UNIVERSITY OF MINNESOTA

VETERINARY DIAGNOSTIC LAB

New rapid semi-quantitative RT-PCR assay developed to detect porcine epidemic diarrhea virus

In light of the porcine epidemic diarrhea virus (PEDV) outbreak in the United States, the University of Minnesota Veterinary Diagnostic Laboratory (UMVDL) realized the necessity to develop a specific, sensitive, rapid, and high throughput real-time PEDV RT-PCR to detect PEDV in swine samples. Initially, the UMVDL implemented a gel-based RT-PCR test to detect PEDV in porcine samples using a conserved region of the spike protein (S) gene (Li et al., 2012). The UMVDL sought and received Minnesota Rapid Agricultural Response Fund support to develop a rapid, semi-quantitative, high-throughput viral diagnostic test for PEDV. To design an appropriate PEDV real-time RT-PCR detection assay for use in the United States, the complete glycosylated membrane (M), unglycosylated RNA-binding nucleocapsid (N), and partial S gene segments were sequenced from 10 US PEDV positive samples using published primers (Li et al., 2013, Li et al., 2012) and subsequently, three semi-quantitative real-time RT-PCR (sqRT-PCR) TaqMan tests were designed using PrimerExpress®, one for each gene (Table 1). Complete PEDV assay details are available in the UMVDL protocol MOL.SOP.337.

To evaluate the UMVDL PEDV assays, 455 porcine samples (e.g. fecal swabs, saliva, serum, feed, and fecal, intestinal, and lung homogenates), across all pig age groups, from both clinically affected and normal pigs were tested with the partial S gel RT-PCR and M, N and S sqRT-PCRs. Of the 455 samples, 107 samples were positive for PEDV on all 4 RT-PCR assays. Any samples with conflicting results between any of the 4 assays were re-extracted and re-run on all four assays again. There were 22 samples with conflicting results; 15 were only positive on the partial S gel RT-PCR assay, 4 were positive on the N sqRT-PCR and the S gel assay, and 3 were only positive on the S sqRT-PCR. For the samples in question, the Ct values for N sqRT-PCR were above 40 while the S Ct values were above 35 (Ct cutoff for positives is <35). The 22 samples did not amplify when re-extraction and re-PCR with the 4 RT-PCR assays.

The 3 PEDV sqRT-PCRs are specific and did not amplify known porcine viral and bacterial pathogens including North American PRRSV, European PRRSV, porcine cytomegalovirus, Pseudorabies virus, influenza A viruses H1N1, H1N2, H3N2, H3N1, and H2N3, transmissible gastroenteritis virus, porcine respiratory coronavirus, porcine hemagglutinating encephalomyelitis virus, porcine encephalomyocarditis virus, porcine enterovirus, hog cholera, porcine parvovirus type I, porcine adenovirus, porcine circoviruses type I and II, porcine picornavirus, porcine rotaviruses A, B, and C, porcine lymphotrophic gamma herpes viruses I and II, porcine hokovirus, hemolytic and non-hemolytic *E. coli, Pasteurella mutlocida, Salmonella choleraesuis, S. typhimurium, Actinobacillus pleuropneumoniae, A. suis, A. rossi, A. minor, A. indolicus, A. equuli, A. pyogenes, Bordetella bronchiseptica, Clostridium perfringens type A* and *C, Brachyspira hampsonii, B. hyodysenteriae, B. pilosicoli, B. murdochii, B. intermedia, B. innocens, Streptococcus suis, Staphylococcus aureus,*

Enterococcus durans, Yersinia enterocolitica, Camplyobacter coli, C. jejuni, Mycoplasma hyorhinis, M. hyosynoviae, M. hyopneumonie, Haemophilus parasuis, and *Erysipelothris rhusiopathiae.*

To compare the Ct values obtained from the three sqRT-PCR assays, pairwise paired *t*-tests with the Bonferroni correction were conducted between the PEDV N, M, and S sqRT-PCR assays. The Ct values obtained from the S sqRT-PCR were significantly different from both the N and the M sqRT-PCR ($p<0.0001$ for both); the N and M sqRT-PCR assay Ct values were not significantly different from each other ($p=0.47$). The mean for S sqRT-PCR Ct was 19.82 while the means for N and M sqRT-PCR Ct were 22.97 and 23.39, respectively.

