

Real-Time NASBA Detection of SARS-Associated Coronavirus and Comparison With Real-Time Reverse Transcription-PCR

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Severe acute respiratory syndrome (SARS) exhibits a high mortality rate and the potential for rapid epidemic spread. Additionally, it has a poorly defined clinical presentation, and no known treatment or prevention methods. Collectively, these factors underscore the need for early diagnosis. Molecular tests have been developed to detect SARS coronavirus (SARS-CoV) RNA using real time reverse transcription polymerase chain reaction (RT-PCR) with varying levels of sensitivity. However, RNA amplification methods have been demonstrated to be more sensitive for the detection of some RNA viruses. We therefore developed a real-time nucleic acid sequence-based amplification (NASBA) test for SARS-CoV. A number of primer/beacon sets were designed to target different regions of the SARS-CoV genome, and were tested for sensitivity and specificity. The performance of the assays was compared with RT-PCR assays. A multi-target real-time NASBA application was developed for detection of SARS-CoV polymerase (Pol) and nucleocapsid (N) genes. The N targets were found to be consistently more sensitive than the Pol targets, and the real-time NASBA assay demonstrates equivalent sensitivity when compared to testing by real-time RT-PCR. A multi-target real-time NASBA assay has been successfully developed for the sensitive detection of SARS-CoV. **J. Med. Virol.** 77:602–608, 2005.

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KEY WORDS: SARS; NASBA; RT-PCR; coronavirus

INTRODUCTION

In November, 2002, the first cases of a severe respiratory disease emerged in the Guangdong province

of China, and by March, 2003, this translated into a worldwide outbreak of severe acute respiratory syndrome (SARS). The ensuing search for the cause of the disease led to identification of a novel coronavirus [Drosten et al., 2003; Ksiazek et al., 2003]. This was closely followed by genomic characterization of the virus [Marra et al., 2003; Rota et al., 2003], and subsequent confirmation that the new SARS-associated coronavirus (SARS-CoV: order Nidovirales, family Coronaviridae, genus *Coronavirus*) fulfills Koch's postulates for SARS disease [Fouchier et al., 2003].

Several diagnostic assays were developed in response to the outbreak to determine the most sensitive and accurate method for early detection of SARS. Culture detection of live SARS-CoV requires biosafety level three facilities and expertise, which severely limits the number of facilities or institutions in which it can be performed. In addition, cell culture isolation of SARS-CoV is not as sensitive as detection by real-time RT-PCR [Emery et al., 2004]. Conversely, serological methods have been shown to be sensitive for confirmation of infection but detection of antibodies occurs late in the course of illness, which limits their efficacy for early detection and disease control [Peiris et al., 2003]. The focus of early diagnosis has therefore centered on the development of molecular-based assays including conventional as well as real-time reverse

Abbreviations used: SARS-CoV, SARS coronavirus; SARS, severe acute respiratory syndrome; NASBA, nucleic acid sequence-based amplification; RT-PCR, reverse transcriptase-polymerase chain reaction; nt, nucleotide.

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transcription-polymerase chain reaction (RT-PCR) methods. It has been suggested that these provide the greatest sensitivity for testing within the first 2 weeks of infection [Wu et al., 2004]. Studies have found that real-time RT-PCR is more sensitive [Emery et al., 2004; Jiang et al., 2004; Poon et al., 2004], and is less likely to produce cross contamination of samples compared to end-point RT-PCR. Nested PCR assays have also been described [Drosten et al., 2003; Yam et al., 2003; Jiang et al., 2004] but they carry an inherently high risk of sample cross contamination.

Given the very low viral load at disease onset [Peiris et al., 2003], maximum assay sensitivity is crucial for early SARS-CoV detection. Nucleic acid sequence-based amplification (NASBA) has been developed specifically to detect RNA and is more sensitive than RT-PCR for the detection of some RNA viruses [Lanciotti and Kerst, 2001; Ginocchio et al., 2003]. We therefore developed a real-time NASBA SARS-CoV assay and evaluated it in comparison with the real-time RT-PCR assays developed at the Centers for Disease Control (CDC) [Drosten et al., 2003; Emery et al., 2004].

