Indirect transmission and stability of porcine epidemic diarrhea virus on fomites

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Abstract

Porcine epidemic diarrhea virus (PEDV) is a causative agent of diarrhea in pigs. It recently caused significant economic damage worldwide. The disease is characterized by severe diarrhea, vomiting and dehydration, resulting in up to 100 % mortality in young piglets. While vaccines are available in market, low to moderate efficacy of vaccines have been a concern. One of the reasons implicated for vaccine failures has been genetic differences between vaccine and field strains. Thus, finding effective ways to prevent transmission of PEDV is critical to minimize outbreaks of this disease. However, very little is known about indirect transmission of the virus via personnel movement, and virus stability on fomite materials under different environmental condition. The work described in this dissertation, aims to assess contribution of fomites in indirect transmission events, and study PEDV stability on different fomites at varying temperatures.

Personnel movement across pig rooms caused rapid transmission of the PEDV when low biosecurity (LB) procedures were practiced. Donning contaminated personal protective equipment (PPE) was sufficient to transmit infection to naïve pigs resulting in virus shedding, and providing proof of the contagious nature of PEDV. Also, compared to direct-contact transmission which occurred within 24 hours, indirect transmission via personnel movement happened surprisingly rapidly, similar to direct contact. In addition, we found that PEDV transmission through contaminated body surfaces on personnel in medium biosecurity (MB) group may be possible based on detection of viral RNA on the surfaces of their face and hands. This indicates that given adequate exposure, there is an increased risk for PEDV transmission when procedures like taking a shower or changing

PPE between rooms are not followed.

On further investigation of contaminated fomites under different environmental conditions, we tested the stability of cell cultured PEDV on various fomite materials at two different temperatures, 4ºC and room temperature (25ºC). PEDV remained viable at 4°C, for over 20 days post application on fomites, specifically Styrofoam, Tyvek® coverall, metal and plastic. However, PEDV viability decreased rapid when stored at room temperature on the fomite surfaces we tested, rendering it undetectable using infectious virus assays. However, viral RNA copy numbers could be detected on all surfaces by real-time RT-PCR, which do not correlate well with cell-based assays demonstrating presence of infectious virus. This data reinforces the fragile nature of this enveloped virus, suspecting effect changes of envelope without RNA degradation over time.

These studies are the evidences that indirect transmission of PEDV through contaminated personnel, which occurs rapidly, and how fomite material and temperature impact viral stability over time. These observations provide an increased understanding of indirect transmission of PEDV through personnel movement on contaminated PPEs and fomites. This information can help improve the implementation of biosecurity procedures in controlling PEDV transmission and to prevent new outbreaks.

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General introduction

Porcine epidemic diarrhea (PED) is a highly contagious viral disease which causing acute diarrhea in pigs and inducing more severe clinical sign in young piglets (Pensaert and de Bouck, 1978). In May 2013, the porcine epidemic diarrhea virus (PEDV) was first reported in the US and inflicted catastrophic economic losses to the swine industry due to rapid transmission and high mortality. Over 8 million pigs died because of PEDV infection and it led to an estimated total economic loss of more than $1.8 billion US dollars (Paarlberg, 2014).

Since preventing transmission of virus is central to control outbreak and adequate to eradicate viral disease in pig herds, it is essential to investigate transmission of PEDV. The main transmission route of PEDV is fecal to oral by which contaminated feces with infectious PEDV from infected, viral shedding pigs. PED virus contaminated feed, aerosolized PEDV, and contaminated transportation equipment are also considered as potential sources of infection and spread. The route of PEDV transmission have been studied including direct transmission to pigs by an infected pig (Crawford *et al.*, 2015), PEDV contaminated transport vehicles (Lowe *et al.*, 2014), and feed (Canadian Food Inspection, 2014; Gerber *et al.*, 2014; Opriessnig *et al.*, 2014; Pasick *et al.*, 2014; Bowman *et al.*, 2015a; Dee *et al.*, 2015; Dee *et al.*, 2016; Scott *et al.*, 2016). However, indirect transmission routes by movement of contaminated personnel have not been covered. In addition, PEDV stability on personal protective equipment (PPE) over time has also not been studied. The purpose of this study, therefore, was to evaluate the PEDV transmission by personnel movement changing biosecurity procedures and assess PEDV stability over time on fomite PPE materials in different environment to understand PEDV transmission

and prevent and eradicate PEDV outbreak.

This thesis aims for 1) assessing the indirect transmission route by personnel movement along with evaluating biosecurity procedures regarding PEDV transmission, and for 2) evaluating the stability of PEDV on different fomite materials at different temperatures. To assess indirect transmission, we examined the effect of changing the outer layer of PPE with or without taking a shower compared with applying the contaminated outer layer of PPE. And to assess the stability of PEDV over time, we examined PEDV infectivity on fomites over time, changing temperature and materials (surface of nitrile glove, aluminum material, cloth material, rubber, Styrofoam, cardboard, metal and plastic).

Chapter 1: Literature review

Coronaviruses causing enteric disease in swine

Coronaviruses are spherical shaped, enveloped, RNA viruses causing enteric and respiratory diseases in human and animals. Among the coronaviruses, porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), and porcine deltacoronavirus (PDCoV) cause enteritis in pigs. These viruses infect the intestinal epithelium leading to marked villous atrophy due to acute necrosis of infected enterocytes (Chen *et al.*, 2015; Jung *et al.*, 2015b; Ma *et al.*, 2015). Disruption of villi eventually causes clinical signs including diarrhea and enteritis. All the viruses above that infect pigs belong to the *Coronaviridae* family, and *Coronavirinae* subfamily. The family *Coronaviridae* is subdivided into four genera according to the characteristics of the viral genome: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* (Woo *et al.*, 2012). PEDV and TGEV is classified as *Alphacoronavirus* genus but PDCoV is in the genus *Deltacoronavirus* (Gonzalez *et al.*, 2003). The clinical signs and pathological features of PEDV infection are similar to those of TGEV and PDCoV, requiring differential diagnosis through laboratorial tests (Saif *et al.*, 2012; Jung *et al.*, 2014; Jung and Saif, 2015). Various methods are routinely used for PEDV detection, such as immunofluorescence (IF) or immunohistochemistry (IHC), *in situ* hybridization, electron microscopy, virus isolation, enzyme-linked immunosorbent assays (ELISA), and reverse transcription polymerase chain reaction (RT-PCR) (Lee, 2015). Conventional RT-PCR and real-time RT-PCR diagnostic assays are widely used these days due to their rapid turnaround and high sensitivity (Song *et al.*, 2006; Kim *et al.*, 2007; Zhao *et al.*, 2014; Gou *et al.*, 2015). Serological assays such as immunofluorescence assay (IFA), ELISA, and

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virus neutralization tests can also be used for detecting PEDV antibodies (Rodak *et al.*, 2005; Gerber and Opriessnig, 2015; Li *et al.*, 2015; Okda *et al.*, 2015).

History of porcine epidemic diarrhea virus

In 1971, the first PEDV outbreak was reported in the United Kingdom (Wood, 1977). The clinical signs were very similar to a related *Coronavirus*, specifically TGEV. Clinical signs of PEDV were mild and less severe in growing pigs at that time, hence named epidemic viral diarrhea (EVD) (Wood, 1977; Chasey and Cartwright, 1978). However, five years later the disease re-emerged in multiple regions of Europe causing disease in pigs of all ages. After significant diseases were induced the EVD, then, was classified as EVD type 2 as its severity (Lee, 2015). Meanwhile, in 1978, a research group in Belgium identified the causative agent of EVD was distinct from TGEV and described it as a coronavirus like agent (CV777) (Pensaert and de Bouck, 1978). They provided differences in clinical signs as an evidence that the virus was different from TGEV (Debouck and Pensaert, 1980). Since then the EVD was re-named as porcine epidemic diarrhea (PED). In 1990s, PED outbreaks became silent in adult pigs and showed comparatively mild clinical signs in young suckling piglets in European countries (Saif *et al.*, 2012). The first report of PEDV in Asia was in 1982 and since then the outbreak has been observed in Japan, Korea, Philippines, Vietnam and China with enormous economic losses to pig industries suggesting transmission of European PEDV to Asia (Takahashi *et al.*, 1983; Kweon *et al.*, 1993; Chen *et al.*, 2008; Puranaveja *et al.*, 2009; Li *et al.*, 2012).

Since its initiation in Europe, PEDV has been spreading steadily through various continents resulting in severe, economically devastating outbreaks. In April 2013, a variant PEDV, which was closely related to a Chinese strain, was introduced in the United States and caused high mortality and rapid transmission across the country (Huang *et al.*, 2013). The source of the initial PEDV introduction to the United States remains unknown, but the initial outbreak resulted in over 8 million pigs dead in 2013 with an estimated economic loss of about 1.8 billion US dollars (Mole, 2013; Stevenson *et al.*, 2013; Vlasova *et al.*, 2014; Ojkic *et al.*, 2015).

Genomic structure of porcine epidemic diarrhea virus

The length of the PEDV RNA genome is ~28 kilobase pairs (kb), containing a 5′ cap and a 3′ polyadenylated tail, which serves as both the messenger RNA for viral transcription and the viral genome (Kocherhans *et al.*, 2001). The genome consists of at least seven open reading frames (ORFs), including ORF1a, ORF1b, and ORF 2-6. The proximal two-thirds of the genome encodes for ORF1a and 1ab polypeptide, which is a product of ribosomal frameshifting to produce two unique polyproteins by a single base shift during translation. These two poly proteins are processed post translationally to yield 16 non-structural proteins, including several proteases, an RNA-dependent RNA polymerase, helicase, and endonucleases that are involved in RNA replication (Racaniello, 2013). The other ORFs encode four major structural proteins (spike [S] protein, membrane [M] protein, nucleocapsid [N] protein, and envelope [E] protein) (Duarte and Laude, 1994; Bridgen *et*

al., 1998; Kocherhans *et al.*, 2001). Among the major structural proteins, S protein mediates viral entry, including fusion and entry of the virus into host cells (Bosch *et al.*, 2003) and is the antigenically dominant protein in the virus (Wang *et al.*, 2016). The S protein is cleaved into S1 and S2 by host cell proteases where S1 is responsible for receptor binding and the S2 protein is involved in membrane fusion (Spaan *et al.*, 1988; Cavanagh, 1995; Jackwood *et al.*, 2001). The S protein has been implicated as a pathogenicity determinant region (Vlasova *et al.*, 2014) and shows significant genetic diversity among coronaviruses (Woo *et al.*, 2009). Bernand and Laude (1995) showed significant changes in pathogenicity due to small mutations in S protein of TGEV. Although the parental virus (Purdue-115 strain) showed 100% mortality in new born piglets, a vaccine strain with point mutations or a small deletion in the S gene showed 1000-fold reduced enteropathogenicity in newborn piglets. A mutation in S gene is common in cell culture adaptation of the virus, whereas viral M and N genes are strongly conserved during serial passages (Sato *et al.*, 2011). The most abundant component of the virion, which is the M protein, has a critical role in viral assembly (de Haan *et al.*, 2000). For viral budding, the small, minor membrane protein E is required (Baudoux *et al.*, 1998). The N protein has multiple roles, including interaction between viral mRNAs, increasing stability of viral genome, and in immune evasion strategies (McBride *et al.*, 2014). PEDV ORF3 gene is an accessory gene, and it is believed to function as an ion channel and to influence virus virulence and virus production (Song *et al.*, 2003; Wang *et al.*, 2012).

PEDV-host interactions

Coronaviruses can be found in the variety of animals and mainly infecting respiratory and enteric systems. Viruses belonging to this family cause important diseases like severe acute respiratory syndrome (SARS) and middle east respiratory syndrome (MERS) in humans, feline infectious peritonitis (FIP) in cats, enteritis in dogs, avian infectious bronchitis (IB) in birds, enteritis in turkeys, epizootic catarrhal enteritis (ECE) in ferrets, enteritis in cows, and TGE in pigs (Kahn and Line, 1962; Buonavoglia *et al.*, 2006; Forgie and Marrie, 2009). Coronaviruses have a tropism for specific cells, like the respiratory epithelial cell, intestinal epithelial cell, hepatic cells, and macrophages (Woo *et al.*, 2012). TGEV infects small intestinal epithelial cells and causes severe enteritis in piglets, and has an ability to replicate in the porcine respiratory tract, however it does not induce primary respiratory disease (Kemeny *et al.*, 1975; Enjuanes *et al.*, 1995; Saif, 2004b, a). PEDV has specific tissue tropism for small and large intestinal epithelial cells causing villous atrophy resulting in malabsorption and diarrhea (Debouck and Pensaert, 1980).

