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SARS-CoV-2 saliva testing is a useful tool for Covid-19 diagnosis

Cristina Mendes de Oliveira [a,*], Leila Brochi [b], Luciano Cesar Scarpelli [a], Annelise Correa Wengerkievicz Lopes [b], José Eduardo Levi [a]

[a] Dasa, Barueri, Brazil
[b] Santa Luzia, Florianópolis, Brazil

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ABSTRACT

SARS-CoV-2 is the etiologic agent of coronavirus disease 2019 (COVID-19) and is mainly detected by RT-PCR methods from upper respiratory specimens, as recommended by the World Health Organization. Oro/nasopharyngeal swabbing can be discomfortable to the patients, requires trained healthcare personnel and may generate aerosol, increasing the risk of nosocomial infections. In this study, we describe two SARS-CoV-2 RNA extraction-free single RT-PCR protocols on saliva samples and compared the results with the paired oro/nasopharyngeal swab specimens from 400 patients. The two saliva protocols demonstrated a substantial agreement when compared to the oro/nasopharyngeal swab protocol. Moreover, the positivity rate of saliva protocols increased according to the disease period. The 95 % limit of detection of one of the therefore implemented saliva protocol was determined as 9441 copies/mL. Our results support the conclusion that RNA extraction-free RT-PCR using self-collected saliva specimens is an alternative to nasopharyngeal swabs, especially in the early phase of symptom onset.

1. Introduction

Coronavirus disease 2019 (COVID-19), a life-threatening viral respiratory infection, was first identified in China in December 2019, rapidly spread globally and the World Health Organization (WHO) declared it a pandemic on March, 2020 (World Health Organization, 2020a). By April 12th, 2021 more than 135 million confirmed cases and almost 3 million deaths were reported to WHO (WHO, 2021).

COVID-19 is caused by a novel coronavirus (SARS-CoV-2) of probably bat origin, which is related to the virus responsible to the Severe Acute Respiratory Syndrome (SARS) outbreak in 2002/2003 in humans (Wu et al., 2020; Zhou et al., 2020). The transmission occurs mainly by respiratory droplets and close contact and can be accelerated by populational migration movements and agglomeration of people (Adhikari et al., 2020). Therefore, the most important approach to control the pandemic is the detection and isolation of infected people. The gold standard for SARS-CoV-2 diagnosis is the reverse transcription polymerase chain reaction (RT-PCR) of swab specimens collected from the upper respiratory tract of suspected patients (World Health Organization, 2020b). Routinely, the RT-PCR is preceded by the RNA extraction, which is a major bottleneck due to lack of supplies since it is being used by large amounts all over the world. To circumvent the shortage of RNA extraction reagents, researchers investigated the performance of RT-PCR avoiding the extraction step. Successful results were obtained (Smyrlaki et al., 2020) and are indeed used routinely. However, the collection of the oro/nasopharyngeal swab requires trained personnel with intrinsically exposure risk, is difficult to perform in young children and can cause discomfort. Besides that, nasopharyngeal collection is unfeasible in some medical situations like post-operative of nasal or sinus surgery and neoplastic conditions of the face. These caveats led to the evaluation of RT-PCR assays using saliva specimens as an alternative of oro/nasopharyngeal swab (Bastos et al., 2021; Butler-Laporte et al., 2021; Caulley et al., 2021; Yokota et al., 2020) and RNA extraction-free RT-PCR protocol were also developed using self-collected saliva specimen (Vogels et al., 2021a), as well as, a commercially available assay which is widely used in Japan (Fukumoto et al., 2020). Here, we describe two SARS-CoV-2 RNA extraction-free single RT-PCR testing on saliva samples and compared the results with the paired oro/nasopharyngeal swab specimens performed as the gold standard test. In addition, we evaluate the positivity rate of saliva protocols according to the time of symptoms onset and determined the 95 % limit of detection of one of the assays.

* Corresponding author at: Av. Juruá 548, Alphaville, Barueri, SP 06455-010, Brazil.
E-mail address: cristina.mendes@dasa.com.br (C.M. de Oliveira).

