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Quantitation of severe acute respiratory syndrome coronavirus genome by real-time polymerase chain reaction assay using minor groove binder DNA probe technology

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The ability to rapidly recognize severe acute respiratory syndrome coronavirus (SARS-CoV) as a cause of infections is critical to quickly limiting further spread of the disease. A rapid, sensitive, and specific laboratory diagnostic test is needed to confirm outbreaks of SARS-CoV infection and distinguish it from other diseases that can cause similar clinical symptoms. An improved TaqMan technology using minor groove binder (MGB) probes was used to detect and quantify SARS-CoV in suspected patients. SARS-CoV primers and probe were designed based on the open reading frame 1b sequence, which encodes coronavirus replicase protein. A linear standard curve with $R^2 >$ 0.99 was obtained, and the threshold sensitivity was 10 genome equivalents per reaction. Interassay coefficients of variation were 1.73 to 2.72%, indicating good reproducibility of this method. A total of 228 specimens from 151 suspected patients were quantified by this method, 13 specimens from 6 patients were positive with viral load range from 362 to 36,240,000 genome equivalents/mL. In conclusion, the high sensitivity and reproducibility of the real-time polymerase chain reaction SARS-CoV RNA quantitation using MGB probe allowed the screening of large numbers of clinical samples.

Key words: Diagnosis, DNA probes, polymerase chain reaction, SARS virus

Severe acute respiratory syndrome coronavirus (SARS-CoV) is an enveloped, single-stranded, positive-sense RNA virus that belongs to the family *Coronaviridae* [1-4]. It is a newly identified coronavirus responsible for the pandemic outbreak of SARS [5-8]. Infection with SARS-CoV has a highly contagious nature and results in rapid disease progression. One of the major routes of transmission of this virus is cough and sneeze droplets from SARS patients [9]. Consequently, those at greatest risk for contracting the disease are health care workers and families, who may be the primary care providers for infected patients. Because of the mode of transmission, high fatality rates, and the need to institute proper barrier nursing precautions and public health measures during outbreaks, it is essential to establish a diagnosis early in the course of the disease.

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Although no specific treatment exists for SARS-CoV infection, a fast and specific diagnosis is needed to prevent further spread by quarantine. Even though virus isolation is the gold standard, it is very time-consuming, has a low sensitivity, and needs to be performed under biosafety level 3 conditions [1,2]. Rapid diagnosis by sensitive and virus-specific polymerase chain reaction (PCR)-based methods would permit the timely implementation of prevention and control measures. Detection of the SARS-CoV RNA by conventional reverse transcriptase-PCR (RT-PCR) has been successful in a variety of clinical specimens including sputum, feces, urine, and plasma from infected patients [1,2]. In addition, several rapid nucleic acid analysis systems using a fluorogenic probe-based assay to monitor the accumulation of PCR products in real time have been applied to the detection of feline coronavirus [10], avian infectious bronchitis virus [11], and SARS-CoV [2]. With these assays, it is possible to detect a positive sample within half a day.

In this study, we report the evaluation of SARS-CoV real-time PCR assay by using a TaqMan minor groove binder (MGB) probe recently developed by Applied Biosystems (Foster City, CA, USA). In the TaqMan MGB assay, the standard 3' quencher fluorophore (6-carboxy-tetramethyl-rhodamine; TAMRA) is replaced by a non-fluorescent quencher (NFQ) and an MGB molecule that stabilizes the oligoprobe-target duplex by folding into the minor groove of the double stranded DNA [12,13]. This allows the use of very short oligoprobes (13-18 nucleotides), which makes it ideal for designing specific oligoprobes for the detection of SARS-CoV. In comparison with unmodified DNA probes, MGB probes have a higher T_m for the same length and an increased specificity. The short length and unique structure of the TaqMan MGB probes also gives a very low fluorescent background and excellent specificity, and this performance is demonstrated in the real-time PCR application of gene expression analysis, single nucleotide polymorphism [14], and viral load determination [15].

Here we describe a rapid and reproducible method that allows the quantitation of SARS-CoV RNA genome in clinical samples by real-time PCR assay using MGB-DNA probe technology. This method presents a good sensitivity and reproducibility for the quantitation of SARS-CoV.

