RESEARCH REPORT
SWINE HEALTH

Title: Characterization of the pathogenesis of porcine epidemic diarrhea virus (PEDv) in neonates and weaned pigs and generation of reference diagnostic specimens – **NPB #13-244**

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Industry summary
Porcine epidemic diarrhea virus unexpectedly entered the North American swine population in 2013 resulting in widespread and devastating disease. Understanding this virus and its infection characteristics has proven difficult worldwide. Experimental inoculation of three-week-old pigs with PEDV isolate US/Iowa/18984/2013 induced severe clinical disease with stalled weight gain for approximately 10 days post inoculation. Diarrhea with measurable differences in villous to crypt ratios were first observed on dpi 3; however, viral shedding and PEDV antigen detection within infected cells can be as early as 24 hrs post-infection leading to possible transmission of virus prior to the onset of clinical signs. Porcine epidemic diarrhea virus shedding, under the conditions of this wean-pig study, was much longer than previously reported as viral shedding was detected up to dpi 24 which was approximately two weeks post resolution of clinical disease. Accordingly, an absence of clinical diarrhea does not eliminate the risk of PEDV transmission in weaned pigs. In neonatal piglets PEDV infection was severe resulting in diarrhea, dehydration, and death. Viral shedding was confirmed by 12 hr post infection. Comparing neonate study results with previous reports, our data confirms that clinical differences can exist among PEDV isolates and PED can be as severe as TGE in neonates.

Keywords
Porcine epidemic diarrhea virus, immunohistochemistry, neonate, swine, pathogenesis, shedding, antibody

Scientific Abstract
Weaned pig study:
Porcine epidemic diarrhea virus (PEDV) is associated with clinical diarrhea in naïve swine of all ages. This report describes timing of antibody generation and disease progression following infection with a US PEDV isolate by assessing fecal viral shedding, morphometric analysis of intestinal lesions, and magnitude of immunohistochemical staining. Sixty-three, three-week-old pigs were randomly allocated into control (*n*=27) and challenged (*n*=36) groups. Challenged pigs were administered 1 ml of $1x10^3$ PFU/ml of US/Iowa/18984/2013 PEDV isolate by oro-gastric gavage. Three control and four challenged pigs were necropsied on days post inoculation (dpi) 1, 2, 3, 4, 7, and weekly thereafter, until study termination on dpi 35. Clinical disease, fecal shedding, body weight and temperature were monitored during the study period. Diarrhea was observed in challenged pigs beginning for some on dpi 2, affecting a majority of pigs by dpi 6 and

subsiding by dpi 10. Average daily gain was significantly lower (P<0.001) for one week post infection in challenged pigs. PEDV was detected in feces by PCR on dpi 1 and continued in a subset of pigs until dpi 24. PEDV-specific antigen was detected in villous enterocytes of challenged pigs by immunohistochemistry (IHC) on dpi 1, 2, 3, 4, 7, and 14. Microscopic lesions included severe diffuse atrophic enteritis with significantly reduced (P<0.001) villous length observed on dpi 3, 4, and 7. Oral fluid detection of PEDV RNA was similar to fecal swab detection, and reinfection does can occur, but clinical signs can be unapparent and shedding is of short duration. Under the conditions of this study, fecal shedding of PEDV and IHC staining can precede and continue beyond the observation of clinical signs, thus increasing the risk of viral transmission.

Neonate study

Porcine epidemic diarrhea virus (PEDV) was first recognized in North America in April of 2013 and has since caused devastating disease. The objective of this study was to characterize disease and viral detection associated with an original North American PEDV isolate inoculated in neonatal piglets. Thirty-six one-day-old caesarian derived and colostrum deprived piglets were randomly assigned to control (n=16) or challenged groups (n=20) which were oro-gastrically inoculated with 1 ml of US/Iowa/18984/2013 PEDV isolate titered at $1x10^3$ PFU/ml. Rectal swabs were collected from all piglets prior to inoculation and every 12 hrs post-inoculation (hpi) thereafter with four control and five challenged piglets euthanized at 12, 24, 48, and 72 hpi. One piglet had a positive rectal swab PCR at 12 hpi, and all remaining piglets were positive thereafter with highest viral quantities detected at 24 and 36 hpi. All challenged pigs had diarrhea at 24 hpi with 30% diarrhea at 18 hpi. Viral antigen was detected in enterocytes by immunohistochemistry in the upper small intestine of piglets necropsied at 12 hpi and remained in all subsequent necropsies. Lesions first appeared in the duodenum and ileum at 12 hpi and were evident throughout the small intestine of all piglets thereafter with villus height to crypt depth ratios consistently below 4:1. Viremia was confirmed in 18/20 pigs at euthanasia. Clinical disease with an original North American PEDV isolate is rapid and severe with lesions, viremia and antigen detection possible by 12 hpi.

Introduction

Porcine epidemic diarrhea virus (PEDV), an enteric pathogen recently introduced into the United States (US) swine industry, is an enveloped, single-stranded RNA virus and member of the *Coronaviridae* family, genus *Alphacoronavirus* (de Groot et al., 2011). Following fecal-oral transmission, the virus infects and replicates in mature, small intestinal enterocytes resulting in villous atrophy that causes a malabsorptive, watery diarrhea with vomiting and anorexia in swine of all ages (Saif et al., 2012). Clinical signs and histopathologic lesions associated with PEDV infection are indistinguishable from those of transmissible gastroenteritis virus (TGEV); however, these alphacoronaviruses are antigenically distinct and do not demonstrate serological cross-reactivity (Pensaert et al., 1981).

