Coronaviruses with Special Emphasis on First Insights Concerning SARS
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Performance evaluation of a Bayer Healthcare Diagnostics research-based SARS coronavirus assay

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Introduction

Severe acute respiratory syndrome (SARS) is an emerging infectious disease caused by a highly contagious coronavirus (SARS-CoV) that is transmitted through direct or indirect contact with secretions from mucous membranes [1, 2]. The disease first appeared in southern China in late 2002, and rapidly spread to 31 countries within the first 6 months of 2003 [3–5]. At the end of this epidemic, the World Health Organization (WHO) estimated the number of probable SARS cases at 8,098 with 774 deaths [6]. Since the initial epidemic was declared over by the WHO in July 2003, there have been several new laboratory confirmed cases of SARS in late 2003 and 2004 resulting from accidental research laboratory exposure (in Singapore, Taiwan and China) and animal or environmental exposure (in China) [7–10].

A recent Centers for Disease Control and Prevention (CDC) guideline for clinical specimen collection, diagnostic testing and an interpretation algorithm for SARS-CoV infection has been developed [11]. According to these guidelines, respiratory tract, blood and stool are the preferred specimens for serological and molecular diagnostic testing through the clinical course of the disease. Several enzyme immunoassays and immunofluorescent assays for serological diagnosis and reverse-transcriptase PCR (RT-PCR) assays for detection of SARS-CoV RNA in clinical specimens have been described [12–15].

Bayer Healthcare Diagnostics developed a research based RT-PCR assay for detection and quantification of SARS-CoV in clinical specimens during the 2002–2003 outbreak of SARS. (This assay is for research use only, not for use in diagnostic procedures.) This article describes this assay, together with the assay's validation and performance and compares it with the

RealArt™ HPA-Coronavirus LC RT PCR Kit (Artus GmbH, Hamburg, Germany) for detection of SARS-CoV RNA in clinical specimens.

SARS coronavirus detection assays

In an astounding research accomplishment, the full-length genome sequence of the SARS-CoV was available within weeks after the identification of the pathogen and the initial global WHO alert [16, 17]. The availability of the nucleotide sequence allowed for the development of specific molecular diagnostic assays to detect SARS-CoV RNA in clinical specimens.

Bayer Healthcare Diagnostics developed a one-step, real-time quantitative RT-PCR assay for SARS-CoV RNA quantification. Full genomic sequences of SARS-CoV were used to design homologous forward and reverse primers and fluorescent labeled TaqMan probe targeting a 67-base pair nucleocapsid genomic region. Primer and probe design included a BLAST search with other human coronaviruses and human genomic DNA to exclude sequence cross reactivity [18]. A second heterologous amplification system including forward and reverse primers, fluorescent labeled TaqMan probe and target was included as an internal control for the assay process including sample preparation and PCR amplification.

One-step amplification reactions were performed using the Qiagen® OneStep RT-PCR kit (Qiagen, Valencia, CA, USA) in a reaction volume of 25 μl containing 5 μl of target (extracted patient tissue RNA and heterologous internal control RNA). Reactions were first incubated at 55ºC for 30 min to complete the reverse transcriptase step followed by incubation at 95ºC for 15 min to inactivate the RT enzyme. Reactions were then thermocycled with the following parameters: denaturation at 95ºC for 30 sec followed by 40 cycles of 60ºC for 60 sec and 72ºC for 30 sec. The Stratagene MX3000P™ Real-Time PCR System (Stratagene, La Jolla, CA, USA) was used to analyze the emitted fluorescence during amplification. Positive and negative controls, containing standardized SARS viral culture RNA extract from cell culture supernatants of VeroE6 cells (National Center for Infectious Diseases, CDC, Atlanta, GA, USA) and nuclease-free RNA diluent, respectively, were included in each run. External standards for SARS-CoV RNA quantification were prepared with serial dilutions of quantified SARS-CoV RNA culture extract, with concentrations ranging from 10 to 10^6 copies/5 μl reaction. The quantification standards were value assigned in comparison with three lots of purified RNA transcripts generated from the nucleocapsid region cloned into a plasmid (pCR-N9). The plasmid was provided through a material transfer agreement with the CDC. The RNA transcripts were prepared from the T7 promoter using the MEGAscript™ In Vitro Transcription Kit (Ambion, Austin, TX, USA). The concentration of the transcripts was determined by measuring the absorbance at 260 and

280 nm. These quantified viral RNA standards were treated as purified samples, tested in 5 μl volumes in an amplification reaction and used to calculate SARS-CoV viral load in clinical specimens.

