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Alternative SARS-CoV-2 detection protocol from self-collected saliva for mass diagnosis and epidemiological studies in low-incoming regions

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ABSTRACT

Until mass vaccination befalls, control of the new betacoronavirus-associated severe acute respiratory syndrome pandemic (SARS-CoV-2) is based on decreasing virus circulation by social distancing and blocking transmission foci after diagnosis. Globally adopted SARS-CoV-2 diagnostic criteria embrace viral RNA detection by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) on nasopharynx secretions, which requires healthcare facilities and specialized personnel for sample collection. To develop an alternative protocol, hydrophilic cotton as the material and saliva as the source for biological sample collection in qRT-PCR/RT-endpoint-PCR SARS-CoV-2 diagnostic methods prepared with local consumables were evaluated using 99 archived nasopharynx samples previously diagnosed as positive for SARS-CoV-2 and 111 prospective saliva samples pared with nasopharynx samples from patients attending the local reference ABC Medical School diagnostic laboratory. The kappa agreement coefficient between the SARS-CoV-2 qRT-PCR and RT-endpoint-PCR was k = 0.97 (95 % CI 0.92−1.00) and k = 0.90 (95 % CI 0.81−0.99), respectively, on SARS-CoV-2-positive archived samples, with the initial qRT-PCR C_T under 25. The agreement coefficient of the SARS-CoV-2 alternative saliva diagnostic protocol, when used to test the paired nasopharynx samples, was k = 0.79 (95 % CI 0.56–1,00). These data support that the SARS-CoV-2 diagnostic assay based on self-collected saliva on cotton represents an alternative protocol for mass diagnosis and epidemiological studies in low-income regions.

1. Introduction

A new etiological agent for severe acute respiratory syndrome, first described in the Chinese city of Wuhan in December 2019, is a novel coronavirus (SARS-CoV-2) belonging to the group of betacoronaviruses. SARS-CoV-2 officially reached Brazil in February 2020 and was notified by the World Health Organization (WHO) as a pandemic in March 2020, producing in one year, thousands of fatal cases worldwide (Wu et al., 2020a, b; Wu et al., 2020c).

The single-stranded RNA-based genome of SARS-CoV-2 is approximately 30 kilobases and encodes viral proteins from 14 positively-oriented open reading frame gene transcripts (Chen et al., 2020; Lu et al., 2020). The replication and transcription of the viral genome are carried out by a complex of proteins including RNA-dependent RNA polymerase, endonucleases, and exonucleases, corresponding to 16 non-structural proteins (Nsp1-16) generated by the auto-proteolytic

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Table 1
Primers and probes.

Name	5′ sequence 3′	PCR condition	Gene	Amplicon
WuhanCoV-spk1-f (S)#	TTGGCAAAATTCAAGACTCACTTT	**1 cycle** 95 °C 3 min **40 cycles** 95 °C 30 seg 56 °C 30 seg 72 °C 30 seg **1 cycle** 72 °C 7 min	Spike	557 bp
WuhanCoV-spk2-r (AS)#	TGTGGTTCATAAAAATTCCTTTGTG			
NIID_WH-1_F24381 (S)#	TCAAGACTCACTTTCTTCCAC			492 bp
NIID_WH-1_R24873 (AS)#	ATTTGAAACAAAGACACCTTCAC			
2019-nCoV_N1-F	GACCCCAAAATCAGCGAAAT	**1 cycle** 95 °C 3 min **45 cycles** 95 °C 3 seg	Nucleocapsid	209 bp
2019-nCoV_N1-R	TCTGGTTACTGCCAGTTGAATCTG			
2019-nCoV_N1-P*	FAM-ACCCCGCATTACGTTT GGTGGACC-BHK			
2019-nCoV_N2-F	TTACAAACATTGGCCGCAAA			110 bp
2019-nCoV_N2-R	GCGCGACATTCCGAAGAA			
2019-nCoV_N2-P*	Hex-ACAATTTGCCCCCAGC GCTTCAG - BHK	55 °C 30 seg		
RNAse P (RP-F)	AGATTTGGACCTGCGAGCG		Human RNAse P	62 bp
RNAse P (RP-R)	GAGCGGCTGTCTCCACAAGT			
RNAse P (RP-P)	TxRed-TTCTGACCTGAAGG CTCTGCGCG - BHK			

activity of a polyprotein encoded in the single open reading frame synthesized from the 5′ end of the viral genome (Chen et al., 2020). The other 13 open reading frame gene products are expressed from the 3′ end of the viral genome, including the four structural proteins: Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N). These structural proteins underlie capsid formation, the encapsulation of the viral genome, and host cell invasion. Specifically, the virus infects human cells through the major virus entry receptor, human angiotensin-converting enzyme (ACE2), which is present in several tissues and organs including the lungs, kidneys, and at particularly high concentrations in salivary glands (Xu et al., 2020).

