Sensitive and Quantitative Detection of Severe Acute Respiratory Syndrome Coronavirus Infection by Real-Time Nested Polymerase Chain Reaction

Shih Sheng Jiang,[1] Tsan-Chi Chen,[1] Jyh-Yuan Yang,[3] Chao A. Hsiung,[2] Ih-Jen Su,[3] Ying-Lan Liu,[1] Po-Cheng Chen,[1] and Jyh-Lyh Juang[1]

Divisions of [1]Molecular and Genomic Medicine and [2]Biostatistics and Bioinformatics, National Health Research Institutes, and [3]Division of Laboratory Research and Development, Center for Disease Control, Department of Health, Taipei, Taiwan

(See the editorial commentary by Poon et al. on pages 297–9)

A quantitative, real-time, nested polymerase chain reaction (PCR) method, combining the high sensitivity of nested PCR with time-saving real-time instrumentation, was developed for large-scale screening for severe acute coronavirus (SARS) coronavirus. Forty-six clinical specimens were analyzed by this method, and results were compared with those obtained by conventional, single-round, real-time reverse-transcriptase PCR (RT-PCR) performed in parallel. Of the 17 positive results, 2 identified by our method were not detected by single-round, real-time RT-PCR, which suggests that real-time nested PCR has the potential for increased sensitivity, leading to earlier detection of SARS.

The outbreak of severe acute respiratory syndrome (SARS) in Southeast Asia and on other continents reached serious global epidemic proportions in >30 countries [1–3]. In the absence of a vaccine or effective therapeutic drugs, the key to preventing and controlling future epidemics is to block transmission of infection through a strict quarantine policy. Therefore, rapid, sensitive, and specific diagnostic methods are essential for the assessment of patients suspected of being infected and for prevention of spread to the larger community.

Real-time RT-PCR detection is currently favored for the detection of SARS coronavirus (SARS-CoV) because of its advantages as a rapid and quantitative assay [3, 4]. Unfortunately, results obtained with this method vary on the basis of which detection method is used as part of a real-time, high-throughput clinical screening protocol. In particular, because the concentration of extracted viral RNA from samples obtained during the early phase of infection is exceptionally low, the aforementioned problems would usually worsen. In most of the cases, we and others have found that the single-step real time RT-PCR methods (as suggested by the World Health Organization [WHO]; available at http://www.who.int/csr/sars/diagnostictests/en/) could specifically detect SARS-CoV but were unable to proficiently detect <10 copies of virus per test, suggesting that the conventional RT-PCR assay may actually yield false-negative results. Failure of early detection of SARS has meant that "super-spreaders" of SARS have not been isolated and quarantined, thus highlighting the importance of a more sensitive early diagnostic method.

To solve this problem of diagnostic methods, several nested PCR methods have been reported [5–9] to enhance both the specificity and sensitivity of the assay. However, those nested RT-PCR methods are either unfavorable for quantitative assay or require lengthy and labor-intensive procedures, making these protocols impractical for large-scale screening. In the present report, we describe an innovative 2-round, real-time PCR method for detection of SARS-CoV. We utilized the on-line detection method of the LightCycler (Roche) instrument to optimize the first-round PCR conditions for linear amplification, and then used real-time nested PCR for sensitive and quantitative virus detection. We show that this method is rapid and highly sensitive and could be scaled up for routine screening for early diagnosis of SARS-CoV infection in a laboratory setting.

Methods. Forty-six clinical throat swab specimens (1 each from 46 patients with suspected or reported SARS), which had been obtained during the period of April through May 2003 by contracted hospitals of the Taiwan Center for Disease Control, were randomly selected for this study. According to the WHO's definition (http://www.who.int/csr/sars/casedefinition/en/), a patient with a suspected case of SARS has high fever (temperature, >38°C), the symptoms of cough or breathing difficulty, and a history of exposure to or contact with a person with suspected or probable SARS. In our study, reported case patients are people who had symptoms similar to those of patients with suspected cases of SARS, except that a history of contact was not clear. Viral RNA was extracted from 200 μL

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Reprints or correspondence: Dr. Jyh-Lyh Juang, Div. of Molecular and Genomic Medicine, National Health Research Institutes, 128 Yen-Chiu-Yuan Rd., Sec. 2, Taipei, Taiwan (juang@nhri.org.tw).

