FATE OF PORCINE EPIDEMIC DIARRHEA VIRUS (PEDV) AND PORCINE DELTACORONAVIRUS (PDCOV) IN THE ENVIRONMENT UNDER MANITOBA CLIMATIC CONDITIONS

BY

ZHANGBIN CAI

A Thesis

Submitted to the Faculty of Graduate Studies

In Partial Fulfillment of the Requirements for the Degree of

MASTERS OF SCIENCE

Department of Animal Science

University of Manitoba, Winnipeg, Manitoba

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ABSTRACT

Porcine epidemic diarrhea virus (PEDv) and porcine deltacoronavirus (PDCoV) have been a major challenge for the North American swine industries recently. As these viruses are transmitted via the fecal-oral route, their survival in the environment can impact the spread and re-occurrence of associated diseases. This study determined the viability of the viruses in the earthen manure storages (EMS) and in the soil under Manitoba climate. Two PEDv and PDCoV co-infected farms were monitored. PDCoV was monitored over the Fall and found to survive in the EMS for the whole period while maintaining a stable population. PEDv was monitored from Fall into Winter and Spring and found to survive –and potentially replicate– in the EMS for at least 9 months. Once the infected manure was surface applied on the land, PEDv were found in 95% of soil samples three weeks following land application, including the soils from off-band manure applied locations.

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FOREWORD

This dissertation is written in manuscript style and is composed of three manuscripts. The first manuscript is published in Frontiers in Microbiology. The second and third manuscripts have not yet been submitted to a journal.

The manuscripts include:

Manuscript I: Monitoring Survivability and Infectivity of Porcine Epidemic Diarrhea Virus (PEDv) in the Infected On-Farm Earthen Manure Storages (EMS). Hein Min Tun, Zhangbin Cai, and Ehsan Khafipour

Manuscript II (short communication): Monitoring Survivability of Porcine Deltacoronavirus (PDCoV) in Earthen Manure Storages Co-Infected with Porcine Epidemic Diarrhea Virus (PEDv). Zhangbin Cai, Shadi Sepehri, Hein Min Tun, and Ehsan Khafipour

Manuscript III: Monitoring Survivability of Porcine Epidemic Diarrhea Virus (PEDv) Following Surface Application of PEDv-Positive Manure to the Soil. Zhangbin Cai, Shadi Sepehri, Mario Tenuta, and Ehsan Khafipour

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LIST OF ABBREVIATIONS

AIV = Avian influenza virus

BC = Benzalkonium chloride

BtCoV = Bat coronavirus

$CaCl_2$ = Calcium chloride

CAT = Chloramine-T

CPC = Cetylpyridin chloride

Cq = Quantitative cycle

Deoxyribonucleic acid = DNA

ELISA = Enzyme linked immunosorbent assay

EMS = Earthen manure storages

FIPv = Feline infectious peritonitis virus

HCoV = Human coronavirus

HEV = Hepatitis E virus

HO = Hydroxy radicals

HOCl = Hypochlorous acid

HP = Hydrogen peroxide

IgY = Immunoglobulin Y

MDR = Multi-drug resistant

MEM = Minimum essential medium

MeOH = Methanol

MRSA = Methicillin-resistant *Staphylococcus aureus*

ORF = Open reading frame

pAPN = Porcine aminopeptidase N

PCR = Polymerase chain reaction

PCV2 = Porcine circovirus type 2

PDCoV = Porcine deltacoronavirus

PEDv = Porcine epidemic diarrhea virus

PG = Propylene glycol

PRRSV = Porcine reproductive and respiratory syndrome virus

PRV-A = Porcine group A rotavirus

qPCR = Quantitative polymerase chain reaction

RBD = Receptor-binding domain

RdRp = RNA-dependent RNA polymerase

Ribonucleic acid = RNA

RNAi = RNA interference

RNP = Ribonucleoprotein

RT-qPCR = Reverse transcriptase quantitative PCR

SC = Stearalkonium chloride

SDPP = Spray-dried porcine plasma

SeCoV = Swine enteric coronavirus

Sg = Subgenome

TCID50/ml = 50% tissue culture infective dose per ml

TD = Terminal domain

TGEV = Transmissible gastroenteritis coronavirus

WSP = Waste Stabilization Pond

GENERAL INTRODUCTION

Coronaviruses are single-stranded, positive-sense, enveloped RNA viruses under the Coronaviridae family, which is subdivided into four genera including Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus (Woo et al., 2010).

Porcine epidemic diarrhea virus (PEDv) is a swine enteric coronavirus belonging to Alphacoronavirus, which causes severe diarrhea, vomiting and dehydration among all ages of pigs but it can be deadly to suckling piglets (Song and Park, 2012). The mortality rate for the infected suckling pigs could reach 100% (Song and Park, 2012). The virus has first been documented in Europe during the 1970s, thereafter it spread into Asia, and recently has frequently been reported in Americas (Carvajal et al., 2015). Since the initial outbreak in the USA in 2013, PEDv quickly developed into a major challenge for the North American swine industry. Millions of pigs have been culled out with a sudden decrease in the USA swine litter rate within a year. In 2014, PEDv was first introduced in Ontario, Canada (Pasick et al., 2014); subsequently, it was found in other provinces including Manitoba. This RNA virus is highly mutable and as such the vaccines developed in Asia during early 2000s are no longer effective to control the new virulent strains (Song et al., 2015). Recently several vaccines have been developed in North America; however, their effectiveness remains to be evaluated (Ouyang et al., 2015). Before the effective treatments or vaccines become available, strict biosecurity practices are critical to control the spread of the disease. Fecal-oral route is considered the main route of transmission. Contaminated fomites such as vehicles (Lowe et al., 2014) and

feedstuff (Pasick et al., 2014) could be abiotic carriers. Still, elimination of PED virus from the environment could be a challenging due to its high survivability and low infectious dose (Goyal, 2013; Pujols and Segales, 2014).

Followed by PEDv, another novel coronavirus, porcine deltacoronavirus (PDCoV, genus Deltacoronavirus) was identified in the USA for the first time in early 2014 (Wang et al., 2014a), and has been frequently reported to co-infect with other swine enteric viruses including PEDv (Lee and Lee, 2014a; Marthaler et al., 2014a; Marthaler et al., 2014b; Song et al., 2015; Wang et al., 2014a). PDCoV alone may also cause infection, and the clinical signs are similar to PEDv, including diarrhea, vomiting, dehydration, and fecal shedding of the virus (Jung et al., 2015).

Swine manure is considered a valuable fertilizer for crops. Thus, it is important to properly treat the infected manure before they are applied on land as a fertilizer. Under the experimental settings, PEDv could survive in inoculated manure at room temperature for 14 days and at temperatures ranging from -20°C to 4°C for more than 28 days (Goyal, 2013). Earthen manure storages (EMS) are engineered structures for storage and treatment of liquid livestock manure, and have been widely used in Western Canada. Although it has been suggested that EMS is able to reduce pathogen load including bacteria, viruses and parasites in the manure (Hill and Sobsey, 2001), concerns have been risen that some viruses may survive in the EMS for prolonged period if no proper treatment is conducted (Costantini et al., 2007).

This research aims to investigate the survivability of PEDv and PDCoV in the EMS under the Manitoba climate conditions by longitudinally monitoring the viral copy

in the infected EMS. It further aims to determine the infectivity of the survived PEDv using cell culture bioassays. Lastly, it also aims to test the survivability and infectivity of PEDv in the soil following the surface application of the infected manure on the land.

LITTERATURE REVIEW

1.0 PEDv Genome Structure and Morphology

Coronaviruses have the largest genome size among the known RNA viruses, encoding 4 structural proteins, and 16 nonstructural proteins (enzymes) that are required for the RNA genome synthesis (Brian and Baric, 2005; Sawicki et al., 2007). It is a single-stranded, positive-sense RNA genome with a 5' cap and a 3' polyadenylated tail and seven open reading frames (ORF1a, ORF1b, and ORF2 through 6), which is roughly 28 kb if the poly A- tail is excluded (Kocherhans et al., 2001). ORF 1a and 1b are the largest part of the genome coding for nonstructural proteins, and take up two-thirds of the genome (Lee, 2015). The unglycosylated nucleocapsid (N) protein (55-58 kDa) encapsulates the single-stranded RNA and offers structural foundation for the helical nucleocapsid (Laude et al., 1992).

PEDv is generally round-shaped, enveloped and pleomorphic with a diameter range between 95 and 190 nm, including the 18nm club-shaped projections (Pensaert et al., 1978). The lipid envelope contains 3 structural proteins including spike (S), envelope (E) and membrane (M) proteins (Song and Park, 2012). The spike glycosylated peplomers helps the virus to bind the host receptor and mediate the viral envelope fusion with cell membranes (Bosch et al., 2003). Unlike other coronaviruses, the S protein (180–220 kDa) in PEDv is uncleaved due to the absence of furin cleavage site. The E protein (7 kDa), located in the viral membrane, is related to the viral envelope formation (Maeda et al., 2001). The glycosylated M protein (27–32 kDa) is the most abundant envelope element, which is involved in virus particle formation especially in the assembly of the

nucleocapsid and membrane (Narayanan et al., 2000).

2.0 PEDv Replication

During the infection, PEDv first binds porcine aminopeptidase N (pAPN) via S protein, followed by the S protein-mediated membrane fusion (Lee, 2015). The genome replication starts after the uncoating. The genome functions as mRNA for the synthesis of two polyproteins including ppla and pplab. Subsequently, the polyproteins are cleaved into 16 proteins including RNA-dependent RNA polymerase (RdRp), which act as replication and transcription complex. Then, full-length strand and 9 kinds of subgenome(sg)-length minus (antisense) strands are synthesized from the genome by the transcription complex, which are used to replicate full-length genomic RNA and sg mRNAs (positive sense). Each sg mRNA is translated to proteins, including the four structural S, E, N, and M proteins. The full-length genomic RNA is usually used as the viral progeny's genome. The N protein binds to the full-length genomic RNA to form helical ribonucleoprotein (RNP) complexes, while S, E, and M proteins are inserted in the ER and anchored in the Golgi apparatus. Finally, the progeny is assembled at the ER-Golgi intermediate compartment and released (Lee, 2015).

3.0 PEDv Pathogenesis

3.1 Host Range

Coronaviruses affect a wide range of hosts, covering avian and mammal species including humans, bats, dogs and many other species, causing respiratory, enteric, hepatic and neurological diseases (Hu et al., 2015).

PEDv, PDCoV and Transmissible Gastroenteritis Coronavirus (TGEV) are known as pig viruses. However, PEDv is genetically more related to bat coronavirus 512/2005 (BtCoV/512/2005), suspecting that PEDv originated from bats (Huang et al., 2013). On the other hand, PEDv may not only infect porcine cells. For example, PEDv is able to grow in African green monkey kidney cell lines (VERO) with the presence of trypsin, which has been used for isolation of this virus in vitro for decades (Hofmann and Wyler, 1988). Recently, PEDv has also been successfully isolated from duck intestinal epithelial cell (MK-DIEC) (Khatri, 2015). Moreover, PEDv has been found to bind to human and bat cell lines (Liu et al., 2015a).

3.2 Tissue Tropism

Spike proteins of coronaviruses play an important role in binding proteinaceous and carbohydrate cell surface molecules. The spike protein S1 domain contains an N-terminal domain (S1-NTD) and a C-terminal domain (S1-CTD), both of which are considered as receptor-binding domains (RBDs) (Li, 2015). The characteristics of coronavirus RBDs determines the viral host range and tropism (Li et al. 2006; Liu et al. 2015a).

Porcine aminopeptidase N (pAPN), a 150-kDa glycosylated transmembrane protein, has been identified as the cellular receptor for PEDv (Li et al., 2007). Porcine aminopeptidase N is largely expressed on the surface of small intestinal epithelial cells (Li et al., 2007), thus, PEDv mainly infects small intestinal cells of pigs. However, a recent study indicated that the virus could be found outside the intestinal tract and in lung, liver, kidney and spleen of experimentally infected piglets (Park and Shin, 2014).

Moreover, some tissues such as VERO cell lines do not express APN, suggesting that other receptors may be involved in PEDv entry (Li et al. 2016).

3.3 Pathophysiology

PEDv is cytolytic, causing acute necrosis of small intestinal cells and hence leads to villous atrophy (Jung et al., 2014). Reabsorption of water and electrolytes is disturbed due to ultrastructural changes and mild vacuolation in the infected colonic epithelial cells. Massive degeneration of mitochondria in infected enterocytes results in a shortage of transport energy for nutrients absorption (Jung and Saif, 2015). Thus, the infected pigs undergo malabsorption because of the massive loss and functional disorders of absorptive enterocytes, leading to acute diarrhea (Jung and Saif, 2015). In some cases, vomiting also occurs after the infection, however its mechanisms have not been fully understood (Jung and Saif, 2015). Gut microbiota could be interfered by the disease. Studies have observed that the abundance of some dominant phyla could be affected greatly by the infection (Koh et al., 2015; Liu et al., 2015b). The disturbance of the microbiota could lead to metabolic and immunological disorders (Bomba et al., 2014). Further, weak gut integrity caused by PEDv infection poses a high risk of co-infections by luminal bacteria (Jung and Saif, 2015).

3.4 Clinical Signs

PEDv can infect pigs at all ages, resulting in watery diarrhea, vomiting, dehydration and usually co-occurs with anorexia and depressive symptoms. The incubation period is usually about 2 days but the length may vary up to10 days depending on the field or experimental conditions (Debouck and Pensaert, 1980; Jung et al., 2014;

Madson et al., 2014; Pensaert and De Bouck, 1978; Song and Park, 2012).

The severity of the disease and mortality rates are age-dependent. In piglets, the infection causes greater disease severity and mortality rate (up to 100%) in suckling piglets (normally under one or two weeks old) compared with weaned pigs (Jung and Saif, 2015; Song and Park, 2012). PEDv infection in suckling piglets induces severe watery diarrhea and vomiting for 3–4 days, leading to severe dehydration and electrolyte imbalance. Suckling piglets can hardly tolerate this imbalance and thus die easier from this infection. In older pigs including weaners to finishers, mortality rate decreases to 10% but morbidity is usually high. The infection impacts the growth performance; thus, infected growers require additional time to achieve the market weight. The period between the onset and cessation of clinical presentations could vary between 3 and 4 weeks (Debouck and Pensaert, 1980; Jung et al., 2014; Madson et al., 2014; Pensaert and De Bouck, 1978). The age-dependent resistance to PEDv may be related to several anatomical, physiological, and immunological factors. For example, neonatal piglets have slower turnover rate of enterocytes (5–7 days) compared with 3-week-old weaned pigs (2–3 days) (Jung and Saif, 2015).

As for sows, some may not suffer from diarrhea but they may experience anorexia and depression (Olanratmanee et al., 2010). Sow's reproductive performance may also be affected. In one study, pregnant sows infected with PEDv had a decreased farrowing rate and an increased abortion and return rates (Olanratmanee et al., 2010). Most importantly, the impact of the infection on the reproductive performance is highly associated with parity number and the period of pregnancy when the sows or gilts are exposed to the virus. The exposure of the pathogen during the early stages of pregnancy may result in

more severe reproductive disorders than the later stages. Also, the infection in pregnant gilts can more severely impact reproductive performance than in sows. The reason might be that the gilts still utilize nutrients for both growth and reproductive function, and hence, nutrients absorbed by the PEDv-infected pregnant gilts could be much below the demand level in order to meet the extra energy requirements of the immune system (Olanratmanee et al., 2010). Farrowing sows might also undergo reproductive disorders including agalactia or delayed estrus due to the loss of suckling piglets during the lactation period (Lee, 2015; Olanratmanee et al., 2010).

4.0 Transmission of PEDv

4.1 Animal to Animal and Fecal – Oral Route

Fecal-oral route is considered the main route of transmission of PEDv. Infected pigs excrete high levels of the virus in feces and vomitus (Authie et al., 2014; Sun et al., 2014). Within two days following infection, fecal shedding of PEDv initiates even before the presentation of clinical signs, and normally lasts for several weeks. In some cases, fecal shedding could extend for months even after clinical symptoms had completely resolved (Authie et al., 2014; Madson et al., 2014; Steinrigl et al., 2015; Sun et al., 2014). These features increase the risk of transmission. On the other hand, the virus is highly contagious and even small amounts of it can make the animals sick. Thomas et al. (2015a) reported that 10ml of inoculum containing titers ranging from 560 to 0.056 TCID50/ml [stands for 50% tissue culture infective dose per ml and is a measure of infectivity of the virus. The higher TCID50/ml value, the more infective is the virus. The reported range in Thomas et al. study is equivalent to quantitative PCR (qPCR) quantitative cycle (Cq)

values ranging from 24.2 to 37.6 of a US virulent PEDv isolate (USA/IN19338/2013) could cause infection in 100% of 5-day-old inoculated neonatal pigs. Thus, a small amount (couple of grams) of infected manure could be a potential transmission source of PEDv. Contaminated transport trailers may play an important role in spread of the virus between farms in the USA. Lowe et al. (2014) reported that 6.6% of trailers were contaminated with PEDv before unloading, and 5.2% of trailers were not contaminated at arrival but became contaminated during the unloading process.

In addition to feces and vomitus, researchers argue that the transmission of PEDv through other animal fluids cannot be ruled out. For example, PEDv RNA has been found in sow milk samples with a detection rate ranging from 23% to 77% of samples (Chen et al., 2013; Li et al., 2012b; Sun et al., 2012). Sun et al. (2014) reported that semen samples from 80% of boars in PEDv-infected farms were positive for PEDv. Unfortunately, neither of these studies conduct bioassays using cell culture or animal models, so it cannot be confirmed whether or not the detected virus was infective. Hence, the transmission of PEDv through other fluids remains to be determined.

4.2 Feed Supplement

Processed porcine whole blood is a valuable animal by-product. Spray-dried porcine plasma (SDPP) has been widely incorporated in weaning pigs' diet to improve growth rate, feed intake and gut health of piglets (Van Dijk et al., 2001). However, low levels of PEDv RNA have been detected in serum fraction of whole blood samples. Contaminated SDPP might be a potential vehicle of transmission of the virus, which has been suspected as the source of the initial introduction of PEDv into Eastern Canada

(Pasick et al., 2014). However, it is hard to determine when and how the virus was introduced into the feed. The blood could be cross-contaminated with PEDv during the collection processes (Authie et al., 2014). Indeed, cross-contamination might also take place at any point during the manufacturing, packaging, storage and transportation of the product (Authie et al., 2014).

