Available online at www.sciencedirect.com
SCIENCE DIRECT®
Diagnostic Microbiology and Infectious Disease 53 (2005) 47–55
DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE
www.elsevier.com/locate/diagmicrobio

Virology

Comparison of 9 different PCR primers for the rapid detection of severe acute respiratory syndrome coronavirus using 2 RNA extraction methods

Linda Chui[a,*], Michael Drebot[b], Anton Andonov[b], Astrid Petrich[c], Martin Glushek[a], James Mahony[c]

[a]*Provincial Laboratory for Public Health (Microbiology), University of Alberta Hospital, Edmonton, Alberta, Canada T6G 2J2*
[b]*National Microbiology Laboratory, Winnipeg, Manitoba, Canada R3E 3R2*
[c]*St. Joseph's Healthcare, McMaster University, Hamilton Ontario, Canada L8N 4A6*

Received 6 October 2004; accepted 25 March 2005

Abstract

The sensitivity and specificity of various severe acute respiratory syndrome coronavirus (SARS-CoV) PCR primer and probe sets were evaluated through the use of commercial kits and in-house amplification formats. Conventional and real-time PCR assays were performed using a heat-block thermocycler ABI 9600, the Roche LightCycler™ version 1.2, or the ABI 7000 Sequence Detection System. The sensitivity of all primers was between 0.0004 and 0.04 PFU with viral cell lysate and between 0.004 and 0.4 PFU in spiked stool specimen per PCR assay. The primer sets for real-time PCR assays were at one least 1 log more sensitive than the primer sets used in the conventional PCR. A panel of viruses including swine gastroenteritis virus, bovine coronavirus, avian bronchitis virus (Connecticut strain), avian bronchitis virus (Massachusetts strain), human coronaviruses 229E and OC43, parainfluenza virus (type III), human metapneumovirus, adenovirus, respiratory syncytial virus, and influenza A were tested by all assays. All real-time PCR assays used probe-based detection, and no cross-reactivity was observed. With conventional PCR, analysis was performed using agarose gel electrophoresis and multiple nonspecific bands were observed. Two commercial extraction methods, magnetic particle capture and silica-based procedure were evaluated and the results were comparable. The former was less laborious with shorter time for completion and can easily be adapted to an automated system such as the MagNa Pure-LC, which can extract nucleic acid from clinical samples and load it into the sample capillaries of the LightCycler™. As exemplified by this study, the continued refinement and evaluation of PCR procedures will greatly benefit the diagnostic laboratory during an outbreak of SARS.
© 2005 Elsevier Inc. All rights reserved.

Keywords: SARS-CoV; Real-time and conventional RT-PCR; Sensitivity and specificity

1. Introduction

Severe acute respiratory syndrome (SARS) first manifested as an outbreak of severe pneumonia in the province of Guangdong in China in November 2002 (WHO, 2003). The illness was introduced into Hong Kong in February 2003 and spread across 5 continents over a period of weeks because of international travel. Approximately 8500 people worldwide were diagnosed with probable SARS during the epidemic with over 900 deaths (National Advisory Committee on SARS and Public Health, 2003). In Canada, the outbreak of SARS occurred mostly by means of nosocomial transmission in acute care settings. Culture, electron microscopy, serological tests, and RT-PCR were all used during the initial investigation of SARS outbreaks. The etiological agent has been identified as a novel coronavirus, SARS-CoV (Drosten et al., 2003; Guan et al., 2003; Ksiazek et al., 2003; Lee et al., 2003; Peiris et al., 2003; Tsang et al., 2003). Because of the severity and communicability of the illness, rapid and sensitive diagnostic methods are essential for timely and effective institution of infection control measures to prevent further transmission.

In this study, we have used several sets of primers directed to the polymerase and nucleocapsid gene sequences of SARS-CoV for the in-house assays by conventional and real-time amplification and compared with 2 commercial kits, Artus RealArt™ HPA-Coronavirus LightCycler™ RT PCR Reagents Assay (Artus BioTech USA Inc, San

* Corresponding author. Tel.: +1-780-407-8951; fax: +1-780-407-8253.
E-mail address: l.chui@provlab.ab.ca (L. Chui).

0732-8893/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.diagmicrobio.2005.03.007

Francisco) and Roche LightCycler™ SARS-CoV quantification kit (Roche Diagnostics, Laval, ON) in terms of their specificities and sensitivities. Stool specimens have been submitted for testing during the SARS outbreak (Lee et al., 2003; Peiris et al., 2003; Yam et al., 2003; Zhai et al., 2004), and because they are complex and heterogeneous and most likely to contain different types of inhibitors, this specimen type was chosen for all the spiking experiments in this study.

2. Material and methods

2.1. Virus culture and determination of virus titer

The SARS-CoV was grown in Vero E6 cells in Level III biohazard containment (Timani et al., 2004). After complete cytopathic effect (CPE) was observed, the infectious materials were frozen at −70 °C and kept as stock virus until used. The titer of the virus was determined by plaque assay with dilutions from 10^{-1} to 10^{-9} using a standardized protocol. Briefly, viral dilutions were added to Vero E6 cell monolayer prepared in 6-well cluster plates and incubated for an hour at 37 °C. A nutrient-agar overlay was then added and the plates were placed in a CO_2 incubator for approximately 2 days. A second overlay was then applied, which contained neutral red as a vital stain, and the plates were then checked for plaque formation over the next 12–24 h.

