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Evaluation of two singleplex reverse transcription-Insulated isothermal PCR tests and a duplex real-time RT-PCR test for the detection of porcine epidemic diarrhea virus and porcine deltacoronavirus

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Recent outbreaks of porcine epidemic diarrhea virus (PEDV) and porcine deltacoronavirus (PDCoV) in multiple countries have caused significant economic losses and remain a serious challenge to the swine industry. Rapid diagnosis is critical for the implementation of efficient control strategies before and during PEDV and PDCoV outbreaks. Insulated isothermal PCR (iiPCR) on the portable POCKIT™ device is user friendly for on-site pathogen detection. In the present study, a singleplex PEDV RT-iiPCR, a singleplex PDCoV RT-iiPCR, and a duplex PEDV/PDCoV real-time RT-PCR (rRT-PCR) commercial reagents targeting the M gene were compared to an N gene-based PEDV rRT-PCR and an M gene-based PDCoV rRT-PCR that were previously published and used as reference PCRs. All PCR assays were highly specific and did not cross react with other porcine enteric pathogens. Analytical sensitivities of the PEDV RT-iiPCR, PDCoV RT-iiPCR and duplex PEDV/PDCoV rRT-PCR were determined using *in vitro* transcribed RNA as well as viral RNA extracted from ten-fold serial dilutions of PEDV and PDCoV cell culture isolates. Performance of each PCR assay was further evaluated using 170 clinical samples (86 fecal swabs, 24 feces, 19 intestines, and 41 oral fluids). Compared to the reference PEDV rRT-PCR, the sensitivity, specificity and accuracy of the PEDV RT-iiPCR were 97.73%, 98.78%, and 98.24%, respectively, and those of the duplex PEDV/PDCoV rRT-PCR were 98.86%, 96.34%, and 97.65%, respectively. Compared to the reference PDCoV rRT-PCR, the sensitivity, specificity and accuracy of the PDCoV RT-iiPCR were 100%, 100%, and 100%, respectively, and those of the PEDV/PDCoV duplex rRT-PCR were 96.34%, 100%, and 98.24%, respectively. Overall, all three new PCR assays were comparable to the reference rRT-PCRs for detection of PEDV and/or PDCoV. The PEDV and PDCoV RT-iiPCRs are potentially useful tools for on-site detection and the duplex PEDV/PDCoV rRT-PCR provides a convenient method to simultaneously detect the two viruses and differentiate PEDV from PDCoV.

1. Introduction

Coronaviruses (CoVs) are enveloped, single-stranded, positive-sense RNA viruses in the order Nidovirales and the family Coronaviridae. Four genera, *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*, have been described (Woo et al., 2010, 2012). In pigs, five CoVs have so far been identified and these include: porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV) in the *Alphacoronavirus* genus; porcine hemagglutinating encephalomyelitis virus (PHEV) in the *Betacoronavirus* genus; and porcine deltacoronavirus (PDCoV) in the *Deltacoronavirus* genus. PEDV, TGEV and PDCoV primarily cause enteric

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infections in pigs; PRCV has a predilection for the respiratory tract; PHEV infection produces encephalomyelitis rather than enteritis.

PEDV was first identified in England in the 1970s and has since spread to other European and Asian countries (Song and Park, 2012). In October 2010, the PEDV strains circulating in China were reported to have become more virulent than classical PEDV strains, with some causing up to 100% morbidity and high mortality in suckling piglets (Li et al., 2012; Sun et al., 2012). In April 2013, PEDV emerged in United States (U.S.) swine and caused severe illness and high mortality in piglets (Stevenson et al., 2013). Sequence analyses revealed that the U.S. virulent PEDVs (U.S. PEDV prototype strain) are most genetically similar to the Chinese virulent PEDV strain AH2012 (Chen et al., 2014; Huang et al., 2013; Stevenson et al., 2013). In January 2014, a mildly virulent PEDV variant was identified in the U.S. that had insertions and deletions in the spike gene (U.S. S-INDEL-variant strain) compared to the virulent U.S. PEDV prototype strain (Chen et al., 2016a; Vlasova et al., 2014; Wang et al., 2014b). Since its emergence in the U.S. in April 2013, PEDV has spread rapidly across the country and resulted in the estimated death of 8 million pigs in the first year, causing economic losses of $900 million to $1.8 billion (Paarlberg, 2014). In addition, PEDV has recently also emerged or re-emerged in China, Japan, South Korea, Philippines, Thailand, Vietnam, Canada, Mexico, Germany, Belgium, France and Portugal (Grasland et al., 2015; Mesquita et al., 2015; Pasick et al., 2014; Puranaveja et al., 2009; Song and Park, 2012; Stadler et al., 2015; Theuns et al., 2015; Vlasova et al., 2014; Vui et al., 2015). Currently, PEDV remains a significant threat to the global swine industry.

Porcine deltacoronavirus was first detected in pig samples collected in 2009 by a Hong Kong group (Woo et al., 2012). In the U.S., PDCoV was first reported in early 2014 (Li et al., 2014; Marthaler et al., 2014; Wang et al., 2014a) but PCR-based retrospective testing indicated that PDCoV was present in U.S. swine at least from August 2013 (Sinha et al., 2015). Pathogenicity of PDCoV has been experimentally confirmed in pigs (Chen et al., 2015; Jung et al., 2015; Ma et al., 2015). Recently PDCoV has also been reported in South Korea and China (Lee and Lee, 2014; Song et al., 2015).

