Viral RNA load quantification as helpful tool to arbitrate SARS–CoV-2 detection results in respiratory samples

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Abstract

Purpose: The increasing COVID-19 widespread has created the necessity to assess the diagnostic accuracy of newly introduced (RT-PCR based) assays for SARS–CoV-2 RNA detection in respiratory tract samples.

Methods: We compared the results of the Allplex™ 2019-nCoV assay with those of the Simplexa™ COVID-19 Direct assay, both performed on 125 nasal/oropharyngeal swab samples of patients with COVID-19 suspicion.

Results: Fifty-four samples tested positive (C_T below 40) and 71 negative (C_T above 40) with the Allplex™ 2019-nCoV assay, whereas 47 of 54 samples were also positive with the Simplexa™ COVID-19 Direct assay. Eight results were discordant, resulting in 93.6% agreement between the assays. We used the Quanty COVID-19 assay—developed to detect and quantify SARS–CoV-2 in respiratory tract samples—to arbitrate these results. One Allplex™ 2019-nCoV negative (but Simplexa™ COVID-19 positive) and seven Simplexa™ COVID-19 negative samples were truly false negative. Interestingly, a Spearman's negative association was found between the viral RNA loads quantified by the Quanty COVID-19 assay and the C_T values of RT PCRs performed with either the Allplex™ 2019-nCoV assay or the Simplexa™ COVID-19 Direct assay. However, the strength of this association was higher for the Allplex™ 2019-nCoV assay (N gene, $\rho = -0.92$; RdRP gene, $\rho = -0.91$) than for the Simplexa™ COVID-19 Direct assay (ORF1ab gene, $\rho = -0.65$; S gene, $\rho = -0.80$).

Conclusion: The Allplex™ 2019-nCoV and Simplexa™ COVID-19 Direct assays yielded comparable results. However, the role these assays might play in future clinical practice warrants larger comparison studies.

Introduction

Since first isolation on December 2019 [1], the severe acute respiratory syndrome coronavirus 2 (SARS–CoV-2)—initially called 2019-nCoV—which causes the illness referred to as coronavirus disease 2019 (COVID-19) has increasingly spread worldwide. By 29 April 2020, the number of confirmed cases reported by the World Health Organization (WHO) had reached 3,023,788 (<https://covid19.who.int/>), hence representing an unprecedented viral pandemic. To prevent virus transmission and/or ensure appropriate management of COVID-19 patients [2], clinical microbiology laboratories are constantly requested to implement relatively quick and sensitive diagnostic assays for SARS–CoV-2 RNA detection in clinical samples [3].

Nowadays, real-time reverse-transcription–polymerase chain reaction (RT-PCR)-based assays performed on upper respiratory tract (URT) samples (e.g., nasopharyngeal and/or oropharyngeal swabs) is the current diagnostic strategy to confirm COVID-19 cases [4], regardless of clinical disease manifestation [5]. In general, diagnosis relies upon the *in vitro* amplification of one or more molecular targets within the positive-sense, single-stranded SARS–CoV-2 RNA, including the envelope (E), RNA-dependent RNA polymerase (RdRP), and nucleocapsid (N) genes among others [6,7]. In particular, the assay developed by the Centers for Disease Control and Prevention (CDC)—the most widely used in the United States—utilizes two N gene regions (N1 and N2) as targets [4].

As soon as the WHO published protocols for RT-PCR assays [8], Seegene launched the Allplex™ 2019-nCoV assay—approved for emergency use authorization (EUA) from U.S. Food and Drug Administration (FDA) on 21 April 2020. This single-tube assay identifies E, RdRP, and N genes, as established by the WHO

(<https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance>). Later, DiaSorin Molecular developed the Simplexa™ COVID-19 Direct assay, for which the FDA granted a EUA on 19 March 2020 [4]. The assay targets two regions within the SARS–CoV-2 genome, one encoding the spike (S) protein (i.e., the S gene) and the other well-conserved non-structural proteins (i.e., the open reading frames ORF1a and ORF1b) of SARS–CoV-2. Remarkably, both assays received CE (Conformité Européenne) marking. Compared to the Allplex™ 2019-nCoV assay, the Simplexa™ COVID-19 Direct assay has the advantage of quicker turnaround test results (75 minutes vs 4 hours, respectively). However, the true sensitivity of currently available assays is unknown [9]. In particular, few studies so far have compared the results obtained with different commercial assays in routine laboratory practice [10–12].

