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Time and temperature requirements for heat inactivation of pathogens to be applied to swine transport trailers

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Summary

Objective: Biosecurity in swine transport trailers is of concern for spreading pathogens between premises, and as such, they require extensive cleaning and disinfection between loads. Our goal in this study was to find the optimal time and temperature required to heat inactivate swine pathogens of high concern to producers in a laboratory setting to then be extrapolated to transport trailers.

Materials and methods: Using standard microbiological techniques for growth and purification, 5 bacterial and 5 viral pathogens important in swine health were produced and tested. Heat inactivation of these pathogens were tested in the lab using several time and temperature combinations. Fecal matter was added to test the effect of biological material on the time and temperatures required for inactivation.

Results: Inactivation was complete for viruses and bacteria tested when heated to 75°C for 15 minutes. The presence of fecal matter resulted in increased time and temperature needed for pathogen inactivation.

Implications: Heat baking of transport trailers is now being applied as a useful tool to reduce the transmission of pathogens commonly associated with swine disease. However, operators must ensure consistent heating to 75°C for a minimum of 15 minutes in all areas of the trailer for reliable inactivation. Cleaning trailers plays an important role prior to heat treatment, as the presence of fecal contamination will insulate the pathogens and inactivation may not be complete even at 75°C for 15 minutes.

Keywords: swine, biosecurity, pathogen inactivation, heating

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Resumen - Requisitos de tiempo y temperatura que se deben aplicar para la inactivación por calor de patógenos

Objetivo: La bioseguridad en los remolques de transporte de cerdos es motivo de preocupación para la propagación de patógenos entre las instalaciones y, como tales, requieren una limpieza y desinfección exhaustivas entre cargas. Nuestro objetivo en este estudio fue encontrar el tiempo y la temperatura óptimos necesarios para inactivar por calor a los patógenos porcinos de gran preocupación para los productores en un entorno de laboratorio, para luego extrapolarlos a los remolques de transporte.

Materiales y métodos: Utilizando técnicas microbiológicas estándar para el crecimiento y la purificación, se produjeron y probaron 5 patógenos bacterianos y 5 virales importantes para la salud porcina. La inactivación por calor de estos patógenos se probó en el laboratorio utilizando varias combinaciones de tiempo y temperatura. Se agregó materia fecal para probar el efecto del material biológico en el tiempo y las temperaturas requeridas para la inactivación.

Resultados: La inactivación fue completa para los virus y bacterias probados cuando se calentó a 75°C durante 15 minutos. La presencia de materia fecal resultó en un aumento del tiempo y la temperatura necesarios para la inactivación de patógenos.

Implicaciones: El horneado térmico de los remolques de transporte se está aplicando ahora como una herramienta útil para reducir la transmisión de patógenos comúnmente asociados a enfermedades porcinas. Sin embargo, los operadores deben garantizar un calentamiento constante a 75°C durante un mínimo de 15 minutos en todas las áreas del remolque para una inactivación confiable. La limpieza de los remolques juega un papel importante antes del tratamiento térmico ya que la presencia de contaminación fecal aislará a los patógenos y la inactivación puede ser incompleta incluso a 75°C durante 15 minutos.

Résumé - Exigences de temps et de température pour l'inactivation d'agents pathogènes par la chaleur

Objectif: La biosécurité dans les remorques de transport des porcs est

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une préoccupation pour la transmission d'agents pathogènes entre les sites, et comme tel, elles nécessitent un nettoyage et une désinfection minutieuse entre les chargements. Notre but dans la présente étude était de trouver le temps et la température optimums requis pour inactiver par la chaleur des agents pathogènes porcins hautement préoccupants pour les producteurs dans un environnement de laboratoire et par la suite l'extrapoler aux remorques de transport.

Matériels et méthodes: En utilisant des techniques microbiologiques standards pour la croissance et la purification, cinq bactéries pathogènes et cinq virus pathogènes d'importance en santé porcine furent produits et testés. L'inactivation par la chaleur de ces agents pathogènes fut testée en laboratoire en utilisant plusieurs combinaisons de temps et température. Des matières fécales furent ajoutées pour tester l'effet de matériel biologiques sur le temps et la température requis pour l'inactivation.

Résultats: L'inactivation fut complète pour les virus et bactéries testés lorsque chauffés à 75°C pendant 15 minutes. La présence de matière fécale a résulté en une augmentation du temps et de la température requis pour l'inactivation des agents pathogènes.

Implications: L'exposition à la chaleur des remorques de transport est présentement appliquée comme un outil utile pour réduire la transmission d'agents pathogènes fréquemment associés avec des maladies porcines. Toutefois, les opérateurs doivent s'assurer un chauffage constant à 75°C pour un minimum de 15 minutes dans toutes les parties de la remorque pour une inactivation fiable. Le nettoyage des remorques joue un rôle important avant le traitement à la chaleur, étant donné que la présence de contamination fécale isolera les agents pathogènes et l'inactivation pourrait ne pas être complète même à 75°C pour 15 minutes.

Biosecurity has become an essential part of modern farm management with the aim of reducing exposure of animals to disease both within and between farms.[1] Heat treatment of transport trailers is a relatively new method being incorporated by transport companies to minimize spread of pathogens in the wake of several porcine epidemic diarrhea virus (PEDV) outbreaks in eastern Canada. Heat treatment also results in faster turnaround time as well as less risk to sanitation personnel as compared to fumigation of trailers with formaldehyde or quaternary glutaraldehyde. Thermo-assisted drying and decontamination (TADD) is one way of controlling the spread of PEDV and other porcine pathogens in transport trailers.[2-12]

The question remains for TADD, however, how long should the trailer be heated and to what temperature? Our objective was to determine a reasonable time and temperature that would effectively kill both bacterial and viral pathogens relevant to the swine industry.

