Comparison of Two Real-Time Quantitative Assays for Detection of Severe Acute Respiratory Syndrome Coronavirus

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The new severe acute respiratory syndrome (SARS) coronavirus (CoV), described in February 2003, infected a total of 8,439 people. A total of 812 people died due to respiratory insufficiency. Close contact with symptomatic patients appeared to be the main route of transmission. However, potential transmission by blood transfusion could not be definitely excluded. Two real-time SARS-specific PCR assays were assessed for their sensitivities, agreement of test results, and intra-assay variabilities. Both assays rely on reverse transcription and amplification of extracted RNA. Dilutions of gamma-irradiated cell culture supernatants of SARS CoV-infected Vero E6 cells were prepared to determine the precisions, linear ranges, and accuracies of the assays. The linear range for the Artus RealArt HPA-Coronavirus assay (Artus assay) was 1×10^2 to 1×10^7 copies/ml, and that for the Roche LightCycler SARS CoV Quantification kit (Roche assay) was 1×10^4 to 2×10^8 copies/ml. The detection limit of the Roche assay was 3,982.1 copies/ml, whereas that of the Artus assay was 37.8 copies/ml. Detection limits were calculated with a standard preparation that was recommended for use by the World Health Organization. However, quantification of CoV in this preparation may be imprecise. In summary, both assays are suitable for quantitative measurement of SARS CoV at the high concentrations expected in sputum samples. The Artus assay is also suitable for detection of SARS CoV at the low concentrations found in serum samples.

In early 2003 cases of a newly emerged infectious disease, later named severe acute respiratory syndrome (SARS), were described (3, 6). In April 2003, a real-time PCR-assay for specific detection of the causative novel SARS coronavirus (CoV) was developed at the Bernhard Nocht Institute, Hamburg, Germany (9). The assay was subsequently marketed as the RealArt HPA-Coronavirus assay by the Artus Company, Hamburg, Germany (referred to here as the Artus assay), and facilitated diagnosis of the infection during the peak of the epidemic.

Before the PCR assay for the SARS CoV was available, diagnosis of this atypical pneumonia was based solely on clinical and epidemiological findings: acute febrile illness with respiratory symptoms not attributed to another cause and a history of exposure to an individual with a suspect or probable case of SARS or the individual's respiratory secretions or other bodily fluids (15).

The introduction of specific PCR testing led to the faster and more reliable confirmation of infections with the new strain of coronavirus, thus helping to gain control over the epidemic (20). Even though no new infections have been reported for several months, a renewed outbreak in colder seasons seems likely because the reservoir of the virus has not been definitely identified (1, 18).

In the present study we investigated two reverse transcription (RT)-PCR assays using the Roche LightCycler apparatus: the new Roche SARS CoV Quantification kit (referred to here as the Roche assay), codeveloped by Roche Diagnostics, Mannheim, Germany, and the Genome Institute of Singapore, was compared with the first commercially available assay, the Artus assay. The sensitivities and the ranges of validity of both assays were assessed.

MATERIALS AND METHODS

Standard RNA preparation. A gamma-irradiated, purified SARS CoV-infected Vero cell culture supernatant was used as an external quantification standard. This standard preparation was recommended by the World Health Organization network for use in the quantification of SARS CoV (19). The viral RNA concentration of 9.4×10^6 RNA copies/ml had been determined in an external laboratory by multiple quantitative real-time PCR measurements (C. Drosten, unpublished data). The preparation was shown to be noninfectious in cell culture (M. Niedrig [Robert Koch Institute, Berlin, Germany], personal communication).

RNA was prepared from the viral standard with a Qiagen viral RNA kit according to the instructions of the manufacturer. Purified RNA (external standard RNA) was diluted to final concentrations of 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 copies/ml.