The transmission of SARS-CoV-2 occurs through contact with saliva and other secretions from the upper airways of an infected individual, which can contaminate the eyes and mouth of a susceptible exposed individual. Also, contamination can occur through the inhalation of the viral particles present in aerosols expelled by an infected individual when speaking, sneezing, or coughing (Patel, 2020). The clinical manifestations of SARS-CoV-2 infection have a wide spectrum ranging from a total absence of symptoms to fever, body aches, headache, conjunctivitis, cardiovascular symptoms, changes in liver function, loss of smell and taste, gastrointestinal discomfort, diarrhea, and may even include more severe manifestations such as heart failure and severe respiratory distress including heart disease and severe respiratory distress (Archer et al., 2020; Chate et al., 2020; Fried et al., 2020; Jin et al., 2020; Lovato and de Filippis, 2020; Qi et al., 2020; Seah and Agrawal, 2020; Zheng et al., 2020). Individuals with symptoms associated to upper airway involvement are more likely to transmit the virus (Yang et al., 2020).

Serological surveys conducted in cities across Brazil show that the percentage of individuals infected with SARS-CoV-2 is low in the population but high and concentrated in transmission foci (Hallal et al., 2020). Asymptomatic individuals can transmit the virus for up to 14 days, and the number of asymptomatic cases in an affected population can potentially reach 40–45% (Oran and Topol, 2020). The identification of asymptomatic individuals associated with transmission foci enables health management teams to establish the flow of the spreading virus and identify the entire affected population, thereby preventing virus dissemination. Mass diagnostic tests that utilize affordable and straightforward self-collection methods are essential toward achieving this goal.

SARS-CoV-2 diagnostic tests hinge on detecting the viral genome, mainly in nasopharynx secretions, by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Li et al., 2020), RT-endpoint-PCR (Shirato et al., 2020), RT- loop-mediated isothermal amplification (Uchida et al., 2020), or by detecting antigens by immunochromatography (rapid test) (Linares et al., 2020), which has reasonable but lower sensitivity compared with the PCR-based methods (Matsuda et al., 2021). The WHO recommends several commercial kits for the diagnosis of SARS-CoV-2 by qRT-PCR and rapid testing that are certified in different regions of the world performed largely in public health laboratories. Rapid tests also use nasopharyngeal sampling, which requires trained health professionals to avoid the propagation of aerosols and further virus dissemination during sample collection.

The barriers hindering SARS-CoV-2 qRT-PCR mass diagnosis include the availability of biosafety-equipped laboratories to handle samples before virus inactivation, specialized technicians to perform the qRT-PCR assays, and trained health professionals to collect biological samples. Also, in low-income regions, WHO recommended consumables needed for biological collection and tests are expensive, depending on importation from other regions, and due to the high global demand, are not always available. Therefore, this study aimed to develop an alternative protocol for SARS-CoV-2 genomic detection on a large scale, adapted to low-income regions. The method described uses self-collected saliva in hydrophilic cotton and was tested by qRT-PCR and RT-endpoint-PCR with local consumables on archived and prospectively collected biological samples from the local reference clinical laboratory of the ABC Medical School.

2. Material and methods

2.1. Patients and biological samples

Two groups of patient samples obtained in the local reference SARS-CoV-2 diagnostic laboratory, the ABC Medical School clinical laboratory, were employed in this study: 99 archived nasopharynx SARS-CoV-2 positive samples; and 111 nasopharynx samples paired with self-collected saliva in hydrophilic cotton from patients attended prospectively. The archived 99 nasopharynx secretions were obtained from patients attended in November 2020. The detection of SARS-CoV-2 in these samples was performed through qRT-PCR using a kit from IDT, USA, based on Taqman method with primers and probes N1 and N2, complementary to the virus nucleocapsid protein (Table 1), according to the Center for Disease Control protocol (CDC, 2020). In a flow hood, the archived samples were thawed and a sterile ball of hydrophilic cotton with a diameter of 0.5 cm^2 was submerged in each tube. Afterward, with the help of disposable forceps, the ball of cotton was transferred to a 2-mL snap crew tube containing 1 mL of RNA extraction solution (0.8 M of guanidine thiocyanate; 0.4 M of ammonium thiocyanate; 0.1 M sodium acetate; 5 % glycerol and 38 % of acid buffered phenol) (Rodriguez-Ezpeleta et al., 2009) and sent to the molecular biology laboratory of the Universidade Federal do ABC at room temperature.

