RESEARCH REPORT
SWINE HEALTH

Title: Porcine Epidemic Diarrhea (PED): Development of tests and reagents for diagnosis and disease management **- NPB #13-209**

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Industry Summary:

Porcine epidemic diarrhea (PED), a coronavirus-mediated enteric disease, emerged in US swine unexpectedly in 2013. While introduction pathway of disease to the US was unknown, the US swine industry had to deal with the new disease. Although the pan-coronavirus PCR test with sequencing at Iowa State University Veterinary Diagnostic Laboratory (ISUVDL) allowed the initial identification of PED virus (PEDV), this test was considered inadequate for routine high-throughput diagnostic testing. There was then an urgent need to develop an array of validated high-throughput tests as well as other reagents for PEDV for use in diagnosis of disease outbreaks, epidemiological investigations, and the management and/or elimination of PED. That was the objective of the study. Efforts were made on developing various diagnostic tools for PEDV such as virus isolation, PCR assay, immunohistochemistry (IHC) assay, antibody assays through literature review, collaboration with foreign scientists with appropriate expertise in PEDV and continuous monitoring of test performance once implemented. As a result, ISUVDL was able to offer a variety of laboratory tests fitting swine veterinarians and producers' needs (e.g., diagnosis, monitoring, surveillance, etc). At the same time, virus isolates and laboratory methods became valuable tools for research endeavor to understand the pathogenesis and control of PEDV. These studies in turn will produce additional scientific information which are critical for effective control of PED in the US.

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Scientific Abstract:

Porcine epidemic diarrhea (PED) is a coronavirus-associated enteric disease of swine which has been well known in many parts of the world, particularly Europe and Asia since 1970's, although the disease has been absent from the US. The disease emerged in US swine unexpectedly in early 2013 and devastated the entire industry due to extremely high pre-weaning mortality and substantial productivity loss, not to mention rapid spreading through the entire country. More importantly it was a puzzle how the virus was entered the US, which still has not solved.

Although the pan-coronavirus PCR test with sequencing at Iowa State University Veterinary Diagnostic Laboratory (ISUVDL) allowed the initial identification of PED virus (PEDV) for the first time in the US, this test was considered inadequate for routine diagnostic testing since it was labor intensive test with low-throughput. Then, it became critical and urgent to develop an array of validated high-throughput tests for PEDV for use in diagnosis of disease outbreaks, epidemiological investigations, and control of PED. That was the objective of the study.

Coordinated efforts were made in a collective way on developing various diagnostic tools for PEDV such as virus isolation technique, nucleic acid-based assays (e.g., PCR, sequencing), tissue assays (e.g., immunohistochemistry), antibody assays (e.g., IFA, ELISA, VN) through literature review, collaboration with foreign scientists with appropriate expertise in PEDV and continuous monitoring of test performance once implemented. Necessary reagents were procured via importation or self-production. At a later stage of the study, all assays were re-validated once samples from pigs with known infection status (e.g., experimental animals) were available.

As a result, ISUVDL was able to offer a variety of laboratory tests fitting swine veterinarians and producers' needs (e.g., diagnosis, monitoring, surveillance, etc). At the same time, virus isolates and laboratory methods became valuable tools for research endeavor to understand the pathogenesis and control of PEDV.

Introduction:

