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Evaluation of Advanced Reverse Transcription-PCR Assays and an Alternative PCR Target Region for Detection of Severe Acute Respiratory Syndrome-Associated Coronavirus

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First-generation reverse transcription-PCR (RT-PCR) assays for severe acute respiratory syndrome-associated coronavirus (SARS-CoV) gave false-negative results in a considerable fraction of patients. In the present study, we evaluated two second-generation, replicase (R) gene-based, real-time RT-PCR test kits—the RealArt HPA coronavirus LC kit (Artus, Hamburg, Germany) and the LightCycler SARS-CoV quantification kit (Roche, Penzberg, Germany)—and a real-time RT-PCR assay for the nucleocapsid (N) gene. Detecting the N-gene RNA might be advantageous due to its high abundance in cells. The kits achieved sensitivities of 70.8% (Artus) and 67.1% (Roche) in 66 specimens from patients with confirmed SARS (samples primarily from the upper and lower respiratory tract and stool). The sensitivity of the N-gene assay was 74.2%. The differences in all of the sensitivities were not statistically significant ($P = 0.680$ [analysis of variance]). Culture cells initially contained five times more N- than R-gene RNA, but the respective levels converged during 4 days of virus replication. In clinical samples the median concentrations of R- and N-gene RNA, respectively, were 1.2×10^6 and 2.8×10^6 copies/ml (sputum and endotracheal aspirates), 4.3×10^4 and 5.5×10^4 copies/ml (stool), and 5.5×10^2 and 5.2×10^2 copies/sample (throat swabs and saliva). Differences between the samples types were significant but not between the types of target RNA. All ($n = 12$) samples from the lower respiratory tract tested positive in all tests. In conclusion, the novel assays are more sensitive than the first-generation tests, but they still do not allow a comprehensive ruling out of SARS. Methods for the routine sampling of sputum without infection risk are needed to improve SARS RT-PCR.

Severe acute respiratory syndrome (SARS) involves an initial febrile phase, followed by interstitial pneumonia, which leads to respiratory distress syndrome and death in a fraction of patients. The case fatality proportion has been calculated to range ca. 13.2% in patients younger than 60 years of age and 43.3% in those more than 60 years old (1). The first outbreak of SARS started in southern China in autumn 2002 and spread to several countries of the northern hemisphere until it came to rest in July 2003. A novel coronavirus (CoV), SARS-CoV, has recently been identified as the causative agent of SARS (3–5, 8). Irrespective of its low (zero) prevalence in a nonepidemic situation, a diagnosis of SARS has to be considered in patients with compatible symptoms because the disease is highly contagious and can involve large numbers of secondary cases. Ideally, in this situation it would be possible to rule out the disease by a laboratory test.

Since antibodies are detectable only late in the disease (7), virus detection by reverse transcription-PCR (RT-PCR) is most promising for this purpose. Because of the low sensitivity of current methods, however, it is not possible to categorically rule out SARS on the basis of RT-PCR results (7, 10). This situation will become a major problem in case management in upcoming influenza epidemics.

So far only first-generation (in-house) RT-PCR methods have been studied, but now there are two second generation real-time RT-PCR test kits available, both targeting the replicase (R) gene of SARS-CoV, that might have superior sensitivity. Switching the RT-PCR target to the nucleocapsid (N) gene might increase the sensitivity even further due to the higher abundance of subgenomic N RNA in cultured cells (9). Experimental animal data support this rationale (6), but the situation in patients has not been studied yet. We evaluate here both commercial tests (the RealArt HPA coronavirus LC kit [Artus, Hamburg, Germany] and the LightCycler SARS-CoV quantification kit [Roche, Penzberg, Germany]) and an in-house real-time RT-PCR assay for the N gene.

