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COVID-19 laboratory diagnosis: comparative analysis of different RNA extraction methods for SARS-CoV-2 detection by two amplification protocols

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ABSTRACT

The gold standard for the laboratory diagnosis of COVID-19 is the reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) assay, which searches for SARS-CoV-2 target genes in nasopharyngeal/oropharyngeal (NP/OP) samples, and its performance depends on the quantity and quality of the RNA input. This study compared the performance and cost-effectiveness of three different kits/reagents for RNA extraction used in COVID-19 diagnosis in Sao Paulo, Brazil. A total of 300 NP/OP samples belonging to suspected cases of COVID-19 stored in a biorepository were randomly selected, and RNA was extracted using (i) automated extraction (Loccus, Extracta Kit FAST), (ii) manual extraction (BioGene Kit, Bioclin, Quibasa), and (iii) quick extraction methods (Lucigen, Quick DNA Extract Kit). Next, the samples were tested using RT-qPCR for SARS-CoV-2 with the Allplex 2019-nCoV modified assay and the Charité-Berlin protocol. All assays/kits were used according to the manufacturer's instructions. For the Allplex kit, the sensitivity in detecting SARS-CoV-2 with previously extracted RNA by different procedures was 100.0% for Loccus, 100.0% for BioGene and 91.9% for Quick. Using the Charité-Berlin protocol, the sensitivities were 81.4% for Loccus, 81.2% for BioGene and 60.7% for Quick. The least sensitive target gene and the gene most affected by RNA extraction procedures was the RNA-dependent RNA polymerase gene (Charité-Berlin protocol). No false-positive SARS-CoV-2 results were detected using RNA obtained from any of the different protocols. In conclusion, Loccus and BioGene RNA extractions were efficient for RT-qPCR assays, and although the BioGene procedure is less expensive, Loccus is the best choice because it allows the rapid handling of hundreds or thousands of samples, a desirable feature during pandemics. Although less sensitive, the Quick extraction is useful during outbreaks coupled with the Allplex amplification kit for SARS-CoV-2 diagnosis ($\kappa = 0.925$).

KEYWORDS: COVID-19. SARS-CoV-2. SARS-CoV-2 RT-PCR. SARS-CoV-2 molecular diagnosis. RNA extraction methods. Assays performances. Cost-effectiveness. RT-qPCR.

INTRODUCTION

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) for SARS-CoV-2, the new coronavirus that causes severe acute respiratory syndrome, is the gold standard for the laboratory diagnosis of COVID-19. It is preferably performed soon after the onset of using nasopharyngeal/oropharyngeal (NP/OP) secretions obtained with the help of swabs. RT-qPCR for SARS-CoV-2 is carried out using primer-probe sets targeting the highly conserved sequences of the envelope (*E*),

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nucleocapsid (*N*), and RNA-dependent RNA polymerase (*RdRP*) viral genes[1-4]. RT-qPCR performance for SARS-CoV-2 depends on the quality of the specimen, the date of collection, the presence of PCR inhibitors, the handling and quick delivery of specimens, and the quantity and quality of extracted RNA[1-4].

Since the beginning of the SARS-CoV-2 pandemic, several approaches/kits and molecular diagnostic reagents have been described in the literature and are available in the market[1-4]; however, because of the speed at which the virus has spread worldwide and the scarcity of kits and supplies, various government entities, such as the Brazilian Ministry of Health, have granted authorization for the use of these kits and protocols without a previous and accurate evaluation of their performance.

Brazil is ranked third in the number of COVID-19 cases worldwide[5], and on March 25, 2021, 14,340,787 confirmed cases and 390,797 deaths were reported to the Ministry of Health through the Health Surveillance Secretariat[6]. Sao Paulo State, located in Southeast Brazil, has the highest number of confirmed COVID-19 cases: 2,834,738 cases and 61,463 deaths[6], and the city of Sao Paulo (the largest in Latin America) accounts for 991,683 cases and 26,532 deaths[7]. Thus, this State and its capital city has received a large number of samples for analysis, from suspected cases of COVID-19 in a daily basis.

The Institute Adolfo Lutz (IAL) is a public health laboratory located in Sao Paulo city and is the reference laboratory for respiratory viruses, receiving many samples daily for diagnosis and surveillance. In a previous study, we compared the performance and cost-effectiveness of seven assays/kits available for COVID-19 routine molecular diagnosis, and found that, although there were some sensitivity and specificity differences among the assays, all assays/kits showed good performances (all of the Cohen's Kappa index were above 0.893). We confirmed that the assays/kits employing multiplex RT-qPCR methods had the best cost-benefit parameters[8]. In the present study, we decided to compare the diagnostic performance and cost-effectiveness of three different RNA extraction methods because this is another bottleneck in COVID-19 diagnosis due to the shortage of reagents and supplies, the fact that the procedure is time-consuming, and thousands of samples must be daily processed.