Table 2
Comparative sensitivity of RT-qPCR and RT endpoint PCR according to the amplification cycle (C_T) obtained in the qRT-PCR applied in the routine of diagnostic of archived nasopharynx samples, using commercial kit.

	RTqPCR			RT-endpoint PCR		
C_T*	Detected	Non-detected	K (IC 95 %)	Detected	Non-detected	k (IC 95 %)
< 25 N = 41	**40**	**1**	0,97 (0.92–1.00)	**37**	**4**	0,90 (0,81–0,99)
26–30 N = 21	**18**	**3**	0.86 (0.72–1.00)	**14**	**7**	0,66 (0,46–0.86)
31–35 N = 27	**13**	**14**	0.48 (0.29–0.67)	**10**	**17**	0,37 (0,18–0.56)
36–40 N = 10	**1**	**9**	0.10 (0 – 0.28)	**1**	**9**	0.10 (0 – 0,28)
Total N = 99	**72**	**27**	0,72 (0,63–0,80)	**62**	**37**	0,63 (0,54–0,72)

* C_T – correspond to the cycle of amplification by qRT-PCR as a result of the positive diagnostic using the commercial IDT kit for N1 and N2 detection.

Nasopharynx secretion samples matched with cotton self-collected saliva samples were collected from 111 patients consecutively attending the outpatient clinic for the diagnosis of SARS-CoV-2 of the ABC Medical School from 6th to 13th January 2021. Nasopharynx-based SARS-CoV-2 diagnostic tests were routinely performed in the outpatient clinic for symptomatic and asymptomatic individuals, mainly health workers and traveling people, to monitor the occurrence of infection. Each patient provided their informed consent and completed a form containing enquires about demographic information (e.g., gender, age), symptoms associated with COVID-19 and their period of its occurrence. From all patients attending the ABC Medical School, a health care professional collected samples from each nose and throat using a swab. These nasopharynx samples were combined in a conical tube containing saline and transported to the clinical laboratory of ABC Medical School where SARS-CoV-2 diagnostic tests were mainly performed with GeneFinder™ COVID-19 PLUS RealAmp Kit (OSANG Healthcare Co., Ltd, Gyeonggi-do, Korea) based on Taqman technology using primers and probes to detect SARS-CoV-2 RNA-dependent RNA polymerase (RdRp) and envelope (E)-encoding genes.

The experimental protocol used for human sample collection was approved by the Ethical Human Committee on Human Research of the ABC Medical School and of the Universidade Federal do ABC under number 33800620.0.3001.0082.

2.2. Self-collection of saliva

All patients who agreed to participate in the research received a kit for the self-collection of a saliva sample containing: a sterile cotton ball with a diameter of 0.5 cm; a sterile 9-mL white-capped tube for vacuum blood collection without additive; a 15 × 10 cm 70 % isopropanol wipe; plastic gloves and a seal type envelope containing a form for patient identification. On a clean bench, after handwashing with soap and put on plastic gloves, the patients placed the cotton in their mouths and immersed it in saliva for 1 min. Afterward, the cotton was removed and placed in a vacuum blood collection tube with a screw cap. The gloves were then removed, and the tube was wrapped in a wipe moistened with 70 % isopropanol and placed in a sealed envelope labeled with the patient's identifying information (Video 1).

2.3. RNA extraction and SARS-CoV-2 diagnostic methods

One mL of RNA extraction buffer was added to the vacuum blood collection tube containing the self-collected saliva in cotton, as previously described (Rodriguez-Ezpeleta et al., 2009), through the rubber cap using a syringe. Then, the tube containing inactivated virus was opened in a fume hood and the RNA extraction buffer was transferred to a 1.5-mL tube containing 200 µL of chloroform and mixed vigorously. After centrifugation for 5 min at 4°C and 20,000 × *g*, the aqueous phase containing the nucleic acids was transferred to a 1.5-mL tube with 900 µL of absolute ethanol, followed by centrifugation for 15 min at 4 °C and 20,000 × *g*. After that, the ethanol solution was discarded, and 1 mL of 70 % ethanol was added to the pellet and centrifuged for 5 min at 4 °C and 20,000 × *g*. The 70 % ethanol solution was discarded, and the pellet was dried at room temperature for 15 min and resuspended in 40 µL of RNAse-free 10-mM Tris−HCl at pH 7.8.

cDNA was synthesized with the reverse transcriptase (RT) Script® (Cellco Biotec do Brasil Ltda, São Carlos, São Paulo, Brazil) according to the manufacturer's instructions using 100 ng of random hexamers (ThermoFischer Scientific, Waltham, MA, USA), 100 units of RT, and 8.5 µL of total nucleic acids extracted as described above. The reaction conditions for cDNA synthesis were 10 min at 42 °C, 50 min at 50 °C, and 5 min at 85 °C.