PEDV utilizes a specific receptor to enter the host cell. It is porcine aminopeptidase N (pAPN) which is highly expressed on the surface of the epithelium of the small intestine. It has been identified as the port of viral entry for PEDV (Li *et al.*, 2007; Nam and Lee, 2010). The PEDV spike protein S1 domain binds pAPN on the host cell (Cha and Lee, 2011), followed by direct fusion of the viral envelope with the cell membrane resulting in internalization of the viral genome. Subsequently, the viral genome is released into the cytosol to start genome replication. A recent study showed that heparan sulfate on the cell surface functions as an attachment factor for PEDV entry (Huan *et al.*, 2015). In addition, trypsin was identified to be indispensable for growth of PEDV in Vero cells, *in vitro*

(Hofmann and Wyler, 1988; Chen *et al.*, 2014; Oka *et al.*, 2014; Lee *et al.*, 2015). Trypsin cleaves the PEDV S protein into its constituent S1 and S2 subunits, which facilitate viral entry and subsequent replication within the cell (Shirato *et al.*, 2011; Wicht *et al.*, 2014). However, some cell-adapted PEDV strains have been shown to possess the ability to replicate *in vitro* in the absence of trypsin (Nam and Lee, 2010).

Clinical signs and pathogenesis of porcine epidemic diarrhea virus

PEDV is a highly contagious virus and pigs of all ages are susceptible. The clinical signs and mortality caused by PEDV infection can vary based on the virus strain, but it generally causes severe diarrhea accompanied with vomiting, depression and anorexia. The incubation period of PEDV is approximately 1 to 2 days (Pensaert and de Bouck, 1978; Saif *et al.*, 2012; Madson *et al.*, 2014; Jung *et al.*, 2015a). Viral shedding through feces can be detected within 24 hours after infection and persists for about 30 days post infection (Saif *et al.*, 2012; Thomas *et al.*, 2015). Suckling piglets are the most susceptible to disease, resulting in severe diarrhea and vomiting, followed by dehydration, eventually leading to death. Average mortality rate is around 50 % particularly in neonatal piglets, but some virulent strains are capable of causing 100 % mortality in piglets (Lee, 2015). In growing pigs, from wean to finish, clinical signs are usually mild, and mortality rates are below 10%. However, it affects the growth rate leading to substandard growth performance resulting in significant decrease in average daily gain (ADG) and feed conversion ratio (FCR) in pig ranging from from 23 to 90 days of age (Alvarez *et al.*, 2015).

PEDV targets the epithelium on the small intestine villi and mucosa of the large intestine for entry. Infection results in a characteristic pathology showing thin and transparent intestinal walls, with accumulation of yellowish fluid (Shibata *et al.*, 2000; Jung *et al.*, 2014). Histologically, PEDV infection is characterized as severe diffuse atrophic enteritis, reduced villous length, vacuolation of superficial epithelial cells, subepithelial edema in cecum and colon, and contraction of subjacent villous in the lamina propria with apoptotic cells and scattered lymphocytes (Debouck *et al.*, 1981; Shibata *et al.*, 2000; Jung *et al.*, 2014; Madson *et al.*, 2014).

Biological characteristics of PEDV

PEDV has a buoyant density of 1.18 in a linear sucrose density gradient (Hofmann and Wyler, 1989). PEDV loses infectivity when treated with lipid solvents, such as ether and chloroform, and it remains stable at temperatures ranging from 4-50°C. The virus completely lost infectivity when incubated at 60°C for 30 min (Hofmann and Wyler, 1989). According to Hofmann and Wyler, the virus was stable at 4°C in pH ranges between 5-9, however, it was completely inactivated at pH of under 4 or over 9. These data suggest that PEDV can be effectively controlled using acidic or alkaline disinfectants. Similarly, SARS-coronavirus (SARS-CoV) lost infectivity with formaldehyde, 2-propanol, and glutaraldehyde solution and was inactivated at 56° and 60°C for 30 min (Rabenau *et al.*, 2005). SARS-CoV can stay viable for up to 5 days at 22 to 25°C at 40 to 50% relative humidity (RH) and the virus losses viability rapidly when the temperature and humidity

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are increased (Chan *et al.*, 2011).

The German isolate of the PEDV V215/78 strain and the reference strain CV777 showed no serological cross-reactivity with Belgian transmissible gastroenteritis virus (TGEV) and feline infectious peritonitis virus (FIPV) when tested under immune-electron microscopy and by immunofluorescence (Debouck *et al.*, 1981; Hofmann and Wyler, 1989). However, subsequent studies found cross-reactivity between PEDV and other coronaviruses using enzyme linked immunosorbent assay (ELISA), immunoblotting and immune-precipitation (Yaling *et al.*, 1988; Have *et al.*, 1992). A recent study also reported that the prototype CV777 and recent US PEDV strains (PC22A, S Indel strain Iowa 106, and S 197del strain PC177) and TGEV (Miller strain) share at least one conserved epitope on the N protein (Lin *et al.*, 2015). Cross-reactivity of PEDV strains also can be explained by amino acid similarity in the S1 region, which harbors neutralizing epitopes (Chen *et al.*, 2016).

Global Epidemiology of PEDV infection

Epidemiology of PEDV in Europe

PEDV was first identified as a unique pig disease in 1977 in the United Kingdom (Wood, 1977) and Belgium in 1978 (Pensaert and de Bouck, 1978). At that time, the disease in Europe was reported as causing mild watery diarrhea with insignificant economic impact on the pig industry, compared to what was seen when it spread into Asia and the United States (Lee, 2015). Hence, the disease and its pathological agent were not studied

extensively for a few decades since its identification. Farm outbreaks of PEDV were infrequent in Europe since its first identification through the early 1990s. Some studies determined that the seroprevalence of PEDV was relatively low in European pigs (Van Reeth and Pensaert, 1994; Carvajal et al., 1995). However, the virus remained endemic in the swine population as evidenced by sporadic outbreaks reported in some European countries in the early 1990s (Song and Park, 2012).

In the early part of the 21st century, several PEDV outbreaks were reported in Europe exhibiting the typical epidemic form and affecting all ages of pigs. These outbreaks were reported in Hungary, Netherlands, England (all in the late 1990s) (Pijpers et al., 1993; Nagy et al., 1996; Pritchard et al., 1999), later in Italy in 2006 (Martelli et al., 2008), and Germany in 2014 (Hanke et al., 2015). The German outbreak occurred in a fattening farm and reported PEDV infection in pigs of all age groups, and similar outbreaks in a farrow-finish farm in France, and in a fattening farm in Belgium occurred around the same time (Grasland et al., 2015; Hanke et al., 2015; Theuns et al., 2015). Molecular analysis of the PEDV strains showed 99.9% sequence identity to the strains from outbreaks that occurred in China, South Korea, and the United States around the same time (Lee, 2015). This information indicates that PEDV was rapidly transmitted and its global expansion indicates not only the possibility of transmission between countries but also that the virus could be re-introduction to the endemic countries. The transmission route and direction of these recent outbreaks remain unexplored, however, it is clear that PEDV has become a global emerging and re-emerging disease causing major financial impact for the swine industry worldwide.

Epidemiology of PEDV in Asia

Swine diarrhea outbreaks, where no specific etiological agent was identified, have been prevalent in China for over 30 years (Carvajal *et al.*, 2015). PEDV was first identified in 1982 as the causative agent for diarrheal infections in China (Xuan *et al.*, 1984) and Japan (Takahashi *et al.*, 1983). The virus spread quickly through neighboring countries, South Korea and east Asia (Kweon *et al.*, 1993; Jinghui and Yijing, 2005). According to a retrospective study using *in-situ* hybridization in South Korea, it was determined that the first PED case occurred there in 1987 (Park and Lee, 1997). Since the PED outbreaks became common and reoccurred every year, with high mortality in piglets, it resulted in significant economic losses to the swine industry in that region. A serological survey in 2007 reported 91.8 % seroprevalence from 159 farms tested, suggesting that the majority of farms were endemic for PEDV (Park and Pak, 2009). PED incidences declined in the early 2000s, with the increased use of both modified attenuated DR-13 and inactivated SM98-1 vaccines. Nevertheless, PED continued to break in vaccinated farms, indicating an inefficiency of vaccination programs implemented to prevent the spread of infection. This low efficiency of vaccination can be explained by the emergence of novel genogroups of PEDV in South Korea.

PEDV strains can be classified into two distinct genogroups, genogroup 1 (G1) and genogroup 2 (G2), according to the phylogenetic trees created using PEDV whole genome sequence (Huang *et al.*, 2013). G1 includes classical strains such as, CV777, LZC

and SM98 strains. The PEDV variant strain isolated in 2010s, which caused outbreaks in China and the United States, were classified as G2. They are closely related to AH2012 strain and strains that are recently isolated in the United States (Lee *et al.*, 2010; Huang *et al.*, 2013). After a short period of PED outbreaks from 2010 to 2012, perhaps a consequence of severe outbreaks of foot-and-mouth disease (FMD) in 2010, PEDV re-emerged and spread rapidly throughout South Korea in 2013 (Lee and Lee, 2014). In November 2013, severe PED epidemics arose again resulting in more than 40 % of pig lost in the country (Lee and Lee, 2014). The re-emergent PEDV isolates in South Korea isolated between 2013 and 2014 belonged to subgroup G2, and are closely related to the US PEDV strains (Lee *et al.*, 2014).

PEDV outbreaks continue in China leading to serious losses since the first identified PED case in the 80's. Vaccines containing the prototype of CV777 strain have been widely used from early 1990s, however, incidence of PED outbreaks increased in late 2010 (Li *et al.*, 2012). The viral agents associated with these outbreaks in China were identified as both G1 variants and G2 subgroup (Sun *et al.*, 2012; Wang *et al.*, 2013; Tian *et al.*, 2014).

The first PED outbreaks in Thailand occurred in late 2007 and the viral strains isolated from these were classified as G2 subgroup, which cluster adjacent to contemporary Chinese and Korean strains (Temeeyasen *et al.*, 2014). These G2 subgroup strains were also observed in Vietnam in 2013 (Vui *et al.*, 2014). During the end of 2013, PEDV G2 subgroup strains were isolated from Taiwan and Japan causing severe and re-emerging PED outbreaks in these countries (Lin *et al.*, 2014; Yamamoto *et al.*, 2015). These

observations not only show various PEDV genotypes present across the world with increased incidence, but also suggest that global transmission of PEDV is taking place.

Epidemiology in the United States

Prior to May 2013, PED had never been reported in the United States. However, in May 2013, a PEDV strain genetically related to the Chinese AH2012 strain, emerged and rapidly spread across the United States (Huang *et al.*, 2013; Stevenson *et al.*, 2013). However, how these viruses are rapidly transmitted between herds and rapidly across the country is still unknown (Jung and Saif, 2015). Other novel US PEDV strains were isolated in January 2014, which had 89 % or even lower nucleotide identity in the S gene compared to AH2012 strain, suggesting the virus has undergone multiple deletions and insertions during the course of its spread in the US (Wang *et al.*, 2014). These observations indicate that the virus has begun to adapt through mutations while still spreading to naive herds rapidly.

Transmission of porcine epidemic diarrhea virus

PEDV is shed in feces of infected pigs and can be transmitted via the fecal-oral route through direct contact, by fomites, and on farm personnel (Scott *et al.*, 2016). Contaminated feed has been considered as a potential source of infection in the USA, although its contribution to the 2013 outbreak is still controversial (Dee *et al.*, 2014; Opriessnig *et al.*, 2014; Pasick *et al.*, 2014; Pujols and Segales, 2014; Bowman *et al.*, 2015a; Gerber and Opriessnig, 2015). Some researchers have detected the presence of PEDV RNA

in feed, especially in spray dried porcine plasma (SDPP) using real-time RT-PCR. However, Pujols and Segalés (2014) conducted a study by spiking cell cultured PEDV in spray dried bovine plasma (SDBP) and found it was negative for PEDV RNA after processing procedures. A similar study performed by Gerber et al. (2014), mimicking spray drying procedures, could not detect PEDV RNA and could not observe pathophysiological signs in the challenge group fed with virus contaminated feed after the spray drying process. Recently, aerosolized PEDV has been suspected as a source of disease transmission based on the detection of PEDV RNA in air samples (Alonso *et al.*, 2014). Further investigation using bioassay confirmed the presence of infectious PEDV associated with contaminated air but more studies are needed to assess the likelihood of aerosol transmission under field conditions.

