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Project Title and Project identification number: #20-178 SHIC: Understanding the role of feed manufacturing and delivery within a series of porcine deltacoronavirus investigations
Principal Investigator: Cassandra Jones
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Industry Summary: Two feed mills and three breed-to-wean facilities were investigated after being diagnosed with porcine deltacoronavirus (PDCoV) with initial suspicion that feed manufacture and delivery processes were involved in disease transmission. Both feed mills were audited and environmental samples collected in areas that were deemed high risk for virus contamination. All breed-to-wean facilities had PDCoV detected as would be expected, while the only positive samples for enteric coronaviruses associated with feed mills were feed delivery trucks. These results indicate that feed delivery surfaces can help spread virus during an ongoing disease outbreak and must be considered when determining the outbreak origin

Keywords: swine, epidemiology, feed safety, porcine deltacoronavirus

Scientific Abstract:
Two feed mills and three breed-to-wean facilities were investigated after being diagnosed with porcine deltacoronavirus (PDCoV) with initial suspicion that feed manufacture and delivery processes were involved in disease transmission. Both feed mills were audited and environmental samples collected in areas that were deemed high risk for virus contamination. All breed-to-wean facilities had PDCoV detected as would be expected, while the only positive samples for enteric coronaviruses associated with feed mills were feed delivery trucks. These results indicate that feed delivery surfaces can help spread virus during an ongoing disease outbreak and must be considered when determining the outbreak origin.

Introduction:
The swine industry has made advancements in biosecurity practices since the introduction of porcine epidemic diarrhea virus (PEDV) and porcine deltacoronavirus (PDCoV) in 2013 and 2014. Both diseases spread quickly through US swine production systems due to naïve herd status and fomites playing a large role in disseminating these viruses. Both PEDV and PDCoV rely on fecal-oral transmission; therefore, these viruses can be prevented if fecal contamination is limited.[1] The US swine industry quickly applied this concept to our animal transportation system and how workers and veterinarians enter and exit facilities. Practices adopted during this time, such as truck washing, disinfection, and heat treating or the usage of shoe covers, are now considered normal day-to-day practices for swine production settings.

Within the last decade, feed safety became heavily emphasized once it was hypothesized that a contaminated batch of feed ingredients imported from Asia was responsible for bringing PEDV and PDCoV to the US.[2] Prior to the realization that feed can serve as a vector for virus transmission, feed safety concerns primarily focused on controlling *Salmonella*, other bacteria,

and mycotoxins in feed mills. Since then, scientists have proven that PEDV-contaminated feed can cause clinical disease and once in the feed mill environment, impractical methods such as wet cleaning and disinfection are required to successfully remove PEDV from the feed mill.[3,4] Most feed safety research has focused on PEDV, but this research opened the door to the idea that a feed mill could serve as a transmission source of any virus. Currently, feed safety has a focus on bioexclusion of endemic pathogens as well as prevention of potential foreign animal disease introduction through feed and feed ingredients. The industry has also begun to further understand the epidemiological role the feed delivery supply chain has on feed mills and production sites. Taking what is known about fomites, such as people and trucks, feed safety research is working to understand the interaction between the feed mill and these moving pieces. Therefore, the authors conducted an investigation where multiple isolated facilities were diagnosed with PDCoV.

Objectives:
The goals were to 1) understand if the feed mill was the origin of disease, and 2) determine if trucks or people, either coming from the infected farms or coming from the feed mills, served as vectors to spread this virus.

