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Review

Molecular Diagnosis of Severe Acute Respiratory Syndrome

The State of the Art

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Severe acute respiratory syndrome (SARS) first appeared in Guangdong Province, China, in November 2002. Although virus isolation and serology were useful early in the SARS outbreak for diagnosing new cases, these tests are not generally useful because virus culture requires a BSL-3 laboratory and seroconversion is often delayed until 2 to 3 weeks after infection. The first qualitative reverse transcriptase-polymerase chain reaction tests for SARS-coronavirus (CoV) were sensitive and capable of detecting 1 to 10 genome equivalents. These assays were quickly supplemented with quantitative real-time assays that helped elucidate the natural history of SARS, particularly the initial presence of low viral loads in the upper respiratory tract and high viral loads in the lower respiratory tract. The unique natural history of SARS-CoV infection dictates the testing of both respiratory and nonrespiratory specimens, the testing of multiple specimens from the same patient, and sending out positives to be confirmed by a reference laboratory. Commercially available reverse transcriptase-polymerase chain reaction tests for SARS have recently appeared; however, meaningful evaluations of these assays have not yet been performed and their true performance has not been determined. These and other issues related to diagnosis of SARS-CoV infection are discussed in this review. ***(J Mol Diagn 2005, 7:551–559)***

Before 2003 human coronaviruses (CoVs) were the interest of only a few select virologists, and CoVs were not even on the radar screen of most clinical virology laboratories. This changed dramatically in March of 2003 with the emergence of severe acute respiratory syndrome (SARS) from China. SARS first appeared as a potentially fatal cause of pneumonia in Guangdong Province of China in November 2002. The first human case was identified on November 16, and within 6 months SARS had spread to 29 countries and had infected 8098 and killed 774 individuals (World Health Organization: Summary of probable SARS cases with onset of illness from 1 November 2002 to 31 July 2003. *http://www.who.int/cst/sars/country/talbe2004_04_21/en/*). The last known case in North America was identified on June 22, 2003, in Toronto and globally on July 15, 2003, in Taiwan. Beyond public health the outbreak had a profound effect on economics worldwide with an estimated $100 billion lost from global economies. SARS has arguably been the most significant event in medical virology since the emergence of human immunodeficiency virus and acquired immune deficiency syndrome in the early 1980s.

CoVs have been identified in a wide range of animals including mice, rats, chickens, turkeys, swine, dogs, cats, rabbits, horses, cattle, and humans, causing a variety of severe diseases including gastroenteritis and respiratory tract diseases. Until 2002, there were only two CoVs know to infect man namely CoV-OC43 and CoV-229E. CoV-OC43 and CoV-229E were identified in the mid-1960s as a cause of mild self-limiting upper respiratory infection and were subsequently shown to cause approximately one third of common cold-like illnesses in adults. Overall, they account for between 5 and 30% of respiratory tract infections, and outbreaks may occur at 3- to 4-year intervals.[1] Based on genotypic and serological characterization, CoVs have been divided into three distinct groups, with CoV-229E in group 1 and CoV-OC43 in group 2.[2]

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Since 2002, three additional CoVs have been discovered, bringing the total number of CoVs infecting man to five. SARS emerged from China in the fall of 2002, and SARS-CoV was identified as the causative agent.[3] On the basis of genome analysis, SARS-CoV was originally assigned to a fourth CoV group but more recently has been recognized as distantly related to the group 2 CoV and has been considered as a group 2 subgroup.[4] In 2004, a novel group 1 CoV associated with respiratory tract infections, CoV-NL63, was discovered in The Netherlands and its genome sequenced.[5] In 2005, a fifth human CoV, a group 2 CoV, was discovered in patients with pneumonia in Hong Kong.[6]

All CoVs belong to the genus *Coronavirus* within the family *Coronaviridae* and are enveloped viruses that possess a positive-strand RNA genome of 27 to 32 kb in size, representing the largest known genome among all RNA viruses. The genomic RNA is capped and polyadenylated and comprises several genes encoding both structural and nonstructural proteins. The genome organization of CoV has the characteristic order 5′-replicase, spike (S), envelope (E), membrane (M), nucleocapsid (N)-3′.[2] The GC content ranges from a low of 32% for CoV-HKU1 to a high of 41% for SARS-CoV. In addition to the S, E, M, and N structural proteins, the genome also encodes a chymotrypsin-like protease, replicase (polymerase), helicase, and hemagglutinin-esterase. For the replicase gene a frame shift interrupts the protein coding regions and separates open reading frames 1a and 1b. Both the 5′ and 3′ ends contain short untranslated regions. The RNA genome is translated as a polyprotein that is subsequently cleaved by the chymotrypsin-like protease. The S gene encodes the spike glycoprotein, which binds to the host cell receptor [ACE-2 in the case of SARS-CoV and aminopeptidase N (CD13) for human HCoV-229E] and leads to membrane fusion and viral entry.

