How to choose the right real-time RT-PCR primer sets for the SARS-CoV-2 genome detection?

Ahalieyah Anantharajah (ahalieyah.anantharajah@uclouvain.be)
Cliniques universitaires Saint-Luc, Université Catholique de Louvain
Raphael Helaers
Human Molecular Genetics, de Duve Institute, Université catholique de Louvain
Jean-Philippe Defour
Cliniques universitaires Saint-Luc, Université Catholique de Louvain
Nathalie Olive
Cliniques universitaires Saint-Luc, Université Catholique de Louvain
Florence Kabera
Cliniques universitaires Saint-Luc, Université Catholique de Louvain
Luc Croonen
Cliniques universitaires Saint-Luc, Université Catholique de Louvain
Françoise Deldime
Cliniques universitaires Saint-Luc, Université Catholique de Louvain
Jean-Luc Vaerman
Cliniques universitaires Saint-Luc, Université Catholique de Louvain
Cindy Barbée
Cliniques universitaires Saint-Luc, Université Catholique de Louvain
Monique Bodéus
Cliniques universitaires Saint-Luc, Université Catholique de Louvain
Anais Scohy
Cliniques universitaires Saint-Luc, Université Catholique de Louvain
Alexia Verroken
Cliniques universitaires Saint-Luc, Université Catholique de Louvain
Hector Rodriguez-Villalobos
Cliniques universitaires Saint-Luc, Université Catholique de Louvain
Benoît Kabamba-Mukadi
Cliniques universitaires Saint-Luc, Université Catholique de Louvain

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Abstract

The SARS-CoV-2 pandemic has created an unprecedented need for rapid large-scale diagnostic testing. Currently, several quantitative reverse-transcription polymerase chain reaction (RT-qPCR) assays recommended by the World Health Organization are being used by clinical and public health laboratories and typically target regions of the RNA-dependent RNA polymerase (RdRp), envelope (E) and nucleocapsid (N) coding region. However, it is currently unclear if results from different tests are comparable.

The present study demonstrates substantial differences in SARS-CoV-2 RNA detection sensitivity among the primer/probe sets recommended by the World Health Organization especially for low-level viral loads. The alignment of thousands of European SARS-CoV-2 sequences against the primers/probe highlights single mismatches which might also contribute to false negatives.

An understanding of the limitations depending on the targeted genes and primer/probe sets may influence the selection of molecular detection assays by clinical laboratories.

Introduction

Efforts to control SARS-CoV–2, the novel coronavirus causing COVID–19 pandemic, depend on accurate and rapid diagnostic testing. The reverse transcription real-time polymerase chain reaction (RT-qPCR) assay has become the gold standard for the diagnosis of SARS-CoV–2 infection. The European Commission recently advised to follow one of the World Health Organization protocols of RT-qPCR assays, published as early as January 2020 [1,2]. Among them, the United States Center for Disease Control (US CDC) recommended two nucleocapsid gene targets (*N1* and *N2)* [3] while the German Consiliary Laboratory for Coronaviruses hosted at the Charité in Berlin (Charité/Berlin) recommended first line screening with the envelope (*E)* gene assay followed by a confirmatory assay using the RNA-dependent RNA polymerase (*RdRp)* gene, even before the first COVID–19 cases appeared in Europe [4,5]. At the time of data submission 295 molecular assays are commercially available or in development for the diagnosis of COVID–19 (https://www.finddx.org/) and most of them use these recommended gene targets alone or in combination. However, there has been no indication that any one of these sequence regions offer an advantage for clinical diagnostic testing, especially as the number of samples from patients with confirmed COVID–19 has been relatively small in the preliminary evaluations. Recent studies reported that the RT-qPCR assays have limited sensitivity, while chest computed tomography (CT) may reveal pulmonary abnormalities consistent with COVID–19, including ground-glass opacities, multifocal patchy consolidation, and/or interstitial changes with a peripheral distribution, even in patients with negative RT-qPCR results [6–8]. RT-qPCR assays with higher sensitivity might help to reduce the false-negative rate.

