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FULL PAPER

Development of one-step real-time reverse transcriptase-PCR-based assays for the rapid and simultaneous detection of four viruses causing porcine diarrhea

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Abstract

Porcine diarrhea caused by viruses is a major problem of the pig farming industry and can result in substantial losses of revenue. Thus, diagnosing the infectious agents is important to prevent and control diseases in pigs. We developed novel one-step real-time quantitative RT-PCR (qPCR) assays that can detect four porcine diarrheal viruses simultaneously: porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine deltacoronavirus (PDCoV), and porcine group A rotavirus (PRVA). The qPCR analysis takes only 75 minutes to detect the presence of the four viruses. The limits of detection of our new assays for PEDV, TGEV, PDCoV, and PRVA were 100, 10, 10 and 10 copies per reaction, respectively. The sensitivity of qPCR was 1–1000 times higher than that of published gel-based RT-PCR. We used our qPCR method to successfully diagnose clinical samples from infected pigs, and no false positive results were obtained. In conclusion, qPCR can drastically reduce the diagnostic time to detect viruses compared to currently employed methods. We predict that the qPCR assays will become a useful tool for detecting viral infections that cause diarrhea and other complications in pigs.

Key Words: one-step real-time RT-PCR, rapid diagnosis, swine, viral diarrhea

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Introduction

Acute diarrhea caused by viruses in piglets can stunt their growth and even lead to death, causing economic losses to the pig farming industry. Porcine epidemic diarrhea (PED) caused by porcine epidemic diarrhea virus (PEDV) is characterized by serve enteritis, watery diarrhea, vomiting, and dehydration in swine[15]. Although pigs of all ages are at risk of acquiring the infection, PED has the highest mortality rate (sometimes up to 100%) among suckling piglets. In October 2013, outbreaks of PED reemerged after a seven year hiatus in Japan. As reported by the Ministry of Agriculture, Forestry and Fisheries (http://www.maff.go.jp), PED occurred at 1,049 farms throughout Japan and killed about 490,000 pigs (mainly piglets) from October 2013 to July 2015. Another virus called transmissible gastroenteritis virus (TGEV) causes severe gastroenteritis similar to PEDV and belongs to the alphacoronavirus family together with PEDV[17]. Transmissible gastroenteritis has also occurred sporadically in pigs from Japan[13]. A third virus that affects pigs is the group A rotavirus and is the most common causative agent of diarrhea not only in pigs but in other livestock and human. Porcine group A rotavirus (PRVA) is most often detected in suckling pigs presented with diarrhea, and the clinical signs are sometimes similar to those associated with PEDV infection[6,16]. Porcine deltacoronavirus (PDCoV), a novel genus of *Coronaviridae*, was first reported in Hong Kong in 2009 and 2010[24]. Recently, PDCoV was detected in South Korea[11]. We have also detected PDCoV in diarrhea fecal samples of pigs in Iwate Prefecture in Japan (manuscript in preparation). PDCoV infection is not limited to the eastern hemisphere since it was also detected in pigs showing clinical symptoms, such as watery diarrhea in sows and death in piglets, in the United States in February 2014[23]. The pathogenicity of PDCoV was investigated following oral inoculation of piglets with PDCoV strains isolated in the US. The investigation revealed that PDCoV, similar to PEDV and TGEV, was enteropathogenic in pigs because diarrhea and vomiting was observed in PDCoV-infected piglets[2,5].

Diagnostic tools are needed to detect pathogens from clinical samples since it is extremely difficult to identify the viruses that cause porcine diarrhea based solely on clinical symptoms. Methods including virus isolation and serological assays (e.g. virus neutralizing tests) are time-consuming and laborious, but conventional RT-PCR (gel-based) assays are relatively quicker and more widely used as diagnostic tools. However, real-time quantitative RT-PCR is even more accurate and sensitive, allowing high-throughput detection and quantitation of viral loads using small volumes[3,7]. Additionally, one-step assays are more convenient and have less risk of contamination compared to two-step assays. For these reasons, we developed one-step real time quantitative RT-PCR (qPCR) assays for the simultaneous detection of four viruses and evaluated its application for field samples collected from outbreaks of PED, TGE, PRVA and PDCoV infections.