Epidemiology

SARS-CoV is believed to be zoonotic in origin, having crossed into humans most likely from an unidentified animal host in the wild game markets in Guangdong, China. Many of the first infected individuals in November and December of 2002 had contact with the live-game trade. The disease was first called "infectious atypical pneumonia" because it caused clusters of disease in families and health care workers. The etiological agent of SARS was identified as a new CoV by Peiris and coworkers[3] at the University of Hong Kong, and the viral genome was fully sequenced, in a record time of 3 weeks, by researchers at the University of British Columbia Center for Disease Control in Canada.[4] Genomic analysis revealed that SARS-CoV was a new CoV not previously found in humans. The absence of antibody in healthy humans suggested that SARS-CoV had recently emerged in the human population and that animal to human interspecies transmission seemed the most probable explanation for its emergence. Specimens collected from apparently healthy animals in the wild-game animal markets in China indicated that a number of animals, including the Himalayan palm civet cat and raccoon dogs, yielded a SARS-CoV-like virus with more than 99% nucleotide homology to human SARS-CoV.[7] Many animal handlers with no previous history of a SARS-like illness were later found to have serum antibodies to SARS-CoV. Taken together with the fact that a number of SARS cases had an epidemiological link to wild-game animals, SARS-CoV was most probably introduced into humans from wild animals sold in wet markets in Guangdong, China.

At the beginning of the SARS outbreak, nosocomial infections played an important role in the outbreak. The first major outbreak in Hong Kong occurred in the Prince of Wales Hospital approximately March 10, 2003, and resulted in 138 SARS cases, 69% of which were hospital workers. By the end of the outbreak, there were 1755 SARS cases in Hong Kong, 339 of which involved workers in 16 hospitals. In Canada, the first large outbreak also occurred in a community hospital, affecting 128 patients, 37% of which were hospital staff. The major sources of transmission in humans are droplets, aerosolization, and fomites. Deposition of droplets onto the respiratory epithelium probably initiates infection. Whether infection can occur through the oral or conjunctival epithelium remains unknown, but SARS-CoV has been detected in tears. Asymptomatic infection of humans appears to be a rare event. Despite the rapid spread of SARS worldwide, household transmission of SARS is relatively inefficient,[8] and the average number of secondary infections caused by any one case is low (2.2 to 3.7) compared with influenza (5 to 25). Superspreading events in which a few infected individuals disproportionately contribute to transmission (ie, a difficult intubation in the intensive care unit resulting in infection of a number of house staff) were characteristic of the outbreak. Factors associated with superspreading events are not well understood but may include co-infection with other viruses, host factors such as immunosuppression, and/or environmental factors. The number of infected staff in hospitals was strongly correlated with the number of admitted SARS patients, the length and type of exposure, and the use of personal protective equipment such as gloves and masks. Later in the outbreak it was shown that personal protective equipment significantly decreased nosocomial infections and played a significant role in outbreak management.[9]

Natural History

The natural history of SARS-CoV has been documented in several studies. The initial symptoms are unremarkable and common to all viral infections of the upper respiratory tract. A few days of cough and low-grade fever progress rapidly to full-blown pneumonia requiring hospitalization, often including mechanical ventilation. Fever, malaise, lymphopenia, and elevated liver enzymes, together with infiltrates and consolidation on chest X-ray, are typically present.[10] Quantitative polymerase chain reaction (PCR) studies have shown that the viral load is high in the lower respiratory tract but low in the upper respiratory tract. Viral load in the upper respiratory tract and feces is low during the first 4 days and peaks at approximately day 10 of illness.[10] This is in marked contrast to other respiratory viral infections, such as influenza, that peak soon after the

onset of symptoms. This unusual feature of SARS-CoV infection explains its low transmissibility early in the illness and perhaps explains why outbreaks in some countries were limited to only a few cases. More importantly, it explains the poor sensitivity of early reverse transcriptase (RT)-PCR tests on nasopharyngeal (NP) specimens collected early in the illness.

Although the main clinical symptoms are those of severe respiratory tract disease, the virus also infects other organs. Approximately a quarter of SARS patients had watery diarrhea, and virus can be cultured from the feces and urine as well as the respiratory tract.[10] Virus can also be detected by RT-PCR in serum, plasma, and peripheral blood leukocytes; however, the viremia might be short lived. Patients also have a pronounced peripheral T-cell lymphocytopenia with reduced CD4 and CD8 cell counts and with a third of individuals having a CD4 count less than 200 cells/mm^3. Infected individuals with high serum viral loads have a poor prognosis. Between days 10 to 15 of illness, high viral loads in NP aspirates, feces, and serum are independent predictors of adverse clinical outcome. SARS-CoV is invariably found in the lungs of individuals dying of SARS, but viral load is usually higher in those dying earlier in the course of illness ($<$21 days). Approximately one quarter of patients with SARS require management in intensive care units and the overall case fatality rate is $\sim$11%. Disease severity and mortality correlates with age, the highest mortality rates (52%) occurring in those $>$65 years of age, and the lowest rate in the 0- to 24-year-old group. Children acquiring SARS seldom require intensive care or mechanical ventilation.[11] Although SARS-CoV can be found for months in the feces of an infected individual, there is no evidence that the virus persists after resolution of the illness.

