RESEARCH REPORT
SWINE HEALTH

Title: Feasibility of viability PCR and ex-vivo bioassay to detect viable PED virus in feed. Identification - **NPB# 14-153**

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Industry Summary:
Porcine epidemic diarrhea virus (PEDV) was first detected in the United States in April 2013, spread rapidly, affected most of the swine producing states and caused significant economic losses. In this study, we developed a PCR-based tool to differentiate between viable and non-viable PEDV to assist in the study of risk factors associated with PEDV transmission. PMA (propidium monoazide) is a dye capable of penetrating cell membranes of non-viable organisms. PMA intercalates into the DNA/RNA of structurally damaged viruses, resulting in a higher cycle threshold (Ct) PCR value indicative of non-infectious virus. In this study, we show that RT-PCR in combination with PMA can be used to differentiate between infectious and heat inactivated PEDV. However, the ability to differentiate infectious from heat inactivated PEDV depends on the conditions set for sample incubation and pre-treatment, type of sample, quantity of genetic material in the sample. In summary, this report demonstrates the differentiation between infectious and heat inactivated PEDV using PCR based methods in combination with intercalating DNA/RNA dyes in an experiment setting. However, more research is needed to evaluate the feasibility of this technique in field samples.

Keywords: porcine epidemic diarrhea virus, viability PCR, propidium monoazide, PMA, intercalating dyes

Scientific Abstract:

Porcine epidemic diarrhea virus (PEDV) was first detected in the United States in April 2013. During the first year, PEDV spread rapidly, affecting most of the swine producing states and causing significant economic losses. In this study, a viability PCR (v-PCR) was developed to differentiate viable and non-viable PEDV to assist in the study of risk factors associated with PEDV transmission. Propidium monoazide (PMA) is a dye capable of penetrating cell membranes of non-viable organisms. PMA intercalates into the DNA/RNA through penetration of structurally damaged or compromised capsids of inactivated viruses limiting the replication of the viral DNA/RNA during the PCR reaction. In this study, we report various v-PCR experiments to test the effects of PMA concentration, incubation temperature and time, and sample pre-treatment conditions in infectious (viable) and heat inactivated (non-viable) samples. We also evaluated the performance of the v-PCR in a solution containing infectious and heat treated PEDV, and determined the impact and limit of detection of infectious and heat-treated PEDV concentration using this method. Lastly, the feasibility of this technique was evaluated in fecal samples from experimentally infected animals, feed samples spiked with PEDV, and slurry and feed-samples of known PEDV status based on pig bioassay data.

Infectious virus samples treated with PMA had a change in Ct value (ΔCt) of ~<3. In contrast, heat inactivated samples treated with PMA had a ΔCt ~>5. In the suspension of infectious and heat inactivated PEDV, differentiation was detected in the ratio of alive:dead PEDV equal or greater than 50:50. Differences in Ct values for infectious PEDV samples treated with PMA increased as the concentration of PEDV decreased, with ranges of ΔCt of 2.4 to 3.89 for 10^0 to 10^{-5} PEDV dilutions. The change in Ct values for heat- inactivated samples ranged from 5.86 to 12.19 for10^0 to 10^{-4} PEDV dilutions. ΔCt >~5 of heat inactivated samples was shown in 4 out of6 fecal samples collected from experimentally infected animals (ΔCt value could not be estimated in two samples

because starting Ct was >35), compared to ΔCt < 5 for 3 out of 6 samples in non-heat treated samples. A ΔCt of <3.70 was shown in infectious PEDV spiked feed when incubated in solution but not as a solid paste. In contrast, a ΔCt of 5.98-8.90 was shown in heat inactivated PEDV spiked feed samples incubated in solution but not as a solid paste. While samples from the bioassay studies and vPCR were in agreement 8 out 9 in slurry samples, agreement was poor or unobtainable in feed samples and plasma protein samples that had high Ct values in the starting material. Overall, this study differentiates between infectious and heat inactivated PEDV by combining RT-PCR with PMA treatment. However, further research is needed to evaluate the feasibility of this technique in field samples.

Introduction:

PEDV was first detected in the United States in April 2013. Since then, the disease spread rapidly with 33 states reporting cases by January 2015. PEDV is shed in very high concentrations in feces and is transmitted through the oral-fecal route. PEDV causes diarrhea in pigs of all age groups with elevated mortalities in piglets, leading to significant economic losses. Significant resources and efforts have been directed to prevent and control the spread of PEDV across the North American swine industry.

