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Evaluation of saliva as an alternative to standard collection for SARS-CoV-2 detection

Fernando Ferreira [1,2], Daniela Alves [2], Telma Oliveira [2], Sílvia Coelho [2], Inês Oliveira [2] and Paula de Melo Alves [2,3,*]

[1] University Institute of Health Sciences (IUCS), CESPU, Rua Central da Gandra, 1317, 4585-116, Gandra, Paredes, Portugal; fernando.ferreira@cespu.pt
[2] CESPU Diagnóstico, SA company, Rua Central de Gandra, 1317, 4585-116, Gandra, Paredes, Portugal; danielaalves_25@hotmail.com; telma.sofia@cespu.pt; silvia.coelho@cespu.pt; ines.oliveira@cespu.pt
[3] Polytechnic Institute of Health of the North (IPSN) CESPU, Rua Central da Gandra, 1317, 4585-116, Gandra, Paredes, Portugal

* Correspondence: paulaapct@gmail.com

Abstract: In December 2019, in Wuhan, an outbreak of a new coronavirus (SARS-CoV-2) began to spread rapidly, resulting in a potentially fatal viral respiratory disease. Since August 23, 2020, more than 679 million cases of COVID-19 and nearly 6.8 million deaths have been confirmed in more than 200 countries. This, in turn, had a severe impact on public health and the world economy. The SARS-CoV-2 pandemic also resulted in a shortage of viral transport medium and in the need to find different diagnostic means, such as saliva. To fill this gap in the market, a new viral transport medium was created and tested with samples collected from the nasopharynx and/or oropharynx using swabs and saliva samples. The specificity, sensitivity, and threshold cycles (Cts) of Real-Time PCR (RT-PCR) testing of the samples were compared, revealing concordant results with approximate sensitivity and specificity. Our study highlighted the need to optimize saliva sample collection and its potential use as a substitute for standard collection.

Keywords: SARS-CoV-2; saliva; viral transport medium

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Introduction

It was in the Wuhan province, China, that in December 2019 an outbreak of atypical pneumonia emerged, with a high transmission rate and associated mortality. Its rapid spread forced the World Health Organization (WHO) to declare a Pandemic status in March 2020. The outbreak had been caused by the emergence of a new and unknown virus, initially named N-CoV19 (New Coronavirus 2019), a name later changed to Severe Acute Respiratory Syndrome – Coronavirus 2 (SARS-CoV-2). According to the WHO, the genomic sequence identified in the new virus is very similar to that of other already known and studied viruses, which made it possible to state that it was a new strain of coronavirus [1].

Classified as belonging to the *Coronaviridae* family, *Coronavirinae* subfamily, and *β-coronavirus* genus, SARS-CoV-2 is thought to be a zoonotic virus (possibly with the bat as its usual host) that has crossed the species barrier and infected humans through intermediate hosts [2]. Coronaviruses (CoVs) are a large family of viruses, responsible for causing numerous respiratory diseases in humans, ranging from the common cold to severe, potentially lethal, respiratory infections such as coronavirus disease 2019 (COVID-19), the name given to the disease caused by the new coronavirus [2]. Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and Middle East Respiratory Syndrome Coronavirus (MERS-CoV) are examples of other coronaviruses that cause severe, highly deadly cases of pneumonia [1].

Round in shape, with a diameter of between 65 and 125 nm, and surface loaded with crown-like spicules, SARS-CoV-2 is composed of four major structural proteins and multiple accessory proteins [1,2] (Fig. 1).