In the context of lift confinement restrictions where large scale COVID–19 testing should be needed, this study aimed to clarify the clinical performances of the primer/probe sets designed by US CDC and Charité/Berlin to help guide assay selection by clinical laboratories for SARS-CoV–2 routine detection.

Results

Oligonucleotide binding regions alignments

No significant homologies of SARS-CoV–2 sequences with MERS-CoV and seasonal human coronaviruses HKU1, OC43, NL63, 229E were observed suggesting a low risk of potential false positive RT-qPCR results with other circulating human coronaviruses. However, due to the relative paucity of positive control materials at the time these assays were developed, the primers and probe were designed such that they would also cross-react with the SARS-CoV [4,9]. Indeed, probe and primers sequences of *E* gene assay showed high sequence homology with other related betacoronaviruses such as SARS coronavirus and bat SARS-like coronavirus genomes [4]. Although the forward and reverse primer sequences of *RdRp* and *N2* assays showed also high sequence homology with SARS coronavirus, the combination of primers and probe would allow the specific detection of SARS-CoV–2. The assay targeting *N1* gene was found to be specific to SARS-CoV–2.

Very little is known about how rapidly the SARS-CoV–2 genome mutates but its genetic diversity has been reported with a high rate of mutation [10–12]. To assess if these recommended primers and probes covered the circulating strains in Europe, 6750 SARS-CoV–2 sequences were downloaded from GISAID [13], and compared to the primers and probe binding regions [14] *(Table 1).* Over 99% of analyzed sequences of SARS-CoV–2 showed exact identity to *N1, N2* and *E* primers and probe sequences. The remaining sequences of SARS-CoV–2 exhibited 95% of identity corresponding to single base-pair mismatches. It appeared that two types of single mismatches were observed close to the 3' end of the *N1* reverse primer in 31 deposited sequences. A single nucleotide mismatch was found in RdRP_SARSr-R2 reverse primer with all SARS-CoV–2 analyzed genomes. The primer (CARATGTTAAASACACTATTAGCATA) contained an incorrect degenerate base S at position 12 that binds with G or C while all SARS-CoV–2 analyzed sequences encoded for a T at this position *(Table 1).* This mismatch would not be derived from a new variant but rather due to the initial oligonucleotide design allowing to amplify SARS-CoV, bat-SARS-related CoV and SARS-CoV–2 genomes.

Clinical performances

We collected nasopharyngeal samples from one hundred patients who presented Chest CT abnormalities consistent with COVID–19 (median age, 63.5 years; 51% female). The average length of stay in COVID–19 care units was 12 days with 15 deaths as of April 30th 2020. The nasopharyngeal specimens were obtained upon hospital admission corresponding to an average of 6.6 days after symptoms onset.

Mean Ct values of the sample cohort detected by all RT-qPCR assays were significantly lower in *N1* and *N2* gene assays than in *RdRp*-P2 and *E* gene assay (One way ANOVA, Tukey post-test p<0.001). Compared to CT-Scan, *N1* and *N2* primer/probe sets showed the highest positive rate (73 and 74% respectively) followed by *E* primer/probe set (58%) and then *RdRp* primer/probe sets (44%) *(Table 2).* The use of Genesig commercial kit with optimized target region and primer/probe sequences in the *RdRp* gene exhibited a slightly increased sensitivity (53%) compare to *RdRp*-P2 assay recommended by Corman et al [4].

Interestingly, the combination of *N1* and *N2* assays allowed an increase in the sensitivity (84%) compared to *N1* or *N2* alone, including SARS-CoV–2 RNA detection in 5 additional specimens (viral load range: 519 - 1007 copies/mL) that were tested negative by the others assays (*Figure 2).* Among these patients, we could exclude 3 false positive results as patients had positive SARS-CoV–2 IgG (MAGLUMI assay, Snibe) 7–12 days after molecular testing. Serological control could not be performed for the 2 other patients as they died within a short time spanning.