The aim of this study was to perform a comparative evaluation of the Allplex™ 2019-nCoV (Arrow Diagnostics S.r.l., Genova, Italy) and Simplexa™ COVID-19 Direct (DiaSorin Molecular, Saluggia, Vercelli, Italy) assays on nasal/oropharyngeal swab (NOS) samples of patients screened for SARS–CoV-2 infection. To resolve potential discrepancies, the results obtained with both assays were arbitrated using the CE-marked Quanty COVID-19 assay (Clonit S.r.l, Milan, Italy) that was performed according to CDC guidelines (<https://www.cdc.gov/>) to detect and, importantly, quantify SARS–CoV-2 RNA in clinical samples using three N gene regions (N1, N2, and N3) as targets.

Materials And Methods

Study design and samples

This retrospective study was performed on NOS samples collected from patients admitted to the Fondazione Policlinico Universitario A. Gemelli (FPG) IRCCS hospital's emergency department with COVID-19 suspicion during a two-week period in May 2020. NOS samples were collected together within a single tube of universal transport medium (UTMâ; Copan Italia S.p.A., Brescia, Italy) to prevent viral RNA degradation and/or bacterial/fungal overgrowth. We considered all samples tested for SARS–CoV-2 RNA by the Allplex™ 2019-nCoV assay (see below) eligible for inclusion. Among SARS–CoV-2 positive samples, we randomly selected samples that were representative of differing target(s) positive levels, as assessed by their cycle threshold (C_T) values (see below). We also selected negative samples to reach a number of 125 samples in total. Aliquots of primary samples were immediately frozen and kept at -70°C until further analysis. Before testing, aliquots were thawed at room temperature and briefly vortexed.

SARS-CoV-2 molecular detection

Testing of NOS sample aliquots using SARS–CoV-2 molecular assays were performed in accordance with the manufacturer's instructions.

Allplex™ 2019-nCoV assay

Briefly, 200 μl of sample was processed with a Seegene Nimbus automated system (Arrow Diagnostics), which performs both RNA extraction—using STARMag Universal Cartridge kit—and PCR assay setup. A reaction microplate with therein-extracted RNA was loaded onto a real-time PCR CFX96 Touch™ system (Bio-Rad

Laboratories, Hercules, CA, USA). Positive and negative controls were included in each run. After assay's completion, the Seegene Viewer 2019-nCoV software allowed automated analysis and interpretation of results. A positive result (i.e., a C_T less than 40) for at least one of two viral targets (i.e., RdRP and N genes) or for the E gene alone indicates, respectively, the certain or presumptive presence of SARS-CoV-2 RNA in the patient sample. An invalid result (e.g., due to internal control failure) indicates inconclusive determination of the SARS-CoV-2 RNA presence or absence in the patient sample, thus requiring sample retesting.

Simplexa™ COVID-19 Direct assay

Briefly, 50 μ l of sample and 50 μ l of reaction mixture were separately loaded into Direct real-time PCR amplification-disc wells and onto a LIAISON® MDX instrument (DiaSorin Molecular) and allowed to react for a 75 min run. Positive and negative controls were included in each run. After assay's completion, the instrument's Studio software automatically calculated and displayed results. A positive result (i.e., a C_T less than 40) for at least one of two viral targets (i.e., S and ORF1ab genes) indicates the presence of SARS-CoV-2 RNA in the patient sample. As with the Allplex™ 2019-nCoV assay, an invalid result requires sample retesting.