Materials and Methods

Patients and samples

Patients presenting to our hospital with atypical pneumonia or symptoms mimicking SARS between April 30 and June 26, 2003 were included in this study. In total, 228 samples from 151 patients were tested: 137 sputum, 53 throat swabs or throat wash, 17 nasopharyngeal swabs, 19 stool specimens, 2 pleural fluids, 2 urine samples, and 1 serum sample. Fifty nine patients were reported cases to Taiwan Center for Disease Control who had symptoms similar to those of patients with suspected case of SARS. Nineteen among the 59 patients fulfilled the definition of World Health Organization (WHO) of probable cases of SARS (http://www.who.int/csr/sars/casedefinition/en/), 28 were excluded as SARS cases, and 12 could not be completely excluded. Sputum was the specimen of choice; however, if patients could not expectorate an adequate amount of sputum, throat swab, throat wash, or nasopharyngeal aspirates were acceptable. A small number of samples from stool, urine, pleural fluid, and serum were also tested. Swabs were collected in viral transport medium (VTM) [containing, per liter, 2 g of sodium bicarbonate, 5 g of bovine serum albumin, 200 μg of vancomycin, 18 μg of amikacin, and 160 U of nystatin in Earl's balanced salt solution]. Stool and urine were collected in sterile containers.

Sample processing

Sputum samples were shaken for 5 min with equal volume of N-acetyl-L-cysteine (10 g/L) and 0.9% sodium chloride. Nasopharyngeal and rectal swabs in VTM were vortexed. For stool samples, a 10% (w/v) suspension was prepared in VTM, vortexed, and centrifuged at 2000 × *g* for 15 min and the clarified supernatant was used.

RNA extraction

Viral RNA was extracted from 140 μL of each processed sample by the QIAamp viral RNA kit (Qiagen GmbH, Germany) and was dissolved in 60 μL of RNase-free water. RNAs were used immediately or stored at –80°C until use.

Detection of SARS-CoV by 2-step TaqMan RT-PCR

Oligonucleotide primers and TaqMan MGB probe

The 2-step TaqMan RT-PCR was performed with one set of PCR primer and probe complementary to the region of SARS-CoV open reading frame (ORF) 1b gene [3,4]. The set consisted of forward primer (nucleotide 15343-15367), 5'-AGTGTGCGCAAGTATTAAGTGA GAT-3', the reverse primer (nucleotide 15416-15393), 5'-GGATGATGTTCCACCTGGTTTAAC-3'; and the TaqMan MGB probe (nucleotide 15382-15367), 5'-CCGCCACACATGACC-3' (Applied Biosystems). The TaqMan MGB probe contained a 5' reporter, 6-carboxyfluorescein (FAM), and a 3' dark quencher conjugated with MGB.

Two-step TaqMan RT-PCR

Real-time RT-PCR was done in 2 steps (RT and PCR in different tubes). SARS-CoV RNA standard (provided by Taiwan's Center for Disease Control, equivalent to 10^5 genome equivalents/mL) was reverse transcribed, and then 10-fold diluted as the starting point for each experiment.

Random reverse transcription: An aliquot (10 μL) of the extracted RNA with 1 μL of 0.5 μg/μL random hexamers (Promega Corp., Madison, USA) and 4.5 μL

of diethylpyrocarbonate-treated H_2O was first denatured at 72°C for 10 min, then quenched on ice; and subsequently added to an RT mixture (25 μL) consisting of 75 mM potassium chloride, 50 mM Tris-HCl (pH 8.3), 3.0 mM magnesium chloride, 250 μM concentrations of each of the 4-deoxynucleoside triphosphates, 10 mM dithiothreitol, 8 U of Moloney murine leukemia virus reverse transcriptase (Promega), and 1.6 U of RNAsin (Promega). RT was conducted at 37°C for 60 min, followed by 70°C for 15 min, then held at 4°C.