The performances of the RT-qPCR assays were highly dependent on the viral load. In our study, positive SARS-CoV–2 clinical samples exhibited median Ct values > 30 corresponding to low viral load which made the detection challenging. Indeed, both *RdRp* and *E* assays reliably detected specimens with Ct values < 30, but did not detect 40–60% of specimens with Ct values ≥ 30 (*Table 2, Figure 2, Figure S1)*..

Discussion

The survey published on February 11, 2020 reported [5] that the *E-*,, *RdRp-* and *N-* gene assays had rapidly been implemented by the European laboratories. Very few studies have been published to date on the relative performance characteristics of these assays recommended by the WHO [3,4,15]. One of the key factors determining detection sensitivity is how efficiently primers and probes bind target genes. Our findings highlight substantial differences in sensitivity for the primer/probe sets when comparing under the same conditions. Indeed, *N1* and *N2* assays stand out in comparison with the *E* and *RdRp* assays for the detection of low-level viral loads. Furthermore, positive *E* and negative *RdRp* results were obtained in 15 cases. We may therefore question the need of confirmatory testing following an initial positive test according to the Charité/Berlin protocol, resulting in turnaround time delay and increased workload. The cross-reactivity of the primers and probes with SARS-CoV should not cause any diagnostic ambiguity as SARS-CoV is no longer detected since the resolution of the epidemic in 2004 [16]. We observed notable mismatches in regions targeted by the primers/probe sets which might affect RT-qPCR assays performance depending on their location and the nature of the substitution [17,18].Based on our preliminary observations, multiplexing CDC *N1* and *N2* assays within a single PCR mixture could allow a reliable SARS-CoV–2 detection.

At present, in the context of large-scale screening, RT-qPCR testing remains the standard for the diagnosis of COVID–19 despite the false-negative rate. Indeed, normal chest CT scan cannot exclude COVID–19, especially for patients with early symptoms [19] and conversely an abnormal CT scan is not specific for COVID–19 diagnosis [20]. We believe our results would encourage the laboratory staff to be aware of certain limitations depending on the targeted genes and to evaluate the clinical performances of COVID–19 molecular diagnostic tests across a wide range of viral concentrations before their implementation. Due to the emergence of genetic diversity in the SARS-CoV–2 genome and the extensive transmission of this virus, we also encourage the detection of SARS-CoV–2 by targeting at least two distinct regions and oligonucleotide binding regions should be monitored continuously with the circulating virus strains.

Methods

In silico analysis of primer and probe sequences

The amplified regions of SARS-CoV–2 by the recommended primers (E_Sarbeco_F1, E_Sarbeco_R2, E_Sarbeco_P1; RdRP_SARSr-F2, RdRP_SARSr-R1, RdRP_SARSr-P2; 2019-nCoV_N1-F, 2019-nCoV_N1-R, 2019-nCoV_N1-P; 2019-nCoV_N2-F, 2019-nCoV_N2-R, 2019-nCoV_N2-P) were aligned with SARS-Coronavirus, MERS-Coronavirus and seasonal human coronaviruses genome using MUSCLE [21], and formatted using MSAViewer [22]. Phylogenies have been inferred using MetaPIGA 3.1 [23] with “Human CoV 229E” selected as an outgroup. Resulting consensus trees have been formatted using iTOL.

In addition, 6750 SARS-CoV–2 sequences from 33 European countries have been downloaded from Global Initiative on Sharing All Influenza Data (GISAID, as available on 2020 April 24th) [13], and aligned against *N1, N2, RdRp* and *E* primers/probe sequences using the Smith–Waterman algorithm [14].