MATERIALS AND METHODS

SARS-CoV RNA

High titer whole genome material (Urbani strain) was kindly provided by Dean Erdman, CDC, Atlanta, GA in Trizol. Ten microlitre aliquots were transferred to 0.9 ml NucliSens Lysis Buffer (bioMérieux, Durham, NC) and stored at -80°C prior to extraction. The 200 nt in vitro transcribed SARS-CoV RNA fragment was kindly provided by Christian Drosten, Bernhard-Nocht Institute for Tropical Medicine (BNITM), Hamburg, Germany [Drosten et al., 2003], reconstituted at an estimated 10^8 copies/ml, and aliquots stored at -80°C . Hong Kong and Frankfurt isolates of SARS-CoV were kindly provided by Mathias Niedrig as part of a

European Quality Assurance (EQA) study. Three Vietnamese SARS-CoV isolates were kindly provided by Dean Erdman as extracted nucleic acid material. 229E and OC43 human respiratory coronaviruses were obtained from the American Type Culture Collection (Manassas, VA).

RNA Preparation

Samples in 0.9 ml of lysis buffer (bioMérieux) were extracted on a NucliSens Extractor (bioMérieux) according to the manufacturer's instructions, yielding an approximate eluate volume of 50 μl , and 5 μl of total nucleic acid was used per amplification reaction.

Real-Time NASBA

Multiple upstream and downstream primers targeting the SARS-CoV (Urbani strain AY278741) polymerase gene (Pol), the nucleocapsid (N), 5'-Leader (5'-L), and 3'-non-coding (3'-NCR) regions, along with corresponding beacons, 5'-6-FAM labeled and quenched by 3'-Dabcyl, were designed for real-time detection of SARS-CoV RNA (Table I). Different combinations and concentrations of the primers were tested to optimize the assay using NucliSens Basic Kit reagents (bioMérieux) according to the manufacturer's instructions. Briefly, a reagent sphere was dissolved in diluent to which KCl (80 mM final concentration) and water were added. Primers and beacon were added to provide a final optimized concentration of 0.2 and 0.1 μM , respectively. Five microlitres of nucleic acid was added to 10 μl of this reagent mix and incubated at 65°C for 2 min then 41°C for 2 min. Five microlitres of reconstituted enzymes was then added, and samples were transferred to the NucliSens EasyQ Analyzer (bioMérieux). The isothermal real-time NASBA reaction continued at 41°C for 90 min, and fluorescence was measured every 45 sec.

TABLE I. Primers and Molecular Beacons for SARS-CoV Real-Time NASBA Assays

Target	Oligonucleotide	Sequence
Pol 1	P1 primer	5'-aattctaatacagactcactatagggAACATAACCAGTCGGTACAGCTACTA-3'
	P2 primer	5'-GAAGCTATTTCGTCACGTTTCG-3'
	Molecular beacon	5'-[6-FAM]-ccatgggCTGTTCATGCAACTAGAGATGCTGTccatgg-[Dabcyl]-3'
Pol 2	P1 primer	5'-aattctaatacagactcactatagggAAGATGTTTAAACTGGTACCTGGTGGGA-3'
	P2 primer	5'-TGCCTGGATTGGCTTTGATGT-3'
	Molecular beacon	5'-[6-FAM]-ccatgggCTGTTCATGCAACTAGAGATGCTGTccatgg-[Dabcyl]-3'
N1	P1 primer	5'-aattctaatacagactcactatagggAGAAGTACCATCTGGGGCTGA-3'
	P2 primer	5'-AGATTCCCTCGAGGCCAGGGCGT-3'
	Molecular beacon	5'-[6-FAM]-ccatgggCTACTACCGAAGAGCTACCCGACGAccatgg-[Dabcyl]-3'
N2	P1 primer	5'-aattctaatacagactcactatagggAAGGTGTGACTTCCATGCCAA-3'
	P2 primer	5'-CAGAACAACCCAAAGGAAATT-3'
	Molecular beacon	5'-[6-FAM]-ccatggACCAAGACCTAATCAGACAAccatgg-[Dabcyl]-3'
5'-L	P1 primer	5'-aattctaatacagactcactatagggAGATCACACCCGGACGAAACCTA-3'
	P2 primer	5'-ATGCCTAGTGCACCTACGCAGT-3'
	Molecular beacon	5'-[6-FAM]-ccatgggCGTCCCTCTTCTGCAGACTGCTTAccatgg-[Dabcyl]-3'
3'-NCR	P1 primer	5'-aattctaatacagactcactatagggAAGCTATTTAAATCACATGGGGGA-3'
	P2 primer	5'-TAACTAAACAGCACAAAGTAGGT-3'
	Molecular beacon	5'-[6-FAM]-ccatggGCCACCACATTTTCATCGAGgcatgg-[Dabcyl]-3'

P1 primers contain a T7 RNA polymerase promoter sequence in lower case, and the stem sequence for the molecular beacons is also shown in lower case.

