applied biosystems

Detecting Porcine Coronaviruses PEDV, PDCoV and TGEV by Reverse Transcriptase Real-Time PCR

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ABSTRACT

We designed a reverse transcriptase-real-time PCR (RT-qPCR) assay to detect three coronaviruses, porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), and porcine delta coronavirus (PDCoV), in a single reaction. This test can be performed with environmental samples taken off the floors and walls of barns, swabs taken from trucks, or swabs from farm personnel. The multiplex RT-qPCR assay uses a different fluorescent dye for each target for pathogen ID, and includes an internal positive control. Bioinformatic tests show that the assay will detect all known stains of PEDV, TGEV, and PDCoV based on sequences from around the world that were entered into GenBank.
We have found that the multiplex coronavirus assay worked with all environmental samples tested, including samples collected from pigs at the individual and herd level. The sensitivity and specificity of the multiplex coronavirus assay was determined on field samples consisting of oral fluids, feces, and diverse types of environmental samples. Over 400 field samples were tested. The workflow for testing field samples includes RNA isolation, RT-qPCR and data analysis. Results from testing field samples showed a sensitivity of ≥97% and a specificity of 100% for all three targets.

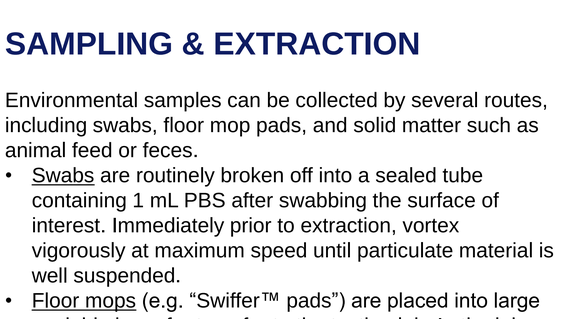
INTRODUCTION

Porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), and porcine delta coronavirus (PDCoV) are viral infections that can cause gastrointestinal diseases in pigs. Clinical signs can include diarrhea, vomiting, dehydration and anorexia. Histological examination of the intestinal lining often shows villous atrophy. Current recognition of the spread of porcine coronaviruses and the importance of early detection has led to the need for regular environmental testing that includes checking trucks, barn floors, walls and incoming feed components to prevent new outbreaks in naive herds. Using environmental samples for detection of these pathogens will give information on herd levels of these diseases. It is also an animal-friendly sampling method to quickly get information about the actual situation in the farm and can lead to quick actions, if needed, or additional sampling at the animal level to confirm. Environmental samples cannot be used to diagnose single animals in the herds.

SAMPLING & EXTRACTION

Environmental samples can be collected by several routes, including swabs, floor mop pads, and solid matter such as animal feed or feces.

- Swabs are routinely broken off into a sealed tube containing 1 mL PBS after swabbing the surface of interest. Immediately prior to extraction, vortex vigorously at maximum speed until particulate material is well suspended.
- Floor mops (e.g. “Swiffer™ pads”) are placed into large sealable bags for transfer to the testing lab. In the lab 100 mL of an isotonic solution is added to the pad in the bag, the bag is massaged to wring contents into the solution, and either 100 µL is immediately extracted or 15 mL of the resultant solution is removed for longer storage.
- Solid matter, like fecal samples or feed samples. Environmental fecal samples can be scraped from the floors of the pen; feed samples can be taken from several feed boxes in the pens or from several pens in the barn or from the feed mills. These samples are collected in a suitable container to which an isotonic solution is added at a ratio of 1:9 (g of sample to mL of PBS). This is agitated sufficiently to maximize disruption/suspension/dissolution of the sample, and either 100 µL is immediately extracted or 15 mL of the resultant suspension is removed for longer storage.



Upon receipt of the samples in the laboratory, the samples are agitated in an isotonic solution such as PBS to suspend particulates, and 100 µL of this was used in an extraction with the MagMAX™ Pathogen RNA/DNA Kit as per instructions. An Internal Positive Control (**IPC**), “Xeno ™ RNA,” is spiked into the lysate mixture for each sample.

Real-Time PCR (qPCR)

Real Time PCR is used to find RNA or DNA of the viruses in the samples. Test results of a PCR will be shown as C_T values. Laboratories will report results as positive/ negative or as positive/ negative with C_T values mentioned. TaqMan™ real-time PCR (qPCR) utilizes the dual primers of a standard PCR, and adds a third oligonucleotide, called the TaqMan probe, which hybridizes to the region between the primers and cannot serve as a primer itself. When the polymerase extends into it, 5’→3’exonuclease activity in the enzyme clips this oligo up. This releases a fluorescent tag, causing a huge increase in fluorescent signal. With each amplification cycle, there is ideally a doubling of the amount of fluorescent tag released.

