

**Efficacy of two commercial disinfectants against porcine epidemic diarrhea virus (PEDV)  
in livestock trailers under freezing conditions**

by

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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## **DEDICATION**

This thesis is dedicated to my husband, Kyle Baker, dogs, Mildred and Mabel, and all of the pigs that participated in my research projects.

## TABLE OF CONTENTS

	Page
LIST OF FIGURES .....	v
LIST OF TABLES .....	vi
ACKNOWLEDGMENTS .....	vii
ABSTRACT.....	viii
CHAPTER 1. GENERAL INTRODUCTION .....	1
Thesis Introduction .....	1
Thesis Organization.....	2
Thesis Objectives.....	3
CHAPTER 2. A REVIEW OF METHODS SUFFICIENT TO INACTIVATE SWINE ENTERIC CORONAVIRUSES .....	4
Introduction .....	4
Materials and methods.....	5
Definition of the review question.....	5
Scope (PICO) .....	5
Indexes searched.....	6
Search terms .....	6
Screening criteria.....	7
Results .....	7
The effect of pH on swine enteric coronavirus survival .....	7
The effect of temperature and time on swine enteric coronavirus survival .....	8
Description of the swine enteric coronaviruses.....	10
The effect of chemical disinfection on swine enteric coronavirus survival .....	12
Discussion.....	13
References .....	18
CHAPTER 3. EVALUATION OF AN ACCELERATED HYDROGEN PEROXIDE DISINFECTANT TO INACTIVATE PORCINE EPIDEMIC DIARRHEA VIRUS IN SWINE FECES ON ALUMINUM SURFACES UNDER FREEZING CONDITIONS .....	22
Abstract.....	22
Introduction .....	23
Methods .....	26
Experimental design .....	26
Coupons.....	27
Treatment groups.....	28
Contamination and disinfection procedures .....	28
Swine Bioassay.....	32
Results .....	35
Pre-trial diagnostic screening .....	35

Coupon Swabs .....	35
Swine bioassay .....	36
Discussion.....	36
Conclusions .....	39
Tables.....	40
Figures .....	43
Declarations .....	45
Ethics approval and consent to participate .....	45
Competing interests .....	45
Funding.....	45
Authors' contributions.....	45
Acknowledgements .....	45
References .....	46
 CHAPTER 4. EVALUATION OF A PEROXYGEN-BASED DISINFECTANT FOR INACTIVATION OF PORCINE EPIDEMIC DIARRHEA VIRUS AT LOW TEMPERATURES ON METAL SURFACES .....	49
Abstract.....	49
Introduction .....	50
Methods .....	52
Experimental design .....	52
Coupons.....	53
Treatment groups.....	54
Peroxygen-based disinfectant.....	54
PEDV propagation and titration .....	55
Contamination material .....	55
Contamination and disinfection of aluminum coupons.....	56
Swine bioassay procedures.....	61
Results .....	64
Pre-trial diagnostic screening .....	64
Swine bioassay .....	65
Virus isolation .....	66
Discussion.....	67
Tables.....	70
Figures .....	73
Acknowledgements .....	73
Conflict of interest .....	74
References .....	74
 CHAPTER 5. GENERAL CONCLUSION .....	78
Summary of thesis conclusions .....	78
Clinical application of findings .....	81
Implications .....	84
 REFERENCES .....	86

**LIST OF FIGURES**

	Page
Figure 3.1 Fecal contamination of aluminum coupons.....	43
Figure 3.2 Collection of feces / AHP disinfectant homogenate used to inoculate a pig for the bioassay.. ..	44
Figure 4.1 Schematic of overall experimental design.....	73

## LIST OF TABLES

		Page
Table 3.1	Description of treatment groups (4 replicates per treatment group).....	40
Table 3.2	Summary of PEDV RT-qPCR results for the pre-treatment and post-treatment swabs. ....	41
Table 3.3	Post-inoculation rectal swab PEDV N-gene RT-qPCR and swine bioassay results.....	42
Table 4.1	Summary of treatment groups .....	70
Table 4.2	PEDV RT-qPCR results for environmental samples collected from the contaminated coupons before and after the application of the peroxygen-based disinfectant .....	71
Table 4.3	Post-challenge rectal swab PEDV RT-qPCR and swine bioassay results.....	72

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**ABSTRACT**

Since its emergence in 2013, porcine epidemic diarrhea virus (PEDV) spread rapidly throughout the United States due, in part, to the mobile nature of the United States swine industry and contaminated livestock trailers. The mobile nature of the swine industry cannot easily be changed, but the risk associated with contaminated livestock trailers can be mitigated by performing proper trailer sanitation and decontamination protocols.

The number of studies investigating methods for inactivating PEDV in livestock trailers is small, but growing. Current literature shows that multiple sanitation and disinfection procedures can inactivate PEDV including the industry-standard high-pressure wash with detergent, disinfection, and dry either naturally or with a thermo-assisted drying and decontamination facility. Additionally, a commonly used accelerated hydrogen peroxide disinfectant inactivated PEDV in the presence of swine feces at room temperature. Additional disinfectants need to be evaluated for efficacy against PEDV under cold temperatures because PEDV outbreaks tend to be more prevalent in the fall and winter months and the cold temperatures make a complete wash, disinfect, and dry of the livestock trailer between every load of pigs more difficult to complete.

The first study investigated if the accelerated hydrogen peroxide disinfectant was capable of inactivating PEDV in swine feces on aluminum surfaces at -10°C. PEDV inactivation was assessed using a swine bioassay. Both the 1:16 and 1:32 dilutions of the accelerated hydrogen peroxide disinfectant, mixed with 10% propylene glycol as an anti-freezing agent, successfully inactivated PEDV in swine feces on metal surfaces at -10°C with a minimum of 40 minutes of contact time.



A second study tested a peroxygen-based disinfectant for the ability to inactivate PEDV on aluminum surfaces at 4°C or -10°C. Swine bioassay was used to determine if PEDV remained infective after disinfection. Both the 1:100 and 1:600 dilutions of peroxygen-based disinfectant successfully inactivated PEDV in swine feces on metal surfaces at 4°C and -10°C with a minimum of 10 minutes of contact time.

The results of the literature review and the original research in this thesis suggest that trailer sanitation and decontamination protocol involving washing to remove fecal and organic material, followed by chemical disinfection with an accelerated hydrogen peroxide, peroxygen-based, quaternary ammonium glutaraldehyde, phenol, sodium hypochlorite, or quaternary ammonium disinfectant product, and drying either naturally or through the use of a thermo-assisted drying and decontamination system are efficacious for inactivating PEDV under field conditions. While the studies conducted in this thesis demonstrate that PEDV can be inactivated by chemical disinfection alone in the presence of some organic matter, performing the three steps (wash, disinfect, and dry) together is synergistic and will provide greater protection against PEDV transmission.

## **CHAPTER 1. GENERAL INTRODUCTION**

### **Thesis Introduction**

Porcine epidemic diarrhea (PED) was first described in England in 1971 as a case of profuse watery diarrhea affecting all ages of swine on the premises (Oldham, 1972). In 1978, a novel coronavirus, porcine epidemic diarrhea virus (PEDV) was determined to be the causative agent of PED (Chasey and Cartwright, 1978; Pensaert and deBouck, 1978). Since PEDV emerged in the United States during May 2013, it spread rapidly across the country due to the ease of transmission via pig-to-pig contact or fecal-oral routes and the mobile nature of the swine industry (Popischil et al., 2002; Thomas et al., 2015a). Livestock trailers were implicated as mechanical vectors for PEDV and are in part responsible for the rapid dissemination of the virus across the United States (Alvarez et al., 2016; Lowe, et al., 2014; O’Dea et al., 2016).

Contaminated livestock trailers pose a significant risk for transmitting PEDV between swine premises. One way to mitigate this risk is to identify efficient and cost-effective methods for trailer sanitation and decontamination that can be applied to trailers once they have visited a PEDV positive premises or high-risk area, such as a swine harvest facility, buying station, or truck wash, before they return to another swine site. Many of the current trailer sanitation and decontamination procedures used by United States pork producers were developed for porcine reproductive and respiratory syndrome virus (PRRSV). The industry standard trailer sanitation and decontamination procedure for PRRSV includes trailer washing, disinfection, and drying, either naturally or with a thermo-assisted drying and decontamination (TADD) system (Dee et al., 2004a; Dee et al., 2004b; Dee et al., 2005a; Dee et al., 2005b; Dee et al., 2006). Commonly used disinfectants need to be evaluated for their efficacy against PEDV.

This thesis will serve as a summary of what is currently known about the properties of PEDV and methods effective to inactivate the virus. It will also summarize specific trailer sanitation and decontamination procedures that are efficacious at inactivating PEDV and preventing it from infecting subsequent groups of live pigs placed on that trailer. The objective of this thesis is to provide United States pork producers with practical, effective, and cost-efficient methods for livestock trailer sanitation and decontamination to stop the continued spread of PEDV between swine premises.

### **Thesis Organization**

This thesis is comprised of 5 chapters. Chapter 1 provides a brief summary of the organization and objectives of the thesis. Chapter 2 is titled “A review of methods sufficient to inactivate porcine enteric coronaviruses,” and is a literature review of the chemical and physical parameters that are sufficient to inactivate coronaviruses that cause enteritis in swine. The viruses included in the literature search include PEDV, transmissible gastroenteritis virus (TGEV), porcine deltacoronavirus (PDCoV), and porcine enteric alphacoronavirus (PEAV). The information gathered from the literature review was used as a guide to design the research documented in chapter 3 and chapter 4. Chapter 3 “Evaluation of an accelerated hydrogen peroxide disinfectant to inactivate porcine epidemic diarrhea virus in swine feces in aluminum surfaces under freezing conditions,” was published in *BMC Veterinary Research* in December of 2017. Chapter 4 “Evaluation of a peroxygen-based disinfectant for inactivation of porcine epidemic diarrhea virus at low temperatures on metal surfaces,” was published in the January 2018 issue of *Veterinary Microbiology*. Chapter 5 includes a summary of the results of the thesis and conclusions on what decontamination and sanitation procedures are effective against PEDV.

### **Thesis Objectives**

The objective of Chapter 2 was to identify and describe conditions that could be used to inactivate PEDV present in swine livestock trailers, specifically the types of disinfectants and other physical parameters that are effective against PEDV. Chapter 3 looked at the ability of an accelerated hydrogen peroxide (AHP) disinfectant to inactivate PEDV in swine feces under cold environmental temperatures. The specific objective of chapter 3 was to evaluate two concentrations of an AHP disinfectant in a 10% PG solution to determine if the mixture was sufficient to inactivate PEDV in the presence of swine feces on metal surfaces at  $-10^{\circ}\text{C}$ . Chapter 4 looked at a peroxygen-based disinfectant for its ability to inactivate PEDV under freezing conditions since PEDV is more prevalent during the winter months in the United States. The specific objective of chapter 4 was to test two concentrations (1:100 and 1:600) of a peroxygen-based disinfectant for the ability to inactivate PEDV on aluminum surfaces under freezing conditions.

## **CHAPTER 2. A REVIEW OF METHODS SUFFICIENT TO INACTIVATE SWINE ENTERIC CORONAVIRUSES**

### **Introduction**

Porcine epidemic diarrhea virus (PEDV) was first detected in the United States during May 2013 (Stevenson et al., 2013). The infectious dose of PEDV is very low (56 TCID<sub>50</sub> per pig for 3-week-old pigs and <1 TCID<sub>50</sub> per pig for neonatal piglets) and is easily transmitted via the fecal-oral route or by contact with contaminated fomites which makes contaminated livestock trailers a significant risk to the continued spread of PEDV (Thomas et al., 2015a; Popischil et al., 2002; Alvarez et al., 2016; Lowe, et al., 2014; O'Dea et al., 2016). After its introduction in 2013, PEDV proceeded to spread rapidly across the United States due, in part, to the mobile nature of the United States swine industry and contaminated livestock trailers. The mobile nature of the swine industry will not change, but trailer sanitation and decontamination can be improved to help prevent the continued spread of PEDV from positive premises to negative premises.

The risk of porcine reproductive and respiratory syndrome virus (PRRSV) transmission via contaminated livestock trailers was mitigated by developing stringent trailer sanitation and decontamination protocols. The industry standard trailer sanitation and decontamination procedure for PRRSV includes trailer washing, disinfection, and drying, either naturally or with a thermo-assisted drying and decontamination (TADD) system (Dee et al., 2004a; Dee et al., 2004b; Dee et al., 2005a; Dee et al., 2005b; Dee et al., 2006).

The number of studies investigating methods for inactivating PEDV in livestock trailers is small, however; much has been learned about PEDV in recent years. This review is an attempt to summarize what is known about the means for effective inactivation of coronaviruses that cause enteritis in swine, especially PEDV, to help make the best recommendations for swine producers and swine transport companies for future sanitation and disinfection protocols.

## **Materials and methods**

### **Definition of the review question**

What are the physical and chemical methods required to inactivate swine enteric coronaviruses that are relevant to conditions found in swine livestock trailers?

Specifically, the parameters required for inactivation by a chemical disinfectant that are commonly used in the swine industry and how these parameters might change under various temperatures and the presence of organic matter (feces).

### **Scope (PICO)**

#### **Participants**

Participants in this literature review were limited to viruses that are in the family *Coronaviridae* that cause enteritis in pigs. The four viruses that met this description were porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine deltacoronavirus (PDCoV), and porcine enteric alphacoronavirus (PEAV). All four viruses are single-stranded RNA (ssRNA) enveloped viruses that are susceptible to similar methods and parameters for inactivation.

#### **Intervention**

Application of chemicals or other physical methods to inactivate the virus including:

- Chemical disinfection
- Heat
- Changes in pH
- Time-dependent virus inactivation

## **Comparisons**

Only studies that compare one or more of the aforementioned interventions to no intervention under the same environmental conditions were included in this review.

## **Outcomes**

- Changes in the cycles to threshold (Ct) value
- Bioassay outcomes
- Reduction in 50% tissue culture infective dose (TCID<sub>50</sub>)
- Reduction in plaque-forming units (PFU)
- Reduction in focus forming units (FFU)
- Other objective quantifiable measures of virus reduction or inactivation

## **Indexes searched**

The Swine Information Library maintained by the American Association of Swine Veterinarians was searched to find peer-reviewed articles published in the Journal of Swine Health and Production that are not indexed in other article databases. Additionally, the National Library of Medicine (PubMed) was searched to find articles that met the aforementioned criteria.

## **Search terms**

The search terms used to search both the Swine Information Library and PubMed were: (chemical\* OR disinfect\* OR temperature\* OR thermal OR heat) AND (PEDV OR “porcine epidemic diarrhea virus” OR “porcine epidemic diarrhea” OR TGEV OR “transmissible gastroenteritis virus” OR “transmissible gastroenteritis” OR PEAV OR “porcine enteric alphacoronavirus” OR SeACoV OR “swine enteric alphacoronavirus OR SADS-CoV OR “swine acute diarrhea syndrome coronavirus” OR PDCoV OR “porcine deltacoronavirus”) AND (inactivat\* OR surviv\* OR destr\*).

### **Screening criteria**

- Study must have evaluated a method for inactivating a swine enteric coronavirus
- Study must be a full-length, peer-reviewed article
- Study must use objective measurable outcomes
- Study must be published in English but can be from any year or country

### **Results**

The search terms returned 44 results from PubMed. Of the 44 articles, only 36 of them were full-length peer-reviewed articles published in English. Of the remaining 36 articles, 17 were excluded because they did not objectively evaluate a specific intervention compared to no intervention on the inactivation of swine enteric coronaviruses. Two additional studies were excluded from this review because they are included in their entirety in a later chapter of this thesis.

The search terms returned 36 results from the Swine Information Library. Of the 36 results, many were duplicate listings and only 2 studies were full length peer-reviewed publications related to the inactivation of swine enteric coronaviruses.

### **The effect of pH on swine enteric coronavirus survival**

Laude (1981) showed that TGEV was inactivated by temperature at an increased rate when present in an alkaline environment (pH 8). The alkaline environment decreased the infective titer of TGEV by 1-2 log<sub>10</sub> PFU compared to a neutral pH. The effect of pH was more pronounced at higher temperatures (51°C) than lower temperatures (31°C). No studies that examined the effect of pH alone on TGEV infectivity were identified in this literature search.

Stevens et al. (2018) evaluated the infectivity of PEDV via swine bioassay in swine feces after treatment with hydrated lime to alkalinize the feces. When the pH of the manure was raised



to pH 10 for one hour, the amount of PEDV detected in the fecal sample by RT-qPCR decreased and lost infectivity. Holding manure at pH 12 for 12 hours resulted in no detection of PEDV in the fecal sample by RT-qPCR and inactivation of PEDV (Stevens et al., 2018).

In contrast to Stevens et al. (2018), Quist-Rybachuck et al. (2015) demonstrated that a pH of 10.2 alone did not reduce the TCID<sub>50</sub> of PEDV incubated in either porcine plasma or minimum essential media at 4°C for 120 minutes. However, when combined with heat treatment, an alkaline pH potentiated inactivation of PEDV (Quist-Rybachuck et al., 2015).

### **The effect of temperature and time on swine enteric coronavirus survival**

Early research on TGEV demonstrated that it persists for a long time in the environment at low temperatures. Laude (1981) demonstrated that TGEV was still infectious after 80 hours when heated to 38°C as determined by plaque formation on pig kidney cells. When evaluated at 54°C, TGEV lost infectivity in 50 minutes (Laude 1981). Laude (1981) observed two distinctly different thermodynamic curves in this study. Below 45°C, Laude (1981) suggested that RNA hydrolysis was responsible for the inactivation of TGEV. Above 45°C, Laude (1981) suggested that viral protein degradation was responsible for the inactivation of TGEV.

Haas et al. (1995) demonstrated that TGEV present in swine feces was inactivated (determined by tissue culture) more readily at warmer temperatures than cool temperatures. TGEV was inactivated within 30 minutes when heated to 55°C compared to 2 weeks at 20°C. At 5°C, the TGEV in swine feces was still infectious 8 weeks after the feces were spiked with TGEV (Haas et al., 1995).