Animal transportation is especially a risk for introducing disease to naïve animals and as such, transport trailers require extensive cleaning, washing, and disinfection after each load. In addition, the industry has incorporated the use of heating bays which expose trailers to hot air for varying amounts of time. Current protocols involve heating trailers to 70°C for 10 to 15 minutes. This regimen is based on an extensive amount of studies that have determined the necessary temperatures to inactivate both porcine viruses and bacteria.[2-10,12-14]

Several pathogens have been identified as high risk to swine producers in the last few years, including but not limited to those listed in Table 1. As an added complication, current protocols for disinfection have proven time consuming and costly. This, along with regional trailer shortages, have resulted in increased noncompliance with biosecurity practices.[3,27] Several recent outbreaks of porcine reproductive and respiratory syndrome virus (PRRSV) and PEDV have prompted calls for new methods to sanitize livestock trailers that are robust and cost effective in inactivating targeted porcine pathogens.[2,4-6,8,9] The objective of this study was to find the optimal time and temperature required to inactivate important swine pathogens. For this purpose, we selected a number of common swine pathogens including PEDV, PRRSV, swine influenza virus (SIV), transmissible gastroenteritis virus (TGEV), porcine rotavirus, *Streptococcus suis*, *Salmonella* Typhimurium, *Escherichia coli*, *Actinobacillus pleuropneumoniae* (APP), and *Brachyspira hampsonii*. We chose three different experimental settings for our analysis. First, purified pathogens were inactivated in cell culture. Second, fecal matter was examined for insulating capacity of biological material. Thirdly, the pathogens were incubated inside fecal matter to be more representative of field conditions. Bacteria are differently susceptible to environmental conditions, but generally most bacteria are heat labile (apart from thermophiles which are not of concern in this study).[10,12,14,22,28-30] Although they exist ubiquitously in the environment, pathogenic bacteria usually require certain conditions to make humans and animals sick. Many species of bacteria can survive for years in soil and on fomites unlike viruses. For example, *Salmonella* are normally killed by heat and disinfectants[14,22,28-30] except when they form biofilms on biotic and abiotic surfaces.[28,29] Similarly, *E coli* has been reported to be viable on fomites for more than 14 months, but are also able to form biofilms, and thus pose a risk to naïve animals through direct fomite contact.[10,23,30,31] *Streptococcus sui*s is part of the normal pig microflora but occasionally can mutate into a more pathogenic form or can make animals sick if it gets into an unusual site within the body or as a confounding coinfection with viruses.[22,26,31] Pathogenic species of *S suis* are of particular concern as they can make both humans and animals sick.[31]

Materials and methods

Propagation of viruses

PEDV. Porcine epidemic diarrhea virus is an emerging animal disease in Saskatchewan and as such, all PEDV experiments were performed at VIDO-Intervac in containment level 3 as required by Canadian Food Inspection Agency. The PEDV virus was the US/Colorado/2013 isolate and was obtained from the US Department of Agriculture's National Veterinary Services Laboratories (Lot 025 PDV 1303). Vero76 cells (ATCC) were subcultured 24 hours prior to infection in Dulbecco's Modified Eagles Essential Medium (DMEM; Sigma Aldrich; D5796) supplemented with complete 10% fetal bovine serum (FBS; gamma-irradiated and Australian sourced; PAA Laboratories; A15-503), 0.1M Hepes (Gibco; 15630-080), and 0.05 mg/mL gentamicin (Gibco; 15750078). Cells were 99% confluent at the time of infection. The PEDV inoculum containing tosyl phenylalanyl chloromethyl ketone (TPCK)-treated

Table 1: Important disease pathogens in swine herds and previously reported inactivation conditions in vitro and in environmental studies

Pathogen	Temperature, °C	Time	References
PEDV	60	30 min	2, 9, 15
	71	10 min	
	21	7 d	
TGEV	56	30 min	16
	60	10 min	
PRRSV	56	20 min	17, 18
	37	24 h	
SIV	56	30 min	13, 19
	60	10 min	
	70	<1 min	
Rotavirus	50	5 min	20
	20	7-9 mo	
PCV	70	6 h	21
Salmonella Enteritidis H2292, Heidelberg 21380 Typhimurium avirulent strain 8243	71	< 1 min	14, 22
	70	80-100 min (feces)	
Escherichia coli	70	<1 min	10
Escherichia coli 0157:H7 biofilm forming	22	6 h	23
Swine Brachyspiral colitis	56	10 min	11, 24
	37	60 min	
Mycoplasma hypopneumoniae	60	10 min	25
	45	30 min	
Actinobacillus pleuropneumoniae	42	< 4 h	12
	37	8 h	
Streptococcus suis	60	10 min	26
	25	24 h (dust)	
	20	8 d (feces)	

PEDV = porcine epidemic diarrhea virus; TGEV = transmissible gastroenteritis virus; PRRSV = porcine reproductive and respiratory syndrome virus; SIV = swine influenza virus; PCV = porcine circovirus.