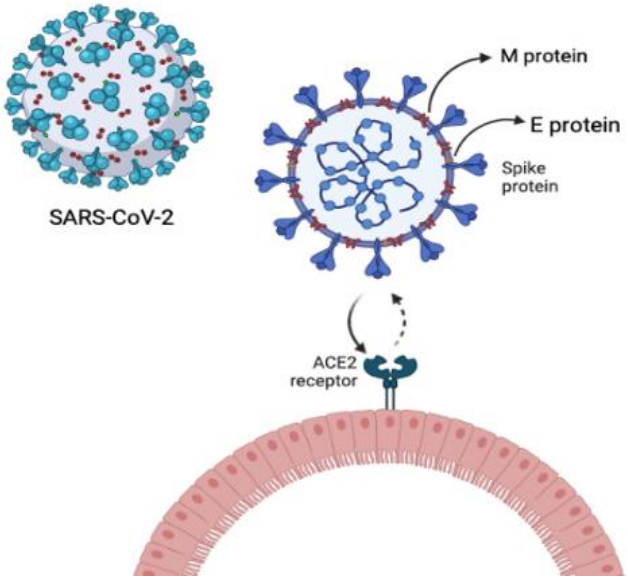

Figure 1. SARS-CoV-2 virus shape and structure. The SARS-CoV-2 spherical particle consists of four structural proteins: the spike proteins, the membrane protein (M), the envelope protein (E), and the nucleocapsid protein. The spike protein, M protein, and E protein are incorporated in the virus membrane, while the nucleocapsid protein resides inside the particle and is associated with virus genomic RNA. Upon binding to the cellular receptor angiotensin-converting enzyme 2 (ACE2), the spike protein is activated by protease cleavage. Created by the authors with BioRender.com.

The four main structural proteins are Glycoprotein S (Spike), Glycoprotein E (similar to a small envelope), Glycoprotein M (membrane glycoprotein), and Nucleocapsid (N) protein. Each of these proteins has different functions, but they are equally essential for the survival, stability, and replication of the virus. For Glycoprotein S (Spike) to become functional, the S protein needs to be cleaved into 2 subunits (S1 and S2), thus increasing its connection to the host cells. The cleavage of the Spike protein occurs through the action of furin, an enzyme present in host cells of different organs such as the liver, lungs, kidneys, or intestines. This mechanism may explain the high rate of lung infection and contagion among humans [3]. The S1 and S2 subunits have a high affinity for the angiotensin-converting enzyme 2 (ACE2) produced by cells of the lower respiratory tract, epithelial cells of the upper digestive tract, enteric absorptive cells, myocardial cells, and cells of the urinary tract, including those of the kidneys and bladder. The expression of ACE2 by all these cells explains the clinical manifestations that can arise in patients, from acute respiratory illness to potential cardiac, renal, and digestive tract complications that can lead to the failure of the infected individual's system [3,4].

Glycoprotein E is the smallest structural protein with an active role in the production and maturation of these viruses. Glycoprotein M corresponds to a membrane protein and is decisive for the shape of the virus envelope. It allows it to become structurally robust by protecting the Nucleocapsid-RNA complex inside the virion. Nucleocapsid protein, or N protein, is the structural component linked directly to the genome and is therefore a determining protein in the replication cycle of the virus, as well as in the cellular response of host cells to viral infection [4].

Due to the highly infectious and pathogenic nature of SARS-CoV-2, the best way to fight this pandemic and prevent COVID-19 is to identify and break the chains of contagion, thus preventing the continuous spread of the virus. It is only possible to break the chains of contagion by using laboratory diagnostic tests that are highly sensitive and accurate in detecting the viral load. There are different laboratory tests with different functions (serological tests and diagnostic tests – Real Time-PCR tests (RT-PCR) and antigen tests) [5].

Serological tests are based on the identification of anti-SARS-CoV-2 antibodies (immunoglobulins IgA, IgG, and IgM) produced by the immune system. They are important in epidemiological studies of the prevalence of the disease in the population, as well as in the assessment of the state of "herd immunity" [5]. Immunochromatography tests are less complex and can be performed by a healthcare professional or as a self-test, but tend to be less sensitive than RT-PCR [5]. The detection of molecular components of the virus (either by the expression of certain proteins or by the identification of specific sequences of its genome) is the only way to make a precise and concrete diagnosis. As such, molecular biology plays an extremely important role in the fight against this pandemic [5].

Reverse transcriptase is an enzyme that uses a specific sequence of single DNA (primer) to pair with the RNA in the sample, generating the complementary strand of DNA (cDNA), to which the RT-PCR technique is then applied [6]. RT-PCR is a technique used for the quantification of specific DNA or RNA sequences in a sample. If there is a strong presence of the sequence that is intended to be amplified,

amplification begins in earlier cycles. If, on the other hand, there is no abundant presence of the genetic material, amplification occurs in later cycles [7].
There are 3 main steps in the RT-PCR: denaturation (high temperatures cause the separation of the DNA double chain), annealing (pairing of specific primers with each of the single DNA chains), and extension (synthesis of the new sequences of the target DNA by a DNA polymerase) [6,7] (Fig. 2).