Infection with PEDV, PDCoV and TGEV can lead to similar clinical symptoms including diarrhea, dehydration, variable vomiting, and high mortality especially in neonatal piglets. These clinical diseases and lesions are indistinguishable and specific laboratory diagnostic testing is imperative to differentiate PEDV, PDCoV and TGEV infection. However, prevalence of TGEV in the swine population has been very low in recent years based on data from various veterinary diagnostic laboratories (unpublished) while detection and differentiation of emerging PEDV and PDCoV have become critical. Polymerase chain reaction (PCR) is a rapid, sensitive and specific tool and is currently most commonly used for detecting PEDV and PDCoV from clinical samples. A number of real-time reverse transcription PCR (rRT-PCR) assays have been developed for the detection of PEDV (Alonso et al., 2014; Jung et al., 2014; Kim et al., 2007; Lowe et al., 2014; Opriessnig et al., 2014; Thomas et al., 2015) and PDCoV (Chen et al., 2015; Marthaler et al., 2014). A spike gene-based real-time RT-PCR has also been described to differentiate the U.S. prototype and S-INDEL-variant PEDV strains (Wang et al., 2014c). However, implementation of these rRT-PCR assays requires trained technicians and sophisticated and expensive instruments and therefore these rRT-PCR assays are not suitable for on-site applications. In recent years, a point-of-need PCR detection platform integrating the insulated isothermal PCR (iiPCR) technology and a field-deployable device (POCKIT™ Nucleic Acid Analyzer) has been developed and is now commercially available for automatic detection and interpretation of PCR results within one hour (Tsai et al., 2014, 2012b). The iiPCR or RT-iiPCR assays have been demonstrated to have excellent sensitivity and specificity for the detection of various targets, including classical swine fever virus in pigs (Lung et al., 2015) and various pathogens in shrimp, dogs, cats, and horses (Balasuriya et al., 2014; Tsai et al., 2012a, 2014; Wilkes et al., 2015a,b, 2014a).

In the present study, commercial RT-iiPCR on POCKIT™ methods (POCKIT™ PEDV Reagent Set and POCKIT™ PDCoV Reagent Set, GeneReach USA, Lexington, MA, USA) available for on-site detection of PEDV and PDCoV, respectively, were evaluated. In addition, a commercial duplex rRT-PCR test (IQ REAL PEDV/PDCoV Quantitative System, GeneReach USA) available for simultaneous detection and differentiation of PEDV and PDCoV was evaluated as well. Analytical sensitivity, specificity, and clinical performance of the three newly established assays were compared with the previously published singleplex PEDV rRT-PCR (Lowe et al., 2014; Madson et al., 2014; Thomas et al., 2015) and singleplex PDCoV rRT-PCR assays (Chen et al., 2015).

2. Materials and methods

2.1. Viruses and other enteric pathogens

Isolation and characterization of the U.S. PEDV prototype isolate USA/IN19338/2013, U.S. PEDV S-INDEL-variant isolate USA/IL20697/2014, and U.S. PDCoV isolate USA/IL/2014 were previously described (Chen et al., 2015, 2014, 2016b). For analytical sensitivity analysis, PEDV and PDCoV cell culture isolates were 10-fold serially diluted in minimum essential medium and subjected to RNA extraction and PCR testing. In order to determine the specificity of the PEDV and PDCoV RT-PCR assays, other porcine enteric pathogens were included in the study: TGEV Purdue strain (ATCC VR-763), TGEV Miller strain (ATCC VR-1740), PRCV ISU-1 strain, PHEV Mengling strain (NVSL 001-PDV), porcine rotaviruses (groups A–C), *Escherichia coli*, *Salmonella typhimurium*, *Clostridium difficile*, *Clostridium perfringens*, *Brachyspira hyodysenteriae* (B204), and *Lawsonia intracellularis*.

2.2. Clinical samples

A total of 170 clinical samples (86 fecal swabs, 24 feces, 19 small intestines and 41 oral fluids), submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) and tested for PEDV and PDCoV by virus-specific RT-PCRs, were selected to determine the performance of various PEDV and PDCoV PCRs evaluated in this study.

2.3. Nucleic acid extraction

Nucleic acids were extracted from virus isolates and their dilutions (50 μl), bacterial isolates (50 μl), small intestine homogenates (50 μl), oral fluids (100 μl), processed feces or fecal swabs (100 μl) using a MagMAX™ Pathogen RNA/DNA Kit (Thermo Fisher Scientific, Waltham, MA, USA) and a Kingfisher-96 instrument (Thermo Fisher Scientific) following the instructions of the manufacturer. Nucleic acids were eluted into 90 μl of Elution buffer.

Nucleic acids were also extracted from clinical samples (fecal swab, feces, small intestine, and oral fluid) using taco™ mini Nucleic Acid Automatic Extraction System (taco™ mini, GeneReach USA). Briefly, 200 μl of the samples were added into the first well of a taco™ Preloaded DNA/RNA Extraction plate (GeneReach USA) and subjected to the extraction steps as described in the manufacturer's user manual. Nucleic acids were eluted into 200 μl of Elution buffer.

2.4. In vitro *transcribed (IVT) RNA*

To prepare the PEDV and PDCoV RNA standards, plasmids artificially synthesized to contain a fragment of the membrane (M) gene

of PEDV USA/IN19338/2013 strain (nucleotides [nt] 25978–26066; GenBank accession no. KF650371) (Chen et al., 2014) or a fragment of the M gene of PDCoV USA/IA/2014/8734 (nt 23356–23461; GenBank accession no. KJ567050) (Li et al., 2014) were linearized and subjected to *in vitro* transcription (IVT) using the MEGAscript T7 Kit (Thermo Fisher Scientific). RNA transcripts were produced, treated with DNase I, and purified by LiCl precipitation following the manufacturer's instructions. Copy numbers of RNA transcripts were calculated based on concentrations determined by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Serial dilutions of RNA were prepared in 40 ng/μl yeast tRNA. Aliquots were frozen at −80 °C for single use of each aliquot.

2.5. PEDV and PDCoV reference real-time RT-PCRs

A singleplex PEDV nucleocapsid (N) gene-based rRT-PCR (Lowe et al., 2014; Madson et al., 2014; Thomas et al., 2015) and a singleplex PDCoV M gene-based rRT-PCR (Chen et al., 2015) previously developed and routinely used at the ISU VDL for diagnostic testing were included in this study as the reference PCRs for the detection of PEDV and PDCoV. Each PCR was set up in a 20 μl total reaction using TaqMan® Fast 1-Step Master Mix (Thermo Fisher Scientific): 5 μl of 4× Master Mix, 0.4 μl of forward primer at 20 μM, 0.4 μl of reverse primer at 20 μM, 0.24 μl of probe at 10 μM, 1 μl XENO Internal Control Reagent (Thermo Fisher Scientific), 7.96 μl nuclease-free water, and 5 μl nucleic acid extract. Amplification reactions were performed on an ABI 7500 Fast instrument (Thermo Fisher Scientific) with the following conditions: 1 cycle of 50 °C for 5 min, 1 cycle of 95 °C for 20 s, and 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Any cycle threshold (Ct) values <40 were reported as positive.

