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Alternative RNA extraction-free techniques for the real-time RT-PCR detection of SARS-CoV-2 in nasopharyngeal swab and sputum samples

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ABSTRACT

Standard diagnoses of SARS-CoV-2 infections are done by RNA extraction and real-time RT-PCR (rRT-PCR). However, the need for RNA extraction complicates testing due to increased processing time, high cost, and limited availability of commercial kits. Therefore, alternative methods for rRT-PCR detection of SARS-CoV-2 without RNA extraction were investigated. Nasopharyngeal and sputum samples were used to compare the sensitivity of three techniques: Trizol RNA extraction, thermal shock, and the direct use of samples with an RNase inhibitor. Direct, extraction-free use of primary samples plus the RNase inhibitor produced diagnostic values of 100 % sensitivity and specificity compared to standard protocols, and these findings were validated in a second, independent laboratory.

1. Introduction

Coronaviruses are RNA viruses belonging to the *Coronaviridae* family and are widely distributed in humans and other mammals (de Groot et al., 2012; Huang et al., 2020). Two betacoronaviruses had previously caused severe respiratory syndromes in humans: the severe acute respiratory syndrome-related human coronavirus 1 (SARS-CoV; species *Severe acute respiratory syndrome-related coronavirus*) (Drosten et al., 2003) and the Middle East respiratory syndrome-related coronavirus (MERS-CoV; species *Middle East respiratory syndrome-related coronavirus*) (de Groot et al., 2013; Ziebuhr et al., 2015). In December 2019, a second member of the *Severe acute respiratory syndrome-related coronavirus* species (SARS-CoV2) was identified in China as the cause of another severe coronavirus disease (COVID-19), which has spread throughout the world (Won et al., 2020). In March 2020, the World Health Organization (WHO) declared COVID-19 a pandemic, registering over 228 million confirmed cases of COVID-19 as of September 20th, 2021, including over 4,690,000 deaths (WHO, 2021). After more than a year of this pandemic, the Americas region reports 40 % of global confirmed cases (WHO, 2021). Ecuador had been hard-hit particularly, with over 505,000 confirmed COVID-19 cases, according to the report of the Ministry of Public Health (MSP) dated September 12th, 2021 (MSP, 2021).

The high transmission rate and case counts demand fast, affordable, and efficient diagnostic tests that provide reliable results at low cost to patients who are suspected of having the disease (Smyrlaki et al., 2020). Real-time reverse transcription PCR (rRT-PCR) is the standard diagnostic test for SARS-CoV-2 (WHO, 2020). Samples collected for rRT-PCR are processed by a number of methods to extract the viral RNA prior to converting it to cDNA with reverse transcriptase, and then PCR for specific SARS-CoV-2 targets using various primer-probe sets (Liu et al., 2020; WHO, 2020). Currently, the time required for SARS-CoV-2 molecular diagnosis and the shortage of reagents are limiting factors in large-scale COVID-19 diagnosis in the population. Diagnosing the virus without RNA extraction and purification steps reduces the time required for sample preparation and amplification, the cost for molecular testing, and the potential for human error. Several studies have detected the virus in saliva, nasopharyngeal, and oropharyngeal swab samples without RNA extraction, some of which have noted a decreased sensitivity (Beltrán-Pavez et al., 2020; Fomsgaard and Rosenstierne, 2020; Smyrlaki et al., 2020; Vogels et al., 2020). This could be due to the high content of RNases in these samples that degrade the genetic material of interest (Pandit et al., 2013). To address these limitations, this study described the standardization and validation of protocols for alternative

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sample processing techniques prior to SARS-CoV-2 rRT-PCR, using detection methods with and without RNA extraction from nasopharyngeal and sputum samples. Specifically, we evaluated two different extraction protocols, two extraction-free protocols, and two different rRT-PCRs using nasopharyngeal and sputum samples, and the best-performing protocol was then evaluated at a second laboratory. Our aim was to obtain a protocol that could be used regardless of the type of sample (nasopharyngeal swabs or sputum samples), and that proved to be reproducible.