Laboratory Diagnosis

The approach to diagnosing specific viral infections is delineated by the understanding of the natural history of infection. The natural history of a viral infection indicates the most appropriate clinical specimens for diagnosis, ie, where the virus will be secreted and detected and when in the course of disease different specimen types would be expected to be positive for virus, and SARS-CoV is no exception. The evolution of diagnostics for SARS has been different from that of most viruses. The need for a BSL-3 facility for virus isolation and difficulty in culturing the virus from infected individuals late in the outbreak (possibly as a result of genetic drift of the virus) precluded the use of culture for diagnosis in most outbreak settings. Furthermore, the late seroconversion, 2 to 4 weeks after infection for most patients, all but eliminates a serological diagnosis for detecting recent infections. The first serological tests used SARS-CoV-infected cells on microscope slides and immunofluorescent staining or enzyme-linked immunosorbent assay tests of crude cell lysates containing SARS-CoV antigens to detect serum antibodies. When used together with multiple specimens, virus isolation and serology proved useful for identifying patients at the beginning of the outbreak; however, they proved less satisfactory for diagnosing new cases in the first few days after onset of symptoms. Initial culturing of a CoV from patients in Hong Kong enabled the early identification of SARS-CoV by electron microscopy and led to the development of the first nucleic acid amplification tests (NAATs) for detecting SARS-CoV RNA. Identification of a CoV by electron microscopy led to the search for conserved regions of the CoV genome and the development of RT-PCR assays for SARS-CoV RNA. Molecular diagnostic testing using NAAT quickly became the mainstay of SARS diagnosis.

The first RT-PCR assays described for SARS-CoV RNA targeted the polymerase (*pol*) 1b region of the 5′-replicase gene. The first PCR primers were developed as a collaborative effort between 17 laboratories participating in the World Health Organization SARS Laboratory Consortium. These primers have been used for the development of both nested and nonnested, one-step or two-step (combined or separate reverse transcriptase reaction) conventional heat block RT-PCR, as well as real-time PCR assays (Table 1). Drosten and colleagues[12] used two sets of primers in a nested PCR format to amplify a 109-bp fragment. This assay had an analytical sensitivity of 10 genome equivalents (GE) per reaction. They also used the same primers in a real-time TaqMan assay and showed that the real-time assay had equivalent sensitivity to the nested assay in detecting SARS-CoV RNA in 18 clinical specimens. This study reported PCR results for only 49 specimens obtained from 3 patients in Frankfurt, Germany, and 18 patients in Hanoi, Vietnam. Ksiazek and colleagues[13] used different *pol* 1b gene primers and described a two-step assay with a sensitivity of 100 GE. Using specimens from six countries, they reported that RT-PCR was more sensitive than culture for diagnosing SARS. It is not clear, however, how many specimens were tested by both methods. Poutanen and colleagues[10] also described a two-step *pol* 1b assay for detecting SARS-CoV RNA in clinical specimens obtained from 10 patients in the Toronto SARS outbreak. These first three assays were used to test only a small number of clinical specimens and were not compared to any other NAATs, precluding an assessment of their performance.

Lau and colleagues[14] described an enhanced RT (ERT)-PCR that involved target preamplification by using two separate amplifications of a region of the *pol* 1b gene. The first PCR was a conventional heat block assay followed by a TaqMan real-time amplification using product from the first reaction. The resulting product of the ERT-PCR was a 68-bp amplicon. The assay was used to test 120 specimens from 80 patients in Hong Kong and China. The enhanced assay detected 28 of 120 positives versus 21 of 120 positives for the TaqMan assay.[14] However, the analytical sensitivity of this assay was not provided, and additional testing of the discordant specimens was not performed to allow comparative assay performance.