Artus assay. The Artus assay is a ready-to-use system for the detection of SARS CoV RNA. The assay contains reagents and enzymes for the specific amplification of an 80-bp region of the SARS CoV genome, and additionally, the assay contains a second heterologous amplification system to identify possible PCR inhibition. Primer and probe sequences were described by Drosten et al. (9). Internal SARS CoV standards, which allow the determination of the pathogen load, are supplied with the assay kit.

Real-time quantitative amplification of SARS CoV RNA was performed according to the instructions of the manufacturer. A total of 5 µl of RNA extract was transferred into reaction tubes containing 15 µl of PCR reagents. RT was performed at 50°C for 10 min; and amplification was performed for 1 cycle of

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TABLE 1. Precisions of the Roche and Artus assays for detection of SARS CoV[a]

Nominal concn (no. of copies/ml)	Roche assay				Artus assay			
	Mean measured concn (no. of copies/ml [range])	Mean C_t	SD	% CV	Mean measured concn (no. of copies/ml [range])	Mean C_t	SD	% CV
10^6	4,974,250 (3,686,000–5,992,000)	26.38	0.22	0.82	3,401,750 (2,531,000–4,409,000)	25.52	0.21	0.83
10^5	514,925 (340,000–689,000)	29.58	0.25	0.84	301,950 (222,800–365,500)	28.86	0.19	0.66
10^4	76,688 (52,080–107,720)	32.28	0.37	1.15	30,684 (23,740–37,480)	32.00	0.18	0.55
10^3	22,057 (14,586–28,540)	33.98	0.15	0.45	3,716 (2,822–5,763)	34.92	0.25	0.71
10^2	13,661 (8,584–20,520)	34.73	0.22	0.62	439 (256–766)	37.95	0.57	1.49
10^1	17,460	34.33			220 (85–165)	39.14	0.88	2.24

[a] External standard RNAs (a gamma-irradiated, purified Vero cell culture supernatant was used as a full-virus quantification standard) were tested at six different concentrations. Each concentration was analyzed in eight PCR runs. C_t represents the PCR cycle at which the probe-specific fluorescent signal can be detected against the background.

95°C for 10 s and 50 cycles of 95°C for 2 s, 55°C for 12 s, and 72°C for 10 s. Finally, cooling was performed at 40°C for 30 s.

Roche assay. The Roche assay contains a reaction mixture that amplifies a 180-bp target sequence of the replicase 1AB/polymerase gene of SARS CoV. Specific probes emit fluorescent light after hybridization to the target sequence. An internal control sequence (180 bp) is amplified by the same pair of primers that amplify the target sequence, but the internal control sequence is detected with different hybridization probes.

Real-time quantitative amplification of SARS CoV RNA was performed according to the instructions of the manufacturer. The company does not publish the primer sequences. A total of 5 μl of RNA extract was transferred into reaction tubes containing 15 μl of PCR reagents. RT was performed at 61°C for 20 min; and amplification was performed for 1 cycle of 95°C for 30 s and 45 cycles of 95°C for 5 s, 55°C for 15 s, and 72°C for 10 s. Finally, cooling was performed at 40°C for 30 s.

Statistical analysis. The standard deviation (SD) and coefficient of variation (CV) of the real-time PCR test were calculated by using Excel 2000 software. Sensitivity was estimated by probit analysis with SPSS (version 11.5) software. Student's unpaired t test was performed with the data from the probit analysis. Statistical significance was considered if P was <0.05.

RESULTS

Precision. Precision is defined as the degree of scatter within a series of measurements. It is expressed as the SD, percent CV, and the range (the lowest and the highest measured values). Each external standard RNA concentration (10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 copies/ml) was measured eight times by both assays (Table 1). The SD of the threshold cycle (C_t) by the Roche assay ranged from 0.15 to 0.37, whereas the SD of the C_t by the Artus assay ranged from 0.18 to 0.88. No significant differences with regard to SD or CV were observed between the two assays. The Roche assay detected the lowest external standard RNA concentration of 10^1 molecules/ml in only one of eight PCR runs. Therefore, SD and CV could not be calculated for the assay at this external standard RNA concentration.