Real-time RT-PCR assay: Real-time RT-PCR assay conditions were optimized in an ABI Prism 7000 SDS (Applied Biosystems). 5 μL of reverse transcribed cDNA was added to the reaction mix, resulting in a final concentration of 900 nM forward primer, 900 nM reverse primer, 250 nM FAM-labeled TaqMan MGB probe, 1 × TaqMan universal master mix (Applied Biosystems). The amplification conditions were as follows: 95°C for 10 min to activate AmpliTaq Gold DNA polymerase; 50 cycles of 95°C for 10 sec (denaturation step), and 60°C for 1 min (a combined annealing-extension step). Fluorescence data were collected during each annealing-extension step and analyzed by using ABI Prism 7000 sequence detector system.

Detection of real-time RT-PCR products on agarose gels: Ten-μL aliquots of the real-time RT PCR products were resolved electrophoretically in a 2% agarose gel in 1 × Tris-borate-ethylenediamine tetraacetic acid (EDTA) buffer (0.5 × 0.045 M Tris-borate, 0.001 M EDTA [pH 8.0]), stained with ethidium bromide, and visualized by ultraviolet (UV) transillumination. Each gel was electrophoresed initially for 90 min at 50 V. A 100-bp ladder (Protech) was used as a molecular size marker in each gel.

Confirmation of SARS-CoV positive samples by nested PCR and sequencing

Oligonucleotide primers

Nested PCR detection was combined with reverse transcription and 2 rounds of PCR reactions to increase the sensitivity and specificity for SARS-CoV detection. Two sets of PCR primers complementary to sequences located in the ORF 1b region were used [8]. Set 1 consisted of the forward primer SARS-1 (nucleotide 15239-15257), 5'-CAGA-GCCATGCCTAACATG-3'; the reverse primer SARS-2 (nucleotide 15627-15608), 5'-AATGTTTACGCAGGTAAGCG-3'. Set 2 consisted of the forward primer SARS-3 (nucleotide 15392-15409), 5'-TGTTAAACCAGGTGGAAC-3' and the reverse primer SARS-4 (nucleotide 15548-15531), 5'-CCTGTGTTGTAGATTGCG-3'. Primers were purchased from Purigo Biotech (Taipei, Taiwan).

Nested PCR

The first round of PCR was performed with 1 μL of the cDNA added to 25 μL of a PCR mixture consisting of 50 mM potassium chloride, 10 mM Tris-HCl (pH 9.0), 2.45 mM magnesium chloride, 0.01% gelatin, 0.1% Triton X-100, 200 μM concentrations of each of the 4 deoxynucleoside triphosphates, 0.4 μM concentrations each of the primers SARS-1 and SARS-2, and 1.5 U of *Taq* DNA polymerase (ProTaq; Protech Technology Co., Taipei, Taiwan). The amplification conditions for the first round of PCR were as follows: 95°C for 3 min; 10 cycles of 95°C for 10 sec; 55°C for 15 sec (decreasing by 1°C per cycle), 72°C for 40 sec; 40 cycles of 95°C for 10 sec; 50°C for 10 sec, 72°C for 40 sec, and 72°C for 10 min.

The second round of PCR involved a nested PCR in which 2 μL of the amplified product from the first round of PCR served as the template. The reaction mixture for the nested PCR was prepared under sterile conditions in a 50-μL reaction volume by using the same PCR buffer described above, except that the *Taq* DNA polymerase concentration was increased to 2.5 U per reaction mixture. In addition, the reaction mixture contained 0.4 μM each of nested primers SARS-3 and SARS-4. The amplification conditions for the nested reaction were as follows: 95°C for 3 min; 30 cycles of 95°C for 10 sec, 45°C for 15 sec, 72°C for 40 sec, and 72°C for 10 min. Both the RT and nested PCR were performed in a thermocycler (Gene Amp PCR System model 9700; Perkin-Elmer Applied Biosystems, Foster City, CA, USA). 10 μL of the nested PCR products were electrophoresed and visualized as described earlier. To reduce the risk of contamination, we prepared the solutions and performed PCR in a laboratory space that was never used for SARS-CoV extraction or electrophoresis of amplified products. All mixtures were prepared under a UV-irradiated, PCR-dedicated biosafety cabinet. All solutions were pipetted with sterile pipettor tips containing an aerosol barrier (Molecular Bio-Products). Gloves were changed after each step. Water was used as a negative control template in the RT-PCR and nested PCR tests. The nested PCR products (12 μL) were resolved electrophoretically as described earlier.

