ORIGINAL ARTICLE

Use of Saliva-PCR as an alternative diagnostic tool for COVID-19 positive cases.

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ABSTRACT... Objective: To find the presence of SARS-CoV-2 in saliva and oropharyngeal secretions in confirmed cases of COVID 19 to establish them as potential diagnostic alternatives. **Study Design:** Cross Sectional study. **Setting:** Hayatabad Medical Complex and Public Reference Lab of Khyber Medical University, Peshawar. **Period:** April 2022 to September 2022. **Material & Methods:** Study included SARS-CoV-2 patients confirmed by a positive RT-qPCR diagnosed through nasopharyngeal swab. Data was collected using a digital questionnaire. Saliva was collected using "passive drool" procedure. RNA was extracted from saliva samples and virus was detected through one-step-RT-qPCR using Universal qPCR Master Mix. The data obtained was presented using graphical representation in the form of proportions. **Results:** The study included 211 individuals with a wide range from 12 to 75 years. There were 146 (69%) males and 65 (31%) females.17% reported being hospitalized or received medical treatment recently. Only six individuals reported regular use of medications for hypertension and diabetes. 30% of participants reported a positive response when asked about their pervious respiratory allergic conditions. Results showed that out of 211 participants 75 (36%) were positive for COVID-19 using saliva-PCR, while the remaining 136 (64%) were negative. **Conclusion:** The results of saliva-PCR diagnosis showed lower sensitivity than nasopharyngeal-PCR in detecting COVID-19. Additional research is required to substantiate the effectiveness of PCR using saliva as an alternative diagnostic option.

Key words: Covid-19, Nasopharyngeal PCR, RT-qPCR.

INTRODUCTION

During December 2019, an acute respiratory tract infection named Coronavirus Disease 2019 (COVID-19) started spreading in the city of Wuhan, China.[1,2] Shortly after its emergence, the causal agent responsible for the acute respiratory syndrome was identified as a new coronavirus, which is currently referred to as SARS-CoV-2.[3] The WHO categorized it as an airborne pathogen that can spread through contact with infected droplets and aerosols via close contact, even by those who are asymptomatic, pre-symptomatic, or symptomatic.[4] Although it shares 80 % sequence homology with the virus that caused SARS epidemic in 2003, SARS-CoV-2 spread rapidly due to its heightened transmissibility across the globe.[5,6] In March 2020, the World Health Organization declared COVID-19 as the second worldwide pandemic of the 21st century,

.Globally, as of 16 March 2023, there have been 760,360,956 confirmed cases of COVID-19, including 6,873,477 deaths, reported to W.H.O.[7]

Talking, breathing, coughing, sneezing, and even singing have been identified as activities that can transmit SARS-CoV-2. It was initially ambiguous if SARS-CoV-2 can directly penetrate and reproduce in the tissues of the oral cavity. The virus has been documented to infect cells in the upper airways, lungs, and nose.[8-10] If mouth tissues are involved in the initial infection, they may aid in the virus's spread to the digestive or pulmonary systems. High levels of SARS-CoV-2 can be found in the saliva of people with COVID-19. Researchers thought that, at least occasionally, the virus-filled saliva might originate from infected mouth tissues as opposed to nasal discharge or material coughed up from. The

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SARS-CoV-2 infection initiates with the interaction of receptor-binding domain (RBD) of its spike protein with the angiotensin-converting enzyme 2 (ACE2) receptor expressed at surface of the epithelial cells in the upper respiratory tract of the host.[11,12] An essential first line of defense is thus the immune response in the nasal and oral mucosa. Saliva can reveal details about the SARS-CoV-2 antibody responses at these mucosal sites.[13]

The nasopharyngeal swab is a commonly utilized method for sampling for the reverse transcription quantitative real-time PCR (RT-qPCR) analysis, that is regarded as the primary method for detecting SARS-CoV-2.[14] Unfortunately, nasopharyngeal sample collection brings its share of disadvantages: including its highly invasive nature, increased risk of spreading infection, and the requirement of technically skilled personnel. In addition, developing countries like Pakistan face shortage of medical supplies, including personal protective equipment (PPE), sterile swabs, and virus transportation medium (VTM), leading to unfavorable conditions. For these reasons, biomedical industries are striving towards the development of new diagnostic solutions that are rapid, efficient, and cost-effective. PCR-based or in vitro immunochromatography-based assays have been suggested as methods to identify specific antibodies in blood samples. Despite the obvious rapid detection, the major limitation is their feasibility during mass screening as well as blood samples at designated places.[15,16]