Two microliters of synthesized cDNA were used in each endpoint-PCR using Hot-Star Taq DNA Polymerase (Cellco Biotec do Brasil Ltda, São Carlos, São Paulo, Brazil) in a thermocycler Biometra® (Analytik Jena, Jena, Thuringian, Germany) and multiplex quantitative TaqMan PCR (qPCR) in the CFx96 real-time PCR equipment (Bio-Rad, Hercules, CA, USA). The endpoint-PCR was performed by PCR and nested-PCR based on the protocol described by Shirato et al. (2020) and recommended by the Center for Disease Control (CDC), which amplifies a 492-base pair (bp) fragment complementary to the SARS-CoV-2 spike protein (Shirato et al., 2020). Nested PCR was performed from 2 µL of 20 µL of the first PCR reaction after treatment for 20 min at 37 °C with one unit of exonuclease I (Exo) and one unit of shrimp alkaline phosphatase (SAP) (Cellco, São Carlos, São Paulo, Brazil), followed by the inactivation of the enzymes for 5 min at 85 °C. Eight µL of the nested PCR reaction was evaluated by agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light using the L-Pix® Touch image analyzer (Loccus, Cotia, São Paulo, Brazil). The fragments were sequenced using the Sanger method after purification with Exo and SAP, as described above. Thirty nanograms of PCR fragment with 5 mM of the specific primer were sent to the Sanger sequencing service of ACTGene, a company located in Rio Grande do Sul, Brazil.

For multiplex qPCR, primers and probes used were based on the SARS-CoV-2 nucleocapsid encoding gene recommended by the CDC, N1 and N2. Human RNAse P primers and probes were used as positive controls for the qPCR reaction. The probes were synthesized by GenOne Biotech and the primers by ThermoFisher Scientific. The qPCR reactions were performed by using the GoTaq® qPCR probe (Promega, Madison, WI, USA) according to the manufacturer's instructions. The sequences of the primer and probes used, in addition to the reaction amplification conditions, are described in Table 1. The concentration of each primer and probe used in the multiplex reaction was 1 µM and 0.4 µM, respectively.