On the other hand, it has not been entirely agreed upon that the contaminated feed product is a notable route of PEDv transmission because spray-drying process and storage conditions may be sufficient to inactivate the infectious virus (Carvajal et al., 2015). Spray-drying process usually involves high temperatures (e.g. 166°C inlet and 80°C outlet temperatures) (Gerber et al., 2014). In one study, PEDv was artificially introduced in bovine plasma before the spray-drying process. After spray-dried, the samples were subjected to bioassay using the VERO cell line. Results indicated the spray-dried samples did not contain infective virus, suggesting that spray-drying conditions possibly inactivated the virus (Pujols and Segalés, 2014). A swine bioassay study also confirmed that porcine plasma products contaminated with PEDv did not contain infective virus after the spray-drying process (Gerber et al., 2014).

4.3 Airborne

A recent study proposed that airborne dissemination might be an additional transmission route because the virus has been found to be aerosolized and transported long distances (up to 10 miles downwind) by air (Alonso et al., 2014). However, the air samples collected under field conditions were negative on bioassay, while pigs received experimental air samples had PED symptoms (Alonso et al., 2014). Thus, it is still

unclear if airborne transmission is a critical route.

5.0 Diagnostics and Study Tools

Since several swine viruses' infections usually lead to very similar clinical presentations, PED confirmation cannot rely on clinical findings. Laboratory testing is required for the identification of PEDv.

5.1 Polymerase Chain Reaction (PCR)

Conventional polymerase chain reaction (PCR) and reverse transcriptase quantitative PCR (RT-qPCR) have been most commonly used in PED diagnosis. The PCR primer design is generally within the M, N, or S genes (Carvajal et al., 2015). The cost of gel based conventional PCR may be lower than qPCR, however, qPCR assays are more appropriate in analysis of varies environmental samples to trace carriers of infection, or the survivability of PEDv due to its sensitivity and capacity to calculate the quantity (Lowe et al., 2014; Pasick et al., 2014; Thomas et al., 2015b; Tun et al., 2016). The assays can also be used to distinguish different strains of PEDv or different enteric porcine viruses. For example, qPCR assays recently developed by Wang et al. (2014c) and Zhao et al. (2014) are able to simultaneously discriminate virulent and variant strains or recent and classical variants. qPCR assays developed by Kim et al. (2007) could detect TGEV and PEDv. Moreover, a multiplex nested PCR assay has been developed to detect multiple different enteric porcine viruses including PEDv, Porcine Group A rotavirus (PRV-A), and TGEV (Salem et al., 2010). However, it is important to note that primers developed in early years or different geographical locations might not be suitable for detection of the new variants PEDv in North America.

5.2 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA is another important tool for the virus detection, which can be based on either the whole virus or viral protein antigens, such as monoclonal antibodies against PEDv (Carvajal et al., 2015; Lee, 2015; Wang et al., 2015). Considering their high-throughput and fast turnaround time features, ELISA is well suited for screening larger herds (Choudhury et al., 2016). The analytical sensitivity of ELISA, however, may be lower than PCR assays (Sozzi et al., 2010). Wang et al. (2015) reported that antigen-capture ELISA's based on the monoclonal antibody 5D7 may have a sensitivity and specificity close to PCR targeted M gene.

5.3 Survivability Assay

The survivability of a virus refers to presence or absence of its genetic materials within an environment without considering its ability to enter and replicate within a host. This survivability concept is sometime referred to as being live, so before describing how to measure the survivability of PEDv it is important to have a clear understanding of the definition of live vs. dead in living organisms compared to viruses.

5.3.1 Live or Dead? Living Organisms Compared to Viruses

In living organisms, the cell is a single fundamental unit of life (O'Connor et al., 2010). Within the cell membrane, the cell's interior environment (cytoplasm) is liquid filled with functional and structural elements and intracellular organic molecules consist of nucleic acids, proteins, carbohydrates, and lipids. Nucleic acids including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) contain genetic codes for biological activities. DNA contains information required for protein synthesis, while

RNA is associated with expression of the information in DNA, and proteins dictate cell function and metabolism. Therefore, in a living organism (dormant cells as well as growing or non-growing metabolically active cells), the cell functions are maintained through the flow of information from DNA to RNA to protein (O'Connor et al., 2010). A living organism is considered dead when above-mentioned biological functions and activities are terminated (Taylor et al., 2008). As RNA normally have a very short life time in the cell and also in the environment (less than 15 minutes; Chen et al., 2008; Chen et al., 2015; Dubelman et a., 2014), the presence or absence of RNA can be considered as an indicator of a cell being living or dead.

In contrast to living cells, viruses are in a gray area of determination between living and nonliving. Viruses are submicroscopic infectious agents that are biologically active only when they are inside living cells, because their structure is too simple to conduct the biological activities on their own. A single virus particle, which is called virion, is simply composed of the genetic materials (either DNA or RNA) captured by a protein shell (capsid). Some viruses have an additional lipoprotein outer layer (envelope) enclosing the protein shell (Modrow et al., 2013). While capsid and envelope help virion resist change in the environment, viruses have no other mean to maintain a steady-state internal environment. Viruses also cannot grow, reproduce, and generate/consume energy (ATP) by their own (Modrow et al., 2013). As such, it is difficult to consider viruses as either live or dead. Instead, other terminologies such as survivability (refers to presence of virion's genetic materials in the environment) and infectivity (refers to the ability of a virion to infect its host) are used to describe viruses' fate.

5.3.2 Testing the Survivability of PEDv

To test the survivability of PEDv in the environment, total RNAs from the environment can be extracted followed by determination of presence of the virus genetic material using PCR. PEDv is an RNA virus. As mentioned above, RNA normally has a very short life time in the cell and also in the environment. Inside the cell, mRNA may be degraded within 10-15 minutes by RNases activities (Chen et al., 2008; Chen et al., 2015). In the environment, RNA could also be immediately degraded by RNases which is from direct metabolism of the resident microbial community (Dubelman et a., 2014). When PCR results of extracted environmental RNAs are PEDv positive, it is an indicator that the virion has kept its structure intact and protected its RNA, therefore survived in the environment. That, however, does not imply that PEDv can infect its host. To test the infectivity of PEDv, a cell culture or an animal bioassay must be performed.

5.4 Cell Culture

Viral isolation is an important step to study the biological features of viruses for investigating pathogenesis and developing diagnostic assays and vaccines (Chen et al., 2014). As early as 1988, Hofmann and Wyler have successfully isolated the PEDv from VERO cells (African green monkey kidney cells). Trypsin is essential for the culturing due to its ability to cleave the virus S protein to induce membrane fusion between the cell and virus (Park et al., 2011). Unfortunately, PEDv is still difficult to isolate and propagate based on this protocol (Chen et al., 2016; Chen et al., 2014; Choudhury et al., 2016). For example, Chen et al. (2016) failed to culture a PEDv S-INDEL-variant strain on VERO cells from 68 clinical samples (27 fecal swabs, 24 feces, 13 small intestines and 4 oral fluids). The authors had to inoculate the virus into 3-week-old pigs and used

the new fresher small intestine tissues and cecum contents to culture the virus. Other than VERO cell lines, recently PEDv cell culture assays have been developed either in non-swine originated cells such as MARC-145 (African green monkey kidney cells; Sun et al., 2017) and MK-DIEC (duck intestinal epithelial cell; Khatri, 2015), or in swine cells IPEC-J2 (porcine intestinal epithelium cells; Lin et al., 2017). However, most laboratories still use the VERO cell lines as a standard protocol.

6.0 Evolution of PEDv

Coronaviruses are known to have high mutation and recombination rates. The genetic replication relying on RdRp has a high error rate (Hu et al., 2015). Moreover, coronaviruses are able to recombine between different strains, even different species of coronaviruses during coinfections (Boniotti et al., 2016; Huang et al., 2013). Interestingly, a new swine enteric coronavirus generated by recombination of TGEV and PEDv, tentatively named swine enteric Coronavirus (SeCoV), may have been circulating in Italy from 2009 to 2012 (Boniotti et al., 2016). Then a virus closely related to SeCoV was found in Germany's archived fecal samples from 2012 (Akimkin et al., 2016). The severity of coronaviruses as well as their tropism and host range may be changed due to mutations, deletions, and recombination events, especially in S gene (Lau and Chan, 2015; Su et al., 2016).

7.0 Epidemiology of PEDv

PEDv (CV777 strain) has first been reported in the UK (Wood, 1977) and Belgium (Pensaert and De Bouck, 1978) in the 1970s. From the 1970s to 1980s, PEDv has been wide spread in the European continent and caused epidemics with severe losses

in neonatal pigs (Pensaert and Martelli, 2016). PED outbreaks then markedly decreased, and PEDv did not persist in Europe except for some sporadic outbreaks (Lin et al., 2016). In the 1980s and 1990s, PEDv has spread into Asia with severe epidemics in some Asian swine producing countries, resulting in a significant economic impact on the Asian swine industry. Subsequently in 2010, the non-S INDEL PEDv strains emerged (referred to as "highly virulent" PEDv strains), which contributed to devastating outbreaks causing up to 100% mortality in suckling piglets (Li et al., 2012b; Sun et al., 2012). Although vaccines have been developed and widely used in Asia based on classical CV777 strain, those vaccines failed to control the epidemics caused by the highly virulent strains of PEDv (Lin et al., 2016). In 2013, a virulent PEDv has been identified in the US for the first time, which has been tested to be genetically highly similar to the Chinese non-S INDEL strains isolated in 2012 (Stevenson et al., 2013). Soon after, a variant OH851 strain designated as S INDEL PEDv, was found in Ohio, USA. Compared with the virulent strains, S INDEL PEDv has sequence deletions, insertions, and mutations in S gene, which was reported with reduced disease severity (Wang et al., 2014b). After the initial introduction, PEDv spread across the USA within a year, killing about 10% of American swine population, accounting for deaths of 7 million pigs (Lin et al., 2016). During this period, virulent PEDv was introduced into other countries in Americas, including Canada, Mexico, and Columbia (Lin et al., 2016; Pasick et al., 2014; Vlasova et al., 2014). Meanwhile, the US-like virulent PEDv emerged rapidly in East Asian countries including Japan, Thailand, South Korea, and Taiwan (Cheun-Arom et al., 2015; Lee and Lee, 2014b; Suzuki et al., 2015). Further, the US-like S INDEL PEDv has been reported frequently in European countries such as Germany, Belgium, Portugal, and Austria

(Mesquita et al., 2015; Stadler et al., 2015; Steinrigl et al., 2015; Theuns et al., 2015). However, in Europe, only Ukraine has been exposed to virulent PEDv so far (Dastjerdi et al., 2015).

8.0 Porcine deltacoronavirus (PDCoV)

PDCoV is a novel coronavirus under genus Deltacoronavirus, which was initially discovered in Hong Kong in 2012 (Woo et al., 2012). PDCoV was first detected in the USA in February 2014 (Wang et al., 2014a), afterwards it was introduced into Canada concurrently with PEDv (Marthaler et al., 2014b). However, it is speculated that PDCoV might have been circulating in the USA at least since August 2013 (Sinha et al., 2015). Currently, PDCoV has only been reported in Eastern Asia and North America, and several strains have been successfully isolated in the USA (Chen et al., 2015; Hu et al., 2015; Jun et al., 2016). Since the discovery, PDCoV has been frequently reported to co-infect with other enteric viruses including PEDv (Lee and Lee 2014a; Marthaler et al. 2014a; Marthaler et al. 2014b; Song et al. 2015; Wang et al., 2014a). However, it has been confirmed that PDCoV infection alone can also induce enteropathogenic lesions in gnotobiotic pigs (Jung et al., 2015). Like PEDv, pigs infected with PDCoV may experience diarrhea, vomiting, and atrophic enteritis, however, the clinical disease may be less severe (Jung et al., 2015; Jung et al., 2016).

9.0 Control Programs

9.1 Vaccines and Therapy

Although PEDv first emerged in Europe, no vaccines have been developed in Europe due to the disease not causing significant losses (Authie et al., 2014). In contrast,

PEDv has caused remarkable economic losses in Asia, and therefore vaccination has been used for many years in several Asian countries such as China, Japan and South Korea (Authie et al., 2014). In 2014, new vaccines have been granted conditional licenses from the US Department of Agriculture (Authie et al., 2014; Ouyang et al., 2015). However, detailed efficacy studies of the vaccines are lacking. Moreover, it has been suggested that available vaccines may not be fully effective against American strains (Crawford et al., 2016; Ouyang et al., 2015).

In addition to vaccination, passive immunization by oral administration of egg yolk antibodies (Immunoglobulin Y, IgY) obtained from immunized chickens has been proposed as an alternative method, which could provide supportive immunity to PEDv infected neonatal piglets (Jeon et al., 2015). A combined use of extracts of medicinal herbs named MYCI have been reported to improve average daily gain and intestinal health in 3-day old piglets challenged with PEDv (Kim et al., 2015). RNA interference (RNAi) has been shown to significantly inhibit the PEDv replication in VERO cells (Shen et al., 2015). However, these studies still require field observations to further evaluate their efficacies.

9.2 Intentional Exposure (feedback)

Before the effective vaccines or treatments are available, intentional exposure (feedback) of the virus to sows to stimulate maternal immunity has shown variable success in preventing reinfection. The feedback materials include the feces or/and the intestinal tracts of infected piglets. After the feedback, sows' serum neutralizing antibodies could be transferred to milk, which is likely to persist for months and thus

constantly protect their neonatal piglets (Liu et al., 2015c; Morrison and Goede, 2014; Ouyang et al., 2015). However, there are several considerations for this approach. For example, there is a potential risk to spread other viral pathogens shed in the intestinal or fecal contents such as Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) or Porcine Circovirus Type 2 (PCV2) (Allan et al., 2000; Jung et al., 2006; Lee, 2015). The large amount of infectious PEDv shed in feces of sows after intentional exposure is also considered a hazard for PEDv transmission between farms (Lee, 2015).

9.3 Biosecurity Measures

9.3.1 Viruses in The Environment

Viruses may be released into the environment from infected hosts. Although viruses do not have biological activities outside their hosts, they are likely to survive in the environment and get transferred to the susceptible hosts via various routes (Pirtle and Beran, 1991). Viruses in the environment can be considered as biochemical complexes, which interact with various environmental media and matrices and likely to be degraded. Once a viral particle is damaged in the environment, it may no longer be able to infect and replicate when introduced to the hosts. Thus, not all viruses in the environment are successful in surviving and reaching a susceptible host to infect; even though the virus has survived, it might still lose the infectivity due to the damage of its proteins (Sobsey and Meschke, 2003).

Many physical, chemical, and biological factors such as temperature, pH, moisture, biocides, UV light and microbial antagonism affect a virus's survival in the environment (Alonso et al., 2014; Polo et al., 2015; Sobsey and Meschke, 2003). An early

study suggests that PEDv is sensitive to heat, but it is stable under low temperatures (Hofmann and Wyler, 1989). The virus could be inactivated at 60℃ for 30 min. The virus exhibited active at 4℃with the pH range between 5 and 9; at 37℃ the infectivity of PEDv was limited to the pH range from 6 to 8 and the infectivity was completely lost at pH<4 or >pH 9 (Hofmann and Wyler, 1989). A recent study shows that PEDv could survive up to 3 weeks at 4℃, 2 weeks at 12℃, 1 week at 22℃ in spray-dried bovine plasma (Pujols and Segalés, 2014). In manure slurry, preliminary lab-based study suggests that PEDv could survive for 14 days at room temperature (~25°C) and more than 28 days at -20 to 4°C (Goyal, 2013).

Based on these features, the US swine industry suggested to heat animal hauling trailers to 71°C for 10 min to avoid spreading the virus through the contaminated trailers (Thomas et al., 2015b). A recent study indicates that PEDv could be completely inactivated after a ten sec or longer exposure to water heated to ≥ 76°C (Zentkovich et al., 2016). However, temperature treatment may not be applicable in many field conditions. To minimize introduction of the virus by any material or person, the viral elimination programs may focus on chemical disinfectants. Several factors including application method, type of the chemical, contact time, and surface type could significantly affect the viral inactivation (Yeargin et al., 2016). Thus, the efficacy of different disinfectants needs to be evaluated under various field conditions with different application methods. In one study, PEDv could be fully inactivated by several commercially available disinfectants such as quaternary ammonium, phenol, Synergize, Virkon S, Clorox Regular-Bleach (Bowman et al., 2015). Several commercially available virucides are discussed below.

9.3.2 Chlorine

Chlorine plays an important role in disinfecting microorganisms in public water supplies and swimming pools. Chlorine is a strong oxidative chemical leading to cellular membrane disruption or extensive protein degradation, especially at a low pH level (McDonnell and Russell, 1999). Chlorine can destruct the capsid and the genetic material in viruses, so it possesses a strong virucidal activity (McDonnell and Russell, 1999; Simonet and Gantzer, 2006). Chlorination has been evaluated to eliminate many kinds of enteric viruses, including poliovirus, hepatitis A virus, coxsackievirus, echovirus, norovirus and adenovirus (Bigliardi and Sansebastiano, 2006; Cromeans et al., 2010; Kahler et al., 2010; Kitajima et al., 2010; Li et al., 2002; Xue et al., 2013). For example, one study showed the RNA of poliovirus was degraded into fragments by chlorine (Simonet and Gantzer, 2006).

However, chlorination could be limited by corrosiveness and instability (Rutala et al., 1998). Adverse factors of the disinfecting function include light, temperature, organic matter, catalysts, and pH (Rutala et al., 1998; Sisti et al., 1998). Chlorine might not perform well above pH 8 (Taylor and Butler, 1982). In some cases, the virucidal activity could be inhibited even at pH 5 and 6 (Junli et al., 1997; Li et al., 2004; Taylor and Butler, 1982). Importantly, chlorine is irritative to skin and respiratory tract, which is a hazard to humans and animals (Nemery et al., 2002).

9.3.3 Virkon S

Virkon S is an oxidative disinfectant that has been used for disinfecting a wide variety of bacteria, spores, fungi, and viruses. Virkon S is described as a balanced,

stabilized blend of peroxygen compounds, surfactant, organic acids, and inorganic buffer (McCormick and Maheshwari, 2004). Virkon S contains potassium peroxymonosulfate as an active oxidizing ingredient, which has been used in a variety of industrial and consumer applications (Anipsitakis et al., 2008). Virkon S forms hypochlorous acid (HOCl) in solution, which is also a strong oxidizing agent (McCormick and Maheshwari, 2004).

