Effect of porcine epidemic diarrhea virus infectious doses on infection outcomes in naïve conventional neonatal and weaned pigs

by

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NOMENCLATURE

PEDV	Porcine Epidemic Diarrhea Virus
TGEV	Transmissible Gastroenteritis Virus
PRCoV	Porcine Respiratory Coronavirus
PDCoV	Porcine Deltacoronavirus
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus
ORF	Open Reading Frame
RTC	Replication Transcription Complex
NSP	Non-Structural Protein
PP1ab	Polyprotein 1ab
PP1a	Polyprotein 1a
ssRNA	Single Stranded RNA
dsRNA	Double Stranded RNA
sgRNA	Sub-Genomic RNA
RBD	Receptor Binding Domain
pAPN	Porcine Aminopeptidase N
S	Spike
N	Nucleocapsid
M	Membrane
E	Envelope
CD13	Cluster of Differentiation 13
PAMP	Pathogen Associated Molecular Pattern

RIG-1	Retinoic Acid-Inducible Gene 1
MDA-5	Melanoma Differentiation Associated Protein 5
TLR	Toll Like Receptor
IRF3	Interferon Regulatory Factor 3
NF-κβ	Nuclear Factor κβ
ATF-2/c-Jun	Activating Transcription Regulatory Factor c-Jun
IFN-α/β	Interferon α/β
CBP	CREB Binding Protein
PLP2	Papain Like Protease 2
STING	Stimulator Interferon Gene
NEMO	NF- κβ Essential Modulator Protein
IL-8	Interleukin 8
ER	Endoplasmic Reticulum
NKC	Natural Killer Cell
IgG	Immunoglobulin Gamma
IgA	Immunoglobulin Alpha
ADCC	Antibody Dependent Cellular Cytotoxicity
EM	Electron Microscopy
VI	Virus Isolation
FA	Fluorescent Antibody
IFA	Indirect Fluorescent Antibody
ELISA	Enzyme Linked Immunosorbent Assay
IHC	Immunohistochemistry
ISH	*in situ* Hybridization
PCR	Polymerase Chain Reaction

RT-PCR	Reverse Transcription Polymerase Chain Reaction
VN	Virus Neutralization
MID	Minimum Infectious Dose
CT	Cycle Threshold
$TCID_{50}$	50% Tissue Culture Infectious Dose
PFU	Plaque Forming Unit
CPE	Cytopathic Effect
DPI	Days Post Inoculation

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ABSTRACT

Porcine epidemic diarrhea virus (PEDV) has been an important pathogen in Europe and Asia for several decades. It was identified in the United States (U.S.) swine population for the first time in April 2013, and spread rapidly across country and into Canada and Mexico. However, no information has been published regarding the minimum infectious dose (MID) of PEDV in different pig models. The main objective of this study was to determine the oral minimum infectious dose of PEDV in naïve conventional neonatal piglets and weaned pigs. A U.S. virulent PEDV prototype isolate (USA/IA19338/2013) with known infectious titer was serially ten-fold diluted in virus-negative cell culture medium, and inoculated into 5-day-old and 3-week-old pigs. Our data showed that PEDV is infectious in an age-dependent manner with a significantly lower MID for neonatal pigs compared to weaned pigs. Furthermore, it showed that, once an infection was established in pigs, the initial dose of PEDV administered did not affect the extent of fecal viral shedding, severity of histopathologic lesions, or magnitude of antibody titer subsequently developed. This information should be taken into consideration when interpreting clinical relevance of PEDV PCR results, designing a PEDV bioassay model, as well as emphasizes the importance of strict biosecurity and thorough cleaning/disinfection on sow farms.

CHAPTER 1: INTRODUCTION

Background

Porcine epidemic diarrhea virus (PEDV) is the causative agent for Porcine epidemic diarrhea (PED), a disease which was first described in England in the 1970's. It subsequently spread through much of Europe and Asia, before being introduced to the United States in April of 2013. Within one year of introduction to the US, the virus had been detected in 29 states and resulted in the death of over 7 million pigs. Factors that contribute to the ease of introduction and spread of a virus through a population include the immune status of the host population, the inherent ability of the virus to resist environmental degradation, and the dose of virus necessary to infect an individual animal. Practical investigation into these factors should be framed in the context of relevant diagnostic assays. Doing so allows for better application of scientific data to real life clinical scenarios.

Objectives

The objectives of this research were to: 1. Correlate PEDV RT-PCR Ct values to infectious titers of the virus. 2. Determine the minimum infectious dose of PEDV in naïve neonatal piglets. 3. Determine the infectious dose of PEDV in naïve weaned pigs and to assess their corresponding antibody responses.

Thesis Organization

This thesis consists of an introduction, literature review, one original research chapter that has been published in PLOS ONE, and a conclusion. Chapter 1 serves as the introduction and chapter 2 serves as the literature review. Topics included in the literature include PEDV classification and genome organization, genetic diversity and molecular epidemiology, transmission, infection and pathogenesis, immune response, diagnosis of infection, and

prevention and control. Chapter 3 consists of original research regarding the effects of PEDV infectious doses on outcome of infection in neonatal and weaned pigs. The author's role in this study included development of study design, titration of the viral isolate, execution of animal studies, necropsy and sample collection from test subjects, execution of laboratory testing, analysis and interpretation of results, and manuscript writing. This chapter is presented as it appears in publication. Chapter 4 consists of a discussion/conclusion.

CHAPTER 2: LITERATURE REVIEW

Classification and Genome Organization

Taxonomy

Porcine epidemic diarrhea virus (PEDV) is a spherical, enveloped, positive sense and single stranded RNA virus. It is a member of the genus *Alphacoronavirus* along with two other important swine pathogens: transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCoV), as well as numerous other mammalian coronaviruses. This genus along with the 3 others: *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* make up the subfamily *Coronavirinae*. *Coronavirinae* together with *Torovirinae* make up the *Coronaviridae* family, so named because of member viruses' crown-like appearance on electron microscopy. Members of this family along with the members of 3 other families: *Arteriviridae*, *Roniviridae*, and *Mesonivirinae* comprise the relatively newly described order *Nidovirales* (1). Membership to this order is based on genomic organization, replication/transcription strategy, and characteristics of the replicase/transcriptase polyprotein (2). It derives its name from the Latin word *nido* meaning "nest" due to the fact that members of this order replicate via transcription of a set of nested subgenomic messenger RNA's at the 3'end of their genome. Porcine reproductive and respiratory syndrome virus (PRRSV), perhaps the most economically important swine pathogen in the US, is a member of the *Arteriviridae* family. This means that 2 of the most devastating swine diseases in the United States (PRRS and PED) are caused by Nidoviruses.

Genome Structure and Protein Function

The genome of PEDV is approximately 28 KB in length, and is encompassed by a 5' cap and 3' polyadenylated tail. It encodes both 5' and 3' untranslated regions along with 7 open reading frames (ORFs): 1a, 1b, and 2-6 (3, 4).

ORFs 1a and 1b comprise the 5' two-thirds of PEDV's genome and encode what is known as the "replicase" gene. Expression of this gene is made possible by a "-1 ribosomal frameshift" occurring during some constant proportion of translation events. This frame shift occurs due to the presence of a "slippery sequence", 5'-UUUAAAC-3', and a downstream RNA pseudoknot structure, which together result in a change in "reading frame" and no termination at the ORF 1a stop codon (5). The end result of this translation strategy is production of a polyprotein 1a (pp1a) most of the time, and production of the longer polyprotein 1ab (pp1ab) some smaller proportion of the time. Proposed reasons for this mechanism include production of the non-structural proteins (nsps) encoded by ORFs 1a and1b at a constant proportion necessary for assemblage into the replicase-transcriptase complex (RTC) (6), and/or to delay the production of the enzymatic nsps processed from pp1ab until those processed from pp1a have altered the cellular environment to better allow for RNA synthesis (7).

These two polyproteins are post translationally processed to form 16 non-structural proteins (nsp1-16), with cleavage of pp1a forming nsp1-11, and cleavage of pp1ab forming nsp1-10, and nsp12-16. These proteolytic products then assemble to form the replication transcription complex (RTC). While the function of the individual nsps has not been extensively studied in PEDV specifically, they have been described for others in the coronavirus family. Nsp1 has been demonstrated to aide in evasion of the host immune system through inhibition of Type 1 IFN production (8). The large nsp3 protein has been shown to have a variety of

functions, including: autoprotolytic cleavage of nsp1, nsp2, and nsp3 from pp1a and pp1ab via "papain like proteases" contained within nsp3 (9), interaction with the N protein to help initiate RNA synthesis (10), several mechanisms to aid in avoiding host immunity (11, 12), as well as others not listed here. Nsp4, nsp6, and nsp3 contain transmembrane helices that bind the RTC to cellular membranes (13, 14). Nsp5 also referred to as the main protease (M^{pro}) or 3C-like protease ($3CL^{pro}$) is responsible for the 11 cleavages of pp1a/pp1ab not performed by nsp3 (15, 16). Nsp7 and nsp8 form a structure with a central pore that has been proposed as the RNA clamp for the RNA Dependent RNA Polymerase encoded by nsp12 (17, 18). Nsp9 binds single stranded RNA (ssRNA), while nsp10 binds zinc and may also play a role in the processing of nsp5 (16, 19-22). Nsp13, nsp14, and nsp16 are involved in the production of the 5'-termainal cap found on the viral genome and sgRNA (16, 23-26). Finally, both nsp14 and nsp15 serve as ribonucleases, with nsp14 apparently playing a role in the "proof reading" of viral RNA during replication (16, 27-29).

Expression of ORFs 2-6 is accomplished via the production of corresponding sgRNAs. Cis-acting elements at the 5' and 3' end of the genome interact during replication allowing for discontinuous extension of the replicating RNA strand, leading to the synthesis of sgRNA via negative stranded RNA intermediates. Each sgRNA subsequently serves as a template for the protein encoded by the ORF at its 5' end (5).

ORF2 encodes a class 1 fusion protein called the spike protein (S protein). This 128-160 kilodalton (kDa) S protein monomer consists of a highly variable S1 domain, and highly conserved S2 domain (30-32). These monomers trimerize to form the 18-23nm club like projections seen on electron micrographs of coronaviruses, with the S1 and S2 domains forming the bulbous and stalk like portions of this structure respectively (33). Glycosylation of the S

monomers occurs cotranslationally, as well as after the S trimer is formed (33). The S1 portion contains the receptor binding domain (RBD), which binds to porcine aminopeptidase N (pAPN) found on host enterocytes (34). This allows for entry into the host cell, although the exact mechanism of entry varies among coronaviruses and has not yet been described for PEDV. Additionally, the S protein is postulated to harbor neutralization epitopes and is also associated with viral virulence/attenuation (35-39).

The accessory protein encoded by ORF3 may be associated with cell culture adaptation and may also have an influence on cell cycle and subcellular structure (40, 41).

ORF4 encodes the 8-12 kDa envelope protein (E protein). This small unglycosylated protein appears to interact with the membrane protein (M protein) during envelope formation (42). The E protein also appears to play a role in virion release (43).

ORF5 encodes the M protein. This 25-30 kDa protein contains a large globular endodomain, 3 transmembrane domains, and a small extensively glycosylated ectodomain (44). It is the most abundant structural protein found in coronaviruses, and appears to be responsible for their spherical shape as well as contact with their nucleocapsid (45, 46).

The 43-50 kDa nucleocapsid protein (N protein) is encoded by ORF6. This protein binds to viral RNA, forming the protein component of the helical nucleocapsid (5, 7). It also binds the globular endodomain of the M protein (47, 48). The N protein has also been shown to antagonize interferon-β production (49).

Genetic Diversity and Molecular Epidemiology of PEDV

Phylogenetic analysis of global PEDV isolates has been performed based on full length genome, full length S gene, S1, S2, E, M, N, and ORF 3 gene nucleotide sequences. Of these

analyses, those based on full length S, S1, and S2 most closely resembled the genetic diversity seen with whole genome analysis, demonstrating their utility as targets for studying genetic relatedness of PEDV isolates (50). PEDV isolates can be phylogenetically separated into 2 genogroups: G1 and G2 based on variations in the N-terminal domain of the S protein (51). These can be further separated into subgroups G1a, G1b, G2a, and G2b. CV777 the prototype PEDV isolate, first described in Europe in 1978, belongs to G1a (32) . The first Asian country that reported a PEDV outbreak was Japan in 1982 (52). Since then PEDV epidemics have been described in many Asian countries including China, South Korea, Thailand, and Vietnam (53-59). Many PEDV isolates responsible for the PEDV epidemics in Asia have a specific set of small insertions and deletions within the S protein when compared to CV777, placing them in G2 (60). Since October 2010, a large-scale and severe PED epizootic had occurred in multiple provinces in China, characterized by high mortality rates among suckling piglets (61, 62).

PEDV was detected in US swine for the first time in April 2013 with severe clinical presentations (63) and the virus spread rapidly across the country, resulting in the estimated death of over 7 million pigs in the first year and substantial economic losses (64). Isolates initially identified in the 2013 US outbreak, termed “US PEDV prototype strains,” were most genetically similar (>99.5% nucleotide identity) to the Chinese isolate AH2012 (50, 51, 65). Subsequent to initial US PEDV outbreak, US prototype-like PEDV has been identified in Canada, Mexico, Taiwan, South Korea, and Japan (66-70).

In the spring of 2014 a new so called “variant” strain of PEDV (OH851) was identified in US swine with mild clinical signs based on field observations (71). When compared to the US PEDV prototype strains, the US PEDV variant strain has 3 deletions (a 1-nt deletion at position 167, a 11-nt deletion at positions 176-186, and a 3-nt deletion at positions 416-418) and one 6-nt

insertion between positions 474 and 475 in the spike gene; thus this variant strain was also called S INDEL strain (67). However, some people have argued with calling the US PEDV variants as 'S INDEL' strain because they think genetic comparisons of all PEDV strains should be made to the global prototype strain CV777 (32). But since a number of publications have used 'S INDEL' to describe the US PEDV variants (67, 72, 73), in order not to create further confusions, we have proposed to refer US PEDVs as 'US PEDV prototype strain' and 'US PEDV S-INDEL-variant strain' (65, 74). Phylogenetic analysis based on the first 1170 nucleotides of the S1 region indicated that the US PEDV S-INDEL-variant strain was most closely related to the Chinese G1b isolate CH/HBQX/10 (71). It is likely that G1b strains arose from recombination events between G1a and G2 strains in China in or before 2010 (32, 61). Retrospective testing demonstrated that the US PEDV prototype strain was present in the US from April 15, 2013 and the US PEDV S-INDEL-variant strain was present in the US from May 16, 2013 (Chen et al, unpublished data). Two PEDV strains were likely introduced into the US concurrently. Interestingly, US S-INDEL-variant-like PEDVs have also been detected in South Korea, Japan, Germany, Belgium, France, Portugal, Austria and Slovenia (75-82).