Enteric disease associated with PEDV was first recorded in England in the early 1970s and has since spread to other European and Asian countries.(Song et al., 2012) After the initial introduction and outbreak of PEDV in the United States (April 2013), sequencing of PEDV isolates revealed similar nucleotide homology (>99%) with a Chinese strain entered into GenBank in 2012 (Huang et al., 2013; Stevenson et al., 2013). The source of PEDV introduction into the US swine population has not yet been determined; nonetheless, there have been over 6,000 PEDV-positive accessions to US veterinary diagnostic facilities originating from 30 different states as of May 2014 (http://www.aasv.org).

Because PEDV was a foreign animal disease in the US until 2013, its emergence generated numerous scientific questions from veterinarians, diagnosticians, swine researchers and the US swine industry. The intent of these studies were to characterize the pathogenicity of PEDV isolate US/Iowa/18984/2013 in post-weaned and neonatal pigs by 1) assessing clinical disease progression and gross lesions, 2) quantifying fecal viral shedding and antibody production, and 3) analyzing morphometric intestinal lesions and magnitude of immunohistochemical staining.

Stated Objectives from original proposal

- Study 1: Compare viral fecal titers, duration of fecal shedding, antibody development, oral fluid detection, intestinal microscopic lesions with antigen detection, and temporal tissue distribution of post-weaned pigs infected with porcine epidemic diarrhea virus (PEDv).
- Study 2: Measure the extent of intestinal microscopic lesions with antigen detection, viral fecal titers, and fecal shedding in neonatal caesarian derived-colostrum deprived (CDC) pigs infected with PEDv.
- Study 3: Determine if PEDv reinfection can occur by monitoring duration of fecal shedding, clinical signs and microscopic lesions in previously exposed pigs.

Materials and methods

Weaned pig study

Animals

Sixty-three, 3-week-old weaned pigs of mixed sex and breed were sourced from a single commercial, cross-bred farrow-to-wean herd with no known prior exposure to PEDV. Pigs were free of porcine reproductive and respiratory syndrome virus and TGEV antibodies

PEDV inoculum

Porcine epidemic diarrhea virus isolate US/Iowa/18984/2013 (GenBank accession #KF804028) was propagated in Vero cells (ATCC® CCL-81) as previously described (Hofmann et al., 1988). Briefly, this isolate was obtained from a case submission to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) on May 16, 2013 from a 2,500 head farrow-to-wean farm in Northern Iowa experiencing acute onset of diarrhea in suckling pigs. Virus isolation, plaque cloning and propagation were performed as previously documented (Hoang et al., 2013).

Study design and housing

Pigs were randomly allocated into control (*n*= 27) and challenged (*n*=36) groups. PEDV-challenged pigs received a 1 ml dose of $1x10^3$ plaque-forming unit (PFU)/ml via oro-gastric gavage using a 12 gauge French catheter flushed with 10 ml of 0.01M phosphate-buffered saline on days post inoculation (dpi) 0. Three control and four challenged pigs were randomly selected for necropsy on dpi 1, 2, 3, 4, 7, 14, 21, 28, and 35. The experimental design was approved by the Iowa State University Institutional Animal Care and Use Committee (protocol log #6-13-7593-S). A subset of pigs were (re)challenged at 56 days and then euthanized at 77 days.

Clinical assessment

Body weights were recorded for each pig prior to inoculation and weekly thereafter until study termination. Rectal temperature was recorded for a subset of pigs within each group (9 controls and 12 challenged pigs) that remained alive for the first 7 days post challenge. In addition, the total number of pigs with clinical diarrhea were subjectively scored for fecal consistency using the following criteria: 1) normal, 2) semi-liquid without a formed consistency (cow-pie feces), and 3) watery/liquid contents. Fecal scores were recorded daily for the first week, dpi 10 and 14, and weekly thereafter.

Biological sample collection and necropsy

Fecal swabs were collected prior to inoculation (dpi 0), daily for the first week post-inoculation, dpi 10, 14, 17, 21, 24, 28, 31 and 35 from all remaining pigs. Serum was collected on dpi 0, 5, 7, 10, 14, 21, 28 and 35. Fresh and formalin fixed sample collection at necropsy included: small intestine, cecum, colon, mesenteric lymph node, tonsil, stomach, lung, heart, liver, spleen, kidney and feces. Five sections of formalin fixed small intestine were collected and included: 1) duodenum, 2) proximal jejunum, 3) mid jejunum, 4) distal jejunum, and 5) ileum.

Histopathology and morphometry

In each of the five small intestinal sections, three perceived full length villi and crypts, based on tissue orientation from each of four serial sections, were measured using a computerized image system (Olympus DP72 camera, cellSens® digital imaging software). The mean villous length and crypt depth from each intestinal

segment was used to determine statistical differences. Villous height to crypt depth ratios were also determined using these calculated means.

PCR

Real-time RT-PCR was performed as previously described (Chen et al., 2014) with minor modifications to include viral standards with known infectivity titers for quantification.

Immunohistochemistry

Immunohistochemistry (IHC) slides were prepared for all five evaluated sections of small intestine. Antigen detection was semi-quantitatively scored based on the following criteria: 0=no signal, 1= 1-10% of villous enterocytes within the section showing a positive signal, 2= 11-50% of villous enterocytes showing a positive signal, and 3= greater than 50% of villous enterocytes showing a positive signal. Semi-quantitative scores were recorded by a single blinded veterinary pathologist.