Virucidal features of Virkon S have been evaluated in many viruses including both enveloped and non-enveloped viruses (McCormick and Maheshwari, 2004; Muhammad et al., 2001; Royer et al., 2001). For example, adenovirus types 5 and 6 copies were significantly reduced after five min exposure to 0.9% of Virkon S (McCormick and Maheshwari, 2004). Avian influenza viruses (AIV) was completely inactivated after 90 min contact to 0.5% of Virkon S, while at 1% and 2% concentrations only 30 min was required to fully inactivate the AIV (Muhammad et al., 2001). Remarkably, Virkon S has shown effective in killing viruses below the freezing point with the presence of antifreeze agent supplements such as propylene glycol (PG), methanol (MeOH), or calcium chloride ($CaCl_2$) (Guan et al., 2014; Guan et al., 2015). However, some studies indicated that certain species of viruses or microorganisms might not be easily eliminated by Virkon S. Even more than 1% of Virkon S was inefficient in disinfecting mycobacteria (Broadley et al., 1993) and poliovirus (Tyler et al., 1990).

9.3.4 Quaternary Ammonium Compounds (QACs)

The basic structure of QACs could be shown as $N^+ R_1R_2R_3R_4 X^-$, of which R is regarded as a hydrogen atom, a plain alkyl group or an alkyl group substituted with other

functional groups, and X is an anion (Buffet-Bataillon et al., 2012). Common QACs include benzalkonium chloride (BC), stearalkonium chloride (SC), cetrimonium chloride/bromide (cetrimide) and cetylpyridin chloride (CPC) (Hegstad et al., 2010). The most commercially popular product, BC, is a mixture of alkyl-benzyl-dimethyl-ammonium chlorides with various, even-numbered, straight alkyl chains which usually ranges from C_8 to C_{18} in length (Hegstad et al., 2010). Based on the product formulation, QACs might be effective against non-lipid-containing viruses and spores (Gerba, 2015).

However, QACs could potentially induce antimicrobial resistance. When the environment is exposed to low chemical reactive QACs, microbial communities are treated with sub-inhibitory levels, and thus more resistant clones would emerge (Hegstad et al., 2010). On the other hand, some researchers argued that the multi-target action of QACs makes the development of resistance unlikely (Gerba, 2015).

9.3.5 New composite biocide with both N-chloramine and quaternary ammonium (QA) moieties

Recently, a new composite biocide combined an oxidant, hydantoin-based organic N-chloramide, with QA moiety in one molecule (termed as “composite” biocide) (Li et al., 2012a). This novel biocide may possess improved virucidal activity. Organic N-chloramines are a group of organic compounds bearing N-Cl bonds. The bactericidal efficacy of N-chloramines is comparable to hypochlorites, however, they are safer, more stable and more resistant to organic matter (Williams et al., 1985). Inactivation of microorganisms by N-chloramines occurs in 3 steps. The initial attack leads to the chlorination of external protein matrix of microorganisms, forming a moderate chlorine

cover. Meanwhile, N-chloramines penetrates into microbial cells. After penetration, N-chloramines attack many vitally important constituents. Thiols and thioethers are destroyed by oxidation, and proteins are denatured by transchlorination (Gottardi et al., 2013). These actions will eventually cause the inactivation and destruction of microorganisms. More importantly, it is unlikely to induce bacterial resistance against the QA, as N-chloramines have nonspecific interactions with protein sites (De Silva et al., 2015; Li et al., 2012a).

N-chloramines virucidal activity has been witnessed in the environment and in anti-infective drug ingredients. For example, chloramine-T (CAT) has suggested to be an effective virucide against poliovirus type 2 (Gowda et al., 1981). NVC-422 (N, N-dichloro-2, 2-dimethyltaurine) has been proposed for the treatment of adenoviral conjunctivitis (Yoon et al., 2011).

To date, this new biocide has shown enhanced antibacterial activity against two clinically retrieved bacteria: methicillin-resistant *Staphylococcus aureus* (MRSA) and multi-drug resistant (MDR) *Pseudomonas aeruginosa* (Ning et al., 2015). Since this composite biocide is unlikely to induce antimicrobial resistance, extensive use of the compounds in the field would not create concerns in regards to inducing resistance in animal pathogens. So far, the new composite biocides have not been evaluated in disinfecting viruses. Given the virucidal nature of QA and N-chloramines, it is believed that the composite biocides can be promising products in inactivating viruses.

9.3.6 Accel

Accel contains the oxidizing agent hydrogen peroxide (HP) which demonstrates

broad-spectrum efficacy against viruses, bacteria, yeasts, and bacterial spores (Linley et al., 2012). HP acts by forming free hydroxy radicals (HO) leading to oxidizing thiol groups in proteins, lipids, and nucleic acids (McDonnell and Russell, 1999). HP is considered environmentally sustainable because it eventually breaks down into water and oxygen (Linley et al., 2012).

Studies suggest that vaporized HP could significantly inactivate viruses from variety of families including avian and mammalian species, containing both lipid and nonlipid viruses (Heckert et al., 1997; Pottage et al., 2010; Vickery et al., 1999; Zonta et al., 2016). However, the role of Accel in disinfecting PEDv in particular has not been evaluated.

10.0 Manure Storage System in Western Canada

Earthen manure storages (EMS; previously referred to as lagoon) are a common livestock manure storage and treatment system in Western Canada, which includes aerobic and anaerobic forms.

Naturally aerobic EMS are designed based on large surface area with shallow depth (up to 5 feet) because of the need for oxygen diffusion across the surface (Barker, 1996). Further, when sunlight penetrates the EMS, algae may also generate oxygen through photosynthesis. Compared with anaerobic EMS, bacterial digestion in aerobic EMS may be more complete and hence generate less odorous end products (Barker, 1996).

The majority of swine EMS are anaerobic, in which the anaerobic bacteria are easily preserved to decompose organic materials (Cole et al., 2000). Since anaerobic

EMS is not relied on oxygen, the EMS can be much deeper（from 6 to 20 feet） and require less surface area (Barker, 1996). However, the oxygen may still penetrate into the EMS up to 50 cm, depending on temperature, wind and organic loading rate (Zhang, 2001).

Beside bacteria, EMS may harbor some other higher life forms microorganisms including non-mammalian eukaryotes (e.g. protozoa or amoeba), which can also survive in both anaerobic and aerobic environments (Fenchel and Finlay, 1990; Sobsey et al., 2006). Based on concentration of dissolved oxygen in anaerobic EMS, distribution of the microorganism's species at different layer may be different, as aerobic species may be at the top while anaerobic species may be at the bottom (Fenchel and Finlay, 1990). Moreover, it has been noticed that some of the bacteria, viruses, and even eukaryotes can live and replicate within the free-living amoeba, as such the amoeba may serve as hosts of and vehicle for replication of many viruses (Scheid, 2014; Balczun and Scheid; 2017). Viruses including mimiviruses, pandoraviruses and pithoviruses have been suggested to be able replicate in the amoeba, and some species may also be human pathogens (Balczun and Scheid; 2017).

11.0 Summary

PEDv and PDCoV are swine coronaviruses leading to severe diarrhea in all ages of the infected pigs. PEDv is particularly deadly to suckling pigs, with the mortality rate up to 100%. The disease spreads through fecal-oral route with a two-day incubation period. These viruses have been introduced to North America in 2013 and PEDv outbreaks continue to be a major challenge for the North American swine producers. No

effective vaccine and therapy are currently available for the US-like S INDEL PEDv. Strict biosecurity measures are the major strategy to control the spread of the disease. However, PEDv is a highly contagious virus with low infectious dose and high survivability.

Microorganisms may be better preserved in the low temperature environment due to low biological activities and decomposition rates (Sobsey and Meschke, 2003). Manitoba has a cold continental climate, and thus the persistence length of PEDv in Manitoba environment is an important question to answer in order to allow the swine farmers and manure applicators make necessary precautions. EMS is a livestock manure storage and treatment system that have been widely used in Western Canada. However, some viruses may survive in the EMS for prolonged period if no proper treatment is conducted (Costantini et al., 2007). This research aims to investigate the viability of PEDv and PDCoV in the EMS and soil under the Manitoba environment. The survivability of PEDv and PDCoV in the EMS will be longitudinally monitored in the infected farms using RT-qPCR to determine the viral RNA copy number over time. The infectivity of PEDv will be further tested using the cell culture bioassay. If PEDv would survive in the EMS, the survivability and infectivity of PEDv in the soil will be further determined following similar procedures.

HYPOTHESES

1. PEDv survives more than 28 days –reported previously under laboratory conditions– after the initial outbreak in the farm in the infected earthen manure storages (EMS) under Manitoba climatic condition.
2. Survived PEDv is viable and able to replicate when introduced to its host.
3. PDCoV co-infected with PEDv survives in the EMS for prolonged period.
4. PEDv survives in the soil after the land application of PEDv-infected manure.
5. Survived PEDv in the soil is viable and able to replicate when introduced to its host.

OBJECTIVES

1. To observe the on-farm status of PEDv and PDCoV in the infected Manitoba farms after the outbreak of diarrhea.
2. To determine the survivability of PEDv and PDCoV in the infected EMS under the Manitoba climatic conditions.
3. To determine if survived PEDv is infective.
4. To determine the survivability of PEDv in the soil after the land application of infected manure under the Manitoba climatic conditions.
5. To determine if survived PEDv in the soil is infective.

MANUSCRIPT I

Monitoring Survivability and Infectivity of Porcine Epidemic Diarrhea Virus (PEDv) in the Infected On-Farm Earthen Manure Storages (EMS)

ABSTRACT

In recent years, porcine epidemic diarrhea virus (PEDv) has caused major epidemic outbreaks, which have been a burden to North America's swine industry. Low infectious dose and high viability in the environment are major challenges in eradication of this virus. To further understand the viability of PEDv in the infected manure, we longitudinally monitored survivability and infectivity of PEDv in two open earthen manure storages (EMS; previously referred to as lagoon) from two different infected swine farms identified in the province of Manitoba, Canada. Our study revealed that PEDv could survive up to 9 months in the infected EMS after the initial outbreak in the farm. The viral load varied among different layers of the EMS with an average of 1.1×10^5copies/ml of EMS, independent of EMS temperature and pH. In both studied EMS, the evidence of viral replication was observed through increased viral load in the later weeks of the samplings while there was no new influx of infected manure into the EMS, which was suggestive of presence of potential alternative hosts for PEDv within the EMS, which are likely to be non-mammalian eukaryotes (e.g. protozoa or amoeba). Decreasing infectivity of virus over time irrespective of increased viral load suggested the possibility of PEDv evolution within the EMS and perhaps in the new host that negatively impacted virus infectivity. Viral load in the top layer of the EMS was low and mostly non-infective suggesting that environmental factors, such as UV and sunlight, could diminish the replicability and infectivity of the virus. Thus, frequent agitation of the EMS that could expose virus to UV and sunlight might be a potential strategy for reduction of PEDv load and infectivity in the infected EMS.

Keywords: Swine, porcine epidemic diarrhea virus (PEDv), earthen manure storages (EMS), survivability, infectivity

INTRODUCTION

Porcine epidemic diarrhea virus (PEDv), a highly contagious virus that causes severe diarrhea, vomiting, dehydration and high mortality particularly in piglets, is an enveloped, single-stranded RNA virus under the Coronaviridae family (Pensaert and de Bouck, 1978; Debouck and Pensaert, 1980; Song and Park, 2012). In late 1970, PEDv was first identified in the UK and Belgium (Wood, 1977; Pensaert and de Bouck, 1978). It has been reported in other European countries and Asia over the past four decades (Song and Park, 2012). In 2013, PEDv was first identified in the US, which genetically was highly similar to the Chinese virulence prototype isolated in 2012 (Stevenson et al., 2013). Subsequently, the virus has spread rapidly within North America, and the first introduction into Canada was reported in early 2014 (Kochhar, 2014; Pasick et al., 2014; Ojkic et al., 2015). Fecal-oral route presents the major transmission route for PEDv, however, airborne dissemination has been proposed as a potential additional transmission route because the virus can be aerosolized and transported over long distances (up to 10 miles downwind) by air (Alonso et al., 2014). In addition, fecal contamination of PEDv can cause fomites, for instance farm equipment such as transport trailers (Lowe et al., 2014) or feed supplements (Pasick et al., 2014) that act as potential abiotic carriers for PEDv. Fecal shedding of PEDv in pigs appears prior to clinical presentations, and hence increases the risk of transmission (Madson et al., 2014).

Environmental factors especially temperature and pH influence the survivability of virus in contaminated environments (Pesaro et al., 1995; Pujols and Segales, 2014).

PEDv is able to survive up to 3 weeks at 4°C, 2 weeks at 12°C, 1 week at 22°C in spray dried plasma (Pujols and Segales, 2014), which was epidemiologically recognized as the source of virus infection for the first introduction into Canada (Pasick et al., 2014). Due to significant fecal shedding and the highly persistent nature of PEDv, proper storage, treatment and utilization of infected manure are important to prevent further contamination of uninfected environments.

Earthen manure storages (EMS; previously referred to as lagoon) are engineered structures for storage and treatment of liquid livestock manure. Swine EMS are mostly anaerobic, allowing anaerobic bacteria to decompose organic materials (Cole et al., 2000). Anaerobic EMS can significantly reduce pathogen concentrations including bacteria, viruses and parasites (Hill and Sobsey, 2001). However, the persistence of viruses in EMS may be prolonged if no proper treatment is employed (Costantini et al., 2007). Dee et al. (2005) found that porcine reproductive and respiratory syndrome virus (PRRSv) could survive in the infected EMS at 4°C for up to 8 days. In addition to PEDv, swine hepatitis E virus (HEV) has been reported to survive in infected EMS (Kase et al., 2009). Viruses in EMS can be expected to contaminate the uninfected environment via soil, ground and surface water and bioaerosols, from which they may infect other susceptible hosts (Cole et al., 2000). In western Canada, open farm anaerobic EMS are commonly used to store and treat the manure in most swine farms. To date (January 2016) and in Manitoba, five cases of PEDv outbreaks have been reported since February 2014 with an evidence of recurrent infection in some farms. However, the exact length of PEDv survivability in infected EMS is still unknown. This is an important question to answer in order to allow farmers and manure applicators to take necessary precautions to

avoid further disease spread between infected farms and farms without prior exposure.

In this study, we monitored the survivability and infectivity of PEDv in two infected on-farm EMS over a period of 9 months. Based on the survivability and infectivity results, we determined that PEDv is highly viable in the infected EMS beyond 6 months, but environmental factors, such as UV and sunlight, could perhaps diminish its infectivity. In the present study, the ability of PEDv to replicate in EMS provided a clue that the virus may find an alternative host(s) to replicate and evolve within the EMS. Further studies are warranted to confirm this finding.

MATERIALS AND METHODS

Study Design

Two PEDv infected farms identified in the province of Manitoba in 2014 participated voluntarily in this study. An on-farm EMS in each infected farm was selected for sampling: the farm-1 EMS had an area of 78 m × 46 m with an average depth of 1.5 m whereas the farm-2 EMS had an area of 79 m × 73 m and average depth of 0.7 m. The PEDv outbreak at farm-1 was confirmed in May 2014 by Veterinary Diagnostic Services of Manitoba. Samples tested at the time included saliva, fecal swabs (live animal and manure) and environmental swabs from load out, entrance, and pits. All representative samples from these areas were positive. However, no testing was done on EMS samples. No other testing was done following these samplings and no other visits to the site occurred after May until sampling started for the current study in September of 2014. No more pigs entered the barn between May and the end of the project. At the time of site

visit in September, pigs looked healthy, active without any signs of scouring.

The outbreak at farm-2 occurred in September 2014. Similarly, initial tests were done at Veterinary Diagnostic Services of Manitoba. Samples included saliva, fecal swabs (live animal and manure) and environmental swabs (load out, entrance). All representative samples from these areas were positive. However, all samples were again collected in-barn, and no EMS sampling/testing was performed.

Before barns were depopulated, the status of active viral shedding was examined in fresh fecal and pit samples collected from each barn. Samplings from fresh fecal and pits were started late September for farm-1 and early October 2014 for farm-2. Sampling was terminated if animals showed high viral shedding, however, continued for another two consecutive weeks if samples were negative or showed low positivity. Farm-1 was completely depopulated on September 28, 2014, and farm-2 was depopulated on October 8, 2014.

To monitor the survivability and infectivity of PEDv, EMS samples were taken weekly before EMS were emptied. Details of the study design were illustrated in Figure 1.

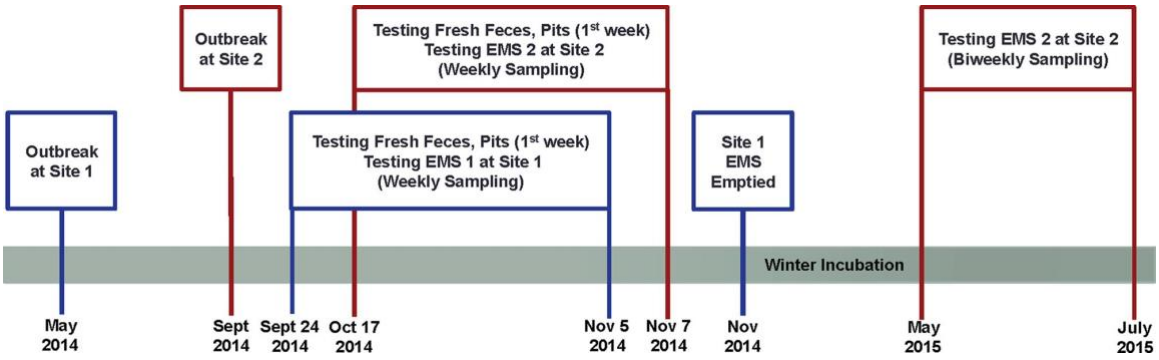

Figure 1. Schematic diagram of the study design indicating porcine epidemic diarrhea virus (PEDv) outbreak and sampling schedule for monitoring survivability and infectivity of the virus in two on-farm Manitoba earthen manure storages (EMS).

Biosecurity Measures

Strict biosecurity procedures were followed during planning, working on the site, transporting samples off-site, leaving the site, and during decommissioning to eliminate the risk of PEDv transmission. All protocols were established prior to accessing the sites to prevent further spread of the virus. A change tent was set up on the initial site visit to provide a location to add additional protective gear and site-specific footwear at a crossover point near the farm entrance. A research tent was also located to provide shelter during the sampling days and provide a location to store sampling and cleaning equipment. Specific route was identified to access the EMS, and separate vehicles were used for each farm to add an additional level of biosecurity. After sampling, samples were kept on dry ice and transferred to the vehicle. A specific biosecurity protocol for washing vehicles at the site as well as in the city was implemented as part of the sample hand off to the Gut Microbiome Laboratory (Department of Animal Science, University of Manitoba, Winnipeg, Manitoba, Canada) where further analyses were performed.