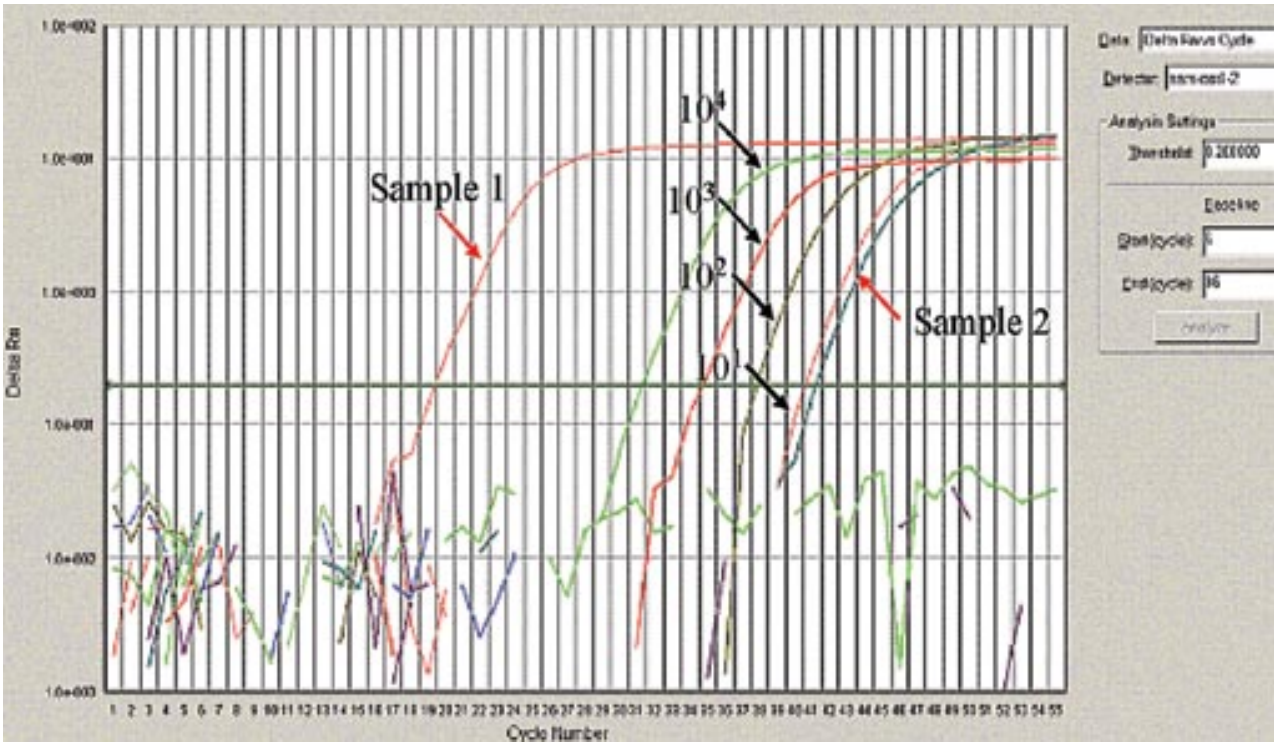

Fig. 1. Amplification plot of severe acute respiratory syndrome coronavirus real-time reverse transcriptase-polymerase chain reaction assay. Ten-fold serial dilutions of cDNA were prepared, amplified using TaqMan minor groove binding probe, and detected using ABI Prism 7000 sequence detector. For each dilution, Delta Rn, fluoresence units of normalized reporter, is plotted against each cycle number.

Sequencing of nested PCR products

To confirm the identities of the PCR products, amplicons from RT-PCR were sequenced with dye-labeled terminators and by cycle sequencing (Taq Prism kit; Applied Biosystems). The products from the sequencing reactions were analyzed on an Applied Biosystems 377 sequencer. The sequences were then identified with the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/).

