RESEARCH REPORT
SWINE HEALTH

Title: Environmental stability of PEDV (porcine epidemic diarrhea virus) – **NPB #13-215**

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Objective: The overall objective of this project was to determine the environmental stability of PEDV. There were four specific aims as follows:

Aim 1. To determine survival of PEDV in fresh feces that represents the risk posed by transport.
Aim 2: To determine survival of PEDV in slurry (old feces in the pit) that reflects the risk of manure spreading.
Aim 3. To study PEDV survival in animal feed.
Aim 4. To study PEDV survival in drinking and recycled water (truck washes).

Extra aim: To determine the MID_{50} of PEDV.

All studies have been completed and the results are given in the attached sheets. In most instances, we examined the survival of TGEV (transmissible gastroenteritis virus) in addition to that of PEDV for comparative purposes.

General Methodology

1. Source of PEDV: A stock of PEDV was prepared from the small intestines collected from a pig infected experimentally with a field strain of PEDV. The jejunum was cut into 5-10 cm long sections and washed with sterile PBS (pH 7.2). The mucosa was scraped with sterile glass slides followed by homogenization in PBS. After centrifugation at 2000xg for 15 min, the supernatant was collected, aliquoted, and stored at -80^{0}C until used.

2. Source of TGEV: The stock of TGEV (Purdue strain) was prepared by growing the virus in swine testicle (ST) cells

3. Real time RT-PCR: The presence of PEDV in the samples was confirmed by rRT-PCR using PEDV spike (S) gene primers (Forward 1910: ACGTCCCTTTACTTTCAATTCACA and Reverse 2012: TATACTTGG TACACACATCCAGAGTCA) and probe 1939: FAM-TGAGTTGATTACTGGCACGCCTAA ACCAC-BHQ) using One-Step RT-PCR Reagents.

4. Experimental plan:

4a: Survival in fresh feces: Aliquots of fresh feces in 3.5 g amounts were placed in sterile glass tubes. To each aliquot were added 100 µL of 3X antibiotics (150 µg/mL gentamicin, 450µg/mL neomycin sulfate, 4.5 µg/mL fungizone, and 1.36 mg/mL streptomycin) to inhibit the growth of bacteria during the experiment. A set of aliquots was spiked with 0.5 mL of PEDV and another set with 0.5mL of TGEV. The tubes were placed in nine different water baths; three each at 40ºC, 50ºC and 60ºC. The 40ºC water baths were maintained at 30%, 50%, or 70% levels of RH (Relative Humidity). The same was done with water baths at 50ºC and 60ºC. The levels of 30%, 50%, and 70% RH were attained and maintained by using saturated solutions of $MgCl_2$, NaBr and $NaNO_3$, respectively (Rockland et al., 1960; Greenspan et al., 1997). Three tubes from each water bath were removed at 0, 1, 3, 7, and 14 days of incubation. To elute the residual virus, 5 mL of an elution buffer (3% beef extract-0.05M glycine, pH 7.5) was added to each vial. After thorough mixing, the tubes were centrifuged at 1,200xg for 10 min. The supernatants obtained from triplicate tubes at each time point were pooled together. Serial 10-fold dilutions of each pool were tested for PEDV and TGEV by rRT-PCR. In addition, PEDV pools were inoculated in 11-day-old piglets to determine virus survival and TGEV pools were inoculated in ST cells for virus titration.

4b: Survival in slurry: This experiment was done at room temperature (~25ºC), 4ºC, and -20ºC. The experiments at ~25ºC and 4ºC were done at three levels of RH (30%, 50%, and 70%) while experiment at -20^0C was done only at ambient RH of the freezer. Aliquots of slurry (in 5 mL amounts) contained in sterile tubes were treated with antibiotics as above followed by the addition of 0.5 mL of PEDV or TGEV. Three tubes from each set were removed at various times after incubation and the virus eluted with 5 mL of elution buffer. After mixing, the tubes were centrifuged at 1,200xg for 10 min. The three supernatants obtained were pooled from each set of samples (at each time point). Serial 10-fold dilutions were prepared from each pool and tested for PEDV and TGEV by rRT-PCR. In addition, PEDV pools were inoculated in 11-day-old piglets to determine virus survival and TGEV pools were inoculated in ST cells for virus titration.