2.6. PEDV RT-iiPCR and PDCoV RT-iiPCR

The RT-iiPCR methods for PEDV (POCKIT™ PEDV Reagent Set, GeneReach, USA) and PDCoV (POCKIT™ PDCoV Reagent Set, GeneReach, USA) were designed according to the hydrolysis probe-based POCKIT™ method described previously (Tsai et al., 2012b). As shown in Table 1, the primers and probe for PEDV were designed to target a highly conserved region in M gene among PEDV strains; the PDCoV RT-iiPCR primers and probe also targeted a highly conserved region in M gene among PDCoV strains. Both PEDV and PDCoV RT-iiPCR probes were labeled with FAM fluorescent reporter dye at the 5′ end. The PEDV probe was labeled with an internal ZEN quencher and Iowa Black FQ (IWBkFQ) quencher at the 3′ end. The PDCoV probe was labeled with a minor groove binder group (MGB) with a non-fluorescent quencher (NFQ) at the 3′ end. After adding 5 μl of the nucleic acid sample, the final Premix/sample mixture was transferred to an R-tube™ (GeneReach USA). The tube was spun briefly in a Cubee™ mini centrifuge (GeneReach USA) and placed into the reaction chamber of POCKIT™ Nucleic Acid Analyzer. The default program of the POCKIT™ device includes an RT step at 50 °C for 10 min and an iiPCR step at 95 °C for about 30 min. The reaction completed in less than one hour. POCKIT™ device collects optical signals through an integrated circuits controlled-regulated sensor. Signal-to-noise (S/N) ratios were calculated by dividing light signals collected after iiPCR by those from before iiPCR (Tsai et al., 2012b). Based on the default thresholds, S/N ratios of <1.2 and >1.3 were assigned as "+" and "−", respectively. A "?" result was assigned to those with an S/N ratio between 1.2 and 1.3, indicating that the signals were ambiguous and the sample should be tested again.

2.7. Duplex PEDV/PDCoV real-time RT-PCR

Primers and probes of the duplex rRT-PCR for PEDV and PDCoV (IQ REAL PEDV/PDCoV Quantitative System, GeneReach USA) were designed to target two different highly conserved regions in the M gene among PEDV strains from all known clades and among all known PDCoV strains, respectively (Table 1). An internal control (IC) containing an artificial nucleotide sequence was also included in this multiplex rRT-PCR. The expected size of the IC amplicon was 100 bp. The PEDV, PDCoV, and IC amplicons were individually detected by a FAM-, NED-, and Cy5-labeled TaqMan probe, respectively. The rRT-PCR amplification was carried out in 25 μl reaction mixtures containing 20 μl of master mixture, 2.5 μl of 200 nM ROX and 2.5 μl of the extracted nucleic acids. The duplex PEDV/PDCoV rRT-PCR was performed on an ABI 7500 Fast instrument (Thermo Fisher Scientific). The conditions were set as follows: 30 min at 42 °C and 15 min at 93 °C, followed by 45 cycles of denaturation at 93 °C for 15 s and annealing-elongation at 60 °C for 1 min. Fluorescence signals were captured at the 60 °C step. The threshold for the Ct analysis was manually adjusted to 0.05, together with an automatic baseline. The Ct of the internal control was designed to be about 30 under these conditions. In addition to clinical samples, the serial dilutions of PEDV IVT RNA, PDCoV IVT RNA, PEDV isolates and PDCoV isolates were also tested by the duplex PEDV/PDCoV rRT-PCR. The linear correlation coefficient (R^2) and slope of the curve were determined automatically with the 7500 Real-Time PCR System software. The slope was used to calculate amplification efficiencies ($E = 10^{1/-\text{slope}} - 1$) for each primer/pair set. Based on statistical analysis of analytical sensitivity with IVT RNA of known copies, cutoff Ct of the duplex PEDV/PDCoV rRT-PCR for PEDV (FAM) and PDCoV (NED) detection was both set at 40 to achieve >50% detection at 10 copies per reaction (Caraguel et al., 2011).

2.8. Statistical analyses

For limit of detection 95% ($LoD_{95\%}$) calculation, probit regression analysis was performed to calculate the concentration of the template that could be measured with 95% probability using SPSS Statistics 14.0 (SPSS Inc., Chicago, IL). Kappa's tests were performed to determine the agreement between different RT-PCR assays and between different extraction methods.

3. Results

3.1. Analytical specificity of PEDV RT-iiPCR, PDCoV RT-iiPCR, and duplex PEDV/PDCoV rRT-PCR assays

As shown in Table 2, the PEDV RT-iiPCR and duplex PEDV/PDCoV rRT-PCR (reporter dye for PEDV detection) as well as the reference PEDV rRT-PCR only specifically reacted with PEDV and did not cross-react with other enteric pathogens including PDCoV, TGEV Purdue strain, TGEV Miller strain, PHEV, porcine rotaviruses (Groups A, B and C), PRCV, *E coli*, *S. typhimurium*, *C. difficile*, *C. perfringens*, *B. hyodysenteriae*, and *L. intracellularis*. Similarly, the PDCoV RT-iiPCR and duplex PEDV/PDCoV rRT-PCR (reporter dye for PDCoV detection) as well as the reference PDCoV rRT-PCR only specifically reacted with PDCoV and did not cross-react with other enteric pathogens (Table 2).

3.2. Analytical sensitivity of PEDV RT-iiPCR and duplex PEDV/PDCoV rRT-PCR assays for PEDV detection

The analytical sensitivities of the PEDV RT-iiPCR and duplex PEDV/PDCoV rRT-PCR (FAM-labeled TaqMan probe) for PEDV detection were determined by testing 10-fold serial dilutions of an IVT RNA containing partial PEDV M gene sequence. For the PEDV RT-iiPCR, testing various replicates of 100, 50, 20, 5 and 0 copies of standard RNA per reaction revealed that 10/10 (100%), 20/20 (100%), 18/20 (90%), 9/20 (45%), and 0/24 (0%) produced positive results on these RNA copies, respectively. The $LoD_{95\%}$ of the PEDV

Table 1
Primers and probes of various PEDV and PDCoV RT-PCRs evaluated in this study.