As respiratory droplets represent the primary mode of SARS-CoV-2 transmission, the use of sputum and oropharyngeal secretions has been suggested as a potential option for diagnosing COVID-19 infections.[17,18] Saliva, as a diagnostic sample, presents many advantages as previously reported including easy provision and collection without any specialized equipment or personnel.[19] Additionally, it is much more comfortable alternative for the nasopharyngeal swabbing. However, our understanding of the potential utility of those secretions in detecting SARS-CoV-2 is currently quite limited. Consequently, it is essential to verify the existence of SARS-CoV-2 in saliva before considering it as a possible diagnostic alternative.

AIMS AND OBJECTIVES
The aims of the present study were: To investigate the presence of SARS-CoV-2 in the saliva of RT-qPCR confirmed COVID-19 patients.

MATERIAL & METHODS
It was a cross-sectional study carried out in 211 SARS-CoV-2 infected patients who were recruited after obtaining informed consent for participation. Sample size was calculated using EPIinfo calculator with confidence interval (CI) of 90%. Saliva samples were collected at Hayatabad Medical Complex, Peshawar during April 2022 to September 2022. Followed by RT-qPCR diagnosis, which was performed at Public Health Reference Lab, Khyber Medical University, Peshawar. The data was collected using a digital questionnaire for each patient. Patients with COVID-19 infection confirmed by the nasopharyngeal swab followed by RT-qPCR diagnosis were included in the study. Patients with a history of obstructive sleep apnea syndrome (OSAS), thymoma, or other upper airway illnesses were excluded from the study. The data regarding oral symptoms and medical history including age, gender and previous infections, comorbidities, drugs, allergies were collected using a digital questionnaire for each patient for subsequent analysis and interpretation under the supervision of professional doctors. Saliva was collected using "passive drool" procedure, considered as the gold standard in collecting saliva for biological testing because of the purity of obtained samples.[14,15] Passive drooling is usually performed by asking the subject to let the saliva drop into plastic tubes (e.g. polypropylene tubes to avoid sample retention or contamination). Saliva specimens were resuspended in PBS (2ml) and used for extraction of RNA by viral RNA mini kit as per the manufacturer recommendations. One-step RT-qPCR, Universal qPCR Master Mix was used with the extracted RNA serving as the template. Both direct samples and extracted RNA were used for RT-qPCR targeting the conserved region of the SARS-CoV-2 genome, the N and ORF1ab genes. No interventive procedure was involved in our study. The primer and probe sequences and their

final concentration in the reaction mixture are given in Table-I. The final reaction volume of 20 µl was prepared as follows:
PCR reaction mix (10 µl) (Vazyme Ltd, China), primer/probe mix (1.5 µl),
Template (5 µl; sample, negative or positive control), and
Nuclease-free H2O (to make the final volume upto 20 µl).

The thermocycling protocol is given in Table-II.
A signal at Ct < 40 for any gene indicated a positive sample. The data obtained from the survey of COVID-19 positive patients was presented using graphical representation in the form of proportions. The use of proportions helped to convey the percentage of participants who answered each question with a "Yes" or "No" response. All data was entered in Microsoft Excel. Research study was approved by Institutional Review Board of Peshawar Medical College and Hospital Research and Ethical Committee (IREB), Hayatabad Medical Complex, Peshawar (Ref no.649/HEC/B&PSC/2022).

