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Development and Evaluation of a Multitarget Real-Time Taqman Reverse Transcription-PCR Assay for Detection of the Severe Acute Respiratory Syndrome-Associated Coronavirus and Surveillance for an Apparently Related Coronavirus Found in Masked Palm Civets

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Severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) is the etiological agent of SARS. It is believed that SARS-CoV originates from wild animals. We have developed a multitarget real-time Taqman reverse transcription-PCR (RT-PCR) assay for the quantitative detection of SARS-CoV. The sequences of the Taqman probes with a minor groove binder and the corresponding primers were based on the sequences of the N gene, open reading frame (ORF) 3, and ORF 8. The overall linear range of this assay was from at least 10^1 to 10^6 copies per reaction, and the detection limit could reach less than 10 copies per reaction. The quantification results for SARS-CoV from cell culture correlated well with those of the RT-PCR by using any two of the three sets of primer and probe used in this assay. However, the results of quantification of SARS-CoV obtained by using a few available throat swab specimens from SARS patients and the N gene as the target were almost 10 times higher than those obtained by using ORF 3 and ORF 8. Using this assay, we also detected an apparently SARS-CoV-related coronavirus in the throat swab specimens from masked palm civets in the west part of Hubei Province, People's Republic of China.

The outbreak of severe acute respiratory syndrome (SARS) in 2003 severely harmed public health and the global economy (11). The etiologic agent of SARS was identified as a novel coronavirus, the SARS-associated coronavirus (SARS-CoV) (3, 11, 18), an enveloped, positive-strand RNA virus with a genome size of 29 to 30 kb (7, 14). Although the SARS outbreak passed with time (22), two problems remain unsolved: the early diagnosis of SARS-CoV infections and the origin of SARS-CoV.

In the absence of effective drugs and vaccines, the placement of patients with SARS-CoV infection under quarantine is the only effective way to prevent the spread of SARS. However, the early diagnosis of SARS is difficult because its general symptoms are shared by many other kinds of atypical pneumonias, such as those caused by *Chlamydia* species and mycoplasmas. At present, PCR testing holds more promise for the early diagnosis of SARS-CoV infection than serologic testing and virus isolation (21). Since the SARS outbreak, many reverse transcription-PCR (RT-PCR) and real-time RT-PCR assays have been established for the early diagnosis of SARS (2, 4, 8, 10, 19, 23). Almost all these assays, however, use open reading frame (ORF) 1b of the RNA polymerase gene or the nucleocapsid (N) gene, or both, as their targets.

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There appears to be a reservoir for the SARS-CoV in wild animals. A high proportion of early SARS patients were food handlers with likely animal contact in Guandong Province, People's Republic of China (16), and the genome sequence of SARS-CoV is distinct from those of other known human coronaviruses (14, 20), suggesting an animal origin of SARS-CoV. Furthermore, during the outbreak of SARS in 2003, a research team identified an apparently SARS-CoV-related coronavirus from Himalayan palm civets (*Paguma larvata*) and detected that cornavirus in a raccoon dog (*Nytereutes procyonoides*) in a live-animal market in Guandong, People's Republic of China (5).

In the study described here, we developed and evaluated a multitarget real-time RT-PCR assay for the quantitative detection of SARS-CoV. In this assay, we chose the N gene, ORF 3, and ORF 8 from the SARS-CoV Tor2 strain (GenBank accession number AY274119) as targets and used Taqman probes with a minor groove binder (MGB) in place of traditional Taqman probes. This assay was performed with both human clinical specimens and specimens from masked palm civets (*P. larvata*) raised in the western part of Hubei Province, which was unaffected by the SARS outbreak.

MATERIALS AND METHODS

Virus culture. The SARS-CoV strain used for standard titration in this study was isolated from SARS patient W20 from Wuhan, Hubei, People's Republic of China. Vero E6 cells were inoculated with throat swab specimens from the patient, and the supernatant was harvested after a clear cytopathic effect was observed, which was about 60 h postinfection. The 50% tissue culture infective dose ($TCID_{50}$) was titrated by an endpoint dilution assay.