4c. Survival in dry feed: Aliquots of animal feed (5g amounts) were placed in sterile tubes. To each vial 0.5 mL of PEDV (set A) or TGEV (set B) was added. After mixing well, the tubes were stored at RT (approx. 25ºC). Three tubes from each set were removed at weekly intervals from 0 to 5 weeks. Elution buffer was added at 10 mL/vial followed by thorough mixing and centrifugation at 1,200xg for 10 min. Eluates from each of the 3 tubes were pooled and serial 10-fold dilutions of the pools were tested by rRT-PCR (for PEDV) In addition, PEDV pools were inoculated in 11-day-old piglets to determine virus survival and TGEV pools were inoculated in ST cells for virus titration.

4d. Survival in wet feed: For wet feed 5g aliquots of animal feed were placed in sterile tubes. To each vial 5 mL of PBS (pH 7.2) was added followed by the addition of 0.5 mL of PEDV (set A) or TGEV (set B). After mixing well, the tubes were stored at RT (approx. 25ºC). Three tubes from each set were removed weekly from 0 to 4 weeks. The virus was eluted by adding 5 mL of elution buffer and triplicate supernatants were pooled. Serial 10-fold dilutions of the pools were tested by rRT-PCR (for PEDV). In addition, PEDV pools were inoculated in 11-day-old piglets to determine virus survival and TGEV pools were inoculated in ST cells for virus titration.

4e. Survival in drinking water: Aliquots of drinking water (5 mL amounts) were prepared in sterile tubes. To each vial was added 0.5 mL of virus (either PEDV or TGEV). The tubes were stored at room temperature

(25^0C). Three tubes were removed at weekly intervals from 0 to 7 weeks. The replicate samples were pooled and tested for PEDV and TGEV as detailed above.

4f. Survival in recycled water: Aliquots of truck wash water (5 mL amounts) were seeded with 0.5 mL of virus per tube (either PEDV or TGEV). The tubes were stored at room temperature (25^0C). Three tubes were removed at weekly intervals from 0 to 7 weeks. The replicate samples were pooled and tested for PEDV and TGEV as detailed above.

4g. Effect of water chlorination: For this experiment, we used PEDV grown in Vero cells (passage 14; initial cT value 19.84); in all other experiments we used PEDV present in intestinal scrapings. To determine the effect of chlorine on PEDV, we prepared 5 mL aliquots of drinking and recycled water samples with 5 different levels of chlorine (0, 10, 20, 30 and 50ppm). We then added 0.5 mL of virus (either PEDV or TGEV) to all tubes. Three tubes each were removed at 0, 30 and 45 min. The residual chlorine was neutralized by using 10% sodium thiosulphate solution. After pooling the three tubes, we conducted titration of PEDV and TGEV in Vero-76 and ST cells, respectively.

4h: MID_{50}: To determine the infectious dose of PEDV, we conducted two experiments. Serial 10-fold dilutions of PEDV (clarified homogenate of intestinal mucosa from infected piglet) were prepared in PBS and were tested by rRT-PCR. Two days after virus inoculation, the pigs were killed, their intestinal mucosa collected, and tested for PEDV by PCR.

5. Bioassays: Piglets were obtained from PEDV-negative farms at the age of 10 days and housed in separate rooms (one pig per room). After acclimatization for a day in animal isolation facilities, the piglets were inoculated by inserting 14 gauge 16-inch rubber esophageal tube directly into the pig's mouth without using a gag or a laryngoscope. The tube was introduced into the piglet's mouth over the back of the tongue and gently pushed in feeling for a swallowing reflex. After confirmation by negative pressure that the tube was inserted into the stomach, we introduced 20 mL of virus inoculum. Clinical signs were noted daily for two days post inoculation. The clinical signs were scored according to the following criteria:

Pigs showing no clinical signs and presenting as being active with normal flat lying hair and well-formed solid feces were assigned a score of 0.