Clinical specimens

From April 1 to April 30, 2020, we retrospectively collected one hundred nasopharyngeal swab specimens from patients who were clinically diagnosed with COVID-19 according to the chest CT image and hospitalized in COVID–19 care units of the Cliniques universitaires Saint-Luc, in Brussels, Belgium. An additional 30 “pre-intervention screening” samples from patients who were not suspected of COVID–19 were also included in this study. Nucleic acids from nasopharyngeal swabs were extracted by the Abbott m2000sp following manufacturer’s magnetic microparticle-based protocol (Abbott molecular, IL, USA).

Real-time RT-PCR assays

The reaction mix (20 µL) consisted of 4x TaqPath™ 1-Step RT-qPCR Master Mix, CG (ThermoFisher), 5 µL of extracted nucleic acid and primers/probes at concentrations recommended by Charité/Berlin and CDC protocols [1]. A multiplexing assay with 2019-nCoV_N1 and 2019-nCoV_N2 was also evaluated in parallel. We used human RNAse P as control for RNA extraction, and as indicator of the sample quality. The RT-qPCR was performed on LightCycler 480 II (Roche Diagnostics, Mannheim, Germany) with the following amplification parameters: 10 minutes at 55 °C for reverse transcription, 3 minutes at 95 °C for

activation followed by 45 cycles of 15 seconds at 95 °C and 30 seconds at 58 °C [4]. We also tested samples for SARS-CoV–2 using the RdRp genesig® Real-Time PCR COVID–19 CE IVD assay (Primerdesign Ltd, Chandler's Ford, UK) according to the manufacturer's instructions.

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Declarations

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Authors contributions

AA, BKM, JPD and RH contributed to the conception and design of the study. AA, FD, NO, FK, LC performed the experiments. RH performed the bioinformatics analysis. JLV and CB contributed to the molecular analysis. AA and BKM analysed all experiments. AA and BKM wrote the manuscript. RH, JLV, MB, AS, AV, and HRV provided critical feedback and contributed to the final version of the manuscript. All authors approved the manuscript.

Competing interests

The authors declare no competing interests

Tables

RT-qPCR assays	Number of analyzed sequences*	Average Position Start sequence	Primer	Primer sequence 5'->3'	Primer size bp	Identity 100%	Identity ~ 95 % Single nucleotide mismatch		
						Number of sequences	Number of sequences	Number of countries	Number of variants
N1	6736	28267	2019-nCoV_N1-F	GACCCCAAAATCAGCGAAAT	20	6730	6	5	5
			2019-nCoV_N1-P	ACCCCGCATTACGTTTGGTGGACC	24	6713	23	8	9
			2019-nCoV_N1-R	TCTGGTTACTGCCAGTTGAATCTG	24	6672	64	7	12
N2	6724	29144	2019-nCoV_N2-F	TTACAAACATTGGCCGCAAA	20	6703	21	6	6
			2019-nCoV_N2-P	ACAATTTGCCCCCAGCGCTTCAG	23	6706	18	5	6
			2019-nCoV_N2-R	GCGCGACATTCCGAAGAA	18	6715	9	5	6
E	6727	26249	E_Sarbeco_F2	ACAGGTACGTTAATAGTTAATAGCGT	26	6716	11	4	8
			E_Sarbeco_P1	ACACTAGCCATCCTTACTGCGCTTCG	26	6710	17	7	5
			E_Sarbeco_R1	ATATTGCAGCAGTACGCACACA	22	6725	2	2	1
RdRp	6740	15411	RdRP_SARSr-F1	GTGARATGGTCATGTGTGGCGG	22	6727	13	8	6
			RdRP_SARSr-P2	CAGGTGGAACCTCATCAGGAGATGC	25	6737	3	1	2
			RdRP_SARSr-R2	CARATGTTAAASACACTATTAGCATA	24	6740	6740	33	1