Sampling Procedures, and Measurement of Temperature and pH of the EMS

Based on the EMS size, manure samples from farm-1 EMS were collected at 12 different locations and at three depths including top, middle and bottom layers (n = 36/week). The top layer samples were collected from the surface; middle layer samples were collected at 91 cm below the surface; bottom samples were collected from the bottom by pushing the sampler to the bottom of the sludge layer before it was opened. From farm-2 EMS, samples were collected at 16 different locations at two depths (top and bottom; n = 32/week; Figures 2A,B). In farm-1 EMS, data loggers were placed at three different locations, at three depths to continuously record the temperature and pH throughout the sampling period. However, in farm-2 EMS, data loggers were only placed at a single depth at three different locations due to shallow depth of the EMS (Figures 2C,D).

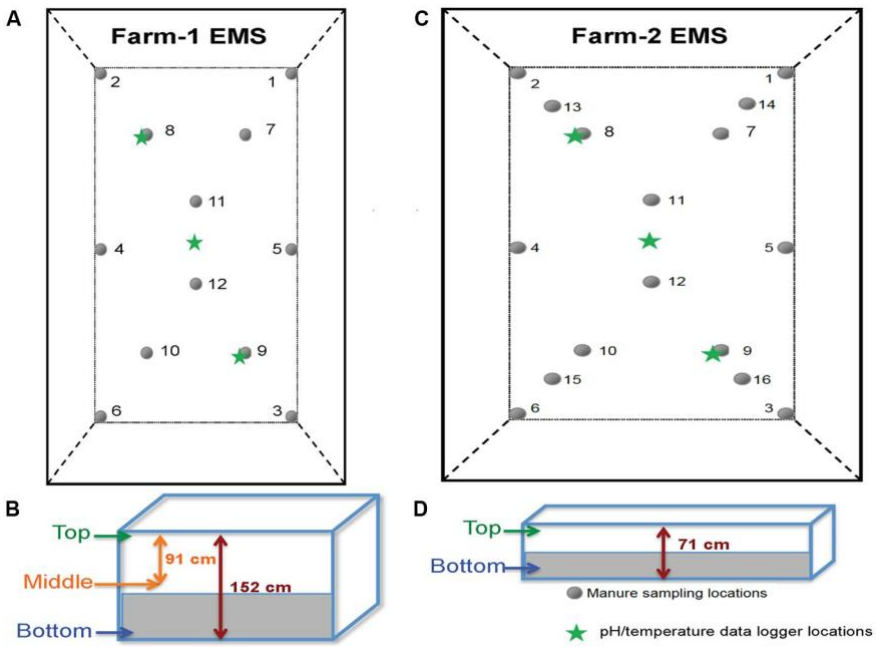

Figure 2. The layout of sampled EMS in this study. (A) The layout of farm-1 EMS indicating the locations of 12 sampling points and pH/temperature data loggers. (B) Three sampling depths at farm-1 EMS. (C) The layout of farm-2 EMS indicating the locations of 16 sampling points and pH/temperature data loggers. (D) Two sampling depths at farm-2 EMS.

At the initial sampling, buoys tied to weights were used to mark the sampling locations in each EMS. Ropes were set up in a grid to mark the relative buoy location and allow researchers to maneuver the boat on the EMS with minimal agitation of the liquid. Samples were retrieved from the site in a double tote system to maintain biosecurity. A primary containment tote was taken to the change tent and left in the change tent while a cooler was taken to the EMS. The sample cooler and primary containment tote were placed in a secondary containment tote that stayed in the vehicle. Stainless steel samplers were custom fabricated for the EMS sampling, ranging in length from 2.1, 2.7, and 3.9 m. Samplers were made with a sample cup attached to shaft that slid through a pipe with sealing face. At the sampling location, the desired depth was measured prior to the

sampling of each layer. Spring pressure held the sample cup closed until manually opened at the desired depth. Once opened, the manure flowed into the sample cup and then manually closed. The sample cup was retrieved and transferred the sample to a disposable sample jar for subsampling into vials. Manure samples were collected from two locations at a time before the samplers were brought back to shore for cleaning prior to the next set of samples being collected. The samplers were disassembled and the exteriors/interiors of all components were pressure washed prior to collecting the next set of samples. The manure samples were subdivided into vials and placed in the cooler with dry ice for transport back to the laboratory for testing. The gross appearances of EMS samples from different layers showed more liquid in the top layer while the bottom layer had more solid fraction.

Nucleic Acid Preparation and Real-Time RT-PCR Assay

The liquid manure samples were vortexed to be homogenized before the RNA extraction. The viral genomic RNA was extracted from 50 µl of sample using the MagMAXTM-96 Viral RNA Isolation Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. RNA was then eluted in 90 µl of elution buffer. Viral RNA extracted from fecal, pit and EMS samples were subjected to molecular detection for both virulence (subgenogroup 2a) and variant-INDEL strains of PEDv using duplex real-time RT-PCR as described previously (Wang et al., 2014c), which used spike gene primers (Forward S1F: AGG CGG TTC TTT TCA AAA TTT AAT G and Reverse S1R: GAA ATG CCA ATC TCA AAG CC) and specific probes targeted to virulent PEDv and new variant PEDv (Virulent S1P: 5Cy5-TAT TGG TGA AAA CCA GGG TGT CAA T-3BHQ-2, and Variant S1P: 56-FAM-TGG TTA TCT ACC TAG TAT GAA CTC CTC

TAG C-3IABkFQ). The primer and hydrolysis probe utilized the AgPath-IDTM One-Step RT-PCR Reagents (Life Technologies, Grand Island, NY, USA) and 2 µL of RNA with the CFX348 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) under the following thermal cycling conditions, reverse transcription, 30 min at 50°C; Taq activation, 15 min at 95°C; followed by 40 cycles of 10 s at 94°C and 30 s at 54°C. A 500 bp of spike gene fragment that included sequence variation between PEDv strain OH1414 (virulent PEDv) and PEDv strain OH851 (variant PEDv) was chosen to use as both standard and positive control. A gBlocks Gene Fragments (Integrated DNA Technologies, Inc., Coralville, IA, USA) containing the PEDv spike gene targets was synthesized. Stock concentration of 109 copies/µl were made and a 10-fold serial dilution was run on the real-time RT-PCR to generate a standard curve for each genotype of PEDv, which was used to transform the Cq values into estimated copies of PEDv RNA per ml of EMS. The sensitivity of the duplex real-time RT-PCR assay was validated through serial dilutions of both gene fragments, and triplicate of each dilution were run in the assay. The detection limit was two copies for both variant and virulent strains of PEDv. As shown in Figure 3, there is a strong linear correlation ($r2 > 0.99$) between Cq values and the corresponding amount of gene fragment copy numbers for both virulent and variant PEDv. The standard curves of virulent and variant PEDv were plotted with slopes of -3.358 and -3.363, respectively. The amplification efficiencies of the assays for both virulent and variant PEDv were 98.5 and 98.3%, respectively (Figures 3A,B). Triplicate for each EMS sample were also subjected for duplex real-time RT-PCR assay, and generated Cq values were transformed into copy numbers based on the slopes of respective standard curves. Subsequently, the resulted copy numbers were further transformed into copy numbers in 1ml of EMS

sample.

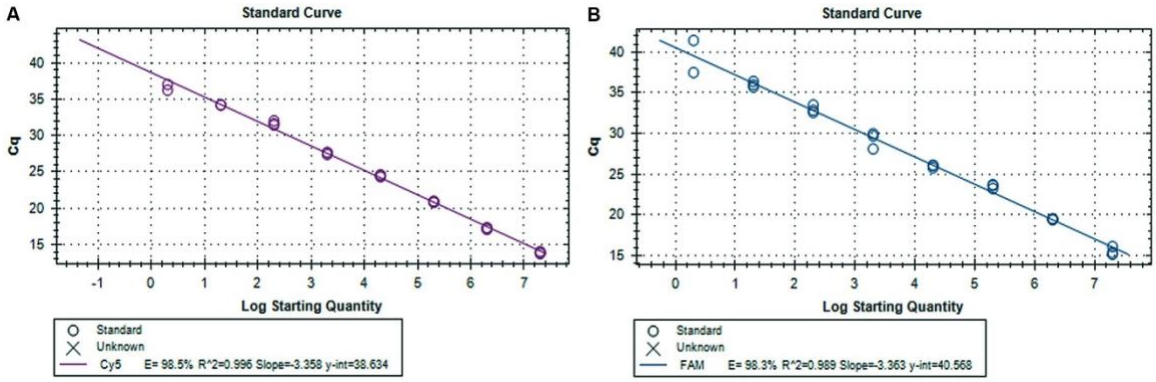

Figure 3. Standard curves for the duplex RT-qPCR assay. (A) Synthetic DNA standard curve for virulent PEDv strain. (B) Synthetic DNA standard curve for variant-INDEL PEDv strain.

PEDv Infectivity in Cell Culture

To evaluate the infectivity of PEDv, EMS samples were centrifuged and filtrated using 0.2 micron syringe filter (VWR International Inc., Radnor, PA, USA; cat. no. 194-2520), then subjected to 10-fold serial dilution with phosphate buffer saline (PBS). The serially diluted samples were inoculated in VERO cells as previously described (Hofmann and Wyler, 1989; Chen et al., 2014). Briefly, VERO cells (ATCC CCL-81) in 96-well plate were washed twice with 100 µl PBS then inoculated with 200 µl of sample. Triplicate for each sample were tested in the presence of known positive control. After a 1.5 h incubation at 37°C with 5% CO2, 100 ml PBS was used to wash the cells once. Then, cells were incubated for 5 days with the post-inoculation medium. The post-inoculation medium was composed of MEM supplemented with tryptose phosphate broth (0.3%), yeast extract (0.02%), and trypsin 250 (5 µg/ml). The cytopathic effect (CPE) in the cell culture plates was monitored daily. After 5 days, the plate was frozen-and-thawed

to detach cells from the plates. Cells were then subjected to viral RNA extraction to examin PEDv's replicability using the duplex real-time RT-PCR assay (Wang et al., 2014c). The virus titers were determined according to the Reed and Muench (1938) method and expressed as the 50% tissue culture infective dose (TCID50)/ml. Higher TCID50 values were indicative of higher infectivity of virus.

Statistical Analyses

Data from the quantitative RT-PCR analysis, sampling date, different sample types and pathogen were consolidated in a spreadsheet (Microsoft EXCEL; Microsoft Corporation, Redmond, WA, USA) and organized for analysis. Means, standard deviations, and minimum and maximum values for quantitative variables, and positive samples counts and percentages for qualitative variables were calculated for descriptive analysis. Statistical analysis was performed on real-time RT-PCR results using chi-square, two-way ANOVA with Sidak's multiple comparison test, and linear regression in Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). P-values < 0.05 were considered significant.

RESULTS

The Status of PEDv Shedding in the Farms

To evaluate active viral shedding in the farms, we verified the presence of PEDv in both fresh fecal and pit samples using duplex qualitative RT-PCR assay. Table 1 presents the status of virus shedding in fresh fecal and pit samples collected from barns before they were depopulated. In farm-1 (which contained two barns), minor PEDv

shedding (only virulence PEDv strain) occurred in the barn A, 1 and 2 weeks before barn was depopulated (5 and 15%, respectively), whereas no viral shedding was detected in barn B. Pit samples in farm-1 showed higher PEDv positivity in barn A (100 and 90%) than in barn B (20 to 30%). In farm-2, pigs were actively shedding PEDv and 100% of tested pit and fecal samples were positive to virulence PEDv strain with average viral loads of 2.4×10^6 copies/ml of manure mix in the pit and 3.2×10^6 copies/g of feces, respectively. No variant-INDEL strain was detected in this study.

Table 1. Status of porcine epidemic diarrhea virus (PEDv) shedding before depopulation of barns by examining the presence of PEDv in fresh feces and pit samples.

Farm	**Barn**	**Sample type**	**% of PEDv positive samples (Number of PEDv positive samples/total number of tested samples)**			
			September 24th	**October 1st**	**October 8th**	**October 17th**
1	A	Fresh feces	15 (3/20)	5 (1/20)	Barn was emptied	
		Pit	100 (10/10)	90 (9/10)	Barn was emptied	
	B	Fresh feces	0 (0/10)	0 (0/10)	0 (0/10)	Barn was emptied
		Pit	20 (2/10)	30 (3/10)	20 (2/10)	Barn was emptied
2	NA[1]	Fresh feces	NS[2]	NS	NS	100 (12/12)
		Pit	NS	NS	NS	100 (12/12)

[1]NA, Not Applicable, one barn at site#2 was examined.
[2]NS, No Sampling. The first sampling week was on October 17th.

Dynamics of pH and Temperature in Earthen Manure Storages

Figure 4A presents the temperature dynamics in farm-1 EMS during Fall sampling. Data were retrieved from nine temperature/pH loggers that were set up at three different locations in three different depths were presented in Figures 2A,B. In all three layers of the EMS, the temperature was in the range 16–19°C at the beginning of the study in late September. However, the temperature of the top and middle layers of the EMS declined to 4–8°C by Oct 25th, followed by the second decline by mid-November to 0–2°C. The temperature of the bottom layer of the EMS remained at the range of 6–10.5°C until mid-November and was less impacted by the environmental temperature. Figure 4B presents the pH dynamics in farm-1 EMS where pH was relatively more stable in the bottom layer compared to the top and middle layers throughout the monitoring period. The bottom layer of the EMS also had the lowest pH ranging from 6.8 to 7.2. The top layer had the highest pH ranging from 7.5 to 8.4 while the middle layer had a pH of 7.2–8.3.

Figures 4C,D show the temperature and pH dynamics in farm-2 EMS during the Fall and Spring/Summer sampling periods, respectively. Due to the shallow depth, data were retrieved from three loggers that were set up at three different locations of only at a single layer presented in Figure 2D. The temperature dynamic in farm-2 EMS followed a similar trend like in farm-1 EMS. The pH ranged from 6.6 to 6.95 which was similar to the pH observed in the bottom layer of farm-1 EMS. However, the temperature in farm-2 EMS steadily increased during Spring/Summer sampling (Figure 4E). Similarly, the pH showed an increase in Spring and gradually decreased to 6.8–7.2 by the end of the sampling period (Figure 4F).

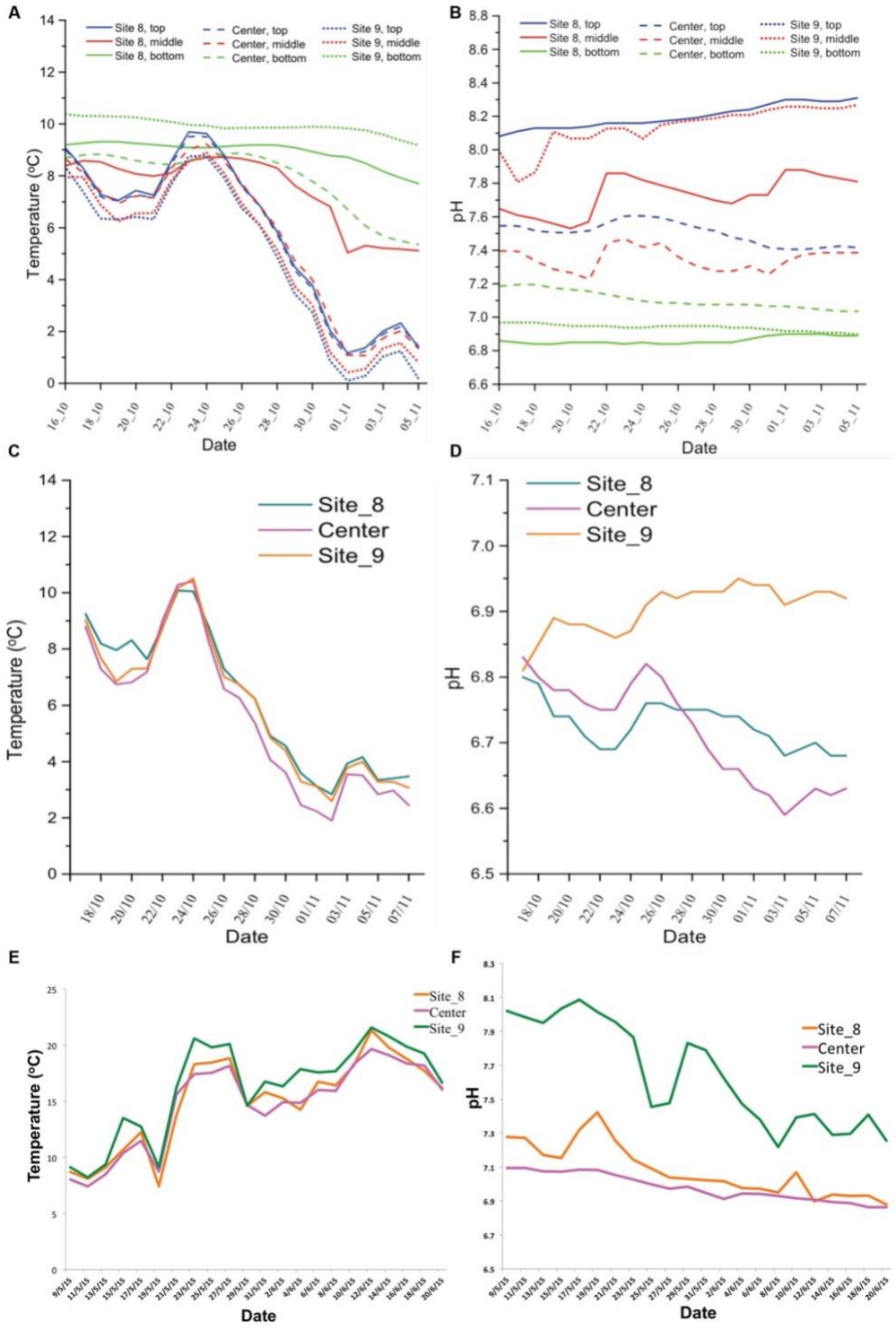

Figure 4. Temperature and pH dynamics in the studied EMS. The temperature and pH records for farm-1 EMS (A,B) and for farm-2 EMS (C,D) during Fall sampling. The temperature and pH records for farm-2 EMS (E,F) during Spring/Summer sampling.