On April 29, 2013, the Iowa State University Veterinary Diagnostic Laboratory (ISUVDL) received the first of several cases with similar histories. A severe transmissible-gastroenteritis (TGE)-like disease was reported in breed-to-wean sites ranging from 500-to-6000 sows in size in Iowa, Illinois and Indiana. Disease was first recognized in either sows in gestation or suckling pigs and rapidly spread to all ages on the site. Severe watery diarrhea, anorexia, dehydration and vomiting were reported with mortality of 70-90% in suckling pigs. Lesions in small intestines of suckling pigs were of severe atrophy of villi typical of severe villus-epitheliotropic viral enteritis and compatible with TGE virus (TGEv) or rotavirus. Standard tests for TGEV and Rotavirus were negative, including polymerase chain reaction (PCR), immunohistochemistry (IHC) and indirect fluorescent antibody (IFA) tests. Electron microscopy of feces revealed coronavirus particles. A PCR test targeting a highly conserved portion of the polymerase gene designed to detect all viruses in the coronavirus family (pan-coronavirus PCR) was positive on samples from these farms. The PCR products were sequenced and, in all cases, were determined to be porcine epidemic disease virus (PEDV), a virus not previously reported in the U.S. and known to be endemic in Southeast Asian countries. These findings were corroborated by National Veterinary Services Laboratories (NVSL) using a nested PCR test that amplifies 2 segments of the spike gene and one of the nucleoprotein gene of PEDv. Subsequently, additional farms in Iowa, Illinois, Minnesota, and other states are reporting similar disease that is being confirmed as PED. Although the pan-coronavirus PCR test with sequencing at ISU VDL allowed the initial identification of PEDV, this test is inadequate for routine high-throughput diagnostic testing. There is an urgent need to develop an array of validated high-throughput tests as well as other reagents for PEDv for use in diagnosis of disease outbreaks, epidemiological investigations, and the management and/or elimination of PEDv.

Objectives:

Our long-term goal was to understand the pathogenesis and epidemiology of PEDV and to develop effective prevention and control strategies against PED. The immediate goal of the proposed work was to establish reliable diagnostic tools/capability for the virus/disease and necessary reagents which can assist not only disease diagnosis but also epidemiological studies and field-based control effort. Specific aims were:

1. Develop nucleic acid based assay(s) to rapidly detect and differentiate PEDV and TGEV in a high throughput manner and evaluate its test performance on various sample matrices such as fecal material, intestinal tissue, feeds, environmental/product samples, and oral fluids.
2. Establish virus isolation capability and obtain PEDV isolate(s).
3. Evaluate PEDV-specific monoclonal antibodies which are currently available in Southeast Asian countries on Tissue FA test and IHC with the goal to establish an immunohistochemical technique for PEDV.
4. Assess the diagnostic performance of ELISA kits commercially available in PEDv-endemic areas and implement a serologic assay for serodiagnosis and retrospective studies.
5. Establish sequencing capability for the S gene of PEDV and conduct molecular epidemiologic studies.
6. Produce PEDV-specific antibodies against US PEDV.

Materials & Methods:

***Specific aim #1*:** A thorough literature review was conducted for available nucleic acid based assays for PED virus in various clinical specimens. Input from research scientists and diagnosticians in Asian countries were also sought on selection of PCR assays. A few PCR-based assays were selected for evaluation on clinical cases. After brief evaluation, the test was put in work and offered by Iowa State University Veterinary Diagnostic Laboratory (ISUVDL) to swine practitioners. After that, effort was made to develop a real-time RT-PCR which targets N gene using limited sequence data of PEDV identified in the US since the N gene of PED viruses was known to be highly conserved. The assay was evaluated on various clinical specimens procured through submissions to ISUVDL and eventually on samples collected from experimentally infected pigs.

Specific aim #2: Based literature review, virus isolation technique using a monkey kidney derived continuous cell line VERO and trypsin treatment was established and evaluated on selected PEDV nucleic acid positive clinical specimens from earlier cases for its feasibility. Then the method was adapted to testing case materials submitted to ISUVDL for evaluating test performance with the goal of obtaining a PEDV isolate. At the same time, other cell lines were evaluated for their usefulness in virus isolation.

Specific aim #3: Efforts were made to obtain PEDV-specific monoclonal antibodies (MAbs) which were commercially and privately available in Southeast Asian countries. Once MAbs were obtained, those were applied to frozen and/or fixed tissues collected from clinically affected and unaffected pigs in order to establish immunohistochemical technique (i.e., IHC) for PEDV using routine protocol used at ISUVDL. Microscopic examination along with PCR testing for PEDV was done on the samples employed for IHC to establish expected status with PEDV infection.