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of viral transport medium using the QIAamp viral RNA mini kit (Quiagen) and eluted in 50 μL of RNase-free water, which was frozen in aliquots at −70°C until use. For the control experiment, viral RNA was extracted from the supernatant of viral culture medium. The concentration of this control viral RNA was calibrated using the RealArt HPA-coronavirus RT-PCR reagents kit (Artus), in accordance with the manufacturer's instructions. The titer of calibrated viral RNA was then adjusted to 5.2×10^5 copies/mL as high-titer RNA stock.

Two pairs of PCR primers made available by the WHO (BNIoutS/BNIoutAs and BNIinS/BNIinAs; see http://www.who.int/csr/sars/primers/en/) were used for the first round of RT-PCR and the subsequent nested PCR, respectively. The first-round PCR amplification was performed using LightCycler (Roche) with 2 μL of viral RNA diluted to a volume of 20 μL with PCR mix (LightCycler RNA Master SYBR Green I kit; Roche) containing 2 mmol/L $Mn(OAC)l_2$, and 0.5 μmol/L of BNIoutS/BNIoutAs primers at the following settings: 61°C for 20 min and 95°C for 30 s, followed by 25 cycles of 95°C at 1 s, 55°C for 10 s, 72°C for 8 s. The nested PCR was then conducted using 1 μL of the first-round amplicon as template diluted to a volume of 20 μL with PCR mix (LightCycler FastStart DNA Master SYBR Green I kit; Roche) containing 2 mmol/L $MgCl_2$ and 0.5 μmol/L BNIinS/BNIinAs primers, with the following settings: 95°C for 10 min, followed by 25–35 cycles of 95°C for 10 s, 56°C for 5 s, and 72°C for 5 s. After amplification, melting curve analysis at temperatures of 65°C–95°C, with a temperature transition rate of 0.1°C/s, was performed to mark out the presence of PCR-amplified product. The sizes of the product were analyzed by gel electrophoresis analysis in 2% agarose. Furthermore, the sequence of nested PCR amplicon was subjected to sequence analysis with an ABI 3700 auto-sequencer (ABI) to confirm whether it was a SARS-CoV sequence. To reduce the risk of random or carry-over contamination of nested PCR [10], sample preparation, reagent preparation, and PCR amplification were performed in different buildings or rooms with separated air-conditioning using different sets of the pipette system. All samples and reagents were transferred via filter tips to protect the PCR from aerosol contamination.

For real-time nested PCR data analysis, the threshold cycle was calculated with a "fit points" algorithm using 2-points calculation, and the crossing point was then determined automatically. For quantification of the SARS-CoV RNA load in the samples, a standard curve was generated using various dilutions of calibrated control viral RNA, as mentioned previously.

To confirm positive PCR results, occurrence of seroconversion was determined by ELISA using available serum samples obtained during the convalescent phase of infection (>28 days after illness) [11]. The SARS ELISA antigen was kindly provided by the US Centers for Disease Control and Prevention (Atlanta). The optimal dilution (1:1000) for the use of this antigen was determined by checkerboard titration against human serum samples obtained during the convalescent phase. The control antigen, prepared from uninfected Vero E6 cells, was used to control for the specific reactivity of tested serum. The conjugates used were goat anti-human IgG, IgA, and IgM conjugated to fluorescein isothiocyanate and horseradish peroxidase for the indirect fluorescence antibody test and ELISA, respectively.

Approval for this study was obtained from the Center for Disease Control, Department of Health, Taiwan.

Results. Using the single-step RT-PCR protocol, our first-round PCR yielded a minor amplification signal, although the nonspecific fluorescence signal background frequently occurred after 20 cycles of amplification (data not shown). In contrast, the second-round amplification by nested real-time PCR proficiently generated a signal of SARS-CoV DNA without apparent background, compared with no detectable signal for the