Trypsin-(MJS BioLynx; UB22725S2), with a final concentration of 2 µg/mL, was added to Vero76 cells. Flasks were incubated for 72 hours, harvested by scraping, supernatants frozen at -80°C, thawed and concentrated by ultracentrifugation. The PEDV virus stock was titered and found to be 2.4×10^8 viral copies/mL as determined by quantitative polymerase chain reaction standard curve. Pelleted virus was aliquoted and stored at -80°C.

PRRSV. The PRRSV virus was purchased from ATCC (LN 14832 MARC145 cell line [monkey kidney]) and was subcultured in Eagle's Minimal Essential Medium (MEM; Sigma Aldrich M4655) with complete 10% FBS, 1× non-essential amino acids, and 1× antibiotic/antimycotic for 24 hours prior to infection. Cells were 80% confluent at the time of infection. Monolayers were washed once with phosphate-buffered saline (PBS; Mg^{2+} and Ca^{2+} free) and PRRSV inoculum strain VR2385 was added at a multiplicity of infection (MOI) of 0.1 for 2 hours at 37°C. Inoculum was removed and replaced with MEM with complete 10% FBS. Flasks were incubated for 72 hours until complete cytopathic effect (CPE) was observed. Virus was harvested and subsequently titered. The final titer of viral lysates was 5.0×10^6 median tissue culture infectious dose ($TCID_{50}$)/mL. Virus was aliquoted and stored at -80°C.

SIV. At 24 hours prior to infection, MDCK cells were subcultured in DMEM supplemented with 10% FBS. Cells were 99% confluent at the time of infection. Monolayers were rinsed with PBS prior to infection. An A/SW/SK/02 (H1N1) strain of SIV was isolated from an infected animal in Saskatchewan, was added at an MOI of 0.1 in the presence of TPCK-trypsin and incubated for 48 hours until complete CPE was observed. Flasks were harvested by scraping, supernatants frozen at -80°C, and then thawed before virus was concentrated by ultracentrifugation on a sucrose cushion. Titer of the SIV stock was 4×10^7 plaque-forming units (PFU)/mL. Virus aliquots were stored at -80°C.

TGEV. The TGEV virus was purchased from ATCC. Swine testicular cells (ST cells) were subcultured in MEM with 10% FBS and antibiotic/antimycotic 24 hours prior to infection. Virus was adsorbed onto the cells for 1 hour in low volume MEM (no FBS) and then additional MEM (no FBS) was added. Cytopathic effect was complete in 24 hours and flasks were scraped into the surrounding medium. The cell/virus/medium mixture was frozen at -80°C and then thawed to lyse cells. Cellular debris was spun out of the supernatant by centrifugation and supernatant containing virus was stored at -80°C. The virus titer was 1×10^7 PFU/mL.

Porcine rotavirus. For 24 hours prior to infection, MA-104 cells (ATCC; CRL-2378) were subcultured in MEM supplemented with 10% FBS. Trypsin was added at 10 µg/mL to stock virus and monolayers were washed with MEM alone prior to infection. Virus was diluted 1:10 and adsorbed onto cells for 1 hour in low volume. Additional MEM containing 5 µg/mL trypsin was added and the virus was allowed to replicate for 3 to 4 days. When CPE was 80% to 90% complete, cells were harvested into the medium and both cells and supernatant were stored at -80°C. The virus titer was 6.8×10^5 $TCID_{50}$/mL.

Heat inactivation of viral pathogens

Using 1.5 mL centrifuge tubes, 100 µL of MEM without serum was preheated in digital heating blocks to individual temperatures being tested. For each tube, 20 µL of supernatants containing specified amounts of infectious virus was added and incubated for specific times (Table 2). After the time, 900 µL of ice-cold MEM was immediately added to the tube to rapidly cool the sample and stop any further heat inactivation. Samples were then kept on ice until titrating on fresh tissue culture cells to quantify viable virus. A non-heat-treated sample was used as a positive control. Cells were kept for 3 to 5 days after infection to monitor for evidence of CPE.

Growth and heat inactivation of bacteria

The bacteria of interest were *S* Typhimurium SL1344, *E coli* 0157:H7 (EC1647), *S suis* strain 89-1591, APP AP37 (serotype 1), and *B hampsonii* clade 2 clinical isolate. Each bacterial strain was grown according to established protocols. Briefly, an agar plate (Luria broth agar for *S* Typhimurium and *E coli*; tryptone soya agar with 5% sheep blood for *S suis*; and PPLO agar + 1% IsoVitaleX for APP) was streaked out with bacteria of interest from a -80°C glycerol stock and incubated overnight at 37°C. A single colony was picked from the agar plate and grown in 10 mL of selective growth media (Luria broth for *S* Typhimurium and *E coli*; Todd Hewitt broth for *S suis*; and PPLO broth for APP) overnight (approximately 16 hours) at 37°C and shaken at 200 rpm. The bacteria were subcultured at 1:100 or 1:50 into 10 mL fresh media and grown at 37°C and shaken at 200 rpm for 1.5 to 3 hours until the desired optical density of 600 nm (OD600) was achieved. *Brachyspira hampsonii* bacteria were propagated by streaking out approximately 10 µg of feces or intestinal contents onto BJ and CVS agar plates. Plates were then incubated anaerobically using a commercial system (Anaerogen, Oxoid Limited) at 42°C for 48 hours.

