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ORIGINAL ARTICLE

Simultaneous detection of severe acute respiratory syndrome, Middle East respiratory syndrome, and related bat coronaviruses by real-time reverse transcription PCR

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Abstract Since severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) coronaviruses (CoVs) share similar characteristics with respect to clinical signs, etiology, and transmission, methods for a rapid and accurate differential diagnosis are important. Therefore, the aim of this study was to develop a duplex real-time reverse transcription (RT)-PCR method for the simultaneous detection of these viruses. Primers and probes that target the conserved spike S2 region of human SARS-CoV, MERS-CoV, and their related bat CoVs were designed. The results of real-time RT-PCR showed specific reactions for each virus with adequate detection limits of 50–100 copies/mL and 5–100 copies/mL using pUC57-SARS-pS2 (a template for SARS-CoV) and pGEM-MERS-S2 (a template for MERS-CoV), respectively. In addition, this real-time RT-PCR system was able to detect the target viruses SARS-like bat CoV and MERS-CoV in bat fecal samples and sputum of MERS patients, respectively. Therefore, this newly developed real-time RT-PCR method is expected to detect not only SARS-CoV and MERS-CoV in humans but also several bat CoVs that are closely related to these viruses in bats.

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Introduction

The recent emergence of human coronaviruses (CoVs) causing severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) with global spread represents a significant threat to public health. Both SARS-CoV and MERS-CoV cause severe respiratory diseases and belong to the genus *Betacoronavirus* [1]. Phylogenetic analysis has shown that SARS-CoV belongs to lineage B, which includes SARS-like bat CoVs and some other bat-derived CoVs, whereas MERS-CoV belongs to lineage C, which also includes bat-derived CoVs [1].

SARS-CoV and MERS-CoV share similar transmission characteristics. Both viruses are thought to have originated from bats, which are the primary reservoir of diverse CoVs [2]. Cross-species transmission of these viruses to palm civets and dromedary camels has increased the chance of their zoonotic transmission to humans [3]. Nosocomial transmission is thought to be the main cause of human-to-human transmission of SARS-CoV and MERS-CoV [4].

Although there have been no SARS cases reported since 2004, MERS cases have been reported continuously worldwide [3]. In addition, a recent analysis of the SARS-like bat CoVs that use the ACE2 receptor highlighted the need for preparedness for their potential zoonotic transmission [5, 6]. Bat CoVs related to SARS-CoV and MERS-CoV have been reported continuously worldwide [7–10].

Given the high similarity between SARS-CoV and MERS-CoV with respect to clinical signs, etiology, and transmission, establishment of a simultaneous detection method for these viruses would be useful for their accurate

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diagnosis and monitoring in humans as well as their reservoir, bats. Therefore, in this study, a duplex real-time reverse transcription (RT)-PCR method was developed based on primers and probes that target the conserved spike S2 region of SARS-CoV, SARS-like bat CoVs, MERS-CoV, and MERS-related bat CoVs.

Materials and methods

Primers and probes

For the universal detection of SARS-CoV and SARS-like bat CoVs, consensus primers and probes (Fig. 1a) were designed based on the conserved sequences of the spike S2 region by aligning the following reference sequences: human SARS-CoVs Sino1 (GenBank no. AY485277), Tor2 (AY274119), and Urbani (AY278741); SARS-like bat CoVs B15-21 (KU528591), RP3 (DQ071615), RsSHC014 (KC881005), Rf1 (DQ412042), HKU3 (DQ084199), and 273 (DQ648856).

For the detection of MERS-CoV and possible related bat CoVs, consensus primers and probes (Fig. 1b) were designed based on the conserved sequences of the spike S2 region by aligning the following reference sequences: human MERS-CoVs THA-CU (KT225476), Jeddah (KF958702), and KOR-NIH (KT029139); MERS-related bat CoVs A434 (DQ648790), PML-PHE1 (KC869678), GX2012 (KJ473822), HKU5 (EF065512), SC2013 (KJ473821), and HKU4 (EF065508). The designed primers and probes are shown in Table 1.

