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SARS diagnosis, monitoring and prognostication by SARS-coronavirus RNA detection

Key Messages

1. We demonstrated the utility of a test for SARS-CoV RNA in serum/plasma in the diagnosis and prognostication of patients and its possible role in serial monitoring of treatment efficacy.
2. An automated viral RNA extraction procedure was found to be less effective than manual extraction.
3. The experience gained in developing the SARS diagnostic test was used to develop rapid methods for genotyping the SARS-CoV.

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Introduction

The 2003 SARS epidemic affected 29 countries around the world.[1,2] The early identification and isolation of infected individuals appeared important for the effective control of such an epidemic.[3] We reported the development of a diagnostic test based on the detection of the SARS-CoV RNA in serum/plasma by real-time quantitative reverse transcriptase–polymerase chain reaction (RT-PCR)[4,5]; 80% of infected individuals were shown to be positive on the first day of hospital admission with no false-positive results.[4,5] The serum SARS-CoV RNA concentration detected upon admission was predictive of the requirement for subsequent intensive care.[4] Further developments to SARS-CoV RNA detection from serum/plasma may improve our preparedness for future epidemics.[6]

Systematic analysis of SARS-CoV sequence information demonstrated that characteristic viral genotypes predominated at certain periods during the course of the outbreak.[7-10] Furthermore, characterisation of viral sequences is useful for confirming epidemiological associations between infected individuals as suspected from conventional epidemiological investigations.[9-11] In-depth analysis of the available sequence data on SARS-CoV also revealed that the viral isolates could be readily sub-classified into several major genotypes based on nucleotide variations at specific genomic positions.[8,12] Phylogenetic analysis of SARS-CoV sequences revealed a 5-nucleotide motif (GenBank Accession: AY390556; comprising reference nucleotide residues 17,564, 21,721, 22,222, 23,823, and 27,827) that was identified to be most useful for distinguishing the major SARS-CoV genotypes.[8] These major viral genotypes predominated at different periods of the epidemic.[8] Thus, it is evident that viral sequence and molecular epidemiological data provide valuable information to combat infectious diseases. However, direct sequencing of viral isolates from a large number of clinical samples is cumbersome and time consuming. A rapid system for the characterisation and screening of viral genotypes, such as for SARS-CoV, could be useful.

Aims and objectives

Our collaborative group has a long-standing history in the development of novel diagnostic and monitoring tests using plasma and serum. Following the outbreak of SARS, we applied this expertise to the development of a novel plasma/serum RNA test for SARS-CoV infection. Using this assay, we demonstrated, for the first time, the existence of SARS-CoV RNA in the cerebrospinal fluid of a SARS patient with neurological manifestations.

We aimed to (1) enhance our understanding of the scope for applying this plasma/serum RNA-based test, (2) explore ways to further enhance the throughput of the test, and (3) develop assays based on the new technology to rapidly genotype strains of SARS-CoV. Experience gained in developing the serum SARS-CoV RNA test for the diagnosis of SARS may provide valuable insights for the investigation of other emerging infectious diseases.

Methods

Three issues related to SARS-CoV RNA detection were explored:

(1) The role of SARS-CoV RNA detection in plasma/serum for the monitoring of treatment efficacy.
(2) The potential for shortening the turnaround time of the diagnostic assay based on SARS-CoV RNA detection in plasma/serum through automation.
(3) The development of rapid methods for the genotyping of SARS-CoV isolates.

Regarding issue 1, we compared the plasma SARS-CoV RNA concentrations in ribavirin-treated patients who received early hydrocortisone therapy with those who received placebo. Serial plasma SARS-CoV RNA concentrations measured in the setting of a prospective, randomised double-blinded, placebo-controlled trial designed to assess the efficacy of 'early' (before day 7 of illness) hydrocortisone use in previously healthy SARS patients were analysed. SARS-CoV RNA was quantified using a one-step real-time RT-PCR assay targeting the nucleocapsid gene.