Transmission of PEDV

Transmission of PEDV, as with most enteric pathogens, occurs primarily via a fecal-oral route. Aerosol transmission has been described for other alphacoronaviruses in pigs and may represent a potential transmission route for PEDV as well (83). The geographical spread of PEDV from infected premises has been demonstrated to mirror prevailing wind patterns, suggesting airborne dissemination of the virus (84). Alonso et al. detected airborne PEDV RNA within positive premises and as far as 10 km downwind from those same sites. While they were

able to demonstrate the infectivity of airborne PEDV RNA within a positive premise (via oral gavage into a 10 day old pig bioassay), they were not able to demonstrate infectivity of PEDV RNA collected downwind from those sites (85). It is important to note that while this demonstrates that PEDV is able to remain infectious while aerosolized, it does not demonstrate the ability of aerosolized PEDV to remain infectious when exposed to environmental factors such as heat and UV radiation. Furthermore, it does not conclusively demonstrate aerosol transmission within a site, as positive air samples were orally gavaged into pigs. Other studies have successfully housed PED positive pigs in very close proximity to negative pigs and did not cause viral transmission as long as direct/indirect contact between the positive and negative pigs was avoided (86, 87), suggesting that aerosolization of PEDV may not play a large role in transmission.

The exact route of PEDV introduction into the US is not known, and likely will remain unknown. However, its simultaneous introduction into several premises with excellent biosecurity during the initial outbreak led many to look to feed as the vehicle of transmission for the virus (88). A proof of concept of contaminated complete feed as a fomite for PEDV transmission was demonstrated (89, 90), and a report from an early case in Ohio implicated a pelleted piglet diet as the source of infection (91). Spray dried porcine plasma (SDPP), a component of hog feed, was implicated as a potential source of PEDV introduction into Canada (66, 92). However, other reports have demonstrated the efficacy of the spray drying process in inactivating PEDV (93, 94). Another study showed the contamination of livestock trailers with PEDV at bottlenecks in production such as harvest facilities and livestock auctions, demonstrating their potential as fomites in the spread of PEDV (95). Regardless of what the route of initial introduction into a previously unaffected area is, once PEDV is introduced, a

strong spatio-temporal clustering pattern of infected premises emerges. This suggests that indirect transmission, whether through fomites or aerosol, is responsible for the local transmission of PEDV (96, 97).

Infection and Pathogenesis

Host Range and Cellular Tropism

There are many coronaviruses affecting both mammalian and avian species. Some infect multiple species, while others are limited to one host species. To date, PEDV has only been shown to infect swine (98); however, intestinal epithelial cells derived from other species have been infected with PEDV *in vitro* (99). Coronaviruses generally target host respiratory and/or enteric epithelium. PEDV primarily targets porcine enterocytes. However, recent reports have shown the replication of PEDV in porcine alveolar macrophages as well as porcine monocyte-derived dendritic cells (100, 101).

Cell Entry

The S protein of coronaviruses is a class I fusion protein. The S1 domain is responsible for receptor binding, while the S2 domain comprises the membrane fusion machinery. In an effort to prevent early inappropriate triggering of this fusion mechanism, the S protein exists in a "locked conformation" (102). For the exposure of the fusion peptide and subsequent fusion of viral envelope and cell membrane to occur, a site at the junction of the S1 and S2 domains must be proteolytically cleaved (103). For some coronaviruses, such as Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), this cleavage event is performed by intracellular endosomal proteases prior to viral release (104). For PEDV, cleavage is only accomplished with

exogenous proteases i.e. trypsin. Interestingly, for this to occur in PEDV, the S1 domain must first bind to its receptor, which thereby theoretically exposes the cleavage site (102). However, trypsin independent cell entry has been accomplished by both serial passage in Vero cells as well as a single point mutation in the S2 domain of the S protein (40, 105).

The functional receptor of most alphacoronaviruses is Aminopeptidase N (APN), also designated as cluster of differentiation 13 (CD13). This is the case for several important swine alphacoronaviruses including TGEV, PRCoV, and PEDV (34, 106). APN is a zinc binding protease involved in pain management, vasopressin release, cell proliferation and immunity in host tissues (107), however the enzymatic properties of APN do not appear necessary for virus entry (108). Animals previously resistant to PEDV infection are rendered susceptible after transgenic expression of porcine APN (pAPN) (109). Similarly, introduction of soluble pAPN into non-susceptible animals increased their anti-PEDV antibody production (110). Intestinal epithelial cells mature in a polarized manner (111), resulting in pAPN localization onto the apical surface of the plasma membrane (112). As a result, PEDV entry and release is restricted to this area (112). The use of sugar co-receptors has been suggested to play a role in the enteric tropism of other swine coronaviruses (113). PEDV's recognition of N-acetylneuraminic acid had been suggested to perform a similar function (103).

Viral replication and release

After membrane fusion and nucleocapsid delivery into the cell cytoplasm, pp1a and pp1ab are translated from ORFs 1a and 1b. These are proteolytically cleaved and assemble to form the RTC as previously described. The RTC subsequently transcribes negative sense full length genomic RNA along with 5 negative sense subgenomic RNAs corresponding to ORF 3

and proteins S, E, M, and N. These negative sense RNA transcripts are used as templates by the RTC to form positive sense genomic and subgenomic RNA. The subgenomic RNAs are translated to synthesize the protein coded by the ORF at their 5' most end. Structural proteins S, E, and M insert into the endoplasmic reticulum, while N proteins binds to the full length positive sense genomic RNA to form a helical nucleocapsid. This nucleocapsid then buds through the Endoplasmic Reticulum-Golgi intermediate compartment (ERIC) forming virion filled smooth walled vesicles. These vesicles fuse to the plasma membrane, thereby releasing virions from the host cell (7, 32).

Gross and Histopathologic Lesions

Gross lesions observed with PEDV infection are typical for an atrophic enteritis, including transparent thin walled small intestines with watery content (114, 115). Immunohistochemical (IHC) staining shows PEDV primarily targets mature villous enterocytes, with occasional staining in replicating crypt cells (63). Madson et al. described microscopic changes as "superficial villous enterocyte swelling with minimal cytoplasmic vacuolation and karyomegaly, necrosis of scattered enterocytes, enterocyte attenuation, sloughing of degenerate and necrotic enterocytes, contraction of subjacent villous lamina propria containing apoptotic cells admixed with scattered neutrophils and lymphocytes, and crypt cell hyperplasia" (115). The contraction of the villous lamina propria and hyperplasia of crypt cells in response to PEDV infection are evidenced structurally as reduced villous height and increased crypt depth. This is often quantified as the villous height to crypt depth ratio. Decreases in this ratio are observed in duodenum, jejunum, and ileum tissue sections from infected pigs (65, 86, 115-117). This pattern of microscopic lesions is typical of a viral enteritis, and is not distinguishable from those caused

by TGEV or a severe rotavirus infection. Although PEDV viral antigen could be detected by IHC or immunofluorescence staining in small intestine, cecum, colon, mesenteric lymph node, and some spleen tissues, PEDV-related microscopic lesions were only apparent in small intestines (65, 116-118). Microscopic lesions or IHC staining were not observed in other non-enteric tissues (such as tonsil, heart, lung, liver, kidney, and muscle) of PEDV-infected pigs although low level of PEDV RNA could be detected from these tissues (65, 117).

Clinical Signs/viral shedding

Virulent PEDV infection results in anorexia, lethargy, and severe watery diarrhea as early as 1 day post inoculation (DPI) in conventional neonatal piglets or in 1-day-old cesarean-derived colostrum-deprived piglets (117). Diarrhea is likely a result of several factors including: loss of enterocytes and dysfunction of remaining enterocytes leading to malabsorption, reduction in membrane bound digestive enzymes leading to maldigestion, and decreased gut integrity due to loss of junction proteins leading to increased water loss into the gut lumen (114, 115, 117, 119, 120). Vomiting is also often described in the early stages of infection, but mechanisms behind this has not been elucidated. Electrolyte derangements observed in PEDV infected pigs include hypernatremia, hyperkalemia, hyperchloremia, with hypocalcemia and decreased bicarbonate (114). Body temperatures are reduced during the acute stages of infection, resulting in heat seeking behavior. In the field this is often seen as suckling pigs lying on top of sows. A significant reduction in average daily gain (ADG) have been demonstrated in weaned pigs in the first week of infection, but not thereafter (115). It is unclear whether or not this translates to an increase in time to market weight. Electrolyte derangement and dehydration due to diarrhea often lead to death in affected pigs. Mortality rates may approach 100% in naïve neonatal pigs,

and decreases generally with age. Virus is detectable in feces as early as 12 hours post inoculation by RT-PCR, and is generally cleared by 21-28 DPI (86, 114, 115).

Age dependent susceptibility

Early reports of PEDV in Europe described suckling pigs as unaffected by the disease (121). However, over time researchers began to observe more severe clinical disease in suckling pigs (115, 117, 122, 123). The reason/mechanism for this adaptation over time to younger hosts has not been described. Possible reasons for this increased susceptibility of neonatal pigs includes: longer intestinal villi comprised of more mature permissive enterocytes (124), increased susceptibility to electrolyte derangement/water loss (125), and a less mature immune system as demonstrated by lower phagocytic capabilities and decreased IFN-α levels (125-128). Another likely contributing factor is the time it takes for a replicating crypt cell to become a mature enterocyte: 7-10 days in neonatal pigs vs 2-4 days in weaned pigs as described by Moon et al. in 1971 (129). This idea was recently supported by Jung et al. (123) who demonstrated decreased markers of crypt cell proliferation (LGR5+ and Ki67) in neonatal pigs compared to weaned pigs.

Virus strain dependent virulence differences

The genetic diversity of PEDV strains likely results in differences in virulence. Global PEDV strains have not been head-to-head compared for their virulence differences in experimentally infected pigs. However, since PEDV emerged in the US in 2013, a number of studies have been conducted to evaluate pathogenicity of US PEDVs. The US PEDV prototype strain has been experimentally confirmed as highly virulent in gnotobiotic (116), cesarean-

derived-colostrum-deprived (CDCD) (117, 130), and conventional pigs (65, 86, 115, 123). Recent experimental studies demonstrate that the US S-INDEL-variant PEDVs overall had lower pathogenicity compared to the US prototype strains in 3-4 day old pigs or 5-day old pigs or 1-week old pigs (65, 73, 76). However, the virulence of S-INDEL PEDVs observed in the field varies among farms and countries (71, 78). Further investigations of pathogenicity of S-INDEL PEDV variants in pigs at different ages are warranted.

Immune Response to PEDV Infection

Innate Immunity

Innate immunity plays a very important role in the pathology of viral infections. The interplay between this first line of defense and a virus's ability to evade it will set the stage for the development of clinical disease caused by a particular virus. Viral components are largely host derived, which can make recognition by the host difficult. Recognition of viral nucleic acid as a pathogen-associated molecular pattern (PAMP) is one method utilized by a host's innate immune system to determine that a viral invasion has occurred and to initiate an antiviral response. Recognition of double stranded RNA (dsRNA, a replicative intermediate of RNA viruses) by cytoplasm associated sensor proteins such as retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation associated protein 5 (MDA-5), or membrane associated Toll Like Receptors (TLRs), is often the first step in this process (131). This signal is then relayed through specific downstream kinases leading to the activation of transcription factors: interferon regulatory factor 3 (IRF3), activating transcription regulatory factor c-Jun (ATF-2/c-Jun), and nuclear factor κβ (NF-κβ). These transcription factors work in concert to increase expression of type 1 interferons (IFN-α/β), which work through many mechanisms to create an antiviral state

in the host (132). Coronaviruses as a group generally do not induce high levels of IFN- α/β in their hosts (133). Many different mechanisms have been described by which these viruses inhibit IFN-α/β production via both non-structural and structural viral proteins (134-136). PEDV's NSPs 1, 3, 5, 7, 14, 15, 16, as well as structural proteins E, M, N, and the accessory protein coded by ORF 3 have all been demonstrated to inhibit IFN- β production(137, 138). The mechanism by which this occurs has been well described for NSPs 1, 3, 5, and N. NSP1 has been shown to degrade CREB binding protein (CBP) and thereby inhibit activation of the transcription factor IRF3 (137). Inhibition of IRF3 has also been demonstrated by PEDV N protein, which prevents its phosphorylation and nuclear translocation (49). Xing et. al. demonstrated that the papain like protease 2 (PLP2) domain on NSP3 of PEDV functioned as a deubiquitinase (DUB), and was thereby able to inhibit RIG-1 and the stimulator of interferon genes (STING) protein, effectively blocking the expression of IFN-β (139). The papain like protease 1 (PLP1) domain of NSP3 of PEDV, however, was shown not to share this function. NSP5, also known as the 3-C like protease ($3CL^{Pro}$), inhibits activation of NF-κβ through proteolytic cleavage of the NF-κβ essential modulator (NEMO) protein (138). PEDV does not completely inhibit NF-κβ, as it is still activated by the N protein via TLR's 2, 3, and 9 (140). NF-κβ regulates the expression of Interleukin 8 (IL-8) (141), which serves as a chemotactic factor for neutrophils as well as other granulocytes to move to the site of infection. Both the N and E protein of PEDV have been demonstrated to upregulate IL-8 expression through endoplasmic reticulum (ER) stress in host cells (142, 143). Interestingly the N protein was also shown to prolong the S phase of the host cell cycle, although the exact mechanism behind this has not been elucidated (143).

Apoptosis of virally infected cells is often employed by a host's innate immune system to limit viral replication. In response some viruses have developed mechanisms to inhibit apoptosis, while others have developed systems to promote apoptosis and thereby abet the release of progeny virions (144). The immunomodulation of PEDV appears to promote apoptosis through mitochondrial apoptosis inducing factor (AIF) (145, 146). Blocking this pathway *in vitro* was found to reduce viral replication by 90%, demonstrating its importance in the pathophysiology of PEDV infection (145).

Natural Killer cells (NKC), a type of lymphocyte, play an important role in innate immune responses to viral infections. These cells as well as other components of the innate immune system of neonates are not fully functional at birth (147-150). Annamalai et. al. demonstrated that PEDV infected suckling pigs had fewer NKCs, less NKC activity, and fewer IFN-λ producing NKCs than infected weaned pigs (151). This data correlates well with the difference in susceptibility of suckling pigs and weaned pigs to PEDV demonstrated by our group (86).

