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Early diagnosis of SARS Coronavirus infection by real time RT-PCR

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Abstract

Background: A novel coronavirus was recently identified as the aetiological agent of Severe Acute Respiratory Syndrome (SARS). Molecular assays currently available for detection of SARS-coronavirus (SARS-Cov) have low sensitivity during the early stage of the illness. **Objective:** To develop and evaluate a sensitive diagnostic test for SARS by optimizing the viral RNA extraction methods and by applying real-time quantitative RT-PCR technology. **Study design:** 50 nasopharyngeal aspirate (NPA) samples collected from days 1–3 of disease onset from SARS patients in whom SARS CoV infections was subsequently serologically confirmed and 30 negative control samples were studied. Samples were tested by: (1) our first generation conventional RT-PCR assay with a routine RNA extraction method (Lancet 361 (2003) 1319), (2) our first generation conventional RT-PCR assay with a modified RNA extraction method, (3) a real-time quantitative RT-PCR assay with a modified RNA extraction method. **Results:** Of 50 NPA specimens collected during the first 3 days of illness, 11 (22%) were positive in our first generation RT-PCR assay. With a modification in the RNA extraction protocol, 22 (44%) samples were positive in the conventional RT-PCR assay. By combining the modified RNA extraction method and real-time quantitative PCR technology, 40 (80%) of these samples were positive in the real-time RT-PCR assay. No positive signal was observed in the negative controls. **Conclusion:** By optimizing RNA extraction methods and applying quantitative real time RT-PCR technologies, the sensitivity of tests for early diagnosis of SARS can be greatly enhanced.

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Keywords: Early diagnosis; SARS Coronavirus; Real time RT-PCR

1. Introduction

Severe acute respiratory syndrome (SARS) is a new emerging viral disease that has affected many countries (Poutanen et al., 2003; Tsang et al.,

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2003). A novel coronavirus (SARS-CoV) was isolated from patients with SARS (Peiris et al., 2003a; Drosten et al., 2003; Ksiazek et al., 2003). Seroconversion to SARS-CoV was found in the majority of SARS patients (Peiris et al., 2003a,b). By contrast, none of the patients with other respiratory disease and healthy blood donors had detectable antibody (Peiris et al., 2003a,b). Furthermore, experimental infection of cynomolgus macaques (*Macaca fascicularis*) confirmed the hypothesis that this newly discovered SARS-CoV is the aetiological agent of SARS (Fouchier et al., 2003; Kuiken et al., 2003). Thus, this novel virus fulfills all Koch's postulates as the primary aetiological agent of SARS.

The clinical case definition of SARS is essentially one of fever and pneumonia, with or without a contact history. There are many causes of pneumonia and in the absence of a definite history of contact with other patients with SARS, it is not easy to differentiate SARS from other causes of pneumonia. Laboratory tests that can confirm a diagnosis of SARS-CoV infection early in the course of the illness are therefore a critical clinical need. It allows prompt patient management, isolation and quarantine, thereby minimizing the risk of having large-scale outbreaks in hospitals or in local communities (Riley et al., 2003). Serology is a sensitive and specific diagnostic approach but seroconversion can only be detected around day 10 of illness and in some patients, especially if they have been treated with immunomodulator drugs

such as steroids, may be delayed until the 3rd or 4th week of the disease. In any event, they only provide a retrospective diagnosis. Detection of virus by RT-PCR in clinical specimens offers the option of diagnosis in the early stage of the disease (Poon et al., 2003a). However, in contrast with many other acute respiratory infections (e.g. influenza) (Kaiser et al., 1999), our previous studies demonstrated that viral loads in NPA are low in the first few days of illness and peak around day 10 of the disease (Peiris et al., 2003b). This profile of viral activity poses a challenge for the diagnosis of SARS during the first few days of the illness, the period when such a diagnosis would be most useful, both for patient management and public health. The clinical sensitivity of first generation RT-PCR methods during the first few days of disease has been low and much better sensitivity is obtained after day 6 of the illness (Peiris et al., 2003b). On the other hand, the gradual increase in viral load implies that the window for effective therapeutic intervention with an antiviral is wider than that with other respiratory viral diseases such as respiratory syncytial virus or influenza infections.

