Development of a SYBR green-based real-time RT-PCR assay for rapid detection of the emerging swine acute diarrhea syndrome coronavirus

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ABSTRACT

Swine acute diarrhea syndrome coronavirus (SADS-CoV) is a novel coronavirus which was associated with severe diarrhea disease in pigs. SADS-CoV was first detected and identified as the causative agent of a devastating swine disease outbreak in southern China in 2017. Routine monitoring and early detection of the source of infection is therefore integral to the prevention and control of SADS-CoV infection. In this study, a SYBR green-based real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) technique was established for rapid detection and monitoring of this emerging virus. Specific primers were designed based on the conserved region within the M gene of the viral genome. The lowest detection limit of the RT-qPCR assay was 10 copies/µL. This assay was specific and had no cross-reaction with other 11 swine viruses. The positive rate of 84 clinical samples for the SYBR green-based RT-qPCR and the conventional RT-PCR was 73.81% (62/84) and 53.57% (45/84), respectively. These results demonstrated that the SYBR green-based RT-qPCR technique was an effectively diagnostic method with higher sensitivity than probe-based RT-qPCR and gel-based RT-PCR for detection and epidemiological investigations of SADS-CoV.

Swine acute diarrhea syndrome coronavirus (SADS-CoV), a new member of the genus Alphacoronavirus, contributed to a devastating swine disease outbreak in Guangdong province, China, which caused death to more than twenty thousand piglets across four farms and huge economic losses (Zhou et al., 2018b). The piglets infected with SADS-CoV mainly exhibited the clinical manifestations of acute vomiting and diarrhea and death (Gong et al., 2017; Xu et al., 2018; Zhou et al., 2018b). It was notable that the genome sequence of the causative agent of this large-scale outbreak shares remarkable homology with a bat coronavirus detected in a cave near the pig farm, implying SADS-CoV most likely originated from bats (Gong et al., 2017; Lau et al., 2007; Zhou et al., 2018b). Considering the high lethality of this new emerging virus and the clinical signs of the infected pigs that are similar to that caused by other porcine enteric coronaviruses, there is an urgent need to establish a sensitive and reliable diagnostic method that will be of help to early discovery and identification of SADS-CoV infection.

Cytopathic effect (CPE) could be observed only after serial passages of SADS-CoV in Vero cells, so isolation and detection of the virus by cell culture are time-consuming (Pan et al., 2017; Zhou et al., 2018b).

Immunofluorescence (IF) staining has been used for the detection of SADS-CoV (Pan et al., 2017; Zhou et al., 2018b), however virus-specific antibody and fluorescently-labeled antibody are required to react with the SADS-CoV antigen, this procedure makes the IF method complex and time-consuming. Recently, a reverse transcription loop-mediated isothermal amplification method (RT-LAMP) was established for the detection of SADS-CoV, however it couldn't be used for quantitative analysis of the viral copies in the samples (Wang et al., 2018). Real-time quantitative PCR (qPCR) is a powerful diagnostic method and has been widely used for detection and quantitation of pathogenic microorganism because of high sensitivity and reproducibility (Jensen et al., 2018; Yang et al., 2018; Yuan et al., 2016; Zhou et al., 2018a). Recently, a study reported a probe-based real time RT-qPCR for the detection of SADS-CoV, with a detection limit of 30 DNA copies/µL (Zhou et al., 2018a). Whereas, the SYBR green-based qPCR assay does not require an expensive probe, and is more cost-effective and easier to design than probe-based RT-qPCR. However, up to now, there is no report about SYBR green-based real-time RT-qPCR for the detection of SADS-CoV.

