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ORIGINAL ARTICLE

Conventional Naso-Oropharyngeal Sampling Versus Self-Collected Saliva Samples in COVID-19 Testing

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Abstract Comparing the diagnostic utility of salivary specimen samples with conventional nasopharynx-oropharynx (NP-OP) specimen samples to identify COVID-19 cases by reverse transcription-polymerase chain reaction (RT-PCR). Eighty COVID-19 suspects enrolled for the paired sampling. In addition to conventional sampling, suspects were asked to follow stepwise pictorial instructions for self salivary sampling. Separate nylon swab stick was used for taking the samples from NP-OP and the floor of the oral cavity. The data were analyzed for sensitivity, specificity, concordance of COVID-19 status, and limits of agreement for cycle threshold (ct) values by either method. Forty-nine suspects (61.3%) were males, the mean age was 36.4 years. To determine the diagnostic test performance of the saliva, RT-PCR results of the NP-OP samples were used as the reference standard. Out of 80 suspects, 41 showed positivity by NP-OP swabs and 12 by salivary samples. The salivary samples showed significantly lesser positivity rate. The sensitivity and specificity of salivary samples against conventional reference standards are 24.4%, 94.9% respectively. Concordance of these two types of samples in terms of agreement kappa statistics is estimated as K = 0.252 (0.09–0.42). Median ct values of both the E and ORF1ab gene for the salivary samples were higher compared to the corresponding NP-OP sample. This study showed lesser sensitivity with salivary swab samples as compared to conventional NP-OP sampling for RT-PCR, COVID-19 detection. Hence, we are of opinion that more studies are required to establish the utility of salivary sampling in COVID-19 diagnostics.

Keywords Nasopharyngeal swab · RT-PCR · Saliva · Severe acute respiratory syndrome coronavirus 2 · Throat swab · Self-sampling · Diagnostic tests

Introduction

The Corona Virus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has swept the globe and continues to add newly infected cases [1]. As of mid-January 2021, globally 93.2 million cases and near to 2 million deaths have been reported. Next to the United States of America (USA), India has the maximum number of COVID-19 cases in the world.

The vaccines are getting rolled out now however their efficacy is under evaluation [2]. The three "T's" 'Test, Trace and Treat' are heavily relied upon in the majority of the countries. Currently, the COVID-19 diagnosis is largely based on reverse transcription-polymerase chain reaction (RT-PCR) testing on combined nasopharynx-oropharynx (NP-OP) specimen swabs. However, this sampling procedure not only needs some expertise but also is discomforting for the health care worker (HCW) and the suspect respectively. In line with these, since the beginning of the pandemic alternate effective sample collection strategies with the lessened threat of transmission and more comfort are actively looked at by the health care system [3]. Studies

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evaluating validation of diagnostic accuracy of saliva as biological fluid by RT-PCR testing are at the forefront [4].

The diagnostic and research opportunities of saliva in relation to COVID-19 explored from various studies have demonstrated, saliva collection as a non-invasive, self-collection method hence considered an alternative to conventional NP-OP sampling. Besides, it is believed that various salivary biomarkers including the salivary metabolomics offer a higher promise which will be useful for an overall better understanding of COVID-19, such as in the case of triaging of infection from severe to asymptomatic carriers [5].

However, the clarity on labelling the sample as salivary secretions in most of these studies is not uniform. Various means to generate the saliva mentioned in these studies include direct spitting, deep coughing, OP gargles, or more invasive stenting of the salivary duct as in few instances [6, 7].

Hence, we believe that evidence collected under the heading of saliva sample encompasses a wide range of specimens from oral or oropharyngeal secretions. Our study was aimed to evaluate the utility of true salivary samples for detection of COVID-19 infection with minimum to negligible contamination by an oral cavity or oropharyngeal respiratory secretions. The paired samples of conventional NP-OP swab and salivary secretions swab among the suspects presented with a different spectrum of COVID-19 illnesses were evaluated for agreement on RT-PCR, COVID-19 status, and virological outcomes.

Material and Methods

Study Design

We conducted a cross-sectional analytical study that involved parallel testing of COVID-19 from a paired sample of saliva and conventional NP-OP swabs. Suspects aged 8 years and above presenting with a varied spectrum of illness of the COVID-19 as per Indian Council of Medical Research (ICMR) criteria to the screening area were included in the study after consent.