The RealArt™ HPA-Coronavirus LightCycler® RT Reagents Assay (Artus GmbH, Hamburg, Germany) with a LightCycler® real-time PCR instrument (Roche Diagnostics, Laval, Canada) was performed according to the manufacturer's instructions. Viral load was calculated from a standard curve based on four external positive controls, ranging from 10 to 10^4 copies/μl, included in the assay kit. Assay performance was defined in the User Manual provided with the RealArt™ HPA-Coronavirus LC RT PCR Kit (Artus).

The performance of the Bayer Healthcare Diagnostics research-based SARS-associated coronavirus assay (Bayer Assay) and the Artus RealArt™ HPA-Coronavirus LC RT PCR assay (Artus Assay) were compared for detection of SARS-CoV RNA in clinical specimens. Identical parallel RNA extracts from clinical specimens were used in this performance evaluation.

Bayer Healthcare research-based SARS-associated coronavirus assay performance

Assay sensitivity is defined as the lowest concentration of SARS virus that can be detected 95% of the time, and is expressed in terms of the limit of detection (LoD). Assay sensitivity was determined by testing a serial dilution panel of SARS viral culture RNA extract, consisting of 9 levels ranging from 0.5 copies/5 μl to 20 copies/5 μl and analyzed with the Stratagene MX3000P™ Real-Time PCR System (Stratagene). Each level of the dilution panel was tested in a total of 27 replicates (9 replicates per run over 3 runs).

In a real-time PCR assay, the threshold cycle is defined as the cycle at which the system begins to detect the increase in signal associated with an exponential growth of PCR product during the log-linear phase. The percent detected, that is the percentage of results with the threshold cycle (CT) value less than 40, at each level of the dilution panel was calculated. The percent detected was then fitted against the log concentration using a logistic regression model. The LoD was estimated by the concentration where the model predicted percent detected was 95%.

Table 1 shows the observed and predicted percent detected for each dilution level of the SARS viral culture RNA extract panel. The LoD was estimated by the concentration where the predicted % detected was 95% (Fig. 1). Based on this panel, the estimated LoD was 9.30 copies/5 μl (0.974 log copies/5 μl).

In a real-time quantitative RT-PCR assay, the mean CT value is inversely proportional to the log input concentration. In practice, one way to assess

Table 1: Observed and predicted % detected for the viral RNA culture extract panel

Concentration (copies/5 µl)	Log concentration	N	Observed % detected	Predicted % detected (from logistic regression model)
0.5	-0.301	27	7.4%	6.2%
1	0.000	27	22.2%	20.2%
2	0.301	27	44.4%	49.2%
3	0.477	27	66.7%	68.0%
4	0.602	27	81.5%	78.8%
5	0.699	27	85.2%	85.1%
7.5	0.875	27	92.6%	92.6%
10	1.000	27	92.6%	95.7%
15	1.176	27	100.0%	98.0%
20	1.301	27	100.0%	98.8%

the linearity of such an assay is to plot the mean CT value against the log input concentration and fit a simple linear regression. Assay linearity is established if the regression plot has a slope close to the theoretical value of –3.32.

Assay precision (reproducibility) is a measure of assay variability in repeated testing of replicates of an identical input under varying test conditions, such as within-run and between-run replications. Assay precision is commonly expressed in terms of the standard deviation (SD) or coefficient of variation (% CV) associated with the test condition.

To assess the linearity and precision of the Bayer Assay, a dilution panel, made of SARS viral culture RNA, was constructed and tested across multiple runs, by two operators on three instruments. The virus panel consisted of 6 levels, ranging from 10 to 10^6 copies. The dilution levels of 10 to 10^4 copies were each tested for a total of 32 replicates (8 replicates per run over 4 runs); the dilution levels of 10^5 and 10^6 copies were each tested for a total of 20 replicates (5 replicates per run over 4 runs). The heterologous amplification system that serves as an internal control was added to each well of all dilution levels. Signal generated from the internal control increased confidence in the real-time PCR result and helped to eliminate the occurrence of false negatives.