Survivability of PEDv in the Infected on-Farm Earthen Manure Storages

The survivability of PEDv in the infected EMS was determined by the presence of viral RNA for both virulent PEDv (subgenogroup 2a) and variant-INDEL strains. In farm-1 EMS, 97% of tested EMS samples were PEDv positive only for virulent PEDv (subgenogroup 2a), whereas no sample was detected for the variant-INDEL strain. The viral load significantly increased after the third week of sampling in all three layers of the EMS. On average, the viral load ranged from 6.3×10^3 to 3.3×10^4 copies/ml of EMS during the first 3 weeks, however, that significantly increased ($P < 0.05$) to 4.3×10^4–1.4×10^5 during the last 4 weeks (Figure 5A).

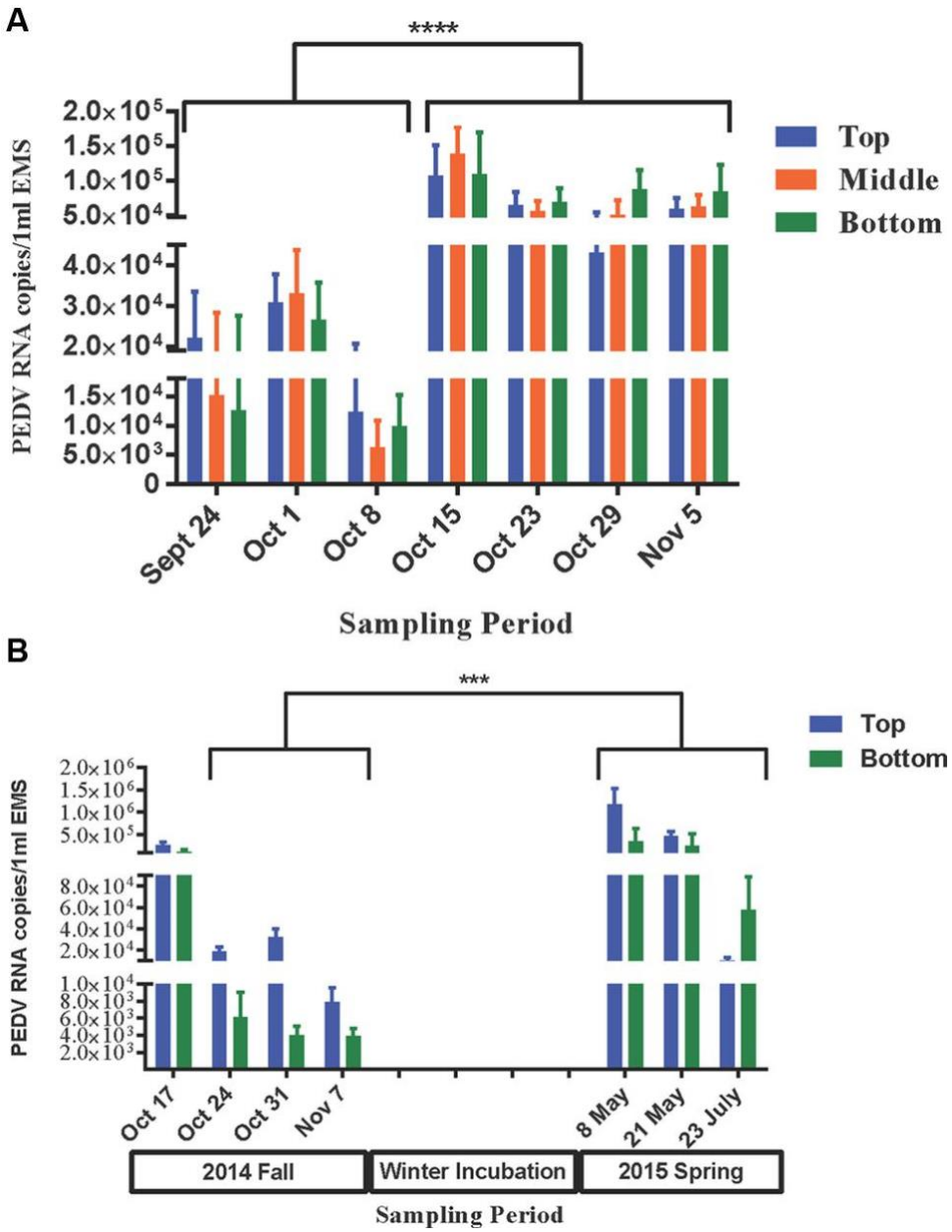

Figure 5. Survivability of PEDv in two infected on-farm EMS. Using real-time RT-PCR targeted to S gene, the survivability of PEDv over time was tested based on the detectable viral RNA copies number in 1 ml of EMS at each sampling time point during Fall 2014 sampling for farm-1 EMS (A), and from Fall 2014 to Summer 2015 sampling for farm-2 EMS (B). The bar shows average RNA copy number of PEDv in the respective layer of EMS. The error bars show the standard deviation based on 12 replicates per layer of the EMS in farm-1 and 16 replicates in farm-2. Each biological sample was analyzed in triplicate using real-time RT-PCR. ***$P < 0.001$ and ****$P < 0.0001$.

In farm-2 EMS, 76% of tested EMS samples were positive for virulent PEDv (subgenogroup 2a), and no variant-INDEL strain was observed like in farm-1 EMS. During the Fall sampling, viral load was higher ($P < 0.05$) at the first week of sampling (on average 1.26×10^5–2.69×10^5 copies/ml of EMS) compared to the following 3 weeks (on average 3.9×10^3–7.8×10^3 copies/ml of EMS on week 4; Figure 5B). After a long winter incubation, the viral load increased ($P < 0.05$) significantly in the early Spring (May 2015) ranging, on average, from 3.53×10^5 to 1.12×10^6 copies/ml of EMS in the bottom and top layers of the EMS, respectively. However, the viral load significantly declined ($P < 0.05$) by mid-Summer (July 2015) ranging, on average, from 1.02×10^4 to 5.76×10^4 copies/ml of EMS in the top and bottom layers, respectively (Figure 5B).

Infectivity of PEDv in the Infected on-farm Earthen Manure Storages

To examine the infectivity of PEDv in the infected EMS, sets of samples from selected weeks were inoculated in VERO cell culture and their ability to replicate was examined. Samples from both weeks 5 and 7 of farm-1 EMS, and weeks 2 and 4 from Fall sampling and weeks 1, 3, and 5 from Spring/Summer sampling of farm-2 EMS were selected for infectivity analysis (Table 2). In farm-1 EMS, PEDv was detectable in the top layer and its number increased during the last 4 weeks of sampling in the Fall, however, the viruses were not infective (Table 3), whereas samples from both middle and bottom layers of the EMS were infective. In week 5, the percentage of infectivity in both middle and bottom layer were the same (8.3%), whereas the infectivity was higher in the bottom layer (108 TCID50) compared to the middle layer (105 TCID50) although no statistical differences were observed ($P = 0.58$). In week 7, a higher number of samples showed positive infectivity in the middle layer when compared to the bottom layer ($P = 0.01$;

Table 3).

Table 2. Sampling information and analyses performed in this study.

Farm	Sampling weeks	Season	Sampling date	Performed tests
1	Week 1	Fall	September 24, 2014	Survivability
	Week 2	Fall	October 1, 2014	Survivability
	Week 3	Fall	October 8, 2014	Survivability
	Week 4	Fall	October 15, 2014	Survivability
	Week 5	Fall	October 23, 2014	Survivability and infectivity
	Week 6	Fall	October 29, 2014	Survivability
	Week 7	Fall	November 5, 2014	Survivability and infectivity
2	Week 1	Fall	October 17, 2014	Survivability
	Week 2	Fall	October 24, 2014	Survivability and infectivity
	Week 3	Fall	October 31, 2014	Survivability
	Week 4	Fall	November 7, 2014	Survivability and infectivity
	Week 1	Spring	May 8, 2015	Survivability and infectivity
	Week 3	Spring	May 21, 2015	Survivability and infectivity
	Week 5	Summer	July 23, 2015	Survivability and infectivity

Table 3. Infectivity of PEDv in the farm-1 EMS.

Sampling week/date	EMS layer	% of PEDv infective (number of PEDv infective samples/ total number of tested samples)	Infective titer (TCID50)[1]	Fisher's exact test[2]	
				X^2	*P*-value
Week 5/23 October 2014	Top	0 (0/12)	0	1.06	0.58
	Middle	8.3 (1/12)	1.00E + 05		
	Bottom	8.3 (1/12)	1.00E + 08		
Week 7/05 November 2014	Top	0 (0/12)	0	8.4	0.01
	Middle	41 (5/12)	1.00E + 06 to 1.00E + 09		
	Bottom	8.3 (1/12)	1.00E + 08		

[1]TCID50: the 50% tissue culture infective dose. Greater values are indicative of greater infectivity of virus.

[2]Statistical comparisons were made between different layers of the EMS within each sampling time point.

Table 4. Infectivity of PEDv in the farm-2 EMS.

Sampling week/date	EMS layer	% of PEDv infective (number of PEDv infective samples/ total number of tested samples)	Infective titer ($TCID_{50}$)[1]	Fisher's exact test[2]	
				X^2	*P*-value
Week 2 Fall/24 October 2014	Top	12.5 (2/16)	1.00E + 02 to 1.00E + 06	0	1
	Bottom	12.5 (2/16)	1.00E + 01 to 1.00E + 07		
Week 4 Fall/07 November 2014	Top	6.25 (1/16)	1.00E + 02	1.14	0.28
	Bottom	18.75 (3/16)	1.00E + 02 to 1.00E + 04		
Week 1 Spring/08 May 2015	Top	0 (0/16)	–	1.03	0.31
	Bottom	6.25 (1/16)	1.00E + 02		
Week 2 Spring/21 May 2015	Top	0 (0/16)	–	6.36	0.01
	Bottom	31.25 (5/16)	1.00E + 02		
Week 3 Summer/23 July 2015	Top	0 (0/16)	–	–	–
	Bottom	0 (0/16)	–		

[1]TCID50: the 50% tissue culture infective dose. Greater values are indicative of greater infectivity of virus.
[2]Statistical comparisons were made between different layers of the EMS within each sampling time point.

In farm-2 EMS, the infective virus was found in both top and bottom layers during the Fall sampling. However, only samples from the bottom layer of the EMS were found to be infective with a low titer of 102 TCID50 during the Spring samplings on May 8th and 21st. Both infectivity percentage and titer showed no significant difference between top and bottom layer of the EMS in the Fall samples and the first week (May 8th) of Spring sample. However, there was a significant difference in the second week (May 21st) of Spring sample ($P = 0.01$; Table 4).

DISCUSSION

The primary route of infection for PEDv is oral-fecal transmission through direct contact with infected pigs or the infected manure (Crawford et al., 2015). Other potential routes of infection have been proposed (Alonso et al., 2014; Lowe et al., 2014; Pasick et al., 2014). Transport vehicles for swine, contaminated air and feed or feed ingredients have been shown to contain the genetic material of PEDv, which are indicative of highly persistence nature of PEDv outside the host. In general, most viruses cannot survive long in the environment, outside their host. However, viruses under the Coronaviridae family show high survivability in the contaminated environment (Geller et al., 2012). PEDv is an enveloped, single-stranded, positive-sense RNA virus belonging to the genus Alphacoronavirinae in the family Coronaviridae and is related to transmissible gastroenteritis virus (TGEV; Hofmann and Wyler, 1989). Persistence of viruses in the environment varies with the type of virus. Two coronaviruses including a swine pathogen, TGEV and mouse hepatitis virus (HMV) remain infectious in water and sewage from several days to weeks. At 25°C, TGEV survives up to 22 days and MHV survives up to 17 days in the water, whereas the survivability for TGEV is 9 and 7 days for MHV in

settled sewage. At 4°C, both viruses can survive up to 4 weeks in water and sewage (Casanova et al., 2010). In case of canine coronavirus (CCV), it has lower survivability and loses its infectivity at 20°C and 4°C after 24 h, whereas complete loss of its infectivity is observed at -20°C or -70°C by 3 months (Tennant et al., 1994). Hepatitis A virus (HAV) is another example that is more stable than most enteroviruses at elevated temperatures (Deng and Cliver, 1995).

Experimentally, it was demonstrated that PEDv has high survivability in the infected manure (Goyal, 2013). However, so far there has been no epidemiological investigation of PEDv survivability in the infected manure over time in the environment. PEDv is a highly contagious virus with low infective dose (Liu et al., 2015c). PEDv infected manure can contaminate the uninfected environment, making the manure storage methods and treatments critical to the control of this pathogen (Song and Park, 2012). In most swine farms, EMS are traditional manure storage and treatment system, and the manure is applied to the agricultural land as a valuable fertilizer source (Hunt et al., 2010; Ducey and Hunt, 2013). However, viruses shed in EMS are likely to contaminate the environment through soil, ground and surface water and bioaerosols, and therefore, application of infected manure could be a potential disease transmission source (Cole et al., 2000).

This study is the first field monitoring which examines the survivability of the PEDV in the infected EMS. In the lab-based observations, PEDv survives more than 7 days in the inoculated fresh feces, while the virus can survive up to 14 days at room temperature and up to 28 days at -20 to 4°C in inoculated manure slurry (Goyal, 2013). In another landmark study, PEDv has been experimentally reported to survive and be

infectious up to 3 weeks at 4°C, 2 weeks at 12°C, and 1 week at 22°C in spray dried bovine plasma (Pujols and Segales, 2014). However, the experimental periods observed in both lab-based studies were limited and the observations were done only up to 28 and 21 days, respectively. Thus, the maximum duration PEDv can survive and be infectious in the environment was still unknown. Our current findings showed that PEDv could survive up to 9 months in the infected EMS (according to farm-2 EMS data) under Fall, Winter, Spring and Summer temperatures in Manitoba (range of -30 to 23°C according to 2014–2015 weather records). The stability of coronaviruses at various temperatures appears to be dependent on the nature of surrounded environmental conditions. In general, coronaviruses can survive at 56°C for 10–15 mins, at 37°C for several days, and at 4°C for several months whilst virus at a frozen temperature (-60°C) survives for many years without loss of infectivity (Casanova et al., 2010). The survival time of most viruses in infected manure is highly variable but should be considered in terms of days, weeks, or months as opposed to minutes or hours. Enteroviruses are reported to survive for 3–170 days in the soil of various compositions at various temperatures and for 1–23 days on crops (Deng and Cliver, 1995). Survivability of viruses is substantially longer at cold temperatures. The average daily temperature of EMS would not be stable as in experimental setting, and is influenced by the ambient environmental temperature and other factors. Thus, PEDv survivability and infectivity in experimental settings cannot directly represent the exact environmental conditions. Based on our study, in particular, the results from farm-2 EMS, PEDv has the ability to survive and be infective up to 9 months after the outbreak in the farm. In addition, temperature fluctuations within a single day should also be considered to evaluate the impact on the survivability of PEDv

in EMS.

Beside the temperature, pH of the environment has an impact on the survivability of virions. Generally, PEDv favors neutral pH with a wide range between 5 and 9 (Hofmann and Wyler, 1989). However, the combined effect of pH and temperature plays a critical role in PEDv survivability. Although PEDv is active in the pH range between 5 and 9 at 4°C, the range narrows down with increased temperature to between 6 and 8 at 37°C. Regardless of temperature, the virion completely loses its replicability at pH < 4 and >pH 9 (Hofmann and Wyler, 1989). In this study, we observed that the variation of pH over time in both studied EMS were not significant, with the pH ranging between 6.6 and 8.3. Among three layers of EMS, pH was the lowest at the bottom layer and the highest in the top layer (Figure 4). The pH of both EMS in this study fell within the range that PEDv could actively replicate indicating that the EMS are favorable environment for PEDv replication. However, there was no significant direct correlation among pH, temperature, and viral copy numbers.

In both studied EMS, the viral load was numerically higher in the top layer during the first 3 weeks, however, the trend switched toward the end of the sampling period with the higher number observed in the bottom layer (Figure 5). This was most likely due to the decomposition of PEDv by sunlight from the top layer, as being exposed to sunlight could cause destruction of virions (Eischeid et al., 2009; Wilhelm et al., 1998). In the waste water, usually lower layers contain higher solids, where sunlight may not easily penetrate and inactivate microorganisms. In a Waste Stabilization Pond (WSP) system study, the researchers analyzed *Cryptosporidium parvum* oocyst viability in surface and at depth (Reinoso and Bécares, 2008). The depth of anaerobic pond was 2.5 m and was

1.5 m in facultative pond, while estimated dark zones (Secchi disk visibility) were 0.6 m and 0.44 m, respectively. The survival of oocysts was significant longer in the bottom layer compared with the top layer in sunlight conditions (Reinoso and Bécares, 2008). Similarly, higher die-off rates of bacteria were observed near the surface than at depth in waste treatment systems (Davies-Colley et al., 2000; Mayo, 1989). In both EMS, the top layer had the least amount of solids (<1.5% in Site #1 and <1% in Site #2) while the bottom layer had the most solids (6.5% – 8.5% in both sites). Thus, PEDv in the lower layers may receive less effect from sunlight compared to the top layer.

In farm-1 EMS of this study, the viral load significantly increased after the third week of sampling in all three layers of the EMS. In farm-1 minor or no viral shedding was found in the fresh fecal samples from both barns during the first 3 weeks of sampling (Table 1). However, higher percentage of PEDv positive samples were detected from the pit samples. The reason might be that it had been 4 months after the initial outbreak of PEDv when we accessed the farm; therefore, the pigs had been recovering from the PEDv infection at the beginning of this study. In the Fall sampling of farm-2 EMS, viral load was higher at the first week of sampling compared to the following 3 weeks. This was most likely due to active viral shedding, which was confirmed by fresh fecal samples (Table 1) at the beginning of the study as the barn was depopulated about 2 weeks after EMS sampling was started. After a long-winter incubation, the viral load significantly increased in the early Spring (May 2015), but significantly declined in mid Summer (July 2015). Beyond the existing knowledge that PEDv significantly survives longer in contaminated environments, the apparent increased viral copies in both studied EMS provides evidence for the ability of the virus to replicate outside its typical host, the

swine. Although PEDv is believed to be a genuine pig virus, its ability to replicate in cells of non-swine origin has been reported since late 1980. In 1988, Hofmann and Wyler (1989) were the first to report replication of PEDv in kidney cells of monkey origin (VERO cells). Subsequently, Utiger et al. (1995) presented serological evidence that PEDv may circulate in humans and cats. A recent report verified that PEDv could replicate in duck intestinal cell line (Khatri, 2015). To our knowledge, reservoirs of PEDv have not yet been discovered. Likewise, other swine viral pathogens could replicate in hosts of non-swine origin. However, in the current scenario, potential alternative hosts for PEDv in EMS are favored to be non-mammalian eukaryotes (e.g. protozoa or amoeba). EMS are natural inhabitants for most amoeba and protozoa in which a variety of viruses are able to replicate (Baron et al., 1980; Miles, 1988; Diamond, 1991; Wang and Wang, 1991a,b; Kasprzak and Majewska, 1995; Truong et al., 2013). Thus, further research is needed to investigate the ability of PEDv to replicate in non-mammalian eukaryotic hosts.