2.4. Statistical analysis

The statistical calculation of the obtained data was achieved through

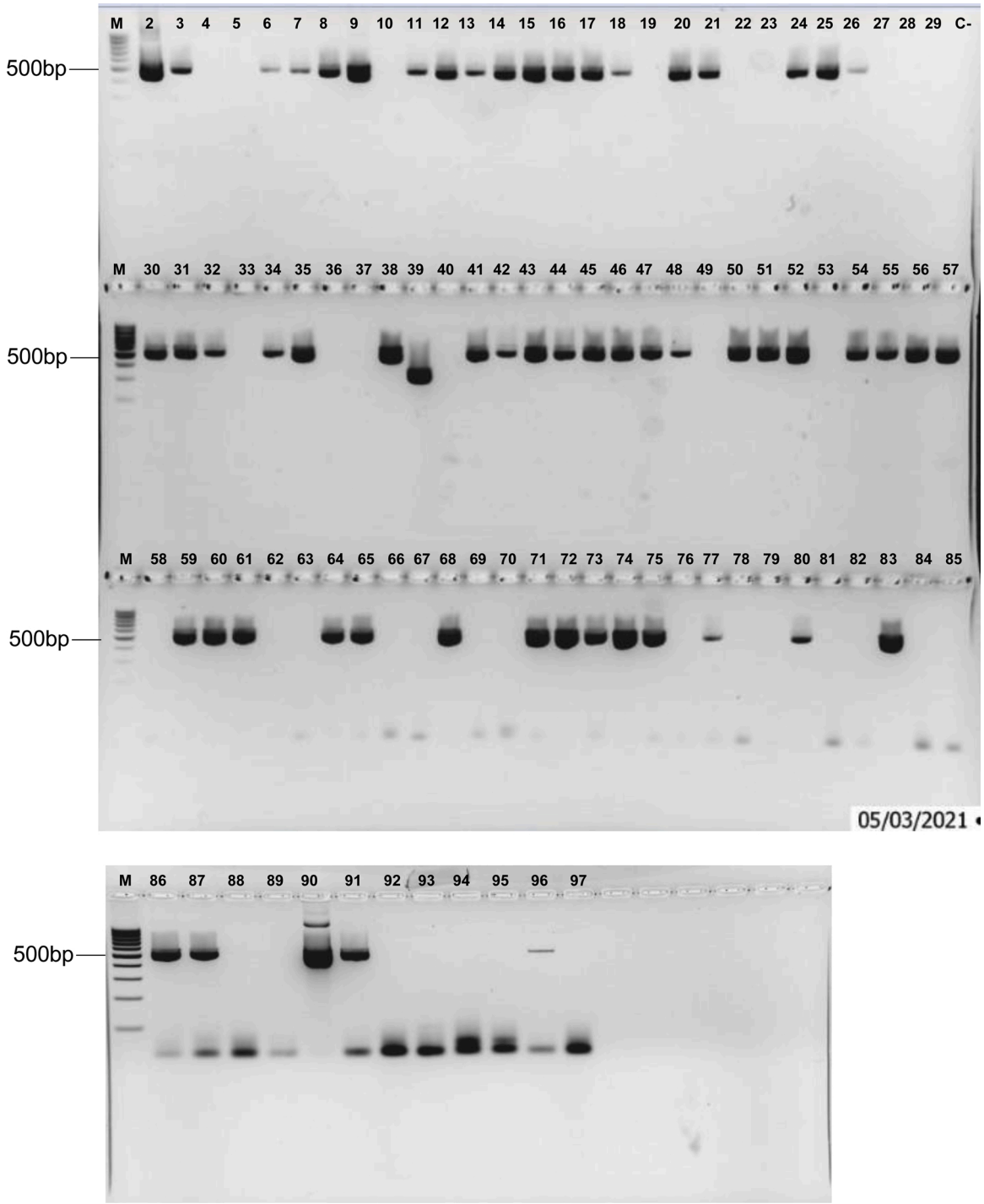

Fig. 1. Electrophoresis in 2% agarose gel stained with ethidium bromide containing the products of RT-endpoint nested PCR directed to amplify a 492 bp fragment of the Spike SARS-CoV-2 encoding gene. **C-,** negative control; **M,** 100 bp ladder; **2-97**, represent different nasopharynx archived samples.

Fischer's exact test for the categorical variables according to sample size. The evaluation of the diagnostic tests was performed using the kappa (k) correlation coefficient (McHugh, 2012). The applicability of saliva for SARS-CoV-2 diagnosis by qRT-PCR was evaluated by calculating sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Significance was considered with a 95 % confidence interval (CI) and a p-value < 0.05. All statistical analyses were achieved using the free statistical software available from Social Science Statistics (socscistatistics.com).

3. Results

To validate the use of hydrophilic cotton for saliva collection and to standardize the SARS-CoV-2 qRT-PCR and the RT-endpoint-PCR diagnostic methods, 99 archived nasopharynx samples with SARS-CoV-2 qRT-PCR diagnosis with N1 and N2 primers and probes, were employed. The quality of the nucleic acid extracted from cotton embedded in the nasopharynx secretion was confirmed for 96 samples, which presented positive results for the primers and probes to human RNAse P (Table 1) after cDNA synthesis and multiplex qPCR (Table_1_suppinfo).

Table 3
Characteristics of patients attended at ABC Medical School submitted for matched nasopharynx secretion and cotton self-collected saliva prospective study for SARS-CoV-2 RT-PCR diagnostic.

	Overall (n = 111)	SARS-CoV-2 positive	SARS-CoV-2 negative	*P- value*[#] IC 95%
		Nasopharynx[*] (n = 10)/ Saliva[$] (n = 7)	Nasopharynx (n = 101)/ Saliva (n = 104)	Nasopharynx/ saliva
Age (years) median	34 (16–80)	41.5 (32–80)/48 (36–80)	33 (16–71)/ 33 (16–71)	**0.01325**[$]/ **0.0035**
Male	37	3	34	1.000
	37	3	34	0.681
Symptoms at presentation	39	6	33	0.1611
	39	4	35	0.2385
Fever	6	3	3	**0.0093**
	6	3	3	**0.0029**
Cough	16	2	14	0.6352
	16	1	15	1.000
Sore throat	12	4	8	**0.0119**
	12	2	10	0.1658
Headache	16	4	12	**0.0119**
	16	3	13	0.0605
Dizziness/ nausea/ vomiting	6	0	6	1.0000
	6	0	6	1.0000
Runny nose	13	3	10	0.0932
	13	1	12	0.5927
Adynamia	17	3	14	0.1800
	17	1	16	1.0000
Dyspneal	8	1	7	0.5423
	8	1	7	0.4166
Diarrhea	6	1	5	0.4403
	6	1	5	0.3299
Asymptomatic	72	4	68	0.1611
	72	3	69	0.2385

[*] SARS-CoV-2 positive diagnostic by qRT-PCR using nasopharynx samples; $SARS-CoV-2 positive diagnostic by RT-qPCR using saliva samples.
[#] *p-value* for all variables except age was calculated using Fisher hypothesis test.
[$] Statistical test used for age was *T*-test for two independent means.