MATERIALS AND METHODS

Clinical samples. Sixty-six samples were available from 29 retrospectively confirmed SARS patients from Singapore, Hong Kong, and Germany (more details are given in Table 1). Twenty-five patients showed seroconversion in immunofluorescence assay; four patients had positive results in three independent RT-PCR assays (equivalent World Health Organization criteria for labo-

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TABLE 1. Clinical sensitivities of three different SARS-CoV RT-PCR assays

Sample origin[a]	Median day of disease (range)[b]	Sensitivity[c] in confirmed patients as determined by various methods (target gene):		
		Artus kit (R)	Roche kit (R)	In-house (N)
Upper respiratory tract[d]	10.5 (3–25)	11/19	10/18[g]	11/19
Lower respiratory tract[e]	12 (3–34)	12/12	12/12	12/12
Stool	12.5 (8–37)	18/22[g]	18/23	20/23
Plasma	9 (3–14)	1/7	2/7	3/7
Other[f]	31 (2–54)	4/5	1/4	3/5

[a] $n = 66$ samples. The samples were from 29 laboratory-confirmed SARS patients (25 patients with seroconversion in an immunofluorescence assay and 4 patients with a positive RT-PCR result in three independent assays [equivalent to World Health Organization criteria for laboratory confirmation of SARS]).

[b] That is, the day at which the sample was obtained.

[c] Expressed as the number of positive samples/total number of samples. The total percent sensitivities (95% confidence intervals) for the Artus, Roche, and in-house methods were 70.8% (59 to 82%), 67.1% (55 to 79%), and 74.2% (63 to 85%), respectively.

[d] Three saliva samples and sixteen nasopharyngeal swabs.

[e] Eight sputum samples, two endotracheal aspirate specimens, and two bronchoalveolar lavage samples.

[f] One jejunectomy sample, one dialysis fluid specimen, and three urine samples.

[g] There was no result for one sample due to a failed internal control. The sample was therefore omitted from the sensitivity evaluation for this assay.

ratory confirmation of SARS). The samples were taken after a median duration of 12 days (range, 2 to 54 days) from the onset of symptoms. The median durations from symptoms to sampling in five different categories of specimens are given in Table 1 (samples from the upper and lower respiratory tract, stool, plasma, and others). These values were not significantly different from each other (Kruskal-Wallis test, $P = 0.092$).

RNA extraction. RNA was extracted from the samples as described earlier (3), by using a DNA stool kit (Qiagen, Hilden, Germany) for stool specimens and a viral RNA minikit (Qiagen) for all other samples. Sputum samples were pretreated with 2× sputum lysis buffer (10 g of *N*-acetylcysteine/liter, 0.9% sodium chloride) for 30 min in a shaking incubator.

Second-generation R-gene RT-PCR assays. The RealArt HPA coronavirus LC kit and LightCycler SARS-CoV quantification kit were used for amplification and quantification of the R gene according to each manufacturer's instructions. Both assays yielded quantitative data in terms of RNA copy numbers per reaction that were converted into RNA copy numbers per milliliter of liquid sample (sputum, stool, urine, plasma, and saliva) or total of solid sample (swabs), based on correction factors derived from the manipulations in the sample preparation procedures. Both assays contained internal positive controls to detect RT-PCR inhibitors.

N-gene real-time RT-PCR assay. The N gene was amplified and quantified in a 25-μl reaction containing 5 μl of RNA, 12.5 μl of reaction buffer, and 0.6 μl of reverse transcriptase-*Taq* mixture (Superscript II RT/Platinum One-Step RT-PCR kit; Invitrogen), 3.6 mM magnesium sulfate, 1 μg of bovine serum albumin (Sigma), 200 nM concentrations (each) of primer SANS1 (TGGACCCACAGA TTCAACTGA) and probe SANP1 (6-carboxyfluorescein-TAACCAGAATGG AGGACGCAATGG-6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine), and a 400 nM concentration of primer SANPAs2 (GCTGTGAACCAAGACGCAGTAT). Thermal cycling involved 50°C for 10 min, followed by 95°C for 3 min and then 50 cycles of 95°C for 2 s, 55°C for 12 s, and 72°C for 10 s. Fluorescence (F1/F2) was read at 55°C on the LightCycler. Quantification of RNA was done by using cloned and in vitro-transcribed RNA standards as described earlier (2, 3). Inhibition control was performed by parallel testing of a second aliquot of each sample, spiked with in vitro-transcribed RNA standard in a concentration 10-fold above the sensitivity limit of the assay. No cross-reaction of the test was observed with human coronaviruses 229E and OC43, as well as with various animal coronaviruses. No positive results were obtained in tests of 54 stool samples from diarrheic patients and 30 respiratory samples (sputum and throat swab specimens) from non-SARS patients with respiratory disease symptoms (mostly atypical pneumonia).

