

A one step quantitative RT-PCR for detection of SARS coronavirus with an internal control for PCR inhibitors

Leo L.M. Poon^{a,*}, Bonnie W.Y. Wong^a, Kwok Hung Chan^b,
Cynthia S.W. Leung^a, Kwok Yung Yuen^a,
Yi Guan^a, Joseph S.M. Peiris^a

^a Department of Microbiology, Queen Mary Hospital, University of Hong Kong, Pokfulam, Hong Kong SAR, PR China

^b Department of Microbiology, Queen Mary Hospital, Hong Kong SAR, PR China

Accepted 18 December 2003

Abstract

In this study, we report a one step quantitative RT-PCR assay for severe acute respiratory syndrome (SARS) diagnosis. The overall detection rate for clinical samples collected from Days 1 to 9 of disease onset is 86.2% and the specificity of the assay is 100%. To detect false negative results due to PCR inhibitors or faulty RNA extraction, we have further modified this one step RT-PCR assay for simultaneous detection of severe acute respiratory syndrome (SARS) coronavirus (CoV) and 18S ribosomal RNA (rRNA) as an internal positive control.
© 2003 Elsevier B.V. All rights reserved.

Keywords: SARS coronavirus; Quantitative RT-PCR; Early diagnosis

1. Introduction

A novel coronavirus (CoV) is recently identified as etiology of severe acute respiratory syndrome (Dristan et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003). The initial clinical presentation of SARS in many ways very similar to atypical pneumonia caused by other viral infections. It is therefore critical to have a reliable rapid laboratory SARS test to allow prompt patient management and isolation.

By increasing the initial sample volume for RNA extraction and utilizing real-time quantitative PCR technology, we have recently developed a sensitive and accurate two step RT-PCR assay for the prompt identification of SARS-CoV (Poon et al., 2003a). Here, we report a one step quantitative RT-PCR assay for SARS diagnosis. In addition, we have further modified this assay to duplex with 18S ribosomal RNA (rRNA) to serve as an internal positive control for PCR inhibitors.

2. Materials and methods

2.1. Patients and sample collection

Nasopharyngeal aspirates (NPA) from patients who presented with clinical SARS with serological evidence of SARS-CoV infection (Peiris et al., 2003) were studied. NPA samples were collected as described previously (Poon et al., 2003b). The study was approved by the Institutional Review Board of The University of Hong Kong. NPA samples from patients with unrelated respiratory diseases (adenovirus, $N = 5$; respiratory syncytial virus, $N = 5$; human metapneumovirus, $N = 4$; influenza A virus, $N = 5$) or normal individuals ($N = 5$) were recruited as controls.

2.2. RNA extraction and quantitative RT-PCR

Five hundred and sixty microliter of clinical sample was extracted by QIAamp virus RNA mini kit (Qiagen) as described before (Poon et al., 2003a). RNA was reverse transcribed and amplified by a TaqMan EZ RT-PCR Core Reagents kit in a 7000 Sequence Detection System (Ap-

* Corresponding author. Fax: +852-2855-1241.

E-mail address: llmpoon@hkucc.hku.hk (L.L.M. Poon).

plied Biosystems). The ORF1b region of SARS-CoV was the target for SARS detection and 18S rRNA sequence derived from infected host was used as internal positive control. Briefly, 4 μ l of RNA was amplified in a 25 μ l reaction containing 2.5 U rTth DNA polymerase (Applied Biosystems), 5 μ l of 5 \times TaqMan EZ buffer, 300 μ M of dNTPs (except 1200 μ M for dUTP), 3 mM of manganese acetate and 2.5 U of AmpErase UNG. For the multiplex assay for ORF1b sequence, 800 nM of primers (5'-CAGAA-CGCTGTAGCTTCAAAAATCT-3' and 5'-TCAGAACCC-TGTGATGAATCAACAG-3'), and 400 nM of probe [5'-(FAM)TCTGCGTAGGCAATCC(NFQ)-3'; FAM, 6-carboxyfluorescein; NFQ, nonfluorescent quencher] were used.