Results

Sensitivity, dynamic range and reproducibility of real-time quantitative RT-PCR

In order to determine the sensitivity and dynamic range of the real-time quantitative RT-PCR, we generated standard curves with 10-fold serial dilutions of cDNA, synthesized from SARS-CoV RNA (10^4 genome equivalents/mL) by reverse transcription (Fig. 1). In this quantitative RT-PCR assay, we chose a highly specific set of primers and TaqMan MGB probe complementary to SARS-CoV ORF 1b gene, for encoding the coronavirus replicase proteins [3, 4]. A standard curve showed linearity over a range of 5 orders of magnitude, with correlation coefficient R^2>0.99, slope –3.39, and y-axis intercept point 44.24 (Fig. 2). The detection limit was determined at 10 genome equivalents per reaction mixture, equivalent to 850 genome equivalents per mL. To evaluate the interassay reproducibility, we compared the threshold cycle (C_T) value for each of the four 10-fold dilutions (10^1-10^4 genome equivalents per reaction) in 5 independent experiments. As Table 1 shows, the mean coefficients of variation (CV) of C_T values and cDNA input between

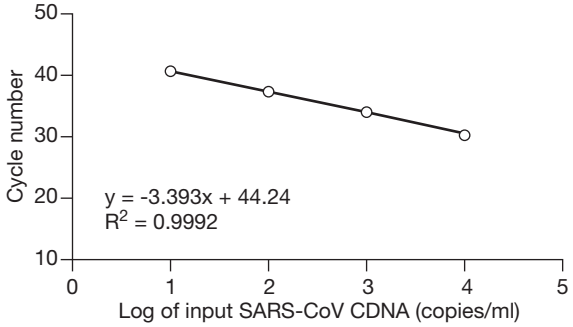

Fig. 2. The standard curve of 10-fold serial dilutions of severe acute respiratory syndrome coronavirus cDNA control with a correlation coefficient (R^2) of 0.9992. The curve was generated based on the data shown in Table 1 and the C_T (threshold cycle) values are the mean of 5 runs.

Table 1. Interassay reproducibility of severe acute respiratory syndrome coronavirus real-time reverse transcriptase-polymerase chain reaction

Run	Threshold cycle			
	$10^{1,a}$	$10^{2,a}$	$10^{3,a}$	$10^{4,a}$
1	39.21	37.69	33.34	29.53
2	41.18	37.73	34.69	31.26
3	41.87	38.37	34.69	31.40
4	40.09	36.56	34.03	29.90
5	41.29	37.68	33.77	30.86
Mean ± SD	40.73 ± 1.06	37.61 ± 0.65	34.10 ± 0.59	30.59 ± 0.83
CV	2.61	1.74	1.73	2.72

Abbreviations: SD = standard deviation; CV = coefficient of variation

[a]Input numbers of severe acute respiratory syndrome coronavirus cDNA control.

different runs indicated a high level of reproducibility for the assay. The standard deviations ranged from 0.59 to 1.06 and the CV of the C_T ranged from 1.75% to 2.72%.

Detection of SARS-CoV in clinical specimens

Thirteen samples from 6 patients, included 10 from sputum, 2 from throat swabs, and 1 from urine, were positive (Table 2). The viral loads ranged from 360 to 3.83×10^9 genome equivalents per mL, equal to 4.24 to 4.5×10^7 genome equivalents per reaction. The time of detected was range from 5-24 days after disease onset. One female patient was tested 4 times, with gradual decline of viral loads from sputum (7.66 log on day 11, 4.66 log on day 13, 3.42 log on day 14, and 3.14 log on day 15). The first of her amplification plots is shown as sample 1 in Fig. 1. Among these 13 positive samples, 3 had less than 10 genome equivalents per reaction, i.e., below the low detection limit. The identities of positive samples from these 3 samples were confirmed by nested PCR reaction and gel electrophoresis in 1 case. This male patient came from Guangdong province, China and fulfilled the WHO case definition of probable case of SARS. His amplification plot is shown as sample 2 in Fig. 1.