2.2. Preparation of viral cell lysate and spiked stool samples with SARS-CoV

Viral cell lysate was diluted in PBS (pH 7.4) from 10^{-1} to 10^{-9}. A volume of 0.3 mL of each dilution was added to 1.0 mL of 5.0 M guanidinium hydrochloride and 200 μL of the mixture was used for RNA extraction. Twenty randomly selected stool specimens submitted for other viral investigations were pooled and used in the spiking experiments. Approximately 0.2 g of stool was weighed and a 10% (w/v) suspension was made with PBS at pH 7.4. The suspension was vortexed, and after the large particulates had settled, 200 μL of the stool suspension was added to 300 μL of each of the viral cell lysate dilutions. The mixtures were resuspended by pipetting up and down, and a 200-μL aliquot from each spiked dilution was removed and added into 400 μL of 5.0 M guanidinium hydrochloride. The suspensions were vortexed and an aliquot of 200 μL was used for the RNA extraction.

2.3. RNA extraction

Extraction of RNA was performed on all dilutions of viral cell lysates and spiked stool samples by 2 commercial kits; one kit used a silica-based procedure (NucliSens®, bioMérieux, St-Laurent, Quebec) and the other used a magnetic bead based technology (MagaZorb™, Cortex BioChem, San Leandro). Extraction of RNA was performed as per manufacturers' specifications. The RNA was resuspended in 100 μL of DEPC-treated water and 5 μL of the purified material was used for the RT-PCR. Both methods were compared in terms of time requirement for completion and efficiency of RNA extraction.

2.4. Sensitivities and specificities of different sets of primers

The primer/probe sequences, amplicon sizes, conditions for RT-PCR, instrumentation used for the amplification, and the detection limits are summarized in Table 1. Sequences of all the primers and their respective locations within the SARS-CoV genome used in the in-house assays are shown in Fig. 1. The Qiagen One Step RT-PCR kit (Qiagen, Valencia, CA) was used for the standard in-house RT-PCR assays with primer sets 1 and 4. The reaction for reverse transcription was as follows: 10.0 μL of 5× PCR buffer, 2.0 μL of 10 mmol/L dNTPs, 1.5 μL of each primer at 10 μM, 2.0 μL of polymerase mix, and 5.0 μL of the purified RNA. The reverse transcription assay was performed at 50 °C for 30 min RT followed by 15 min of inactivation at 95 °C. The PCR parameters are described in Table 1. Supercript II RT PCR kit (Invitrogen Life Technologies, Burlington, ON) was used for primer set 2 for the first round PCR and the AmpliTaq Gold (Roche Diagnostics) was used for the nested PCR. As for primer set 3, SuperScript II RTA (Invitrogen Life Technologies) was used for the reverse transcription followed by PCR with AmpliTaq Gold (Roche Diagnostics). The amplification conditions were carried out as specified by Yam et al. (2003). After amplification, 10 μL of each amplified product was subjected to 1.5% (w/v) agarose gel electrophoresis running at 150V for 1 h in the presence of ethidium bromide (0.07 μg/mL). DNA size marker (1 kbp) was included in each gel (Invitrogen Life Technologies). The bands were visualized under UV illumination and captured using Polaroid film (Polaroid, Cambridge, MA).

Real-time TaqMan® RT-PCR was performed with primer sets 5 and 6 (Table 1) using a 1-step RT-PCR master mix kit (Applied Biosystems, Branchburg, NJ) with 5 μL of RNA sample. Primers and probes were used at concentrations of 900 and 200 nmol/L, respectively. For the in-house real-time LightCycler™ RT-PCR assay, the LightCycler™-RNA Master Hybridization Probe (Roche Diagnostics) was used as per manufacturer's instructions.

Along with these 7 sets of primers, 2 commercial assays, Artus RealArt™ HPA-Coronavirus LightCycler™ RT PCR Reagents Assay and Roche LightCycler™ SARS-CoV quantification kit (referred as Artus and Roche assays, respectively, throughout this study) were also examined. The Artus assay is a ready-to-use kit containing reagents and enzymes to amplify a 92-bp region of the SARS-CoV genome. The assay is multiplexed with an internal control to identify the presence of inhibitors. SARS-CoV standards are also provided to measure the viral load of the specimen. An aliquot of 5 μL of extracted RNA was added to the LightCycler™ capillary containing 15 μL of PCR reagents and the amplification parameters are listed in Table 1.