Study Setting

This study was conducted in one of the tertiary care teaching hospitals in central India which is designated COVID-19 care facilities in the city of Nagpur. During the study period of October–November 2020, around sixty COVID-19 suspects presented daily to the screening area.

Sample Size and Sampling

We required a minimum of 35 patients with COVID-19 assuming the sensitivity of salivary samples against conventional NP-OP swab as 90%, laboratory positivity rate of 10%, and 10% error. However, as the laboratory positivity rate in the study setting has reached more than 30% along with the resource constraints for doing dual sampling for the same individual, we decided to recruit around eighty COVID-19 suspects for this study.

Recruitment of Suspects

Among eligible suspects who are aged 8 years and above and willing to undergo sample collection of both saliva and NP-OP were selected for the study. Consent from parent/guardian is obtained in case he/she is a minor. If the participant is not willing the next eligible suspect was approached to meet the sample size.

Data Collection

Details on the suspect's demographic information were extracted from the sample registration form. Category of COVID-19 in accordance to ICMR was extracted from COVID-19 triage register and tracked from sample ID number. Initially, conventional NP-OP swabs of suspects were collected by trained HCW using Nylon swab stick and transferred into viral transport medium (VTM) as per guidelines laid by statutory bodies. After completion of this procedure, self-sampling of saliva was done by the suspect himself in stepwise manner as shown in pictorial representation provided to them. The ongoing procedure was supervised by the HCW and intervened if deemed necessary. Before collecting the salivary sample, the suspect was asked to give a gentle external message to the submandibular gland region from outside for a period not less than 60 s. A broad-ended flocked Nylon swab stick was rolled in the floor of the oral cavity for 20–40 s by the suspect himself/herself. (Fig. 1) The suspect was instructed not to touch the swab in any other parts of the oral cavity. After collecting the sample, it was transferred in separate labeled VTM for RT-PCR qualitative testing in the diagnostic molecular laboratory.

Specimen Processing and Workflow of RT-PCR

The team of microbiologists who performed specimen processing and RT-PCR were unaware of the details of the suspects and the nature of samples. The NP-OP swabs in a VTM and saliva samples in another VTM were treated with lysis buffer to inactivate the SARS-CoV-2. Viral RNA and

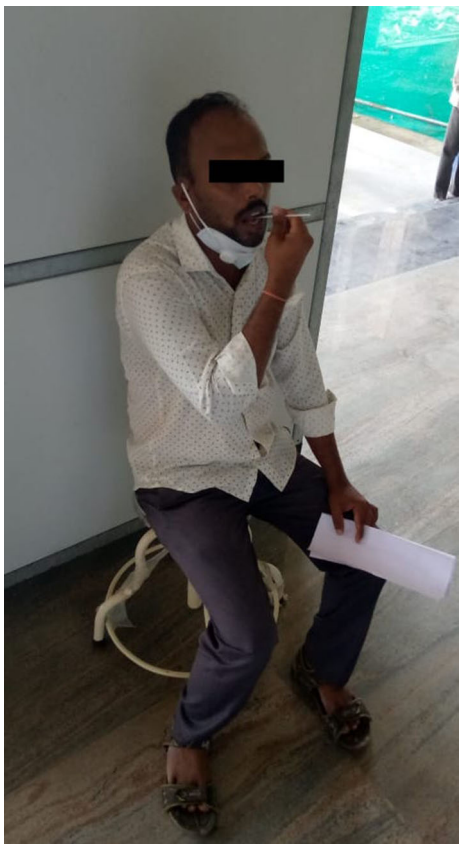

Fig. 1 Suspect collecting saliva using a nylon swab after massaging the submandibular gland

was extracted from 200 µL of the samples within 26 min using Labsystem RTPCR kit.

The detection of SARS-CoV-2 in the specimen samples was performed by RT-PCR amplification of the SARS-CoV-2 *ORF1ab* gene and *E* gene fragments, using a SARS-CoV-2 Nucleic Acid Diagnostic Kit. The lower limit of detection of the test was 200 copies/sample. The detection of the human *RNase P* gene was included in the kit as a control. The result was considered positive if the cycle threshold (ct) values of both target genes were ≤ 38, and negative when ct values of both targets were > 38. Retesting was carried out among the samples with discordancy of the ct values; i.e. samples with one target gene with a ct value of ≤ 38 and another showing a ct value of > 38. The COVID-19 suspects who got Inconclusive / indeterminate findings for COVID-19 were informed to undergo repeat sample collection within 24 h. The repeat sample was collected only for the method either saliva or NP-OP swab in which the indeterminate findings were obtained. The turnaround time of the results was approximately 4 h.

