Evaluating the impact of organic matter and sample processing techniques on RNA detection using environmental samples

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Summary

This study evaluated sample processing methods and the presence of organic matter on detection of porcine epidemic diarrhea virus (PEDV) from environmental samples using real-time reverse transcriptase-polymerase chain reaction (qRT-PCR). Steel coupons were inoculated with PEDV and different types of organic material contamination. Surface samples were collected and processed in one of four ways: none, centrifugation, syringe filtration, or combination of centrifugation and syringe filtration, then submitted for PEDV qRT-PCR. There was a surface inoculation type by processing method interaction ($P < .001$) that impacted the sample cycle threshold value. Centrifugation resulted in the most consistent detection of PEDV RNA.

Keywords: swine, environmental samples, feed safety

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Resumen - Evaluación del impacto de la materia orgánica y las técnicas de procesamiento de muestras en la detección de ARN utilizando muestras ambientales

Este estudio evaluó los métodos de procesamiento de muestras y la presencia de materia orgánica en la detección del virus de la diarrea epidémica porcina (PEDV) a partir de muestras ambientales utilizando la reacción en cadena de la polimerasa con transcriptasa inversa en tiempo real (qRT-PCR). Unas superficies de acero se inocularon con el PEDV y con diferentes tipos de contaminantes de material orgánico. Posteriormente estas superficie se recolectaron y procesaron con uno de cuatro procedimientos: ninguno, centrifugación, filtración con jeringa, o una combinación de centrifugación y filtración con jeringa, y posteriormente se enviaron para la qRT-PCR del PEDV. Hubo un tipo de inoculación superficial por interacción del método de procesamiento ($P < .001$) que afectó el valor del umbral del ciclo de muestreo. La centrifugación dio como resultado la detección más consistente del ARN del PEDV.

Résumé - Évaluation de l'impact de la matière organique et des techniques de manipulation de l'échantillon sur la détection d'ARN lors de l'utilisation d'échantillons environnementaux

La présente étude visait à évaluer les méthodes de traitement des échantillons et la présence de matière organique sur la détection du virus de diarrhée épidémique porcine (PEDV) à partir d'échantillons environnementaux par réaction d'amplification en chaîne par polymérase en temps réel utilisant la transcriptase réverse (qRT-PCR). Des échantillons d'acier ont été inoculés avec du PEDV et contaminés avec différents types de matériel organique. Des échantillons de surface ont été prélevés et traités par l'un des quatre procédés suivants: aucun, centrifugation, filtration à la seringue, ou combinaison de centrifugation et filtration à la seringue, puis testé pour PEDV par qRT-PCR. Il y avait une interaction entre le type d'inoculation de surface et la méthode de traitement ($P < .001$) qui influençait la valeur-seuil de cycles de l'échantillon. La centrifugation a permis la détection la plus constante d'ARN de PEDV.

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Swine veterinarians have come to heavily rely on polymerase chain reaction (PCR) assays for viral detection in samples like oral fluids, tissues, and environmental samples. The advantages of using PCR assays are that it is fast, sensitive, and can be used across multiple sample types.[1] Typically, oral fluids and tissue samples are used to diagnose clinical disease and help guide health decisions within populations of pigs. Environmental samples can help swine veterinarians detect pathogens on a variety of surfaces and address gaps in biosecurity practices for swine production systems or feed mills. Unfortunately, environmental samples can be heavily contaminated with dirt, feces, dust, feed, or a combination of these organic substances that naturally occur in the sample. This wide variety of contamination is an important factor when considering the accuracy of the PCR assay. The organic materials present in the environmental sample can inhibit the PCR reaction, resulting in decreased sensitivity or false-negative results.[1] There are multiple ways to approach sample handling to account for the potential of inhibitory substances depending upon which step of the PCR reaction is inhibited.[1,2]

When considering veterinary diagnostic laboratories, most PCR assays are validated for blood, tissue, and other clinical samples but environmental samples have yet to be validated. This is due to the fact that environmental samples can often contain different types of substances or a combination of substance that could inhibit the PCR assay. Thus, if a validated and standardized protocol for environmental samples would be created, these protocols would have to account for all of the potential inhibitory substances but also be time efficient. Ideally, the protocol could also be done relatively quickly in a laboratory so samples would still have the same turnaround time for submission. Therefore, the objective of this project was to evaluate different surface contamination types commonly found in environmental samples and if different processing techniques conducted prior to real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis would impact sample porcine epidemic diarrhea virus (PEDV) detection.

