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Saliva: An Economic and Reliable Alternative for the Detection of SARS-CoV-2 by Means of RT-qPCR

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ABSTRACT

The current pandemic has generated the search for new reliable and economic alternatives for the detection of SARS-CoV-2, which produces the COVID-19 disease, one of the recommendations by the World Health Organization, is the detection of the virus by RT-qPCR methods from upper respiratory tract samples. The discomfort of the pharyngeal nasopharyngeal swab described by patients, the requirement of trained personnel, and the generation of aerosols, are factors that increase the risk of infections in this type of intake. It is known that the main means of transmission of SARS-CoV-2 is through aerosols or small droplets, which is why saliva is important as a relevant means of detecting COVID-19. In this study, a modified method based on SARS-CoV-2 RNA release from saliva is described, avoiding the isolation and purification of the genetic material and its quantification of viral copies; the results are compared with paired pharyngeal/nasopharyngeal swab samples (EF/EN). Results showed good agreement in saliva samples compared to EF/EN samples. On average, a sensitivity for virus detection of 80% was demonstrated in saliva samples competing with EF/EN samples. The use of saliva is a reliable alternative for the detection of SARS-CoV-2 by means of RT-PCR in the first days of infection, having important advantages over the conventional method. Saliva still needs to be studied completely to evaluate the detection capacity of the SARS-CoV-2 nucleic acid, however, the described process is viable, due to the decrease in materials and supplies, process times, the increment in the sampling and improvement of laboratory performance.

Keywords: *SARS-CoV-2, COVID-19, Saliva, swab, RT-qPCR.*

1. Introduction

The current pandemic has generated a high demand for medical supplies, due to the rapid spread of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the fight for timely diagnosis is vital, this has resulted in a shortage of supplies worldwide in the area of molecular diagnostics. The process for molecular identification of SARS-CoV-2 involves sample collection by pharyngeal and nasopharyngeal swab, viral RNA extraction, and detection by polymerase chain reaction (RT-qPCR) [1], that is why alternatives are currently being sought for the mass detection of infected individuals at low cost and with the least consumption of supplies by means of the gold standard (RT-qPCR). Saliva is a vital fluid for the digestion of food, it is known that the main composition of saliva is water (98%-99%), in addition contains both organic and inorganic molecules in a smaller proportion [2]. At least 700 microbial species are currently known thanks to saliva, in molecular biology, it is considered an important biological material for the diagnosis of various pathogens [3]. This fluid has been described in the literature for the detection of the coronavirus that causes porcine epidemic diarrhea (PEDV) in pigs, it has been detected in saliva by means of RT-qPCR in higher concentrations than serum [4],[5]; along the same lines, the literature reports the detection of cytomegalovirus in saliva in high concentrations [6], besides this fluid is important for the detection of Zika disease caused by a flavivirus detected in concentrations even higher than serum [7].

Saliva samples for the detection of SARS-CoV-2 have multiple advantages, since they provide a similar option to swab samples with similar sensitivity to these tests, but with obvious advantages for salivary samples [13]. Saliva has multiple advantages, these consist of reducing possible infections for health personnel, besides of eliminating

of the discomfort perceived by the patient at the time of sample collection, highlighting that the patients could collect their own sample, the cancellation of materials (swabs and viral transport media) for sample collection and storage, since only collection tubes are required for saliva. One of the main attributes of the release of genetic material in saliva consists in the elimination of commercial extraction and purification supplies of viral RNA, different methods have currently been described for the extraction and/or release of genetic material in saliva, however the presented method is based on the addition of an enzymatic medium (proteinase K) and heat for the release of viral RNA (Fig.1), reducing the response time in the determination of results for possible diagnosis.

