Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Short communication

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

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ABSTRACT

Objectives: The SARS-CoV-2 pandemic has created an unprecedented need for rapid large-scale diagnostic testing to prompt clinical and public health interventions. 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. This study aimed to clarify the clinical performances of the primer/probe sets designed by US CDC and Charité/Berlin to help clinical laboratories in assay selection for SARS-CoV-2 routine detection.

Methods: We compared the clinical performances of the recommended primer/probe sets using one hundred nasopharyngeal swab specimens from patients who were clinically diagnosed with COVID-19. An additional 30 "pre-intervention screening" samples from patients who were not suspected of COVID-19 were also included in this study. We also performed sequence alignment between 31064 European SARS-CoV-2 and variants of concern genomes and the recommended primer/probe sets.

Results: 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 SARS-CoV-2 sequences reveals that the genetic diversity remains relatively low at the primer/probe binding sites. However, multiple nucleotide mismatches might contribute to false negatives.

Conclusion: 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.

1. 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 advised to follow one of the World Health Organization protocols of RT-qPCR assays, published as early as January 2020 (WHO, 2020; European Commission, 2020). Among them, the United States Center for Disease Control (US CDC) recommended two nucleocapsid gene targets (*N1* and *N2)* (Holshue et al., 2020) 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

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assay using the RNA-dependent RNA polymerase (*RdRp*) gene, even before the first COVID-19 cases appeared in Europe (Corman et al., 2020; Reusken et al., 2020). At the time of data submission 437 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 (Huang et al., 2020; Ai et al., 2020; Xie et al., 2020). The genomic evolution of SARS-CoV-2 and specifically, the emergence of three new viral variants, at the end of 2020, associated with extensive transmission started to raise concerns (van Dorp et al., 2020; Stefanelli et al., 2020; Wang et al., 2020; Gonzalez-Candelas et al., 2021). These variants of concern were first reported in the UK (B.1.1.7), South Africa (B.1.351) and Brazil (P.1). Their designation as VOCs was determined by an increase of local cases and by the high number of amino acid substitutions harboured by these lineages, which can lead to increased infectivity and potentially decreased vaccine effectiveness. Genetic variations in the viral genome at primer/probe binding regions could result in potential mismatches and false-negative results. RT-qPCR assays with higher sensitivity targeting conserved regions 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.

2. Material and methods

2.1. 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 (Madeira et al., 2019), and formatted using MSAViewer (Yachdav et al., 2016). Phylogenies have been inferred using MetaPIGA 3.1 (Helaers and Milinkovitch, 2010) with "Human CoV 229E" selected as an outgroup. Resulting consensus trees have been formatted using iTOL.

In addition, 31064 SARS-CoV-2 sequences from 32 European countries and 4078 well-characterized SARS-CoV-2 sequences from 173 countries in the World have been downloaded from Global Initiative on Sharing All Influenza Data (GISAID) (Shu and McCauley, 2017), and aligned against *N1, N2, RdRp* and *E* primers/probe sequences using the Smith–Waterman algorithm (Smith and Waterman, 1981).

2.2. 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. The nasopharyngeal swabs were eluted in 1 mL of universal viral transport media (UTM, Copan, Italia). Nucleic acids were extracted from 650 µL of nasopharyngeal swab medium by the Abbott m2000sp following manufacturer's magnetic microparticle-based protocol (Abbott molecular, IL, USA). Samples were eluted in 60 µL of elution buffer.

2.3. Real-time RT-PCR assays

The reaction mix (20 µL) consisted of 4x TaqPath™ 1-Step RT-qPCR Master Mix, CG (ThermoFisher, Waltham, MA, USA), 5 µL of extracted nucleic acid and primers/probes at concentrations recommended by Charité/Berlin and CDC protocols (Integrated DNA Technologies, Coralville, IA, USA) (WHO, 2020). 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 min at 55 °C for reverse transcription, 3 min at 95 °C for activation followed by 45 cycles of 15 s at 95 °C and 30 s at 58 °C (Corman et al., 2020). 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. RT-qPCR efficiency, linearity and viral load were determined using a 10-fold dilution standard curve of SARS-CoV-2 RNA transcripts standard (Bio-Rad, Hercules, CA, USA) with three replicates at each concentration. Statistical analysis was performed using GraphPad Prism software (San Diego, USA).

3. Results

3.1. 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 (Corman et al., 2020; Chu et al., 2020). 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 (Corman et al., 2020). 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 (Fig.1).