Agreement of tested multiplex qRT-PCR was obtained for 72 from 99 nasopharynx samples (k = 0.72 CI 95 % 0.63 – 0.80), which presented positive results for both N1 and N2 primers and probes (Table 2, Table_1_suppinfo). The endpoint-nested-PCR results using primers directed to amplify a fragment of 492 bp of the SARS-CoV-2 spike encoding gene was positive for 62 samples (k = 0.63 CI 95 % 0.52 – 0.72) (Fig. 1, Table 2). Agreement of both SARS-CoV-2 diagnostic methods tested presented a significant decrease in virus genomic detection, according to the initial N1 and N2 qRT-PCR amplification cycle (C_T) numbers of the positive samples (Table 2). The agreement kappa coefficient of the multiplex qPCR and the endpoint-nested PCR were k = 0.97 (95 % CI 0.92−1.00) and k = 0.90 (95 %CI 0.81−0.99), respectively, when compared with samples with initial C_T value under 25 (Table 2).

Performance of SARS-CoV-2 qRT-PCR in 111 self-collected saliva samples was investigated through comparative analysis with matched diagnosis by qRT-PCR on nasopharynx samples. Nine nasopharynx samples and 7 saliva samples were positive for SARS-CoV-2; three samples were positive only in nasopharynx sample and one was positive only in the saliva sample. The detection of SARS-CoV-2 RNA only in saliva was confirmed in a second nasopharynx sampling and testing in the outpatient clinic of ABC Medical School. The saliva nucleic acids from the three patients with SARS-CoV-2 detected only in their nasopharynx samples were evaluated with qRT-PCR used in the clinical laboratory of ABC Medical School, which confirmed the negative result for all three samples. The quality of the extracted nucleic acids of all saliva samples was confirmed by the detection of human Rnase P (Table_2_Suppinfo).

Correlation of the patient's characteristics, including age, gender, and independent COVID-19 symptoms with SARS-CoV-2 positive diagnostic is presented in Table 3. Statistical analysis showed that age and fever were significantly associated with positive diagnostic in both types of biological samples, where sore throat and headache were only significantly associated with qRT-PCR SARS-CoV-2 positive diagnostic on nasopharynx samples. The sensitivity and specificity of saliva sampling were 70 % and 100 %, respectively, for the SARS-CoV-2 diagnostic test presented in this study, when compared with results from the nasopharynx samples using the methodology of qRT-PCR routinely applied. The PPV and NPV were 1 and 0.97, respectively. The SARS-CoV-2 positive diagnostic agreement of saliva with nasopharynx-matched samples was 97 % (κ coefficient 0.79, 95 % CI 0.56–1.00). The individual results of a comparative study of nasopharynx with saliva samples are presented in Table_2_ Suppinfo.

The specificity of the SARS-CoV-2 492 bp fragment of the spike encoding gene, obtained by RT-endpoint, and nested-PCR from 22 nasopharynx archived samples, was confirmed by Sanger sequencing. One sample with a fragment of approximately 320 bp also confirmed the specificity to the SARS-CoV-2 spike encoding gene, however presented a deletion of 172 bp (Fig. 2). All sequences obtained showed 100 % similarity to sequences from SARS-CoV-2 isolated in the USA. From the 22 nested-PCR spike PCR fragment of 492 bp, only one presented a substitution in one base, which did not result in an amino acid change (Fig. 1_Suppinfo).

4. Discussion

Based on the experience in the management of the COVID-19 pandemic, public global health institutions, including the WHO, Pan American Health Organization (PAHO), and the CDC, recommend social distancing to decrease virus circulation and mass diagnostic testing to detect and isolate infected people to prevent SARS-CoV-2 transmission foci. The detection of genomic viral RNA through the qRT-PCR method with diverse commercial kits based on different virus genomic targets, also recommended by public global health institutions, is the most commonly used worldwide. The main biological specimen type used for genomic RNA virus detection corresponds to nasopharynx secretions, whose collection method with a swab is invasive and uncomfortable for the patient, requires a trained professional to collect and poses a risk of contamination to the person responsible for collection. Toward designing a molecular SARS-CoV-2 diagnostic protocol for low-income regions, in this work, we adapted the recommended diagnostic methods using self-collected saliva as the biological source associated with viral genomic detection through RT followed by qPCR and/or endpoint nested-PCR with consumables locally available.

