RESEARCH REPORT
SWINE HEALTH

Title: Evaluation of an accelerated hydrogen peroxide disinfectant to inactivate porcine epidemic diarrhea virus in swine feces on metal surfaces under freezing conditions – **NPB #14-272**

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INDUSTRY SUMMARY

In May of 2013, porcine epidemic diarrhea virus (PEDV) was detected in swine for the first time in the United States and spread quickly across much of the country, partly due to the movement of contaminated livestock trailers. The objective of this study was to evaluate two concentrations of an accelerated hydrogen peroxide® (AHP) disinfectant in a 10% propylene glycol (PG) solution to determine if the mixture was sufficient to inactivate PEDV in the presence of swine feces on metal surfaces at -10ºC. Conditions were chosen to mimic those found in commercial livestock trailers in winter-like conditions when a traditional wash is unavailable.

The AHP disinfectant was mixed in a 10% PG solution to prevent it from freezing at -10^{0}C. PEDV positive feces (PEDV negative feces for the negative control group) were spread evenly across the bottom of a 6 inch by 6 inch aluminum coupon with 1 inch sides designed to replicate the floor of a commercial livestock trailer. Eight treatment groups representing two AHP disinfectant concentrations (1:16 and 1:32) in a 10% PG solution, two contact times in a -10^{0}C freezer (40 minutes and 60 minutes), and two concentrations of feces (5mL and 10mL) in addition to a negative and positive control group were evaluated on the coupons. Following treatment, the contents of the coupons were recollected and administered to 3 week old pigs via gastric tube. These pigs served as a bioassay to determine the infectivity of virus following treatment. Infectivity was determined by detection of virus with reverse transcriptase polymerase chain reaction (RT-PCR) on fecal swabs collected from the inoculated pigs on days 3 and 7 post-inoculation. Each treatment was performed in 4 replicates (4 coupons and 4 pigs per treatment). Pigs in each treatment group were housed separately in raised tubs within the same room.

Bioassay results were PEDV negative in 100% of the pigs in the negative control group as well as all eight treatment groups. Bioassays were PEDV positive in 100% of the pigs in the positive control group.

Additionally, bioassay outcomes for the negative control (Neg) and all of the AHP disinfectant treatment groups were significantly different (p-value < 0.05) than the bioassay outcomes for the positive control group via Fishers Exact Test.

All AHP disinfectant treatment groups were 100% effective at inactivating PEDV in swine feces under winter-like conditions. Both concentrations (1:16 and 1:32) of AHP disinfectant in a 10% PG solution and both contact times tested (40 minutes and 60 minutes) inactivated PEDV in the presence of light and heavy fecal contamination. These results suggest that when washing, disinfecting, and drying the trailer are not possible; using a 1:16 or 1:32 concentration of AHP disinfectant in a 10% PG solution with 40 minutes of contact time is an effective alternative to reduce the risk of PEDV transmission between groups.

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KEYWORDS

Swine, PEDV, disinfection, temperature, accelerated hydrogen peroxide

SCIENTIFIC ABSTRACT

In May of 2013 porcine epidemic diarrhea virus (PEDV) was detected in swine for the first time in the United States and spread quickly across much of the country, partly due to the movement of contaminated livestock trailers. The objective of this study was to evaluate two concentrations of an accelerated hydrogen peroxide (AHP) disinfectant in a 10% propylene glycol (PG) solution to determine if the mixture was sufficient to inactivate PEDV in the presence of swine feces on metal surfaces at -10ºC. 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. Eight treatment groups representing two AHP disinfectant concentrations (1:16 and 1:32) in a 10% PG solution, two contact times in a -10^{0}C freezer (40 minutes and 60 minutes), and two concentrations of feces (5mL and 10mL) in addition to a negative control group and a positive control group were evaluated. PG was combined with the AHP disinfectant to prevent it from freezing at -10^{0}C. PEDV positive (Positive control group and all treatment groups) or negative (negative control group) feces were applied to an aluminum coupon and subjected to one of the ten groups described above. Post-treatment, contents from the aluminum coupons were administered to 3-week old commercial pigs. These pigs served as a bioassay to determine the infectivity of virus following treatment. Infectivity was determined by detection of virus with reverse transcriptase polymerase chain reaction (RT-PCR) on fecal swabs collected from the inoculated pigs on days 3 and 7 post-inoculation. Each treatment was performed in 4 replicates (4 coupons and 4 pigs per treatment). Pigs in each treatment group were housed separately in raised tubs within the same room. Bioassay results were PEDV negative in 100% of the pigs in the negative control group as well as all AHP disinfectant treatment groups. Bioassays were PEDV positive in 100% of the pigs in the positive control group. Bioassay outcomes for all of the AHP disinfectant treatment groups were significantly different (p-value < 0.05) than the bioassay outcomes for the positive control group (Pos) via Fishers Exact Test. These results suggest that that it may be possible to inactivate PEDV in the presence of feces on metal surfaces under freezing conditions (-10^{0}C) by applying a 1:16 or 1:32 concentration of an AHP disinfectant prepared in a 10% PG solution to a scraped commercial trailer for 40 minutes of contact time. All