After the desired OD600 was achieved, the bacteria of interest were exposed to 5 temperatures (5° increments from 50°C to 70°C). For each temperature point, 11 tubes (one for each time point), each containing 90 µL of media, were preheated in a heating block set at the desired temperature. After 10 µL of bacteria was added to each tube, the set temperature point was applied for 1, 2, 3, 4, 5, 10, 15,

Table 2: Summary of pathogen inactivation in vitro using known concentrations of virus and bacterial stocks

	Time to inactivate, min						
Pathogen	**80°C**	**75°C**	**70°C**	**65°C**	**60°C**	**55°C**	**50°C**
PEDV	< 1	15	15	45	> 60	> 60	> 60
PRRSV	< 1	< 1	< 1	5	10	60	> 60
SIV	< 1	< 1	< 1	< 1	< 1	3	60
TGEV	< 1	< 1	< 1	< 1	10	30	30
Rotavirus	< 1	< 1	< 1	< 1	< 1	15	15
Streptococcus suis	--	--	< 1	3	3	10	45
Actinobacillus pleuropneumoniae	--	--	< 1	< 1	< 1	< 1	4
Escherichia coli	--	--	< 1	2	3	10	60
Salmonella Typhimurium	--	--	< 1	10	NA*	15	60
Brachyspira hampsonii	--	--	< 1	< 1	< 1	2	15

* No colonies were seen at this temperature.

PEDV = porcine epidemic diarrhea virus; PRRSV = porcine reproductive and respiratory syndrome virus; SIV = swine influenza virus; TGEV = transmissible gastroenteritis virus.

20, 30, 45, or 60 minutes, respectively. At completion, 900 µL of ice-cold medium was added to each tube to rapidly cool the contents. The tubes were incubated on ice until plating. Each suspension was serially diluted 1/10 in medium and then 100 µL of each dilution was spread onto agar. Plates were incubated at 37°C overnight and colonies were counted and recorded. For *B hampsonii*, positive cultures were indicated by zones of strong β-hemolysis. A non-heat-treated sample was also included for each temperature point to serve as a reference sample.

Heat treatment of PEDV samples from clinically infected piglets

In a previous study performed at VIDO-Intervac containment level 3 laboratory, fecal samples from PEDV-infected piglets were collected post mortem, so no additional animal ethics protocol was required for this study. The samples were diluted 1:1 with sterile PBS to make them pipettable. Clinical samples were then diluted 1:5 in cell culture medium and incubated in the heating block for each time point as previously described. Temperatures tested were in 5° increments from 55°C to 80°C for a total of 6 temperatures. Samples were incubated with Vero76 cells in the presence of TPCK-Trypsin as previously outlined and virus was allowed to attach for 1 hour at 37°C before inoculum was removed and replaced with fresh culture medium. Cells were incubated for 2 days and then scraped and frozen. Viral lysate was then passaged once more on fresh Vero76 cells to monitor for CPE. The clinical samples were pooled and diluted as previously described for use as positive control without heat treatment.

Assessing insulating capacity of fecal matter

Fecal material was collected from healthy animals. Thirty grams were formed into a large mass and packed into a fabricated aluminum corner, similar to a normal corner in a transport trailer. This was termed an "exposed environment." The aluminum corner (30 × 30 × 30 cm) was fabricated by the Department of Engineering at the University of Saskatchewan. An OM-EL-USB-TC thermocouple data logger (Omega Engineering Inc) was inserted into the center of the fecal mass and a second logger was mounted on the exterior of the corner to monitor ambient air temperature. The aluminum corner was added to the prewarmed 80°C oven and the temperature rise was monitored overnight for 14 hours.

To account for the thermodynamic effect of water in the feces, a more closed system was used to better monitor the insulating capacity. This was termed a "covered environment." For this, 30 g of feces was packed into a 50 mL conical tube and the thermocouple was inserted into the center of the mass. The 50 mL conical tubes were tightly wrapped in aluminum foil to minimize evaporation. The oven was again set to 80°C and the ambient and internal fecal temperature was monitored for 14 hours.

Assessment of survival of pathogens inside fecal matter

Fecal matter was serially diluted alone and in the presence of 100 ug/mL of Baytril for testing in tissue culture, but even at very low concentration, it was found to be toxic to the Marc145 cells (data not shown). Thus, in order to assess survival of viral pathogens inside fecal matter, 10 µL of PRRSV at 5.0×10^6 $TCID_{50}$/mL was added to MEM for a total of 100 µL volume inside a thin walled 0.2 mL polymerase chain reaction (PCR) tube. The tube was inserted into the center of a 10-g fecal mass. A thermocouple was inserted beside the tube and the feces packed down to minimize air pockets. The conical outer tube was sealed to prevent evaporation. The oven was heated to 80°C from room temperature and samples were removed at 15, 30, 60, and 120 minutes. Following heating, the PCR tube was removed from the fecal mass and kept on ice until serially diluted on tissue culture cells. Tissue culture cells were monitored for 5 days post infection for CPE. This experiment was repeated three times.

To assess survival of bacterial pathogens inside fecal matter, 10 µL of an *S suis* suspension concentrated at approximately 1×10^8 colony forming units/mL was added to 90 µL 0.1M PBS (Mg^{2+} and Ca^{2+} free) for a total of 100 µL volume inside a thin walled 0.2 mL PCR tube. A thermocouple was inserted in the center of the 10-g fecal clump, as well as the PCR tube containing the bacteria, and was covered and packed down to minimize air pockets. The conical outer tube was covered tightly with aluminum foil to prevent evaporation. The oven was heated to 65°C, 70°C, 75°C, or 80°C before the addition of the samples and the rise in temperature within the sample was tracked for 15 minutes. Following heating, the PCR tube was removed from the fecal mass and kept on ice until serial dilution on selective agar growth plates. Bacterial colonies were counted the next day. Triplicate samples were used for each temperature point so that the bacterial counts could be monitored after 5, 10, and 15 minutes of heat treatment (the 65°C temperature point was not in triplicate and only had a 15-minute sample). The experiment was later repeated using a 45-minute heat treatment with bacterial counts being examined after 5, 10, 15, 30, and 45 minutes of heat treatment. A non-heat-treated sample was also included for each temperature point to serve as a reference sample.