Assay name	Primer & Probe	Nucleotide sequence (5′–3′)[a]	Nucleotide Position	Target gene	Amplicon	Reference
PEDV RT-iiPCR	PEDV iiF	AATAGCATTCGGTTGTGGCG	25978–25997	M	89 bp	This paper
	PEDV iiR	CGGCCCATCACAGAAGTAGT	26066–26047			
	PEDV iiP	FAM-CATTCTTGG/ZEN/TGGTCTTTCAATCCTGA-IABkFQ	26005–26030			
PDCoV RT-iiPCR	PDCoV iiF	GAGAGTAGACTCCTTGCAGGGATTAT	23356–23381	M	106 bp	This paper
	PDCoV iiR	GCTTGCCATGCTTAACGACTG	23461–23441			
	PDCoV iiP	FAM-AATGCACCTCCATGTACC-MGB	23409–23392			
PEDV/PDCoV duplex real-time RT-PCR[a]	PEDV rF	GGTTGTGGCGCAGGACA	25988–26004	M	79 bp	This paper
	PEDV rR	CGGCCCATCACAGAAGTAGT	26066–26047			
	PEDV rP	FAM-CATTCTTGG/ZEN/TGGTCTTTCAATCCTGA-IABkFQ	26005–26030			
	PDCoV rF	TGAGAGTAGACTCCTTGCAGGGA	23355–23377	M	105 bp	
	PDCoV rR	GAGAATTGGAGCCATGTGGT	23436–23417			
	PDCoV rP	NED-TGTACCCATTGGATCCATAA-MGB	23397–23378			
PEDV real-time RT-PCR	PEDV-F	CGCAAAGACTGAACCCACTAACCT	26684–26707	N	198 bp	Lowe et al. (2014)
	PEDV-R	TTGCCTCTGTTGTTACTTGGAGAT	26881–26858			
	PEDV-P	FAM-TGTTGCCAT/ZEN/TACCACGACTCCTGC-IABkFQ	26847–26824			
PDCoV real-time RT-PCR	PDCoV-F	CGACCACATGGCTCCAATTC	23415–23434	M	70 bp	Chen et al. (2015)
	PDCoV-R	CAGCTCTTGCCCATGTAGCTT	23484–23464			
	PDCoV-P	FAM-CACACCAGT/ZEN/CGTTAAGCATGGCAAGC-IABkFQ	23436–23461			

[a] Nucleotide positions of PEDV and PDCoV primers and probes are based on GenBank accession no. KF650371 and KJ567050, respectively.

RT-iiPCR was estimated to be 21 RNA copies/reaction by probit regression analysis. For the duplex PEDV/PDCoV rRT-PCR, each dilution of the PEDV IVT RNA (10^6, 10^5, 10^4, 10^3, 10^2, 50, 20, 10, 5 and 0 copies) was run in two to eight replicates. The standard curve had an $r^2 = 0.99$ and a slope of −3.18 in the range of 10^6 to 10 copies of the PEDV IVT RNA. The $LoD_{95\%}$ of the duplex PEDV/PDCoV rRT-PCR for PEDV detection was calculated to be about 7 RNA copies/reaction by probit analysis.

The analytical sensitivities of the PEDV RT-iiPCR and duplex PEDV/PDCoV rRT-PCR assays for PEDV detection were also evaluated by testing RNA extracts from 10-fold serial dilutions (triplicate for each dilution) of PEDV cell culture isolates (U.S. PEDV prototype isolate and S-INDEL-variant isolate) and compared to that of an N gene-based singleplex PEDV rRT-PCR reference assay. The 100% detection endpoints of the PEDV RT-iiPCR and duplex PEDV/PDCoV rRT-PCR to detect U.S PEDV prototype isolate and S-INDEL-variant isolate were both at 10^{-5} dilutions (Table 3). In contrast, the 100% detection endpoints of the reference singleplex PEDV rRT-PCR were 10^{-7} dilution for the U.S. PEDV prototype isolate and 10^{-6} dilution for PEDV S-INDEL-variant isolate (Table 3), indicating that the PEDV RT-iiPCR and duplex PEDV/PDCoV rRT-PCR were about 10–100 fold less sensitive than the reference singleplex PEDV rRT-PCR in detecting PEDV RNA.

3.3. *Analytical sensitivity of PDCoV RT-iiPCR and duplex PEDV/PDCoV rRT-PCR assays for PDCoV detection*

The analytical sensitivities of the PDCoV RT-iiPCR and duplex PEDV/PDCoV rRT-PCR (NED-labeled TaqMan probe) for PDCoV detection were determined first by testing 10-fold serial dilutions of an IVT RNA containing the target PDCoV M gene sequence. The testing results of the PDCoV RT-iiPCR were 10/10 (100%), 24/24 (100%), 46/46 (100%), 13/16 (81%), 41/46 (89%) and 0/29 (0%) positive on reactions containing 100, 50, 20, 10, 5 and 0 PDCoV IVT RNA copies per reaction, respectively. The $LoD_{95\%}$ of the PDCoV RT-iiPCR was about 9 RNA copies/reaction by probit regression analysis. For the

Table 2
Specificity of various PEDV and PDCoV RT-PCRs evaluated in this study. (For interpretation of the references to color in this table legend, the reader is referred to the web version of this article.)

Pathogen	PEDV						PDCoV					
	RT-iiPCR		duplex rRT-PCR (Ct)		Reference rRT-PCR (Ct)		RT-iiPCR		duplex rRT-PCR (Ct)		Reference rRT-PCR (Ct)	
	Result	S/N[‡]	Target	IC[‡]	Target	IC[‡]	Result	S/N[‡]	Target	IC[‡]	Target	IC[‡]
Porcine epidemic diarrhea virus	+	4.79	17.8	30.3	12.9	34.1	-	0.94	Neg	30.3	Neg	34.6
Porcine deltacoronavirus	-	1.08	Neg	31.2	Neg	34.4	+	2.32	20.7	31.2	16.6	35.3
Transmissible gastroenteritis virus (Purdue strain)	-	1.04	Neg	30.2	Neg	34.7	-	0.99	Neg	30.2	Neg	35.1
Transmissible gastroenteritis virus (Miller strain)	-	1.02	Neg	29.8	Neg	34.4	-	0.98	Neg	29.8	Neg	35.3
Porcine hemagglutinating encephalomyelitis virus	-	1.08	Neg	30.1	Neg	34.2	-	1.00	Neg	30.1	Neg	34.8
Porcine rotaviruses*	-	1.02	Neg	30.2	Neg	34.8	-	0.97	Neg	30.2	Neg	35.2
Porcine respiratory coronavirus	-	1.02	Neg	30.8	Neg	34.1	-	0.99	Neg	30.8	Neg	35.3
Escherichia coli	-	1.06	Neg	30.4	Neg	34.4	-	0.98	Neg	30.4	Neg	35.0
Salmonella typhimurium	-	1.15	Neg	30.3	Neg	34.4	-	0.95	Neg	30.3	Neg	35.2
Clostridium difficile	-	1.05	Neg	30.5	Neg	34.9	-	0.98	Neg	30.5	Neg	35.0
Clostridium perfringens	-	1.01	Neg	30.1	Neg	34.5	-	0.95	Neg	30.1	Neg	35.1
Brachyspira hyodysenteriae (B204)	-	1.06	Neg	30.4	Neg	34.6	-	1.00	Neg	30.4	Neg	34.3
Lawsonia intracellularis	-	1.00	Neg	30.6	Neg	33.4	-	0.97	Neg	30.6	Neg	34.4
Phosphate buffered saline (PBS)	-	1.04	Neg	30.9	Neg	34.0	-	0.99	Neg	30.9	Neg	35.3