The presence of viral RNA in EMS simply indicated the presence of virion, while its replicability that reflects the infectivity, has been determined in vitro using cell culture bioassay in this study. To examine the replicability (infectivity) of PEDv in the studied EMS, samples were selectively monitored using VERO cell culture, which is a standard in vitro model for PEDv infectivity (Hofmann and Wyler, 1989; Khatri, 2015), however, the results obtained from such method may provide an underestimation compared to bioassays. In this study, the PEDv in farm-1 EMS showed no infectivity in the top layer, whereas certain infectivity was observed in the top layer of farm-2 EMS during Fall, but not in Spring/Summer. These contradictory results could be explained by the differences in viral shedding status in two farms at the beginning of sampling. The virus shedding

was active at the beginning of sampling in farm-2, which probably contributed to the infectivity of PEDv in the top layer of its EMS in the Fall. Mostly, the top layer of both EMS had low or no infective PEDv, probably due to direct exposure of the environmental UV and sunlight that perhaps effectively reduced the infectivity of the virus.

Generally, in both studied EMS, the infectivity titer of PEDv showed a gradual decrease in the later weeks of sampling. Combining survivability and infectivity data led us to hypothesize that although PEDv may have the ability to find an alternative host(s), and replicate in EMS, the virulence property of the virus might not stay the same when virus is replicating and evolving in a different host than swine. Therefore, further studies are needed to monitor the evolution of PEDv in infected EMS.

CONCLUSION

In summary, this study furthers our existing knowledge of PEDv, indicating high survivability of this virus in the environment, especially in on-farm earthen manure storage system typical of western Canada. In practice, the non-infected farms may share manure applicators with other farms that have been infected with PEDv. Machines used for manure application are potential physical vectors (fomites), which can easily spread the virus if no proper disinfection is practiced. The handling and managing of infected manure is a critical component to reduce the risk of further virus transmission from one farm to others. Frequent agitations of EMS may allow direct exposure of infectious virions from the lower layers to environmental UV and sunlight, and reduce the infectious viruses in EMS, thus, probably decreasing the risk for recurrent infection

within the farm and further spread of viruses. More research should be carried out to better understand the life cycle of PEDv in the environment, as well as its survivability outside the host. Disinfectants and treatment strategies for infected EMS should be reviewed in order to eradicate the PEDv from the environment. Additional studies are needed to monitor the survivability of PEDv in contaminated soils after application of infected manure onto agricultural land.

MANUSCRIPT II (SHORT COMMUNICATION)

Monitoring Survivability of Porcine Deltacoronavirus (PDCoV) in Earthen Manure Storages co-infected with Porcine Epidemic Diarrhea Virus (PEDv)

ABSTRACT

Porcine deltacoronavirus (PDCoV) has been frequently reported to co-infect swine herds with porcine epidemic diarrhea virus (PEDv). Both viruses are transmitted via fecal-oral route. While our recent studies suggest that PEDv survives in the infected on-farm earthen manure storages (EMS) in Manitoba, Canada for more than 9 months from fall to spring, no data is available on the survivability of PDCoV in the environment. Parallel to that study, we evaluated the survivability of PDCoV in the two PEDv infected EMS by determining the viral shedding and its quantitative changes over time in the Fall period. Our data suggest that PDCoV survives in infected EMS for more than two months after the initial shedding in the farm. In farm-1 EMS, the highest amount of PDCoV was found in the first sampling week. During the following weeks the viral copy significantly decreased but it remained constant over time. In farm-2 EMS, the viral copy was stable over the sampling period, while a higher PDCoV detection rate was found at the bottom layer of EMS.

Key words: Swine, porcine deltacoronavirus (PDCoV), earthen manure storage (EMS), virus survivability

INTRODUCTION

Coronaviruses, belonging to Coronaviridae family, are single-stranded, positive-sense, enveloped RNA viruses. Coronaviridae is subdivided into four genera including Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus (Woo et al., 2010). In 2013, a swine enteric coronavirus, porcine epidemic diarrhea virus (PEDv), under genus Alphacoronavirus, was introduced to the USA for the first time, although it was initially detected in UK and Belgium in late 1970s. Subsequent to the 2013 PEDv outbreak, another novel coronavirus, porcine deltacoronavirus (PDCoV; genus Deltacoronavirus) was identified in the USA in February 2014 although it was suggested that PDCoV has been circulated in the USA at least since August 2013 (Sinha et al., 2015). PDCoV is a newer virus compared to PEDv and was discovered for the first time in Hong Kong in 2012 (Wang et al., 2014a; Woo et al., 2012). Both PEDv and PDCoV has been spread into Canada since 2014 (Marthaler et al., 2014b). Interestingly, PDCoV has been frequently found to co-infect with other enteric viruses especially PEDv and rotavirus (Lee and Lee, 2014a; Marthaler et al., 2014a; Marthaler et al., 2014b; Wang et al., 2014a; Song et al., 2015). A study by Jung et al. (2015) confirmed that PDCoV alone could result in enteropathogenic lesions in gnotobiotic pigs. Similar to PEDv, PDCoV induces severe diarrhea, vomiting, fecal shedding of virus, and severe atrophic enteritis (Jung et al., 2015; Song and Park, 2012). Compared to PEDv, however, little is known about PDCoV especially its epidemiology, infectivity and pathogenesis (Chen et al., 2015).

Several studies have demonstrated the strong survivability of PEDv in the environment, emphasizing the importance of biosecurity measures to prevent the spread

of this virus (Pujols and Segalés, 2014; Tun et al., 2016). However, no data is available to date on the survivability of PDCoV. Since PDCoV can contaminate the environment through fecal shedding, the handling of infected manure is critical. In Western Canada, swine manure is usually treated or stored in open on-farm earthen manure storages (EMS or lagoons). Studies have shown that pathogens including viruses might persist in EMS for prolonged periods of time if EMS is not properly treated (Costantini et al., 2007; Dee et al., 2005; Kase et al., 2009; Tun et al., 2016). Our previous report suggests that PEDv could survive in EMS for at least 9 months under the cold climate (Tun et al., 2016). Parallel to that study, here, we investigated the co-infection of PDCoV in the PEDv infected farms and monitored the survivability of the PDCoV in the EMS over time.

MATERIALS AND METHODS

Study Design

Two previously reported PEDv-infected swine farms (farm-1 and farm-2) located in Manitoba, Canada were involved; the outbreak histories of those farms were described previously (Tun et al., 2016). The Veterinary Diagnostic Services of Manitoba confirmed the onset of PEDv outbreak at farm-1 in May 2014 and at farm-2 in September 2014. The farm-1 EMS had an area of 78 m × 46 m with an average depth of 1.5 m, while the farm-2 EMS had an area of 79 m × 73 m with average depth of 0.7 m (Tun et al., 2016). Manure entrance pipe to the EMS was sealed in both farms so the level of manure was stable for the duration of sampling and no wash water and disinfectant were released into the EMS while sampling was ongoing. Samples were taken from different locations at different depth based on the EMS size. From farm-1 EMS, samples were collected from

12 different locations at three depths (n=36/EMS), and samples were collected weekly from September 24 to November 5 in 2014. Farm-2 EMS samples were collected from 16 different locations at two depths (n=32/ EMS), and samples were collected from October 17 to November 7 in 2014.

Real-Time RT-PCR Assay

Both PDCoV and PEDv were detected using reverse transcriptase quantitative PCR (RT-qPCR) assay. RNA was extracted from samples using the MagMAX™-96 Viral RNA Isolation Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. A specific primer set targeting membrane (M) gene of PDCoV (Forward 67F: ATC CTC CAA GGA GGC TAT GC and Reverse 560R: GCG AAT TCT GGA TCG TTG TT) was used to detect this virus. As described previously (Tun et al. 2016), PEDv was detected using primers targeting spike (S) gene (Forward S1F: AGG CGG TTC TTT TCA AAA TTT AAT G, Reverse S1R: GAA ATG CCA ATC TCA AAG CC, Probe 5Cy5-TAT TGG TGA AAA CCA GGG TGT CAA T-3BHQ-2). A gBlocks Gene Fragments (Integrated DNA Technologies, Inc., Coralville, IA, USA) containing the PDCoV M gene target was used with 10-fold serial dilution as described previously (Tun et al., 2016) to transform the cycle threshold (Ct) values into estimated copies of PDCoV RNA per ml of EMS.

Means and standard deviations were calculated. Statistical analysis was performed on RT-qPCR data using chi-square, two-way ANOVA with Sidak's multiple comparison test, and linear regression in Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). P-values < 0.05 were considered significant.

RESULTS AND DISCUSSION

The on-farm status of PDCoV and PEDv was verified by analyzing the presence of virus RNA in fresh feces and pit samples. As summarized in Table 5, the co-infection of PDCoV and PEDv was identified in both farms at the time of testing. In farm-1 (which contained two barns A and B), fresh fecal samples (n=20/barn) were continually collected for three weeks. In pit samples of both barns, PDCoV was not present in the first week. Perhaps there was no prior introduction of PDCoV in that farm. However, the viral shedding in barn-B started in the first week of testing with a low percentage (10%) and increased in later weeks, indicating a recent introduction of PDCoV. In the second week, fresh feces and pit samples from both barns were positive for PDCoV, with the barn-A generally showing higher positivity rates compared to the barn B. Perhaps, the virus transmitted from primary infected barn to the other barn within few days following the onset of the infection, highlighting the importance of biosecurity measures within a farm to prevent the virus spread from barn to barn. Interestingly, in farm-1, over time, and particularly in barn-B, the increasing trend of PDCoV shedding in the fresh feces were negatively associated with the PEDv shedding, which indicated that the infected pigs probably developed the specific immunity against PEDv but not yet for PDCoV. In farm-2, PDCoV was not present in pit samples but it was shed in fresh feces, which implied the recent introduction.

Table 5. The presence of PDCoV and PEDv in pit and fecal samples in farms 1 and 2.

Farm	Barn	Sample type	% of PDCoV positive samples (Number of PEDv positive samples/total number of tested samples)			
			September 24th	**October 1st**	**October 8th**	**October 17th**
1	A	Fresh feces	0(0/20)	75%(15/20)	Barn was emptied	
		Pit	0(0/10)	90%(9/10)	Barn was emptied	
	B	Fresh feces	10%(2/20)	45%(9/20)	100%(20/20)	Barn was emptied
		Pit	0(0/10)	30%(3/10)	10%(1/10)	Barn was emptied
2	NA[1]	Fresh feces	NS[2]	NS	NS	80%(4/5)
		Pit	NS	NS	NS	0(0/5)
Farm	**Barn**	**Sample type**	**% of PEDv positive samples (Number of PEDv positive samples/total number of tested samples)**			
			September 24th	**October 1st**	**October 8th**	**October 17th**
1	A	Fresh feces	15 (3/20)	5 (1/20)	Barn was emptied	
		Pit	100 (10/10)	90 (9/10)	Barn was emptied	
	B	Fresh feces	0 (0/10)	0 (0/10)	0 (0/10)	Barn was emptied
		Pit	20 (2/10)	30 (3/10)	20 (2/10)	Barn was emptied
2	NA[1]	Fresh feces	NS	NS	NS	100 (12/12)
		Pit	NS	NS	NS	100 (12/12)

[1]NA, Not Applicable, only one barn at site#2 was examined.
[2]NS, No Sampling. The first sampling week was on October 17th.

As shown in Table 6, PDCoV was detected in all layers of farm-1 EMS with

generally high positivity rate (25% to 100%) over the study period. However, in farm-2 EMS, PDCoV was less prevalent (0 to 37.5%; Table 7). This may be due to the fact that pigs in farm-2 were infected with PDCoV for a shorter period of time, thus less infected manure was excreted into the EMS. Unlike farm-1 EMS, PDCoV was more prevalent in the bottom layer of farm-2 EMS (Table 7). Particularly in week#1 and week#3, none of the 32 collected manure samples from the top layer of farm-2 EMS were positive. Perhaps, being exposed to the sunlight may have led to destruction of virions, and hence reduced detection rate (Wilhelm et al., 1998).

Table 6. The positivity of PDCoV for each layer of farm-1 EMS at each sampling week.

Sampling Time (Week #, Date)	PDCoV Positivity % (n/N) EMS Layer		
	Top	Middle	Bottom
Week #1 24-Sep-2014	66.7(8/12)	58.3 (7/12)	33.3 (4/12)
Week #2 1-Oct-2014	91.7 (11/12)	100 (12/12)	41.7 (5/12)
Week #3 8-Oct-2014	50 (6/12)	41.7 (5/12)	25 (3/12)
Week #4 15-Oct-2014	91.7 (11/12)	83.3 (10/12)	66.7 (8/12)
Week #5 23-Oct-2014	83.3 (10/12)	83.3 (10/12)	75 (9/12)
Week #6 29-Oct-2014	66.7 (8/12)	75 (9/12)	83.3 (10/12)
Week #7 5-Nov-2014	83.3 (10/12)	83.3 (10/12)	75 (9/12)

Table 7. The positivity of PDCoV for each layer of farm-2 EMS at each sampling week.

Sampling Time (Week #, Date)	PDCoV Positivity % (n/N) EMS Layer	
	Top	Bottom
Week #1 17-Oct-2014	0 (0/16)	12.5 (2/16)
Week #2 24-Oct-2014	12.5 (2/16)	37.5 (6/16)
Week #3 31-Oct-2014	0 (0/16)	12.5 (2/16)
Week #4 7-Nov-2014	18.75 (3/16)	6.25 (1/16)

Figures 6 and 7 present the viral loads of PDCoV in each EMS layer during each sampling week. As shown in Figure 6, in farm-1 EMS, the highest number of PDCoV was detected during the first week (ranging on average from 12,500 to 16,200 copies/ml), but the viral load decreased sharply in the subsequent weeks and became stable over time

(approximately 2,000 copies/ml). In farm-2 EMS, the viral load remained stable from the beginning (1,600 to 2,900 copies/ml; Figure 7). Stable viral loads in both EMS suggests that PDCoV could remain viable in EMS for a long period after shedding. Our previous study on PEDv showed that virus copy numbers increased significantly after the Winter incubation in farm-2 EMS, speculating that PEDv may have found alternative host(s) in the EMS allowing the virus to replicate (Tun et al., 2016). In this study, although we did not observe an increase of PDCoV copy numbers during the Fall months, the virus maintained its population in the EMS. As we did not evaluate the infectivity of the virus, it is not clear whether or not PDCoV particles remained infective in EMS.

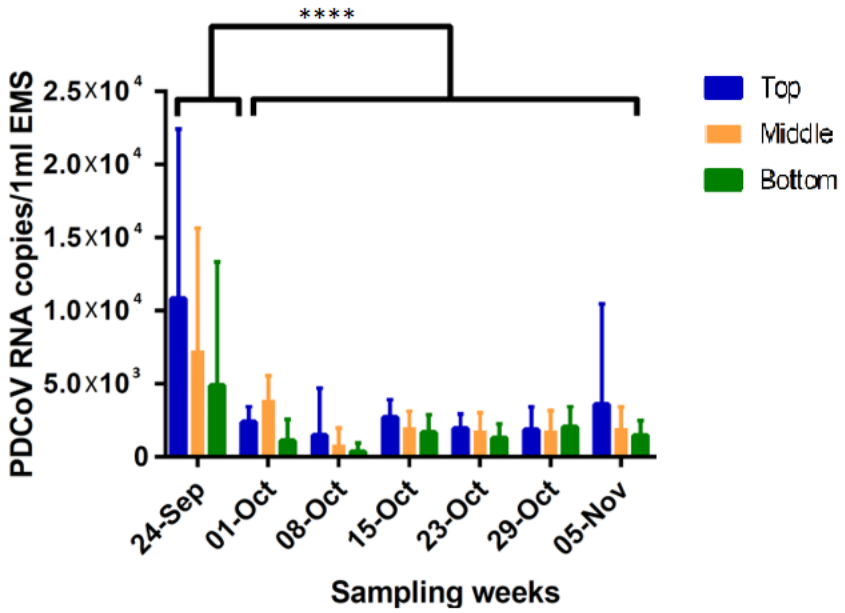

Figure 6. Viral loads of PDCoV for each layer of EMS in farm-1 at each sampling week during Fall 2014. The survivability of PDCoV over time was tested using RT-qPCR assay targeting M gene. The assay was based on the detectable viral RNA copies number in 1 ml of EMS, and each biological sample was analyzed in triplicate. The error bars represent the standard deviation generated from 12 replicates for each layer of the EMS. ****P < 0.0001.

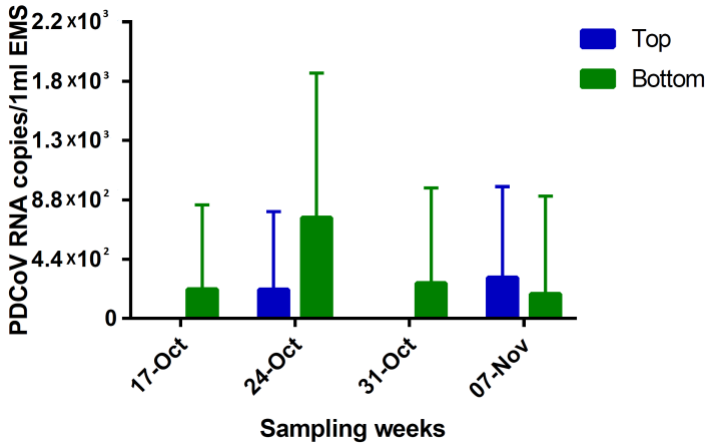

Figure 7. Viral loads of PDCoV for each layer of EMS in farm-2 at each sampling week during Fall 2014. The survivability of PDCoV over time was tested using RT-qPCR targeting M gene. The assay was based on the detectable viral RNA copy number in 1 ml of EMS, and each biological sample was analyzed in triplicate. The error bars represent the standard deviation generated from 16 replicates per layer of the EMS.