Table 1
Conditions and data summary for the different PCR assays

Primer set	Sequence	Amplicon size (bp)	PCR conditions	Detection limit	
				Lysate	Stool
A. Conventional block PCR					
Primer set 1 (Poutanen et al., 2003)	Forward 5′-CAGAGCCATGCCTAACATG-3′ Reverse 5′-AATGTTTCAGCAGGTAAGCG-3′	389	RT: 50 °C/30 min, 95 °C/15 min PCR: 95 °C/30 s, 55 °C/30 s, 72 °C/30 s 50 cycles	10^{-6}	10^{-4}
Primer set 2 (Drosten et al., 2003)	Forward 5′-ATGAATTACCAGTCAATGGTTAC-3′ Reverse 5′-CATAACCAGTCGGTACAGCTAC-3′	189	RT: 45 °C/30 min, 95 °C/3 min PCR: 10 cycles of 95 °C/10 s, 60 °C/10 s (decrease by 1 °C/ cycle), 72 °C/30 s 40 cycles of 95 °C/10 s, 56 °C/10 s, 72 °C/30 s	10^{-6}	10^{-6}
	Nested forward 5′-GAAGCTATTCGTCACGTTCG-3′ Nested reverse 5′-CTGTAGAAAATCCTAGCTGGAG-3′	108	Nested PCR: 95 °C/5 min 10 cycles of 95 °C/10 s, 60 °C/10 s (decrease 1 °C/cycle), 72 °C/20 s: 20 cycles of 95 °C/10 s, 56 °C/10 s, 72 °C/20 s		
Primer set 3 (Peiris et al., 2003)	Forward 5′-TACACACCTCAGCGTTG-3′ Reverse 5′-CACGAACGTGACGAAT-3′	182	RT: 42 °C/50 min, 95 °C/15 min PCR: 94 °C/1 min, 50 °C/1 min, 72 °C/1 min 50 cycles	10^{-5}	10^{-4}
Primer set 4 (WHO, 2003)	Forward 5′-CACCGTTTCTACAGGTTAGCTAACGA-3′ Reverse 5′-AAATGTTTACGCAGGTAAGCGTAAAA-3′	313	RT: 50 °C/30 min, 95 °C/15 min PCR: 95 °C/30 s, 55 °C/30 s, 72 °C/30 s 50 cycles	10^{-6}	10^{-4}
B. Real-time PCR					
Primer set 5 (ABI 7000 SDS)	Forward 5′-CACACCGTTTCTACAGGTTAGCT-3′ Reverse 5′-GCCACACATGACCATCTCACTTAAT-3′ MGB probe 5′-6FAM—ACTTGCGCACACTCGTT-3′	64	RT: 48 °C/30 min, 95 °C/10 min PCR: 95 °C/15 s, 60 °C/1 min 50 cycles	10^{-7}	10^{-5}
Primer set 6 (ABI 7000 SDS)	Forward 5′-ACCAGAATGGAGGACGCAATG-3′ Reverse 5′-GCTGTGAACCAAGACGCAGTATTAT-3′ MGB probe 5′-6FAM—ACCCCAAGGTTTACCC-3′	85	RT: 48 °C/30 min, 95 °C/10 min PCR: 95 °C/15 s, 60 °C/1 min 50 cycles	10^{-7}	10^{-5}
Primer set 7 (Roche LightCycler™)	Forward 5′-TGAATACACCCAAAGACCAC-3′ Reverse 5′-TGATGAGGAGCGAGAAGAG-3′ Probe 5′-6FAM—CCTAATAACAATGCTGCCACCGT-3′	148	RT: 61 °C/20 min, 95 °C/30 s PCR: 95 °C/1 s, 59 °C/15 s 50 cycles	10^{-6}	10^{-4}
RealArt CoV kit (Roche LightCycler™)	Proprietary	92	RT: 50 °C/10 min, 95 °C/10 min PCR: 95 °C/2 s, 55 °C/12 s, 72 °C/10 s 50 cycles	10^{-7}	10^{-5}
Roche CoV kit (Roche LightCycler™)	Proprietary	180	RT: 61 °C/20 min, 95 °C/30 s PCR: 95 °C/5 s, 55 °C/15 s, 72 °C/10 s 45 cycles	10^{-7}	10^{-5}

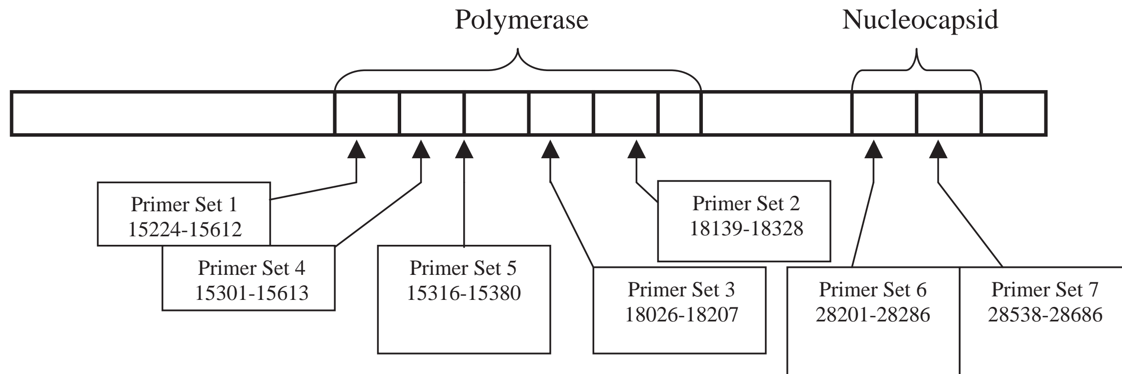

Fig. 1. Schematic diagram of the SARS-CoV viral genome demonstrating the location of primer/probe sets used for amplification. Numbers denote the positions of the forward and reverse primers used for the different assays.

The Roche assay amplifies a 180-bp target sequence of the replicase 1 AB/polymerase gene of SARS-CoV. An internal control using the same set of primers but with a different internal probe is also included to monitor the presence of inhibitory effect. Standards are also provided to quantify the viral load of the test sample if required. A total of 5 μL of extracted RNA was added to 15 μL of PCR reaction mix, and the conditions of amplification are listed in Table 1. Both Artus and Roche assays were performed on the Roche LightCycler™ (LC) Version 1.2.

A panel of viruses (including representatives from all 3 major coronaviridae group species) was included to test for the specificities of all the primer sets: swine gastroenteritis virus, bovine coronavirus, avian bronchitis virus (Connecticut strain), avian bronchitis virus (Massachusetts strain), and human coronaviruses (229E, OC43), as well as parainfluenza virus (type III), metapneumovirus, adenovirus, respiratory syncytial virus, and influenza A. RNA was extracted from the viral lysate directly using both silica- or magnetic bead–based technology extractions. For stool spiking experiments, viral lysate from the above viral panel was added to the stool suspension as described in Section 2.2.

3. Results

3.1. Sensitivity and specificity of different primer sets by real-time and conventional RT-PCR

The titer of SARS-CoV stock used in this study was 2×10^6 PFU/mL. CPE was observed with rounding as early as 48-h postinfection at 10^{-1} to 10^{-5} dilutions of the virus and progressed rapidly. Finally, the cells detached from the surface of the tubes. Aliquots of diluted infectious material were used for the determination of specificity and sensitivity in both viral cell lysate and stool spiking experiments. Detection limits for each of the assays are summarized in Table 1.

