

Molecular Diagnosis of SARS

Enders K. O. Ng and Y. M. Dennis Lo

Abstract

The etiologic agent of severe acute respiratory syndrome (SARS) has been identified as a new type of coronavirus, known as SARS coronavirus (SARS-CoV). Although the severe acute respiratory syndrome (SARS) epidemic was subsided, many authorities including the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) have warned on the possible re-emergence of this highly infectious disease. Although antibody-based diagnosis of SARS has been demonstrated to be a reliable proof of SARS infection, it is not sensitive enough for detection during the early phase of the disease. To date based on the publicly released full genomic sequences of SARS-CoV, various molecular detection methods based on reverse transcriptase polymerase chain reaction (RT-PCR) have been developed. Although most of the assays have been initially focused on RNA extracted from nasopharyngeal aspirates, urine and stools, subsequently developed plasma/serum-based assays allow the precise and standardized quantitative expression of viral loads demonstrated to be potentially used for early SARS diagnosis. In this chapter, two real-time quantitative RT-PCR systems based on our recent development of the quantifications of SARS-CoV RNA in serum are discussed. The two RT-PCR systems, one towards the nucleocapsid region and the other towards the polymerase region of the virus genome have detection rate of 80% during the first week of illness. These quantitative systems not only be potentially used in early diagnosis of SARS but also provide the viral load information allowing clinicians to make a prognostic evaluation of the infected individual.

Keywords: serum RNA, SARS-CoV RNA, viral RNA extraction, RNA quantification, real-time quantitative reverse transcriptase PCR

1. Introduction

The recent identification of SARS-coronavirus (SARS-CoV) as the etiologic agent of severe acute respiratory syndrome (SARS) has led scientists to develop rapid and sensitive diagnostic tests (1-6). However, the progress in the development of sensitive and early diagnostic tests for SARS has been slower than originally expected. Although the severe acute respiratory syndrome (SARS) epidemic was subsided, many authorities including the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) have warned on the possible re-emergence of this highly infectious disease. Thus, the development of better diagnostic tests for SARS is urgently needed. Most of the diagnostic tests are not sensitive enough for detection during the early phase of the disease. For instance, the use of nasopharyngeal aspirates has a sensitivity of only 32% on day 3 of the disease (7). This severe limitation has restricted our ability to identify patients in a prompt manner and to institute isolation and treatment.

Based on the publicly released full genomic sequences of SARS-CoV (8-10), various molecular detection methods based on reverse transcriptase polymerase chain reaction (RT-PCR) have been developed. These PCR-based diagnostic tests are used to detect SARS-CoV RNA in patients' specimens in which viral RNA is reverse-transcribed into DNA and then different regions of the SARS-CoV genome are specifically amplified by PCR. Several RT-PCR protocols developed by members of the WHO laboratory network are available on the WHO website (<http://www.who.int/csr/sars/primers/en/>).

RT-PCR is mainly divided into qualitative (conventional) and quantitative approaches. Conventional RT-PCR approaches are normally qualitative in nature and require time-consuming and contamination-prone post-PCR analysis. Real-time quantitative RT-PCR has overcome many of these shortcomings and has been increasingly adopted by various laboratories for SARS diagnosis (2,11-15). With suitable instrumentation, this technology allows data to be recorded and analyzed during PCR cycling. Furthermore, it runs as a closed-tube system and post-amplification manipulation can be eliminated. Thus, this methodology reduces the risk of contamination and minimizes hands-on time. The entire amplification process requires only 3 hours and allows such technology to be used for high-throughput application.

After SARS outbreak, the PCR-based testing for SARS has mainly been focused on the analysis of nasopharyngeal aspirates, urine and stools (7,11). An early study has reported that SARS-CoV RNA was detected in 32% of nasopharyngeal aspirates from SARS patients studied at a mean of 3.2 days after the onset of illness and the detection rate increased to 68% at day 14 (7). In the same study, SARS-CoV RNA was detected in 97% of stool samples collected at a mean of 14.2 days after symptom onset. Similarly, viral RNA was detected in 42% of urine samples collected from the SARS patients at a mean of 15.2 days after onset (7). Despite the high sensitivity of stool sample testing, early detection of SARS-CoV still suffers from a lack of high