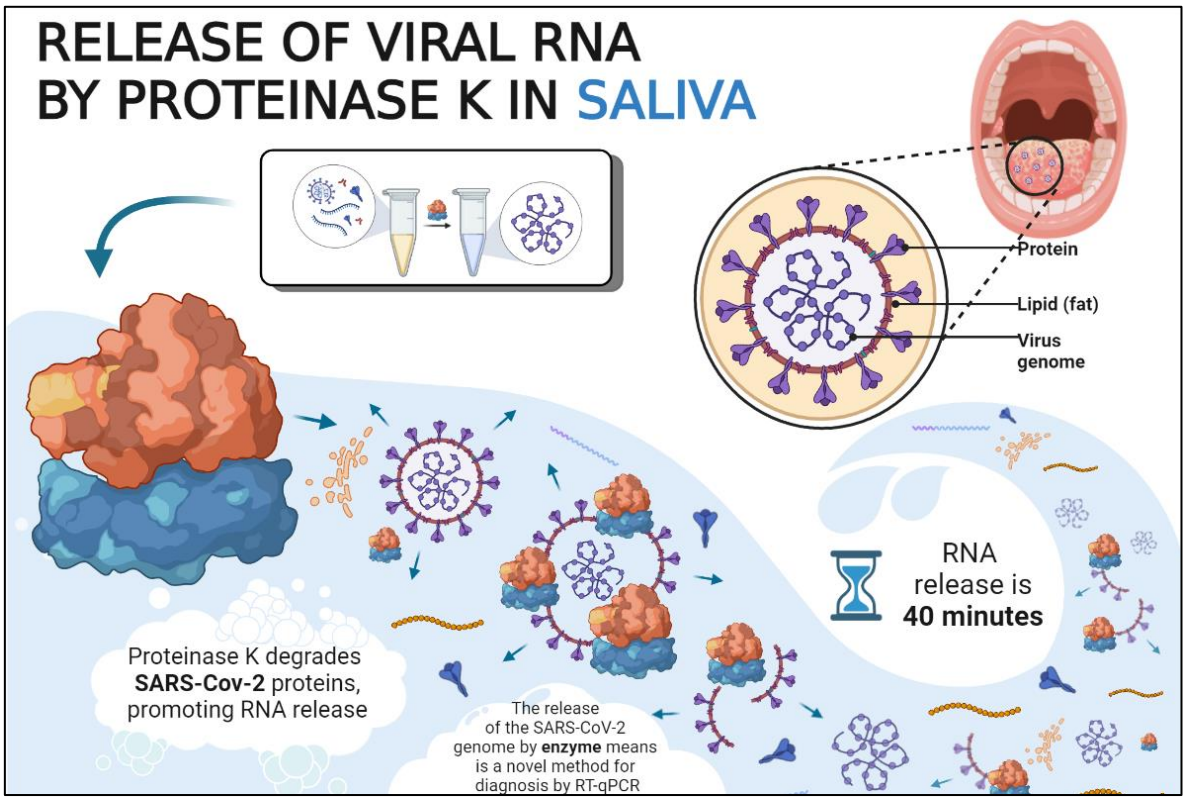

Fig.1. General scheme of the SARS-CoV-2 nucleic acid release method in saliva samples by means of Proteinase K

Different authors refer to the combination of saliva samples as a viable and economical alternative for sampling many people, screening asymptomatic individuals with low levels of the SARS-CoV-2 virus [14],[15]. The United States of America Food and Drug Administration (FDA) has recently approved the detection of SARS-CoV-2 in saliva samples [16]. Currently, there are projects such as SalivaClear from Mirimus Clonical Labs where the sample amalgamation strategy is used for monitoring asymptomatic individuals [17]. In this document the effectiveness of the modified analytical method used for the release of SARS-CoV-2 nucleic acid in saliva samples is studied, with the prospect that it could be used for higher sampling in places with scarce supplies.

2. Materials and Methods

2.1. Sample Collection

A total of 90 samples of pharyngeal and nasopharyngeal swabs (EF/EN) and saliva were collected over a period of 90 days (April 30 to July 30, 2021) by health workers of the State Laboratory of Public Health , from the state of Coahuila, Mexico (Coahuila Health Services). The samples were from outpatients.

2.2. Swab Sampling

Pharyngeal and oropharyngeal exudates were collected from 90 patients. After collection, the swabs were placed in 2.5 mL of viral transport medium (MTV).

2.3. Saliva Collection

The saliva samples were self-collected by the patients who were asked to deposit them in 50 mL wide-mouth sterile containers, until completing 2-3 mL of saliva. It should be noted that no type of stabilizing agent or viral transport medium was added, respectively.

After collection, both the swab and saliva samples were stored at 4°C until analysis, which was within 24 h after sample collection, and were transferred to the Molecular Biology Laboratory of the same institution.