Virus culture. Virus replication was monitored by inoculating SARS-CoV onto subconfluent Vero cell cultures at a multiplicity of infection of 0.01. After 1 to 4 days of incubation at 37°C and 5% CO_2, the supernatant was removed and cleared by centrifugation at 10,000 × *g* for 10 min.

Determination of analytical sensitivities of RT-PCR methods. To make sure that the analytical sensitivity of the in-house N-gene assay was generally equivalent to that of commercial test kits, its lower detection limit was compared to that of one of the R-gene assays (RealArt HPA coronavirus LC kit) by probit analysis as previously described (2) with cloned and in vitro-transcribed RNA calibrators. A 95% detection chance was achieved with 2.8 or 3.0 copies per reaction in the Artus and N assays, respectively. According to the Poisson distribution formula, this reflects a true detectability of one copy of RNA per reaction in both tests [i.e., $P(a) = e^{-m}(m^a/a!)$, where P is the probability of a positive occurrences in an 100% efficient test at an average of m objects per volume unit; set $P = 0.05$, $a = 0$].

Statistical analysis. All statistical calculations were done with the StatgraphicsPlus software package (version 5.0; Statistical Graphics Corp.).

RESULTS

All 66 samples from 29 confirmed SARS patients were tested with the RealArt HPA coronavirus LC kit and the LightCycler SARS-CoV quantification kit. Quantitative results were recorded for all samples that tested positive. Samples with negative test results for SARS-CoV but no amplification of the internal positive control were excluded from the evaluation due to PCR inhibition. Inhibition occurred in one sample in the Artus assay and in two samples in the Roche assay. All samples were also tested in an N-gene real-time RT-PCR assay to determine whether detecting this gene of SARS-CoV might further increase the sensitivity of RT-PCR. The data were recorded as described above. No RT-PCR inhibition occurred with this test.

Table 1 summarizes the qualitative test results by sample type. Virus RNA was detected in only ca. 70% of samples depending on the test used. No significant difference in sensitivity between the individual assays could be observed as determined by an analysis of variance ($P = 0.680$; F ratio = 0.39). Although all three assays detected SARS-CoV in all (100%) samples from the lower respiratory tract, only 56 to 58% of upper respiratory tract samples and 78 to 87% of stool samples tested positive. When all three RT-PCR test results per sample were taken into account, 71.2% of samples were positive in at least two of three tests, and 80.3% of samples were positive in at least one of three tests. In the 11 patients from whom more than one sample was available (three to seven samples per patient; median, four samples), each assay detected the virus in at least one sample per patient.

Figure 1 shows that the results of RNA quantification in both commercial R-gene kits correlated well with those of the N-gene test. There was no significant difference of N- and R-gene copy numbers in the samples in a Wilcoxon matched-pairs signed-rank test ($\log_{10}$ copies in the N assay versus $\log_{10}$ copies in the Artus kit [$P = 0.5865$]; N assay versus the Roche kit [$P = 0.3897$]). As depicted in Fig. 2, none of the three main categories of samples commonly taken from SARS patients (specimens from the deep respiratory tract, the upper respiratory tract, and stool) yielded significantly more N-gene than R-gene RNA. The median concentrations of R- and N-gene RNA, respectively, were 5.5×10^2 and 5.2×10^2 copies/sample in throat swabs and saliva, 1.2×10^6 and 2.8×10^6 copies/ml in sputum and endotracheal aspirates, and 4.3×10^4 and 5.5×10^4 copies/ml in stool. This suggests that the N assay also would not yield a better sensitivity with a particular sample type.