Transmission of PEDV occurs by direct contact with infected animals, indirectly through ingestion of contaminated feed (Dee et al., 2014) and inoculation of air samples collected from infected animals (Alonso et al., 2014). Transmission of PEDV during transport is also highly suspected due to the number of contaminated trailers reported at harvest plants (Lowe et al., 2014). Among all these potential sources of infection, contamination of feed with PEDV appears to have had the highest impact on the spread of PEDV in the US and the introduction of PEDV in Canada. Contamination of feed can happen by various mechanisms, ranging from the introduction of contaminated raw materials such as spray dried plasma (SDP) in the feed, gross fecal contamination in feed-mills or cross-contamination during the manufacturing process. Due to the documentation of feed in the transmission of PEDV, producers and veterinarians have tested large

numbers of feed samples and feed ingredients for PEDV. Interestingly, a number of feed samples have tested positive for PEDV by PCR, indicating the presence of PEDV genetic material. Unfortunately, conventional PCR tests do not differentiate between viable and non-viable organisms; thus, the development of methods is urgently needed to differentiate between viable and non-viable PEDV to better estimate the risk of PEDV transmission. Currently, swine bioassay is the only reliable method to assess the infectivity of PEDV. However, this method is expensive, time consuming, uses animal resources and is limited to a small number of samples.

In this study, we developed a viability PCR (vPCR) to differentiate between viable and non-viable PEDV. This vPCR uses propidium monoazide (PMA) dye to penetrate the membrane of non-viable organisms. PMA intercalates into the genetic material of dead viruses binding covalently the PEDV RNA upon exposure to bright light, and limiting the replication of viral RNA during the PCRreactions.

Objectives:

1) To evaluate the feasibility of viability-PCR (v-PCR) to identify viable PEDV in feed samples.

2) To evaluate the feasibility of an ex-vivo bioassay (intestine explants and IPEC J2 cells) to identify viable PEDV in feed.

Materials & Methods:

i. Virus, cells and virus inactivation conditions

A PEDV strain obtained from the National Veterinary Services Laboratory, Ames, IA was propagated and cultured in VERO 76 cells in Minimum Essential Medium (MEM) cell culture medium supplemented with 10% tryptose phosphate broth, 1X antibiotics-antimycotic and 4.5 µg/ml porcine trypsin. Infectious viruses were enumerated by determining the 50% tissue culture infectious dose ($TCID_{50}$) and the concentration of the stock virus was $10^{5.5}$ $TCID_{50}$/ml. In order to generate the inactivated inoculum, PEDV was killed by heat treatment at 82°C for 30 minutes. The rest of the inoculum was processed at 4°C.

ii. Propidium monoazide (PMA) treatment and photoactivation conditions

In order to determine the appropriate reaction conditions, the following parameters were tested: a) propidium monoazide (PMA) (Biotium, Hayward, CA) concentration, b) PMA incubation time and, c) PMA incubation temperature. PMA was dissolved in nuclease-free water to a working concentration of 1 mM. PMA was further diluted in 1X PBS and added to infectious and inactivated PEDV suspensions to obtain final PMA concentrations of 50, 100, 150, 200, 500, 750 and 900 µM. After the addition of PMA, incubation in the dark, at either 4°C or room temperature (RT) for 60 or 120 minutes was performed. After that, samples, in duplicates, were exposed to light for 15 minutes using the PMA-Lite LED photolysis device (Biotium, Hayward, CA).

Once the optimal PMA concentration and incubation conditions were identified, the remaining experiments were performed in duplicate with 200 µM of PMA, an incubation period of 60 minutes at room temperature and photoactivation of 15 minutes.

iii. Live:dead PEDV mixtures

In order to evaluate the sensitivity of the vPCR in a solution of infectious and inactivated PEDV, different ratios of live and dead PEDV were made (live:dead ratios): 100:0, 90:10, 50:50, 10:90, 0:100. The effect of PMA concentrations of 100, 150 and 200 µM was also tested on mixed viability solutions in duplicate.

iv. Effect of PEDV inoculum concentration

In order to assess the effect of PEDV inoculum concentration on the masking effect of PMA, eight 10-fold dilutions of the PEDV stock solution were made and processed as infectious or heat inactivated as outlined above and below. Samples were run in duplicate.

v. RNase, DNase and RNase+DNase treatments

For the RNase treatment, PEDV suspensions were treated with RNase as follows: 1 ml of PEDV suspension was mixed with 140 µl RNase ONE 10X buffer, 14 of µl RNase ONE Ribonuclease (Promega Corp., Madison, WI), and 246 µl of PEDV growth media. After incubation at 37°C for 30 minutes, RNase treated PEDV suspensions were divided into two equal aliquots. Then, an aliquot was placed at 4°C while the other aliquot was inactivated by incubation at 82°C for 30 minutes. Following RNase treatment and heat inactivation, samples proceeded to PMA treatment and RNA extraction as outlined below.