Following these original assays, additional tests have been developed that amplify alternative gene targets. Mahony and colleagues[15] introduced one-step and two-step real-time assays that amplify a 149-bp fragment of the nucleocapsid (*nuc*) gene. The one-step TaqMan as-

Table 1. Description of 13 RT-PCR and 1 LAMP Nucleic Acid Amplification Assays for Detection of SARS CoV RNA

Target	Primers		Amplicon (bp)	Format*
pol 1b	BNIoutS2	5′-ATG AATTACCAAGTCAATGGT TAC-3′	190	Two-step nested
	BNIoutAS	5′-CATAACCAGTCGGTACAGCTAC-3′		One-step TaqMan
	BNIinS	5′-GAAGCTATTCGTCACGTTCG-3′	109	Real-time
	BNIAs	5′-CTGTAGAAAATCCTAGCTGGAG-3′		
pol 1b	Cor-p-F2	5′-CTAACATGCTTAGGATAATGG-3′	368	Two-step
	Cor-p-R1	5′-CAGGTAAGCGTAAAACTCATC-3′		
Pol 1b		5′-TGATGGGATGGGACTATCCTAAGTGTGA-3′	NP	Two-step
		5′-TTGCATCACCACTAGTTGTGCCACCAGGTT-3′		
nuc	APNF	5′-TGAATACACCCAAAGACCAC-3′	149	One-step
	APNR	5′-TGATGAGGAGCGAGAAGAG-3′		
	Sense	5′-TTATCACCCGCGAAGAAGCT-3′	123	Two-step
	Anti-sense	5′-CTGTAGAAATCCTAGCTGGAG-3′		
		FAM-TCGTGCGTGGATTGGCTTTGATGT-TAMRA		
pol 1b consensus	Coro1	5′-TGATGGGTTGGGACTCTAAATGTGA-3′	220	One-step
	Coro2	5′-GTAGTTGCATCACCGGAAGTTGTGCCACC-3′		
pol 1b consensus		5′-GGTTGGGACTATCCTAAGTGTGA-3′	440	Two-step
		5′-CCATCATCAGATAGAATCATCATA-3′		
3′-NCR	Sense	5′-GGACCTGAAAGAGCCACCACA-3′	NP	Two-step
	Anti-sense	5′-CATTATTCACTGTACCCTCGATCG-3′		
		FAM-TTTCATCGAGGCCACGCGGAG-TAMRA		
NP	Cor-1 Sense	5′-CACCGTTTCTACAGGTTAGCTAACGA-3′	286	Two-step
	Cor-2 Anti-sense	5′-AAATGTTTACGAAGGTAAGCGTAAAA-3′		
Nuc	SANS1	5′-TGGACCCCACAGATTCAACTGA-3′	NP	One-step
	SANPAs	2 5′-GCTGTGAACCAAGACGCAGTAT-3′		
		FAM-TAACCAGAATGGAGGACGCAATGG-TAMRA		
nuc	Forward	5′-GGAGCCTTGAATACACCCAAAG-3′	66	Two-step
	Reverse	5′GCACGGTGGCAGCATTG-3′		
		FAM-CCACATTGGCACCCGAATCCTAATA-TAMRA		
pol 1b		5′-CAGAACGCTGTAGCTTCAAAAATCT-3′	NP	One-step
		5′-TCAGAACCCTGTGATGAATCAACAG-3′		
		FAM-TCTGCGTAGGCAATCC-TAMRA		
pol 1b	Anti-sense	5′-AGTTGCATGACAGCCCTCTACA-3′	68	Two-step
	Sense	5′-CCCGCGAAGAAGCTATTCG-3′		Two PCR reactions
		FAM-CGTTCGTGCGTGGATTGGCTTTG-TAMRA		
LAMP *pol* 1b		Six primers used included two outer, two inner, and two loop primers that recognize eight distinct regions on target zone	196	One-step

*Format refers to whether the RT step is combined (one-step) or separate (two-step) from the PCR reaction.
†Sensitivity refers to the number of SARS-CoV RNA genome equivalents (GE) per reaction.
pfu, virus plaque forming units; NP, not provided.

(*Continued on next page*)

say had a sensitivity of 10 GE and was evaluated by testing 68 specimens.[15] The *nuc* assay had similar analytical sensitivity to that of the *pol* 1b assay.[12] Bressler and Nolte[16] described a two-step real-time TaqMan assay that amplified a 66-bp fragment of the *nuc* gene with an analytical sensitivity of 90 GE. This assay was evaluated only for analytical sensitivity using armored RNA spiked specimens and did not involve testing any SARS specimens. They showed that the *nuc* gene assay had similar sensitivity to their *pol* 1b assay. Drosten and colleagues [17] also showed that a one-step TaqMan assay targeting the *nuc* gene detected a similar number of positive specimens when compared with two other *pol* 1b assays. They evaluated these assays by testing 66 clinical specimens.

Real-time PCR assays have been introduced for SARS-CoV using both *pol* 1b and *nuc* gene targets. Real-time assays offer increased turnaround time and the ability to quantitate viral load. Wang and colleagues[18] tested 116 SARS specimens using a two-step assay amplifying a 123-bp fragment of the *pol* 1b gene. Although they tested a significant number of specimens they did not indicate the analytical or clinical sensitivity of the assay. Houng and colleagues[19] introduced a two-step real-time assay that amplified a region of the 3′-noncoding region (NCR) of the genome. This assay had an excellent analytical sensitivity of 6 to 8 GE but was not evaluated with SARS specimens.