In this study, a SYBR green-based real-time RT-qPCR assay targeted

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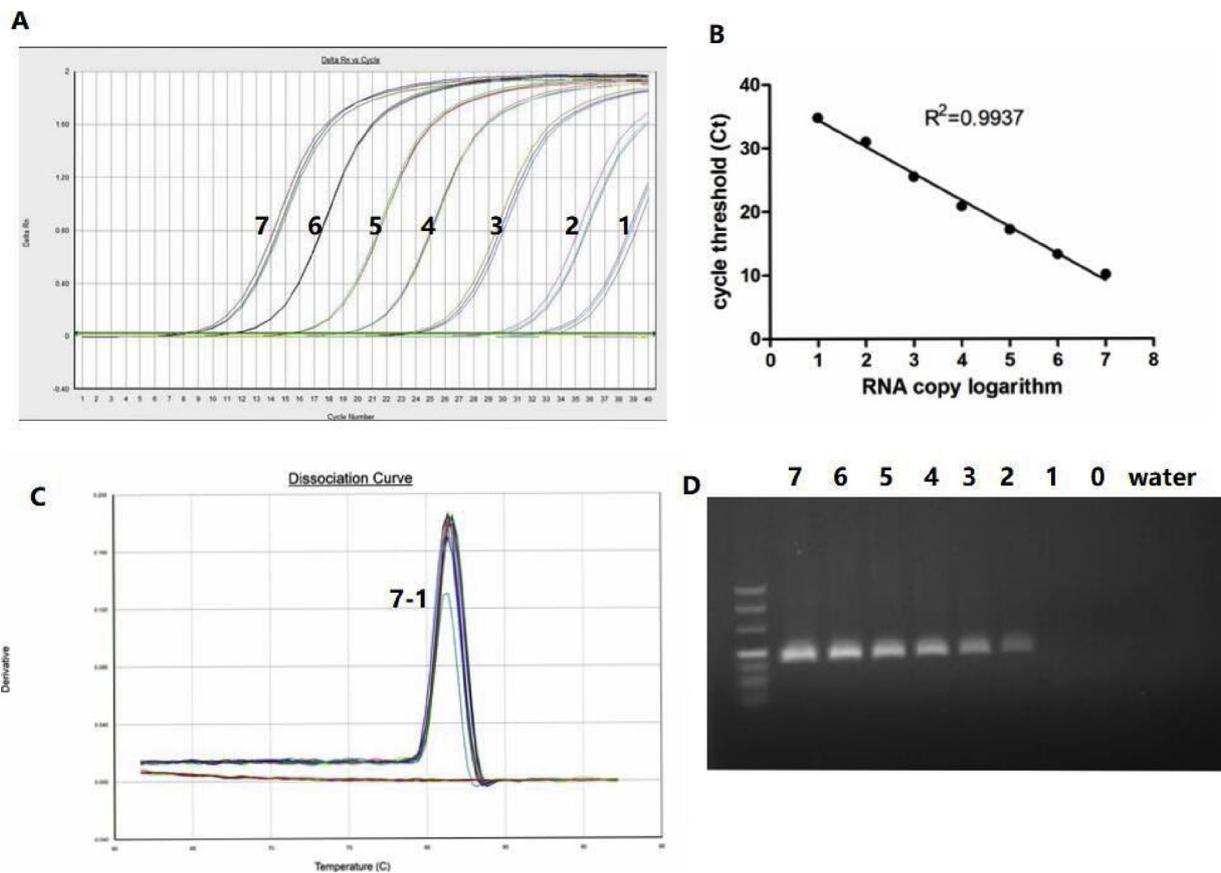
Fig. 1. Sensitivity of SYBR green-based RT-qPCR. A: Amplification of ten-fold dilutions of transcribed RNA by SYBR green-based RT-qPCR in triplicates. B: A standard curve of RT-qPCR, which was generated by plotting mean Ct values *versus* RNA copy number in triplicates. C: Dissociation curve analysis of RT-qPCR products. D: The detection limit of gel-based RT-PCR. 0–7: a serial of ten-fold RNA dilutions (1×10^0–1×10^7 copies/μL).

