Evaluating the Impact of Presence of Organic Matter on Environmental Samples and Sample Processing Technique on RNA Detection of PEDV

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Evaluating the Impact of Presence of Organic Matter on Environmental Samples and Sample Processing Technique on RNA Detection of PEDV

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Summary

Environmental sampling has become a commonly accepted diagnostic sampling technique for a means of identifying breaks in biosecurity. However, environmental samples have yet to be validated for reverse transcriptase real-time PCR (qRT-PCR) analysis and there is no standardization for environmental sample processing. Therefore, the objective of this project was to evaluate different types of environmental samples, and whether processing the samples prior to qRT-PCR analysis would impact results. Steel coupons were inoculated with PEDV in different types of environmental conditions, then were environmentally swabbed using cotton gauze. Treatments were arranged as a 5 × 4 factorial with five treatments for the different types of contamination and four treatments for the types of sample processing. Samples were processed in four different ways: no pre-qRT-PCR processing, centrifuging, syringe filtering, and centrifuging then syringe filtering to determine if pre-sample processing impacted the cycle threshold (Ct) value. Once samples were processed, they were submitted for PEDV qRT-PCR analysis. Results were reported as proportion of qRT-PCR positive and the resulting Ct value. If samples had no detectable RNA, they were assigned a Ct value of 45. For the Ct values, there was an inoculated surface × sample processing ($P < 0.0001$) interaction indicating that the type of environmental sample and the way the sample was processed impacted the Ct value of the sample. For pure virus and virus with PBS, there was no difference in Ct values between different sample processing techniques ($P<0.05$). For virus and dirt contamination, samples that were centrifuged had greater amounts of PEDV RNA detected compared to samples that were not processed ($P < 0.05$). For virus and fecal contamination, samples that were not processed or samples that were processed with centrifuging only had greater amounts of PEDV RNA detected compared to syringe filtered samples or centrifuged and syringe filtered samples ($P < 0.05$). For virus and organic matter contamination, samples that were centrifuged had greater amounts of PEDV RNA detected compared to all other sample processing techniques ($P < 0.05$). Main effects of inoculated surface ($P < 0.0001$) and sample processing ($P < 0.0001$)

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were also significant. For surface inoculation type, pure virus inoculation and virus with PBS inoculation had greater amounts of PEDV RNA compared to virus with feces inoculation or virus with organic matter inoculation, while virus with dirt was intermediate. For sample processing type, centrifuged samples had the greatest amount of PEDV RNA compared to syringe filtered and centrifuged then syringe filtered samples with unprocessed samples being intermediate. In summary, if environmental samples are particularly dirty, processing prior to qRT-PCR analysis will impact the results.

Introduction

Feed safety sampling relies heavily on environmental samples to identify potential breaks in biosecurity at the feed mill. Unfortunately, these environmental samples can sometimes be extremely dirty due to the amount of dust, dirt, animal fecal material, and/or other contaminants because of the sampling environment. While the usage of environmental samples has become accepted as a means for diagnostics, reverse transcriptase real-time PCR (qRT-PCR) analysis has yet to be validated for environmental samples. Due to this, there is a lot of variation between veterinary diagnostic laboratories and how they handle environmental samples.[3] Therefore the objective of this project was to evaluate various kinds of contamination of environmental samples and whether processing the samples prior to qRT-PCR analysis would impact sample results.

Materials and Methods

General

To achieve the objective, treatments were arranged as a 5 × 4 factorial with five treatments for the different types of contamination and four treatments for the types of sample processing. The five treatments for the inoculation of steel coupons were: inoculation with 1 mL of PEDV, inoculation with 1 mL of PEDV and 5 mL of phosphate buffered solution (PBS), inoculation with 1 mL of PEDV and 5.0 ± 0.2 grams of dirt, inoculation with 1 mL of PEDV and 5.0 ± 0.2 grams of feces, or inoculation with 1 mL of PEDV and 5.0 ± 0.2 grams of organic matter mixture. The four treatments for the processing of samples prior to qRT-PCR analysis were: no processing prior to submission, centrifuging the sample prior to submission, syringe filtering prior to submission, and centrifuging then syringe filtering the sample prior to submission. Each treatment was replicated three times.