Results

Time and temperature required for pathogen inactivation in cell culture

The results from these experiments are summarized in Table 2. When tested, certain viruses proved more heat labile than others. For example, SIV was inactivated in culture after only 3 minutes at 55°C, while TGEV and PRRSV were inactivated after 5 minutes at 65°C. The hardiest virus proved to be PEDV, surviving 15 minutes at 75°C in cell culture (Table 2). All tested viruses were completely inactivated after 2 minutes at 80°C. As bacteria are more sensitive to heat inactivation in general, all bacteria were inactivated after 1 minute at 70°C (Table 2). Lower temperatures could inactivate the bacteria if the heating time was increased. For example, all bacteria were inactivated after 15 minutes at 55°C. Under laboratory conditions and using purified pathogen stocks, 15 minutes at 75°C or 2 minutes at 80°C were sufficient to inactivate all selected pathogens in cell culture.

Heat treatment of PEDV samples from clinically infected piglets

In order to look at more field-relevant specimens, we used frozen fecal samples from PEDV clinically infected piglets. These samples were not separated before freezing and contained typical intestinal content including fecal matter, dead enterocytes, microbiota, gut enzymes, etc. The PEDV titers in these piglets was unknown and samples were obtained after all piglets succumbed to challenge. Using the same temperatures and heating times as described above, all viral particles in these samples were completely inactivated after 15 minutes at 75°C. Positive-control (non-heated) samples were positive for CPE.