Linear range. Figure 1A shows the relation between the nominal and measured external standard RNA concentrations. The results presented in Fig. 1 demonstrate significant differences between the two assays. The Roche assay revealed linear measurements only between 10^4 and 10^6 copies of the external standard RNA preparation per ml, whereas the Artus assay was linear between 10^2 and 10^6 copies/ml. This indicates that the Roche assay measures low SARS CoV concentrations only qualitatively, whereas the Artus assay is able to determinate low SARS CoV RNA concentrations quantitatively. Each kit contains assay-specific SARS CoV RNA standards (internal

standards) at different concentrations. The Roche assay includes internal standard concentrations between 2×10^4 and 2×10^8 copies/ml, and the Artus-assay includes internal standard concentrations between 1×10^4 and 1×10^7 copies/ml. Figure 1B shows the linear range of each assay with its own internal standards. Both assays showed comparable and highly significant correlations between the nominal and the measured SARS CoV concentrations ($R^2 = 0.9731$ and 0.998 for the Roche and Artus assays, respectively). The results in Fig. 1A and B demonstrate a linear range from 1×10^4 to 2×10^8 copies/ml and 1×10^2 to 1×10^7 copies/ml for the Roche and Artus assays, respectively.

Sensitivity. Each of six different dilutions of SARS CoV external standard RNA preparations (10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 copies/ml) were tested eight times by each assay. To eliminate the effects of the different numbers of PCR cycles (the Roche assay uses 45 cycles, whereas the Artus assay uses 50 cycles), we defined positive results as a fluorescent signal below the 45th cycle. The results are shown in Table 2. Probit analysis of these data revealed a hit rate of 95% in parallel tests when an average of at least 3,982 copies/ml (range, 2,986 to 4,978 copies/ml) was amplified by the Roche assay and an average of at least 37.8 copies/ml (range, 1.7 to 50.4 copies/ml) was amplified by the Artus assay ($P < 0.05$). Figure 2 shows the results of real-time PCR runs by both assays with 10^6 to 10^1 input copies of the external standard RNA preparation. Whereas the Artus assay identified 10^1 copies/ml, the lowest standard concentration (10^1 copies/ml) was not detectable by the Roche assay.

Accuracy. Accuracy is defined as the closeness of the agreement between the nominal value and the mean measured value and is expressed as the absolute difference between the two. Since no nominal standards are available for SARS CoV, we analyzed the accuracies of the assays with internal (assay-specific) and external (9) standards. The accuracies of the internal and external standards ranged from 0.02 to 0.13 and 0.7 to 3.2, respectively, for the Roche assay. The accuracies of the internal and external standards ranged from 0.004 to 0.03 and 0.48 to 1.34, respectively, for the Artus assay (data not shown). There were no statistically significant differences between the two assays on the basis of accuracy.

Agreement between both assays. To assess the level of agreement between the two assays, we plotted the data on a logarithmic scale (Fig. 3) and drew a line of equality on