Quanty COVID-19 assay

Briefly, separate wells on each real-time PCR microplate was filled with 5- μ l derivative sample (i.e., derived from the Nimbus RNA extraction step), positive control, negative control, and standards in. For SARS-CoV-2 RNA qualitative detection, the instrument's software automatically analyzed and interpreted the results. A positive result (i.e., a C_T less than 40) for all three viral targets (N1, N2, and N3 genes) indicates the presence of SARS-CoV-2 RNA in the patient sample. Otherwise, the software defines the result as inconclusive, requiring sample retesting. For SARS-CoV-2 RNA quantitative detection, the software built a standard curve with the C_T values obtained following amplification of the aforementioned standards (which contain 10^1 , 10^2 , 10^3 , 10^4 , and 10^5 copies/ μ l of synthetic viral N1-encoding RNA, respectively). This allowed calculating the viral load in the patient sample by interpolation of the corresponding C_T value with the standard curve. Then, the actual viral load of the sample (expressed in copies/ml) was determined multiplying the calculated number of viral copies by $1000/V_e$ and E_v/E_a ratios, where V_e is the extracted sample volume (200 μ l), E_v is the eluted sample volume during the extraction step (100 μ l), and E_a is the extracted sample volume used for amplification (5 μ l). To validate the manufacturer's standards, we generated a standard curve using the Quantitative Synthetic SARS-CoV2 RNA: ORF, E, N (ATCC® VR3276SD™), which was diluted at the same concentrations as the standards used in the Quanty COVID-19 assay. In preliminary experiments, each of the ATCC® VR3276SD™ RNA samples was quantified in triplicate with the Quanty COVID-19 assay, and results were in the expected C_T values ranges (data not shown).

Data analysis

No sample retesting was performed due to the absence of invalid results; consequently, we analyzed the first testing results for all study samples. We calculated sensitivity, specificity, and positive and negative predictive values, together with their respective confidence intervals (CIs), for the Allplex™ 2019-nCoV assay and Simplexa™ COVID-19 Direct assay using the Quanty COVID-19 assay as an arbitrator. Analysis was performed with Stata software version 11.1 (StataCorp, College Station, TX, USA). Differences between the C_T values in

sample groups were assessed using the Student's *t*-test. Two-sided *P* values of <0.05 were considered statistically significant. We used Cohen's kappa to assess the strength of agreement between the assays [13]. Values greater than zero indicated none to slight (0.01–0.20), fair (0.21–0.40), moderate (0.41–0.60), substantial (0.61–0.80), or almost perfect (0.81–1.00) levels of agreement, and values lower than/equal to zero indicated the absence of agreement. To assess the relationship between the viral load levels determined by the Quanty COVID-19 assay and the *C_T* values determined by the Allplex™ 2019-nCoV or Simplexa™ COVID-19 Direct assays, we performed a Spearman correlation on all samples where the concentration of the SARS-CoV-2 N1 gene was within a range of 10¹ to 10⁷ copies per ml.

Results

To evaluate the performance of the Simplexa™ COVID-19 Direct assay—a *quasi*-point-of-care SARS-CoV-2 detection assay—we compared the results from 125 cases that first tested either positive (*n* = 54) or negative (*n* = 71) with the Allplex™ 2019-nCoV assay—the first implemented SARS-CoV-2 detection assay in our laboratory. As shown in Table 1 (for details, see Table S1 in the supplemental material), *C_T* values of Allplex™ 2019-nCoV positive samples ranged from 17.9 to 39.3 for E, RdRP, and N genes (33 samples), 28.4 to 39.3 for RdRP and N genes (9 samples), 33.7 to 39.4 for the N gene (11 samples), and 35.6 to 37.1 for E and N genes (1 sample). In particular, the mean ($\pm$ SD) *C_T* value for the E gene (26.4 $\pm$ 3.9) was lower than the values for RdRP (28.0 $\pm$ 3.6; *P* = 0.09) or N (28.9 $\pm$ 4.4; *P* = 0.02) genes in 33 samples and the value for the N gene (37.1) in 1 sample. Forty-seven of 54 positive samples by the Allplex™ 2019-nCoV assay had also positive results with the Simplexa™ COVID-19 Direct assay. The *C_T* values of positive Simplexa™ COVID-19 samples ranged from 17.5 to 39.7 for S and ORF1ab genes (40 samples), 21.0 to 35.6 for the ORF1ab gene (4 samples), and 29.3 to 34.9 for the S gene (4 samples). In particular, the mean ($\pm$ SD) *C_T* value for the S gene (27.9 $\pm$ 5.1) was lower than the value for the ORF1ab gene (27.9 $\pm$ 3.9; *P* = 0.99) in 40 samples.