2.4. Extraction and release of total rna and detection of SARS-CoV-2 by means of RT-qPCR

Total RNA extraction in swab samples was performed using the QIAamp Viral RNA Qiagen Minikit following the protocol established by Qiagen, 140 µL of viral transport medium was used, and purified RNA elution was performed in 60 µL of buffer of elution.

Saliva samples were treated with Qiagen Proteinase K (20 mg/mL) by mixing 50 µL of saliva with 6.25 µL of Proteinase K, vortexing for 3 minutes and heating for 5 minutes at 95°C with vigorous orbital shaking, again the mixture was vortexed for 3 seconds and then incubated at 95°C for 30 minutes with vigorous orbital shaking, then the mixture was centrifuged at 6,000 RPM and kept at 4°C until later use.

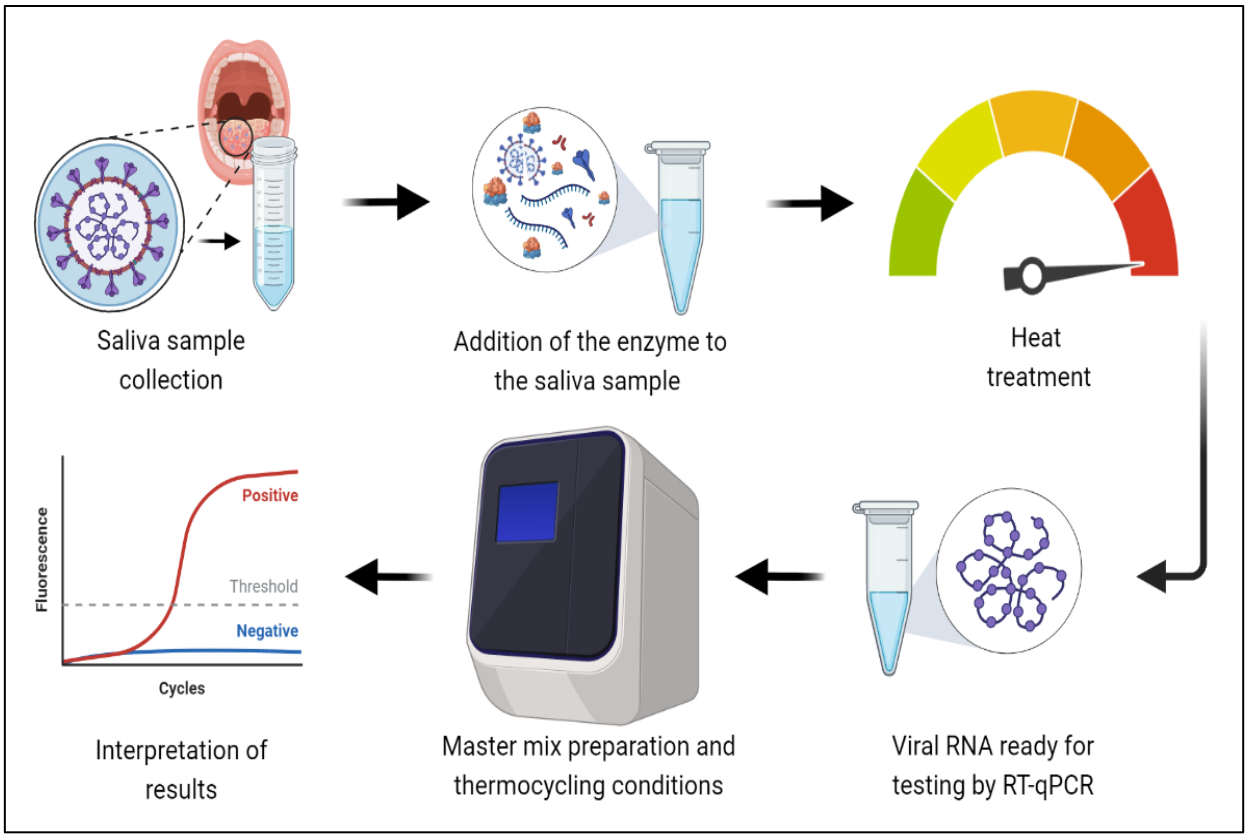

Fig.2. Representative outline for the detection method for SARS-CoV-2 in saliva samples