2. Materials and methods

2.1. Clinical samples

A total of 192 clinical samples were used in this study, 153 clinical samples were collected and processed in Ecuador and 39 samples were collected and processed in USA. The 153 clinical samples were sent for respiratory virus testing and SARS-CoV-2 rRT-PCR assays at the National Reference Laboratory for Influenza and other Respiratory Viruses at the National Institute of Research for Public Health (Quito-Ecuador) and the test results from this laboratory were used as the reference for the set-up of the current study. This first group consisted of 123 samples that tested positive for SARS-CoV-2 (nasopharyngeal n = 73, and sputum n = 50) and 30 samples that tested negative for SARS-CoV-2 (nasopharyngeal n = 20 and sputum n = 10). The remaining group of 39 clinical samples were nasopharyngeal samples that were sent to Emory University for respiratory virus testing; these included 19 samples that tested positive for SARS-CoV-2 and 20 samples that tested negative for SARS-CoV-2 but were positive for another respiratory pathogen.

Nasopharyngeal samples were collected using FLOQswabs (Copan Diagnostics Inc). Each swap sample was added to a 4-ml tube containing three ml of virus transport media (VTM) or PBS. For sputum samples, each patient was asked to cough up a deep sputum sample from the lower respiratory tract, which was collected in a sterile 50-ml plastic tube. Around three ml of sputum were collected per patient. Before processing, two ml of sterile PBS were added to each sample and mixed by vortex for one minute to disrupt any aggregates. Given the important impact that data from this study could have on public health, all samples from COVID-19 positive patients were de-identified, and this was not considered human subjects research at INSPI or Emory University.

2.2. RNA extraction protocols

Two methods were used for RNA extraction: a commercial kit for the extraction of viral RNA (Qiagen Viral RNA Mini Kit), and the Trizol LS Reagent (Invitrogen) for total RNA extraction. Both methods were performed according to the manufacturers' instructions for RNA extraction from 200 µL of sample and nucleic acids elution in 60 µL. RNA extraction with the Qiagen Viral kit was used as the reference technique for the initial evaluation of the sample processing protocols used in this study, including the extraction-free methods described below.

2.3. Extraction-free protocols

Two different sample treatment methods were tested to replace the RNA extraction step: thermal shock and direct use of the sample (direct technique). For thermal shock, 100 µL of the collected sample was heat-treated either at 99 °C for 5 min (TS1) or 95 °C for 15 min (TS2) (Fomsgaard and Rosenstierne, 2020; Smyrlaki et al., 2020). At the end of each heat-treatment, the samples were incubated at 4 °C for 5 min. For the direct technique, 5 µL of the untreated sample was used in the rRT-PCR reaction.

2.4. rRT-PCR protocols for the detection of SARS-CoV-2

Two rRT-PCR protocols were used to detect SARS-CoV-2. The first protocol involved a commercial detection kit with targets in the *ORF1ab* and nucleocapsid (*N*) genes (DA0930; Da An Gene Co. Ltd. of Sun Yat-sen University, https://www.who.int/diagnostics_laboratory/eual/eul_0493_141_00_detection_kit_for_2019_ncov_rna_pcr_flourescence_probing.pdf?ua=1). Reactions were prepared according to the manufacturer's specifications: 20 µL of the master mix and 5 µL of each RNA, extraction-free sample, or control were dispensed into each well. Samples were run in duplicate. For the direct technique, 1U of RNase inhibitor (M0314S, New England BioLabs) was added to the master mix. Cycling conditions were the following: 50 °C for 15 min, 95 °C for 15 min, and 45 cycles of 94 °C for 15 s and 55 °C for 45 s. The rRT-PCR was done on a Bio Rad CFX96.

The second rRT-PCR protocol used an in-house assay with a target in the *N* gene. Briefly, the SuperScript™ III Platinum™ One-Step RT-qPCR Kit (Invitrogen) was used. Each reaction contained 1X reaction mix, 800 nM magnesium sulfate, 500 nM of each primer for the gene *N* (HKU-NF 5'- TAATCAGACAAGGAACTGATTA-3', HKU-NR 5'- CGAAGGTGT-GACTTCCATG -3'), 250 nM probe (HKU-NP 5'-[FAM]GCAAATTGTG-CAATTTGCGG[TAM]-3'), and 1 µL of SuperScript enzyme mix. Twenty µL of the master mix and 5 µL of RNA or extraction-free sample were dispensed into each well. Samples were run in duplicate. For the direct technique, 0.6 U of RNase inhibitor was added to the master mix. The thermal cycling profile was 50 °C for 15 min, 95 °C for 15 min, 45 cycles of 95 °C for 15 s and 60 °C for 45 s.