Preparation of standard plasmids

The partial sequences of the S2 region (nucleotides 3221–3620) of the spike gene of the SARS-CoV Sino1 strain (GenBank no. AY485277), including the primer- and probe-binding regions, were synthesized and inserted in the pUC57 vector at CosmoGenetech Co., Ltd. (Seoul, Korea). The nucleotide sequence of the MERS-CoV S2 domain (amino acids 765–1288, EMC strain, GenBank no. JX869059) was cloned in the pGEM vector by Bioneer (Daejeon, Korea). Competent *Escherichia coli* cells (DH5α) were transformed with the recombinant plasmid vectors (pUC57-SARS-pS2 and pGEM-MERS-S2), and the amplified plasmid was extracted. These plasmids were then used as templates for the positive control as well as in the detection limit test after calculating their copy numbers.

In vitro transcription

A portion of the S2 region of pUC57-SARS-pS2 was amplified using primers containing a T7 promoter sequence: SARS-Tem-F (5'-tcg gta ccT AAT ACG ACT CAC TAT AGG Gaa gaG CCA CCA TGA AGG TGT TTT TGT GTT TAA TGG-3') and SARS-Tem-R (5'-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT tta ATT AGT CCA GCA ATG AAG CC-3'). The amplified PCR product was used as template for *in vitro* transcription using an mMESSAGE mMACHINE T7 kit (Ambion, USA) following the manufacturer's manual. The *in vitro*-transcribed mRNA was treated with Turbo DNase (2 U/μL) and purified with lithium chloride precipitation solution (7.5 M lithium chloride, 50 mM EDTA). The final concentration of SARS-pS2-based mRNA was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

Real-time RT-PCR

A SensiFAST Probe No-ROX One-Step Kit (BIOLINE, Taunton, MA, USA) was used for the real-time RT-PCRs. Each 20-μL reaction consisted of 4 μL of template, 0.8 μL of each primer (SCOV-F/R and MCOV-F/R, 10 μM each), 10 μL of 2X SensiFAST Probe No-ROX One-Step Mix, 0.2 μL of each probe (SCOV-probe and MCOV-probe,

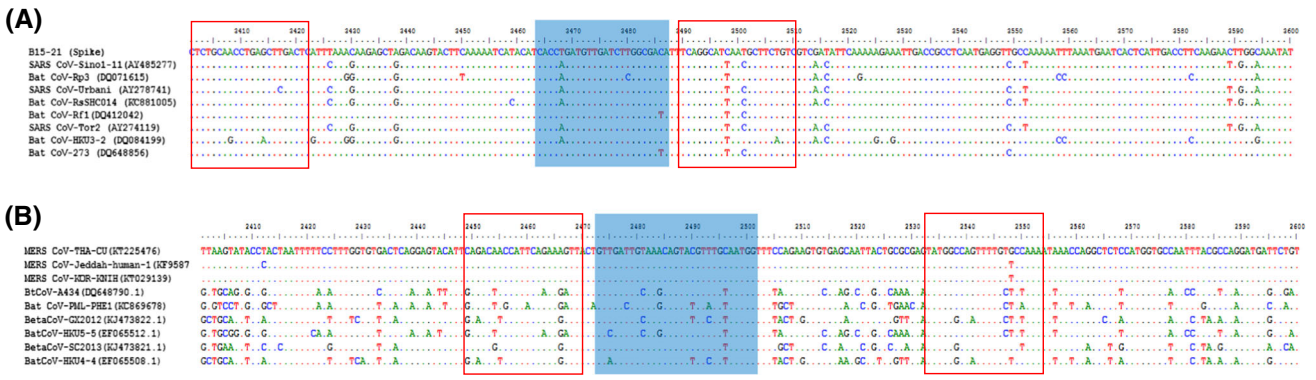

Fig. 1 Multiple sequence alignment of the spike S2 region of SARS-CoV and SARS-like bat CoVs (a) and MERS-CoV and MERS-like bat CoVs (b). Primer-binding sites are indicated by unshaded boxes, and probe-binding sites are indicated by shaded boxes