Most coronaviruses are transmitted through either airborne or fecal-oral route and have been found to survive outside of their host (Gundy et al., 2009). The survival period varies depending on the type of virus and the environmental conditions. For instance, transmissible gastroenteritis virus (TGEV) and mouse hepatitis virus (MHV) could remain viable in water and sewage from days to weeks (Casanova et al., 2009); while PEDv has been found to survive in different environments from weeks to months (Pujols and Segalés, 2014; Tun et al., 2016); and feline infectious peritonitis virus (FIPv), and human coronavirus 229E (HCoV) could survive in water at 4°C for more than three months (Gundy et al., 2009). In this study, the initial introduction of PDCoV at farm-1 perhaps happened in September 2014. The virus was consistently detected in the EMS throughout October and November 2014. As manure entrance pipe to the EMS was sealed and no new infected manure was released into the EMS while sampling was ongoing, we can conclude that the PDCoV can survive in the EMS for at least 2 months.

The role of EMS in promoting or reducing pathogen load is controversial. While some studies have suggested that EMS could reduce pathogen load over time, others have shown otherwise (Hill and Sobsey, 2001; Costantini et al., 2007; Tun et al., 2016). For instance, swine hepatitis E virus (HEV) has been shown to survive under EMS conditions (Kase et al., 2009), and PEDv was reported to remain viable in EMS for 9 months. In the latter case, PEDv numbers even went up following Winter incubation in EMS while manure entrance pipe to the EMS was sealed and there was no new introduction of infected manure into the EMS (Tun et al., 2016). This suggests that EMS might provide an alternative host(s), such as non-mammalian eukaryotes, to the virus, resulting in an increased viral copy number after a given incubation period (Tun et al., 2016). Hence,

without proper treatment, both PDCoV and PEDv may survive in the EMS for prolonged periods.

CONCLUSION

To our knowledge, this is the first study evaluating survivability of PDCoV in the environment. Although EMS has been used for decades for livestock manure storage and treatment, our results suggests that the natural inactivation of PDCoV in EMS could take long, as two months after the initial introduction our samples were still PDCoV positive. Considering swine manure is a valuable fertilizer for crops, application of infected manure poses a potential risk to the naïve environments. Further studies are required to determine the infectivity of PDCoV, particularly after long incubation periods from Fall over the Winter to Spring. Effective and timely treatment of manure is also critical in preventing the spread of this virus.

MANUSCRIPT III

Monitoring Survivability of Porcine Epidemic Diarrhea Virus (PEDv) Following Surface Application of PEDv-Positive Manure to the Soil

ABSTRACT

Outbreaks of virulent variants of porcine epidemic diarrhea virus (PEDv) remain a great threat to the North American and global pig industry. Research done in Manitoba has shown that not only can the PED virus survive Manitoba winters, the virus can potentially replicate throughout winter in the earthen manure storages (EMS). However, epidemiological investigation of PEDv survivability in the soil following surface application or injection of PEDv-positive manure is still lacking. Here, we tested the survivability of PEDv in the soil after the surface application of PEDv-positive manure. PEDv positive manure from an infected farm in Manitoba were surface applied in a perennial field on Nov 5, 2016 at a rate of 7-8 k gallons per acre. Soil samples were collected from 20 different field locations (10 on manure application band and 10 off-band) on Nov 25, 2016, three weeks after the manure application. During this period, daytime high temperatures averaged 8°C, and overnight low temperatures averaged -0.6°C. Samples were kept at -80°C until Feb 2017 when RNA were extracted from two subsets of 2 g soil samples. RT-qPCR was performed to determine the presence and concentration of PED virus. For each soil subset, 3 qPCR replications were included. The qPCR quantification cycle (Cq) values were transformed into estimated copies of PEDv RNA per gram of original soil using a serially diluted gBlocks Gene Fragments containing the PEDv gene target. PEDv was detectable in 19 out of 20 soil samples (95%). The average PEDv copy numbers per gram of soil for on-band and off-band samples were 125 and 95, respectively. For on-band samples, five out of ten (50%) were considered positive (>100 copies /g soil), while four samples were weak positive (2<copies<100 /g soil). Three off-band (30%) samples were positive, while the rest of

them (70%) were weak positive. In summary, PED virus was detectable in soil samples collected three weeks after surface application of PEDv-positive manure in a perennial field from both on- and off-band locations. Our data were indicative of the presence of virion (PED RNA) in 95% of soil samples in relatively low amounts. Confirming virus infectivity (ability to replicate) is critical in understanding the risk posed by soils when surface-applied by the PEDv positive manure.

Key words: Porcine epidemic diarrhea virus (PEDv), soil, virus survivability

INTRODUCTION

Outbreaks of virulent variants of porcine epidemic diarrhea virus (PEDv) remain a great threat to the North America and global pig industry. Fecal-oral route is considered the main route of PEDv transmission (Song and Park, 2012). However, it is the capability of PEDv to survive in the environment that leads to prolonged outbreaks (Lowe et al., 2014; Pasick et al., 2014). Research done in Manitoba, Canada has shown that PED virus not only could survive in the earthen manure storages (EMS) for at least 9 months (from Fall into Winter and Spring), but possibly found alternative host(s) and replicated over that period (Tun et al., 2016). The improperly treated manures may introduce the pathogen to the environment after manure is land applied. It has long been noticed that viruses shed in animal or human wastes can be reserved in soil for prolonged periods of time (Hurst et al., 1980; Tierney et al., 1977; Yeager and O'Brien, 1979). Environmental factors such as temperature and moisture can significantly affect viral persistence in soil (Sobsey and Meschke, 2003). Certain types of soil, particularly farming soils, contain high organic matters, salts and moisture that may support the persistence of viruses (Sobsey and Meschke, 2003). Additionally, Manitoba has many cold months year around where the average temperature decreases to minus Celsius degrees, which favor the viral survival in the environment (Tun et al., 2016). Following application of infected manure, the affected areas could be larger than the applied surfaces, as microorganisms can be transported via the water and human/animal activities (Cole et al., 2000).

There are concerns about PED virus surviving in the soil after the manure infected with PEDv is applied to land and the potential for this PEDv contaminated soil to become a vector for infecting pigs. However, no published data exist regarding the survivability

and infectivity of PEDv in soils under the field conditions. In this study, we first evaluated methods for PEDv RNA preparation from soil and viral infectivity testing using cell culture. Further we investigated the survivability of PEDv in the field soil after the surface application of infected manures in Manitoba.

MATERIALS AND METHODS

Study Design

PEDv positive manures from an infected farm in Manitoba were land applied in a perennial field in November 2016 at the rate of 7-8 k gallons per acre. Eight manure samples were collected prior to the application to test the status of the viral shedding in the manure. Soil samples were collected from 20 different field locations [10 within manure application band (on-band) and 10 outside the manure application band (off-band)] on Nov 25, 2016, three weeks after the manure application.

Soil properties analysis

A 500g dry equivalent soil was pooled from 10 off-band samples. The sample was subjected to AgVise (Northwood, North Dakota, USA) for measurement of biological characteristics and pH.

Evaluation of PEDv RNA extraction from the soil and viral survivability

PEDv negative soil and manure slurry (manure in liquid form used for agriculture) from the PEDv infected barn was obtained. The slurry was spiked to soil samples at increasing rates from 0, 0.36, 0.9, 1.19, 1.8, 2.7, 3.6, and 4.5 ml per 100 g dry equivalent soil with four replicates for each rate. The addition rate of 3.6 ml was

equivalent to slurry addition increasing soil moisture by 0.05 g H2O/g dry soil which represents a scenario of slurry application of 11,000 US gal/ac (~103,000 L/Ha) and thorough incorporation into the top 7" (~18 cm) of soil –the industry standard.

The viral genomic RNA was extracted from 2g of spiked soil samples using the MoBio Power Soil RNA isolation kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instruction. Additionally, spiked soil samples were incubated at 10°C for 30 days, and then the viral survivability assay was repeated following the same procedures to determine the viral survivability under the experimental setting.

For the field samples, viral genomic RNA was extracted from the infected manure before land application (8 replicates) and from the field soil samples (two extractions per sample each on a 2g subsample). Thus, the total number of soil extractions included: (10 soil samples on manure application band + 10 off-band) × 2 extraction/sample = 40 extractions. Two negative controls (NC; PEDv-free field soil) were also included in the analyses.

Real-Time RT-PCR Assay

Viral RNA extracted from infected manure as well as soil samples were subjected to molecular detection for virulence (subgenogroup 2a) strain of PEDv using real-time RT-PCR as described previously (Wang et al., 2014c; Tun et al., 2016), which used spike gene primers (Forward S1F: AGG CGG TTC TTT TCA AAA TTT AAT G and Reverse S1R: GAA ATG CCA ATC TCA AAG CC) and a specific probe targeted to virulent PEDv (Virulent S1P: 5Cy5-TAT TGG TGA AAA CCA GGG TGT CAA T-3BHQ-2, and). The primer and hydrolysis probe utilized the AgPath-IDTM One-Step RT-PCR Reagents

(Life Technologies, Grand Island, NY, USA) and 2 µl of RNA with the CFX348 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) under the following thermal cycling conditions, reverse transcription, 30 min at 50°C; Taq activation, 15 min at 95°C; followed by 40 cycles of 10 s at 94°C and 30 s at 54°C. A 500 bp of spike gene fragment of PEDv strain OH1414 (virulent PEDv) was chosen to use as both standard and positive control. A gBlocks Gene Fragments (Integrated DNA Technologies, Inc., Coralville, IA, USA) containing the PEDv spike gene target was synthesized. Stock concentration of 10^9 copies/µl were made and a 10-fold serial dilution was run on the RT-qPCR to generate a standard curve, which was used to transform the Cq values into estimated copies of PEDv RNA per gram of soil. The sensitivity of the RT-qPCR assay was validated through serial dilutions of the gene fragment, and triplicate of each dilution were run in the assay. The detection limit was two copies of PEDv. As shown in Figure 8, there is a strong linear correlation ($r^2 > 0.99$) between Cq values and the corresponding amount of gene fragment copy numbers for PEDv. The standard curve of PEDv was plotted with slopes of -3.358. The amplification efficiencies of the assay was 98.5. For each soil subset, 3 qPCR replications were included, and generated Cq values were transformed into copy numbers based on the slope of standard curve. Subsequently, the resulting copy numbers were transformed into copy numbers in 1g of soil sample.

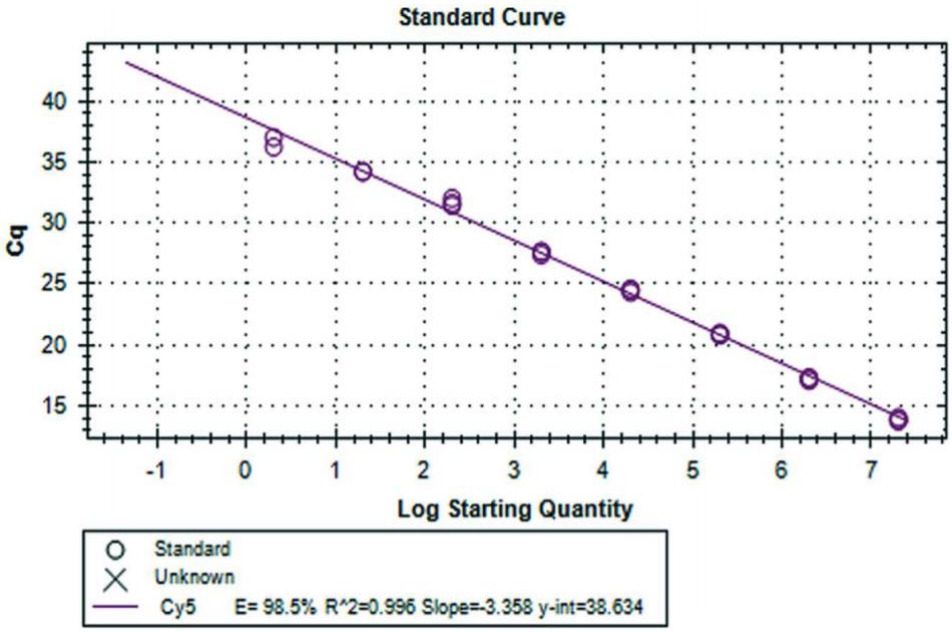

Figure 8. Standard curve of the RT-qPCR assay for synthetic DNA virulent PEDv strain.

Determination of the infectivity of PEDv

The level of infectious virus in 8 manure slurry and 20 field soil samples was determined in triplicate by titration in 96-well microtiter plates seeded with VERO cells (ATCC CCL-81). One gram of soil sample was mixed with 1ml phosphate buffer saline (PBS) to elute the virus. One milliliters of vial PEDv culture (10^3 $TCID_{50}$) was mixed with 1g soil to be used as the positive control. After the mixture was centrifuged at 10,000 × *g* for 10 min, the supernatant was filtrated first using a 0.45 micron syringe filter followed by a 0.22 micron syringe filter (VWR International Inc., Radnor, PA, USA). The filtrate was 10-fold serial diluted with PBS then inoculated in Vero cells as previously described with minor changes (Hofmann and Wyler, 1989; Chen et al., 2014; Tun et al., 2016). Briefly, VERO cells were washed twice with 100 µl PBS then inoculated with 200 µl of sample. After incubation at 37°C with 5% CO2 for an hour, the

cells were washed with 100 µl PBS twice. Then, 200 µl post-inoculation medium was added to the cells. The medium was Minimum Essential Medium (MEM) supplemented with 50µl/ml Gentamicin Sulfate, 20mM GlutaMax, 0.33% Tryptose Phosphate Broth, and 1.0ug/ml TPCK Trypsin. The assay was performed in the presence of pure viable PEDv (Colorado 2013 Isolate, National Veterinary Services Laboratories, Ames, IA) as a positive control. After a strong syncytia formation was witnessed, the plates were subjected to RNA extraction and RT-qPCR. The virus titters were determined according to the Reed and Muench method and expressed as the 50% tissue culture infective dose ($TCID_{50}$)/ml as described previously (Tun et al., 2016).

RESULTS AND DISCUSSION

Our previous study suggested that PEDv could survive in the EMS for a prolonged period of time (Tun et al., 2016). To further explore the survivability of PEDv in the field, we monitored the fate of PEDv in the soil that was subjected to the PEDv-infected manure.

Before the field soil samples were tested, we analyzed the efficiency of PEDv RNA extraction by spiking the PEDv positive manure slurry to the soil at increasing rates to generate a standard curve. To simulate the field conditions in this study, the spiked soils were incubated at 10°C for 30 days following which the survivability assay was repeated. Figure 8 presents the viral copies in the soil after the soil was spiked with the manure slurry containing PEDv concentration of 4.45E+05 copies/ml at different rates as was described above. Each spiked rate included 4 replicates. A regression line was

generated based on the spiked rates and the viral copies in the soil. The virus was detected at the lowest spike rate (0.36 mL, 81.92 copies). After the incubation at 10°C for 30 days, the slope of regression equation was similar to the original samples, however, the constant number increased from 194.65 to 525.36. Thus, a slight decrease of PEDv was witnessed after a 30-day incubation under the experimental setting, however, the virus still survived over that period in the soil. The R^2 of the original samples and incubated samples were 0.65 and 0.56, respectively. There were a few factors which may cause the variation of the viral concentration at each spike rate replicates. Complete homogeneous mixing of the manure with 100g dry equivalent soils is difficult by hand, especially at lower spike rates; further, RNA extraction from soil is considered challenging due to the co-extraction of humic acids and other organic compounds, which may inhibit downstream PCR analyses (Mettel et al., 2010). There was no previous study optimizing and evaluating the PEDv RNA extraction from soils, so there was no reference data that can be compared with. However, R^2 cannot suggest whether a regression is biased, as such we could not conclude if the results over or under-estimated the viral concentration in the soil. While our data present the degree of accuracy of the PEDv RNA extraction from the soil that can be used as a reference for future research.

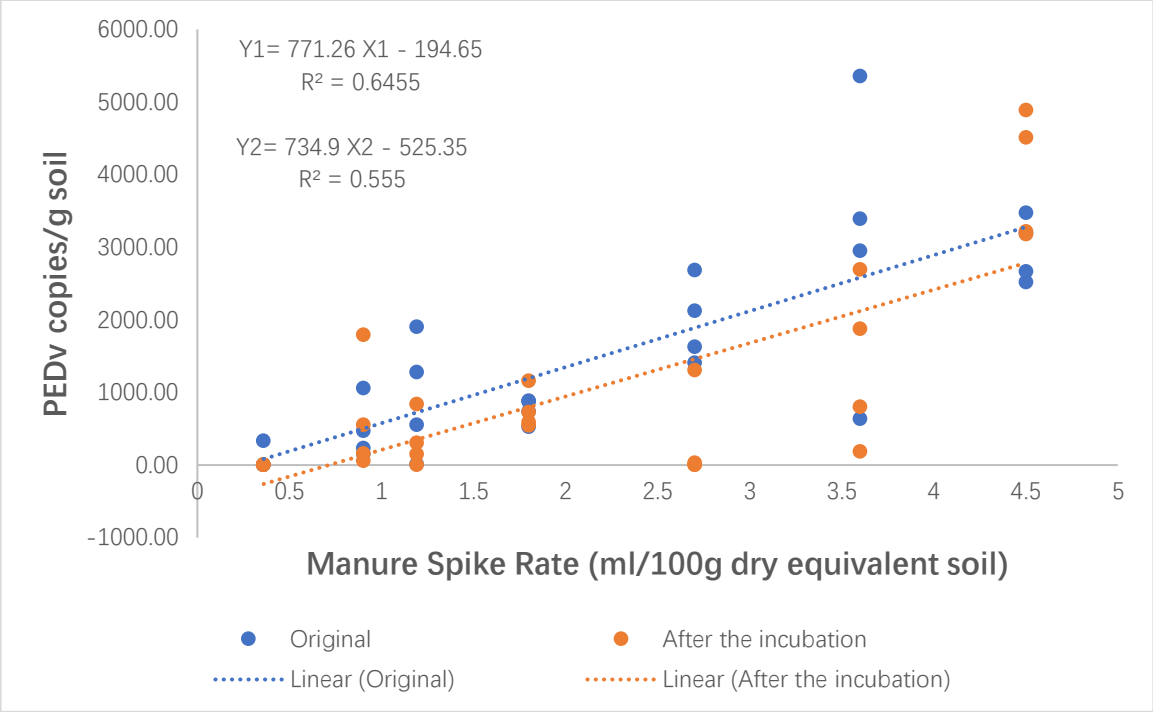

Figure 9. The regression lines of PEDv copy numbers (copies/g soil) in the 100g dry equivalent soil following different manure slurry spiked rates. The blue line (Y1) was generated from the original spiked soil samples, and the orange line (Y2) was generated from the spiked soil after the incubation at 10°C for 30 days. Each spiked rate included 4 replicates. For each replicate, 2 g of soil sample was RNA extracted using the MoBio Power Soil RNA isolation kit. Each biological sample was analyzed in triplicate using RT-qPCR to calculate the viral copies in 1g of soil.