At a neutral pH, temperature inactivation of PEDV takes longer than when combined with an alkaline pH. Quist-Rybachuk et. al. (2015) demonstrated that when PEDV was heated to 40°C with a neutral pH of 7.2 it took 21.7 hours to inactivate PEDV present in minimum essential media (MEM) compared to only 2.9 hours when the sample was held at a pH of 10.2.

Heating to 48°C alone at a neutral pH required 6.1 hours to inactivate PEDV compared to 15.2 minutes when heat plus a pH of 10.2 was used. PEDV in porcine plasma is more sensitive to heat treatment than PEDV present in MEM. At 48°C, PEDV in MEM required 6.1 hours to inactivate, while PEDV in porcine plasma was inactivated in only 0.9 hours (Quist-Rybachuk et al., 2015).

Gerber et al. (2014) demonstrated that PEDV is also highly susceptible to temperature inactivation. Porcine plasma spiked with PEDV and heated via a commercial spray drying process with 74°C inlet and 80°C outlet temperatures was no longer infectious via swine bioassay (Gerber et al., 2014). Pujols and Segales (2014) support these results. In that study, bovine plasma was spiked with PEDV and heated via a commercial spray drying process (200°C inlet temperature and 70°C – 80°C outlet temperatures) which successfully inactivated PEDV, as determined by virus isolation on VERO cells (Pujols and Segales, 2014). In contrast, Hulst et al. (2019) demonstrated the infectious PEDV was still detected by virus isolation in citrate treated porcine plasma after the spray drying process (190°C inlet and 80°C outlet) in 5% of the samples tested. When this spray-dried porcine plasma was stored at 20°C for 2 weeks, no infectious PEDV was recovered via virus isolation indicating that temperature plus time is synergistic (Hulst et al., 2019).

Pujols and Segales (2014) spiked spray-dried bovine plasma with PEDV after the spray drying process and held the spray-dried plasma at various temperatures for 21 days to assess PEDV infectivity as determined by tissue culture. PEDV in spray-dried bovine plasma held at 22°C was no longer infectious at 7 days. PEDV in spray-dried bovine plasma held at 12°C and 4°C was no longer infectious at 14 days and 21 days respectively (Pujols and Segales, 2014).

Thomas et al. (2015b) contaminated aluminum coupons with PEDV present in swine feces and demonstrated that heating the aluminum coupon to 71°C for 10 minutes was sufficient

to render PEDV inactive, as determined by swine bioassay. Additionally, holding the PEDV contaminated aluminum surface at room temperature (20°C) for 7 days was sufficient to inactivate PEDV. Heating the PEDV contaminated aluminum coupons to 63°C for 10 minutes, 54°C for 10 minutes, 38°C for 12 hours, and 20°C for 24 hours were not able to inactivate PEDV (Thomas et. al., 2015b).

The type of material PEDV is present on plays a role in its susceptibility to time-dependent inactivation. Kim et al. (2018) showed that infectious PEDV persisted on styrofoam, aluminum foil, Tyvek, cloth, and plastic for 20 days (as determined by virus isolation and immunoplaque assay) when held at 4°C. At 4°C, PEDV persisted on nitrile gloves, cardboard, and metal surfaces for 15 days and rubber for 10 days. In contrast, when these same materials were evaluated at 20°C (room temperature), PEDV survival decreased drastically and all were PEDV negative within 2 days of contamination (as determined by virus isolation and immunoplaque assay) (Kim et. al., 2018). When held at room temperature, PEDV titers decreased by 4-5 log<sub>10</sub> within 24 hours compared to only 1-2 log<sub>10</sub> reduction in titers when held at 4°C for 5 days.

### **Description of the swine enteric coronaviruses**

The studies summarized in this review contain information PEDV and TGEV. No studies pertaining to the inactivation of PDCoV or PEAV were discovered during the literature review process.

Transmissible gastroenteritis virus (TGEV) is an alphacoronavirus that causes atrophic enteritis in swine. Clinical disease is characterized by vomiting and diarrhea that is more severe in neonatal pigs. Disease is limited in adult swine and immune populations. TGEV is present worldwide.

Porcine epidemic diarrhea virus (PEDV) is an alphacoronavirus that also causes atrophic enteritis in swine. Clinical disease is similar to TGEV. Mortality can reach 100% in naïve pigs less than 7 days of age (Popischil et al., 2002; Thomas et al., 2015a, Chen et al., 2016). PEDV emerged in the United States during 2013 and is currently present worldwide (Stevenson et al., 2013). PEDV has a very low infectious dose and infected pigs shed a large amount of the virus in their feces. An infectious dose of 56 TCID<sub>50</sub>/pig is sufficient to infect most 3-week-old pigs and <1 TCID<sub>50</sub>/pig could cause infection in 5-day-old neonatal piglets (Thomas et al., 2015a). PEDV is transmitted to pigs via the fecal-oral route, aerosol, and fomites (Popischil et al., 2002).

Porcine deltacoronavirus (PDCoV) was initially reported in Hon Kong in 2012 and was first detected in the United States in 2014 (Li et al., 2014; Wang et.al, 2014). PDCoV causes similar disease as PEDV and TGEV, but is thought to be milder than PEDV. Common clinical signs include vomiting and diarrhea with high morbidity and mortality in suckling pigs (Jung et al., 2015). PDCoV is widespread across the United States and the world and is transmitted in similar fashion to PEDV (Zhang J, 2016).

Porcine enteric alphacoronavirus (PEAV) is an enveloped single-stranded positive-sense RNA virus in the *Alphacoronavirus* genus that was discovered in China in 2017 (Gong et al, 2017). The same virus was also reported by other research groups and given different names, such as swine enteric alphacoronavirus (SeACoV) (Pan et al, 2017) and swine acute diarrhea syndrome coronavirus (SADS-CoV) (Zhou et al, 2018). This coronavirus is highly similar to an alphacoronavirus found in bats (HKU2 strain) (Lau et al, 2007). Case reports and clinical studies have demonstrated that this emerging coronavirus can cause vomiting, diarrhea, and dehydration in neonatal pigs similar to both PEDV and TGEV (Xu et al. 2019). There are no reports of PEAV in the United States to date.

### **The effect of chemical disinfection on swine enteric coronavirus survival**

Multiple commonly used disinfectants have demonstrated efficacy against TGEV when no organic matter was present. Five minutes of contact time with a 2% concentration of a glutaraldehyde disinfectant, 1:64 dilution of a 15.36% solution of a quaternary ammonium disinfectant, or a 1:200 dilution of a 24% quaternary ammonium disinfectant resulted in a 4.5  $\log_{10}$  TCID<sub>50</sub> reduction of TGEV that was dried on a disc (Brown, 1981). Goyal et al. (2014) demonstrated that exposing TGEV to 25mL of hydrogen peroxide vapor was efficacious at contact times of greater than or equal to 16 minutes and resulted in a 5.05  $\log_{10}$  reduction in TCID<sub>50</sub> post-treatment.

Other chemical disinfectants showed poor efficacy against TGEV when no organic matter was present. Exposure of TGEV to a 6% sodium hypochlorite disinfectant for 1 minute resulted in  $< 1 \log_{10}$  PFU reduction in infectivity titer (Hulkower et al., 2011). Similarly, when exposed to a 0.55% aldehyde disinfectant, the TCID<sub>50</sub> of TGEV only reduced by 2.27  $\log_{10}$  (Hulkower et al., 2011). A 1:128 dilution of a 16.75% phenol disinfectant did not significantly reduce the infectivity of TGEV with only a 2.03  $\log_{10}$  PFU reduction in infectivity titer (Hulkower et al., 2011).

Similar to TGEV, multiple disinfectants are effective against PEDV under experimental conditions with no organic matter contamination. Exposure of PEDV (dried on a steel disc) to a 0.5% w/v solution of a peracetic acid disinfectant for 15 minutes reduced the TCID<sub>50</sub> of the virus from 320 TCID<sub>50</sub>/mL to  $< 1$  TCID<sub>50</sub>/mL (Dagher et al., 2017). Immersion of PEDV in a solution of superoxide water with a pH of 6.0 at 20°C resulted in loss of infectivity of PEDV after 10 minutes of contact time, as determined by virus isolation (Chen et al., 2017). Bowman et al. (2015) demonstrated that 5 commonly used disinfectants in the swine industry (1.5:128 dilution of a quaternary ammonium disinfectant; 1:256 dilution of a phenol disinfectant; 1:256 dilution of

a quaternary ammonium and glutaraldehyde combination disinfectant; 1:200, 1:100, and 1:150 dilutions of an oxidizing disinfectant; and 1:50, 1:16, 1:8, and 1:4 dilutions of sodium hypochlorite) were able to inactivate PEDV (dried on a petri dish) in the absence of organic matter with 60 minutes of contact time at 37°C, 4°C, and -20°C. Exposure of PEDV on aluminum surfaces to a 0.5% oxidizing agent and a 2.06% solution of sodium hypochlorite for 60 minutes of contact time rendered PEDV inactive as determined by bioassay (Bowman et al., 2015).

Bowman et al. (2015) also demonstrated that a phenol disinfectant, quaternary ammonium disinfectant, sodium hypochlorite, oxidizing agent, and quaternary ammonium and glutaraldehyde combination disinfectant were all effective at inactivating PEDV (dried in a petri dish) in the presence of swine feces when given 60 minutes of contact time. An accelerated hydrogen peroxide disinfectant at 1:16 and 1:32 dilutions were also effective at inactivating PEDV in the presence of swine feces on aluminum surfaces when given 30 minutes of contact time at 20°C (Holtkamp et al., 2017).

### **Discussion**

The virucidal efficacy of any of the methods of inactivation reviewed here is hard to accurately apply to field conditions. The efficacy of an intervention against any virus is highly dependent on the environmental conditions the intervention is applied in, the amount of organic material present around the virus, the infectious dose of the virus, the immune status and age of the host exposed to the virus, and route of transmission. The infectious dose of PEDV is extremely low and depends on the age of the pig exposed. Neonatal pigs are susceptible to an infectious dose of 0.056 TCID<sub>50</sub>/pig, while older swine require an infectious dose of 56 TCID<sub>50</sub>/pig to become infected (Thomas et. al., 2015a). Therefore, methods that only reduce the amount of virus in the sample post-intervention by only 3 or 4 log<sub>10</sub> TCID<sub>50</sub>/mL may not be

sufficient to inactivate PEDV in the field even though that is the standard used to evaluate virucidal efficacy of products in Europe and North America (Geller et al., 2012).

Swine enteric coronaviruses are sensitive to drastic changes in pH, but the application of pH changes in the field as a method for livestock trailer sanitation and decontamination is limited due to the caustic nature of substances with an alkaline pH. pH changes inactivate coronaviruses by causing an irreversible change in the spike protein which is the protein required for the virus to enter the host's enterocytes (Darnell et. al., 2004). PEDV and TGEV appear to be more sensitive to alkaline pH than acidic pH changes. Stevens et al, (2018) showed that PEDV loses infectivity when held at a pH of 10 for one hour. However, another study by Quist-Rybachuck et al. (2015) demonstrated that PEDV remained infectious after exposure to a pH of 10.2 for 2 hours. This makes interpreting the use of pH for PEDV inactivation difficult. However, combining heat and an alkaline pH results in a synergistic relationship leading to a shorter time required to inactivation of PEDV and TGEV than with heat or alkaline pH alone (Laude, 1981; Quist-Rybachuck et al., 2015). This should be taken into account when designing protocols for inactivating swine enteric coronaviruses. If the pH of the substance is neutral, higher temperatures or longer contact times may be needed to inactivate the coronavirus. If the pH is alkaline ( $\geq 10$ ), lower temperatures or shorter duration of high temperatures may be sufficient to inactivate PEDV or the other swine enteric coronaviruses. This modality could be very useful in inactivating PEDV present in effluent from PEDV positive sites or even run-off from truck wash sites. pH manipulation would not be appropriate for livestock trailer sanitation and decontamination as the alkaline pH would likely damage the aluminum on livestock trailers and may cause employee safety concerns.

Using various combinations of heat and time to inactivate porcine enteric coronaviruses is very effective. The rate of viral infectivity loss is directly related to the amount of heat applied. Swine enteric coronavirus infectivity is lost rapidly at high temperatures and more slowly at lower temperatures. Temperatures less than 45°C do not result in rapid viral inactivation and coronaviruses such as TGEV can survive for up to 8 weeks when held at 5°C (Hass et al., 1995). Therefore, when rapid viral inactivation is desired heating to higher temperatures is preferred. In the context of livestock trailer sanitation and decontamination, if rapid PEDV inactivation is required, the trailer should be heated to at least 71°C and maintained at 71°C for 10 minutes (Thomas et al., 2015b). This study was conducted with a mild amount of fecal contamination on the aluminum surface, a higher temperature or longer time at 71°C may be necessary if a moderate to heavy level of fecal contamination is present on the trailer at the time of heating. If rapid inactivation is not required, holding livestock trailers or other potential fomites at room temperature (20°C) for 2 days if not contaminated with feces (Kim et al., 2018) or for one week if contaminated with feces (Thomas et al, 2015b) may be an effective means to inactivate the various swine enteric coronaviruses in the field. Holding trailers or other potential fomites at lower than room temperature is not indicated because infectious PEDV can be isolated from materials such as Styrofoam, cloth, and plastic for 20 days after contamination when held at 4°C (Kim et al., 2018).

It is difficult to directly compare the efficacy of disinfectants against swine enteric coronaviruses based on the large variability in study design, dilution of the disinfectant used, presence or absence of organic matter during the study, contact time, and temperature the disinfectant was tested under. Research suggests that swine enteric coronaviruses, specifically PEDV, are susceptible to most commonly used commercial disinfectants under experimental



conditions. Swine enteric coronaviruses can be inactivated by the following disinfectants: phenols, quaternary ammonium, sodium hypochlorite, oxidizing agents (hydrogen peroxide, accelerated hydrogen peroxide, peroxygen-based compounds), and quaternary ammonium and glutaraldehyde combination disinfectants under conditions specific to the study they were evaluated in. Disinfectants are less effective against viruses at low temperatures, with high amounts of organic material present, at lower concentrations, and at shorter contact times. For example, a glutaraldehyde based disinfectant was ineffective against TGEV with a one minute contact time (Hulkower et al., 2011), but was effective against PEDV at longer contact time and higher temperature (Bowman et al., 2015).

Unfortunately, low temperatures, presence of organic material, and short contact times are all constraints frequently encountered when attempting to disinfect livestock trailers that are contaminated with PEDV since PEDV is more common in the winter. To reduce the effects of these constraints, swine veterinarians and producers can implement a few additional steps to ensure their disinfectant protocol is as effective as possible. Thoroughly washing the livestock trailer, or other contaminated fomites, prior to disinfection is paramount to have an effective sanitation and decontamination process. Washing reduces the amount of organic material (feces and shavings) present when the disinfectant is applied. If temperatures less than 20°C are expected, disinfect the trailer in a heated area or increase the concentration and contact time of the disinfectant. Always check EPA regulation when increasing the concentration of disinfectants from the labeled rate. If all organic material (feces, dirt, wood shavings) cannot be removed from the trailer, increase the contact time and/or concentration of the disinfectant used.

In summary, a successful strategy for swine enteric coronavirus elimination will include heat, time, and chemical disinfection. In the context of a livestock trailer, the first step in the

sanitation and decontamination protocol should be a through high-pressure wash to remove all feces, dirt, shavings, and other material from the inside of the trailer. Second, the livestock trailer should be placed in an area that is at 20°C or above and a chemical disinfectant (phenol, quarternary ammonium, accelerated hydrogen peroxide, peroxygen-based, sodium hypochlorite, or quarternary ammonium glutaraldehyde combinations) should be applied. The efficacy of chemical disinfectants decreases as the temperature decreases, so ensuring that disinfection is applied in an area heated to ambient room temperature is essential for the best success. Finally, the trailer should be heated to 71°C and held at 71°C for at least 10 minutes. This time and temperature combination alone is sufficient to inactivate PEDV, and the increased temperature will likely enhance the efficacy of the disinfectant.

This ideal strategy is in line with the current industry standard wash, disinfect, and dry trailer sanitation and decontamination protocols. However, due to the mobile nature of the swine industry, a full wash, disinfect, and dry between every load of pigs hauled especially on market hog trailers is next to impossible. For this reason, additional research needs to be conducted on alternative trailer sanitation and decontamination procedures effective against swine enteric coronaviruses, specifically PEDV, that could be applied in situations when a full wash, disinfect, and dry is not feasible. This information could be applied to disinfecting livestock trailers after hauling loads of market pigs, which are at an increased risk of transmitting PEDV but are often asked to turn around too quickly to perform a full wash, disinfect, and dry. While a full wash, disinfect, and dry should always be performed, the research described in Chapters 3 and 4 in this thesis would give swine veterinarians and producers another option to protect themselves against PEDV.

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### CHAPTER 3. EVALUATION OF AN ACCELERATED HYDROGEN PEROXIDE DISINFECTANT TO INACTIVATE PORCINE EPIDEMIC DIARRHEA VIRUS IN SWINE FECES ON ALUMINUM SURFACES UNDER FREEZING CONDITIONS

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#### Abstract

**Background:** Since its emergence in 2013, porcine epidemic diarrhea virus (PEDV) spread rapidly throughout the country due, in part, to contaminated livestock trailers. The objective of this study was to test the efficacy of an accelerated hydrogen peroxide (AHP) disinfectant for inactivating PEDV in swine feces on metal surfaces under freezing conditions. One 15.24 X 15.24 X 2.54 cm aluminum coupon, contaminated with swine feces, and randomly matched to one pig was the experimental unit. Eight treatment groups representing two AHP concentrations (1:16 and 1:32) in a 10% propylene glycol solution, two contact times in a -10°C freezer (40 minutes and 60 minutes), and two levels of fecal contamination (5mL and 10mL) in addition to negative and positive control groups were evaluated. Forty 3-week-old pigs,

intragastrically inoculated with the contents of the coupons after treatment, were used as a bioassay to determine the infectivity of PEDV after treatment. Infectivity was determined by detection of virus with a nucleocapsid (N) gene-based quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) on rectal swabs collected from the inoculated pigs on days three and seven post-inoculation.