Transmission by contaminated transportation equipment has also been reported as a risk factor for PEDV infection (Lowe *et al.*, 2014; O'Dea *et al.*, 2016). Arruda et al. suggested that all swine sites, approximately 94%, were indirectly connected through their service providers' networks which means viral transmission can be easily happened by these networks. This inference was made using data from swine sites in a porcine reproductive and respiratory syndrome (PRRS) risk analysis study (Arruda *et al.*, 2016). These associations within swine production networks observed during PRRS outbreaks would be similar for PEDV. Even after an outbreak, PEDV may persist within the farm unit, especially when inadequate hygiene management measures are practiced, such as poor biosecurity procedures or inappropriate disinfections are done. Then break of biosecurity is happened, and contaminated materials can be placed into these networks triggering

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PEDV outbreak as consequences. Therefore, preventing transmission into networks is critical in developing control and eradication strategies.

PEDV may also persist in pigs at weaning or at grow-finishing age enabling the virus to circulate within the herd causing mild post-weaning diarrhea with low mortality (Lee, 2015). In this situation, maternal derived immunity provided through colostrum and milk becomes an important component of the defense against PEDV. However, piglets not receiving sufficient levels of maternal immunity from the sow, due to incomplete/inefficient sow vaccination, may easily become infected with circulating virus by direct contact with shedding pigs or from the contaminated environment. This may cause recurrence of epidemic outbreaks, leading to sudden and explosive emergence of virus (Park and Lee, 2009).

Interaction between host innate immune system and PEDV infection

A virus infected cell generates innate immune responses against the viral infection by secretion of type 1 interferon (IFN). IFN is a cytokine that stimulates resistance to viral infection and modulates the innate and adaptive immune response in the host (Roberts *et al.*, 1991; Lefèvre *et al.*, 1998; Stark *et al.*, 1998). However, PEDV infection of intestinal epithelial cells (*in vitro*) inhibited dsRNA-mediated IFN-β production by blocking the activation of IFN-β promoter stimulator-1 (IPS-1) activated by the retinoic acid-inducible gene I (RIG-I) mediated pathway (Cao *et al.*, 2015), whereas IFN was produced in the lung, intestine and other several organs in porcine respiratory coronavirus infection (PRCV) and

TGEV infection (La Bonnardiere and Laude, 1981; Bernard and Laude, 1995; Riffault *et al.*, 2001). These *in vitro* study results suggest host innate protection against PEDV infection may unmanageable in infected pig itself. The mechanism by which PEDV blocks this innate immune response is through viral papain like protease 2 (PLP2) that acts as a deubiquitinase to inhibit IFN-signaling (Xing *et al.*, 2013). PEDV nucleocapsid (N) protein also impedes the activation of the IFN-β promoter targeting interaction between IFN regulatory factor 3 (IRF3) and TANK binding kinase1 (TBK1) (Ding *et al.*, 2014). These studies not only indicate insufficient innate immunity of host responses against PEDV but also suggest requirements of aggressive action on prevention methods.

Vaccination and prevention strategies of PEDV

Vaccines

Although PEDV was first identified in Europe, PEDV vaccines have been developed and used in Asian countries because of the significant economic impact the disease had in this geographic area. The commercially available vaccines that are widely used include live attenuated vaccine and killed-inactivated vaccine (Song and Park, 2012). In China, PEDV CV777 strain is used for both live attenuated and killed-inactivated vaccine (Pan *et al.*, 2012). In Japan, PEDV 83P-5 strain is used as a live attenuated vaccine. 83P-5 was attenuated by passaging the virus 100 times in Vero cells (Sato *et al.*, 2011). In South Korea, PEDV Korean isolate SM98-1 strain is used for both live attenuated and killed inactivated vaccine, while the isolate DR-13 is only used as a live attenuated vaccine (Kweon *et al.*,

1999; Song *et al.*, 2007). However, recently some researchers questioned the efficacy and safety of these PEDV vaccines (Ayudhya *et al.*, 2012; Luo *et al.*, 2012; Pan *et al.*, 2012; Sun *et al.*, 2012; Tian *et al.*, 2013). These concerns are based the genetic (G1 and G2) differences between the classical vaccines and recent field virulent strains that emerge during outbreaks (Lee *et al.*, 2010; Lee and Lee, 2014; Oh *et al.*, 2014; Kim *et al.*, 2015; Lee *et al.*, 2015). This argument is controversial because studies have showed PEDV G2 strain can cross protect against G1 strain (Oh *et al.*, 2014). It is possible that cross-reactivity may provide partial protection against disease against a heterologous strain, but could lead to a subclinical infection that facilitated viral emergence. But, to effectively break the transmission of virus within a herd in such situations, practicing strict biosecurity procedure might be the only current available option.

Mucosal immunity

For PEDV infection, induction of localized immune response in the gut is critical since PEDV targets the intestinal villi epithelium (Song and Park, 2012). Localized secretory immunoglobulin A (sIgA) antibodies serve to prevent attachment and invasion of the virus in the intestine (Huy *et al.*, 2012). Since PEDV only targets intestine, gut-associated lymphoid tissue (GALT) is crucial for the defense of PEDV viral infection (Song *et al.*, 2015). The Peyer's patches and mesenteric lymph nodes are major components of GALT in swine and are responsible for gut immune responses (Andersen *et al.*, 1999). The Payers patches serve as principal location for proliferation and maturation of B cells after an

infection in the gut (Butler *et al.*, 2009). In early 1970s, researchers found that oral inoculation with live, virulent TGEV showed high rates of protection in suckling pigs. These sows showed high titers of sIgA antibodies in colostrum and milk, and were associated with high rates of protection in their piglets (Bohl *et al.*, 1972; Saif *et al.*, 1972). Recently, a research study on pregnant sows determined that oral inoculation of mildly pathogenic PEDV resulted in high protection rates for their piglets, showing the importance of lactogenic immunity against PEDV infection (Goede *et al.*, 2015). Studies also demonstrated a gut-mammary gland-sIgA axis, suggesting IgA plasmablasts stimulated in the gut, following TGEV or PEDV infection migrate from the gut lamina propria, into the mammary glands (Roux *et al.*, 1977; Saif *et al.*, 1994). Several highly attenuated TGEV vaccines, which failed to induce high milk sIgA antibody titers were due to poor viral replication in the gut, which resulted in lower protective immunity in suckling piglets compared to when virulent TGEV was given to induce a higher level of milk sIgA antibodies (Moxley and Olson, 1989; Saif, 1999). Similar to TGEV, IgA is more effective in providing protection against PEDV due to its stability against proteolytic enzymes in the intestine and higher neutralization ability compared to IgG and IgM (Offit and Clark, 1985; Song *et al.*, 2015). Therefore, a vaccine that can induce high amount of IgA in the gut may be required to confer successful protection against PEDV in pigs.

Prevention

Following an outbreak of PED, the virus may persist in susceptible pigs, or in the

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environment leading to maintenance of the virus within the farm (Scott *et al.*, 2016). Endemic PEDV generally affects young piglets, causing severe dehydration due to intensive diarrhea. To control outbreaks, therefore, vaccinating sows for transferring maternal antibodies such as IgA to piglets via colostrum and milk should be considered an effective control for endemic PEDV infection (Nam and Lee, 2010; Saif *et al.*, 2012; Lee, 2015). Given the low efficacy of commercial vaccines, many are killed vaccines and given parenterally, one of the most effective options for controlling a PEDV outbreak is to implement strict biosecurity to break the transmission cycle of PEDV in pig herds. In addition, to prevent introduction of PEDV, fomites capable of transmitting the virus should be thoroughly disinfected. For optimal management to minimize outbreaks, contaminated air, transport vehicles and by-products of pigs must be considered as potential avenues to spread infection (Alonso *et al.*, 2014; Canadian Food Inspection, 2014; Dee *et al.*, 2014; Lowe *et al.*, 2014; Pasick *et al.*, 2014; Bowman *et al.*, 2015a; Dee *et al.*, 2015; O'Dea *et al.*, 2016). There is also a concern that PEDV contaminated personnel may move the virus across pens and farms which could lead to an outbreak (Scott *et al.*, 2016).

To control the transfer of contaminated fomites strict biosecurity procedures should be followed. A study in 2013, showed that nearly 60% of producers did not request employees to wash their hands and/or shower before entering the pig facility suggesting required improvement (Bottoms *et al.*, 2013) even though some of the producers followed high biosecurity practices including thorough cleaning and disinfection of transport vehicles,. Also, transportation to slaughter plant facilities and to livestock auction markets can be an effective source of vehicle contamination which could have played an important role in the

rapid dissemination of PEDV (Lowe *et al.*, 2014). Overall, the observations provided to date indicate that improved sanitation, hygiene and segregation practices are needed to minimize transmission of PEDV.

To develop strategies to control PEDV, understanding the impact of indirect transmission of PEDV particularly via personnel movement, and evaluating the stability of PEDV on fomites is needed.

Chapter 2: Evaluation of biosecurity measures to prevent indirect transmission of porcine epidemic diarrhea virus

Introduction

Porcine epidemic diarrhea (PED) is a highly contagious viral disease that causes severe diarrhea in pigs (Saif *et al.*, 2012). In May 2013, the porcine epidemic diarrhea virus (PEDV) was first reported in the US, causing significant economic losses to the swine industry due to high mortality rates in piglets. Over 8 million pigs died due to PEDV, leading to an estimated total industry economic loss of more than 1.8 billion US dollars (Paarlberg, 2014). PEDV is an enveloped, single-stranded, positive-sense RNA virus belonging to the *Coronaviridae* family in the genus *Alphacoronavirus* (Hofmann and Wyler, 1989; Saif *et al.*, 2012). The virus is shed in feces of infected pigs and transmitted via the fecal-oral route. PEDV can be transmitted either by direct contact between infected and susceptible pigs or indirectly through contaminated fomites. Transmission via pig transportation has been reported as a major risk factor for the spread of PEDV (Lowe *et al.*, 2014; O'Dea *et al.*, 2016). Five percent of PEDV negative trailers became contaminated during the unloading process at slaughterhouse facilities handling infected pigs (Lowe *et al.*, 2014). Contaminated feed has also been implicated in the spread of PEDV, and both food ingredients (viz. dried spray plasma) and cross-contamination at the feed mill or from other sources have been implicated in the spread of PEDV (Canadian Food Inspection, 2014; Dee *et al.*, 2014; Gerber *et al.*, 2014; Pasick *et al.*, 2014; Bowman *et al.*, 2015a; Scott *et al.*, 2016). PEDV has also been detected in air samples and aerosol transmission has been suspected as a potential source of disease transmission in high pig density areas (Alonso *et al.*, 2014).

In order to mitigate transmission of PEDV within and between farms, producers employ a range of biosecurity practices. These practices include disinfecting footwear and changing clothing of visitors or personnel prior to entering farm premises, washing and sanitizing delivery trucks or vehicles entering the farm, and controlling insects. Controlling transmission via feed can be done by using feed additives such as formaldehyde (Dee *et al.*, 2016) and transmission via transport can be minimized by implementing proper cleaning and disinfection methods (Bowman *et al.*, 2015b). However, these implementations are not always practical or cost effective. The effectiveness of methods to mitigate transmission via farm personnel or contaminated fomites are less understood given that intervention strategies at the farm level have not been properly investigated. Furthermore, Stevenson et al. indicated that even shower-in/shower-out facilities with excellent biosecurity protocols also reported PEDV outbreaks (Stevenson *et al.*, 2013). Given the limited knowledge available on how biosecurity procedures may disrupt the transmission cycle of PEDV, the present study was undertaken to evaluate the effectiveness of biosecurity procedures directed at minimizing transmission via personnel following different biosecurity protocols using a controlled experimental setting.

Materials and Methods

Animals and animal housing

Forty-eight, 3-week-old pigs were obtained from a farm with no history of PEDV

infection and were housed at the St. Paul animal research isolation units at the University of Minnesota. After arrival (2 days before the start of the study), the rectal swabs from all pigs were collected and tested by real-time reverse transcription polymerase chain reaction (rRT-PCR) for PEDV, transmissible gastroenteritis virus (TGEV) and porcine delta coronavirus (PDCoV) at the University of Minnesota Veterinary Diagnostic Laboratory (St. Paul, MN, USA).

The pigs were housed in 17 separate rooms that were independently operated from each other as described below. All individual rooms had anterooms with footbaths, a sink for hand and face washing, a storage area of 2.08 m^2, and an animal housing area of 7.28 m^2. Rooms were connected through a clean common hallway as shown in Fig. 1. The floor of the animal housing area was constructed with solid concrete and each animal housing area had a single water line with two water nipples as a source of drinking water. Prior to introducing the pigs into the rooms, environmental swabs were collected from the floors and confirmed PEDV negative by rRT-PCR. Ventilation for all rooms was kept under negative differential pressure to the main corridor, having one air inlet and one exhaust vent per room. The air supply was conditioned with a 3 ply panel filter (TRI-DEK® 15/40, TRI-DIM Filter Corp., Louisa, VA, USA) and 100 % of exhaust air was filtered through a HEPA filter (XH Absolute HEPA filter, Camfil, Stockholm, Sweden).