Primer/ Probe	Sequence (5' – 3')	Final Conc.
ORF-F	CCCTGTGGGTTTTACACTTAA	2 µM
ORF-R	ACGATTGTGCATCAGCTGA	2 µM
ORF-P	FAM−CCGTCTGCGGTATGTG-GAAAGGTTATGG-BHQ1	0.5 µM
N-F	GGGAGCCTTGAATACACCAAAA	2 µM
N-R	TGTAGCACGATTGCAGCATTG	2 µM
N-P	HEX-AT CACATTGGCACCCGCAATCCTG-BHQ2	0.5 µM

Table-I. Sequences of primers and probes used for RT-qPCR

F = Forward Primer; R = Reverse Primer; P = Probe

Cycle Step	Temperature	Time	Cycles
Reverse Transcription	55oC	10 mins	1
Initial Denaturation	95 oC	1 min	1
Denaturation	95oC	10 secs	40-45
Extension	60oC	30 secs	
Melt Curve	72oC	2 mins	1

Table-II. The thermocycling protocol

RESULTS

Characteristics of the study sample
Upon satisfaction of the established inclusion and exclusion criteria, a total of 211 individuals were included in the study. The study sample included individuals with a wide range of age with the youngest participant was 12 years old and the oldest was 75 years old.

Gender Distribution
Out of 211 patients in our study, 146 (69%) were male, while only 65 (31%) of individuals were female. (Table-III)

Medical History
The study participants were asked about their past medical history from the past year. Specifically, they were asked if they were hospitalized or received any medical treatments during that time. Analysis of the data collected revealed that 35 (17%) patients reported being hospitalized or were receiving medical treatment in the past year, while the remaining 176 (83%) patients did not report any such history (Figure-1).

Gender	Frequency	Percent
Female	65	30.8
Male	146	69.2
Total	211	100.0

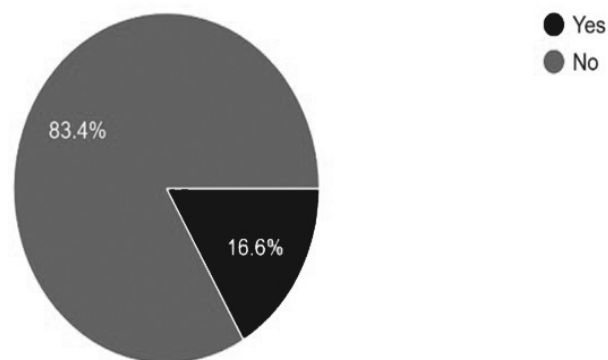**Table-III. Gender distribution of study population**

Figure-1. Medical history of the study participants in past one year

MEDICATIONS
As part of the data collection process, the study participants were asked about their current medication use. Specifically, they were asked if they were currently taking any medications on a

regular basis. Analysis of the responses revealed that the majority of the study participants were not taking any long-term medications, with only six individuals reporting regular medication use for hypertension (04) and diabetes (02).

The study participants were further asked regarding the use of specific class of medications such as anti-depressants, anti-coagulants, or pain killers. 1% of the participants were taking antidepressants, 9% anti-coagulants, while 93% had recently taken pain killers in the past month (Figure-2).
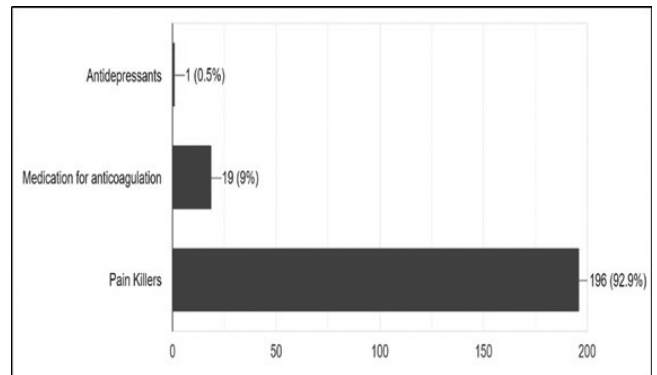
Asthma, Hay fever, and similar allergies
To evaluate the prevalence of respiratory allergic conditions such as asthma, hay fever, and similar allergies, study participants were asked a specific question regarding their medical history. Participants were asked whether they had ever experienced any of these conditions, which are known to be common respiratory and allergic disorders affecting individuals of all ages. Of the 211 individuals included in the study, a total of 64 (30%) reported a positive response, indicating that they had experienced one or more of these conditions in their lifetime (Figure-3). In contrast, a majority of the study participants, i.e., 147(70%), responded negatively to the question, indicating that they did not have a history of asthma, hay fever, or similar allergies (Figure-3, Table-IV). These findings provide important insights into the prevalence of allergic conditions among the study population and may have important implications for public health interventions and clinical management strategies.
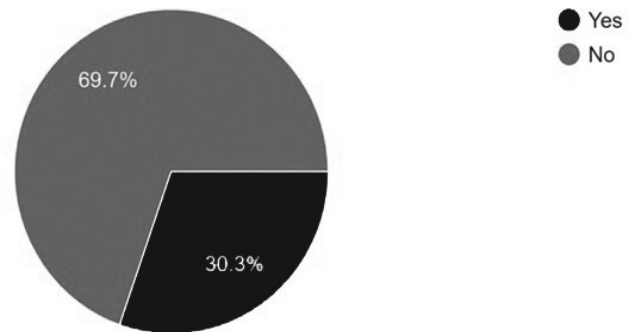
Prevalence of Co-Morbidities in the Study Population	Percentages
Heart or vascular diseases	0.5%
Asthma, hay fever, or other allergies	30%
Respiratory diseases	62%
Liver diseases	0.5%
Diabetes	6%
Kidney diseases	0.5%