Based on the titer obtained from the plaque assay, the input virus at neat per 200 μL of extraction for viral cell lysate and fecal spiked samples was 8.0×10^4 PFU. Using end-point titration analysis, both Artus and Roche kits demonstrated a detection limit at the dilutions of 10^{-7} corresponding to 0.0004 PFU per amplification assay (Fig. 2a and b, A top panels). As for the spiked stool samples, the end point was at 10^{-5} dilution PFU (0.04) per assay (Fig. 2a and b, B top panels). There was approximately a 2-log decrease in sensitivity when the target RNA was extracted from the spiked stool samples by both assays. The internal controls present in both kits were added to each dilution of viral cell lysate spiked with stool for extraction and amplification to monitor the presence of inhibition. The size of the amplicons generated by the internal control primers as well as the target primers were the same. No inhibition was observed with any of the extracts derived from spiked stool samples. These 2 kits also provide sets of known standards that can be incorporated into the runs to quantify the number of RNA copies if required. The detection levels demonstrated by Artus and Roche kits were 0.5–1.5 and 25–30 copies of RNA per reaction, respectively, calculated based on the standards provided by the 2 kits. The bottom panels of Fig. 2a and b are gel analyses of the amplified products generated by the 2 real-time RT-PCR commercial assays. The size of the amplicon from the Artus and Roche kits are 92 and 180 bp, respectively. The amplicon generated by the internal control primers from the Roche kit has the same molecular weight as the target amplicon; therefore, the products could not be differentiated using agarose gel electrophoresis analysis. Multiple amplicon bands were produced from RT-PCR with RNA template extracted from spiked stool samples (Fig. 2A and B, B bottom panels) and viral RNA from the specificity panel (Fig. 2A and B, C top panels). However, no cross-reactivity was observed when the real-time curves were analyzed (Fig. 2A and B, C top panels) because of the specificities of the probes used in both assays. Besides the 2 commercial kits described above, 3 other sets of primers (primer sets 5, 6 and 7) were designed for real-time amplification using the Roche LightCycler™ version 1.2 and the ABI 7000 Sequence Detection System (SDS) instruments. Primer sets 6 and 7 targeted the nucleocapsid gene sequence whereas primer set 5 was specific for the polymerase gene. With viral cell lysate, PCR end-point

