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Abstract. Porcine reproductive and respiratory syndrome virus (PRRSV) is a single-stranded and positive-sense RNA virus which can cause severe clinical disease and economic problem for worldwide pig industry. Droplet digital PCR (ddPCR) is an absolutely quantification method that can be used in viral detections. Here a one-step reverse transcription ddPCR was established for detection of highly pathogenic PRRSV (HP-PRRSV) and classical PRRSV. The assay was able to quantitatively detect HuN4 from 10^3 to 10^{-1} $TCID_{50}$/mL. Through comparing with real-time quantitative reverse transcriptase PCR, the one-step RT-ddPCR was demonstrated more sensitive and can be used for the accurate quantification of PRRSV.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a severe clinical disease of swine which causes economic problem for worldwide pig industry [1]. Initially the disease was referred to as "mystery swine disease" and "mystery reproductive syndrome", and it was firstly reported in the late 1980s in both North America and Central Europe [2-7]. The etiological agent of the disease, porcine reproductive and respiratory syndrome virus (PRRSV), is a single-stranded and positive-sense RNA virus of the genus *Arterivirus*, family *Arteriviridae*, order *Nidovirales* [8-10]. The characterization of European (Type 1) and North American (Type2) genotype of PRRSV were found to be genetically divergent by ~40%, although the two stains cause similar clinical symptoms [2]. Herds of pigs in the Mid-Eastern region of China were confirmed to be infected by the highly pathogenic PRRSV (HP-PRRSV) in 2006, which caused a high proportion of deaths in different age pigs and had spread widely in China [11-14]. Genomic sequencing of one of the HP-PRRSV(HuN4 strain) showed amino acid mutations and deletions in GP5 and Nsp2 protein comparing with two classical PRRSV in China, CH-1a and BJ-4 [12].

Diagnosis method of PRRSV initially was viral culture to confirm the presence of virus in serum or in tissue samples. This method often requires long time and has relatively low diagnostic sensitivity [15]. In 1995, nested PCR was developed to detect PRRSV and found to be more sensitive [16]. Later on, real-time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) method have been developed that allows the quantification of RNA more rapid without the need of gel electrophoresis [17-19]. Nevertheless, absolute quantification and interlaboratory reproducibility of

qPCR has been a big challenge because of the interdependence of samples and standards (based on quantification cycle, C_q) [20, 21].

Droplet Digital PCR (ddPCR) is a technology which partitions PCR samples into 20,000 nanodrops and acquires amplification data of each unit at the end point [21-23]. Based on Poisson statistics, ddPCR enables the absolute quantification of nucleic acid owe to the independence of reaction efficiency and standard calibration curve [24]. And because of that, ddPCR has been used in many viral detections [21, 25-27]. Here, a one-step RT-ddPCR method has been optimized and established based on the conserved regions of PRRSV genomes. The optimized assay was then used to detect one of the PRRSV strains and the performance characteristics of the assay was validated.

2. Materials & methods

2.1. Virus strains

Classical swine fever virus (CSFV), porcine pseudorabies virus (PRV), and porcine circovirus-2 (PCV-2) were commercial vaccines. PRRSV HuN4 and CH-1a virus strains were provided by Harbin veterinary research institute. Tissue culture infective doses of HuN4 and CH-1a were determined to be 10^5 $TCID_{50}$/mL.

2.2. Nucleic acid extraction

All Nucleic acids were extracted using the QIAamp Viral RNA Mini Kit (QIAGEN, USA) according to the manufacturer's instructions. 140 μL of samples were lysed and eluted with 60 μL of elution buffer. Isolated RNA was stored at -80℃.

2.3. Primers and probe

The primers and probe used for assays were designed based on the conserved regions within open reading frame 6 (ORF6) of PRRSV. Forward and reverse primers used were F1 (5'-CTAGGCCGCAAGTACATTCT-3') and R1 (5'- GACGACAAATGCGTGGTTATC-3'), respectively. The TaqMan probe was P1 (5'-FAM-ATTTGCCGCAATCGGATGAAAGCC-BHQ1-3'). Primers and probe concentrations were optimized in subsequent experiments.