fecal loads (5 mL and 10 mL), AHP disinfectant dilutions (1:16 and 1:32), and contact times (40 minutes and 60 minutes) evaluated were sufficient to inactivate PEDV.

INTRODUCTION

Porcine epidemic diarrhea (PED) was first described in England in 1971 in growing pigs and the causative agent, porcine epidemic diarrhea virus (PEDV), was identified in 1978. The virus spread to the rest of Europe where it caused outbreaks of diarrhea and significant losses throughout the 1970s and 1980s. PEDV is considered endemic to Europe today, but does not cause widespread significant disease. In parts of Asia, outbreaks were recognized first in 1982 and have continued to occur since. Until recently, the virus was considered absent from North America. In May of 2013, PEDV was identified in swine for the first time in the United States. The virus caused severe diarrhea in sows and piglets, with near 100% mortality in piglets across a wide geographical area of the US. Outbreaks of PED continue to occur in the US, with over 6,000 PEDV-positive accessions reported from 29 states as of May 2014. Genetic analysis of PEDV isolates from affected farms in the US found the virus to be 99% genetically similar to isolates from China suggesting both isolates originated from China, but efforts to determine the source of entry to the US have been unsuccessful.

Although the original route of entry of PEDV into the US remains unknown, contaminated livestock trailers certainly represent a significant risk for movement of the virus between and within herds. This is true of other swine diseases as well including porcine reproductive and respiratory syndrome virus (PRRSV) and transmissible gastroenteritis virus (TGEV). Recent PEDV research demonstrated that holding a metal surface contaminated with PEDV positive feces at 71ºC for 10 minutes or 20ºC for 7 days was efficacious at inactivating PEDV. In another study, an accelerated hydrogen peroxide (AHP) disinfectant (Accel®, Ogena Solutions LLC, Stoney Creek, Ontario, Canada) inactivated porcine epidemic diarrhea virus (PEDV) at 20ºC after 30 minutes of contact time in the presence of significant amounts of fecal material. The concentrated form of the AHP disinfectant was efficacious at dilutions of 1:16 and 1:32 in the presence of feces. 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. AHP, like most disinfects, is aqueous and freezes at around 0ºC. 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 0ºC. Considering the effectiveness of diluting the quaternary ammonium and glutaraldehyde combination disinfectant, mixing accelerated hydrogen peroxide disinfectant (AHP) with propylene glycol (PG) may be an efficacious means of inactivating PEDV under freezing conditions (-10ºC) in the presence of feces on metal surfaces found in livestock trailers.

OBJECTIVES

The objective of this study was to evaluate two concentrations of an AHP disinfectant in a 10% propylene glycol solution to determine if the mixture was sufficient to inactivate PEDV in the presence of swine feces on metal surfaces at -10ºC. 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.

MATERIALS AND METHOD

The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee prior to the initiation of any experimental activity.

Source of animals and housing.

Forty (40), 3-week old, clinically healthy, barrows were sourced from a private commercial producer. Seventy-two hours after arrival, blood and fecal samples were collected from each pig. Serum and fecal samples were submitted to Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) for diagnostic testing to confirm that pigs were negative for PEDV, TGEV and PRRSV. On arrival, each pig was identified with a unique plastic ear tag and weighed. Following a 72 hour rest period and initial screening as described above, pigs were randomly assigned to one of ten groups (n=4) and each group was housed in a separate room in the Iowa State University Veterinary Medical Research Institute for the duration of the study. The four pigs within each group were housed individually in elevated tubs. Each tub was constructed of solid transparent dividers completely separating pigs from one another. Each divided portion of the tub had dedicated water and feed sources. Pigs were fed an age-appropriate corn and soybean meal based diet ad libitum that did not contain porcine derived products. Feces fell through the plastic, slatted flooring of the tub into a common collection area below the pigs, where it fell into a holding container that contained water and disinfectant to inactivate any PEDV particles and minimize the potential for environmental contamination.