The specificity of this primers-probe set was demonstrated previously (Poon et al., 2003a, 2004). In vitro transcribed T7 RNA polymerase transcripts or plasmid DNA containing the target viral sequence was used positive controls to generate calibration curves. Reactions were first incubated at 50 $^{\circ}$ C for 2 min, followed by 60 $^{\circ}$ C for 30 min. After a 5 min denaturation at 95 $^{\circ}$ C, reactions were then thermal-cycled for 50 cycles (94 $^{\circ}$ C for 15 s, 58 $^{\circ}$ C for 1 min). For simultaneous detection of SARS-CoV and 18S rRNA, 2.5 nM of primers and 10 nM of VIC-labeled probe specific for 18S rRNA molecules (TaqMan Ribosomal RNA Control Reagents, Applied Biosystems) was added into a reaction as instructed (Applied Biosystems).

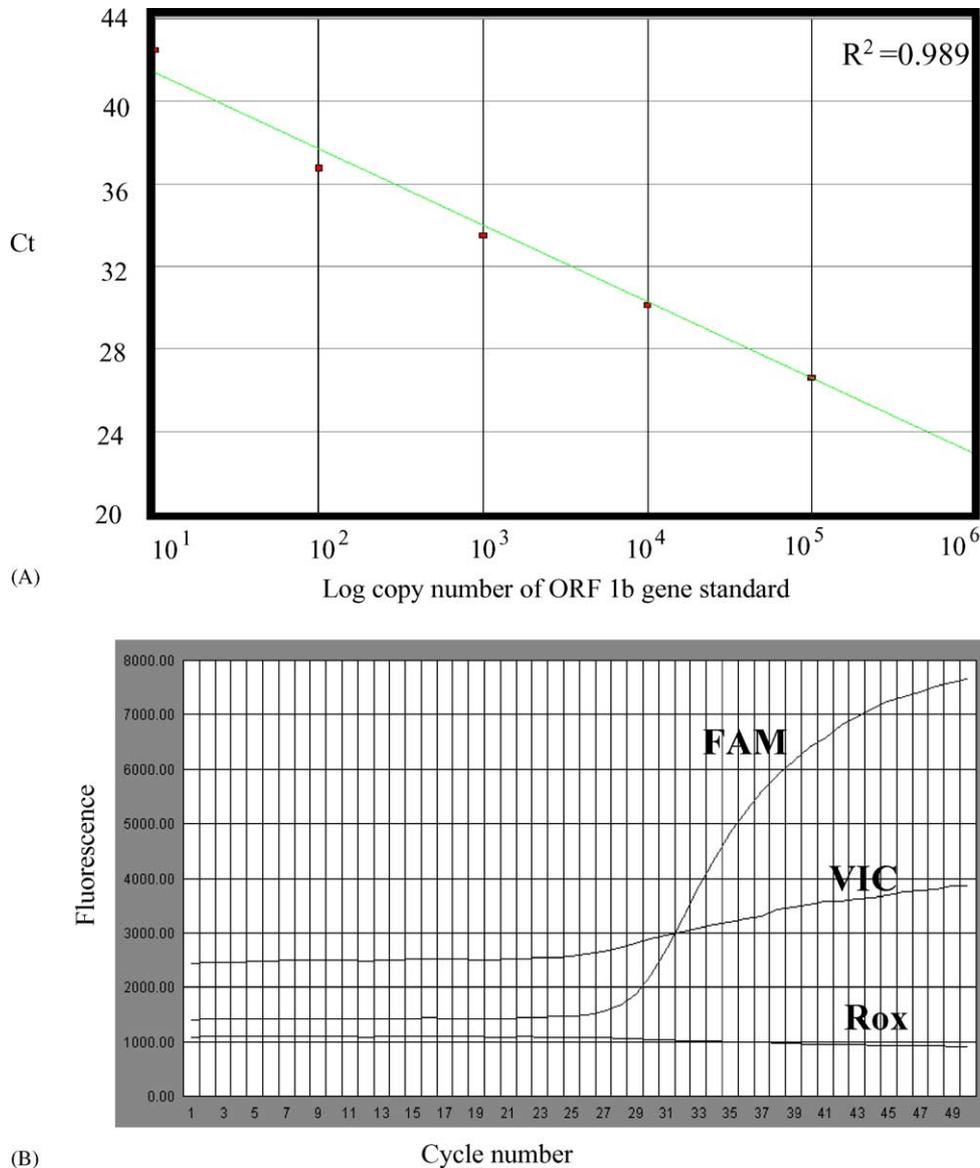


Fig. 1. Duplex quantitative RT-PCR assay for SARS-CoV and 18S rRNA detection. (A) Standard curve for quantitative analysis of ORF 1b of SARS-CoV. The threshold cycle (Ct) is the number of PCR cycles required for the fluorescent intensity of the reaction to reach a pre-defined threshold. The Ct is inversely proportional to the logarithm of the