*Porcine rotaviruses include groups A–C porcine rotaviruses.
[‡]S/N: Signal-to-noise ratio.
[‡]IC: Internal Control.
Nucleic acids were extracted from all samples using the MagMAX™ Pathogen RNA/DNA Kit and Kingfisher-96 instrument from Thermo Fisher Scientific.

Table 3
Analytical sensitivity of PEDV RT-iiPCR and duplex PEDV/PDCoV real-time RT-PCR for detection of PEDV RNA and comparison with the reference singleplex PEDV real-time RT-PCR using viral RNA from the serially diluted PEDV cell culture isolates. (For interpretation of the references to color in this table legend, the reader is referred to the web version of this article.)

Virus strain	Dilution	Theoretical titer ($TCID_{50}$/ml)	PEDV RT-iiPCR						Duplex PEDV/PDCoV rRT-PCR (Ct) – FAM dye			Reference singleplex PEDV rRT-PCR (Ct)		
			Result 1	S/N	Result 2	S/N	Result 3	S/N	Result 1	Result 2	Result 3	Result 1	Result 2	Result 3
US PEDV Prototype strain	10^{-3}	2000	+	4.94	+	4.9	+	4.9	24.6	26.4	25.3	22.3	22.2	22.3
	10^{-4}	200	+	4.81	+	4.84	+	4.91	29	29.5	28.9	26.2	26.3	26.2
	10^{-5}	20	+	3.69	+	4.51	+	3.16	32.5	32.3	32.1	29.2	29.3	29.2
	10^{-6}	2.0	-	0.98	-	1.09	-	1.08	Neg	37	34.6	33.2	33.1	33.1
	10^{-7}	0.2	-	1.01	-	1.02	-	0.97	37	Neg	Neg	36.3	37.4	35.9
	10^{-8}	0.02	-	1.01	-	0.98	-	0.96	Neg	Neg	Neg	Neg	Neg	Neg
	10^{-9}	0.002	-	1.06	-	1.03	-	1.01	Neg	Neg	Neg	Neg	Neg	Neg
US PEDV S-INDEL-variant strain	10^{-3}	100	+	4.93	+	4.95	+	4.87	27.3	27.2	27.3	24.2	24.3	24.3
	10^{-4}	10	+	4.54	+	4.97	+	4.82	31.2	30.7	31.4	28.1	28.2	28.1
	10^{-5}	1	+	4.44	+	4.74	+	4.48	34.1	34.1	34.7	31.3	31.5	31.4
	10^{-6}	0.1	-	1.01	-	1.08	-	1.03	36.6	Neg	Neg	37.5	35.4	36.4
	10^{-7}	0.01	-	0.99	-	0.99	-	0.97	Neg	Neg	Neg	37.3	Neg	38.6
	10^{-8}	0.001	-	1.04	-	1	-	1.01	Neg	Neg	Neg	Neg	Neg	37.1
	10^{-9}	0.0001	-	0.98	-	0.99	-	0.98	Neg	Neg	Neg	Neg	Neg	Neg

Note: Nucleic acids were extracted from all samples using the MagMAX™ Pathogen RNA/DNA Kit and Kingfisher-96 instrument from Thermo Fisher Scientific.

duplex PEDV/PDCoV rRT-PCR, each dilution of the PDCoV IVT RNA (10^6, 10^5, 10^4, 10^3, 10^2, 50, 20, 10, 5 and 0 copies) was run in two to eight replicates. The standard curve had an $r^2 = 0.99$ and a slope of −3.13 in the range of 10^6 to 20 copies of the PDCoV IVT RNA. Accordingly, probit analysis indicated that $LoD_{95\%}$ of the duplex rRT-PCR for PDCoV was about 14 copies per reaction.

Analytical sensitivities of the PDCoV RT-iiPCR and duplex PEDV/PDCoV rRT-PCR were also determined by testing RNA extracts from 10-fold serial dilutions (triplicate for each dilution) of PDCoV cell culture isolate and compared to that of an M gene-based singleplex PDCoV rRT-PCR reference assay. The 100% endpoints to detect PDCoV were at 10^{-5} dilution for both PDCoV RT-iiPCR and singleplex reference PDCoV rRT-PCR, and at 10^{-4} dilution for the duplex PEDV/PDCoV rRT-PCR (Table 4), indicating that the duplex PEDV/PDCoV rRT-PCR was 10-fold less sensitive than the PDCoV RT-iiPCR and reference PDCoV rRT-PCR for PDCoV RNA detection.

3.4. Performances of PEDV RT-iiPCR, PDCoV RT-iiPCR and duplex PEDV/PDCoV rRT-PCR in detecting PEDV and PDCoV in clinical samples

To evaluate the performances of the PEDV RT-iiPCR, PDCoV RT-iiPCR and duplex PEDV/PDCoV rRT-PCR assays in detecting PEDV and PDCoV in clinical samples, previously published singleplex PEDV rRT-PCR and PDCoV rRT-PCR were used as the reference assays in a side-by-side comparison study testing a panel of 170 archived clinical specimens, including 86 fecal swabs, 24 feces, 19 intestines, and 41 oral fluid samples. Distributions of 170 clinical samples based on specimen types and Ct ranges are summarized in Table 5. Among 170 samples, 88 samples were positive by the reference PEDV rRT-PCR with Ct ranges of 12.2-35.7; 82 samples were positive by the reference PDCoV rRT-PCR with Ct ranges of 15–37.1; 16 samples were positive for both PEDV and PDCoV.