Humoral Immunity

Humoral immunity involves the production of several classes of immunoglobulins by B lymphocytes. The IgA class of immunoglobulins plays an important role in the immune response to enteric pathogens such as PEDV. Its production and secretion onto mucosal surfaces allows for virus neutralization, opsonization, and antibody dependent cellular cytotoxicity (ADCC) against virally infected cells (152). IgA is detectable in serum as early as 12-15 days post PEDV challenge, with peak levels reached at 21 days post exposure. IgA secreting lymphocytes are detectable in the lamina propria of the small intestine by 21 days as well (153).

The presence of both of these has been correlated to the development of a protective immune response against a homologous PEDV challenge (154). IgG titers appear in pigs at nearly the same time as IgA (153). Serum neutralizing titers, however, have been demonstrated as early as 7 days post challenge (86). This correlates well to the earliest detection of an IgM titer demonstrated by de Arriba et al. (153). PEDV infection, however, is most severe in neonatal pigs whose immune systems are immature and who are too young to have had time to develop an antibody response against PEDV. In addition, due to the histological structure of swine placenta, *in utero* transfer of immunoglobulins from dam to fetus does not occur (155). Thus, maternal immunity especially the production of IgA in milk has been the focus of providing protection to neonatal pigs. Producers have tried various strategies to increase maternal immunity in sows, including attenuated live vaccines, killed vaccines, subunit vaccines, and "feeding back" of infected tissues from clinically affected animals on the same farm (114). The route by which dams are exposed affects the characteristics of the immune response they subsequently develop. Exposure via a mucosal surface (i.e. oral or intranasal exposure) will lead to increased IgA production (152). However, there is evidence of compartmentalization of the mucosal immune response, such that oral exposure to enteric pathogens stimulates lymphocytes associated with the GI mucosa better than intranasal exposure (156). Parenterally/systemically immunized sows will also deliver IgG to their offspring through colostrum for a short period immediately after parturition, which may offer some protection if titers are high enough.

The neutralizing epitope(s) for PEDV are thought to locate in the spike protein, just as they do for Transmissible Gastroenteritis Virus (TGEV), another swine enteric coronavirus (35). Several potential epitopes have been identified in the highly variable S1 region (39, 157, 158). Others have been identified in the endodomain of the more conserved S2 region (36, 159).

Cell Mediated Immunity

Viral replication and assembly occurs within host cells, which limits exposure to antibody. Other mechanisms of immunity are often necessary to clear viral infections. While cell mediated immunity is likely involved in the immune response to PEDV, it has not been described as extensively as the innate and humoral responses. However, De Arriba et al did show an increase in T helper cells (CD4+ cells) in the mesenteric lymph nodes of pigs 4 days after challenge with a virulent CV777 strain of PEDV (160). CD4+ cells work in concert with dendritic cells to activate cytotoxic T cells (CD8+ cells), macrophages, and stimulate antibody production by B lymphocytes. Peak levels of CD4+ cells in mesenteric lymph nodes occurred at 21 days post infection, correlating with peak IgG and IgA antibody production. This suggests that cell mediated immunity may play an important role in the development of a protective titer against PEDV (160). PEDV is able to infect monocyte derived dendritic cells (Mo-DCs) *in vitro.* Doing so resulted in increased IL-12 production by Mo-DCs and IFN-γ production by CD4+ cells, further demonstrating the importance of cell mediated immunity as a component of the host's immune response to PEDV (101).

Diagnosis of Infection

Clinical signs of PEDV include watery diarrhea, vomiting, heat seeking behavior, as well as others. These signs, however, are not unique to PEDV, making diagnosis by clinical presentation impossible. Multiple swine diarrheal diseases also cause an atrophic enteritis, making diagnosis by histopathology impossible as well. Electron microscopy (EM) has been used in the identification of PEDV, especially early on in outbreaks when other methods of

detection are not yet available (63, 161, 162). Two other common swine enteric pathogens, TGEV and Porcine Deltacoronavirus (PDCoV), are also coronaviruses, making definitive diagnosis by EM alone impossible.

Specific methods of detection are available for PEDV and include virus isolation (VI), immunofluorescence assays (FA and IFA), enzyme linked immunosorbent assays (ELISA's), immunohistochemistry (IHC), *in situ* hybridization (ISH), and reverse transcription polymerase chain reactions (RT-PCR). Virus isolation of PEDV traditionally has a very low rate of success (50), making it a poorly sensitive diagnostic assay. Immunofluorescence assays can be used to identify PEDV in tissue culture as well as clinical samples (86, 116). Commercial antigen ELISA kits are available in other countries, and may have potential as a rapid pen-side detection method for PEDV although the sensitivity could be lower than real-time RT-PCRs (163, 164). ISH has been used experimentally, but is not a commonly used method of detection on clinical samples (165). IHC is widely used to identify PEDV within specific histopathologic lesions (65, 115, 117). This allows to definitively link PEDV to the clinical disease observed. PEDV RT-PCRs are the most common method for viral detection. They are highly sensitive, high-throughput assays with a rapid turnaround time, making them ideal for identification of PEDV from clinical samples. Various genes have been used as targets for this assay including the N, M, and S2 genes and so on. While all target highly conserved genes, a recent study showed that an N gene based RT-PCR was slightly more sensitive than a S2 based RT-PCR for PEDV detection (166).

PEDV serological assays can be used to determine a herd's recent/past exposure to the virus. They can also be used as a metric to determine the immune response/protection after exposure to vaccine or wild type virus. These assays include immunofluorescence antibody

(IFA) assays, various ELISAs based on different viral proteins, virus neutralization (VN) tests or fluorescent focus neutralization (FFN) tests, and fluorescent microsphere immunoassays (FMIA).

VN and FFN tests have been used in some studies (74, 86, 167-169) to assess levels of PEDV neutralizing antibodies in serum, milk or colostrum samples and have the added benefit of quantifying the functional neutralizing ability of an animal's antibody titer. They are however a fairly time consuming assay, and are unable to differentiate specific isotypes of antibody. IFA and ELISA have a much more rapid turnaround time and allow for isotype differentiation. This is particularly important for PEDV as the detection of IgA specifically is thought to be an important metric of mucosal/maternal immunity. In addition, ELISA is a high-throughput assay making it suitable for testing a large number of samples. The whole virus-based ELISA (86), recombinant S1 protein-based ELISAs (74, 170-172), recombinant membrane protein-based ELISA (173), and recombinant nucleocapsid protein-based ELISA (167) have been developed for the detection of PEDV antibody. The FMIA is a bead-based high-throughput assay and is useful for simultaneous detection of antibodies against multiple pathogens (167).

In summary, each PEDV serological assay has its advantages and limitations. Selection of appropriate serological assays depends on the objectives of a particular study. IFA, ELISA and FMIA assays are useful in determining whether pigs were previously exposed to PEDV infection or whether piglets have acquired maternal antibodies from their dams. In addition, these assays could determine the isotype of antibodies. A study showed that IFA, ELISA and FMIA had strong correlations in determining PEDV antibodies (167). However, another report indicated that a whole virus-based PEDV ELISA could detect PEDV IgG antibodies longer than an IFA assay in infected pigs (174). VN and FFN tests measure neutralizing antibodies that

could provide protection against virus infection. Although serum neutralizing antibodies may not provide direct protection against an enteric pathogen such as PEDV, a study showed correlations of neutralizing antibodies detected in the serum and in milk and colostrum of previously exposed or vaccinated sows (167). It has been demonstrated that IFA, VN or FFN, and various ELISA assays can detect antibodies against both US prototype and S-INDEL-variant PEDVs (74, 175).

Prevention and Control

Control of PEDV in commercial herds is accomplished in the field through a variety of methods including: strict biosecurity, vaccination, and controlled exposure.

Strict biosecurity protocols were in place in most US commercial barns prior to the introduction of PEDV to prevent the spread of other pathogens especially PRRSV. These include shower-in/shower-out facilities, disinfection of all fomites coming onto the property, limiting traffic on and off farm, heat treatment/disinfection of livestock trailers, as well as others. However, farms with seemingly strong and effective biosecurity protocols were affected by the PEDV outbreak (63). PEDV has previously been shown to be sensitive to ether and chloroform disinfectants and to inactivate at a pH outside the range of 4-9 (32, 176). More recent studies have found that accelerated hydrogen peroxide (Accel©) is an effective disinfectant against PEDV even while feces were present (177). It was also found that time and temperature combinations of 71º C for 10 minutes or 20º C for 1 week were capable of inactivating PEDV in the presence of feces (87, 177). All of this data provides valuable information for trailer baking and disinfection processes on commercial farms.

On sow farms, vaccination strategies typically involve vaccination of females prior to farrowing to stimulate lactogenic immunity to PEDV that is transferred via colostrum and milk to suckling piglets. In weaned pigs, maternally-derived antibodies gradually wane and vaccination of weaned pigs may boost PEDV antibody production and provide active protection (178). Several cell culture attenuated virus strains have been used as vaccines in Asia including CV777, 93P-5, DR-13, and SM98-1 to varying degrees of success (32, 38, 179, 180). Numerous killed vaccines are also in use across Asia. Interestingly one study found that a killed SM98 resulted in a higher neutralizing titer than a modified live vaccine based on the same strain (181). In the U.S. two conditionally licensed vaccines are available, a killed virus vaccine from Zoetis, and an RNA particle vaccine from Harris Vaccines. Studies have shown that these PEDV vaccines induced good IgG and IgA immune responses in herds previously exposed to PEDV but did not induce good IgA responses in naïve pigs after vaccination (168, 169). Modified live virus vaccines based on the contemporary US PEDV strains are currently under development.

In the event of an outbreak on a sow farm, with absence of a safe and effective PEDV vaccine, most of the control strategies involve controlled exposure (feedback) of pregnant sows to wild type virus. This is generally accomplished by feeding back infected tissues and/or feces from piglets to pregnant sows at some interval prefarrowing. This practice has a great deal of variability in how it is executed, which leads to a great deal of variability in its effectiveness in providing protective immunity to piglets (182). It is important to note that materials used for feedback may contain other pathogens such as PRRSV and/or PCV2 and may pose a potential risk of unintentionally infecting pigs with these pathogens.

Conclusions and Objectives of This Thesis

Tremendous knowledge on PEDV has been learned especially since PEDV emergence in the US in 2013. However, before the project described in this thesis was started, no information had been published regarding the minimum infectious dose of PEDV in pigs at different stages of production. Correlations of PEDV infectious titers to the real-time RT-PCR cycle threshold (Ct) values had not been described either. Thus, the objectives of this research were to: 1) correlate PEDV RT-PCR Ct values to infectious titers of the virus; 2) determine the minimum infectious dose of PEDV in naïve neonatal piglets; 3) determine the infectious dose of PEDV in naïve weaned pigs and to assess their corresponding antibody responses. Applications of these research data to the interpretation of the clinical relevance of PEDV diagnostic results and to development of a sensitive swine bioassay model for PEDV are discussed.

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CHAPTER 3: EFFECT OF PORCINE EPIDEMIC DIARRHEA VIRUS INFECTIOUS DOSES ON INFECTION OUTCOMES IN NAÏVE CONVENTIONAL NEONATAL AND WEANED PIGS

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Abstract

Porcine epidemic diarrhea virus (PEDV) was identified in the United States (U.S.) swine population for the first time in April 2013 and rapidly spread nationwide. However, no information has been published regarding the minimum infectious dose (MID) of PEDV in different pig models. The main objective of this study was to determine the oral minimum infectious dose of PEDV in naïve conventional neonatal piglets and weaned pigs. A U.S. virulent PEDV prototype isolate (USA/IA/2013/19338) with known infectious titer was serially ten-fold diluted in virus-negative cell culture media. Dilutions with theoretical infectious titers from 560 to 0.0056 $TCID_{50}$/ml together with a media control were orogastrically inoculated (10ml/pig) into 7 groups of 5-day-old neonatal pigs (n=4 per group) and 7 groups of 21-day-old weaned pigs (n=6 per group). In 5-day-old pigs, 10ml of inoculum having titers 560 – 0.056 $TCID_{50}$/ml, corresponding to polymerase chain reaction (PCR) cycle threshold (Ct) values 24.2 – 37.6, resulted in 100% infection in each group; 10ml of inoculum with titer 0.0056 $TCID_{50}$/ml (Ct>45) caused infection in 25% of the inoculated pigs. In 21-day-old pigs, 10ml of inoculum with titers 560 – 5.6 $TCID_{50}$/ml (Ct 24.2 – 31.4) resulted in 100% infection in each group while 10ml of inoculum with titers 0.56

– 0.0056 $TCID_{50}$/ml (Ct values 35.3 – >45) did not establish infection in any pigs under study conditions as determined by clinical signs, PCR, histopathology, immunohistochemistry, and antibody response. These data reveal that PEDV infectious dose is age-dependent with a significantly lower MID for neonatal pigs compared to weaned pigs. This information should be taken into consideration when interpreting clinical relevance of PEDV PCR results and when designing a PEDV bioassay model. The observation of such a low MID in neonates also emphasizes the importance of strict biosecurity and thorough cleaning/disinfection on sow farms.

Keywords: porcine epidemic diarrhea virus; PEDV; quantitative PCR; infectious dose; neonatal pigs; weaned pigs

Introduction

Porcine epidemic diarrhea virus (PEDV), the causative agent of porcine epidemic diarrhea (PED), is an enveloped positive-sense, single-stranded RNA virus belonging to the order *Nidovirale*, family *Coronaviridae*, subfamily *Coronavirinae*, genus *Alphacoronavirus* [1]. PEDV was identified in 1978 [2, 3] although the disease was first recognized in England in 1971 [4]. PEDV caused widespread epidemics in several European countries during the 1970s and 1980s [5, 6]; however, since the 1990s PED has become rare in Europe with occasional outbreaks [7]. PED was first reported in Japan in 1982 and has since been confirmed in other Asian countries such as South Korea, China, Thailand, and Vietnam [5, 8, 9]. PEDV was detected in the United States (U.S.) for the first time in April 2013 [10]. So far, at least two genetically different PEDV strains have been identified in the U.S. (U.S. PEDV prototype strain and S-INDEL-variant strain) [11, 12]. After the PED outbreak in the U.S., detection of U.S. prototype-like PEDV has been reported in Canada, Mexico, Taiwan, and South Korea [11, 13-15]; detection of U.S. S-INDEL-variant-like PEDV has been reported in South Korea, Germany, Belgium and France [16-20].

Since its emergence in the U.S., PEDV has spread rapidly across the country and resulted in the estimated death of over 7 million pigs in the first year [21], causing substantial economic losses. PED in U.S. swine is characterized by watery diarrhea, dehydration, variable vomiting, high mortality in neonatal piglets, and high morbidity but low mortality in weaned pigs [10]. The rapid spread of PEDV suggests that the virus is highly transmissible. However, no information has been published regarding the minimum infectious dose (MID; the smallest quantity of virus to establish infection) of PEDV in pigs at different stages of production.