TABLE 1. Primer and probe sets for real-time RT-PCR[a]

Target gene	Primer or probe	Sequence (5′→3′)	T_m (°C)	Location
N gene	FP	AGGAACTGGCCCAGAAGCTT	59	28461–28480
	RP	AACCCATACGATGCCTTCTTTG	59	28500–28521
	Probe	ACTTCCCTACGGCGCTA	69	28482–28498
ORF 8	FP	ACTACTAAAAGAACCTTGCCCATCAG	59	27359–27384
	RP	TATTGTCAGCAAGAGGGTGAAATG	59	27406–27429
	Probe	TGAATTGCCCTCGTATGTT	70	27386–27404
ORF 3	FP	TCTCCTGCAAGTACTGTTCATGCT	59	25337–25360
	RP	GCCATCCGAAAGGGAGTGA	60	25385–25403
	Probe	CAACGATACCGCTACAAG	69	25365–25382

[a] Abbreviations: FP, forward primer; RP, reverse primer; T_m, melting temperature. The target gene name and location are based on the SARS-CoV Tor2 strain (GenBank accession number AY274119).

RNA extraction. RNA was extracted from 200 μl virus culture supernatant or specimens with TRIzol reagent (Invitrogen), according to the manufacturer's instructions. The RNAs extracted from the supernatants of infected cells, human clinical specimens, and masked palm civet specimens were dissolved in 20, 30, and 45 μl diethyl pyrocarbonate-treated water, respectively, and were stored at −80°C until use.

Preparation of quantitative standards. The RNA transcripts of the N gene, ORF 3, and ORF 8 were used as quantitative standards in the real-time RT-PCR assay. The N gene, ORF 3, and ORF 8 were amplified from viral RNA by RT-PCR with a OneStep RT-PCR kit (QIAGEN), according to the manufacturer's instructions. The RT-PCR primers were designed according to the sequence of the SARS-CoV Tor2 strain (GenBank accession number AY274119) and had the following sequences: N-forward, 5′-ATGTCTGATAATGGACCC CAA-3′; N-reverse, 5′-TTATGCCTGAGTTGAATCAGC-3′; ORF 8-forward, 5′-CATATGAAAATTATTCTCTTCCTGAC-3′; ORF 8-reverse, 5′-GGATCC TCATTCATTCTGTCTTTCTC-3′; ORF 3-forward, 5′-CATATGGATTTGTT TATGAGATTTTTT-3′; and ORF 3-reverse, 5′-GGATCCTCATTCATTCTG TCTTTCTC-3′. The three amplicons were cloned to the pT-Easy vector (Promega, Madison, WI) and oriented under the control of the T7 promoter. Each plasmid DNA was linearized by digestion with Spe to provide the template for RNA transcription. The RNA transcripts of these three genes were synthesized in vitro by using Riboprobe System—T7 (Promega), according to the manufacturer's protocols. Plasmid DNA was removed with RQ1 RNase-free DNase (PromegaWI) after in vitro transcription was performed, and the RNA transcripts were purified by phenol-chloroform extraction and ethanol precipitation. The RNA transcripts of the N gene, ORF 3, and ORF 8 were 1333, 889, and 443 nucleotides, respectively, and all RNA transcripts were positive sense. The RNA concentrations were determined by using a Lambda 25 UV/VIS spectrometer (Perkin-Elmer, Inc.), and the copy number was calculated by using the molecular weight of the RNA for each of the RNA transcripts of the three genes. The RNA transcripts were serially diluted 10-fold, and each diluted standard was divided into aliquots for single use and were stored at −80°C until use.

Primer and probe sets. The primer and probes sets (Table 1) were designed from the sequences of the N gene, ORF 3, and ORF 8 of the Tor2 strain of SARS-CoV by using Primer Express software (version 2.0; Applied Biosystems). Taqman-MGB probes were labeled at the 5′ end with the reporter dye 6-carboxyfluorescein and with the quencher dye 6-carboxy-*N*,*N*,*N*,*N*-tetramethylrhodamine and with MGB at the 3′ end. The three sets of primers and probes were synthesized by Genecore BioTechnologies Co. (Shanghai, People's Republic of China). The amplicon sizes were 61 bp, 71 bp, and 67 bp for the N gene, ORF 8, and ORF 3, respectively. The regions on the SARS-CoV genome (Tor2 strain; GenBank accession number AY274119) covered by the three primer and probe sets were positions 28461 to 28521, positions 27359 to 27429, and positions 25337 to 25406 for the N gene, ORF 8, and ORF 3, respectively.