Comparisons of the PEDV RT-iiPCR and the reference PEDV rRT-PCR on testing 170 clinical samples for PEDV detection are summarized in Table 6a. Kappa analysis of the 2 × 2 contingency table showed that the sensitivity of the PEDV RT-iiPCR was 97.73% (CI 95%: 93.50–100%) and specificity was 98.78% (CI 95%: 94.86–100%) when compared to the reference PEDV rRT-PCR. Overall agreement between the reference PEDV rRT-PCR and the PEDV RT-iiPCR was 98.24% (CI 95%: 95.74–100%) with a kappa value of 0.96. As shown in Table 6b, when compared to the reference PEDV rRT-PCR, the sensitivity, specificity and accuracy of the duplex PEDV/PDCoV rRT-PCR for PEDV detection was 98.86% (CI 95%: 95.20–100%), 96.34% (CI 95%: 91.31–100%), and 97.65% (CI 95%: 94.92–100%; kappa = 0.95), respectively.

As shown in Table 6c, 100% agreement (CI 95%: 98.90–100%; kappa = 1) was found between the PDCoV RT-iiPCR and the reference PDCoV rRT-PCR with the sensitivity of 100% (CI 95%: 96.80–100%) and specificity of 100% (CI 95%: 97.01–100%) for the

Table 4
Analytical sensitivity of PDCoV RT-iiPCR and duplex PEDV/PDCoV real-time RT-PCR for detection of PDCoV RNA and comparison with the reference singleplex PDCoV real-time RT-PCR using viral RNA from the serially diluted PDCoV cell culture isolate. (For interpretation of the references to color in this table legend, the reader is referred to the web version of this article.)

Dilution	Theoretical titer ($TCID_{50}$/ml)	PDCoV RT-iiPCR						Duplex PEDV/PDCoV rRT-PCR (Ct) - NED dye			Reference singleplex PDCoV rRT-PCR (Ct)		
		Result 1	S/N	Result 2	S/N	Result 3	S/N	Result 1	Result 2	Result 3	Result 1	Result 2	Result 3
10^{-3}	2000	+	2.6	+	2.84	+	2.42	32.3	32.1	32.4	27.6	27.4	27.6
10^{-4}	200	+	2.69	+	2.65	+	2.23	36.3	36.1	35.7	30.7	30.8	30.6
10^{-5}	20	+	2.54	+	2.5	+	2.68	Neg	Neg	38.5	34	35.3	35.1
10^{-6}	2	-	1.01	+	2.75	-	0.97	Neg	Neg	Neg	Neg	Neg	Neg
10^{-7}	0.2	-	0.96	-	1	-	0.95	Neg	Neg	Neg	Neg	Neg	Neg
10^{-8}	0.02	-	1.02	-	1.01	-	1.01	Neg	Neg	Neg	Neg	Neg	Neg
10^{-9}	0.002	-	0.98	-	1.01	-	1.01	Neg	Neg	Neg	Neg	Neg	Neg

Note: Nucleic acids were extracted from all samples using the MagMAX™ Pathogen RNA/DNA Kit and Kingfisher-96 instrument from Thermo Fisher Scientific.

Table 5
Specimen types of 170 clinical samples and Ct ranges of positive samples tested by the reference PEDV rRT-PCR and PDCoV rRT-PCR.

Specimen type	Number	Positive by the reference PEDV rRT-PCR		Positive by the reference PDCoV rRT-PCR		Positive by both the reference PEDV and PDCoV rRT-PCRs
		Number	Ct ranges	Number	Ct ranges	Number
Fecal swab	86	47	14.1–30.6	37	16.7–35.6	5
Feces	24	9	12.2–30.8	17	15.0–28.3	2
Intestine	19	10	16.2–34.1	9	17.2–31.7	0
Oral fluid	41	22	14.6–35.7	19	20.3–37.1	9

Note: Nucleic acids were extracted from all samples using the MagMAX™ Pathogen RNA/DNA Kit and Kingfisher-96 instrument from Thermo Fisher Scientific.

PDCoV RT-iiPCR when compared to the reference PDCoV rRT-PCR. The sensitivity, specificity and accuracy of the duplex PEDV/PDCoV for PDCoV detection was 96.34% (CI 95%: 91.31–100%), 100% (CI 95%: 97.01–100%), and 98.24% (CI 95%: 95.74–100%; kappa = 0.96), respectively, when compared to the reference PDCoV rRT-PCR (Table 6d).

Discrepant results were observed on three samples between the PEDV RT-iiPCR and the reference PEDV rRT-PCR (Table 6a), four samples between the duplex PEDV/PDCoV rRT-PCR and the reference PEDV rRT-PCR (Table 6b), and three samples between the duplex PEDV/PDCoV rRT-PCR and the reference PDCoV rRT-PCR (Table 6d). These discrepancies are summarized in Table 7.

3.5. *Performance comparisons between taco™ mini extraction/POCKIT™ PCR system and MagMAX™ extraction/real-time PCR system in detecting PEDV and PDCoV in clinical samples*

To evaluate the performance of the portable system combining the taco™ mini extraction and POCKIT™ amplification/detection method for PEDV and PDCoV detection in clinical samples, the taco™ mini extraction/PEDV RT-iiPCR system was compared to the MagMAX™ extraction/reference singleplex PEDV rRT-PCR system (Table 8a) and the taco™ mini extraction/PDCoV RT-iiPCR system was compared to the MagMAX™ extraction/reference singleplex PDCoV rRT-PCR system (Table 8b), based on testing 170 clinical samples.

Five of the 88 positive samples identified by the MagMAX™ extraction/reference PEDV rRT-PCR system (#43Ct = 34.1; #45Ct = 32.6; #63Ct = 30.8; #75Ct = 35.1; and #146Ct = 35.7) were negative by the taco™ mini extraction/PEDV RT-iiPCR system (Table 8a). All 82 samples negative by MagMAX™ extraction/reference PEDV rRT-PCR system were also negative by the taco™ mini extraction/PEDV RT-iiPCR system. Total agreement between the two systems was 97.06% (CI 95%: 94.12–100%; kappa = 0.94).