sensitivity. Although most of the assays have been predominantly focused on RNA extracted from nasopharyngeal aspirates, urine and stools, the quantitative interpretation of these data is difficult due to the inability to standardize such data as a result of the influence of numerous factors, such as sampling technique for nasopharyngeal aspirates, urine volume, variations of bowel transit time (e.g. during diarrhoea) or stool consistency. On the other hand, plasma/serum-based assays may allow the precise and standardized quantitative expression of viral loads, thus, enabling the assessment of disease severity and prognosis. Detection of viral nucleic acids in plasma/serum has been well-established for viral load studies for numerous other viruses (16,17). At the beginning of the SARS outbreak, there has been a single report showing the relatively low sensitivity of detecting SARS-CoV RNA in plasma using an ultracentrifugation-based approach, with low concentrations of SARS-CoV detected in the plasma of a patient 9 days after disease onset (2). Subsequently, together with the improvement of viral RNA extraction in which plasma or serum required no ultracentrifugation, two real-time quantitative RT-PCR assays, one towards the polymerase region and the other towards the nucleocapsid region of the virus genome (see **Figure 1**), were developed for measuring the concentration of SARS-CoV RNA in serum/plasma samples from SARS patients (13,14). In these assays, the absolute calibration curves are constructed by serial dilutions of high performance liquid chromatography (HPLC)-purified single stranded synthetic DNA oligonucleotides specifying the studied amplicons (see **Figure 2**). Previous studies have shown that such single stranded oligonucleotides reliably mimic the products of the reverse transcription step and produce calibration curves that are identical to those obtained using T7-transcribed RNA (18,19). The use of such calibration methodology significantly simplifies the process of obtaining a calibration curve when compared with the labour-intensive preparation of calibration curve involves amplicon subcloning and *in vitro* transcription.

The sensitivities of the amplification steps of these assays are sufficient to detect 5 copies of the targets in the reaction mixtures, corresponding to 74 copies/mL of serum (13). Using these RT-PCR assays SARS-CoV RNA was detected in 75% to 78% of serum samples from SARS patients during the first week of illness (13). In the same study, data showed that median concentrations of serum SARS-CoV RNA in patients who required ICU admission during the course of hospitalisation was significantly higher than those who did not require intensive care (13) (see **Figure 3**). This quantitative test thus provides the viral load information allowing clinicians to make a prognostic evaluation of the infected individual.

Recent reports revealed that the clinical course of paediatric SARS patients was less severe in comparison with adult SARS patients (20,21). On the whole, the outcomes of paediatric SARS patients were favorable. With the use of the plasma/serum-based quantitative RT-PCR assay, SARS-CoV RNA has recently been shown to be detectable in the plasma samples of paediatric patients during different stages of SARS (see **Figure 4**) (14). No significant difference in plasma SARS-CoV

viral load has been observed between paediatric and adult SARS patients taken within the first week of admission and at day 7 after fever onset (*14*). Overall, viraemia appears to be a consistent feature in both paediatric and adult SARS patients.

The relatively high detection rate of SARS-CoV in plasma and serum during the first week of illness suggests that plasma/serum-based RT-PCR should be incorporated into the routine diagnostic workup of suspected or confirmed SARS patients both in adult and paediatric populations. This approach opens up numerous interesting research opportunities. For example, this assay can be used to monitor the effect or lack of effects of anti-viral agents. Also, it would be valuable to explore the potential damaging effect of giving steroids at a time when the viral load is still relatively high. We are aware that many of these questions might not be answerable with retrospectively collected samples. Nonetheless, the development of animal models (*5,6*) might allow the testing of some of these hypotheses in a controlled manner.

In this chapter, the two systems discussed below are based on our recent development of the quantifications of SARS-CoV RNA in serum. The SARSN RT-PCR system towards the nucleocapsid region and SARSpoll1 towards the polymerase region of the virus genome aim to measure the concentration of SARS-CoV RNA and demonstrated to have detection rate of 80% during the first week of illness. These SARS-CoV RT-PCR systems not only are used in early diagnosis of SARS but also provide the viral load information allowing clinicians to make a prognostic evaluation of the infected individual.

2. Materials

2.1. Sample Collection

1. Plain collection tubes for serum collection.
2. RNase Away (Invitrogen, Carlsbad, California).

2.2. RNA Extraction

1. QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany).
2. Absolute ethanol

2.3. Real-Time Quantitative RT-PCR

2.3.1. Amplification Reagents

1. Primers (*see Note 1*):
 - a. SARSpol1:
 - i. Forward: 5'-GAGTGTGCGCAAGTATTAAGTGA-3'.
 - ii. Reverse: 5'-TGATGTTCCACCTGGTTTAAACA-3'.
 - b. SARSN:
 - i. Forward: 5'-TGCCCTCGCGCTATTG-3'.
 - ii. Reverse: 5'-GGCCTTTACCAGAACTTTGC-3'.
2. Dual-labelled Fluorescent probes (*see Note 1*):
 - a. SARSpol1:

5'-(FAM)ATGGTCATGTGTGGCGGCTCACTA(TAMRA)-3'.
 - b. SARSN:

5'-(FAM)TGCTAGACAGATTGAACCAGCTTG(TAMRA)-3'.

where FAM is 6-carboxy-fluorescein; TRMRA is 6-carboxy-tetramethylrhodamine.