For the detection of the SARS-CoV-2 genome by means of RT-qPCR, the US CDC protocol was used, using probes and oligonucleotides of 2019-nCoV_N1 and 2019-nCoV_N2 and RNase P (RP) as internal control. For the RT-qPCR, an analysis of 5µL RNA from both matrices was performed with 25 µL of reaction mixture. The classification of positive samples for SARS-CoV-2 were detected with a threshold cycle value (Ct) less than 40 in N1 and N2 [18]. Testing was performed in the Applied Biosystems QuantStudio 5 model real-time thermal cycler with the following protocol: 52°C for 10 minutes, 95°C for 2 minutes, then 45 cycles of 95°C for 10 seconds and 55 °C for 30 seconds. The flow diagram of the detection of SARS-CoV-2 in Saliva is shown in Fig.2.

2.5. Viral Copy Number Analysis

The quantification of viral copies of SARS-CoV-2 was determined by performing a standard curve with 10-fold serial dilutions using the positive control recognized by the probes and oligonucleotides synthesized by Integrated DNA Technologies [18], reporting the N1 and N2 target genes. The concentration of each dilution was plotted against the Ct, and these values were extrapolated to calculate viral copy number using the Ct of the unknown samples.

2.6. Statistic Analysis

All experiments were carried out independently at least three times. The mean and standard deviation for the data set are presented. Data sets were compared using one-way analysis of variance (ANOVA). The difference in the means was verified with Least significant difference (LSD) test usign the Origin 2017 software, and statistical significance was considered at the $p < 0.05$ level.

3. Results and Discussion

3.1. Detection of SARS-COV-2 IN EF/EN Wall and Saliva Samples

In this study, the identification of the SARS-CoV-2 genome in saliva followed by RT-qPCR was evaluated, a total of 90 paired samples of pharyngeal and nasopharyngeal (NP/NP) swabs and saliva were collected from outpatients. As a gold standard (control), the pharyngeal and nasopharyngeal swab (EF/EN) was used by means of RT-qPCR for the identification of the virus. The sampled patients had 2 to 4 clinical symptoms associated with the common flu, the most predominant being cough and fever. Of the samples taken, 44/90 (48.8%) were women and 46/90 (51.2%) were men.

For a timely detection of SARS-CoV-2 in the aforementioned matrices, probes and oligonucleotides synthesized by Integrated DNA Technologies (IDT) were used for monitoring by RT-qPCR that detect the N1 and N2 target genes, coinciding with what various authors have expressed, where it is shown that the cells infected by SARS-CoV-2 abound in the N protein of the coronavirus [19],[20], that is why the protocols that report the RNA-dependent RNA polymerase gene (RdRP) were excluded, since in the literature has found a weak detection of this gene for SARS-CoV-2 [12].

The extraction and purification of the RNA by means of pharyngeal and nasopharyngeal swabs was carried out from the QIAamp viral RNA minikit, on the other hand, the RNA released from the saliva was obtained thanks to Proteinase K, from Qiagen, applying different heat treatments, as reported in the previous section.

Of the 90 samples collected from EF/EN, 61/90 samples were positive for SARS-CoV-2, which were used as a comparison with saliva samples to obtain the percentage of positivity in these, the positive samples of EF/EN were used. EN as 100% (61 samples) to compare with positivity in saliva, being 80% with 49/61 of these saliva samples positive for the virus (Table 1). In total, there were 49/61 paired samples which obtained a concordant result in the detection of viral RNA, for the saliva and EF/EN samples, these concordant in their positivity in the N1 and N2 genes, respectively with the Ct values ranged between 16.2 and 30, because the patients were in the first days of infection, containing high viral titers within their body. On the other hand, the samples that were positive only in the swab (EF/EN), the Ct values were higher than 34 respectively, being 12 samples in which the viral RNA was present in the EF/EN and not in the saliva, these results coincide and support the findings obtained at the United States CDC, where encouraging results were not obtained for virus culture from upper respiratory samples, when the values of the cycle of treshold (Ct) were greater than 34 [21]. It is important to emphasize that the failure to identify SARS-CoV-2 in saliva samples is attributed to the absence of viruses present, as well as some unknown problems during the release of genetic material, where it is believed that saliva could still contain some inhibitors that could be affecting RT-qPCR, however some authors have described that the presence of proteinase K, presents hydrolysis on Taq polymerase, being a fundamental component for PCR [22]. On the other hand, in the study presented, it was detected that 9 saliva samples presented high viscosity, after the PCR they obtained lower Ct values compared to that of the EF/EN, it is important to emphasize that authors have described the presence of materials deep in the respiratory tract, which implies higher viral yields in saliva samples with higher viscosity [23]. The EF/EN samples were slightly more efficient in the detection of SARS-CoV-2 at any time of the patient's infective evolution (Fig.3), however, it should be taken into account that RNA purification and specialized extraction kits are needed, coupled with the scarcity of medical supplies, saliva as a biosample has important benefits, obtaining results with saliva is faster since it does not require a multi-step processing (viral RNA extraction kit) as in samples of EF/EN. Simply, the virus is inactivated in the laboratory and goes directly to the RT-qPCR test. It is important to emphasize that the virus continues to evolve genetically, different types of variants of SARS-CoV-2 are currently known, with the Omicron variant some researchers have described that it lodges in the upper airways, saliva being a diagnostic target in the first days of the patient's infection [24].