Regarding issue 2, we compared the quantitative performance of a manual (Qiagen Viral mini kit) and automated (MagNA Pure LC instrument) protocol for SARS-CoV RNA extraction. We determined the optimal nucleic acid extraction kit to be adopted by the automated system, assessed the possibility of contamination and carry-over by the automated system, and compared the quantitative performance between the optimised automated and manual protocols for the extraction of inactivated SARS-CoV spiked in transport medium and in human serum.

Regarding issue 3, sequence analysis of SARS-CoV isolates revealed that specific genotypes predominated at different periods of the epidemic. This information can be used as a footprint for tracing the epidemiology of infections and monitor viral evolution. However, direct sequence analysis of a large number of clinical samples is cumbersome and time consuming. We aimed to develop a simple and rapid assay for the screening of SARS-coronavirus genotypes based on the use of fluorogenic oligonucleotide probes for allelic discrimination. In a large-scale phylogenetic analysis of SARS-CoV sequences, a 5-nucleotide motif was identified to be most useful for distinguishing the major SARS-CoV genotypes. We focused on the development of allelic discrimination assays for these five characteristic single nucleotide variations (SNV). Each patient's RNA was extracted from viral isolates cultured from clinical specimens using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Eleven microliters of the extracted viral RNA was reverse-transcribed by Superscript III (Invitrogen, Carlsbad [CA], USA) with random hexamer according to manufacturer's instructions. Genotyping of the five SNVs was determined using TaqMan (Applied Biosystems, Foster City, CA, USA) allelic discrimination assays on an ABI Prism 7900HT sequence detection system (Applied Biosystems). Each assay consisted of two allele-specific minor groove binding probes associated with either 6-carboxyfluorescein (FAM) or VIC™ as the fluorescent label. These were to discriminate between the two respective alleles at each SNV locus. One assay was designed for each of the 5 SNVs. The primer and probe sequences were designed using the Primer Express 2.0 software (Applied Biosystems).

Results

In issue 1, among 16 non–intensive-care-unit patients, SARS-CoV RNA was detected in the plasma at day 3-4 after fever onset; viral concentration peaked in the first week, which then rapidly declined in the second week. On days 8, 12, 16 and 20, the cumulative proportion of patients with undetectable virus in plasma was 31%, 69%, 92% and 100%, respectively. Plasma SARS-CoV RNA concentrations in the second and third weeks of illness were significantly higher in patients who received initial hydrocortisone treatment (n=9), as compared to those who received placebo (n=7) (AUC; Mann-Whitney, P=0.023). Their respective median time for SARS-CoV to become undetectable in plasma was 12 (range, 11-20) days and 8 (range, 8-15) days.

In issue 2, the detection sensitivity of the MagNA Pure LC total nucleic acid large volume kit was compared with the MagNA Pure LC total nucleic acid kit. The former kit had superior sensitivity and was therefore adopted for further comparison against the manual extraction method. Samples of viral transport medium spiked with and without inactivated SARS-CoV were arranged in a sequential manner for the assessment of carry-over contamination in the automated system. None of the plain samples revealed positive detection of SARS-CoV RNA, which therefore suggested that the MagNA Pure LC instrument was not prone to carry-over contamination. Median SARS-CoV RNA concentration in transport medium was 3.8-fold higher when extracted by the manual method in contrast to the automated method (Wilcoxon P=0.002). A constant negative bias was also noted in serum SARS-CoV RNA concentrations when extracted by the automated in comparison to the manual protocol (Wilcoxon P=0.002). The detection sensitivities for serum SARS-CoV RNA of both protocols were comparable.

In issue 3, TaqMan allelic discrimination assays for the five SNVs were tested on synthetic templates (Sigma Genosys, Australia) and verified using two viral isolates, CUHK-W1 (GenBank Accession: AY278554) and CUHK-Su10 (GenBank Accession: AY282752). We confirmed that the newly developed allelic discrimination assays were able to differentiate the two viral isolates and genotype each SNV correctly. Following initial development and optimisation, the allelic discrimination assays were used to genotype SARS-CoV in clinical samples. We were able to successfully determine the SARS-CoV genotypes in all the 30 samples studied. The SARS-CoV genotypes isolated from the 30 patients were also confirmed by direct sequencing. The sequencing results were fully concordant

with those based on the allelic discrimination assays at all five SNVs.