Table 1 Primers and probes

Target	Oligo name	Sequence	Amplicon size
Spike S2 region of SARS-CoVs and SARS-like bat CoVs	SCOV-F	5′-TCT GCA ACC TGA GCT TGA CT-3′	105 bp
	SCOV-R	5′-ACA GAA GCR TTA ATG CCT GA-3′	
	SCOV-Probe	5′ 6-FAM-CAC CWG ATG TTG ATC TTG GCG ACA-3′ BHQ-1	
Spike S2 region of MERS-CoVs and MERS-related bat CoVs	MCOV-F	5′-CAG ACA ACC ATT CAG AAR GTT A-3′	108 bp
	MCOV-R	5′-TTT AGA ACA AAA CTG GCC ATA-3′	
	MCOV-Probe	5′ 6-HEX-GTT GAT TGT AAA CAG TAC GTT TGC AAT GG-3′ BHQ-1	

10 μM each), 0.2 μL of reverse transcriptase, 0.4 μL of RiboSafe RNase Inhibitor, and 1.8 μL of distilled water. The real-time RT-PCR was performed with reverse transcription at 45 °C for 10 min followed by 95 °C for 2 min and cycling 35 times at 95 °C for 10 s and 60 °C for 20 s. Thermocycling was performed using a LightCycler 96 System (Roche, USA), and positive results were estimated according to analysis of the fluorescent curves originating from each probe within 40 cycles.

Estimation of the detection limits of the real-time RT-PCR

The purified recombinant plasmids were prepared at a concentration of 1×10^8 copies/mL for the templates of SARS-CoV and MERS-CoV. Each template was diluted tenfold in nuclease-free distilled water (Ambion, USA). The diluted plasmids were then used as templates for the real-time RT-PCR to evaluate the detection limits of the primers and probes. The *in vitro*-transcribed mRNA of SARS-CoV pS2 and RNA extracted from MERS-CoV isolate (KOR/KNIH/002_05_2015) were prepared at a concentration of 1×10^4 pg/μL. They were then diluted tenfold with nuclease-free distilled water and used as template for the real-time RT-PCR to evaluate the detection limits of the primers and probes.

Specificity test

To evaluate the specificity of the primers and probes, several RNA viruses, including dengue virus types 2, 3, and 4 (KBPV-VR-29, -30, and -31, respectively; Korea Bank for Pathogenic Viruses, Seoul, Korea), porcine reproductive and respiratory syndrome virus (PRRSV) strain CP401-9, porcine epidemic diarrhea virus (PEDV) strain DR13 (Green Cross Veterinary Products, Yongin, Korea), and influenza A virus (H1N1) strain sk14 from our laboratory, were tested using the newly developed real-time RT-PCR protocol. In addition, RNA extracted from a MERS-CoV isolate (KOR/KNIH/002_05_2015), which was kindly provided by the Korea Center for Disease Control, was tested using the real-time RT-PCR system. A recombinant plasmid including partial sequences of the S2 region (nucleotides 2231–2630 of the spike gene, MERS-like bat CoV strain HKU4 [GenBank no. EF065508]) was synthesized (pUC57-MERS-like-CoV-pS2) and used as a template for the real-time RT-PCR. Agarose gel electrophoresis was performed to observe the target bands as well as for fluorescent detection.

Bat samples

RNA was extracted from 47 bat samples (feces, urine, and oral swabs) collected between July 2015 and April 2016 in Korea. Among them, samples B15-8, B15-40, B15-41, B16-6, and B16-40, and B15-21 were determined to be positive for CoVs according to consensus primers-based RT-PCR, which can detect diverse CoVs by targeting conserved sequences of the RNA-dependent RNA polymerase gene [11]. The positive samples were found to have RNA sequences that are closely related to those of alphacoronaviruses or SARS-like bat CoV [7]. Real-time PCR was performed with the extracted RNAs and compared to the previous RT-PCR results.

Human samples

MERS-CoV-positive RNA samples extracted from the sputum of MERS-CoV-infected patients were kindly provided by the Department of Laboratory Medicine of Seoul National University Hospital, Korea. According to the regulations of Category B UN 3373, the samples were packaged with triple-packaging shells following the P620 UN packaging guideline and transported to the animal biosafety-level 3 facility of the National Primate Research Center of the Korea Research Institute of Bioscience and Biotechnology by the Green Cross Labcell company, a specialist in the transport of clinical samples. The single (MERS-CoV-specific primers and probe only) and duplex real-time RT-PCR were performed with these RNAs and

compared with the results of UpE-based real-time RT-PCR as a reference [12].