Materials and Methods

Primers and probes design: We designed primer sets for PRVA and TGEV based on the nucleotide sequences of the VP6 and nucleocapsid protein coding regions, respectively, using a multiple alignment analysis of reference strains including Japanese strains. Primers and probes were designed using a consensus sequence on PrimerQuest software (Integrated DNA Technologies, Inc, Iowa, USA). One parameter used for the designing was set to allow simultaneous amplification under the same reaction condition. Primer and probe sequences were adjusted with minor modifications by visual inspection. The specificity of each nucleotide sequence was conformed *in silico* using NCBI BLAST. Primers and probes of PEDV and PDCoV were described in previous reports[8,12]. Nucleotide information of

Table 1. The nucleotide information of primers and probes used in this study

Virus	Desig-nation	Target gene	Sequence(5'-3')	Nucleotide position	Amplicon size(bp)	Reference No.
PEDV	Forward	Nucleocapside	CGCAAAGACTGAACCCACTAATTT	26,679–26,876[a)]	198	8
	Reverse		TTGCCTCTGTTGTTACTTGGAGAT			
	Probe		TGTTGCCATTGCCACGACTCCTGC			
TGEV	Forward	Nucleocapside	AGCTATTGGACTTCAAAGGAAGATG	27,787–27,904[b)]	118	This study
	Reverse		CATAGGCATTAATCTGCTGAAGGAA			
	Probe		TCACGTTCACACACAAATACCACTTGCCA			
PDCoV	Forward	Membrane	ATCGACCACATGGCTCCAA	23,413–23,484[c)]	72	12
	Reverse		CAGCTCTTGCCCATGTAGCTT			
	Probe		CACACCAGTCGTTAAGCATGGCAAGCT			
PRVA	Forward	VP6	GCAAGCGCCACCATTTATATTTC	950–1,085[d)]	136	This study
	Reverse		TGCATACTCCTGACGTACYGAT			
	Probe		TGTGAATCTGTGCTTGCGGAYGCTTC			

Probes were labeled with 6-carboxyfluorescein(FAM) at the 5' end and with tetramethylrhodamine(TAMRA) at 3' end
a) The position was based on strain CV777 (GenBank accession number: AF353511.1)
b) The position was based on strain Purdue (GenBank accession number: AJ271965.2)
c) The position was based on strain HKU15-44 (GenBank accession number: JQ065042.1)
d) The position was based on strain CMP107/02 (GenBank accession number: EU372775.1)

each assay is summarized in Table 1. All probes were labeled with the fluorescent reporter dye FAM (6-Carboxyfluorecein) at the 5' end and with the fluorescent quencher dye TAMRA (6-Carboxytetramethylrhodamine) at the 3' end. Primers for all qPCR and probes for the detection of PEDV, TGEV, and PDCoV were purchased from Sigma Aldrich. The probes used to detect PRVA were purchased from Integrated DNA Technologies (IDT) because mixed bases were included in its sequence.

qPCR cycling condition: One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) (TAKARA Bio Inc., Otsu, Japan) was used in these assays. The reaction mixture contained the following: 10 µl of 2× One Step RT-PCR Buffer III, 2.8 µl of RNase-free H_20, 0.4 µl of TaKaRa Ex Taq HS (5 U/µl), 0.4 µl of PrimeScript RT enzyme Mix II, 1 µl of a forward and reverse primer and probe mix (from a 4 µM stock), 0.4 µl Rox Reference Dye, and 5 µl of template RNA (final volume of 20 µl per reaction). Amplification was performed with the ABI 7300 real-time PCR system (Life Technologies Inc., Carlsbad, CA, U.S.A.). Cycling conditions were as follows: reverse transcription at 42°C for 5 min, inactivation at 95°C for 10 s, followed by 40 cycles of denaturing at 95°C for 5 s and annealing and extension at 60°C for 34 s. The fluorescence signals were acquired during the annealing and extension steps. The baseline and threshold were set using the auto-baseline feature in the ABI7300 Software v1.4 (Life Technologies Inc.).

Evaluation of qPCR sensitivity and analytical performance: To validate the performance of each assay under the same reaction conditions, their sensitivities were evaluated using synthesized target DNA. The template DNAs were designed to include the qPCR target region in their sequence. The synthesized target DNA (gBlocks) was purchased from IDT. Ten-fold serial dilutions in the range of 2×10^{-1} to 2×10^{5} copies per µl were prepared and subjected to real time PCR. Since 5 µl of each dilution was added per well, this created a range of 1×10^{0} to 1×10^{6} copies per well. Each dilution was tested in two wells per run. In addition, the sensitivity assay was repeated twice in separate runs. Limit of detection (LOD) was defined as the lowest concentration that fluorescence signals could be detected in all

reaction including the separate runs.