**Results:** All post-treatment swabs from the negative control coupons were negative for PEDV via RT-qPCR. All post-treatment swabs collected from coupons in the AHP disinfectant treatment groups and the positive control group were positive for PEDV via RT-qPCR. For the bioassay, no rectal swabs from pigs in the negative control (0 of 4) or the AHP disinfectant treatment groups (0 of 32) were positive for PEDV. Rectal swabs from all pigs within the positive control group (4 of 4) were positive for PEDV by RT-qPCR.

**Conclusions:** Under the conditions of this study, 1:16 and 1:32 dilutions of the AHP disinfectant successfully inactivated PEDV in swine feces on metal surfaces when applied at -10°C with 40 or 60 minutes of contact time. This study also suggests that a positive RT-qPCR result for PEDV on an environmental sample should be expected when the AHP disinfectant is applied under freezing conditions, but does not necessarily indicate that an infectious dose of PEDV remains after disinfection.

## Introduction

Porcine epidemic diarrhea (PED) was first described in 1971 as cases of profuse, watery diarrhea affecting all ages of pigs in England [1]. In 1978, a novel coronavirus, porcine epidemic diarrhea virus (PEDV), was determined to be the causative agent [2, 3,]. The first cases of PEDV in the United States were confirmed during May 2013 from farms in Iowa and Indiana [5]. PEDV spread rapidly throughout the United States after its introduction mainly due to the ease of



transmission via fecal-oral and pig-to-pig contact [4]. Livestock trailers that haul pigs to and from collection points such as livestock auctions or harvest facilities have been implicated as mechanical vectors for PEDV [6]. Contaminated livestock trailers likely pose a significant risk for PEDV transmission and movement throughout the country.

This risk has historically been mitigated through trailer sanitation and decontamination procedures developed for porcine reproductive and respiratory syndrome virus (PRRSV) when high pressure washing alone was found insufficient to inactivate PRRSV [7, 8, 9, 10]. Because disinfection and drying are more effective when applied to a clean trailer with little or no remaining organic matter, the industry standard for sanitation and decontamination of livestock trailers includes trailer washing, disinfection, and drying, either naturally or with a thermo-assisted drying and decontamination (TADD) system [7, 8, 9]. Similar research on PEDV demonstrated that the industry standard wash, disinfection, and dry successfully inactivated PEDV on metal surfaces when detergent and a combination of quaternary ammonium and glutaraldehyde disinfect were used [11].

The industry standard wash, disinfect, and dry is always the best method for livestock trailer sanitation and decontamination. For this reason, swine producers should always strive to use the industry standard sanitation and decontamination protocols on all livestock trailers between loads. However, a complete wash, disinfect, and dry requires an investment in time, logistics, and specialized facilities which deters some swine producers and contract haulers from performing the industry standard protocols between every load of pigs hauled. Consequently, a significant number of pigs, especially market pigs, are hauled on trailers that are not subjected to any sanitation or decontamination procedures between loads, posing a significant risk. Successfully decreasing the risk of PEDV transmission from contaminated livestock trailers may

depend on the development of cost-effective sanitation procedures, as an alternative to doing nothing, which can be completed in a short period of time without specialized facilities. In addition, identification of disinfectants and other decontamination processes that work in the presence of some organic matter may decrease the risk of viral transmission from livestock trailers that are washed, but sanitation and decontamination procedures are not closely monitored or performed poorly.

Recent PEDV research demonstrated that holding a metal surface contaminated with PEDV positive feces at 71°C for 10 minutes or 20°C for 7 days was efficacious at inactivating PEDV [12]. An accelerated hydrogen peroxide® (AHP®) disinfectant (Intervention®, Virox Technologies Inc., Oakville, Ontario, Canada) successfully inactivated PEDV in the presence of fecal contamination on metal surfaces with a 30-minute contact time at 20°C. The concentrated form of the AHP disinfectant was efficacious at dilutions of 1:16 and 1:32 in the presence of feces [13]. Intervention® is labeled as virucidal at dilution rates of 1:16 to 1:64 in the presence of 200 ppm hard water and 5% serum load with a 5-minute contact time. It contains anionic surfactants, nonionic surfactants, and stabilizers that help improve the stability and microbicidal action of hydrogen peroxide [14, 15]. PEDV outbreaks, however, tend to be more prevalent in the cooler winter months where a complete wash, disinfect, and dry is more difficult to complete due to freezing temperatures. Water along with most aqueous disinfectants freeze around 0°C; making trailer sanitation and decontamination difficult. It has been previously demonstrated that diluting a quaternary ammonium and glutaraldehyde combination disinfectant (Synergize; Preserve International, Atlanta, Georgia) in either a 10% propylene glycol (PG) or 40% methanol solution prevented freezing and allowed the disinfectant to inactivate PRRSV at temperatures below 0°C [10].

The objective of this study was to evaluate two concentrations of an AHP disinfectant in a 10% PG solution to determine if the mixture was sufficient to inactivate PEDV in the presence of swine feces on metal surfaces at -10°C. Conditions were chosen to mimic those found in commercial livestock trailers in winter months after most of the fecal and organic matter has been removed by scraping and a traditional wash is unavailable.

## **Methods**

### **Experimental design**

The experimental unit was a single aluminum coupon contaminated with swine feces matched to an individual 3-week old pig. The pig was intragastrically inoculated with the contents of the coupon post treatment, as a bioassay to determine if the treatment applied to the contaminated coupon effectively inactivated PEDV. Three-week old pigs were used in this study because they are relatively susceptible to infection with PEDV, but mortality is rare. Previous work reported that 100% of 21-day-old pigs inoculated with 10 mL of a virulent PEDV prototype isolate with titers of 5.6-560 TCID<sub>50</sub>/ml were infected, but pigs inoculated with lower titers (0.0056-0.56 TCID<sub>50</sub>/ml) of PEDV were not infected [16].

The primary outcome variable was the proportion of pigs in each treatment group that were PEDV-positive by bioassay to determine if infectious PEDV was present after the disinfectant treatment. The null hypothesis was that there was no difference between the positive control group and the disinfectant treatment groups in the proportion of pigs infected with PEDV after being inoculated with the material collected from the coupons. The bioassay result was determined by nucleocapsid (N) gene-based quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) performed at the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) on rectal swabs collected from each pig on days three and

seven post-inoculation. The primers and probe of the PEDV RT-qPCR were previously described [6, 16, 17]. Each PCR was set up and performed in accordance with previously described procedures [13, 16, 17]. Current viral culture methods make it difficult to culture wild-type PEDV outside of an animal model. Therefore, to determine if live infectious PEDV is present in a sample, a bioassay using an animal model remained the best alternative. The use of a bioassay also eliminated questions about the cytotoxic impact feces and disinfectant may have had on the outcome of virus isolation.

Personnel performing disinfectant treatments, necropsies, and collecting samples were not blinded to the treatment groups. Blinding these individuals was not possible because all procedures were performed in a specific order, starting with the negative control group and ending with the positive control group, to minimize the risk of transmitting PEDV between treatment groups. Laboratory personnel that performed the RT-qPCR testing for PEDV and personnel performing the statistical analyses were blinded to the treatment groups.

### **Coupons**

Forty 15.24 cm X 15.24 cm X 2.54 cm aluminum coupons were manufactured using aluminum with a material thickness of 0.32cm, resembling the type of material found in livestock trailers. These coupons were used in previous studies evaluating the efficacy of other sanitation and decontamination procedures for PEDV inactivation [11, 12, 13]. To simulate the cleaning action of the AHP disinfectant and runoff seen in commercial livestock trailers as the AHP disinfectant is transformed from foam to a liquid, six 8 mm diameter holes were drilled at the junction of the bottom and sidewall of the coupon.

## **Treatment groups**

Two volumes of fecal contamination (5 mL and 10 mL); two concentrations of AHP disinfectant (1:16 and 1:32) prepared in a solution that was 10% PG by volume; and two contact times (40 minutes and 60 minutes) were evaluated. A positive control and negative control group were also included (Table 1). Forty minutes of contact time was chosen by applying the Arrhenius equation which in previous work led to the conclusion that for every 10°C decrease in temperature the contact time of a disinfectant doubles [18]. PEDV-positive feces were used to contaminate coupons in the positive control group (B) and all treatment groups (C through J). PEDV negative feces were used to contaminate coupons in the negative control group (A). The negative (A) and positive (B) control groups were not sham disinfected. The AHP disinfectant used in this study was applied as a thick foam which persisted for the duration of the contact time and had minimal rinsing and diluting effects. The best candidate for a sham disinfectant would be a non-disinfecting solution that produced a persistent foam similar to that of the AHP disinfectant; however, after extensive research and pre-trial work, a suitable non-disinfecting foam was not identified by the investigators. Using a non-foam liquid for sham disinfection would result in a greater rinsing and diluting effect as the liquid would run out of the holes in the coupons at a faster rate than the persistent foam produced by the AHP disinfectant would; therefore, sham disinfection was not done. Four replicates of aluminum coupons were included for each treatment group.

## **Contamination and disinfection procedures**

The feces used to contaminate the coupons were obtained from a previous experiment where 3-week-old pigs were inoculated with PEDV isolate US/Iowa/18984/2013 [17]. Feces were collected from pigs, confirmed to be positive for PEDV by RT-qPCR, seven days post inoculation, which was within the peak viral shedding timeframe [17]. After collection, feces

from individual pigs were stored at  $-80^{\circ}\text{C}$ . On study day 0, the feces from individual pigs were thawed and pooled into a single fecal homogenate to ensure that the amount of PEDV and composition of the feces was uniform for each replicate. Samples from each replicate were tested at the ISU VDL by RT-qPCR. The quantitative genomic copies/mL ranged from  $10^{8.00}$  to  $10^{9.06}$  genomic copies/mL across all replicates (Table 2 and Additional file 1). PEDV negative feces were obtained from the negative control pigs in a previous study [13]. Fecal collection and storage procedures were the same as those for the PEDV positive feces. Prior to freezing, a sample of the PEDV negative feces was submitted to the ISU VDL to confirm its PEDV negative status. Diagnostic testing confirmed that the sample was negative for PEDV by RT-qPCR.

The in-vivo portion of the study was initiated on study day 0. Prior to contamination and treatment of the coupons, 2 mm thick plastic sheeting was placed on the floor. The plastic sheeting was changed and the floor under the plastic sheeting was disinfected with Virkon™ S disinfectant (Lanxess, Wilmington, DE, USA) between each treatment group to reduce the risk of cross contamination. For the negative control group (A), 5 mL of PEDV negative feces were applied to four aluminum coupons. Five mL of PEDV positive feces was applied to all coupons in groups B, C, E, G, and I. Ten mL of PEDV positive feces were applied to all coupons in groups D, F, H, and J (Figure 1). For all study groups (A through J), contamination of the coupons with feces was performed using a disposable hard plastic spreader sold in hardware stores to spread adhesive on floors. A new adhesive spreader was used on each coupon to prevent cross-contamination between replicates. Five mL and 10 mL of feces, when spread evenly over the floor of each coupon, resulted in an even layer that was  $\leq 2$  mm and were chosen to reflect the range of organic matter remaining in the interior of a commercial livestock trailer after it has been manually scraped to remove bedding and feces. Following contamination with

feces, all coupons were individually sampled using a commercial swab and transport system. The pre-treatment swabs were submitted to the ISU VDL to test for the presence of PEDV by RT-qPCR.

Following fecal contamination, coupons, except those assigned to the negative control group A, were pre-cooled in a commercial refrigerator, set to 4°C for 30 minutes. This pre-cooling period was designed to mimic conditions in a scraped livestock trailer after transporting pigs during the winter months. Plastic sheeting was placed on the refrigerator's shelves and changed between each treatment group to prevent cross-contamination.

After the pre-cooling period, AHP disinfectant solution was applied to the contaminated coupons in the treatment groups C through J. The AHP disinfectant was prepared by diluting a 4.25% concentrate of the AHP disinfectant (Intervention<sup>®</sup>, Virox Technologies Inc., Oakville, Ontario, Canada) with tap water from a municipal water source and PG. The final AHP disinfectant solution contained 10% PG and a ratio of AHP concentrate to final solution of 1:16 for treatment groups E, F, I, and J and 1:32 for treatment groups C, D, G, and H. PG is an organic solvent that can be used as a safe anti-freezing agent when mixed with phenol, quaternary ammonium, and quaternary ammonium formaldehyde disinfectants without reducing their efficacy [19]. Coupons in the negative control (A) and positive control (B) groups were not sham disinfected. A liquid volume of approximately 30 mL of AHP disinfectant solution was applied as a foam to all 4 coupons in each treatment group (C through J) using a 5.7 L pump-up foamer (model #A8020A, Ogena Solutions, LLC, Stoney Creek, ON, Canada). To disinfect a 15.8 m double-decked livestock trailers, 189 L of AHP disinfectant would be applied over a 10-minute period using a proportioning foamer with a flow rate of 18.9 L per minute. Based on the area of the coupons used in this study, 30 mL of AHP disinfectant was determined proportionally

equivalent to the 189 L used on livestock trailers. Using the same 5.7 L foamer, a series of timed applications were performed prior to study initiation to establish that a 3 second application time was required to apply the 30 mL of AHP disinfectant solution.

Following treatment with AHP disinfectant solution, the coupons were placed in a freezer set at  $-10^{\circ}\text{C}$  for their allotted contact time as described in Table 1. To prevent cross contamination between treatments, the freezer drawers were lined with 2 mm plastic sheeting and a folded bath towel was placed on top of the plastic to absorb any liquid runoff from the coupons. To prevent replicates within the treatment group from cross-contaminating each other, each coupon was placed into a 16 cm X 16 cm X 3 cm pan crafted from aluminum foil. New plastic, towels and aluminum foil pans were used for every treatment group. Coupons in the positive control group (B) were also placed in the freezer for 40 minutes to confirm that the time at  $-10^{\circ}\text{C}$  alone was not responsible for PEDV inactivation. Coupons in the negative control group (A) were not placed in the freezer.

Ten minutes after contamination of the coupons in the negative control group (A), a post-treatment swab was taken using a commercial swab and transport system. For all other study groups (B through J), the swabs were collected following the treatment described in Table 1. All swabs collected post-treatment were submitted to the ISU VDL and tested for the presence of PEDV RNA by RT-qPCR.

After the post-treatment swab was collected, the coupon was tipped away from the holes and 10 mL of sterile 0.9% sodium chloride (saline) solution was added using a new 12 mL syringe for each coupon. A coupon dedicated toothbrush was used to re-suspend the feces / AHP disinfectant / PG / saline mixture; creating a homogenate sample suitable for recollection as inoculum. The resultant homogenate was collected using a 20-mL syringe (Figure 2). During



recollection of the inoculum sample, nitrile gloves were changed between each coupon to prevent cross contamination between replicates and 2 mm plastic sheeting was placed under the coupons and changed between treatment groups to prevent cross-contamination between treatments.

## **Swine Bioassay**

### **Source of animals and housing**

The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee (Log Number: 11-14-7904-S) and the Iowa State University Institutional Biosafety Committee (Log Number 14-I-0040-A) prior to initiation of any experimental activity. Forty 3-week-old commercial pigs, 17 barrows and 23 gilts, were obtained from WeSearch, LLC in Iowa and housed in the Iowa State University Veterinary Medical Research Institute for the duration of the study. Upon delivery (study day -4), all pigs (40/40) were individually identified with a unique ID number on a plastic ear tag and weighed. Pigs were blocked by weight and then randomly assigned within each weight group to one of the ten treatment groups (Table 1) using the RAND function in Microsoft Excel (version 2010).

On study day -1, serum and rectal swabs were collected from the pigs to confirm their PEDV negative status. Eight mL of whole blood was collected from each pig via jugular venipuncture and transferred to an 8.5 mL serum separator tube and centrifuged at 3100g for 8 minutes. Serum was submitted to the ISU VDL for diagnostic testing. Serum samples were pooled (5 samples per pool) and tested for porcine reproductive and respiratory virus (PRRSV) via RT-qPCR. Individual serum samples were tested for antibodies to PRRSV using a commercial PRRS X3 Ab Test (IDEXX Laboratories, Inc., Westbrook, Maine), and for antibodies to transmissible gastroenteritis virus (TGEV) and porcine respiratory corona virus (PRCV) by a commercial TGEV/PRCV-Ab differential enzyme-linked immunosorbent assay

(ELISA, SVANOVA Biotech AB, Uppsala, Sweden). Individual serum samples were also screened for antibodies to PEDV by indirect fluorescent antibody (IFA) assay following previously described procedures [16]. Rectal swabs were obtained via the same commercial swab and transport system used to swab the coupons. Individual rectal swabs were tested for TGEV, porcine rotaviruses (groups A, B and C), and PEDV by real time RT-PCRs at the ISU VDL.

Each treatment group was designated one room. The four pigs in each group were housed individually in a raised tub described in detail in previous studies [12, 13]. Individual housing was accomplished using solid transparent dividers, creating four compartments within one tub. The transparent dividers allowed visual and auditory contact between the pigs, but prevented nose-to-nose and fecal-oral contact. Urine and feces fell through a grate at the bottom of the tub and drained into a bucket to minimize the risk of contaminating the room's floor. Each section of the tub had designated water and feed sources. During the study, each pig was assigned its own bag of feed and feed cup to minimize cross contamination. Previous studies demonstrated that this housing method successfully prevented transmission of PEDV between pigs in a group [12]. Pigs were fed an age appropriate, pelleted, starter diet ad libitum. The diet consisted primarily of corn and soybean meal and did not contain porcine derived products.