The following analyses were performed on the data from the dilution panel:

- The percent detected (percentage of results with CT value less than 40) was calculated for each level of the dilution.
- The mean CT was calculated for each dilution level, based on all CT values that were less than 40. The mean CT was plotted against the $\log_{10}$ nominal concentration to assess linearity of the assay response. A linear regression was fitted on the plot and the regression equation and R^2 were also calculated.

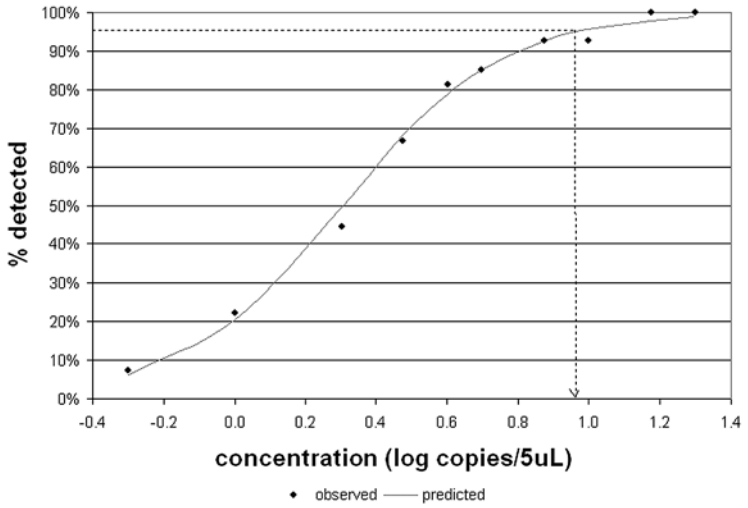

Figure 1. Assay sensitivity: percent detected (percentage of results with the threshold cycle (CT) value less than 40) fitted against the log concentration using a logistic regression model.

- A linear mixed model was used to estimate the component of variations of the CT values due to between-run and within-run variability, based on CT values that were less than 40. The assay precision was expressed in terms of SD due to between-run, within-run, and total components. The corresponding % CV was also calculated. The total SD of the CT values for the dilution panel was plotted against the nominal concentration.
- The CT values of the internal control (IC) were analyzed to assess the detection of the IC and the stability of the IC signal relative to the concentration of the panel. Additionally, the variability of the CT values of the IC, expressed in terms of the total SD was also evaluated.

Table 2 shows the percent detected, mean CT, SDs and % CV of CT, for the viral culture RNA extract panel.

Figure 2 shows the plot of the mean CT value against the $\log_{10}$ nominal concentration for the viral culture RNA extract dilution panel. The regression plot has a slope of –3.535. Furthermore, the R^2 value shown on the plot indicates that the linear regression fit the data very well, thus showing the highly linear response of the assay.

Figure 3 shows the precision profile of the assay. The total SD of the CT values for the viral culture RNA extract dilution panel was plotted against $\log_{10}$ nominal concentration. The plot shows that the precision was fairly constant from 10^2 copies to the upper limit of the dynamic range (10^6 copies). The highest SD was observed at 10 copies, with a total SD of 1.21, however, this corresponds to only about 3.3% CV.

Table 2. Linearity and precision of the viral culture RNA extract panel

Nominal concentration (copies/5 μl)	N	% detected	Mean CT	Between-run SD	Within-run SD	Total SD	Between-run %CV	Within-run %CV	Total %CV
10	32	96.9%	36.23	0.75	0.95	1.21	2.1%	2.6%	3.3%
10^2	32	100%	32.50	0.44	0.34	0.55	1.4%	1.0%	1.7%
10^3	32	100%	29.05	0.43	0.36	0.56	1.5%	1.2%	1.9%
10^4	32	100%	25.46	0.44	0.24	0.50	1.7%	0.9%	2.0%
10^5	20	100%	21.97	0.48	0.14	0.50	2.2%	0.6%	2.3%
10^6	20	100%	18.52	0.47	0.33	0.57	2.5%	1.8%	3.1%