Table-IV. Prevalence of co-morbidities in the study population

Figure-2. Use of certain medications in the past one month

Figure-3. Prevalence of respiratory allergies in the patients

Respiratory or lung diseases
To assess the prevalence of respiratory illnesses in the study population, such as pulmonary hypertension, occupational lung diseases, lung cancer, and chronic obstructive pulmonary disease (COPD), participants were asked whether they had ever experienced these conditions. Of the 211 individuals included in the study, 149 (71%) reported that they had not experienced any of these respiratory diseases, while 62 (29% reported that they had (Figure-4). These findings suggest that respiratory diseases are relatively common among the study population and highlight the need for effective prevention and treatment strategies for these conditions.

Among the 29% of participants who reported a history of respiratory diseases, the most commonly reported conditions were COPD and occupational lung diseases, with 11% and 9% of participants reporting these conditions, respectively. In addition, 6% of participants reported a history of lung cancer, while 3%

reported a history of pulmonary hypertension.

History of Infectious Diseases
Upon questioning the study participants about their medical history, we inquired about any past history of infectious diseases. Out of the 211 individuals included in the study, 161 (89%) reported having experienced infectious diseases while 24 (11%) did not, (Figure-4). This indicates that infectious diseases were relatively common among the study population.
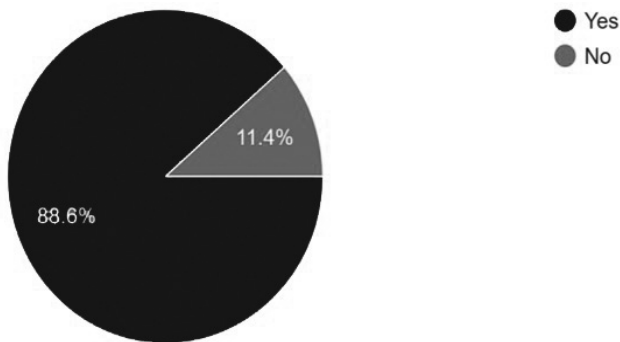

Figure-4. History of infectious diseases in the participants

Saliva based PCR
The present study aimed to evaluate the efficacy of saliva-based PCR testing for COVID-19 among 211 individuals with nasopharyngeal positive PCR. Out of the total participants, 36% tested positive for COVID-19 on the saliva-based PCR test while the remaining 64% tested negative (Figure-5, Table-V). These results suggest that saliva-based PCR testing may not be as sensitive as nasopharyngeal PCR testing for detecting COVID-19.
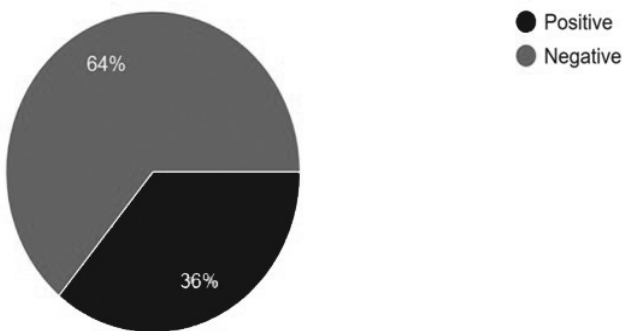

Figure-5. Saliva-based PCR results of the study population

Sensitivity Analysis
According to the current study, sensitivity is the number of positive cases diagnosed by Saliva PCR / Total number of positive cases by standard method (Nasopharyngeal PCR).