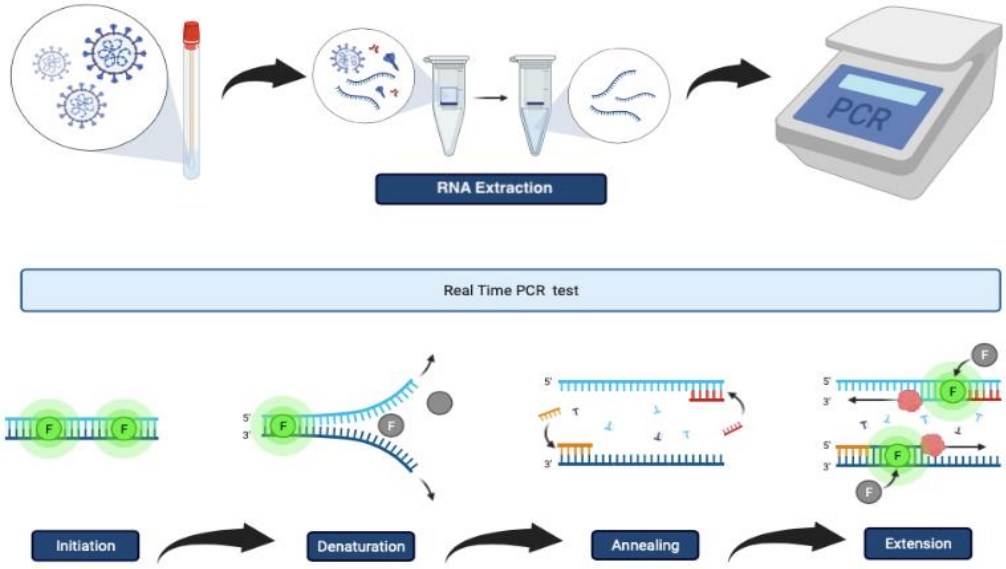

Figure 2. The procedure used for COVID-19 testing involves the collection of patient material and deposition of potential SARS-CoV-2 viral particles in a transport medium, RNA extraction, and transfer of samples to the PCR-plate in which RT-PCR may take place. The PCR is based on three simple steps required for any DNA synthesis reaction: denaturation of the template into single strands, annealing of primers to each original strand for new strand synthesis, and extension of the new DNA strands from the primers. Created by the authors with BioRender.com.

Molecular detection methods such as PCR do not require a virus to be able to replicate its genetic material, but the preservation of its nucleic acids is essential [8]. To obtain genetic material (RNA) from SARS-CoV-2, the sample is collected by exfoliating the mucosa of the nasopharynx using a swab. The means of transport chosen is of great importance because it can condition or even make an accurate analysis unfeasible. The choice of means of transport must be appropriate to the type of sample. Thus, if the sample is collected due to suspected bacterial infection, the transport medium must be different from that used for a sample collected due to suspected viral infection. However, whatever the transport medium, its function is to maintain the stability of the sample and to preserve both the genetic material of the host cells and the genome of the suspected microorganism. Most commercial transport media are based on a buffer solution, antimicrobials, proteins or amino acids, and a solidifying agent like gelatin and/or a cryoprotectant agent like sucrose (e.g., Universal Transport Media, M4RT, M4) [9]. Some commercial transport media may also have a lysis solution in their composition (e.g., CyMol, PrimeStore MTM, eNat).
Based on several literature reviews, it was possible to create an in-house viral transport medium with freezing potential, for cases where there is a long waiting time between sample collection and laboratory processing, to avoid degradation of the genetic material of the virus and the host [8,9].
Fetal Bovine Serum (FBS) gives the viral transport medium characteristics that are as close as possible to those of the host cells, since it is rich in growth factors, hormones, amino acids, proteins, vitamins, inorganic salts, and antibodies. The high protein concentration in the serum also acts as a cryoprotectant during the freezing process. Antimicrobials – antibiotics (gentamicin) and antifungals (amphotericin B) – prevent the proliferation of bacteria and fungi that may contaminate the sample. The agar solution provides consistency to the medium and acts as a cryoprotectant [9].
Saliva is a hypotonic liquid secreted by the parotid, submandibular, sublingual, and minor salivary glands that are distributed throughout the oral cavity. These glands are very permeable and have plenty of blood capillaries, allowing the exchange of molecules and biomarkers, which can be secreted along with saliva. These salivary biomarkers have been analyzed and used to detect local and systemic diseases such as caries, periodontitis, oral and lung cancer, diabetes, cardiovascular diseases, and viral infections [10].
Oral fluid samples can indicate the presence of viral infection by analyzing viral nucleic acids, antigens, and antibodies [11].
The use of saliva for the diagnosis of viral infections has attracted interest in recent years, mainly because it is a non-invasive technique, easier to perform, reduces transportation and procurement costs, reduces the use of personal protective equipment (PPE) by not requiring collection by a healthcare professional, and therefore reducing the risk of infection, and has a low cost [12]. In addition, saliva is seen as a positive detection medium for coronavirus nucleic acids associated with severe acute respiratory syndrome and,