Score 1= soft poorly formed feces

Score 2= presence of watery liquid feces

Score 3= watery diarrhea and dehydration

6. Necropsy: All animals were euthanized two days post inoculation with an injection of 2mL of pentobarbital intravenously via the external jugular vein. The intestines were removed immediately and pieces of jejunum from four different areas were collected, pooled, homogenized, and tested for the presence of PEDV by rRT-PCR.

7. Interpretation of rRT-PCR results for PEDV based on serial 10-fold dilutions of samples: To add value to bioassay results, we prepared 10-fold serial dilutions of samples containing PEDV and subjected them to rRT-PCR. Based on cT values, we found lower and higher dilutions to be positive and negative by rRT-PCR, respectively. To simplify the Tables, we have shown the highest dilution of the pooled sample that was positive by rRT-PCR. Please note that the cT values for the inoculum (sample pool) and homogenates of jejunum samples from the necropsied pigs are for undiluted samples.

<u>Results</u> - Aim 1. To determine the survival of PEDV in fresh feces

Table 1: Survival of PEDV in fresh feces at 40^0C

RH level	Time (days)	Highest dilution of sample pool positive by rRT-PCR	Diarrhea Score in inoculated pigs	cT value of:		TGEV $TCID_{50}$/mL
				Inoculum (undiluted sample pool)	Jejunum of inoculated pigs	
30%	0	10^4	3	24.13	16.48	$3.2x10^4$
	3	10^3	1	27.29	32.00	$1.4x10^3$
	7	10^1	0	30.54	-	$1.5x10^2$
	14	0	0	-	-	$6.8x10^1$
50%	1	10^4	3	27.59	16.35	$1.5x10^3$
	3	10^2	0	28.70	-	$3.2x10^2$
	7	10^2	0	31.09	-	$6.8x10^1$
	14	0	0	36.21	-	$6.8x10^1$
70%	0	10^5	3	27.36	13.79	$6.8x10^4$
	3	10^5	2	32.74	13.30	$6.8x10^4$
	7	10^1	1	37.92	15.16	$3.2x10^3$
	14	0	0	-	-	$1.5x10^1$

Conclusions:

1. rRT-PCR results showed that PEDV RNA could be detected in fresh feces for 7 days.
2. Bioassay results showed that PEDV survives for 1-7 days at 40^0C.
3. PEDV survives longer at 70% RH at 40^0C.
4. TGEV survives for up to 14 days at 40^0C.

Table 2: Survival of PEDV in fresh feces at 50^0C.

RH level	Time (days)	Highest dilution of sample pool positive by rRT-PCR	Diarrhea Score in inoculated pigs	cT value of:		TGEV $TCID_{50}$/mL
				Inoculum (undiluted sample pool)	Jejunum of inoculated pigs	
30%	0	10^4	3	22.68	15.65	$6.8x10^3$
	1	10^3	2	26.99	15.75	$1.4x10^3$
	3	10^2	0	_	-	$6.8x10^2$
	7	0	0	_	-	$3.2x10^1$
50%	0	10^5	3	21.85	16.63	$3.2x10^4$
	3	10^4	2	31.63	19.08	$3.2x10^4$
	7	10^1	1	37.85	_	$1.5x10^4$
	14	0	0	_	_	$3.2x10^1$
70%	0	10^5	3	20.24	16.12	$3.2x10^4$
	3	10^4	1	32.30	14.99	$6.8x10^3$
	7	10^1	0	33.79	35.33	$6.8x10^3$
	14	0	0	_	_	$1.5x10^1$

Conclusions:

1. rRT-PCR results show that PEDV RNA could be detected in fresh feces for 3-7 days.
2. Bioassay results show that PEDV survives for 1-7 days at 50^0C.
3. PEDV survives longer at 70% RH at 50^0C.
4. TGEV survives for up to 14 days at 50^0C.