Poon and colleagues[20] introduced a real-time multiplex assay that targets both the *pol* 1b gene and 18S rRNA as an internal control. This one-step TaqMan assay had an analytical sensitivity of 10 GE and was evaluated using 86 nasopharyngeal specimens from SARS patients. The use of an internal control was used to identify specimens that contained amplification inhibitors or that lacked specimen RNA resulting from a failed extraction. In their study 0 of 86 NP specimens failed to amplify the internal control indicating success-

Table 1. Continued

Sensitivity†	Specimens	Comparisons	Reference
10 GE	49	Nested and real-time assays had equivalent sensitivity for 18 specimens	Drosten et al., 2003[12]
100 GE	19 patients	PCR more sensitive than culture	Ksiazek et al., 2003[13]
NP	10 patients	5 of 6 patients were PCR-positive	Poutenen et al., 2003[10]
10 GE	68	CBH nested and nonnested TaqMan RT-PCR Artus assay	Mahony et al., 2004[15]
NP	116	No	Wang et al., 2004[18]
1 to 10 GE	44	No-Detects other CoV as well as SARS-CoV	Adachi et al., 2004[21]
NP	No	No-Detects other CoV as well as SARS-CoV	Woo et al., 2005[6]
6 to 8 GE	16	No	Houng et al., 2004[19]
NP	No	RT-PCR more sensitive than culture	Chan et al., 2004[32]
NP	66	Artus, compared Roche and in-house assays	Drosten et al., 2004[17]
90 GE	No	*pol* Ib and *nuc* RT-PCR had similar sensitivities	Bressler and Nolte, 2004[16]
10 GE	86	No 18S-rRNA as internal control	Poon et al., 2004[20]
NP	120	One CHB PCR followed by second TaqMan PCR assay	Lau et al., 2003[14]
0.01 pfu	59	LAMP detected seven additional positives	Thai et al., 2004[22]

ful RNA isolation and absence of amplification inhibitors.[20]

Two recent reports have described consensus *pol* 1b gene primers for the detection of CoV RNA including SARS-CoV. Adachi and colleagues[21] used consensus primers to amplify a 220-bp fragment of *pol* 1b in a one-step assay with an analytical sensitivity of 10 GE. They tested 44 SARS specimens as well as cultured CoV-229E and CoV-OC43 virus and turkey infectious bronchitis virus and showed that this consensus RT-PCR assay could detect all four CoVs. Woo and colleagues[6] described a two-step conventional heat block, nonreal-time assay that uses consensus *pol* 1b primers to amplify a 440-bp fragment of the *pol* 1b gene. This assay was used to detect a novel human respiratory CoV, HKU1, in a 71-year-old with non-SARS pneumonia during the SARS outbreak in Hong Kong. This assay, which uses consensus primers, should be able to detect SARS-CoV although these authors did not show that the assay was able to do so.

Isothermal amplification formats including nucleic acid sequence-based amplification or loop-mediated isothermal amplification (LAMP) have also been used to detect SARS-CoV RNA. Thai and colleagues[22] used six primers, including two outer, two inner, and two loop primers that recognize eight distinct regions of the *pol* 1b gene, to amplify SARS-CoV RNA using LAMP. The one-step LAMP assay amplified a 196-bp fragment and had an analytical sensitivity of 0.01 plaque forming units. They evaluated the assay by testing 59 specimens from Vietnamese patients by both LAMP and conventional RT-PCR. LAMP detected 13 positive specimens, compared to 6 for RT-PCR, and had a sensitivity of 100% and a specificity of 87%. Discordant analysis to determine whether the additional positive specimens were true positives or false-positives was not performed. A nucleic acid sequence-based amplification test for SARS-CoV RNA has been described, but this test has not yet been evaluated with clinical specimens.[23] Juang and colleagues[24] recently de-

Table 2. Performance of One Nested, Two Nonnested, and Two Real-Time RT-PCR Assays for Detecting SARS-CoV RNA in 68 Clinical Specimens Using a Combined Reference Standard

Assay	RT-PCR type	Assay format	Target	Primers	% Sensitivity	% Specificity	% Positive predictive value	% Negative predictive value
Nested	One-step	HB	*pol* Ib	BNIoutS2/AS BNIin S/AS	94.4 (17/18)	100 (50/50)	100 (17/17)	98.0 (50/51)
Nonnested	Two-step	HB	*pol* Ib	BNIout S2/AS	100 (18/18)	94.0 (47/50)	85.7 (18/21)	100 (47/47)
Nonnested	Two-step	HB	*pol* Ib	Cor-p-F21R1	94.4 (17/18)	100 (50/50)	100 (17/17)	98.0 (50/51)
Real-time	One-step	LC TaqMan	*nuc*	APNF/APNR	83.3 (15/18)	100 (50/50)	100 (15/15)	94.3 (50/53)
Artus	One-step	LC TaqMan	NP	NP	94.4 (17/18)	100 (50/50)	100 (17/17)	98.0 (50/51)

True positives are defined as positive in at least two assays (n = 18). Values in parentheses represent the number of specimens positive or negative for the specified assay/the total number of true positives (sensitivity) or true negatives (specificity). Sixty-eight specimens included 17 NP, 29 urine, and 22 fecal specimens.