For the DNase treatment, PEDV suspensions were treated with DNase as follows: 1 ml of PEDV suspension was mixed with 140 µl of 10X TURBO DNase buffer, 84 µl of TURBO DNase (Ambion by Life Technologies, Carlsbad, CA), and 176 µl of PEDV growth media. After incubation at 37°C for 30 minutes, the reaction was inactivated by adding 140 µl of DNase inactivation reagent and incubated for 5 minutes at room temperature. The DNase treated PEDV suspension was centrifuged at 10,000 x g for 1.5 minutes, and the supernatant was aliquoted into 2 new tubes. Then, an aliquot was placed at 4°C while the other aliquot was inactivated by incubation at 82°C for 30 minutes. Following DNase treatment and heat inactivation, samples proceeded to PMA treatment and RNA extraction as outlined below.

For the RNase/DNase treatment combination, PEDV suspensions were treated with RNase and DNase as follow: 1 ml PEDV suspension was mixed with 70 µl of RNase ONE 10X buffer, 70 µl of 10X TURBO DNase buffer, 14 µl of RNase ONE Ribonuclease (Promega Corp., Madison, WI), 84 µl of TURBO DNase (Ambion by Life Technologies, Carlsbad, CA), and 162 µl of PEDV growth media. After incubation at 37°C for 30 minutes, the reaction was inactivated by adding 140 µl DNase inactivation reagent and incubated for 5 minutes at room temperature. The RNase and DNase treated PEDV suspension was centrifuged at 10,000 x g for 1.5 minutes, and the supernatant was aliquoted equally into 2 new tubes. Then, an aliquot was placed at 4°C while the other aliquot was

inactivated by incubation at 82°C for 30 minutes. Following RNase and DNase treatment and heat inactivation, samples proceeded to PMA treatment then RNA extraction as outlined below.

vi. RNA extraction and real time PCR (RT-PCR)

After incubating the samples for 60 minutes at room temperature with a PMA concentration of 200 µM, the RNA was extracted using the MagMAX™-96 Viral RNA Isolation Kit (Thermo, Austin, TX) according to manufacturer's instructions. Real time RT-PCR was carried out in a 25 µl mixture using the AgPath-ID One-Step RT-PCR reagent kit (Thermo) containing 5 µl RNA, 12.5 µl 2X buffer, 1.0 µl 25X enzyme mix, 1.67 µl detection enhancer, 18.8 pmol of each primer and 5 pmol of probe in an ABI 7500 Fast thermocycler (Thermo). The reaction was carried out at 45°C for 10 min, followed by 95°C for 10 min, then subsequent 45 cycles at 95°C for 15 sec then 60°C for 45 sec, with data collection occurring at 60°C. Samples with Ct values > 40 were considered negative.

vii. Calculation of virus inactivation

The masking effect of PMA was calculated as average cycle threshold (Ct) value of sample with PMA minus average Ct value of sample without PMA for infectious or unknown status samples, and heat inactivated samples. We refer to this value as change in Ct value (ΔCt).

viii. Feed samples

Three feed/PEDV virus combinations were tested as follows: a) 1g of feed was spiked with 5 ml of PEDV ($10^{5.5}$ $TCID_{50}$/ml); b) 10 g of feed was spiked with 1 ml of PEDV in 9 ml PBS; c) 10 g of feed was spiked with 10 ml of PEDV. Spiked feed samples were vortexed and tested immediately or incubated overnight at 4°C. For the 10 g of feed samples, 30 ml of PBS were added to facilitate elution of the sample. From each of the experiments, 1 ml of the PEDV spiked feed mix was aliquoted into 2 microcentrifuge tubes. Then, one microcentrifuge tube was kept at 4°C for 30 minutes, serving as the infectious sample while the other microcentrifuge tube was heat

inactivated at 82°C for 30 minutes and moved to 4°C for 30 minutes to cool. All microcentrifuge tubes were centrifuged at 10,000 x g for 10 minutes to pellet the feed, and 20 µl of supernatant from each tube was obtained in duplicate for experimentation at 0 or 200 µM PMA concentrations. The volume of all tubes was equalized to a final volume of 200 µl with PBS, then incubated in the dark for 1 hour at room temperature. Afterwards, one set of tubes was photoactivated with PMA-Lite LED Photolysis device while the remaining set of tubes was not photoactivated. Finally, both sets of tubes were processed for RNA extraction and RT-PCR.