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2. Materials and methods

2.1. Sample collection

Four hundred and three adults (>18 years) attended at the Santa Luzia Laboratory in Florianópolis, Santa Catarina State, Brazil, from October to November 2020 seeking for routine RT-PCR for SARS-CoV-2 were invited to participate in the study. Two oro/nasopharyngeal swabs (ONS) were collected: one was introduced into the throat and scraped for 10 s and the second was into the nose and scraped for 10 s in each nostril. Then, both, were deposited in 3 mL of 0.9 % saline solution, immediately frozen at −20 °C and sent to the Molecular Biology Department of Dasa Laboratories in Barueri, Brazil.

Saliva samples were self-collected by the patients after the swabs collection. They were previously instructed to avoid drinking water 10 min and refrain to other drinks, food, and nasal sprays for half an hour before sample collection (Vogels et al., n.d.). Patients received a dry sterile container and were asked to concentrate the saliva in mouth and spit it (~1 mL) in the container and close it. The specimens were sent to the Molecular Biology Department of Dasa Laboratories in Barueri, Brazil at room temperature. At the laboratory, the specimens were stored at −20 °C until processed (4 h to 3 days).

2.2. Ethics statement

The local Research Ethics Committee approved the study (CAAE 39648020.6.0000.0068). All participants provided written informed consent, and all the personal information was maintained encrypted in a database to ensure participants' data confidentiality.

2.3. RNA isolation and one-step RT-PCR assay from ONS

After the samples arrived at the laboratory, they were submitted to one of the routine diagnostic assays available: a lab-developed test (LDT) described below (n = 361), the Cobas® SARS-CoV-2 test in a Cobas 6800 system (Roche Diagnostics, USA) (n = 32) or the Abbott RealTime SARS-CoV-2 assay (Abbott, USA) (n = 10).

LDT: samples were vortexed and a 300 µL aliquot was RNA extracted using Chemagen magnetic bead technology on the Chemagic™ 360 instrument (PerkinElmer, USA). Total nucleic acids were eluted in 90 µL and 7 u L submitted to amplification in a duplex combination targeting the Sars-CoV-2 E gene in addition to the human RNAseP as described (Corman et al., 2020). The protocol comprises 400 nM of gene E forward primer, 400 nM of gene E reverse primer, 200 nM of gene E probe-FAM, 1X PrimeTime® RNAseP-VIC, (Integrated DNA Technologies IDT, USA), and 1x TaqMan Fast Virus (ThermoFisher, USA). Cycling/detection was performed in a QuantStudio 12k™ instrument (ThermoFisher Scientific, USA).

2.4. Pre-processing and one-step RT-PCR saliva assays

Prior to the RT-PCR assay, saliva specimens were thawed at room temperature, vortexed and a 50 µL aliquot was transferred to a 96 wells plate and 60 µg of proteinase K (New England Biolabs, USA) were added. The plate was sealed, vortexed for 1 min at 3000–5000 rpm, briefly spun down, and heated at 37 °C for 5 min, followed by 95 °C for 5 min. This processed saliva was then submitted to two RT-PCR assays:

The first RT-PCR assay (protocol L) was performed using an *in house* protocol that comprises 1,5x Assay N1 (IDT, USA), 1x Assay RNAse P (IDT, USA), 1x Luna® Universal Probe qPCR Master Mix (New England Biolabs, USA) and 7 µL of pre-processed saliva in a total reaction volume of 20 µL. The RT-PCR was performed in a QuantStudio 5™ instrument (ThermoFisher Scientific, USA) under the following conditions: 55 °C for 20 min, 95 °C for 1 min, and 40 cycles of 95 °C for 20 s followed by 60 °C for 1 min.

The second RT-PCR assay (protocol T) was performed using the TaqPath™ Covid-19 CE-IVD RT-PCR kit (ThermoFisher Scientific, USA), able to detect 3 SARS-CoV-2 targets (gene S, gene N and ORF1ab) with some modifications.

The reaction mixture comprised 1x TaqPath 1-Step multiplex mastermix, 1x COVID-19 Real Time PCR Assay Multiplex, 12.5 µL of nuclease free water and 5 µL of pre-processed saliva with a total reaction volume of 25 µL. The RT-PCR was performed in a QuantStudio 5™ instrument (ThermoFisher Scientific, USA) under the following conditions: 25 °C for 2 min, 53 °C for 10 min, 95 °C for 2 min, and 40 cycles of 95 °C for 3 s followed by 60 °C for 30 s.