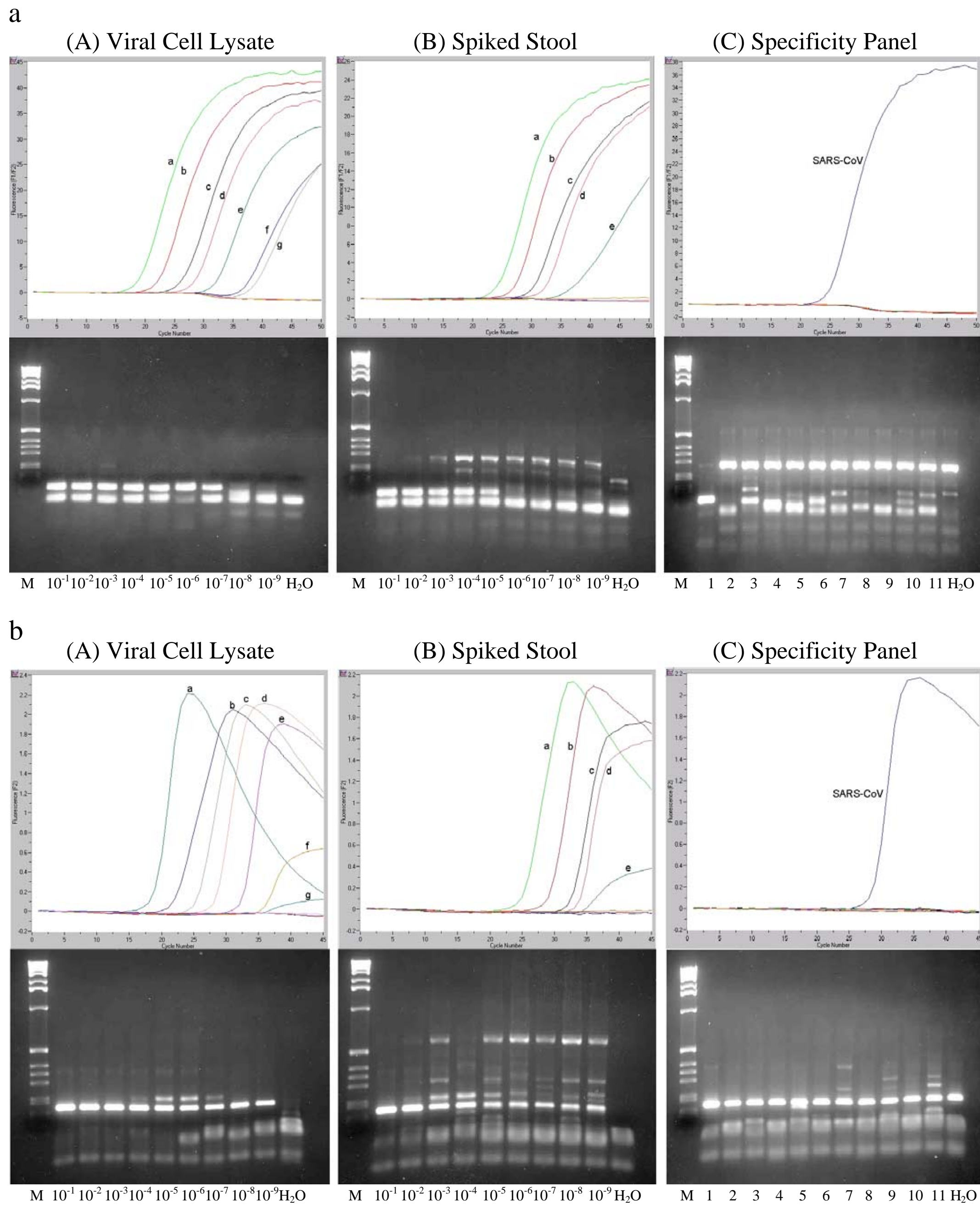

Fig. 2. (a) Comparative evaluation of RealArt™ HPA-Coronavirus LightCycler™ RT PCR Reagents Assay. Top panel: Software output of real-time PCR using extracted RNA from (A) viral cell lysate, (B) spiked stool, (C) viruses from the specificity panel. Bottom panel: Gel analysis of PCR amplicons. Dilutions from 10^{-1} to 10^{-7} are shown as a to g on the curves. Specificity panel from 1 to 11: SARS-CoV, human coronavirus 229E, bovine coronavirus, avian bronchitis virus (Connecticut strain), avian bronchitis virus (Massachusetts strain), swine gastroenteritis virus, parainfluenza virus (type III), adenovirus, human metapneumovirus, respiratory syncytial virus, and influenza A. The last sample of each panel is the water control and M is the 1-kbp DNA size marker. (b) Comparative evaluation of Roche LightCycler™ SARS-CoV Detection kit. Top panel: Software output of real-time PCR using extracted RNA from (A) viral cell lysate, (B) spiked stool, (C) viruses from the specificity panel. Bottom panels are gel analysis of PCR amplicons. Dilutions from 10^{-1} to 10^{-7} are shown as a to g on the curves. Specificity panel from 1 to 11: SARS-CoV, human coronavirus 229E, bovine coronavirus, avian bronchitis virus (Connecticut strain), avian bronchitis virus (Massachusetts strain), swine gastroenteritis virus, parainfluenza virus (type III), adenovirus, human metapneumovirus, respiratory syncytial virus, and influenza A. The last sample of each panel is the water control and M is the 1-kbp DNA size marker.

titration was at 10^{-7} for both primer sets 5 and 6 and 10^{-6} with primer set 7 (Table 1). A similar 2-log drop in sensitivity with the above primers was observed when RNA extracted from the spiked stool samples was used with in-house real-time assays. No positive result was seen with non–SARS-CoV RNA from the specificity panel. However, when PCR products were subjected to agarose gels electrophoresis analysis, multiple bands were present, which

indicated that the hybridization probes had differentiated the products from the nonspecific amplicons (data not shown). Because no internal control has been designed to be included in these reactions, the presence of inhibitors causing a failure in the amplification would not be detected.

The sensitivities of conventional block PCR assays for dilutions of viral cell lysate and viral cell lysate spiked stool samples with the different sets of primers are shown in Fig. 3, panels A and B. The detection limit by agarose gel electrophoresis analysis of primer sets 1 and 4 was at 10^{-6} (0.004 PFU/PCR) with viral cell lysate and at 10^{-4} (0.4 PFU/PCR) With fecal spiked samples. The end-point titrations of viral cell lysate with primers sets 2 and 3 were at 10^{-6} and 10^{-5}, respectively. There was no change in sensitivity by PCR end-point titration with primer set 2 with fecal spiked viral cell lysate samples. As for primer set 3, there was a 1-log drop in sensitivity with fecal spiked samples, and the PCR end-point detection was at 10^{-4}. There was also weakly stained band observed slightly lower than the desired amplicon from 10^{-5} to 10^{-9} dilutions in Fig. 3. The band was excised from the gel, and DNA was purified, sequenced, and compared with the SARS-CoV and other coronavirus sequences in GenBank. The sequence data showed no resemblance to the coronavirus genome. If the identification of the presence of virus was mainly based on agarose gel electrophoresis analysis without any further confirmation, this could have been mistaken as a weak-positive, assuming that it was because of a low viral load in the specimen, and thus a false-positive result would be reported. Primer set 4 was the only exception that amplified one specific product with no other nonspecific products even in the presence of fecal material.

As for target specificity (Fig. 3C), PCR amplification using primer set 1 resulted in many nonspecific products with sizes ranging from 100 bp to over 1 kbp for all of the viruses in the specificity panel, and some of these bands also had similar migration rates as the amplicon of SARS-CoV. For primer set 3, multiple nonspecific amplified products were also produced using RNA extracted from bovine coronavirus, avian bronchitis virus (strain C), swine gastroenteritis virus, and parainfluenza virus. No cross-reactivity was observed when primers 2 and 4 were tested against different viruses in the specificity panel, and positive result was obtained only with SARS-CoV (Fig. 3, panel C). RNA from OC43 was also tested using all the primers described above, and no cross-reactivity was observed (data not shown).

3.2. Comparison of 2 RNA extraction methods

Because nucleic acid extraction can be one of the factors that determine the efficiency of PCR amplification,