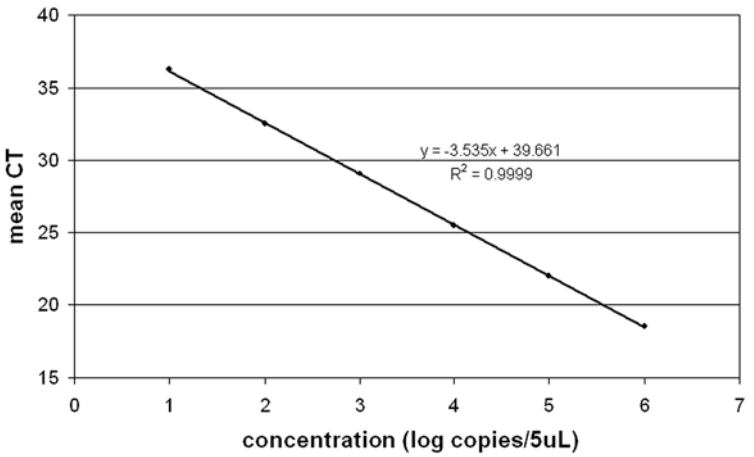

Figure 2. Assay viral culture RNA extract linearity panel: mean CT *versus* concentration. The assay response is highly linear ($R^2 = 0.9999$).

Table 3 shows the summary statistics of the internal control CT value. For the viral culture panel runs, the internal control was detected in all wells up to a target concentration of 10^6 copies. Furthermore, the variability of the CT values, expressed in terms of the total SD, was very small and stable across the dynamic range of the assay. Thus the internal control provides a reliable signal for the assay process as well as for detecting amplification inhibition.

Figure 4 shows the plot of the mean CT of the IC against the nominal concentration of the viral RNA extract panel.

Analytical specificity was assessed by testing nucleic acid extracts from a control panel of common viral and bacterial respiratory pathogens acquired from the American Type Culture Collection (ATCC, Manassas, Va, USA.). Clinical specificity was assessed by testing nucleic acid extracts

Table 3. Summary statistics of the CT values of the internal control (IC)

Panel	Panel nominal concentration (copies/5 μl)	Total N of IC	N detected	% detected	IC CT mean	Total SD
virus	10	32	32	100%	35.3	0.33
virus	10^2	32	32	100%	35.1	0.29
virus	10^3	32	32	100%	34.9	0.34
virus	10^4	32	32	100%	34.8	0.36
virus	10^5	20	20	100%	35.2	0.49
virus	10^6	20	20	100%	35.3	0.43

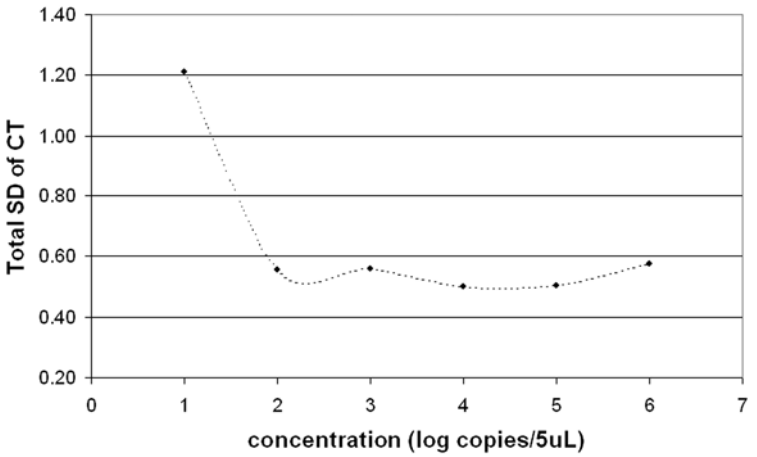

Figure 3. Assay precision profile: total standard deviation (SD) *versus* concentration. The precision was fairly constant form 10^2 copies to the upper limit of the dynamic range (10^6 copies).

from lung tissue, nasopharyngeal swabs (NPS) and whole blood. Lung was derived from patients who died during the SARS outbreak in Toronto, but who did not have SARS as defined by the CDC and WHO definitions. Permission to use the lung tissues was obtained by the Chief Coroner's Office of Ontario. NP swabs were from non-SARS patients with respiratory symptoms who did not meet the criteria for SARS. Permission to use these samples was from the IRBs at Mount Sinai Hospital and University Health Network. Whole blood samples were purchased from the Sacramento Blood Bank (Sacramento, CA, USA).

Table 4 shows the results of the specificity study. The Bayer Assay showed high specificity, in that there was no amplification signal from any nucleic acid target in the control panel or the SARS negative clinical specimens.