The study protocol was reviewed and approved by the Institutional Ethics Committee (vide letter no. IEC/Pharmac/2020/182).

Statistical Analysis

Characteristics of the suspects and category of COVID-19 illness were summarized as mean (SD) or frequencies with percentages. The sensitivity, specificity, and predictive value of salivary samples were estimated against the NP-OP reference standard and the accuracy measures of salivary samples were described as percentages with 95% CI. Differences in proportions of COVID-19 positive status between salivary and NP-OP swabs were analyzed using the McNemar Chi-Square test. The COVID-19 status was categorized into positive, negative, or inconclusive. Concordance of the COVID-19 status between salivary and NP-OP specimens was estimated using Weighted Kappa (Agreement) statistics with 95% CI. Similarly, ct values of E gene and *ORF1ab* genes were compared using Wilcoxon signed-rank test and plotted using Bland Altman plot with 95% limits of Agreement.

Results

Participant Characteristics

Eighty participants consented to concurrent conventional NP-OP and self-salivary sampling procedure for the study. Forty-nine (61.3%) were males, the mean age of the participant was 36.4 years (Table 1).

To determine the diagnostic test performance of RT-PCR of the saliva, RT-PCR results of the NP-OP swabs were used as the reference standard. Details of patients undergoing each type of swab and the COVID-19 findings of these samples are shown in (Fig. 2).

Out of 80 suspects who underwent paired samples for COVID-19 detection, 41 showed positivity by conventional NP-OP swabs, and 12 suspects showed positivity in salivary samples. The salivary samples had shown a significantly lesser positivity rate compared to the conventional NP-OP specimen samples standards (Fig. 3, $p = 0.002$).

The sensitivity and specificity of salivary samples against conventional reference standards are 24.4%, 94.9% respectively. Out of 80 paired samples 44 (55%) paired samples had similar findings (Positive Vs Positive; Negative Vs Negative; Inconclusive Vs Inconclusive). In two instances, where COVID-19 positivity was missed by the conventional NP-OP swab was picked by the salivary samples. Concordance of these two types of samples in terms of agreement kappa statistics is estimated as K = 0.252 (0.09–0.42) (Table 1).

Median ct values of both the E gene and *ORF1ab* gene for the salivary samples were higher than ct values for the conventional NP-OP swabs. However, when the difference

Table 1 Concordance of COVID-19 status between Naso-Oropharyngeal swabs and salivary samples

Saliva swab sample	Nasopharyngeal-Oropharyngeal (NP-OP) swab sample			
	Negative	Positive	Inconclusive	Total (%)
Negative	32	28	2	62 (77.5)
Positive	2	10	0	12 (15)
Inconclusive	1	3	2	6 (7.5)
Total (%)	35 (43.7)	41 (51.2)	4 (5)	80 (100)
Weighted kappa (95% CI): 0.252 (0.09–0.42)				