Surfaces for inoculation were 15, steel, 4 × 4 in. coupons that were autoclaved and placed within a BSL-2 biosafety cabinet. Dirt and feces were collected prior to the start of this experiment and aliquoted into 5 ± 0.2 grams. For the organic matter mixture, 10 grams of the same dirt and 10 grams of the same feces were mixed together with 3 mL of deionized water. Once the mixture was completely mixed, it was aliquoted into 5 ± 0.2 gram aliquots. Dirt, feces, and organic matter mixture was frozen at -112°F until the experiment was conducted. Virus utilized was PEDV virus isolate USA/Co/2013 with a titer of $1.33 \times 10^5 \, TCID_{50}/mL$.

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[3] Schumacher LL. *Evaluation of porcine epidemic diarrhea virus in feed manufacturing.* Dissertation. Kansas State University; 2016.

Inoculation of surface

Steel coupons were inoculated with the assigned treatment as stated earlier in this report. After inoculation, the coupon sat for 15 min within the BSL-2 biosafety cabinet. After the 15 min time limit, each steel coupon was environmentally swabbed as previously described.[4] Once the environmental sample was taken, 20 mL of PBS was added to the environmental sample, inverted for 5–10 s, and allowed to incubate at room temperature for 1 hr. At the end of incubation time, the sample was vortexed for 15 s and then processed for qRT-PCR analysis.

Processing of samples

For each environmental sample, 4 samples were taken directly from the conical tube after vortexing. For Sample A, 1 mL was taken from the environmental sample and placed in a cryovial and submitted for qRT-PCR analysis without further processing. For Sample B, 1 mL was taken from the environmental sample, placed into a new conical tube, and centrifuged for 10 min at $706 \times g$. Following centrifugation, the supernatant was pipetted into a cryovial then submitted for qRT-PCR analysis. For Sample C, 1 mL was taken from the environmental sample, then filtered through a 0.45 μm 25 mm syringe filter into a cryovial then submitted for qRT-PCR analysis. For Sample D, 1 mL was taken from the environmental sample, placed into a new container, centrifuged as previously described, then filtered through a 0.45 μm 25 mm syringe filter into a cryovial then submitted for qRT-PCR analysis.

Reverse transcriptase real-time PCR analysis

Reverse transcriptase real time PCR was conducted at the Molecular Research and Development Laboratory within the Kansas State Veterinary Diagnostic Laboratory. Fifty microliters of supernatant from each sample was loaded into a deep-well plate and extracted using a Kingfisher Flex magnetic particle processor (Fisher Scientific, Pittsburg, PA) with the MagMAX-96 Viral RNA Isolation kit (Life Technologies, Grand Island, NY) according to manufacturer's instructions with one modification, reducing the final elution volume to 60 μL. One negative extraction control consisting of all reagents, but replaced the sample with PBS, was included in the extraction. Positive controls of each stock virus were also included with each extraction. Extracted RNA was frozen at -112°F until assayed by qRT-PCR. Analyzed values represent cycle threshold (Ct) at which virus was detected. If a sample had no detectable PEDV RNA, the sample was assigned a value of 45 as a total of 45 cycles were run for each sample. It is important to note that the smaller Ct value of the sample indicates greater amounts of PEDV RNA in the environmental sample.

Statistical analysis

Statistical analysis for the Ct value of samples was done through the aov function utilizing R programming language (R Foundation for Statistical Computing, Vienna, Austria). Fixed effects included the inoculation treatment, sample processing treatment, and the associated interaction. Results of Ct data are reported as least squares means ± standard error of the mean. Samples not containing detectable PEDV were

[4] Elijah, C. G.; Trujillo, J. D.; Jones, C. K.; Gaudreault, N. N.; Stark, C. R.; Cool, K. R.; Paulk, C. B.; Kwon, T.; Woodworth, J. C.; Morozov, I.; Gebhardt, J. T.; and Richt, J. A. (2020) "Evaluating the Distribution of African Swine Fever Virus Within a Feed Mill Environment Following Manufacture of Inoculated Feed," Kansas Agricultural Experiment Station Research Reports: Vol. 6: Iss. 10. https://doi.org/10.4148/2378-5977.8012.

assigned a value of 45 because that was the greatest number of cycles the qRT-PCR assay performed before concluding a sample did not have detectable virus. All statistical models were evaluated using visual assessment of studentized residuals, and model assumptions appeared to be appropriate. A Tukey multiple comparison adjustment was incorporated when appropriate. Results were considered significant at $P \leq 0.05$ and marginally significant between $P > 0.05$ and $P \leq 0.10$.