Serology

Seroconversion was assessed using an indirect fluorescent antibody (IFA) test. Serum samples were diluted 1:40 in PBS prior to testing, and 50µl of each diluted sample was inoculated into each well.

Statistical analysis

Mean intestinal villous length and crypt depth data were analyzed with SAS 9.3 code (SAS Institute, Cary, NC) using linear mixed models, with group, necropsy, location, and their interactions as fixed effects and pig as random effect. Difference in mean response was assessed among groups by location and necropsy, and also between necropsy by group and location. One way analysis of variance (ANOVAs) was used for weight and body temperature evaluation. Data calculations were considered statistically significant at the level of $P < 0.05$.

Neonate study

Neonatal piglets used in this experiment were caesarian derived and colostrum deprived (CDCD) on day 113 of gestation.

Experimental design and housing

Thirty-six CDCD piglets were randomly divided into two groups: 1) negative control (n=16) and 2) challenged (n=20). Piglets were individually housed in 18 gallon plastic totes (Rubbermaid®, Port Washington, NY) for the study period and groups were kept in a BSL-2 animal facility separated by room, entry, and ventilation system. Four control and five challenged piglets were randomly selected and euthanized at 12, 24, 48, and 72 hrs post-inoculation (hpi).

PEDV inoculum

Plaque-cloned isolate US/Iowa/18984/2013 (GenBank accession #KF804028) was propagated in Vero cells (ATCC® CCL-81) and titrated by plaque assay as previously reported as reported above.

Sample collection and clinical observations

Fecal swabs were collected from all piglets prior to inoculation and at 12, 24, 36, 48, 60 and 72 hpi. Diarrhea, vomiting, lethargy, loss of condition and dehydration was recorded for each piglet prior to inoculation and every 12 hrs thereafter until euthanasia. Tissues collected at necropsy included fresh and formalin-fixed stomach, small intestine, colon, cecum, mesenteric lymph node, tonsil, lung, heart, liver, spleen, and kidney. Intestinal contents were also obtained from all pigs at necropsy. Sections of fixed small intestine for microscopic evaluation were collected as previously described and included standardized locations of: 1) duodenum, 2) proximal jejunum, 3) mid jejunum, 4) distal jejunum, and 5) ileum as previously described above.

Real-time RT-PCR

Swabs, tissue homogenates, and sera were processed on the day of collection and PCR was as decribed previously.

Histology, intestinal morphometry, and immunohistochemistry

Mean villus length and crypt depth was used for statistical analysis and for determining villus height to crypt depth ratios as previously described above. Immunohistochemistry was also has previously described.

Statistical analysis

Mean intestinal villus height and crypt depth values and ratios were analyzed by a commercial statistical software program (SAS version 9.3,SAS Institute, Cary, NC) using linear mixed models. Treatment group,

necropsy time point, small intestinal location, and their interactions were fixed effects while piglet was the random effect. Mean measurements were evaluated between groups by location and necropsy time point, and also between necropsy time points by group and location. Statistical significance was set at $P < 0.05$ for all analyses.

Results

The following tables summarize clinical disease (table 1), fecal PEDV shedding (table 2), villus height (table 3) and crypt depth (table 4) measures, and IHC staining in intestines (table 5) from the weaned study.

Table 1

		Fecal Consistency		
DPI	Clinical Observations	Normal	Semi-solid	Watery
0	All active and eating well; normal	100%	0%	0%
1	All active and eating well; normal	90%	10%	0%
2	10% lethargy and anorexia minimal vomiting	45%	30%	25%
3	10% lethargy and anorexia; minimal vomiting	30%	40%	30%
4	40% lethargy and anorexia	20%	50%	30%
5	75% lethargy and anorexia; gaunt	10%	40%	50%
6	85% lethargy and anorexia; gaunt	0%	30%	70%
7	60% lethargy and anorexia; gaunt	0%	50%	50%
10	All active and eating; filled out; normal	85%	15%	0%
14	All active and eating well; normal	95%	5%	0%
21	All active and eating well; normal	95%	5%	0%
28	All active and eating well; normal	100%	0%	0%
35	All active and eating well; normal	100%	0%	0%

Table 2. Fecal PEDV shedding in challenged pigs by days post-inoculation (DPI)

DPI	*N*	# positive	% positive	Mean PCR CT value	Mean PFU¥	Max PFU¥	Min PFU¥	Standard error (SEM)
0	36	0	0	>40	N/A	N/A	N/A	N/A
1	36	8	22%	29.3	7.41×10^{3}	6.67×10^{4}	3.60×10^{-1}	2.62×10^{3}
2	32	32	100%	20.4	1.42×10^{4}	7.87×10^{4}	5.50×10^{-1}	2.52×10^{3}
3	28	28	100%	14.1	5.12×10^{4}	1.78×10^{5}	3.69×10^{2}	9.67×10^{3}
4	24	24	100%	13.6	3.99×10^{4}	1.32×10^{5}	1.89×10^{3}	8.14×10^{3}
5	20	20	100%	14.8	7.29×10^{3}	1.97×10^{4}	1.96×10^{2}	1.63×10^{3}
6	20	20	100%	15.1	1.60×10^{4}	5.61×10^{4}	8.02×10^{2}	3.57×10^{3}
7	20	20	100%	18.6	4.39×10^{3}	2.12×10^{4}	1.05×10^{2}	9.83×10^{2}
10	16	11	69%	28.1	8.08×10^{1}	2.29×10^{1}	1.60×10^{1}	2.44×10^{1}
14	16	14	88%	26.3	6.28×10^{2}	3.18×10^{3}	6.00×10^{-2}	1.68×10^{2}
17	12	9	75%	30.2	3.44×10^{1}	1.29×10^{2}	8.60×10^{-1}	1.15×10^{1}
21	12	5	42%	33.3	8.21×10^{1}	1.36×10^{1}	3.50×10^{-1}	3.67×10^{-1}
24	8	5	63%	32.9	1.22×10^{1}	1.92×10^{1}	2.20×10^{-1}	5.44×10^{-1}
28	8	0	0	>40	N/A	N/A	N/A	N/A
31	4	0	0	>40	N/A	N/A	N/A	N/A
35	4	0	0	>40	N/A	N/A	N/A	N/A