2.4. One step RT-qPCR

One step RT-qPCR was carried out using the AgPath-IDTM One-Step RT-PCR Kit (Life Technologies, USA) on LightCycler® 480 Instrument (Roche, Switzerland). The reaction mixture comprised 2 μL of template combined with 12.5 μL of 2×RT-PCR buffer, 1μL of RT-PCR Enzyme mix, the primers and probe with final concentrations of 100 nM and 400 nM, respectively, additional RNase-free water to a final volume of 25 μL. All samples were analyzed in triplicate on 96-well plate. Cycling conditions were as follows: 45℃ for 10 min (reverse transcription); 95℃ for 10 min; and 40 cycles of 95℃ for 15 sec, and 60℃ for 45 sec. Data were analyzed automatically using the LightCycler® 480 software (Roche, Switzerland).

Plasmid containing ORF6 (nt 14630~14787) of CH-1R (GenBank No. EU807840.1 was commercially synthesized (Sangon Biotech, China) to generate standard calibration curve for RT-qPCR. Plasmid was tenfold serially diluted and analyzed in triplicates.

2.5. One step RT-ddPCR

Purified viral RNA was quantified using the One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad, USA). The reaction mixture in a final 20 μL volume included 5 μL of supermix, 2 μL of reverse transcriptase, 1 μL of 300 mM DTT, 11 μL of mixture of forward and reverse primers, probe and RNase-free water and 1 μL of template. The final concentrations of primers and probe were 400 nM and 240 nM, respectively. Each reaction mix was converted to droplets using the QX200 Droplet generator (Bio-Rad, USA). After generation, the emulsion of droplets was transferred to a 96-well plate, heat sealed and amplified in a GeneAmp System 9700 thermal cycler (Applied Biosystems,

USA). The thermal cycling conditions were as follows: 45℃ for 10 min (reverse transcription); 95℃ for 10 min; and 40 cycles of 95℃ for 15 sec, and 54℃ for 45 sec. The cycled plate was then transferred to the QX200 reader (Bio-Rad, USA) and analyzed using the Quanta Soft droplet reader softer ware (Bio-Rad, USA).

3. Results

3.1. Optimization of primer/probe concentration and annealing temperature of RT-ddPCR

Typically, RT-qPCR would be performed for the optimization of primer/probe set. In this experiment, three sets of primers and probe were firstly designed within ORF6 of PRRSV. The concentrations of the primers and probe was further optimized with orthogonal design. In order to obtain higher amplification efficiency and better amplification plot, the first set was chosen to be used and the sequences were listed in 2.3. The concentrations of primers and probe used in the RT-ddPCR were 400 nM and 240 nM, respectively. Then thermal gradient optimization test was carried out with annealing temperature ranging from 50℃ to 60℃. Nevertheless, no obvious differences were observed among the results. 54℃ was chosen in the subsequent experiments for the comparatively greater difference between the negative and positive fluorescence amplitude.

3.2. Specificity of RT-ddPCR

The nucleic acids of HP-PRRSV (HuN4), C-PRRSV (CH-1a), CSFV, PRV, and PCV-2 were extracted by the same method and tested in the RT-ddPCR. The results were negative when using CSFV, PRV and PCV-2 as templates, whereas it was positive when using all the PRRSV strains (Fig.1). It suggested that the RT-ddPCR assay has a high specificity for detecting PRRSV strains.