Table 1
The specific primers.

| Primers	Primer sequence (5′–3′)
SADS-CoV-F	ATGACTGATTCTAACAACAC
SADS-CoV-R	TTAGACTAAATGCAGCAATC
PRRSV-F	ATTTGAATGTTCGCACGGTCTC
PRRSV-R	CCGCTGAAACTCTGGTTAAAGG
PEDV-F	AACACGGCGACTACTCAGC
PEDV-R	GCCTTCTTTAGCAACCCAG
PRV-F	CCACGCGGTCGATGTCGTAG
PRV-R	GGTGCGCGGCGGCGTGTACG
CSFV-F	ATGCCCATAGTAGGACTAGCA
CSFV-R	TCAACTCCATGTGCCATGTAC
PCV2-F	AAGAAGCGGACCCCAAC
PCV2-R	AGGTGGCCCCACAATGA
TGEV-F	GTGGTGTTAGGTGATTATTTTCC
TGEV-R	TATGGTTTAACCTGCACTCACTA
RV-F	TATGCAATACCAGTAGGACCAG
RV-R	GCTCTACGTAGCGAGTATGAAATC
PPV-F	AGCCAAAAATGCAAACCCCAATA
PPV-R	CTTTAGCCTTGGAGCCGTGGAG
FMDV-F	GACAAAGGTTTTGTTCTTGGTCA
FMDV-R	TGCGAGTCCTGCCACGGA
PDCoV-F	TTTCAGGTGCTCAAAGCTCA
PDCoV-R	GCGAAAAGCATTTCCTGAAC
SIV-F	GGACATGTTACCCAGGAGA
SIV-R	ACTGGTAGGTGGATGGTGAA

the mostly conserved gene of SADS-CoV was established for rapid, sensitive and quantitative detection of the virus infection.

Complete genome sequences of 34 SADS-CoV isolates in total retrieved from GenBank were aligned using MegAlign7.1.0 (DNAStar, USA), and Matrix (M) gene sequence was found to be highly conserved

among all the strains of SADS-CoV in the database. The primers named as SADS-F (5′-ATGACTGATTCTAACAACAC-3′) (covering nucleotides 24,784–24,803) and SADS-R (5′-TTAGACTAAATGCAGCAATC-3′) (complementary to nucleotides 25,451–25,470) were designed and used to amplify the M gene. Viral RNA was isolated from the cells infected with SADS-CoV isolate SADS-CoV/CN/GDWT/2017 (Accession number: MG557844) by TGuide virus DNA/RNA kit (TIANGEN, China) following the manufacturer's instruction. The viral RNA was transcribed and amplified by using PrimeScript™ One Step RT-PCR Kit (Takara, China) with the primers SADS-F and SADS-R. The amplicon was subjected to gel electrophoresis and purified by using DNA Gel Extraction Kit (Beyotime, China). The purified amplicon was inserted into pGEM-T vector and transformed to *Escherichia coli* DH5α competent cells. The insert of positive clone was confirmed by sequencing and named as SADS-N. SADS-N plasmid was linearized by endonuclease digestion and transcribed *in vitro* by RiboMAX™ Large Scale RNA Production System (Promega, USA) according to the instruction of the kit manual. The *in vitro* transcribed RNA was purified, quantified and converted into copy number as the previously reported method (Yuan et al., 2016). Ten-fold serially diluted RNA ranged from 1×10^0–1×10^7 copies/μL were prepared with DEPC-treated water and used as the molecular standards in the following assays.