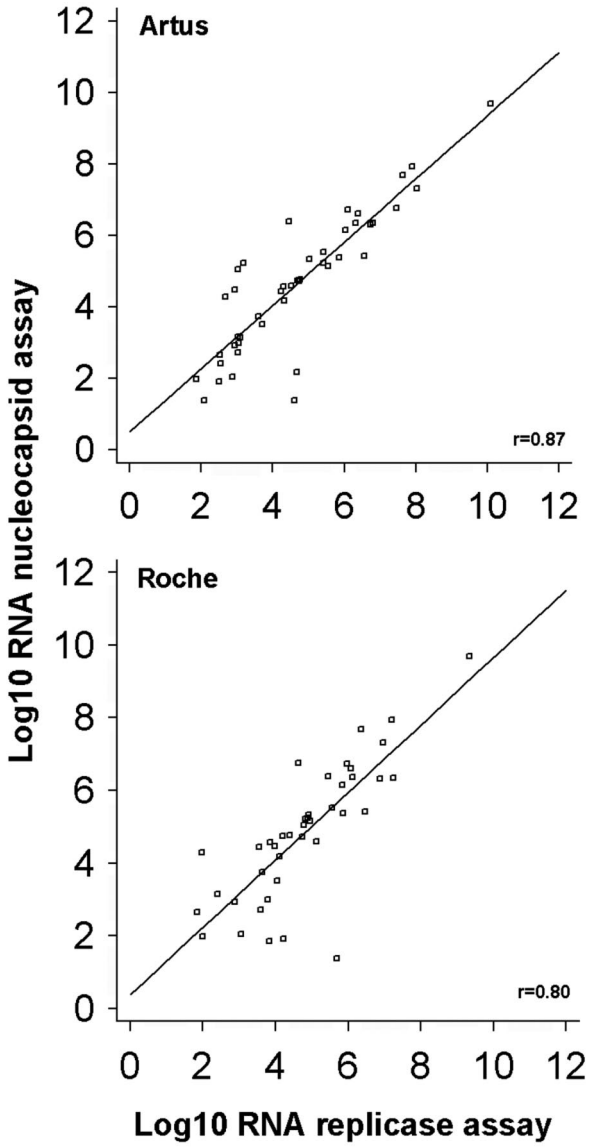

FIG. 1. Linear regression of the virus RNA concentrations determined with the N assay (*y* axis) and each of the two commercial R assays (*x* axis; suppliers of R assays are identified in the panels) in clinical specimens of SARS patients from Singapore, Hong Kong, and Germany that yielded positive results in both assays (r = correlation coefficient). For swab samples, virus concentrations are expressed in terms of copies per swab; for all other samples, the virus concentration is given as copies per milliliter of sample.

RNA concentrations between the categories of samples differed significantly: for both RNA species measured, Wilcoxon two-sample tests comparing the virus concentrations in the whole cohort by category of sample yielded *P* values from 0.001 to $\leq$0.00001. Thus, successful detection of SARS-CoV depended more on the type of sample tested than on the type of test used (note that the time of sampling was equivalent in all categories of samples). As shown previously, samples from the lower respiratory tract contained the highest concentration of RNA (3). From four patients, samples from the upper and lower respiratory tract were obtained on the same day. The virus RNA concentrations in the lower respiratory tract were clearly higher than in the upper respiratory tract in each patient (means of three quantitative tests per sample); in the four patients, the lower respiratory tract samples contained 9×10^5, 6×10^7, 3.2×10^5, and 1.8×10^7 more RNA copies/ml than the respective upper respiratory tract samples.

Virus concentrations in plasma and urine were very low with either assay (ranges, 7.2×10^1 to 5.6×10^3 copies/ml [plasma] and 1.5×10^2 to 4.7×10^4 copies/ml [urine]).

To appreciate why the previously reported higher intracellular abundance of the N gene was not reflected in clinical diagnostic results, we quantified SARS-CoV N- and R-gene RNA in Vero cell cultures 1 to 4 days after infection (Fig. 3). The cytoplasm of the cells yielded about five times more N than R RNA after 1 day. On subsequent days, however, levels of both RNAs gradually converged and approximated each other. In the supernatant analyzed as a control, the abundance of both RNAs was always equivalent.