ix. Fecal samples

Rectal swabs were obtained from experimentally infected animals. After collection, rectal swabs were suspended in 2.0 ml of DMEM cell culture media supplemented with 2% bovine serum albumin, 1X antibiotics-antimycotic and 1.5 µg/ml Trypsin-TPCK, and stored at -80°C until use. Each fecal swab sample was thawed, and two aliquots containing 400 µl of suspended fecal swab solution were created. One tube was kept at 4°C for 30 minutes while the second tube was heat inactivated at 82°C for 30 minutes and placed at 4°C for 15-30 minutes to cool. 20 µl of infectious and heat inactivated samples were aliquoted into appropriate tubes, in duplicate, for experimentation at 0 or 200 µM PMA. The volume of all tubes was equalized to a final volume of 200 µl with PBS and incubated in the dark for 1 hour at room temperature. Afterwards, one set of tubes was photoactivated with PMA-Lite LED Photolysis device while the remaining set of tubes was not photoactivated. Finally, both sets of tubes were processed for RNA extraction and RT-PCR.

x. Bioassay samples

Dr. Goyal kindly provided samples tested by using a bioassay model consisting of inoculating 10 day old pigs. Each bioassay sample was thawed and aliquoted into 2 tubes containing 200 µl of sample. One tube was kept at 4°C for 30 minutes while the second tube was heat inactivated at 82°C for 30 minutes and placed at 4°C for 15-30 minutes to cool. 20 µl of heat inactivated and non-

heat inactivated samples were aliquoted into appropriate tubes, in duplicate, for experimentation at 0 or 200 µM PMA. The volume of all tubes was equalized to a final volume of 200 µl with PBS and incubated in the dark for 1 hour at room temperature. Afterwards, one set of tubes was photoactivated with PMA-Lite LED Photolysis device while the remaining set of tubes was not photoactivated. Finally, both sets of tubes were processed for RNA extraction and RT-PCR.

Methods for objective 2. Two attempts were tried: a) IPEC cells were grown following published procedures and inoculated with PEDV; b) intestinal explants of 1-3 day old piglets were obtained and inoculated with PEDV. Explants were fixed with formalin after 24 h post inoculation and assessed for PEDV lesions.

VIII. Results:

Objective 1.

i. Incubation conditions

Table 1 summarizes the results of different PMA dye concentrations obtained at incubation conditions of 4°C and room temperature, and 60 and 120 minutes with and without photoactivation. 200 µM of PMA offered the larger Ct value difference (ΔCt) for the heat inactivated samples. There was <3 Ct value difference for the infectious PEDV samples. Overall, samples incubated at room temperature had higher Ct value differences than samples incubated at 4°C. Differences in incubation time of 60 and 120 minutes were less clear and both incubation times provided similar discriminatory power. There were no differences in results from dye concentration >200 µM (results not shown). Based on these results, we selected the incubation conditions of 60 minutes at room temperature. Table 1 also shows the results of the samples without photoactivation as a measure of controls indicating that PMA is not activated and does not have a masking effect when it is not activated with light.

ii. Effect of live:dead PEDV mixtures

A summary of the Ct values and ΔCt obtained of live:dead mixtures with and without PMA at the 200 μM PMA concentration are shown in Table 2. Results for concentrations 100 and 150 are not shown for simplicity. Ct differences of live:dead PEDV mixtures with and without PMA are shown for PMA 200 μM concentration (Figure 1). The sample containing 100% dead PEDV, when treated with PMA, shows a ΔCt of ~5 when compared with its untreated counterpart. In contrast, the sample containing 100% infectious PEDV shows ~2 Ct value difference, which agrees with the result (Ct 2.48) shown in Table 1.

iii. Effect of PEDV inoculum concentration

Table 3 summarizes the impact of the PEDV inoculum concentration on the discriminatory effect of PMA in RT-PCR reactions. For infectious PEDV, inoculum concentration had some effect on ΔCt values obtained with and without PMA. ΔCt value ranged between 2.41 and 3.89 for concentrations ranging between 10^0 and 10^{-5}. The ΔCt in the 10^{-6} dilution could not be assessed confidently. Ct value of the infectious sample without PMA was 35.46 which is close to the upper limit of detection for the PCR used in this study, and Ct value with PMA was negative. In contrast, the ΔCt value difference with and without PMA increased significantly as the concentration of PEDV decreased for the heat inactivated PEDV samples, with a ΔCt value range between 5.65 and 12.19 for concentrations between 10^0 and 10^{-5}.