The pigs were randomly distributed as described below and treated with a single

intramuscular dose of enrofloxacin (0.5 mL/pig; Baytril®, Bayer HealthCare AG, Leverkusen, Germany) one day prior to infection. In the first iteration of the study, forty eight 3-week-old piglets were randomly assigned to 5 experimental groups: (i) ten pigs were infected with PEDV and 2 contact sentinel pigs were housed together to serve as the source of infection (INF group); (ii) 10 pigs (5 replicates of 2 each) were assigned to low biosecurity (LB) sentinel groups; (iii) 10 pigs (5 replicates of 2 each) were assigned to medium biosecurity (MB) sentinel groups; (iv) 10 pigs (5 replicates of 2 each) were assigned to high biosecurity (HB) sentinel groups and (v) six pigs were assigned to a negative control (NC) group, which were uninfected and handled separately.

In the 2nd iteration of the study, twenty-three 6-week-old pigs were assigned to 4 different groups: (i) 3 pigs were infected with PEDV and 1 contact sentinel pig were assigned to the INF group; (ii) 4 pigs were assigned to LB sentinel group; (iii) 12 pigs (3 replicates of 4 each) were assigned to MB sentinel groups; and (iv) 3 pigs (1 replicate) were assigned to NC group.

Study personnel

Study personnel were exclusively assigned to handle pigs in this study and had no direct contact with other pigs or with PEDV-infected pigs from another source for the entire duration of this study. Personnel entering LB, MB, and HB sentinel rooms had direct contact with infected pigs in the INF room only and performed all necessary procedures

(e.g. pig fecal swab collection, pig blood collection, feeding of pigs, cleaning of room) in their designated rooms as assigned during the deputed sampling and movement days.

Clothing and personal protective equipment

All study personnel showered in the research animal facility prior to donning the facility-dedicated clothing and personal protective equipment (PPE). After showering, personnel put on clean scrubs, a pair of disposable plastic boots and entered the animal isolation corridor after stepping through an iodine footbath. In the animal isolation clean hallway, personnel put on disposable Tyvek® coverall (DuPont, Wilmington, DE, USA), nitrile gloves, and a bouffant cap for the first iteration of the study. In the 2nd iteration, a cloth coverall was used instead of Tyvek® coverall. Upon entry into the anterooms through another iodine footbath, personnel put on face shield and room-specific rubber boots. Before entering the animal housing area, personnel put on another pair of disposable plastic boots over their rubber boots.

Experimental Design:

Infected group (INF)

For the first iteration of the experiment, ten PEDV negative 3-week-old piglets were inoculated with PEDV (USA/Colorado/2013) strain via the intra-gastric route. Each pig was infected with 10 mL of the virus inoculum containing 3.6×10^4 50% tissue culture

infective dose (TCID$_{50}$) per mL. Two uninfected PEDV negative 3-week-old piglets were housed with infected animals to serve as sentinels to assess transmission by direct contact. During the 2nd iteration, three PEDV negative 6-week-old pigs were inoculated with gastrointestinal mucosal scrapings obtained from animals infected with the PEDV virulent strain, by intra-gastric route. An uninfected 6-week-old piglet was added to this group as a direct contact sentinel. All study personnel interacted with infected pigs for the movements.

Movement between experimental groups

A movement was defined as the process when study personnel moved from the INF room to either the LB, MB, or HB sentinel rooms (Fig. 2). The first movement started approximately 44 hours following the experimental inoculation of pigs in the INF group.

Exposure of study personnel to INF pigs

All study personnel who participated in movements between experimental groups were in contact first with pigs in the INF group for 45 minutes. Personnel interacted directly with the pigs by handling the pigs, collecting samples from them, and allowing pigs to come in contact with personnel clothes and PPE, e.g. biting, sniffing, and rubbing. Accordingly, potential infectious secretions and feces could be transferred to clothing and PPE worn by the study personnel.

Movement from infected room to LB rooms

Following the interaction period with pigs in the INF group, study personnel who were designated to LB rooms placed their used nitrile gloves, disposable plastic boots, bouffant cap and coveralls into a clean plastic bag while in the storage area of the INF room. LB room study personnel exited INF room through a soiled (outside) corridor and entered directly into the LB sentinel holding room through an exit door in the soiled corridor, without stepping into iodine footbaths (Fig. 1). The LB room study personnel re-donned their used PPE, including nitrile gloves, disposable plastic boots, bouffant cap and coveralls, in the LB anteroom area. Prior to initiating contact with LB sentinel pigs, each person collected four separate swab samples from their (i) used coveralls, (ii) used disposable plastic boots, (iii) used nitrile gloves, and (iv) used bouffant cap in the LB storage area. After collecting the swab samples, personnel collected rectal swab samples from LB room sentinel pigs and interacted with LB room sentinel pigs for 45 minutes as previously described. All study personnel designated to LB rooms did not wash their hands and face prior to contact with LB sentinel pigs. These movements were scheduled once a day for 9 consecutive days and terminated after LB sentinel pigs tested positive for PEDV.

Movement from infected room to MB rooms

Following interaction with INF pigs, study personnel collected four separate swabs from

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the surface of used (i) coveralls, (ii) disposable plastic boots, (iii) nitrile gloves, and (iv) bouffant caps in the INF room storage area. All MB room study personnel exited the INF room through the anteroom and removed their used coveralls, disposable plastic boots, latex gloves, and bouffant cap, and washed their hands and face with soap and water for approximately 20-40 seconds according to the Centers for Disease Control and Prevention (CDC) guidelines [18] prior to exiting the room into the clean corridor (Fig. 2). In the clean hallway, study personnel donned new coveralls and bouffant cap and collected four separate fomite swab samples from the new PPE, including (i) coveralls, (ii) disposable plastic boots, and from their (iii) hands, and (iv) hair prior to entering the anteroom of MB sentinel rooms. Here, study personnel washed again their hands and face and then, put on gloves, protective eyewear and room-specific rubber boots. Before entering the MB room animal housing area, personnel put on another pair of disposable plastic boots over their rubber boots. MB study personnel collected rectal swab samples from sentinel pigs and interacted with them, as described above. These movements were completed once a day over nine consecutive days.

Movement from infected room to HB rooms

The HB animals were housed in a separate building located approximately 10 m away. After interacting with pigs in the LB or MB treatment group, study personnel showered with soap and shampoo for approximately 10 minutes before donning a new set of facility-dedicated scrubs and a pair of new disposable plastic boots. Study personnel

donned new PPE, interacted again with pigs in the INF group, and took a full shower again before donning a new set of facility-dedicated scrubs and a pair of new disposable plastic boots before entering the isolation unit where the HB animals were housed. Study personnel entered the animal isolation hallway through an iodine footbath, then donned new coveralls and bouffant cap. Each of the study personnel collected four separate fomite swab samples from the new PPE, hands, and hair as described above. In the anteroom, study personnel washed their hands and face again, donned gloves, protective eyewear, and room-specific rubber boots with disposable plastic boots over them before entering the animal housing area. All study personnel collected rectal swab samples from HB room sentinel pigs and interacted with them as described above.

Collection of rectal and fomite swabs

Fomite and rectal swab samples were collected using a sterile rayon-tipped swab (BD CultureSwab™, liquid Stuart medium, single plastic applicator, Becton Dickinson and Co., Sparks, MD, USA). Fomite swabs were collected from coveralls, disposable plastic boots, hands or nitrile gloves, face and hair areas using a zigzag pattern to cover maximum surface area. Following collection, each swab was suspended in 2 mL transport media solution of Dulbecco's minimal essential medium (Gibco® DMEM, Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 2 % Bovine Albumin Fraction V 7.5 % solution (Gibco® BSA, Thermo Fisher Scientific Inc., Waltham, MA, USA), 1 % Antibiotic-Antimycotic, 100x solution (Gibco® Anti-Anti, Thermo Fisher Scientific Inc.,

Waltham, MA, USA), 0.15 % Trypsin-TPCK, 1 mg/mL (Sigma-Aldrich, St. Louis, MO, USA) and 0.1 % Gentamicin-Sulfate, 50 mg/mL (Lonza Inc., Walkersville, MD, USA). An aliquot (50 μL) of the swab suspension sample was used to extract RNA for rRT-PCR, and the remainder of the samples were stored at -80℃. Swab samples were tested for the presence of PEDV Spike (S) gene by rRT-PCR. Briefly, RNA was extracted from eluent using the MagMAX™-96 Viral RNA Isolation Kit (ThermoFisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. A primer pair was designed to amplify a portion of the PEDV S gene with the following sequences: Forward 1910: ACGTCCCTTTACTTTCAATTCACA and Reverse 2012: TATACTTGGTACACACATCCAGAGTCA. PCR amplification was quantified using a FAM labeled probe 1939: FAM-TGAGTTGATTACTGGCACGCCTAAACCAC-BHQ. The primers and hydrolysis probe set were added to the AgPath-ID^{TM} One-Step RT-PCR Reagents (ThermoFisher Scientific, Waltham, MA, USA) with 5 μl of extracted total RNA and amplified with the ABI 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using the following condition: reverse transcription at 48 °C for 10 min; denaturation at 95 °C for 10 min; 45 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 45 s. Gene scanning was carried out on an ABI Prism 3130Xl Sequencer using GeneMapper Software (version 4.0; Applied Biosystems, Foster City, CA, USA). Cut-off cycle threshold (Ct) value for the rRT-PCR was determined to be ≤35 for positive and >35 for negative samples.

Results

Fecal shedding

In both studies, PEDV RNA was detected by rRT-PCR from rectal swabs of pigs in the INF group at 1-day post infection (dpi), indicative of virus shedding from inoculated pigs. Rectal swabs of direct contact sentinel pigs, co-housed with the INF group in both trials, tested rRT-PCR positive at 2 dpi (Table 1 and 2), one day after virus was detected in inoculated pigs. Virus shedding in pigs of the INF group, measured as viral RNA copies per rectal swab, peaked at 1 dpi and viral RNA levels remained elevated through 19 dpi. During the 2nd trial, rectal swabs of INF pigs remained positive until 12 dpm when that experiment was terminated (Fig. 3 and 4).

Movements were started at 2 dpi of the INF group. Sentinel pigs in the LB group tested PEDV positive on rectal swabs 24 h after the first movement. Viral RNA was detected in 10 out of 10 sentinel pigs during the 1st trial and 4 out of 4 sentinel pigs in the 2nd trial (Table 1 and 2). Viral shedding in the LB group of 1st trial was undetectable after 21 dpm (Fig. 3). Rectal swabs from pigs in the MB and HB groups tested rRT-PCR negative during the 10 consecutive days of movement and remained negative through 24 dpm, when the first trial was terminated. Rectal swabs from pigs in the NC group remained negative for the entire duration of the study.

Fomite swabs

Fomite swab samples collected on 1, 2, and 3 dpm from hair/face, hands, coverall, and

boots prior to contact with each group of sentinel pigs were tested by rRT-PCR to determine where PEDV was carried on each person that could potentially contribute to virus transmission (Table 3). Viral RNA was detected at 1 dpm through 3 dpm from all LB group PPE fomite swab samples during the first trial. PEDV was only detected in one coverall swab in the 2nd study and it was positive at 1 dpm. In addition, at 1 dpm, 2 hair/face swabs from MB personnel were positive in the 1st study, even though transmission of virus was not detected. All fomite swabs from HB study personnel tested negative.

Discussion

Although several aspects of PEDV transmission have been examined, the efficiency by which biosecurity measures prevent indirect transmission of PEDV has been largely unexplored. In the current study, we sought to address this by modifying biosecurity measures in a controlled experimental design, using study personnel to simulate movements between rooms that reflect situations within swine farms around the country. Graded biosecurity stringency was designed into movements made by study personnel between a known infected room and sentinel rooms. As expected, direct-contact sentinel pigs showed signs of PEDV infection 24 h after viral shedding was detected in infected pigs supporting the view that PEDV is highly contagious (Dee *et al.*, 2015). Movements between INF and sentinel rooms were designed to begin when viral shedding peaked in the source group at 2 dpi. Movements to the LB rooms simulated indirect transmission in

the absence of biosecurity protocols. Interestingly, transmission to the LB sentinel groups happened surprisingly rapidly. Virus shedding in the LB sentinels was detected 24 h after the first movement into the room again providing proof of the contagious nature of PEDV. Samples from PPE of all study personnel in contact with experimentally infected pigs were found to be contaminated with PEDV by rRT-PCR, and transmitted infection to the LB sentinel pigs even though virus infectivity on PPE was not tested. This information is relevant since it helps explain the rapid spread of PEDV within populations even in the absence of direct contact pig transmission.