Saliva samples have been successfully used to diagnose SARS-CoV-2 infection by qRT-PCR, presenting the advantage of being non-invasive permitting collection by the patients themselves, thereby avoiding nosocomial infection risks during sample collection (Azzi et al., 2020; Pasomsub et al., 2021; Takeuchi et al., 2020; Wyllie et al., 2020). There are several commercial disposables for saliva collection, however, generally, the biological sample is transported in the liquid state in screw cap flasks, which in case of accidental occurrence can spill SARS-CoV-2 contaminated saliva during transport and manipulation. This drawback was tracked using humid heat sterilized balls of hydrophilic cotton 0.5 cm in diameter as a disposable material for saliva self-collection, which showed excellent results regarding the obtention of nucleic acids used in RT followed by qPCR and endpoint-PCR (Table 2 and Fig. 1). A decrease in the detection of SARS-CoV-2 in samples according to the increase in C_T values is expected since, in −80 °C archived biological samples, the detection of viral RNA after thawing is less efficient.

The detection of SARS-CoV-2 through endpoint- and nested-PCR

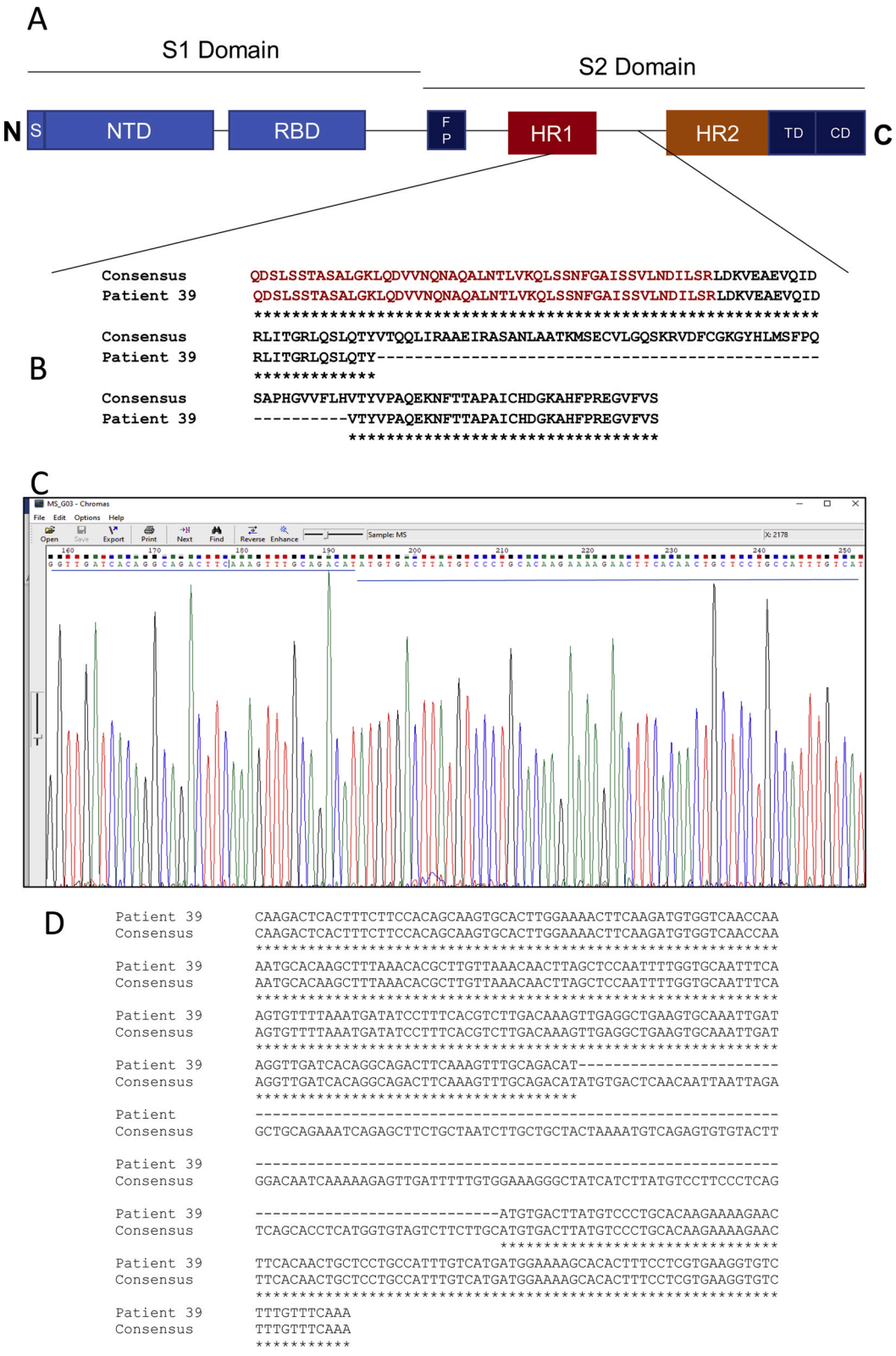

Fig. 2. Detection of SARS-CoV-2 with a deletion of 172 bp of the spike encoding gene, including the partial domain of heptanucleotide repeat 1 (HR1) obtained directly from a patient sample. **A.** Representative domains of the SARS-CoV-2 spike protein based on Huang et al. (2020): S - signal peptide; NTD - N-terminal domain; RBD - receptor binding domain; FP - fusion peptide; HR1 - heptanucleotide repeat 1; HR2 - heptanucleotide repeat 2; TD - transmembrane domain; CD - C-terminal domain. **B.** Amino acid sequence comparison from the consensus SARS-CoV-2 spike 492 bp fragment and the 320 bp fragment obtained from patient 39. **C.** Sanger's sequence chromatogram of the SARS-CoV-2 320 bp fragment obtained from Patient 39; inset – bars indicate deletion position. **D.** Nucleotide sequence comparison between the SARS-CoV-2 spike 492 bp consensus fragment and the 320 bp fragment obtained from Patient 39.

showed lower sensitivity when compared with qPCR (Table 2). However, in low-income regions that lack real-time PCR equipment, this method can serve as an alternative by using a conventional thermocycler. Also, endpoint-PCR can serve to investigate gene polymorphisms and detect viral genetic variants by directly sequencing the PCR fragments obtained from patient samples. Patient 39, who was positive with qPCR and endpoint-PCR, presented a smaller fragment than expected (Fig. 1). After sequencing, this fragment showed specificity to the SARS-CoV-2 spike genomic region with a deletion of 172 bp (Fig. 2). The deleted sequence is a part of the repetitive heptapeptide domain 1 (HR1) of the SARS-CoV-2 spike protein, which is involved in the virus' cellular fusion and entry processes (Huang et al., 2020) (Fig. 2). The detection