MATERIALS AND METHODS

Clinical specimens

In the present study, 300 NP/OP samples collected between September and October 2020 and sent to IAL for COVID-19 molecular diagnosis. They were used for the comparative analysis of three different RNA extraction methods for SARS-CoV-2 RNA target gene detection. The samples were collected, placed in saline transport solution and stored at -70 °C in a biorepository. The only criterion for selecting these samples was a minimum volume of 800 µL. The samples were divided into three aliquots: 200 µL for automated RNA extraction, 400 µL for manual RNA extraction, and 150 µL for quick RNA extraction. The assays were conducted blindly, and the authors did not know the SARS-CoV-2 result of the samples at the time of diagnosis and the cycle threshold (Ct) values of these samples were also unknown.

Methods

For the automated RNA extraction, the Loccus kit/equipment, the Extracta Fast kit (Cod. MVXA-P016 Fast) and the Loccus Extracta 32 equipment (Sao Paulo, Brazil) were employed. Briefly, the Loccus extraction procedure involves four steps: sample lysis, nucleic acid binding to electromagnetic beads covered with silicon dioxide, washing and elution. All steps were conducted according to the manufacturer's instructions in 32 deep well plates for approximately 1 h.

For the manual RNA extraction, the BioGene DNA/RNA viral kit (Bioclin Quibasa, MG, Brazil) was used, and the extraction was conducted according to the manufacturer's instructions. Briefly, the BioGene extraction has the same steps as the Loccus extraction (lysis, binding, washing and elution), however, it differs in the type of handling and the time expended in the procedure. It was conducted in a biosafety cabin, employed tubes, one of which contained a column of silica for nucleic acids binding, and required a vortex, a dry thermoblock and a centrifuge equipment. The number of handling steps and hazards reduced the number of extractions; for 32 samples, the time required was 2.5 h.

To ascertain the quantity of RNA was sufficient for RT-qPCR analyses in all extraction procedures (automated and manual), two extractions were performed and the RNA pool obtained was maintained at -70 °C and used up to one day after the extraction.

For the quick RNA extraction, the Lucigen QuickExtract™ DNA Extraction Solution (Lucigen Corporation, Middleton, WI, USA) was employed. Briefly, for each Quick extraction, 150 µL of sample was mixed by vortexing in 150 µL of Lucigen buffer, subjected to a heat shock at 95 °C for 5 min, cooled in a freezer or in ice bath for 5 min, and tested for RT-qPCR SARS-CoV-2 immediately, or maintained at -70 °C and used up to one

day after the extraction. This procedure saves time and sample handling, taking approximately 30 min to extract 32 samples.

For SARS-CoV-2 RNA detection, two RT-qPCR assays were employed: Allplex 2019-nCoV modified assay, Seegene, Korea[8] (provided by the Coordenacao Geral de Laboratorios de Saude Publica, CGLAB, Ministry of Health and used as a routine procedure at IAL and other public health laboratories in Brazil), and the Charité-Berlin protocol (employed in public health laboratories in Brazil at the beginning of the pandemic) using a P2 probe[9]. These RT-qPCR assays diverge in their composition, target genes, labeled probes, methods, interpretation of results and both were conducted according to the respective manufacturer's instructions, using 8 µL and 5 µL of RNA input for each assay, respectively.

All RT-qPCR reactions were performed in duplicate using the QuantStudio 5 Real-Time PCR System (Applied Biosystems, CA, USA).

Briefly, the Allplex kit employs as target genes and labeled probes *E* (FAM), *N* (Cal Red 610), *RdRP* (Quasar 670), and an internal control (IC; HEX) in one multiplex RT-qPCR, whereas the Charité-Berlin protocol employs the *E*- and *RdRP* target genes and probes labeled with FAM in two single RT-qPCR assays[9].

We employed primers/probes of the human *RnaseP* gene, as previously described[10], as the IC for both kit/assay. We did not use the IC of the Allplex kit because it did not work well in our laboratory (Ct values more than 35 and 20% of false-negative results), probably because this IC has been standardized for the conditions and the equipment Nimbus/STARlet of the Seegene manufacturer (NIMBUS/STARlet equipment, Franklin, MA, USA), which are not available in our laboratory. The Seegene manufacturer recently described four kits/equipment for RNA extraction that can be used with the Allplex kit: two automatized (SEEPREP 32, from Seegene Inc. Korea, and NucliSENS easyMAG from BioMérieux SA, Marcy l'Etoile France), and two manual RNA extraction kits (Ribospin vRD kit from GeneAll Biotechnology Co., Ltd, Seoul, Korea, and QIAmp DSP Virus Spin Kit, Qiagen Inc., Hilden, Germany). None of these are available in our laboratory.

The Ct limit (cut-off) of 40 was adopted for both kits/assays. In samples with Ct values close to the cut-off value (one Ct under the cut-off), the reactions were repeated in duplicate and the characteristics of the exponential/linear curves were observed.

The criteria established by the manufacturer for considering SARS-CoV-2 positive samples using the Allplex kit is the positivity of at least one of the RNA SARS-CoV-2 gene targets, except when only the *E* gene is detected, when the sample is considered "presumptive positive." The Charité-Berlin protocol considers SARS-CoV-2 positive samples when both *E* and *RdRP* are detected.