Real-time RT-PCR (rRT-PCR) has been widely used for PEDV detection and diagnosis. However, correlations of PEDV infectious titers to the rRT-PCR cycle threshold (Ct) values have not been described. Virus isolation has generally been used to assess if a live virus is present in samples. But for PEDV, the success rate of virus isolation in cell culture has been quite low [22]. Currently, swine bioassay remains the most reliable means to determine if infectious PEDV is present in a clinical specimen or if "X" treatment will inactivate the virus. However, there remains inconsistency in selecting swine models for bioassay.

The objectives of this study were to 1) determine the minimum infectious dose of PEDV in naïve conventional neonatal piglets and weaned pigs; 2) determine the correlation of PEDV PCR Ct values to the infectious titers. Applications of these research data to the interpretation of the clinical relevance of PEDV diagnostic results and to development of a sensitive swine bioassay model for PEDV are discussed.

Materials and Methods

Virus and cells

A U.S. PEDV prototype strain cell culture isolate USA/IA/2013/19338 was isolated and propagated in Vero cells (ATCC CCL-81) in our lab as previously described [22]. A virus stock at the 7th passage in cell culture was prepared and used in this study. The virus stock was 10-fold serially diluted in post-inoculation media (Minimum Essential Medium supplemented with 0.3% tryptose phosphate broth, 0.02% yeast extract, 5 µg/ml of trypsin 250, 10 unit/ml penicillin, 10 µg/ml streptomycin, 0.05 mg/ml gentamicin, and 0.25 µg/ml amphotericin B) to obtain 10^{-1} to 10^{-10} dilutions (Table 3.1). The post-inoculation medium without virus was used as a negative control. Multiple aliquots of each dilution were prepared and stored at -80°C so that quantification of these

virus dilutions and inoculation into pigs could be performed under the same number of freeze/thaw cycles.

Virus titration

The virus stock and serial dilutions were back titrated in Vero cells to determine their infectious titers in the unit of median tissue culture infective dose per milliliter ($TCID_{50}$/ml) and plaque forming unit per milliliter (PFU/ml). To determine $TCID_{50}$/ml, each sample was serially 10-fold diluted in post-inoculation media and inoculated into Vero cells grown in 96-well plates, 100µl per well, triplicate wells per dilution. The plates were incubated at 37°C with 5% CO_2 for 5 days. Viral cytopathic effects (CPE) were recorded daily. After 5-day inoculation, the plates were subjected to immunofluorescence staining using a monoclonal antibody conjugate SD6-29 against the PEDV nucleocapsid protein (SD-1F-1, Medgene Labs, Brookings, South Dakota, USA). The virus titers were determined according to the method described by Reed and Muench [23] and expressed as $TCID_{50}$/ml. To determine PFU/ml, each sample was serially 10-fold diluted in post-inoculation media and inoculated into Vero cells grown in 6-well plates, 500µl per well, duplicate wells per dilution. After incubation at 37°C for 1 h, 4ml of overlay medium (post-inoculation media supplemented with 0.75% carboxymethyl cellulose) was added to each well. The plates were then incubated undisturbed at 37°C with 5% CO_2 for 4 days. The media were discarded and the plates were stained with crystal violet in 10% buffered formalin. The bottoms of the plates were then illuminated. Wells containing 20-200 plaques were counted and titers expressed as PFU/ml.

RNA extraction

Nucleic acids were extracted from virus dilutions, rectal swabs, and tissue homogenates using a MagMAX Pathogen RNA/DNA Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and a Kingfisher-96 instrument (Thermo Fisher Scientific) following the instructions of the manufacturer.

Quantitative real-time RT-PCR for PEDV

A PEDV nucleocapsid (N) gene-based real-time RT-PCR was previously developed at the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) [24, 25]. On the basis of the PEDV N gene-based rRT-PCR, a quantitative rRT-PCR to determine genomic copies/ml of PEDV in a sample was developed in this study.

To generate *in vitro* transcribed RNA standards, a fragment covering the PEDV N gene-based RT-PCR products (nucleotide positions 26,679-26,885 of the PEDV USA/IN/2013/19338, GenBank # KF650371) flanked by restriction sites *EcoR*I and *Hind*III at its 5' and 3' end, respectively, was cloned into the plasmid vector pIDTBlue to obtain the plasmid pIDTBlue:PEDV_N_IVT (IDT, Coralville, Iowa, USA) in which the PEDV N gene products were located downstream of the bacteriophage T7 RNA polymerase promoter. The recombinant plasmids were transformed into One Shot® TOP10 chemically competent *Escherichia coli* cells (Thermo Fisher Scientific) and propagated following the instruction manual. The plasmids were extracted using a QIAprep® Spin Miniprep Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. The plasmid DNA was linearized with *Hind*III, treated with Proteinase K, purified with QIAquick® PCR purification kit (Qiagen), and resuspended in nuclease-free water. The linearized DNA was subject to run-off *in vitro* transcription into RNA

using a MEGAshortscript™ T7 Transcription Kit (Thermo Fisher Scientific) followed by purification using a MEGAclear™ Transcription Clean-up Kit (Thermo Fisher Scientific) according to the instruction manuals provided with the kit. The *in vitro* transcribed RNA was quantified using a BioSpectrometer (Eppendorf, Enfield, Connecticut, USA).

The *in vitro* transcribed RNA was 10-fold serially diluted in nuclease-free water and used as standards in the PEDV N gene-based rRT-PCR to generate standard curves and quantify viral loads in test samples. Five µl of each RNA template was used in PCR setup in a 25µl total reaction using Path-ID™ Multiplex One-Step RT-PCR Kit (Thermo Fisher Scientific) as previously published [24, 25]. Amplification reactions were performed on an ABI 7500 Fast instrument (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with the following conditions: 1 cycle of 48°C for 10 min, 1 cycle of 95°C for 10 min, and 45 cycles of 95°C for 15 sec and 60°C for 45 sec. After generating 22 standard curves, the Ct values were averaged (means determined) and an equation of $X = 10^{(Ct-47.262)/-3.3969}$, where X=genomic copies/ml, was developed to transform the Ct values into estimated genomic copies of PEDV RNA per ml in test samples under the PCR conditions of this study.

Ethics statement

The experimental protocol for the pig studies was approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC, Approval No. 4-14-7777-S; approved on 24th of April 2014).

Neonatal pig study design

Twenty-eight 5-day-old piglets purchased from a conventional breeding farm were tested at the ISU VDL and confirmed negative for PEDV, porcine deltacoronavirus (PDCoV), transmissible gastroenteritis virus (TGEV), and porcine rotaviruses (groups A, B, & C) by virus-specific PCRs on rectal swabs and negative for PEDV antibody by a virus-specific indirect fluorescent antibody (IFA) assay on serum samples. Upon arrival at the Iowa State University Laboratory Animal Resources (LAR) facilities, all pigs were administered an intramuscular injection of Excede® (Zoetis, Florham Park, New Jersey, USA) per label instructions. Pigs were randomized by weight into 7 groups of 4 pigs each, one room per group. Within each group, pigs were housed in tubs with solid dividers completely separating each of the 4 pigs from one another. Each divided portion of the tub had dedicated water sources (Fig 3.1). Pigs were fed a mixture of Esbilac liquid milk replacer and yogurt and had free access to water.

After 1 day acclimation (piglets were 6 days old), pigs in groups 1-7 were orogastrically inoculated with 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} dilutions of the PEDV stock and the virus-negative cell culture media, respectively, using a size 8 French feeding tube and 60ml syringe (10ml/pig) (Table 3.2). Piglets were evaluated daily for clinical signs of vomiting, diarrhea, lethargy, and body condition. Diarrhea severity was assessed with the following criteria by both visual observation and rectal swabbing: 'normal'=no diarrhea, 'mild diarrhea'=soft (cowpie), 'moderate diarrhea'=liquid with some solid content, 'watery diarrhea'=watery with no solid content. Rectal swabs were collected daily from each pig from 0 day post inoculation (DPI) until necropsy and were submerged into 1ml phosphate buffered saline (PBS, 1× pH 7.4) immediately after collection. Serum was collected before inoculation and at necropsy. Duration of the study was designed to be 0-7 DPI. However, per IACUC protocol, pigs with severe clinical signs were

euthanized and necropsied at 4 DPI and the remaining pigs were kept through 7 DPI for necropsy (Table 3.2). At necropsy, intestinal tissues and contents were grossly evaluated. Fresh proximal jejunum, middle jejunum, distal jejunum, ileum, cecum and colon were collected, along with cecum content. Additionally, a portion of the three sections of jejunum, and ileum were fixed in 10% neutral buffered formalin. Rectal swabs and cecum contents were tested by aforementioned quantitative PEDV N gene-based rRT-PCR. Formalin-fixed jejunum and ileum were submitted to the ISU VDL for histopathology and immunohistochemistry examinations.

Weaned pig study design

Forty-two 3-week-old pigs purchased from a conventional breeding farm were tested at the ISU VDL and confirmed negative for PEDV, PDCoV, TGEV, and porcine rotaviruses (groups A, B, & C) by virus-specific PCRs on rectal swabs and negative for PEDV by a virus-specific indirect fluorescent antibody assay. All pigs were administered an intramuscular injection of Excede® (Zoetis) per label instructions. Pigs were randomized by weight into 7 groups of 6 pigs each, one group per room, with the 6 pigs within each group housed together in one room on a solid floor.

After 3 days acclimation (pigs were 24 days old), pigs in groups 1-7 were orogastrically inoculated with 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} dilutions of the PEDV stock and the virus-negative cell culture media, respectively, using a size 10 French feeding tube and 60ml syringe (10ml/pig) (Table 3.2). Pigs were evaluated for clinical signs of vomiting, diarrhea, lethargy, and body condition daily for the first week, every other day for the second week and then once weekly until 28 DPI. Rectal swabs were collected at 0-7, 10, 14, 21, and 28 DPI. Serum was collected and body weight was recorded on 0, 7, 14, 21, and 28 DPI. Three of the six pigs in each group were randomly selected for necropsy at 7 DPI with the remaining 3 pigs being necropsied at 28

DPI. Sample collections at necropsy and post-mortem diagnostics were identical to those in the neonatal pigs study. In addition, serum samples were tested for PEDV antibody using virus-specific IFA assay, virus neutralization (VN) test, and a whole virus-based enzyme linked immunosorbent assay (ELISA).

Histopathology and immunohistochemistry

All sections of formalin-fixed jejunum and ileum were microscopically evaluated by a veterinary pathologist blinded to individual animal identifications and treatment groups. Three representative villi and crypts with integrated longitudinal sections were randomly selected from ileum of each pig for measurement of villus heights and crypt depths using a computerized image system following previously described procedures [24]. Villus-height-to-crypt-depth ratio of each tissue was calculated as the quotient of the average villus length divided by the average crypt depth.

Serial sections of ileums were evaluated for PEDV antigen by immunohistochemistry (IHC) using a PEDV-specific monoclonal antibody (BioNote, Hwaseong-si, Gyeonggi-do, Korea) as previously described [24]. The IHC antigen detection was semi-quantitatively scored as previously described [26] with the following criteria: 0 = no staining; 1 = approximately 1-10% enterocytes with positive staining; 2 = approximately 10%-25% enterocytes with positive staining; 3 = approximately 25%-50% enterocytes with positive staining; 4 = approximately 50%-100% enterocytes with positive staining.

Indirect fluorescent antibody (IFA) assay

Serum samples (N=147) collected at 0, 7, 14, 21, and 28 DPI from the weaned pig study were tested for antibodies by the PEDV IFA assay following the previously described procedures [24] with modifications. For IFA plate preparation, confluent Vero cells grown in 96-well plates were inoculated with 100 µl/well of the U.S. PEDV prototype strain USA/IA/2013/19338 at 1000 $TCID_{50}$/ml and incubated at 37°C, with 5% CO_2. Twenty-four hours later, the inocula were removed and cells were fixed with cold 80% acetone for 10 min. Plates were air dried, sealed and stored at -80°C until use. Serum samples were serially two-fold diluted from 1:40 to 1:5120 in PBS, and 100µl of each diluted serum was added to each well of the IFA plates. After one-hour incubation at 37°C, the sera were removed and plates rinsed twice with PBS. Then 60µl/well of 1:50 diluted goat anti-swine IgG (γ) antibody conjugated with FITC (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland) was added to each well and incubated at 37°C for 1 h. Plates were then rinsed twice with distilled water and cell staining was examined under a fluorescent microscope. A positive signal at a serum dilution of 1:40 or higher was considered to be IFA antibody positive.

Virus neutralization (VN) test

Serum samples (N=147) from the weaned pig study were tested for PEDV neutralizing antibodies. Serum samples were first inactivated at 56°C for 30 min, then 2-fold serially diluted from 1:4 to 1:512 dilutions in 96-well plates with a volume of 75µl per well after dilution. Subsequently 75µl of 4×10^3 $TCID_{50}$/ml of PEDV strain USA/IA/2013/19338 was mixed with the equal volume of diluted sera and incubated for 1 h at 37°C with 5% CO_2. Then 100µl of the serum-virus mixture (containing 200 $TCID_{50}$ of virus) was transferred to 96-well plates with a Vero cell

monolayer, and the plates were incubated for 1 h at 37°C. The cells were washed twice with post-inoculation media and incubated with 100µl/well of such media for 48 h at 37°C with 5% CO_2. Then cells were fixed with cold 80% acetone and stained with PEDV N protein-specific monoclonal antibody SD6-29 conjugated to FITC (Medgene, Brookings, South Dakota) at a 1:100 dilution for 40 min. The staining was examined under a fluorescent microscope. The reciprocal of the highest serum dilution resulting in >90% reduction of staining as compared to the negative serum control was defined as the VN titer of the serum sample. A VN titer of ≥8 was considered positive.