of SARS-CoV-2 in the nasopharynx samples of patient 39 by qRT-PCR and qRT-PCR showed C_Ts under 25 for both Taqman primers and probes N1 and N2 (Table_1_Suppinfo), indicating high viral gene expression, and consequently, a high capability of infection. Amplification of the 492 bp fragment specific to SARS-CoV-2 from nucleic acids obtained with the use of hydrophilic cotton as a disposable biological sample collection medium establishes the proof of concept for an alternative protocol that would greatly facilitate epidemiological studies.

Saliva and nasopharynx matched samples prospectively collected from patients attending the outpatient clinics of ABC Medical School in January 2021 showed 97 % agreement for the alternative protocol, confirming that saliva is an excellent biological source for SARS-CoV-2 diagnosis that is easily accessible using local consumables. The sample size was too small to thoroughly investigate individual characteristics and symptoms associated with differential positive diagnoses according to the biological sample used, as indicated by a κ coefficient of 0.79 and a wide 95 % CI of 0.56−1.00. Also, as most of the patients attending prospectively were asymptomatic or oligosymptomatic, without clinical and physical exams, it was difficult to investigate the relationship between the initial infection and symptom onset with the differential detection of SARS-CoV-2 according to biological sample source. However, sore throats and headaches were significantly associated with nasopharynx over saliva samples positive for SARS-CoV-2. Future studies with a higher number of positive SARS-CoV-2 samples would help to resolve these remaining unknowns.

The stability of RNA in saliva samples without the addition of transport liquid medium or toxic reagents to inactivate RNAses has been described in the literature (Ott et al., 2020, 2021) and confirmed here by the results obtained with the SARS-CoV-2 qRT-PCR diagnostic test with saliva self-collected on cotton. These findings provide a basis for establishing a methodology for the non-supervised self-collection of samples for SARS-CoV-2 diagnosis by qRT-PCR, thereby alleviating demands for specific collection disposables, health care personnel, and individual protective equipment supplies, in addition to allowing the samples to be transported at room temperature in simple boxes and nucleic acid extraction to be executed up to 24 h post collection. The alternative protocols described in the present study are well suited even in low-income regions and enable the inclusion of basic structured laboratories in SARS-CoV-2 mass testing.

Authors' contributions

All authors contributed to the study, commented on previous versions of the manuscript, 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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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.2021.114382.

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