Real-time RT-PCR. The real-time RT-PCR assays were performed on an Opticon DNA engine (MJ Inc.) by using a OneStep RT-PCR kit (QIAGEN). Each 50-μl reaction mixture contained 2 μl of enzyme mix, 10 μl of 5× buffer, 10 μl of 5× Q solution, 1 μl of 10 mM deoxynucleoside triphosphates (final concentration, 200 μM), 1 μl of 10 μM probe (final concentration, 200 nM), 1 μl each of 50 μM forward and reverse primers (final concentrations, 1 μM each for the forward and the reverse primers), 5 μl of RNA template, and 19 μl of RNase-free water. The thermal cycling conditions were 50 min at 50°C for reverse transcription, 15 min at 95°C for hot activation, and 45 cycles of 15 s at 94°C and 30 s at 59°C. Each run included SARS-CoV genome RNA as a positive control and three negative controls. The three negative controls were used for the monitoring of cross-contamination of the RNA extracted from human clinical specimens, animal specimens, and the sample loading step. The test results were considered valid only if the three negative controls and the positive control gave the expected results. Fluorescence measurements were taken after each cycle, and the threshold cycle (C_T) was defined as the cycle number in which there was an increase in fluorescence above the calculated background levels. The result was considered positive if the C_T value was less than 45. A specimen was considered positive if the results for two or more sets of primer and probe were positive. The different procedures of the experiment (processing of RNA, mixing of the real-time RT-PCR reagents, and loading of the samples) were conducted in different biosafety cabinets with aerosol-resistant tips to avoid cross-contamination.

Human clinical specimens. Nine clinical specimens, which consisted of throat swab specimens from nine individuals, were used for testing. Among these nine individuals, five had been confirmed to have SARS-CoV infections, while the other four were non-SARS patients. Human clinical specimen processing was performed in a biosafety level 3 laboratory. The use of human clinical specimens complied with the relevant national guidelines of the People's Republic of China.

Masked palm civet specimens. Specimens were collected from masked palm civets on a farm located in the western part of Hubei Province, People's Republic of China, a location unaffected by the 2003 SARS epidemic. The farmer raised about 260 masked palm civets, with each animal housed in a small wire cage. The first generation of these masked palm civets had been captured from the surrounding mountain area. Forty throat swab specimens from 40 randomly selected masked palm civets were screened by an RT-PCR targeting the membrane (M) gene of SARS-CoV by using the following primer sequences: 5′-CTTTGCTAG TACAGTAAGTG-3′ and 5′-GGATCCACTTACTGTACTAGCAAAG-3′. This study used seven throat swab specimens collected from seven distinct masked palm civets between 23 April 2004 and 25 April 2004, and the animals were positive for SARS-CoV by screening for the SARS-CoV M gene. The throat swab specimens were dissolved in 500 μl phosphate-buffered saline containing 0.5% bovine serum albumin and were stored at −80°C until use. The masked palm civet specimens were processed separately from the human clinical specimens in the biosafety level 3 laboratory. The use of the masked palm civet specimens complied with the relevant national guidelines of the People's Republic of China.

Statistical analysis. The coefficient of variation of the real-time Taqman RT-PCR assay was calculated by using Excel 2000 software. The linear relationship of the curves generated in this study was analyzed by using Origin (version 7.5) software (OriginLab Corporation).

RESULTS

Analytical sensitivity and dynamic range of the Taqman-MGB real-time RT-PCR assay for SARS-CoV. To assess the detection limit and dynamic range of the Taqman-MGB real-time RT-PCR assay for SARS-CoV in terms of the copy number, the RNA transcripts of the N gene, ORF 3, and ORF 8 were serially diluted 10-fold and tested nine times with the corresponding primer and probe sets in three different runs,