When the 170 samples were tested for PDCoV, the MagMAX™ extraction/reference PDCoV rRT-PCR system and the taco™ mini extraction/PDCoV RT-iiPCR systems yielded the same results (Table 8b) with 100% agreement (CI 95%: 98.43–100%; kappa = 1) between the two systems.

4. Discussion

Compared to the epidemic phase (April 2013–April 2014) of PEDV in the U.S., the incidence of PEDV has now subsided, but there are still 34 U.S. states positive for PEDV, 14 states positive for PDCoV, and 14 states with premises positive for both PEDV and PDCoV, according to the USDA report on October 22, 2015 (www.aasv.org). PEDV and PDCoV have also emerged or re-emerged in other countries. Rapid diagnosis is critical for the implementation of efficient control strategies before and during PEDV and PDCoV outbreaks.

Table 6
Sensitivity and specificity of PEDV RT-iiPCR, PDCoV RT-iiPCR, and duplex PEDV/PDCoV rRT-PCR as compared to the reference singleplex PEDV rRT-PCR and PDCoV rRT-PCR based on testing 170 clinical samples.

		Reference PEDV rRT-PCR or reference PDCoV rRT-PCR		
		Positive	Negative	Total
(a) PEDV RT-iiPCR compared to the reference singleplex PEDV rRT-PCR				
PEDV RT-iiPCR	Positive	86	1	87
	Negative	2	81	83
	Total	88	82	170
Sensitivity: 97.73%; Specificity: 98.78%; Accuracy: 98.24%				
(b) Duplex PEDV/PDCoV rRT-PCR compared to the reference singleplex PEDV rRT-PCR				
Duplex PEDV/PDCoV rRT-PCR	Positive	87	3	90
	Negative	1	79	80
	Total	88	82	170
Sensitivity: 98.86%; Specificity: 96.34%; Accuracy: 97.65%				
(c) PDCoV RT-iiPCR compared to the reference singleplex PDCoV rRT-PCR				
PDCoV RT-iiPCR	Positive	82	0	82
	Negative	0	88	88
	Total	82	88	170
Sensitivity: 100%; Specificity: 100%; Accuracy: 100%				
(d) Duplex PEDV/PDCoV rRT-PCR compared to the reference singleplex PDCoV rRT-PCR				
Duplex PEDV/PDCoV rRT-PCR	Positive	79	0	79
	Negative	3	88	91
	Total	82	88	170
Sensitivity: 96.34%; Specificity: 100%; Accuracy: 98.24%				

Notes: (1) Nucleic acids were extracted from all samples using the MagMAXTM Pathogen RNA/DNA Kit and Kingfisher-96 instrument from Thermo Fisher Scientific. (2) The reference PEDV rRT-PCR and PDCoV rRT-PCR: Ct < 40 positive. (3) The duplex PEDV/PDCoV rRT-PCR: Ct < 40 positive; Ct ≥ 40 negative.

Table 7
Discrepancies on clinical samples by various PEDV and PDCoV RT-PCRs. (For interpretation of the references to color in this table legend, the reader is referred to the web version of this article.)

Sample ID	Specimen type	PEDV RT-iiPCR		duplex PEDV/PDCoV rRT-PCR (for PEDV)		Reference PEDV rRT-PCR		PDCoV RT-iiPCR		duplex PEDV/PDCoV rRT-PCR (for PDCoV)		Reference PDCoV rRT-PCR	
		Result	S/N	Result	Ct	Result	Ct	Result	S/N	Result	Ct	Result	Ct
#25	Fecal swab	(+)	3.69	(+)	30.7	(-)	Neg	(-)	1.04	(-)	Neg	(-)	Neg
#46	Oral fluid	(-)	1.18	(+)	34.5	(+)	35.6	(-)	0.95	(-)	Neg	(-)	Neg
#47	Oral fluid	(-)	1.01	(+)	36.1	(-)	Neg	(-)	0.99	(-)	Neg	(-)	Neg
#75	Oral fluid	(-)	1.01	(+)	33.5	(+)	35.1	(-)	0.94	(-)	Neg	(-)	Neg
#76	Fecal swab	(-)	0.93	(-)	Neg	(-)	Neg	(+)	2.47	(-)	40.9	(+)	35.6
#77	Fecal swab	(-)	1.11	(-)	Neg	(-)	Neg	(+)	2.63	(-)	40.9	(+)	34.3
#94	Fecal swab	(-)	1.04	(+)	39.7	(-)	Neg	(+)	2.99	(+)	22.9	(+)	18.1
#109	Oral fluid	(-)	1.05	(-)	Neg	(-)	Neg	(+)	2.65	(-)	Neg	(+)	37.1
#146	Oral fluid	(+)	3.47	(-)	Neg	(+)	35.7	(+)	2.96	(+)	26.3	(+)	22

Note: (1) Nucleic acids were extracted from all samples using the MagMAX™ Pathogen RNA/DNA Kit and Kingfisher-96 instrument from Thermo Fisher Scientific. (2) Samples with discrepancy results by PEDV RT-iiPCR and the reference PEDV rRT-PCR or by duplex PEDV/PDCoV rRT-PCR and the reference PEDV rRT-PCR are highlighted in light blue color. (3) Samples with discrepancy results by duplex PEDV/PDCoV rRT-PCR and the reference PDCoV rRT-PCR are highlighted in light brown color.