Table 1. Summary of the results obtained from patients with suspected COVID-19 from the paired tests of saliva and pharyngeal and nasopharyngeal swabs

RT-qPCR	n=90	RESULTS
EF/EN	POSITIVE	61
	NEGATIVE	29
SALIVA	POSITIVE	49
	NEGATIVE	41

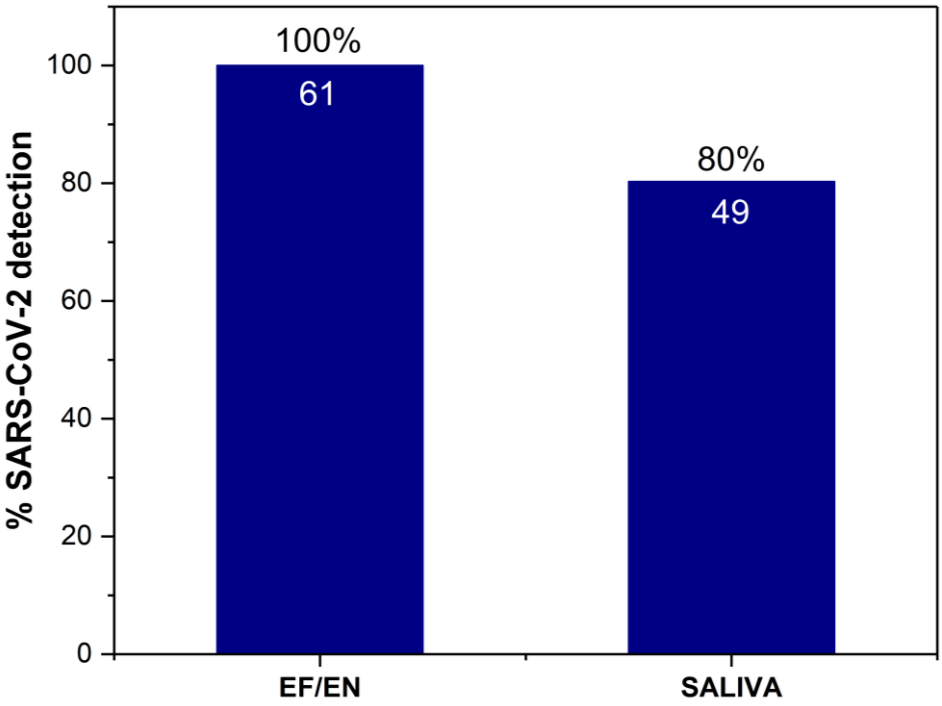

Fig.3. Detection of SARS-CoV-2 in paired EF/EN and saliva samples

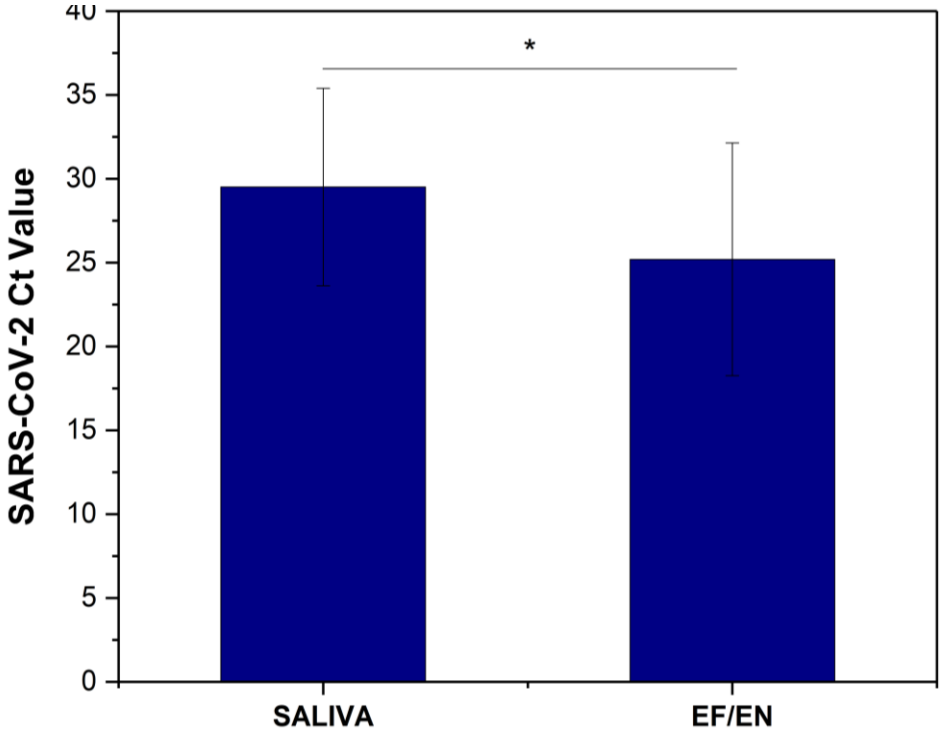

Fig.4. Detection of SARS-CoV-2 in paired EF/EN and saliva samples

3.2. Viral RNA Quantification

Copies of the viral genome in the swab (EF/EN) and saliva samples were quantified, statistical significance was detected between both studies, with saliva samples having slightly lower values than the swab samples ($p < 0.04281$), in addition the highest viral copy numbers were obtained in the EF/EN samples (Figs.3-4). The change in viral copy numbers among both matrices can be associated with a difference of approximately ±7 Ct units,

indicating that the techniques for obtaining genetic material from SARS-CoV-2 differ from each other, however, it is important to emphasize that the presence of an infectious virus in saliva that causes COVID-19 is of the utmost importance for health personnel to determine the relationship of the infectious particles of the virus on the copies of the viral genome, in saliva these variables will play a important role in understanding the transmission dynamics of COVID-19.