The amplicon locations of the SARSN and SARSpol1 RT-PCR systems are showed in Figure 1.
3. Calibrators
 - a. Synthetic DNA oligonucleotides specifying the polymerase gene of SARS-CoV genome, HPLC purified (Proligos, Singapore) (*see Note 2*):

5'-AACGAGTGTGCGCAAGTATTAAGTGAGATGGTCATGTGTGGCGGCTCACTATATGTAAACCAGGTGGAACATCATCCGG-3'.
 - b. Synthetic DNA oligonucleotides specifying the nucleocapsid gene of SARS-CoV genome, HPLC purified (Proligos, Singapore) (*see Note 2*):

5'-GAAACTGCCCTCGCGCTATTGCTGCTAGACAGATTGAAC CAGCTTGAGAGCAAAGTTTCTGGTAAAGGCCAACAA- 3'
4. RNase-free water.
5. EZ *rTth* RNA PCR reagent set (Applied Biosystems, Foster City, CA).

2.3.2. Instrumentation for Quantitative Analysis

1. ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA).

3. Methods

3.1. Prevention of contamination

Due to the high sensitivity of RT-PCR-based approaches, strict precautions should be applied to prevent the RT-PCR assay from contamination (**22**). These precautions include:

1. Aerosol-resistant pipette tips should be used for all liquid handling.
2. Separate areas should be used for the RNA extraction step, the setting up of amplification reactions, the addition of template and the carrying out of amplification reactions.
3. Real-time PCR approaches obviate the need for post-PCR processing and further reduce the risk of contamination.
4. The assay should include a further level of anticontamination measure in the form of pre-amplification treatment using uracil N-glycosylase which destroys uracil containing PCR products (**23**).
5. Multiple negative water blanks should be included in every analysis so as to eliminate the possibility of reagent contamination.

3.2. Sample Collection

1. Collect blood samples in plain tubes.

To ensure a sufficient amount of serum for RNA analysis, at least 3 mL of blood should be taken for each sample (*see Note 3*). The blood samples should be processed as soon as possible to guarantee good quality of the viral RNA in plasma. If the processing procedures cannot take place immediately, the blood samples should be stored with extra cares (*see Note 4*).

2. Centrifuged the blood samples at 1,600g for 10 min at 4°C.
3. Transfer serum into new tubes.
4. Store the serum at -80°C until RNA extraction (*see Note 5*).

3.3. RNA Extraction

The RNA extraction should be performed in a clean and separated area to minimize cross contamination.

1. Add 1.12 mL of AVL buffer to 0.28 mL of serum, mix and incubate at room temperature for 10 min.
2. Add 1.12 mL of absolute ethanol to the mixture and mix.
3. Apply the mixture to an RNeasy Mini column and wash the column according to the manufacturer's recommendations.
4. To elute RNA, add 50 µL of AVE buffer onto the silica-gel membrane and incubate for 1 min at room temperature. Centrifuge the RNeasy column for 1 min at 6,000g.

5. Stored the extracted RNA at -80°C until use.

3.4. Real-Time Quantitative RT-PCR

Serum SARS-CoV RNA is quantified by using one-step real-time quantitative RT-PCR (24). In this method, the *rTth* (*Thermus thermophilus*) DNA polymerase functioned both as a reverse transcriptase and a DNA polymerase (25) (see Note 7). Instead of using a two-step RT-PCR approach, one-step RT-PCR, incorporating both the reverse transcription and PCR steps in a single tube, should be used to reduce both hands-on time and the risk of contamination. In this protocol, the quantifications of SARS-CoV RNA are described as follows:

3.4.1. Quantification of *pol* and *N* genes of SARS-CoV genome

1. Prepare calibration curves by serially diluting the synthetic DNA oligonucleotides specifying the amplicons with concentrations ranging from 1.0×10^7 copies to 5 copies (see Note 3).
2. Set up the RT-PCR reaction mixture for *pol* and *N* genes according to Table 1.
3. Add 11.25 μL of sample RNA, synthetic DNA oligonucleotides (for calibration curve) or RNase-free water (for negative blanks) into the reaction mixture.
4. Perform the real-time RT-PCR reactions in the ABI Prism 7700 Sequence Detector with cycling conditions shows in Table 2.