Materials & Methods:
Three swine breed-to-wean herds, designated as sites A, B, and C, were diagnosed with PDCoV within one week in November 2020, with reports of initial clinical signs in the gestation area of the respective facilities (Figure 1). All 3 sites were located in the Midwestern United States and operate in accordance with Pork Quality Assurance Plus guidelines. All diagnostic samples confirming clinical disease within the production sites were collected under standard veterinary oversight procedures. All environmental swabs were collected from surfaces with no animal contact and environmental sampling personnel did not enter the production facilities. Site A and B were operated by the same production system, whereas site C did not share any management oversight with the other two sites. Workers for site A reported clinical signs of PDCoV in the gestation barn on November 9, 2020 and the diagnosis of PDCoV was confirmed that afternoon via polymerase chain reaction (qPCR) from samples sent to Kansas State University Veterinary Diagnostic Laboratory (KSU VDL). Workers from site B reported clinical signs on November 9, 2020 and with the diagnosis confirmed by Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) on November 11, 2020. Veterinarians from site B instructed workers to collect 1 feed sample from the gestation barn after confirmation of clinical signs of PDCoV. The sample was placed in the freezer, and submitted it to ISU VDL on November 30, 2020. Workers from site C reported 60 animals with scours in the gestation barn on November 11, 2020. Site C receives gilts from sites A and B, but gilts are raised in off-site gilt development units (GDU) and the timeline of animal deliveries did not indicate an epidemiological link between site C and sites A and B. A clinical diagnosis of PDCoV for site C was confirmed by laboratory evaluation the evening of November 11, 2020. Once PDCoV was diagnosed, all sites conducted controlled oral exposure with infected fecal material.

Feed mill 1 supplies site C and 12 to 15 other sow farms and only makes swine diets. Prior to the outbreak on site C, this feed mill monitored high risk areas such as boot soles, foot pedals, reclaim trucks, and office space every week. When clinical signs were first observed in gestation, the company reviewed their diets and determined that wheat middlings was the only ingredient unique to the gestation diet. Environmental samples were collected from all major ingredient bins, as it was believed that samples of accumulated dust would be more representative over a longer period compared to subsamples of feed or feed ingredients. The mill investigated the transport and handling of the wheat middlings and determined that the trucks used for transportation were not used for any other purpose, such as transporting ingredients other than wheat coproducts.

Feed mill 2 supplied feed to sites A and B and also supplied the same gestation feed to three other sites that also were infected with PDCoV but were not part of this investigation. Our investigation was focused on understanding the potential link between feed manufacture and delivery with acute outbreaks, so these additional three sites were excluded from this investigation because a significant amount of time had elapsed since clinical signs were noted at the farms. Feed delivery records reported that feed mill 2 delivered diets to site A and B from November 9-12, 2020, but what type of diet, how much, and what bin diets were delivered to are not recorded. Previous to this investigation, this feed mill had collected and submitted 7 environmental samples to the KSU VDL following initial clinical signs at a farm and suspicion of a potential link to the feed mill. All 7 samples were free of detectable PDCoV RNA and a link between the feed mill and farm outbreak was not found.

Investigations of the production sites and feed mill locations took place on November 14, 2020; approximately one week after observing clinical signs and confirming clinical diagnosis within production sites. Samples from sites A, B, and C focused on feed contact and nonfeed contact surfaces outside of the barn. Environmental sampling was limited to feed bins of gestation, lactation, and GDU unit barns and areas of high foot traffic or potential for high viral load. No feed samples or environmental samples were collected interior to the entry shower because all sites conducted controlled oral exposure once confirming PDCoV on site, so environmental samples would knowingly test positive for PDCoV. Site A had 12 sampled locations including feed bins, entry benches, and barn exhaust fans. Site B had 22 sampled locations including feed bins, spilled feed under feed bins, and areas of high foot traffic like barn entries, visitor log sign in, and areas around the crossover benches before the entry shower. Site C had 13 sampled locations including feed bins, netting surrounding exhaust fans near feed bins, and fan exhaust shrouds. Feed mill sampling locations included high-risk ingredients like porcine derived ingredients, areas of high foot or vehicle traffic (receiving and load out bay and warehouse floor), feed trucks going from farm to feed mill, and bulk feed bins. Feed delivery surfaces were those within the feed delivery trucks including dashboards, foot mats, truck steps, and driver seats. Feed mill 1 had 42 samples and feed mill 2 had 44 samples.