¥ Plaque-forming unit (PFU) equivalent/ml in feces of PCR positive challenged pigs. N/A = not applicable

Table 3.

	Duodenum			Prox jejunum			Mid jejunum			Distal jejunum			Ileum		
DPI	Con.[¥]	Chall.[£]	*P*	Con.	Chall.	*P*	Con.	Chall.	*P*	Con.	Chall.	*P*	Con.	Chall.	*P*
1	729	786	0.34	610	655	0.45	532	641	0.07	532	575	0.47	464	569	0.08
2	716	681	0.55	673	565	0.07	618	557	0.30	585	572	0.82	446	488	0.48
3	805	412	**<0.01**	675	363	**<0.01**	670	357	**<0.01**	583	351	**<0.01**	488	325	**<0.01**
4	777	536	**<0.01**	705	414	**<0.01**	718	371	**<0.01**	656	365	**<0.01**	526	347	**<0.01**
7	736	414	**<0.01**	719	403	**<0.01**	702	375	**<0.01**	619	356	**<0.01**	489	287	**<0.01**
14	750	685	0.28	667	651	0.79	597	652	0.35	551	657	0.08	517	555	0.52
21	889	740	**0.01**	773	696	0.19	656	632	0.68	609	617	0.89	490	540	0.39
28	901	846	0.35	753	759	0.91	671	720	0.41	638	669	0.60	541	531	0.86
35	784	804	0.74	726	727	0.99	647	687	0.50	632	645	0.82	541	596	0.35

[¥] Control pigs
[£] Challenged pigs

Table 4.

	Duodenum			Prox. jejunum			Mid jejunum			Distal jejunum			Ileum		
DPI	Con.[¥]	Chall.[£]	*P*	Con.	Chall.	*P*	Con.	Chall.	*P*	Con.	Chall.	*P*	Con.	Chall.	*P*
1	135	172	**<0.01**	138	161	**0.05**	132	147	0.18	138	154	0.18	115	142	**0.03**
2	140	165	**0.03**	145	150	0.67	152	149	0.79	154	142	0.33	136	138	0.83
3	180	210	**<0.01**	149	212	**<0.01**	159	204	**<0.01**	160	208	**<0.01**	138	180	**<0.01**
4	155	157	0.85	164	164	0.99	176	171	0.64	164	163	0.91	143	149	0.59
7	144	185	**<0.01**	158	186	**0.02**	166	178	0.31	155	188	**<0.01**	140	156	0.18
14	169	207	**<0.01**	167	180	0.29	174	180	0.59	154	195	**<0.01**	150	165	0.19
21	181	170	0.36	159	167	0.50	170	162	0.45	181	162	0.10	164	143	0.07
28	158	165	0.55	167	165	0.85	164	171	0.60	164	162	0.81	159	163	0.74
35	179	176	0.79	180	178	0.86	178	186	0.53	170	171	0.93	173	172	0.94

[¥] Control pigs
[£] Challenged pigs

Table 5.

DPI	PCR		Immunohistochemistry		
	# fecal shedding pigs[Ω]	Mean CT	# positive pigs[£]	Mean positive sections/pig[¥]	Mean score/positive section[*]
1	2	21.9	2	4	2.25
2	3	23.6	2	5	2.90
3	4	14.1	4	5	2.80
4	4	13.7	4	5	2.55
7	4	16.2	4	4	2.13
14	4	27.1	2	3	1.17
21	2	>40	0	N/A	N/A
28	0	>40	0	N/A	N/A
35	0	>40	0	N/A	N/A

[Ω] Fecal PCR positive pigs at necropsy for a particular day post inoculation (maximum=4)
[£] Positive IHC pigs at necropsy for a particular day post inoculation (maximum=4)
[¥] Duodenal, proximal, mid, and distal jejunal, and ileal sections were stained; 5 total sections. This column is the mean number of positive intestinal sections out of five for IHC positive pigs (maximum=5)
[*]Mean IHC score for all positive small intestinal sections (maximum=3)
N/A= not applicable

The following graphs show IFA antibody response with time (figure 1), oral fluid vs feces detection (figure 2) and reinfection (figure 3).