A

B

LightCycler SARS-CoV Quantification Kit

y = 1,1987x - 931919
R² = 0,9731

RealArt HPA-Coronavirus
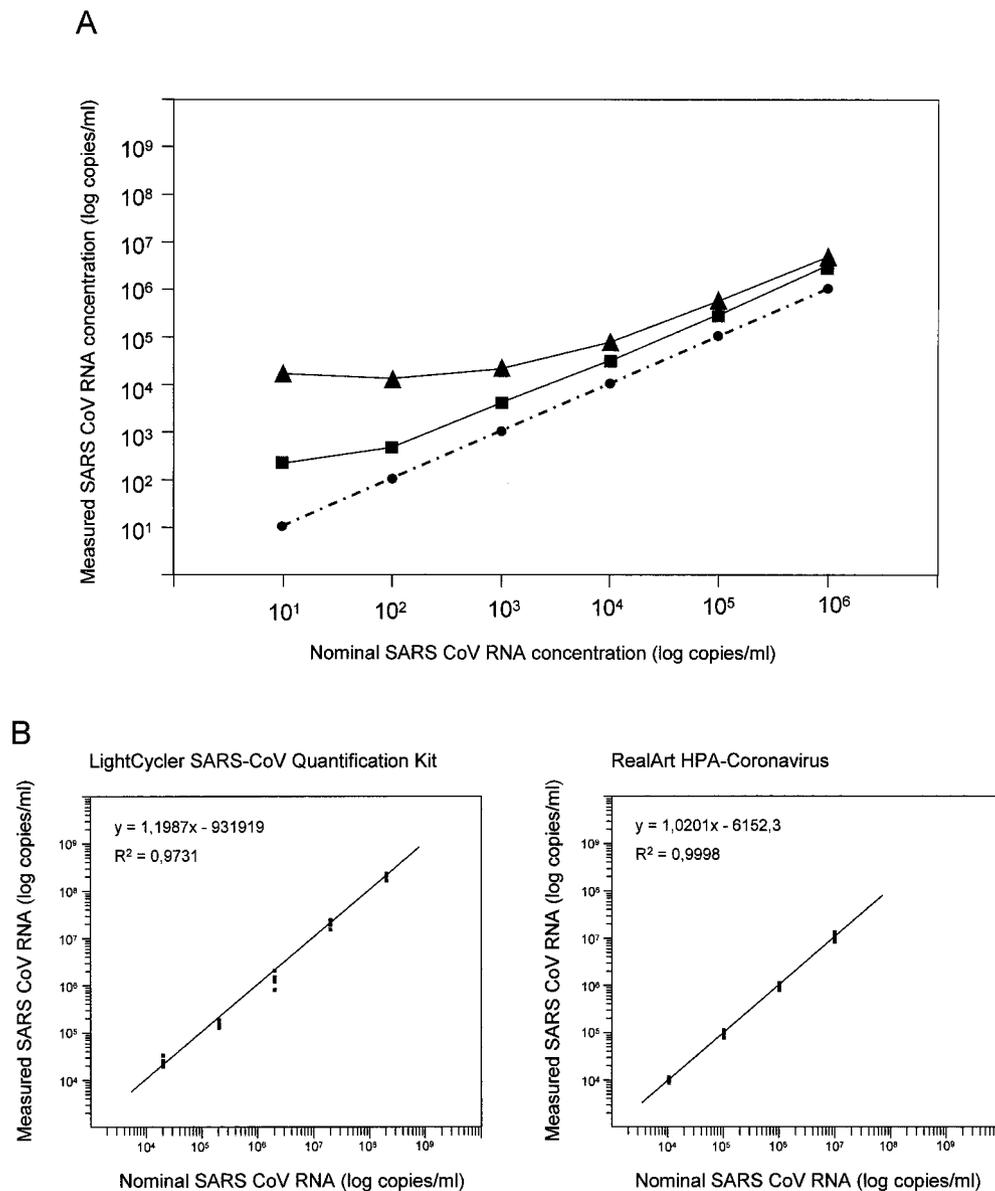
y = 1,0201x - 6152,3
R² = 0,9998

FIG. 1. Linear ranges of SARS CoV assays. (A) Correlation between nominal SARS CoV RNA concentrations and measured SARS RNA concentrations analyzed with the external standard RNA. ▲, Roche assay; ■, Artus assay; ●, line of equality. (B) Correlation between nominal and measured SARS CoV RNA concentrations analyzed with kit-specific internal standards. The correlation factors (R^2 values) showed no significant differences.