Recent technology of the iiPCR on the portable POCKIT™ device provides a simple, convenient and inexpensive method for on-site detection of pathogens in clinical samples (Tsai et al., 2012b). For example, iiPCR methods have been used in small animal clinics and shelters (*e.g.* canine distemper virus and canine influenza virus), on livestock farms (*e.g.* porcine circovirus type 2), on shrimp farms (*e.g.* white spot syndrome virus and *Enterocytozoon hepatopenaei*), and by government agencies (*e.g.* classical swine fever virus and *Vibrio parahaemolyticus*-acute hepatopancreatic necrosis disease) for surveillance and diagnostic purposes in various countries (Lung et al., 2015; Tsai et al., 2014; Wilkes et al., 2014b). In the current study, we developed and evaluated PEDV RT-iiPCR and PDCoV RT-iiPCR assays which were highly specific and did not cross react with other enteric pathogens. The PEDV RT-iiPCR and PDCoV RT-iiPCR had high analytical sensitivity with $LoD_{95\%}$ of 21 copies of PEDV RNA molecules per reaction and 9 copies of PDCoV RNA molecules per reaction, respectively. Analysis of viral RNA extracted from 10-fold serial dilutions of PEDV cell culture isolates (a U.S. PEDV prototype strain and a U.S. PEDV INDEL-variant strain) showed that the PEDV RT-iiPCR was 10–100 fold less sensitive for endpoint detection compared to the reference PEDV rRT-PCR (Lowe et al., 2014; Madson et al., 2014; Thomas et al., 2015). However, it is noteworthy that the reference PEDV rRT-PCR was run 40 cycles in this study and all Ct values <40 were reported as positive without an established cutoff Ct. The endpoint dilutions negative by the PEDV RT-iiPCR but positive by the reference PEDV rRT-PCR had very high Ct values (35.4–37.5). When testing 10-fold serial dilutions of a PDCoV cell culture isolate, the PDCoV RT-iiPCR had equal sensitivity for endpoint detection to the reference PDCoV rRT-PCR (Chen et al., 2015). Overall, the PEDV RT-iiPCR and PDCoV RT-iiPCR had analytical sensitivities comparable to the reference PEDV rRT-PCR and PDCoV rRT-PCR.

For evaluation of performances on clinical samples, nucleic acids were first extracted from 170 clinical samples using the MagMAX™ Pathogen RNA/DNA Kit and Kingfisher-96 instrument and then tested by the PEDV RT-iiPCR, PDCoV RT-iiPCR, and the reference PEDV rRT-PCR and PDCoV rRT-PCR. Comparing various PCRs using the same nucleic acid extracts eliminates variations on nucleic acid extractions and truly reflects the performance differences on each PCR itself. There were discrepant results on three samples between the PEDV RT-iiPCR and the reference PEDV rRT-PCR (Tables 6 a and 7) and zero discrepant results between the PDCoV RT-iiPCR and the reference PDCoV rRT-PCR (Tables 6 c and 7), demonstrating excellent agreements between these PCR assays.

However, it is impossible to extract nucleic acids from samples using the MagMAX™ Pathogen RNA/DNA Kit and Kingfisher-96 instrument in field situations. A portable POCKIT™ package includes reagents and Cubee™ mini centrifuge for taco™ mini nucleic acid extraction, lyophilized iiPCR reagents, and a POCKIT™ Nucleic Acid Analyzer. This ensures nucleic acid extraction and iiPCR testing can be completed within 1.5 h in field. Since under field conditions, taco™ mini extraction and iiPCR are combined for testing, we further compared the clinical performances of the taco™ mini extraction/RT-iiPCR system to MagMAX™

Table 8
Sensitivity and specificity of PEDV RT-iiPCR and PDCoV RT-iiPCR as compared to the reference singleplex PEDV rRT-PCR and PDCoV rRT-PCR based on testing 170 clinical samples with different nucleic acid extraction methods.

(a) Comparison of PEDV RT-iiPCR and the reference singleplex PEDV rRT-PCR

		MagMAX™ extraction and reference PEDV rRT-PCR		
		Positive	Negative	Total
taco™ mini extraction and PEDV RT-iiPCR	Positive	83	0	83
	Negative	5	82	87
	Total	88	82	170

Sensitivity: 94.32%; Specificity: 100%; Accuracy: 97.06%

(b) Comparison of PDCoV RT-iiPCR and the reference singleplex PDCoV rRT-PCR

		MagMAX™ extraction and reference PDCoV rRT-PCR		
		Positive	Negative	Total
taco™ mini extraction and PDCoV RT-iiPCR	Positive	82	0	82
	Negative	0	88	88
	Total	82	88	170

Sensitivity: 100%; Specificity: 100%; Accuracy: 100%

extraction/reference rRT-PCR system based on testing 170 clinical samples. Overall, 97.06% agreement was observed for the two PEDV PCR systems and 100% agreement for the two PDCoV PCR systems (Table 8).

Due to prevalence and co-circulation of PEDV and PDCoV in some areas, there are advantages of detecting and differentiating PEDV and PDCoV in a single sample using a multiplex rRT-PCR. In the present study, a duplex PEDV/PDCoV rRT-PCR including an internal control was evaluated. The duplex PEDV/PDCoV rRT-PCR was also highly specific and sensitive with $LoD_{95\%}$ of 7 copies per reaction for PEDV and 14 copies per reaction for PDCoV. Based on testing 170 clinical samples, the duplex rRT-PCR showed 97.65% agreement for PEDV detection compared to the reference PEDV rRT-PCR and 98.24% agreement for PDCoV detection compared to the reference PDCoV rRT-PCR. Sixteen samples that were positive for both PEDV and PDCoV as determined by the reference PEDV rRT-PCR and PDCoV rRT-PCR were successfully identified and distinguished by the duplex PEDV/PDCoV rRT-PCR.

In this study, 170 clinical samples including fecal swabs, feces, intestines, and oral fluids were used to evaluate various PEDV and PDCoV PCRs. Occasionally, there is a need to test other specimen types such as feed and environmental samples for presence of PEDV and/or PDCoV. In fact, the reference PEDV rRT-PCR (Lowe et al., 2014; Madson et al., 2014) and the reference PDCoV rRT-PCR (Chen et al., 2015) have been successfully used to detect PEDV and PDCoV RNA from feed and environmental samples at the ISU VDL. Although not evaluated in the current study, it is expected that the PEDV RT-iiPCR, PDCoV RT-iiPCR, and duplex PEDV/PDCoV rRT-PCR should be able to detect PEDV and/or PDCoV RNA present in feed and environmental samples as well. Studies to validate the on-site application of the RT-iiPCR/POCKIT™ system to help monitor the presence of PEDV in the environment is currently underway.

In summary, the PEDV RT-iiPCR, PDCoV RT-iiPCR and duplex PEDV/PDCoV rRT-PCR assays developed and evaluated in the current study were overall comparable to the reference rRT-PCRs for detection of PEDV and/or PDCoV. The PEDV and PDCoV RT-iiPCRs are potentially useful tools for on-site detection and the duplex PEDV/PDCoV rRT-PCR provides a convenient method to simultaneously detect the two viruses and differentiate PEDV from PDCoV.

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