Results and Discussion

There was a contamination type × sample processing ($P < 0.0001$; Table 1) interaction in Ct values, indicating that the contamination type and its processing method impacted the Ct value of the sample. For pure virus and virus with PBS, there was no difference in Ct values between different sample processing techniques ($P < 0.05$). For virus and dirt contamination, samples that were centrifuged had greater amounts of PEDV RNA detected compared to samples that were not processed ($P < 0.05$). For virus and fecal contamination, samples that were not processed, or samples that were processed with centrifuging, only had greater amounts of PEDV RNA detected compared to syringe filtered samples or centrifuged and syringe filtered samples ($P < 0.05$). For virus and organic matter contamination, samples that were centrifuged had greater amounts of PEDV RNA detected compared to all other sample processing techniques ($P < 0.05$). It is important to note, that this investigation sought to investigate sample processing that would maximize our ability to identify viral contamination in dirty samples, so methods that had a greater amount of PEDV RNA (or a lower Ct value) were considered more successful at identifying PEDV RNA in an environmental sample.

There were also statistically significant main effects of inoculated surface ($P < 0.0001$) and sample processing ($P < 0.0001$; Table 2). For surface inoculation type, pure virus inoculation, and virus with PBS inoculation had greater amounts of PEDV RNA compared to virus with feces inoculation and virus with organic matter inoculation, while virus with dirt was intermediate. For sample processing type, centrifuged samples had the greatest amount of PEDV RNA compared to syringe filtered and centrifuged then syringe filtered samples, with unprocessed samples being intermediate.

In summary, if environmental samples are particularly dirty, processing prior to qRT-PCR analysis will impact the results. As samples became progressively more dirty, the PEDV RNA decreased within the sample suggesting that dirt, feces, or a combination of both inhibit the viral targets during qRT-PCR analysis. Centrifuging processing of samples was able to maximize qRT-PCR assay's ability to identify PEDV RNA across all environmental types.

Table 1. Interactive means of qRT-PCR reactions based on different surface inoculation types with PEDV and different types of sample processing[1]

Item	No processing (Sample A)	Centrifuge (Sample B)	Syringe filter (Sample C)	Centrifuge and syringe filter (Sample D)	SEM
Proportion qRT-PCR positive					--
Pure virus[2]	3/3	3/3	3/3	3/3	
Virus and PBS[3]	3/3	3/3	3/3	3/3	
Virus and dirt[4]	2/3	3/3	3/3	3/3	
Virus and feces[5]	3/3	3/3	0/3	0/3	
Virus and organic matter[6]	1/3	3/3	0/3	2/3	
Cycle threshold					1.41
Pure virus	24.5^a	24.6^a	28.9^{abcd}	27.5^{abc}	
Virus and PBS	24.8^{ab}	24.7^{ab}	28.0^{abc}	28.4^{abc}	
Virus and dirt	35.9^{de}	28.2^{abc}	32.0^{cd}	30.8^{abcd}	
Virus and feces	31.8^{bcd}	32.5^{cd}	45.0^f	45.0^f	
Virus and organic matter	42.4^{ef}	31.3^{abcd}	45.0^f	40.9^{ef}	

[1]Steel coupons, measuring 4 × 4 in., were inoculated with PEDV, isolate USA/Co/2013 with a titer of 1.33×10^5 TCID$_{50}$/mL, and different amounts of dirt, feces, and organic matter to simulate different environmental conditions. After surfaces were allowed to sit for 15 min, the steel coupon was environmentally swabbed with aseptic technique. Environmental samples were inverted for 5–10 s, incubated for an hour, vortexed for 10–15 s, and then processed according to designated sample pre-processing. Sample A aliquot was not processed any further and submitted as is, Sample B aliquot was centrifuged for 10 minutes at 706 × g, Sample C aliquot was syringe filtered with a 0.45 μm 25 mm syringe filter, and Sample D aliquot was centrifuged for 10 min at 706 × g then syringe filtered through a 0.45 μm 25 mm syringe filter. After processing, samples were submitted for PEDV qRT-PCR assay. Results are reported as proportion qRT-PCR positive and in cycle threshold (Ct) value. If there was no detectable RNA in the sample, the sample was assigned a Ct value of 45. Inoculated surface × sample processing $P < 0.0001$.