To assess if these recommended primers and probes covered the circulating strains in Europe, 31064 SARS-CoV-2 sequences were downloaded from GISAID (Shu and McCauley, 2017), and compared to the primers and probe binding regions (Smith and Waterman, 1981) (Table 1A). A single nucleotide mismatch was found in RdRP_SARSr-R2 reverse primer binding region with 99.9 % of 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. 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. Over 98 % 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 mainly single base-pair mismatches. *N1* and *N2* binding regions appeared to carry more mutations than *E* and *RdRp* binding regions. At least one mutation in last five nucleotides of 3' primer regions (forward, probe or reverse) were observed in 615 (2 %) deposited sequences which could affect sensitivity. The alignment of the variants of concern B.1.1.7, B.1.351, P.1 sequences (Table 1B) and the variants of interest B.1.525, B.1.427/B.1.429, P.2 and P3 sequences against the sets of primer/probe

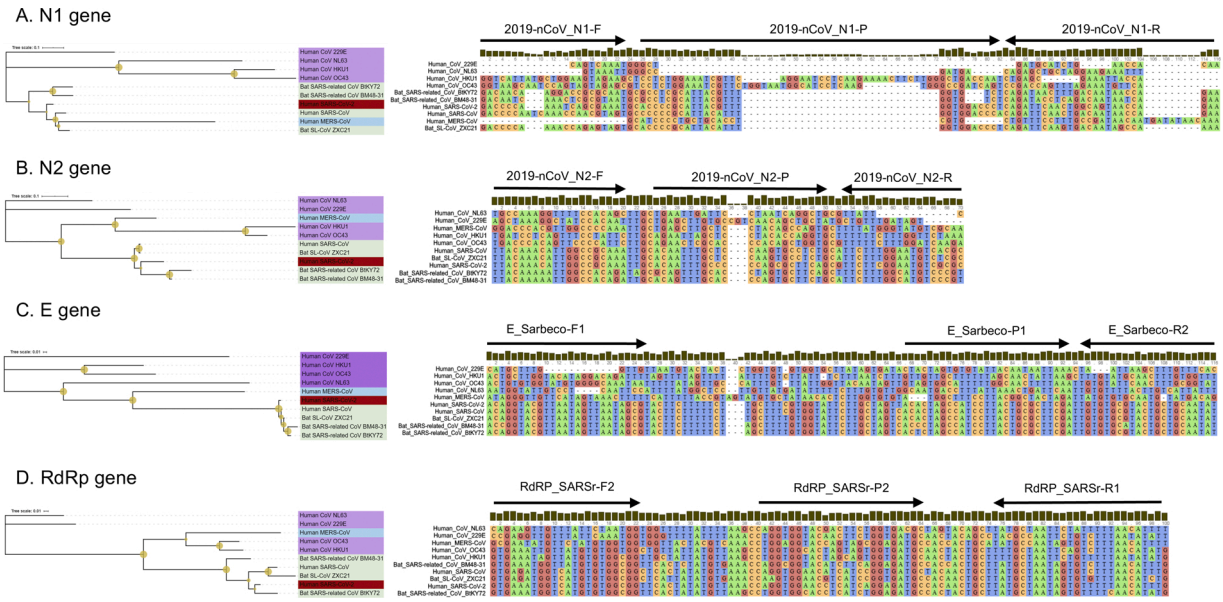

Fig. 1. Alignments of N1 (A), N2 (B), E (C), RdRp (D) regions of SARS-CoV-2 with SARS-Coronavirus, MERS-Coronavirus and seasonal human coronaviruses genomes.

The arrows indicate the regions targeted by the sets of primer/probe. 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.

has revealed that the genetic diversity remains relatively low at the binding sites. Of note, the probe primer of *N2* assay showed one nucleotide mismatch with 13.9 % of B.1.351 sequences.

3.2. Clinical performances

The RT-qPCR efficiencies of the recommended primer/probe sets, calculated using 10-fold serial dilution of RNA transcripts standard were above 90 % which match the criteria for an efficient RT-qPCR assay (Svec et al., 2015). The limit of detection was 5 copies per reaction for the *N1* and *N2* gene assays, 10 copies per reaction for the *E* gene assay and 25 copies per reaction for the *RdRp-P2* assay (Table 2)

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 5.6 days (range: 1–16) 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. (2020).

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 (Fig. 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, Figs. 2, S1).