#### **Inoculation of pigs with contents of coupon post-treatment**

On study day 0, the homogenate of feces, AHP disinfectant, PG and saline, collected from each coupon as described above, was immediately used to inoculate pigs for the bioassay via oral gastric gavage with a 14 French rubber catheter as previously described [12]. Each pig was inoculated with the contents of its designated coupon, with PEDV RT-qPCR results ranging from  $10^{3.70} - 10^{8.88}$  genomic copies / mL for coupons assigned to treatment groups B – J (Table 2 and Additional file 1). To prevent cross contamination between treatment groups and replicates

within the same treatment, study personnel followed a strict biosecurity protocol used successfully in previous work [12]. Disposable Tyvek coveralls (DuPont, USA) and respirators were worn by personnel at all times and were changed between each treatment group. Coveralls were inspected after each inoculation and were changed between pigs if inoculum or fecal material was present. Furthermore, nitrile gloves and arm-length disposable obstetrical sleeves were worn and changed between each pig to prevent cross-contamination between replicates.

Pigs were monitored daily, on study days 0 through 7, for clinical signs consistent with PEDV by the same study investigator. On days 3 and 7 post-inoculation, rectal swabs were collected from each pig using a commercial swab and transport system. To avoid cross contamination between replicates, pigs were not removed from their compartment and personnel used the same biosecurity procedures as previously described for inoculation. A bioassay was considered positive if the rectal swab was PEDV-positive by RT-qPCR (Ct value less than 35) on study day 3 or study day 7. A bioassay was considered negative if both rectal swabs (study days 3 and 7) were PEDV-negative by RT-qPCR (Ct > 35).

On study day 7, all pigs were humanely euthanized using a penetrating captive bolt gun and necropsied. During necropsy, all organ systems were evaluated and any gross lesions or abnormal pathology was noted. Fresh and 10% formalin-fixed samples of mesenteric lymph nodes, ileum, and jejunum and fresh cecal and spiral colon contents were collected from each pig. All fresh samples were placed in a -80°C freezer and all samples were held in the event further testing might be required to confirm the results of the swine bioassay.

All statistical analyses were performed using SAS<sup>®</sup> (Enterprise Guide 5.1; SAS Institute, Cary, NC, USA). A Fisher's Exact Test was used to evaluate pairwise differences in the proportion of pigs positive by bioassay between all ten treatment groups. Ct values were

analyzed using two way analyses of variance (ANOVA) models with treatment, time (pre versus post) and their interaction. Pre-treatment Ct values were compared between groups using an F-test. Differences in Ct values between pre- and post-treatment were assessed for each study group using a two-sided T-Test. A  $p$  value  $<0.05$  was considered statistically significant.

## **Results**

### **Pre-trial diagnostic screening**

Serum and fecal samples obtained on study day -1 confirmed that all 40 pigs were negative for PEDV by RT-qPCR as well as negative for antibodies to PEDV via IFA. Additionally, all pigs were negative for PRRSV, porcine rotaviruses (groups A, B, and C), and TGEV by RT-qPCR and for antibodies to TGEV via differential ELISA. Fifteen pigs were positive, 23 were suspect, and 2 were negative for antibodies to PRCV by differential ELISA. Three pigs were positive for antibodies to PRRSV via ELISA.

### **Coupon Swabs**

PEDV RT-qPCR results from swabs taken from fecal contaminated aluminum coupons before and after treatment with AHP disinfectant are displayed in Table 2 and Additional file 1. In the negative control (A), all pre-treatment swabs, taken immediately after PEDV negative feces were applied to the four coupons, were negative for PEDV RNA by RT-qPCR. All pre-treatment swabs, taken immediately after PEDV positive feces were applied to coupons, from the positive control (B) were positive for PEDV RNA by RT-qPCR with quantitative results ranging from  $10^{8.17}$ - $10^{8.56}$  genomic copies/mL. Likewise, all swabs obtained after fecal contamination but prior to treatment from the coupons in groups C – J were PEDV-positive via RT-qPCR with quantitative results from  $10^{8.00}$ - $10^{9.06}$  genomic copies/mL. There were no significant differences in pre-treatment Ct values from the coupons assigned to the eight disinfectant (C – J) and the positive control (B) groups ( $p$ -value = 0.9435).

Post-treatment swabs (4 of 4) from the negative control (A) coupons, taken ten minutes after PEDV negative feces were applied to the coupon, were negative for PEDV RNA by RT-qPCR. All (4 of 4) post-treatment swabs from the positive control (B) coupons, taken after pre-cooling and 40 minutes in a  $-10^{\circ}\text{C}$  freezer, were positive for PEDV RNA via RT-qPCR with quantitative results ranging from  $10^{8.38}$ - $10^{8.88}$  genomic copies/mL. All (32 of 32) post-treatment swabs collected from coupons in the AHP disinfectant treatment groups C – J were positive for PEDV RNA via RT-qPCR with quantitative results ranging from  $10^{3.76}$ - $10^{8.26}$  genomic copies/mL. The difference in Ct values between pre- and post-treatment were significantly different than zero (p value < 0.0001) for all of the AHP disinfectant treatment groups (C – J).

### **Swine bioassay**

PEDV RT-qPCR results for rectal swabs taken 3 and 7 days post inoculation and the final swine bioassay results by treatment group are displayed in Table 3 and Additional file 1. Rectal swabs collected from all pigs in the negative control (A) and AHP disinfectant treatment groups C – J on study days 3 and 7 were negative for PEDV by RT-qPCR. Rectal swabs from all four pigs in the positive control (B) collected on study days 3 and 7 were positive for PEDV by RT-qPCR. The proportion of pigs positive by bioassay for the negative control (A) and all of the AHP disinfectant treatment groups (C – J) were significantly different than proportion of pigs positive in the positive control (B) group via Fishers Exact Test (p-value < 0.05).

### **Discussion**

Under freezing conditions ( $-10^{\circ}\text{C}$ ) an AHP disinfectant prepared in a 10% PG solution inactivated PEDV in the presence of feces on metal surfaces. Both AHP disinfectant dilutions (1:16 and 1:32), and contact times (40 minutes and 60 minutes) evaluated were sufficient to inactivate PEDV at either fecal load (5 mL and 10 mL), under the conditions of this study. Bioassay results from this study support previous work where the same AHP disinfectant

successfully inactivated PEDV in swine feces on metal surfaces at 20°C [13]. Findings from this study demonstrate that manually removing the organic material from a trailer via scraping and then applying an AHP disinfectant may be an effective alternative to doing nothing between loads, year round. Additionally, mixing the AHP disinfectant in a 10% PG solution prevented the disinfectant solution from freezing during 40 or 60 minutes of contact time in a freezer set to -10°C, supporting previously published data on utilizing PG as an anti-freezing agent [10, 19].

In contrast to previous work [13], all post-treatment swabs (32 of 32), from the coupons contaminated with PEDV positive feces and then subjected to one of eight AHP disinfectant treatments (groups C-J) at -10°C, were positive for the presence of PEDV RNA by RT-qPCR. However, none of the coupons (0 of 32) contained infectious virus as demonstrated by swine bioassay. While swine veterinarians sometimes use RT-PCR to evaluate the success of sanitation and decontamination protocols in practice, its major limitation is that it does not differentiate between infectious virus and noninfectious fragments of PEDV RNA. These results indicate that the AHP disinfectant inactivated the virus while leaving a sufficient amount of genetic material intact to interact with the primers used in the RT-qPCR assay. Hydrogen peroxide is an oxidizing agent that inactivates viruses by denaturing viral proteins, lipids and nucleic acids [20, 21]. These results suggest that denaturation of nucleic acid by the AHP disinfectant occurred to a lesser degree at lower temperatures than at higher temperatures, but this difference did not affect its ability to inactivate PEDV as demonstrated by the swine bioassay results. These results are consistent with those from previous studies where disinfection of contaminated metal surfaces with oxidizing disinfectants inactivated PEDV but did not consistently produce negative PEDV RT-qPCR results after disinfection [22, 23]. Therefore, PEDV-positive RT-qPCR results on environmental samples should be expected when the AHP disinfectant is utilized under freezing

conditions, but this does not necessarily indicate that an infectious dose of PEDV remains in the trailer.

Livestock trailers have many non-smooth surfaces and are more complex than the coupons utilized in this study. Trailers contain many channels, grooves, rough surfaces, hinges, latches, and corners that organic material can build up on and provide areas for a virus to be missed by a disinfectant. While, it was understood the smooth aluminum coupons do not replicate all of the surfaces found within livestock trailers, performing a study of this magnitude would not have been feasible with full-size trailers, so the coupons were utilized as a model. The ease with which the 15.24 X 15.24 X 2.54 cm aluminum coupons could be handled made it possible to contaminate the coupons, perform the treatments, collect the inoculum and inoculate pigs for the bioassay for all study groups in less than one day. The model also enabled the investigators to stagger the start time for each treatment group so that the pigs could be inoculated immediately after the inoculum was collected, thereby eliminating the need to attempt to neutralize the AHP disinfectant after the 40 minute or 60 minute contact time.

Livestock trailers are frequently bedded with wood shavings prior to transporting pigs. Incorporation of wood shavings into the model was considered however, the size and type of shavings used for bedding varies widely across the industry and previous work demonstrated that some wood has virucidal properties [24]. Therefore, the aluminum coupons were contaminated with swine feces alone to avoid the possibility of confounding the results with the effect of choice of wood shavings.

While the AHP disinfectant utilized in this study is labeled as virucidal in 5 minutes with dilutions of 1:16 to 1:64, this study only evaluated two dilutions (1:16 and 1:32) and used considerably longer contact times (40 minutes and 60 minutes). The longer contact times were

chosen because the conditions in this study were less favorable than those used in determining the label and in a previous study which evaluated its efficacy at 20°C [13]. Further research on the efficacy of an AHP disinfectant under other adverse conditions such as shorter contact times, greater dilution rates, and on perpendicular surfaces to simulate trailer sidewalls is warranted.

### **Conclusions**

In cold weather months when a complete wash, disinfection, and dry cannot be accomplished, due to lack of resources or other logistical constraints, the results of this study suggest that scraping livestock trailers to remove as much organic material as possible followed by disinfection with at minimum a 1:32 concentration of AHP disinfectant in a solution with 10% PG with at least 40 minutes of contact time, may be used, as an alternative to doing nothing, to reduce the risk of PEDV transmission associated with livestock trailers.

The results also suggest that a PEDV-positive RT-qPCR result on an environmental sample should be expected after 60 minutes of contact time when the AHP disinfectant is applied under freezing conditions, but does not necessarily indicate that an infectious dose of PEDV remains in the trailer. Obtaining a negative real-time RT-qPCR result on an environmental sample after disinfection is largely dependent on the type of disinfectant used and the conditions under which it was applied.



## Tables

**Table 3.1** Description of treatment groups (4 replicates per treatment group)

<b>Table 1</b> Description of treatment groups (4 replicates per treatment group)			
Treatment group	Volume and PEDV status of feces	Disinfectant and concentration	Contact time at -10 °C
(A) Negative Control	5 mL PEDV-negative feces	None	None
(B) Positive Control	5 mL PEDV-positive feces	None	40 min
(C) Light, 1:32, 40 min	5 mL PEDV-positive feces	AHP at 1:32	40 min
(D) Heavy, 1:32, 40 min	10 mL PEDV-positive feces	AHP at 1:32	40 min
(E) Light, 1:16, 40 min	5 mL PEDV-positive feces	AHP at 1:16	40 min
(F) Heavy, 1:16, 40 min	10 mL PEDV-positive feces	AHP at 1:16	40 min
(G) Light, 1:32, 60 min	5 mL PEDV-positive feces	AHP at 1:32	60 min
(H) Heavy, 1:32, 60 min	10 mL PEDV-positive feces	AHP at 1:32	60 min
(I) Light, 1:16, 60 min	5 mL PEDV-positive feces	AHP at 1:16	60 min
(J) Heavy, 1:16, 60 min	10 mL PEDV-positive feces	AHP at 1:16	60 min

Table 3.2 Summary of PEDV RT-qPCR results for the pre-treatment and post-treatment swabs.

<b>Table 2</b> Summary of PEDV RT-qPCR results for the pre-treatment and post-treatment swabs				
Treatment group	Pre-treatment Ct Value and (genomic copies/mL)	Percentage positive or suspect for PEDV	Post-treatment Ct Value and (genomic copies/mL)	Percentage positive or suspect for PEDV
(A) Neg Control	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)
(B) Pos Control	18.2 ( $10^{8.56}$ ) 19.0 ( $10^{8.32}$ ) 19.5 ( $10^{8.17}$ ) 19.2 ( $10^{8.26}$ )	100% (4 of 4)	18.6 ( $10^{8.44}$ ) 17.1 ( $10^{8.88}$ ) 18.8 ( $10^{8.38}$ ) 18.8 ( $10^{8.38}$ )	100% (4 of 4)
(C) Light, 1:32, 40 mins	17.2 ( $10^{8.85}$ ) 17.4 ( $10^{8.73}$ ) 17.9 ( $10^{8.64}$ ) 17.6 ( $10^{8.73}$ )	100% (4 of 4)	27.1 ( $10^{5.94}$ ) 21.0 ( $10^{7.73}$ ) 29.1 ( $10^{5.35}$ ) 28.6 ( $10^{5.49}$ )	100% (4 of 4)
(D) Heavy, 1:32, 40 mins	16.8 ( $10^{8.97}$ ) 18.0 ( $10^{8.61}$ ) 17.8 ( $10^{8.67}$ ) 18.1 ( $10^{8.58}$ )	100% (4 of 4)	24.6 ( $10^{6.67}$ ) 19.2 ( $10^{8.26}$ ) 24.0 ( $10^{6.85}$ ) 20.6 ( $10^{7.85}$ )	100% (4 of 4)
(E) Light, 1:16, 40 mins	18.7 ( $10^{8.41}$ ) 17.9 ( $10^{8.64}$ ) 18.2 ( $10^{8.56}$ ) 18.7 ( $10^{8.41}$ )	100% (4 of 4)	33.6 ( $10^{4.02}$ ) 31.1 ( $10^{4.76}$ ) 28.9 ( $10^{5.41}$ ) 30.7 ( $10^{4.88}$ )	100% (4 of 4)
(F) Heavy, 1:16, 40 mins	17.2 ( $10^{8.85}$ ) 17.8 ( $10^{8.67}$ ) 18.5 ( $10^{8.47}$ ) 18.2 ( $10^{8.56}$ )	100% (4 of 4)	26.1 ( $10^{6.23}$ ) 23.0 ( $10^{7.14}$ ) 23.9 ( $10^{6.88}$ ) 23.0 ( $10^{7.14}$ )	100% (4 of 4)
(G) Light, 1:32, 60 mins	18.0 ( $10^{8.61}$ ) 17.6 ( $10^{8.73}$ ) 17.0 ( $10^{8.91}$ ) 18.2 ( $10^{8.56}$ )	100% (4 of 4)	27.5 ( $10^{5.82}$ ) 26.1 ( $10^{6.23}$ ) 23.6 ( $10^{6.97}$ ) 27.3 ( $10^{5.88}$ )	100% (4 of 4)
(H) Heavy, 1:32, 60 mins	17.6 ( $10^{8.73}$ ) 17.9 ( $10^{8.64}$ ) 18.7 ( $10^{8.41}$ ) 17.0 ( $10^{8.91}$ )	100% (4 of 4)	26.1 ( $10^{6.23}$ ) 23.7 ( $10^{6.94}$ ) 21.5 ( $10^{7.59}$ ) 24.8 ( $10^{6.61}$ )	100% (4 of 4)
(I) Light, 1:16, 60 mins	17.6 ( $10^{8.73}$ ) 17.5 ( $10^{8.76}$ ) 17.8 ( $10^{8.67}$ ) 17.9 ( $10^{8.64}$ )	100% (4 of 4)	34.5 ( $10^{3.76}$ ) 33.4 ( $10^{4.08}$ ) 34.7 ( $10^{3.70}$ ) 32.3 ( $10^{4.40}$ )	100% (4 of 4)
(J) Heavy, 1:16, 60 mins	16.5 ( $10^{9.06}$ ) 18.0 ( $10^{8.61}$ ) 17.3 ( $10^{8.82}$ ) 20.1 ( $10^{8.00}$ )	100% (4 of 4)	28.0 ( $10^{5.67}$ ) 24.9 ( $10^{6.58}$ ) 24.7 ( $10^{6.64}$ ) 24.9 ( $10^{6.58}$ )	100% (4 of 4)

Table 3.3 Post-inoculation rectal swab PEDV N-gene RT-qPCR and swine bioassay results

<b>Table 3</b> Post-inoculation rectal swab PEDV N-gene RT-qPCR and swine bioassay results					
Treatment Group	Day 3 Rectal Swab; Individual CT results (genomic copies / mL)	Day 3 Rectal Swab; Percentage positive for PEDV RNA	Day 7 Rectal Swab; Individual CT Results (genomic copies / mL)	Day 7 Rectal Swab; Percentage positive for PEDV RNA	Swine Bioassay Result; Percentage positive for PEDV RNA
(A) Negative Control	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	0% (0 of 4) <sup>a</sup>
(B) Positive Control	16.2 (10 <sup>9.14</sup> ) 15.1 (10 <sup>9.46</sup> ) 16.8 (10 <sup>8.96</sup> ) 15.0 (10 <sup>9.49</sup> )	100% (4 of 4)	22.2 (10 <sup>7.37</sup> ) 15.2 (10 <sup>9.43</sup> ) 19.3 (10 <sup>8.23</sup> ) 17.4 (10 <sup>8.79</sup> )	100% (4 of 4)	100% (4 of 4) <sup>b</sup>
(C) Light, 1:32, 40 mins	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	0% (0 of 4) <sup>a</sup>
(D) Heavy, 1:32, 40 mins	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	0% (0 of 4) <sup>a</sup>
(E) Light, 1:16, 40 mins	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	0% (0 of 4) <sup>a</sup>
(F) Heavy, 1:16, 40 mins	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	0% (0 of 4) <sup>a</sup>
(G) Light, 1:32, 60 mins	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	0% (0 of 4) <sup>a</sup>
(H) Heavy, 1:32, 60 mins	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	0% (0 of 4) <sup>a</sup>
(I) Light, 1:16, 60 mins	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	0% (0 of 4) <sup>a</sup>
(J) Heavy, 1:16, 60 mins	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	>35 (0) >35 (0) >35 (0) >35 (0)	0% (0 of 4)	0% (0 of 4) <sup>a</sup>