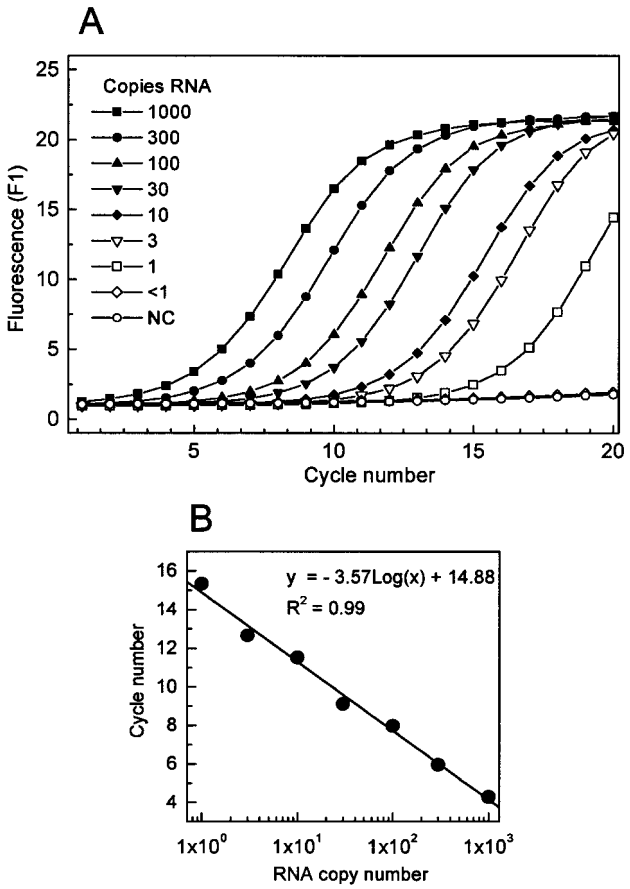

Figure 1. *A,* Results of the real-time nested PCR with indicated starting RNA copy number in each assay. ◇, <1 RNA copy; □, 10-fold dilution of the control RNA sample used for the single-copy RNA test; NC, negative control, in which input RNA was replaced by deionized water. *B,* Linear amplification by real-time nested PCR. The starting viral RNA copy number was plotted against the threshold cycle number of each dilution and fitted with a linear regression model.

Table 1. Comparison of results of real-time nested PCR and single-round RT-PCR test for clinical samples from 46 patients.

Test	Positive result[a]		Negative result	Total
	⩾10 copies per test	<10 copies per test		
Real-time nested PCR				
No. of results	14	3	29	46
Seroconversion[b]	5	2	0	7
Single-round RT-PCR[c]				
No. of results	14	1	31	46
Seroconversion[b]	5	0	2	7
Shared results				
No. of results	14	1	29	44
Seroconversion[b]	5	0	0	5

NOTE. Data are no. of RNA samples. RNA samples were extracted from 46 clinical throat swab specimens and analyzed by real-time nested PCR and single-round RT-PCR in parallel.

[a] Results of 3 independent tests.

[b] Data are no. of samples obtained from patients for whom seroconversion was also noted, as determined on the basis of ELISA results for available serum samples obtained during the convalescent phase of illness (i.e., >28 days after onset of illness).

[c] Results obtained by hybridization probe–based detection using the RealArt HPA-coronavirus RT-PCR reagents kit (Artus).

negative control samples (figure 1*A*). In addition, by melting curve analysis, we could clearly define an average melting temperature of 84.2°C for the nested amplicon of SARS-CoV, in contrast to no indication of melting temperature for the negative control (data not shown). The size (110 bps) of every nested PCR amplicon was confirmed by the agarose gel analysis, and the sequence was further validated by direct sequencing, to verify the specific detection of SARS-CoV.

To determine the detection limit for this developed method, samples of various dilutions of control SARS-CoV RNA were subjected to the assay. After 25 cycles of first-round amplification and 25 cycles of nested PCR amplification, our assay could detect a theoretical single copy of extracted viral RNA (figure 1*A*), suggesting its superior sensitivity for detection of SARS-CoV. Nonetheless, for the quantitative assay, the cycle number of the first-round RT-PCR should be controlled to <30 cycles to prevent nonlinear amplification, as reflected by saturation of the fluorescence signal. We found that, for most cases, 25 cycles was the optimized condition for the first-round PCR to produce the most adequate amplicon level as the template for subsequent nested PCR. Under such conditions, the linear relationship between the copy number of input RNA and the threshold cycle number of nested PCR was accurately held within the range of 10^3–10^0 in the semilog plot (figure 1*B*). In general, our developed assay is highly sensitive and specific for detection of trace amounts of virus.

To further validate this method for real clinical diagnoses, we then examined RNA samples from 46 patients with suspected or reported SARS using this method and another commercial single-round RT-PCR kit (Artus) in parallel for comparison. We found that the single-round PCR detected 15 of 46 positive cases, comparison with 17 of 46 cases for our 2-round, real-time PCR. Of the 17 positive cases, 15 were those identified by single-round PCR, and only 2 were missed by the single-round PCR (table 1). For those 2 possible false-negative results for the single-round PCR, we used direct sequencing analysis to confirm the positive results detected by real-time nested PCR. Furthermore, we carefully avoided the possibility of carry-over contamination by following the protocols described in Methods and by conducting 3 independent tests for all assays. Most importantly, we also confirmed seroconversion for the convalescent-phase serum samples for these 2 cases (table 1), further supporting the result obtained by the nested real-time PCR method. It is interesting to note that virus titers for the aforementioned 2 possible false-negative cases were actually quantified and shown to contain <10 copies of viral genome in the test. Of the 15 cases that yielded positive results by both assays, only 1 contained <10 copies of viral genome in the assay, indicating that the single-round PCR method may be insensitive for the low-titer virus assay.