Coupons

In this study, forty (40) 15.24 cm by 15.24 cm by 2.54 cm aluminum trays were used as coupons to mimic the surface of commercial hog trailers. The coupons were manufactured using 0.32 cm thick aluminum. To simulate the cleaning action of the AHP disinfectant and runoff seen in a commercial livestock trailer during disinfection, six 8 mm diameter holes were drilled at the junction of the bottom and one sidewall of the coupon.

Study Design

Two concentrations of fecal contamination (5 mL and 10 mL); two concentrations of AHP disinfectant (1:16 and 1:32) in a 10% PG solution; and two contact times (40 minutes and 60 minutes) at -10^{0}C were evaluated. A negative control and positive control group were also included. The negative and positive control were not sham disinfected. The negative group used PEDV-negative feces; all other groups used PEDV-positive feces (See Table 1).

The individual pig was the experimental unit and a single pig was matched to a single coupon. Each treatment group had 4 replicates. Therefore, four coupons and four pigs were used for each treatment group.

Challenge Material

PEDV positive feces, used for fecal contamination of the coupons, was obtained from a previous experiment where three week old pigs were challenged with PEDV positive feces via oral-gastric gavage. Feces was collected from clinically affected pigs 7 days post inoculation. After collection, feces was stored in sterile fecal cups at -80^{0}C. Prior to freezing, one sample was submitted to the ISU VDL to confirm its PEDV positive status by real time RT-PCR. Diagnostic results confirmed that the sample was PEDV positive with a cycle threshold (Ct) value of 17.5.

PEDV negative feces was obtained from the negative control pigs in the same study. Fecal collection and storage procedures were the same as described 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 PEDV negative with a Ct value > 40.

Contamination and Disinfection Procedures

Five (5) mL and 10 mL of feces were chosen to represent the range of organic matter remaining in the interior of a commercial livestock trailer after a manual scraping. For all study groups, contamination of the coupons with fecal material was performed using a disposable hard plastic spreader sold in hardware stores to spread adhesive on floors. For the negative control group (A), 5 mL of PEDV negative feces were applied to four

aluminum coupons in an even ≤ 2 mm layer. Five (5) mL of PEDV positive feces were applied to all coupons in groups B, C, E, G, and I. Ten (10) mL of PEDV positive feces were applied to all coupons in groups D, F, H, and J. Following contamination with feces, all coupons were individually sampled using a commercial swab and transport system. The swabs were submitted to the ISU VDL to test for the presence of viral RNA by real-time RT-PCR prior to treatment.

All coupons, except the negative control (group A), were then pre-cooled in a 4^0C refrigerator for 30 minutes. This pre-cooling period was designed to drop the temperature of both the feces and the metal surface; representing the cooler temperatures of feces left in a scraped commercial livestock trailer after transporting pigs during the winter months.

Following pre-cooling, the AHP disinfectant solution was applied to the contaminated coupons in the treatment groups (C- J). The AHP disinfectant solution was prepared using a 4.25% concentrate of AHP disinfectant. The solution applied 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. Propylene glycol is an organic solvent that can be used as a safe anti-freezing agent when mixed with disinfectants, without reducing their efficacy. Water from a municipal water source was used to prepare the AHP disinfectant solution. The positive control group (B) and negative control group (A) 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 - J) using a 1.5 gallon pump-up. The volume of AHP disinfectant applied to the coupons was proportionally equivalent to the volume of AHP disinfectant typically used on a full-sized, double-deck, 15.8 meter livestock trailer.

Following treatment with AHP disinfectant, coupons were placed in a freezer set to – 10^0C. All coupons in groups C, D, E, and F were held in the freezer for 40 minutes of contact time. Coupons in groups G, H, I and J were held in the freezer for 60 minutes of contact time. Coupons in the positive control group (B) were also placed in the freezer for 40 minutes to confirm that the additional freeze/thaw cycle did not inactivate the PEDV present in the feces. Coupons in the negative control group (A) were not placed in the freezer.