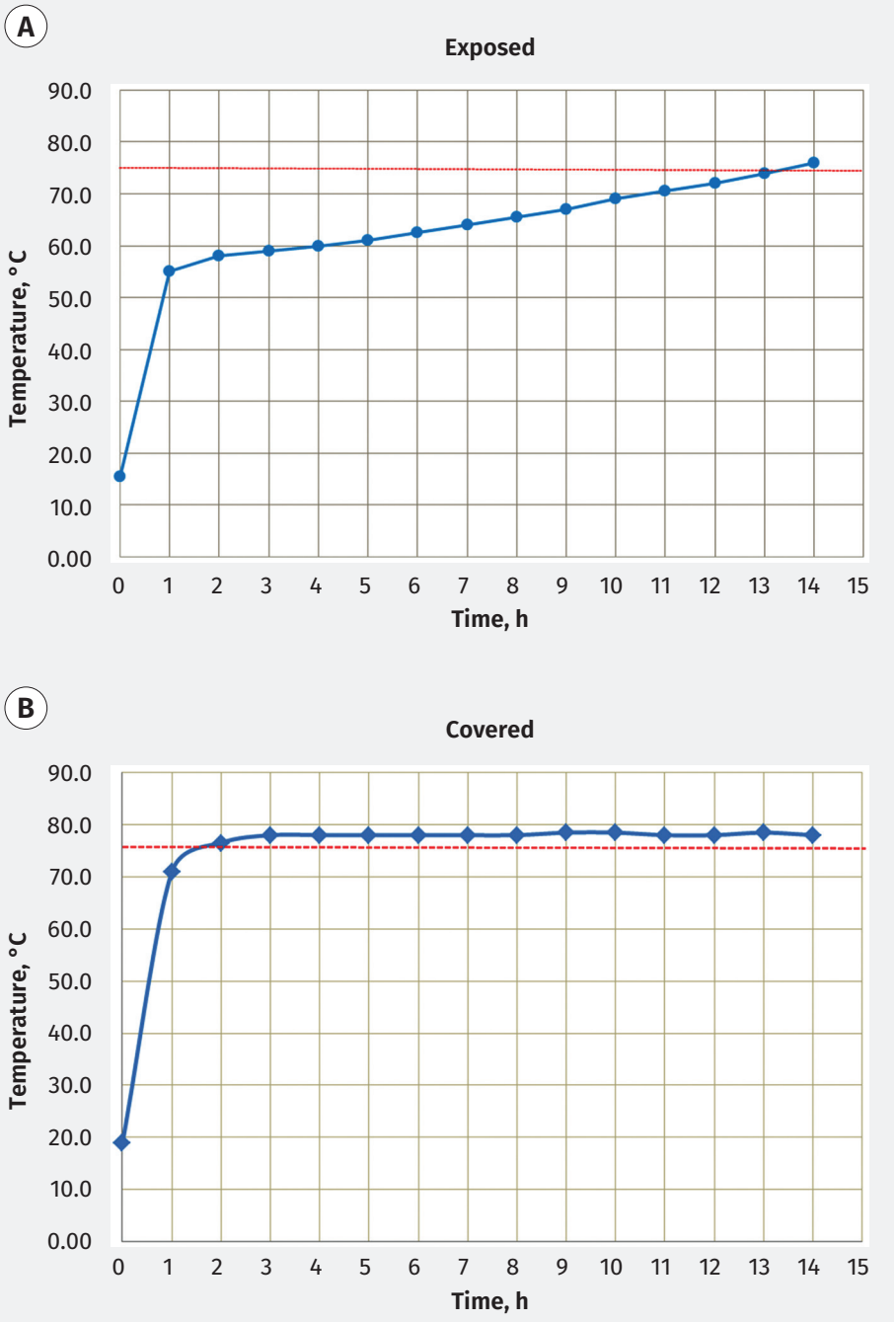
Insulating capacity of fecal matter

To simulate field conditions as much as possible, we added biological material to our experiments. Such material is usually present on commercial transport trailers in the form of feces and animal bedding. If trailers are not properly cleaned, biological material remains hidden behind lights, gates, or in corners. To determine the insulating effect of such material, a 30-g fecal mass was exposed to 80°C temperatures, in open air, for 14 hours with thermocouple data loggers monitoring temperature both inside the mass as well as ambiently. The internal temperature in this exposed sample rose very slowly and reached the desired temperature of 75°C after 14 hours (Figure 1A). The dried outer layer acted as very effective insulation and prevented a rise in the internal temperature. In contrast, the ambient temperature in the oven was able to reach 80°C within approximately 22 minutes from a cold start.

To further investigate the effect of fecal drying, we tested the same amount of fecal matter, 30 g, in a more closed system. The fecal matter was placed in a sealed tube to prevent water evaporation. As expected, the temperature rose internally much faster than in the exposed system. Beginning with a cold oven, internal temperature reached 78°C within 2.33 hours and was maintained until the oven was turned off (Figure 1B). We then reduced the amount of fecal matter to 10 g, as this may represent a more feasible amount of manure left behind post trailer cleaning. It was found that after 15 minutes at 80°C, the internal fecal mass temperature was not above 67.5°C. This temperature would not be high enough to inactivate some of the viruses tested in this study. Next, we placed a small thin-walled PCR tube containing a viral PRRSV suspension inside the fecal matter. When heated from room temperature up to 80°C, total virus inactivation only happened after 30 minutes, which corresponded to an internal temperature > 70°C (Table 3).

In contrast, inactivation of bacteria in these samples occurred faster and at lower temperatures. Heating the samples for 15 minutes (blue line, Figure 2) in an oven set to 75°C or 80°C was sufficient for inactivation of the bacteria, as the internal temperature within the fecal matter was able to briefly reach temperatures above 60°C. This matches our previous data where *S suis* needed 3 minutes at 60°C for inactivation (Table 2). However, when samples were heated for 15 minutes in the oven set to 65°C or 70°C, the bacteria were not inactivated. The internal temperature within the fecal matter never reached 60°C. The fecal matter temperature reached the required minimum threshold of 55°C, but not for the necessary 15 minutes for inactivation to occur (lower red line, Figure 2).

Figure 1: A) Time for internal temperature of 30 g of porcine feces to reach 75°C (red dash line) in an exposed environment. B) Time for internal temperature of 30 g of porcine feces to reach 75°C (red dash line) in a covered environment.



Since we did not expect that the addition of fecal matter would provide so much heat protection, we repeated the experiments with *S suis*, the most heat stable bacterium. This time, we included a 45-minute heat treatment. After 45 minutes (grey lines, Figure 2), all bacteria were inactivated regardless of ambient temperature studied. It is interesting to note that temperatures within the fecal matter only reached 75°C when the ambient temperature was at 80°C for

Table 3: Heat inactivation of PRRSV at 15, 30, 60, and 120 minutes inside 10 g fecal matter*

		Internal fecal matter temperature, °C			Positive CPE,† %		
Time, min	**Ambient air temperature, °C**	**Exp 1**	**Exp 2**	**Exp 3**	**Exp 1**	**Exp 2**	**Exp 3**
15	75	45	45.5	47	100	100	100
30	80	64	69.5	63.5	0	100	0
60	80	75	77	75.5	0	0	0
120	80	76.5	77.5	77.5	0	0	0

* The PRRSV titer was 5.0×10^6 $TCID_{50}$/mL.

† Positive CPE is reported as the percent of positive wells out of 8. The experiment was repeated 3 times.

PRRSV = porcine reproductive and respiratory syndrome virus; CPE = cytopathic effect; Exp = experiment.

Figure 2: Temperature logs tracking the rise in temperature within the 10 g fecal sample when it is placed in an oven and heated at A) 65°C, B) 70°C, C) 75°C, or D) 80°C respectively for 15 (blue line) or 45 (grey line) minutes. The red lines at 55°C and 75°C represent the threshold temperatures that the sample would need to reach for bacterial and viral inactivation, respectively.