[2]The surface was inoculated with 1 mL of PEDV, environmentally swabbed with aseptic technique, inverted for 5–10 s, incubated at room temperature for 1 h, vortexed for 10–15 s, and then had four aliquots pulled for different processing techniques.

[3]The surface was inoculated with 1 mL of PEDV and 5 mL of phosphate buffered solution (PBS), environmentally swabbed with aseptic technique, inverted for 5–10 s, incubated at room temperature for 1 h, vortexed for 10–15 s, and then had four aliquots pulled for different processing techniques.

[4]The surface with inoculated with 5 g of dirt and 1 mL of PEDV, environmentally swabbed with aseptic technique, inverted for 5–10 s, incubated at room temperature for 1 h, vortexed for 10–15 s, and then had four aliquots pulled for different processing techniques.

[5]The surface was inoculated with 5 g of feces and 1 mL of PEDV, environmentally swabbed with aseptic technique, inverted for 5–10 s, incubated at room temperature for 1 h, vortexed for 10–15 s, and then had four aliquots pulled for different processing techniques.

[6]The surface was inoculated with 5 g of organic matter mixture and 1 mL of PEDV, environmentally swabbed with aseptic technique, inverted for 5–10 s, incubated at room temperature for 1 h, vortexed for 10–15 s, and then had four aliquots pulled for different processing techniques.

^{abc}Means lacking common superscripts differ, $P < 0.05$.

Table 2. Main effects of qRT-PCR reactions based on different surface inoculation types with PEDV and different types of sample processing[1]

	qRT-PCR Proportion positive	Cycle threshold	SEM
Surface inoculation[2]			0.80
Pure virus	12/12	26.4[a]	
Virus and PBS	12/12	26.5[a]	
Virus and dirt	11/12	31.7[b]	
Virus and feces	6/12	38.6[c]	
Virus and organic matter	6/12	39.9[c]	
Sample processing[3]			0.74
No processing (Sample A)	12/15	31.9[b]	
Centrifuge (Sample B)	15/15	28.2[a]	
Syringe filter (Sample C)	9/15	35.8[c]	
Centrifuge and syringe filter (Sample D)	11/15	34.5[c]	

[1]Steel coupons, measuring 4×4 in., were inoculated with PEDV, isolate USA/Co/2013 with a titer of 1.33×10^5 $TCID_{50}$/mL, and different amounts of dirt, feces, and organic matter to simulate different environmental conditions. After surfaces were allowed to sit for fifteen minutes, the steel coupon was environmentally swabbed with aseptic technique. Environmental samples were inverted for 5–10 s, incubated for 1 h, vortexed for 10–15 s, then processed according to designated sample processing treatments. Results are reported as proportion qRT-PCR positive and in cycle threshold (Ct) value. If there was no detectable RNA in the sample, the sample was assigned a Ct value of 45.
[2]Pure virus inoculation was the steel coupon inoculated with 1 mL of PEDV. Virus and phosphate buffered solution (PBS) was the steel coupon inoculated with 1 mL of PEDV and 5 mL of PBS. Virus and dirt inoculation was the steel coupon inoculated with 1 mL of PEDV and 5.0 ± 0.2 grams of dirt. Virus and feces was the steel coupon was inoculated with 1 mL of PEDV and 5.0 ± 0.2 grams of feces. Virus and organic matter inoculation was the steel coupon inoculated with 1 mL of PEDV and 5.0 ± 0.2 grams of organic matter mixture. Main effect of surface, $P < 0.0001$.
[3]Sample A aliquot was not processed any further and submitted as is, Sample B aliquot was centrifuged for 10 minutes at $706 \times g$, Sample C aliquot was syringe filtered with a 0.45 μm 25 mm syringe filter, and Sample D aliquot was centrifuged for 10 minutes at $706 \times g$ then syringe filtered through a 0.45 μm 25 mm syringe filter. Main effect of sample processing technique, $P < 0.0001$.
[abc]Means lacking common superscripts differ, $P < 0.05$.