Artus represents the RealArt HPV Coronavirus RT-PCR kit from Artus (Hamburg, Germany).

NP, not provided; HB, conventional heat block assay; LC, LightCycler assay. Data from Mahony et al.[15]

scribed a multiplex RT-PCR that amplifies two SARS-CoV genes and uses a gene chip detection platform.

Performance of Various RT-PCR Assays

The first RT-PCR assays for SARS were not evaluated for performance. At the time these assays were developed, little was known of the natural history of SARS, including when the virus was present in various body compartments. For this reason, care must be taken when reading early publications especially when the words sensitive or sensitivity are used. Early publications often used the word sensitive in the context of the rate of positivity; when articles said that PCR was only 40 to 60% sensitive for NP specimens in the first few days after infection, they really meant that the virus was present in only 40 to 60% of NP specimens. The assay may have had a clinical sensitivity higher than 60%, but the natural history of the infection dictated that the NP specimen would be positive at best only 60% of the time. Determining the performance (sensitivity and specificity) of a test involves comparing it to an appropriate gold standard or reference standard. To determine the sensitivity and specificity of any one RT-PCR assay, the test in question must be compared to the best possible reference standard that defines the true status of specimens. When one test is compared to another test, the discordant specimens (positive in one test and negative in another) should be examined by a third test that acts as an arbitrator to determine the true status of the specimen. In this way a true positive can be defined as being positive in two or more tests, and this can be used as the reference standard.[25] As new assays are developed, evaluation using three or more tests helps determine the performance of each individual test.

Few of the NAATs described for SARS in Table 1 have been evaluated by comparison with other amplification tests. Only two reports in Table 1 involved comparison of three or more assays and only one study used a combined reference standard to determine sensitivity and specificity of specific tests. In the first report, Drosten and colleagues[17] tested 66 SARS specimens by three different tests including an in-house RT-PCR and two first generation commercial tests, RealART HPA coronavirus kit from Artus (Hamburg, Germany) and the SARS Coronavirus LightCycler kit from Roche (Branchburg, NJ). They indicated sensitivities of 70.8% for Artus and 67.1% for Roche, calculated by the number of positive samples divided by the number of samples tested.[17] They did not however indicate the results for each specimen in the three different tests or use a combined reference standard to calculate performance. In the second publication, Mahony and colleagues [15] tested 68 SARS specimens in seven different assays (six in-house and the Artus test) and used a combined standard of positivity in two or more tests to calculate performance. As shown in Table 2, based on a small number of positive specimens, the sensitivities of the tests ranged from 83.3 to 100% while the specificities ranged from 94 to 100%. Of interest, the commercially available Artus test had a sensitivity of 94.4% (17 of 18) and a specificity of 100% (50 of 50). In

Table 3. Performance of Three Commercial and One In-House Real-Time RT-PCR Assays for Detecting SARS-CoV RNA in 60 Clinical Specimens Using a Combined Reference Standard

	% Sensitivity	% Specificity	% Positive predictive value	% Negative predictive value
Artus	81.8 (18/22)	86.8 (33/38)	78.3 (18/23)	89.2 (33/37)
Roche	36.4 (8/22)	100 (38/38)	100 (8/8)	73.1 (38/52)
EraGen	81.8 (18/22)	86.8 (33/38)	78.3 (18/23)	89.2 (33/37)
In-House	77.3 (17/22)	89.5 (34/38)	81.0 (17/21)	87.2 (34/39)

Sixty specimens included 12 NP, 28 urine, and 20 fecal specimens. True positives were defined as positive in at least two assays (n = 18). Values in parentheses represent the number of specimens positive or negative for the specified assay/the total number of true positives (sensitivity) or true negatives (specificity). Artus represents the RealArt HPV Coronavirus RT-PCR kit from Artus (Hamburg, Germany). Roche represents the Coronavirus RT-PCR kit from Roche Diagnostics (Montreal, Canada). EraGen represents the GeneCode SARS Coronavirus POL kit from EraGen Biosciences (Madison, WI). The in-house RT-PCR assay represents the one-step real-time LightCycler assay with a *nuc* gene target described in Mahony et al.[15] NP, not provided. Data from Mahony et al.[26]

a second evaluation of three different commercially available tests from Artus, Roche, and EraGen Biosciences (Madison, WI), a total of 60 SARS specimens were tested by all three commercial assays plus two in-house assays (Table 3). Using a combined reference standard of positivity in two or more assays, there were 22 positives and 38 negatives. The sensitivities of the Artus, EraGen, and Roche assays were 81.8% (18 of 22), 81.8% (18 of 22), and 36.4% (8 of 22) while the specificities were 86.8% (33 of 38), 86.8% (33 of 38), and 100% (38 of 38), respectively.[26] Additional evaluations involving multiple tests and larger numbers of specimens will be required to determine the true performance of these commercial assays. The poor clinical sensitivity of these first generation assays is a concern if SARS returns and should be a directive for the manufacturers to reformulate these assays.