Table 3: Survival of PEDV in fresh feces at 60^0C.

RH level	Time (days)	Highest dilution of sample pool positive by rRT-PCR	Diarrhea Score in inoculated pigs	cT value of:		TGEV $TCID_{50}$/mL
				Inoculum (undiluted sample pool)	Jejunum of inoculated pigs	
30%	1	10^2	3	23.58	13.26	$3.2x10^3$
	3	0	0	29.08	35.10	$3.2x10^2$
	7	0	0	-	37.24	$1.5x10^1$
50%	0	10^5	2	22.16	17.94	$6.8x10^3$
	3	10^2	3	31.03	16.31	$6.8x10^2$
	7	10^1	0	32.00	-	$1.5x10^2$
	14	0	0	_	-	$1.5x10^1$
70%	3	10^4	0	33.34	33.93	$3.2x10^4$
	7	10^2	0	34.50	35.61	$3.2x10^4$
	14	0	0	_	_	$6.8x10^3$

Conclusions:

1. rRT-PCR results show that PEDV RNA can be detected in fresh feces for 1-7 days.
2. Bioassay results show that PEDV survives for 1-7 days at 60^0C.
3. PEDV survives longer at 30% and 70% RH at 60^0C.
4. TGEV survives for up to 14 days at 60^0C.

Aim 2: To determine survival of PEDV in slurry (old feces in the pit) that reflects the risk of manure spreading.

Table 4. Survival of PEDV in slurry at -20^{0}C.

Time (days)	Highest dilution of sample pool positive by rRT-PCR	Diarrhea Score in inoculated pigs	cT value of:		TGEV $TCID_{50}$/mL
			Inoculum (undiluted sample pool)	Jejunum of inoculated pigs	
0	10^6	3	20.29	16.77	$1.5x10^6$
3	10^3	2	26.61	15.79	$6.8x10^3$
7	10^3	2	26.95	16.27	$1.5x10^4$
14	10^2	1	27.55	15.51	$6.8x10^3$
28	10^2	1	28.56	14.81	$1.5x10^3$

Conclusions:

1. rRT-PCR results show that PEDV RNA can be detected in slurry for $\geq$28 days.
2. Bioassay results show that PEDV survives for $\geq$28 days at -20^{0}C.
3. TGEV also survives for $\geq$28 days at -20^{0}C.

Table 5: Survival of PEDV in slurry stored at room temperature.

RH level	Time (days)	Highest dilution of sample pool positive by rRT-PCR	Diarrhea Score in inoculated pigs	cT value of:		TGEV $TCID_{50}$/mL
				Inoculum (undiluted sample pool)	Jejunum of inoculated pigs	
30%	0	10^5	3	20.30	17.01	$3.2x10^4$
	7	10^4	2	21.17	15.81	$3.2x10^3$
	14	10^3	1	22.17	36.86	$1.5x10^2$
	28	10^3	0	23.54	_	$3.2x10^3$
50%	7	10^4	3	21.83	17.60	$6.8x10^3$
	14	10^4	2	23.51	17.56	<1
	28	10^3	0	24.08	_	<1
70%	7	10^4	3	21.01	15.75	$1.5x10^3$
	14	10^4	1	21.41	35.86	$6.8x10^2$
	28	10^4	0	21.51	_	$1.5x10^2$

Conclusions:

1. rRT-PCR results show that PEDV RNA can be detected in slurry for ≥28 days at room temperature.
2. Bioassay results show that PEDV survives for 14 days in slurry at room temperature.
3. The survival of PEDV in slurry was similar at all 3 levels of RH.
4. TGEV survives for up to 28 days in slurry.

Table 6: Survival of PEDV in slurry stored at 4^0C.