A standard curve was obtained from the results of the serial dilution tests to estimate the analytical assay performance. The efficiency (E) of each primer and probe set was calculated by standard curve slope (The formula is $E = (10^{-1/\text{slope}}) - 1$). The correlation co-efficient (R^2) was also calculated. The repeatability (intra-assay variance) and reproducibility (inter-assay variance) was assessed by co-efficient value (CV) calculated a basis of quantification cycle (Cq) values.

Sensitivity comparison of qPCR vs. gel-based PCR: PEDV and PRVA were isolated from suckling pigs in Tottori Prefecture using Vero cells and MA104 cells, respectively. Fecal samples including TGEV and PDCoV were obtained from pigs in Tottori and Iwate Prefectures. RNAs were extracted from virus-infected cell culture supernatants or fecal suspensions using QIAamp viral RNA mini kit (QIAGEN K.K., Tokyo, Japan). The sensitivity of gel-based PCR and qPCR assays was compared by ten-fold serial dilutions of four viral RNAs. Gel-based PCR for PED, TGE, and PRVA was performed using primers previously described[4,9]. In the case of PDCoV, primers for detection of pancoronavirus were used[14]. All gel-based PCR assays were performed using OneStep RT-PCR kit (QIAGEN K.K.).

Field samples: The assays were applied to clinical samples obtained from 8 farms with the history of either virus infection. All samples used as positive were proven to be positive for viral infection by clinical symptoms and gel-based PCR. Furthermore, samples deemed as PEDV and TGEV positive samples were confirmed by antigen immunostaining methods (expect for sample No.13 and 14). PRVA positive samples were also shown to be positive with a commercial antigen detection immunochromatographic assay kit (Dipstick'Eiken' Rota (Eiken Chemical Co., Ltd, Tokyo, Japan)). PDCoV positive sample were confirmed by direct sequencing (data not shown). As a negative control, fecal samples from 3 farms were used that were confirmed to be negative for the four viruses by gel-based PCR. Details of the field samples are shown in Table 3. Fecal and homogenate suspensions were diluted 1 : 10 in Eagle's MEM (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan). The samples were centrifuged at 3,000 rpm for 10 min and the supernatants were collected. RNA was extracted from the supernatants by using QIAamp viral RNA mini kit (QIAGEN K.K) and used in the qPCR assays.

Results

Limit of detection

The sensitivity of our real-time RT-PCR with each primer and probe set was determined using synthesized DNAs that have a target amplification region. The LOD of PEDV, TGEV, PDCoV, and PRVA were 100, 10, 10, and 10 copies per reaction, respectively.

Linearity of a standard curves

A standard curves were constructed for each separate run on the basis of the 10-fold serial dilutions of the synthesized DNAs. Dynamic range, R^2 and PCR efficiency are shown in Fig. 1. and Table 4. All assays displayed a broadly dynamic range of at least a 5-order scale. The standard curve formula, R^2 value and E value of each primer set were as follows: (A) PEDV; $y = -3.3668x + 40.622$, $R^2 = 0.9984$, $E = 0.9816$. (B) TGEV; $y = -3.3577x + 37.497$, $R^2 = 0.9997$, $E = 0.98528$. (C) PDCoV; $y = -3.7063x + 37.944$, $R^2 = 0.9953$, $E = 0.86127$. (D) PRVA; $y = -3.5924x + 40.825$, $R^2 = 0.9993$, $E = 0.8983$. The PCR efficiency viruses in each of the detection assays was sufficient to quantify the copy number within the dynamic range (PEDV: 98.16%, TGEV: 98.52%, PDCoV: 86.12%, PRVA: 89.83%) with an R^2 of at least 0.995.

