) of the TC PC22A was used to inoculate one germfree pig. This pig developed watery diarrhea at 21 hour-post-inoculation (hpi) and was euthanized on the same day for the collection of intestinal contents. Another germfree pig was housed together with the PEDv-inoculated pig. It showed watery diarrhea one day after the first pig broke with diarrhea, and was euthanized for intestinal contents on the day of onset of diarrhea.

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-TGYYACCAYYACCACGACTCCTGC-BHQ3) were designed (4) based on the partial N gene sequences of new US PEDv strains and previous publication (6). The RT-PCR products of primer pair PEDN219/557 was used to generate a standard curve for the calculation of template RNA titer.

Immunofluorescence assay (IFA) for the detection of PEDv antigens or isotype antibodies (IgA and IgG) in Vero cells. PEDv-infected Vero cells in 24-well plates were fixed with acetone/methanol (20:80, v/v) at -20°C for 20 min, and then the fixed cells were washed by PBS, and blocked with 5% bovine serum albumin (BSA) at room temperature for 1 h. Mouse anti-PEDv N or S protein monoclonal antibody and Fluorescence isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Serotec) were used as first and second antibodies, respectively, for the detection of PEDv antigens. For the detection of antibodies in pig serum samples, 100 $TCID_{50}$ of TC PEDv PC22A will be used to infect Vero cells in 96-well plates, and 4-fold serially diluted serum samples and goat anti-pig IgA/IgG antiserum (KPL) will be added as the primary and secondary antibodies, respectively (8). The reciprocal of the highest serum dilution at which PEDv antigen is detected will be the antibody titer of the serum sample.

Plaque assay for the titration of PEDv infectivity and plaque reduction assay for detection of PEDv virus neutralizing (VN) antibodies in serum samples of inoculated pigs. We developed a plaque assay for PEDv as described previously (3). Confluent cell monolayers in 6-well plates were inoculated with 10-fold serially diluted virus, in duplicate per virus dilution. After inoculation, virus inoculum was removed and cell monolayers were washed with PBS (-). The cell monolayer was covered by 1.5% SeaPlaque in the PEDv growth medium. At 3 PID, 0.01% neutral red in PBS (-) was added for staining. After incubation, neutral red solution was removed and plaques were visualized and counted. Infectious viral titers were described as plaque forming units (PFU)/mL. All viruses to be used in Objectives 1&2 produced plaques (titers shown in Table 1). For the detection of VN antibodies, a serum sample will be serially diluted 4-fold and will be incubated with 60 PFU PEDv for 60 min before inoculation into cell monolayers. The reciprocal of the highest serum dilution resulting in a 50% reduction in plaques will be defined as the VN titer of the serum sample (3).

Pathology of US PEDv strain PC21A in Gnotobiotic Pigs (4). By using germfree animals, the precise role of a single pathogen in the disease process can be evaluated. Five 10- to 35-day-old germfree pigs were inoculated orally and/or intranasally with 6.3-9.0 log10 genomic equivalents (GE) of a US PEDv strain (PC21A). We found that the germfree pig-passaged PC21A were free of other bacteria and viruses, as determined by immune electron microscopy and RT-PCR/PCR for TGEV/PRCV, rotavirus groups A, B and C, porcine enteric caliciviruses (noroviruses, sapoviruses and St-Valerien-like viruses),

astroviruses, circoviruses, enterovirus, kobuvirus, and bocavirus. Pigs were euthanized when onset of clinical signs for histopathological exams.

Results:

<u>Obj 1.1</u> Use multiple sources of Vero cells and primary pig kidney cells with various concentrations of porcine trypsin or pancreatin, and intestinal content preparations (ICP) from germfree piglets to propagate PEDv.

Out of the field diarrheic samples tested, it was determined that only 9, 6 and 2 were exclusively positive for PEDv, Rota-A and Rota-C, respectively. 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.

We have successfully isolated one US PEDv strain PC22A in Vero cells, but not from primary porcine kidney cells. Currently the tissue culture-adapted (TC) PC22A strain replicates in Vero cells consistently and infectious virus titers reach 5-6 $\log_{10}$ 50% tissue culture infectious dose ($TCID_{50}$) or plaque forming units (PFU) per mL.

<u>Obj 1.2.</u> Development of standardized reference samples that can be utilized by Veterinary Diagnostic Labs (VDLs) for diagnostic test validation

The above TC PEDv PC22A strain can be utilized by Veterinary Diagnostic Labs (VDLs) for diagnostic test validation. The plaque-purified and germfree pig-passaged PC22A pool has a high infectious titer (7.7 $\log_{10}$ PFU/mL) and will be used as challenge pool in future vaccine studies.

<u>Obj 1.3.</u> Develop and validate antibody-based diagnostic tests for serologic monitoring and surveillance

TC PEDv-based **immunofluorescent assays (IFA)** and **plaque reduction virus neutralization assay (VN)** were developed for the detection of isotype (IgA and IgG) and virus neutralizing antibodies, respectively, from pig serum samples.

Extra study: We have investigated the pathogenesis of US PEDv in nursing germfree pigs (4). 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 titers in feces and serum, ranging from 9.6-12.3 and 4.8-7.6 $\log_{10}$ GE/ml, respectively. Overall, our data suggest that the US PEDv PC21A strain acutely infects the entire intestine, leading to severe atrophic enteritis, and is highly enteropathogenic.

Discussion:

We have completed the proposed studies on the set time course. The successful isolation of PEDv in cell culture is the first step toward the development of an attenuated vaccine. This is based on the observation that most viruses including PEDv and TGEV will accumulate mutations and become attenuated after serial passage in cell culture (7, 9). Our 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 develop a live attenuated PEDv vaccine for use in naïve PEDv seronegative sows to protect their nursing piglets against

epidemic PED. The immunoassays developed in this study 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 whether a vaccine induces protective immunity.

Any publications, presentations or abstracts

1. **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:**668-671.
2. **Wang, Q., T. Oka, M. Esseili, S. Sommer-Wagner, T. Meulia, Y. Zhang, and 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.
3. **Oka, T., L.J. Saif, M.A. Esseili, T. Meulia, C.M. Lin, A.N. Vlasova, K. Jung, D. Marthaler, Y. Zhang, and Q. Wang.** Successful isolation and characterization of diverse US porcine epidemic diarrhea virus strains in cell culture. (In preparation)

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