RH level	Time (days)	Highest dilution of sample pool positive by rRT-PCR	Diarrhea Score in inoculated pigs	cT value of:		TGEV $TCID_{50}$/mL
				Inoculum (undiluted sample pool)	Jejunum of inoculated pigs	
30%	0	10^5	3	21.96	16.26	$6.8x10^4$
	7	10^4	2	20.50	16.41	$6.8x10^4$
	14	10^3	2	21.08	17.19	$3.2x10^4$
	28	10^3	1	22.13	16.08	$1.5x10^4$
50%	7	10^5	2	20.77	17.03	$3.2x10^3$
	14	10^5	3	21.29	16.04	$3.2x10^3$
	28	10^4	2	21.95	17.90	$6.8x10^3$
70%	7	10^5	2	21.60	16.19	$3.2x10^4$
	14	10^5	3	22.26	17.08	$6.8x10^4$
	28	10^4	0	22.04	36.69	$6.8x10^3$

Conclusions:

1. PEDV RNA was detected in slurry for $\geq$28 days by rRT-PCR.
2. Bioassay results show that PEDV survives in slurry for $\geq$28 days.
3. The survival of PEDV was similar at all 3 levels of RH.
4. TGEV survives for up to $\geq$28 days at 4^0C.

Aim 3. To study PEDV survival in animal feed at room temperature.

Table 7: Survival of PEDV in wet feed.

Time (days)	**Highest dilution of sample pool positive by rRT-PCR**	**Diarrhea Score in inoculated pigs**	**cT value of:**		**TGEV $TCID_{50}$/mL**
			Inoculum (undiluted sample pool)	**Jejunum of inoculated pigs**	
0	10^6	3	19.13	15.52	$6.8x10^5$
7	10^4	2	21.95	15.21	$3.2x10^4$
14	10^4	1	23.36	27.63	$3.2x10^4$
28	10^4	0	24.24	29.67	$3.2x10^3$

Conclusions:

1. By rRT-PCR, PEDV RNA was detected for $\geq$28 days.
2. Bioassay results show that PEDV was infectious for $\geq$28 days.
3. TGEV was also infectious for $\geq$28 days.

Table 8: Survival of PEDV in dry feed.

Time (weeks)	Highest dilution of sample pool positive by rRT-PCR	Diarrhea Score in inoculated pigs	cT value of:		TGEV $TCID_{50}$/mL
			Inoculum (undiluted sample pool)	Jejunum of inoculated pigs	
1	10^4	2	30.39	16.52	$3.2x10^3$
2	10^3	0	33.41	-	$1.5x10^3$
3	10^3	0	33.63	-	$6.8x10^3$
4	10^2	0	30.45	-	$3.2x10^2$
5	10^3	0	27.62	-	$6.8x10^1$

Conclusions:

1. By rRT-PCR, PEDV RNA was detected for $\geq$5 weeks.
2. Bioassay results show that PEDV was infectious for 1 week.
3. TGEV was also infectious for 5 weeks.

Aim 4. To study PEDV survival in drinking and recycled water (truck washes).

Table 9: Survival of PEDV and TGEV in drinking water.

Time (weeks)	Highest dilution of sample pool positive by rRT-PCR	Diarrhea Score in inoculated pigs	cT value of:		TGEV $TCID_{50}$/mL
			Inoculum (undiluted sample pool)	Jejunum of inoculated pigs	
0	10^6	3	22.20	13.06	$6.8x10^5$
1	10^4	2	23.65	13.11	$6.8x10^4$
2	10^4	0	22.72	37.40	$3.2x10^3$
3	10^3	0	24.18	-	$1.5x10^3$
4	10^3	0	23.90	-	$3.2x10^3$
5	10^4	0	23.41	-	$1.5x10^3$
6	10^4	0	24.86	-	$1.5x10^3$
7	10^3	0	23.99	-	$1.5x10^2$

Conclusions:

1. By rRT-PCR, PEDV RNA was detected for $\geq$7 weeks.
2. Bioassay results show that PEDV was infectious for 2 weeks.
3. TGEV was also infectious for 7 weeks.

Table 10: Survival of PEDV in recycled water (truck washes).