We have continued to develop and evaluate specimen extraction methods and conventional RT-PCR and quantitative real-time PCR assays for SARS detection (Peiris et al., 2003a; Poon et al., 2003a,b) with the aim of closing the diagnostic gap in the first 5 days of illness. In this study, we evaluate an assay based on a modified RNA

Table 1

Detection of SARS CoV in clinical specimens by different conventional and real-time RT-PCR protocols in relation to time after onset of disease

Day of onset	Sample Size	Number of positives		
		Conventional RT-PCR assay	Conventional RT-PCR assay with a modified RNA extraction protocol*	Real-time RT-PCR assay with a modified RNA extraction protocol***
1	8	0 (0%)	2 (25%)	5 (63%)
2	16	3 (19%)	8 (50%)	14 (88%)
3	26	8 (31%)	12 (46%)	21 (81%)

* The overall detection rate of the assay is statistically different from that of the conventional RT-PCR assay (McNemar's test, $P < 0.001$).

** The overall detection rate of the assay is statistically different from that of the conventional RT-PCR assay with a modified RNA extraction protocol (McNemar's test, $P < 0.0001$).

extraction protocol and quantitative real-time RT-PCR assay for early SARS diagnosis.

2. Materials and methods

2.1. Patients and sample collection

Stored clinical specimens from 50 patients fulfilling the clinical WHO case definition of SARS (<http://www.who.int/csr/sars/casedefinition/en/>) in whom the diagnosis was subsequently confirmed by seroconversion (Peiris et al., 2003a) were used in this study. NPA samples were collected from days 1–3 of disease onset as described previously (Poon et al., 2003a). NPA samples from patients with unrelated diseases were recruited as controls.

2.2. RNA extraction and reverse transcription

RNA from clinical samples was extracted using the QIAamp virus RNA mini kit (Qiagen) as instructed by the manufacturer. In our previously published conventional RT-PCR assay, 140 μ l of NPA was used for RNA extraction. In the revised RNA extraction protocol, 540 μ l of NPA was used for RNA extraction. Extracted RNA was finally eluted in 30 μ l of RNase-free water and stored at -20°C . Complementary DNA was generated as described (Poon et al., 2003a).

2.3. Conventional PCR for SARS-CoV

Conventional PCR assay for was performed as described (Peiris et al., 2003a).

2.4. Real-time quantitative PCR assays for SARS-CoV

A real-time quantitative PCR specific to the 1b region of the SARS-CoV was used in this study (Poon et al., 2003b). Complementary DNA was amplified by a TaqMan PCR Core Reagent kit in a 7000 Sequence Detection System (Applied Biosystems). Briefly, 4 μ l of cDNA was amplified in a 25 μ l reaction containing 0.625 U AmpliTaq Gold polymerase (Applied Biosystems), 2.5 μ l of $10\times$ TaqMan buffer A, 0.2 mM of dNTPs, 5.5 mM of