DISCUSSION

In the present study we have determined whether the clinical detection of SARS-CoV RNA can be improved by novel commercial RT-PCR kits or by using an alternative target gene for detection (N gene instead of R gene). The rationale for using commercial tests was that these are highly optimized and contain improved technical features, e.g., internal controls. Both kits detected the virus in ca. 70% of all samples, which appears to be better than what has been observed in an earlier study (7). The sensitivity in stool samples reached 78 to 87% versus

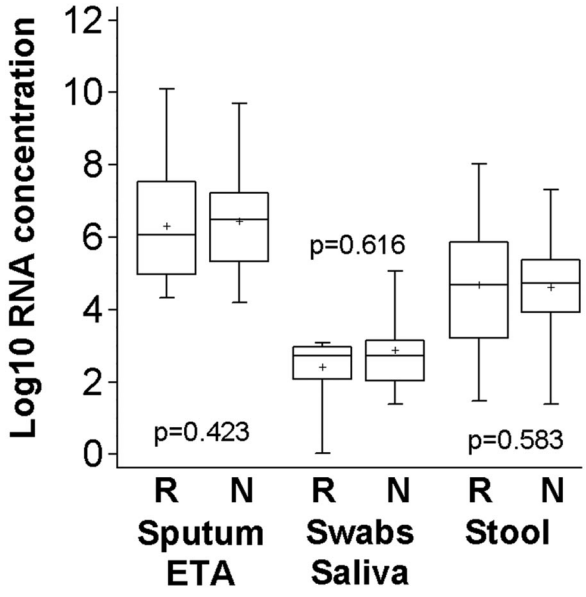

FIG. 2. Box plot analysis of concentrations of R- and N-gene RNA (*y* axis) in three different categories of clinical samples (*x* axis; ETA = endotracheal aspirate). The upper and lower limits of the boxes represent the innermost two quartiles of the ranked datasets, whereas the lines represent the outermost quartiles. Horizontal lines within boxes represent the medians; crosses depict the means of the datasets. The *P* values associated with each category of samples are derived from Wilcoxon matched-pair signed-rank tests comparing the observed concentrations of the two different RNA species in each category of samples.

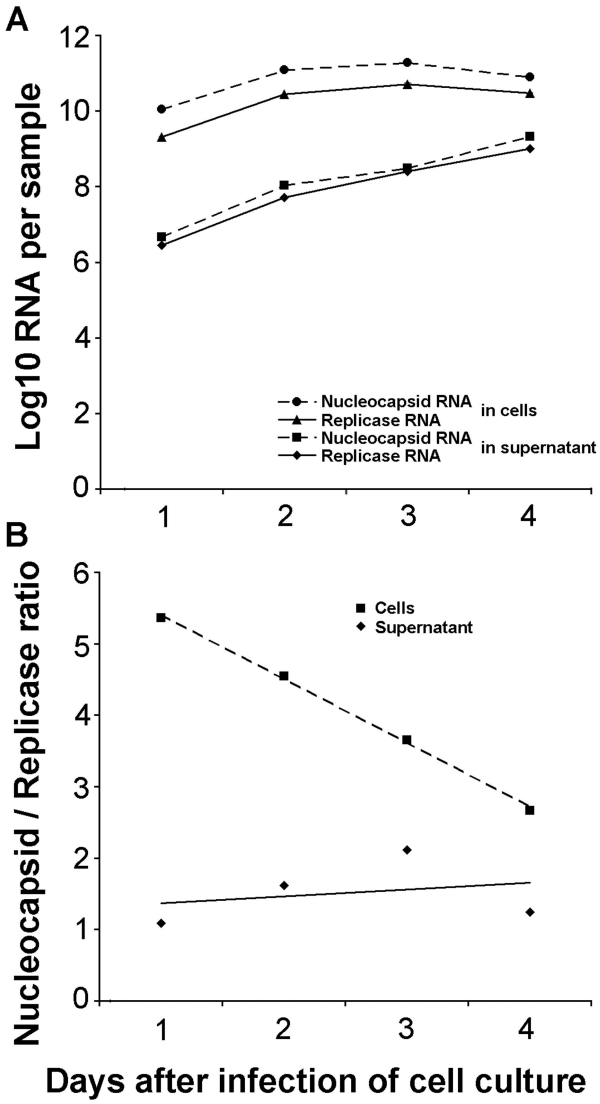

FIG. 3. (A) Absolute concentrations of R- and N-gene RNA in cell culture during the first 4 days after infection (*x* axis) in cytoplasm (1:100 dilutions of lysates of 10^6 cells each) and supernatant (copies per 100 μl). (B) Relative amount of N-gene per R-gene RNA in cells and supernatant over time (linear regressions).