2.5. Comparative experiments

Three comparative experiments were performed using the 123 clinical samples (73 nasopharyngeal and 50 sputum samples) that tested positive for SARS-CoV-2 (reference positive) at the National Institute of Research for Public Health (Quito-Ecuador).

In the first experiment, 23 of the nasopharyngeal samples were processed using the two RNA extraction methods and the two extraction-free protocols. SARS-CoV-2 assays of these methods were performed with the commercial rRT-PCR protocol as described above.

The second experiment compared SARS-CoV-2 detection using either thermal shock or direct technique samples as template for the rRT-PCR assays. Here, another 50 nasopharyngeal and 50 sputum samples (reference positives, n = 100) were selected based on their cycle threshold (Ct) values obtained for SARS-CoV-2 detection by the National Reference Lab. Twenty-five samples of each sample type (50 %) had a Ct value <30 (high virus levels; Ct range, 17.0–29.77), and the remaining half of the samples had Ct values >30 (low virus levels; Ct range, 30.14–37.80). Detection of SARS-CoV-2 was done using the in-house rRT-PCR protocol and the results were compared to those obtained at the National Reference Lab.

Finally, a third experiment was done to determine the need for the addition of RNAse inhibitor in the detection of SARS-CoV-2. This was done by comparing the amplification results of 20 of the reference positive samples from previous experiments (10 nasopharyngeal and 10 sputum samples) with and without RNAse inhibitor. Detection of SARS-CoV-2 was done using the in-house rRT-PCR protocol as previously described.

2.6. Specificity determination of the direct technique

A total of 50 clinical samples were used to evaluate the specificity of the direct technique. Thirty clinical samples (20 nasopharyngeal and 10 sputum samples) that tested negative for SARS-CoV-2 (reference positive) at the National Institute of Research for Public Health (Quito-Ecuador) were tested at INSPI using the in-house rRT-PCR protocol for detection of SARS-CoV-2. Additionally, 20 clinical samples that were positive for another respiratory pathogens were tested at Emory University using the in-house rRT-PCR protocol.

2.7. Independent inter-laboratory evaluation of the direct technique

The second group of clinical nasopharyngeal samples (positive n = 19, and negative n = 20) were collected and processed at the Emory University to validate the direct technique for the detection of SARS-CoV-2 (see Section 2.1). For this evaluation, the direct technique was compared to results from a laboratory developed protocol for SARS-CoV-2 detection, which is distinct from that described in Section 2.4 (Waggoner et al., 2020). Briefly, the laboratory-developed protocol involved total nucleic acid extraction from a 500 µL of sample on an eMAG instrument (bioMerieux) with a 50 µL elution volume, followed by rRT-PCR assay for the *N2* target in the SARS-CoV-2 genome and using the *RNase P* gene as a specimen control.

2.8. Statistical analysis

Data were expressed as mean ± SEM, and r^2. The data were tested for normality of distribution using the D'Agostino & Pearson test with a confidence interval of 95 %. Statistical comparison between two groups were performed by paired *t*-test. Two-tailed Pearson's correlation statistic was used for correlation between Ct values of the different techniques. All statistics and graphs were performed with GraphPad Prism 8.4.3 (La Jolla, USA); $p < 0.05$ was considered statistically significant.

3. Results

3.1. Sensitivity of detection of the direct technique compared to other sample processing systems

The reference SARS-CoV-2 positive nasopharyngeal samples (n = 23) which were subjected to the four different extraction techniques, were tested by the commercial detection kit that targets the *ORF1ab* and *N* genes. The direct technique yielded 100 % sensitivity compared to RNA extracted with the Viral RNA Mini Kit. Trizol RNA extraction gave the second-best sensitivity results (96 %), while both thermal shocks techniques yielded a sensitivity below 90 % (Table 1; Fig. 1).