The agreement between the Allplex™ 2019-nCoV assay and the Simplexa™ COVID-19 Direct assay results was 93.6% (Table 1). Of eight samples with discordant results, seven samples tested positive with the Allplex™ 2019-nCoV assay (the N gene was detected alone or in combination with E and/or RdRP genes) but negative with the Simplexa™ COVID-19 Direct assay. The remaining one sample tested negative with the Allplex™ 2019-nCoV assay but positive with the Simplexa™ COVID-19 Direct assay (both S and ORF1ab genes were detected). As detailed in Table S1, the mean ($\pm$ SD) *C_T* value of the N gene in the seven samples with discordant results was 34.7 $\pm$ 5.9, and this value differed from that of the 47 remaining Allplex™ 2019-nCoV positive samples (31.2 $\pm$ 5.0; *P* = 0.09).

Table 1

Overall results of 125 NOS samples tested by two molecular SARS-CoV-2 detection assays.^a

	Value for the following assays expressed as number (C_T range):		Between-assay value
	Allplex™ 2019-nCoV	Simplexa™ COVID-19 Direct	
Positive results			
All	54 (17.9–39.4)	48 (17.5–39.7)	
By target(s)			
E, RdRP, and N genes	33 (17.9–39.3)		
E and N genes	1 (35.6–37.1)		
RdRP and N genes	9 (28.4–39.3)		
N gene	11 (33.7–39.4)		
S and ORF1ab genes		40 (17.5–39.7)	
S gene		4 (21.0–35.6)	
ORF1ab gene		4 (29.3–34.9)	
Negative results	71 (0.0–0.0)	77 (0.0–0.0)	
No. of concordant results			117
No. of discordant results			8
% agreement			93.6
Cohen's kappa (95% CI)			0.86 (0.78–0.96)

^aNOS, nasal/oropharyngeal swab; C_T , threshold cycle; E, envelope; RdRP, RNA-dependent RNA polymerase; N, nucleocapsid protein; S, spike; ORF, open reading frame.

Analytic performance of molecular assays

We used the Quanty COVID-19 assay to arbitrate the results of the two molecular assays under comparison. Table S1 provides an overview of testing results. Fifty-five samples, including Allplex™ 2019-nCoV (n = 54) and Simplexa™ COVID-19 (n = 48) positive samples, tested positive and the remaining 70 samples tested negative for all the N gene regions targeted by Quanty COVID-19 assay. In summary, the Simplexa™ COVID-19 Direct assay failed to detect seven positive samples and the Allplex™ 2019-nCoV assay failed for one positive sample. Interestingly, this one sample tested positive also with the Simplexa™ COVID-19 Direct assay.

As shown in Tables 2 and 3, sensitivity and negative predictive value (NPV) of the Allplex™ 2019-nCoV assay were 98.2% and 97.2%, respectively, and those of the Simplexa™ COVID-19 Direct assay were 87.3% and 90.9%, respectively. When analyzing the results according to single assay's targets, we found lower sensitivities and NPVs for RdRP (76.4% and 84.3%, respectively) and E (61.8% and 76.9%, respectively) genes in one assay (Table 2) and for both S and ORF1ab (80.0% and 86.4%, respectively) genes in the other assay (Table 3).

Table 2

Performance of the Allplex™ 2019-nCoV assay according to the Quanty COVID-19 assay used as comparator assessed by get.^a

	Allplex 2019-nCoV results by target			
	E gene	RdRP gene	N gene	Total
no. matched positives	34	42	54	54
no. matched negatives	70	70	70	70
no. Allplex 2019-nCoV misses	21	13	1	1
agreement	83.2	89.6	99.2	99.2
sensitivity (95% CI)	61.8 (47.7–74.6)	76.4 (63.0–86.8)	98.2 (90.3–100.0)	98.2 (90.3–100.0)
specificity (95% CI)	100.0 (94.9–100.0)	100.0 (94.9–100.0)	100.0 (94.9–100.0)	100.0 (94.9–100.0)
PPV (95% CI)	100.0 (89.7–100.0)	100.0 (91.6–100.0)	100.0 (93.3–100.0)	100.0 (93.3–100.0)
NPV (95% CI)	76.9 (66.9–85.1)	84.3 (74.7–91.4)	97.2 (90.3–99.7)	97.2 (90.3–99.7)

envelope; RdRP, RNA-dependent RNA polymerase; N, nucleocapsid; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