3.4.2. Data Analysis

Amplification data were analyzed and stored by the Sequence Detection System Software (Version 1.9; Applied Biosystems). The SARS-CoV RNA concentration is expressed as copies per milliliter of serum (copies/mL). The calculation is shown as follows:

$$C = Q \times \frac{V_{RNA}}{V_{serum}}$$

in which C represents the SARS-CoV RNA concentration in serum (copies/mL), Q represents the target quantity (copies/ μL) determined by a sequence detector in a PCR, V_{RNA} represents the total volume of RNA obtained after extraction (typically 50 μL), V_{Serum} represents the volume of serum extracted (typically 0.28 mL).

The validations of the two real-time RT-PCR systems are described in previous publications (13).

5. Notes

1. Primers and probes are designed with the use of *Primer Express*[®] Software v2.0 (Applied Biosystems). Certain precautions for the design are listed as follows:
 - i. The amplicon length should be less than 100 bp, ideally no longer than 80 bp. Short amplicon length is preferable due to several reasons: (i) The synthetic oligonucleotides specifying the amplicon used as a calibration curve is commercially available with size only up to 100 bp. Thus, the amplicon length is limited to 100 bp. (ii) Amplification with shorter amplicon length are more efficient than that of longer amplicon length (**18**).
 - ii. To avoid false positive results arising from co-amplification of genes with high homology, it is necessary to perform a BLASTN search with the primer and probe sequences against the NCBI GenBank. The result of such search will provide information regarding the specificity of the amplification.
2. Generally, *in vitro*-transcribed RNA is used as a calibration curve for an absolute RNA quantification. This *in vitro*-transcribed RNA is usually generated by subcloning the amplicon behind a T7 RNA polymerase promoter in a plasmid vector. However, this procedure is labor intensive and time consuming, which is unsuitable when a large number of RT-PCR systems need to be constructed in a short period of time. An alternative method is increasingly used to construct a calibration curve with the use of synthetic single-stranded oligonucleotides specifying the amplification amplicons. Previous data have shown that such single stranded oligonucleotides reliably mimic the products of the reverse transcription step and produce calibration curves that are identical to those obtained using T7-transcribed RNA (**18**). Due to commercial availability of the synthetic oligonucleotides with size only up to 100 bp, the amplicon length should be less than 100 bp.
3. Our studies have revealed that 0.28 mL of plasma is the minimal sample volume for the viral RNA detection in plasma. Thus, 3 mL of blood samples should be sufficient for testing.
4. When the blood samples are left unprocessed, their corresponding plasma RNA concentrations will fluctuate over time (**26**). This artifactual fluctuation may due to several factors, such as release of RNA from necrotic and/or apoptotic blood cells, and the stability of the original and the newly released RNA. To guarantee a reliable plasma/serum RNA concentration, we recommend all blood samples, including EDTA blood and clotted blood, store at 4°C and process within 1 hours.
5. According to our pre-analytical studies, no significant differences in viral RNA concentrations were observed between serum stored at -20°C and -80°C (unpublished data). However, serum samples stored at -80°C is preferred.
6. The use of one-step one-enzyme RT-PCR with *rTth* polymerase has several advantages over the two-enzyme RT-PCR: (i) It has been reported that *Tth*

polymerase is more resistant to inhibitors present in biological specimens than *Taq* polymerase (27). (ii) The *rTth* polymerase is thermostable and thus allows the reverse transcription to perform at high temperature (60°C). This minimized the secondary structures present in the RNA. (iii) As both reverse transcription and PCR carry out in a single tube, this reduces both hands-on time and the risk of contamination.