Table 2. Result of real-time reverse transcriptase-polymerase chain reaction of 228 clinical specimens from 151 patients

Source	No. of specimens	Positive no.
Sputum	137	10
Throat wash	53	2
NPA	17	0
Pleural fluid	2	0
Urine	2	1
Stool	16	0
Serum	1	0
Total	228	13

Abbreviation: NPA = nasopharyngeal aspirate

Confirmation of the positive results by sequencing nested PCR products

Virus isolation is the gold standard for detection of viral infections. Nevertheless, due to lack of biosafety level 3 tissue culture facilities, we use another alternative, nested PCR and sequencing, to confirm positive results from clinical specimens. Using 2 different sets of primers complementary to ORF 1b gene [8], we were able to amplify SARS-CoV-specific nested PCR products (Fig. 3). Subsequently, the sequences of all positive bands were verified by sequencing. However, the detection limit was about 10^3 genome equivalents in the standard cDNA control, indicating that the nested PCR reaction was less sensitive than real-time RT-PCR reaction.

Discussion

The worldwide outbreak of SARS originated from south China and spread to several countries, including Hong Kong, Vietnam, Singapore, Canada, USA, and Taiwan. According to the WHO, the cumulative number of reported probable cases of SARS from 1 November 2002 to 4 July 2003 amounted to 8439 individuals and resulted in 812 deaths. Transmission in health care settings has been documented in several countries. Transmission to health care workers appears to have occurred primarily after close contact with symptomatic persons before recommended infection control precautions for SARS were implemented. Understanding the epidemiology of respiratory pathogens such as those that cause SARS is challenging [16,17]; approximately 40 to 60% of persons with pneumonia do not have a defined etiology, even when extensive testing for known respiratory pathogens

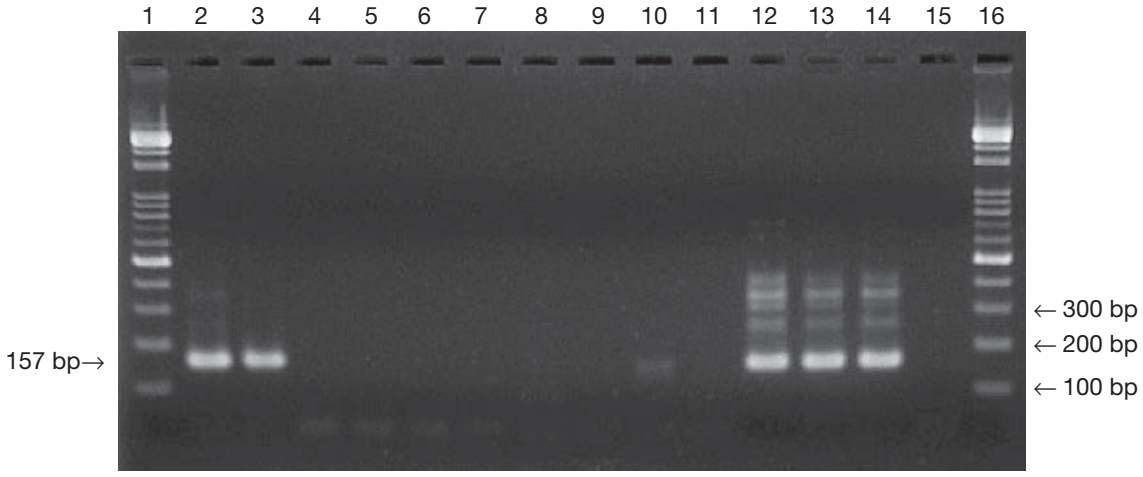

Fig. 3. Results of nested polymerase chain reaction to detect severe acute respiratory syndrome coronavirus (SARS-CoV). Lanes 1 and 16 = molecular weight marker (100-bp ladder); lanes 2 to 6 = 10^4 to 10^0 copies of SARS-CoV cDNA; lanes 7 to 15 = clinical samples; lane 15 = negative control.

is attempted [18]. The development of laboratory testing techniques to identify infected persons rapidly will be an important step towards understanding and reducing transmission of SARS.