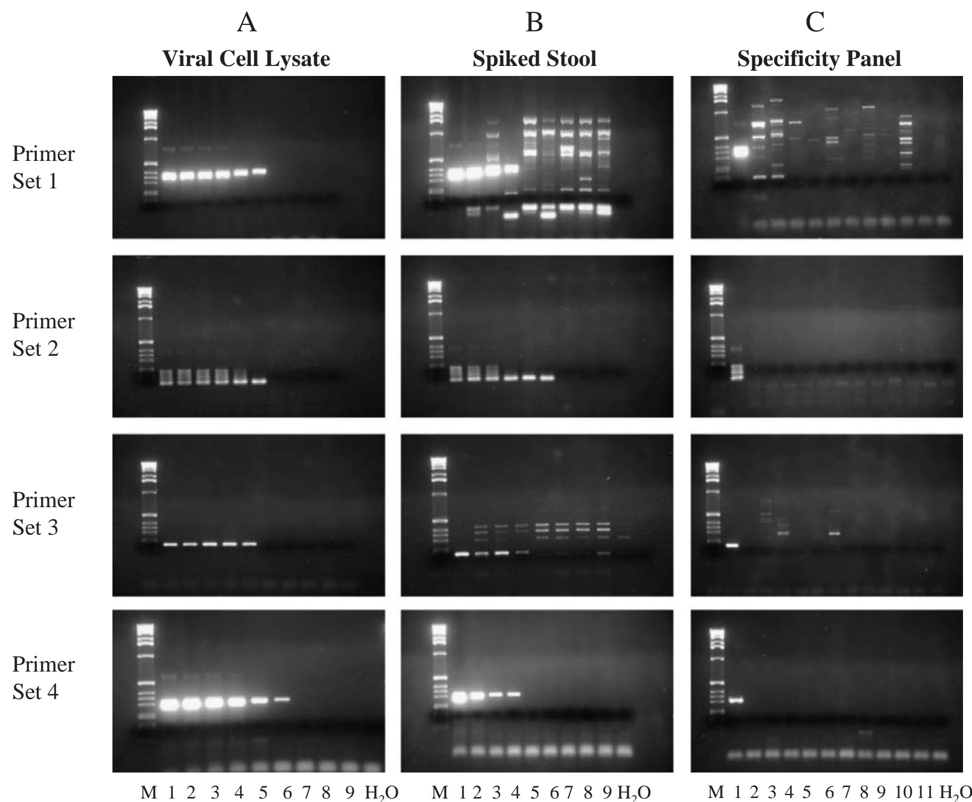

Fig. 3. Agarose gel analysis of PCR amplicons generated by conventional PCR using RNA from (A) viral cell lysate, (B) spiked stool, (C) viruses from the specificity panel with primer sets 1–4. Lanes 1–9 of panels A and B are dilutions from 10^{-1} to 10^{-9}. Specificity panel from 1 to 11: SARS-CoV, human coronavirus 229E, bovine coronavirus, avian bronchitis virus (Connecticut strain), avian bronchitis virus (Massachusetts strain), swine gastroenteritis virus, parainfluenza virus (type III), adenovirus, human metapneumovirus, respiratory syncytial virus, and influenza A. The last sample of each panel is the water control and M is the 1-kbp DNA size marker.

2 commercially available extraction methods were used in this study. One of the methods used a silica-based format (NucliSens®, bioMérieux) and the other was a magnetic-based extraction technology (MagaZorb™). Our sensitivity results indicated that end-point titrations using RNA extracted by the MagaZorb™ and NucliSens® were comparable using conventional and real-time PCR. The total time for extraction was 1.5 h with MagaZorb™ kit compared with 2.5 h with the NucliSens® kit (based on 10 samples per run) and the amount of hands-on time was higher with the latter kit.

4. Discussion

Although, no cases of SARS have been documented in Canada in the past year, future outbreaks are still a possibility because of the frequency of international travel. Countries affected by SARS reported an attack rate of greater than 56% among healthcare workers caring for SARS patients (WHO, 2003). Because of risk of infection among the general population and particularly healthcare workers who are in contact with suspect of SARS, it is of the utmost importance that every laboratory has the capability to provide a sensitive, accurate, and rapid assay for early diagnosis of SARS and implementation of a timely and cost-effective infection control measures.

In our investigation, we have compared different sets of primers targeting various sequences of the SARS-CoV genome for the in-house assays and 2 commercial kits. The commercial kits from Artus and Roche contain internal controls that allow the monitoring of extraction and detection of inhibitory factors. This is extremely important in a diagnostic laboratory because clinical samples may contain inhibitors that might interfere with PCR resulting in amplification failure and the reporting of a false-negative result. PCR inhibition is not the only contributory factor to a false-negative report. The type and quality of the specimen and time of collection during the course of the disease play critical roles. Stool was chosen for the spiking experiments because of its heterogeneity, complexity, and that it is likely to contain the most diverse inhibitory factors; it was also one of the specimen types collected from SARS-CoV patients for diagnosis. Although no inhibition was observed with fecal samples spiked with SARS-CoV, it does not mean that inhibition problems associated with clinical samples have been eliminated by using silica- or magnetic-based types of RNA extraction. In Drosten et al.'s (2004) study, inhibition was seen with one PCR assay but not the other using the same extracted RNA; this might because of the types of enzyme and reagents used in the amplification assay (Wiedbrauk et al., 1995). There was no available clinical sample in this investigation, but both Artus and Roche kits have improved detection of SARS-CoV, and the sensitivity in stool samples have reached 78–87% (Drosten et al., 2004) versus 58–63% reported earlier (Yam et al., 2003).

The Artus assay includes a set of internal standards with concentrations between 1×10^4 and 10^7 copies/mL and the Roche assay's internal standard concentrations are between 2×10^4 and 2×10^8 copies/mL. By using an external preparation of standards recommended by WHO, Hourfar et al. (2004) found that linear measurement by the Roche assay was between 2×10^4 and 2×10^8 copies of the external standard RNA preparation per milliliter and that the Artus assay has a broader linear range from 1×10^2 to 1×10^7 copies of RNA/mL (Hourfar et al., 2004). With the wide dynamic range of the Artus assay, even low concentrations of SARS-CoV can be measured quantitatively. Sensitivity calculated based on 95% probability limits was >40,000 copies/mL for Roche assay and >380 copies/mL for Artus Assay (Hourfar et al., 2004). The Roche assay is less sensitive as compared with the Artus assay and this might be because of the amplicon size and the enzymes used in these 2 assays. The amplified product of the Artus assay is 92 bp as compared with 180 bp in the Roche assay. A combination of 2 enzymes, Moloney murine leukemia virus reverse transcriptase and *Taq* DNA polymerase are used in the Artus assay, whereas the Roche assay applies the 1-enzyme rTth (*Thermus thermophilus*) DNA polymerase assay in combination with manganese ions for RT-PCR. The enzyme rTth is less susceptible to GC-rich genomes and inhibitors; however, the lack of sensitivity at low concentration of virus is most likely because of insufficient reverse transcriptase activity (Cusi et al., 1994). Contrary to the observation by Hourfar et al. (2004), in our study, results of the PCR end-point titration by the 2 kits were identical, and the detection level was at 0.004 PFU/PCR for viral cell lysate and 0.4 PFU/CPR with fecal spiked samples.