TABLE II. Sequences for the Primers and Probes Used in the RT-PCR Assays [Emery et al., 2004]

Target	Oligonucleotide	Sequence 5'-3'
SARS1 RNA polymerase	Forward	CAT GTG TGG CGG CTC ACT ATA T
	Reverse	GAC ACT ATT AGC ATA AGC AGT TGT AGC A
	Probe	TTA AAC CAG GTG GAA CAT CAT CCG GTG
SARS2 Nucleocapsid	Forward	GGA GCC TTG AAT ACA CCC AAA G
	Reverse	GCA CGG TGG CAG CAT TG
	Probe	CCA CAT TGG CAC CCG CAA TCC
SARS3 Nucleocapsid	Forward	CAA ACA TTG GCC GCA AAT T
	Reverse	CAA TGC GTG ACA TTC CAA AGA
	Probe	CAC AAT TTG CTC CAA GTG CCT CTG CA

CDC Real-Time RT-PCR

Real-time RT-PCR was performed with a first generation primer/probe set (Qiagen, Germantown, MD), for which sequences were obtained from the CDC, targeting the SARS-CoV polymerase gene (see Table II). In accordance with the protocol, 5 μ l sample was added to a mix containing a final concentration of 1 \times RT-PCR reaction mix (Invitrogen, Carlsbad, CA), 5 mM MgSO₄, 250 nM forward and reverse primers, 200 nM 5'-FAM labeled Taqman probe quenched with 3'-TAMRA, and 0.5 U SS-RT/Taq (Invitrogen, Carlsbad, CA). An initial primer and probe titration was performed to confirm primer/probe concentrations (results not shown). Cycling conditions were as follows: (50°C, 30 min; 94°C, 5 min) \times 1 followed by (95°C, 15 sec; 60°C, 1 min) \times 40, and reactions were carried out on an ABI Prism 7000 (Applied Biosystems, Foster City, CA). A second generation multi-target assay (kindly provided by Dean Erdman, CDC, Atlanta) including N-target primer/probe sets [see Table II; Emery et al., 2004] was carried out as directed (CDC) with Taqman One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Foster City, CA) under the following conditions: (48°C, 30 min; 95°C, 10 min) \times 1 followed by (95°C, 15 sec; 60°C, 1 min) \times 45.

RESULTS

Molecular testing for SARS-CoV was developed using real-time NASBA and was compared to a real-time RT-PCR assay developed by the CDC. The first generation Pol 1 NASBA primer set was tested on whole genome Urbani strain and BNITM SARS-CoV transcript as well as the 229E and OC43 human respiratory coronaviruses to determine assay specificity. Data in Figure 1 demonstrate that the NASBA-based test is specific, and does not cross-react with the non-SARS 229E and OC43 coronavirus materials, whereas both Urbani and BNITM control RNAs are positive. Whole blood and bronchoalveolar lavage (BAL) samples from a suspected SARS case that presented at the University of Pittsburgh Medical Center in May, 2003 were also screened by this assay (Fig. 1). The patient was negative for SARS-CoV by NASBA testing and subsequently shown to be negative for serum antibodies by the CDC (data not shown).

Because of the potential mutation rate of SARS-CoV and possible corresponding difficulties in viral detection, several primer/beacon sets were designed to target a number of different regions in the genome. These sets were optimized by evaluating different combinations for each target, and optimized primer/beacon combinations were then tested on a 10-fold dilution series of cultured SARS-CoV RNA (derived from the Frankfurt SARS index case) (Fig. 2). Initial analysis indicated the Pol 1 and N2 primer sets gave the greatest sensitivity followed by the Pol 2 set as defined by detection of these targets at the highest dilution of SARS-CoV RNA. None of the primer sets demonstrated cross-reactivity with other clinically relevant viruses [Zhang et al., 2004]. Differences in signal amplitude between the various targets are a function of the individual kinetics of a given primer/probe set interaction with its target and do not relate to sensitivity since the assay is not quantitative. In order to establish a negative cut-off for the assay, nine SARS-CoV negative respiratory samples were tested, and a fluorescence value greater than the average of the negative controls plus four standard deviations was defined as positive for the assay.