In addition, these results also indicate that samples with Ct values around 35 prior to heat inactivation or PMA treatment cannot reliably be assessed.

iv. RNase and DNase treatments

Results from the RNase and DNase treatments can be seen in Table 4 and Table 5. ΔCt values for PEDV infectious inoculum in PMA and RNase treated samples was similar to ΔCt seen in non-treated RNase samples and ΔCt values were <3. In contrast, ΔCt for PEDV heat inactivated inoculum in PMA RNase treated samples increased to ~8 -9 ΔCt compared to the value of ~5 obtained in non-treated

RNase samples. There were no differences in ΔCt between RNase, DNase or RNAse/DNase combined treatments.

v. PEDV spike feed

A summary of the results obtained with the various PEDV incubation conditions in feed can be seen in Table 6. Incubation conditions of PEDV in feed overnight appeared to matter for volumes of 10 gr of feed with 10 ml of PEDV solution. There was an increase of ~ 9 ΔCt for four out of six samples that were not heat treated, suggesting inactivation of PEDV occurred when mixing the virus with the feed. For the remaining two samples, ΔCt ranged between 3.00 and 3.33. For the 1 g feed samples inoculated with 5 ml PEDV stock, ΔCt ranged between 2.45 to 3.70. In contrast, all heat inactivated feed samples had a ΔCt values ranging from 6.24 to 8.90.

vi. Rectal fecal swabs

A summary of the results obtained from the fecal rectal swabs collected from experimentally infected animals is available in Table 7. ΔCt from non-heat treated samples ranged between 2.97 and 10.66 with 3 out 6 samples having ΔCt < 5. All heat inactivated samples with a starting Ct value of less than 35 had ΔCt ranging from 5.87 to >6.98 (ΔCt value could not be estimated in two samples because starting Ct was >35).

vii. Bioassay results

A summary of the RT-PCR results obtained from bioassay samples with known viable PEDV status can be seen in Table 8.

Agreement between vPCR with status of samples obtained from bioassay studies was 8 out of 9 in slurry samples, but it could not be determined for the liquid plasma or the feed samples, in particular when the Ct values were >35 in the original material.

We calculated sensitivity and specificity comparing the vPCR results to the bioassay, using only the slurry samples (Table 9). Our results indicated that vPCR had 85% sensitivity while specificity was 100%.

Objective 2. To evaluate the feasibility of an ex-vivo bioassay (intestine explants and IPEC J2 cells) to identify viable PEDV in feed.

We could not show permissivity of IPEC cells to PEDV under the culture conditions used.

Furthermore, intestinal explants obtained from young piglets and inoculated with PEDV did not show lesions suggestive of PEDV replication.

We decided not to further pursue this objective and focused on objective 1, given that the results were more promising.

Discussion:

In this study, we investigated the feasibility of combining RT-PCR and PMA dye, which intercalates into RNA of viruses with damaged capsids, to discriminate between infectious (viable) and heat inactivated (non-viable) PEDV samples. We reported various experiments testing the effect of PMA concentration, incubation temperature, incubation time, and sample pre-treatment conditions. We also evaluated the performance of vPCR and determined the impact of PEDV concentration and limit of detection in a solution containing viable and non-viable PEDV. . Lastly we evaluated the feasibility of this technique in suspensions of fecal samples, feed and feed plasma protein. The results indicated differentiation between infectious and inactivated PEDV obtained by heat treatment is possible by combining RT-PCR with PMA. However, we also point out limitations of this technique and potential problems that can be encountered when testing field samples. Therefore, further research is needed to evaluate the feasibility of this technique in field samples of different natures.

First of all, we established the conditions of incubation temperature, incubation time and PMA dye concentration. Heat inactivated samples consistently had higher ΔCt values than the infectious virus inoculum and ΔCt was greater for dye concentrations of 200 μM. There were no striking differences between incubation conditions, but overall room temperature offered slightly better ΔCt value differentiation. In addition, we also observed that ΔCt for infectious samples had a Ct value <3, when in theory, it should be zero. This difference is attributed to the presence of non-viable PEDV mixed within the infectious inoculum. However, when the sample was heat inactivated, this ΔCt was enhanced (ΔCt ~>5) as expected.

The combination of RT-PCR and PMA was able to identify different ratios of infectious/inactivated PEDV. We could not differentiate between the presence of 100% infectious and 90% infectious, but a more clear differentiation was seen when there was <50% infectious PEDV (we did not estimate the effect between 90 and 50% infectious). In the same regards, we could identify presence of inactivated PEDV when there was >50% heat inactivated PEDV in the solution.

We also identified that the discriminatory effect of RT-PCR and PMA depended on the concentration of PEDV in the starting solution. ΔCt for infectious PEDV PMA treated samples increased as the concentration of PEDV decreased. This ΔCt was seen for both infectious and heat inactivated samples, but the magnitude was greater for heat inactivated samples. Thus, interpretation of ΔCt should be made based on the concentration of PEDV presence in the solution. In this study, incubation periods and dye concentrations were only evaluated with the PEDV stock of $10^{5.5}$ $TCID_{50}$/ml, with an average Ct value of 14.31. Additional studies are needed to elucidate the effect of PEDV concentration in the methods proposed and in particular to evaluate the effect of PEDV concentration in mixed solutions containing infectious and heat inactivated virus.