The design of the in-house primer sets included in this study was based on both the polymerase and nucleocapsid genes. The sensitivities of all these assays are comparable and confirm an earlier observation by Drosten et al. (2004) indicating the nucleocapsid gene is only in abundance in the early stage of viral infection in cell culture. Therefore, PCR assays with nucleocapsid gene as target would not likely to increase the sensitivity of detection in symptomatic patients. The conventional PCR assays were performed using the heat block format (ABI 9600, Applied BioSystem), and the real-time amplification assays were conducted on the Roche LC version 1.2 and ABI 7000 SDS instruments. Because the primers selected for conventional PCR and real-time PCR were not identical, no direct comparison of sensitivity could be made between the 2 types of assays. However, the primer sets for real-time PCR included in this study seemed to be at one least 1 log more sensitive than the primer sets used in the conventional PCR. According to Emery et al. (2004), using real-time PCR with their published primers, the potential detection limit is less than 10 transcript copies per PCR and has a greater sensitivity than cell culture isolation and conventional PCR; lesser efficiency of amplification in the presence of clinical specimens was also observed. Our data also showed a drop in the sensitivity by

1–2 orders of magnitude with the addition of fecal material. The sensitivity of the amplification assays has shown improvement by increase in volume of specimen for extraction and real-time PCR coupled with modified extraction method (Poon et al., 2003a, 2003b). The turnaround time for the conventional PCR required a minimum of 6 h because the cycling time was longer and also required gel analysis for detection. There were multiple nonspecific bands with variable sizes observed in the agarose gels after electrophoresis with some of the primer sets. Using primer set 4, we did not observe other nonspecific PCR bands with RNA extracted from spiked stool samples but this might occur with other clinical specimens submitted for diagnosis. There was insufficient data to show that there would not be any cross-reactivities with other submitted stool samples or any other clinical samples. Without probe hybridization, restriction digestion or sequencing of the amplicon as means of confirmation, there is the potential of reporting a false-positive, which has an important implication especially in an outbreak setting such as a SARS epidemic.

Data from PCR end-point titrations showed that RNA extracted by the MagaZorb™ kit was comparable with the results obtained with RNA extracted by the NucliSens® kit. Both kits performed equally well in the removal of inhibitors and was shown by the successful amplification of the internal control in the PCR assays of the Roche and Artus Assay kits. The completion and hands-on time for extraction is less, and fewer consumables are required for the MagaZorb™ kit as compared with the NucliSens® kit. If the basic kit cost is identical for both, the choice of one over the other will depend on expenditures for labor and consumables. We have also found that specimens that were extracted by the MagaZorb™ kit could easily be adapted to the MagNa Pure-LC, an automated nucleic acid extractor (Roche Diagnostics). Because this instrument can also dispense the extracted nucleic acid into the sample holders (capillaries) of the Roche LC Version 1.2, the extraction of clinical specimens for SARS can be moved to this automated platform with minimal technical requirement. This is extremely important in outbreak situation when automation and rapid turnaround time are parts of the critical factors for isolation of and treatment of patients.

SARS has great impact on clinical management; therefore, it is important to use different gene target such as the nucleocapsid gene for confirmation. Another alternative is to refer an unopened aliquot of specimen to another laboratory that perform SARS-CoV amplification with target primers of equal sensitivity and specificity. For any positive results, it is also recommendable to request for follow up specimens not only for amplification but also for serology and culture. Although much emphasis has been on the utilization of RT-PCR as one of the diagnostic tool for SARS because of its sensitivity, the results obtained should be interpreted with caution because of the possibility of contamination. Therefore, it is important for laboratory personnel conducting these tests to have meticulous technique as well as physical separation for the different steps in the manipulation of clinical specimens.

Our study presented 7 sets of primers using real-time RT-PCR or conventional RT-PCR with gel-based detection and 2 commercial kits for detecting SARS-CoV (Artus and Roche assays). For laboratories that do not have a real-time instrument but only perform conventional PCR, an internal probe specific for SARS-CoV amplicons or sequencing of amplicons is a necessity to avoid a false-positive result. Further testing on different clinical specimens is required to confirm our observations of the performance of all these primer sets in their respective PCR assays. However, this study provides important comparative data on the sensitivities and specificities of the primers that are currently being used in different parts of the world for the rapid diagnosis of SARS-CoV.

Acknowledgments

The authors thank B. Leblanc, Lisa Lin, Elsie Grudeski, Sylvia Chong, and Sean Song for their excellent technical assistance and Dr. B. Lee and Dr. X. Pang for their valuable comments. Appreciation is also extended to Roche Diagnostics System and Artus BioTech for supplying the kits for this study.

References

Cusi MG, Valassina M, Valensin PE (1994) Comparison of M-MLV reverse transcriptase and Tth polymerase activity in RT-PCR of samples with low virus burden. *Biotechniques* 17:1034–1036.

