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Evaluation of filter paper to transport oro/nasopharyngeal samples to detect SARS-CoV-2 by RT-qPCR

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ABSTRACT

Purpose: To evaluate filter paper as a means to transport oro/nasopharyngeal samples from laboratories with few resources for SARS-CoV-2 detection by RT-qPCR in a central laboratory that usually performs this technique as routine.
Methods: A total of 40 specimens were evaluated in parallel by RT-qPCR carried out after RNA extraction using two different protocols: direct RNA extraction (Protocol A - reference method) and RNA extraction after impregnation in filter paper (Protocol B).
Results: The RT-qPCR for SARS-CoV-2 using Protocol B presented 97.22% (35/36) of agreement for SARS-CoV-2-positive samples when compared to the reference method (Protocol A), even for specimens with low viral load (increased Ct values). Noteworthy, three clinical specimens which were categorized as inconclusive by Protocol A presented amplification of both N1 and N2 targets using Protocol B, presenting positive results for SARS-CoV-2.
Conclusion: The use of filter paper to transport oro/nasopharyngeal clinical samples presented very satisfactory results to detect SARS-CoV-2 by RT-qPCR. In addition, it proved to be a feasible and sensitive approach, being able to generate the detection of SARS-CoV-2 even at low concentrations, without presenting false-negative results.

1. Introduction

The rapid propagation of the SARS-CoV-2 virus significantly increases the demand on health care systems. An important concern is represented by the need for tests that provide rapid diagnosis of COVID-19 cases. SARS-CoV-2 detection by Reverse Transcription Polymerase Chain Reaction in real time (RT-qPCR) using oral/nasopharyngeal swab presents good sensitivity and high specificity and has been considered the gold standard for the diagnosis of COVID-19 (Goudouris, 2021; Shen et al., 2020). Several countries have used RT-qPCR on a large scale and had to expand their routine. However, many laboratories, especially in underserved areas, became overloaded and new approaches to cope with this ever increasing and long-lasting challenge are needed (Volpato et al., 2021). In this context, SARS-CoV-2 diagnostic tests are extremely important to surveillance and outbreak management and should be used to establish infection prevention measures. Moreover, the development of effective diagnostic strategies is needed to limit the risk of contagion and to avoid serious consequences for individuals (Falzone et al., 2021; Nguyen et al., 2020; Vandenberg et al., 2021).

Nonetheless, some laboratories with few resources perform point of care tests or usually send the respiratory specimens to central laboratories for SARS-CoV-2 virus identification using RT-qPCR. Therefore, a proper transportation of clinical materials is important as these specimens may present a high biological risk. Furthermore, the transport of biological specimens is usually expensive and must be performed with a specific packaging system, then it is important to evaluate cheap and easy techniques for the transportation of clinical samples from laboratories with low resources to reference centers (Carneiro et al., 2020).

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The use of filter paper as a means of transportation of inactivated clinical samples would be much simpler than sending a viable sample collected of oral/nasopharyngeal swab. The aim of this study was to evaluate the filter paper as a means to transport oro/nasopharyngeal samples for detection of SARS-CoV-2 by RT-qPCR.

2. Methods

2.1. Clinical samples and RNA extraction

A total of 40 clinical samples from different patients were obtained by swabbing of oro/nasopharyngeal regions by a professional trained in a single institution. Individual swabs were mixed with 3 mL of 0.9 % NaCl solution. Two different protocols were evaluated in parallel: Protocol A and Protocol B. For both protocols the virus RNA was extracted from clinical specimens using Maxwell® RSC equipment (Promega Corporation, Wisconsin, USA) with Maxwell® RSC Viral TNA kit (Promega Corporation, Wisconsin, USA). Protocol A: the virus RNA was extracted directly from 150 μL of clinical respiratory specimens according to the manufacturer instruction; Protocol B: the virus RNA was extracted after impregnation of clinical samples in a sterile filter paper (6.4 mm diameter, 0.63 mm thickness - KAJ LAB, Brazil). A volume of 750 μL of respiratory specimens was centrifuged at 2800 *g* for 10 min in a 1.5 mL sterile Eppendorf tube. Afterwards, the supernatant was discarded with a sterile pasteur pipette and the resulting pellet (25 μL) was used to saturate the whole surface of the sterile paper disks. After the preparation of the disks, virus inactivation was performed in an ultraviolet light for 15 min (spectral range of 200–370 nm.). The disks were left at room temperature for 24 h to simulate the transport time between laboratories. The RNA obtained from both protocols was eluted in water for a final volume of 50 μL.