The UMVDL ordered an engineered gBlock® Gene Fragment from Integrated DNA Technologies (Coralville, IA) incorporating the N, M, and S sqRT-PCR primers and probes and partial S gel primers to estimate the assays' detection limits. Each of the sqRT-PCRs detects 40 RNA copies per reaction (approximately 600 copies of PEDV RNA per mL) while the S gel RT-PCR detects 400 RNA copies per reaction (approximately 6,000 copies of PEDV RNA per mL). Since the detection limit of all 3 sqRT-PCR was equivalent, the UMVDL is currently running the S sqRT-PCR as the routine PEDV screening assay, due to its generation of consistently lower Ct values. To date, over 1,220 samples have been tested by the PEDV S-gene real-time RT-PCR. Any sample with no amplification are reported as negative while samples with a Ct value < 35 is reported as positive. Samples with Ct values $\geq$ 35 are re-extracted and retested again on the S real-time RT-PCR and also via N sqRT-PCRs to verify the initial retest result. If re-extractions do not have a Ct value on the N and S sqRT-PCR assays, the sample is reported as negative. If the re-extracted sample is positive on the N sqRT-PCR but the S sqRT-PCR is negative or vice versa, the sample is reported as suspect. If the re-extraction is positive on both the N and S sqRT-PCR, the sample is reported as positive.

To reduce the cost of diagnostics for the producer, the UMVDL is validating a multiplexed UMVDL transmissible gastroenteritis virus (TGEV) sqRT-PCR assay with the new USA PEDV sqRT-PCR. Preliminary results indicated the TGEV/PEDV multiplex assay will be available within a month.

Table 1. S, N and M sqRT-PCR primers and probes

S gene sqRT-PCR	
PED_S_Forward-1910	ACG TCC CTT TAC TTT CAA TTC ACA
PED_S_Probe-1939, FAM-BHQ	TGA GTT GAT TAC TGG CAC GCC TAA ACC AC
PED_S_Reverse-2021	TAT ACT TGG TAC ACA CAT CCA GAG TCA
N gene sqRT-PCR	
PED_N_Forward-941	GAA TTC CCA AGG GCG AAA AT
PED_N_probe-963, FAM-BHQ	CGT AGC AGC TTG CTT CGG ACC CA
PED_N_Reverve-1028	TTT TCG ACA AAT TCC GCA TCT
M gene sqRT-PCR	
PED_M_Forward-393-TG	TTT GTC AAT AGC ATT CGG TTG T
PED_M_Forward-392-CA	TTT GTC AAC AGC ATT CAG TTG T
PED_M_probe-397, FAM-BHQ	GCG CAG GAC ACA TTC TTG GTG GTC T

PED_M_Reverse-458-T	CAG AAG TAG TGA GAA GCG CGT
PED_M_Reverse-458-G	CAG ACG TAG TGA GAA GCG CGT

For more information contact the University of Minnesota Veterinary Diagnostic Laboratory at vdl@umn.edu or call (612) 625 8787.

Reference:

Li, Z., Chen, F., Yuan, Y., Zeng, X., Wei, Z., Zhu, L., Sun, B., Xie, Q., Cao, Y., Xue, C., Ma, J., Bee, Y., 2013. Sequence and phylogenetic analysis of nucleocapsid genes of porcine epidemic diarrhea virus (PEDV) strains in China. Arch.Virol. 158, 1267-1273.

Li, Z.L., Zhu, L., Ma, J.Y., Zhou, Q.F., Song, Y.H., Sun, B.L., Chen, R.A., Xie, Q.M., Bee, Y.Z., 2012. Molecular characterization and phylogenetic analysis of porcine epidemic diarrhea virus (PEDV) field strains in south China. Virus Genes. 45, 181-185.