4. Discussion

The survey published on February 11, 2020 reported (Reusken et al., 2020) 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 (Holshue et al., 2020; Corman et al., 2020; Konrad et al., 2020). 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 (Wang et al., 2005). Based on our preliminary observations, multiplexing CDC *N1* and *N2* assays within a single PCR mixture could allow a reliable SARS-CoV-2 detection. For now, the low variability in the primer/probe binding regions of the SARS-CoV-2 sequences analysed, in particular of the B.1.1.7 VOC sequences can be considered reassuring. However, we observed notable mismatches in regions targeted by the primer/probe sets which might affect RT-qPCR assays performance depending on their location and the nature of the substitution (Klungthong et al., 2010; Stadhouders et al., 2010).

At present, in the context of large-scale screening, RT-qPCR testing

Table 1A
Primers alignments with SARS-CoV-2 target sequences.

	Average Position Start sequence	Primer	Primer sequence 5'->3'	Primer size bp	Identity 100 % Number sequences	1 nucleotide mismatch			≥2 nucleotide mismatches			% sequences with ≥ 2 nucleotide mismatches	Number of sequences with at least one mutation in last 5 nucleotides of 3' primer region
						Number sequences	Number countries	Number variants	Number sequences	Number countries	Number variants		
N1	28267	2019-nCoV_N1-F	GACCCCAAAATCAGCGAAAT	20	30755	304	19	27	5	5	4	0,016	37
		2019-nCoV_N1-P	ACCCCGCATTACGTTTGGTGGACC	24	30623	437	22	26	4	4	3	0,013	8
		2019-nCoV_N1-R	TCTGGTTACTGCCAGTTGAATCTG	24	30864	196	16	28	3	3	2	0,010	66
N2	29144	2019-nCoV_N2-F	TTACAAACATTGGCCGCAAA	20	30821	162	18	13	79	3	4	0,254	135
		2019-nCoV_N2-P	ACAATTTGCCCCCAGCGCTTCAG	23	30798	182	17	16	84	6	5	0,270	109
		2019-nCoV_N2-R	GCGCGACATTCCGAAGAA	18	30858	119	15	10	87	5	1	0,280	114
E	26249	E_Sarbeco_F2	ACAGGTACGTTAATAGTTAATAGCGT	26	30978	85	16	13	1	1	1	0,003	3
		E_Sarbeco_P1	ACACTAGCCATCCTTACTGCGCTTCG	26	31002	55	10	9	7	4	3	0,023	26
		E_Sarbeco_R1	ATATTGCAGCAGTACGCACACA	22	31039	17	8	8	8	5	3	0,026	15
RdRp	15411	RdRP_SARSr-F1	GTGARATGGTCATGTGTGGCGG	22	30903	160	15	12	1	1	1	0,003	93
		RdRP_SARSr-P2	CAGGTGGAACCTCATCAGGAGATGC	25	31030	32	8	7	2	1	2	0,006	6
		RdRP_SARSr-R2	CARATGTTAAASACACTATTAGCATA	26	3	31045	32	1	16	6	7	0,052	3

31064 SARS-CoV-2 sequences from 32 European countries (Austria, Belgium, Belarus, Bosnia and Herzegovina, Croatia, Czech Republic, England, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Lithuania, Netherland, North Macedonia, Northern Ireland, Norway, Poland, Romania, Russia, Scotland, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey, Wales) have been downloaded from GISAID (as available from 19th to 24th April 2021), and aligned against the sets of primer/probe. R is G/A and S is G/C.*Only complete human SARS-CoV-2 genomes were included in the analysis.

Table 1B
Primers alignments with variants of concern target sequences.

	Primer	All sequences (n=4078)			B.1.1.7 [GRY/20I/501Y.V1] (n=1169)			B.1.351 [GH/20H/501Y.V2] (n=163)			P.1 [GR/20J/501Y.V3] (n=46)		
		Number sequences with 100 % identity	Number sequences with 1 mismatch	Number sequences with ≥2 mismatches	Number sequences with 100 % identity	Number sequences with 1 mismatch	Number sequences with ≥2 mismatches	Number sequences with 100 % identity	Number sequences with 1 mismatch	Number sequences with ≥2 mismatches	Number sequences with 100 % identity	Number sequences with 1 mismatch	Number sequences with ≥2 mismatches
	2019-nCoV_N1-F	4009	60	9	1158	8	3	157	6	0	44	2	0
N1	2019-nCoV_N1-P	3949	121	8	1167	3	2	144	19	0	44	2	0
	2019-nCoV_N1-R	4042	26	9	1165	2	2	161	2	0	46	0	0
	2019-nCoV_N2-F	4010	34	34	1159	1	9	159	0	4	46	0	0
N2	2019-nCoV_N2-P	3994	50	33	1140	20	9	159	0	4	46	0	0
	2019-nCoV_N2-R	4023	18	37	1159	0	10	159	0	4	46	0	0
	E_Sarbeco_F2	4062	7	9	1166	3	0	162	0	1	46	0	0
E	E_Sarbeco_P1	4062	4	12	1167	0	2	162	0	1	46	0	0
	E_Sarbeco_R1	4056	4	18	1164	1	4	162	0	1	46	0	0
	RdRP_SARSr-F1	4039	36	3	1163	4	2	161	2	0	46	0	0
RdRp	RdRP_SARSr-P2	4060	12	6	1167	0	2	163	0	0	46	0	0
	RdRP_SARSr-R2	1	4073	4	1	1168	0	0	163	0	0	46	0