which all points would lie if both assays gave exactly the same values at the same concentrations. At higher external standard RNA concentrations ($>10^4$ copies/ml) the level of agreement between the two both methods approached an asymptote along the line of equality. However, measurements below 10^4 copies/ml disagreed considerably. Therefore, the best way to estimate the intermethod differences would be to take the mean values obtained by both methods and plot those values against the differences in the means. According to Bland and Altman (4), limits of agreement are defined as the mean of differences ± two times the SD. It is

assumed that 95% of the data lie between these limits if the differences are normally distributed. As demonstrated in Fig. 4A, there was good agreement between the two assays with high external standard RNA concentrations (10^6 copies/ml). The mean of the differences was close to zero (0.17), as expected for good agreement, and 95% of the data were between 0.36 and -0.02 after logarithmic transformation. However, for low external standard RNA concentrations (10^2 copies/ml; Fig. 4C), the mean of the differences was 1.45 and the limits of agreement (mean ± two times the SD) were 1.72 and 1.18, respectively.

TABLE 2. Sensitivities of Roche and Artus assays for
detection of SARS CoV[a]

No. of SARS CoV copies/ml	Roche assay		Artus assay	
	No. of samples positive/ no. tested	% Positive	No. of samples positive/ no. tested	% Positive
10^6	8/8	100	8/8	100
10^5	8/8	100	8/8	100
10^4	8/8	100	8/8	100
10^3	8/8	100	8/8	100
10^2	6/8	75	8/8	100
10^1	1/8	12.5	4/8	50

[a] Both assays were quantified with an external standard RNA. Eight runs were performed with each concentration. Statistical probit analysis yielded 95% detection limits of 3,982 copies/ml (range, 2,986 to 4,978 copies/ml) for the Roche assay and 37.8 copies/ml (range, 1.7 to 50.4 copies/ml) for the Artus assay.

DISCUSSION

In the present study we compared the Roche assay with the Artus assay. The sensitivities of both assays was evaluated by using the inactivated and quantified SARS CoV external standard RNA recommended for use by the World Health Organization network. External standard RNA was extracted and diluted in 10-fold steps from 10^6 to 10^1 copies/ml. The linearity of each kit was assessed on the basis of assay-specific internal standard RNAs.

Our results demonstrate that the Roche assay shows a linear range from 1×10^4 to 2×10^8 copies/ml, whereas the Artus assay is linear from 1×10^2 to 1×10^7 copies/ml.

The 95% detection limits were shown to be 3,982 copies/ml for the Roche assay and 38 copies/ml for the Artus assay.

According to the instructions of the manufacturers, 5 µl of extract is used for each PCR. Therefore, in the case of the Artus assay, 0.19 copies per PCR mixture could be detected with 95% probability. Nevertheless, no false-positive measurements were obtained.
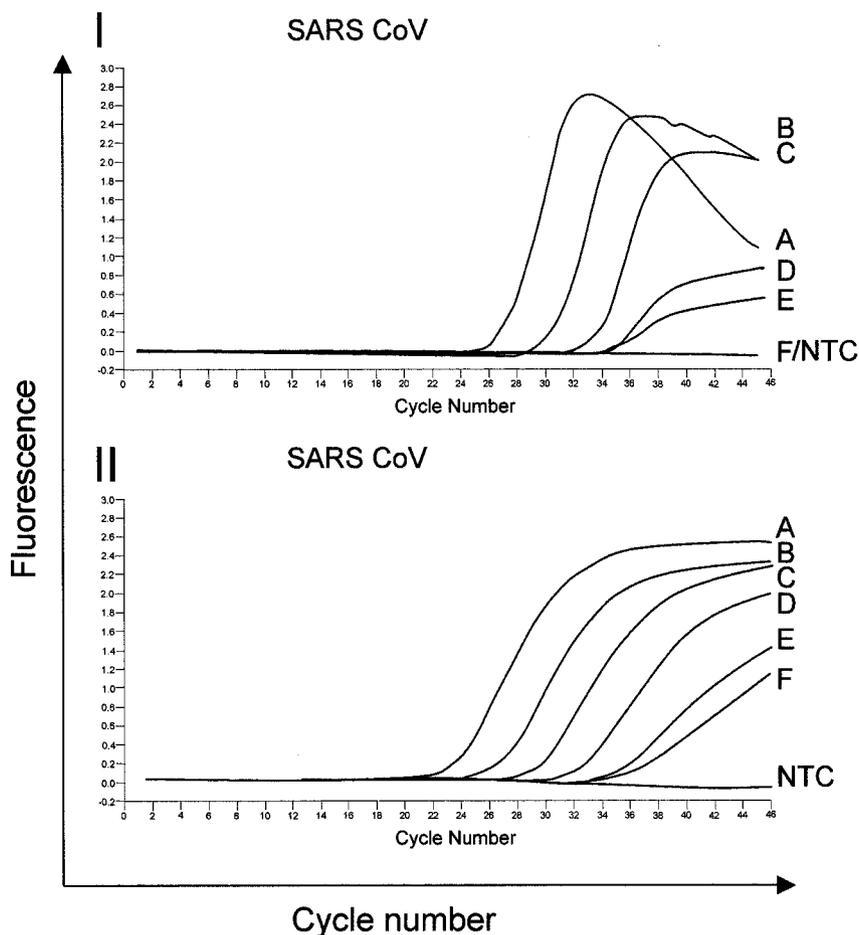