After contamination of the coupons in the negative control group (A), a post-treatment swab was taken of each coupon. For all other study groups, the swabs were collected following treatment. All swabs collected post-treatment were submitted to the ISU VDL and tested for the presence of PEDV RNA by real time RT-PCR.

Swine Bioassay

After the post-treatment swab was collected, coupons were tipped away from the holes and 10 mL of sterile 0.9% sodium chloride saline solution was applied to each coupon to maximize fecal collection and facilitate oral gavage. A new toothbrush for every coupon was used to re-suspend the feces / AHP disinfectant / PG / saline mixture; creating a homogenate sample suitable as inoculum. The resulting homogenate was recollected using a 20 mL syringe. The syringe was labeled with the coupon identification number; matched to a single pig for bioassay purposes. Three (3) mL of the inoculum were transferred to a snap-cap tube and stored at -80^0C in the event that further diagnostic testing of the inoculum was required.

The homogenate of feces / AHP disinfectant / PG / saline was immediately used to inoculate pigs for the bioassay via oral gastric gavage with a 14 French rubber catheter. Each pig was inoculated with the contents of its designated coupon. Personnel wore disposable TyVek® coveralls and a N95 respirator while performing inoculation. This PPE was changed between each treatment group. TyVek coveralls were changed between pigs if they became contaminated during the inoculation process. To further prevent cross-contamination between replicates, personnel wore nitrile gloves and arm-length disposable obstetrical (OB) sleeves. Gloves and sleeves were discarded and changed between each pig.

Pigs were monitored daily following inoculation for clinical signs consistent with PED. Rectal swabs were collected from each pig 3 and 7 days post-inoculation and were tested for PEDV by real-time RT-PCR.

Personnel collecting fecal samples used the same biosecurity procedures as those used for inoculation. Pigs were not removed from their quadrant of the tub during sampling.

All pigs were humanely euthanized and necropsied 7 days post inoculation. Gross evaluation was performed on all organ systems and any gross lesions or abnormal pathology was noted. From each pig; fresh cecal and spiral colon contents and sections of fresh and 10% formalin-fixed mesenteric lymph nodes, ileum, and jejunum were collected. Fresh samples were immediately frozen and stored at -80^{0}C in the event further testing was required and for use as contamination material in future studies.

A bioassay was considered positive if the fecal swabs were PEDV positive by real-time RT-PCR on day 3 or day 7 (Ct value less than 35). A bioassay was considered negative if both fecal swabs (day 3 and day 7) were PEDV negative by real-time RT-PCR (Ct value greater than 35).

Statistical Analysis

All statistical analysis was performed using Fisher's Exact Test. The test evaluated difference in the proportion of pigs bioassay positive between the positive control (Group B) and the eight treatment groups (C-J). A P-Value of less than 0.05 was considered statistically significant. To ensure validity of statistical analysis, all personnel performing statistical analysis were blinded to the treatment group.

RESULTS

Swabs from all coupons (36/36) to which PEDV-positive feces were applied (Positive control group and all eight AHP disinfectant treatment groups) were found to be PEDV-positive by RT-PCR before and after exposure to the designated AHP disinfectant treatment. Swabs from all coupons to which PEDV-negative feces were applied (4/4, Negative control group) were found to be PEDV-negative by RT-PCR. Mean RT-PCR Ct values of coupons pre- and post-treatment are summarized in Table 2.

Swine bioassay results were PEDV negative in 100% (4/4) of pigs in the negative control group as well as 100% of pigs in the AHP treatment groups (32/32). Swine bioassay results were PEDV positive in 100% (4/4) of pigs in the positive control group (Table 3).

A Fisher's Exact test ran to evaluate the overall effect of treatment on bioassay outcome found that treatment did have a significant effect on bioassay status ($P<.05$). More specifically, bioassay outcomes for all AHP disinfectant treatment groups (C-J) were significantly different than the positive control group (B) with a p-value of less than 0.05. These results are summarized in Table 3.

DISCUSSION

The results of this study suggest that it may be possible to inactivate PEDV in the presence of feces on metal surfaces under freezing conditions (-10ºC) by applying a 1:16 or 1:32 concentration of an AHP disinfectant prepared in a 10% PG solution to a scraped commercial trailer for 40 minutes of contact time. All fecal loads (5 mL and 10 mL), AHP disinfectant dilutions (1:16 and 1:32), and contact times (40 minutes and 60 minutes) evaluated were sufficient to inactivate PEDV.