Figure 1

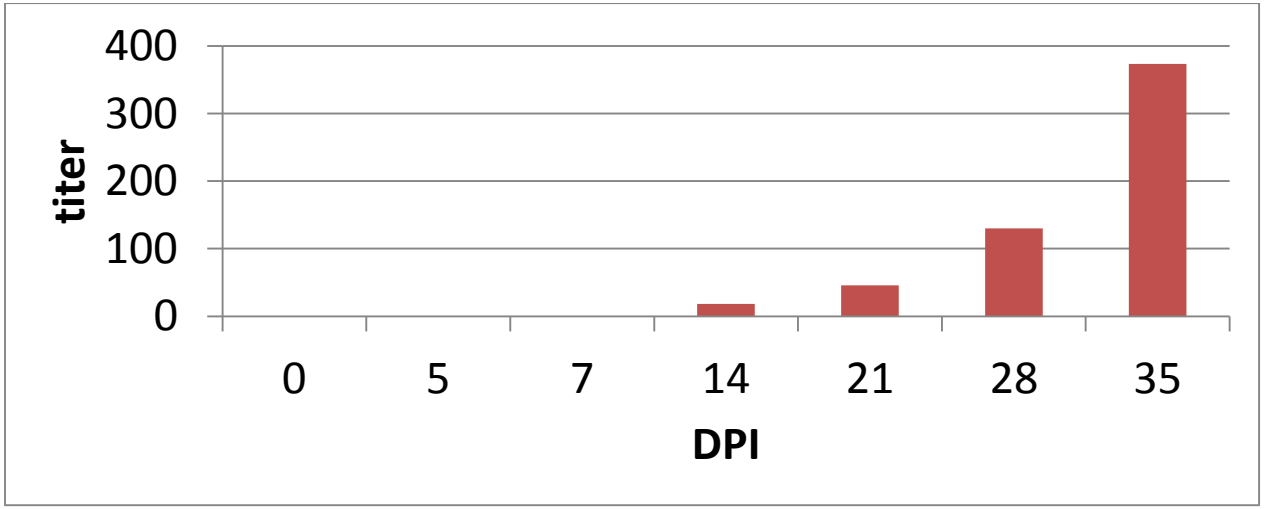


Figure 2

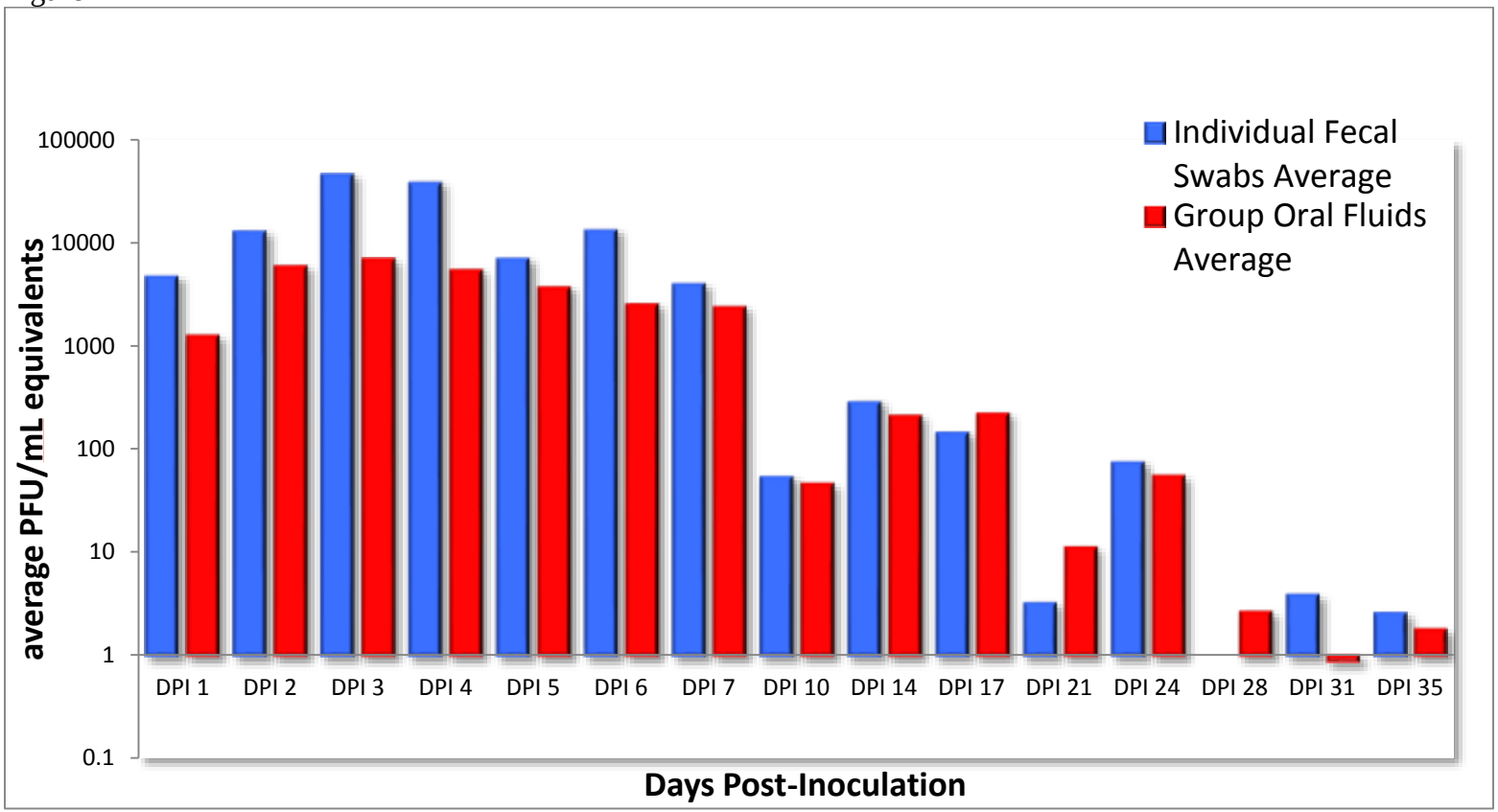
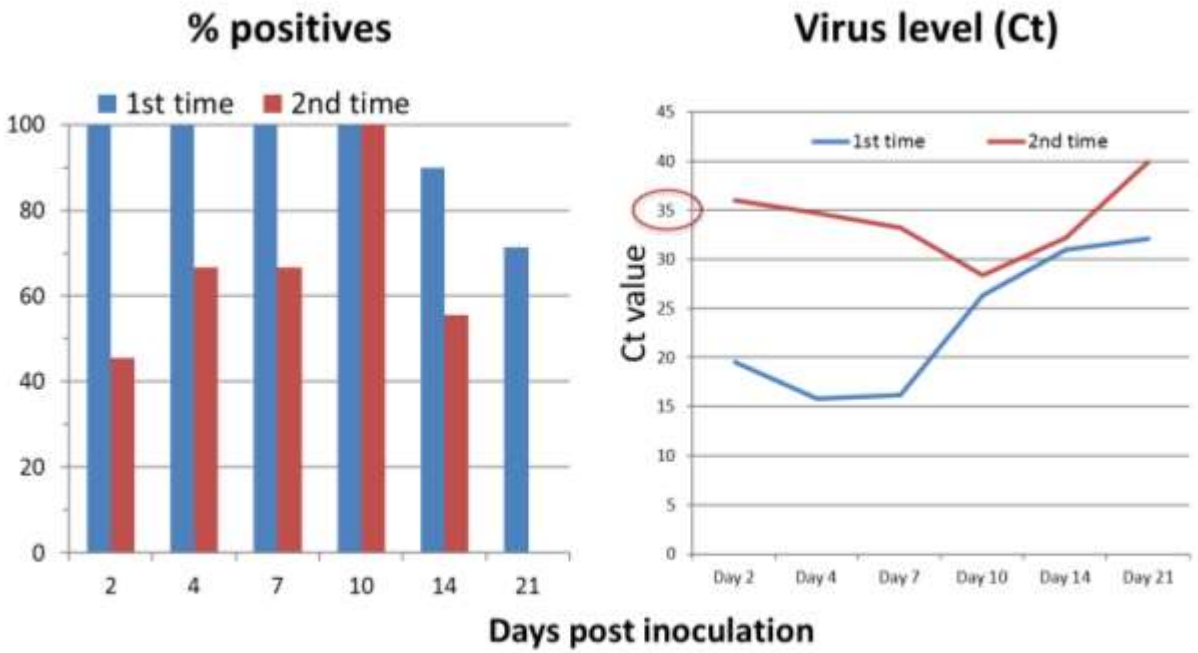