<sup>a,b</sup> Different superscripts indicate statistically significant differences ( $p$ -value <0.05) by a Fishers Exact Test

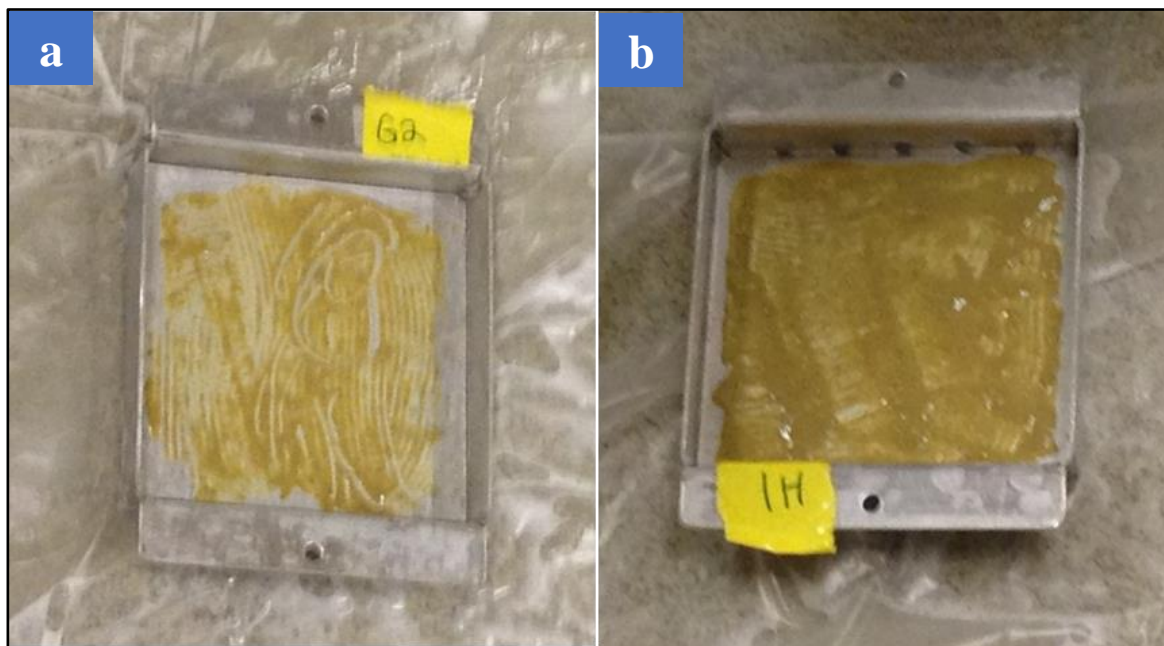
**Figures**

Figure 3.1 Fecal contamination of aluminum coupons. *Feces were applied in an even layer to aluminum coupons using an adhesive spreader. Five mL of feces were applied to coupons in treatment groups A, B, C, E, G, and I. Ten mL of feces were applied to coupons in treatment groups D, F, H, and J. One representative coupon in group G (a) and group H (b) is shown.*

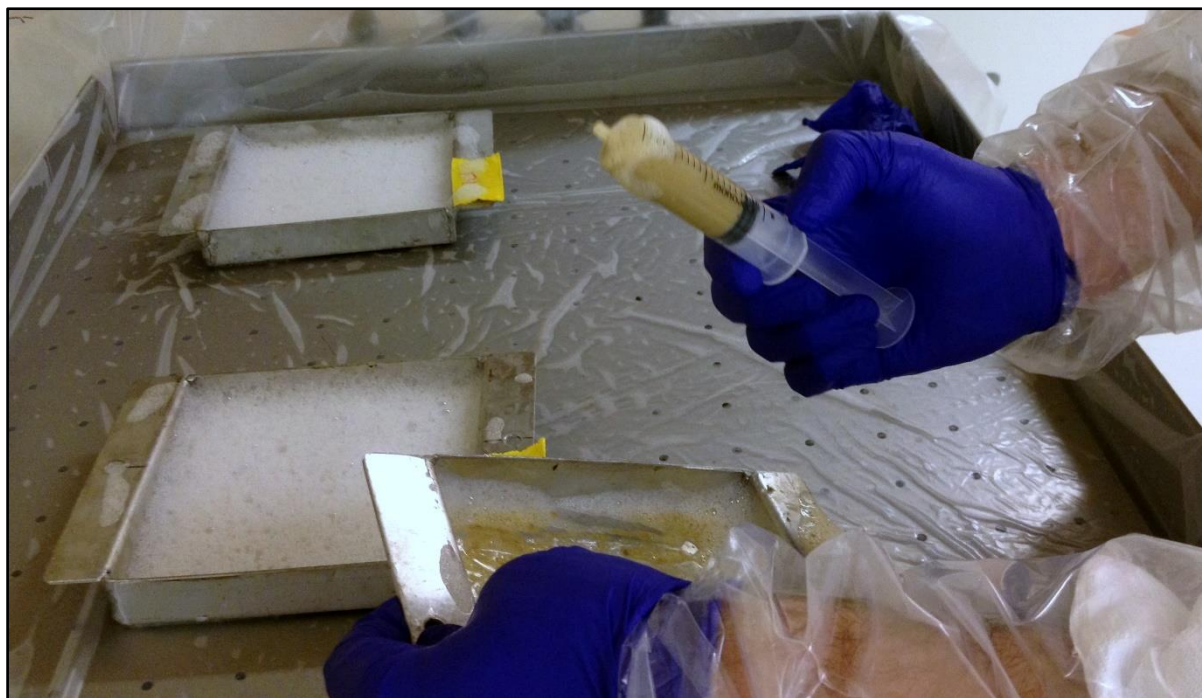


Figure 3.2 Collection of feces / AHP disinfectant homogenate used to inoculate a pig for the bioassay. *A mixture of feces, AHP disinfectant, PG, and saline was recollected from the coupon using a 20 mL syringe. This syringe was labeled with the coupon identification number and matched to a single pig for the bioassay*

## **Declarations**

### **Ethics approval and consent to participate**

The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee (log number# 11-14-7904-S) and the Iowa State University Institutional Biosafety Committee (log number # 14-I-0040-A) prior to study initiation. The study was carried out in strict adherence to Institutional Animal Care and Use Committee guidelines regarding humane use of animals.

### **Availability of data and material**

All data generated or analyzed during this study are included in this article and additional file.

### **Competing interests**

The author's report no competing interests for this study.

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### **Authors' contributions**

KLB, DJH, PRT, LAK, AR designed, organized and conducted the trial. JZ assisted with study design and performed the diagnostic testing. CW conducted the statistical analysis. KLB drafted the manuscript with significant contributions from DJH. All authors read, revised, and approved the final manuscript.

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## **CHAPTER 4. EVALUATION OF A PEROXYGEN-BASED DISINFECTANT FOR INACTIVATION OF PORCINE EPIDEMIC DIARRHEA VIRUS AT LOW TEMPERATURES ON METAL SURFACES**

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### **Abstract**

Porcine epidemic diarrhea virus (PEDV) spread rapidly across the United States in part due to contaminated livestock trailers. The objective of this study was to test a peroxygen-based disinfectant for the ability to inactivate PEDV on aluminum surfaces at 4<sup>0</sup>C or -10<sup>0</sup>C. Forty 3-week-old individually housed barrows were used as a bioassay to determine the infectivity of PEDV after treatment with either a 1:100 or 1:600 dilution of a peroxygen-based disinfectant with 10 or 30 minutes of contact time. One coupon matched to one pig was the experimental unit. Coupons in the positive control and disinfectant treatment groups were contaminated with 2 mL of feces spiked with PEDV. A negative control group was contaminated with PEDV-negative feces. Following treatment, the feces and disinfectant remaining in the coupons was collected and administered to pigs intragastrically. Rectal swabs were collected from pigs 3 and

7 days post-inoculation (DPI) and tested for PEDV by RT-qPCR. Samples from all coupons, except the negative control, were positive by RT-qPCR for PEDV before and after treatment. All rectal swabs from the pigs in the negative control and the seven disinfectant treatment groups were RT-qPCR negative for PEDV on 3 and 7 DPI. All pigs in the positive control at 4°C and 3 of 4 pigs in the positive control conducted at -10°C were RT-qPCR positive for PEDV on 3 and 7 DPI. Both the 1:100 and 1:600 dilutions of peroxygen-based disinfectant successfully inactivated PEDV under the conditions of this study.

### **Introduction**

PEDV is an enveloped RNA virus belonging to the family *Coronaviridae*, genus *Alphacoronavirus* (Saif, 2012). Porcine epidemic diarrhea (PED) was first described in England in sows and fattening swine in 1971 (Oldham, 1972). In 1978, porcine epidemic diarrhea virus (PEDV) was determined to be the causative agent (Chasey and Cartwright, 1978; Pensaert and deBouck, 1978). Thenceforth, PEDV caused epidemic and endemic outbreaks of diarrhea in Europe and Asia and most recently North America (Song et al., 2015). PEDV was first detected in the United States in April 2013 from swine farms in Iowa and Indiana and is characterized by dehydration, profuse watery diarrhea, vomiting, high mortality in neonatal pigs, and high morbidity but low mortality in weaned pigs (Stevenson et al., 2013).

After its introduction, PEDV spread rapidly across the United States due to its relatively low minimum infectious dose (Thomas et al., 2015a) and ease of transmission via pig-to-pig or fecal-oral contact (Popischil et al., 2002). Because of the mobile nature of the United States swine industry, the rapid and continued spread of PEDV was likely due in part, to contaminated livestock trailers. Analysis of spatial and temporal epidemiology of PED in the United States showed that swine transport was one of the major routes of PEDV transmission (Alvarez et al., 2016; O'Dea et al., 2016). Livestock trailer associated transmission of PEDV is supported by

previous work that implicated livestock trailers that haul pigs to and from harvest facilities, livestock auctions, or other swine collection points as mechanical vectors for PEDV (Lowe et al., 2014).

The risk associated with contaminated livestock trailers can be mitigated by performing proper trailer sanitation and decontamination protocols. In response to porcine reproductive and respiratory virus (PRRSV), the swine industry developed standard trailer sanitation and decontamination protocols. Washing a trailer with a high pressure power washer followed by disinfection and drying the trailer, either naturally or with a thermo-assisted drying and decontamination (TADD) system, has proven effective against PRRSV under both warm and freezing conditions (Dee et al., 2004a; Dee et al., 2004b; Dee et al., 2005a; Dee et al., 2005b; Dee et al., 2006).

More recent work on PEDV demonstrated that multiple sanitation protocols are effective at inactivating PEDV on metal surfaces similar to those found in commercial livestock trailers. The industry standard trailer sanitation and decontamination protocol is effective against PEDV at room temperature when detergent and a combination of quaternary ammonium and glutaraldehyde disinfectant (Synergize, Preserve International, Atlanta, Georgia) were used (Holtkamp et al., 2016). An accelerated hydrogen peroxide disinfectant (Intervention, Virox Technologies Inc., Oakville, Ontario, Canada) inactivated PEDV in the presence of swine feces at room temperature (Holtkamp et al., 2017) and under freezing conditions (Baker et al., In Review). TADD was also effective at inactivating PEDV when the metal surface contaminated with PEDV positive feces is held at 71<sup>0</sup>C for 10 minutes (Thomas et al., 2015b). However, additional disinfectants need to be evaluated for efficacy against PEDV under cool temperatures

because PEDV outbreaks tend to be more prevalent in the fall and winter months and the cooler temperatures make a complete wash, disinfect, and dry more difficult to complete.

The objective of this study was to test two concentrations of a peroxygen-based disinfectant for the ability to inactivate PEDV on aluminum surfaces under freezing conditions.

## **Methods**

### **Experimental design**

The experimental unit was one diamond plate aluminum coupon randomly matched to one 3-week-old barrow. The diamond plate coupon was contaminated with swine feces spiked with PEDV and then subjected to one of 10 treatment groups (Table 1). Following treatment, the pig was inoculated with the contents of its assigned coupon as a swine bioassay to determine if the treatment applied to the coupon inactivated the PEDV present in the swine feces. Three-week-old pigs were utilized in this study because previous work demonstrated that 21-day-old pigs inoculated with 10 mL of a virulent PEDV prototype isolate with titers of  $5.6 \times 10^6$  TCID<sub>50</sub>/mL were infected, but mortality was rare (Thomas et al., 2015a).

The primary outcome variable in this study was the swine bioassay measured by the proportion of pigs in each treatment group that became infected and shed PEDV in their feces within 7 days post inoculation. The null hypothesis was that there was no difference between the proportion of pigs infected with PEDV in the positive control groups and the seven peroxygen-based disinfectant treatment groups. The infection of pigs for the swine bioassay was determined by PEDV nucleocapsid (N) gene-based quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) on rectal swabs collected from each pig on days three and seven post-inoculation. The RT-qPCR tests were performed at the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL). The primers and probe used for the PEDV RT-qPCR were previously described (Lowe et.al., 2014; Madson et. al., 2014). Current viral culture methods

make it difficult to culture wild-type PEDV. Therefore, to determine if live infectious PEDV is present in a sample, a bioassay using an animal model remained the best model. However, post-treatment samples from each coupon (the inoculum administered to each pig) were also tested for live PEDV via PEDV virus isolation at the ISU VDL to compare the virus isolation results with those from the swine bioassay under the conditions of this study.

Laboratory personnel that performed the PEDV RT-qPCR and virus isolation and personnel performing the statistical analysis were blinded to the treatment groups. However, personnel performing the sample collection from the pigs, necropsies, and disinfectant treatments were not blinded to the treatment groups. In order to minimize the risk of transmitting PEDV between treatment groups via human vectors or fomites associated with humans, all procedures were performed in a specific order, starting with the negative control group, then the disinfectant treatment groups, and ending with the two positive control groups.

### **Coupons**

Forty 15.24cm × 15.24cm × 2.54cm aluminum coupons were manufactured using diamond plate aluminum flooring to accurately represent the type of material and surface found on the floors of commercial livestock trailers. To simulate the runoff seen in commercial livestock trailers as the peroxygen-based disinfectant transforms from a foam to a liquid, five 6 mm diameter holes were drilled at the junction of the bottom and one sidewall of the coupon. Environmental samples from the coupons were tested for PEDV by RT-qPCR prior to study initiation to confirm that the coupons were not previously contaminated with PEDV RNA.

### **Treatment groups**

Two concentrations of the peroxygen-based disinfectant (1:100 and 1:600), two temperatures (4°C and -10°C), two contact times (10 minutes and 30 minutes) and one volume of fecal contamination (2 mL) were evaluated. Treatment groups for all combinations of disinfectant concentration, temperature and contact time were evaluated except for the 1:600 concentration of disinfectant at -10°C for 10 minutes of contact time which was not evaluated due to pig facility constraints. A negative control group and two positive control groups (one for each temperature) were also included in this study (Table 1). For treatment groups conducted at -10°C, the disinfectant was prepared in a solution that was 10% propylene glycol (PG) to prevent freezing. PEDV-negative feces were used to contaminate coupons in the NegCtrl group. Feces spiked with PEDV were used to contaminate the coupons assigned to all other treatment groups. The NegCtrl group was not sham disinfected. The 4C/PosCtrl and -10C/PosCtrl groups were sham disinfected with phosphate buffered saline (PBS).

### **Peroxygen-based disinfectant**

The peroxygen-based disinfectant used in this study was Virkon™S Disinfectant and Virucide Powder (Lanxess, Wilmington, DE, USA). The concentrated peroxygen-based disinfectant powder contained 21.41% potassium peroxymonosulfate and 1.5% sodium chloride as active ingredients and 77.09% other ingredients and was labeled as virucidal at a 1:100 dilution with 10 minutes of contact time. This disinfectant was selected because of its commercial availability and degree of usage in commercial swine facilities and commercial truck washes in the United States. Additionally, when used as the disinfect step of a wash, disinfect, and dry trailer sanitation and decontamination protocol this peroxygen-based disinfectant was able to inactivate PRRSV, another single-stranded RNA virus (Dee et al., 2006). Furthermore,

previous work indicated that this peroxygen-based disinfectant remained effective in low levels of organic matter (Chandler-Bostock and Mellitis, 2015).

### **PEDV propagation and titration**

A U.S. PEDV isolate (USA/IN19338/2013) was used in this study. This PEDV isolate was isolated in 2013 at the ISU VDL from a piglet small intestine submitted from a swine farm in Indiana. Isolation, propagation and titration of this PEDV isolate have been previously described (Chen et al, 2014; Thomas et al, 2015a). Briefly, PEDV was isolated and propagated in Vero cells (ATCC CCL-81) using post-inoculation medium which included minimum essential medium (MEM) supplemented with tryptose phosphate broth (0.3%), yeast extract (0.02%), and trypsin 250 (5 $\mu$ g/ml), as well as 2 mM L-glutamine and antibiotics (0.05 mg per ml gentamicin, 100 units/ml penicillin, 100  $\mu$ g per ml streptomycin, and 0.25  $\mu$ g/ml amphotericin). Virus titration was performed in 96-well plates with 10-fold serial dilutions, triplicate per dilution. After 5 days of inoculation, the plates were subjected to immunofluorescence staining and the virus titers determined according to the Reed and Muench method (Reed and Muench, 1938) and expressed as the 50% tissue culture infective dose per ml (TCID<sub>50</sub>/ml). This isolate has proven highly virulent in previous experimental infection studies (Chen et al, 2016; Thomas et al, 2015a). The final PEDV isolate used to contaminate coupons in this study had an infectious titer of  $3.9 \times 10^6$  TCID<sub>50</sub>/mL which is within the range of infectious titers that successfully infected 3-week-old pigs in a previous experiment (Thomas et al., 2015a).