Commercial transport vehicles hauling market hogs often transport multiple loads, from the same or different sites, to the harvest facility each day without a complete wash, disinfect, and dry between loads. Previous research indicates that PEDV can be transferred from PEDV positive trailers to PEDV negative trailers at swine harvest facilities; making market hog trailers a substantial risk for virus transmission. When a complete wash, disinfection, and drying of the trailer are not possible, scraping the trailer after unloading and then applying an AHP disinfectant before they return to the site may greatly reduce the risk of PEDV transmission associated with market hog trailers. The same protocol could be applied to trailers dedicated to hauling high health animals or known PEDV negative pig flows to reduce the risk of the trailer becoming contaminated with PEDV at a commercial trailer wash facility. Additionally, this alternative trailer sanitation protocol could be used on PEDV

positive trailers before they enter a commercial trailer wash facility to reduce the risk of contaminating the facility and other trailers with PEDV. This work suggests that scraping commercial livestock trailers and then applying an AHP disinfectant in a 10% PG solution may be a viable alternative if a traditional wash, disinfect, and dry cannot be completed due to the sub-freezing temperatures or other extenuating circumstances.

It is noteworthy that all post-treatment swabs, from the 32 coupons contaminated with PEDV positive feces and then subjected to one of eight AHP disinfectant treatments (groups C-J) at -10^0C, were positive for the presence of PEDV RNA by real-time RT-PCR post-treatment. However, none of the 32 coupons (0%) contained infectious virus as demonstrated by swine bioassay. The post-treatment Ct results suggest that the AHP disinfectant inactivated the virus by an alternative mode of action; leaving a sufficient amount of genetic material to interact with the primers in a real-time RT-PCR assay, but no infectious virus. Oxidizing agents such as the AHP disinfectant are known to inactivate virus through interactions with viral proteins and lipids by oxidation, hydrolysis, and denaturation.

While real-time RT-PCR is a useful tool for rapid PEDV detection after sanitation its major limitation is that it cannot differentiate between infectious virus and fragments of RNA from inactivated virus. If the disinfectant used did not completely denature the viral genetic material, an environmental sample could be PCR positive for PEDV RNA, but contain no infectious virus determined through sentinel animals, bioassay, cell culture, or virus isolation as demonstrated in this and other previous studies. Previous work demonstrated that the disinfectant classes most likely to produce negative environmental real-time RT-PCR results when used against enveloped RNA viruses are oxidizing agents and sodium hypochlorite solutions, while phenolic and quaternary ammonia based disinfectants are the least likely to produce negative real-time RT-PCR results, but this is not always the case. In fact, the AHP disinfectant used in this study is classified as an oxidizing agent and did not produce any negative real-time RT-PCR results when used under freezing conditions. On the other hand, when the same AHP disinfectant was tested at room temperature, only 12.5% of post-treatment swabs were PEDV positive by real-time RT-PCR, but none were infectious. These results suggest that obtaining a negative real-time RT-PCR result after disinfection is largely dependent on the type of disinfectant used and the conditions it was applied under. Real-time RT-PCR is not a reliable method for sanitation protocol evaluation. For this reason, a sanitation protocol or disinfectant should not be dismissed based on the presence of a PEDV positive real-time RT-PCR result because this does not necessarily indicate that an infectious dose of live virus remains.

This study demonstrated that applying an AHP disinfectant to metal surfaces in the presence of swine feces is effective against PEDV in temperatures below freezing and reinforced the use of disinfectants in a 10% PG solution to prevent freezing. Finally, the results suggested that using real-time RT-PCR as the sole method of sanitation protocol evaluation is not reliable and should not be the basis for protocol rejection. Further research should be done to evaluate the efficacy of an AHP disinfectant under other adverse conditions such as shorter contact times, higher dilution rates, and on perpendicular surfaces to simulate trailer sidewalls.

Tables

Table 1: Description of Treatment Groups

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	None
(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 2: Summary of PEDV N-gene real time RT-PCR results for the pre-treatment and post-treatment swabs of forty 15.24 cm by 15.24 cm by 2.54cm aluminum coupons.