Pretreatment of samples with RNase also appeared to increase the discriminatory effect of PMA when we used the stock infectious PEDV inoculum. However, the advantage was less clear when

RNase was used in suspended fecal samples (results not shown). The effect of RNase needs to be further evaluated in fecal samples of known PEDV status.

The methods proposed here might be applicable to field samples. We tested suspensions of fecal samples collected from experimentally infected animals. Our results suggest that infectious samples contain a mixture of infectious and inactivated PEDV, but how much of each remains to be elucidated to properly interpret the results. In addition, spiked fecal samples with known quantity of PEDV are needed to further evaluate the impact of fecal material on the methods proposed here. Furthermore, information from slurry samples tested using bioassay (i.e inoculation of the sample in susceptible piglets), showed slightly lower sensitivity (85%) but had 100% specificity for the vPCR compared to the bioassay. However, these values need to be interpreted carefully given the limited number of samples used.

Processing of spiked feed samples in terms of the volume of PEDV solution in relation to the mass of feed inoculated appeared to impact the vPCR results. A higher than expected ΔCt was observed in pasty feed samples compared to samples where feed remained in solution. Incubation time conditions may also have impacted the results. Bioassay results from feed samples were difficult to interpret. All feed bioassay samples that were heat inactivated had starting Ct values > 35, thus the impact of inactivation was difficult to evaluate because PCR results were considered negative at Ct 40. Furthermore, ΔCt for the samples (prior to heat inactivation) were >5 even in a sample that was considered infectious by the bioassay, although we cannot be sure of the sample infectivity status at the time of testing.

In addition, we also evaluated 4 plasma protein samples with bioassay status data. Interpretation of results was difficult because all samples in the heat inactivated group had Ct values >35 in the starting material, and heat inactivation changed the physical characteristics of the sample. ΔCt for the samples that had not been heat treated and were bioassay negative were low compared to the results obtained during the vPCR validation and not different from the bioassay positive results.

Overall, more research needs to be done to evaluate the suitability of vPCR for PEDV detection in feed.

Lastly we only tested the conditions of inactivation by heat treatment. However, other methods of inactivation such as use of disinfectants, UV and others may disrupt the virus structure , but it is unknown if these inactivation methods would yield similar results as obtained by heat treatment. Thus, additional work reflecting a broader range of inactivation conditions is needed.

In summary, to our knowledge, this is the first report to successfully differentiate between infectious and inactivated PEDV obtained by heat treatment by combining RT-PCR with PMA treatment. The results presented in this report are encouraging but further research is needed to evaluate the feasibility of this technique in field samples.

Tables and Figures

Table 1. Summary of time and temperature incubation conditions for various PMA dye concentrations with and without photoactivation on the RT-PCR for PEDV.

		60 min, 4°C				60 min, Room temperature			
		Photoactivation*		No photoactivation		Photoactivation		No photoactivation	
	PMA	Ct	ΔCt[&]	Ct	ΔCt	Ct	ΔCt	Ct	ΔCt
Infectious	0	17.72		17.74		17.73		17.66	
	50	19.17	1.45	17.98	0.23	19.12	1.40	17.92	0.25
	100	19.24	1.52	17.94	0.20	19.11	1.38	17.87	0.21
	150	19.80	2.09	17.91	0.17	19.58	1.85	17.95	0.29
	200	20.19	2.48	17.90	0.16	20.00	2.27	17.83	0.16
Heat inactivated	0	19.63		19.75		19.89		19.81	
	50	22.63	3.00	19.78	0.03	22.61	2.73	19.93	0.12
	100	22.90	3.27	19.91	0.15	22.99	3.11	19.89	0.08
	150	23.80	4.17	19.80	0.04	23.88	4.00	19.98	0.16
	200	24.55	4.92	19.61	-0.15	24.97	5.08	19.89	0.08
		120 min, 4°C				120 min, Room temperature			
		Photoactivation		No photoactivation		Photoactivation		No photoactivation	
	PMA	Ct	ΔCt	Ct	ΔCt	Ct	ΔCt	Ct	ΔCt
Infectious	0	17.76		17.75		17.77		17.67	
	50	19.34	1.58	17.93	0.18	19.08	1.31	17.83	0.15
	100	19.14	1.38	17.99	0.24	19.52	1.76	18.08	0.40
	150	19.69	1.93	17.94	0.19	19.81	2.05	17.87	0.19
	200	20.24	2.47	17.95	0.19	20.40	2.64	17.74	0.07
Heat inactivated	0	19.34		19.26		19.39		19.40	
	50	21.53	2.19	19.39	0.13	21.97	2.58	19.37	-0.03
	100	22.71	3.37	19.57	0.31	22.78	3.39	19.35	-0.05
	150	22.82	3.48	19.42	0.16	24.00	4.61	19.34	-0.06
	200	23.52	4.18	19.27	0.01	25.48	6.10	19.31	-0.09

*Photoactivation is required to activate the PMA dye

[&]ΔCt: Difference in Ct value for samples with and without PMA for infectious and heat inactivated samples

Table 2. Effect of PMA on PEDV RT-PCR from solutions including different ratios of infectious (live) and heat inactivated (dead) PEDV.