### **Contamination material**

PEDV-negative feces were obtained from the cecum and colon of PEDV-negative pigs during necropsy in an unrelated experiment conducted at Iowa State University's College of Veterinary Medicine. Following collection, feces from individual pigs were stored in a freezer set to -80°C. On study day -1, the feces were thawed, homogenized, and tested for the presence



of PEDV (Madson et al., 2014), porcine deltacoronavirus (PDCoV) (Chen et al., 2015) and transmissible gastroenteritis virus (TGEV) (Schneider et al., 2015) via virus specific RT-qPCR at the ISU VDL to confirm their negative status. The feces were then split into forty 2 mL aliquots. The 2 mL aliquots of PEDV-negative feces were stored in a refrigerator until needed on study day 0.

### **Contamination and disinfection of aluminum coupons**

Fecal contamination and disinfection of the coupons occurred on study day 0. Two mL of PEDV-negative feces were applied evenly across the surface of the diamond plate aluminum coupons assigned to the NegCtrl group using a new wooden craft stick for each coupon. For the remaining nine treatment groups (4C/PosCtrl, -10C/PosCtrl, 4C/1:600/10M, 4C/1:600/30M, 4C/1:100/10M, 4C/1:100/30M, -10C/1:600/30M, -10C/1:100/10M, -10C/1:100/30M), a 2mL aliquot of PEDV-negative feces was spiked with 1mL of the PEDV isolate and mixed together with a wooden craft stick. PEDV-positive feces were applied evenly to the surface of the aluminum coupons using a new wooden craft stick for each coupon. Two mL of feces over the 15.24 cm × 15.24 cm area of the coupon emulated the level of organic material remaining in the interior of a livestock trailer that might be found after an unsatisfactory washing. Following fecal contamination but prior to disinfectant treatment (pre-treatment), an environmental sample was taken from each coupon using a commercial swab and transport system (StarSwab II, Starplex Scientific, Etobicoke, Ontario, Canada). Pre-treatment swabs were submitted to ISU VDL and tested for the presence of PEDV by RT-qPCR.

After fecal contamination, coupons were pre-cooled for 30 minutes in a 4°C commercial refrigerator. Pre-cooling dropped the temperature of the aluminum coupons and allowed the feces to dry and adhere tightly to the aluminum, simulating conditions in a poorly washed

livestock trailer during winter months. Two mm thick plastic sheeting was placed on the refrigerators shelves and changed between each treatment group to prevent cross contamination between groups. Only one treatment group was placed on each shelf of the refrigerator.

Immediately after pre-cooling, the peroxygen-based disinfectant solution was applied to coupons in the disinfectant treatment groups (4C/1:600/10M, 4C/1:600/30M, 4C/1:100/10M, 4C/1:100/30M, -10C/1:600/30M, -10C/1:100/10M, -10C/1:100/30M) and the sham disinfectant was applied to the two positive control groups (4C/PosCtrl, -10C/PosCtrl). The peroxygen-based disinfectant mixture was prepared by dissolving concentrated peroxygen-based disinfectant powder in water from a municipal water source for all disinfectant treatment groups. For treatments conducted at -10°C (-10C/1:600/30M, -10C/1:100/10M, -10C/1:100/30M), PG was added to the disinfectant solution. PG is an organic solvent that is used as an anti-freezing agent that is not toxic to pigs. Previous work demonstrated that PG did not change the efficacy of phenol, quaternary ammonium, quaternary ammonium formaldehyde, and accelerated hydrogen peroxide disinfectants when added as an anti-freezing agent (Davison et.al.,1999). In a similar study, PG did not reduce the efficacy of an accelerated hydrogen peroxide disinfectant when applied to PEDV positive swine feces on aluminum surfaces (Baker et. al., In Review). The peroxygen-based disinfectant solution contained 10% PG and a ratio of concentrated peroxygen-based disinfectant powder to final solution of 1:100 for the -10C/1:100/10M and -10C/1:100/30M treatment groups and a ratio of 1:600 for the -10C/1:600/30M treatment group. For the 4C/1:100/10M and 4C/1:100/30M groups, the peroxygen-based disinfectant solution had a ratio of concentrated peroxygen-based disinfectant powder to final solution of 1:100. For the 4C/1:600/10M and 4C/1:600/30M groups the peroxygen-based disinfectant solution had a ratio of concentrated peroxygen-based disinfectant powder to final solution of 1:600.

Thirty mL of the peroxygen-based disinfectant solution was applied as a foam to coupons in all disinfectant treatment groups (4C/1:600/10M, 4C/1:600/30M, 4C/1:100/10M, 4C/1:100/30M, -10C/1:600/30M, -10C/1:100/10M, -10C/1:100/30M) using a 5.7 L handheld pump-up foamer (model #A8020A, Ogena Solutions, LLC, Stony Creek, ON, Canada). In commercial trailer washes, 189 L of disinfectant would be applied to a 15.8 m double-decked livestock trailer over a 10 minute time-period using a proportioning foamer with a flow rate of 18.9 L per minute. Thirty mL of disinfectant applied to a diamond plate aluminum coupon measuring 15.24 cm × 15.24 cm × 2.54 cm is equivalent to 189 L applied to a 15.8 m livestock trailer. Pre-trial work demonstrated that a 2.5 second application time was required to apply 30 mL (liquid volume) of peroxygen-based disinfectant solution to the coupon.

The coupons assigned to the 4C/PosCtrl group were sham disinfected with 30 mL phosphate buffered saline (PBS) using a 5.7 L handheld pump-up foamer (model #A8020A, Ogena Solutions, LLC, Stony Creek, ON, Canada). A new foamer was used for the positive control groups to ensure that no peroxygen-based disinfectant residue would alter the results. The coupons assigned to the -10C/PosCtrl group were sham disinfected with 30 mL of a PBS solution that was 10% PG by volume to prevent freezing. The same foamer was used for both positive control groups. Pre-trial work indicated that a 1 second application time was required to apply 30 mL of the sham disinfectant to the coupon.

Following application of the peroxygen-based disinfectant or sham disinfectant solution, the coupons were placed in either a refrigerator set to 4°C or a freezer set at -10°C for their allotted contact time as described in Table 1. Plastic sheeting was placed on the shelves of the refrigerator and inside the drawers of the freezer and changed between groups to prevent cross contamination between treatment groups. To further prevent cross contamination in the freezer,

the freezer drawer was lined with a folded bath towel to absorb liquid runoff from the disinfected coupons, as described in previous work (Baker et al., In Review). To prevent cross-contamination between replicates within each treatment, coupons assigned to the 4C/1:600/10M, 4C/1:600/30M, 4C/1:100/10M, 4C/1:100/30M, and 4C/PosCtrl groups were placed into a 34.29 cm × 24.13 cm × 7.47 cm aluminum foil pan lined with a hand towel to absorb liquid runoff from the coupon prior to placement in the refrigerator. Two coupons were placed as far apart as possible in each aluminum foil pan, with two aluminum foil pans used per treatment group. Coupons assigned to the -10C/1:600/30M, -10C/1:100/10M, -10C/1:100/30M were placed into individual 16 cm × 16 cm × 3 cm aluminum foil pans prior to placement in the freezer as previously described (Baker et.al., In Review). New plastic sheeting, hand towels, bath towels, and aluminum foil pans were used for every treatment group.

Following 30 minutes of pre-cooling in the 4°C refrigerator, feces were re-collected from the coupons assigned to the NegCtrl group. The coupon was tilted away from the holes and 10 mL of 0.9% sodium chloride (sterile saline) was added to the coupon to aid in fecal recollection. A new toothbrush dedicated to each coupon was used to suspend the feces in the saline; creating a homogenate solution. The resulting feces / saline homogenate was collected from the coupon using a 20 mL syringe. One mL of the homogenate was placed into a snap cap tube and placed on dry ice to serve as the post-treatment environmental sample from that coupon. The remainder of the feces / saline homogenate served as the inoculum for the swine bioassay portion of this study.

For the disinfectant treatment groups (4C/1:600/10M, 4C/1:600/30M, 4C/1:100/10M, 4C/1:100/30M, -10C/1:600/30M, -10C/1:100/10M, -10C/1:100/30M), similar procedures to the NegCtrl group were used to collect post-treatment samples. The only difference is that, for these

peroxygen-treated groups, one mL of the feces / saline / peroxygen based disinfectant homogenate was placed into a snap cap tube filled with 3 mL of Dey-Engly neutralizing broth (MilliporeSigma, Massachusetts, USA) and placed on dry ice to serve as the post-treatment environmental sample from the coupon. A 1:3 ratio of peroxygen-based disinfectant solution to Dey-Engly neutralizing broth was sufficient to neutralize the virucidal action of the peroxygen-based disinfectant in the tube to obtain a more accurate post-treatment sample (personal communications). The remaining 15 - 20 mL of the feces / saline / peroxygen-based disinfectant homogenate served as the inoculum for the swine bioassay portion of this study.

The feces / PBS / saline homogenate from the coupons assigned to the 4C/PosCtrl and the feces / PBS / PG / saline homogenate from the coupons assigned to the -10C/PosCtrl group were obtained as described above for the disinfectant treatment groups. One mL of the homogenate was placed into a snap cap tube and placed on dry ice to serve as the post-treatment environmental sample from that coupon. The remainder of the feces / saline homogenate served as the inoculum for the swine bioassay portion of this study. To prevent cross contamination between replicates during re-collection of coupon contents, nitrile gloves were changed between each coupon. To prevent cross contamination between treatment groups, feces were re-collected from the negative control group first and then from the other treatment groups ending with the two positive control groups. Plastic sheeting was placed under the coupons and changed between treatment groups as an additional precaution.

Post-treatment environmental samples were collected from each coupon, after the designated contact time expired, and tested for the presence of PEDV RNA via RT-qPCR and the presence of live PEDV by PEDV virus isolation at the ISU VDL. To overcome the potential cytotoxicity of post-treatment samples, virus isolation was conducted using the following

procedures. Post-treatment samples were 10-fold serially diluted ( $10^{-1}$  to  $10^{-4}$ ) and inoculated into Vero cell monolayer grown in 96-well plate that had been pre-washed twice with the post-inoculation medium, 100  $\mu$ l inoculum per well, triplicate wells per dilution. The plates were incubated at 37°C with 5% CO<sub>2</sub> and viral cytopathic effect (CPE) development was recorded daily through 5 days post inoculation (DPI). On 5 DPI, the plates were frozen at -80°C. After one freeze-thaw cycle, the cell lysates were passed one more passage in Vero cells. The CPE was recorded every day. On 5 DPI of the 2nd passage, the plates were subjected to immunofluorescence staining using a monoclonal antibody conjugate SD6-29 against the PEDV nucleic acid protein (SD-1F, Medgene Labs, Brookings, South Dakota, USA). If one or more wells inoculated with any dilution had CPE and was immunofluorescence staining positive, virus isolation on that sample was considered positive.

### **Swine bioassay procedures**

The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee (Log Number: 7-16-8310-S) and the Iowa State University Institutional Biosafety Committee (Log Number 16-I-0025-A) prior to initiation of any experimental activity. Forty 3-week-old barrows were sourced from a commercial producer in Iowa and housed in the Iowa State University Livestock Infectious Disease Isolation Facility for the duration of the study. Pigs arrived on study day -4 and were weighed and identified with a unique ID number on a plastic ear tag upon arrival. Arrival weight was used to divide the pigs into 4 blocks with 10 pigs per block. One pig from each block was randomly assigned to one of the ten treatment groups using the RAND function in Microsoft Excel (version 2016).

Pigs were allowed to acclimate to their new housing for 72 hours. A rectal swab and blood were obtained from each pig on study day -1 to confirm that they were negative for PEDV, PDCoV, and TGEV. Rectal swabs were collected from each pig using a commercial swab and

transport system. Individual rectal swabs were tested for PEDV and PDCoV by a duplex PEDV/PDCoV RT-qPCR at the ISU VDL. The primers and probes used in the PEDV/PDCoV RT-qPCR were described previously (Zhang et. al., 2016). Rectal swabs were pooled (5 swabs per pool) and tested for the presence of TGEV by RT-qPCR at ISU VDL (Schneider et al., 2015). Eight mL of whole blood was obtained from each pig by venipuncture of the external jugular vein and transferred to an 8.5 mL serum separator tube. The whole blood was centrifuged at 3100 g for 8 minutes and the resulting serum was individually screened for antibodies to PEDV by indirect fluorescent antibody (IFA) in accordance with previously described procedures (Thomas et al., 2015a).

Pigs in each treatment group (n=4) were housed individually in a raised tub that allowed visual and auditory contact between pigs but prevented nose-to-nose and fecal-oral contact. Tubs used in this study successfully prevented PEDV transmission between pigs within the same treatment group in previous studies (Thomas et al., 2015a; Thomas et al., 2015b; Holtkamp et al., 2017, Baker et al., In Review). Each pig had its own source of water and feed and was fed a pelleted starter diet ad libitum. The diet was a corn and soybean meal based diet without porcine derived products. A sample of the feed was tested for PEDV via RT-qPCR at the ISU VDL prior to study initiation and was confirmed PEDV-negative.

On study day 0, the contents remaining in the coupon after treatment (described above) were immediately administered to the pig assigned to that coupon via oral gastric gavage with a 14 French rubber catheter as previously described (Thomas et. al., 2015b). To prevent cross contamination both within and between treatment groups study personnel followed a strict biosecurity protocol previously demonstrated to prevent transmission of PEDV between pigs (Thomas et al., 2015b, Holtkamp et al., 2017, Baker et al., In Review). Study personnel changed

disposable TyVek coveralls (DuPont, USA) and disposable boots between treatment groups. To prevent cross contamination between replicates, study personnel changed arm-length disposable obstetrical sleeves and nitrile gloves between each pig. Furthermore, the disposable coveralls were inspected after each pig was inoculated; if inoculum or fecal material was present, the coveralls were changed before the next pig was inoculated.

The same investigator monitored the pigs for clinical signs consistent with PEDV for the next seven days (study day 0 to study day 7). A rectal swab was collected from each pig on days 3 and 7 post-inoculation using the same biosecurity procedures as described above for inoculation to prevent cross contamination within or between treatment groups. The pig was considered bioassay positive if its rectal swab was positive for PEDV RNA by RT-qPCR (Ct value less than 36) on 3 or 7 days post inoculation. The pig was considered bioassay negative if rectal swabs collected on both 3 and 7 days post inoculation were negative for PEDV RNA by RT-qPCR (Ct value equal to or greater than 36).

Seven days post inoculation (study day 7), all pigs were euthanized via penetrating captive bolt gun and necropsied. Gross evaluation of all organ systems was performed and any gross lesions or abnormal pathology was noted. Fresh and 10% formalin-fixed samples of mesenteric lymph nodes, ileum, and jejunum and fresh cecal and spiral colon contents were collected and were held in the event that further testing might be required to confirm the results of the swine bioassay.

SAS® (Enterprise Guide 5.1; SAS Institute, Cary, NC, USA) was used to perform the statistical analyses. A Fisher's Exact Test was used to evaluate pairwise differences in the proportion of pigs positive by bioassay between all ten treatment groups. Ct values were analyzed using two way analyses of variance (ANOVA) models with treatment, time (pre vs



post) and their interaction. Pre-treatment Ct values were compared between groups using an F-test. Differences in Ct values between pre- and post-treatment were assessed for each study group using a two-sided t-test. A *p* value <0.05 was considered statistically significant.

## Results

### Pre-trial diagnostic screening

Fecal samples obtained on study day -1 confirmed that all pigs were negative for PEDV, PDCoV, and TGEV by RT-qPCR. Serum samples collected on day -1 confirmed that all pigs were negative for antibodies to PEDV via IFA prior to the initiation of the study.

### Environmental samples

PEDV RT-qPCR results from environmental samples collected from each contaminated aluminum coupon before and after the application of the peroxygen-based disinfectant or the sham disinfectant are presented in Table 2. For the negative control group (NegCtrl), all environmental samples collected pre-treatment, immediately after contamination with PEDV-negative feces, were negative for PEDV RNA via RT-qPCR. Pre-treatment environmental samples, obtained immediately after PEDV-positive feces were applied to the coupons, from the coupons in both positive control groups (4C/PosCtrl and -10C/PosCtrl) were positive for PEDV RNA via RT-qPCR with quantitative results ranging from  $10^{7.60}$  to  $10^{8.01}$  genetic copies/mL. Correspondingly, all pre-treatment environmental samples obtained from the coupons in the peroxygen-based disinfectant treatment groups (4C/1:600/10M, 4C/1:600/30M, 4C/1:100/10M, 4C/1:100/30M, -10C/1:600/30M, -10C/1:100/10M, -10C/1:100/30M) tested positive for PEDV RNA by RT-qPCR with quantitative results ranging from  $10^{7.37}$  to  $10^{8.08}$  genetic copies/ml. There were no significant differences in pre-treatment Ct values from the coupons assigned to the two positive control (4CPosCtrl, -10CPosCtrl) and the seven peroxygen-based disinfectant

(4C/1:600/10M, 4C/1:600/30M, 4C/1:100/10M, 4C/1:100/30M, -10C/1:600/30M, -10C/1:100/10M, -10C/1:100/30M) groups (p-value > 0.05).

Post-treatment environmental samples from the negative control (NegCtrl) coupons, obtained after the coupons spent 30 minutes in a 4°C fridge, were negative for PEDV RNA via RT-qPCR. All (8 of 8) post-treatment environmental samples, collected from the coupons in both positive control groups (4C/PosCtrl and -10CPosCtrl) after 30 minutes of pre-cooling in a 4°C fridge and treatment with the sham disinfectant as described in Table 1, were positive for PEDV RNA via real-time RT\_PCR with quantitative results ranging from  $10^{7.74}$  to  $10^{8.84}$  genetic copies/mL. All post-treatment environmental samples (28 of 28) from the coupons assigned to the seven peroxygen-based disinfectant groups (4C/1:600/10M, 4C/1:600/30M, 4C/1:100/10M, 4C/1:100/30M, -10C/1:600/30M, -10C/1:100/10M, -10C/1:100/30M) were positive for PEDV RNA via RT-qPCR with quantitative results ranging from  $10^{7.01}$  to  $10^{8.38}$  genetic copies/mL. The difference in Ct values between pre-treatment and post-treatment environmental samples were significantly different than zero (p value < 0.05) for all of the peroxygen-based disinfectant groups (4C/1:600/10M, 4C/1:600/30M, 4C/1:100/10M, 4C/1:100/30M, -10C/1:600/30M, -10C/1:100/10M, -10C/1:100/30M).