Table 3
Performance of the Simplexa™ COVID-19 Direct assay according to the Quanty COVID-19 assay used as comparator assessed by target.^a

	Simplexa COVID-19 results by target		
	S gene	ORF1ab gene	Total
no. matched positives	44	44	48
no. matched negatives	70	70	70
no. Simplexa COVID-19 misses	11	11	7
agreement	91.2	91.2	94.4
sensitivity (95% CI)	80.0 (67.0–89.6)	80.0 (67.0–89.6)	87.3 (75.5–94.7)
specificity (95% CI)	100.0 (94.9–100.0)	100.0 (94.9–100.0)	100.0 (94.9–100.0)
PPV (95% CI)	100.0 (92.0–100.0)	100.0 (92.0–100.0)	100.0 (92.6–100.0)
NPV (95% CI)	86.4 (77.0–93.0)	86.4 (77.0–93.0)	90.9 (82.2–96.3)

spike; ORF, open reading frame; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

Relationship between samples' C_T values and viral loads

Table 4 shows the viral loads determined by the Quanty COVID-19 assay (expressed as $\log_{10}$ N1 copies per ml) in positive samples, which were stratified by the Allplex™ 2019-nCoV (E, RdRP, and N) or the Simplexa™ COVID-19 Direct (S and ORF1ab) assays' targets. We found highest proportions of E (29.4% and 26.5%, respectively), RdRP (23.8% and 26.2%, respectively), and N (22.2% and 20.4%, respectively) gene detections, as well as S (27.3% and 22.7%, respectively) and ORF1ab (25.0% and 22.7%, respectively) gene detections in samples with viral load levels ranging from >3.0 to ≤ 4.0 or >4.0 to ≤ 5.0 $\log_{10}$ copies per ml.

To determine if there was relationship between viral load and C_T value, we performed a Spearman's correlation analysis. Before that, samples with C_T values ≥ 40 by the Allplex™ 2019-nCoV assay or the Simplexa™ COVID-19 Direct assay were assigned a value of 40. Analyzing all 55 samples that tested positive or negative by the assays, we found a strong (negative) association between the C_T values of N (Spearman's $\rho = -0.92$; $P < 0.001$) and RdRP ($\rho = -0.91$; $P < 0.001$) genes—detected by the Allplex™ 2019-nCoV assay—and viral loads (Fig. 1). Conversely, we found a less strong (negative) association between the C_T values of ORF1ab ($\rho = -0.65$; $P < 0.001$) and S ($\rho = -0.80$; $P < 0.001$) genes—detected by the Simplexa™ COVID-19 Direct assay—and viral loads (Fig. 2).

Table 4
Detection results of Allplex™ 2019-nCoV and Simplexa™ COVID-19 Direct assays' targets according to viral load levels : positive NOS samples.^a

Viral load levels (log ₁₀ copies/ml)	No. (%) of detections by Allplex 2019-nCoV targets ^b			No. (%) of detections by Simplexa COVID-19 targets ^c	
	E gene	RdRP gene	N gene	S gene	ORF1ab gene
	n = 34	n = 42	n = 54	n = 44	n = 44
≤1.0	0 (0.0)	0 (0.0)	1 (1.9)	0 (0.0)	0 (0.0)
>1.0–≤2.0	3 (8.8)	5 (11.9)	12 (22.2)	7 (16.0)	10 (22.7)
>2.0–≤3.0	2 (5.9)	6 (14.3)	8 (14.8)	6 (13.6)	5 (11.4)
>3.0–≤4.0	10 (29.4)	10 (23.8)	12 (22.2)	12 (27.3)	11 (25.0)
>4.0–≤5.0	9 (26.5)	11 (26.2)	11 (20.4)	10 (22.7)	10 (22.7)
>5.0–≤6.0	7 (20.6)	7 (16.7)	7 (13.0)	6 (13.6)	5 (11.4)
>6.0–≤7.0	3 (8.8)	3 (7.1)	3 (5.5)	3 (6.8)	3 (6.8)

^aThe Quanty COVID-19 assay was used to determine the SARS-CoV-2 RNA level in nasal/oropharyngeal swab (NOS) samples.