Individual Fecal Swabs Average
Group Oral Fluids Average
average PFU/mL equivalents
100000
10000
1000
100
10
1
0.1
DPI 1
DPI 2
DPI 3
DPI 4
DPI 5
DPI 6
DPI 7
DPI 10
DPI 14
DPI 17
DPI 21
DPI 24
DPI 28
DPI 31
DPI 35
Days Post-Inoculation

Figure 3


% positives
1st time
2nd time
100
80
60
40
20
0
2
4
7
10
14
21
Virus level (Ct)
1st time
2nd time
45
40
35
30
25
20
15
10
5
0
Ct value
Day 2
Day 4
Day 7
Day 10
Day 14
Day 21
Days post inoculation

Neonate study

The following table report fecal PEDV shedding (table 6), tissue distribution (table 7), villus height to crypt depth ratios (table 8).

Table 6

hpi	*n*	# positive	% positive	Mean PCR CT value	Mean PFU[¥]	Max PFU[¥]	Min PFU[¥]	Standard error (SEM)
0	20	0	0	N/A[†]	N/A	N/A	N/A	N/A
12	20	1	5%	22.4*	4.34×10^{1}	N/A	N/A	N/A
24	15	15	100%	13.4	3.08×10^{4}	9.64×10^{4}	2.42×10^{1}	6.88×10^{3}
36	15	15	100%	16.6	3.81×10^{3}	1.12×10^{4}	1.33×10^{2}	1.03×10^{3}
48	10	10	100%	21.8	1.56×10^{2}	8.33×10^{2}	1.38×10^{1}	7.46×10^{1}
66	5	5	100%	22.8	1.59×10^{2}	5.08×10^{2}	4.40×10^{-1}	8.14×10^{1}
72	5	5	100%	21.2	3.20×10^{2}	1.18×10^{3}	3.94×10^{0}	1.95×10^{2}

¥

Plaque-forming unit (PFU) equivalent/ml in feces of PCR positive challenged pigs

* Cycle threshold (Ct) value reported for one piglet positive at 12 hpi.

[†]N/A = not applicable

+

Table 7.