Among the graded biosecurity measures designed to break the virus transmission cycle, movements into the MB sentinel groups showed no evidence of transmission even though swabs from MB study personnel's hair and face were PEDV rRT-PCR positive. Transmission of PEDV with MB protocols may have been limited by low dose of virus, presence of non-infectious virus, inadequate interaction of pigs with contaminated PPE/surfaces, or the decreased efficiency of fecal oral transmission route from these contaminated areas. Similar experiments using an influenza virus transmission model showed a breakdown of medium biosecurity measures after 10 consecutive movements (Allerson *et al.*, 2013). Swabs from HB group study personnel tested PEDV rRT-PCR negative even on the hair and face. Similarly, HB sentinel pigs were rRT-PCR negative for 10 consecutive days. These results together indicate that taking a shower and changing PPE before contacting pigs is an ideal way to completely prevent indirect viral transmission in conditions generally seen in farms. Although our results also support that

only changing PPE and washing skin exposed areas is beneficial to decrease the risk of PEDV transmission, there may still be an inherent risk of PEDV transmission from contaminated body surfaces on personnel. Hence, only changing PPEs might not be the most effective way to protect against spread of PEDV.

Our results also indicate that breaches in biosecurity procedures can very rapidly transmit PEDV to naïve herds through indirect means (viz. contaminated fomites). These findings also suggest that contact with PEDV contaminated fomites for a sufficient time is an efficient source of infection and likely plays a role in the rapid transmission of PEDV when there is adequate contact with fomites. Previous studies have suggested that fomites may be an effective mode of PEDV transmission (Alonso *et al.*, 2014; Canadian Food Inspection, 2014; Gerber *et al.*, 2014; Bowman *et al.*, 2015a; Dee *et al.*, 2015; Dee *et al.*, 2016; Scott *et al.*, 2016). These previous studies rely on PCR detection of viral RNA particles or demonstration of infectious PEDV in cell culture assays to suggest the possibility of transmission by fomites. For example, airborne transmission (Alonso *et al.*, 2014), vehicles (Lowe *et al.*, 2014), feed (Canadian Food Inspection, 2014; Pasick *et al.*, 2014; Dee *et al.*, 2015), storage bags (Scott *et al.*, 2016), personnel working with pigs (Scott *et al.*, 2016) and other fomites have tested positive for PEDV indicating their possible role in viral transmission and should be considered as a source for virus spread. However, these studies lack a tangible demonstration of the ability of contaminated fomites to infect pigs, either in an experimental setting or in a farm, except for the role of contaminated feed. The present study provides evidence that personnel exposed to

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infected pigs can transmit the virus to a naïve population, when basic biosecurity procedures are not followed.

The experimental design in the present studies allowed a 45-minute contact time with animals under the assumption that most routine activities in a farm, based on the size of the pen and number of pigs housed, may be completed within that time frame. However, one cannot rule out the possibility that transmission may occur with medium biosecurity, if longer interaction periods or a larger infected source groups were used in the design. Contact with infected pigs for more than 45 minutes and/or 10 movements could have increased the probability of PEDV transmission to naïve sentinel pigs. However, we observed that with low biosecurity procedures, transmission and infection of PEDV was both efficient and rapid. This data provides evidence that spread of PEDV within farms may occur efficiently with failures in biosecurity procedures.

Results presented here should be considered carefully as many factors, including contact time, exposure time, viral dose, time after exposure to virus and other experimental conditions may influence the outcome of transmission studies. However, this experimental study highlights the main advantages of good biosecurity procedures in breaking the transmission cycle between rooms. The fact that PEDV transmission occurred under low biosecurity procedures indicated that the virus could spread easily through contaminated fomites worn by personnel. These results provide critical

information to develop effective biosecurity procedures and will have potential applications for the development and implementation of transmission control policies in swine production systems. Our results are also relevant to design biosecurity measures to control the spread of other pathogens of similar characteristics and transmission routes than PEDV such as transmissible gastroenteritis virus.

In conclusion, these results indicate the indirect transmission of PEDV through contaminated personnel PPEs occurs rapidly under modeled conditions and to prevent transmission between groups of pigs, taking a shower and changing PPE is recommended as an effective option to lower the risk of virus spread.

Table 2.1. Numbers of porcine epidemic diarrhea virus positive sentinel pigs (1st trial).

Days Post Infection	-1	0	1	2	3	4	5
Infection group	0/10	0/10	10/10	10/10	10/10	10/10	10/10
Infection group sentinel	0/2	0/2	0/2	2/2	2/2	2/2	2/2
Days After Movement				0	1	2	3
Low biosecurity				0/10	9/10	10/10	10/10
Medium biosecurity				0/10	0/10	0/10	0/10
High biosecurity				0/10	0/10	0/10	0/10

Table 2.2. Numbers of porcine epidemic diarrhea virus positive sentinel pigs (2nd trial).

Days Post Infection	-1	0	1	2	3	4	5
Infection group	0/3	0/3	3/3	3/3	3/3	3/3	3/3
Infection group sentinel	0/1	0/1	0/1	1/1	1/1	1/1	1/1
Days After Movement				**0**	**1**	**2**	**3**
Low biosecurity				0/4	4/4	4/4	4/4
Medium biosecurity				0/12	0/12	0/12	0/12

Table 2.3. Number of porcine epidemic diarrhea virus positive fomite swab prior to contact with pigs in the respective groups and mean (±SD) cycle threshold RT-PCR values for positive samples (1st trial)

Group	Swab	Movement day 1	2	3
Negative	Hair/face	(0/5)*	(0/5)	(0/5)
	Coverall	(0/5)	(0/5)	(0/5)
	Hands	(0/5)	(0/5)	(0/5)
	Boots	(0/5)	(0/5)	(0/5)
LB	Hair/face	(3/5) (31.58±1.03)†	(2/5) (33.62±0.16)	(5/5) (32.66±1.58)
	Coverall	(5/5) (26.16±3.17)	(5/5) (29.28±2.22)	(5/5) (27.96±3.96)
	Hands	(5/5) (28.81±3.83)	(4/5) (28.01±2.98)	(5/5) (28.76±2.21)
	Boots	(5/5) (26.30±4.44)	(5/5) (27.42±6.22)	(5/5) (24.51±3.94)
MB	Hair/face	(2/5) (30.75±0.93)	(0/5)	(0/5)
	Coverall	(0/5)	(0/5)	(0/5)
	Hands	(0/5)	(0/5)	(0/5)
	Boots	(0/5)	(0/5)	(0/5)
HB	Hair/face	(0/5)	(0/5)	(0/5)
	Coverall	(0/5)	(0/5)	(0/5)
	Hands	(0/5)	(0/5)	(0/5)
	Boots	(0/5)	(0/5)	(0/5)

*: Number of positive

†: Ct value (avg.±S.D)

Table 2.4. Number of porcine epidemic diarrhea virus positive fomite swab prior to contact with pigs in the respective groups and mean (±SD) cycle threshold RT-PCR values for positive samples (2nd trial)

Group	Swab	Movement day 1	Movement day 2	Movement day 3
Negative	Hair/face	(0/4)	(0/4)	(0/4)
	Coverall	(0/4)	(0/4)	(0/4)
	Hands	(0/4)	(0/4)	(0/4)
	Boots	(0/4)	(0/4)	(0/4)
LB	Hair/face	(0/1)	(1/1) (31.62)	(1/1) (33.58)
	Coverall	(1/1) (33.40)	(1/1) (29.27)	(1/1) (24.60)
	Hands	(0/1)	(1/1) (28.03)	(1/1) (30.01)
	Boots	(0/1)	(1/1) (28.74)	(1/1) (28.54)
MB	Hair/face	(0/3)	(0/3)	(0/3)
	Coverall	(0/3)	(0/3)	(0/3)
	Hands	(0/3)	(0/3)	(0/3)
	Boots	(0/3)	(0/3)	(0/3)
HB	Hair/face			
	Coverall			
	Hands			
	Boots			

*: Number of positive

†: Ct value (avg.±S.D)

Figure 2.1. Movement from infected source group (INF) to low biosecurity group (LB) and INF to medium biosecurity group (MB).
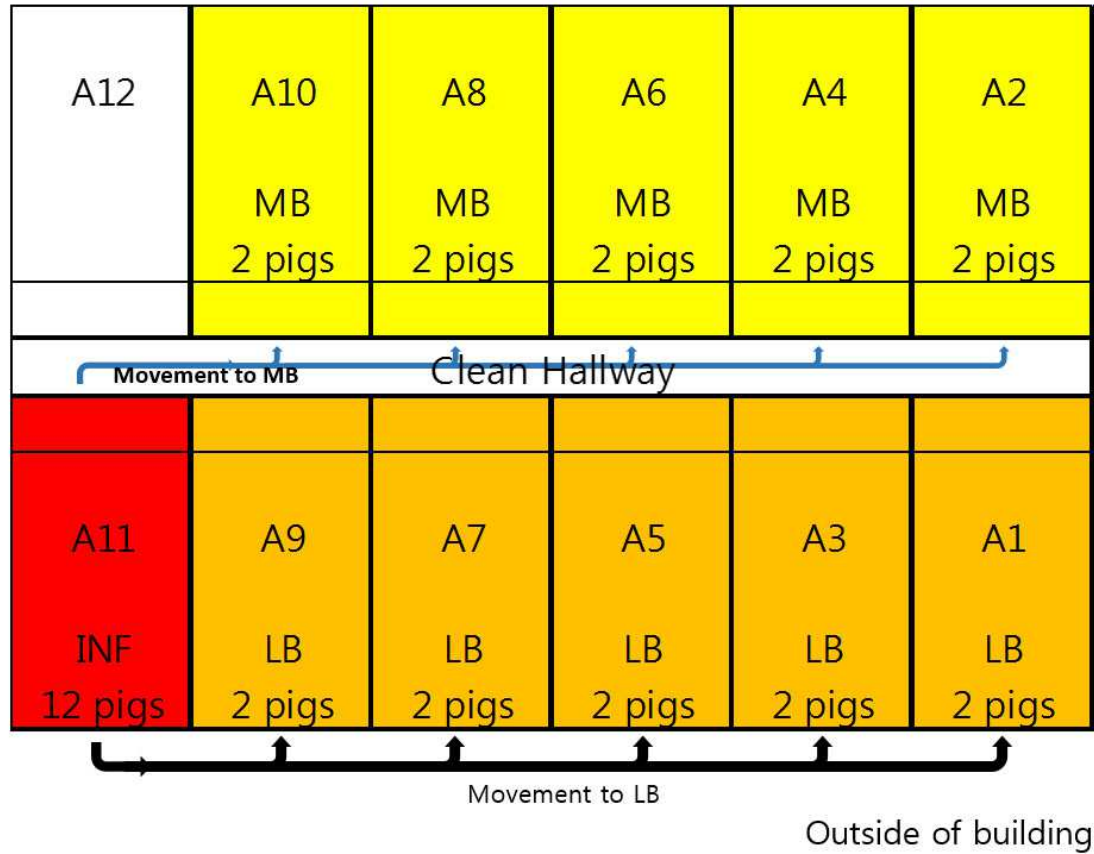
Figure 2.2. Experimental design. Movements of study personnel to low biosecurity, medium biosecurity, and high biosecurity rooms.
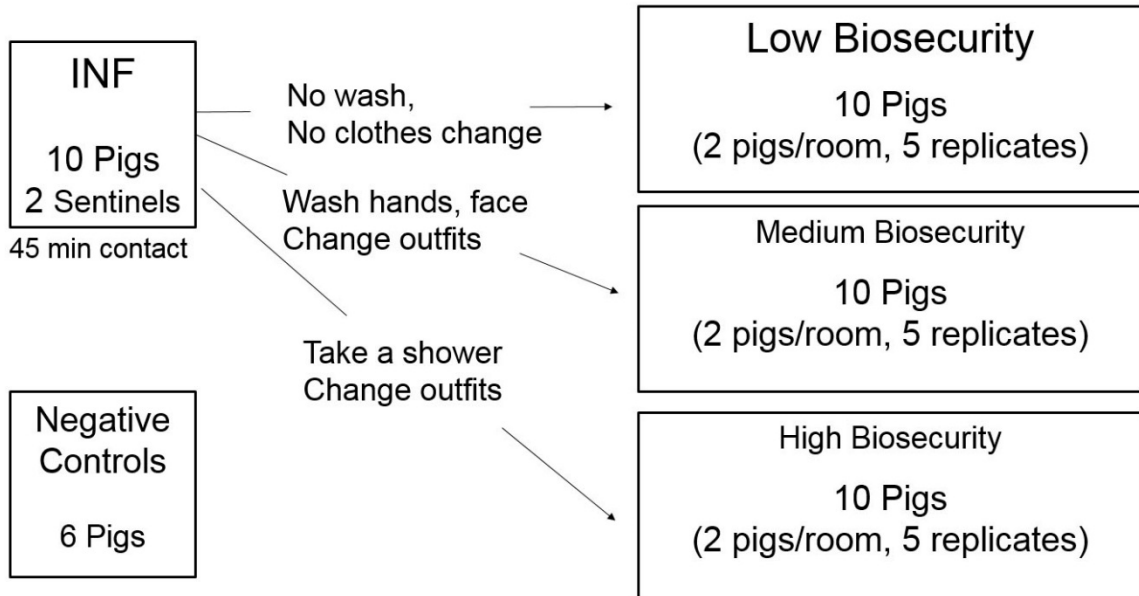
Figure 2.3. Viral shedding of pigs (1st trial).
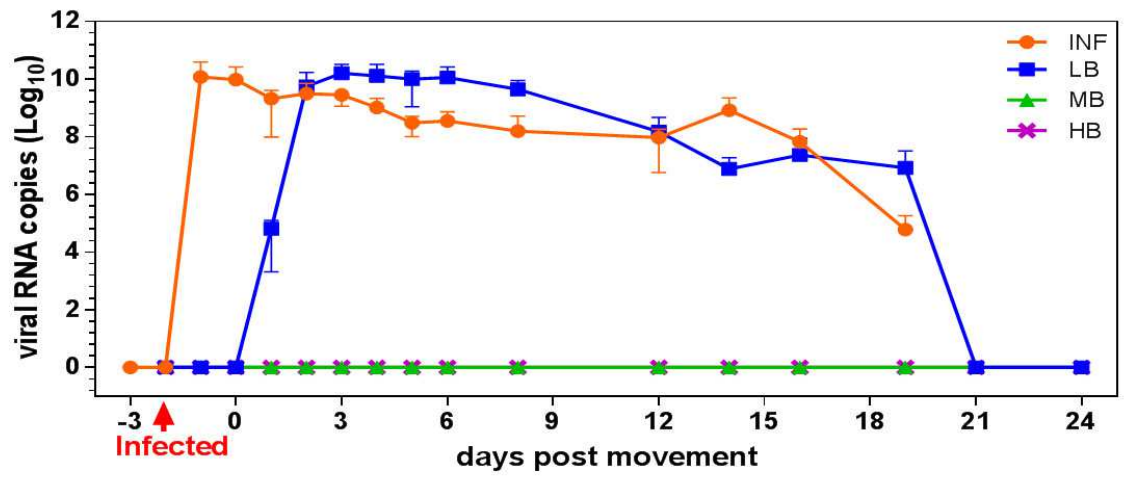
Data presented are average values of viral RNA copies (± S.E.M) of infected source group
(INF) (n=12), low biosecurity group (LB) (n=10), medium biosecurity group (MB) (n=10),
and high biosecurity group (HB) (n=10) groups.