In a comparison of Ct values, Trizol RNA extraction showed a mean increase in Ct of 1.92 (SEM 0.90) for the *ORF1ab* target ($p < 0.05$) but no significant difference in Ct values for the *N* target (Fig. 1A). Both thermal shock techniques exhibited a mean increase in Ct values for both targets of more than 2.88 cycles (TS1: *ORF1ab* SEM 0.75, *N* SEM 0.95; TS2: *ORF1ab* SEM 0.81, *N* SEM 0.85; $p < 0.01$; Fig. 1C and D). The direct technique yielded a mean increase in Ct values for the *N* and *ORF1ab* targets of 1.41 (SEM 0.50) and 1.87 (SEM 0.62) cycles, respectively, compared to the reference technique ($p < 0.05$; Fig. 1B). Furthermore, the quantitative detection of *N* and *ORF1ab* genes was positively correlated between the reference viral RNA extraction technique and each technique our study tested (Pearson's correlation, $p < 0.001$).

Table 1
Sensitivity of RNA extraction and extraction-free techniques using nasopharyngeal and sputum samples. Results of amplification with detection kit of Sun Yat-sen University and in-house rRT-PCR targeting *N* gene for all samples.

Sample	Technique	Total (n)	Positive samples	False negative samples	Sensitivity (%)
Detection kit of Sun Yat-sen University					
Nasopharyngeal	Trizol	23	22	1	95.7
	TS 1	23	19	4	82.6
	TS 2	23	19	4	82.6
	Direct	23	23	0	100.0
In-house rRT-PCR					
Nasopharyngeal	TS 1	50	44	6	88.0 %
	Direct	50	50	0	100 %
Sputum	TS 1	50	45	5	90.0 %
	Direct	50	50	0	100.0

Abbreviations: TS, thermal shock.

3.2. Extraction-free high-sensitivity detection technique for nasopharyngeal and sputum samples

Additional SARS-CoV-2-positive samples were tested using the TS1 and direct technique, including 50 nasopharyngeal and 50 sputum samples, and results were compared to test results obtained at the National Reference Laboratory. The direct technique demonstrated 100 % sensitivity with amplification of a target in the *N* gene (Table 1; Fig. 2). The TS1 technique yielded a sensitivity of 88 % for nasopharyngeal and 90 % for sputum. Furthermore, mean Ct values were 2.4 (SEM 0.62) and 3.1 (SEM 0.71) cycles higher when nasopharyngeal or sputum samples, respectively, were treated with TS1 compared to the direct technique ($p < 0.001$; Fig. 2).

Moreover, the comparison between the use of the direct technique with and without RNAse inhibitor showed that the mean Ct value was 3.47 (SEM 0.47) cycles higher in samples without RNAse inhibitor ($p < 0.0001$; Fig. 3).

3.3. Specificity of extraction-free detection

To determine the specificity of the direct technique, 50 clinical samples were tested for targets in the *N* gene. No false-positive detection was observed with these samples using the direct technique (100 % specificity; Table 2).

3.4. Independent direct technique evaluation

Finally, the direct technique was independently evaluated in a second laboratory. The direct technique detected 19/19 (100 %) nasopharyngeal samples that had tested positive for SARS-CoV-2 across a range of Ct values (22.0–36.2) in a reference, laboratory-developed protocol. Ct values were, on average, 4.79 cycles (SEM 0.51) later with the direct method for this comparison ($p < 0.001$, Supplementary Fig. S1). However, the comparator method for this protocol utilized an automated extraction instrument with 500 µL of sample volume (100-times the volume added to each reaction for the direct technique).

4. Discussion

Due to the rapid spread of SARS-CoV-2, there is a critical need for maximizing detection while reducing test costs, reagent use and turn-around-time to minimize the impact of this pandemic on global health. Here, we present an alternative RNA-extraction-free technique for the detection of SARS-CoV-2 by rRT-PCR that can be implemented at a lower cost than conventional techniques and demonstrated equivalent qualitative performance to reference SARS-CoV-2 rRT-PCR protocols at two independent institutions.