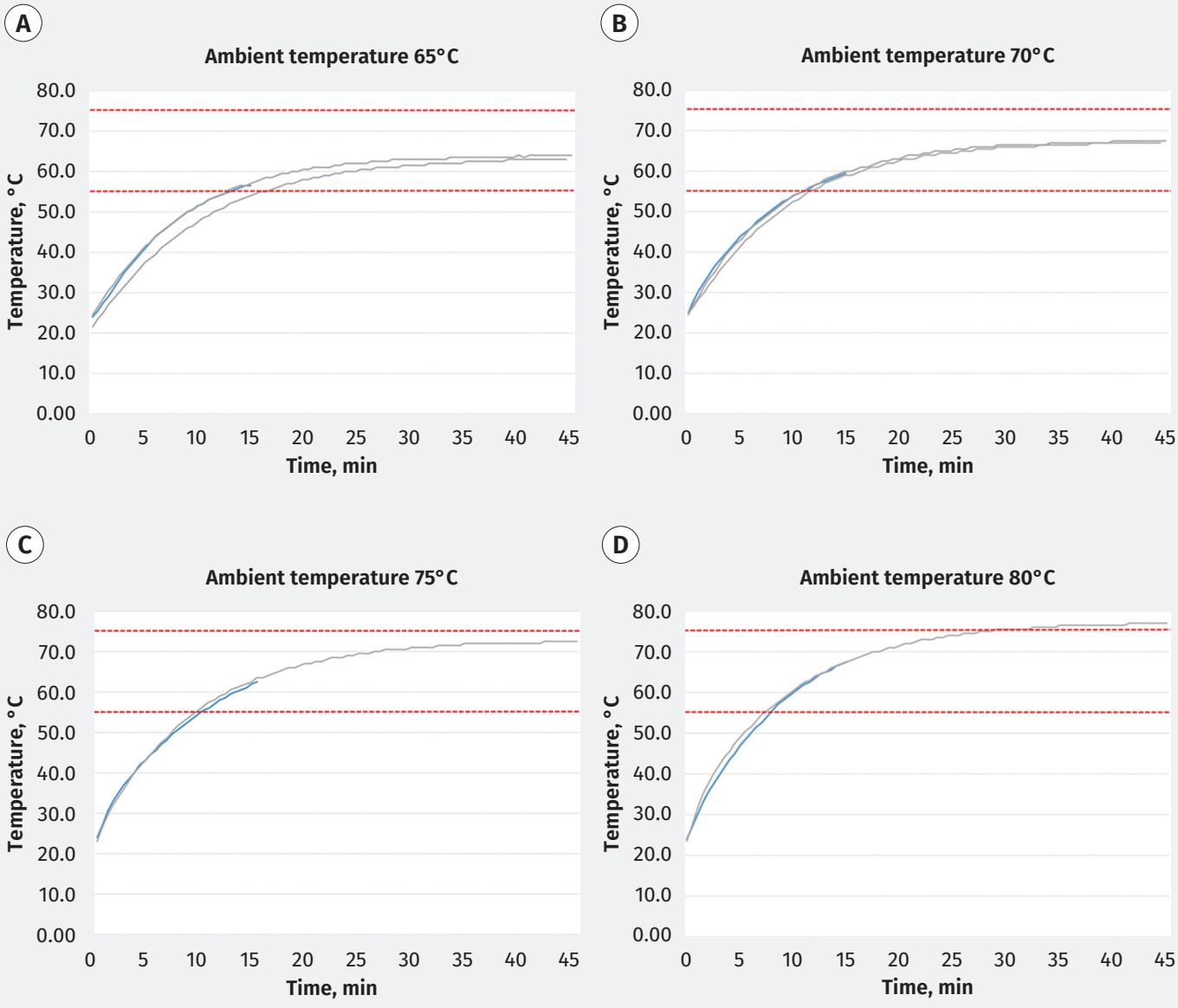


Table 4: Heat inactivation of *Streptococcus suis* at 5, 10, 15, 30, and 45 minutes inside 10 g of fecal matter*

		Fecal matter temperature,† °C			Bacterial growth‡		
Ambient temperature, °C	**Time, min**	**Exp 1**	**Exp 2**	**Exp 3**	**Exp 1**	**Exp 2**	**Exp 3**
80	45	NA	77.0	NA	NA	-	NA
	30	NA	75.5	NA	NA	-	NA
	15	67.5	67.5	NA	-	+	NA
	10	60.0	60.5	NA	+	NA	NA
	5	47.0	49.0	NA	+	NA	NA
75	45	NA	72.5	NA	NA	-	NA
	30	NA	71.0	NA	NA	-	NA
	15	62.5	63.5	NA	-	+	NA
	10	55.5	56.5	NA	+	NA	NA
	5	44.5	44.5	NA	+	NA	NA
70	45	NA	67.0	67.5	NA	-	-
	30	NA	66.0	66.5	NA	-	-
	15	59.5	59.0	60.0	+	+	+
	10	54.0	52.5	54.0	+	NA	NA
	5	44.0	41.5	43.0	+	NA	NA
65	45	NA	63.0	64.0	NA	-	-
	30	NA	61.5	63.0	NA	+	-
	15	56.5	54.0	57.0	+	+	+
	10	51.5	47.5	51.5	NA	NA	NA
	5	41.0	37.5	41.5	NA	NA	NA

* The concentration of *S suis* was 4×10^6 to 2×10^7 colony forming units/mL.

† The experiment was repeated up to 3 times for certain temperature points.

‡ Positive growth is reported as the presence of bacterial colonies on the plate.

NA = this time and temperature combination was not assessed.

30 minutes. This is critical for the complete inactivation of virus (upper red line, Figure 2). Table 4 outlines the fecal matter temperature in comparison to the ambient temperature at the time points of interest. It also shows whether the bacteria were inactivated or not. At the 80°C 15-minute, 75°C 15-minute, and 65°C 30-minute intervals, there is inconsistency in whether the bacteria were completely inactivated. This illustrates that the amount of fecal matter present can significantly affect the inactivation of bacteria. Both adequate time and temperature must be reached, and the introduction of fecal matter greatly impacts this.