Sample	Necropsy											
	12 hpi			24 hpi			48 hpi			72 hpi		
	Mean CT	Mean PFU[Ω]	SEM	Mean CT	Mean PFU	SEM	Mean CT	Mean PFU	SEM	Mean CT	Mean PFU	SEM
Serum	21.60*	9.68×10^{1}	3.56×10^{1}	27.85	1.66×10^{1}	1.00×10^{1}	26.90	4.86×10^{0}	1.77	30.22*	6.14×10^{-1}	2.34×10^{-1}
Stomach	31.98*	7.31×10^{-1}	3.27×10^{-1}	26.56	6.16×10^{0}	2.76×10^{0}	30.75	2.98×10^{-1}	1.33×10^{-1}	29.77	2.81×10^{0}	1.26×10^{0}
Intestine	10.58	1.43×10^{5}	6.40×10^{4}	12.87	7.41×10^{4}	3.31×10^{4}	19.00	6.15×10^{2}	2.75×10^{2}	19.78	1.43×10^{3}	6.39×10^{-2}
Colon	23.24	2.29×10^{2}	1.02×10^{2}	14.91	9.59×10^{3}	4.29×10^{3}	20.54	1.63×10^{2}	7.29×10^{1}	21.37	2.74×10^{2}	1.22×10^{-2}
Mes. LN	25.12	1.22×10^{2}	5.48×10^{1}	22.92	8.83×10^{1}	3.95×10^{1}	25.35	1.58×10^{1}	7.06×10^{-2}	27.15	4.55×10^{0}	2.04×10^{0}
Lung	32.64	1.06×10^{-1}	4.73×10^{-2}	29.51*	1.05×10^{0}	4.71×10^{-1}	34.44	4.87×10^{-2}	2.18×10^{-2}	33.17[¥]	9.56×10^{-2}	4.27×10^{-2}
Spleen	28.58	1.59×10^{0}	7.10×10^{-1}	23.40	8.76×10^{1}	3.92×10^{1}	26.03	8.67×10^{0}	3.88×10^{0}	27.46	2.85×10^{0}	1.28×10^{0}
Kidney	32.02	1.54×10^{-1}	6.90×10^{-2}	28.86	2.21×10^{0}	9.90×10^{-1}	31.60	2.19×10^{-1}	9.80×10^{-2}	30.29*	7.73×10^{-1}	3.46×10^{-1}
Heart	32.91	1.11×10^{-1}	4.97×10^{-2}	32.68	2.65×10^{-1}	1.19×10^{-1}	35.03	2.86×10^{-2}	1.28×10^{-2}	35.85[¥]	2.21×10^{-2}	9.90×10^{-3}

[Ω]Plaque-forming unit (PFU) equivalent/ml in feces of PCR positive challenged pigs

Table 7.

hpi*	Duodenum			Prox jejunum			Mid jejunum			Distal jejunum			Ileum		
	Con.¥	Chall.£	*P*	Con.	Chall.	*P*	Con.	Chall.	*P*	Con.	Chall.	*P*	Con.	Chall.	*P*
12	939	639	**<0.01**	818	753	0.06	789	663	0.33	719	650	0.30	704	522	**<0.01**
24	1060	228	**<0.01**	975	225	**<0.01**	936	223	**<0.01**	857	221	**<0.01**	737	312	**<0.01**
48	989	199	**<0.01**	929	162	**<0.01**	893	171	**<0.01**	835	175	**<0.01**	698	191	**<0.01**
72	977	185	**<0.01**	939	187	**<0.01**	952	163	**<0.01**	985	170	**<0.01**	901	185	**<0.01**

*Hours post infection (hpi)

¥ Control pigs

£ Challenged pigs

I. Discussion

Weaned pig study

Porcine epidemic diarrhea virus infection can occur in swine of any age with suckling pigs being most severely affected. Reported clinical disease within a population typically occurs 4-5 days after shipping or receiving animals into a facility (Saif et al., 2012), although clinical diarrhea can occur as early as 24 hrs post inoculation in neonatal pigs (Coussement et al., 1982; Kim et al., 2003). Resolution of clinical signs, if uncomplicated by other factors, is often within 7-10 days post infection (Pospischil et al., 2002). Our current study showed a similar trend in the progression of clinical disease (Table 2). Clinical signs were first observed on dpi 2 with occasional vomiting and diarrhea. Surprisingly, there was a rather unexpected resolution of clinical signs by dpi 10 with only a minority of challenged pigs passing semi-solid feces in spite of 70% of challenged pigs shedding virus in feces at that time.

Porcine epidemic diarrhea virus nucleic acid was detected in feces in 22% (8/36) of challenged pigs on dpi 1 and all challenged pigs were shedding virus in feces from dpi 2-7. Onset of viral fecal shedding was similar to a recent report using a different US PEDV strain in gnotobiotic pigs (Jung et al., 2014). Peak quantities of PEDV in the current study were detected on dpi 3. Interestingly, this was three days prior to peak clinical signs indicating a significant biosecurity risk if infection is not recognized early. Fecal shedding slowly diminished thereafter and was intermittent in a few PEDV-challenged pigs with variable detection up to dpi 24. To our knowledge, this is the longest duration of PEDV shedding reported in pigs. Shedding was previously reported up to 9 days post infection (Song et al., 2005). This finding should be taken into consideration for herd management practice particularly related to pig movement as part of prevention and control of PEDV.

Viral antigen in villous enterocytes was observed on dpi 1 in multiple PEDV-challenged pigs. There was no apparent association between virus replication and a particular segment of intestine as antigen was detected in all segments that were evaluated. Porcine epidemic diarrhea virus IHC correlated with fecal shedding from dpi 1-7. In this study, overall PCR fecal detection was more sensitive than IHC as the duration of infection progressed. However, IHC is a highly sensitive and specific test in the acute phase of disease.

Atrophic enteritis was observed in all sections of small intestine. The degree of villous atrophy and fusion has been reported to be similar to TGEV, but somewhat less severe (Pospischil et al., 1981; Coussement et al., 1982). However, microscopic lesions associated with infection by this North American PEDV strain were indistinguishable from TGEV lesions (Stevenson et al., 2013). In this study, no particular segment or segments of small intestine appeared to be more severely affected. There was a trend that indicated villous height was decreased in the upper small intestine sections of challenged pigs relative to controls at dpi 2 although no significant differences were observed.