Specific aim #4: Due to the lack of available virus isolate or genetic information of US PEDV, PEDV antibody ELISA kits which were commercially available in PEDV-endemic areas (Korea, China) were purchased with USDA import permission and evaluated for its test performance on serum samples collected from swine operations undergoing PED. Archived serum samples collected from experimental pigs before 2012 were used as negative control in the evaluation. At a later stage of the project, indirect fluorescent antibody (IFA) test was established as a cell culture adapted PEDV isolate became available in the lab and used for comparison.

Specific aim #5: Based on sequence information of historical PED viruses available in a public domain (e.g., GenBank) and sequence data of a limited number of US PED viruses (4), a sequencing protocol was established for the Spike (S) and nucleoprotein (N) genes. The protocol combined PCR amplification and purification at ISUVDL and sequencing at the ISU Nucleic Acid Facility. Raw sequence data was assembled and compared to the sequence of a prototype US PEDV. At least one case from a swine operation in different state was selected for sequencing and sequence analysis for molecular epidemiological study.

Specific aim #6: With separate funding, a pig trial using 3 to 4-week-old weaned pigs was conducted to assess the pathogenicity of a US PEDV. Serum samples were collected from those pigs over time and at the end of the study and used for additional validation of assays and as source of PEDV-monospecific polyclonal antibody.

Results and Discussion:

***Specific aim #1*:** A S-gene based gel-based RT-PCR was quickly developed with help from research scientists in Asian countries where PEDV has been endemic. After brief evaluation, the test was put in

work and offered by ISUVDL to swine practitioners. After that, effort was made to develop a real-time RT-PCR which targets N gene. The N-gene based real-time RT-PCR was successfully developed and made available to clients for rapid detection of PEDV in fecal materials. It became a standard PCR test for PEDV at ISUVDL (1, 2, 6). While efforts continued to develop a PCR-based assay for rapid detection and differentiation of PEDV and TGEV, commercial manufacturers had developed a multiplex PCR for the same purpose. ISUVDL evaluated those commercial assays and adapted one with the best performance.

Once a real-time PCR was available, a semi-retrospective study was conducted to assess how soon PEDV was introduced to US swine using archived fecal samples from diarrheic pigs which were negative for TGEV or rotaviruses. It was found that a case from a grower-finisher farm in OH back in early April 2013 was the first case positive for PEDV based on the samples available in the ISUVDL (7).

***Specific aim #2*:** Effort was made to establish virus isolation technique using various cell lines (Vero, MA104, ST, PK15, BHK21). One with Vero cells and trypsin treatment was adapted since Vero cell was the only cell line yielding successful isolation of PEDV (4). Then the method was then applied to a large number of archived samples (n=250) which were positive for varying level of PEDV as determined by PCR. Overall success rate of isolation was extremely poor (less than 5%), requiring further refinement on virus isolation technique or alternative method. At the time of report, a total of 7 isolates are available (2).

***Specific aim #3*:** A few PEDV-specific MAb available abroad were imported from several sources and applied to tissue assay for PEDV via frozen tissue section FA test and immunohistochemistry (IHC). Overall one MAb specific for N protein was found work the best without cross reaction with other coronavirus such as TGEV. This MAb was employed to establish PEDV-specific IHC. After brief evaluation, IHC was offered swine practitioners at ISUVDL. At a later stage of study period, the IHC was evaluated on various tissues collected from experimental pigs, demonstrating good test performance and correlation with disease progress (6). Efforts continue to develop MAb against US PEDVs in ISUVDL as well as other institutes such as South Dakota State University, Kansas State University and University of Minnesota.

***Specific aim #4*:** A few ELISA kits for detection of PEDV antibody which were commercially available abroad were imported and evaluated on a set of serum samples from known clinical status of PED. All those kits were found not to be suitable for serology purpose with unknown reasons. The decision was made to develop own ELISA-based assay for antibody detection (5).