Statistical analysis

The GraphPad Prism software, version 5.03 (GraphPad, San Diego, CA, USA) was used for comparisons of Ct values among three or more assays using the Kruskal–Wallis analysis of variance, complemented with the Dunn's multiple comparison test, and the Mann-Whitney U-test for the comparison of two assays. Results with a p-value of ≤ 0.05 were considered statistically significant. Analysis of repeatability and reproducibility were performed by calculating the mean Ct value, the standard deviation (SD), and the coefficient of variation in percentage (CV). The Ct total values considered the mean, SD and CV obtained in each reaction.

The Epi Info, version 3.5.4 software (Atlanta, GA, USA) was employed for the comparative analysis of the sample final result by each RNA extraction protocol and assay/kit, and determined the sensitivity, specificity, and Cohen's Kappa values, which were interpreted as follows: perfect ($\kappa = 1.0$), almost perfect ($1.0 < \kappa > 0.80$), substantial ($0.80 \leq \kappa > 0.60$), moderate ($0.60 \leq \kappa > 0.40$), fair ($0.40 \leq \kappa > 0.20$), slight ($0.20 \leq \kappa > 0$), or poor ($\kappa = 0$). Differences in sensitivity of the assays were statistically evaluated using the Chi-square test or Fisher's exact test, as appropriate. Statistical significance was set at $p \leq 0.05$.

For the cost-effectiveness evaluation of the three RNA extraction protocols both, cost and consequences were considered (cost in American dollars of each assay/kit acquired in May 2020, number of samples analyzed in each protocol, time consumption, ease of performing, and the assay sensitivity and specificity)[11].

Ethical approval

All procedures were performed according to the principles established in the Declaration of Helsinki of 1975, as revised in 1975, 1983, 1989, 1996, and 2000. The study was approved by the IAL Ethics Committee for Research CTC Nº 21M-2020 under the protocol Nº CAAE – 33282820.8.0000.0059.

RESULTS

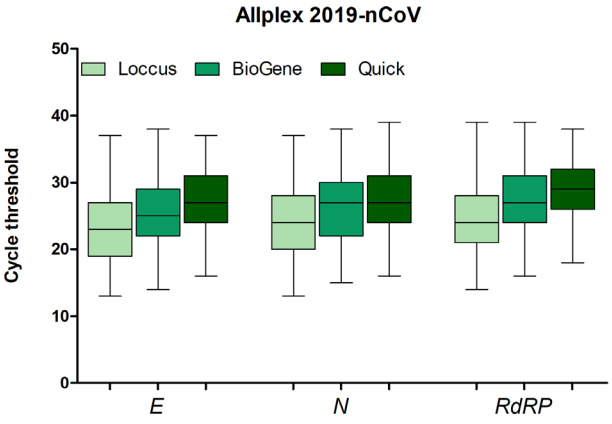
Table 1 presents the final SARS-CoV-2 results in 300 NP/OP samples using three different RNA extraction procedures and tested with two different molecular kits/protocols. These results considered the manufacturer's

criteria, and showed that, despite the same efficiency of Loccus and Biogene RNA extraction procedures, more SARS-CoV-2 positive samples were found using the Allplex kit compared to the Charité-Berlin protocol: 272/300 (90.7%) vs. 210/300 (70.0%), respectively. The ability to detect SARS-CoV-2 positive samples was lower with the Quick RNA extraction procedure, particularly when the Charité-Berlin protocol was employed: 96/300 (32.0%) (Table 1).

When analyzing the Ct values of the positive samples for the RNA extraction procedure and diagnostic kit/protocol, low median Ct values (higher SARS-CoV-2 viral load) were detected using the Loccus RNA extraction procedure, irrespective of the target gene analyzed and kit/protocol employed for diagnosis: *E*, *N*, and *RdRP* in the Allplex kit (Figure 1), and *E* and *RdRP* in the Charité-Berlin protocol (Figure 2). In contrast, high Ct values were detected when using the Quick RNA extraction procedure, except for the *RdRP* target gene using the Charité-Berlin protocol, which resulted in Ct values similar to those detected by the BioGene extraction procedure (Figure 2). Overall, almost all comparative analyses of Ct values revealed statistically significant differences ($p \leq 0.001$) (Figures 1 and 2).

Statistically significant differences were detected when the Ct values of *E*- and *RdRP* target genes were analyzed using the Allplex kit and the Charité-Berlin protocol. For the *E* gene, irrespective of the RNA extraction procedure, low Ct values were detected using the Allplex kit (Figure 3A). In contrast, when the Ct values for the *RdRP* gene were analyzed, low Ct values were detected using the Charité-Berlin protocol, showing significant differences in samples in which RNA was extracted using the BioGene and the Quick procedures (Figure 3B).