Procedures

General

Dirt and finishing pig feces were collected before this experiment and aliquoted into 5-g samples. For the organic matter mixture, 10 g of the same dirt and 10 g of the same feces were mixed together with 3 mL of deionized water. Once the organic matter was thoroughly mixed, it was aliquoted into 5-g samples. Dirt, feces, and organic matter were confirmed to have no detectable PEDV or porcine deltacoronavirus (PDCoV) RNA via PCR prior to the start of the experiment. Once confirming dirt, feces, and organic matter mixture had no detectable PEDV or PDCoV RNA, all material was frozen at -80°C until the experiment was conducted. Virus used was PEDV isolate USA/Co/2013 with a titer of 1.33×10^5 median tissue culture infectious dose/mL.

Surface inoculation

Fifteen autoclaved, steel, 10 × 10 cm coupons were placed within a biosafety level (BSL)-2 cabinet. A coupon was inoculated with one of the 5 surface inoculation types: 1 mL of PEDV; 1 mL of PEDV and 5 mL of phosphate buffered saline (PBS); 1 mL of PEDV and 5 g of dirt; 1 mL of PEDV and 5 g of feces; or 1 mL of PEDV and 5 g of organic matter mixture. Each treatment was replicated 3 times using 3 separate steel coupons.

Surface sample collection

After inoculation, the coupon sat for 15 minutes within the BSL-2 cabinet. After the 15-minute time limit, each steel coupon was environmentally swabbed as previously described.[3] Once the environmental sample was taken, 20 mL of PBS was added to the sample, it was inverted for 5 to 10 seconds, and then allowed to incubate at room temperature (24°C) for 1 hour. At the end of incubation, the sample was vortexed for 15 seconds and then processed for qRT-PCR analysis.

Sample processing

For each environmental sample, 4 samples were taken directly from the conical tube after vortexing and processed using 4 different techniques. For sample A, 1 mL was taken from the environmental sample, 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 minutes at 706*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, filtered through a 0.45-µm, 25-mm syringe filter into a cryovial, and then submitted for qRT-PCR analysis. For sample D, 1 mL was taken from the environmental sample, placed into a new conical tube, centrifuged as previously described, filtered through a 0.45-µm, 25-mm syringe filter into a cryovial, and then submitted for qRT-PCR analysis.

qRT-PCR analysis

The Molecular Research and Development Laboratory within the Kansas State University Veterinary Diagnostic Laboratory conducted the qRT-PCR analysis. 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) with the MagMAX-96 Viral RNA Isolation kit (Life Technologies) according to manufacturer instructions with one modification, reducing the final elution volume to 60 µL. One negative extraction control consisting of all reagents and PBS in place of the sample was included in the extraction. Positive controls of each stock virus were also included with each extraction. Extracted RNA was frozen at -80°C until assayed by qRT-PCR. Analyzed values represent cycle threshold (Ct) at which virus was detected. A total of 45 cycles were ran for each sample, so if a sample had no detectable PEDV RNA for the qRT-PCR assay, the sample was assigned a value of 45.

Statistical analysis

Statistical analysis of variance for the sample Ct values was performed using the *aov* function utilizing R programming language (R Foundation for Statistical Computing; version 4.1.1). Fixed effects included the inoculation treatment, sample processing treatment, and the associated interaction. Results of Ct data are reported as least squares means (SEM). 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 $P \leq .05$ and marginally significant between $P > .05$ and $P \leq .10$.