Whole virus-based ELISA for PEDV antibody detection

A U.S. PEDV prototype strain whole virus-based ELISA was developed and validated at the ISU VDL for detection of PEDV-specific IgG antibody. Each batch of one liter of PEDV propagated in Vero cells (infectious titers ranging from 10^5 - 10^6 TCID50/ml) were subjected to one freeze-thaw and then centrifuged at 4,000 × *g* for 15 min to remove cell debris. The virus was then pelleted by ultracentrifugation at 140,992 × *g* for 3 h. The virus pellet was resuspended in sterile PBS (1× pH 7.4) at a ratio of 1:100 of the original volume and stored at -80°C until use. Polystyrene 96-well microtitration plates (Thermo Fisher Scientific) were coated with the viral antigen solution (100µl/well) and incubated at 4°C overnight. Plates were washed 5 times, blocked (300µl/well) with PBS containing 1% bovine serum albumin (Jackson ImmunoResearch Inc., West Grove, Pennsylvania), and incubated at 25°C for 2 h. Plates were then dried at 37°C for 4 h and stored at 4°C in a sealed bag with desiccant packs until use. Serum samples were 1:50 diluted and added to the coated plates (100µl/well). Plates were incubated at 25°C for 1 h and then washed 5 times with PBS (1× pH 7.4). Subsequently 100µl of peroxidase-conjugated goat anti-pig IgG (Fc)

antibody (Bethyl Laboratories Inc., Montgomery, Texas, USA) at 1:25,000 dilution was added to each well and the plates were incubated at 25°C for 1 h. After a washing step, 100µl of tetramethylbenzidine-hydrogen peroxide substrate solution (TMB, Dako North America Inc., Carpinteria, California, USA) was added to each well. The plates were incubated for 5 min at room temperature and the reaction was stopped by adding 50µl of stop solution (1 M sulfuric acid) to each well. Reactions were measured as optical density (OD) at 450 nm using an ELISA plate reader operated with commercial software (Biotek® Instruments Inc., Winooski, Vermont, USA). The antibody response in serum samples was represented as sample-to-positive (S/P) ratios calculated as: S/P ratio = (sample OD – blank well control mean OD) / (positive control mean OD – blank well control mean OD). After multiple optimizations, the S/P ratios of >0.8 were considered antibody positive, 0.6-0.8 as suspect, and <0.6 as negative [27, 28].

Statistics

The villus height, crypt depth, and villus/crypt ratio were compared with a generalized linear mixed (GLIMMIX) model. IHC scores were compared using a Kruskal-Wallis Test. All tests were performed using JMP software (SAS Institute, Cary, North Carolina, USA), with $P<0.05$ considered significant.

Results

Infectious titers and PCR results of the 10-fold serially diluted PEDV stock

A PEDV virus stock (USA/IN/2013/19338 passage 7) with a known infectious titer of 5.62×10^5 TCID50/ml was 10-fold serially diluted into virus-negative cell culture medium, giving rise to theoretical infectious titers of 5.62×10^5 to 5.62×10^{-5} $TCID_{50}$/ml for the 10^0 to 10^{-10} dilutions,

respectively (Table 3.1). Infectious titers of each virus dilution were back-titrated in Vero cells by end-point titration assay ($TCID_{50}$/ml) and plaque assay (PFU/ml), with results summarized in Table 3.1. The stock virus and 10^{-1} to 10^{-4} dilutions all had the same logarithms of titers as the theoretical titers based on the dilution factor, regardless of titration assays in the units of $TCID_{50}$/ml or PFU/ml. The 10^{-5} virus dilution had an infectious titer of 5 PFU/ml but remained undetected by the end-point titration assay in the unit of $TCID_{50}$/ml. The 10^{-6} to 10^{-10} virus dilutions were undetected by either the end-point titration assay or the plaque assay during back titration.

Each virus dilution was also tested in triplicate by a quantitative PEDV N gene-based rRT-PCR. The virus stock had an average Ct of 12.2 and the Ct values increased by roughly 3-4 for every 10-fold dilution until the 10^{-7} dilution that had a Ct value of 37.62, beyond which all dilutions had Ct>45 (Table 3.1). This range of Ct values correlated to 2.1×10^{10} genomic copies/ml in the virus stock through 689 genomic copies/ml in the 10^{-7} dilution, and an undetected number of genomic copies/ml in all dilutions beyond that (Table 3.1).

The PEDV dilutions 10^{-3} to 10^{-8}, having theoretical infectious titers of 5.62×10^{2} to 5.62×10^{-3} $TCID_{50}$/ml and PCR Ct values of 24.22 to >45, together with virus-negative culture medium control, were selected to determine the infection outcomes in 5-day-old piglets and 3-week-old pigs (Table 3.2).

Infection outcomes in 5-day-old piglets

All four 5-day-old piglets in the groups 1 to 5 (inoculated with virus dilutions 10^{-3} to 10^{-7}) and one pig (pig ID 9) in group 6 (inoculated with virus dilution 10^{-8}) developed mild to watery diarrhea at 1 DPI and diarrhea lasted through the study period. Dehydration and lethargy were also observed in these piglets at 2 DPI through the end of the study period. Vomiting was only

observed in piglets inoculated with the 10^{-3} dilution (5.62×10^{2} $TCID_{50}$/ml, 10ml/pig) at 1 DPI. Due to severity of clinical signs, these piglets were euthanized and necropsied at 4 DPI. In contrast, 3 pigs (pig IDs 23, 13 and 8) in the group 6 inoculated with virus dilution 10^{-8} and all 4 pigs in the negative control group remained active and clinically unaffected throughout the 7-day study period.

Fecal virus shedding of the inoculated 5-day-old piglets is summarized in Table 3.3 and Fig. 3.2A. One pig each in groups 1, 2, and 3 (10^{-3}, 10^{-4} and 10^{-5} dilutions) were PCR positive on rectal swabs at 1 DPI with Ct values 22.98-39.97. All 4 pigs in each of groups 1-5 (10^{-3} – 10^{-7} dilutions) were PCR positive on rectal swabs by 2 DPI and remained positive through the necropsy day 4 DPI. One pig (pig ID 9) in group 6 (10^{-8} dilution) shed virus in rectal swabs starting from 3 DPI with a Ct value of 14.2 (Table 3.3). The quantitative genomic copies/ml of PEDV RNA in rectal swabs is shown in Fig. 3.2A. The average virus shedding in groups 1-5 (10^{-3} to 10^{-7} dilutions) at 2-4 DPI and virus shedding in pig ID 9 from group 6 (10^{-8} dilution) at 3-4 DPI were similar, having $10^{7.6}$ to $10^{9.2}$ genomic copies/ml. All rectal swabs from the 3 unaffected pigs (IDs 23, 13 and 8) in group 6 (10^{-8} dilution) and all 4 pigs in the negative control group were negative by PEDV PCR. Cecum contents obtained at necropsy were also tested by PEDV PCR and the results were consistent with those of the rectal swabs collected on the corresponding necropsy days (data not shown).

Thin-walled small intestine and soft to watery contents in small intestines, ceca and colons were observed at necropsy in pigs in groups 1-5 (10^{-3} – 10^{-7} dilutions) and one pig (ID 9) of group 6; similar villous atrophy consistent with viral infection was observed in these pigs. Villus height and crypt depth were measured, villus-height-to-crypt-depth ratios were calculated, and the magnitude of IHC staining was scored and compared on ileums of different groups (Table 3.4).

Pigs in groups 1-5 (10^{-3} – 10^{-7} dilutions) were all necropsied at 4 DPI and there were no significant differences between groups in villus height, crypt depth, villus/crypt ratio, or IHC scores. Only one pig (ID 9) from group 6 was necropsied at 4 DPI and was thus not included in the statistical analysis. Microscopic intestinal lesions consistent with viral enteritis were not observed in the 3 remaining pigs in group 6 and the negative control pigs (group 7); villus height, crypt depth, and villus/crypt ratio of ileums of these pigs were not significantly different from each other; IHC staining was negative on these pigs. Compared to the pigs in group 6 (IDs 23, 13 and 8) and group 7 (negative control), the pigs in groups 1-5 had decreased villus heights, increased crypt depth, and lower villus/crypt ratios; however, statistical differences were not analyzed because they were necropsied at different DPI.

Infection outcomes in 3-week-old pigs

In groups 1-3 (10^{-3} to 10^{-5} dilutions), 1/6, 1/6, and 6/6 pigs in each group developed mild diarrhea at 2, 3, and 4-7 DPI; mild dehydration or lethargy was observed in a few pigs in these groups during 3-7 DPI. No diarrhea, dehydration, or lethargy was observed in pigs in groups 4-7 (10^{-6} to 10^{-8} dilutions and negative control) throughout the duration of the study.

Fecal virus shedding from the inoculated 3-week-old piglets is summarized in Table 3.5 and Fig. 3.2B. Pigs in all groups were PCR negative on rectal swabs collected before inoculation and at 1 DPI. One pig each in groups 1 and 2 along with two pigs in group 3 were PCR positive on rectal swabs by 2 DPI. All pigs in groups 1-3 were PEDV PCR positive by 4 DPI and remained positive through at least 7 DPI although there were variations on PCR Ct values between individual pigs. Most of the remaining pigs in groups 1-3, after necropsy at 7 DPI, continued to shed virus in rectal swabs during 10-21 DPI but all pigs in these groups ceased shedding virus at 28 DPI

(Table 3.5). Based on the quantitative genomic copies/ml of PEDV RNA in rectal swabs, the average virus shedding in groups 1-3 (10^{-3} to 10^{-5} dilutions) had similar patterns: increasing virus shedding during 2-6 DPI and decreasing virus shedding after 7 DPI (Fig. 3.2B). However, pigs in group 2 (10^{-4} dilution) had a peak virus shedding during 10-14 DPI (Fig. 3.2B). All rectal swabs from pigs in groups 4-7 (10^{-6} to 10^{-8} dilutions and negative control) were negative by PEDV PCR through the study period. Cecum contents obtained at necropsy were also tested by PEDV PCR in all pigs and the results were consistent with those of the rectal swabs collected on the corresponding necropsy days (data not shown).

For pigs necropsied at 7 DPI, microscopic lesions consistent with viral infection were observed in 2/3 pigs of group 1 (10^{-3} dilution), 1/3 pigs of group 2 (10^{-4} dilution), 1/3 pigs of group 3 (10^{-5} dilution), and 0/3 pigs of groups 4-7 (10^{-6} to 10^{-8} dilutions and negative control); IHC staining results were consistent with the presence or absence of microscopic lesions. However, villus heights, crypt depths, villus/crypt ratios of pigs in all groups 1-7 were not significantly different from each other at 7 DPI (data not shown). For pigs necropsied at 28 DPI, no microscopic lesions or IHC staining were observed in any pigs of groups 1-7.

Seroconversion was first observed at 14 DPI for pigs in groups 1-3 (10^{-3} to 10^{-5} dilutions) by both IFA (Fig. 3.3A) and whole virus-based ELISA (Fig. 3.3C) assays and then remained through 28 DPI. PEDV neutralizing antibodies was first detected by VN test at 7 DPI in 1/3 pigs of groups 1 and 2, and in 2/3 pigs of group 3 (Fig. 3.3B). Serum anti-PEDV antibody was not detected in pigs from groups 4-7 (10^{-6} to 10^{-8} dilutions and negative control) throughout the study period by any of the 3 antibody assays.

Discussion

One objective of this study was to determine the correlation of PEDV PCR Ct values to the infectious titers. A U.S. PEDV prototype strain cell culture isolate USA/IA/2013/19338 was 10-fold serially diluted and tested in this study. Since there are variations among laboratories in reporting virus titer units, we determined the infectious titers of the PEDV dilutions using both end-point titration assay and plaque assay. It was found that one $TCID_{50}$/ml is almost equivalent to one PFU/ml for PEDV. Based on a quantitative PEDV N gene-based rRT-PCR we developed, PEDV with an infectious titer of 5 $TCID_{50}$/ml or 5 PFU/ml had a Ct value of approximate 31 corresponding to about 4.8×10^4 genomic copies/ml. An equation of $X = 10^{(Ct-47.262)/-3.3969}$, where X=genomic copies/ml, was used to transform the Ct values into estimated genomic copies of PEDV RNA per ml in samples. However, these results need to be interpreted with caution. First, a PEDV N gene-based primers and probe and reagents from the Path-ID™ Multiplex One-Step RT-PCR Kit (Thermo Fisher Scientific) were used in this study. PEDV rRT-PCRs using other primers, probe, and master mix buffer reagents may generate results somewhat different from what we obtained in this study. Second, correlations of the infectious titers, Ct values and genomic copies/ml in this study were obtained using a PEDV cell culture isolate diluted in cell culture medium. For PEDV present in other sample matrices, this correlation could have some variations.

Another objective of this study was to determine the minimum infectious dose of PEDV in 5-day-old and 3-week-old naïve conventional pigs. In 5-day-old pigs, 100% of pigs (4/4 pigs) became infected with 10ml of inoculum having titers 560 – 0.056 $TCID_{50}$/ml (Ct 24.2 – 37.6); 25% of pigs (1/4 pigs) became infected with 10ml of inoculum having titer 0.0056 $TCID_{50}$/ml (Ct>45) (Table 3.6). In 3-week-old pigs, 10ml of inoculum with titers 560 – 5.6 $TCID_{50}$/ml (Ct 24.2 – 31.4) resulted in 100% (6/6 pigs) infection while 10ml of inoculum with titers 0.56 – 0.0056 $TCID_{50}$/ml

(Ct values 35.3 – >45) could not establish infection in any pigs under these study conditions (Table 3.6). The smallest virus magnitude that can cause infection in at least one pig in a group is considered the minimum infectious dose. In the 5-day-old pig study, 4 pigs in each group were housed separately without direct contact between pigs. The minimum infectious dose of PEDV was 0.056 $TCID_{50}$ or lower in 5-day-old pigs. In the 3-week-old pig study, due to space limitation, it was not possible to house all pigs individually; however, this did not preclude determination of the minimum infectious dose in the current study because the weaned pig groups 4-6 (dilutions 10^{-6} to 10^{-8}) had no single pig positive during 28 days study period. However, if conditions allowed, for minimum infectious dose studies, pigs would ideally be housed separately during long-term study periods to avoid possible pig-to-pig transmission. The MID of PEDV was 56 $TCID_{50}$ in 3-week-old pigs under our study conditions. This suggests that the MID for neonatal pigs is at least 1000-fold lower than it is in weaned pigs.

Our study confirms that 5-day-old pigs are more susceptible than 3-week-old pigs to PEDV infection. PEDV progresses faster, the fecal virus shedding level is higher, and disease is more severe, in 5-day-old pigs than in 3-week-old pigs (Fig 3.2A&B). We also observed that the response among four 5-day-old pigs in each group was comparable as reflected by similar virus shedding levels (Table 3.3) and similar microscopic lesions. In contrast, there was more variation in virus shedding among six 3-week-old pigs in each group (Table 3.3). These observations were consistent with previous reports on age-dependent resistance of pigs to TGEV infection [29]. However, in either 5-day-old or 3-week-old pig models, once pigs were infected and virus replication began, the initial dose of virus appears to have little impact at the group level on the average amount of fecal viral shedding, average severity of microscopic lesions/IHC staining, or the average magnitude of antibody titer that is subsequently developed. It should be noted that the

5-day-old piglets in this study were fed a mixture of liquid milk replacer and yogurt, which were possibly devoid of IgG, IgA and other immunologic ingredients found in sow milk. Thus, the piglets raised under the current study conditions could be more susceptible to PEDV infection than piglets at the corresponding ages that were naturally nursed by sows under field conditions.