Results

Detection limits of the real-time RT-PCR

The tenfold-diluted plasmids (pUC57-SARS-pS2 and pGEM-MERS-S2) were tested by real-time RT-PCR as a single template or as mixed templates. The intensity of the fluorescence (FAM and HEX) emitted from the hydrolyzed probes was measured every cycle. Based on the measured amplification curve, the results were expressed as the quantification cycle value (Cq value). The Cq values obtained from the diluted plasmids were found to be significantly correlated with the copy numbers of each plasmid, with an R^2 value of 0.9847 and 0.9954 for pUC57-SARS-pS2 and pGEM-MERS-S2, respectively (Fig. 2). The negative control (distilled water) did not yield an amplification curve. As shown in Table 2, the detection limits of the real-time RT-PCR for pUC57-SARS-pS2 and pGEM-MERS-S2 were both 1×10^2 copies/mL in the single-template condition and were 5×10^1 and 5×10^0 copies/mL, respectively, in the mixed-template condition. The detection limits of the real-time RT-PCR for the *in vitro*-transcribed mRNA of pUC57-SARS-pS2 and viral RNA of MERS CoV (KOR/KNIH/002_05_2015) were 1×10^{-3} and 1×10^0 ng/mL, respectively, in the single-template condition and were 5×10^{-3} and 5×10^1 ng/mL, respectively, in the mixed-template condition (Table 2).

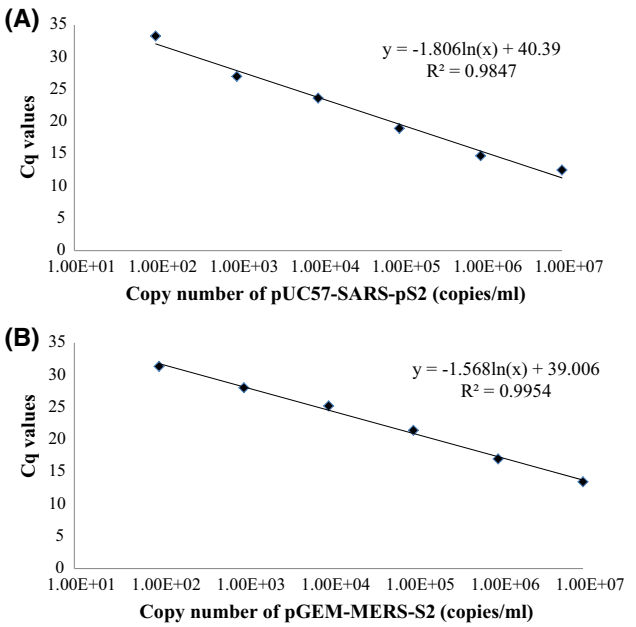

Fig. 2 Correlation curve for plasmids copy number vs. and Cq value. (a) pUC57-SARS-pS2, (b) pGEM-MERS-S2

Specificity of the real-time RT-PCR

The specificity of the real-time RT-PCR method developed in this study was evaluated using RNAs from several RNA viruses, including MERS-CoV (KOR/KNIH/002_05_2015), a recombinant plasmid for the bat CoV HKU4 strain, and RNA from a bat fecal sample containing SARS-like bat CoV. The designed primers and probes were found to be specific for MERS-CoV and SARS-like bat CoV (B15-21) (Table 3). No positive reactions were found for the bat coronavirus strain HKU4, dengue virus, PRRSV, PEDV, or influenza A virus. In the case of the HKU4 strain, although the HEX fluorescence signal was not detected, a target band of primers for MERS-CoV was detected by agarose gel electrophoresis.

Application by the real-time RT-PCR method from bat fecal samples

The extracted RNAs from a total of 47 bat samples that were previously tested using consensus primer-based RT-PCR [11] were re-evaluated by the real-time RT-PCR method developed in this study. As shown in Table 4, six fecal samples were positive in the consensus-primer-based RT-PCR, and these were further sequenced and analyzed using BLASTn (http://www.ncbi.nlm.nih.gov). Among the six positive samples, five were found to be closely related to alphacoronaviruses, while one sample (B15-21) was closely related to SARS-like bat CoV [7]. In the real-time PCR developed in this study, five of the alphacoronavirus-positive samples were negative, but only one SARS-like bat CoV-positive sample was positive.