Treatment Group*	**Pre-Treatment† (Contamination) Ct Value**	**Percentage positive for PEDV RNA**	**Post-Treatment‡ Ct Value**	**Percentage positive for PEDV RNA**
(A) Negative Control	>35 >35 >35 >35	0% (0 of 4)	>35 >35 >35 >35	0% (0 of 4)
(B) Positive Control	18.2 19.0 19.5 19.2	100% (4 of 4)	18.6 17.1 18.8 18.8	100% (4 of 4)
(C) Light, 1:32, 40 mins	17.2 17.4 17.9 17.6	100% (4 of 4)	27.1 21.0 29.1 28.6	100% (4 of 4)
(D) Heavy, 1:32, 40 mins	16.8 18.0 E17.8 18.1	100% (4 of 4)	24.6 19.2 24.0 20.6	100% (4 of 4)
(E) Light, 1:16, 40 mins	18.7 17.9 18.2 18.7	100% (4 of 4)	33.6 31.1 28.9 30.7	100% (4 of 4)
(F) Heavy, 1:16, 40 mins	17.2 17.8 18.5 18.2	100% (4 of 4)	26.1 23.0 23.9 23.0	100% (4 of 4)
(G) Light, 1:32, 60 mins	18.0 17.6 17.0 18.2	100% (4 of 4)	27.5 26.1 23.6 27.3	100% (4 of 4)
(H) Heavy, 1:32, 60 mins	17.6 17.9	100% (4 of 4)	26.1 23.7	100% (4 of 4)

	18.7 17.0		21.5 24.8	
(I) Light, 1:16, 60 mins	17.6 17.5 17.8 17.9	100% (4 of 4)	34.5 33.4 34.7 32.3	100% (4 of 4)
(J) Heavy, 1:16, 60 mins	16.5 18.0 17.3 20.1	100% (4 of 4)	28.0 24.9 24.7 24.9	100% (4 of 4)

*Treatment groups are described in Table 1.

†Pre-treatment swabs were taken immediately following fecal application prior to pre-cooling in a 4^0 C fridge and AHP disinfectant treatment.

‡. Negative Control (Group A): post-treatment swabs were taken directly after the pre-treatment swabs. Positive Control (Group B): post-treatment swabs were taken following pre-cooling and 40 minutes in a freezer set to -10°C. Groups C-J: post-treatment swabs were taken following pre-cooling, AHP disinfectant application, and the allotted contact time in a freezer set to -10°C

Table 3: Summary of study day 3 and 7 rectal swab PEDV N-gene real time RT-PCR and swine bioassay results.

Treatment Group*	Day 3 Rectal Swab†; Percentage positive for PEDV RNA	Day 7 Rectal Swab‡; Percentage positive for PEDV RNA	Swine Bioassay Result♦; Percentage positive for PEDV RNA
(A) Negative Control	0% (0 of 4)	0% (0 of 4)	0% (0 of 4)[a]
(B) Positive Control	100% (4 of 4)	100% (4 of 4)	100% (4 of 4)[b]
(C) Light, 1:32, 40 mins	0% (0 of 4)	0% (0 of 4)	0% (0 of 4)[a]
(D) Heavy, 1:32, 40 mins	0% (0 of 4)	0% (0 of 4)	0% (0 of 4)[a]
(E) Light, 1:16, 40 mins	0% (0 of 4)	0% (0 of 4)	0% (0 of 4)[a]
(F) Heavy, 1:16, 40 mins	0% (0 of 4)	0% (0 of 4)	0% (0 of 4)[a]
(G) Light, 1:32, 60 mins	0% (0 of 4)	0% (0 of 4)	0% (0 of 4)[a]
(H) Heavy, 1:32, 60 mins	0% (0 of 4)	0% (0 of 4)	0% (0 of 4)[a]
(I) Light, 1:16, 60 mins	0% (0 of 4)	0% (0 of 4)	0% (0 of 4)[a]
(J) Heavy, 1:16, 60 mins	0% (0 of 4)	0% (0 of 4)	0% (0 of 4)[a]

[a,b] Different superscripts indicate statistically significant differences (p-value < 0.05) by a Fishers Exact Test

*Treatment groups are described in Table 1.

†Results from rectal swabs taken on study day 3 (3 days post-inoculation) and tested for PEDV by real-time RT-PCR.

‡Results from rectal swabs taken on study day 7 (7 days post-inoculation) and tested for PEDV by real-time RT-PCR.

♦Swine Bioassay was considered positive if rectal swabs on either day 3 or day 7 were PEDV positive by real time RT-PCR. Swine Bioassay was considered negative if rectal swabs on both day 3 and day 7 were PEDV negative by real-time RT-PCR.