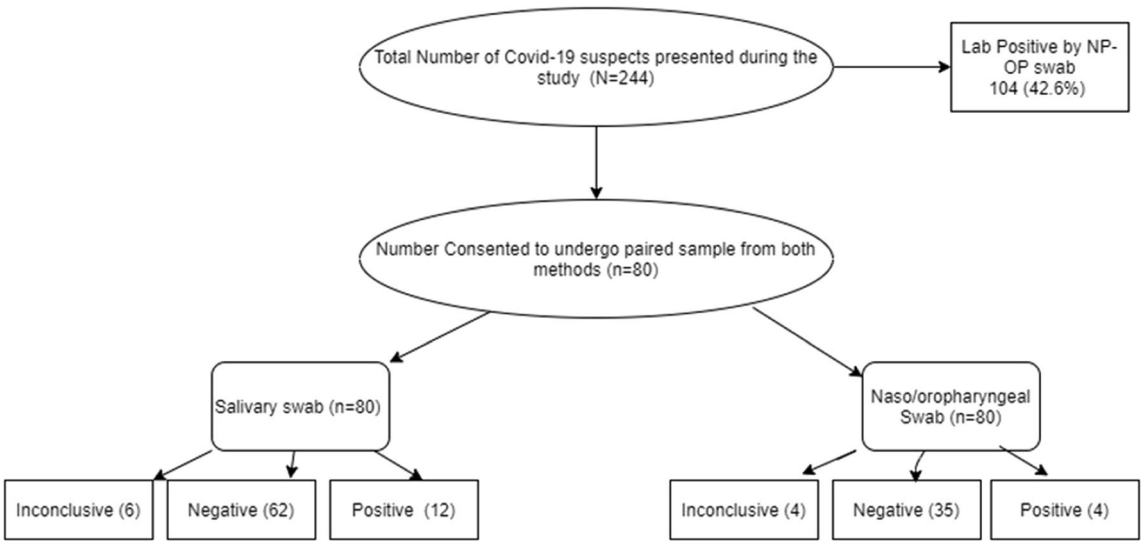

Fig. 2 Flow of COVID-19 suspects participated in parallel testing with saliva and NP-OP sampling for COVID-19

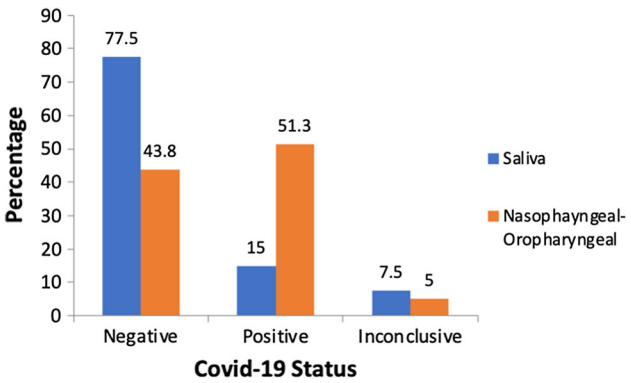

Fig. 3 Comparison of COVID-19 findings between saliva and NP-OP swabs among COVID-19 suspects

in ct values between these two methods was plotted against the average ct values of both salivary and NP-OP sampling methods these differences lied within the limits of agreement (Table 2 and Fig. 4).

Discussion

The saliva as a simple, non-invasive and self-collection method is frequently discussed as an alternative method in COVID-19 diagnosis. This study compared the diagnostic utility of non-contaminated saliva samples with conventional NP-OP sampling. A paired sample from suspects showed the sensitivity and specificity of saliva as 24.4, and 94.9%, respectively.

The access of SARS-CoV-2 to the oral cavity occurs mainly from secretions of the upper and lower respiratory tract. In addition to the secretions of the respiratory tract, the other sources include secretions from oral cavity-specific- crevicular fluid and released particles from infected salivary glands. Saliva is a complex fluid, it acts as a wide resource for genomic, proteomic, and biochemical information which is useful for studying potential diseases. It is produced and secreted from salivary glands. The basic secretary units of salivary glands are clusters of cells called acini. The acinar cells secrete is further altered in composition in its ducts. Demonstration of expression of angiotensin converting enzyme-2 (ACE 2) receptors in the

Table 2 Comparison of viral load [Cycle threshold (ct) values] between salivary and Naso-Oropharyngeal paired samples

Type of sample	(n =)	E gene	ORF1ab
Saliva	12	27.6 (5.8)	27.1 (5.1)
Naso-Oropharyngeal swab	40	22.5 (5.7)	21.2 (4.7)
P value		0.0001	0.0001
Saliva	10	27.6 (6.1)	27.4 (5.3)
Naso-Oropharyngeal swab	10	20.4 (6.5)	19.4 (5.8)
P value		0.01	0.003

epithelial lining of the salivary gland duct in Rhesus Macaques have affirmed that salivary glands could be the source of SARS-CoV-2 in a saliva sample. Apart from the easy and non-invasive method of collection, the temporal profile of salivary specimen is also considered better for serial monitoring of COVID-19 infection [8].