Details concerning the results shown in Figures 1, 2, and 3 are presented in Table 2. Overall, the median Ct values varied from 23 to 29, and the mean Ct values ranged from 23.33 to 29.13. A lower CV was detected for the *RdRP* target gene; nonetheless, this target gene showed fewer positive results in both kits/protocols. In general, the major



Statistic Analysis Allplex Assay		
Gene *E*		
Kruskal-Wallis test	P value	0.0001
Dunn's Multiple Comparison Test:	Significant P < 0.05?	Summary
Loccus vs BioGene	Yes	***
Loccus vs Quick	Yes	*
Gene *N*		
Kruskal-Wallis test	P value	<0.0001
Dunn's Multiple Comparison Test:	Significant P < 0.05?	Summary
Loccus vs BioGene	Yes	***
Loccus vs Quick	Yes	***
Gene *RdRP*		
Kruskal-Wallis test	P value	<0.0001
Dunn's Multiple Comparison Test:	Significant P < 0.05?	Summary
Loccus vs BioGene	Yes	***
Loccus vs Quick	Yes	***
BioGene vs Quick	Yes	**

Figure 1 - Cycle threshold (Ct) values obtained for samples that were positive to different SARS-CoV-2 target genes using the Loccus, BioGene, and Quick RNA extraction procedures, and the Allplex 2019-nCoV assay. Differences statistically significant are depicted as $*p < 0.05$, $**p \leq 0.001$, and $***p \leq 0.0001$ using the Kruskal–Wallis test complemented with Dunn's multiple comparison test.

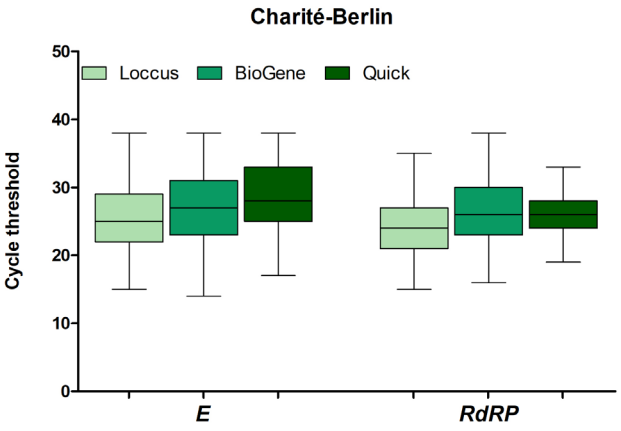
Ct values were detected using the Quick RNA extraction procedure (Table 2).

Concerning the number of target genes detected in SARS-CoV-2 positive samples using the Allplex kit (n = 272), no sample fell in the "presumptive positive" condition (positivity only for the E gene) irrespective of the RNA extraction procedure. The majority of samples were positive for the three target genes (*E*, *RdRP* and *N*), and only

Table 1 - Results of SARS-CoV-2 molecular diagnosis using samples from Sao Paulo, Brazil and different RNA extraction procedures.

RNA extraction procedure	Kit/Protocol	Positive	Negative	Total	Positivity in percentage
Loccus		272	28	300	90.7
BioGene	Allplex (Korea)	272	28	300	90.7
Quick		248	52	300	82.7
Loccus		210	90	300	70.0
BioGene	Charité (Berlin)	209	91	300	69.7
Quick		96	204	300	32.0

Criteria for positivity as described in Materials and Methods



Statistic Analysis Charité Assay		
Gene *E*		
Kruskal-Wallis test	P value	<0.0001
Dunn's Multiple Comparison Test:	Significant P < 0.05?	Summary
Loccus vs BioGene	Yes	*
Loccus vs Quick	Yes	***
BioGene vs Quick	Yes	***
Gene *RdRP*		
Kruskal-Wallis test	P value	<0.0001
Dunn's Multiple Comparison Test:	Significant P < 0.05?	Summary
Loccus vs BioGene	Yes	***
Loccus vs Quick	Yes	***

Figure 2 - Cycle threshold (Ct) values obtained for samples that were positive to different SARS-CoV-2 target genes using the Loccus, BioGene, and Quick RNA extraction procedures, and the Charité-Berlin protocol. Differences statistically significant are depicted as $*p < 0.05$, $**p \leq 0.001$, and $***p \leq 0.0001$ using the Kruskal–Wallis test complemented with Dunn's multiple comparison test.

