Detection of SARS Coronavirus (SARS-CoV) from Stool Specimens
Using Commercially Available Real-Time RT-PCR Assays

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Running title: Real-time PCR assays for SARS-CoV from stool samples
Abstract

The performance of three commercially available real-time RT-PCR assays (Artus RealArt HPA Coronavirus LightCycler; Artus RealArt HPA Coronavirus Rotor-Gene; Eragen SARS Coronavirus Pol Assay) and three RNA extraction methodologies was evaluated for the detection of SARS-CoV RNA from 91 stool specimens. The assays’ sensitivities were highest (58%-75%) for specimens obtained 8-21 days after symptom onset. The assays were less sensitive when specimens were obtained less than eight days or more than 21 days after onset of symptoms. All assays were 100% specific.
The discovery of a novel Coronavirus as the causative agent for severe acute respiratory syndrome (SARS) has led to the development of reverse-transcriptase (RT) PCR-based assays for its detection (1,7,9,15). Although SARS is primarily associated with fever and involvement of the respiratory tract, gastrointestinal symptoms may also occur (2,10). The presence of active viral replication within the large and small intestine as demonstrated in biopsies obtained at colonoscopy or autopsy suggests that stool may be a useful specimen for SARS coronavirus (SARS-CoV) detection (11). The rate of detection of SARS-CoV from stools has been at least as high as that from other clinical specimens using conventional nested RT-PCR (16), and stool specimens have been a useful alternative to respiratory samples for the detection of SARS-CoV (14, 18). Although there have been evaluations of both conventional and real-time RT-PCR based assays (12,19), few studies have evaluated newer, second-generation assays for the detection of SARS-CoV RNA from stool specimens (5). In an outbreak that is short-lived involving an emerging pathogen, evaluation of newer diagnostic tests is difficult. While not ideal, we sought to overcome this problem with the use of frozen, archived stool specimens. In this study, we evaluated three RNA extraction methods and three commercially available second-generation, real-time RT-PCR based assays for detection of SARS-CoV using a collection of archived stool samples. We compared these results to those obtained using a conventional nested RT-PCR assay performed at the time of receipt of these same specimens in 2003. The results of this evaluation may be useful should human infection due to SARS-CoV recur.
Clinical specimens. A total of 91 stool specimens from 57 patients were obtained between April and August 2003, during an outbreak of SARS in Toronto, Ontario, Canada. Thirty-four patients met the WHO case definition for probable or suspect SARS (17) and were seropositive for SARS-CoV antibodies during the convalescent phase of illness as determined by the Euroimmun immunofluorescent assay (IgG; Euroimmun, Lübeck, Germany) (8,13,14). Twenty-three patients with other respiratory illnesses did not meet the case definition for SARS and were seronegative for SARS-CoV antibodies. There were 56 stool specimens available for testing from the seropositive individuals, and 35 specimens from those who were seronegative.

RNA extraction. RNA was extracted from fresh stool samples upon receipt in the laboratory in 2003 using the Viral RNA mini kit (Qiagen Inc., Mississauga, Ont.) as per the manufacturer’s instructions, using 140 µl of a 10% (w/v) stool suspension made in 5 ml of sterile diethylpyrocarbonate (DEPC)-treated water (Invitrogen, Burlington, Ont.). RNA was eluted in a final volume of 50 µl of elution buffer. Stool specimens were then frozen at -80°C until they were re-tested for this evaluation.

For the present evaluation, stool samples were thawed and a 10% (w/v) stool suspension was made in sterile DEPC-treated water. RNA was extracted with three different extraction methods, each performed in accordance with the manufacturer’s instructions: Viral RNA mini kit (Q), miniMag (mM) (bioMérieux, St. Laurent, Que.), and MagaZorb RNA mini-prep kit (Mz) (Cortex Biochem Inc., San Leandro, CA). A sample of 140 µl was used for both the Viral RNA mini kit and miniMag extractions, while 200 µl was used for MagaZorb extraction. RNA was eluted in a final volume of 50 µl elution buffer.
RT-PCR assays. Conventional, nested RT-PCR was performed as per Drosten et al. (6). Real-time RT-PCR with the RealArt HPA Coronavirus (HPAC) LightCycler [ALC] and RealArt HPA Coronavirus (HPAC) Rotor-Gene [ARG] kits (Artus GmbH, Hamburg, Germany) and the SARS Coronavirus Pol Assay (ER) (EraGen Biosciences, Madison, WI) were performed as per manufacturers’ instructions. The target for the real-time RT-PCR assays was the viral polymerase gene, although details regarding the target for each assay are not available as the test kits evaluated are proprietary. All the RNA eluates were tested using the EraGen and Artus kits. The EraGen and the Artus HPAC ALC assays were performed on the LightCycler ver. 1.2 (Roche Diagnostics, Laval, Que.) and the Artus HPAC ARG assay was performed on the Rotor-Gene 3000 (Corbett Life Science, Sydney, Australia). Only the Artus kits provided positive controls to allow generation of a standard curve for viral load quantitation, but this feature of the assays was not evaluated.

Comparison of all methods was performed and their clinical sensitivities and specificities were calculated using serologic evidence for the presence of SARS-CoV antibodies as the gold standard.