### **Swine bioassay**

PEDV RT-qPCR results from rectal swabs obtained on days 3 and 7 post challenge and swine bioassay results by treatment group are summarized in Table 3. One pig in the negative control (NegCtrl) group died on study day 1 due to a severe bacterial septicemia and was removed from the study. The pig was submitted to the ISUVDL for a full necropsy and diagnostic workup which concluded that the cause of death was not PEDV. There are no rectal swab or bioassay results for this pig. All rectal swabs from the pigs in the negative control (NegCtrl) and the seven peroxygen-based disinfectant treatment groups (4C/1:600/10M,

4C/1:600/30M, 4C/1:100/10M, 4C/1:100/30M, -10C/1:600/30M, -10C/1:100/10M, -10C/1:100/30M) were PCR negative for PEDV on both 3 and 7 days post inoculation. All pigs in the positive control group conducted at 4°C (4C/PosCtrl) and 3 of 4 pigs in the positive control group conducted at -10°C (-10C/PosCtrl) were PCR positive for PEDV on both 3 and 7 days post inoculation. The proportion of pigs bioassay positive for PEDV in the seven of the peroxygen-based disinfectant groups (4C/1:600/10M, 4C/1:600/30M, 4C/1:100/10M, 4C/1:100/30M, -10C/1:600/30M, -10C/1:100/10M, -10C/1:100/30M) were significantly different (p-value < 0.05) than the proportion of pigs bioassay positive for PEDV in the positive control group conducted at 4°C (4C/PosCtrl) but not significantly different (p-value > 0.05) than the proportion of pigs bioassay positive for PEDV in the positive control group conducted at -10°C via Fishers Exact Test. The lack of statistical significance to the -10C/PosCtrl group is due to the small sample size in each treatment group, the only swine bioassay results that were statistically significant were 0% (0 of 4 pigs) PEDV positive compared to 100% (4 of 4 pigs) PEDV positive.

### **Virus isolation**

Environmental samples collected from each coupon after the completion of its assigned treatment (Table 1) were tested for the presence of live PEDV via PED virus isolation. Virus isolation results of the individual coupons are described in Table 3. All post-treatment environmental samples from the negative control (NegCtrl) and peroxygen-based disinfectant treatment groups (4C/1:600/10M, 4C/1:600/30M, 4C/1:100/10M, 4C/1:100/30M, -10C/1:600/30M, -10C/1:100/10M, -10C/1:100/30M) were negative for live PEDV by virus isolation. In contrast, 25% (1 of 4) of the coupons in the 4C/PosCtrl group and 75% (3 of 4) of the coupons in the -10C/PosCtrl group were positive for live PEDV by virus isolation. The proportion of coupons with environmental samples positive for PEDV via virus isolation in each

treatment group was not significantly different between any of the 10 groups via Fishers Exact Test ( $p\text{-value} > 0.05$ ).

## **Discussion**

Under winter-like conditions ( $4^{\circ}\text{C}$  and  $-10^{\circ}\text{C}$ ) a peroxygen-based disinfectant inactivated PEDV in the presence of low levels of swine feces on aluminum surfaces like those found in commercial livestock trailers. Both the 1:100 and 1:600 dilutions of the peroxygen-based disinfectant were able to inactivate PEDV with 30 minutes of contact time. Findings from this study suggest that the peroxygen-based disinfectant may be implemented as the disinfect step of a wash, disinfect and dry trailer sanitation and decontamination protocol in the winter. In this study, the peroxygen-based disinfectant remained efficacious in the presence of 2mL of swine feces which supports previous work indicating that this peroxygen-based disinfectant is still effective when applied to low levels of organic matter (Chandler-Bostock and Mellitis, 2015). The ability to remain effective in low levels of organic matter may help the peroxygen-based disinfectant compensate for a subpar wash step which occurs more frequently in the winter. Additionally, preparing the peroxygen-based disinfectant in a 10% PG solution prevented the disinfectant from freezing during the 30 minute contact time at  $-10^{\circ}\text{C}$  supporting previous work that indicated PG can be used as an anti-freezing agent without reducing the efficacy of disinfectants (Davison et al., 1999; Dee et al., 2005b; Baker et al., In Review).

Since PEDV is difficult to culture outside the animal, swine veterinarians and producers sometimes use RT-PCR assays to detect PEDV and determine the success of sanitation and decontamination practices. However, RT-PCR does not differentiate between infectious virus and noninfectious fragments of PEDV RNA. Thus, a positive PCR result following trailer sanitation and decontamination indicates that PEDV RNA was detected but does not mean

infectious virus is still present. In this study, all post-treatment environmental samples (28 of 28) from the coupons contaminated with PEDV-positive feces and then subjected to one of seven peroxygen-based disinfectant treatments (4C/1:600/10M, 4C/1:600/30M, 4C/1:100/10M, 4C/1:100/30M, -10C/1:600/30M, -10C/1:100/10M, -10C/1:100/30M) tested positive for the presence of PEDV RNA by RT-qPCR. However, no infectious virus was detected in these coupons as demonstrated by the swine bioassay results (Table 3). Therefore, under the conditions of this study, the peroxygen-based disinfectant inactivated the PEDV, but left a substantial amount of viral RNA intact to interact with the primers and probes used in the RT-qPCR assay. The peroxygen-based disinfectant is an oxidizing agent that inactivates viruses by denaturing viral proteins, enzymes, lipids by targeting sulfhydryl (-SH) and disulfide (S-S) bonds. This results in cell lysis and exposure of nucleic acids which may also be disrupted by the disinfectant (Block, 2001). These results are consistent with those from previous studies where disinfection of contaminated metal surfaces with oxidizing disinfectants inactivated PEDV but did not consistently produce negative PEDV RT-qPCR results after disinfection (Baker et al., In Review; Bowman et al., 2015; Huss et al., 2017). Therefore, PEDV-positive RT-qPCR results on environmental samples should be expected when the peroxygen-based disinfectant is utilized at 4°C and -10°C, but this does not necessarily indicate that an infectious dose of PEDV remains in the trailer.

The PEDV virus isolation results from this study support conclusions from other studies that PEDV is difficult to grow in cell culture (Chen et al., 2014; Thomas et al., 2015a). For the 4C/PosCtrl group, all of the 4 pigs inoculated with the post-treatment samples became infected with PEDV whereas only 1 of 4 post-treatment environmental samples was positive for live virus via PEDV virus isolation (Table 3). For the 4C/PosCtrl group, the swine bioassay was more

sensitive than virus isolation for detection of infectious PEDV. Seventy-five percent of (3 of 4) pigs in the -10C/PosCtrl group became infected with PEDV and 3 of 4 environmental samples from this treatment group were positive for PEDV via virus isolation. However, the environmental sample from the coupon whose contents were administered to the pig in -10C/PosCtrl group that did not become infected with PEDV was positive for PEDV via virus isolation indicating that this pig's lack of infection was most likely due to an ineffectual gavage. The environmental sample from the -10C/PosCtrl that was PEDV negative via virus isolation caused a PEDV infection in the inoculated pig, indicating that the swine bioassay was a more sensitive method of detection than virus isolation for this sample.

The coupons used in this study were constructed of the same aluminum diamond plate flooring used in most commercial livestock trailers, but livestock trailers also have vertical walls, gates, hinges, latches, and corners that organic material can attach to and provide areas for a virus to hide from a disinfectant. While the diamond plating in the coupons is more representative of the surfaces found in a livestock trailer than the smooth aluminum coupons used in previous studies (Baker et al., In Review; Holtkamp et al., 2016; Holtkamp et al., 2017; Thomas et al., 2015b) they still do not replicate all surfaces found within livestock trailers. However, performing a study of this magnitude with full-size livestock trailers was not feasible, so diamond plate aluminum coupons were used as a model. The small size of the coupons allowed the investigators to contaminate the coupons, perform the disinfectant treatment, collect the inoculum, and inoculate the pigs for the bioassay for all study groups in less than one day. This model also minimized the length of time between inoculum collection and administration to the pigs. Additionally, wood shavings are frequently used as bedding in livestock trailers prior to transporting pigs. Incorporation of wood shavings into the model was considered, however, the

size and type of shavings used for bedding varies widely across the industry and some types of wood may have virucidal properties (Greatest et al., 2011). Therefore, the aluminum coupons were contaminated with swine feces alone to avoid the possibility of confounding the results with the investigators' choice of wood shavings.

The peroxygen-based disinfectant used in this study is labeled as virucidal for swine in 10 minutes with dilutions from 1:50 to 1:200; this study only evaluated two dilutions (1:100 and 1:600) and tested both the labeled contact time of 10 minutes in addition to considerably longer contact time of 30 minutes. The longer contact time was included because the conditions in this study were less favorable than those used in determining the label. Additionally, 30 minutes of contact time is attainable under nearly all circumstances encountered when transporting swine. Further research on the efficacy of a peroxygen-based disinfectant under other adverse conditions such as greater dilution rates, on vertical surfaces to simulate trailer sidewalls, and shorter contact times is warranted.

## Tables

Table 4.1 Summary of treatment groups

Treatment Group	Volume, PEDV status of feces	Disinfectant	Dilution	Temperature (°C)	Contact Time (min)
NegCtrl	2 mL, negative	N/A	N/A	4 °C	30
4C/1:600/10M	2 mL, positive	Peroxygen-based disinfectant	1:600	4 °C	10
4C/1:600/30M	2 mL, positive	Peroxygen-based disinfectant	1:600	4 °C	30
4C/1:100/10M	2 mL, positive	Peroxygen-based disinfectant	1:100	4 °C	10
4C/1:100/30M	2 mL, positive	Peroxygen-based disinfectant	1:100	4 °C	30
–10C/1:600/30M	2 mL, positive	Peroxygen-based disinfectant (10% PG <sup>a</sup> )	1:600	–10 °C	30
–10C/1:100/10M	2 mL, positive	Peroxygen-based disinfectant (10% PG <sup>a</sup> )	1:100	–10 °C	10
–10C/1:100/30M	2 mL, positive	Peroxygen-based disinfectant (10% PG <sup>a</sup> )	1:100	–10 °C	30
4C/PosCtrl	2 mL, positive	Phosphate Buffered Saline	N/A	4 °C	30
–10C/PosCtrl	2 mL, positive	Phosphate Buffered Saline (10% PG <sup>a</sup> )	N/A	–10 °C	30

<sup>a</sup> PG = Propylene glycol. This was added to any disinfectant or sham disinfectant treatment conducted at –10 °C as an anti-freezing agent.

Table 4.2 PEDV RT-qPCR results for environmental samples collected from the contaminated coupons before and after the application of the peroxygen-based disinfectant

<b>Table 2</b> PEDV RT-qPCR results for environmental samples collected from the contaminated coupons before and after the application of the peroxygen-based disinfectant.				
Treatment Group <sup>a</sup>	Pre-treatment <sup>b</sup> Ct value (genomic copies/mL)	Percentage positive for PEDV <sup>c</sup>	Post-treatment <sup>d</sup> Ct value (genomic copies/mL)	Percentage positive for PEDV <sup>c</sup>
NegCtrl	> 36 (0) > 36 (0) > 36 (0) > 36 (0)	0% (0 of 4)	> 36 (0) > 36 (0) > 36 (0) > 36 (0)	0% (0 of 4)
4C/1:600/ 10M	20.2 (10 <sup>7.78</sup> ) 20.7 (10 <sup>7.63</sup> ) 19.7 (10 <sup>7.94</sup> ) 20.6 (10 <sup>7.66</sup> )	100% (4 of 4)	21.8 (10 <sup>7.31</sup> ) 20.1 (10 <sup>7.81</sup> ) 22.5 (10 <sup>7.10</sup> ) 21.4 (10 <sup>7.43</sup> )	100% (4 of 4)
4C/1:600/ 30M	21.6 (10 <sup>7.37</sup> ) 19.6 (10 <sup>7.96</sup> ) 20.4 (10 <sup>7.73</sup> ) 20.1 (10 <sup>7.81</sup> )	100% (4 of 4)	22.0 (10 <sup>7.36</sup> ) 19.3 (10 <sup>8.04</sup> ) 22.3 (10 <sup>7.17</sup> ) 20.9 (10 <sup>7.58</sup> )	100% (4 of 4)
4C/1:100/ 10M	20.0 (10 <sup>7.85</sup> ) 19.2 (10 <sup>8.08</sup> ) 19.2 (10 <sup>8.07</sup> ) 20.0 (10 <sup>7.84</sup> )	100% (4 of 4)	19.5 (10 <sup>7.99</sup> ) 19.8 (10 <sup>7.91</sup> ) 19.5 (10 <sup>7.97</sup> ) 18.2 (10 <sup>8.38</sup> )	100% (4 of 4)
4C/1:100/ 30M	21.6 (10 <sup>7.38</sup> ) 20.1 (10 <sup>7.82</sup> ) 20.6 (10 <sup>7.66</sup> ) 20.6 (10 <sup>7.66</sup> )	100% (4 of 4)	18.9 (10 <sup>8.16</sup> ) 19.3 (10 <sup>8.04</sup> ) 19.1 (10 <sup>8.11</sup> ) 19.6 (10 <sup>7.95</sup> )	100% (4 of 4)
-10C/ 1:600/ 30M	21.2 (10 <sup>7.48</sup> ) 19.7 (10 <sup>7.91</sup> ) 20.2 (10 <sup>7.78</sup> ) 20.2 (10 <sup>7.78</sup> )	100% (4 of 4)	22.1 (10 <sup>7.24</sup> ) 20.8 (10 <sup>7.61</sup> ) 21.5 (10 <sup>7.41</sup> ) 22.8 (10 <sup>7.01</sup> )	100% (4 of 4)
-10C/ 1:100/ 10M	20.1 (10 <sup>7.81</sup> ) 20.1 (10 <sup>7.80</sup> ) 20.2 (10 <sup>7.78</sup> ) 19.9 (10 <sup>7.87</sup> )	100% (4 of 4)	18.5 (10 <sup>8.27</sup> ) 19.1 (10 <sup>8.10</sup> ) 19.7 (10 <sup>7.94</sup> ) 20.4 (10 <sup>7.71</sup> )	100% (4 of 4)
-10C/ 1:100/ 30M	20.6 (10 <sup>7.67</sup> ) 21.1 (10 <sup>7.52</sup> ) 20.4 (10 <sup>7.72</sup> ) 20.2 (10 <sup>7.78</sup> )	100% (4 of 4)	19.0 (10 <sup>8.12</sup> ) 18.6 (10 <sup>8.26</sup> ) 18.3 (10 <sup>8.35</sup> ) 19.0 (10 <sup>8.13</sup> )	100% (4 of 4)
4C/PosCtrl	20.8 (10 <sup>7.60</sup> ) 20.0 (10 <sup>7.85</sup> ) 20.3 (10 <sup>7.74</sup> ) 19.8 (10 <sup>7.91</sup> )	100% (4 of 4)	20.2 (10 <sup>7.77</sup> ) 20.4 (10 <sup>7.74</sup> ) 20.4 (10 <sup>7.74</sup> ) 20.3 (10 <sup>7.75</sup> )	100% (4 of 4)
-10C/ PosCtrl	19.0 (10 <sup>8.14</sup> ) 20.5 (10 <sup>7.69</sup> ) 19.4 (10 <sup>8.01</sup> ) 20.3 (10 <sup>7.74</sup> )	100% (4 of 4)	17.0 (10 <sup>8.71</sup> ) 16.6 (10 <sup>8.84</sup> ) 17.7 (10 <sup>8.52</sup> ) 17.2 (10 <sup>8.67</sup> )	100% (4 of 4)

<sup>a</sup> Treatment groups are described in Table 1.

<sup>b</sup> Results from an environmental swab taken after contamination with feces but before exposure to the peroxygen-based disinfectant or the sham disinfectant.

<sup>c</sup> PEDV Positive, Ct < 36; PEDV Negative, Ct ≥ 36.

<sup>d</sup> Results from an environmental sample collected from the contaminated coupons after treatment with the peroxygen-based disinfectant or sham disinfectant.



Table 4.3 Post-challenge rectal swab PEDV RT-qPCR and swine bioassay results

<b>Table 3</b> Post-challenge rectal swab PEDV RT-qPCR and swine bioassay results.					
Treatment Group <sup>a</sup>	Individual day 3 rectal swab Ct values	Individual day 7 rectal swab Ct values	Swine bioassay; percentage positive for PEDV RNA <sup>b</sup>	Individual PEDV virus isolation results	Virus isolation; percentage positive for PEDV
NegCtrl <sup>c</sup>	> 36	> 36	0% (0 of 3) <sup>d</sup>	Negative	0% (0 of 3) <sup>g</sup>
	> 36	> 36		Negative	
	> 36	> 36		Negative	
4C/1:600/10M	> 36	> 36	0% (0 of 4) <sup>d</sup>	Negative	0% (0 of 4) <sup>g</sup>
	> 36	> 36		Negative	
	> 36	> 36		Negative	
4C/1:600/30M	> 36	> 36	0% (0 of 4) <sup>d</sup>	Negative	0% (0 of 4) <sup>g</sup>
	> 36	> 36		Negative	
	> 36	> 36		Negative	
4C/1:100/10M	> 36	> 36	0% (0 of 4) <sup>d</sup>	Negative	0% (0 of 4) <sup>g</sup>
	> 36	> 36		Negative	
	> 36	> 36		Negative	
	> 36	> 36		Negative	
4C/1:100/30M	> 36	> 36	0% (0 of 4) <sup>d</sup>	Negative	0% (0 of 4) <sup>g</sup>
	> 36	> 36		Negative	
	> 36	> 36		Negative	
-10C/1:600/30M	> 36	> 36	0% (0 of 4) <sup>d</sup>	Negative	0% (0 of 4) <sup>g</sup>
	> 36	> 36		Negative	
	> 36	> 36		Negative	
	> 36	> 36		Negative	
-10C/1:100/10M	> 36	> 36	0% (0 of 4) <sup>d</sup>	Negative	0% (0 of 4) <sup>g</sup>
	> 36	> 36		Negative	
	> 36	> 36		Negative	
	> 36	> 36		Negative	
-10C/1:100/30M	> 36	> 36	0% (0 of 4) <sup>d</sup>	Negative	0% (0 of 4) <sup>g</sup>
	> 36	> 36		Negative	
	> 36	> 36		Negative	
	> 36	> 36		Negative	
4C/PosCtrl	23.9	24.1	100% (4 of 4) <sup>e</sup>	Negative	25% (1 of 4) <sup>g</sup>
	24.1	23.2		Negative	
	19.0	29.2		Positive	
	20.0	24.2		Negative	
-10C/PosCtrl <sup>f</sup>	15.3	20.7	75% (3 of 4) <sup>d,e</sup>	Negative	75% (3 of 4) <sup>g</sup>
	14.0	20.7		Positive	
	21.9	21.0		Positive	
	> 36	> 36 <sup>f</sup>		Positive <sup>f</sup>	

<sup>a</sup> Treatment groups are described in Table 1.