FIG. 2. Real-time PCR of SARS CoV by two real-time PCRs: the Roche assay (I) and the Artus assay (II). Real-time PCR runs for SARS CoV with six external standard RNA concentrations (A, 10^6 copies/ml; B, 10^5 copies/ml; C, 10^4 copies/ml; D, 10^3 copies/ml; E, 10^2 copies/ml; F, 10^1 copies/ml; NTC, no-template control) are shown. The Roche assay demonstrates positive results only for the first five concentrations (A to E), whereas the Artus assay shows positive results for all six concentrations (A to F).
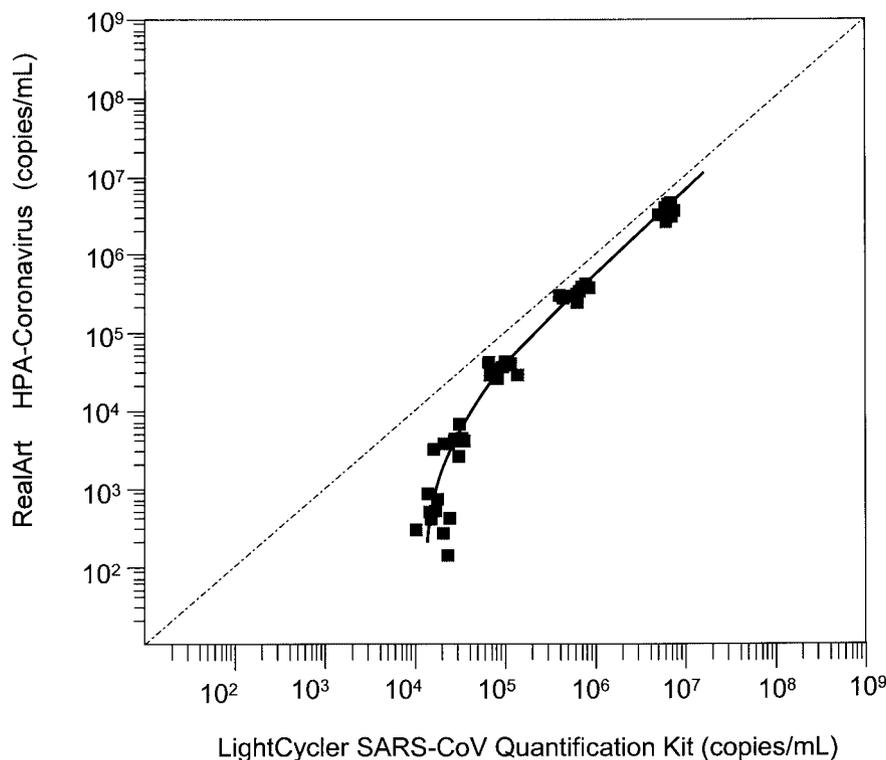
FIG. 3. Agreement between Roche assay and Artus assay. The results for external standard RNAs (10^6 to 10^1 copies/ml) were plotted against each other. The line of equality is represented by a dotted line. ■, measured values.