In addition to sampling the feed mills, audits were conducted using the Kansas State University Swine Feed Mill Biosecurity Audit template (https://www.asi.k-state.edu/research-and-extension/swine/biosecurity%20audit.doc). The audit evaluated the biosecurity practices within the feed mill and the feed delivery system and was completed by one member of the research team by systematically proceeding through the audit document. Feed mill 1 was well kept and clean. Employees had a good understanding of biosecurity and good feed mill practices. Feed delivery trucks were required one night down time between sites and washed once deliveries were finished. However, to prepare for the upcoming holiday season, the warehouse was more crowded than normal resulting in occasional spillage and bag ripping. If spillage occurred, these ingredients are swept up and discarded in the garbage. Feed mill 2 was generally clean and well kept; the receiving pit was covered, warehouse was swept and well maintained, and the mill only manufactured swine diets. When talking with the feed delivery driver, washing trucks and sanitizing wheels and wheel wells were done as biosecurity practices when delivering to various phases of swine production systems. However, there was a porcine-based ingredient on location (choice-white grease) and this facility only had one mixer so all diets went through the same equipment. Truck drivers were allowed to walk through the warehouse without shoe covers and feed trucks were allowed to haul diet ingredients and complete diets in the same trailer. Both the choice-white grease and no clear standard operating procedures (SOPs) for truck drivers had the potential to introduce PDCoV, PEDV, or other diseases within the feed mill and unintentionally contaminate other production sites and animals.

Environmental sampling was performed using one of two methods depending upon accessibility of sampling locations. The first method utilized a premoistened 10-cm square cotton gauze

surgical sponge as previously described.[5] This method was utilized when sample areas were easily accessible and the selected area could be swabbed by hand. The second method utilized premoistened paint roller covers (Marathon 22.9 cm × 0.95 cm nylon/polyester paint roller cover, Purdy North America) and a paint roller extension set (152 cm fiberglass paint roller frame utility pole, Mr. LongArm, Inc) as previously described.[3] The second method was used when sampling was particularly challenging, for example, inside of feed bins. Samples were placed on ice and transported back to Manhattan, Kansas. Before submitting to the lab, surgical gauze environmental swabs had 20 mL of phosphate buffered solution (PBS) added to the conical tube and manually agitated while paint rollers were squeezed inside the transportation plastic bag (Ziploc one-gallon size freezer bags; S.C. Johnson & Son, Inc) and the liquid was poured into a conical tube. If 20 mL could not be extracted from the roller, approximately 20 mL of PBS was added onto the roller and wrung out a second time. Samples were stored at -20°C until shipped to the ISU VDL. All samples were processed at ISU VDL for triplex qPCR for PEDV, PDCoV, and transmissible gastroenteritis virus (TGEV). Extractions from all samples were amplified using two amplification procedures. One amplification sequence used the standard ISU VDL cycle threshold (Ct) cutoff value of 36, and retained sample extractions were amplified using a Ct cutoff value of 45.

Results:
For the first round of qPCR analysis, 17 of 133 samples (12.8%) had detectable PEDV or PDCoV RNA with a Ct cutoff value of 36 (Table 1). Site A had 4 environmental swabs with detectable PDCoV RNA taken from the fans outside the gestation and farrowing barns and on the clean and dirty side of the entrance bench (Table 2). Site B had 6 environmental swabs with detectable PDCoV RNA taken from a feed bin outside the GDU, spilled feed outside the bin, footpath to the barn entrance, beneath shoes on the entrance floor, clean side of the entrance bench, and outside the barn entrance. Site C had 5 environmental swabs with detectable PDCoV RNA taken from exhaust fan netting around 4 different feed bins and a gestation barn fan shroud. Feed mill 2 had 2 environmental swabs with detectable PEDV RNA taken from the feed truck pedals and floor and feed truck steering wheel and dashboard. Feed mill 1 had no samples with detectable PEDV, PDCoV, or TGEV RNA.