Repeatability and reproducibility

Table 2 depicts the repeatability (intra-assay variance) and reproducibility (inter-assay variance)

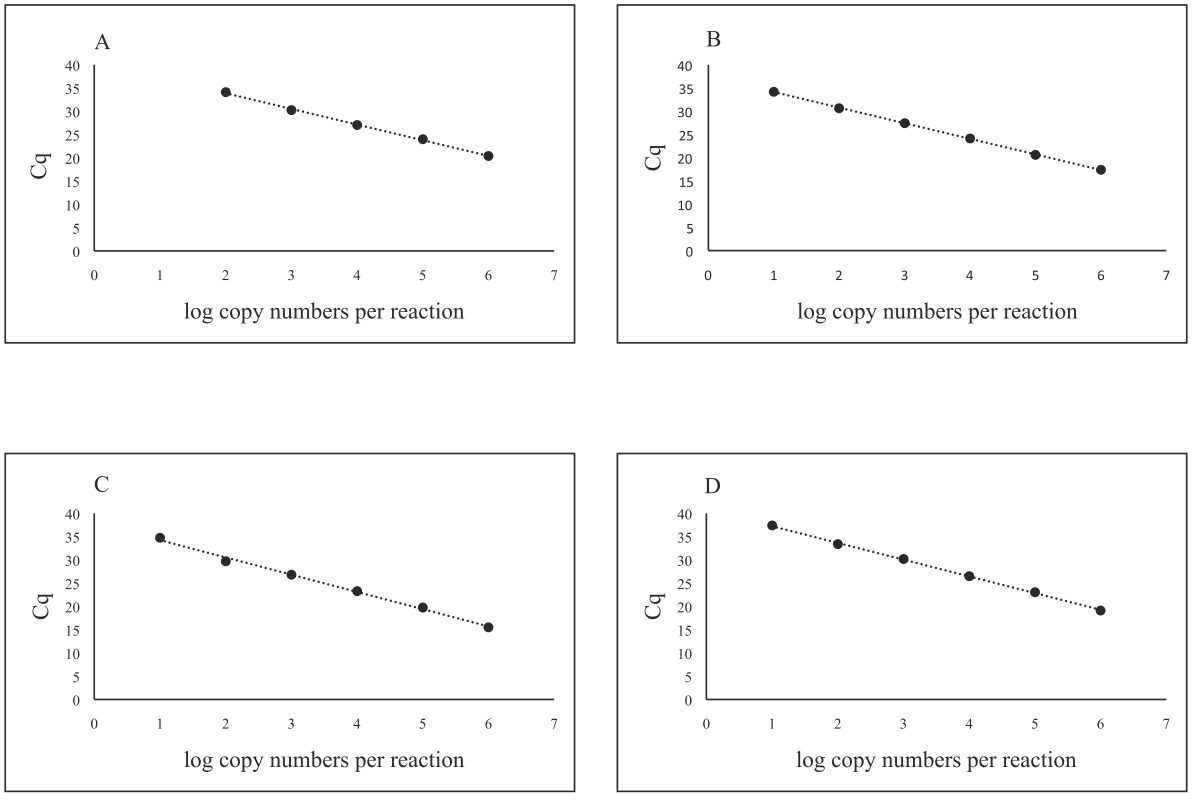


Fig. 1. Representative standard curves of each primer and probe. The quantification cycle (Cq) of each reaction was plotted against the log copy number of the synthesized DNA, including each target genome sequence.

of all primer and probe sets. The repeatability of each qPCR was measured by performing two replicates of the 10-fold serial dilution tests. All dilution point CVs were as follows: PEDV (0.01% to 1.94%), TGEV (0.13% to 4.98%), PDCoV (0.05% to 5.00%) and PRVA (0.01% to 3.64%). Next, reproducibility was estimated with separate runs replicated twice. The inter-assay CVs were as follows: PEDV (1.91% to 4.41%), TGEV (0.03% to 7.15%), PDCoV (0.47% to 2.95%), and PRVA (0.12% to 3.20%).

Sensitivity comparison between qPCR and gel-based PCR

The detection limits of gel-based PCR and our qPCR assays were 10^5 and 10^5 dilution of RNA for PEDV, 10^2 and 10^4 dilution for TGEV, 10^1 and 10^4 dilution for PDCoV and 10^2 and 10^4 dilution for PRVA, respectively (Fig. 2).

Application of qPCR to field samples

Table 3 shows the results of qPCR for field samples, which include 23 positive and 22 negative samples for target viruses. In all positive samples, our qPCR method successfully detected each virus except for sample No.3 that was weakly positive for PEDV on gel-based PCR. Furthermore, sample No.19 was infected with both PEDV and PDCoV and qPCR was able to detect both viruses. No false-positive reactions were observed in any positive samples for either assay and no signal was detected in assays performed with 22 fecal samples that were negative for the target viruses.