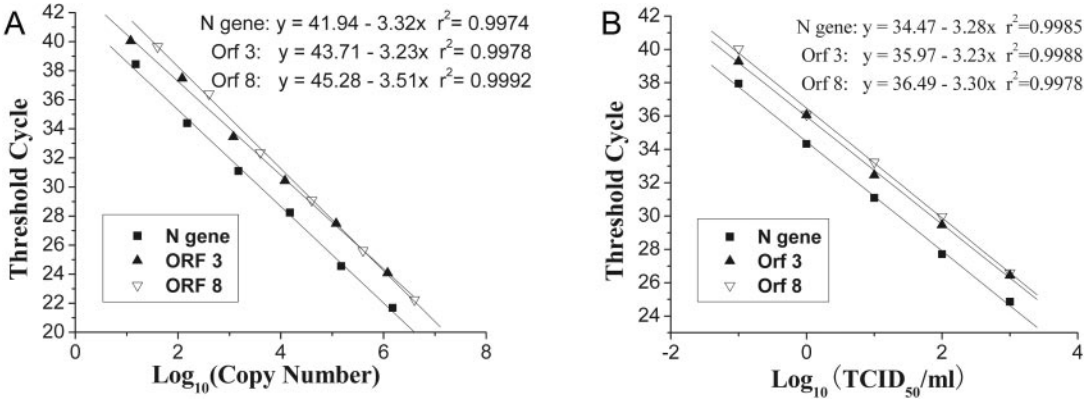

FIG. 1. Standard curves generated for the N gene, ORF 3, and ORF 8. (A) Standard curves generated from the RNA transcripts of the N gene, and ORF 8. Each curve was based on the average of nine replicates. (B) Standard curves generated from the RNA extracted from 10-fold serially diluted live virus. Each curve is based on the average of three replicates.

with each run containing three replicates. Analysis of the RNA transcripts showed that this assay could detect as few as 15 transcript copies per reaction for the N gene, 12 transcript copies per reaction for ORF 3, and 4 transcript copies per reaction for ORF 8. There was a strong linear relationship ($r^2 \geq 0.99$) between the logarithm of the input transcript copy number and the mean C_T value for the nine replicates at least over a 6-log-unit range, from approximately 10^1 to 10^6 copies per reaction, for each of the three primer and probe sets (Fig. 1A). To evaluate the assay's limit of detection of live virus, the RNA extracted from the supernatant of infected Vero E6 cells (isolate W20; 10^7 $TCID_{50}$s/ml) was serially diluted 10-fold and then tested three times with each of the three primer and probe sets. The lower limit of detection of live virus was 5×10^{-5} $TCID_{50}$s per reaction (0.01 $TCID_{50}$/ml) for the N gene and 5×10^{-4} $TCID_{50}$s per reaction (0.1 $TCID_{50}$/ml) for ORF 3 and ORF 8. A strong linear relationship between the logarithm of $TCID_{50}$/ml and the C_T value was obtained over at least 5 log units, ranging from 0.1 to 1,000 $TCID_{50}$s/ml, for all three primer and probe sets (Fig. 1B).

Reproducibility and amplification efficiency. The intra-assay and interassay variabilities over the linear range of this assay were evaluated by testing each dilution from 10-fold serially diluted RNA transcripts in triplicate in three independent runs. The results obtained are summarized in Table 2. At the lowest concentrations for the N gene (1.5 copies transcripts per reaction) and ORF 8 (4 copies transcripts per reaction), the test was reproducible, with seven and nine positive results of nine replicate reactions, respectively; however, the interassay coefficients of variation exceeded 5%. With the lowest concentration for ORF 3 (1.2 copies transcripts per reaction), the reproducibility was less than 50%. The slopes of the standard curves generated from the RNA transcripts and live virus ranged from −3.23 to −3.51 and from −3.18 to −3.30, respectively; therefore, the amplification efficiency was above 90% in all cases.

Specificity. As we had only a limited number of clinical samples and few other kinds of related viruses, the specificity of the assay was determined by testing four human clinical specimens from non-SARS patients and comparing the sequences of the primer and probe sets with those in the GenBank database by using the BLAST program. The four clinical specimens from non-SARS patients and five clinical specimens from patients confirmed to have SARS all gave the expected results when they were challenged by this assay. The BLAST searches of the sequences that covered the three primer and probe sets (positions 28461 to 28521, 27359 to 27429, and 25337 to 25403 in the genome of the SARS-CoV Tor2 strain) indicated that these sequences were identical in all SARS-CoV isolates ($n = 123$) sequenced so far (as of 26 August 2004).

Correlation among the results generated from the three different target genes. To evaluate the correlation of the data generated by targeting each of the three genes, the RNAs extracted from the supernatants of infected Vero E6 cells with