For the second round of qPCR analysis, 30 of 133 samples (22.5%) had detectable PEDV or PDCoV RNA with a Ct cutoff value of 45. Site A had no additional environmental swabs with detectable PDCoV RNA. Site B had 9 additional environmental swabs with detectable PDCoV RNA taken from 4 GDU feed bins, spilled feed by another GDU bin, spilled feed under a lactation feed bin, nursery piglet feed bin, and the floor by the visitor entry and showers. Site C had 2 additional environmental swabs with detectable PDCoV RNA taken from 2 more gestation bin fan shrouds. Feed mill 1 had 2 environmental swabs with detectable PDCoV RNA taken from the feed truck steps and inside the feed truck cab. Feed mill 2 had no additional environmental swabs with detectable PEDV RNA. The site B feed sample submitted on November 30, 2020 was confirmed nondetectable for PEDV, TGEV, and PDCoV on December 2, 2020 at both cutoff values.

Discussion:
For this investigation, nonfeed contact surfaces were the majority of surfaces contaminated with PDCoV and PEDV. Since sites A, B, and C conducted controlled oral exposure once clinical signs appeared, PDCoV quickly dispersed through the environment and could be found on all surfaces including exhaust fans, exhaust fan netting, and fan shrouds. Research done with PEDV has found that once introduced, nucleic acids for the virus can be found throughout the environment.[6] Investigations like this should take into account whether locations have used controlled oral exposure as a disease management strategy because environmental sampling will be of lesser value due to the nature of controlled oral exposure. Interestingly, the only surfaces associated with the feed mill that had detectable RNA for porcine enteric viruses were from the

feed delivery system. These surfaces are freely movable, or transient in nature, and able to travel from one farm to the next which is probably how these surfaces became contaminated with virus. Others have found that surfaces associated with the feed supply chain contributed to the spread of African swine fever virus (ASFV) while feed contact surfaces were negative for ASFV.[7] Another study found that contaminated personal protective equipment and people can contribute to the spread of PEDV.[8] These findings highlight the importance of preventing pathogen introduction into the feed mill and the feed in order to eliminate potential transmission. An important, but not unexpected, takeaway message from the current investigation was that contamination with PDCoV can be found outside of clinically affected farms and that this contamination can be detected in high traffic areas for personnel and trucks. This highlights the need to implement or revisit biosecurity protocols for employees and truck drivers. While these protocols may be labor or cost intensive, it is pivotal that all people and vehicles moving in and out of the supply chain understand the importance of following and maintaining good biosecurity to control the spread of disease.

Another finding of this investigation is that neither feed mill had detectable quantities of enteric coronaviruses in environmental samples. When conducting disease outbreak investigations, particularly those incorporating environmental sampling, collection of appropriate samples in a timely manner is critical to allow for the greatest epidemiological value. Sample collection in the current investigation took place within 48 hours of notification of the desire to conduct sampling by the involved parties. When using environmental sampling to aid in a diagnostic investigation, the sooner the samples can be collected the lower likelihood of secondary epidemiological links causing confounding. A list of sampling locations was generated based on previous feed investigation experience to maximize the likelihood of detecting contamination if present. In this investigation, authors felt our response was timely to collect meaningful diagnostic information. When conducting investigations such as the one described in this manuscript, it is very important that personnel collecting samples are appropriately trained and collect samples in an aseptic manner.