Previous studies also demonstrated that PEDV infection induces greater disease severity in neonatal piglets than in weaned pigs although the minimum infectious dose of PEDV was not determined in those studies [24, 30-32]. The exact reasons for the greater severity of PED in neonatal piglets are unknown but there may be several contributing factors including: 1) an immature immune system [33, 34]; for example, the level of natural IFN-α production by porcine blood mononuclear cells is lower in neonates and the phagocytic cells present in newborn piglets generally have reduced phagocytic activity as compared with adult animals [35, 36]; 2) neonatal piglets are more vulnerable to electrolyte/fluid imbalance and dehydration [33]; 3) the intestinal villi of neonatal piglets are longer and may have more mature permissive enterocytes than weaned pigs [37]; 4) and a slower replacement rate of villous enterocytes (7-10 days) in neonatal pigs compared to 2-4 days in weaned pigs [38]. The virus mainly infects and destroys mature enterocytes lining the villi of small intestine, resulting in shortening and blunting of villi. But the intestinal crypt epithelial cells basically remain uninfected and serve to replace the destroyed villous enterocytes. Regeneration of villous enterocytes in neonatal piglets is not as rapid as that in weaned pigs; this may explain why villous atrophy is more severe in neonatal than in weaned pigs. Recently Jung et al [31, 39] further demonstrated that neonatal pigs have low numbers of LGR5+ (marker for crypt stem cell) cells and low levels of Ki67 staining (marker for crypt proliferation) in contrast to large numbers of LGR5+ cells in the crypts and high proliferation of

intestinal crypt cells in weaned pigs, explaining the rapid turnover or recovery rate of villous enterocytes in weaned pigs.

Success rate of PEDV isolation in cell culture has been low. Currently swine bioassay has remained the most reliable means to determine if infectious PEDV is present in a sample. Data from this study suggests a neonatal pig bioassay is more sensitive than a weaned pig bioassay to assess PEDV infectivity. Regarding duration of the swine bioassay, we observed that one pig (ID 9) in group 6 of the 5-day-old pig study did not shed virus in rectal swabs until 3 DPI; thus 2-day duration of pig bioassay may not be sufficient to evaluate the infection outcome. We propose that 7-day duration would be considered appropriate for PEDV swine bioassay.

In summary, using a U.S. PEDV prototype isolate diluted in culture medium, we determined the correlations of PEDV infectious titers to PCR Ct values and genomic copies per ml. We further demonstrated that PEDV infectious dose is age-dependent with a significantly lower MID for neonatal pigs than for weaned pigs. This information should be taken into consideration when interpreting clinical relevance of PEDV PCR results and when designing a PEDV bioassay model. The observation of such a low MID in neonates also emphasizes the importance of strict biosecurity and thorough cleaning/disinfection on sow farms. In this study, the effect of PEDV infectious doses on infection outcomes was evaluated in naïve conventional neonatal and weaned pigs. It must be noted that infectious dose effects in previously exposed or vaccinated pigs may be different from naïve pigs. In addition, a U.S. PEDV prototype cell culture isolate was used in this study and it remains to be determined whether or not U.S. PEDV S-INDEL-variant isolate has similar minimum infectious dose and pathogenicity.

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Figures

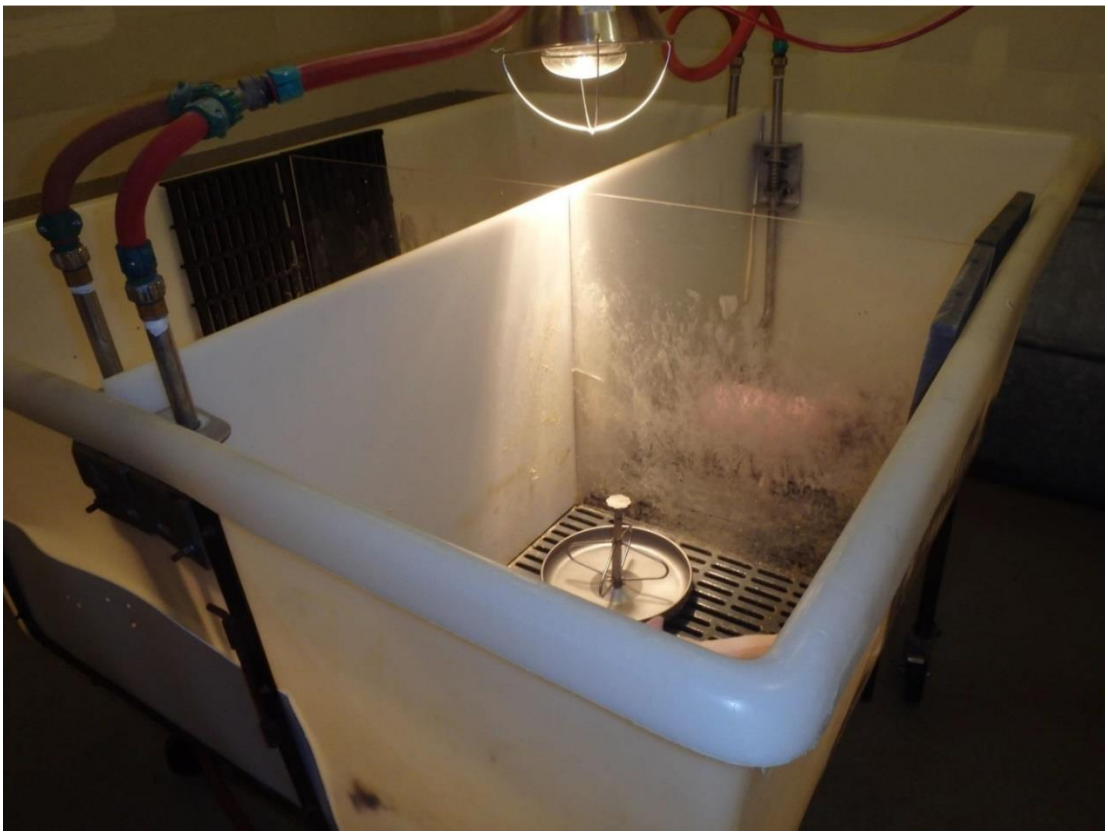

Fig. 3.1. Elevated tubs used to house 5-day-old piglets in this study. One tub was located in each room. Each tub was split into 4 compartments with one pig per compartment. Design of the tub prevented contact between pigs and movement of feces or other waste between tub compartments.

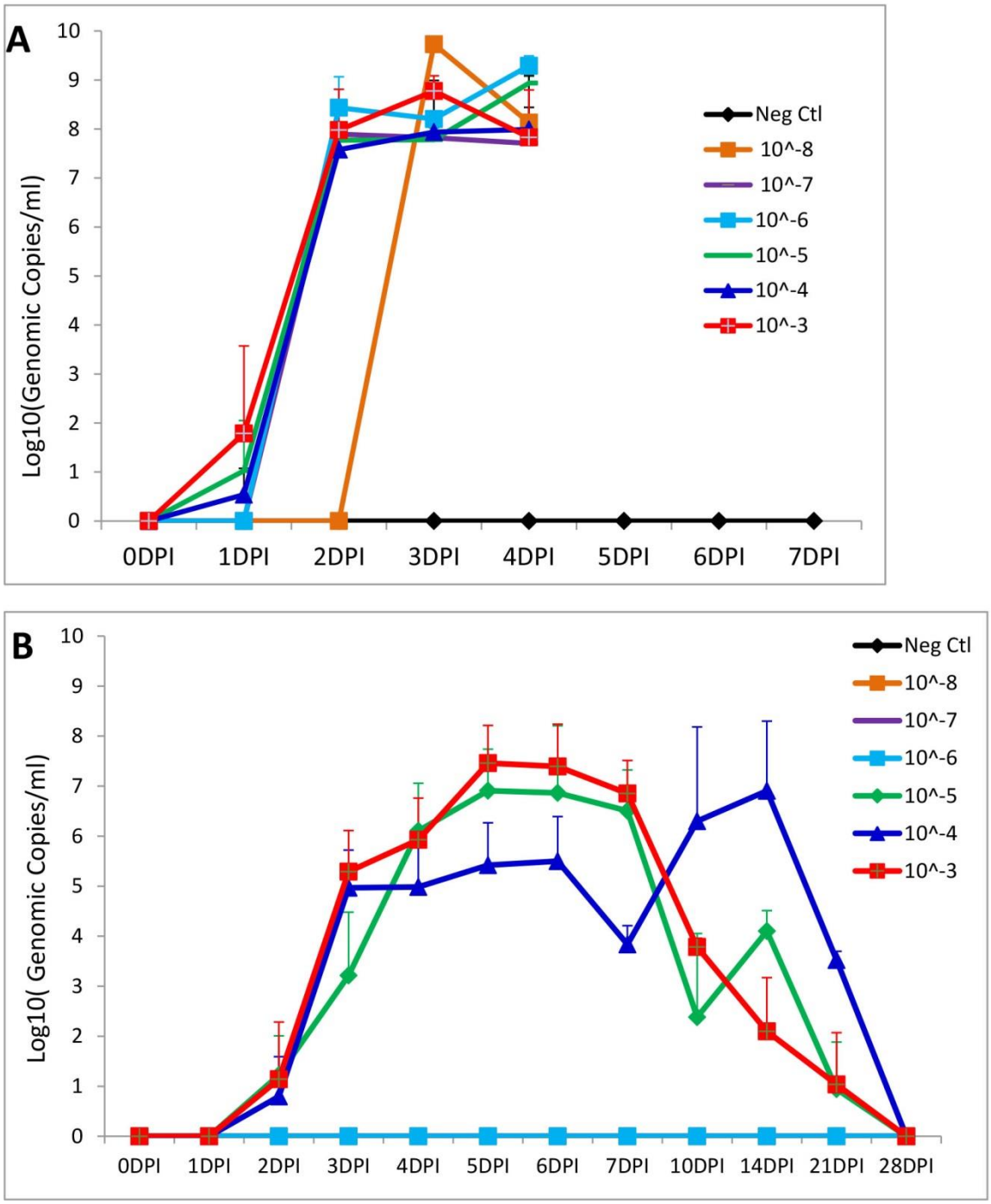

Fig 3.2. Virus shedding in rectal swabs of the 5-day-old pig study (A) and of the 3-week-old pig study (B) as determined by a quantitative PEDV N gene-based rRT-PCR. The virus titers (Log_{10}Genomic copies/ml) at each time points were mean virus titers of all available pigs (both PCR positive and negative pigs) except the 10^{-8} group in the 5-day-old pig study where only one out of 4 pigs was positive in the study period and included in figure (A).

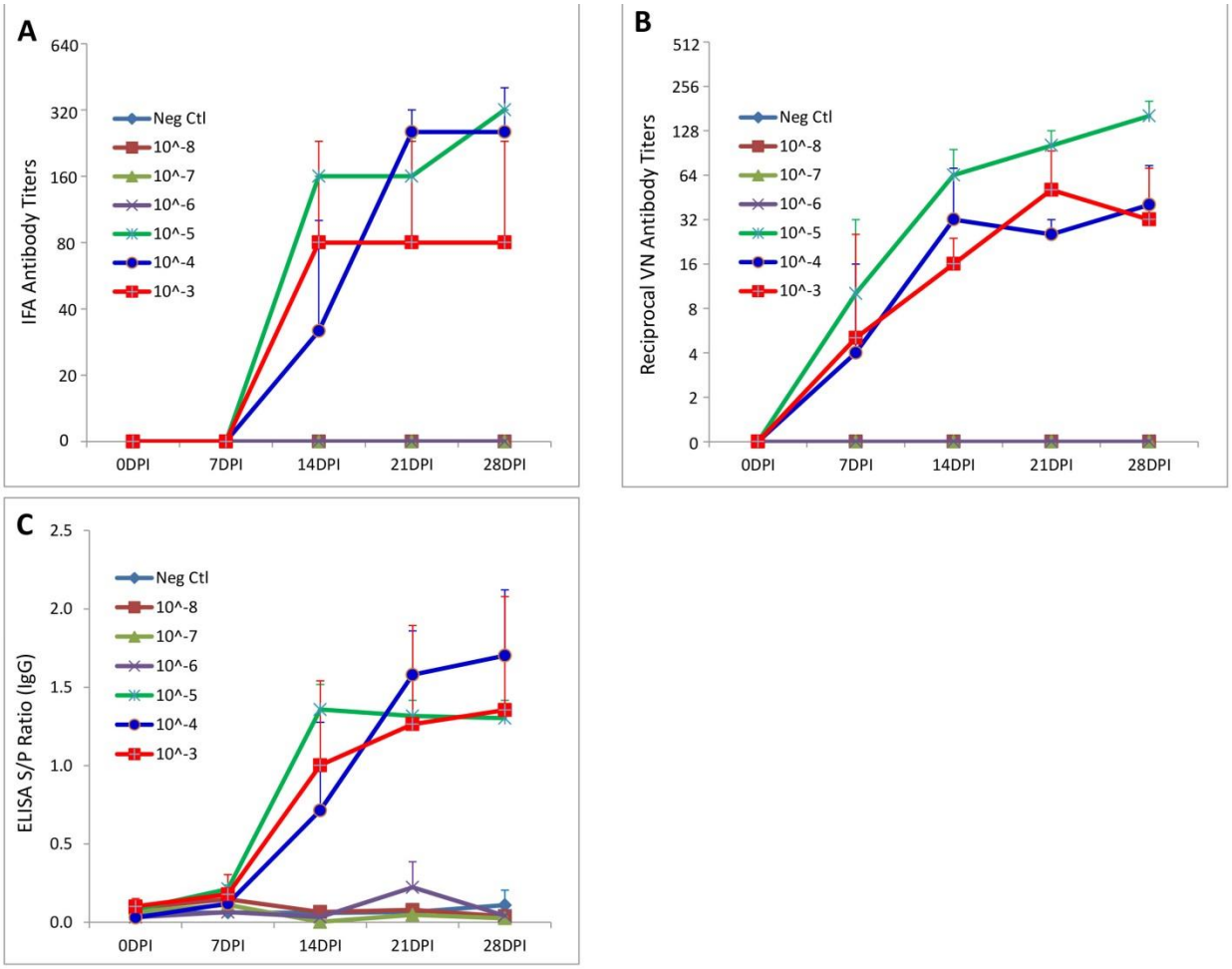

Fig 3.3. Serum antibody responses in 3-week-old pigs inoculated with serial dilutions of PEDV. IFA antibody titers (A), serum virus neutralizing antibody titers (B), and PEDV whole virus-based ELISA antibody titers (C) were shown for 3 pigs in each group that went through 28 days post inoculation.