TABLE 2. Reproducibility of the real-time RT-PCR assay

RNA transcript copy no.			Mean C_T value[a]			Intra-assay CV[b] (%)[c]			Interassay CV (%)[d]		
N gene	ORF 3	ORF 8	N gene	ORF 3	ORF 8	N gene	ORF 3	ORF 8	N gene	ORF 3	ORF 8
1.5×10^6	1.2×10^6	4×10^6	21.68	24.07	22.24	1.62	1.35	0.89	0.37	0.68	0.27
1.5×10^5	1.2×10^5	4×10^5	24.55	27.48	25.62	1.31	0.69	0.71	1.00	0.6	0.68
1.5×10^4	1.2×10^4	4×10^4	28.24	30.43	29.08	1.27	0.69	1.02	1.55	0.73	0.45
1.5×10^3	1.2×10^3	4×10^3	31.09	33.46	32.36	0.68	0.41	0.55	1.01	0.6	1.14
1.5×10^2	1.2×10^2	4×10^2	34.39	37.48	36.41	0.96	0.51	0.73	0.87	0.73	0.22
1.5×10^1	1.2×10^1	4×10^1	38.45	40.05	39.66	0.73	0.49	0.99	0.76	0.52	0.72

[a] Determined from nine replicates.
[b] CV, coefficient of variation.
[c] Determined from three replicates within each dilution.
[d] Determined from three independent assays performed on different days.

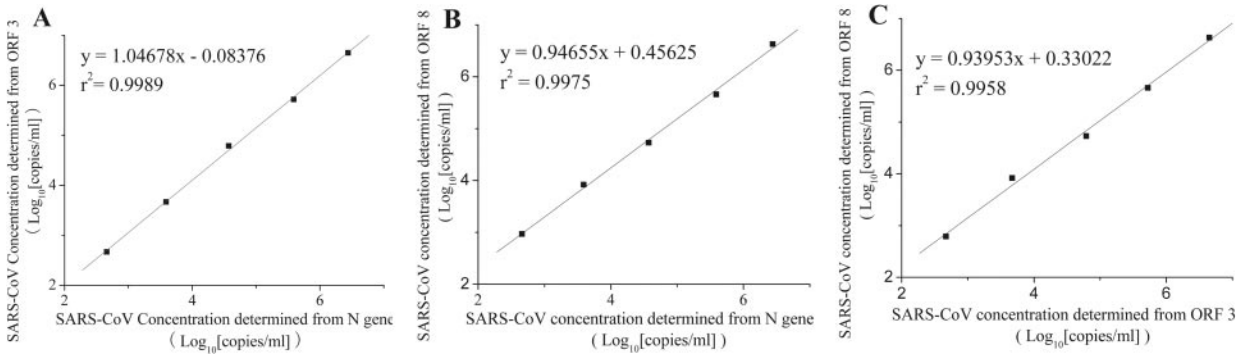

FIG. 2. Correlation of quantification results of 10-fold serially diluted live virus by using real-time RT-PCR targeting the N gene, ORF 3, and ORF 8. Each curve is based on the average results of three replicates. (A) Correlation between the results generated from the N gene and ORF 3; (B) correlation between the results generated from the N gene and ORF 8; (C) correlation between the results generated from ORF 3 and ORF 8.

titers of 10^3, 10^2, 10^1, 10^0, and 10^{-1} $TCID_{50}$s/ml were tested with each of the three primer and probe sets. The results obtained with each of the three genes in terms of the numbers of copies/ml were calculated from the corresponding standard curves generated from the RNA transcripts and then plotted against each other. The results of these plots (Fig. 2) revealed that the concentration of SARS-CoV determined from any of these three genes had a strong correlation ($r^2 \geq 0.99$) with that determined from the other two genes. The slope ranges (0.94 to 1.05) and the *y*-axis intercepts (−0.08 to 0.45) of these three curves indicated that the SARS-CoV concentration from each dilution determined from these three genes were on the same order.

Evaluation with clinical specimens. The RT-PCR assay was used to test nine clinical specimens, all throat swab specimens, from five patients with confirmed SARS-CoV infection and four non-SARS patients (Table 3). Specimens from patients confirmed to have SARS all tested positive, and those from non-SARS patients all tested negative. Specimen H2003004 had the highest virus concentration (in number of copies/ml), according to the results of this assay, in agreement with the fact that this patient had been identified to be a SARS superspreader.

Evaluation with masked palm civet specimens. A total of seven throat swab specimens from seven different masked palm civets, which were selected from about 260 masked palm civets raised on a farm, were tested by the RT-PCR assay (Table 3). Among these specimens, one was positive by using the three primer and probe sets, another two were positive by using two primer and probe sets, and the other four were positive by using one primer and probe set.