Even though no swine enteric viruses were detected in either feed mill, there are multiple preventative strategies both feed mills could implement to mitigate the risk of feed delivery trucks potentially serving as vectors for disease that should remain out of the feed mill. Feed mitigants, like commercially available formaldehyde or medium chain fatty acids, can be expensive but reduce viral contamination in the feed.[9,10] Another solution to help reduce introduction of pathogens into a mill would be to implement truck and visitor SOPs to improve biosecurity within the feed mill. These moving pieces within the feed mill will always be present, but additional training will help to reduce the likelihood of introducing a health hazard into the feed mill.[11] During this investigation, authors would have liked more detailed record keeping and hence recommend all feed deliveries to have detailed records. Feed delivery records were obtained from feed mill 2 to further investigate the presence of PDCoV inside the feed bins at site B but there were not sufficient details within the records to make a definitive link between the feed and outbreak of PDCoV. The records showed supply date and trip location but did not provide details on type of diet transported or what bin was filled. Since there were not enough details present in the delivery records, a link between the PDCoV outbreak and presence of PDCoV RNA in the feed bin can only be speculated. The records did show that feed was unloaded into the bins during a time when PDCoV was intentionally spread through a farm. It is possible these bins were in front of exhaust fans and the bins were unintentionally contaminated with PDCoV from exhaust air. Because the feed sample and feed mill surfaces from feed mill 2 had no detectable RNA for PEDV, PDCoV, or TGEV, a link could not be made between the feed mill and PDCoV farm outbreak. Had there been more information available from the feed records, a possible link between the outbreak and feed mill could have been identified.

Lastly, site B had the largest portion of environmental samples testing positive for PDCoV using a Ct value of 36 and 45. When the Ct cutoff was 36, only 6 of 22 samples were positive but 9 additional samples were positive when the Ct cutoff value was increased to 45. The laboratory performing the analysis, matrix of the sample, and viral load of the sample must all be considered when interpreting diagnostic sample results.[12] There are differences between diagnostic laboratories regarding primers and threshold limit values. Current molecular based diagnostic techniques are not validated for environmental swabs or feed/ingredient samples and consequently care has to be taken when interpreting diagnostic results. In this investigation, using a Ct limit of 45 cycles resulted in a greater number of positive samples. Given where these samples were collected, it was logical there would be virus present, albeit at a low level. Thus, increasing the Ct limit from 36 to 45 within this investigation likely increased the sensitivity of detecting environmental contamination with PDCoV. While increasing the Ct cutoff value to 45 increased the sensitivity of the test results, this practice also may increase the rate of false-positive results. The purpose of this investigation was to identify areas of contamination and make biosecurity recommendations based on results. When interpreted appropriately, having a greater diagnostic sensitivity can help identify areas of concern and the consequences of false positives are outweighed by the value of increased sensitivity in this situation. Individuals must be cautious when interpreting results near the limit of detection for diagnostic assays, but if used appropriately, increasing the Ct limit as demonstrated in the current report can add value to diagnostic investigations using environmental swabs and feed/ingredient matrices.
To further understand the possible connection between the farms with clinical disease, genetic comparison of viruses through sequencing could be a useful tool. However, this was not possible in the current investigation. Additionally, a limitation of the qPCR assay used in the current experiment is that no information is provided regarding the ability for the identified genetic material to be infectious. The assay simply detects a specific sequence of RNA and provides no information regarding potential infectivity. Additional work is necessary to further understand the infectivity characteristics of environmental swabs in diagnostic investigations, but when results are interpreted appropriately qPCR can serve as a rapid, cost-effective diagnostic tool that can provide useful information.

In conclusion, this diagnostic investigation did not find evidence within the feed supply chain indicating feed or feed delivery was associated with outbreaks of PDCoV. Due to the nature of timing, it is believed that the contamination identified at the infected sites was due to the intentional exposure through controlled oral exposure. Furthermore, it is not known what the specific mechanism of transmission was to these farms, although other routes must be considered such as personnel and other possible fomites such as incoming supplies. The goal of this investigation was to evaluate the likelihood of a link between feed manufacturing and delivery with the outbreak of clinical disease, so greater investigation into potential routes of entry were not explored. This investigation highlights the importance of biosecurity during controlled oral exposure because viral contamination can be detected outside of the farm perimeter and common events such as feed delivery may serve as a mechanism for transfer of viral contamination back to the feed mill or to other farms. The current investigation emphasizes the importance of biosecurity in the feed supply chain at both the feed manufacturing and delivery stages, with particular focus needing to be directed towards personnel movement.