2.2. RT-qPCR Reaction

All the RNA specimens were submitted to SARS-CoV-2 RT-qPCR protocol following the CDC guidelines. Two genes of the nucleocapsid protein (N), N1 and N2, were amplified using a set of primers and probes as described by the Centers for Disease Control and Prevention (CDC - USA) in an RT-qPCR assay: 2019-nCoV_N1 Forward Primer (5′-GAC CCC AAA ATC AGC GAA AT-3′), 2019-nCoV_N1 Reverse Primer (5′-TCT GGT TAC TGC CAG TTG AAT CTG-3′), 2019-nCoV_N1 Probe (5′-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1–3′), 2019-nCoV_N2 Forward Primer (5′-TTA CAA ACA TTG GCC GCA AA-3′), 2019-nCoV_N2 Reverse Primer (5′-GCG CGA CAT TCC GAA GAA-3′), and 2019-nCoV_N2 Probe (5′-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1–3′). Primers and probes were purchased from Integrated DNA Technologies (Coralville, IA, USA). The reaction conditions were used as previously described (Wink et al., 2021) and the Superscript III (SSIII) one-step RT-qPCR system (Thermo Fisher Scientific Inc, California, USA) was used for RT-qPCR reactions. The master mix was composed of 5uL of 2X reaction buffer (0.4 mM of each dNTP and 6 mM $MgSO_4$); 0.2 μL of SuperScript™ III RT/Platinum™ Taq Mix; 0.2 μL of ROX (dilution 1:10); 0.75 μL of combined primers/probes mix of nCOV1 (N1 primer) or nCOV2 (N2 primer) or RP (2019-nCoV RUO Kit, IDT, Integrated DNA Technologies Inc, Iowa, USA) and 4 μL of extracted RNA. Thermal cycling was performed at 50 °C for 30 min for reverse transcription, followed by 95 °C for 2 min and then 45 cycles of 95 °C for 15 s, 55 °C for 35 s in QuantStudio® 3 Applied Biosystems™ (Applied Biosystems, Massachusetts, USA).

The result was considered "negative" when neither N1 nor N2 targets were amplified in the RT-qPCR or if the cycle threshold (Ct) was undetectable or greater than 40; "positive" results were defined by amplification of both targets with a Ct value lower than 40 for N1 and N2 targets; and a result was considered inconclusive when only one of the targets (N1 or N2) was amplified (). The values of the (Ct) from each protocol were recorded. The Ct is the number of replication cycles required to produce a fluorescent signal, with lower Ct values representing higher viral RNA loads. The human ribonuclease P gene (RP) was used as internal control to monitor nucleic acid extraction, specimen quality, RNA degradation and presence of reaction inhibitors.

To minimize batch effects, both protocols were performed in parallel and positive and negative controls were included in each plate tested. All samples that presented divergent results were repeated.

3. Results

From the 40 specimens included in this study, 36 were SARS-CoV-2-positive-samples and four presented inconclusive results for the RT-qPCR based on the Protocol A (Table 1). Using Protocol B, a total of 35 specimens were SARS-CoV-2-positive, indicating 97.22% of agreement between the two protocols. Only one specimen presented a discordant result between the two protocols (Sample ID 18), in which the N2 target was not amplified by the RT-qPCR using the Protocol B (Table 1). Noteworthy, three clinical specimens which were categorized as inconclusive by the Protocol A (N1 was not amplified) presented positive results (amplification of both N1 and N2 targets) using the Protocol B. Finally, the Sample ID 40 was classified as inconclusive in both protocols but with different targets amplified in Protocols A and B (Table 1).

From the samples positive for SARS-CoV-2 (n = 36), 12 presented a decrease in Ct values obtained by Protocol B compared to those obtained by Protocol A. These differences varied from 0.1 to 8.79 (median of Ct decrease = 0.75). Twenty-four samples presented an increase in Ct values with Protocol B, with the exception of the Sample ID 18, which presented a Ct increase of 14.97 for the N1 target. The differences in Ct values for the 23 samples varied from 0.12 to 6.21 (median of Ct increase = 2.16). All these differences in Ct values are represented by delta Ct (ΔCt) in Table 1.

4. Discussion

During the pandemic scenario, there was a massive need for laboratories offering COVID-19 diagnostics. However, real-time PCR equipment tended to be restricted to reference centers (Vandenberg et al., 2021). Consequently, a rapid and efficient access to the reference method was necessary to detect SARS-CoV-2 without high investments. Here, we demonstrate that the use of filter paper (Protocol B) to transport clinical samples for the detection of SARS-CoV-2 by RT-qPCR presented satisfactory results, demonstrating that it facilitates the access of any laboratories to RT-qPCR technology.