DISCUSSION

In the multitarget Taqman real-time RT-PCR assay described here, a detection limit of <10 transcript copies was reached with the primer and probe set targeting ORF 8. However, in terms of the $TCID_{50}$, the primer and probe set targeting the N gene was more sensitive than those targeting ORF 3 and ORF 8, since it could detect live virus at levels as low as

TABLE 3. Results of the real-time RT-PCR assay with specimens from patients and masked palm civets[a]

Specimen no.	Real-time RT-PCR C_T value[b]			Log_{10} virus concn (no. of copies/ml)[c]		
	N gene	ORF 3	ORF 8	N gene	ORF 3	ORF 8
H2003001	30.06	37.49	38.34	5.06	3.41	3.46
H2003002	28.46	35.54	36.76	5.54	4.01	3.90
H2003003	13.3	18.42	18.22	10.11	9.31	9.20
H2003004	10.95	15.67	15.63	10.81	10.17	9.93
H2003005	33.99	36.25	37.74	3.87	3.79	3.63
H2003501	ND	ND	ND			
H2003502	ND	ND	ND			
H2003503	ND	ND	ND			
H2003504	ND	ND	ND			
MPC200404081	29.13	ND	ND	5.81		
MPC200404084	ND	ND	ND			2.54
MPC200404085	22.57	41.28	40.34	7.99	2.7	3.36
MPC200404088	28.06	ND	ND	6.12		
MPC200404252	ND	27.72	42.66		6.9	2.70
MPC200404256	ND	41.89	43.29		2.51	2.52
MPC200404258	ND	N	42.16			2.84

[a] Abbreviations: H, human; MPC, masked palm civet; ND, not detected.
[b] The mean of triplicate values.
[c] Calculated from the corresponding C_T values by using standard curves generated from RNA transcripts.

0.01 $TCID_{50}$/ml, whereas the detection limit for the other two sets was 0.1 $TCID_{50}$/ml. There was a strong linear relationship over a wide dynamic range, from 10^1 to 10^6 copies per reaction or from 10^{-1} to 10^3 $TCID_{50}$s/ml, with low intra- and interassay variabilities by use of any of the three primer and probe sets. We chose the Taqman-MGB technology mainly because it has more sequence specificity than the traditional Taqman probes without the MGB molecule (12). In addition, Taqman-MGB probes are more stable than traditional Taqman probes and have an improved signal-to-noise ratio resulting from the presence of the MGB molecule (1). The combined use of the three primer and probe sets in the assay may ensure the assay's specificity.

The lack of sufficient human clinical specimens from patients confirmed to have SARS limits the ability to verify our assay. However, on the basis of its performance with the five specimens available from SARS patients, we noticed that the SARS-CoV concentrations in four specimens in terms of copy number/ml determined in assays with the N gene were about 10 times higher than those determined in assays with ORF 3 and ORF 8, while the SARS-CoV copy numbers in the other specimens determined in assays with these three targets coincided well. Analysis of the correlation among the results obtained by targeting these three genes and by using RNA extracted from the supernatants of infected Vero E6 cells with titers of 10^3, 10^2, 10^1, 10^0, and 10^{-1} $TCID_{50}$s/ml showed that the SARS-CoV concentration determined by use of all three of these genes for a certain dilution were on the same order. Therefore, it is unlikely that the difference of SARS-CoV concentrations of approximately 10 times obtained from the same human clinical specimen by targeting ORF 3, ORF 8, and the N gene is the result of inherent differences in the binding kinetics of the probes to the different target regions. The subgenomic RNAs generated during virus replication are short-lived and are mostly restricted to the intracellular environment (2); furthermore, the sequences for ORF 3, ORF 8, and the N gene are not far away from each other on the virus's genome (the longest distance between these three genes is about 3 kb, between ORF 3 and the N gene on the genome); thus, in the throat swab specimens from SARS patients, the subgenomic RNAs, if there are any, are unlikely to have a strong influence on the overall copy number determined from any of these three genes.

Almost all of the previously established real-time RT-PCR assays for the detection of SARS-CoV infection chose ORF 1b of the RNA polymerase gene or the N gene, or both, as their targets (2, 4, 8, 9, 10, 15, 19). Hourfar et al. (8) showed that the virus copy numbers determined from the N gene and ORF 1b were in agreement with each other by using the virus from cell culture; however, they did not compare the quantitative results for specimens from SARS patients in their paper. Emery et al. (4) published the C_T values determined from ORF 1b and the N gene, respectively, for some specimens from SARS patients; and we calculated the virus copy numbers in the throat swab and throat wash specimens from the standard curves provided in the paper. It revealed that the copy numbers obtained from ORF 1b were all greater than those obtained from the N gene. In two specimens (of seven specimens described in the paper), the difference in the copy number determined from these two genes was greater than 10 times. However, the authors of that paper (4) did not provide enough evidence to rule out the possibility that the inherent differences in binding kinetics of the probes to the different target regions may contribute to this difference.