The sample is considered positive for SARS-CoV-2 if two or more targets amplify or if only gene N amplify at a Ct threshold ≤ 40, as determined during our validation process. Samples in which only gene S or ORF1ab was positive, were considered negative for SARS-CoV-2.

2.5. Determination of the 95 % limit of detection (LoD 95 %) for the protocol T

The 95 % limit of detection for the protocol T was established using a pool of positive SARS-CoV-2 samples, quantified using the AccuPlex™ SARS-CoV-2 (SeraCare, USA). Twenty-four replicates of 7 different concentrations (16,345 copies/mL, 8172 copies/mL, 4086 copies/mL, 2043 copies/mL, 1021 copies/mL, 510 copies/mL, and 255 copies/mL) were submitted to the protocol in 3 different assays. The 95 %LoD was determined using probit analysis.

2.6. Statistical analysis

Statistical analyses were performed using R (http://www.R-project.org/), and the significance level was set at 5% for all tests. All reported p-values are two-sided.

The Kappa coefficient was calculated to evaluate the agreement between the tests. The analysis of sensitivity and specificity rates (and respective confidence intervals) were calculated considering the ONS protocol as the gold standard.

3. Results

A total of 403 patients (>18 years) were invited to participate in the study and provided paired saliva and oro/nasopharyngeal samples. Three were excluded because the saliva sample showed an invalid result (did not amplify the RNAseP target nor any SARS-CoV-2 target). Therefore, the analyses were conducted with the 400 patients that showed valid RT-PCR results in all protocols. At sample collection, patients were on average 106.45 (SD 2) minutes without eating. Seventy-four were asymptomatic and for one patient this information was not available. Among the symptomatic patients (n = 325) the average time since symptoms onset was 5.04 (SD 2.95) days.

3.1. Comparison of results of saliva protocols with the ONS gold standard protocol

The protocol L was conducted using N region as the target of SARS-CoV-2 RNA and RNAseP as the endogenous control. Positive SARS-CoV-2 results for both saliva and ONS were observed in 124 patients, and negative SARS-CoV-2 in both specimens were observed in 242 patients. Six samples were positive in the saliva specimen and negative in the ONS. On the other hand, 28 ONS positive specimens were negative in the saliva (Table 1).

In the protocol T, positive SARS-CoV-2 samples for both materials, saliva and ONS, were observed in 119 patients, and negative on both in 243 patients. Five patients were positive in the saliva specimen and negative in the ONS. On the other hand, 33 ONS positive specimens were negative in the saliva (Table 1). The performance characteristics of both, L and T, protocols are described in Table 2.

Sensitivity rates of both L and T protocols decreased as the elapsed

Table 1
Comparison of SARS-CoV-2 test results on saliva according to RT-PCR protocol (reference group: ONS).

RT-PCR Protocol for saliva specimen	ONS	SARS-CoV-2 test			Kappa	p-value (*)
		Positive	Negative	Total		
Protocol L	Positive	124	6	**130** (32.5 %)	0.81 (**)	<0.001
	Negative	28	242	**270** (67.5 %)		
	Total	**152** (38.0 %)	**248** (62.0 %)	400 (100 %)		
Protocol T	Positive	119	5	**124** (31.0 %)	0.79 (**)	<0.001
	Negative	33	243	**276** (69.0 %)		
	Total	**152** (38.0 %)	**248** (62.0 %)	400 (100 %)		

ONS- oro/nasopharyngeal swabs.
(*)McNemar Test.
(**) p-value < 005 (Kappa).

Table 2
Performance characteristics of the L and T protocols in saliva considering either ONS or saliva result positive as SARS-CoV-2 positive.

	Sensitivity (95 %CI)	Specificity (95 %CI)	PPV (95 %CI)	NPV (95 %CI)
Protocol L	0.81 (0.74–0.87)	1.00 (0.98–1.00)	1.00 (0.97–1.00)	0.89 (0.85–0.92)
Protocol T	0.78 (0.70–0.84)	1.00 (0.98–1.00)	1.00 (0.97–1.00)	0.87 (0.82–0.91)

PPV, positive predictive value; NPV, negative predictive value, ONS- oro/nasopharyngeal swabs.

time increased from de onset of symptoms (Table 3).