<sup>b</sup> PEDV Positive, Ct < 36; PEDV Negative, Ct ≥ 36.

<sup>c</sup> One pig in the negative control group died due to a bacterial septicemia on study day 1 prior to rectal swab sampling.

<sup>d</sup> Values with different letters vary significantly (p-value < 0.05) via Fisher's Exact Test.

<sup>e</sup> Values with different letters vary significantly (p-value < 0.05) via Fisher's Exact Test.

<sup>f</sup> One pig in the positive control group was not bioassay positive for PEDV, but the sample of inoculum given to this pig was PEDV positive by virus isolation.

<sup>g</sup> Values with different letters vary significantly (p-value < 0.05) via Fisher's Exact Test.

## Figures

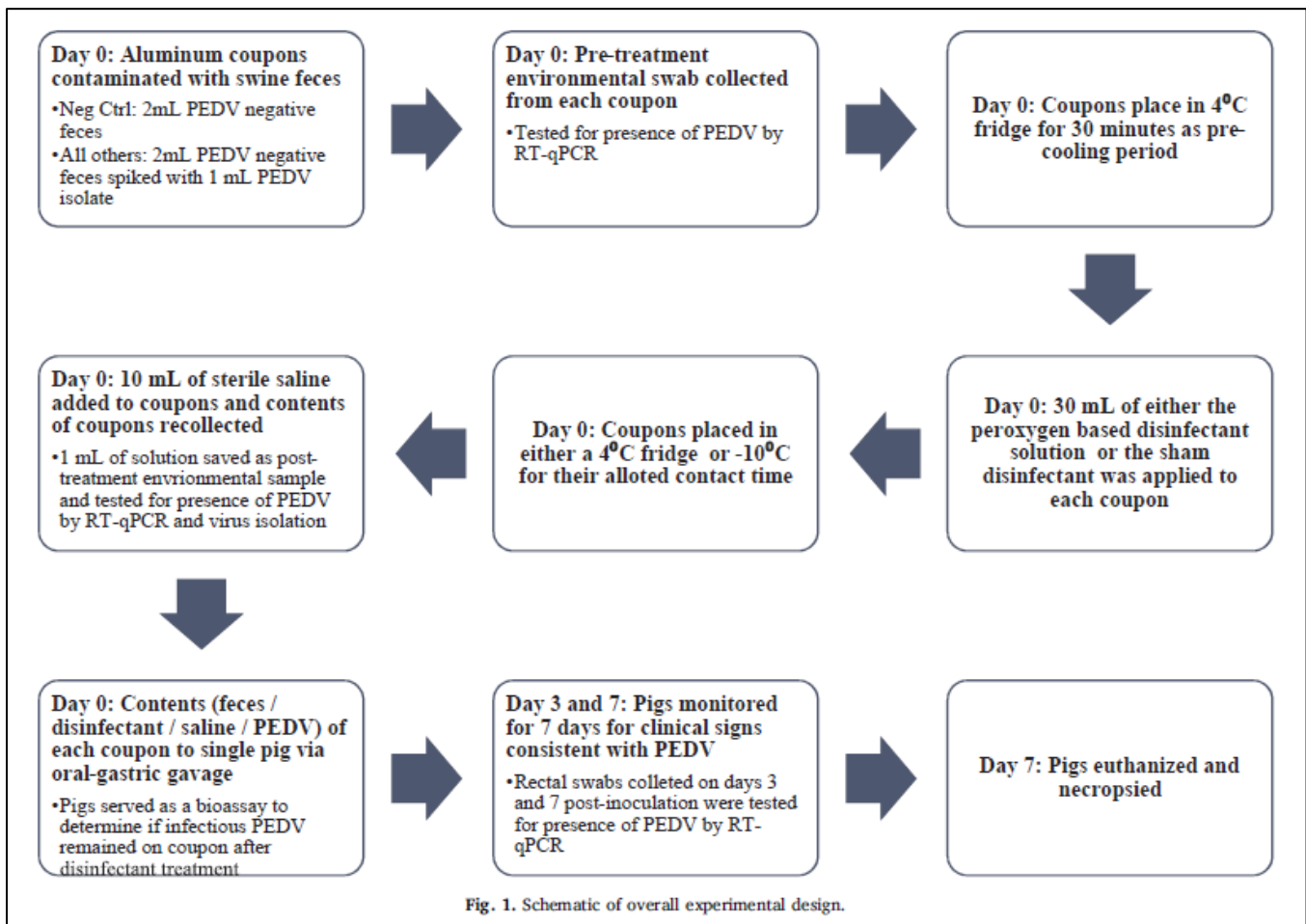


Figure 4.1 Schematic of overall experimental design.

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### Conflict of interest

The study was funded by Lanxess, the company that manufactures Virkon™S Disinfectant and Virucide Powder. The authors declare no other conflicts of interest.

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## CHAPTER 5. GENERAL CONCLUSION

### Summary of thesis conclusions

The original research in this thesis (chapters 3 and 4) was consistent with the findings of the literature review. The literature review suggested that most commonly used disinfectants in the swine industry (phenol, hydrogen peroxide, peroxygen compounds, quaternary ammonium, quaternary ammonium glutaraldehyde, and sodium hypochlorite) were effective at inactivating PEDV under ideal conditions. The original work in this thesis aimed to expand the current research on the efficacy of chemical disinfection under adverse conditions such as cold temperatures and the presence of organic matter which both tend to decrease the efficacy of disinfectants (Dvorak, 2001; Thrash and Robert, 2001).

Holtkamp et al., (2017) demonstrated that an accelerated hydrogen peroxide disinfectant was effective at inactivating PEDV in the presence of swine feces at room temperature when given 30 minutes of contact time at both the 1:16 and 1:32 dilutions. The labeled contact time for the accelerated hydrogen peroxide disinfectant is 5 minutes at the 1:16 to 1:64 dilution rates. In that study, the contact time and concentration of the disinfectant were increased to account for the presence of organic material. Study 1, “Evaluation of an accelerated hydrogen peroxide disinfectant to inactivate porcine epidemic diarrhea virus in swine feces on aluminum surfaces under freezing conditions”, supports these findings. In study 1, the 1:32 dilution of accelerated hydrogen peroxide disinfectant inactivated PEDV when applied at -10°C in the presence of organic matter after 40 minutes of contact time. It is important to note that the accelerated hydrogen peroxide disinfectant used in study 1 was prepared in a solution of 10% propylene glycol to prevent freezing at -10°C. Without the addition of propylene glycol as an anti-freezing agent, the results of this study may differ. The extended contact time used in study 1 was

determined by The Arrhenius equation. This equation established that for every 10°C decrease in temperature, the contact time needed for a disinfectant to be effective doubled (Thrash and Robert, 2001).

Bowman et al. (2015) revealed that the 1:200 dilution of a peroxygen-based disinfectant was able to inactivate PEDV (dried on a petri dish) at 37°C, 4°C, and -10°C when given 60 minutes of contact time. Study 2, “Evaluation of a peroxygen-based disinfectant for inactivation of porcine epidemic diarrhea virus at low temperatures on metal surfaces”, supports these findings. Study 2, established that the 1:100 dilution of the peroxygen-based disinfectant inactivated PEDV in the presence of swine fecal contamination at -10°C with 10 minutes of contact time. The 1:600 dilution of the peroxygen-based disinfectant was able to inactivate PEDV at -10°C with 30 minutes of contact time under the conditions of study 2. Due to facility constraints, the 1:600 dilution was not evaluated at -10°C with a 10 minute contact time. Similar to study 1, the peroxygen-based disinfectant was prepared in a 10% propylene glycol solution to prevent the disinfectant from freezing during the contact time at -10°C. The peroxygen-based disinfectant was labeled as virucidal at the 1:100 dilution with 10 minutes of contact time. Study 2 demonstrated that the labeled dilution and contact time will inactivate PEDV under cold weather conditions when mixed with an anti-freezing agent such a propylene glycol.

Both study 1 and study 2 demonstrated that environmental samples from surfaces contaminated with PEDV may not be RT-qPCR negative after disinfection even if the disinfectant successfully inactivated PEDV. One major limitation of RT-qPCR assays is that the assay cannot differentiate between infectious PEDV viral particles and noninfectious fragments of PEDV RNA. In both study 1 and study 2, 100% of the post-treatment samples collected from the aluminum coupons after disinfectant treatment were positive for PEDV RNA by RT-qPCR,



but did not contain an infectious dose of the virus as evidenced by 0% of the swine bioassay pigs testing positive for PEDV. The results of this thesis supports previous work where disinfectants inactivated PEDV as determined by virus isolation (Bowman et al., 2015) or swine bioassay (Holtkamp et al., 2017), but did not consistently produce negative PEDV RT-qPCR results after disinfection. A positive RT-qPCR result on an environmental sample of a livestock trailer after sanitation and decontamination indicates that RNA from PEDV was present, but cannot determine if the RNA was from an infectious viral particle or not.

Both the accelerated hydrogen peroxide disinfectant used in study 1 and the peroxygen-based disinfectant used in study 2 are oxidizing agents that inactivate viruses by denaturing viral proteins, lipids, and nucleic acids (Block, 2001 and Dvorak, 2008). Oxidizing agents target sulfhydryl (-SH) and disulfide bonds (S-S) within the virus to cause cell lysis and expose viral RNA to the disinfectant where it can also become disrupted (Block, 2001). The results of study 1 and study 2 may suggest that under cold conditions, nucleic acids are denatured to a lesser extent resulting in enough intact viral RNA to cause a positive RT-qPCR result after disinfection. However, enough viral proteins and lipids may still be denatured at low temperatures to render the virus noninfectious after disinfection since there were no PEDV positive swine bioassay results in both study 1 and study 2.

Study 2 demonstrated that PEDV is difficult to grow in cell culture. All (4/4) pigs in the positive control group conducted at 4°C became infected and shed PEDV via their feces, but only 1/4 post-treatment samples from this group were positive for PEDV by virus isolation. This supports the findings of other work that determined that virus isolation is an insensitive test for detecting infectious PEDV compared to swine bioassay (Chen et al., 2014; Thomas et al., 2015a). This should be taken into careful consideration as other studies on PEDV are designed.

Studies that use virus isolation as the sole means of determining if infectious PEDV remains after an intervention may be over-estimating the efficacy of the intervention due to the insensitivity of virus isolation compared to swine bioassay.

### **Clinical application of findings**

The literature review concluded that the commonly used chemical disinfectant agents in the swine industry (phenols, hydrogen peroxide, peroxygen compounds, quaternary ammonium, quaternary ammonium glutaraldehyde, and sodium hypochlorite), alkaline pH, heat, and time are all effective ways to inactivate swine enteric coronaviruses when implemented under very specific parameters. The effects of pH, heat, and time-dependent inactivation of PEDV were not evaluated in this thesis. The effect of pH was not evaluated because this would be difficult to apply to a contaminated livestock trailer in the field. Additionally, the exact pH required to reliably inactivate PEDV is still unknown as the current body of literature is contradicting. One study indicated that a pH of 10 was sufficient to inactivate PEDV without supplemental heat (Stevens et al., 2017) while another study demonstrated that infectious PEDV was still present after being held in an environment with a pH of 10.2 (Quist-Rybachuck et al., 2015). The literature does agree that an alkaline pH ( $\text{pH} \geq 8$ ) and heat work synergistically to inactivate swine enteric coronaviruses (Laude et al., 1981; Quist-Rybachuck et al., 2015). Swine enteric coronaviruses are very susceptible to thermal inactivation. Recent work demonstrated that holding a livestock trailer at 71°C for 10 minutes or at 20°C for one week was sufficient to inactivate PEDV in swine feces (Thomas et al., 2015b).

Based on the literature review findings and the original work in this thesis, the ideal sanitation and decontamination process for a livestock trailer contaminated with PEDV should include: 1) high-pressure wash, 2) chemical disinfection, and 3) drying time either with a thermo-assisted drying and decontamination system or naturally for an extended period of time.

These recommendations match the industry standard trailer sanitation and decontamination protocol that was originally developed for PRRSV (Dee et al., 2006).

The wash step is necessary to reduce the level of organic material (such as wood shavings, dirt, and feces) present on the trailer, which impairs the ability of the disinfectant to inactivate viruses. High-pressure washing also reduces the amount of infectious viral particles present in the trailer at the time of disinfection.

After completion of a high-pressure wash, a chemical disinfectant should be applied to the trailer. Phenols, sodium hypochlorite, hydrogen peroxide, peroxygen-based, quaternary ammonium, and quaternary ammonium glutaraldehyde disinfectants are all expected to have efficacy against PEDV if applied at the correct concentration, for the correct amount of time, and at the correct temperature. Ideally, disinfectants should be applied at temperatures  $\geq 20^{\circ}\text{C}$  for best efficacy; however, that can be hard to accomplish in the winter months in the Midwestern United States. Study 1 established that an accelerated hydrogen peroxide disinfectant is effective against PEDV at  $-10^{\circ}\text{C}$  when used at the 1:32 dilution with 40 minutes of contact time. Study 2 demonstrated that a 1:100 dilution of a peroxygen-based disinfectant inactivated PEDV with 10 minutes of contact time at  $-10^{\circ}\text{C}$ .

The final step of the trailer sanitation process is to allow the trailer to dry completely before another load of pigs is hauled on the trailer. Ideally, a thermo-assisted drying and decontamination (TADD) system would be used to heat the trailer to  $71^{\circ}\text{C}$  for 10 minutes. However, drying the trailer naturally and allowing it to sit for one week at  $20^{\circ}\text{C}$  is also sufficient to inactivate PEDV.

When used in combination, these three steps (wash, disinfect, and TADD) produce a robust trailer sanitation and decontamination protocol that should prevent PEDV transmission to

the next group of pigs transported on the trailer. Swine producers should always aim to perform the industry standard trailer sanitation and decontamination process between every load of pigs hauled on their trailers. However, a complete wash, disinfect, and dry requires a large investment in time, logistics, labor, and specialized facilities which deters some individuals from performing the industry standard protocol between every load of pigs. Additionally, these procedures can be difficult to accomplish in cold weather leading to an increased amount of trailers that remain unwashed or washed poorly between loads.

For swine producers or contract haulers that are unable to perform the industry standard between every load in cold weather months due to a lack of time, facilities, labor, or other constraints, an alternative sanitation and decontamination protocol is suggested by study 1. First, the livestock trailer should be manually scraped to remove as much of the wood shavings, feces, urine, and other organic material present in the trailer as possible. After scraping, an accelerated hydrogen peroxide disinfectant at a minimum concentration of 1:32 should be applied for a minimum of 40 minutes contact time. The accelerated hydrogen peroxide disinfectant must be prepared in a solution that is 10% propylene glycol by volume if temperatures are below 0°C to prevent the disinfectant from freezing during the 40 minutes of contact time. This protocol should only be implemented as an alternative to doing nothing when the industry standard protocol is unachievable.

For swine producers concerned about subpar compliance and execution of the industry standard wash, disinfect, and dry protocol during the winter, both study 1 and study 2 offer guidance. Study 2 demonstrated that the labeled concentration and contact time of a peroxygen-based disinfectant prepared in a 10% propylene glycol solution inactivated PEDV in the presence of low levels of organic material at -10°C. Therefore, using a 1:100 dilution of a peroxygen

based disinfectant prepared in a solution that is 10% propylene glycol by volume with 10 minutes of contact time as the disinfect step of a wash, disinfect, and dry trailer sanitation protocol can compensate for a subpar wash step and cold temperatures. Similarly, study 1 demonstrated that the 1:32 dilution of an accelerated hydrogen peroxide disinfectant would also be a good choice for the disinfect step of the industry standard protocol in the winter to compensate for a subpar wash step and the cold temperatures.

### **Implications**

A robust livestock trailer sanitation and decontamination protocol designed to prevent PEDV transmission during cold weather should include either the 1:100 dilution of a peroxygen based disinfectant with 10 minutes of contact time or the 1:32 dilution of an accelerated hydrogen peroxide-based disinfectant with 40 minutes of contact time as the disinfect step of a wash, disinfect, and dry protocol. Both disinfectants should be prepared in a solution that is 10% propylene glycol by volume to prevent them from freezing. If a complete wash, disinfect, and dry cannot be accomplished; scraping the trailer to remove the organic material followed by application of the 1:32 dilution of the accelerated hydrogen peroxide-based disinfectant prepared in 10% propylene glycol for at least 40 minutes of contact time is a viable alternative to doing nothing between loads of pigs.

Livestock trailers disinfected with either the peroxygen-based disinfectant or the accelerated hydrogen peroxide disinfectant in cold weather are expected to test positive for PEDV RNA by RT-qPCR after disinfection. However, this RT-qPCR result may not indicate that infectious virus is still present on the trailer after disinfectant treatment since RT-qPCR cannot determine between noninfectious fragments of viral RNA or whole infectious viral particles.

The tools for preventing PEDV transmission from contaminated livestock trailers are already in the toolbox of swine veterinarians and producers in the United States. Creating a culture in the industry that prioritizes biosecurity and implementation of robust trailer sanitation and decontamination procedures as outlined above will reduce the risk of PEDV transmission via contaminated livestock trailers.

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