Discussion

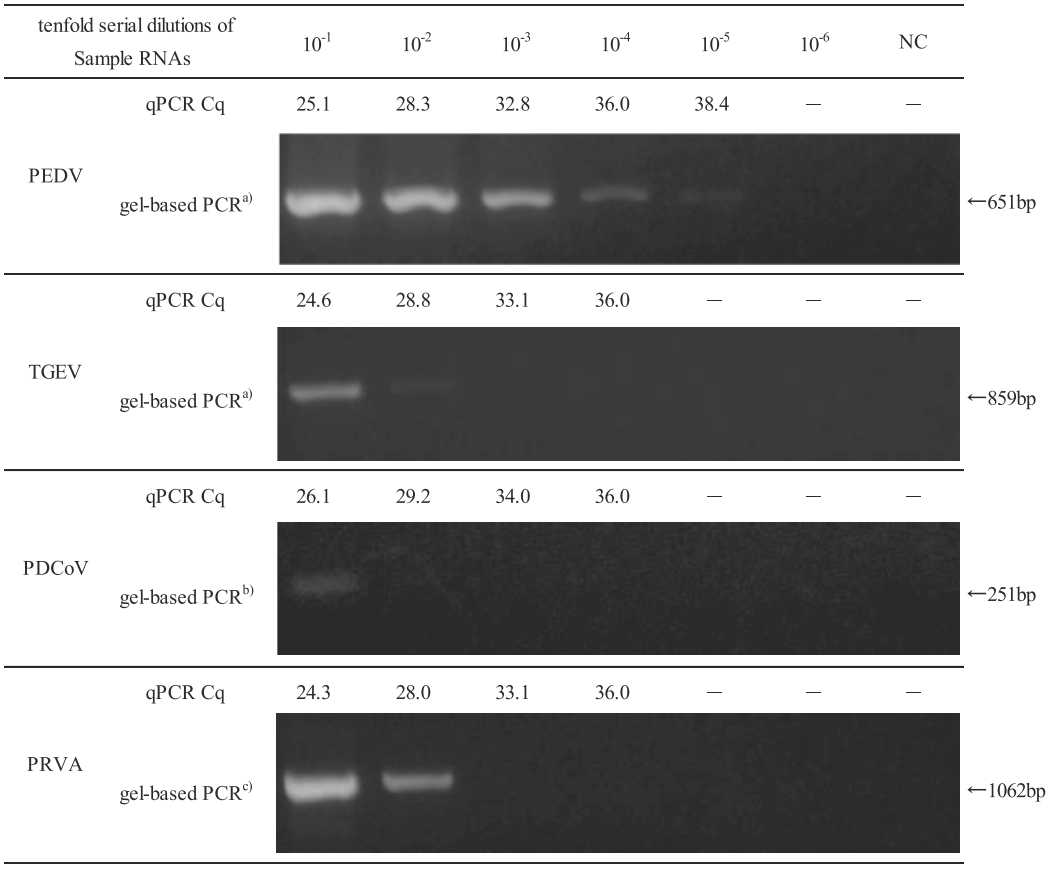
There are several reports that describe multiplex gel-based PCR and qPCR for simultaneous detection of PEDV and TGEV, and

Table 2. (a) Repeatability of each qPCR assays

	PEDV				TGEV				PDCoV				PRVA			
	Run1		Run2		Run1		Run2		Run1		Run2		Run1		Run2	
Copy number per reaction	Average Cq	CV(%)	Average Cq	CV(%)	Average Cq	CV(%)	Average Cq	CV(%)	Average Cq	CV(%)	Average Cq	CV(%)	Average Cq	CV(%)	Average Cq	CV(%)
10^6	20.40	1.08	18.68	1.94	17.39	0.88	15.07	4.98	15.52	1.01	16.47	1.28	19.14	0.89	20.40	1.08
10^5	23.98	0.01	22.18	1.17	20.60	0.73	20.77	0.13	19.73	0.53	20.42	0.60	23.04	0.19	23.98	0.01
10^4	27.06	1.03	25.68	1.04	24.16	0.95	23.21	0.60	23.29	3.29	23.79	0.07	26.47	0.55	27.06	1.03
10^3	30.23	0.36	29.09	0.21	27.47	0.40	26.48	3.44	26.80	1.90	27.06	0.54	30.15	0.09	30.23	0.36
10^2	34.11	0.33	32.73	0.18	30.63	0.22	30.47	0.71	29.71	5.00	30.40	0.05	33.35	0.60	34.11	0.33
10^1	38.48	1.40	—	—	34.22	0.57	34.24	2.26	34.78	0.64	35.18	2.33	37.36	3.64	38.48	1.40
10^0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Table 2. (b) Reproducibility of each qPCR assays