Specimen Selection for Testing

As mentioned above the natural history of SARS-CoV infection determines which clinical specimens will be positive for virus and at what point during the course of infection. NP specimens, which are often negative during the first week of infection, have the highest positivity rates in the second week of illness, peaking at approximately day 10. Quantitative PCR revealed that viral loads were highest in lower tract specimens (bronchoalveolar lavage, sputum, endotracheal aspirates), and bronchoalveolar lavage specimens have been positive more frequently than NP specimens.[17,27,28] The collection of bronchoalveolar lavage specimens, however, has been associated with increased risk of nosocomial transmission and risk to health care workers and was halted in many institutions. The low virus detection rate in NP specimens early in the course of infection underscores the importance of optimal timing and specimen type for diagnosis. The Centers for Disease Control has recommended that multiple specimen sources be tested by nasopharyngeal plus oropharyngeal and serum/plasma during the first week of illness and nasopharyngeal plus oropharyngeal and stool specimens after the first week of illness. For the interpretation of a positive diagnosis based on PCR testing, it is generally recommended that a patient have either two positive clinical specimens from different anatomical sites or positive specimens from the same site on two separate occasions (*www.cdc.gov/ncidod/sars/guidance/f/pdf/app4.pdf*).

Replication of SARS-CoV in the gastrointestinal tract has been confirmed by electron microscopy and RT-PCR testing of biopsy and postmortem specimens. The discovery of SARS-CoV in feces indicated that the virus rapidly disseminates from the NP to other tissues and suggested that the virus could be transmitted by the fecal route. In the case of stool specimens, SARS-CoV RNA could be detected at high levels for several weeks or even months, whereas virus culture was positive only during the first 2 to 3 weeks of illness.[29] Fecal specimens have a high viral load at the end of the first week of illness and are the specimen of choice for diagnosis during the second week of disease. It is possible that virus replication continues for weeks in the gastrointestinal tract but that virus is complexed with antibody and is no longer infective or transmissible. This is consistent with epidemiological findings.

SARS-CoV RNA has been detected in other specimens including blood, cerebrospinal fluid, urine, and tears. Of the nonrespiratory specimens, stool specimens have shown the highest positivity rates and highest viral loads, suggesting that fecal specimens are a good alternative to respiratory tract specimens for identification of SARS patients. Testing of serum or plasma for viral RNA was disappointing at first, but subsequent studies have shown a 50% and 78% positivity rate for serum and plasma, respectively, during the first week of illness.[18] A second study confirmed these results for plasma with a 79% positivity rate during the first 3 days of illness.[30] The demonstration of SARS-CoV RNA in peripheral leukocytes may provide yet another alternative specimen for early diagnosis. Efforts aimed at optimizing RNA extraction from a larger volume of specimens such as stool or plasma should raise the sensitivity of RT-PCR for these nonrespiratory specimens in identifying SARS patients. Real-time PCR assays that provide viral loads may also be useful for identifying patients at increased risk for worse outcomes in terms of survival, requirement for intensive care, and assisted ventilation. Recent studies confirmed that high viral load in NP specimens was associated with the need for intensive care and was an independent predictor of mortality. Quantitative PCR assays may also provide useful prognostic information for clinical management, including the use of antiviral therapy.

RNA Extraction Issues

Other approaches to increasing the sensitivity of SARS-CoV RT-PCR testing have been attempted with varying degrees of success. These include testing multiple or serial specimens, testing for transcripts as well as genomic RNA, and enhancing RNA extraction. Testing multiple aliquots of the same specimen, although not yet evaluated for SARS-CoV, has proven useful for detecting other microorganisms when present in low levels in clinical specimens.[31] Detection of nucleocapsid transcripts by RT-PCR should in theory enhance the sensitivity of PCR for diagnosis because subgenomic RNA transcripts contain nucleocapsid gene sequences following discontinuous transcription. This approach however, has not been the case as RT-PCR assays targeting the *nuc* gene have not been more sensitive than *pol* gene assays and subsequent studies have shown that most of the viral RNA in clinical specimens is genomic RNA. The availability of *nuc* RT-PCR assays however, did provide a confirmatory assay for positive specimens early in the epidemic and assisted with determining the specificity of early tests.[15] Another approach to improving sensitivity involves extraction of a larger volume of the clinical specimen and using more RNA in the RT-PCR assay. By extracting a

larger portion of NP specimens (420 μl versus 140 μl), the investigators increased the sensitivity of RT-PCR from ~50 to 80% for specimens collected from days 1 to 3 after disease onset while retaining the specificity at 100%.[20] In this particular study, there was no increase in the prevalence of amplification inhibitors during RNA purification, but this should be controlled in future studies by using an amplification control in the PCR reaction to rule out false-negatives due to PCR inhibition.