The use of swabs and sponge-like devices is advocated for taking the uncontaminated samples from the oral cavity. This study was conducted using Nylon swab sticks. Rolling of swab head for 20 s in the region of sub-mandibular duct opening in the floor of oral cavity without touching rest of the subsites possibly gives best of the chance for collecting non-contaminated salivary samples. To date, salivary duct intubation is the only secured but potentially invasive method used in saliva collection technique. However, this may not be a feasible way as a sample collection method of choice for mass screening tests as required for COVID-19 [7].

The low sensitivity of salivary samples identified in the current study raises the concern of whether saliva could be used as a reliable alternate biomarker in the detection of COVID-19. The significantly lower median ct values estimated for the E gene and ORF1ab gene also indicate that the saliva may not detect the COVID-19 as equivalent to the conventional NP-OP specimen. This could be attributed to the tiny volumes of secreted molecules which are

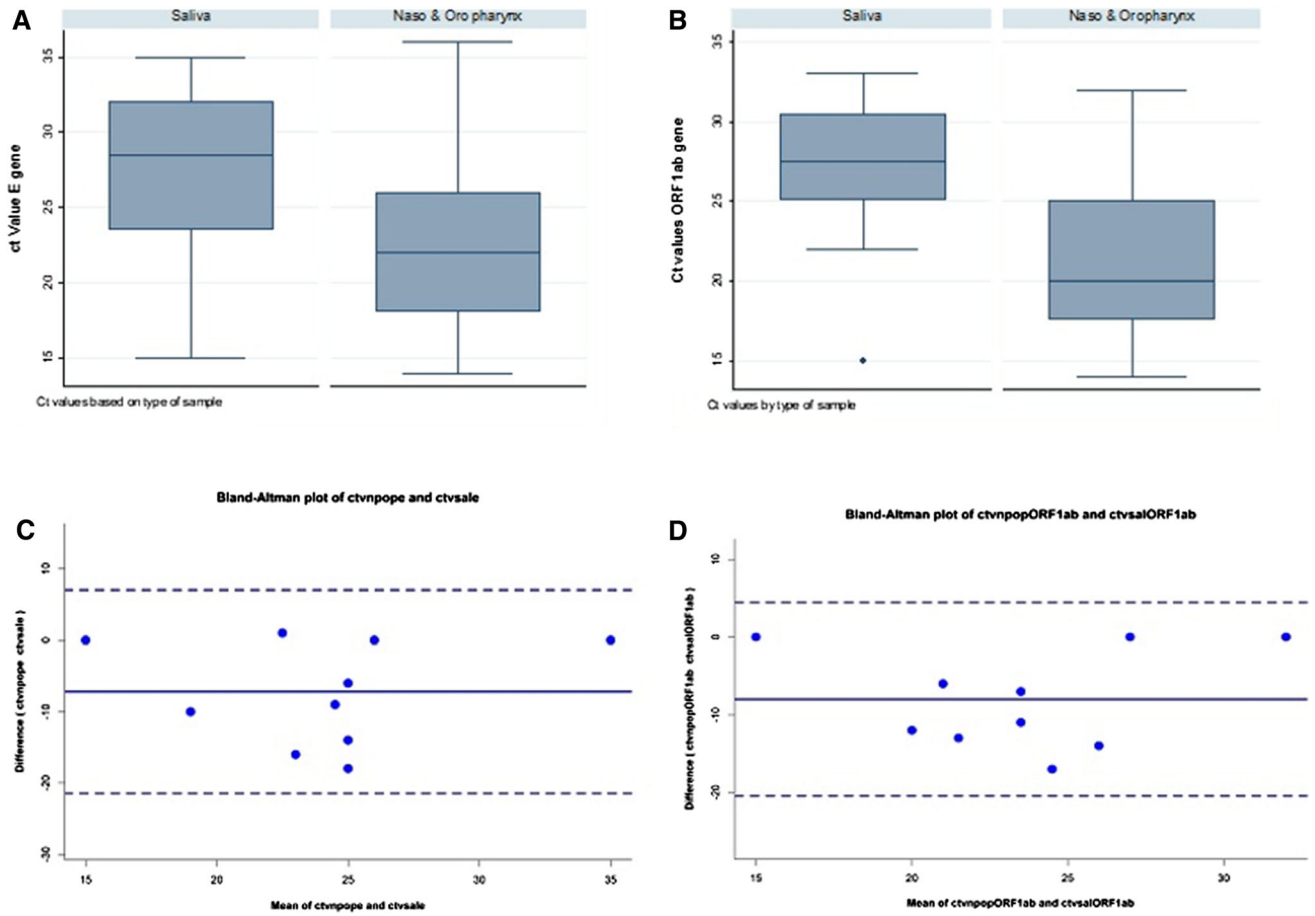

Fig. 4 Difference in cycle threshold (ct) values between saliva and NP-OP sampling plotted against the average ct values of both samples with limits of agreement. **a** Comparison of ct values between NP-OP and saliva sample for E gene. **b** Comparison of ct values between NP-OP and saliva sample for *ORF1ab* gene. **c** Bland Altman plot for ct value difference versus average ct values of NP-OP and saliva sample for E gene. **d** Bland Altman plot for ct value difference versus average ct values of NP-OP and saliva sample for *ORF1ab* gene

measured in nanograms to sometimes even in picograms making it difficult to assess accurately.

A review done by Sapkota D et al. [5] studied seven groups and approved the use of saliva secretion in diagnosis and monitoring of COVID-19. The higher sensitivity of most of the studies advocating saliva could be attributed to the mixing of respiratory secretion collection as saliva. Various methods of sample collection like spit, drool, throat gargles, and deep coughing has chances of contamination or mixing of respiratory secretion with saliva. Further, these studies also lack clarity on the quantity of sample while considering it for COVID-19 testing.

The findings of our study showed significantly lesser sensitivity (24.4%) by saliva sampling. Further, this could be due to the testing among COVID-19 suspects whereas the majority of the previous studies compared the salivary and NP-OP samples of confirmed COVID-19 cases. A systematic review by Sarode et al. [9] concluded non-superiority of saliva in comparison with conventional NP-OP sampling after reviewing nine studies by using Quality Assessment and Diagnostic Accuracy Tool-2. The method of conventional sampling by combining NP-OP region swab samples has an advantage of sampling more than one site and has merit to detect the COVID-19 with better sensitivity [10].