In the absence of access to abundant positive clinical samples and given the inherent differences between transcript and genomic RNA, we tested the performance of the different NASBA primer/beacon sets with several

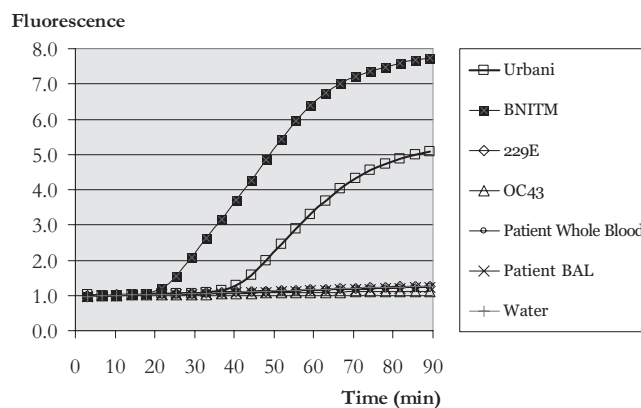


Fig. 1. Real-time NASBA specificity for detection of SARS-CoV. The Pol 1 primer/beacon set was used, and data shown are representative of multiple replicate experiments.

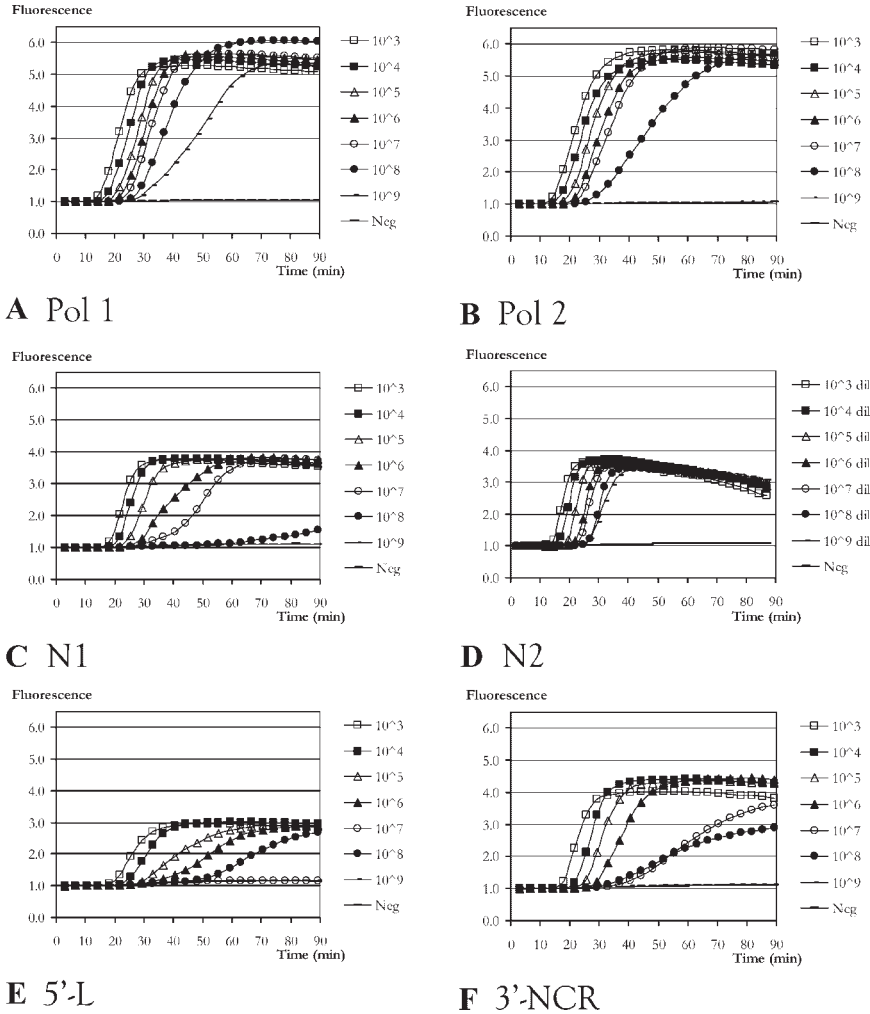


Fig. 2. Analysis of real-time NASBA primer/probe sets on RNA extracted from SARS-CoV from Rotterdam. **A-F:** represent primer/probe sets for: Pol-1, Pol-2, N1, N2, 5'-L, and 3'-NCR regions respectively. Data shown are representative of multiple replicate experiments.