more recently, related to SARS-CoV-2 [13,14]. Saliva can be obtained from: stimulated saliva (accumulating saliva in the oral cavity, as well as any nasal secretion/mucus in the oropharynx) or unstimulated saliva ("drooling" technique) or orally using a swab or a similar tool.
Later oropharyngeal saliva should be differentiated from oral saliva, since the former is part of respiratory secretions that may come from the upper respiratory tract (nasopharynx) and/or lower respiratory tract (bronchi and lungs) and gingival fluid, while the latter is produced by the salivary glands, which are outside the respiratory tract (Fig. 3) [15,16].

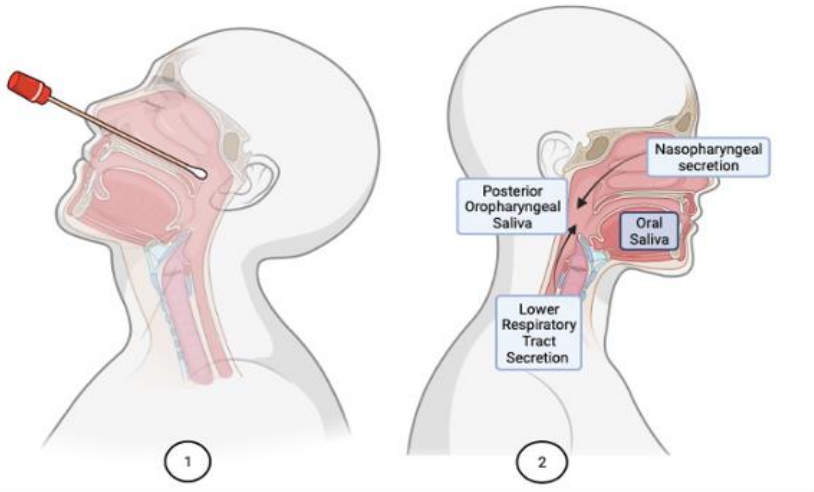

Figure 3. Sample collection from the nasopharynx and/or oropharynx (number 1). Different salivary samples (number 2). Lower oropharyngeal saliva is the secretion produced while coughing or "clearing" the throat, belonging to the respiratory secretions, and mixing secretions from the upper (nasopharynx) and lower (bronchi, lungs) airways. In contrast, oral saliva is produced by the salivary glands. Created by the authors with BioRender.com.

Currently, there is evidence that the salivary collection method performs well in detecting SARS-CoV-2 in both asymptomatic and presymptomatic patients [15,17-21]. Some studies also show that salivary collection samples have greater sensitivity in detecting SARS-CoV-2 than nasopharyngeal samples [15,22-26]. As mentioned before, the SARS-CoV-2 pandemic resulted in a shortage of viral transport medium and in the need to find different diagnostic means, such as saliva. To fill this gap in the market, a new viral transport medium was created and tested with samples collected from the nasopharynx and/or oropharynx using swab and saliva samples.