Drosten C, Günther S, Preiser W, van der Werf S, Brodt HR, Becker S, Rabenau H, Panning M, Kolesnikova L, Fouchier RA, Berger A, Burguière AM, Cinatl J, Eickmann M, Escriou N, Grywna K, Kramme S, Manuguerra JC, Muller S, Rickerts V, Sturmer M, Vieth S, Klenk HD, Osterhaus AD, Schmitz H, Doerr HW (2003) Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med* 348:1967–1976.

Drosten C, Chiu LL, Panning M, Leong HN, Preiser W, Tam JS, Gunther S, Kramme S, Emmerich P, Ng WL, Schmitz H, Koay E (2004) Evaluation of advanced reverse transcription-PCR assays and an alternative PCR target region for detection of severe acute respiratory syndrome-associated coronavirus. *J Clin Microbiol* 42:2043–2047.

Emery SL, Erdman DD, Bowen MD, Newton BR, Winchell JM, Meyer RF, Tong S, Cook BT, Holloway BP, McCaustland KA, Rota PA, Bankamp B, Lowe LE, Ksiazek TG, Bellini WJ, Anderson LJ (2004) Real-time polymerase chain reaction for detecting SARS coronavirus, Beijing, 2003. *Emerg Infect Dis* 10:311–313.

Guan Y, Zheng BJ, He YQ, Liu XL, Zhuang ZX, Cheung CL, Luo WW, Li PH, Zhang LJ, Guan YJ, Butt KM, Wong KL, Chan KW, Lim W, Shortridge KF, Yune KY, Peiris JSM, Poon LLM (2003) Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. *Science* 302:276–278.

Hourfar MK, Roth WK, Seifried E, Schmidt M (2004) Comparison of two real-time quantitative assays for detection of severe acute respiratory syndrome coronovirus. *J Clin Microbiol* 42:2094–2100.

Ksiazek TG, Erdman D, Goldsmith PHC, Zaki SR, Peret T, Emery S, Tong S, Urbani C, Comer JA, Lim W, Rollin PE, Dowell S, Ling AE, Humphrey C, Shieh WJ, Guarner J, Paddock CD, Rota P, Fields B, DeRisi J, Yang JY, Cox N, Hughes J, LeDuc JW, Bellini W, Anderson

LJ, and the SARS Working Group (2003) A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med* 348:1953 – 1956.

Lee N, Hui D, Wu A, Chan P, Cameron P, Joynt G, Ahuja M, Yung A, Leung Y, To B, Lui F, Szeto F, Chung C, Sung S (2003) A major outbreak of severe acute respiratory syndrome in Hong Kong. *N Engl J Med* 348:1986 – 1994.

National Advisory Committee on SARS and Public Health (2003) SARS and Public Health. *Renewal of Public Health in Canada* 1–12.

Peiris JSM, Lai ST, Poon LLM, Guan Y, Yam LYC, Lim W, Nicholas J, Yee WKS, Yan WW, Cheung MT, Cheng VCC, Chan KH, Tsang DNC, Yung RWH, Ng TK, Yuen KY, and members of the SARS study group (2003) Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* 361:1319 – 1325.

Poutanen SM, Low DE, Henry B, Finkelstein S, Rose D, Green K, Tellier R, Draker R, Adachi D, Ayers M, Chan AK, Skowronski DM, Salit I, Simor AE, Slutsky AS, Doyle PW, Krajden M, Petric M, Brunham RC, McGeer AJ, National Microbiology Laboratory, Canada, Canadian Severe Acute Respiratory Syndrome Study Team (2003) Identification of severe acute respiratory syndrome in Canada. *N Engl J Med* 348:1995 – 2003.

Poon LM, Chan KH, Wong OK, Yam WC, Yuen KY, Guan Y, Lo YM, Peiris SM (2003a) Early diagnosis of SARS Coronavirus infection by real time RT-PCR. *J Clin Virol* 28:233 – 238.

Poon LL, Wong OK, Chan KH, Luk W, Yuen KY, Peiris JS, Guan Y (2003b) Rapid diagnosis of a coronavirus associated with severe acute respiratory syndrome (SARS). *Clin Chem* 49:953 – 955.

Tsang KW, Ho PL, Ooi GC, Yee WK, Wang T, Chan-Yeung M, Lam WK, Seto WH, Yam LY, Cheung TM, Wong PC, Lam B, Ip MS, Chan J, Yuen KY, Lai KN (2003) A cluster of cases of severe acute respiratory syndrome in Hong Kong. *N Engl J Med* 348:1977 – 1985.

Timani K, Ye A, Ye L, Zhu L, Wu Y, Gong Z (2004) Cloning, sequencing, expression and purification of SARS-associated coronavirus nucleocapsid protein for serodiagnosis of SARS. *J Clin Virol* 4:309 – 312.

Yam WC, Chan KH, Poon LL, Guan Y, Yuen KY, Seto WH, Peiris JS (2003) Evaluation of reverse transcription-PCR assays for rapid diagnosis of severe acute respiratory syndrome associated with a novel coronavirus. *J Clin Microbiol* 41:4521 – 4524.

WHO (2003) PCR Primers for SARS Developed by WHO Network Laboratories. http://www.who.int/csr/sars/primers/en/.

Wiedbrauk DL, Werner JC, Drevon AM (1995) Inhibition of PCR by aqueous and vitreous fluids. *J Clin Microbiol* 33:2643 – 2646.

Zhai J, Briese T, Dai E, Wang X, Pang X, Du Z, Liu H, Wang J, Wang H, Guo Z, Chen Z, Jiang L, Zhou D, Han Y, Jabado O, Palacios G, Lipkin WI, Yang R (2004) Real-time polymerase chain reaction for detecting SARS coronavirus, Beijing, 2003. *Emerg Infect Dis* 10: 300 – 303.