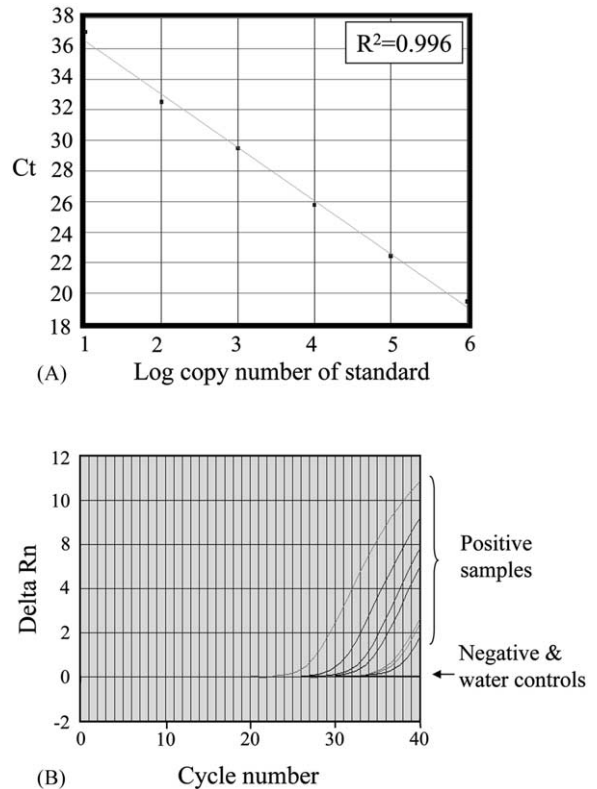


Fig. 1. Real-time quantitative RT-PCR assays for SARS-CoV. (A) Standard curve for quantitative analysis of ORF 1b of SARS-CoV. The threshold cycle (Ct) is the number of PCR cycles required for the fluorescent intensity of the reaction to reach a pre-defined threshold. The Ct is inversely proportional to the logarithm of the starting concentration of the input DNA. (B) An amplification plot of fluorescence intensity against the PCR cycle. The amplification curves of positive clinical samples, negative clinical samples and water controls are indicated. The X axis denotes the cycle number of a quantitative PCR assay. The Y axis denotes the fluorescence intensity over the background.

MgCl_2 , 2.5 U of AmpErase UNG, and $1\times$ primers-probe mixture (Assays by Design, Applied Biosystems). The primer sequences were 5'-CA-GAACGCTGTAGCTTCAAAAATCT-3' and 5'-TCAGAACCCTGTGATGAATCAACAG-3', and the probe was 5'-(FAM)TCTGCGTAGG-CAATCC(NFQ)-3' (FAM, 6-carboxyfluorescein; NFQ, nonfluorescent quencher). Reactions were first incubated at 50°C for 2 min, followed by 95°C for 10 min. Reaction were then thermal-

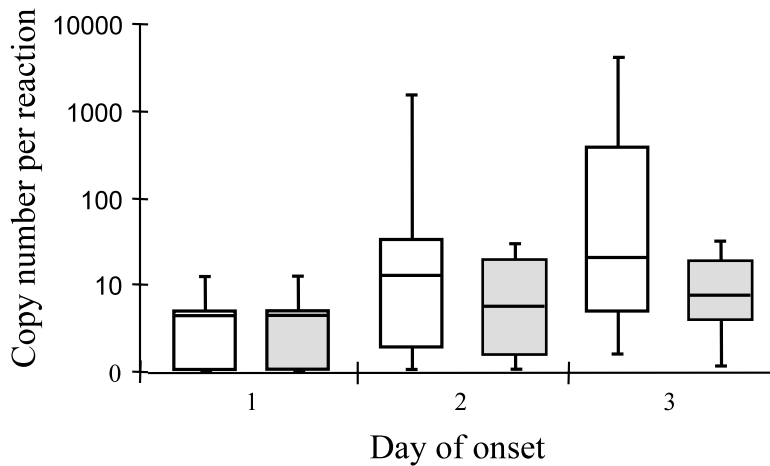


Fig. 2. Changes of viral loads of SARS-CoV in NPA samples collected at day 1-3 of disease onset. Open bar: viral loads of SARS-CoV from samples that were positive in the real-time quantitative PCR assay. Grey bar: Viral loads of SARS-Cov from samples that were positive in the real-time assay, but negative in the conventional RT-PCR assay. The upper and lower limits of the boxes and the lines across the boxes indicate the 75th and 25th percentiles and the median, respectively. The upper and lower horizontal bars indicate the 90th and 10th percentiles, respectively.

cycled for 45 cycles (95 °C for 15 s, 60 °C for 1 min). Plasmids containing the target sequences were used as positive controls. The operating characteristics and specificity of this assay has been validated elsewhere (Poon et al., 2003b).

3. Results

A total of 50 NPA specimens isolated from serologically confirmed SARS patients collected during the first 3 days of illness were studied. Of these, 11 (22%) were positive in our previously reported conventional RT-PCR assay (Peiris et al., 2003a) (Table 1).