Table 1: Primers alignments on SARS-CoV-2 target sequences

6750 SARS-CoV-2 sequences from 33 European countries (21 Austria, 393 Belgium, 2 Belarus, 7 Croatia, 28 Czech Republic, 337 Denmark, 2444 England, 4 Estonia, 40 Finland, 226 France, 86 Germany, 4 Greece, 3 Hungary, 601 Iceland, 13 Ireland, 58 Italy, 10 Latvia, 1 Lithuania, 204 Luxembourg, 585 Netherland, 1 Northern Ireland, 29 Norway, 15 Poland, 100 Portugal, 105 Russia, 396 Scotland, 4 Slovakia, 4 Slovenia, 190 Spain, 53 Sweden, 76 Switzerland, 30 Turkey, 680 Wales) have been downloaded from GISAID (as available on 2020 April 24th), and aligned against the set of primers/probes. R is G/A and S is G/C.*Incomplete sequences on target regions were excluded of the analysis

RT-qPCR assays	PCR efficiency (%)[a,b]/ linearity (R^2)	Limit of detection (copies/ reaction)[c]	Pre-intervention screening (n=30)	Chest CT-Scan positive				
				All samples (n=100)	Positive samples by **all** RT-qPCR assays (n=44)		Positive samples by **at least one** RT-qPCR assay (n=84)	
			Negative agreement % (95% CI)	Positive agreement % (95% CI)	Median Ct (95% CI)	Median Viral Load[a] Log copies/mL (95% CI)	Ct value < 30 (n=19) Positive rate % (95% CI)	Ct value ³ 30 (n=65) Positive rate % (95% CI)
N1	96.81/ 0.99	5	100 (88.6 – 100)	73 (63.6-80.7)	29.59 (29.8-31.8)	5.07 (4.42-5.89)	100 (83.2 – 100)	83.1 (72.2 – 90.3)
N2	91.62/ 1.00	5		74 (64.6-81.6)	30.5 (27.92-32.83)	5.06 (4.40-5.79)		84.6 (73.9 – 91.4)
N1+N2	92.85/ 1.00	5		84 (75.8-89.9)	29.35 (26.82-31.64)	5.11 (4.46-5.83)		100 (94.4 – 100)
RdRp	96.90/ 1.00	10		44 (34.7-53.8)	31.44 (29.77-33.26)	5.46 (4.92-5.95)		38.5 (27.6 – 50.6)
RdRp GenesigÒ	80.27/ 1.00	10		53 (43.3- 62.5)	31.67 (28.87-33.41)	4.47 (4.02-5.18)		52.3 (40.4 – 64.0)
E	95.68/ 1.00	5		58 (48.2-67.2)	31.06 (28.25-33.76)	4.98 (4.19-5.79)		60.0 (47.8 – 71.0)

Table 2: Comparison of the six RT-qPCR assays performances

[a] PCR efficiency, linearity and virus copies were determined using a 10-fold dilution standard curve of positive control plasmids.

N1 : y = -3.4007x + 36.624; N2 : y = -3.5407x + 37.783; N1+N2 : y = -3.506x + 36.74 ;

RdRp : y = -3.3985x + 39.798 ; RdRp GenesigÒ : y=-3.9076x+ 37.404 ; E : y = -3.43x + 37.84

[b] PCR Efficiency E = 100* ($10^{-1/slope}$ – 1).