starting concentration of the input DNA. (B) An amplification plot of fluorescence intensity against the PCR cycle. The fluorescence signals for SARS-CoV (FAM), 18S rRNA (VIC) and internal positive standard (ROX) are indicated. The x-axis denotes the cycle number of a quantitative PCR assay. The y-axis denotes the fluorescence intensity.

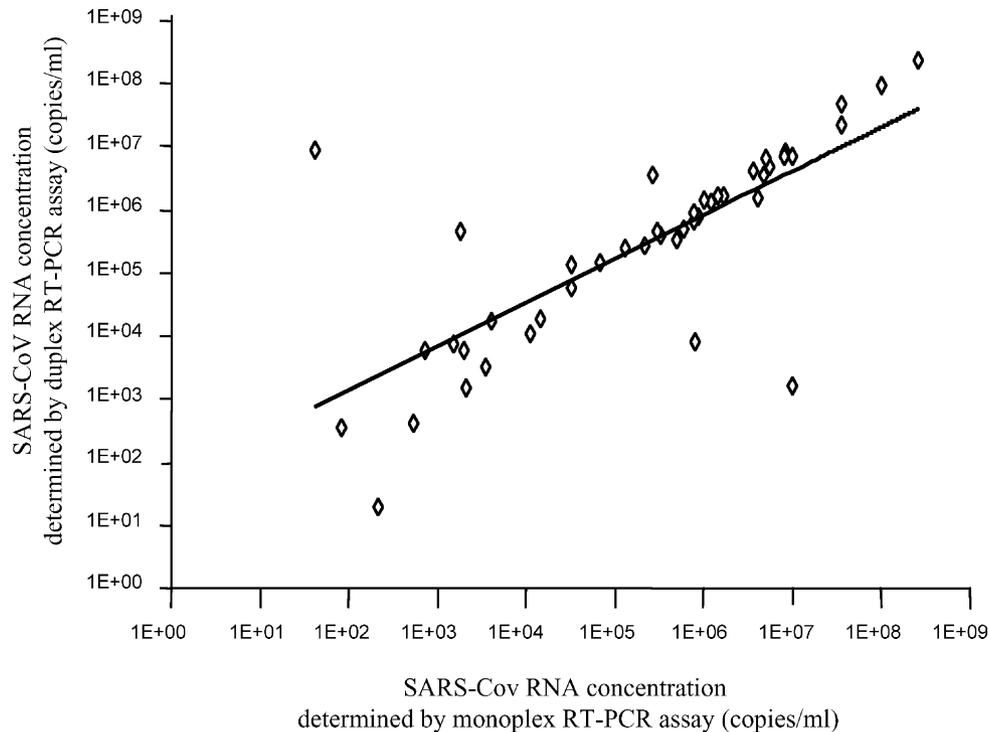


Fig. 2. Correlation of SARS-CoV RNA concentrations in NPA sample between the monoplex and duplex RT-PCR systems.

3. Results

3.1. One step RT-PCR assay for SARS-CoV detection

A total of 86 nasopharyngeal aspirates from clinically confirmed SARS patients were recruited in this study. Twenty-nine of these clinical samples were collected at Days 1–3 of onset (mean = 2.4 ± 0.74 days) and 57 were collected at Days 4–9 of the onset (mean = 6.0 ± 1.39 days). Of the samples collected at the first 3 days of illness, 96.6% ($N = 28$) were positive in the one step RT-PCR assay. For those samples collected at Days 4–9 of the disease, 46 out of 57 of these samples (80.7%) were positive. None of the negative control samples ($N = 24$) and water controls gave a positive reaction.