Application of the real-time RT-PCR method for MERS patient samples

The RNA samples from the sputum of MERS patients were tested by the newly developed real-time RT-PCR method, and the results were compared to those obtained with UpE-based real-time RT-PCR as a reference gold-standard test (Table 5). Among the 20 samples tested, 17 were found to be positive by UpE-based real-time RT-PCR. When the single set of primers and probe for MERS-CoV was used, the newly developed real-time RT-PCR results showed 100% consistency with those of the reference test. However, when mixed sets of primers and probes for SARS-CoV and MERS-CoV were used, 85% of the results were consistent with those of the reference test. Three false-negative samples had Cq values of 33.9, 33.2, and 36.46, respectively, in the reference test.

Table 2 Detection limits of real-time RT-PCR

Condition	Plasmid concentration (copies/mL)		RNA concentration (ng/mL)	
	pUC57-SARS-pS2	pGEM-MERS-S2	SARS-pS2[a]	MERS-CoV[b]
Single template	1×10^2	1×10^2	1×10^{-3}	1×10^0
Mixed template	5×10^1	5×10^0	5×10^{-3}	5×10^1

[a] mRNA obtained by *in vitro* transcription

[b] RNA from MERS-CoV isolate KOR/KNIH/002_05_2015

Table 3 Specificity of the real-time RT-PCR with various templates

Virus	Virus family	Template type	Agarose gel electrophoresis	Cq	
				FAM	HEX
Dengue virus 2 (KBPV-VR-29)	*Flaviviridae*	RNA	-	-	-
Dengue virus 3 (KBPV-VR-30)	*Flaviviridae*	RNA	-	-	-
Dengue virus 4 (KBPV-VR-31)	*Flaviviridae*	RNA	-	-	-
PRRSV (CP401-9)	*Arteriviridae*	RNA	-	-	-
PEDV (DR13)	*Coronaviridae*	RNA	-	-	-
Influenza A virus (sk14)	*Orthomyxoviridae*	RNA	-	-	-
MERS-CoV (KOR/KNIH/002_05_2015)	*Coronaviridae*	RNA	+	-	15.64
MERS-like bat CoV (HKU4)	*Coronaviridae*	Plasmid	+	-	-
SARS-like bat CoV (B15-21)	*Coronaviridae*	RNA	+	12.55	-

Table 4 Real-time RT-PCR results from bat samples

		Positive[a]		Negative
		Alphacoronavirus	SARS-like CoV	
Real-time RT-PCR	Positive	0	1	0
	Negative	5	0	41

[a] Samples that were positive in the RT-PCR (11) were sequenced and analyzed using BLASTn (http://www.ncbi.nlm.nih.gov)

Discussion

The aim of this study was to develop a duplex real-time RT-PCR method for the simultaneous detection of SARS-CoV and MERS-CoV, which are recently emerged pathogens infecting humans. As these viruses are also closely related to several bat CoVs (SARS-like bat CoVs and MERS-related bat CoVs), the primers and probes were designed based on the conserved region of the spike S2 domains of SARS-CoV, MERS-CoV, and their related bat CoVs. In a previous study, two regions within the spike protein of SARS-CoV were identified that showed a high degree of sequence conservation with those of other CoVs [13]. By applying our new approach, we expected to detect not only SARS-CoV and MERS-CoV in humans but also several bat CoVs that are closely related to these viruses.

Indeed, the newly developed real-time RT-PCR method could detect SARS-CoV- and MERS-CoV-specific sequences when the plasmids pUC57-SARS-pS2 and pGEM-MERS-S2 were used as templates. In addition, positive results were obtained when RNA extracted from MERS-CoV (KOR/KNIH/002_05_2015), which was isolated from a patient in Korea in 2015 [14], was used as a template.