^bThe Allplex 2019-nCoV targets the E (envelope), RdRP (RNA-dependent RNA polymerase), and N (nucleocapsid) genes of SARS-CoV-2.

^cThe Simplexa COVID-19 targets the S (spike) and ORF1ab (open reading frame 1ab) genes of SARS-CoV-2.

Discussion

The current speed with which the laboratory-based diagnostic landscape for COVID-19 is changing [3] creates an impelling necessity to assess rigorously the diagnostic accuracy of newly introduced SARS-CoV-2 assays. The DiaSorin Molecular Simplexa™ COVID-19 Direct assay is one of 28 commercially available assays that was EUA granted from the FDA as of 4 April 2020 [4]. One study compared the DiaSorin Molecular assay with the Abbott ID Now assay, using a modified CDC assay as the reference standard [10]. Another study compared the DiaSorin Molecular assay with a modified CDC Diagnostic Panel, the Diagnostics GenMark ePlex SARS-CoV-2 assay, and the Hologic Panther Fusion SARS-CoV-2 assay [11]. In the latter study [11], the authors used a “consensus result”, namely a result obtained by at least three out of four evaluated assays, to establish the reference standard. Both the studies tested URT samples (n = 96 [10] and n = 104 [11], respectively). Independently (albeit concurrently), we assessed the performance of the Simplexa™ COVID-19 Direct assay in comparison with that of the Allplex™ 2019-nCoV assay—one of the first commercialized assays since SARS-CoV-2 had been isolated for the first time [1]. Notably, we determined the agreement of the two assays after arbitrating the discordant results with the Quanty COVID-19 assay, the reference assay in our study. Additionally, we used the Quanty COVID-19 assay to quantitate the SARS-CoV-2 RNA (i.e., the N1 gene) in the 125 NOS samples (Table S1) under consideration.

Our findings show that the Allplex™ 2019-nCoV and the Simplexa™ COVID-19 Direct assays yielded comparable results (Cohen’s kappa value, 0.86). However, while there was 100% agreement between the assays for negative samples (specificity was 100% for both assays), discordant results were found in eight positive samples, i.e., one false negative by the Allplex™ 2019-nCoV assay and seven false negative by the Simplexa™ COVID-19 Direct assay (sensitivity was 98.2% and 87.3%, respectively). Consistently, agreement rates of the Allplex™ 2019-nCoV assay and the Simplexa™ COVID-19 Direct assay with the Quanty COVID-19 assay were 99.2% and 94.4%, respectively. The reasons for the discordant results are unknown. We noticed that the sample testing false negative with the Allplex™ 2019-nCoV assay was true positive with the Simplexa™ COVID-19 Direct assay, and had C_T values (34.5 [S gene] and 34.8 [ORF1ab gene]) comparable with those of the Quanty COVID-19 assay (38.3 [N2 gene] and 37.8 [N3 gene]). The viral load in this sample equated to 6.2 × 10² RNA copies/ml,

and we found a similar value in other five samples (range, 5.3×10^2 to 6.5×10^2 RNA copies/ml) included in this analysis. Except for one (Simplexa™ COVID-19 negative) sample, these samples tested positive with both the Allplex™ 2019–nCoV (two for N gene alone and two for both N and RdRP genes) and the Simplexa™ COVID-19 Direct (two for ORF1ab gene alone and two for both S and ORF1ab genes) assays. The viral loads of seven samples with a false-negative result by the Simplexa™ COVID-19 Direct assay ranged from 3.3×10^1 to 2.8×10^6 RNA copies/ml, and three of these samples were under the limit of detection estimated as 500 RNA copies/ml (<https://www.molecular.diasorin.com>) or reported as 16 to 62 RNA copies/ml [11] for the DiaSorin Molecular assay. Thus, the false-negativity observed, particularly with the Simplexa™ COVID-19 Direct assay, might not be due to a scarce copy number of SARS–CoV-2 RNA in those samples. Consequently, we could not rule out that intrinsic reasons (e.g., virus mutation) have affected the RT-PCR result in our samples. Unfortunately, we did not perform viral sequencing to clarify this issue [14].