The daily temperature during November 2016 at the study field is presented in Figure 10. During this period, daytime high temperatures averaged 8°C, and overnight low temperatures averaged -0.6°C.

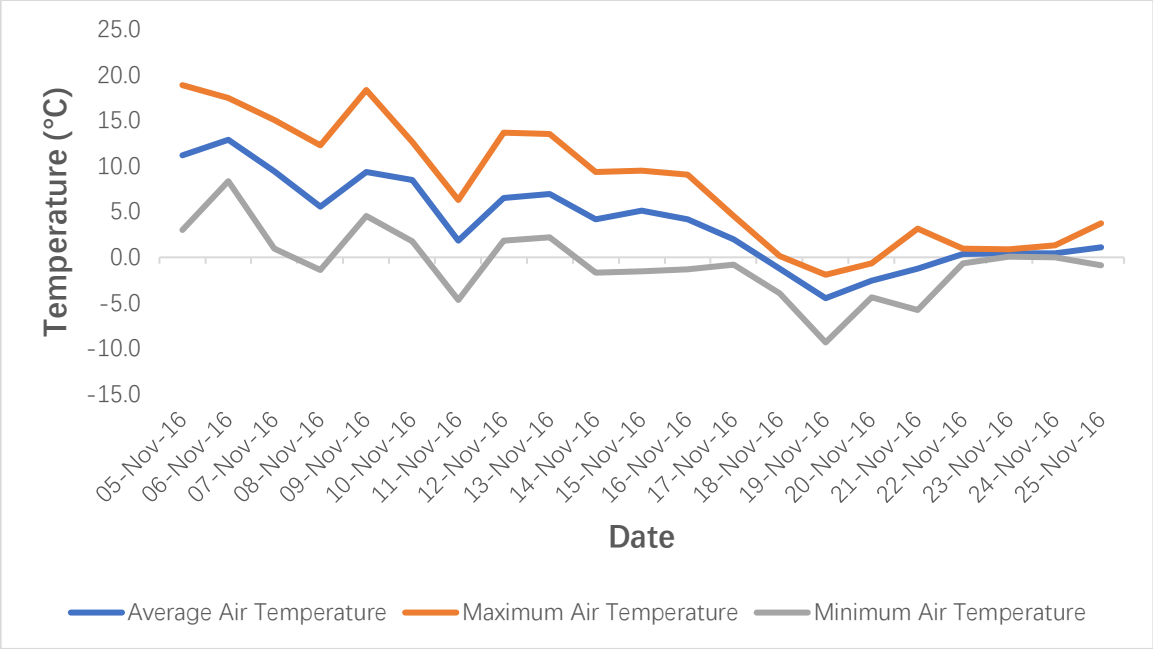

Figure 10. Temperature dynamics at the study filed during November 5th – 25th, 2016.

Off-band soil pH was 8.0 when measured using water. This pH is estimated to be about 0.5 unit higher than 0.01 M $CaCl_2$ method. The soil texture was silt clay loam, containing high organic matter (6.8%) and salts (Figure 11). Such type of soils could offer protection for viruses (Chung and Sobsey, 1993; Jin et al., 2000; Straub et al., 1993). Studies have suggested that viruses adsorbed onto a particle may have lower die-off rates than free viruses (Yeager and O'Brien, 1979; Sobsey and Meschke, 2003). Soils containing high organic matter and salts increase the adsorption force between microorganisms and soil particles (Sobsey and Meschke, 2003). Moreover, the hydrophobic property of the coronaviruses envelope makes them easier to be adsorbed onto soil particles (Sobsey and Meschke, 2003). Clays may further protect viruses by creating a barrier against microbial predators of viruses (Santamaria and Toranzos, 2003). Thus, viruses may easily survive in the clay soils, especially when they are intensely

adsorbed onto soil particles by the effects of organic matter and salts.

Depth	N ppm	P* ppm	K ppm	S ppm	Ca ppm	Mg ppm	Na ppm	B ppm	Cu ppm	Fe ppm	Mn ppm	Zn ppm	Cl ppm	pH	EC dS/m	OM %
0-6	15	84.0	570	8	5600	990	61							8.0	0.42	6.8

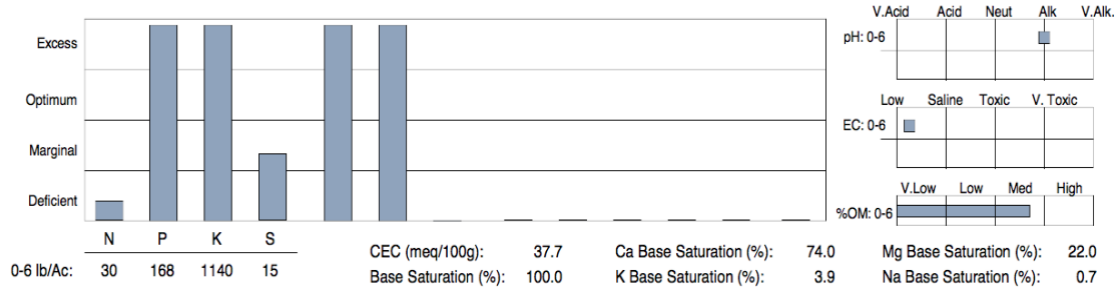

Figure 11. Soil characteristics. *Bicarbonate-Extractable (Olsen) Phosphate. EC: electrical conductivity, OM: organic matter.

Prior to the soil sampling, we collected 8 manure samples to analyze the status of PEDv in the manures before they were land applied. All the samples were positive with PEDv copies ranging from 4.11E+05 to 2.07E+04 copies/ml of manure (Table 8). The average viral load was 1.19E+05 copies/ml, which was similar to our previous report of average 1.10E+05 copies/ml in the EMS (Tun et al., 2016).

Table 8. PEDv viral load in the infected manure slurry.

Sample ID	PEDv load (copies/ml)
16-13352-4	1.78E+05
16-13352-5	4.11E+05
16-13352-6	2.34E+05
16-13386-5	3.85E+04
16-13386-12	2.40E+04
16-13386-13	2.07E+04
16-13386-14	2.13E+04
16-13386-15	2.80E+04

It is important to note that PEDv status of samples reported by majority of diagnostics and research labs is qualitative (negative, weak positive, positive) and is assigned to samples based on their quantitative cycle (Cq) value of the qPCR (negative: Cq > 38; weak positive: 35< Cq <38; positive: Cq < 35). Such descriptive status is not accurate for two reasons. Firstly, Cq values are run-specific and cannot be compared from run to run or across different real-time qPCR equipment and studies. Secondly, recent work by Thomas et al. (2015) has shown that infectious dose of PEDv is age dependent and increases as piglets age. For instance, in 5-day-old piglets, a 10ml inoculum of a US virulent PEDv isolate (USA/IN19338/2013) with titer 0.056 TCID50/ml –which is corresponding to a Cq value of 37.6– resulted in 100% infection (Thomas et al., 2015); whereas, 10ml of inoculum with titer 0.0056 TCID50/ml (Cq > 45) resulted in infection in 25% of the pigs. Based on the Cq value criteria described above, the first inoculum would have been considered weak positive, and the latter, as negative which were both incorrect for 5-day-old piglets. Thomas et al. (2015) further showed that in 21-day-old pigs, 10ml of inoculum with titers ranging from 560 to 5.6 TCID50/ml (Cq range of 24.2

to 31.4) resulted in 100% infection, while 10ml of inoculum with titers ranging from 0.56 to 0.0056 TCID50/ml (Ct range of 35.3 to >45) did not establish infection. Based on the Cq value criteria, the first group of inoculums would have been considered positive, and the second group of inoculums as weak positive or negative. In this case, the assignment of PEDv status based on Cq values was closer to reality for 21-day-old pigs.

To address the above concerns, we transformed the Cq values into PEDv RNA copy numbers according to the slope of standard curve generated by serial dilution of the gene fragment. This approach eliminates run-to-run variability in Cq values and makes data comparable across runs/equipment/studies. The detection limit of our assay is 2 RNA copy of PEDv per ml or g of sample. We next set up a threshold of 100 copies/ml or g to further divide the PEDv positive samples into positive (>100 PEDv copies/g) and weak positive (2< PEDv copies<100 /ml or g). The 100-copy threshold was set based on previous studies reported the infectious dose of PED virus using animal bioassays in different age groups of pigs (Thomas et al., 2015).

In this study, the average PEDv RNA copy number per sample for field soil samples was calculated based on the Cq value of the 6 qPCR data points [2 biological replicates (the subsamples) × 3 qPCR technical replicates per subsample]. Our data indicates that three weeks after manure was surface applied on the land, trace amount of PEDv (28 – 359 copies/g) could be found in 19 out of 20 soil samples (95%), including all of the samples that were taken off-band (Table 9). For on-band samples, five out of ten (50%) were considered positive, while four samples (40%) were weak positive. Three off-band (30%) samples were positive, while the rest of them (70%) were week positive. The average PEDv copies in on-band and off-band samples were 125 and 95 copies/g,

respectively. The result corresponds to our lab observation that PEDv could survive in the soil at 10°C for at least 30 days.

There was no significant difference in the viral copy number between on-band and off-band samples. There is a possibility that human/animal activities contributed to the spread of virus from on-band to off-band locations. We, however, speculate that rainfall was the main contributing factor to the spread of virus as according to the weather records there were 4 rainy days (0.4 – 5.4 mm) during the study period that perhaps resulted in runoff from on-band to off-band locations. The small size of viruses facilitates their infiltration into soil and aids their migration to considerable distances via water and rainfall (Borchardt et al., 2003). We should also keep in mind that soil structure can play an important role in the movement of viruses in the soil (Borchardt et al., 2003). Water saturation state is another factor contributing to the microbial movement as viruses and microorganisms can pass through soil if all pores are filled with water (Santamaria and Toranzos 2003; Azadpour-Keeley and Ward, 2005). This increases the chance of virus penetration to aquifers and transmission via groundwater, allowing them travel long distances (Borchardt et al., 2003; Payment, 1989). Taking into account that PEDv infectious dose is considerably low, this transmissibility could imply a high biosecurity risk (Thomas et al. 2015a).

Table 9. Quantification of PEDv copy number in soil samples that were surface applied with PEDv-positive manure.

Sample ID	location	Avg. copy # in 6 rep /g soil	Avg. copy # in manure applied locations
Neg Ctrl-1*	NA	0	
Neg Ctrl-2*	NA	0	
Sample1	On-band	0	125
Sample2	On-band	108.4	
Sample3	On-band	359.12	
Sample4	On-band	65.86	
Sample5	On-band	115.13	
Sample6	On-band	246.59	
Sample7	On-band	51	
Sample8	On-band	209.88	
Sample9	On-band	29.39	
Sample10	On-band	64.38	
Sample11	Off-band	181.04	95
Sample12	Off-band	87.57	
Sample13	Off-band	287.64	
Sample14	Off-band	62.04	
Sample15	Off-band	102.58	
Sample16	Off-band	56.15	
Sample17	Off-band	28.49	
Sample18	Off-band	45.56	
Sample19	Off-band	47.89	
Sample20	Off-band	46.49	

* Negative control 1 and 2 were PEDv negative soil samples incorporated into the RNA extraction step.

According to our cell culture results, none of the soil samples were infective. The cell culture assay is considered less sensitive compared to the swine bioassays (Choudhury et al., 2016). The detection limit of our cell culture assay was 1.0E+3 TCID50/ml, however, the PEDv titer in the soil were below this threshold. Hence, our negative cell culture results most likely underestimated viral infectivity.

This study has a few shortcomings; only limited field samples were analyzed and the investigation was not longitudinal. Our previous study suggests that PEDv could survive in the EMS for at least 9 months from Fall into Winter and Spring under Manitoba climate condition. Thus, conducting a swine bioassay following by examination of soil from the same manure application locations collected in the Spring could provide additional information regarding the survivability and infectivity of PEDv in soil. The Spring samples have been collected and will be examined in the upcoming studies. Currently, we cannot conclude whether or not the observed PED viruses were infective, as our cell culture assay was not sensitive enough to test the infectivity of such trace amounts of PEDv found in the soil.

CONCLUSION

This study demonstrates that PED virus was detectable in soil samples collected three weeks after surface application of PEDv-positive manure in a perennial field from both on- and off-band locations. Our data were indicative of the presence of virion (PED RNA) in 95% of soil samples in relatively low numbers. Due to low copy number of virus in the soil, and low sensitivity of cell culture assay to PEDv titers below 1.0E+3 TCID50/ml, we cannot confirm if the observed viruses were remained infective (able to replicate). This is critical in understanding the risk posed by soils when surface-applied

by the PEDv positive manure. Performing an animal bioassay can help out with tackling this question.

GENERAL DISCUSSION

Outbreaks of PEDv and PDCoV remain a great threat to the North American and global pig industry. In Manitoba, 90 cases (some farms had repeated cases) have been confirmed up to date (Dec 2017) where most cases were from the current year. The PED outbreak, therefore, is likely to continue to occur if no effective measures are applied. Fecal-oral route is considered the main route of PEDv transmission, and hence the infected manure handling is critical to control the spread of this disease. EMS is a traditional manure storage system that has been widely used in Western Canada. The possibility of pathogens persistence in the EMS has long been recognized (Costantini et al., 2007). When EMS are getting emptied and stored manure is land applied, the survived pathogens can potentially contaminate large areas of soil and the groundwater (Cole et al., 2000). People and animals constantly contact with soils; hence the viruses may enter farms via contaminated vehicles, people who wear contaminated work clothing and footwear, or wild animals such as rodents and birds (Lee, 2015). Although currently, neither mice and sparrows have been found to be infected by PEDv after the experimental or natural exposure, animals interacted with contaminants may still carry the viruses into the farms as physical vectors (Authie et al., 2014). Thus, whether PEDv and PDCoV can survive in the field is an important question to answer in order to allow farmers and manure applicators to take necessary precautions.

In the environment, the PED virus prefers wet and cool conditions. Under an experimental setting, Goyal (2013) observed that PEDv could survive in inoculated manure at room temperature for 14 days and at -20°C to 4°C for at least 28 days. Thomas et al. (2015b) also reported that PEDv could survive at 20°C for 7 days in swine feces on

metal surfaces. Our studies extend the current knowledge about the survivability of PEDv in the field environment. In Manuscript 1, we showed that PEDv was able to survive in the EMS from Fall into Winter and Spring for 9 months under Manitoba conditions (range of -30°C to 23°C according to 2014–2015 weather records). PEDv not only could survive long in the EMS, but also increased in number over time while there was no new influx of infected manure into the EMS, suggesting that the virus may be able to replicate in the EMS. Although PEDv is considered a genuine swine virus, coronaviruses in general are considered highly mutable. The host range of coronaviruses may be changed due to mutations, deletions, and recombinations in the genes, especially S gene (Lau and Chan, 2015; Su et al., 2016). The fact that samples collected on later weeks had less infectivity despite increased viral loads, suggests that the potential evolution and adaptation in the new host(s) leads to decreased viral infectivity in VERO cells.

The farms that infected with PEDv were confirmed to be co-infected with PDCoV (Manuscript 2). In one farm PDCoV was found to be transmitted from the initial infected barn to another barn within a few days, emphasizing the importance of within-farm biosecurity measures to prevent the virus spread between the barns. In the EMS, a strong survivability of PDCoV has also been witnessed as we constantly detected PDCoV for up to 2 months after the outbreak. However, during this period, PDCoV population could remain relatively stable over time, so we speculate that PDCoV could also survive in the EMS.

Knowing the viruses could persist in the EMS, we investigated if PEDv could survive in the soil after the infected manure was land applied (Manuscript 3). Three weeks after the manure application, trace amounts of PEDv was found in 95% of soil

samples including the off-band manure applied locations. Over the period from the manure application to the sampling, the temperature ranged from 18.9°C to -9.3°C according to the weather records, which is a tolerable range for PED virus (Hofmann and Wyler, 1989). In the environment, viruses adsorbed onto a particle may have lower die-off rates than free viruses (Yeager and O'Brien, 1979; Sobsey and Meschke, 2003). Thus, the clay farming soil containing high organic matter and salts may easily preserve viruses, as organic matter and salts increase adsorption rate between viruses and soil particles (Sobsey and Meschke, 2003). Moreover, the hydrophobic envelope of PEDv makes the adsorption force more intense (Sobsey and Meschke, 2003). There were 4 rainy days (0.4 – 5.4 mm) during the study period, which may explain why the virus could be found in off-band locations.

The present research suggests that the natural degradation of PEDv and PDCoV in the environment could be a long process under Manitoba climatic condition. The viruses may spread into the uninfected environment via ground and surface water, bioaerosols and humans/animals' activities, which can result in infecting other susceptible hosts (Cole et al., 2000). To eradicate the viruses from the positive sites, effective disinfectants treatment must be applied. Thus, finding the effective virucides to eliminate the virus from the environment could be the next step of the study.

CONCLUSION

Based on the results of our studies, our original hypotheses can be evaluated as below:

Hypothesis 1: PEDv survives more than 28 days –reported previously under laboratory conditions– after the initial outbreak in the farm in the infected earthen manure storages (EMS) under Manitoba climatic condition.

- ✓ PEDv could persist for at least 9 months in the infected EMS under the Manitoba Fall, Winter, and Spring conditions.

Hypothesis 2: Survived PEDv is viable and able to replicate when introduced to its host.

- ✓ PEDv not only survived in the EMS but also sustained its infectivity.
- ✓ The increase in PEDv copy numbers over the incubation period in the EMS suggests that the virus may have found an alternative host(s) in the EMS.
- ✓ PEDv in Spring samples had less infectivity despite having higher viral loads compared to the Fall samples, suggesting that the evolution and adaptation of the virus may have had negative effect on its infectivity in the VERO cell line.

Hypothesis 3: PDCoV co-infected with PEDv survives in the EMS for a prolonged period.

- ✓ PDCoV was co-infecting with PEDv, and could also persist for at least 2 months in the infected EMS under Manitoba Fall conditions.

Hypothesis 4: PEDv survives in the soil after the land application of PEDv-infected manure.

- ✓ Trace amounts of PEDv could be found in 95% of soil samples 3 weeks following the surface application of PEDv-infected manure on land.

Hypothesis 5: Survived PEDv in the soil is viable and able to replicate when introduced to its host.

- × Due to low copy number of PEDv in the soil, and low sensitivity of VERO cell culture assay to PEDv titers below 1.0E+3 TCID50/ml, it cannot be confirmed whether or not the survived viruses were remained infective and were able to replicate.

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