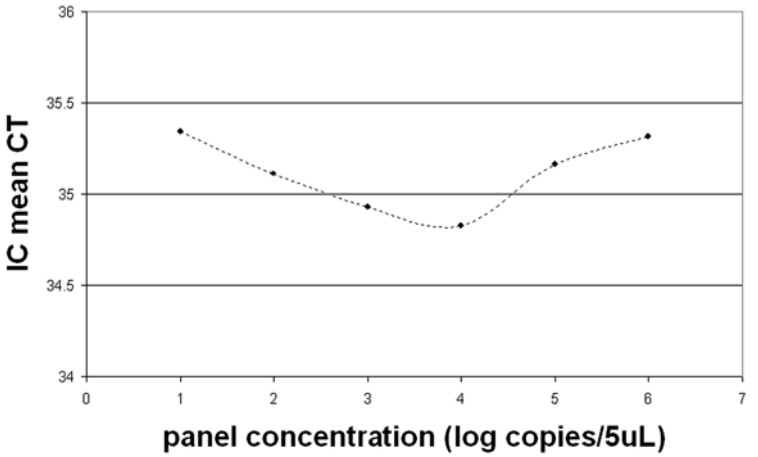

Figure 4. Mean internal control: CT *versus* concentration of the viral culture RNA extract panel. The internal control provides a reliable signal for the assay process as well as for detecting amplification inhibition.

Table 4. Summary of Bayer assay specificity

Pathogens	ATCC number	Total number of samples	SARS-CoV primer/probe set	Internal control primer/probe set
SARS coronavirus	CDC passage # 3	1	Positive	Positive
Influenza A	ATCC VR-1520	1	Negative	Positive
Influenza B	ATCC VR-101	1	Negative	Positive
Parainfluenza 1	ATCC VR-94	1	Negative	Positive
Parainfluenza 3	ATCC VR-93	1	Negative	Positive
Respiratory synocytial virus B	ATCC VR-1401	1	Negative	Positive
Adenovirus type 4	ATCC VR-1081	1	Negative	Positive
Adenovirus type 21	ATCC VR-1098	1	Negative	Positive
Mycoplasma pneumoniae	ATCC 15293	1	Negative	Positive
Human coronavirus 229E	ATCC VR740	1	Negative	Positive
SARS-CoV negative clinical samples		Total number of samples	SARS-CoV primer/probe set	Internal control primer/probe set
Lung tissue extracts		10	Negative	Positive
NPS extracts		10	Negative	Positive
Human genomic DNA extracts		5	Negative	Positive

Clinical performance evaluation of the Bayer Healthcare assay

Clinical specimens were collected from patients who met the CDC and WHO case definitions for probable SARS in Toronto, Canada, between March and June 2003. Permission to use the clinical samples for research purposes was obtained from the Research Ethics Boards at the Mount Sinai Hospital and University Health Network, Toronto and for the tissue samples from the Chief Coroner's Office of Ontario, Canada.

All samples were stored at –70°C until tested. Stool and bronchoalveolar lavage (BAL) samples were collected in a clean, sterile container. NPS specimens were collected using a flexible dacron-tipped swab and placed into viral transport media (Starplex Scientific Inc., Etobicoke, Canada). Tissue samples collected during autopsies were placed into sterile containers and snap frozen immediately in a solution of absolute ethanol and dry ice.

Prior to RNA extraction, mucoid BAL specimens were mixed with an equal volume of physiological salt (0.9% NaCl) containing 1% N-acetylcysteine (Sigma-Aldrich Ltd.,Ontario, Canada) and incubated for 30 min. For non-mucoid BAL specimens and NP specimens and treated BAL specimens, RNA was extracted from 140 μl of specimen using the QIAamp Viral RNA Mini Kit (Qiagen, Mississauga, Canada).

One gram of each stool sample was mixed in 5 ml of distilled RNase-free water and vortexed. It was then allowed to settle at room temperature for 5 min before vortexing again. A volume of 140 μl of stool specimen was then mixed in 560 μl RNeasy Lysis Buffer (RLT buffer, Qiagen) and pipetted directly onto a QIAshredder spin column (Qiagen) placed in a 2 mL collection tube followed by centrifugation for 2 min. The supernatant was then transferred to a new microfuge tube and RNA was extracted using the RNeasy Mini Kit (Qiagen).