Neonate study

Outcomes of this study are similar to other previously published PEDV and TGEV challenge studies in neonatal swine (Debouck et al., 1981; Kim et al., 2002; Kim et al., 2003). Comparing previous studies with results from the current study, it is apparent that 1) virulence can vary

amongst PEDV isolates and 2) PED can be as severe as transmissible gastroenteritis (TGE) in naïve neonates.

Virus isolate CV777 was the first PEDV characterized in Europe and evaluated in pathogenesis studies (Pensaert et al., 1978; Debouck et al., 1981). This particular isolate shares 96.9% nucleic acid homology with US/Iowa/18984/2013 and resulted in diarrhea 22-36 hpi in 2- to 3-day-old CDCD piglets when challenged oro-nasally with 2 ml of 10^4 pig-infectious-doses with antigen positive intestinal cells and villus atrophy noted 18 and 24 hpi, respectively (Debouck et al., 1981). In a more recent investigation, 2 ml of Korean isolate SNUVR971496, $10^{6.5}$ $TCID_{50}$, orally dosed, produced diarrhea at 12 hpi, but initial IHC detection of antigen was not reported until 24 hpi (Kim et al., 2003). Differences in villus measurements between challenge and control piglets were also not observed until 24 hpi with this particular Korean PEDV isolate (Kim et al., 2003). Results from the present study (10^3 PFU) differ in that both villus atrophy and antigen detection was observed 12 hpi with IHC staining being intense in the upper small intestine of all examined piglets (Figure 2). Test sensitivity, antibody affinity, antigen retrieval methods for IHC, and viral dose are all factors that may have impacted these IHC findings as methods differed between studies; however, clinical disease and microscopic lesions both developed earlier in the course of infection with PEDV isolate US/Iowa/18984/2013 compared to either CV777 or SNUVR971496 even though piglets were challenged with a lower viral dose. This heightened replication of the North American PEDV isolate suggests increased virulence compared to the historic CV777 European PEDV or some Asian viruses. In contrast, US/Iowa/18984/2013 induces similar clinical signs and severity of disease as reported with recent Chinese PEDV strains, which have exhibited enhanced disease in endemic regions with increased morbidity and mortality (Li et al., 2012). These observations along with the results from this study suggest there are virulence differences between PEDV isolates, similar to what has been reported previously for TGEV (Kim et al., 2002).

Porcine epidemic diarrhea virus has been previously described as less virulent than TGEV (Pospischil et al., 2002; Kim et al., 2003); however, these studies were conducted prior to the increased virulence of Chinese PEDV strains documented in 2010 (Li et al., 2012). Our results suggest a similarity between severities of clinical disease and microscopic lesions. Specifically, characteristic diarrhea was reported earlier (18 hpi) in the current study compared to what was observed in the study conducted with three different TGEV and all piglets had severe diarrhea by 36 hpi in both studies with anorexia and lethargy.

Coronavirus replication in mature enterocytes is rapid with previous pathogenicity investigations detecting PEDV nucleic acid in feces by 24 hpi (Jung et al., 2014). In the current study, PEDV RNA was first detected in feces from one piglet at 12 hpi with all remaining piglets positive at 24 hpi.

Porcine epidemic diarrhea virus viremia was confirmed in 90% of the challenged pigs at euthanasia in this experiment. This finding is consistent with a recent publication showing PEDV viremia can occur in the acute stage of infection (Jung et al., 2014). Previously unreported prior to this study was viral RNA detection in non-enteric tissue samples. Evaluating tissue and serum PCR findings, detection in heart, kidney, lung, stomach, and spleen are arguably a function of viremia and/or pooling of blood. No PEDV antigen was detected by IHC in these tissues except

for spleen; yet, differences in analytical sensitivity between IHC and PCR are well known. Colon and mesenteric lymph node had lower PCR Ct values and variable IHC positive signaling, suggesting the colon is a potential site of minor viral replication and immune cell phagocytosis followed by antigen presentation in lymphoid tissue. Porcine epidemic diarrhea virus antigen has previously been reported in the colon and mesenteric lymph node (Debouck et al., 1981; Kim et al., 2003).

Summary

Porcine epidemic diarrhea virus unexpectedly entered the North American swine population in 2013 resulting in widespread and devastating disease. Understanding this virus and its infection characteristics has proven difficult worldwide. Experimental inoculation of three-week-old pigs with PEDV isolate US/Iowa/18984/2013 induced severe clinical disease with stalled weight gain for approximately 10 days post inoculation. Diarrhea with measurable differences in villous to crypt ratios were first observed on dpi 3; however, viral shedding and PEDV antigen detection within infected cells can be as early as 24 hrs post-infection leading to possible transmission of virus prior to the onset of clinical signs. Porcine epidemic diarrhea virus shedding, under the conditions of this wean-pig study, was much longer than previously reported as viral shedding was detected up to dpi 24 which was approximately two weeks post resolution of clinical disease. Accordingly, an absence of clinical diarrhea does not eliminate the risk of PEDV transmission in weaned pigs. In neonatal piglets PEDV infection was severe resulting in diarrhea, dehydration, and death. Viral shedding was confirmed by 12 hr post infection. Comparing neonate study results with previous reports, our data confirms that clinical differences can exist among PEDV isolates and PED can be as severe as TGE in neonates.

Thank you for funding this project. If there are any questions please feel free to contact me.
Sincerely

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