Figure 2.4. Viral shedding of pigs (2nd trial).
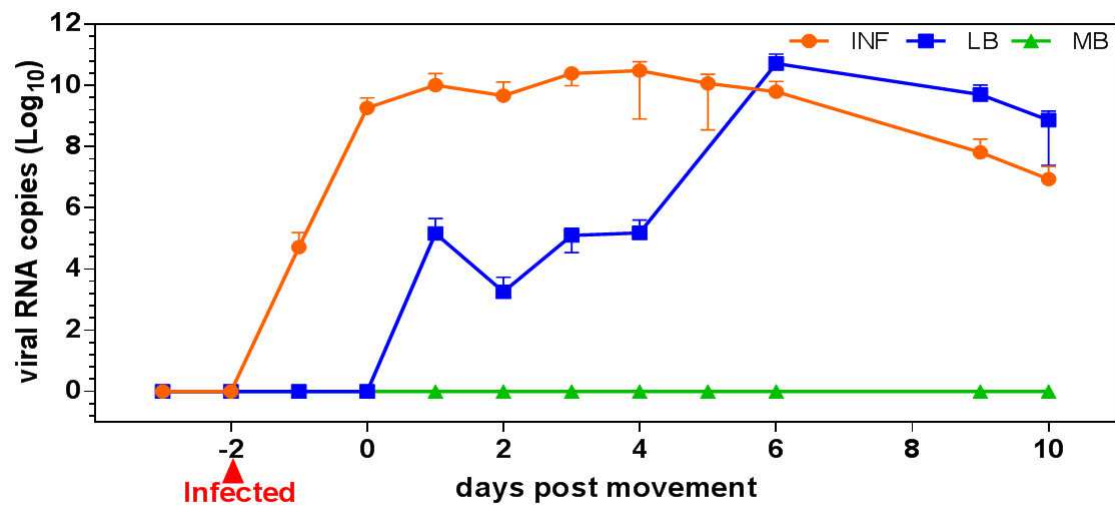
Movements were terminated at 10 dpi. Data presented are average values of viral RNA copies (± S.E.M) of infected source group (INF) (n=4), low biosecurity group (LB) (n=4), and medium biosecurity group (MB) (n=12).

Chapter 3: Stability of PEDV on fomite materials at different temperatures

Introduction

Porcine epidemic diarrhea (PED) is a highly contagious viral enteritis caused by an enveloped RNA virus, classified as a member of the family *Coronaviridae* under genus *Alphacoronavirus* (Pensaert and de Bouck, 1978; Saif *et al.*, 2012). The PED virus (PEDV) was first identified in England in 1971. Since the 1990s, PEDV outbreaks in Europe are sporadic in adult pigs and clinical signs in suckling piglets have been mild in general (Kweon *et al.*, 1993). In Asia, PEDV outbreaks were observed in Japan, Korea, Philippines, Vietnam, and China beginning in 1982. The virus strains in Asia were similar to those found in Europe in the 1980s. However, after 2010, PED outbreaks in Asia increased significantly causing severe clinical signs and high mortality in suckling pigs resulting in significant economic losses to pig industries (Takahashi *et al.*, 1983; Kweon *et al.*, 1993; Chen *et al.*, 2008; Puranaveja *et al.*, 2009; Li *et al.*, 2012). In May 2013, a PEDV strain genetically closely related to a Chinese strain, was introduced in the US possibly importation of contaminated feed (Dee *et al.*, 2016). The virus spread rapidly across the country causing high mortality in piglets (Huang *et al.*, 2013). Over 8 million pigs were killed during the 2013 US outbreak by PEDV, leading to an estimated loss of $ 1.8 billion US dollars (Mole, 2013; Stevenson *et al.*, 2013; Vlasova *et al.*, 2014; Ojkic *et al.*, 2015).

Transmission of PEDV primarily occurs from infected pigs by the fecal-oral route. In addition, contaminated feces can be transferred to susceptible pigs via inanimate objects. Outbreaks frequently occur ahead of fall and winter but the rationale for

seasonality remains unclear (Wang *et al.*, 2007). Previous studies have found that cell-culture adapted PEDV can be stable at 50°C and at pH ranging from 7.2 to 10.2 (Quist-Rybachuk *et al.*, 2015). More recent studies have shown that PEDV can survive up to 9 months at the center of manure lagoons (Tun *et al.*, 2016). While the sensitivity and stability of PEDV under various pH and temperature conditions have been explored, particularly as it relates to contaminated feed and spray-dried porcine plasma (SDPP) (Dee *et al.*, 2014; Pasick *et al.*, 2014; Pujols and Segales, 2014), viral stability on different fomite surfaces under varying temperature conditions have not been studied. Inanimate objects such as rubber boots, gloves, coveralls and other equipment have a potentially high risk of transmitting PEDV by contamination with manure from PEDV infected animals, as they are routinely used in pig farms. Therefore, the objective of this study is to evaluate the survivability of PEDV on fomite materials and at different temperatures.

Methods

Preparation of virus stock and titration

A PEDV strain (PEDV USA/Colorado/2013; GenBank accession number KF272920) was obtained from the National Veterinary Services Laboratory in Ames, IA. The virus was propagated on Vero76 cells (ATCC CRL-1587, Manassas, VA, USA) for 16 passages. Briefly, Vero76 cells were infected with PEDV at 0.01 multiplicity of infection (MOI) and virus was harvested 3 days after infection. The infected cultures were frozen at -80°C, thawed once, and centrifuged at 500×g for 10 minutes, to clarify the culture

supernatant. After clarification, the supernatant was aliquoted and used as crude viral stock. Virus titration was performed in Vero76 monolayers contained in Costar® 96 well cell culture plates (Corning Incorporated, Corning, NY, USA) using 5-fold serial dilutions of samples. Virus titer was calculated using the Spearman-Kärber method [22] and expressed as 50% tissue culture infective dose ($TCID_{50}$)/ml.

Testing virus stability on fomites

To test the ability of virus to survive on fomites, small pieces of fomite materials were cut to fit into individual wells of Costar® 24 well cell culture plates (Corning Incorporated, Corning, NY, USA), approximately 1 cm^2 size. Materials used for the experiment were Styrofoam, nitrile gloves, cardboard, aluminum foil, Tyvek® coveralls, cloth, metal, rubber, and plastic. Crude virus stock (200 μl of stock virus at 2.1×10^6 $TCID_{50}$/ml) was inoculated on each type of material, in triplicate. Virus was also applied on a 24 well plate without any fomite material, to serve as control. The virus applied on fomites was air dried for 2 h in a biosafety cabinet (BSC). At various times thereafter, the virus was eluted in a Corning® 50 ml centrifuge tube (Corning Incorporated, Corning, NY, USA) using 1 ml of 3 % beef extract-0.05M glycine buffer. Tubes were mixed vigorously for 20 seconds, using a vortexer (Scientific Industries Inc., Bohemia, NY, USA). After thorough mixing, the eluate was filtered through a 0.22 μM syringe filter (EMD Millipore, Billerica, MA, USA) and titrated using $TCID_{50}$ assay on confluent Vero cell monolayers. Samples were collected after the initial 2h air-dry period (0 d) and at 1, 2, 5, 10, 15, 20, and 30 days post application in beef extract- glycine buffer, as described above. During

the post virus application period, plates containing fomites were stored either at room temperature or at 4°C prior to elution. Eluted samples were titrated for virus immediately after collection.

PEDV real-time RT PCR

Eluted fomite samples were tested by real-time RT-PCR for the stability of viral RNA on fomites. Briefly, RNA was extracted from eluate using the MagMAX™-96 Viral RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. A primer pair was designed to amplify a portion of the PEDV S gene with the following sequences: Forward 1910: ACGTCCCTTTACTTTCAATTCACA and Reverse 2012: TATACTTGG TACACACATCCAGAGTCA. PCR amplification was quantified using a FAM labeled probe 1939: FAM-TGAGTTGATTACTGGCACGCCTAA ACCAC-BHQ. The primer and hydrolysis probe set were added to the AgPath-ID™ One-Step RT-PCR Reagents (Thermo Fisher Scientific, Waltham, MA, USA) along with 5 μl of extracted total RNA and amplified with the 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using the following condition: reverse transcription at 48°C for 10 min; denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 45 s. Gene scanning was carried out on an ABI Prism 3130Xl Sequencer using GeneMapper Software (version 4.0; Applied Biosystems, Foster City, CA, USA).

Immunoplaque assay

Immunoplaque assays were performed in 24 well tissue culture plate with confluent Vero 76 cells monolayer. To perform the immunoplaque assay, 10-fold serially diluted 200μl of sample eluate or 200μl of crude PEDV stock (positive control) were used to infect Vero 76 cells. Twenty-four hours post-infection, the cells were fixed for 20 min at 4°C in a solution of 4% paraformaldehyde. All subsequent washes and incubations were done in PBS, containing 5% normal goat serum and 0.3% triton X-100. After three washes and blocking an hour in buffer, plates were incubated overnight at 4°C with one of the following primary antibodies: (i) mouse monoclonal antibody for PEDV Spike protein (diluted 1:500; Clone 3F12, Median Diagnostics, Chuncheon, South Korea); or (ii) mouse monoclonal antibody for PEDV Spike protein (diluted 1:500; Clone S1D12, VMRD, Pullman, WA). Plates were then rinsed in wash buffer and incubated for 1 h at room temperature with the anti-mouse IgG alkaline phosphatase conjugated secondary antibody (diluted 1:200; Thermo Fisher Scientific, Waltham, MA, USA). After three washes in buffer, plates were incubated for approximately 20 min at room temperature with 1-Step^{TM} NBT/BCIP substrate solution (Thermo Fisher Scientific, Waltham, MA, USA). Immunostained cells were observed under a light microscope (Nikon, Tokyo, Japan) (Fig. 1).