Studies have tested the use of direct nasopharyngeal samples (Beltrán-Pavez et al., 2020), or the use of a detergent-containing buffer for direct lysis (Smyrlaki et al., 2020) followed by rRT-PCR detection. However, there are several factors that can interfere in the direct detection of SARS-CoV-2. For instance, detection can be compromised as SARS-CoV-2 virus may not be stable in these solutions and the genome can be easily degraded by RNases (Wang et al., 2020). In this regard, our results showed that direct amplification without RNAse inhibitors significantly increased Ct values by an average of 3.59 cycles. Hence, the use of RNAse inhibitor helps protect the viral genome and preserve detection of the virus in samples with low viral loads. Furthermore, our study presents a direct detection technique that can preserve the sensitivity (100 %) and specificity (100 %) of rRT-PCR reactions when performed without dedicated extraction. This technique likely relies on the detection of RNA from cell-free virus in the sample matrices, and it can be used on nasopharyngeal and sputum samples, thereby eliminating the time and reagents necessary for RNA extraction. Additionally, the cost of

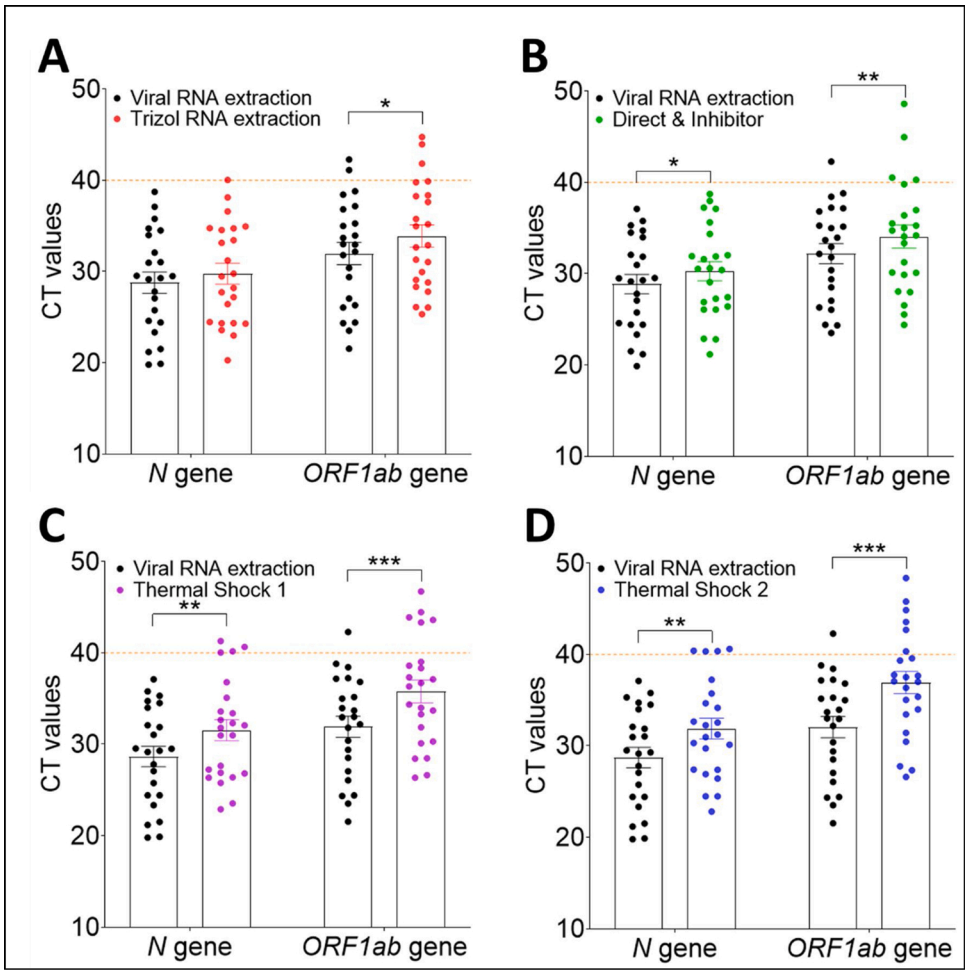

Fig. 1. Comparative distribution of cycle threshold (Ct) values obtained by rRT-PCR of the *N* and *ORF1ab* genes using conventional RNA extraction methods and extraction-free techniques. Nasopharyngeal samples (n = 23) were processed by four different methods prior to rRT-PCR assays. Viral RNA extraction using a commercial kit was used as the reference method (black circle), results were compared to the following: RNA extraction with Trizol (red circle) **(A)**; Direct sample with RNAse inhibitors (green circle) **(B)**, Thermal shock of 99 °C for 5 min (Thermal Shock 1, purple circle) **(C)**, and Thermal shock of 95 °C for 15 min (Thermal Shock 2, blue circle) **(D)**. Ct values ≥ 40 were considered a negative result (dotted orange line). Mean ± SEM. Paired *t*-test student (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