Table 1: Effect of inoculation type and environmental sample processing technique on PEDV detection on steel surfaces*

	Sample processing technique†			
Item	No processing	Centrifuge	Syringe filter	Centrifuge + syringe filter
qRT-PCR proportion, No. positive/No. samples				
Pure virus	3/3	3/3	3/3	3/3
Virus and PBS	3/3	3/3	3/3	3/3
Virus and dirt	2/3	3/3	3/3	3/3
Virus and feces	3/3	3/3	0/3	0/3
Virus and organic matter	1/3	3/3	0/3	2/3
Ct value‡				
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]

* Steel coupons, measuring 10 × 10 cm were inoculated with PEDV, isolate USA/Co/2013 with a titer of 1.33×10^5 $TCID_{50}$/mL. Surfaces were inoculated with 1 mL of pure virus, 1 mL of virus diluted into 5 mL of PBS, 1 mL of virus inoculated with 5 g of dirt, 5 g of feces, or 5 g of organic matter mixture consisting of a 1:1 ratio of dirt and feces. After surfaces were allowed to sit for 15 min, the steel coupon was environmentally swabbed. Environmental samples were inverted for 5-10 s, incubated for 1 hr, vortexed for 10-15 s, and then processed according to designated sample processing technique.

† Sample processing techniques included no processing, centrifuged for 10 min at 706*g* (centrifuge), filtered with a 0.45-µm, 25-mm syringe filter (syringe filter), or centrifuged for 10 min at 706*g* then filtered through a 0.45-µm, 25-mm syringe filter (centrifuge + syringe filter). After processing, samples were submitted for PEDV qRT-PCR assay.

‡ If there was no detectable RNA in the sample, the sample was assigned a Ct value of 45.

a-f Inoculation contamination type by sample processing interaction, $P < .001$; SEM = 1.41. Means lacking common superscripts differ, $P < .05$.

PEDV = porcine epidemic diarrhea virus; qRT-PCR = real-time reverse transcriptase-polymerase chain reaction; PBS = phosphate buffered saline; Ct = cycle threshold; $TCID_{50}$ = median tissue culture infectious dose.

Results

There was an inoculated surface contamination type by sample processing method ($P < .001$) interaction that impacted the sample Ct value (Table 1). For surfaces inoculated with pure virus and virus with PBS, there was no difference in the sample Ct values across the different types of sample processing methods ($P > .05$). For surfaces inoculated with virus and dirt, samples that were centrifuged had greater amounts of PEDV RNA detected (or lower Ct values) compared to samples that were not processed ($P < .05$). For surfaces inoculated with virus and feces, nonprocessed samples or centrifuged samples had greater amounts of PEDV RNA detected (or lower Ct values) compared to syringe filtered samples and centrifuged and syringe filtered samples ($P < .05$). For surfaces inoculated with virus and organic matter mixutre, centrifuged samples had greater amounts of PEDV RNA detected (or lower Ct values) compared to all other types of sample processing ($P < .05$).

There were also statistically significant main effects of surface contamination type ($P < .001$) and sample processing ($P < .001$; Table 2). For surface contamination type, surfaces inoculated with pure virus and virus with PBS had greater amounts of PEDV RNA detected (lower Ct values) compared to surfaces inoculated with virus and dirt ($P < .05$), while surfaces inoculated with virus and feces and virus and organic matter mixture had lower levels of PEDV RNA detected (higher Ct values) compared to all other surfaces ($P < .05$). For sample processing type, centrifugation of samples resulted in a greater amount of PEDV RNA detected (lower Ct values) compared to all other treatments ($P < .05$). Furthermore, syringe filtration or centrifugation and syringe filtration resulted in the lowest amount of PEDV RNA detected (higher Ct values; $P < .05$).