3.3. Linear range, sensitivity and repeatability of RT-ddPCR

To assess the linear range of the RT-ddPCR assay, the nucleic acids of HuN4 were 10-fold diluted and used as templates. As seen in Fig.2, the RT-ddPCR results showed excellent linearity (R^2=0.9988) with the range of at least five orders of magnitude, and the limit of quantification can be 10^{-1} $TCID_{50}$/mL (4±2 copies/20 μL). The copy numbers of the two highest concentrations tested (10^5 and 10^4 $TCID_{50}$/mL) were above the upper range of quantification, because almost 100% of the drops analyzed were positively amplificated and the Poisson law can no longer be applied to calculate the copies of targets [28].

Each dilution was tested in quadruplicate to evaluate the repeatability of RT-ddPCR. The coefficient of variation (CV) of the measured RNA copy numbers decreased with the increase of the input RNA; it met the minimum value (CV% was 1.56%) at 10^2 $TCID_{50}$/mL (4935±77 copies/20 μL). The CV% was below 12% between 10^1 to 10^3 $TCID_{50}$/mL, corresponding to the RNA concentration of 494±58 to 61233±2419 copies/20 μL, indicating the RT-ddPCR showed good measurement repeatability in this range (Fig. 3).

3.4. Linear correlation between RT-ddPCR and RT-qPCR for quantification of HuN4

The decimal dilution series of HuN4 RNA were also tested by RT-qPCR, and a good amplification efficiency (93.7%) and linearity (R^2 is 0.9851) correlation was observed (Fig. 4A). The linear correlation between RT-ddPCR and RT-qPCR was shown in Fig. 4B, with R^2 of 0.9999. Seen from the detailed data showed in table1, the RT-ddPCR assay quantitatively detected HuN4 from 10^3 to 10^{-1} $TCID_{50}$/mL (~10^5 to 10^1 copies/μL), while RT-qPCR from 10^5 to 10^0 $TCID_{50}$/mL (~10^7 to 10^2 copies/μL). So, the RT-ddPCR was one order of magnitude more sensitive than RT-qPCR while the RT-qPCR had the wider detection range.

4. Conclusion

Viral load quantification provides valuable information for viral diagnostics and pathogenesis research [17]. The RT-qPCR technology enables it possible to quantitate RNA viral load more sensitively and

rapidly than viral culture and immunological methods. Whereas as seen from the above data, the RT-qPCR results directly depend on the value of the standard plasmid concentration. Most laboratories now determine the plasmid concentration by spectrum analysis, while the accuracy of this method is impacted largely by the purity of the plasmid.

The one-step RT-ddPCR established here achieved quantitate detection of PRRSV by eliminating the dependence of the outer control and the risk of contamination. Because the primers and probe were designed based on the conserved region of PRRSV, the method could be used for both HP-PRRSV and classical PRRSV. Compared with RT-qPCR, the RT-ddPCR was one log more sensitive although the samples with higher concentrations needed to be diluted before the test. This makes it more suitable when the concentration of the template is relatively low.

For PRRSV detection, a ddPCR method was established before and it differed from our method in some ways [29]. They used "two-steps" ddPCR where PRRSV RNA was reversely transcribed before applying ddPCR and they used primers based on ORF7 and EvaGreen ddPCR Supermix instead of using Taqman probes. The limit detection for BJ-4 and HN07-1 strains was 10 $TCID_{50}$/mL for the "two-steps" ddPCR, and it was 10^{-1} $TCID_{50}$/mL for HuN4 strain in the one-step RT-ddPCR in this study. It was not sure that whether the limit detection can be comparable because totally different samples (different virus strains and the tissue culture infective doses were tested by different laboratories) were used.

In conclusion, the one-step RT-ddPCR developed here is a sensitive and accurate method that would be used in potential PRRSV detections and accurate quantifications.