To reduce the potential risks of cross-reactions with endemic (HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1) or other epidemic (SARS-CoV and MERS-CoV) coronaviruses and SARS–CoV-2 genome mutations, experts advise to include at least two molecular targets when developing a SARS–CoV-2 detection assay [4]. Ideally, these targets should include at least one conserved (e.g., the ORF1ab) and one specific (e.g., the S gene) region within the viral genome. The Simplexa™ COVID-19 Direct assay as well as the Allplex™ 2019–nCoV assay fully satisfies these requirements. In particular, the Seegene assay detects the RdRP (non-structural, conserved) together with N and E (structural, specific) genes of SARS–CoV-2. This was in line with the WHO recommending a first-line sample screening with the E gene followed by confirmation with the RdRP gene [8]. From the Allplex™ 2019–nCoV assay's implementation [15] to current use in our laboratory, Seegene modified the interpretative criteria, so that positivity for one of three assay targets is now sufficient to adjudicate a sample as positive for SARS–CoV-2 RNA. Excluding one sample (negative for all three targets), it is remarkable that in all 54 Allplex™ 2019–nCoV positive samples the N gene was detected. Thus, we are not surprised that the US CDC recommended the N gene as a SARS–CoV-2 assay target alone [16].

As viral dynamics in COVID-19 cases is not fully understood [17], SARS–CoV-2 loads determined by RT PCR assays may not be useful to indicate disease severity [18–20]. However, the viral load in a clinical (primarily URT) sample may be an indication of pathogen transmissibility [21], thereby accounting for the widespread person-to-person transmission that also occurs in patients with mild, limited, or no symptoms [2]. Taking advantage of studies showing that lower C_T values are inversely related to higher viral copy numbers [19,20,22], we explored the relationship between qualitative and quantitative detection results obtained for the 125 study samples. As expected, we found that viral loads were negatively associated with the C_T values of RT PCRs performed with either the Allplex™ 2019–nCoV assay or the Simplexa™ COVID-19 Direct assay. However, we noted a slight difference in the strength of this association between assays, which was in favor of the Allplex™ 2019–nCoV assay.

We and other laboratory scientists acknowledge the importance of comparative evaluation studies to guide implementation and/or correct interpretation of SARS–CoV-2 RNA detection assays. While confirming previously published results (albeit restricted to the Simplexa™ COVID-19 Direct assay) [10,11], we unlike others [11] did not investigate the clinical sensitivity of these assays, but we expanded the general knowledge about performance features of molecular SARS–CoV-2 detection assays, including sample-to-answer platforms [23–25], commercially available to date. As assay target genes are present in equal copy numbers in the SARS–CoV-

2 genome, assay performance is usually not dependent on the target itself [9]. However, the finding that one molecular target would work better than the other is helpful in redesigning such assays (e.g., shifting from multiple targets to a single target) to enhance reagent utilization [3]. Meanwhile, showing the equivalence of assays may aid to promptly redirect our laboratory choice of RNA-based diagnostic assays towards those with less supply chain trouble at that time [3].

In conclusion, the study showed that the Allplex™ 2019–nCoV assay is equivalent to the Simplexa™ COVID-19 Direct assay for the laboratory-confirmed diagnosis of COVID-19, whereas the Quanty COVID-19 assay allows to maximize diagnosis. Additionally, the Quanty COVID-19 assay providing quantitative data may be useful for SARS–CoV-2 infection monitoring purposes. However, further studies are warranted to define the role these assays might play in future clinical practice. Certainly, as testing for COVID-19 increases, these assays or their refinements will contribute to improve the laboratory capacity to identify patients with SARS–CoV-2 infection.

Declarations

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Compliance with ethical standards

Conflict of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Ethics approval and informed consent

The Ethics Committee of our Institution approved the study (reference number 18656/20) and informed consent was obtained from each patient before including his/her samples in the study.

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Figures

Figure 1

Correlation between the viral load levels quantified by the Quanty COVID-19 assay and the CT values obtained with the Allplex™ 2019-nCoV assay. Values are shown for each SARS–CoV-2 gene (E, RdRP, or N) detected by

the assay.

Figure 2

Correlation between the viral load levels quantified by the Quanty COVID-19 assay and the CT values obtained with the Simplexa™ COVID-19 Direct assay. Values are shown for each SARS-CoV-2 gene (S or ORF1ab) detected by the assay.

Supplementary Files

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