Results

Infectious virus stability on surface materials

Infectious PEDV could be recovered from fomite materials for up to 20-day post

application, when stored at 4°C. Viral titers dropped approximately 2 to 4 logs of titer during the first 10 days. On the other hand, PEDV survival decreased precipitously at room temperature (RT) within 1- or 2-day post application, losing 1 to 3 log titers within 24 h (Fig. 2). Infectious PEDV could not be recovered from any fomite material after 2 days in storage at room temperature, using the $TCID_{50}$ assay. Virus recovery from surfaces of Styrofoam, nitrile gloves, aluminum foil, Tyvek® coverall, metal, rubber, plastic, cardboard and cloth showed no significant differences between the materials at room temperature, suggesting the temperature had a substantial influence on virus survival. PEDV, however, could still be recovered after 15 days on Styrofoam, aluminum foil, Tyvek® coverall, cloth, and plastic at 4°C, with detectable titers of 10^2 to 10^3 $TCID_{50}$/ml. However, virus recovery from nitrile gloves, cardboard, metal, and rubber were below assay's detection limit of $2x10^2$/ml $TCID_{50}$ after 15 days at 4°C.

We used a modified plaque assay, employing an immunodetection method to identify PEDV positive plaques (immunoplaque assay) which had greater sensitivity of 24 FFU/ml. Using this method, we tested Styrofoam, Tyvek® coverall, metal, rubber, and plastic fomite materials. At 20-days post storage at 4°C, viable PEDV was identified from Styrofoam, Tyvek® coverall, metal, rubber, and plastic materials using this method (Fig.3). Infectious virus titers of approximately $1x10^3$ FFU/ml were observed in eluates from Styrofoam, metal and plastic, representing a 3-log loss of input virus infectivity over 20 days. Infectious PEDV from Tyvek® coverall and rubber surfaces were observed to be moderately above detection limit (24 FFU/ml), showing approximately 1-3 plaques in wells seeded with 200 µl of eluate. In contrast, fomite materials stored at room

temperature for 48 hours post application produced no plaques from all nine materials tested by immunoplaque assay (data not shown).

To determine if viral RNA could be detected on fomites under these storage conditions, eluates were assessed by quantitative PCR. Viral RNA was detected at 2d and 20d at RT and 4°C respectively, when infectious virus was not detected in fomite samples by $TCID_{50}$. In fact, all materials tested had cycle threshold (Ct) values similar to that of input virus (~16-17), with the exception of eluates from cardboard which showed a Ct value of 21 (Table 1). Quantifiable viral RNA levels were detected in Styrofoam, Tyvek and cardboard materials although infectious PEDV decreased by 4, 5, and 6 logs respectively at 4°C. We could not determine infectious virus titer by $TCID_{50}$ or immunoplaque assay from eluates containing virus infected fecal material due to cell toxicity, but viral RNA was detected at 2d and 20d at room temperature and 4°C respectively at levels similar to input virus indicating no significant changes.

Discussion

Our study demonstrates that cell cultured PEDV remained viable for extended periods when maintained in a cold environment. PEDV remained viable at 4°C, for up to 20-days post application on fomites, such as Styrofoam, metal and plastic, albeit viral titers decreased by 3 logs in 20 days. In contrast, when stored at room temperature, PEDV viability decreased approximately 4 to 5 logs within 48 hours, rendering it undetectable using infectious virus assays. This observation suggests that storage temperature of the

fomite material has major impact on virus stability. Generally, enveloped viruses are most

vulnerable to environmental condition outside their host (Aboubakr *et al.*, 2014).

However, PEDV was found to have higher stability when stored in spray dried bovine

plasma, being stable for up to 3 weeks at 4°C, 2 weeks at 12°C, and 1 week at 22°C

(Pujols and Segales, 2014). In addition, other coronaviruses, like transmissible

gastroenteritis virus (TGEV) and mouse hepatitis virus (MHV), were also found to be

more stable at 4°C under experimental condition, surviving for as long as 28 days at 4°C

and up to 5 days at RT (Casanova *et al.*, 2010). We demonstrated in the present study that

infectious virus decay rate on all fomites tested decreased considerably at RT. This

temperature sensitive feature of PEDV may be applied to routine procedures in the farms

to help eradicate PEDV in the environment and thus prevent transmission via fomites

(Gerber *et al.*, 2014; Opriessnig *et al.*, 2014; Quist-Rybachuk *et al.*, 2015).

Composition of the fomite material has also been implicated in viral stability.

PRRSV was shown to survive differently on various types of materials including solid,

porous, and liquid fomites (Pirtle and Beran, 1996). We found that infectious PEDV

survived more efficiently at 4°C on the surface of Styrofoam, metal and plastic showing a

3 log decrease at 20 d. The rate of loss in infectivity was uniform on Styrofoam,

aluminum, plastic and Tyvek® coverall compared to that of other materials. Viral titers on

nitrile gloves and rubber dropped by 1.0 to 1.5 log at 5 d.p.a and subsequently decreased

rapidly within 5 to 10 days. The virus infectivity on metal showed slow decay rates until

5 d, however, titers declined rapidly between 5 and10 d. At 0 d virus recovery from

cardboard and cloth materials were lower than other non-absorbent materials tested. This result suggests that porosity of materials may influence recovery of virus. Our results are consistent with other studies reporting virus survivability on glass, stainless steel, and plastic for up to 10 days (Pirtle and Beran, 1996; Sattar *et al.*, 2009). Specifically, plastic material provided extensive PEDV stability among the tested materials. Similarly, feed totes made of polypropylene which is used in plastic materials allowed PEDV survival for 10 weeks (Scott *et al.*, 2016). In line with previous findings that temperature is a critical factor influencing stability of PEDV, porosity of surfaces may also alter the ability of fomites to transmit virus. Although the data presented were obtained under experimental conditions, it suggests that long-term persistence of PEDV on contaminated surfaces could have epidemiological impact on disease outbreaks, given the observation that infectious virus was recovered after 20 days and the infectious dose of PEDV has been documented to be as low as 0.056 TCID$_{50}$/ml (Thomas *et al.*, 2015).

PEDV transmission appears to be relatively effective via transportation vehicles (Lowe *et al.*, 2014; O'Dea *et al.*, 2016), and feed (Dee *et al.*, 2014; Pasick *et al.*, 2014; Bowman *et al.*, 2015a; Dee *et al.*, 2015). Our data showed that PEDV can survive on metal for up to 15 days at 4°C. This property of the virus may play a significant role in promoting the spread of PEDV, potentially contaminating vehicles or equipment. Longer survivability of the virus at cold temperature may also explain the occurrence of sporadic outbreak during winter season (Song and Park, 2012). Furthermore, most enveloped viruses generally survive in presence of organic material (Chattopadhyay *et al.*, 2002).

Although we could not recover infectious virus from fecal material applied to fomites, it has been shown that large amount of lagoon manure possibly contributes to survivability of PEDV and be infective up to 9 months after viral shedding of the pigs (Tun *et al.*, 2016).

Finally, we show that viral RNA copy numbers do not correlate well with cell-based assays used to study virus survival and infectivity. It is well known that infectivity of an enveloped virus, which is relatively fragile, may be lost in environmental conditions that affect the envelope without affecting RNA degradation.

In conclusion, our findings give new perspective on how fomite material and temperatures impact viral stability over time, indicating the significance of understanding the nuances of indirect transmission in the epidemiology of PEDV.

Table 3.1. Cycle threshold values and focus forming units (FFU) of porcine epidemic diarrhea virus (PEDV) in fomite materials applied with cultured PEDV or PEDV spiked feces at room temperature and 4C.

|	Cultured		Feces
Input virus	$1.0\pm0.7\mathrm{x}10^6$ FFU/ml	17.06±0.77†	29.65±1.79
	Room Temperature / 2 dpa*		
	Cultured		Feces
Styrofoam	<24 FFU/ml	16.57±0.34	30.04±0.38
Tyvek coverall	<24 FFU/ml	16.65±0.12	28.61±0.49
Cardboard	<24 FFU/ml	21.50±0.39	27.56±0.26
	4C / 20 dpa*		
	Cultured		Feces
Styrofoam	$9.2\pm0.6\mathrm{x}10^2$FFU/ml	17.64±0.51	28.89±0.54
Tyvek coverall	$5.0\pm2.5\mathrm{x}10$FFU/ml	16.93±0.53	27.24±0.75
Cardboard	<24 FFU/ml	17.64±0.22	27.69±0.83

*dpa: day post application

†: cycle threshold (ct) value ± S.D

Figure 3.1. Light microscopic features of immunoplaque assay. Images showing the

distribution and characteristics of PEDV plaque in immunoplaque assay.
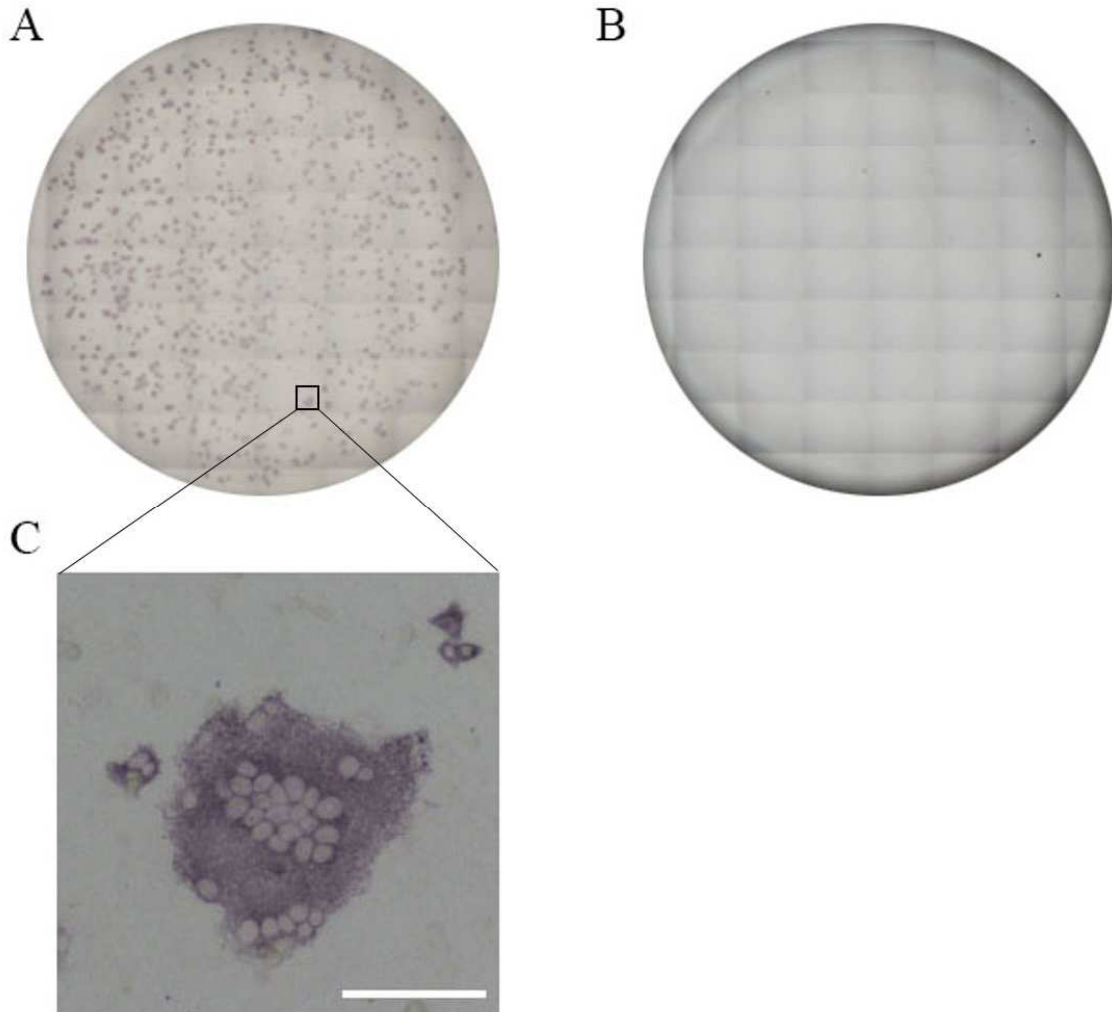


(A) Virus positive well showing distribution and plaques on Vero 76 cells infected with

200μl of 103 TCID50/ml NVSL strain of virus. Stained with anti-PEDV spike protein at

24 h.p.i. (B) Corresponding well without virus stained with anti-PEDV spike protein

showing background. (C) High magnification of a typical plaque, showing syncytium of

virus infected Vero 76 cells. (Scale bar: 100 μm)