5. Figure captions

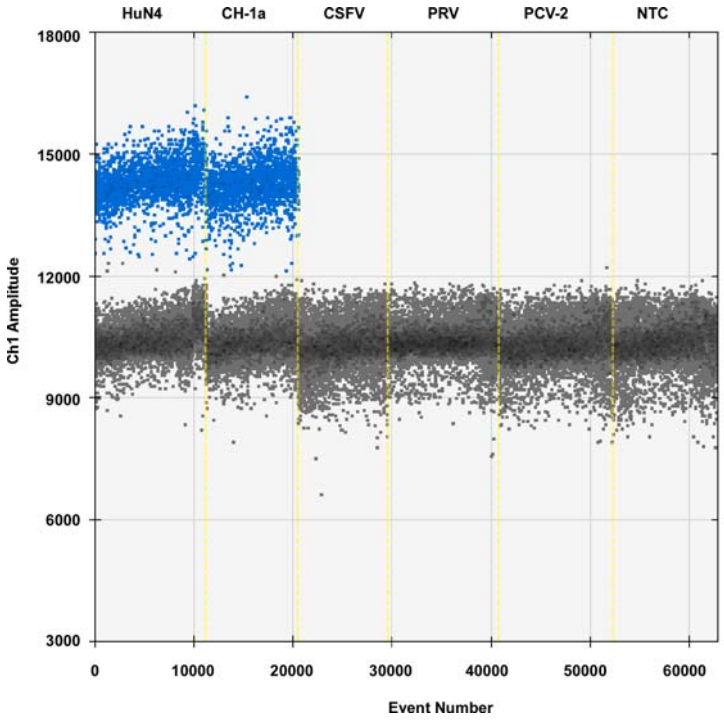

Figure 1. The specificity assay of the RT-ddPCR. The fluorescence amplitude of HuN4, CH-1a, CSFV, PRV, PCV-2 and negative control. NTC: No template control.

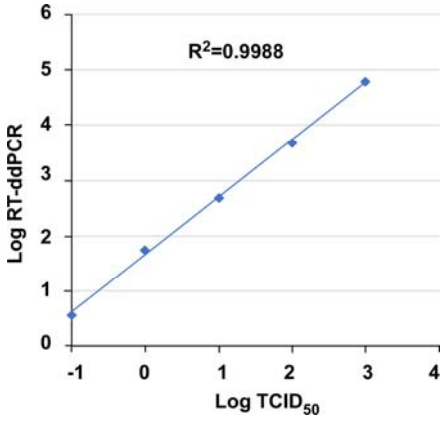

Figure 2. The linearity of the RT-ddPCR. HuN4 were serially 10-fold diluted from 10^5 to 10^{-1} $TCID_{50}$/mL and applied to RT-ddPCR. The quantification correlation was obtained by plotting the log $TCID_{50}$ against the log mean RT-ddPCR concentration.

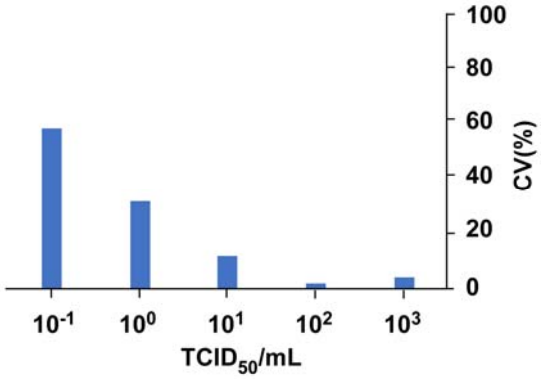

Figure 3. The repeatability of RT-ddPCR. Each concentration was applied in four replicates and the coefficient of variation (CV) is show here.