Discussion

Nucleic acid (NA) extraction and the PCR reaction are the 2 major steps that can influence the test results. For NA extraction, most commercial extraction kits, like the one used in this study, are able to remove most PCR inhibitory materials from the sample and enrich NA content for PCR detections. For a PCR reaction, there are 3 general steps: denaturation (unwind the double helix pattern of DNA), primer annealing (specific primers to attach to the unwound DNA), and extension (polymerase binds to the primer and unwound strand complex to make complimentary strands); then those complimentary strands are amplified and the rate of amplification corresponds with a Ct value.[4] Since primers can be designed for a wide variety of microorganisms and the assay is completed in minutes, PCR is a commonly used diagnostic tool across medical professions.[2,5] For swine veterinarians, PCR

Table 2: Main effects of surface inoculation type and sample processing technique on detection of PEDV on steel surfaces*

Item	qRT-PCR proportion, No. positive/No. samples	Ct†
Surface inoculation		
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		
No processing	12/15	31.9[d]
Centrifuge	15/15	28.2[e]
Syringe filter	9/15	35.8[f]
Centrifuge + syringe filter	11/15	34.5[f]

* Steel coupons, measuring 10 × 10 cm, were inoculated with PEDV, isolate USA/Co/2013 with a titer of 1.33×10^5 $TCID_{50}$/mL. Surfaces were inoculated with 1 mL of pure virus, 1 mL of virus diluted into 5 mL of PBS, 1 mL of virus inoculated with 5 g of dirt, 5 g of feces, or 5 g of organic matter mixture consisting of a 1:1 ratio of dirt and feces. After surfaces were allowed to sit for 15 min, the steel coupon was environmentally swabbed. Environmental samples were inverted for 5-10 s, incubated for 1 hr, vortexed for 10-15 s, and then processed according to designated sample processing technique. Samples were processed as either no processing, centrifuged for 10 min at 706*g* (centrifuge), filtered with a 0.45-µm, 25-mm syringe filter (syringe filter), or centrifuged for 10 min at 706*g* then filtered through a 0.45-µm, 25-mm syringe filter (centrifuge + syringe filter). After processing, samples were submitted for PEDV qRT-PCR assay.

† If there was no detectable RNA in the sample, the sample was assigned a Ct value of 45.

a-c Main effect of surface contamination type on Ct values, $P < .001$; SEM = 0.80. Means lacking common superscripts differ, $P < .05$.

d-f Main effect of sample processing technique on Ct values, $P < .001$; SEM = 0.74. Means lacking common superscripts differ, $P < .05$.

PEDV = porcine epidemic diarrhea virus; qRT-PCR = real-time reverse transcriptase-polymerase chain reaction; Ct = cycle threshold; PBS = phosphate buffered saline; $TCID_{50}$ = median tissue culture infectious dose.

assays are used for many disease syndromes and can include many different sample types like oral fluids, tissues, and environmental samples. However, when considering the 3 steps of a PCR reaction, there are ways for the accuracy of this assay to become compromised and therefore, give inaccurate results. For example, several potential issues that can arise during the PCR analysis process that could lead to false-positive or false-negative results include substances that inhibit any step of the assay, potential contamination during sample collection prior to PCR, or potential laboratory contamination while conducting the PCR assay.[2] There are many sources on how to counteract the potential for problems pertaining to all 3 basic steps of PCR but for the sake of this paper, the rest of the discussion will focus on inhibitory substances.