The analysis of the cycle threshold (Ct) values of positive samples obtained in the three protocols showed statistically significant lower values obtained in the ONS protocol in comparison with both saliva protocols. (Table 4).

False negative results on saliva samples increased with the increase in Ct values on ONS (Table 5).

3.2. LoD 95 % of the protocol T

Twenty-four replicates of 7 different concentrations of a quantified positive pool were submitted to the protocol in 3 different assays (Table 6) and the LoD 95 % was determined as 9441 copies/mL.

Table 3
Performance characteristics of the L and T protocols according to the time of symptoms onset (n = 325).

Time of onset symptoms (days)	protocol L		protocol T	
	Sensitivity (95 %CI)	Specificity (95 %CI)	Sensitivity (95 %CI)	Specificity (95 %CI)
0–3 days (n = 109)	0.92 (0.79–0.98)	0.97 (0.90–1.00)	0.92 (0.79–0.98)	0.97 (0.90–1.00)
4–6 days (n = 134)	0.87 (0.76–0.94)	0.97 (0.90–1.00)	0.84 (0.72–0.92)	0.99 (0.93–1.00)
>7 days (n = 82)	0.63 (0.47–0.78)	0.98 (0.87–1.00)	0.61 (0.45–0.76)	0.98 (0.87–1.00)

Table 4
Cycle threshold (Ct) values of positive samples on the three protocols (n = 113).

	Mean	Standard deviation	Min-max	p*
Ct of ONS	20.34	5.21	5.46–35.08	
Ct of protocol L	27.39	4.61	14.47–38.77	<0.001
Ct of protocol T	29.43	4.74	16.65–38.56	<0.001

ONS- oro/nasopharyngeal swabs.
* Wilcoxon-Mann-Whitney test.

Table 5
Positivity rates in saliva protocols according to Ct values on ONS.

CT values on ONS	Positivity rate in L protocol	Positivity rate in T protocol
< 20	96.8% (61/63)	93.6 % (59/63)
20.1–30	81.7 % (58/71)	81.7 % (58/71)
>30	27.8% (5/18)	5.6 % (1/18)
p*	<0.001	<0.01

ONS- oro/nasopharyngeal swabs.
* chi-squared test for trend in proportions.

Table 6
Positivity rate according to the concentration of SARS-CoV-2.

Concentration (copies/mL)	Positivity rate (%)
16,345	100
8172	96
4086	88
2043	50
1021	13
510	13
255	4

4. Discussion

Rapid and high throughput diagnostic methods could help to control the spread of SARS-CoV-2 and can be achieved through RT-PCR. Moreover, WHO recommends collecting upper respiratory specimens for early-stage infections (WHO, 2020), which can cause discomfort to the patient, requires trained healthcare personnel and may generate aerosol, increasing the risk of nosocomial infections (Hung et al., 2020). In this study, we demonstrated that RNA extraction-free RT-PCR using self-collected saliva specimen when compared to the ONS RT-PCR method, used as reference, can be performed without major sacrifice in accuracy in determining the true positive and negative cases. In comparison to the initial method described (Fukumoto et al., 2020), both protocols use heated method to release viral RNA and take approximately the same time to be performed. Our protocols have the advantage of using widely available proteinase K instead of a commercial pretreatment solution available only in the 2019 Novel Coronavirus Detection kit (nCov-DK; Shimadzu Corporation, Kyoto, Japan). Our protocol L detects N1 region of SARS-CoV-2 simultaneously with an endogenous internal control, while the nCoV-DK has the advantage of detecting two SARS-CoV-2 targets, N1 and N2 regions. Concerning our protocol T, we are able to detect three SARS-CoV-2 targets (ORF1ab, gene N and gene S) simultaneously that diminished the risk of false negative PCR results due to the emergence of a new variant.