Five genes are present on the genomes of all the coronaviruses identified; they are Pol, S, E, M, and N from the 5′ end to the 3′ end on the genomes of coronaviruses. The functions of all five of these genes are similar among all the coronaviruses. Several ORFs lie in the regions between these five genes; and the number, nucleotide sequence, and gene order of these ORFs differ greatly from one coronavirus to another. The functions of most of the ORFs are unknown at present (13). From the results of bioinformatics analysis of the genome sequence of SARS-CoV, the nucleotide sequences of ORF 3 and ORF 8 are unique to SARS-CoV; and the product of ORF 3 is likely to be a transmembrane protein with a C terminus with ATP-binding properties, while ORF 8 may encode a type I membrane protein (14). If these ORFs are translated, their products may be involved in virus replication and pathogenesis or may modulate the immune response to infection (6).

Therefore, when we consider the facts mentioned above and the possibility that some coronaviruses may so far be unidentified, it may be more proper to choose as targets for PCR-based tests for the quantification of SARS-CoV in human clinical specimens the ORFs on the genome of SARS-CoV that are interspersed between the five major genes which are shared by coronaviruses. However, more human clinical specimens from SARS patients and more primer and probe sets designed to be specific for different genome targets are needed to fully evaluate the quantitative test results by targeting different regions of SARS-CoV. Such an evaluation may shed light on which regions on the SARS-CoV genome are more suitable for a PCR-based SARS-CoV-specific quantitative detection assay.

In this study, we also detected a coronavirus apparently related to SARS-CoV in the specimens from masked palm civets, which are believed to be one of the animal reservoirs of SARS-CoV. Since the masked palm civets used in this study were from a remote area in the western part of Hubei Province, People's Republic of China, a location unaffected by the 2003 SARS epidemic, it is unlikely that these masked palm civets got the apparently SARS-CoV-related coronavirus from humans. From the results obtained with the seven specimens collected from seven distinct masked palm civets, which were all positive when they were screened by conventional RT-PCR targeting the M gene of SARS-CoV, we saw that one specimen (specimen MPC200404085) was positive in all tests with the three primer and probe sets, while for the rest of the specimens, different positive or negative results occurred for the same specimen when we used different primer and probe sets. Quantitative analysis of the specimen that was positive by tests with all primer and probe sets (specimen MPC200404085) indicated that the virus copy number determined from the N gene was significantly higher (more than 10^4 times higher) than those determined from ORF 3 and ORF 8. Considering the fact that the Taqman-MGB probes would not generate a signal if the sequences of target regions were not identical to the sequences covered by the primer and probe sets, these results for the masked palm civet specimens may provide us a hint that the SARS-CoV-like coronavirus may consist of more than one

kind of coronavirus or the SARS-CoV-like coronavirus from the masked palm civets used in our study may have sequence variations in the regions of the genome covered by the three primer and probe sets. Since there is insufficient genetic information about the SARS-CoV-like coronavirus from the masked palm civets in the remote region of China and we did not find suitable cell lines for its amplification, more research, such as genome sequencing and serologic tests, is needed to identify the characteristics of this coronavirus. Furthermore, more efforts are needed to establish more suitable and effective assays for surveillance for the SARS-CoV-like coronavirus among wild animals, which may shed light on the origin of SARS-CoV.

It is worthwhile to mention here that although real-time RT-PCR assays have many merits for the early diagnosis of SARS-CoV infection, many factors, such as the type of specimen (23) and the time of infection when the specimens are collected from patients (17), may influence the assays' results; therefore, caution should be taken with the interpretation of the results.

In conclusion, we have developed a multitarget Taqman-MGB real-time RT-PCR assay for SARS-CoV detection. By using this assay, we have detected an apparently SARS-CoV-related coronavirus from masked palm civet specimens, as well as detected SARS-CoV from SARS patients. The assay gives us a sensitive and quantitative platform that enables us to screen more wild animals and identify the animal reservoirs of SARS-CoV in nature, which may help us to have a better understanding of the origin of SARS-CoV.

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