Table 3.1. Infectious titers, PCR Ct values and genomic copies/ml of serial dilutions of PEDV stock

Dilution of stock virus	$TCID_{50}$/ml (Theoretical)	$TCID_{50}$/ml (Back titrated)	PFU/ml (Back titrated)	Ct*	Genomic Copies/ml*
Stock Virus	562,000	562,000	680,000	12.2	2.10E+10
10^{-1}	56,200	31,600	49,000	16.76	9.54E+08
10^{-2}	5,620	5,620	6,600	20.21	9.20E+07
10^{-3}	562	316	430	24.22	6.07E+06
10^{-4}	56.2	56.2	23	28.22	4.03E+05
10^{-5}	5.62	0	5	31.37	4.77E+04
10^{-6}	0.562	0	0	35.28	3.37E+03
10^{-7}	0.0562	0	0	37.62	6.89E+02
10^{-8}	0.00562	0	0	>45	0
10^{-9}	0.000562	0	0	>45	0
10^{-10}	0.0000562	0	0	>45	0
Neg Ctl	0	0	0	>45	0

* Average values of triplicate PCR reactions for each dilution.

Table 3.2. Experimental Design of the neonatal and weaned pig studies

Virus Dilution Used for Inoculation	Inoculum Volume	Neonatal pig study		Weaned pig study			
		Group	Pigs	Group	Pigs	Necropsy 7 DPI	Necropsy 28 DPI
10^{-3}	10 ml	1	N=4	1	N=6	N=3	N=3
10^{-4}	10 ml	2	N=4	2	N=6	N=3	N=3
10^{-5}	10 ml	3	N=4	3	N=6	N=3	N=3
10^{-6}	10 ml	4	N=4	4	N=6	N=3	N=3
10^{-7}	10 ml	5	N=4	5	N=6	N=3	N=3
10^{-8}	10 ml	6	N=4	6	N=6	N=3	N=3
Neg Ctl	10 ml	7	N=4	7	N=6	N=3	N=3

Table 3.3. PEDV shedding in rectal swabs of the neonatal piglets*

Group	Inocula (10ml/pig)			Pig ID	PEDV rRT-PCR Ct values							
	Dilution	$TCID_{50}$/ml	Ct		0 DPI	1 DPI	2 DPI	3 DPI	4 DPI	5 DPI	6 DPI	7 DPI
1	10^{-3}	562	24.22	10	>45	>45	28.63	19.3	29.6	X	X	X
				32	>45	>45	17.15	15.61	21.33	X	X	X
				15	>45	22.98	17.83	19.29	17.06	X	X	X
				6	>45	>45	17.03	15.62	14.65	X	X	X
2	10^{-4}	56.2	28.22	27	>45	>45	15.44	9.96	13.06	X	X	X
				7	>45	>45	27.75	25.58	19.41	X	X	X
				29	>45	39.97	22.24	24.6	17.43	X	X	X
				28	>45	>45	20.64	21.05	30.57	X	X	X
3	10^{-5}	5.62	31.37	16	>45	>45	22.15	19.58	15.87	X	X	X
				3	>45	>45	21.71	21.76	18.08	X	X	X
				5	>45	>45	20.05	19.31	16.46	X	X	X
				4	>45	33.33	19.54	22.67	17.22	X	X	X
4	10^{-6}	0.562	35.28	20	>45	>45	20.19	24.36	15.03	X	X	X
				18	>45	>45	12.28	14.38	15.24	X	X	X
				26	>45	>45	21.86	21.56	17.74	X	X	X
				17	>45	>45	20.12	17.27	14.81	X	X	X
5	10^{-7}	0.0562	37.62	12	>45	>45	21.55	21.24	24.69	X	X	X
				25	>45	>45	18.95	24.61	22.31	X	X	X
				14	>45	>45	21.12	20.07	23.61	X	X	X
				2	>45	>45	20.15	16.87	13.73	X	X	X
6	10^{-8}	0.00562	>45	9	>45	>45	>45	14.2	19.66	X	X	X
				23	>45	>45	>45	>45	>45	>45	>45	>45
				13	>45	>45	>45	>45	>45	>45	>45	>45
				8	>45	>45	>45	>45	>45	>45	>45	>45
7	Neg Ctl	0	>45	21	>45	>45	>45	>45	>45	>45	>45	>45
				24	>45	>45	>45	>45	>45	>45	>45	>45
				1	>45	>45	>45	>45	>45	>45	>45	>45
				31	>45	>45	>45	>45	>45	>45	>45	>45

*All pigs PCR positive on rectal swabs were necropsied on day 4 due to the severity of clinical signs

Table 3.4. Mean villus height (µm), crypt depth (µm), villus/crypt ratio, and IHC scores in ileums of the neonatal piglets

	Group	Dilution	Pigs	Villous Height Mean ± SEM	Crypt Depth Mean ± SEM	Villus/Crypt ratio Mean ± SEM	IHC Score Mean ± SEM
Necropsy at 4 DPI	1	10^{-3}	N=4	218.13 ± 16.33	161.64 ± 6.81	1.35 ± 0.10	2.94 ± 0.68
	2	10^{-4}	N=4	208.56 ± 39.94	171.22 ± 6.59	1.21 ± 0.21	3.81 ± 0.19
	3	10^{-5}	N=4	272.19 ± 22.03	162.21 ± 9.71	1.67 ± 0.04	3.88 ± 0.13
	4	10^{-6}	N=4	230.85 ± 19.33	152.46 ± 4.52	1.51 ± 0.12	3.63 ± 0.24
	5	10^{-7}	N=4	228.18 ± 33.57	158.24 ± 10.89	1.45 ± 0.21	3.94 ± 0.06
	6*	10^{-8}	N=1	222.49	143.12	1.55	4.00
Necropsy at 7 DPI	6	10^{-8}	N=3	423.17 ± 51.35	118.60 ± 16.81	3.81 ± 0.87	0.00 ± .000
	7	Neg Ctl	N=4	450.76 ± 20.05	108.40 ± 12.61	4.31 ± 0.46	0.00 ± 0.00

* Mean and standard error of the mean (SEM) could not be calculated nor could statistical comparison be performed on the one pig from Group 6 necropsied at 4 DPI.

Table 3.5. PEDV shedding in rectal swabs of the weaned pigs*

Group	Inocula (10ml/pig)			Pig ID	PEDV rRT-PCR Ct values											
	Dilution	$TCID_{50}$/ml	Ct		0 DPI	1 DPI	2 DPI	3 DPI	4 DPI	5 DPI	6 DPI	7 DPI	10 DPI	14 DPI	21 DPI	28 DPI
1	10^{-3}	562	24.22	7	>45	>45	>45	34.88	34.87	29.42	31.10	32.94	33.30	>45	>45	>45
				66	>45	>45	>45	32.09	19.24	19.72	15.62	19.17	34.60	35.21	36.71	>45
				89	>45	>45	24.00	16.66	20.13	15.04	20.08	22.26	35.33	37.94	>45	>45
				3	>45	>45	>45	35.01	34.60	29.67	30.79	24.69	X	X	X	X
				10	>45	>45	>45	27.50	24.34	20.99	19.13	26.88	X	X	X	X
				86	>45	>45	>45	29.64	29.63	16.71	16.14	17.97	X	X	X	X
2	10^{-4}	56.2	28.22	18	>45	>45	>45	32.68	34.18	30.13	32.26	35.07	24.21	14.46	34.12	>45
				64	>45	>45	>45	33.44	33.92	31.83	32.25	30.72	15.70	29.83	36.08	>45
				96	>45	>45	31.05	17.61	16.78	14.52	13.78	30.43	37.68	27.13	35.69	>45
				77	>45	>45	>45	33.35	31.69	32.18	31.73	38.46	X	X	X	X
				83	>45	>45	>45	32.14	33.61	32.19	28.42	34.73	X	X	X	X
				93	>45	>45	>45	33.13	31.81	32.29	33.07	36.02	X	X	X	X
3	10^{-5}	5.62	31.37	12	>45	>45	>45	>45	34.16	28.73	30.51	21.96	28.21	30.87	37.67	>45
				74	>45	>45	33.07	19.19	19.22	17.96	17.29	32.14	>45	35.71	>45	>45
				90	>45	>45	36.53	32.05	19.58	15.30	16.47	17.87	34.13	33.42	>45	>45
				2	>45	>45	>45	37.58	33.87	31.46	42.98	35.07	X	X	X	X
				78	>45	>45	>45	34.72	19.07	19.75	13.03	21.25	X	X	X	X
				99	>45	>45	>45	>45	33.23	29.56	23.39	22.69	X	X	X	X
4‡	10^{-6}	0.562	35.28	6; 68; 81	>45	>45	>45	>45	>45	>45	>45	>45	>45	>45	>45	>45
				9; 14; 91	>45	>45	>45	>45	>45	>45	>45	>45	X	X	X	X
5‡	10^{-7}	0.0562	37.62	15; 20; 67	>45	>45	>45	>45	>45	>45	>45	>45	>45	>45	>45	>45
				11; 16; 98	>45	>45	>45	>45	>45	>45	>45	>45	X	X	X	X
6‡	10^{-8}	0.00562	>45	4; 21; 87	>45	>45	>45	>45	>45	>45	>45	>45	>45	>45	>45	>45
				8; 73; 79	>45	>45	>45	>45	>45	>45	>45	>45	X	X	X	X
7‡	Neg Ctl	0	>45	70; 76; 92	>45	>45	>45	>45	>45	>45	>45	>45	>45	>45	>45	>45
				17; 72; 88	>45	>45	>45	>45	>45	>45	>45	>45	X	X	X	X

* 3 pigs were necropsied at 7 DPI with the remaining 3 being necropsied at 28 DPI

‡ For groups 4, 5, 6, and 7, rectal swabs from all pigs were negative by PEDV rRT-PCR. To save space, 3 pigs are shown in one row.

Table 3.6. Summary of PEDV infection outcomes in neonatal and weaned pigs

Group	Inocula (10ml/pig)			Infection Outcomes	
	Dilution	$TCID_{50}$/ml	Ct	Neonatal Piglets (5-day-old)	Weaned Pigs (21-day-old)
1	10^{-3}	562	24.22	100% (4/4)	100% (6/6)
2	10^{-4}	56.2	28.22	100% (4/4)	100% (6/6)
3	10^{-5}	5.62	31.37	100% (4/4)	100% (6/6)
4	10^{-6}	0.562	35.28	100% (4/4)	0 (0/6)
5	10^{-7}	0.0562	37.62	100% (4/4)	0 (0/6)
6	10^{-8}	0.00562	>45	25% (1/4)	0 (0/6)
7	Neg Ctl	0	>45	0 (0/4)	0 (0/6)

CHAPTER 4: GENERAL CONCLUSIONS

PEDV has been an economically devastating disease in Europe and Asia since it was first identified back in 1971. In its first year in the US it resulted in the death of over 7 million pigs not to mention economic losses incurred by disruptions in pig flow/production.

Our research along with the literature review conducted for this thesis yielded a great deal of useful information. More importantly, it underscores areas where our knowledge regarding PEDV is lacking. Innate, humoral, and cell mediated immune responses to PEDV have been partially characterized. Maternal and mucosal immunity are vital to protection of neonatal pigs, the most susceptible group of pigs. Further characterization of the sow's immune response and how it translates to protective antibody titers in both colostrum and milk is needed. Two conditionally licensed vaccines are available in the US; one based on a killed virus, and the other on a replication deficient virus particle. Both vaccines have some issues with efficacy in naïve swine populations. Modified live virus vaccines may serve as more effective vaccine platform and should be actively pursued. The specific route of entry for PEDV into North America has not been definitively identified. Until this route(s) is/are identified, steps cannot be taken to close the holes in our nation's biosecurity. We therefore remain susceptible to the introduction of more exotic diseases.

Specifically, the research work performed in this thesis was to determine the correlation of PEDV infectious titers to the real-time RT-PCR Ct values and to determine the minimum infectious dose of PEDV in naïve conventional neonatal and weaned pigs.

We first demonstrated that the end-point titration assay yields similar PEDV titer ($TCID_{50}$/ml) to the plaque assay (PFU/ml). Five $TCID_{50}$/ml (or 5 PFU/ml) roughly correlates to a Ct value of 31 and 4.8×10^4 genomic copies/ml. However, it is important to note that these correlations are based on a PEDV cell culture isolate diluted in cell culture medium and RT-PCR

conditions described in this thesis. When RT-PCR conditions and sample matrix are different, the correlation described above could differ as well. We also determined that a 10 ml inoculum with a titer of 0.056 $TCID_{50}$/ml and a Ct of 37.62 was able to infect 4/4 (100%) of 5-day-old pigs, and a 10 ml inoculum with a titer of 0.0056 $TCID_{50}$/ml and a Ct >45 was able to infect ¼ (25%) of 5-day-old pigs. Unfortunately, no further viral dilutions were used in this study, so a true MID was not determined for 5-day-old pigs. For 3-week-old pigs we determined that a 10 ml of inoculum with a titer of 5.6 $TCID_{50}$/ml and a Ct of 31.4 was able to infect 6/6 (100%) pigs, and that no further dilutions were able to infect a single pig. This shows at least a 1000-fold difference in MID for 5-day-old pigs compared to 3-week-old pigs, which confirms the greater susceptibility/increased disease severity in neonatal pigs as demonstrated in previous studies. Factors contributing to this difference, as described in Chapter 3 of this thesis, include: 1) an immature immune system, 2) increased vulnerability to electrolyte/fluid imbalance and dehydration, 3) an increased number of permissible enterocytes, and 4) a slower replacement of enterocytes in neonatal piglets. We also showed that an IgG titer was detectable by both IFA and whole virus ELISA in the serum of 3-week-old pigs by 14 DPI, and a virus neutralizing titer was detectable as early as 7 DPI. One possible explanation for this difference is the presence of neutralizing IgM isotype antibodies prior to the formation of IgG antibodies. Interestingly, we also showed that the extent of fecal viral shedding, severity of histopathologic lesions, and the magnitude of antibody titer subsequently developed were not affected by the initial dose of virus administered to pigs once a PEDV infection was established.

This research produced some clear take away points for the swine industry that should have real utility in the field. It showed that PEDV is infectious in an age dependent manner with a significantly lower MID for neonatal pigs than weaned pigs. It also showed that the MID for

neonatal pigs is below the threshold of detection by our PEDV N gene-based real-time RT-PCR. As such it demonstrated that a neonatal bioassay model would be significantly more sensitive than a weaned pig bioassay model, and would likely be more sensitive than an RT-PCR. Most important of all, the results of this study underline the importance of strict biosecurity and thorough cleaning/disinfection on all farms but especially sow farms where neonatal pigs are present.