As a PEDV isolate was made, IFA assay for antibody detection was established quickly. After small scale evaluation of test performance, the test was made available to swine practitioners on experimental basis for field evaluation. The IFA assay was found to be acceptable for both diagnostic specificity (99%) and sensitivity (96%). When serum samples were available form experimental pigs, it was found that IFA antibodies would be relatively short-live after exposure (7). At present the IFA assay is one of the standard serology tests for PEDV available at ISUVDL (5).

Specific aim #5: Based on sequence information of historical PED viruses available in a public domain (e.g., GenBank) and sequence data of a limited number of US PED viruses (4), a sequencing protocol was established for the Spike (S) and nucleoprotein (N) genes. We found the S gene was the good target for molecular epidemiology (8) and US PED viruses were relatively stable with respect to genetic variation over time (3) The protocol combined PCR amplification and purification at ISUVDL and sequencing at the ISU Nucleic Acid Facility.

References

1. Bower L, Hoang H, Sun D, Madson M, Magstadt D, Arruda P, Wilberts B, Gimenez-Lirola L, Harmon K, Johnson J, Main R, Zhang J, Zimmerman J, Yoon K. 2014. Utility of oral fluid sampling and testing for monitoring PEDV in herds. Proceedings, American Association of Swine Veterinarians, pp 61-62.
2. Chen Q, Li G, Stasko J, Thomas JT, Stensland WR, Pillatzki AE, Gauger PC, Schwartz KJ, Madson D, Yoon K-J, Stevenson GW, Burrough ER, Harmon KM, Main RG, Zhang J. 2014. Isolation and Characterization of Porcine Epidemic Diarrhea Viruses Associated with the 2013 Disease Outbreak among swine in the United States. J Clin Microbiol 52:234-243.
3. Chen Q, Chriswell A, Dunn D, Li G, Sinha A, Harmon K, Stensland W, Gauger P, Yoon K-J, Madson D, Schwartz D, Main R, Zhang J. 2014. Molecular Epidemiology of Porcine Epidemic Diarrhea Virus in US Swine. Proceedings, Annual Meeting of American Association of Veterinary Laboratory Diagnosticians, p113.
4. Hoang H, Killian ML, Madson DM, Arruda PHE, Sun D, Schwartz KJ, Yoon KJ. 2013. Full-length genome sequence of a plaque-cloned virulent porcine epidemic diarrhea virus isolate (USA/Iowa/18984/2013) from a US Midwestern swine herd. Genome Announc 1(6):e01049-13.
5. Giménez-Lirola LG, Baum D, Bower L, Chen Q, Dong S, Johnson JK, Kalkwarf E, Madson E, Magtoto R, Yoon KJ, Zhang JQ, Zimmerman JJ, Main R. 2014. Porcine Epidemic Diarrhea Virus: Antibody-based Test Development. Proceedings 22nd Annual Swine Disease Conference for Swine Practitioners, pp. 30-32.
6. Madson DM, Magstadt DR, Arruda PHE, Hoang H, Sun D, Bower LP, Bhandari M, Burrough ER, Gauger PC, Pillatzki AE, Stevenson GW, Wilberts BL, Brodie J, Harmon KM, Wang C, Main RG, Zhang J, Yoon KJ. 2014. Pathogenesis of porcine epidemic diarrhea virus isolate (US/Iowa/18984/2013) in 3-week-old weaned pigs. Vet Microbiol 174:60-68.
7. Yoon KJ. 2014. The emergence of porcine epidemic diarrhea in US swine: Surprises on the road to prevention and control. Proceedings, International Pig Veterinary Society Congress, 23(1):64-66.
8. Zhang J, Chen Q, Gauger PC, Harmon KM, Yoon K-J. 2014. Reply to "Classification of emergent U.S. strains of porcine epidemic diarrhea virus by phylogenetic analysis of nucleocapsid and ORF3 genes". J Clin Microbiol 52:3511-3514.