[c] The Limit of detection was determined as the lowest concentration where ≥ 90% (9/10) of the replicates were positive

Figures

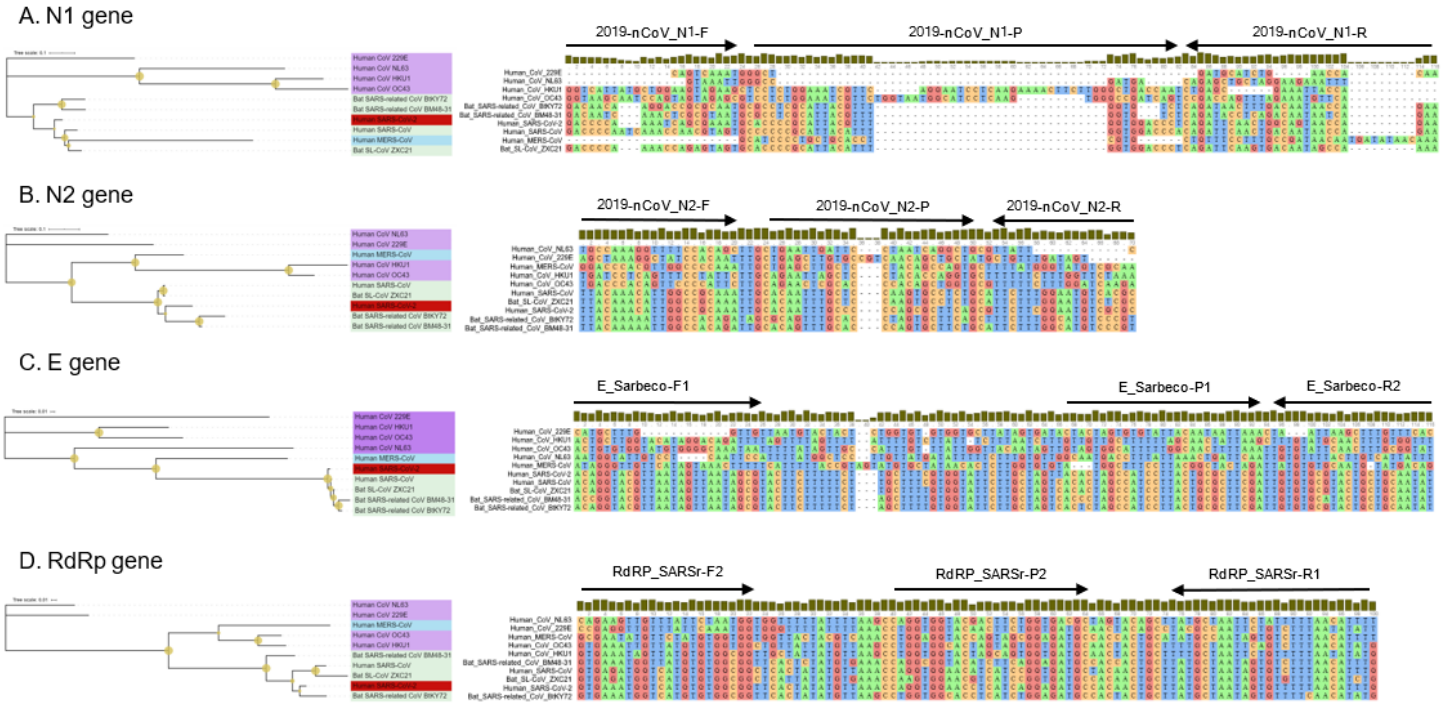

Figure 1

Alignments of N1 (A), N2 (B), E (C), RdRp (D) regions of SARS-CoV-2 with SARS-Coronavirus, MERS-Coronavirus and seasonal human coronaviruses genomes. The arrows indicate the regions targeted by the set of primers/probes. Human SARS-CoV-2: hCoV-19/Belgium/CJM-0323175/2020|EPI_ISL_420432; Human SARS-CoV: SARS coronavirus NC_004718.3; Bat SL-CoV ZXC2: Bat SARS-like coronavirus isolate MG772934.1; Bat SARS-related CoV BM48-3: BGR/2008 GU190215.1 Human MERS-CoV: Middle East respiratory syndrome coronavirus NC_019843.3; Human CoV HKU1: Human coronavirus HKU1 NC_006577.2; Human CoV OC43 : Human coronavirus OC43 strain ATCC VR-759 NC_006213.1; Human CoV NL63: Human Coronavirus NL63 NC_005831.2; Human CoV 229E: Human coronavirus 229E NC_002645.1