Glands and oral mucosae are important sites of SARS-CoV-2 infection and saliva could display a role in the infection transmission (NIH COVID-19 Autopsy Consortium et al., 2021). The general sensitivity of the saliva RNA extraction free RT-PCR in both protocols, L and T, was lower compared with the ONS protocol (81 % and 78 %, respectively), but the value of kappa was in almost perfect or substantial agreement. A meta-analysis that evaluated 5922 samples found a sensitivity of 83.2 % for saliva RT-PCR (Butler-Laporte et al., 2021) similar to our findings.

Studies that used saliva samples achieved sensitivity ranging from 30.8%–86.4% (Chen et al., 2020; Kojima et al., 2020; McCormick-Baw

et al., 2020; Nagura-Ikeda et al., 2020; Pasomsub et al., 2021; Vogels et al., n.d.). These discrepant results could be due to the heterogeneity of the studies, which varies in terms of study population, different SARS-CoV-2 detection strategies (RNA extraction vs RNA extraction-free, different RT-PCR protocols), and timing of testing. In our study, the saliva collection in the early phase of symptoms showed significantly higher detection rates of viral RNA, as previously observed by others (Nagura-Ikeda et al., 2020; Wyllie et al., 2020). It is suggested that timing of testing can affect the diagnostic accuracy of saliva and ONS differentially. Two studies observed that SARS-CoV-2 viral load in saliva decline from symptom onset (To et al., 2020; Wyllie et al., 2020). On the other hand, Butler-Laporte and colleagues suggests that SARS-CoV-2 may remain positive for longer than ONS (Butler-Laporte et al., 2021).

One limitation of our study is that the ONS protocol is imperfect and subjected to false negative and positive results. Therefore, positive results in either saliva or ONS specimens were considered as positive for SARS-CoV-2. We observed eight patients that were positive for SARS-CoV-2 in at least one of the saliva protocols and negative in the ONS specimen. Of those, three were positive in both saliva protocols, being two symptomatic patients (3–5 days) and one asymptomatic, whom 27 days later showed seroconversion. Wyllie and colleagues observed a higher frequency of positive saliva samples than ONS up to 10 days after the Covid-19 diagnosis (Wyllie et al., 2020).

Saliva testing showed a great accuracy in the first days of symptoms (92 % of sensitivity and 97 % of specificity) and could be useful especially for SARS-CoV-2 detection in children (Al Suwaidi et al., 2021). It has the advantages that is an easy and not invasive collection method that can be performed at home, by the patient, avoiding exposure of the health professionals to risk of infection. Besides that, the RNA extraction-free method is cheaper and faster than traditional RT-PCR methods.

Concerning the asymptomatic cases, saliva testing showed lower performance compared to early symptomatic cases (82 % of sensitivity and 98 % of specificity). Most of our false negative results were observed in samples with low SARS-CoV-2 viral load (Ct>30 in ONS protocol). Nonetheless, these false negative results may not have epidemiological importance since Ct>30 were not associated to infectious samples (Jefferson et al., 2020) nor to transmission (Walker et al., 2020). Even with this performance, saliva testing could be a very useful laboratorial tool in the control of the pandemic because of its easy collection procedure, allowing frequent testing (Mina et al., 2020) and even exempting the use of swabs that would be subject to shortage of supplies. Considering this perspective, the importance of the analytical sensitivity of a test for SARS-CoV-2 detection should be rethought, as it was shown that tests with a low analytical sensitivity when applied frequently are more effective than a test with a high analytical sensitivity applied infrequently (Mina et al., 2020).

5. Conclusions

Our results suggest that RNA extraction-free RT-PCR using self-collected saliva specimens shows diagnostic accuracy similar to the ONS and support the use of this protocol as an alternative to nasopharyngeal swab, especially in the early phase of symptom onset.

Author statement

Cristina Mendes de Oliveira – Data curation, formal analysis, investigation, methodology, roles/writing - original draft, writing – review & editing.

Leila Brochi – investigation, methodology, writing – review & editing.

Luciano Cesar Scarpelli – Funding acquisition, resources, writing – review & editing.

Annelise Correa Wengerkievicz Lopes – Conceptualization, data curation, investigation, project administration, supervision, writing – review & editing.

José Eduardo Levi - Conceptualization, data curation, formal analysis, investigation, methodology, project administration, supervision, writing – review & editing.

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Declaration of Competing Interest

The authors report no declarations of interest.

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