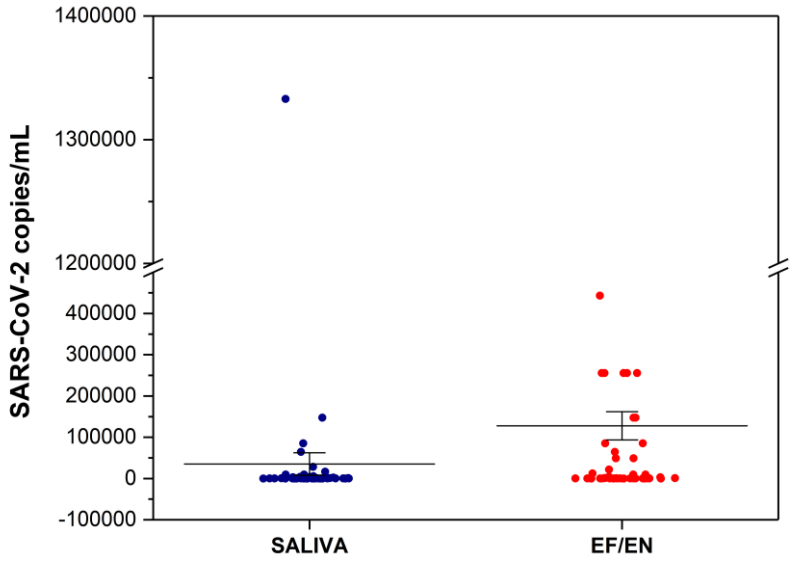

Fig.5. Viral titer detected in paired samples of saliva and EF/EN

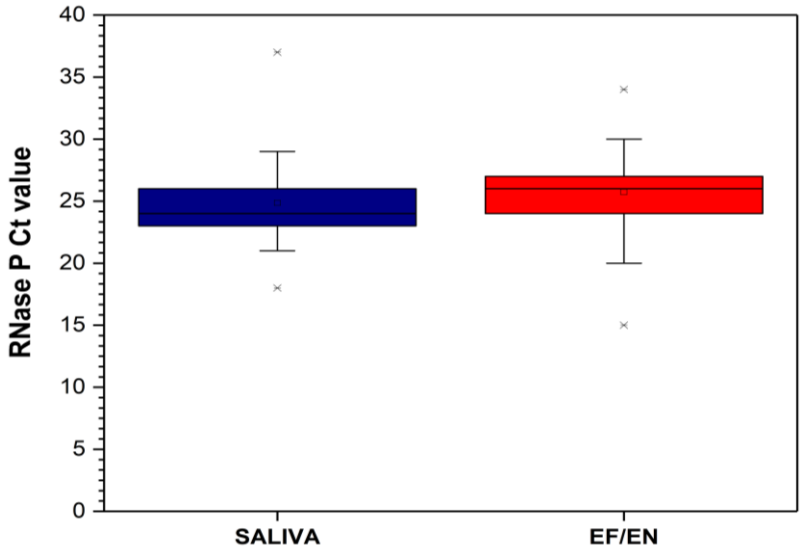

Fig.6. RNase P Ct values in paired samples of saliva and EF/EN

Besides, the quantification of the viral load is essential, to evaluate the severity of the infection, its evolution and its relapses, since in some studies it has been shown that a week after the onset of symptoms, the viral load gradually declines [25],[26], due to that the dynamics of the number of viral copies is important for health personnel, it is important its evaluation of the treatment that each patient will take [27].

On the correlating of the results obtained with respect to the literature, some authors have reported that SARS-CoV-2 is more easily transmitted in the first days of infection and in patients without symptoms, which

indicates a low Ct in the PCR study, and a high viral load at this stage [9],[28],[29]. The difference among the number of viral copies of each of one the studies is still not clear, however, some researchers have refered false negatives in saliva samples derived from unknown problems during sampling, transport and storage [12].

An important parameter to verify that the quality of the sampling was optimal in the extraction and/or release of the genetic material, as the case could be, is the use of human RNase P as a control, the difference among the mean RP in the swab and saliva samples oscillates in 1.8 Ct values, indicating the lowest value in saliva samples (Figs.5-6), so it is ensured that there is a higher number of cells in saliva, as it has been reported in the literature [30].

4. Conclusions

The results obtained in the detection of SARS-CoV-2 among the saliva and (EF/EN) swab samples were 80% concordant, with the (EF/EN) swab samples serving as controls in this study. The study showed a statistically significant differences among the methods (saliva versus (EF/EN) swab) of release and extraction of genetic material followed by RT-qPCR, so that both matrices had differences in virus