Table 1**Composition of RT-PCR Mix for amplification of pol and N genes of SARS-CoV**

Component	Volume for one reaction (μL)	Final Concentration
5X TaqMan EZ Buffer	5	1X
Mn(OAc) ₂ (25 mM)	3	3 mM
dATP (10 mM)	0.75	300 μM
dCTP (10 mM)	0.75	300 μM
dGTP (10 mM)	0.75	300 μM
dUTP (20 mM)	0.75	600 μM
Forward Primer (10 μM)	0.5	300 nM
Reverse Primer (10 μM)	0.5	300 nM
Probe (5 μM)	0.5	100 nM
<i>rTth</i> DNA Polymerase (2.5 U/ μL)	1	0.1 U/ μL
AmpErase UNG (1 U/ μL)	0.25	0.01 U/ μL
Total volume	13.75	

Table 2**Cycling Profile for amplification of pol and N genes of SARS-CoV**

Step	Temperature	Time
UNG Treatment	50 °C	2 min
Reverse Transcription	60 °C	30 min
Deactivation of UNG	95 °C	5 min
40 Cycles	Denaturation	94 °C 20 sec
	Annealing / Extension	56 °C 1 min

Acknowledgements

References

- 1 Ksiazek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J. A. Comer, W. Lim, P. E. Rollin, S. F. Dowell, A. E. Ling, C. D. Humphrey, W. J. Shieh, J. Guarner, C. D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Y. Yang, N. Cox, J. M. Hughes, J. W. LeDuc, W. J. Bellini and L. J. Anderson (2003). A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med* **348**, 1953-66.
- 2 Drosten, C., S. Gunther, W. Preiser, S. van der Werf, H. R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kolesnikova, R. A. Fouchier, A. Berger, A. M. Burguiere, J. Cinatl, M. Eickmann, N. Escriou, K. Grywna, S. Kramme, J. C. Manuguerra, S. Muller, V. Rickerts, M. Sturmer, S. Vieth, H. D. Klenk, A. D. Osterhaus, H. Schmitz and H. W. Doerr (2003). Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med* **348**, 1967-76.
- 3 Peiris, J. S., S. T. Lai, L. L. Poon, Y. Guan, L. Y. Yam, W. Lim, J. Nicholls, W. K. Yee, W. W. Yan, M. T. Cheung, V. C. Cheng, K. H. Chan, D. N. Tsang, R. W. Yung, T. K. Ng and K. Y. Yuen (2003). Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* **361**, 1319-25.
- 4 Poutanen, S. M., D. E. Low, B. Henry, S. Finkelstein, D. Rose, K. Green, R. Tellier, R. Draker, D. Adachi, M. Ayers, A. K. Chan, D. M. Skowronski, I. Salit, A. E. Simor, A. S. Slutsky, P. W. Doyle, M. Krajden, M. Petric, R. C. Brunham and A. J. McGeer (2003). Identification of severe acute respiratory syndrome in Canada. *N Engl J Med* **348**, 1995-2005.
- 5 Fouchier, R. A., T. Kuiken, M. Schutten, G. van Amerongen, G. J. van Doornum, B. G. van den Hoogen, M. Peiris, W. Lim, K. Stohr and A. D. Osterhaus (2003). Aetiology: Koch's postulates fulfilled for SARS virus. *Nature* **423**, 240.
- 6 Kuiken, Thijs, Ron A M Fouchier, Martin Schutten, Guus F Rimmelzwaan, Geert van Amerongen, Debby van Riel, Jon D Laman, Ton de Jong, Gerard van Doornum, Wilina Lim, Ai Ee Ling, Paul K S Chan, John S Tam, Maria C Zambon, Robin Gopal, Christian Drosten, Sylvie van der Werf, Nicolas Escriou, Jean-Claude Manuguerra, Klaus Stohr, JS Malik Peiris and Albert DME Osterhaus (2003). Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. *Lancet* **362**, 263-70.
- 7 Peiris, J. S., C. M. Chu, V. C. Cheng, K. S. Chan, I. F. Hung, L. L. Poon, K. I. Law, B. S. Tang, T. Y. Hon, C. S. Chan, K. H. Chan, J. S. Ng, B. J. Zheng, W. L. Ng, R. W. Lai, Y. Guan and K. Y. Yuen (2003). Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. *Lancet* **361**, 1767-72.
- 8 Tsui, S. K., S. S. Chim, Y. M.D. Lo and and The Chinese University of Hong Kong Molecular SARS Research Group (2003). Coronavirus