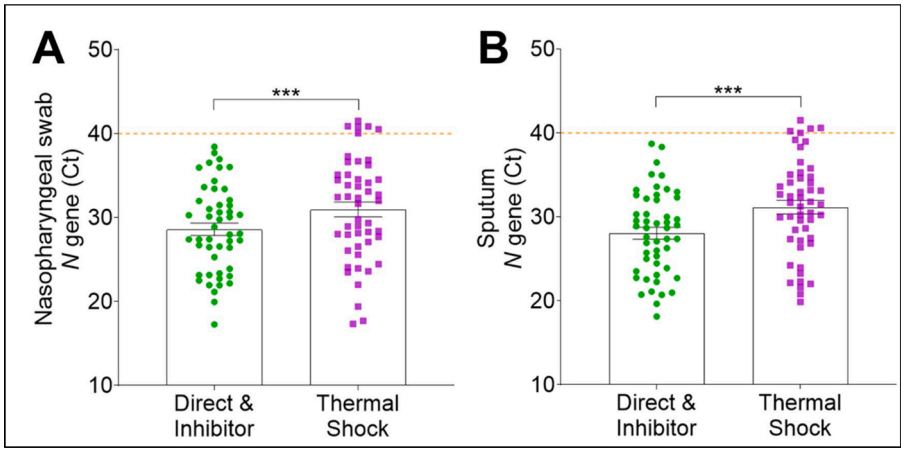

Fig. 2. Cycle threshold (Ct) values obtained for *N* gene using the direct technique or thermal shock (TS1). Nasopharyngeal (n = 50) **(A)** and sputum (n = 50) **(B)** samples were tested directly by rRT-PCR plus RNAse inhibitors (green circle) or processed by thermal shock of 99 °C for 5 min (Thermal Shock 1, purple square) prior to testing. Ct values ≥ 40 were considered a negative result (dotted orange line). Mean ± SEM. Paired *t*-test student (*** $p < 0.001$).

RNA extraction using a commercial kit was 12.42 USD per sample in Ecuador at the time of the study, and the cost of RNase inhibitor was 0.22 USD per sample. Therefore, our direct technique represents a significant reduction in the costs of detection.

Several studies have tried different thermal shock protocols as an extraction-free technique to detect SARS-CoV-2 (Beltrán-Pavez et al., 2021; El-Kafrawy et al., 2021; Fomsgaard and Rosenstierne, 2020; Pearson et al., 2020; Smyrlaki et al., 2020). Based on these data, we tested two different thermal shock techniques. Our results showed that these techniques, regardless of the temperature or the duration, increased the Ct values by more than 2.9 cycles and had reduced sensitivity (below 90 %). Previous studies have reported sensitivity values ranging from 51 % (Beltrán-Pavez et al., 2021) to 91.4 % (Smyrlaki et al., 2020) when nasopharyngeal samples have been treated

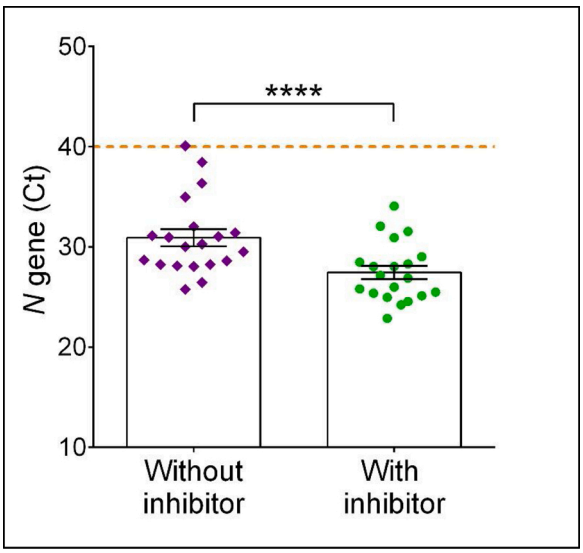

Fig. 3. Cycle threshold (Ct) values obtained for *N* gene using a direct technique with and without RNAse inhibitor. Nasopharyngeal (n = 10) and sputum (n = 10) samples were processed by direct technique without (purple diamond) and with RNAse inhibitors (green circle). Ct values ≥ 40 were considered a negative result (dotted red line). Mean ± SEM. Paired *t*-test student (**** $p < 0.0001$).