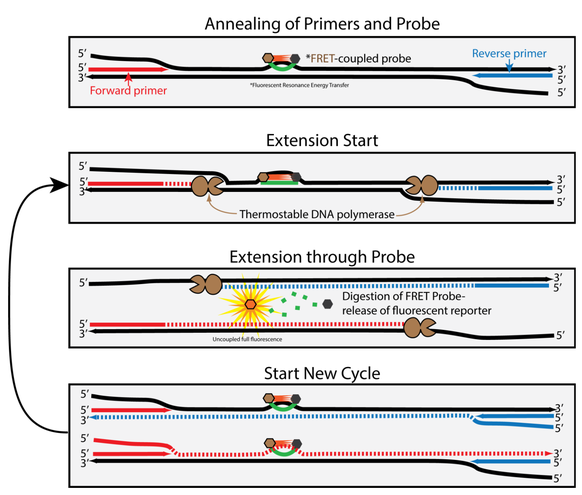


Figure 1. Schematics of Quantitative PCR (qPCR)

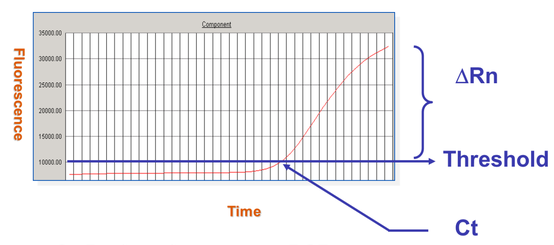


Figure 2. Profile of standard qPCR curve

The above is an idealized representation of fluorescence profile over time. ‘ΔRn’ is the fluorescence signal(normalized to an internal 5th reference dye; ‘Threshold’ is the fluorescence level at which Ct is called; and ‘Ct’ is threshold cycle, which is inversely related to the amount of target present.

ASSAY DESIGN

Target	Number of Oligos/Assay	Target Sequences in GenBank	Predicted Coverage	Top Off-Target Description	Top Off-Target Alignment
PEDV	4	602	100%	Bat coronavirus	7 mismatches
TGEV	6	62	100%	Canine coronavirus	13 mismatches
PDCoV	3	12	100%	Sparrow coronavirus	7 mismatches
Xeno	3	NA	NA	NA	NA

Table 1. Bioinformatics of design for all 3 viruses.
The assays for all 3 viruses were designed using information in GenBank to ensure inclusivity of all desired strains. In some cases this required more than 3 oligos. The closest non-targeted relatives were compared to ensure that the final assays excluded these. The column “Top Off-Target Alignment” compares the best match for one of these off-target relatives in terms of the the number of base-pairs that were still mismatched in the primers and probe sequences.

Function	Dye	Threshold^	LOD
PEDV	LIZ	2%	10
PDCoV	VIC	5%	5
TGEV	FAM	5%	5
IC	NED	10%	N/A
Reference	ROX	N/A	N/A

Table 2. Final Assay Designs
Since the Applied Biosystems real-time PCR instruments read at multiple wavelengths, four non-overlapping spectral dyes can be used in the same assay, so that 3 different targets can be assayed along with an IPC at the same time. Each assay used the fluorophore listed under ‘dye’ and all assay formulations were optimized by DOE statistical analysis.
^Threshold is set based on controls. Data is analyzed as follows: Baseline is determined from cycles 3-15; Ct <37 is classified as positive, and Ct between 37 and 40 is classified as suspect. The Limit of Detection (LOD) is given in copies of target molecule per reaction.

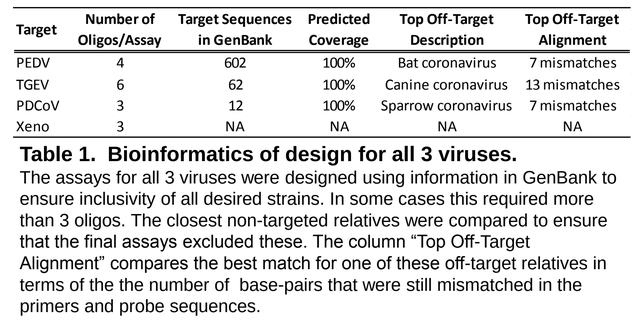


Figure 3. Amplification Performance of Assay
The above graph shows the amplification profiles for all 4 targets at 4 different input levels (shown).

ANALYSIS OF EXTRACTS

•**Analysis of Extracts.** 8 µL of each extract was added to 12 µL of a PCR mixture containing
•5 uL 4X TaqMan® Fast Virus 1-Step Master Mix. This is a one-step ReverseTranscription-qPCR reagent enabling both steps to be perfomed in one thermocycler run.
•1 uL 20X PEDV/TGEV/PDCoV single-well multiplex assay. The primer-probe mixes for all targets and IPC are combined in a single solution in one tube.
•6 uL Water
to generate a final 20 µL PCR mixture.
Along with the test samples, duplicates of each of the following controls were run:
i.Positive control (**PC**). 8 uL of the PEDV/TGEV/PDCoV RNA Control Mix (1000 copies/uL), provided with the assay, to ensure the assay is working and to set the threshold fluorescences for calling C_T’s.
ii.No template control (**NTC**). 8 uL of nuclease-free water, to ensure the assay or lab environment is not contaminated.
iii.Negative extraction control (**NEC**). Extract from a sample of the same with no target, to ensure the reagents are not contaminated and that the extraction succeeded (the IPC is still added to the initial ‘lysate’), and that no inhibitors of PCR are carried over from these samples.
Reactions were run on both the 7500Fast™ & QuantStudio™5 real-time PCR instruments. The thermal profile used was:
1.Reverse transcription: 48°C for 10 min
2.Taq activation: 95°C for 10 min
3.Amplification: 95°C for 15 sec↔ 60°C for 45 sec (40 cycles)
Instrument Run Time: 1 hour 40 minutes.