As a 95% detection limit below 2 to 5 copies is regarded as unrealistic, even for ultrasensitive assays, we assume that the external quantification standard is underestimated by at least one decimal unit. Thus, we calculate 95% probability limits of >40,000 copies/ml for the Roche assay and >380 copies/ml for the Artus assay.

Published concentrations of SARS CoV in different clinical specimens range from 10^8 copies/ml in sputum to 190 copies/ml in plasma (9). Since these concentrations were determined with the same external standard RNA preparations (9), revision is strongly recommended. As testing of sputum (which contains approximately 10^8 copies/ml) represents the main application, the use of both kits is suitable for verification of the disease. Due to high viral levels in sputum, the poor performance of the Roche assay with lower virus concentrations is of minor importance. Nevertheless, one can imagine that other clinical specimens will be used to test for SARS CoV. As reported previously, PCR testing of donated blood for SARS CoV may be used in an epidemic. We previously showed that the Artus assay has sufficient sensitivity to detect the low virus burdens in plasma samples, even in pooled material (17). Our data confirm the previously published high sensitivity of the Artus assay, whereas the Roche assay may not be reliable when viremia levels are below approximately 40,000 copies per ml.

Two possible explanations for the poor performance of the Roche assay with lower virus concentrations can be considered. In addition to the different lengths of the amplified se-

quences used in the Roche and Artus assays, the enzymes used for RT-PCR may be the reason. The Artus assay uses a combination of Moloney murine leukemia virus reverse transcriptase and *Taq* DNA polymerase, whereas the Roche assay applies the one-enzyme *Tth* (*Thermus thermophilus*) DNA polymerase assay, as described by Myers and Gelfand (14). *Tth* polymerase in combination with manganese ions for RT-PCR has been shown to be less susceptible to inhibitors and GC-rich genomes (2, 5, 12, 14, 16). However, the lack of sensitivity at low virus concentrations, presumably due to the insufficient RT activity of *Tth* polymerase, has been reported previously (5, 7, 8, 10, 11, 13).