		Photoactivation		No photoactivation	
Conditions	**PMA (µM)**	**Ct**	**ΔCt***	**Ct**	**ΔCt**
0:100 Dead:Live	0	18.06		18.12	
	200	20.24	2.17	18.07	-0.04
10:90 Dead:Live	0	18.32		18.26	
	200	20.46	2.14	18.36	0.10
50:50 Dead:Live	0	18.80		18.83	
	200	21.59	2.78	18.63	-0.20
90:10 Dead:Live	0	19.16		19.23	
	200	23.54	4.38	19.21	-0.02
100:0 Dead:Live	0	19.12		19.64	
	200	24.14	5.02	19.33	-0.31

ΔCt: Difference in Ct value for samples with and without PMA for infectious and heat inactivated samples

Table 3. Effect of PMA on PEDV RT-PCR in serial dilutions of infectious and heat inactivated stock PEDV.

		Infectious		Heat inactivated	
Dilution	**PMA (µM)**	**Ct**	**ΔCt***	**Ct**	**ΔCt**
10^0	0.00	14.26		16.27	
10^0	200.00	16.67	2.41	21.92	5.65
10^{-1}	0.00	18.33		19.90	
10^{-1}	200.00	20.98	2.65	27.92	8.02
10^{-2}	0.00	22.01		23.35	
10^{-2}	200.00	25.33	3.33	31.66	8.31
10^{-3}	0.00	25.06		26.81	
10^{-3}	200.00	28.36	3.31	36.80	9.99
10^{-4}	0.00	28.56		30.26	
10^{-4}	200.00	32.45	3.89	42.45	12.19
10^{-5}	0.00	32.44		34.70	
10^{-5}	200.00	35.46	3.02	40.00	>5.30
10^{-6}	0.00	35.27		Negative	
10^{-6}	200.00	40.00	>4.73	Negative	
10^{-7}	0.00	Negative		Negative	
10^{-7}	200.00	Negative		Negative	
10^{-8}	0.00	Negative		Negative	
10^{-8}	200.00	Negative		Negative	

*ΔCt: Difference in Ct value for samples with and without PMA for infectious and heat inactivated samples

Table 4. Effect of PMA on PEDV RT-PCR in samples pre-treated with RNase.

		No RNase		RNase	
	PMA (μM)	Ct	ΔCt*	Ct	ΔCt
Infectious	0	17.82		18.03	
	200	20.38	2.56	20.95	2.92
Heat inactivated	0	19.88		19.40	
	200	25.23	5.36	28.17	8.77

* ΔCt: Difference in Ct value for samples with and without PMA for infectious and heat inactivated samples

Table 5. Comparison of pre-treatment samples using RNase, DNase or RNase and DNase combined.

		RNase treatment		DNase treatment		RNase & DNase treatments combined	
	PMA	Ct	ΔCt*	Ct	ΔCt	Ct	ΔCt
Infectious	0	18.53		19.08		18.99	
	100	19.82	1.29	21.38	2.30	21.99	3.00
	150	20.48	1.95	21.10	2.02	21.35	2.36
	200	21.12	2.59	21.64	2.56	21.84	2.85
Heat inactivated	0	19.46		20.06		19.82	
	100	25.90	6.44	26.36	6.30	24.60	4.79
	150	26.67	7.21	26.80	6.74	26.33	6.51
	200	27.21	7.75	27.64	7.58	27.57	7.75

* ΔCt: Difference in Ct value for samples with and without PMA for infectious and heat inactivated samples

Table 6. Effect of PMA on PEDV RT-PCR in feed samples incubated with PEDV.

Feed incubation conditions			Sample		Heat inactivated	
		PMA (µM)	**Ct**	**ΔCt***	**Ct**	**ΔCt**
1 g feed with 5 ml PEDV stock	Feed Sample # 1 (no incubation)[&]	0	19.22		24.48	
		200	21.67	2.45	32.03	7.55
	Feed Sample # 2 (overnight at 4°C)	0	18.90		22.92	
		200	22.60	3.70	29.92	7.00
10 g feed with 10 ml PEDV stock	Feed Sample # 3 (no incubation)	0	22.16		28.16	
		200	31.16	9.00	34.53	6.37
	Feed Sample # 4 (overnight 4°C)	0	21.73		28.91	
		200	24.73	3.00	37.81	8.90
1 g feed with 5 ml PEDV stock	Feed Sample # 5 (no incubation)	0	18.88		22.47	
		200	21.75	2.87	28.45	5.98
	Feed Sample # 6 (overnight 4°C)	0	18.75		23.89	
		200	21.74	2.99	30.13	6.24
10 g feed with 10 ml PEDV stock	Feed Sample # 7 (no incubation)	0	21.59		28.08	
		200	30.53	8.94	36.01	7.93
	Feed Sample # 8 (overnight 4°C)	0	21.87		28.93	
		200	30.84	8.98	36.13	7.20
10 g feed with 1 ml PEDV stock and 9 ml PBS	Feed Sample # 9 (no incubation)	0.00	24.03		31.26	
		200.00	27.36	3.33	38.15	6.89
	Feed Sample # 10 (overnight 4°C)	0.00	24.53		32.17	
		200.00	33.97	9.44	40.00	>8.11