Discussion. Our assay combines the 2-round PCR amplification method with the real-time detection approach to identify SARS-CoV, thus providing an alternative, sensitive means of detection of SARS. These findings reveal several advantages over the conventional real-time RT-PCR and/or nested PCR. First, the assay is simple and rapid. The conventional nested PCR requires arduous processing steps, demanding RT-PCR procedures, performance of nested PCR on regular thermocycler, and use of agarose gel analysis, tests that normally will take at least 4–5 h to complete. This dual real-time PCR method can be easily completed within 2 h using LightCycler, which includes a 45-min, 1-step RT-PCR followed by 40-min real-time nested PCR, making it an ideal routine protocol for high-throughput screening of SARS-CoV. Second, the assay had a detection limit of <10 copies of SARS-CoV, to reduce the rate of false-negative results for trace virus samples. In this report, we show that the developed assay is sensitive enough to detect trace amounts of virus in the sample, suggesting that this test is an excellent alternative to the existing SARS-CoV assay methods, which frequently generate false-negative results for samples obtained during the early phase of infection. Last, this assay allowed for quantification of SARS-CoV RNA in a range over a 3-log span. In theory, the 2-round amplification protocol can be optimized through the on-line detection system for linear amplification of viral RNA without saturation for quantitative analysis. Thus, quantitative analysis for detection of a wider range of values (>4-log span) should be achievable. To our knowledge, this is the first report of a quantitative nested PCR assay for virus detection.

Through analysis of 46 clinical cases using our developed assay in parallel with a commercial, single-round RT-PCR kit, we have demonstrated that our new method has efficacy at least equal to that of the commercial kit while possessing the potential for increased sensitivity for early detection of SARS-CoV infection. In practice, this innovative assay method can be easily adapted to detect the RNA or DNA of other pathogens.

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References

1. Ksiazek TG, Erdman D, Goldsmith CS, et al. A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med **2003**; 348:1953–66.
2. Peiris JS, Lai ST, Poon LL, et al. Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet **2003**; 361:1319–25.
3. Drosten C, Gunther S, Preiser W, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med **2003**; 348:1967–76.
4. Poon LL, Wong OK, Luk W, Yuen KY, Peiris JS, Guan Y. Rapid diagnosis of a coronavirus associated with severe acute respiratory syndrome (SARS). Clin Chem **2003**; 49:953–5.
5. Berg J, Nagl V, Muhlbauer G, Stekel H. Single-tube two-round polymerase chain reaction using the LightCycler instrument. J Clin Virol **2001**; 20:71–5.
6. Bialek R, Weiss M, Bekure-Nemariam K, et al. Detection of *Cryptococcus neoformans* DNA in tissue samples by nested and real-time PCR assays. Clin Diagn Lab Immunol **2002**; 9:461–9.
7. Ratge D, Scheiblhuber B, Landt O, Berg J, Knabbe C. Two-round rapid-cycle RT-PCR in single closed capillaries increases the sensitivity of HCV RNA detection and avoids amplicon carry-over. J Clin Virol **2002**; 24:161–72.
8. Zeaiter Z, Fournier PE, Greub G, Raoult D. Diagnosis of *Bartonella* endocarditis by a real-time nested PCR assay using serum. J Clin Microbiol **2003**; 41:919–25.
9. Koenig M, Kosha S, Hickman M, Heath D, Riddell S, Aldous W. Detection of influenza virus from throat and pharyngeal swabs with a nested duplex light cycler RT-PCR. Diagn Microbiol Infect Dis **2003**; 46:35–7.
10. Porter-Jordan K, Rosenberg EI, Keiser JF, et al. Nested polymerase chain reaction assay for the detection of cytomegalovirus overcomes false positives caused by contamination with fragmented DNA. J Med Virol **1990**; 30:85–91.
11. Peiris JS, Chu CM, Cheng VC, et al. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. Lancet **2003**; 361:1767–72.