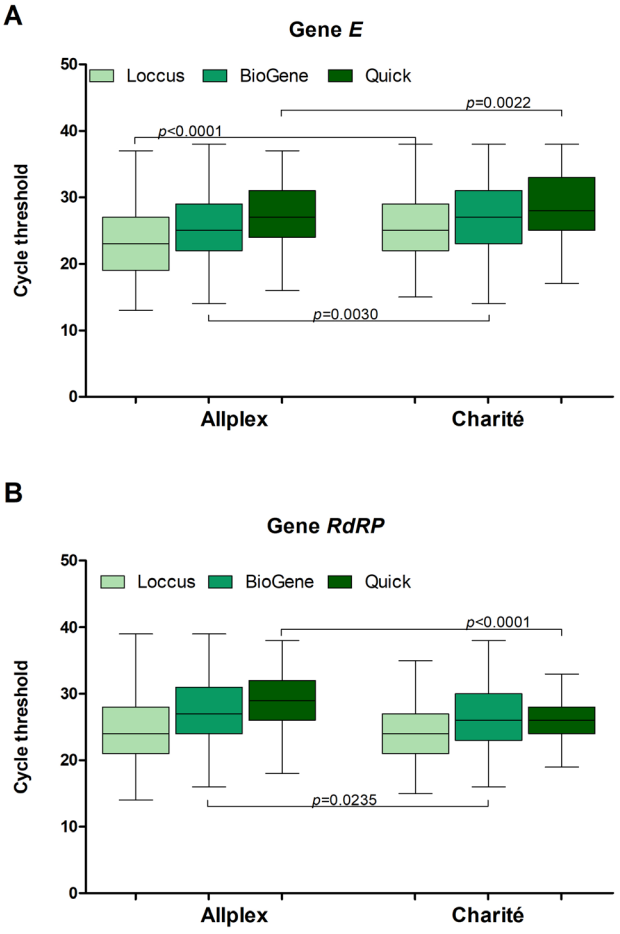


Figure 3 - Cycle threshold (Ct) values obtained for samples that were positive to *E*- and *RdRP* target genes using the Loccus, BioGene, and Quick RNA extraction procedures, tested by the Allplex 2019-nCoV assay and Charité-Berlin protocol. Differences statistically significant were depicted and detected using the Mann-Whitney U-test.

12 samples were positive for two targets [four (*E* and *N*) and eight (*RdRP* and *N*)], and 13 for only one target (*N*) when using RNA obtained with the Loccus extraction procedure. Using the BioGene RNA extraction, the majority of samples were positive for three targets, eight for two targets [six (*E* and *N*) and two (*RdRP* and *N*)], and 14 samples for only one target (*N*). By the Quick extraction, although there were fewer positive cases (n = 248), the majority of samples were positive for three target genes, two samples for two targets [one (*E* and *N*) and one sample for (*RdRP* and *N*)], and 44 samples for only one target (*N*).

When the results were analyzed using the Charité-Berlin protocol, 275, 277, and 229 samples were positive for the *E* gene when using RNA extracted by Loccus, BioGene, and Quick procedures, respectively, whereas only 210, 209, and 96 samples were confirmed SARS-CoV-2 positive for the *RdRP* gene (Table 2), showing a lower sensitivity of the *RdRP* target gene and a possible interference of residues present in the extracted RNA using the Quick protocol.

All samples considered truly positives (n = 272) or negatives (n = 28) for SARS-CoV-2 amplified the IC (*RNaseP* in this study), and low Ct values were detected in SARS-CoV-2 negative samples with statistically significant differences compared to SARS-CoV-2 positive samples (Figure 4). Table 3 shows the Ct values obtained by the IC *RNase* gene protocol in detail, confirming the presence of RNA in such samples.

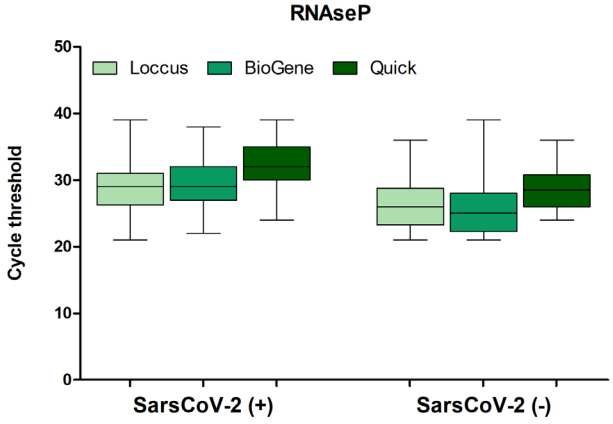
We sequenced SARS-CoV-2 of several samples that presented with discordant results in the diagnostic assays and RNA extraction procedures, as follows: Allplex positive and Quick RNA extraction negative (n = 4), Allplex and Charité positive and Loccus positive but BioGene extraction negative (n = 1), and Allplex positive and Charité negative irrespective of the RNA extraction procedure (n = 29). Sequencing was performed using cDNA samples obtained by the SuperScript IV Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations, and the whole genome sequencing was carried out using an Ion Torrent S5 platform and the AmpliSeq™ SARS-CoV-2 (Thermo Fisher

Table 2 - Analysis of the cycle threshold (Ct) values obtained for each SARS-CoV-2 target gene using three RNA extraction procedures and two RT-qPCR assays.