Assays currently available for the diagnosis of SARS-CoV infection include virus culture, transmission electron microscopy (TEM), immunohistochemistry, antigen detection enzyme-linked immunosorbent assay (ELISA), antibody ELISA, and conventional RT-PCR. Each assay has both advantages and disadvantages; however, few assays that can be used in the field are capable of providing a sensitive, specific, and rapid diagnosis. Although virus isolation is the gold standard, it is very time-consuming, has a low sensitivity, and needs to be performed under biosafety level 3 conditions [1,2]. TEM is only available in well-equipped research facilities. Antibody detection ELISAs are reliable but patients with symptomatic SARS infection often require a seroconversion period to mount a detectable humoral response [19]; therefore, this technique is not suitable for rapid diagnosis. To date, the most rapid and potentially deployable assay that also has good clinical sensitivity is based on RT-PCR.

Currently available nucleic acid amplification techniques such as PCR and RT-PCR are widely applied for the detection of nucleic acid sequences from viruses and bacteria in clinical specimens. Conventional RT-PCR is a sensitive method, but requires more post-PCR manipulations, i.e., analysis of data by gel electrophoresis. However, based on our evaluation, the sensitivity of nest PCR is not adequate to detect samples with low viral loads. The threshold of sensitivity of nest PCR is about 1000 genome equivalents per reaction. Samples with lower viral loads may yield false-negative results. On the other hand, real-time PCR using MGB probe has a higher sensitivity, which allows it to detect as low as 10 genome equivalents per reaction. One probable case that fulfilled the WHO case definition with less than 10 copies per reaction was detected by our method. It revealed that the sensitivity of our method was similar to recent reports from other groups [20,21]. Conventional RT-PCR cannot provide quantitation of viral load. The study of sequential quantitative RT-PCR for SARS-CoV in nasopharyngeal aspirates revealed an inverted V pattern after onset of symptoms and suggested that the lung damage in week 2 is unrelated to uncontrolled viral replication but may be related to immunopathological damage [19]. Thus, quantitation of SARS-CoV is not only important for rapid diagnosis, but also for studies of pathogenesis, new drug and vaccine development.

TaqMan MGB probe is suitable for the quantitative detection of SARS-CoV based on 2 main reasons. (1) Incorporation of an MGB molecule allows the design of a shorter TaqMan probe (about 13-18 bases long) which still meets the high T_m requirement for PCR. Thus, it is easier to design a specific probe to target a shorter region. The MGB, which is a small crescent shaped molecule that fits into the minor groove of duplex DNA, can form a hyper-stabilized duplex with DNA and increase the melting temperature to 49°C for A/T-rich octanucleotides. (2) Using an NFQ to

replace the previous standard TAMRA improves the probe performance. An NFQ is a chromophore that acts as the energy transfer acceptor from the reporter molecule that does not emit a detectable florescent signal of its own. Background fluorescence levels and spectral discrimination are much improved by the NFQ. These 2 innovations of the TaqMan MGB probe allow greater precision and consistency between individual assays [13].

Here we report the development of a SARS-CoV real-time PCR assay by using a TaqMan MGB probe in 2-step RT-PCR procedures. Two-step RT-PCR was chosen due to the following advantages: (1) the 2-step format allows both transcription and PCR to be performed under optimal conditions to ensure efficient and accurate amplification; and (2) 2-step procedures provide flexibility that allows the product of a single cDNA synthesis reaction to be used for analysis of multiple transcripts.

The sensitivity and specificity of this method in the clinical setting is difficult to evaluate due to the absence of a standard to confirm the diagnosis of SARS. We confirmed all of the positive PCR products by nest-PCR and sequenced to ensure that there were no false-positives, except 2 cases whose viral loads were below 10 genome equivalents. This demonstrated that our method has a high specificity.

Most of the positive results were obtained from sputum samples, which is compatible with the results from others. Sputum is easy to collect, and its high sensitivity allows rapid detection of SARS cases and thereby prevents further nosocomial infection of health care workers.

In conclusion, the high sensitivity, simplicity, and reproducibility of this method allows the screening of large numbers of samples, and, with its wide dynamic range, this method is suitable for further studies of the pathogenesis of SARS and for new drug and vaccine development.

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