* ΔCt: Difference in Ct value for samples with and without PMA for infectious and heat inactivated samples

[&]No incubation refers to samples that were processed immediately after PEDV inoculation with feed

Table 7. Effect of PMA on PEDV RT-PCR in rectal fecal samples collected from pigs experimentally infected with PEDV.

		Sample		Heat inactivated	
	PMA (µM)	**Ct**	**ΔCt**	**Ct**	**ΔCt**
	0	19.78		26.62	
Sample 1	**200**	25.58	5.80	32.49	5.87
	0	24.83		28.72	
Sample 2	**200**	28.57	3.74	35.18	6.46
	0	30.95		37.25	
Sample 3	**200**	35.69	4.74	40.00	>2.75
	0	22.12		30.60	
Sample 4	**200**	25.09	2.97	37.05	6.45
	0	27.40		33.02	
Sample 5	**200**	33.95	6.55	40.00	>6.98
	0	32.37		ND	
Sample 6	**200**	43.04	10.66	ND	

Table 8. Effect of PMA on PEDV RT-PCR in samples of known PEDV status based on bioassay results.

	Liquid plasma		Sample		Heat inactivated	
Bioassay results		**PMA (µM)**	**Ct**	**ΔCt**	**Ct**	**ΔCt**
Positive	Bioassay Sample # 1	0	33.30		35.29	
		200	35.22	1.92	38.61	3.33
Positive	Bioassay Sample # 2	0	33.88		35.58	
		200	36.18	2.30	40.00	>4.42
Negative	Bioassay Sample # 4	0	33.32		36.03	
		200	35.08	1.76	38.47	2.44
Negative	Bioassay Sample # 5	0	34.86		37.13	
		200	37.14	2.28	40.00	>2.87
	Slurry		Sample		Heat inactivated	
		PMA (µM)	**Ct**	**ΔCt**	**Ct**	**ΔCt**
Positive	Bioassay Sample # 9	0	26.49		28.57	
		200	27.95	1.46	33.44	4.87
Positive	Bioassay Sample # 13	0	33.88		35.11	
		200	35.72	1.84	38.50	3.39
Positive	Bioassay Sample # 24	0	26.80		29.02	
		200	27.64	0.85	34.87	5.85
Negative	Bioassay Sample # 27	0	27.35		29.45	
		200	30.81	3.45	34.74	5.29
Positive	Bioassay Sample # 28	0	27.55		29.79	
		200	29.21	1.66	35.69	5.89
Negative	Bioassay Sample # 30	0	28.80		30.31	
		200	32.30	3.49	34.78	4.46
Positive	Bioassay Sample # 31	0	26.44		27.56	
		200	27.18	0.74	33.68	6.12
Positive	Bioassay Sample # 32	0	26.54		27.86	
		200	27.71	1.17	33.69	5.83
Negative	Bioassay Sample # 33	0	26.32		27.98	
		200	27.59	1.28	33.96	5.98
	Feed		Sample		Heat inactivated	
		PMA (µM)	**Ct**	**ΔCt**	**Ct**	**ΔCt**
Positive	Bioassay Sample # 34	0	32.49		38.49	
		200	40.00	>7.51	40.00	>1.51
Negative	Bioassay Sample # 35	0	33.43		37.86	
		200	40.00	>6.57	40.00	>2.14
Positive	Bioassay Sample # 36	0	32.54		37.59	
		200	38.43	5.89	40.00	>2.41
Negative	Bioassay Sample # 37	0	32.75		38.06	
		200	40.00	>7.25	40.00	>1.94

Table 9. Comparison of results from the bioassay and the PMA RT-PCR for PEDV for the slurry samples.

		PMA RT-PCR	
		Infectious	Heat inactivated
Bioassay	Infectious	6	0
	Heat inactivated	1	2

Figure 1. Effect of PMA on RT-PCR from solutions including different ratios of infectious (live) and inactivated (dead) PEDV. Results represent the ΔCt values of samples with and without PMA