Sensitivity = 75 / 211
= 0.36 x 100
= 36 %

Saliva sensitivity analysis implies the same as we concluded that it can diagnose only 36% of the patients.

While specificity which is based on the predictive power of negative results i.e. "Number of negative cases diagnosed by saliva-PCR / Total number of negative cases by standard method (nasopharyngeal PCR)". Based on our study's inclusion criteria, individuals with negative nasopharyngeal PCR were not included in the analysis, therefore, specificity could not be calculated.

	Positive Cases	Negative Cases
Saliva Based PCR	75 (36%)	136 (64%)
Nasopharyngeal PCR	211 (100%)	-

Table-V. Saliva based PCR results of study population

DISCUSSION
The present study evaluated the efficacy of saliva-based PCR testing for COVID-19 and found that it may not be as sensitive as nasopharyngeal PCR testing for detecting COVID-19. The results showed that only 36% of the 211 participants who were positive on nasopharyngeal PCR were also positive on the saliva-based PCR test. This showed that the sensitivity of saliva-based PCR testing for COVID-19 may be lower than nasopharyngeal PCR testing.

The age of the participants varied widely, ranging from 12 to 75 years, which allowed for insights into the experiences of different age groups. However, the gender distribution of the study sample was skewed towards males, with 69% of participants being male and only 31% being female in our study. Regarding medical history, only 17% of participants reported being hospitalized or

receiving medical treatment in the past year. The majority of participants (83%) were not taking any long-term medications, with only six individuals reporting regular medication use for hypertension or diabetes. Additionally, 1% of participants were taking antidepressants, 9% anti-coagulants, while 93% had recently taken pain killers in the past month. Concerning the prevalence of certain diseases in the study participants, 30% of participants reported experiencing respiratory allergic conditions such as asthma, hay fever, or similar allergies in their lifetime. 29% of participants reported a history of respiratory diseases, with COPD and occupational lung diseases being the most commonly reported conditions. In contrast, heart and vascular diseases were relatively rare, with only 0.5% of participants reporting a history of these conditions. Similarly, 0.5% of participants reported a history of liver diseases, while 6% reported a history of diabetes. Participants reported no history of rheumatic diseases or joint pain.

The findings of our study are in accordance with a study held in Chicago, which have shown that the sensitivity of saliva-based PCR testing for COVID-19 can vary widely depending on the method of collection, viral load, and time of testing.[20-22] While contrary to our study, a cohort study held in Toronto, Canada, have shown saliva-based PCR testing to be sensitive, convenient and non-invasive alternative to nasopharyngeal PCR testing.[23,24] Our study highlights the importance of considering the limitations of this method when interpreting the results. In our study we assessed the efficacy of saliva-based PCR testing for COVID-19 and found that it is not as sensitive as nasopharyngeal PCR testing for detecting COVID-19. However, the study had several limitations that must be considered. The sample size was relatively small and skewed towards males, with only 31% of participants being female. Moreover, the study did not investigate the impact of viral load on the sensitivity of saliva-based PCR testing. Therefore, further research is needed to confirm these findings and to investigate the potential long-term effects of COVID-19 on oral health.

However, the study has several limitations that must be considered. The sample size was relatively small and skewed towards males, with only 31% of participants being female. Moreover, the study did not investigate the impact of viral load on the sensitivity of saliva-based PCR testing. The study also relied on self-reported medical history, which may be subject to recall bias. Therefore, further research is needed to confirm these findings.

CONCLUSION
Study revealed that only 36% participants who were positive on nasopharyngeal PCR displayed positive results on the saliva-based PCR test, suggesting that the sensitivity of saliva-based PCR testing may be lower than nasopharyngeal PCR testing for detecting COVID-19.
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2	Zain ul Abideen	Data analysis and Final approval.	
3	Sara Maryium	Literature survey and review.	
4	Zia ul Arifeen	Sample collection and Finalization of results.	
5	Muhammad Arsalan	Revision of Manuscript.	
6	Najmush Shakireen	Interpretation of Data and Statistical Analysis	