Thirty milligrams of frozen tissue derived from lung, colon, kidney, skeletal muscle and liver was homogenized in 600 μl of RLT buffer using disposable tissue grinders (Kendall Precision TM, Mansfield, MA, USA). The homogenate was then passed through QIAshredder spin columns as was done for stool specimens. RNA was extracted using the RNeasy Mini Kit (Qiagen). Extracted RNA was then frozen at –70°C prior to testing.

Postmortem lung, liver, colon, kidney, skeletal muscle and liver specimens as well as stool and NPS specimens were tested by the Bayer and/or the Artus assays to assess viral load. Table 5 shows the results comparing the two assays performed on the same RNA extract from the same tissue and Table 6 shows the results of the two assays performed on different extracts from the same tissue. The assays showed perfect qualitative agreement, with 18 specimens positive for SARS-CoV RNA by both assays and 9 specimens negative by both assays. Both assays were able to detect as few as 4 copies of SARS-CoV RNA in a microliter of clinical sample extract.

Table 5. Comparison of Bayer and Artus assays tested on clinical specimens[a]

Sample number	Sample description	Bayer assay[b] Qualitative results	Bayer assay[b] Copies of CoV per ml extract	Artus assay[c] Qualitative results	Artus assay[c] Copies of CoV per ml extract
1	Lung RL	Positive	57,525,000	Positive	25,010,000
2	Lung LUL	Positive	402	Positive	384
3	Lung LL	Negative	0	Negative	0
4	Lung RL	Positive	553,600	Positive	142,700
5	Lung LLL	Positive	1,229	Positive	838
6	Lung RUL	Positive	171	Positive	153
7	Lung LL	Positive	4	Positive	3
8	Lung LUL	Positive	7	Positive	4
9	Lung 9	Positive	21,393	Positive	4,949
10	Lung LLL	Positive	20	Positive	14
11	Lung LL	Positive	17,588,000	Positive	3,269,000
12	Lung RL	Positive	7	Positive	5
13	Small bowel	Negative	0	Negative	0
14	NPS (R)	Negative	0	Negative	0
15	NPS (S)	Negative	0	Negative	0
16	NPS (T)	Positive	7	Positive	53
17	BAL (U)	Positive	6,632	Positive	2,891
18	BAL (V)	Positive	270,400	Positive	174,900
19	BAL (W)	Negative	0	Negative	0
20	BAL (X)	Negative	0	Negative	0
21	BAL #6 right	Positive	4	Positive	27
22	Stool	Negative	0	Negative	0

[a]Quantitative results in both asseys are derived from same extract.
[b]Bayer research-based SARS-associated CoV RT-PCR assay
[c]Artus RealArt™ HPA coronavirus RT-PCR kit
RL, right lobe; RUL, right upper lobe; LUL, left upper lobe; LL, left lobe; LLL, Left lower lobe; BAL, bronchoalveolar lavage; NPS, nasopharyngeal swab

Table 6. Comparison of the Bayer and Artus assays on additional clinical tissues[a]

Sample number	Sample description	Bayer[b] Qualitative results	Bayer[b] Copies of CoV per ml extract	Artus[c] Qualitative results	Artus[c] Copies of CoV per ml extract
1	large bowel	Positive	108	Positive	365
2	kidney	Positive	496	Positive	367
3	skeletal muscle	Negative	0	Negative	0
4	small bowel	Positive	10,804,000	Positive	1,613,000
5	liver	Negative	0	Negative	0

[a]Quantitative results in both assays are derived from different extract from same patient tissue section.
[b]Bayer research-based SARS-associated CoV RT-PCR assay
[c]Artus RealArt™ HPA coronavirus RT-PCR kit