RESULTS

Detection of Multiple Targets Simultaneously

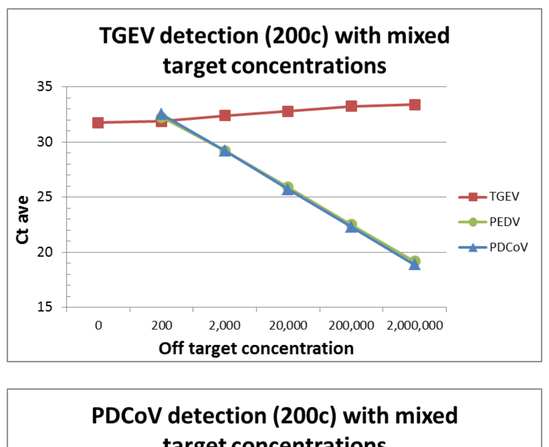


Figure 4. Assaying for Interference Between Assays.
Each target is assayed at a level of 200 copies in the presence of increasing amounts, from 200 to 2,000,000 copies, of the other 2 targets using purified nucleic acid. Each was detected in the presence of the full range of the other 2 targets, although TGEV and PDCoV showed slight increases in Ct of about 0.4 to 0.5 for every 10-fold increase in the excess off-targets.

Inclusion/Exclusion Testing of Various Field Samples

Sample ID	specimen	Thermo Fisher TGEV	Thermo Fisher PDCoV	Thermo Fisher PEDV	Re-test PDCoV	Re-test PEDV
20	environmental	neg	neg	22.9		24.0
21	environmental	neg	neg	22.6		23.9
22	environmental	neg	neg	22.5		23.8
23	environmental	neg	neg	neg		
24	environmental	neg	neg	neg		
25	environmental	neg	neg	neg		
26	fecal swab	neg	neg	17.2		21.6
27	fecal swab	neg	22.7	27.1	27.2	33.7
28	feces	neg	neg	14.2		14.6 (pool)
29	feces	neg	neg	15.4		
30	feces	neg	neg	14.7		
31	oral fluid	neg	neg	neg		
32	oral fluid	neg	neg	16.4		18.2
33	oral fluid	neg	neg	18.3		21.7
34	oral fluid	neg	neg	16.3		18.2
35	oral fluid	neg	neg	27.2		31.8
36	oral fluid	neg	neg	29.8		33.7
37	oral fluid	neg	27.3	neg	29.7	
38	oral fluid	neg	neg	neg		
39	oral fluid	neg	neg	neg		
40	oral fluid	neg	neg	neg		
41	oral fluid	neg	neg	neg		

Figure 5. Performance with Field Samples
Samples were obtained from Iowa State University. Numbers indicate the C_T for a positive result. The positive samples were also tested by the provider with their own qPCR single-target assays for the presence of viruses (none of the samples were positive for TGE). All positive Thermo Fisher assay results also showed positive with the ISU assays as well.

Virus Target	Number of Samples	Median C_t	C_t Range
Environmental Samples			
PEDV	50	16.5	12.5 – 34.0
PDCoV	12	32.3	28.1 – 34.4
Fecal Samples			
PEDV	20	18.7	11.9 – 34.6
TGEV	20	24.7	22.0 – 29.8
PDCoV	20	17.5	14.2 – 35.2
Oral Samples			
PEDV	30	30.8	17.1 – 34.9
TGEV	12	30.0	19.0 – 38.7
PDCoV	30	26.6	16.1 – 37.6

Figure 6. Performance with Isolates from Multiple Sources
Multiple samples from widely distributed regions of the United States were tested with the Thermo Fisher assay. In addition to agreement with the positive samples provided, 80 negative samples from the same sources were also verified as negative with our assay.

CONCLUSIONS

•Environmental samples are a good matrix for indication of the presence of PED/TGE/δ-Corona virus at herd level. It provides a quick, easy method to detect any viral infection in the herd and enables veterinarians and farmers to act quickly.
•We have created a reverse-transcription real-time PCR assay-based workflow which:

- Works for detection of viruses present in various types of environmental samples.
- Detects all three porcine coronaviruses in a single tube, with efficient detection of mixed infections.
- Has a Limit of Detection of ≤10 genomic copies for each target.
- Has a high sensitivity and specificity for each target.

REFERENCES ON VIRUSES

PEDV: D.M. Madson (2014). *Veterinary Microbiology* **174**: 60-68.
TGEV: X.Zhang (2007). *Virology* **358**: 424-435.
PDCoV: Q.Chen et al (2015). *Virology* **482**: 51-59.

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Porcine Field Samples
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TRADEMARKS/LICENSING

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