Materials and Methods

Salivary collection method

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of CESPU (01/CES-CESPU/2022).
202 saliva samples were collected from negative and positive patients aged between 6 and 64 years old, into sterile tubes with 3 ml of the in-house created viral transport medium. For collection, patients were instructed to accumulate saliva in the oral cavity, as well as to gather any nasal secretions/mucus in the oropharynx, and then to deposit these secretions and additional saliva into the collection tube. The amount of saliva collected should be between 0.5 and 1 ml.

Sample collection from the nasopharynx and/or oropharynx

For counter-analysis, samples were collected from the nasopharynx and/or oropharynx using a swab, into sterile tubes with 3 ml of the in-house created viral transport medium. The swab was immediately placed in the vial after collection, immediately coming into contact with the viral transport medium.

Medium preparation

Fetal bovine serum (FBS) was inactivated at 56 °C for 30 minutes. A gentamicin solution was prepared at 100 µg/ml. An agar solution was prepared at 0.5% with purified (non-sterilized) water. At the end, all viral transport medium components were mixed – phosphate-buffered saline (PBS) powder dissolved in distilled water (final dilution with pH 7.4), FBS, gentamicin, amphotericin B and agar solution.

Extraction and amplification

Extraction was performed with the FastPure Viral DNA/RNA Mini Kit (Vazyme) and amplification was performed using the ViroQ SARS-CoV-2 kit (BAG Diagnostics), following the manufacturers' protocols. RT-PCR was performed with a CFX96™ Real-Time System and a C1000 Touch™ Thermal Cycler (Bio-Rad). Human DNA integrity was tested by analyzing the internal control and the integrity of SARS-CoV-

2 RNA. We validated and analyzed data with the advanced analysis modules of the CFX Maestro Software (Bio-Rad).

Statistical analysis
IBM SPSS software was used for statistical analysis. The data analysis for comparison between tests was performed with the McNemar's test and T-test. Data distributed according to the normal are presented according to the mean (standard deviation). Categorical data is represented by n (percentage). Only P values below 0.05 were considered statistically significant.

Results

Over the past few months, the number of studies involving saliva has grown considerably. To centralize our knowledge and increase our understanding of the diagnostic sensitivity of SARS-CoV-2 in saliva samples and in our environment, we analyzed 202 samples from different patients (Table 1).
The RT-PCR results were interpreted according to the following criteria: when fluorescence signal for the specific SARS-CoV-2 gene (RNA-dependent RNA polymerase – RdRP gene) is detected, the result is interpreted as "positive". When the fluorescence for the SARS-CoV-2 specific gene is not detected or its threshold cycle (Ct) is ≥ 39, the result is interpreted as "negative". However, if the fluorescence signal from the internal control is not detected, it is interpreted as "invalid".

Table 1. Results from the detection of SARS-CoV-2 in saliva samples using the RT-PCR technique.

Samples	Positive Saliva Samples	Negative Saliva Samples
202	51(25,25%)	151(74,75%)

In the present study, we compared the potential for detection of SARS-CoV-2 between nasopharyngeal and/or oropharyngeal swab specimens and salivary specimens and the discrepancy between them (Table 2 and Fig. 4).

Table 2. Comparison of SARS-CoV-2 detection in nasopharyngeal and/or oropharyngeal swab specimens and salivary specimens using RT-PCR.

		Nasopharyngeal and/or oropharyngeal swab specimens		
		Negative	**Positive**	**Total**
Salivary specimens	**Negative**	148	3	151
	Positive	7	44	51
Total		155	47	202

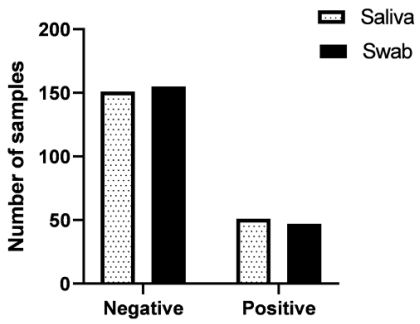

Figure 4. Graphical crossover comparison of SARS-CoV-2 detection in nasopharyngeal and/or oropharyngeal swab specimens and salivary specimens using the RT-PCR technique.