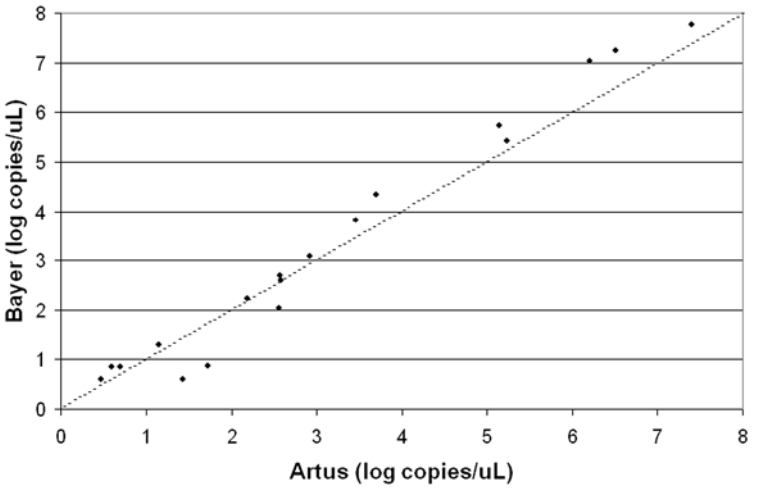

Figure 5. Comparison between Bayer and Artus assay quantification. The correlation coefficient for SARS-CoV RNA concentrations between the assays was 0.98. The Bayer results frequently quantified higher than the Artus results (about 1.4 folds).

The quantitative results of the Bayer and Artus assays given in Tables 5 and 6 were plotted in Figure 5. The correlation coefficient for SARS-CoV RNA concentrations between the Bayer and Artus assays was 0.98. As shown in the plot, the Bayer results frequently quantified higher than the Artus results. On the average, the Bayer results were 0.14 logs (about 1.4 fold) higher than the Artus assay. The higher viral loads detected by the Bayer Assay may be due to the fact that this assay targets the nucleocapsid region of the SARS RNA genome, while the Artus Assay targets the polymerase region. Other investigators have noted that PCR assays targeting the nucleocapsid region achieve higher quantitative values than assays targeting the polymerase region of the SARS-CoV genome [13, 19, 20]. It is speculated that in some tissues, subgenomic fragments that contain the nucleocapsid gene may be present and contribute to the overall signal. Further studies are needed to investigate the mechanism for the higher quantification observed between these two assays.

Discussion

Shortly after the identification of a novel coronavirus (SARS-CoV) as the etiologic agent of SARS, a number of home-brew and commercial assays were developed to aid in the laboratory diagnosis. The most widely used

assays continue to be either molecular amplification assays (e.g. RT-PCR) for the detection of SARS-CoV RNA in clinical specimens or serologic assays for the detection of specific SARS-CoV antibodies in serum. However, due to the urgent need for laboratory tests during the SARS outbreak, many assays were put into clinical use worldwide without undergoing a complete assessment of their performance characteristics. This is reflected in the CDC guidelines for laboratory confirmation of SARS infection that cautions that all assays remain investigational.

The results of this study show that the newly developed RT-PCR assay by Bayer Healthcare Diagnostics performs extremely well for the detection of SARS-CoV RNA in clinical specimens. It is very sensitive, detecting as few as 10 copies of SARS-CoV RNA and has a wide dynamic range (10 to 10^6 copies). Both the analytical and clinical specificities were 100%. There was no cross reactivity with other more common respiratory viruses and testing of clinical samples from non-SARS patients were negative. It should be noted, however, that according to the CDC guidelines, one cannot rule out a diagnosis of SARS based on a negative molecular assay. Early in the course of infection, viral shedding may be too low for detection by many assays. As well, there may be differences in the performance of many assays depending upon the specimen type.

One of the first commercially available PCR assays during the outbreak was the RealArt HPA-Coronavirus LC RT-PCR assay (Artus). Both the Artus and the Bayer assays are based on real-time PCR platforms and thus provide relatively rapid results. By standardizing the specimen processing and RNA extraction methods before performing either the Artus or Bayer assays, we were able to show that both assays were highly correlated. The qualitative results (positive or negative) of the Artus and Bayer assays were identical. The quantitative results of the Bayer assay on the average were 0.14 logs (about 1.4 fold) higher than the Artus assay. The difference in viral load quantification may be due to assay design. The Bayer assay targets the nucleocapsid region while the Artus assay targets the polymerase region. Additionally, although both assays have the same input volume of 5 μl of target into the amplification reaction, the copy number of the Bayer standards ranges between 10 to 10^6 copies/5 μl while the Artus assay standards range between 50 to 5×10^4 copies/5 μl. The excellent correlation between these assays and the fact that the assays target different genomic regions suggests that each can be used to confirm a positive result obtained with the other, fulfilling the CDC requirement for a laboratory confirmed case of SARS.

At the time of writing, there are no known human cases of SARS anywhere in the world. Despite this, the potential for new cases to re-appear remains. The availability of properly validated, sensitive and specific assays is essential if new cases are to be accurately diagnosed particularly in light of the fact that the clinical definition of suspect and probable SARS remain very broad and somewhat non-specific.

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