	PEDV		TGEV		PDCoV		PRVA	
Copy number per reaction	Average Cq	CV(%)	Average Cq	CV(%)	Average Cq	CV(%)	Average Cq	CV(%)
10^6	19.54	4.41	16.23	7.15	16.00	2.95	19.77	3.20
10^5	23.08	3.91	20.68	0.39	20.07	1.72	23.51	2.00
10^4	26.37	2.61	23.68	2.01	23.54	1.08	26.76	1.10
10^3	29.66	1.91	26.98	1.83	26.93	0.47	30.19	0.12
10^2	33.42	2.07	30.55	0.26	30.06	1.16	33.73	1.13
10^1	—	—	34.23	0.03	34.98	0.58	37.92	1.48
10^0	—	—	—	—	—	—	—	—



a) refrence No.9 b) refference No.14 c) refference No.4

Fig. 2. Sensitivity comparison of qPCR and gel-based PCR for PEDV, TGEV, PDCoV and PRVA. Reactions were performed using ten-fold serial dilutions of RNA. RNA of PEDV and PRVA were extracted from virus-infected cell culture supernatant. RNAs of TGEV and PDCoV were extracted from fecal suspensions. Positive and negative reactions of qPCR show Quantification Cycle (Cq) and —, respectively. NC, negative control.

these tests are widely used as rapid diagnostic procedures[1,8,9,26]. However, to our knowledge there are no assays for the simultaneous detection of four porcine diarrheal viruses using qPCR. In this study, we developed new qPCR assays, which can detect four viruses: PEDV, TGEV, PRVA, and PDCoV.

We employed one-step assays in order to minimize the risk of contamination and reduce the time to completion. We used primers and probes for PEDV and PDCoV that were described previously[8,12]. On the other hand, the primers and probes that were used to detect TGEV and PRVA were newly designed based on sequence alignments of conserved genomic region (VP6 and nucleocapsid protein coding regions)[10,18,22]. All primers and probes successfully and specifically detected the target viral genome and no false positive results were observed. In addition, we created an ideal situation for rapid diagnosis since each primer and probe set can amplify their target genomes under the same reaction conditions. The LOD of PEDV, TGEV, PDCoV and PRVA were 100, 10, 10 and 10 copies per reaction, respectively. These were equal to or higher than those of the reported qPCRs[7,8,25]. Several reports have shown that the detection sensitivity of qPCR is higher than gel-based PCR[7,19-21]. In this study, the detection sensitivity using ten-fold serial dilutions of the RNA isolated from clinical samples revealed that the sensitivity of our qPCR assay against TGEV, PDCoV and PRVA were 100–1000 fold higher than that of gel-based PCR under the same reaction

Table 3. The datail of field samples and the results of the qPCR assays[a)]