The Ct values of the non-matching positive samples were analyzed (Table 3). The discrepancies between Ct values for positive samples from different collection procedures were also analyzed for each gene (Fig. 5).

Table 3. Comparison of RT-PCR threshold cycle (Ct) values for SARS-CoV-2 detection in nasopharyngeal and/or oropharyngeal swab specimens and salivary specimens in the 10 non-matching positive samples.

	RT-PCR (Ct)		
Sample	**Internal control**	**RdRP Gene**	**E Gene**
Nasopharynx	29,65	38,56	-
Saliva	27,07	-	-
Nasopharynx	29,93	35,27	37,52
Saliva	23,28	-	-
Nasopharynx	26,04	31,40	32,63
Saliva	32,14	-	-
Nasopharynx	26,86	-	-
Saliva	27,20	38,39	38,66
Nasopharynx	29,03	-	-
Saliva	32,28	38,43	37,95
Nasopharynx	27,68	-	-
Saliva	27,96	38,69	42,95
Nasopharynx	27,38	-	-
Saliva	27,22	38,69	42,95
Nasopharynx	30,30	-	-
Saliva	29,73	38,85	-
Nasopharynx	29,03	-	-
Saliva	27,22	38,75	-
Nasopharynx	29,83	-	-
Saliva	24,45	32,23	31,37

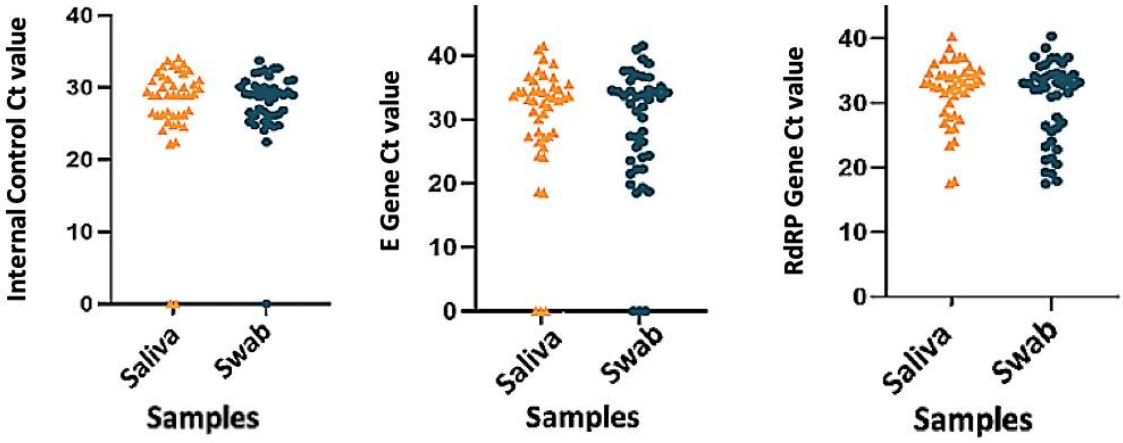

Figure 5. RT-PCR threshold cycle (Ct) values of positive samples from salivary specimen collection *versus* nasopharyngeal and/or oropharyngeal specimen collection using a swab. **(a)** Internal Control Ct comparison; **(b)** E gene Ct comparison; **(c)** RdRP gene Ct comparison.

Discussion

In general, DNA and DNA viruses are more stable than RNA viruses, but both are extremely stable and can be relatively easily preserved. The components of viral transport media are designed to provide an isotonic solution containing protein, antibiotics, and one or more buffers to control pH. Our in-house viral transport medium has all the components required for stability and DNA and RNA preservation [9].

Using RT-PCR molecular testing of salivary specimens and samples collected from the nasopharynx and/or oropharynx using a swab, we conclude that our viral transport medium preserves DNA and viral RNA nucleic acids in different sample types (Table 2).

In our study, we obtained 51 salivary samples with positive detection of SARS-CoV-2 nucleic acids in both asymptomatic and presymptomatic patients. This result evidences that salivary collection samples perform well in detecting SARS-CoV-2 and that they can be used for this purpose. This statement is consistent with the finding that saliva droplets represent the main source of human-to-human transmission of SARS-CoV-2.