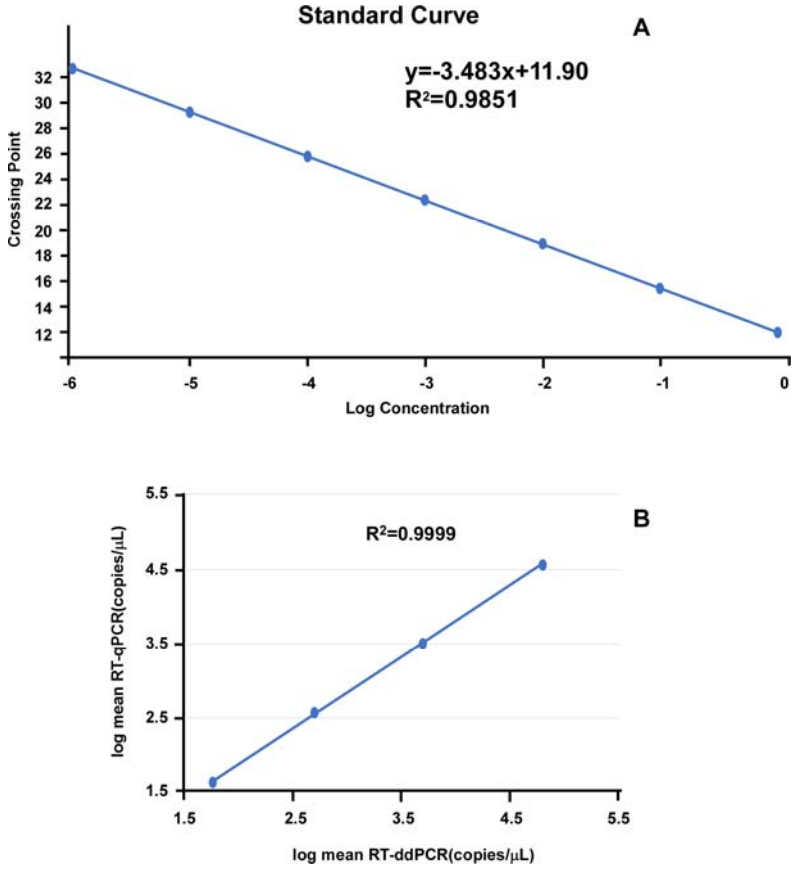

Figure 4. The RT-qPCR standard curve and the linear correlation of RT-ddPCR and RT-qPCR. A. The RT-qPCR standard curve generated by the LightCycler® 480 software (Roche, Switzerland). The slope is -3.483 (amplification efficiency is 93.7%), R^2=0.9851. B. Linear correlation of RT-ddPCR and RT-qPCR for quantification of HuN4 by plotting the log mean RT-ddPCR and RT-qPCR concentration of serially 10-fold diluted HuN4 RNA.

Table 1. Sensitivity of RT-ddPCR and RT-qPCR.

Sample ($TCID_{50}$/mL)	**RT-ddPCR**		**RT-qPCR**	
	Accepted droplets	Normalized Concentration [a] (copies/μL)	Cq values	Normalized Concentration [a] (copies/μL)
10^5	/	/	17.15	1,730,000
	/	/	17.34	1,520,000
	/	/	16.98	1,930,000
10^4	/	/	20.17	234,000
	/	/	19.95	272,000
	/	/	19.89	282,000
10^3	11495	63,000	23.07	34,400
	10219	59,400	23.09	34,000
	11711	64,000	23.15	32,800
	8965[b]	/		
10^2	11364	4,900	26.6	3,330
	12313	4,860	26.89	2,770
	13068	5,040	26.73	3,070
	11609	4,940		
10^1	14309	440	30.24	302
	14688	556	29.93	369
	11567	530	30.01	351
	12424	448		
10^0	12469	50	33.83	28
	14011	36	32.96	50
	15834	62	33.27	41
	12590	78		
10^{-1}	14027	6.8	35	n.d.
	13877	3.4	35	n.d.
	15140	3.2	35	n.d.
	14866	1.6		

[a] Normalized concentration here indicated the initial concentration of the RNA added to the reaction of RT-ddPCR and RT-qPCR.

[b] This assay was not included in the analysis because its number of accepted droplets was below 10,000 which was set as the minimal criterion of the assays.

Accepted droplets: numbers of droplets analyzed in 20μl reaction. *Cq* quantification cycle values for RT-qPCR. *n.d.* not detected.

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The authors contributed equally to this work

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