58 to 63% with earlier methods (10). On the other hand, the sensitivity in samples from the upper respiratory tract was not clearly improved with the refined commercial tests, and the new tests still do not appear to allow ruling out SARS reliably in suspected patients.

Tests for ruling out an infection with SARS-CoV are urgently required because the disease is highly contagious and cannot be distinguished by other means from common respiratory diseases, e.g., influenza. We thus attempted to improve the sensitivity further by switching the RT-PCR target to the N gene, based on the reportedly higher abundance of such subgenomic RNA in infected cells (9). Use of this gene for diagnostic purposes has been encouraged by first animal model data, suggesting that it might improve the detectability of the virus (6). In our patients, however, this approach did not yield the expected benefit, even though our N-gene assay was capable of detecting one RNA molecule per reaction, a sensitivity that cannot be improved further.

Our cell culture data give an explanation for this surprising finding. Although it could be confirmed that the N gene is more abundant than the R gene in freshly infected cells, the excess decreased after a relatively short time. Since it can be assumed that by the time symptoms appear, cells in most clinical specimens would have already been replicating the virus for some days, this explains why the sensitivity of the N assay is not superior in symptomatic patients. When we analyzed the abundance of the N gene by sample type, this assumption was confirmed. No category of samples (specimens from the upper respiratory tract, lower respiratory tract, and stool) contained more N-gene than R-gene RNA.

Apart from the target gene used, time and body compartment of sampling are other possible factors influencing the sensitivity of RT-PCR. The timing of samples in our study was comparable to that in earlier reports (7, 10), representing the acute (febrile) phase of disease when patients are hospitalized, and the probability of detection of RNA is high, especially in respiratory samples (7). The sensitivity of RT-PCR in our SARS patients was significantly influenced by the body compartment a sample was taken from; nasopharyngeal swabs and saliva yielded significantly less viral RNA than other samples. Although they can be easily taken from patients, swabs and saliva thus have to be considered suboptimal material for SARS-CoV RT-PCR. Sputum and endotracheal aspirates can be expected to give better diagnostic sensitivity, but their collection poses the risk of generating infectious aerosols in the hospital. Stool samples appear to be a reasonable alternative, but it has already been shown that they will yield less satisfactory results in the very early phase of the disease (7).

The outcome of the present study for diagnosing acute SARS is rather disappointing. Even refined commercial tests could not detect the virus in all clinical samples, and testing for the N gene also yielded no benefit. The reaction chemistry of these assays cannot be improved further since they already detect one molecule of RNA per reaction. Other possible approaches to technically improve SARS RT-PCR, e.g., by modifying the nucleic acid extraction procedures, are limited since these methods have already been optimized based on experiences with other respiratory diseases. In earlier studies it has been suggested to increase the sensitivity of SARS-CoV RT-PCR by testing several samples per patient (7, 10). In our study, viral RNA could indeed be detected in all patients from whom multiple samples were available, and a slight increase in sensitivity could even be achieved by simply testing the same samples in multiple tests. However, especially when multiple patients have to be tested for SARS-CoV, examination of a sufficient number of samples per patient may not be practicable. A reliable test for ruling out SARS would therefore have to provide optimal sensitivity in single samples.

Our study implicates another possible approach to increase RT-PCR sensitivity. Since specimens from the lower respiratory tract contain far-higher virus concentrations than throat swabs and stool, equipment for aerosol-free sampling of sputum might facilitate the routine use of such samples without infection risk for health-care workers. If these prerequisites can be met, application of RT-PCR might enable ruling out SARS.

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