Figure 3.2. Decrease in viral infectivity on fomites at room temperature and at 4°C.
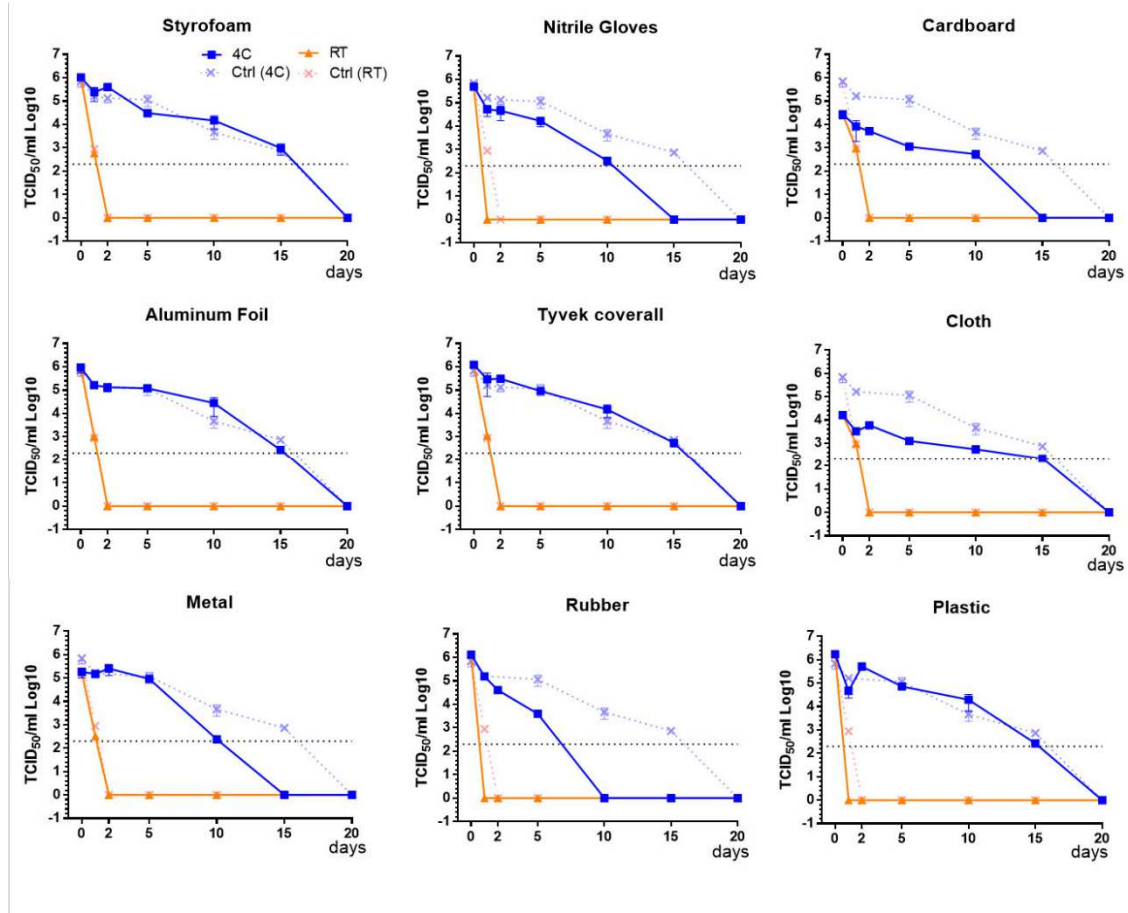
PEDV was applied on fomite materials or in control wells, stored at RT (▲) or at 4°C (■) and assessed by TCID50 for presence of live infectious virus at different days post application. Decay rate of infectious virus was rapid at RT but delayed when stored at 4°C. Detection limit of $TCID_{50}$ assay is $2x10^2$ $TCID_{50}$/ml. Data presented are average values of $TCID_{50}$ (± S.E.M).

Figure 3.3. Viral survivability is different on the surface of materials at 4°C.
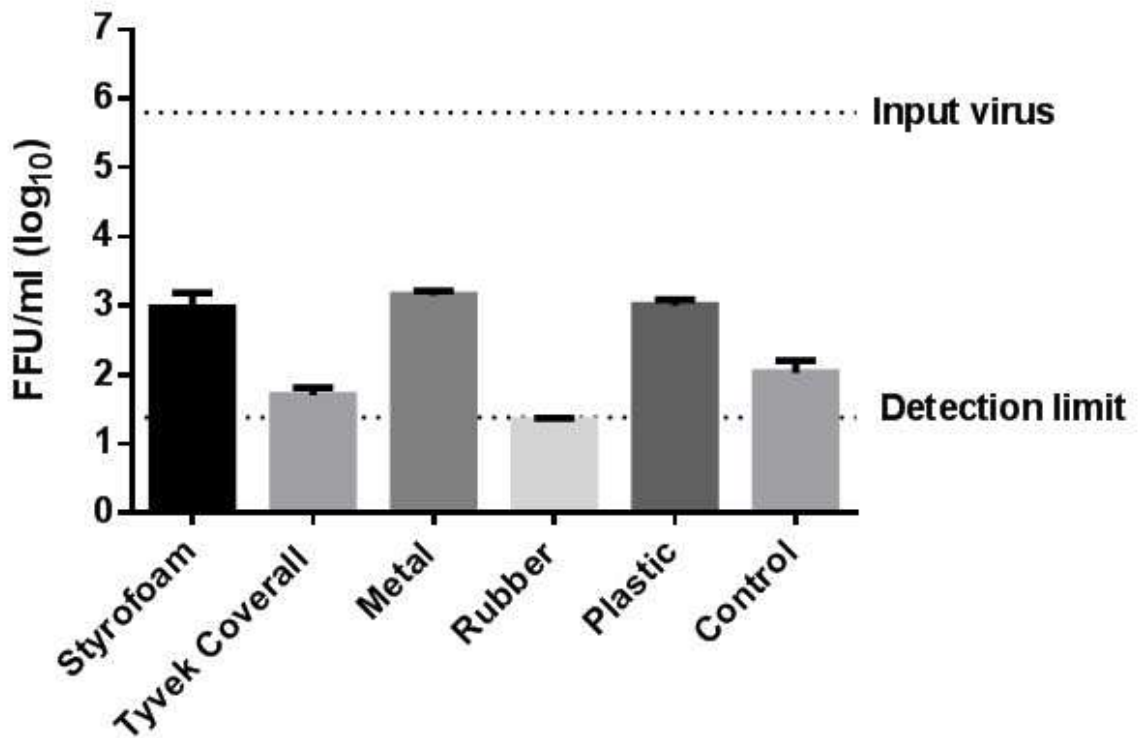
PEDV was applied on fomite materials stored at 4°C for 20 days and titered by immunoplaque assay. Various levels of live infectious virus were detected by immunoplaque assay on different materials. Input virus titer decreased by 3-5 logs over the course of 20 days. Detection limit of the assay is 24 FFU/ml. Data presented are average values of FFU (± S.E.M).

Chapter 4: General discussion and conclusions

General discussion and conclusions

PEDV infection has been a significant viral pathogen to the pork industry for approximately 40 years. In the early 1980s, this virus was first recognized in European countries causing mild clinical signs in pigs. In 1990s, the virus was introduced across the continent in Asia as a mutated but virulent form causing severe disease in pigs. The outbreak of Asia resulted in devastating losses to pig industries, specifically causing high mortality in piglets. Subsequently, PEDV remained "silent" for a few years. However, in the early part of the 21st century, PEDV re-emerged causing significant impact to pig production in Asia. When virus was introduced into the United States in May 2013, the outbreak of PEDV resulted unexpectedly high economical losses to pork industry, reaching approximately $ 1.8 billion US dollars over the course of the year. Episodic outbreaks of viral infection were reported again in Asian countries at the same time due to infection with a virulent form of PEDV, particularly in South Korea, Japan and Taiwan showing several re-occurrences of PEDV infection. PED is now recognized as an emerging and re-emerging global pig disease. Although reports of PEDV infection has reduced significantly over the last year in the US, the phenomena of re-occurrence seen across the globe suggests that the virus may re-emerge in the US again, perhaps as more virulent form, repeating the catastrophic losses in pig industries, seen after its first appearance in 2013.

As described in the literature review (chapter 1), PEDV studies have been ongoing over the last 30 years, however there is a gap in our understanding of viral transmission and the contribution of fomites in facilitating virus spread. Very little is known about indirect transmission of the virus, specifically nothing about personnel movement. Since

most indirect transmission study is about contaminated feed. Virus stability also has been studied regarding to contaminated feed and feed manufacturing process. Thus, nothing has been explored about virus stability on fomite materials in different environmental conditions. Therefore, the objective of this thesis was to fill this knowledge gap. In other words, to assess contributions of fomites in indirect transmission events, and PEDV stability on fomites at different temperatures.

We demonstrated, in chapter 2 of this thesis, that indirect transmission of PEDV between pig herds can take place very rapidly by personnel movement. In-contact sentinel pigs turned PEDV positive within 24 hours after infected pigs began to shed the virus. Sentinel pigs that came into contact with the virus by indirect means also turned positive within 24 hours of first contact, even without direct interaction with infected pigs. In our study, this transmission was effected when study personnel following improper (low) biosecurity measures and donning contaminated PPEs were permitted contact with naïve pigs. It indicates that contaminated fomites such as PPE is sufficient to both carry infectious PEDV across pens, expose pigs to the virus, and infect naïve pigs at a 100 % transmission rate. We also found that in the medium biosecurity group, where contaminated PPE was discarded by personnel, the possibility of viral transmission existed due to retention of virus on the personnel's contaminated hair and face. Yet viral transmission was not affected by the indirect contact afforded by the study design. These results suggest that only changing PPEs between rooms in a farm may not be sufficient to completely prevent transmission of PEDV. An ideal way to completely interrupt the indirect transmission cycle would be to take a shower along with changing PPEs between pig pens and before contact with naive

pigs.

Stability of PEDV was assessed in the present study (see Chapter 3), to evaluate viral survivability on fomites, using common materials from pig operations and those found in equipment used in farms. Surprisingly, infectious virus survived more than 20 days at 4°C, on several fomite materials tested, especially on Styrofoam, aluminum, Tyvek® coverall and plastics. However, the virus remained infectious only for 24 hours at room temperature. Given the low infectious dose (0.056 $TCID_{50}$/ml) of PEDV necessary to infect young pigs, increase in survivability at cold temperatures may explain the seasonality of PEDV transmission in the cooler months (Song and Park, 2012). It indicates that transmission of PEDV may happen more easily at cold temperature rather than warm temperature. This temperature sensitive character of PEDV can be applied to develop virus disinfection processes in pig farms, such as heating up surfaces to destroy enveloped virus on potential fomites thereby preventing transmission. These results indicate that PEDV contaminated fomite material can contribute indirect transmission and the idea will contribute to prevent before entry of PEDV.

An additional finding of chapter 3 was the lack of congruence between real-time RT-PCR data and cell-culture based assay for infectious virus. The Ct values of viral RNA amplification after 48 h were similar to that of input virus even though infectious virus was undetectable by immunoplaque assay. These dissimilar results can be explained by the fact that real-time RT-PCR detects a small part of viral RNA and while it can detect both infectious virus and non-infectious viral particles of virus which is indistinguishable by this assay. However, cell based assay like immunoplaque assay can only detect infectious virus

6 7

and the inherent differences of these two assays warrants paying more attention to interpretation of real-time PCR data and cell based assays for assessing the impact of this virus.

As with most experiments, there are limitations to the studies that are presented in this thesis. The transmission and stability studies were done under experimental settings, trying to imitate general field conditions in a pig farm. Farm environments, however, are not exactly the same as that depicted in these experiments. During the transmission study, our experimental design capped pig contact time at 45 minutes for 10 consecutive days to enable evaluation of biosecurity processes. In a typical grower-finish farm, processes are continued for over 100 days as farm personnel care for pig pens daily basis until the lot is sold. In many situations, farm personnel may have contact with pigs for more than 45 minutes and/or for more than 10 movement counts. These differences can increase exposure to pigs and may increase the probability of transmission. The density of pigs used in the experiment are not similar to the field condition. As of 2010, an average of 26 pigs are typically allocated in 190 ft^2 pen. This dissimilarity may can increase transmission rate of PEDV in real farm environments and was not captured in our experimental design. In addition, survivability of PEDV is greater under higher humidity and balanced pH conditions. The virus also can be stable in organic materials, which helps virus survivability. It is possible that virus in field environments may last more than 20 days in humid organic materials, particularly when pH condition is kept neutral and perhaps increase the risk of transmission.

Nevertheless, we provide evidence of that indirect transmission of PEDV with personnel

movement between infected and naïve pig pens, and stability of PEDV in environment

imitating cold and warm weather. Further investigation on PEDV may take place, such as

evaluating contribution of specific materials in PEDV transmission. In summary, the

findings of this thesis have advanced understanding of indirect transmission of PEDV

through personnel movement on contaminated PPEs and fomites with varying viral

stability in different temperature. This will provide a concept suggesting to minimize the

impact of PEDV infection in pig industries.

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