Curriculum Vitae

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Education

Aug 2013 – Present MS candidate, Veterinary Microbiology major, Iowa State University
Aug 2012 – Present DVM candidate, Iowa State University
Aug 2008 – May 2012 BS, Animal Science major, Iowa State University

Professional and Academic Associations

2012 – Present American Association of Swine Veterinarians (AASV)

Recent Honors and Awards

- 1st place Oral Presentation Award in 2015 American Association of Swine Veterinarians (AASV) Meeting Student Seminar
- 2014 Iowa State University College of Veterinary Medicine Travel Award
- 2014 Wayne and Karen Frees Scholarship in Veterinary Microbiology
- 2012 William T Dunn Memorial Scholarship
- 2010 & 2012 Gamma Sigma Delta Scholarship

Refereed Journal Publications

1. **Thomas JT**, Chen Q, Gauger PC, Gimenez-Lirola LG, Sinha A, Harmon KM, Madson DM, Burrough ER, Magstadt DR, Salzbrenner H, Welch MW, Yoon KJ, Zimmerman JJ, and Zhang J. (2015). Effect of porcine epidemic diarrhea virus infectious doses on infection outcomes in naïve neonatal and weaned pigs. *PLoS ONE*. 10(10): e0139266.
2. Chen Q, **Thomas JT**, Gimenez-Lirola LG, Hardham JM, Gao Q, Gerber P, Opriessnig T, Zheng Y, Li G, Gauger PC, Madson DM, Magstadt DR, and Zhang J. (2016). Evaluation of serological cross-reactivity and cross-neutralization between the United States porcine epidemic diarrhea virus prototype and S-INDEL-variant strains. *BMC Vet Res*. 12(1), 70-79.
3. Chen Q, Gauger PC, Stafne MR, **Thomas JT**, Madson DM, Huang H, Zheng Y, Li G, and Zhang J. (2016). Pathogenesis comparison between the United States porcine epidemic diarrhea virus prototype and S-INDEL-variant strains in conventional neonatal piglets. *J Gen Virol*. 2016 Feb 3. doi: 10.1099/jgv.0.000419. [Epub ahead of print].
4. Chen Q, Gauger P, Stafne M, **Thomas J**, Arruda P, Burrough E, Madson D, Brodie J, Magstadt D, Derscheid R, Welch M, and Zhang J. (2015). Pathogenicity and pathogenesis of a United States porcine deltacoronavirus cell culture isolate in 5-day-old neonatal piglets. *Virology*. 482, 51-59.
5. Chen Q, Li G, Stasko J, **Thomas JT**, Stensland WR, Pillatzki AE, Gauger PC, Schwartz KJ, Madson D, Yoon KJ, Stevenson GW, Burrough ER, Harmon KM, Main RG, and 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(1), 234-243.

3 Co-authored Manuscripts in Preparation

Conference Proceedings and Abstracts

1. Zhang J, Chen Q, **Thomas J**, and Gauger P. (2016). Pathogenicity and cross-protective immunity of the United States PEDVs. *The 24th International Pig Veterinary Society Congress (IPVS).* Dublin, Ireland. June 7-10, 2016.
2. Chen Q, Gauger P, Welch M, Stafne M, Spadaro M, Salzbrenner H, **Thomas J**, Arruda P, Madson D, Gimenez-Lirola L, Magstadt D, Wang C, Sun Y, Ji J, and Zhang J. (2016). Pathogenesis comparison and cross-protection efficacy of the U.S. PEDV Prototype and S-INDEL-variant strains in weaned pigs. *The 24th International Pig Veterinary Society Congress (IPVS).* Dublin, Ireland. June 7-10, 2016.
3. Chen Q, Stafne M, Spadaro M, Salzbrenner H, Gauger P, Welch M, **Thomas J**, Arruda P, Gimenez-Lirola L, Magstadt D, Madson D, and Zhang J. (2016). Evaluation of pathogenesis differences of the U.S. PEDV prototype and S-INDEL-variant strains and examination of the cross-protective immunity of two strains in weaned pigs. *The 47th American Association of Swine Veterinarians Annual Meeting.* New Orleans, Louisiana, USA. February 27-March 1, 2016. Pages 51-53.
4. Chen Q, Stafne M, Spadaro M, Salzbrenner H, Gauger P, Welch M, **Thomas J**, Arruda P, Gimenez-Lirola L, Magstadt D, Madson D, and Zhang J. (2015). Pathogenesis comparison of the U.S. PEDV prototype and S-INDEL-variant strains in weaned pigs and examination of the cross-protective immunity of two virus strains. *The 96th Annual Meeting of the Conference of Research Workers in Animal Diseases.* Chicago, Illinois, USA. December 6-8, 2015.
5. Zhang J, Chen Q, **Thomas J**, and Gauger P. (2015). Characterization of pathogenicity and cross-protective immunity of U.S. PEDVs. *Proceedings of the 2015 ISU James D. McKean Swine Disease Conference.* Ames, Iowa, USA. November 5-6, 2015. Pages 45-49.
6. Chen Q, **Thomas J**, Hunter F, Gauger P, Harmon K, Main R, and Zhang J. (2015). Evaluation of commercial rapid PED, TGE, and Rota Ag Test Kits for detecting virus pathogens in porcine feces. *The 58th Annual Meeting of American Association of Veterinary Laboratory Diagnosticians.* Providence, Rhode Island, USA. October 22-28, 2015. Page 193.
7. Chen Q, Gauger P, Stafne M, **Thomas J**, Arruda P, Burrough E, Madson D, Brodie J, Magstadt D, Derscheid R, Welch M, and Zhang J. (2015). Development of porcine deltacoronavirus (PDCoV) diagnostic tools and investigation of pathogenicity a US PDCoV cell culture isolate in 5-day-old piglets. *The 58th Annual Meeting of American Association of Veterinary Laboratory Diagnosticians.* Providence, Rhode Island, USA. October 22-28, 2015. Page 155.
8. Chen Q, Gauger P, Stafne M, **Thomas J**, Arruda P, Burrough E, Madson D, Brodie J, Magstadt D, Derscheid R, Welch M, and Zhang J. Development of porcine deltacoronavirus (PDCoV) diagnostic tools and investigation of pathogenesis of a US PDCoV cell culture isolate in 5-day-old piglets. *The Iowa State University's College of Veterinary Medicine Annual Research Day.* Ames, Iowa, USA. August 7, 2015.
9. Zhang J, Chen Q, Gauger P, **Thomas J**, Giménez-Lirola LG, Li G, Madson D, Burrough E, Harmon K, Yoon KJ, Zimmerman J, and Main R. (2015). Genetic and phenotypic

characterization of US porcine epidemic diarrhea viruses. *The 7th International Symposium on Emerging and Re-emerging Pig Diseases*. Kyoto, Japan. June 21-24, 2015. Page 48.

10. Chen Q, Gauger P, Stafne M, **Thomas J**, Arruda P, Burrough E, Madson D, Brodie J, Magstadt D, Derscheid R, Welch M, and Zhang J. (2015). Pathogenicity and pathogenesis of a US porcine deltacoronavirus cell culture isolate in 5-day-old piglets. *The 7th International Symposium on Emerging and Re-emerging Pig Diseases*. Kyoto, Japan. June 21-24, 2015. Page 160.
11. Schumacher LL, Woodworth JC, Zhang J, Gauger PC, Chen Q, Welch M, Salzebrenner H, **Thomas J**, Main RG, Dritz SS, Cochrane RA, and Jones CK. (2015). Determining the minimum infectious dose of porcine epidemic diarrhea virus in a feed matrix. *Midwest American Society of Animal Science Meeting*. Des Moines, Iowa, USA. March 15-18, 2015.
12. Schumacher LL, Woodworth JC, Zhang J, Gauger PC, Chen Q, Welch M, Salzebrenner H, **Thomas J**, Main RG, Dritz SS, Cochrane RA, and Jones CK. (2015). Determining the minimum infectious dose of porcine epidemic diarrhea virus in a feed matrix. *Kansas State University College of Veterinary Medicine Phi Zeta Research Day*. Manhattan, Kansas, USA. March 10, 2015.
13. **Thomas JT**, Chen Q, Gauger PC, Madson D, Burrough ER, Magstadt DR, Salzbrenner H, Welch MW, Giménez-Lirola LG, Yoon KJ, and Zhang J. (2015). Effect of porcine epidemic diarrhea virus infectious doses on outcomes of infection in naïve neonatal piglets and weaned pigs. *The 46th American Association of Swine Veterinarians Annual Meeting Student Seminar.* Orlando, Florida, USA. February 28-March 3, 2015. Pages 101-104.
14. Chen Q, **Thomas JT**, Giménez-Lirola LG, Gauger PC, Hardham JM, Rapp-Gabrielson VJ, Madson D, Magstadt DR, Welch MW, Salzbrenner H, and Zhang J. (2015). *In vitro* evaluation of serological cross-reactivity and cross-neutralization between the U.S. PEDV original and variant strains. *The 46th American Association of Swine Veterinarians Annual Meeting.* Orlando, Florida, USA. February 28-March 3, 2015. Pages 53-54.
15. Zhang J, Chen Q, Gauger P, **Thomas J**, Welch W, Madson D, Magstadt D, Burrough E, Arruda P, and Derscheid R. (2015). Comparison of the pathogenesis differences of the U.S. PEDV prototype and variant strains in neonatal piglets. *The 46th American Association of Swine Veterinarians Annual Meeting.* Orlando, Florida, USA. February 28-March 3, 2015. Pages 49-51.
16. Zhang J, Chen Q, Chriswell A, Dunn D, Li G, Sinha A, **Thomas J**, Harmon K, and Gauger P. (2015). PEDV sequencing: Does it really matter? *The 46th American Association of Swine Veterinarians Annual Meeting Seminar #2: Coronavirus Diagnostics and Surveillance*. Orlando, Florida, USA. Feb 28, 2015. Pages 27-32.
17. Magstadt D, Gauger P, Madson D, Burrough E, Arruda P, Harmon K, Pillatzki A, Chen Q, **Thomas J**, and Zhang J. (2015). Cross-protection after challenge with a contemporary, heterologous PRRS virus in pigs vaccinated with Fostera™ PRRSV. *The 46th American Association of Swine Veterinarians Annual Meeting.* Orlando, Florida, USA. February 28-March 3, 2015. Pages 363-364.
18. **Thomas JT**, Chen Q, Gauger PC, Madson D, Burrough ER, Magstadt DR, Salzbrenner H, Welch MW, Giménez-Lirola LG, Yoon KJ, and Zhang J. (2014). Determination of the minimum infectious dose of porcine epidemic diarrhea virus in neonatal and weaned

pigs. *The 95th Annual Meeting of the Conference of Research Workers in Animal Diseases.* Chicago, Illinois, USA. December 7-9, 2014. Oral # 135.

19. Chen Q, **Thomas JT**, Giménez-Lirola LG, Gauger PC, Hardham JM, Rapp-Gabrielson VJ, Madson D, Magstadt DR, Welch MW, Salzbrenner H, and Zhang J. (2014). *In vitro* evaluation of serological cross-reactivity and cross-neutralization between the U.S. PEDV original and variant strains. *The 95th Annual Meeting of the Conference of Research Workers in Animal Diseases.* Chicago, Illinois, USA. December 7-9, 2014. Oral # 096.
20. Zhang J, Chen Q, **Thomas JT**, Gauger P, Giménez-Lirola LG, Yoon KJ, Madson D, Li G, Burrough E, Harmon K, Hoang H, Sun D, Bhandari M, Stevenson G, Zimmerman J, Baum D, and Main R. (2014). PEDV in US: diagnostic tools and research application in diagnostics. *Proceedings of 2014 North American PRRS Symposium: PRRS, PED, PCVAD and Beyond….* Chicago, Illinois, USA. Dec 5-6, 2014. Page 38.
21. Magstadt D, Gauger P, Madson D, Burrough E, Arruda P, Harmon K, Pillatzki A, Chen Q, **Thomas J**, and Zhang J. (2014). Evaluation of cross-protection in Fostera™ PRRSV vaccinated conventional swine challenged with a contemporary, heterologous lineage 9 PRRSV field isolate. *The 95th Annual Meeting of the Conference of Research Workers in Animal Diseases.* Chicago, Illinois, USA. December 7-9, 2014. Oral # 100.
22. Chen Q, Li G, **Thomas JT**, Stensland WR, Pillatzki AE, Gauger PC, Schwartz KJ, Madson D, Yoon KJ, Stevenson GW, Burrough ER, Harmon KM, Main RG, and Zhang J. (2014). Isolation and characterization of US porcine epidemic diarrhea viruses. *Proceedings of the 23rd International Pig Veterinary Society Congress.* Cancun, Mexico. June 8-11, 2014. Volume 2, Page 70.
23. Chen Q, Li G, Stasko J, **Thomas JT**, Stensland WR, Pillatzki AE, Gauger PC, Schwartz KJ, Madson D, Yoon KJ, Stevenson GW, Burrough ER, Harmon KM, Main RG and Zhang J. (2014). Isolation and characterization of US porcine epidemic diarrhea viruses. *Proceedings of the 45th American Association of Swine Veterinarians Annual Meeting.* March 1-4, 2014. Dallas, Texas, USA. Pages 59-60.
24. Chen Q, Li G, **Thomas JT**, Stensland WR, Pillatzki AE, Gauger PC, Schwartz KJ, and Zhang J. (2013). Isolation and characterization of porcine epidemic diarrhea viruses associated with the 2013 disease outbreak in US swine. *Proceedings of the 94th Annual Meeting of the Conference of Research Workers in Animal Diseases.* Chicago, Illinois, USA. December 8-10, 2013. Page 154.
25. Zhang J, Gauger PC, Berkland MK, Chen Q, Haney DM and **Thomas JT**. (2013). Evaluation of Fostera™ PRRSV vaccine shedding in growing pigs following vaccination. *Proceedings of the 2013 Allen D. Leman Swine Conference*. St. Paul, Minnesota, USA. September 14-17, 2013. Page 182.
26. **Thomas J**, Chen Q, Mondal S, Balasuriya U, Gauger P and Zhang J. (2013). Application of equine and porcine chimeric arteriviruses to determine the porcine reproductive and respiratory syndrome virus proteins responsible for the host range and induction of protective immunity. *2013 Iowa State University College of Veterinary Medicine Research Day*. Ames, Iowa, USA. Aug 9, 2013.
27. **Thomas J**, Chen Q, Mondal S, Balasuriya U, Gauger P and Zhang J. (2013). Application of chimeric arteriviruses to decipher determinants of PRRSV host range and protective immunity. *2013 Merial-NIH Veterinary Scholars Symposium*. East Lansing, Michigan, USA. Aug 1-4, 2013.