Virus	Farm No.	Sample No.	Specimen	Pig age or Stage	Clinical signs	PEDV	TGEV	PDCoV	PRVA	Diagnosis
PEDV	1	1	Small intestine	suckling pig	diarrhea, dehydration	+	−	−	−	PED
		2	Small intestine	suckling pig	diarrhea, dehydration	+	−	−	−	PED
		3	Small intestine	suckling pig	diarrhea, dehydration	−	−	−	−	PED
		4	Small intestine	suckling pig	diarrhea, dehydration	+	−	−	−	PED
	2	5	Small intestine	suckling pig	diarrhea, vomitting, dehydration	+	−	−	−	PED
		6	Small intestine	suckling pig	diarrhea, vomitting, dehydration	+	−	−	−	PED
		7	Small intestine	suckling pig	diarrhea, vomitting, dehydration	+	−	−	−	PED
		8	Small intestine	suckling pig	diarrhea, vomitting, dehydration	+	−	−	−	PED
		9	Small intestine	suckling pig	diarrhea, vomitting, dehydration	+	−	−	−	PED
		10	Small intestine	suckling pig	diarrhea, vomitting, dehydration	+	−	−	−	PED
		11	Small intestine	suckling pig	diarrhea, vomitting, dehydration	+	−	−	−	PED
		12	Small intestine	suckling pig	diarrhea, vomitting, dehydration	+	−	−	−	PED
	3	13	Feces	suckling pig	diarrhea, dehydration	+	−	−	−	PED
		14	Feces	suckling pig	diarrhea, dehydration	+	−	−	−	PED
TGEV	4	15	Small intestine	suckling pig	diarrhea, vomitting, dehydration	−	+	−	−	TGE
		16	Small intestine	suckling pig	diarrhea, vomitting, dehydration	−	+	−	−	TGE
		17	Small intestine	suckling pig	diarrhea, vomitting, dehydration	−	+	−	−	TGE
		18	Small intestine	suckling pig	diarrhea, vomitting, dehydration	−	+	−	−	TGE
PDCoV	5	19[b)]	Feces	25 day	diarrhea, dehydration	+	−	+	−	co-infection of PED and PDCoV
		20[b)]	Feces	6 month	diarrhea	−	−	+	−	infection of PDCoV
PRVA	6	21	Feces	4 month	no clinical sign	−	−	−	+	surveillance of salmonellosis
	7	22	Feces	unknown	unknown	−	−	−	+	unknown
	8	23	Feces	2 month	no clinical sign	−	−	−	+	surveillance of salmonellosis
none of these 4 virus	9	24	Feces	suckling pig	diarrhea	−	−	−	−	infection of PRVB[c)]
	10	25	Small intestine	suckling pig	diarrhea, dehydration	−	−	−	−	colibacillosis
		26	Small intestine	suckling pig	diarrhea, dehydration	−	−	−	−	colibacillosis
		27	Small intestine	suckling pig	diarrhea, dehydration	−	−	−	−	colibacillosis
		28	Small intestine	suckling pig	diarrhea, dehydration	−	−	−	−	colibacillosis
	11	29	Feces	suckling pig	diarrhea	−	−	−	−	unknown
		30	Feces	suckling pig	diarrhea	−	−	−	−	unknown
		31	Feces	suckling pig	diarrhea	−	−	−	−	unknown
		32	Feces	suckling pig	diarrhea	−	−	−	−	unknown
		33	Feces	suckling pig	diarrhea	−	−	−	−	unknown
		34	Feces	suckling pig	diarrhea	−	−	−	−	unknown
		35	Feces	suckling pig	diarrhea	−	−	−	−	unknown
		36	Feces	suckling pig	diarrhea	−	−	−	−	unknown
		37	Feces	suckling pig	diarrhea	−	−	−	−	unknown
		38	Feces	suckling pig	diarrhea	−	−	−	−	unknown
		39	Feces	suckling pig	diarrhea	−	−	−	−	unknown
		40	Feces	suckling pig	diarrhea	−	−	−	−	unknown
		41	Feces	suckling pig	diarrhea	−	−	−	−	unknown
		42	Feces	suckling pig	diarrhea	−	−	−	−	unknown
		43	Feces	suckling pig	diarrhea	−	−	−	−	unknown
		44	Feces	suckling pig	diarrhea	−	−	−	−	unknown
		45	Feces	suckling pig	diarrhea	−	−	−	−	unknown

a) Positive and negative reactions are + and −, respectively
b) PDCoV was confirmed by direct sequencing
c) PRVB: Porcine group B rotavirus

Table 4. The correlation co-efficient ($R^{2)}$ and the PCR efficiency (E) of each primer and probe set

Virus	PEDV	TGEV	PDCoV	PRVA
R^2 value	0.9984	0.9997	0.9953	0.9993
E value (%)	98.16	98.52	86.12	89.83

conditions. Furthermore, since our assay consists of a one-step method, experimental turnaround time is 75 minutes.

When applied to field samples, our assays consistently detected four viruses from the samples, which were also diagnosed by standard laboratory methods, and no false positive reactions were observed. However, qPCR of PEDV was negative for field sample No.3 that was weakly positive on a gel-based PCR for PEDV. The sensitivity of qPCR and gel-based PCR for PED were equivalent. Thus, qPCR of field sample No.3 for PEDV might not have detected its presence given the potentially low copy number of PEDV in the sample.

In conclusion, we successfully developed and validated one-step real-time reverse-transcriptase-PCR assays for the detection of four viruses that cause porcine diarrhea. These assays provide a rapid and sensitive detection of viral RNA from field samples and reduce the risk of cross-contamination. Our new qPCR method may be useful for diagnosing the status and prevalence of four common viruses that cause diarrhea in pigs.

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