- genomic-sequence variations and the epidemiology of the severe acute respiratory syndrome. *N Engl J Med* **349**, 187-8.
- 9 Rota, P. A., M. S. Oberste, S. S. Monroe, W. A. Nix, R. Campagnoli, J. P. Icenogle, S. Penaranda, B. Bankamp, K. Maher, M. H. Chen, S. Tong, A. Tamin, L. Lowe, M. Frace, J. L. DeRisi, Q. Chen, D. Wang, D. D. Erdman, T. C. Peret, C. Burns, T. G. Ksiazek, P. E. Rollin, A. Sanchez, S. Liffick, B. Holloway, J. Limor, K. McCaustland, M. Olsen-Rasmussen, R. Fouchier, S. Gunther, A. D. Osterhaus, C. Drosten, M. A. Pallansch, L. J. Anderson and W. J. Bellini (2003). Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* **300**, 1394-9.
 - 10 Marra, M. A., S. J. Jones, C. R. Astell, R. A. Holt, A. Brooks-Wilson, Y. S. Butterfield, J. Khattra, J. K. Asano, S. A. Barber, S. Y. Chan, A. Cloutier, S. M. Coughlin, D. Freeman, N. Girn, O. L. Griffith, S. R. Leach, M. Mayo, H. McDonald, S. B. Montgomery, P. K. Pandoh, A. S. Petrescu, A. G. Robertson, J. E. Schein, A. Siddiqui, D. E. Smailus, J. M. Stott, G. S. Yang, F. Plummer, A. Andonov, H. Artsob, N. Bastien, K. Bernard, T. F. Booth, D. Bowness, M. Czub, M. Drebot, L. Fernando, R. Flick, M. Garbutt, M. Gray, A. Grolla, S. Jones, H. Feldmann, A. Meyers, A. Kabani, Y. Li, S. Normand, U. Stroher, G. A. Tipples, S. Tyler, R. Vogrig, D. Ward, B. Watson, R. C. Brunham, M. Krajden, M. Petric, D. M. Skowronski, C. Upton and R. L. Roper (2003). The Genome sequence of the SARS-associated coronavirus. *Science* **300**, 1399-404.
 - 11 Poon, L. L., O. K. Wong, W. Luk, K. Y. Yuen, J. S. Peiris and Y. Guan (2003). Rapid Diagnosis of a Coronavirus Associated with Severe Acute Respiratory Syndrome (SARS). *Clin Chem* **49**, 953-5.
 - 12 Poon, L. L., K. H. Chan, O. K. Wong, W. C. Yam, K. Y. Yuen, Y. Guan, Y. M. Lo and J. S. Peiris (2003). Early diagnosis of SARS Coronavirus infection by real time RT-PCR. *J Clin Virol* **28**, 233-8.
 - 13 Ng, E. K. O., D. S. Hui, K. C. Chan, E. C. Hung, R. W. Chiu, N. Lee, A. Wu, S. S. Chim, Y. K. Tong, J. J. Sung, J. S. Tam and Y. M. D. Lo (2003). Quantitative Analysis and Prognostic Implication of SARS Coronavirus RNA in the Plasma and Serum of Patients with Severe Acute Respiratory Syndrome. *Clin Chem* **49**, 1976-80.
 - 14 Ng, E. K. O., P. C. Ng, K. L. Hon, W. T. Cheng, E. C. Hung, K. C. Chan, R. W. Chiu, A. M. Li, L. L. Poon, D. S. Hui, J. S. Tam, T. F. Fok and Y. M. D. Lo (2003). Serial Analysis of the Plasma Concentration of SARS Coronavirus RNA in Pediatric Patients with Severe Acute Respiratory Syndrome. *Clin Chem* **49**, 2085-8.
 - 15 Hung, E. C., S. S. Chim, P. K. Chan, Y. K. Tong, E. K. Ng, R. W. Chiu, C. B. Leung, J. J. Sung, J. S. Tam and Y. M. Lo (2003). Detection of SARS Coronavirus RNA in the Cerebrospinal Fluid of a Patient with Severe Acute Respiratory Syndrome. *Clin Chem* **49**, 2108-9.