Assay	Allplex 2019-nCoV - Seegene (N=272 +)									Charité-Berlin protocol (N=210 +)					
Gene	*E*			*N*			*RdRP*			*E*			*RdRP*		
RNA extraction procedure	Loccus	BioGene	Quick	Loccus	BioGene	Quick	Loccus	BioGene	Quick	Loccus	BioGene	Quick	Loccus	BioGene	Quick
N+	251	255	198	266	270	248	255	250	195	275	277	229	210	209	96
Minimum	13	14	16	13	15	16	14	16	18	15	14	17	15	16	19
25% percentile	19	22	24	20	22	24	21	24	26	22	23	25	21	23	24
median	23	25	27	24	27	27	24	27	29	25	27	28	24	26	26
75% percentile	27	29	31	28	30	31	28	31	32	29	31	33	27	30	28
maximum	37	38	37	37	38	39	39	39	38	38	38	38	35	38	33
Mean	23.33	25.45	27.17	24.48	26.48	27.38	24.8	27.51	29.13	25.64	26.8	28.61	24.19	26.52	25.95
Std. deviation	4.955	4.906	4.1	4.97	5,045	4.835	5.224	4.828	4.218	4.96	5.167	4.725	3.906	4.308	3.048
Std. Error	0.313	0.3072	0.2914	0.3047	0.307	0.307	0.3272	0.3054	0.3021	0.2991	0.3105	0.3122	0.2695	0.298	0.3111
Lower 95% CI of mean	22.71	24.85	26.6	23.89	25.87	26.78	24.16	26.91	28.53	25.05	26.19	27.99	23.66	25.93	25.33
Upper 95% CI of mean	23.94	26,.06	27.75	25.08	27.08	27.99	25.44	28.11	29.72	26.23	27.41	29.22	24.72	27.11	26.57
Coefficient of variation	21.24%	19.27%	15.09%	20.30%	19.05%	17.66%	21.07%	17.55%	14.48%	19.35%	19.28%	16.52%	16.15%	16.24%	11.75%
Geometric mean	22.8	24.97	26.85	23.98	25.99	26.95	24.26	27.08	28.82	25.15	26.28	28.21	23.87	26.17	25.77
Lower 95% CI of geo. mean	22.2	24.37	26.27	23.39	25.39	26.35	23.65	26.48	28.23	24.57	25.67	27.59	23.35	25.59	25.15
Upper 95% CI of geo. mean	23.42	25.59	27.44	24.58	26.6	27.56	24.9	27.69	29.42	25.75	26.91	28.83	24.41	26.76	26.4

N+ = number of RT-qPCR positive samples; *E* = envelope gene; *N* = nucleocapsid; *RdRP* = RNA-dependent RNA polymerase; Std. = standard; CI = confidence interval; geo. = geometric

Scientific, Waltham, MA, USA), which allows a complete sequencing of SARS-CoV-2 lineages without gaps. Thereafter, these sequences were sent to the Global Initiative on Sharing Avian Influenza Data, Accession Numbers: EPI_ISL_693215 to EPI_ISL_693243 (Allplex positive and Charité negative), EPI_ISL_693244 to EPI_ISL_693247 (Allplex positive and Quick RNA negative), and EPI_ISL_693248 (Allplex and Charité positive and Loccus positive, but BioGene negative).



Statistic Analysis RNAseP Assay		
Gene *RNAseP* - SarsCoV-2 (+)		
Kruskal-Wallis test	P value	<0.0001
Dunn's Multiple Comparison Test:	Significant P < 0.05?	Summary
Loccus vs Quick	Yes	***
BioGene vs Quick	Yes	***

Figure 4 - Cycle threshold (Ct) values obtained for samples that were positive and negative to SARS-CoV-2 using the Loccus, BioGene, and Quick RNA extraction procedures, and the IC corresponding to the *RNase* target gene amplification. Differences statistically significant are depicted as ***$p \leq 0.0001$ using the Kruskal–Wallis test complemented with Dunn's multiple comparison test.

In summary, considering the results of the Allplex kit as true as it has detected more SARS-CoV-2 target genes and showed the best performance in a previous comparative study of seven kits/assays employed in Sao Paulo, Brazil for COVID-19 diagnosis[8], and was able to detect SARS-CoV-2 by sequencing from several samples that presented discordant results in the diagnostic assays and RNA extraction procedures, we calculated the sensitivity and specificity of the assays for each RNA extraction procedure, and the Cohen's Kappa index (κ) of the assays. Using the Allplex kit, the sensitivity in detecting SARS-CoV-2 using the RNA extracted by different procedures was 100.0% for Loccus, 100.0% for BioGene, and 91.9% for Quick. Using the Charité-Berlin protocol, the sensitivity dropped to 81.4% for Loccus, 81.2% for BioGene, and 60.7% for Quick (all $p < 0.000$). The lowest sensitivity of target gene detection that was most affected by RNA extraction procedures was the *RdRP* gene of the Charité-Berlin protocol. No false-positive SARS-CoV-2 results were detected using the three RNA procedures (specificity of 100.0%). Using the Allplex kit, a perfect agreement of results (κ = 1.0) was detected

Table 3 - Analysis of the cycle threshold (Ct) values using three RNA extraction procedures in the amplification of the *RNAseP* target gene.