Table 2
Specificity percentage of direct technique. Results of the amplification with in-house rRT-qPCR targeting *N* gene for all samples.

Type of sample	Total number of samples	Positive samples	Negative samples	Specificity (%)
Negative for respiratory virus	30	0	30	100 %
Influenza A	4	0	4	100 %
Influenza B	3	0	3	100 %
RSV	3	0	3	100 %
Rhinovirus	2	0	2	100 %
Parainfluenza	1	0	1	100 %
Other Coronaviruses	10	0	10	100 %
TOTAL	50	0	50	100 %

by thermal shock of 95 °C for 5 min. Considering such wide range of sensitivity, our study presented a sensitivity above average for this technique (88 % sensitivity for nasopharyngeal samples amplified by in-house rRT-PCR). Thermal shock cleaves RNA into shorter fragments (Kim et al., 2020; Smyrlaki et al., 2020), and a reduction of RNA copies has been detected after heat inactivation (Pastorino et al., 2020). Hence, samples with lower viral load at baseline suffer from RNA degradation during heating, which could end up reported as false-negative.

There is a direct method to detect SARS-CoV-2 from saliva samples that received Emergency Use Authorization (EUA) from the U.S. Food and Drug Administration (FDA) (Vogels et al., 2020). This method uses a combination of proteinase K and thermal shock to bypass RNA extraction. However, results showed a loss in sensitivity as their Ct value increased 1.8 cycles. As mentioned above, our results from thermal shock protocols showed an increase of more than 2.9 cycles. Conversely, our direct technique presented an increase of 1.4 cycles, while maintaining 100 % sensitivity. These results suggest that proteinase K is somehow helping in the thermal shock reaction. The relative benefits of these two techniques may, therefore, depend on reagent availability and the workflow requirements in each laboratory.

When using the commercial rRT-PCR kit, our study found that Ct values for the target in the *N* gene were lower than for the *ORF1ab* gene. This difference has been previously reported on samples from patients in Wuhan (Liu et al., 2020), and can be explained as a competition between the loci of the target genes (Wang et al., 2020). Therefore, we considered a positive result when at least one target gene presented a Ct value below 40. A separate study with lentivirus compared Trizol RNA extraction and a commercial viral RNA extraction kit (Won et al., 2020). Our study used a similar approach, and our results showed that samples processed with Trizol had similar Ct values and a 96 % sensitivity compared to a commercial RNA extraction kit. Nonetheless, this Trizol technique still requires time and reagents in order to extract RNA.

Working with direct samples can improve laboratory workflow, but this could also represent a risk for the laboratory technicians as plates are being prepared with potentially infectious material. Consequently, dedicated biosafety practices need to be implemented to ensure the safety of laboratory personnel and reduce the risk of contamination.

In conclusion, the direct technique solves a major bottleneck in scaling up nucleic acid extraction, which typically prolongs turn-around-time for rRT-PCR testing and significantly increases cost. The use of the direct sample with RNase inhibitors can also increase the number of samples that can be processed per day. Therefore, this represents an efficient alternative that can speed detection, lower cost and maintain rRT-PCR accuracy to help reduce the spread of SARS-CoV-2 virus.

Authorship

1) The authors confirm that each author has participated sufficiently in the work represented by the article to take public responsibility for the content. Participation has included (i) substantial contributions to the conception and design of the work or the acquisition, analysis or interpretation of data; AND (ii) drafting the article or revising it critically for important intellectual content; AND (iii) final approval of the version to be published.
2) The authors agree to be accountable for all aspects of the work in ensuring that any questions related to the accuracy or integrity of any part of the work are appropriately investigated.
3) The authors confirm that persons who have contributed intellectually to the article but whose contributions do not justify authorship are named in the Acknowledgements and their particular contribution described. Such persons have given their permission to be named.
4) The authors confirm that the given sequence of authors is the consensus of all contributors.

Author contributions

V.C., P.P. and A.O. conceived the study. S.V., V.N., A.C.M., S.H. and J.J.W. performed experiments and analyzed data. All authors revised the manuscript, discussed the results, and contributed to the final manuscript.

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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.2021.114302.

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