Commercially Available Assays

Commercially available tests have recently appeared and include the RealArt HPA CoV RT-PCR assay from Artus GmbH, the GeneCode SARS Coronavirus NP and POL MultiCode-RTx kits from EraGen Biosciences, and the LightCycler SARS-CoV kit from Roche Diagnostics. Meaningful evaluations of these commercial assays have not yet appeared, and their true performance has not yet been determined. These first generation assays appear to have suboptimal performance with low sensitivities (36.4 to 94.4%) and low specificities (87 to 100%) (Tables 2 and 3). We look forward to the introduction of second generation tests with improved sensitivity and specificity. Diagnostic companies are also working on chip-based assays for the detection of SARS-CoV and other respiratory viruses.

Interpretation of Laboratory Results

Our laboratory policy as well as recommendations from the Centers for Disease Control and World Health Organization for a positive test result include the following: for a positive RT-PCR result, repeat the test using a second aliquot of the original specimen, and if positive, confirm the result by sending the specimen to a reference laboratory; or test the positive specimen by a second RT-PCR test targeting a different part of the genome, and if positive, confirm by a reference laboratory (*www.cdc.gov/ncidod/sars/guidance/f/pdf/app2.pdf*). False-positive specimens can occur with poorly designed primers or, alternatively, with specimens collected from a low prevalence population (specificity decreases as prevalence of infection decreases) or if laboratory testing is performed improperly. A negative RT-PCR test on an NP specimen from a suspected SARS patient does not rule out SARS because SARS-CoV is present in low levels in the upper respiratory tract in the first week of infection. A negative result from an infected patient could be due to the presence of PCR inhibitors that co-purify with RNA, a poor quality specimen, or a specimen lacking virus. Because the level of viral RNA in the NP and stool rises in the second week of illness, a follow-up NP and/or fecal specimen should be collected and tested by PCR. Negative PCR results for specimens from the upper respiratory tract could trigger sampling from the lower respiratory tract where the titers of virus are higher, although collection of these invasive specimens are associated with an increased risk of medical staff infection. Detection of SARS-CoV in plasma or peripheral leukocytes should be considered because this approach provides sensitivities of ~80% during the first week of illness. The use of multiple, serial respiratory tract and fecal specimens will improve the sensitivity of PCR diagnostic testing especially when a larger volume of the sample is extracted and a larger proportion of the sample RNA is used for amplification.[20,32] The first generation of commercially available PCR assays for SARS may have suboptimal sensitivity and should not be used alone without sending parallel specimens to an experienced reference laboratory for testing. Recognizing that the first human infections were caused by a zoonotic virus that adapted to its new human host and that mankind faces the continuing possibility for the introduction of new variants of animal SARS-associated viruses, greater vigilance will be required to make a diagnosis of infection if new animal CoVs are introduced into the human population. The use of RT-PCR assays using consensus primers and sequencing that can detect all known CoVs would be recommended in this scenario.

Handling of Specimens For SARS Testing

Given the severity of SARS and recent reports of laboratory-acquired infections,[33,34] appropriate precautions should be followed for handling specimens submitted for SARS investigation. Testing of respiratory or stool specimens must be done using BSL-2 level precautions in a class II biological safety cabinet with laboratory workers wearing personal protective equipment including long-sleeved gowns, gloves, eye protection, and N95 masks. Virus isolation should not be attempted by routine clinical laboratories because SARS-CoV is a level III agent and propagation of virus requires a BSL-3 facility.

Summary

In the event that SARS returns, laboratories will be faced with significant challenges to diagnose SARS when other common respiratory viral infections and possibly new nonconventional agents (ie, avian influenza virus) are co-circulating in the population. Upwards of 20 different NAATs have been described for the detection of SARS-CoV RNA in clinical specimens, the majority being RT-PCR assays. These assays have been evaluated for the most part on small numbers of clinical specimens from infected patients, and their true performance characteristics are poorly defined. This is particularly the case for the first generation of commercially available assays. The unique natural history of SARS-CoV infection dictates the testing of both respiratory and nonrespiratory specimens, the testing of multiple specimens from the same patient and the sending out of positives to be confirmed by a reference laboratory before a positive diagnosis is declared.

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