SARS-CoV isolates. Coronaviruses synthesize eight subgenomic 5' and 3' coterminal RNAs containing the leader sequence at the 5' terminus and the N gene at the 3' terminus. For this reason it was anticipated that an assay targeting the N gene might be more sensitive than those targeting other regions of the viral genome, due to the predicted abundance of N RNA transcripts in SARS-CoV infected cells. Indeed, the N primer/beacon sets outperformed the Pol 2 set and could consistently detect SARS-CoV at a 10^{-6} and frequently at a 10^{-7} dilution of whole genome Urbani strain RNA (Table III). However, interestingly, the Pol 1 primer/probe set was equally as sensitive as either of the N target sets.

The different target sets were also used to detect SARS-CoV in known copy number and high titer isolates from Frankfurt and Hong Kong, respectively. Similar results were obtained for the Urbani strain (data not shown). The Pol and N sets showed highest sensitivity compared to the 5'-L and 3'-NCR targets, for detection of the Frankfurt SARS-CoV (Table IV), and these Pol and N sets were therefore further characterized in the

remaining studies. In order to verify the ability of the assay to detect N and Pol targets in different strains of SARS-CoV, a dilution series of Vietnamese isolates from early and late in the epidemic were also tested. All assays on both of these targets were able to detect all strains (Table V).

The sensitivity of the multi-target real-time NASBA assay for SARS-CoV was directly compared to the three target real-time RT-PCR Taqman assay by testing the same freshly prepared dilution series of SARS-CoV RNA in parallel. While the two multi-target assays appear to have comparable sensitivity overall, there are some interesting differences. The N targets consistently demonstrate higher sensitivity over the Pol target for RT-PCR, while in the NASBA assay, the N targets are more sensitive than the Pol 2 but not the Pol 1. Additionally, the Taqman SARS2 (N) and NASBA N1 targets appear to be equivalent, while the Taqman SARS3 (N) target was at least an order of magnitude more sensitive compared to all the targets (Table III). Furthermore, the NASBA Pol 1 target was more

TABLE III. Comparison of Multi-Target SARS-CoV Real-Time NASBA With Triple-Target CDC Taqman RT-PCR Assay Using the Urbani Strain

	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
NASBA					
Pol 1	+	+	+	+	-
Pol 2	+	+	-	-	-
N1	+	+	+	+	-
N2	+	+	+	-	-
CDC Taqman					
SARS 1 (Pol)	+	+	-	-	-
SARS 2 (N)	+	+	+	+	-
SARS 3 (N)	+	+	+	+	+

Data shown are representative of multiple replicate experiments. All replicate experiments were performed with duplicates for each dilution point. The results were recorded as positive (+) or negative (-) for each duplicate value obtained as defined by the value being above (positive) or below (negative) the cut-off for NASBA (as described in Results) or crossing the signal threshold for RT-PCR. Where both duplicates concur, the result is recorded as either positive (+) or negative (-). In the case of a discrepancy between duplicates, the result was recorded as (-), i.e., the endpoint was determined by the value at which both duplicates were consistently positive.

sensitive than the Taqman SARS 1 (Pol) target by two orders of magnitude. Given that two targets are required to establish positivity by either test it appears that both assays are comparable in sensitivity.

DISCUSSION

A multi-target real-time NASBA application for the detection of SARS-CoV has been developed and shown to be comparable in sensitivity and specificity to a Taqman RT-PCR test [Emery et al., 2004]. The NASBA SARS-CoV assay consistently detected at least four different targets in six different isolates, demonstrating sensitivity across different times and geographical locations of the disease outbreak. The N and Pol targets show greatest sensitivity for real-time NASBA detection of SARS-CoV in the materials tested and this is concordant with results obtained for similar targets in the CDC Taqman test [Emery et al., 2004]. Similar to the N region, the 5'-leader sequence is present in all the subgenomic RNA populations. It was therefore anticipated that this target might also provide increased sensitivity for SARS-CoV detection. This sequence, however, is too short for efficient design of NASBA primer sets, necessitating placement of the downstream

TABLE V. Dilution Series of Vietnamese Patient Isolates With Multiple NASBA SARS-CoV Targets

Target	Patient			
	Dilutions	#1	#2	#3
Pol 1	10^{-5}	+	+	+
	10^{-6}	+	+	-
	10^{-7}	+	+	-
Pol 2	10^{-5}	+	+	+
	10^{-6}	+	+	-
	10^{-7}	+	-	-
N1	10^{-5}	+	+	+
	10^{-6}	+	+	+
	10^{-7}	+	-	-
N2	10^{-5}	+	+	+
	10^{-6}	+	+	+
	10^{-7}	+	+	-

Data shown are representative of multiple replicate experiments.

primer outside the leader region. Consequently, the 5'-L NASBA assay detects only genomic RNA, and exhibits lower sensitivity than assays targeting the N region.