3.2. Duplex one step quantitative RT-PCR assay for SARS-CoV and 18S ribosomal RNA

The occurrence of false negative results might due to the poor recovery of RNA, the poor performance of RT-PCR reaction, the presence of PCR inhibitors or human errors. To minimize and control for these factors, we further modify our test into a duplex RT-PCR assay that allows simultaneous detection of SARS-CoV and endogenous 18S rRNA derived from host cells. This duplex assay had a dynamic range for SARS-CoV from 10^6 to at least 10 copies per reaction (Fig. 1A). We evaluated this duplex assay by using 48 SARS specimens which were tested by the monoplex RT-PCR as described above (Fig. 1B). Of these 48 SARS samples, 44 were positive in the previous assay. Results gen-

erated from the duplex RT-PCR assay correlated completely with the results of the monoplex assay. The correlation coefficient for ORF1b sequence concentrations deduced from these two systems was 0.995 (Pearson correlation analysis; $P < 0.001$; Fig. 2), indicating that the primer-limiting amplification of 18S ribosomal RNA does not affect PCR amplification efficiency for the viral sequence. All of these SARS samples were positive for the 18S rRNA as expected. Furthermore, all of negative control samples ($N = 24$) were negative for SARS-CoV, but positive for the 18S rRNA.

4. Discussion

Since the discovery of SARS-CoV, we and others have developed molecular diagnostic assays for detecting SARS-CoV RNA (Dristan et al., 2003; Ng et al., 2003; Peiris et al., 2003; Poon et al., 2003a, 2003b, 2004). In particular, we recently reported a highly sensitive two step quantitative RT-PCR assay for early SARS diagnosis which has been validated on relevant clinical specimens (Poon et al., 2003a). In order to reduce hands-on processing time and handling procedures, we have modified this assay into a one step RT-PCR assay. The overall sensitivity and specificity of the test is 86.2% and 100%, respectively. In addition, we further modified this assay into a duplex assay that allows simultaneous detection of SARS-CoV and 18S rRNA as an internal positive control. The detection of 18S rRNA enables us to discriminate false-negative due to PCR inhibitors. To avoid interference with the amplification of ORF1b sequence, the concentration of primers specific for

the 18S rRNA was limited to 100 nM, a concentration eight times lower than the one for SARS-CoV detection. Results generated from the duplex assay correlates exactly with that of the monoplex assay, demonstrating the internal positive control could be detected with no discernible effect on target sequence detection. In conclusion, we have reported a one step quantitative RT-PCR assay for simultaneous detection of SARS-CoV and 18S rRNA sequences.

The simplicity of this highly sensitive assay makes it useful for application to large numbers of samples for the detection and quantitation of SARS-CoV in clinical specimens.

Acknowledgements

We acknowledge research funding from Public Health Research Grant A195357 from the National Institute of Allergy and Infectious Diseases, USA, The Research Grant Council of Hong Kong (HKU 7543/03M), The University of Hong Kong (HKU SARS Research Fund) and the Hospital Authority of Hong Kong SAR.

References

- Dristan C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med* 2003;348:1967–76.
- Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, et al. A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med* 2003;348:1953–66.
- Ng EK, Hui DS, Chan KC, Hung EC, Chiu RW, Lee N, et al. Quantitative analysis and prognostic implication of SARS coronavirus RNA in the plasma and serum of patients with severe acute respiratory syndrome. *Clin Chem* 2003;49:1976–80.
- Peiris JS, Lai ST, Poon LL, Guan Y, Yam LY, Lim W, et al. Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* 2003;361:1319–25.
- Poon LLM, Chan KH, Wong OK, Yam WC, Yuen KY, Guan Y, et al. Early diagnosis of SARS coronavirus infection by real time RT-PCR. *J Clin Virol* 2003a;28:233–8.
- Poon LLM, Wong OK, Chan KH, Luk W, Yuen KY, Peiris JS, et al. Rapid diagnosis of a coronavirus associated with severe acute respiratory syndrome (SARS). *Clin Chem* 2003b;49:953–5.
- Poon LLM, Chan KH, Wong OK, Cheung TK, Ng I, Seto WH, Zheng BJ, et al. Detection of SARS coronavirus in SARS patients by conventional and real-time quantitative RT-PCR assays. *Clin Chem*, 2004;50:67–72.