Graphics and figures:

Figure 1: Timeline of events for feed mill investigation. Sites A, B, and C are three breed-wean facilities located in the Midwest. PDCoV = porcine deltacoronavirus; KSU VDL = Kansas State University Veterinary Diagnostic Laboratory; ISU VDL = Iowa State University Veterinary Diagnostic Laboratory; PEDV = porcine epidemic diarrhea virus; TGEV = transmissible gastroenteritis virus.

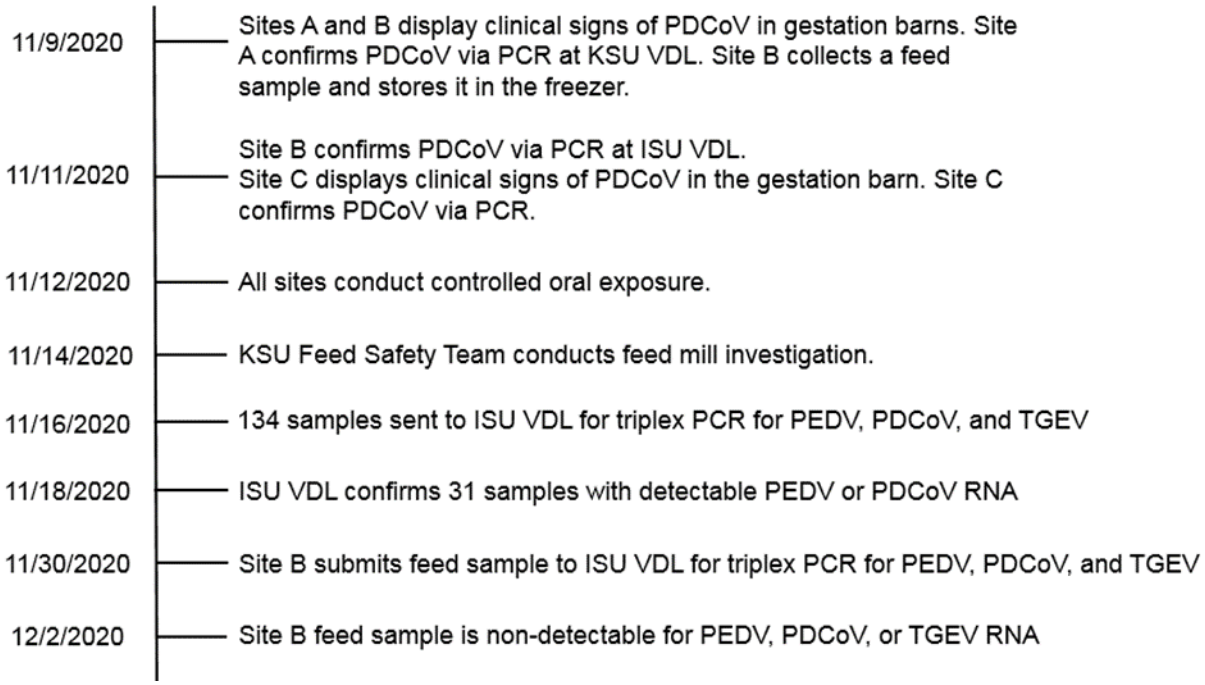

Table 1: Number of environmental swabs positive for viral RNA collected from live animal production sites and feed mills

		qPCR Ct limit					
		PDCoV		**PEDV**		**TGEV**	
Location	**Zone**	**36**	**45**	**36**	**45**	**36**	**45**
Site A	Feed bin - feed contact (n = 8)	0	0	0	0	0	0
	Surfaces exterior facility (n = 2)	2	2	0	0	0	0
	Personnel entry (n = 2)	2	2	0	0	0	0
Site B	Feed bin - feed contact (n =13)	1	6	0	0	0	0
	Feed sample (n = 1)	0	0	0	0	0	0
	Feed spills exterior facility (n = 3	1	3	0	0	0	0
	Personnel entry (n = 6)	4	6	0	0	0	0
Site C	Feed bin - feed contact (n = 6)	0	0	0	0	0	0
	Surfaces exterior facility (n = 7)	5	7	0	0	0	0
Mill 1	Feed contact surface (n = 26)	0	0	0	0	0	0
	Non-feed contact surface (n = 10	0	0	0	0	0	0
	Transient surface (n = 6)	0	2	0	0	0	0
Mill 2	Feed contact surface (n = 29)	0	0	0	0	0	0
	Non-feed contact surface (n = 8)	0	0	0	0	0	0
	Transient surface (n =7)	0	0	2	2	0	0