For the SYBR green-based RT-qPCR, the forward primer (5′-ATGT GGCTCCTATGGCCCTT-3′) (covering nucleotides 24,943–24,962) and the reverse primer (5′-ACGTTCGCGTTCTGCGATAA-3′) (complementary to nucleotides 25,095–25,114) were used to optimize the performance of the assay. The SYBR green-based RT-qPCR was carried out using the one step SYBR PrimeScript RT-PCR Kit (Takara, China) following the kit manual. The RT-qPCR assay in a 20 μL reaction volume contained 10 μL of 2×one step SYBR RT-PCR Buffer, 0.4 μL of
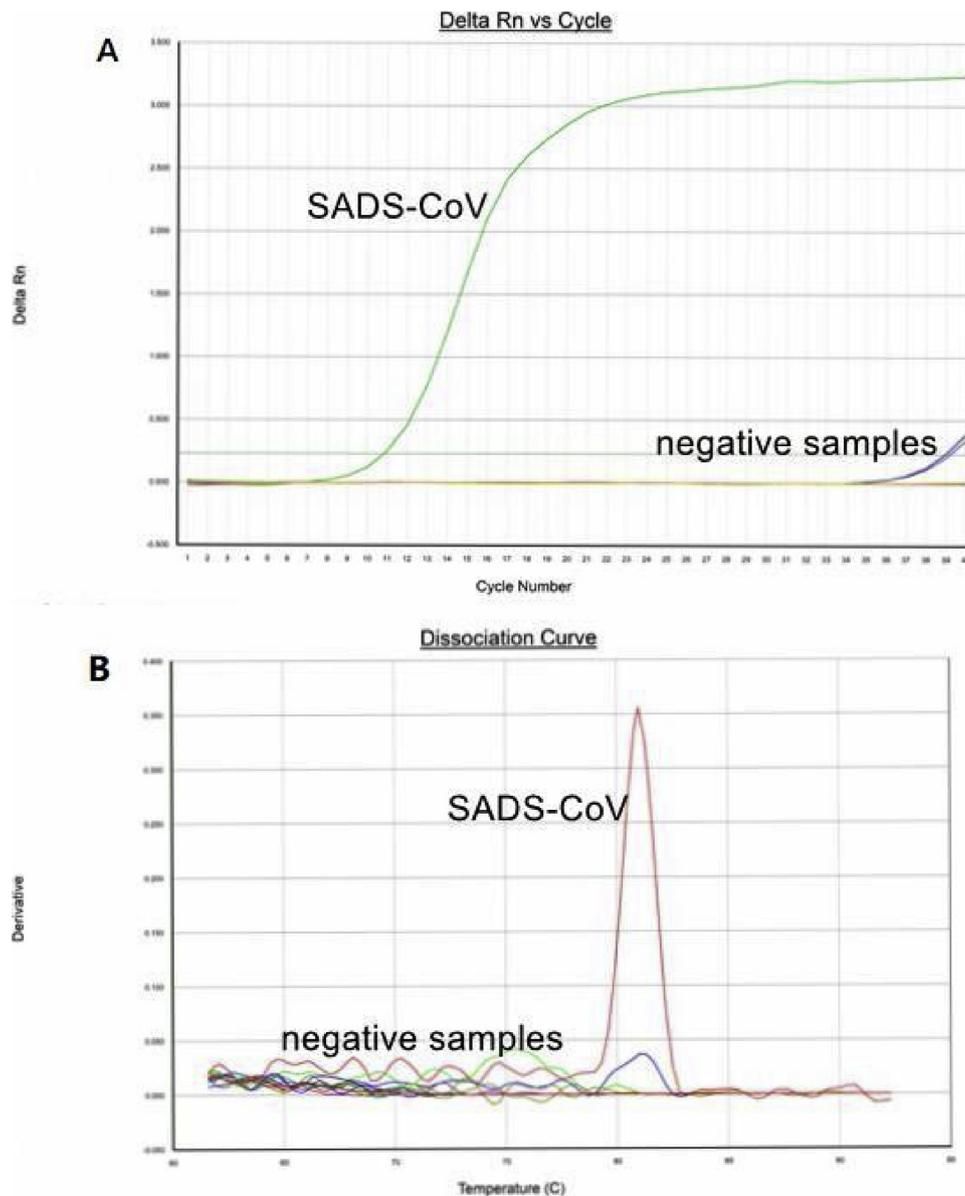
Fig. 2. Specificity of SYBR green-based RT-qPCR. SADS-CoV and negative samples were tested by the RT-qPCR assay. Negative samples included PRRSV, PEDV, PRV, CSFV, PCV2, TGEV, RV, PPV, FMDV, PDCoV, SIV and distilled water. A: Only SADS-CoV produced a strong fluorescence signal under RT-qPCR. B: Melting peak was observed at 81 °C for only SADS-CoV in RT-qPCR.