A summary of all test results is shown in Table 1. Of the 91 specimens tested, 67 were negative and 17 were positive for SARS Co-V RNA by all seven methods. Twenty-three specimens were positive for viral RNA using three or more RT-PCR assays. One specimen was positive in only one of the assay methods (Eragen). Although this may have represented a false-positive test result, the patient met the case-definition for SARS, and was seropositive for SARS-CoV antibodies.
Although all the assays had 100% specificity, the sensitivities were low, ranging from 34 to 40% (Table 2). There were no statistically significant differences between the sensitivities or specificities of the assays evaluated. However, when analyzed by the time of specimen collection from the onset of symptoms, there were certain times that were associated with an improved ability to detect viral RNA (Figure 1). The sensitivities of RT-PCR for the 13 specimens collected ≤7 days from the onset of symptoms ranged from 46-62%, whereas between 8 and 21 days after symptom onset the sensitivities ranged from 58-75% (12 specimens). For the 31 specimens obtained >21 days after symptom onset, the sensitivities were only 13 to 19%. When individual RT-PCR assay results were compared to a modified gold standard of three or more assays yielding a positive result, the sensitivities improved (range: 83% to 96%; data not shown). None of the 35 specimens collected from the 23 non-SARS patient samples was positive for SARS-CoV RNA.

The newer assays performed as well as a conventional nested RT-PCR assay for the detection of SARS-CoV RNA from stool samples even after sample storage at -80°C for approximately two years. This is consistent with a previous report showing that SARS-CoV could withstand freezing and thawing and was stable for many weeks in clinical specimens maintained at 4°C or at −70°C (3). The newer real-time PCR assays produced more rapid results as compared with the conventional method, which was labor-intensive and required five hours to complete.

Our results of RT-PCR testing for SARS-CoV in stool samples are similar to those previously reported (4, 14, 16). The negative RT-PCR results from serologically confirmed SARS patients obtained in this study could have been due to suboptimal
timing of specimen collection (4, 14). Stool samples collected during the first week of illness or more than 21 days after symptom onset often yielded false negative results, possibly due to a decreased viral load. More than half (55%) of the specimens from SARS patients that were available for testing had been collected more than 21 days after onset of symptoms. An additional limitation of this study was the lack of availability of multiple samples collected from a large number of patients over an extended period of time during the course of illness. In the majority of cases, only one stool sample was available for testing. The use of archived stool samples may also have affected the yield, although our data do not appear to suggest that freezing affected RT-PCR assay results.

The results of this evaluation indicate that these commercially available real-time RT-PCR assays with newer extraction methodologies are highly specific, and at least as sensitive as conventional nested RT-PCR assays for the detection of SARS-CoV. A major advantage of the newer tests is their ease of performance and rapidity. Any of these assays would be suitable for use should a need to detect SARS-CoV arise in the future. However, it is clear that should SARS recur in humans, there is still a need for more knowledge about optimal diagnostic test strategies (appropriate specimens and their timing), and for improved diagnostic assays.
Acknowledgments

Additional members of The Ontario Laboratory Working Group for the Rapid Diagnosis of Emerging Pathogens include: Ilene Guglielmi, and Aimin Li, Central Public Health Laboratory, Toronto, Ont.; Donald Low, and Allison McGeer, Mount Sinai Hospital, Toronto, Ont.; Anne Matlow, Hospital for Sick Children, Toronto, Ont.; Kevin Katz, Mona Loutfy, and Barbara Mederski, North York General Hospital, Toronto, Ont.; Marvin Gerson, Reena Lovinsky, David Rose, Zev Shainhouse, The Scarborough Hospital, Toronto, Ont.; Sigmund Krajden, St. Joseph’s Health Centre, Toronto, Ont.; Roslyn Devlin, St. Michael's Hospital, Toronto, Ont.; Anita Rachlis and Mary Vearncombe, Sunnybrook Health Sciences Centre, Toronto, Ont.; James Downey, and Roland Skrastins, Toronto East General Hospital, Toronto, Ont.; Wayne Gold, University Health Network, Toronto, Ont., Elizabeth Phillips, St. Paul's Hospital, Vancouver, BC. Special thanks to Catherine Harlton-Strezov for database management.

This study was funded, in part, by the Ontario Ministry of Health and Long-Term Care, and by the Canadian Institutes for Health Research.
References


Table 1. Summary of SARS-CoV RT-PCR results for seven methods.

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<tr>
<th>No. specimens</th>
<th>Conventional&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LC Artus&lt;sup&gt;b&lt;/sup&gt;</th>
<th>EraGen&lt;sup&gt;c&lt;/sup&gt;</th>
<th>RG Artus&lt;sup&gt;d&lt;/sup&gt;</th>
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a, Conventional, conventional nested RT-PCR; b, LC Artus, Artus RealArt HPA Coronavirus LightCycler; c, EraGen, Eragen SARS Coronavirus Pol Assay; d, RG Artus, Artus RealArt HPA Coronavirus Rotor-Gene.
Table 2. Sensitivities and specificities of seven methods for detection of SARS-CoV RNA from stool samples (as compared to convalescent serology).

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
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<td>Qiagen Viral RNA mini kit</td>
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a, Conventional, conventional nested RT-PCR; b, LC Artus, Artus RealArt HPA Coronavirus LightCycler; c, EraGen, EraGen SARS Coronavirus Pol Assay; d, RG Artus, Artus RealArt HPA Coronavirus
Figure 1. SARS-CoV RT-PCR positivity rates by timing of specimens.
Conventional, conventional nested RT-PCR; Q/ALC, Qiagen Viral RNA mini kit/Artus RealArt HPA Coronavirus LightCycler; Q/ER, Qiagen Viral RNA mini kit/EraGen SARS Coronavirus Pol assay; mM/ER, bioMérieux miniMag/EraGen SARS Coronavirus Pol assay; mZ/ER, Cortex MagaZorb/Eragen SARS Coronavirus Pol assay; mM/ARG, bioMérieieux miniMag/Artus RealArt HPA Coronavirus Rotor-Gene; mZ/ARG, Cortex MagaZorb/Artus RealArt HPA Coronavirus Rotor-Gene.