- 16 White, P. A., Y. Pan, A. J. Freeman, G. Marinos, R. A. Ffrench, A. R. Lloyd and W. D. Rawlinson (2002). Quantification of hepatitis C virus in human liver and serum samples by using LightCycler reverse transcriptase PCR. *J Clin Microbiol* **40**, 4346-8.
- 17 Damond, F., D. Descamps, I. Farfara, J. N. Telles, S. Puyeo, P. Campa, A. Lepretre, S. Matheron, F. Brun-Vezinet and F. Simon (2001). Quantification of proviral load of human immunodeficiency virus type 2 subtypes A and B using real-time PCR. *J Clin Microbiol* **39**, 4264-8.
- 18 Bustin, S. A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* **25**, 169-93.
- 19 Ng, E. K. O., N. B. Tsui, T. K. Lau, T. N. Leung, R. W. Chiu, N. S. Panesar, L. C. Lit, K. W. Chan and Y. M. Lo (2003). mRNA of placental origin is readily detectable in maternal plasma. *Proc Natl Acad Sci U S A* **100**, 4748-53.
- 20 Chiu, W.K., P.C. Cheung, K.I. Ng, P.I. Ip, V.K. Sugunan, D.C. Luk, L.C. Ma, B.H. Chan, K.L. Lo and W. M. Lai (2003). Severe acute respiratory syndrome in children: Experience in a regional hospital in Hong Kong. *Pediatr Crit Care Med* **4**, 279-283.
- 21 Hon, K. L., C. W. Leung, W. T. Cheng, P. K. Chan, W. C. Chu, Y. W. Kwan, A. M. Li, N. C. Fong, P. C. Ng, M. C. Chiu, C. K. Li, J. S. Tam and T. F. Fok (2003). Clinical presentations and outcome of severe acute respiratory syndrome in children. *Lancet* **361**, 1701-3.
- 22 Kwok, S. and R. Higuchi (1989). Avoiding false positives with PCR. *Nature* **339**, 237-8.
- 23 Longo, M. C., M. S. Berninger and J. L. Hartley (1990). Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* **93**, 125-8.
- 24 Gibson, U. E., C. A. Heid and P. M. Williams (1996). A novel method for real time quantitative RT-PCR. *Genome Res* **6**, 995-1001.
- 25 Myers, T. W. and D. H. Gelfand (1991). Reverse transcription and DNA amplification by a *Thermus thermophilus* DNA polymerase. *Biochemistry* **30**, 7661-6.
- 26 Tsui, N. B., E. K. O. Ng and Y. M. D. Lo (2002). Stability of endogenous and added RNA in blood specimens, serum, and plasma. *Clin Chem* **48**, 1647-53.
- 27 Poddar, S. K., M. H. Sawyer and J. D. Connor (1998). Effect of inhibitors in clinical specimens on Taq and Tth DNA polymerase-based PCR amplification of influenza A virus. *J Med Microbiol* **47**, 1131-5.

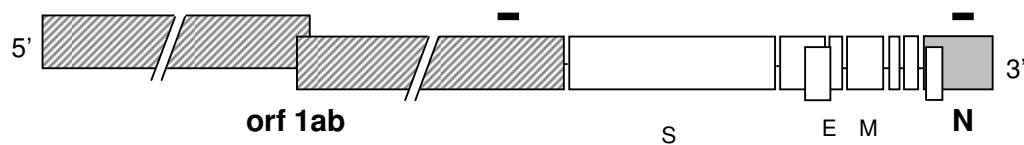


Figure 1. The locations of the PCR amplicons within the genomic organization of SARS Coronavirus. The black bars represent the two PCR amplicons located within the genomic structure of SARS coronavirus. The size of the open reading frames (ORFs) is drawn to scale, except for orf1ab. The size of the genome is 29.3 kb. *Shaded boxes* represent ORFs encoding the viral polymerase while the *filled box (N)* represents the nucleocapsid region. S represents spike protein; E represents envelope protein; M represents membrane protein.

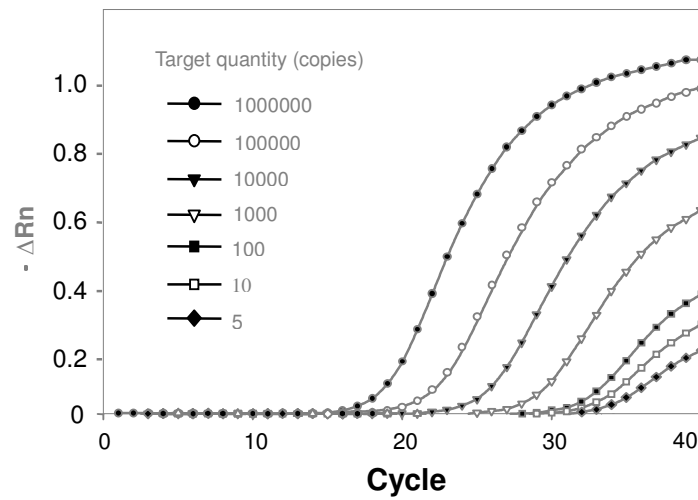


Figure 2. Detection of SARS-CoV RNA by real-time quantitative RT-PCR for the nucleocapsid region of the viral genome. An amplification plot of ΔRn , which is the fluorescence intensity over the background (Y-axis) against the PCR cycle number (X-axis). Each plot corresponds to a particular input synthetic DNA oligonucleotide target quantity marked by a corresponding symbol.