TaKaRa Ex Taq HS, 0.4 μL of PrimeScript RT Enzyme Mix, 0.4 μL of ROX Reference Dye II, 0.4 μL (10 μM) of each primer, 7.0 μL of RNase free water and 1.0 μL of template. Amplification reaction was performed by Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, USA). Amplification condition was set up as follows: reverse transcription (RT) step at 42 °C for 5 min; initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 34 s. After the cycles completed, a dissociation analysis step was added: 95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s. Data were collected and analyzed by the Applied Biosystems 7500 SDS Software (Version 1.4).

The limit of detection of the RT-qPCR was determined by testing the serially diluted RNA standards in triplicate. Meanwhile, the detection limit of gel-based RT-PCR was also determined. As shown in Fig. 1A, RT-qPCR could detect as few as 10 copies RNA molecule, but standard sample containing one RNA molecule or distilled water only didn't generate positive signals. The dissociation curve analysis indicated the RNA dilutions range from 1×10^1–1×10^7 copies/μL showed almost the same melting temperature (Tm) value (mean Tm values ± standard deviation: 81.57 ± 0.15 °C), meanwhile sample containing

one RNA molecule and distilled water as a negative control didn't generate the similar dissociation curve (Fig. 1C). The detection limit of the conventional RT-PCR in this study was 100 copies (Fig. 1D), which was 10 times less sensitive than RT-qPCR. Fig. 1B shows the standard curve generated by plotting the RNA copy number against the cycle (Ct) values of the RNA dilutions tested by RT-qPCR in triplicate using Prism 5.0 software (GraphPad, USA). The Ct values displayed a strong linear correlation ($R^2 = 0.9937$) with the logarithm of the RNA copy numbers (Fig. 1B), indicating robust amplification efficiency of the assay. RNA dilutions ranged from 1×10^7–1×10^2 copies/μL were used to evaluate the reproducibility of the RT-qPCR assay. Intra- and inter-assay coefficients of variation (CV) were calculated as the method previously described (Zhou et al., 2018a). The intra- and inter-assay CVs of the SYBR green-based RT-qPCR ranged from 0.04% to 2.63% and 0.69% to 3.57% respectively, suggesting remarkable reproducibility and high reliability of the assay.

To assess the specificity of this RT-qPCR method, a panel of viruses including porcine reproductive respiratory syndrome virus (PRRSV), porcine epidemic diarrhea virus (PEDV), pseudorabies virus (PRV),

Table 2
Comparative results of clinical samples tested by conventional RT-PCR, SYBR green-based RT-qPCR and probe-based RT-qPCR for SADS-CoV detection.

Samples	Number of samples	Number of SADS-CoV positive samples		
		Conventional RT-PCR	SYBR-based RT-qPCR	probe-based RT-qPCR
Heart	10	3 (13.36–26.16)[a]	5 (13.36–33.67)[b]	5 (18.28–34.41)[c]
Lung	10	4 (14.61–25.23)	6 (14.61–34.45)	6 (20.23–34.98)
Liver	10	4 (15.06–28.63)	7 (15.06–33.23)	7 (20.83–34.04)
Spleen	10	3 (17.23–26.18)	7 (17.23–32.54)	7 (21.35–33.78)
Kidney	10	4 (11.57–27.63)	6 (11.57–31.67)	6 (16.73–32.39)
Jejunum	10	8 (9.36–28.58)	8 (9.36–28.58)	8 (15.28–30.35)
Duodenum	12	9 (8.67–27.78)	11 (8.67–29.67)	11 (14.16–31.41)
Ileum	12	10 (9.17–28.58)	12 (9.17–30.24)	12 (14.79–31.89)
Total	84	45	62	62

[a] Ct values of the positive samples in both SYBR green-based RT-qPCR and conventional RT-PCR.
[b] Ct values of the positive samples in SYBR green-based RT-qPCR.
[c] Ct values of the positive samples in probe-based RT-qPCR.