Discussion

In young, previously healthy adult SARS patients, SARS-CoV RNA was detected in plasma from day 3-4 after fever onset; peak concentration were detected in the first week, and declined rapidly in the second week. 'Early' hydrocortisone treatment initiated within 7 days of the illness was associated with significantly higher subsequent plasma viral concentrations in the second and third weeks. 'Early' initiation of corticosteroid treatment during the viral replication phase in the first week of illness resulted in delayed viral clearance (thus a higher subsequent plasma viral load), which is possibly related to its immunosuppressive effect. The duration of viraemia also appeared to be prolonged (median time to undetectable, 12 vs 8 days), though the difference did not reach statistical significance. Our study was limited by a small sample size. Patients with advanced age, co-morbidity, and those immunocompromised were excluded. Moreover, viral load profiles among more severe SARS cases, and the clinical consequence of a higher plasma viral load in early hydrocortisone treated patients needs further investigation.

To increase the throughput of a previously developed quantitative serum SARS-CoV RNA RT-PCR assay,[4,5] we evaluated the feasibility of automating the RNA extraction procedure through the use of the MagNA Pure LC instrument (Roche Diagnostics). Reagent kits suitable for the extraction of viral RNA from serum and plasma as recommended by the instrument manufacturer were evaluated. As the extraction procedure needed to conform to the biosafety practices recommended by the World Health Organization (WHO), a modified protocol incorporating an external lysis-processing step for the MagNA Pure LC total nucleic acid large volume kit (Roche Diagnostics) was developed. The WHO recommends that nucleic acid extraction procedures for SARS-CoV involving untreated specimens should first be performed under biosafety level-2 facilities, with additional level-3 work practices. After the viral particles had been lysed or inactivated, the specimens could be handled according to standard level-2 practices. We showed that the use of the large volume kit resulted in better analytical sensitivity when compared with the total nucleic acid kit, as evident by higher rates of positive detection among samples containing low concentrations of SARS-CoV. Furthermore, the MagNA Pure LC system was shown to be free from problems of carry-over contamination.

The automated extraction method involving the use of the large volume kit with the external lysis procedure was further compared with the quantitative performance of a previously described manual viral RNA extraction method based on the use of the QIAamp viral RNA mini kit (Qiagen). Across a wide range of SARS-CoV concentrations in both transport medium and serum, viral RNA extracted from the automated method led to SARS-CoV concentrations that were consistently lower than when extracted by the manual method. Furthermore, better detection rates were observed for serum containing low concentrations of SARS-CoV extracted manually than by the automated method. The manual method also contributed to better overall analytical precision as evident by the lower coefficients of variation.

Our study clearly demonstrated the feasibility of using allelic discrimination assays as a method for genetic characterisation of SARS-CoV genotypes in patients. It was particularly useful when extensive sequence information was available. Direct sequencing is still the gold standard for identifying new sequence variations when new infectious disease agents continue to emerge and old ones re-emerge. Once the variations have been identified, allelic discrimination assay is more efficient and suitable for large-scale population investigations. Thus, this approach provides a rapid and simple means to perform accurate genotype screening, making it ideal for epidemiological investigations.

Conclusions

Our study demonstrated that 'early' corticosteroid treatment was associated with a higher subsequent plasma viral load and therefore should be avoided. The automated viral RNA extraction protocol was less sensitive, less precise and produced quantitative results that were consistently lower than those of column-based manual extraction. We have evaluated a rapid approach for characterising SARS-CoV genotypes. The assay is simple, easy to perform and reproducible.

Judicious use of corticosteroid therapy in SARS is advisable. As it has been previously shown that the serum SARS-CoV concentration has prognostic implications and serial assessment is useful for monitoring patient progress, the superior quantitative performance and precision of the column-based extraction are additional reasons for favouring its use rather than the automated protocol. The rapid genotyping method based on TaqMan allelic discrimination can therefore be used as an efficient means to screen for virus genotypes and track the transmission of a particular viral strain during epidemics.

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