Time (weeks)	Highest dilution of sample pool positive by rRT-PCR	Diarrhea Score in inoculated pigs	cT value of:		TGEV $TCID_{50}$/mL
			Inoculum (undiluted sample pool)	Jejunum of inoculated pigs	
0	10^5	3	22.86	14.15	$3.2x10^4$
1	10^3	2	24.37	16.42	$3.2x10^3$
2	10^4	0	25.28	-	$1.5x10^3$
3	10^4	0	23.99	-	$3.2x10^3$
4	10^3	0	23.69	-	$3.2x10^2$
5	10^4	0	23.90	-	$1.5x10^3$
6	10^3	0	26.12	-	$3.2x10^2$
7	10^4	0	26.11	-	$1.5x10^2$

Conclusions:

1. By rRT-PCR, PEDV RNA was detected for $\geq$7 weeks.
2. Bioassay results show that PEDV was infectious for 1 week.
3. TGEV was infectious for 7 weeks.

Table 11. Effect of chlorine on PEDV and TGEV in drinking water.

Virus	Initial Cl_2 conc (ppm)	$TCID_{50}$/mL (residual Cl_2) after indicated contact time:		
		0 min	30 min	45 min
PEDV	10	1.5×10^4 (0)	3.2×10^4 (0)	1.5×10^4 (0)
	20	6.8×10^4 (0)	6.8×10^1 (0)	0 (1)
	30	3.2×10^4 (5)	0 (1)	0 (1)
	50	1.5×10^4 (10)	0 (5)	0 (3)
TGEV	10	3.2×10^5 (0)	6.8×10^4 (0)	3.2×10^4 (0)
	20	1.5×10^5 (0)	1.5×10^2 (0)	0 (1)
	30	1.5×10^4 (5)	0 (1)	0 (1)
	50	3.2×10^4 (10)	0 (5)	0 (3)

Conclusion: An initial concentration of 20 ppm chlorine can kill both viruses within 45 min in drinking water. A residual concentration of 1ppm can kill both viruses after 30 minute contact time.

Table 12. Effect of chlorine on PEDV and TGEV in recycled water.

Virus	Initial Cl_2 conc (ppm)	$TCID_{50}$/mL (residual Cl_2) after indicated contact time:		
		0 min	30 min	45 min
PEDV	10	1.5×10^4 (0)	3.2×10^3 (0)	1.5×10^3 (0)
	20	6.8×10^4 (0)	1.5×10^4 (0)	1.5×10^3 (0)
	30	3.2×10^4 (5)	3.2×10^3 (0)	1.5×10^3 (0)
	50	6.8×10^4 (10)	0 (5)	0 (3)
TGEV	10	3.2×10^5 (0)	1.5×10^5 (0)	3.2×10^4 (0)
	20	3.2×10^5 (0)	3.2×10^4 (0)	3.2×10^4 (0)
	30	1.5×10^4 (5)	6.8×10^3 (0)	1.5×10^3 (0)
	50	6.8×10^4 (10)	0 (5)	0 (3)

Conclusion: An initial concentration of 50 ppm chlorine can kill both viruses within 30 min in recycled water. A residual concentration of 5ppm can kill both viruses in recycled water after 30 minute contact time.

Infectious dose of PEDV:

Table 13: Signs of diarrhea in piglets inoculated with different dilutions of PEDV.

Virus dilution	Initial cT value of indicated virus dilution	Extent of diarrhea	cT value of jejunum of inoculated pigs
Undiluted	16.39	ND*	ND
10^{-1}	17.73	ND	ND
10^{-2}	20.53	3	17.24
10^{-3}	23.55	3	16.92
10^{-4}	27.04	3	15.32
10^{-5}	29.94	2	17.10
10^{-6}	32.06	2	16.02
10^{-7}	35.60	1	15.70
10^{-8}	-	1	16.03
10^{-9}	-	0	30.29
10^{-10}	-	0	-
10^{-11}	-	0	-
10^{-12}	-	0	-

*ND=not done

Conclusion: The results indicate that PEDV is highly infectious and that the MID_{50} is very low.