In conclusion, we show that both the Roche and the Artus assays may be suitable for the verification of SARS by examination of sputum samples. Additionally, the Artus assay could even be used to detect SARS CoV in clinical specimens with low virus loads. Thus, the Artus assay provides a wider range of applications. Furthermore, we believe that the amount of the external standard RNA which was previously used to quantify the virus loads in different clinical specimens has been underestimated and is higher than has been reported previously. Therefore, in our opinion a repeat quantification of the virus load is necessary. Irrespective of the high virus level in sputum, an ultrasensitive PCR test is needed for blood donor services. Each donor receives a brief examination by medical professionals prior to blood donation. However, it is conceivable that a SARS CoV-infected blood donor may not be suspected if, for example, he or she has received
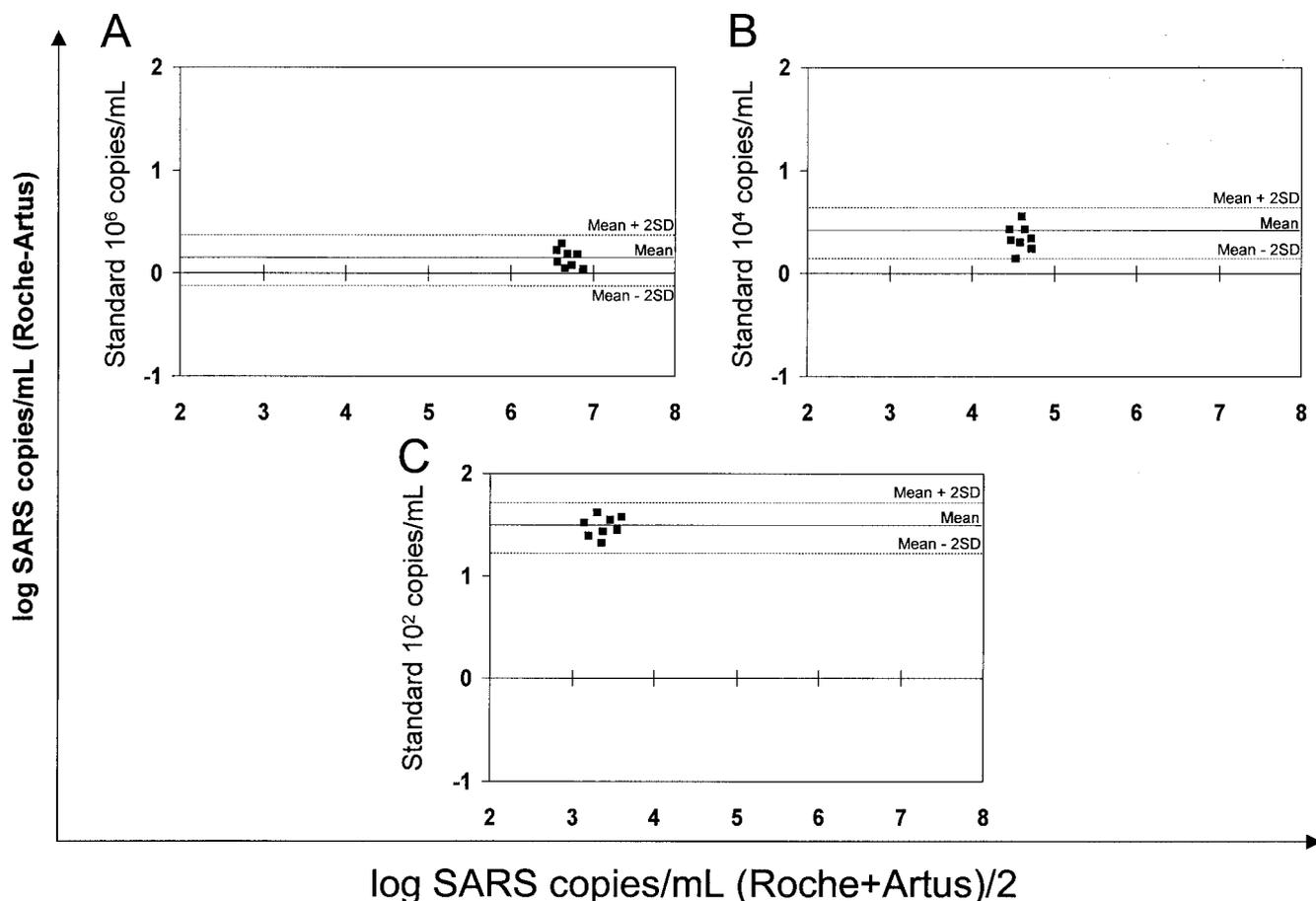
FIG. 4. Limits of agreement according to Bland and Altman (4). The means of both methods (x axis) were plotted against the differences between the means of the two methods (y axis). (A) External standard RNA concentration of 10^6 copies/ml; (B) external standard RNA concentration of 10^4 copies/ml; (C) external standard RNA concentration of 10^2 copies/ml. The mean is indicated by a solid line; the means $\pm$ two times the SD (2SD) are indicated by dotted lines.

antipyretic treatment. An improved PCR-based test would reduce the risk of transmission of SARS CoV by infected blood products.

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