4078 well-characterized SARS-CoV-2 sequences from 173 countries in the World have been downloaded from GISAID (Global Region-specific Auspice source files) and aligned against the sets of primer/probe. 356 different lineages (Pangolin nomenclature) and 12 or 9 different clades (Nextstrain and GISAID nomenclature respectively) were represented including the variants of concern (VOCs) in Europe (B.1.1.7 first detection in United Kingdom; B.1.351 first detection in South Africa; P.1 first detection in Brazil).

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

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 ≥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	25		44 (34.7–53.8)	31.44 (29.77–33.26)	5.46 (4.92–5.95)		38.5 (27.6–50.6)
RdRp Genesig®	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	10		58 (48.2–67.2)	31.06 (28.25–33.76)	4.98 (4.19–5.79)		60.0 (47.8–71.0)

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 Genesig® : y=-3.9076x + 37.404 ; E : $y = -3.43x + 37.84$.

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

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

[c] The Limit of detection was determined as the lowest concentration where 100 % (10/10) of the replicates were positive.

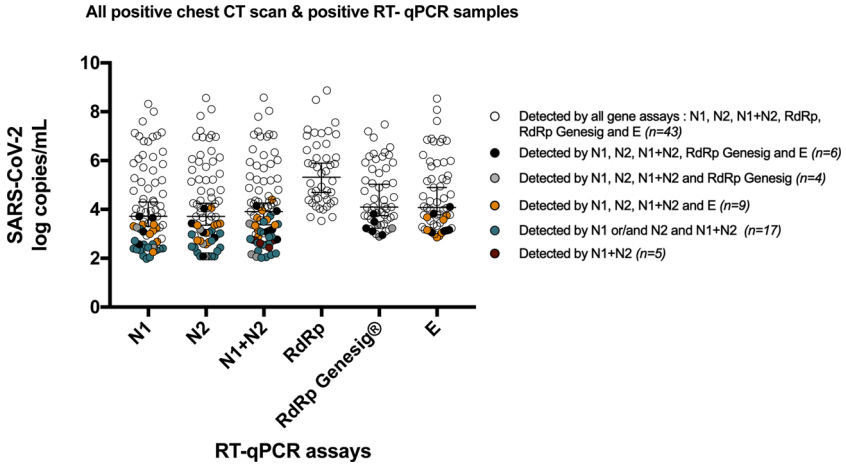

Fig. 2. Comparison of the viral load detected by the six RT-qPCR assays among the positive nasopharyngeal swabs (n = 84).
The viral load is expressed in log copies/mL and each clinical sample is represented by a circle. The white circles represent clinical samples detected by all RT-qPCR assays while colored circles represent samples not detected by the six assays. Bars represent the median and 95 % Confidence Interval.

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 (Yang and Yan, 2020) and conversely an abnormal CT scan is not specific for COVID-19 diagnosis (Hope et al., 2020). 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 extensive transmission of this virus, the genetic diversity in the SARS-CoV-2 genome and the constant emergence of variants of concern, 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.

CRediT authorship contribution statement

Ahalieyah Anantharajah: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Raphaël Helaers:** Conceptualization, Methodology, Software, Formal analysis, Writing - review & editing. **Jean-Philippe Defour:** Conceptualization. **Nathalie Olive:** Investigation. **Florence Kabera:** Investigation. **Luc Croonen:** Investigation. **Françoise Deldime:** Conceptualization, Investigation, Writing - review & editing. **Jean-Luc Vaerman:** Conceptualization, Writing - review & editing. **Cindy Barbée:** Conceptualization, Resources. **Monique Bodéus:** Writing - review & editing. **Anais Scohy:**

Conceptualization, Writing - review & editing. **Alexia Verroken:** Writing - review & editing. **Hector Rodriguez-Villalobos:** Conceptualization, Methodology. **Benoît Kabamba-Mukadi:** Conceptualization, Methodology, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2021.114197.

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