For clinical testing, it is important to determine the RNA population in patient samples, since this could differ from that found in cultured virus material and subsequently affect the choice for the most sensitive SARS-CoV target. In a recent study, the abundance of N and Pol RNA transcripts in virus cultures were found to converge after 4 days in culture [Drosten et al., 2004]. Additionally, detection of N gene transcripts did not provide greater sensitivity over those of the Pol gene in clinical specimens from 29 confirmed SARS patients. However, specimens were collected at a median of 12 days after onset of symptoms; further studies are needed to determine if the N gene may provide greater sensitivity for specimens collected in the earlier days of disease onset.

Rapid and accurate detection of the SARS-CoV was a major concern during the SARS outbreak. That concern continues following occurrences of laboratory-acquired infections and the ongoing possibility of the re-emergence of SARS in the community. SARS-CoV is similar to the more common respiratory influenza viruses in that it is a zoonosis, has dual tropism for respiratory and

TABLE IV. Real-Time NASBA Detection of SARS-CoV Using Multiple Targets on Frankfurt and Hong Kong Strains

SARS-CoV	SARS-CoV (geq)	Pol 1	Pol 2	N1	5'-L	3'-NCR
Frankfurt	9.4×10^5	+	+	+	+	+
Frankfurt	9.4×10^4	+	+	+	+	+
Frankfurt	9.4×10^3	+	+	+	+	+
Frankfurt	9.4×10^2	+	+	+	-	-
Frankfurt	9.4×10^1	-	+	+	-	-
Hong Kong	Unknown	+	+	+	+	+
Hong Kong	Unknown	+	+	+	+	+

These data form part of an unpublished European Quality Assurance study that assessed the ability of laboratories around the world to detect SARS-CoV in blinded samples with molecular tests in use in the respective laboratories. Data for the N2 primer set are not included in these results as this set was not yet developed at the time that the European Quality Assurance was being carried out.

gastrointestinal tissues, and efficient mechanisms for generating genetic variation. While 16 recurring SARS-CoV mutations were identified by Ruan et al. [2003], these probably arose as a result of passaging through Vero cells in culture, and it has been suggested that the virus is in fact fairly genetically stable when passing through humans [Brown and Tetro, 2003]. However, it should not be overlooked that the viral replicase is error prone, and therefore capable of causing rapid, unpredictable genetic changes [Rest and Mindell, 2003].

In direct contrast to influenza viruses where viral titer is highest at onset of the disease, viral load in SARS is initially low and does not typically peak until day 10 of disease [Peiris et al., 2003]. This presents a challenge for early detection and highlights the need for extremely sensitive assays to aid in the early diagnosis of disease; particularly important given the wide variation in clinical presentation and difficulties faced with diagnosing based on symptoms. To date, at least three types of diagnostic laboratory tests are available but all have their limitations [World Health Organization Multicentre Collaborative Network for Severe Acute Respiratory Syndrome Diagnosis, 2003; Chen et al., 2004]. ELISA tests can only reliably detect antigen after a week post onset of symptoms depending on the specimen [Lau et al., 2004], making detection of cases difficult at the time they are most infectious. Likewise, an indirect immunofluorescence assay is only able to detect antibodies reliably after day 10 [World Health Organization Multicentre Collaborative Network for Severe Acute Respiratory Syndrome Diagnosis, 2003]. Any culture-based detection requires specialized biosafety level three facilities, which are beyond the scope of most diagnostic laboratories. A number of RT-PCR tests have been developed and some have been simultaneously evaluated [Yam et al., 2003; Bressler and Nolte, 2004; Mahony et al., 2004], but detection of SARS-CoV RNA by real-time NASBA has not been previously reported in the literature.

The advantage of the real-time NASBA assay platform is that the reaction is isothermal, allowing testing of a range of common respiratory viruses such as influenza virus or human metapneumovirus to be performed simultaneously on the same plate. The assay incubation time is also almost an hour shorter than that for RT-PCR tests, allowing for a faster diagnostic turnaround time. Finally, as is the case for all SARS-CoV molecular assays, more extensive studies are needed to explore the importance of specimen type and timing of collection, in order to achieve maximum reliability for SARS-CoV NASBA diagnostic testing.

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