The current study has the following strengths. The method followed in the current study ensures the collection of pure saliva without contamination from other respiratory secretions. The salivary and NP-OP paired samples collected from the same suspects take care of the influence of other background characteristics. The minimal turnaround time (less than 6 h) took in this study also ensures the difference in positivity is not from other extraneous specimen-related factors such as transport, cold chain issues, etc. These paired samples were collected from COVID-19 suspects which also includes negative and asymptomatic cases. Hence it could reflect the reality when the intended use is for mass self-testing and negate the need for sample collection by HCWs. The person who processed the samples for COVID-19 testing was blinded from knowing the origin/type of sample.

There are few limitations to our study. Though the sample size was adequate for identifying the difference in positivity rate the ct values could be compared only from 12 cases. The sample size is limited as COVID-19 is evolving situation and additional infrastructure procurement is difficult.

The study has the following clinical implications. As the sensitivity of unmixed salivary samples identified in this study is 22% in comparison to NP-OP sampling it raises the concern whether saliva could replace the sampling strategy. The evidences from the past studies claim the sensitivity rate of 75–100% for salivary samples. We are of opinion that these are oral secretion contaminated specimen samples that are labeled as saliva for testing. The majority of these studies did not describe the exact method which is being used for the collection of saliva. Hence, there is a need to review and refine the reporting standards for describing the method of salivary sample collection. Before rolling out these salivary samples for self-collection, further studies have to confirm which type of sample within the given choices such as only saliva or mixed oral secretions or swabs collected from multiple anatomical sites are meant for self-sampling and which can yield similar positivity against the conventional standards.

Conclusion

The salivonomics has proved to be useful and has diagnostic potential for many conditions. However, the current study showed lesser sensitivity with salivary swab samples as compared to conventional NP-OP samples for RT-PCR, COVID-19 detection. Hence, more studies are required to establish the utility of true salivary sampling in COVID-19 diagnostics. There is a need to clearly describe the sampling methods while reporting to avoid mislabeling and to facilitate replication in other settings.

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Declarations

Conflict of interest The authors declared that they have no conflict of interest.

Consent to Participate Verbal informed consent of participant taken.

Consent for Publication All authors provide consent to publish.

Ethical Approval *Institutional Ethical Committee* All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration.

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