Assay	In house					
gene	*RNAseP* SARS-CoV-2 (+)			*RNAseP* SARS-CoV-2 (-)		
RNA extraction procedure	Loccus	BioGene	Quick	Loccus	BioGene	Quick
Minimum	21	22	24	21	21	24
25% percentile	26,25	27	30	23.25	22.25	26
median	29	29	32	26	25	28.5
75% Percentile	31	32	35	28.75	28	30.75
maximum	39	38	39	36	39	36
Mean	29	29.41	31.98	26.25	26.07	28.75
Std. deviation	3.464	3.638	3.253	3,.884	4.396	3.513
Std. error	0.21	0.2206	0.1972	0.734	0.8308	0.6639
Lower 95% CI of mean	28.58	28.98	31.59	24.74	24.37	27.39
Upper 95% CI of mean	29.41	29.85	32.37	27.76	27.78	30.11
Coefficient of variation	11.94%	12.37%	10.17%	14.80%	16.86%	12.22%
Geometric mean	28.79	29.19	31.81	25.99	25.75	28.55
Lower 95% CI of geo. mean	28.38	28.76	31.42	24.58	24.22	27.25
Upper 95% CI of geo. mean	29.2	29.62	32.2	27.47	27.37	29.91

+ samples considered positive for SARS-CoV-2 in the RT-qPCR assays (n = 272, according to Materials and Methods); - samples considered negative for SARS-CoV-2 in the RT-qPCR assays (n = 28, according to Materials and Methods); Std. = standard; CI = confidence interval; geo. = geometric

using Loccus and BioGene RNA extractions, and an almost perfect agreement ($\kappa = 0.925$) using Quick extraction. When the Charité-Berlin protocol was employed, substantial agreement of results was detected using different RNA extraction protocols: Loccus $\kappa = 0.825$, BioGene $\kappa = 0.823$ and Quick $\kappa = 0.621$.

The cost-effectiveness of the three RNA extraction procedures was calculated, and the results are presented in Table 4. Considering the costs of reagents and supplies, ease of performance, time consumption, results obtained (sensitivity of SARS-CoV-2 detection), and the number of tests released during outbreaks, the Loccus protocol was the best choice.

DISCUSSION

In view of the COVID-19 pandemic in Brazil, especially in Sao Paulo[5-7], continuous studies searching for ways to save time and costs in SARS-CoV-2 diagnosis are required.

Therefore, we have studied the performance and cost-effectiveness of assays/kits employed in SARS-CoV-2 RNA gene detections in samples from suspected cases of COVID-19 in Sao Paulo, Brazil, and observed that one of the bottlenecks in such diagnosis is the shortage of RNA extraction kits and supplies, and their time-consuming characteristics because thousands of samples must be processed daily during outbreaks.

Table 4 - Cost-effectiveness of the three RNA extraction procedures employed in SARS-CoV-2 molecular diagnosis using the Allplex 2019-nCoV assay

Parameters	Loccus procedure	BioGene procedure	Quick procedure
Number of steps	4	4	2
Time consumption	1 h	2.5 h	30 min
Ease of performance	high	medium	high
Number of extracted samples	32	32	32
Cost of reagents and supplies (kit) in American dollars ($)	3,429.12	7,021.80	31,020.00
Cost per extraction in American dollars ($)	107.16	28.09	93.15
Sensitivity when applied in SARS-CoV-2 diagnosis	100.0%	100.0%	91.9%
Limitations/risk	Equipment High cost	Handling and hazard	High cost of buffer False-negative results

Since the beginning of the pandemic, we have had the opportunity to use several kits for RNA extraction (national and from abroad), and for this study we selected two of Brazilian kits, which were easier to handle, being one automated and one manual: Loccus, kit Extracta Fast, and Loccus Extracta 32 equipment, SP, Brazil, and BioGene Extração DNA/RNA viral kit, Bioclin Quibasa, MG, Brazil, respectively. In May 2020, a rapid protocol that circumvented the need for RNA extraction, which is compatible with RT-qPCR-based methods, was published[12]; thus, we decided to add this protocol in the comparative analyses. We tested samples from a biorepository with two RT-qPCR kit/protocols, considering the possibility of discontinued supply of the Allplex kit by the Brazilian Ministry of Health.

Herein, we considered the results obtained by Allplex kit and Loccus RNA extraction as truly positive and negative samples, and calculated the sensitivity, specificity, and Kappa index of the assays. We adopted this criteria considering: (i) the laboratory experience and previous results[8], (ii) the majority of SARS-CoV-2 positive samples (more than 90.0%) were positive for the three target genes using this kit and the Loccus extraction procedure, and (iii) the capability of sequencing SARS-CoV-2 in several samples that presented discordant results among diagnostic assays and RNA extraction procedures.

There was a low sensitivity of RT-qPCR assays and an increase in the Ct values when samples were subjected to the Quick RNA extraction procedure. This result can be explained, in part, by the presence of residues (a multitude of nucleic acids and proteins) in the samples or by RNA fragmentation during heating[13]. An increase of more than 3 Ct has been described in samples tested for SARS-CoV-2 without prior RNA extraction compared to samples that underwent RNA extraction by standard protocols[13,14].