qPCR = polymerase chain reaction; Ct = cycle threshold; PDCoV = porcine deltacoronaviru
PEDV = porcine epidemic diarrhea virus; TGEV = transmissible gastroenteritis virus.

Table 2: Summary of qPCR Ct values for positive samples from live animal production sites and feed mills

		qPCR Ct limit					
		PDCoV		PEDV		TGEV	
Site	**Sampling location**	**36**	**45**	**36**	**45**	**36**	**45**
A	Farrowing exhaust fan	31.7	31.1	ND	ND	ND	ND
	Gestation exhaust fan	29.3	28.6	ND	ND	ND	ND
	Dirty side of entrance bench	29.5	29.1	ND	ND	ND	ND
	Clean side of entrance bench	35.5	36.0	ND	ND	ND	ND
B	GDU Bin 1	ND	38.8	ND	ND	ND	ND
	Spilled feed under GDU bins	35.7	36.2	ND	ND	ND	ND
	GDU Bin 2	33.0	32.6	ND	ND	ND	ND
	GDU Bin 3	ND	38.0	ND	ND	ND	ND
	GDU Bin 4	ND	36.9	ND	ND	ND	ND
	GDU Bin 5	ND	37.8	ND	ND	ND	ND
	Spilled feed under gestation bins	ND	38.7	ND	ND	ND	ND
	Spilled feed under lactation bins	ND	38.9	ND	ND	ND	ND
	Nursery holding room feed bin	ND	36.4	ND	ND	ND	ND
	Foot path exterior to facility	33.4	33.0	ND	ND	ND	ND
	Beneath shoe on floor	29.1	28.7	ND	ND	ND	ND
	Clean side of bench	35.2	34.7	ND	ND	ND	ND
	Floor by visitor log	ND	39.1	ND	ND	ND	ND
	Floor by showers	ND	39.0	ND	ND	ND	ND
	Outside near entry door	30.5	30.3	ND	ND	ND	ND
C	Netting by gestation bin 1	34.7	34.3	ND	ND	ND	ND
	Netting by gestation bin 2	30.9	30.2	ND	ND	ND	ND
	Netting by gestation bin 3	32.0	31.5	ND	ND	ND	ND
	Netting by gestation bin 4	34.7	33.6	ND	ND	ND	ND
	Fan shroud 1	ND	37.5	ND	ND	ND	ND
	Fan shroud 2	29.9	29.3	ND	ND	ND	ND
	Fan shroud 3	ND	35.7	ND	ND	ND	ND
Mill 1	Feed truck - steps	ND	37.3	ND	ND	ND	ND
	Feed truck - steering wheel, pedals, floor mat	ND	37.1	ND	ND	ND	ND
Mill 2	Feed truck - floor and pedals	ND	ND	33.4	33.2	ND	ND
	Feed truck - steering wheel and dashboard	ND	ND	35.6	35.0	ND	ND

qPCR = polymerase chain reaction; Ct = cycle threshold; PDCoV = porcine deltacoronavirus; PEDV = porcine epidemic diarrhea virus; TGEV = transmissible gastroenteritis virus; ND = no genetic material detected.

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Any publications, presentations or abstracts of the project results, need to recognize proper funding credit. A statement such as this would be sufficient: "Funding, wholly or in part, was provided by the Swine Health Information Center".

Thank you for your attention to these instructions. Please contact Bev Everitt (phone-515/223-2750 or E-mail: research@swinehealth.org if you have any questions.

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