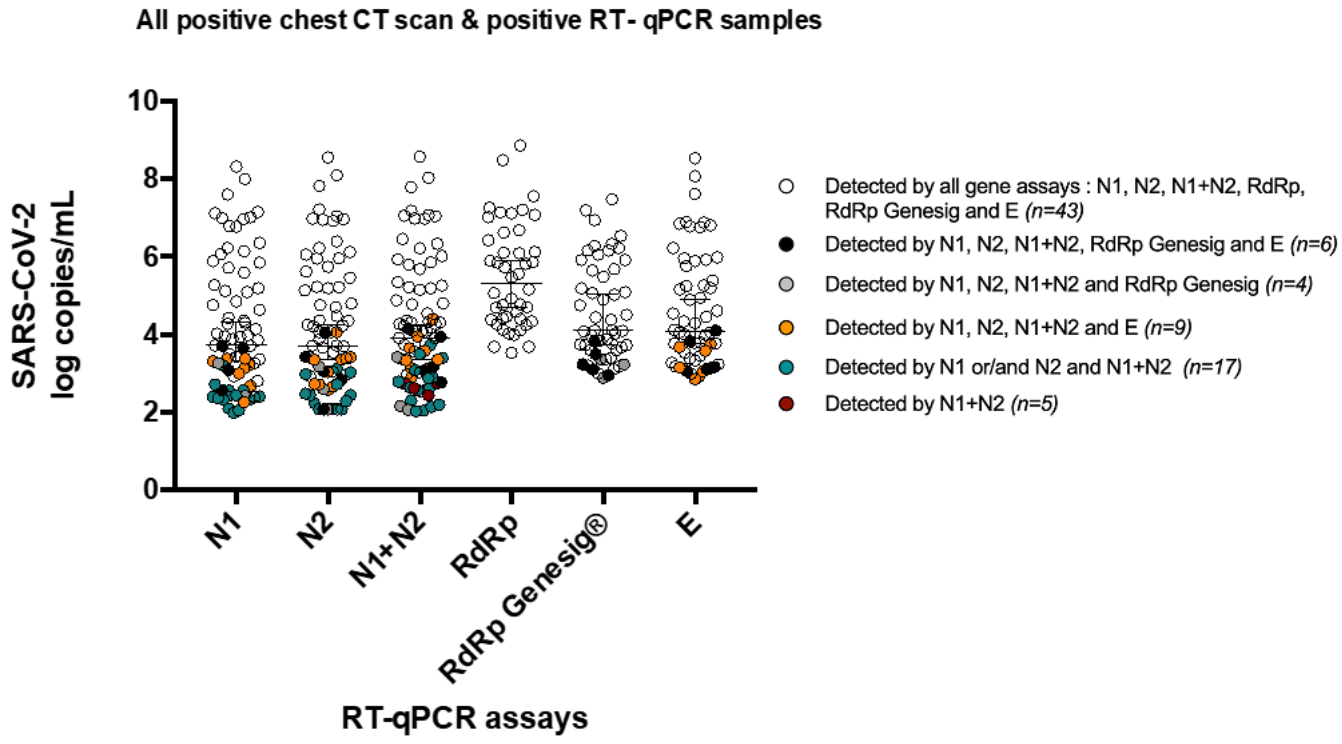

Figure 2

Comparison of the viral load detected by the six RT-qPCR assays among the positive nasopharyngeal swabs (n = 84). The viral load is expressed in log copies/mL and each clinical sample is represented by a circle. The white circles represent clinical samples detected by all RT-qPCR assays while colored circles represent samples not detected by the six assays. Bars represent the median and 95% Confidence Interva

Supplementary Files

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- gisaidhcov19acknowledgementtable2020051916.xls
- FigureS1.pdf