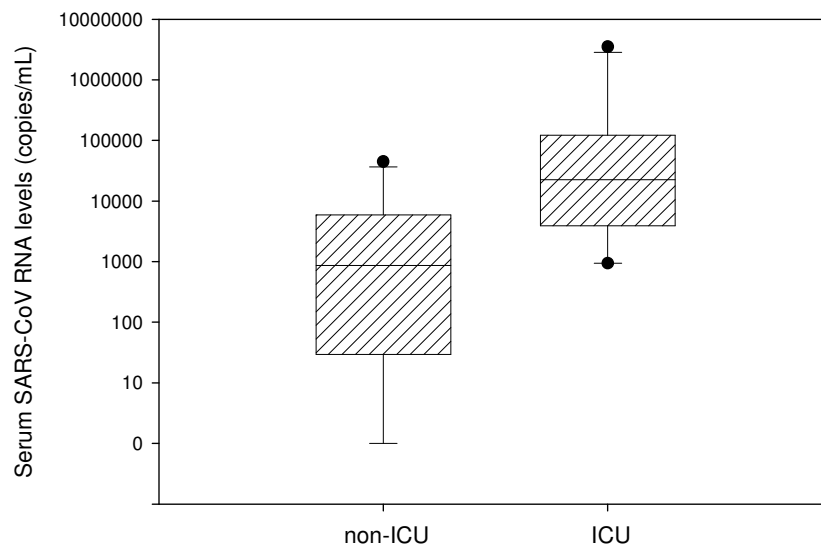


Figure 3. Serum SARS-CoV RNA levels in SARS patients on the day of hospital admission. Box plot of SARS-CoV RNA levels (common logarithmic scale) in sera of SARS patients requiring and not requiring ICU admission. Real-time quantitative RT-PCR assays towards the nucleocapsid region of the SARS-CoV genome was used for quantification. The horizontal lines denote the medians. The lines inside the boxes denote the medians. The boxes mark the interval between the 25th and 75th percentiles. The whiskers denote the interval between the 10th and 90th percentiles. The filled circles mark the data points outside the 10th and 90th percentiles.

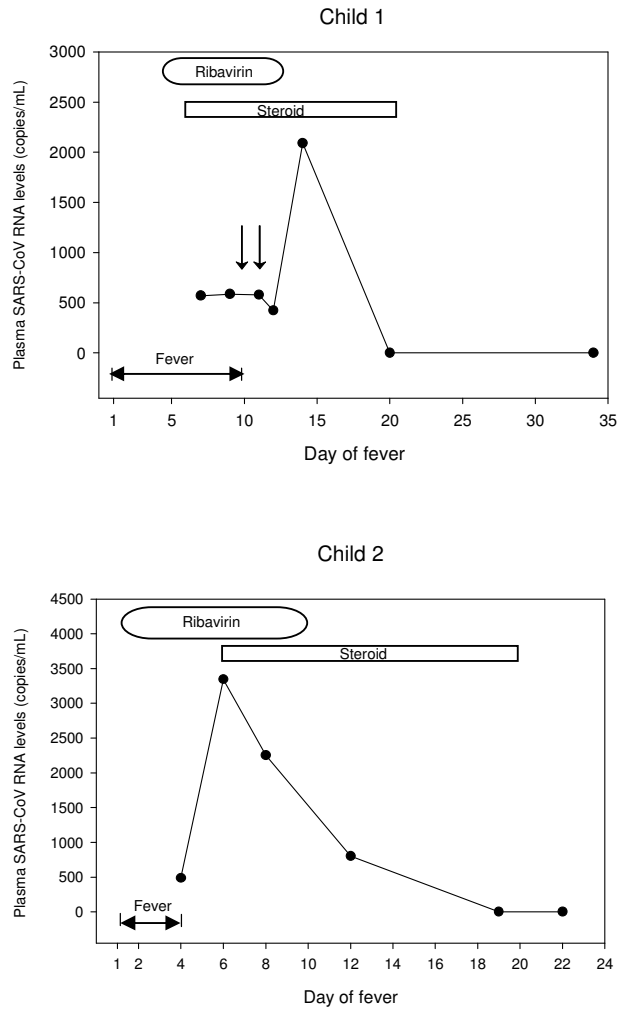


Figure 4. Serial analysis of plasma SARS-CoV RNA levels in paediatric SARS patients. Plots of plasma SARS-CoV RNA levels (Y-axis) against time after the onset of fever (day 1 refers the day of fever onset) (X-axis). The duration of fever and the periods of steroid and ribavirin treatment are indicated for each case. The arrows in patient 1 indicate the time of intravenous methylprednisolone treatment.