The detection sensitivity in both types of samples was also compared. The results obtained were heterogeneous, with a discrepancy in 10 of the results analyzed (Tables 2 and 3). Our results show a 94.55% agreement between the results of both types of collection. The sensitivity of the salivary sampling is 86.27% (95% CI, 81.52 and 91.01), and the sensitivity of the nasopharyngeal and/or oropharyngeal swab samples is 91.66% (95% CI, 87.85 and 95.47).
The detection specificity of the salivary collection samples is 98.01% (95% CI, 96.08 and 99.93), while that of the samples with nasopharyngeal and/or oropharyngeal collection using a swab is 95.48% (95% CI, 92.61 and 98.34).
According to our study, the 3 positive swabs and saliva-negative samples have discrepant Ct values. Two of the samples have values greater than 35 and the other sample has a Ct value of 31.40.
From the samples studied, 7 were positive with salivary collection and negative with swab collection, with only one Ct value lower than 35, and the others with Ct values higher than 38.
According to some studies, the viral load in saliva is higher at the beginning of the infection than at its end, but the viral load in nasopharyngeal and/or oropharyngeal swab specimens is higher after the first week of infection than at the beginning [13,20,24]. These variations in viral load may account for the non-concordant results presented above.
Some patients may not have correctly followed orders to accumulate saliva in the oral cavity, as well as to gather any nasal secretion/mucus in the oropharynx, which may decrease the sensitivity of the test compared to samples collected from the nasopharynx and/or oropharynx, particularly in patients with predominant upper respiratory involvement or mild symptoms.
Recent reports show that “dead” virus particles can remain in the nasopharynx and be detected in molecular biology tests, resulting in a “false positive”. Due to enzymes and their proteolytic activity, saliva can eliminate these particles, reducing “false positives” in testing and helping to determine the elimination of the virus in COVID-19 patients.
The differences in both cases cannot be readily attributed to procedural variations, due to the samples being taken by the same medical team, following the same procedures, in the same place, using the same transport medium, and the same result analysis being used.
In addition, the use of Ct values highlights the viral load but does not allow quantification of viral copies per ml.
In general, the Ct analysis of the concordant positive samples shows that the values are identical for salivary specimens and nasopharyngeal and/or oropharyngeal swab specimens. When we specifically analyze the Cts of the E gene and the RdRP gene, we realize that they show similar values. However, the samples collected from the nasopharynx and/or oropharynx using a swab showed slightly lower Ct values than the salivary collection samples.
The variability in the study performance of tests related to salivary collection for SARS-CoV-2 detection is likely related to differences in the collection technique, the timing of collection (some studies report that first-morning saliva should be collected), the viral or saline transport medium used, whether pre-amplification is performed, and differences in extraction and amplification kits.
Analysis of saliva samples provides reliable and relevant data that can be used to detect “false negatives” revealed by the analysis of nasopharyngeal and/or oropharyngeal swab specimens, thus increasing the sensitivity of the test. Moreover, the data presented allows saliva collection to be confirmed as an alternative to standard collection, showing that it is equally useful and sensitive.
In conclusion, our results suggest that saliva has potential as an alternative to swabbed nasopharyngeal and/or oropharyngeal collection for SARS-CoV-2 detection by RT-PCR and that the in-house created medium is suitable for both collections.
Furthermore, the collection of nasopharyngeal and/or oropharyngeal samples using a swab causes discomfort to patients (such as pain, nausea and even bleeding), there is a higher risk of infection for healthcare workers during collection, as well as the need for technical skill (an improper procedure can lead to a false-negative result).
Standardization of salivary sampling for SARS-CoV-2 detection would, in addition to epidemiological control and self-surveillance, make it possible to reduce the risk of exposure for healthcare workers and the need for PPE, leading to a reduction in costs. Salivary sampling is more advantageous as it does not require any invasive procedures, and is advantageous for patients and medical teams, especially for patients requiring multiple and continuous sampling.

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Author Contributions

PMA, FF, DA and TO conceptualized the work. FF, DA and TO drafted the manuscript. PMA, FF and TO collected the data. SC and IO reviewed and edited the manuscript. All authors agree to be accountable for all aspects of the work. All authors read and approved the final manuscript to be published.

Conflicts of interest

The authors declare no competing interests.

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