In this context, considering the fact that the use of sterile filter paper was already validated to transport inactivated bacteria for its identification as well as for the identification of carbapenemase genes by RT-qPCR (Carneiro el al, 2020; Carneiro et al., 2021), we evaluated this approach for the transport of clinical specimens for SARS-CoV-2 detection. Moreover, the use of filter paper has already been tested to improve the surveillance of dengue virus with the transportation of serum and blood using dried filter paper over thousands of kilometers at ambient temperatures toward reference centers (Aubry et al., 2012).

To the best of our knowledge, this is the first study evaluating the use of sterile filter paper to transport a respiratory RNA virus. Protocol B presented concordant results even for specimens with higher Ct values (Ct> 30), which represents samples with lower viral RNA load. Only one specimen (Sample ID 18) presented a discordant result between both Protocols. Particularly in this clinical sample, the Ct value for the N1 target was higher with Protocol B when compared to Protocol A and the N2 target did not amplify, resulting in an inconclusive result (Table 1). We believe that it could be associated with the RNA degradation as we could observe a slight difference in the RP values for both protocols. This proves the importance of RP association not only as a control of endogenous reactions, but also as a marker of RNA degradation to monitor transport conditions. In addition, it is important to mention that

Table 1
Cycle threshold assessment for SARS-CoV-2 targets obtained by RT-qPCR.

Sample ID	Protocol A•			Protocol B••			ΔCt (N1)	ΔCt (N2)	ΔCt (RP)	RT-qPCR result agreement
	N1	N2	RP	N1	N2	RP				
1	17.40	15.80	26.80	22.40	21.20	32.90	+ 5.00	+ 5.40	+ 6.10	Concordant
2	15.73	14.37	23.10	17.07	15.80	25.19	+ 1.34	+ 1.43	+ 2.09	Concordant
3	22.20	20.74	24.99	26.7	25.12	27.96	+ 4.50	+ 4.38	+ 2.97	Concordant
4	25.90	25.80	25.99	25.00	23.70	29.98	-0.90	-2.10	+ 3.99	Concordant
5	23.80	22.50	25.30	28.90	27.70	30.30	+ 5.10	+ 5.20	+ 5.00	Concordant
6	20.70	19.19	22.90	26.70	25.40	26.90	+ 6.00	+ 6.21	+ 4.00	Concordant
7	17.40	15.80	25.10	16.80	15.05	25.20	-0.60	-0.75	+ 0.10	Concordant
8	27.20	26.40	25.20	30.50	30.06	29.01	+ 3.30	+ 3.66	+ 3.81	Concordant
9	21.20	19.02	24.80	22.50	20.80	27.50	+ 1.30	+ 1.78	+ 2.70	Concordant
10	28.20	27.03	24.70	30.70	29.50	26.30	+ 2.50	+ 2.47	+ 1.60	Concordant
11	21.70	20.80	30.90	21.50	20.10	32.10	-0.20	-0.70	+ 1.20	Concordant
12	19.70	18.20	24.60	22.50	21.20	31.90	+ 2.80	+ 3.00	+ 7.30	Concordant
13	19.40	17.80	23.80	20.80	19.20	26.90	+ 1.40	+ 1.40	+ 3.10	Concordant
14	27.70	26.70	24.40	30.50	29.10	28.10	+ 2.80	+ 2.40	+ 3.70	Concordant
15	16.30	14.50	27.04	19.07	17.40	28.10	+ 2.77	+ 2.90	+ 1.06	Concordant
16	20.55	19.02	26.56	22.78	21.57	28.66	+ 2.23	+ 2.55	+ 2.10	Concordant
17	21.49	20.35	27.45	23.59	22.35	30.43	+ 2.10	+ 2.00	+ 2.98	Concordant
18	21.27	19.85	25.98	36.24	Negative	28.62	+ 14.97	–	+ 2.64	Discordant
19	31.00	29.61	28.74	30.88	29.64	30.64	+ 0.12	+ 0.03	+ 1.90	Concordant
20	16.34	14.87	25.46	17.66	16.01	28.27	+ 1.32	+ 1.14	+ 2.81	Concordant
21	19.80	18.89	21.18	19.26	17.68	22.00	-0.54	-1.21	+ 0.82	Concordant
22	18.67	17.21	25.38	18.95	17.41	27.23	+ 0.28	+ 0.20	+ 1.85	Concordant
23	23.32	22.11	24.63	24.18	23.02	28.26	+ 0.86	+ 0.91	+ 3.63	Concordant
24	18.04	16.45	24.05	19.66	17.84	26.59	+ 1.62	+ 1.39	+ 2.54	Concordant
25	23.25	21.60	27.46	22.74	21.22	28.17	-0.51	-0.38	+ 0.71	Concordant
26	18.66	17.33	25.46	19.52	17.98	27.77	+ 0.86	+ 0.65	+ 2.31	Concordant
27	18.03	16.45	25.14	19.94	18.47	29.62	-1.91	+ 2.02	+ 4.48	Concordant
28	26.11	24.74	24.34	25.11	24.00	27.57	-1.00	-0.74	+ 3.23	Concordant
29	21.83	20.50	22.94	25.57	24.06	29.11	+ 3.74	+ 3.56	+ 6.17	Concordant
30	21.10	18.63	23.24	23.06	20.88	25.20	+ 1.96	+ 2.25	+ 1.96	Concordant
31	35.30	33.00	25.80	33.30	34.90	27.90	-2.00	+ 1.90	+ 2.10	Concordant
32	35.60	36.20	22.60	35.30	36.10	25.70	-0.30	-0.10	+ 3.10	Concordant
33	32.90	31.50	23.60	32.80	34.60	27.30	-0.10	+ 3.10	+ 3.70	Concordant
34	32.21	30.87	28.81	23.44	22.08	28.82	-8.77	-8.79	+ 0.01	Concordant
35	33.93	33.05	26.16	34.28	33.80	26.08	+ 0.35	+ 0.75	-0.08	Concordant
36	35.40	32.76	27.31	33.26	30.49	32.15	-2.14	-2.27	+ 4.84	Concordant
37	Negative	37.31	26.19	35.86	37.74	31.12	–	+ 0.43	+ 4.93	*
38	Negative	35.80	26.50	36.10	35.35	27.60	–	-0.45	+ 1.10	*
39	Negative	35.30	23.50	30.90	29.70	28.30	–	-5.60	+ 4.80	*
40	Negative	35.90	28.20	37.11	Negative	30.18	–	–	–	* *