In general, inhibitory substances can naturally occur in the sample or be introduced into the sample during sample processing.[1] For example, common inhibitor substances can include body fluids or reagents in clinical and forensic sciences like hemoglobin, urea, or heparin; food substances or particles like glycogen, fats, or calcium; and environmental compounds like humic acids, heavy metals, or phenolic compounds.[6] These substances have the potential to interfere with PCR amplification and influence the sensitivity thereby negatively effecting the performance of the PCR assay.[7] There are many potential inhibitory substances and what is present in one sample matrix may be completely different in another sample matrix.[1] When considering common samples submitted for PCR by swine veterinarians, most of those sample types have the potential to include dirt, feces, blood, dust, soil, or a combination of these materials which can potentially inhibit a portion of a PCR reaction. Given this information, it does not mean that veterinarians should stop using PCR for diagnostics, but further reiterates that veterinarians should understand the potential pitfalls associated with their samples. It is important for veterinarians and diagnosticians to consider how best to handle the sample submission to maximize the PCR assay sensitivity. There are multiple methods that can be used to overcome potential inhibitory substances which can include biochemical methods, immunological methods, physical methods, or physiological methods; with the physical methods being the most user friendly.[7] Ideally the method used to process samples prior to PCR analysis would be cost effective, time efficient, and relatively easy to implement. Therefore, this study aimed to evaluate methods of sample processing, specifically physical methodologies, on different surface inoculation contamination types of environmental samples and how that impacted PEDV detection via PCR analysis.

For this study, there was an inoculated surface contamination by sample processing technique interaction indicating that the inoculation contamination type and how that environmental sample was processed prior to qRT-PCR analysis impacted the Ct value of the sample. As samples contained more inhibitory substances like dirt, feces, or a combination of both, how that sample was processed influenced the results of the PCR assay. No one single processing technique was

beneficial across all surface inoculation types. However, when the inoculation type was virus with dirt, feces, or organic matter mixture, the centrifugation methodology consistently identified PEDV RNA across all inoculation types as shown by the lower Ct values and proportion of positive PCR results when compared to other processing methods. Hall et al[8] found similar results when evaluating inhibitor resistance methods for diagnostics in clinical and environmental samples. Specifically, they found that of the 9 possible methods for inhibitor resistant, not a single method performed the best for all the sample matrices, but one method, KAPA blood PCR kit, did produce the most consistent results across the different sample matrices.[8] The current study and Hall et al[8] highlight that the best method for overcoming a variety of inhibitory substances is the method that produces the most consistent results.

Another finding from this study was that the centrifugation processing technique of samples had the lowest Ct values compared to other sample processing techniques. Similarly, one study found that centrifugation of urine samples helped to maximize PCR sensitivity and was also the most time efficient method compared to the traditional dot-plot hybridization method.[9] When considering sample processing techniques, this study and the current study both highlight the importance that the technique should be relatively easy, cost effective, and time efficient. Another finding from the current study was that the more "pure" surface contamination types had lower Ct values when compared to surfaces inoculated with feces or organic matter mixture. There was no statistically significant difference in Ct values for the pure virus inoculation and virus inoculation after dilution with PBS, but the detection of PEDV RNA was generally reduced as dirt, feces, or the combination were included on the environmental surface. This conclusion is similar to another research study that detailed the different ways forensic samples are processed before PCR analysis in order to obtain the purest sample possible to allow for proper PCR amplification.[10] Syringe filtering of samples in the current study reduced the ability to detect RNA in samples, especially those with dirt, feces, or the combination of both. It was hypothesized that the syringe filtering might also be trapping the RNA and not just dirt and feces. To the authors' knowledge, this is the first study to find these results associated with syringe filtration and processing samples prior to RT-PCR.

This study highlight that the best sample for RT-PCR is a sample free of substances that potentially interfere with PCR analysis like dirt, feces, and soil. However, when considering the environment most swine veterinarians acquire their sample from (barns with dirt, feces, and dust; environmental samples containing dirt, dust, and other materials), these findings further highlight the importance of proper sample processing to prevent potential inhibitory substances prior to PCR analysis. Based on the results of the current study, centrifugation of environmental samples at 706*g* for 10 minutes resulted in the most consistent recovery of PEDV RNA across a range of environmental organic material loads.

Implications

Under the conditions of this study:

- Organic material in environmental samples can interfere with qRT-PCR analysis.
- Processing samples before qRT-PCR can improve diagnostic sensitivity.
- Centrifugation maximized qRT-PCR sensitivity for environmental samples.

Acknowledgments

Conflict of interest

None reported.

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