We reasoned that the poor sensitivity of SARS-CoV RT-PCR detection in the early stage of the illness could be enhanced by increasing the initial extraction volume of the NPA sample from 140 to 560 μ l. Using this modified RNA extraction protocol, the sensitivity of the conventional RT-PCR assay doubled from 11/50 to 22/50 (Table 1). The overall detection rate of the modified RT-PCR protocol was statistically different from that of our first generation RT-PCR protocol (McNe-

mar's test, $P < 0.001$, Table 1). Of 30 negative control samples, one false positive result was observed. With the RNA extraction modification, the sensitivity and specificity of the conventional RT-PCR on specimens collected during the first 3 days of illness was 44.0% and 96.6%, respectively.

To further improve the detection of SARS-CoV in samples from early onset, we adopted a highly sensitive real-time quantitative assay for SARS-CoV detection (Poon et al., 2003b) (Fig. 1A). With the modified RNA extraction protocol, 40 out of 50 NPA samples were positive in the real-time assay (Fig. 1B and Table 1). The overall detection rate of the modified RT-PCR protocol was statistically different from the other two assays (McNemar's test, $P < 0.0001$, Table 1). In particular, 63% of the NPA samples isolated on day 1 of disease onset was positive in the real-time quantitative RT-PCR assay. By contrast, none of the specimens isolated on day 1 was positive in the conventional RT-PCR assay. For samples isolated on days 2–3, more than 81% of these samples was positive in the quantitative assay (Table 1). With the modified RNA extraction protocol and real-time PCR technology, the specificity and sensitiv-

ity of the quantitative assay towards early SARS samples were 80% and 100%, respectively.

The real-time assay also allowed one to quantify the viral loads of these clinical specimens (1 copy/reaction = 27.8 copies/ml of a NPA sample). As shown in Fig. 2, the progression of the disease resulted in an increase of viral loads in NPA (open bars). In addition, we further examined the viral loads of clinical samples that were negative ($n = 39$) in our first generation RT-PCR assay (Fig. 2, grey bars). As expected, the viral loads of these samples (grey bars) were much lower than the overall viral loads of the whole cohort (open bars).

4. Discussion

Our objective of this study was to establish a highly sensitive RT-PCR assay for detecting SARS-CoV. In particular, we focused on detecting SARS-CoV RNA in samples isolated on days 1–3 of disease onset. Using our first generation conventional RT-PCR assay (Peiris et al., 2003a,b), only 22% of these samples were shown to have SARS-CoV RNA. In order to establish a more sensitive assay, we modified the RNA extraction method and adapted the quantitative technology in our current study. By increasing the initial volume for RNA extraction from 140 to 540 μ l, the proportion of positive cases was increased to 44%. In addition, by further applying the real-time quantitative PCR technology in the revised assay, 80% of early SARS samples became positive. More importantly, the use of a 5' nuclease probe in the real-time quantitative assay can minimize the false positive rate due to an increased in signal specificity. Taken together, results from this study suggested that our revised RT-PCR assay allows the early and accurate diagnosis of SARS.

The quantitative result of our modified RT-PCR assay provided further information regarding to the viral load of SARS-CoV in these clinical specimens. Our results indicated that the viral load increases as the disease progresses. Of those samples that were negative in the first generation RT-PCR assay, all of these specimens contained very low amounts of viral RNA (Fig. 2b). This observation explained why most of these samples

were negative using our first generation RT-PCR assay. Interestingly, for those specimens that were positive in the first generation assay, some had very high amounts of viral RNA (Fig. 2). Whether this observation has any clinical significance requires further investigation.

In summary, by increasing the initial sample volume for RNA extraction and utilizing real-time quantitative PCR technology, we established a sensitive and accurate RT-PCR assay for the prompt identification of SARS-CoV. It is expected that, with this rapid diagnostic method, a prompt identification of this pathogen will facilitate the control of the disease and the institution of prompt treatment.

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