classical swine fever virus (CSFV), porcine circovirus type 2 (PCV2), transmissible gastroenteritis virus of swine (TGEV), porcine rotavirus (RV), porcine parvovirus (PPV), pig foot-and-mouth disease virus (FMDV), porcine deltacoronavirus (PDCoV) and swine influenza virus (SIV) were tested. All viruses were positive in conventional PCR by their own specific primers and negative by SADS-CoV specific primers. The specific primers were included in Table 1. Distilled water was used as the negative control. The viral genome was extracted by using TGuide virus DNA/RNA kit (TIANGEN, China) following the instruction. When these viruses were tested by the SYBR green-based RT-qPCR assay developed in present study, strong fluorescence signal was only observed for SADS-CoV. No amplification signal was observed throughout the experiment for most of the viruses. Although the fluorescence signal slightly increased after 35 cycles for the PCV2 and PRRSV (Fig. 2A), no specific melting peaks at about 81.57 °C were observed (Fig. 2B), further confirming the specificity of the assay.

To assess the clinical performance of the RT-qPCR for the detection of SADS-CoV, 84 tissue samples from 84 piglets with diarrheal symptoms were collected from a commercial farm in Guangdong, China, and analyzed by the assay. The SADS-CoV-positive status of the samples was determined by the combination of the amplification curve and the melting curve analysis. The samples were also evaluated by conventional RT-PCR and probe-based RT-qPCR which were reported before (Pan et al., 2017; Zhou et al., 2018a). Of 84 samples, 62 samples were SADS-CoV-positive confirmed by SYBR RT-qPCR. When these samples were tested by gel-based RT-PCR, only 45 samples were positive. The positive samples determined by conventional RT-PCR were all positive by SYBR RT-qPCR. Seventeen samples determined negative by conventional RT-PCR were identified as positive samples by SYBR RT-qPCR. When these samples were tested by probe-based RT-qPCR, the detection result was consistent with result by SYBR RT-qPCR. The detection results were summarized in Table 2.

SADS-CoV is a recently discovered virus that has resulted in serious outbreaks and huge financial losses to pig farming industry in south China (Fu et al., 2018; Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018b). Most of these studies applied the conventional RT-PCR to detection of SADS-CoV. However, RT-PCR is time-consuming and cannot determine the amounts of viral copies. A RT-qPCR assay is required for rapid and quantitative detection of SADS-CoV which has been epidemic in the south China.

Recently Zhou et al. reported a probed-based RT-qPCR technique with a detection limit of 30 RNA copies/μL (Zhou et al., 2018a). However the hydrolysis probe increased the experimental cost and the

difficulty of assay design. Thus we established a cost-effective SYBR green-based RT-qPCR assay with high sensitivity and reliability for detection of SADS-CoV. This assay doesn't require a probe and is cost-effective. The limit of detection of the SYBR green-based RT-qPCR is 10 RNA copies/μL, 10 times and 3 times more sensitive than that of the conventional RT-PCR and probed-based RT-qPCR, respectively (Zhou et al., 2018a). The detection limit with 10 RNA copies in this study was theoretical analytical sensitivity, since that the sensitivity experiment was done by serial dilution of RNA standard in water, as the method described in a previous study (Zhou et al., 2018a). To determine more realistic sensitivity of the assay, the sensitivity experiment would be done by spiked RNA from negative tissue homogenate in the future. The positive rate of the clinical samples determined by the SYBR green-based RT-qPCR assay was 73.81%, whereas that of the conventional RT-PCR was merely 53.57%, further demonstrating the higher sensitivity of the SYBR green-based RT-qPCR assay. In the future, it is necessary to detect rectal swabs by the SYBR green-based RT-qPCR assay to evaluate the effect of bacteria in the samples on the detection capacity of the method. The sequences of the primers used in the SYBR RT-qPCR assay are 100% identity with all known SADS-CoV strains and a coronavirus isolated from the bat, suggesting the RT-qPCR assay not only can be used to detect the clinical samples from the swine, but also that from the bats.

In summary, a rapid, sensitive, reliable and cost-effective SYBR green-based RT-qPCR assay was established for the detection of SADS-CoV that poses a huge threat to pig industry. The RT-qPCR not only can be applied to the monitoring of SADS-CoV infection, but also the epidemiology study of the virus. In addition, this assay is of great use for the pathogenicity study of SADS-CoV in pigs. It will prompt the understanding of the tissue tropism of the virus and viral distribution in specific organs.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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