detection rates. The (EF/EN) swabs had slightly earlier values than the saliva samples, this is correlated with the literature where it is stated that the pharyngeal and nasopharyngeal exudate samples (swab) had a higher viral load compared to the samples of saliva studied in other respiratory viruses [31],[32].

Pharyngeal and nasopharyngeal swabs, as well as saliva samples, can provide similar virus detection, the prevalence of COVID-19 remains high in different countries, vaccination coverage has been slow despite having approved and effective vaccines; that is why the priority is the timely detection of the virus, prioritizing massive diagnostic tests that support decisions in epidemiology. In saliva, there is a high concentration of the SARS-CoV-2 virus, so its use for the diagnosis of COVID-19 has shown to have highly reliable results when is combined with the RT-qPCR technique, the main attributes of the test consist of the saving of sampling materials, supplies for the extraction of the viral genetic material, and the time that this process takes. In summary, our analysis showed that the saliva samples treated with proteinase K and heat could be an simple alternative for the detection of SARS-CoV-2 viral RNA, even so, it is necessary to carry out studies with a larger number of samples to be able to really determine the efficiency of saliva as a biosample for COVID-19 using RT-qPCR.

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Declarations

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Competing Interests Statement

The authors declare no competing financial, professional, and personal interests.

Ethical Approval

Based on institutional guidelines.

Consent for publication

Authors declare that they consented for the publication of this research work.

Availability of data and material

Authors are willing to share the data and material according to relevant needs.

Authors' Contributions

All authors equally contributed to data collection, research, and paper drafting.

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