The new real-time RT-PCR method also showed positive results for RNA extracted from a fecal sample containing SARS-like bat CoV (B15-21) [7]. In addition, no nonspecific amplification was found when using RNAs obtained from several RNA viruses belonging to the families *Flaviviridae, Arteriviridae,* and *Orthomyxoviridae,* and the genus *Alphacoronavirus*. The specific binding of the primers and probes used in the real-time RT-PCR was evaluated to determine their detection limits using serially diluted plasmids (pUC57-SARS-pS2 and pGEM-MERS-S2). The detection limits of the real time RT-PCR were 50–100 copies/mL and 5–100 copies/mL for pUC57-SARS-pS2 and pGEM-MERS-S2, respectively. As the previous real-time RT-PCRs were able to detect as few as 1000 copies/mL and 291 copies/mL for SARS-CoV and their related bat CoVs and MERS-CoV, respectively

Table 5 Results of duplex real-time RT-PCR with RNA extracted from MERS patients

Sample no.	UpE-based real-time RT-PCR	Real-time RT-PCR with single primers and probe set for MERS-CoV	Real-time RT-PCR with mixed primers and probe sets for SARS-CoV and MERS-CoV
1	33.9[a]	35.1	-
2	32.39	33.28	31.04
3	30.27	30.88	30.01
4	-[b]	-	-
5	31.64	31.4	29.23
6	30.39	30.52	29.48
7	-	-	-
8	34.63	35.24	33.67
9	-	-	-
10	33.2	34.99	-
11	24.2	23.97	24.15
12	29.44	29.36	29.39
13	29.14	30.44	29.08
14	21.2	21.43	21.74
15	31.54	31.37	31.25
16	36.46	35.63	-
17	30.45	30.01	30.64
18	28.9	29.15	29.83
19	27.93	28.17	29.88
20	33.45	34.51	35.19

[a] Cq value with HEX dye

[b] No HEX signal

[12, 15], the duplex real-time RT-PCR method developed in this study can be applied with an adequate limit of detection.

When the real-time RT-PCR was applied to the bat samples, it could specifically detect a SARS-like bat CoV, and no positive reactions were obtained with the other samples, including alphacoronavirus-positive samples. The real-time RT-PCR method developed in this study could not detect the bat CoV HKU4 strain, which belongs to the same group as human MERS-CoV. Although these viruses are in the same group, the nucleotide sequences were found to be more variable than those of SARS-CoV and its related bat CoVs (Fig. 1). However, as a target band was found in the agarose gel electrophoresis of a PCR product from the HKU4 template, the negative result obtained with HKU4 in the real-time RT-PCR method developed in this study might have been due to inefficient binding of the probe.

The new real-time RT-PCR method was also tested with RNAs extracted from the sputum of MERS patients in Korea. The single set of primers and probe for MERS-CoV could detect the same positive samples detected in the reference test, showing 100% agreement. However, when the mixed sets of primers and probes for SARS-CoV and MERS-CoV were used, the agreement was reduced to 85%. Three samples that tested positive in the reference test were not detected with the newly developed method. This may be due to the low amount of viral RNA in the sputum samples and the reduced sensitivity due to the duplex approach. In addition to using a standard test for the diagnosis of MERS-CoV infection in humans, consideration of additional tests would be helpful to avoid missing true positives.

Collectively, the newly developed real-time RT-PCR method was demonstrated to be applicable for the simultaneous detection of SARS-CoV and MERS-CoV, showing an adequate detection limit and specificity. By testing the new method with bat samples as well as human samples, it could be applicable to survey SARS-CoV, MERS-CoV, and potentially their related bat CoVs in bats and human samples. It could successfully detect SARS-like CoV in bat samples but showed limited detection ability for the bat CoV HKU4 strain, which is related to MERS-CoV. However, according to a recent finding of EMC-like MERS-CoV, which was detected in bats of Saudi Arabia [18], we assume that the new method can be helpful for screening for MERS-CoV in bat samples.

As novel CoVs continue to emerge around the world, real-time-PCR-based detection methods have been developed by pioneering researchers [12, 15–17]. These methods

are applicable with good limits of detection and have been validated using field samples. However, simultaneous detection of SARS-CoV, MERS-CoV, and their related bat CoVs would be particularly useful for the detection of these viruses in humans as well as in bats, which are a known reservoir of the viruses.

Therefore, the duplex real-time RT-PCR approach for the simultaneous detection of SARS-CoV and MERS-CoV developed in this study might allow more-convenient and rapid detection of these viruses in clinical samples. In addition, this new method can be used to closely monitor related bat CoVs in bat populations, which are considered to be a primary reservoir of these viruses.

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Compliance with ethical standards

Conflict of interest The authors have declared that no competing interests exist.

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