N1: Target of nucleocapsid protein 1; N2: Target of nucleocapsid protein 2; RP: Target of human ribonuclease P gene; Negative: absence of N1 and N2 amplification; ΔCt: Ct of Protocol B minus the Ct of Protocol A.
•Protocol A: reference protocol as recommended by CDC (RNA extraction and RT-qPCR directly from the clinical samples).
••Protocol B: RNA extraction and RT-qPCR after the impregnation of the clinical samples in sterile filter papers.
*Protocol A with inconclusive results.
* *Protocol A and B with inconclusive results.

Protocol B presented lower Ct values for 33 % of the samples positive for SARS-CoV-2 (12/36) when compared to Protocol A, reaching a Ct difference as low as 8.79 of those obtained by the Protocol A (Table 1). It can be observed mainly in specimens categorized as inconclusive by Protocol A (Samples ID 37, 38 and 39), which resulted in the amplification of both N1 and N2 targets by RT-qPCR using Protocol B. This may be due to the fact that we used an increased volume of clinical samples in Protocol B and consequently, increased sample concentrations before the impregnation in the filter paper. On the other hand, Protocol B presented higher Ct values for 67 % (24/36) compared to Protocol A, resulting in a Ct difference as high as 6.21 of those obtained by the Protocol A, with the exception of one sample (Sample ID 18) that resulted in an inconclusive result. We believe that the increase in Ct values obtained with Protocol B does not impact in the results of RT-qPCR, as the they are expressed as a qualitative interpretation for the diagnosis of COVID-19 % and 97.22 % of the samples presented concordant qualitative results in comparison to Protocol A.

Our results indicated that the transportation in filter paper could be a reliable approach as it presented 97.22 % (35/36) of agreement with the reference method of extraction for positive samples of SARS-CoV-2, regardless of the viral load presented in clinical specimens. In fact, filter paper disks impregnated with respiratory specimens can be transported without temperature controls, without loss of stability and quality of samples, requiring smaller cargo volumes and reduction of biological waste (Carneiro et al., 2020).

According to Pizzol et al. (2020) less than 20 % of countries have full capacity to detect and report epidemics of potential concern and fewer than 5 % have the ability to respond quickly. In this context, we demonstrated that the use of the filter paper technique to transport clinical samples of the upper respiratory tract is easy to perform, secure and allows the transport of a higher number of samples in a reduced space. It has to be considered that the technique to impregnate the clinical samples in filter paper are not possible to be performed at home, such as the COVID-19 self-tests, as the impregnation procedures require a laboratory structure. Therefore, transporting clinical samples in filter paper would allow to small laboratories to have access to the RT-qPCR technique for SARS-CoV-2 detection.

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Data sharing statement

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval

This work has the approval of the ethics committee under the number C.A.A.E.: 30767420.2.0000.5327 of Porto Alegre Clinical Hospital.

CRediT authorship contribution statement

All authors contributed to the study conception and design. Material preparation and data collection were performed by DCP and LG; analysis was performed by MSC, FCZV and PLW. The first draft of the manuscript was written by FCZV and MSC and reviewer by ALB. All authors read and approved the final manuscript.

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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