Discussion

The aim of this study was to test the most optimal time and temperature for inactivation of a cross section of pathogens (Table 1) to develop improved biosecurity in livestock transport. Current decontamination protocols call for washing for 2 hours followed by overnight (8 hours) drying.[9-12,21,23,32]

It is known that many pathogens, bacterial, viral, and parasitic, can survive outside of a host in various environmental conditions.[11,12,15,17,23,25,26] In general, non-enveloped viruses, as well as most bacterial species, can persist in the environment for longer periods of time.[20,23,27-30] Our study investigated several porcine pathogens to find a time and temperature that would cover most major microbes relevant to swine health. Porcine epidemic diarrhea virus has been of utmost concern to hog producers following recent outbreaks in the United States and Eastern Canada. Being an enveloped RNA virus, PEDV is more fragile in the environment. Under certain conditions, however, the virus can survive on fomites and in organic matter.[2,6-9,15] Current TADD protocols stipulate 10 minutes at 71°C, which may not be enough to fully inactivate PEDV on a transport trailer.[2-6,9] We also investigated TGEV, another enveloped coronavirus and thus does not survive outside the host for long periods of time. It is easily killed by heat and sunlight but is resistant to freezing, thus outbreaks in cold weather are more common.[12] Porcine reproductive and respiratory syndrome virus is also an enveloped virus and does not survive long outside the host unless covered in organic materials.[13,17,18,33] It is quite heat labile but, like TGEV, survives well in temperatures below 20°C.[7] Swine influenza virus is known to be very labile in the environment, as with all enveloped influenza viruses. It

survives longer in cooler temperatures but is rapidly inactivated in sunlight or heat greater than 56°C.[19] Rotavirus, because it is non-enveloped, survives for extended periods in the environment and even longer in manure or manure contaminated environments.[13,20,25] Rotavirus is, however, highly susceptible to heat, and ultraviolet and gamma irradiation.[20,27]

Especially at temperatures below 75°C, PEDV exhibited less consistent inactivation. Because the PEDV stock used in our experiments was a cellular lysate, the variablity in inactivation may be partially explained by the intermittent lysing of cells as the temperature increased, releasing new, previously cell-associated, virus into the medium. It may also be due to heat inactivation of the TPCK-trypsin needed to have an active PEDV infection in vitro. The inactivation of PEDV in our clinical samples appeared to happen at much lower temperatures and less time, ie, 15 minutes at 50°C. Because viral load in these samples was unknown, it may have been significantly lower than that of the previously tested 2.4×10^8 copies/mL. Previous studies have shown 100% PEDV inactivation when surfaces were heated to 71°C for 10 minutes or kept at room temperature (20°C) for 7 days but variable inactivation at all temperatures in between.[2,7-9,15] This would also support our finding that heat inactivation is dose-dependent and as such, trailers contaminated with high amounts of virus from actively shedding animals, would require sufficient heat treatment to ensure all virus is inactivated. Emerging diseases such as African swine fever, Senecavirus A, and foot-and-mouth disease virus have been demonstrated to spread via fomites and infected animal products used as feed. We propose this heating protocol would also be effective against these pathogens in combination with disinfection.[11,34,35] Although heating processes alone in extruding have been demonstrated to kill classical swine fever virus , they may be higher than what is possible in a transport trailer.[11]

Generally, we demonstrated that the bacterial pathogens tested were rendered nonviable at lower temperatures and less time than the viruses. A contributing factor in swine dysentery, *Brachyspira* are anaerobic, but can survive for several days to a few weeks within a permissible environment.[12,25] It is transmitted directly through contaminated feces of both sick and asymptomatic pigs, although indirect contact with contaminated fomites can also be a source of transmission.[25] *Brachyspira* are highly susceptible to high temperatures and drying.[24]

The final bacteria tested in our study was APP. It is a gram-negative bacteria that causes highly contagious respiratory disease with high morbidity and mortality rates making it of particular concern to hog producers.[15,36] It is also a major component of the porcine respiratory disease complex along with *S suis* and PRRSV.[13,17,26,31]

Our data suggest that to effectively kill both bacterial and viral pathogens, the trailers need to reach a consistent temperature of 75°C for 15 minutes in all regions of the trailer. Preliminary data collection using current trailer baking protocols showed that the temperature varied widely across the span of the transport unit leaving "pockets" of potentially infectious material. Swine producers have expressed concerns over the presence of residual biological material (bedding, feces) in the trailers and how that may affect the heat inactivation of pathogens. Indeed, anecdotal reports of fist-sized masses of pig manure falling out of lights after trailers had been washed indicated a need to test the insular capacity of fecal matter. Our findings indicated that presence of large amounts of biological materials, ie, more than 10 g, may reduce the effectiveness of the heat inactivation. As with chemical disinfection, presence of biological material will hamper heat treatment as the insulating properties of feces and bedding are very high. Thorough cleaning of trailers is necessary and must be consistent for the heating process to be effective. Even with intensive cleaning practices, heating to 75°C for 15 minutes should be used to ensure pathogen inactivation. It should be noted that our study did not examine the use of biocides or disinfectants[5,10,19,21,25] routinely used in trailer washing, nor did we examine biofilms as this would have serious implications for cleaning and disinfection practices.[5,22,23,28-30] Our main limitation was not having access to trailers. From a biosafety perspective, we could not take these pathogens out of a laboratory setting to test responses in the field. This work was meant to mimic field conditions as best as possible but may not reflect actual conditions. We also did not examine extreme temperatures found seasonally in Canada. These aspects should be investigated in further studies.

Implications

Under the conditions of this study:

- Heating to 75°C for 15 minutes was sufficient to kill pathogens tested in cell culture.
- All areas of the trailer need to reach 75°C for 15 minutes to be compliant.
- Disinfectant use was not examined, nor was presence of biofilms.

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Conflict of Interest

None reported.

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