The present study disclosed an overall increase of at least 2 Ct when the Quick procedure was employed, except for the *RdRP* gene using the Charité-Berlin protocol. However, only 96 samples were *RdRP*-positive; thus, only samples with high viral loads can be amplified using this protocol.

Another point to consider for discordant results is the length of the segment to be amplified (long segments are more prone to thermal shock degradation), and the presence of mutations affecting regions harboring primer-probe binding sites, mostly in the *RdRP* gene. The length of and the genomic segments amplified by the Allplex kit are not provided by the manufacturer, however, they differ from the segments amplified by the Charité-Berlin protocol (see differences in sensitivities and Ct values of *E*- and *RdRP* target genes using both protocols).

The literature refers to different sensitivities of assays for SARS-CoV-2 detection using standard RNA extraction protocols and protocols without RNA extraction, mostly when buffers are added to the samples[15]. In addition, low sensitivity of RT-qPCR assays has been associated with higher Ct values, i.e., samples that had Ct > 30 using standard RNA extraction protocols are probably going to be negative using quicker procedures[14-16].

Concerning the presence of mutations affecting regions harboring primer-probe binding sites, an *in silico* analysis of SARS-CoV-2 genomes from South America indicated abundant genomic diversity in the *RdRP* and *N* genes with respect to the *E* gene[17]. This finding highlighted a possible effect on the false-negative results when employing the *RdRP* target gene, and supports the results obtained here. To corroborate this finding, when comparing the analytical efficiencies and sensitivities of the primer-probe sets of the four most common SARS-CoV-2 qRT-PCR assays developed by CDC-China, CDC-USA, Charité-Berlin and Hong Kong University, the *E* gene Sarbeco (Charité) was the most sensitive, and the *RdRP*-SARS gene (Charité) was the least sensitive[18], again corroborating the results of the present study.

Future analysis of the 29 sequences that were positive using the Allplex kit and negative using the Charité-Berlin protocol could solve this issue, and confirm whether there are mutations in the regions harboring the primer/probes employed to amplify *RdRP* target gene corroborating the low sensitivity of the Charité-Berlin protocol when samples from Brazil were tested. A revised version of the PAHO/WHO laboratory guidelines recommended an algorithm using only the *E* target gene for the diagnosis of COVID-19 as only SARS-CoV-2 is infecting humans, and also due to the low sensitivity of the *RdRP* target gene used in the Charité-Berlin protocol[19].

Considering the Allplex kit results as the true results, the Cohen's Kappa index using RNA extracted by Loccus and BioGene procedures showed a perfect concordance ($\kappa = 1.000$), and using the Quick procedure, an almost perfect concordance ($\kappa = 0.925$), allowing the quick protocol to be used.

However, when analyzing the cost-effectiveness of RNA extraction procedures, the Quick RNA extraction, although having few steps and a slightly reduced time-consumption, it employs an expensive buffer (Lucigen) and has a lower sensitivity when compared to automated and manual procedures. The Loccus procedure, although presenting with the best performance, is expensive, and should be used when the demand for tests is very high and the result must be released in a short time during outbreaks and pandemics. The Loccus manufacturer, located in Sao Paulo, Brazil,

offers two Extracta types of equipment: Loccus Extracta 32 and Loccus Extracta 96, allowing RNA extraction of 32 or 96 samples in 1 h. In contrast, although the BioGene extraction procedure has more handling time and is more time-consuming, its high performance and lower cost (26% of the cost of the Loccus procedure) makes it a good choice, especially during a time of increasing health care expenditure and limited resources when outbreaks are under control.

Several recent studies have been conducted to bypass RNA extraction using only hot shock procedures without buffer addition[13-16,20-23]. Thus, we are now interested in comparing the performance of the Quick RNA extraction procedure and direct approaches with no buffer in order to save time and expenses during pandemics.

In conclusion, although less sensitive, the Quick RNA extraction procedure is useful during outbreaks with the Allplex kit. Loccus and BioGene RNA extraction protocols were accurate for RT-qPCR assays, and although the BioGene procedure is the least expensive, the Loccus extraction protocol is the best choice because it allows the rapid handling of hundreds or thousands of samples during pandemics.

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AUTHORS' CONTRIBUTIONS

KRC: designed the study, performed the laboratory experiments, acquisition and analysis of data, revision and editing of the manuscript; CTS: was responsible for the conception and design of the study, administration of the project, analysis of data, revision and editing of the manuscript; CRG, EVRGP and ASD: performed the laboratory experiments, revision and editing of the manuscript; LOF: was also responsible for the conception of the study, data analysis, revision and editing of the manuscript; ACA: helped in the acquisition and analysis of the data, manuscript writing, revision and editing of the manuscript.

CONFLICT OF INTERESTS

The authors declare that they do not have any commercial or other associations that might pose a conflict of interests to the funding sources reported.

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