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## Simple and low-cost nucleic acid extraction methods for detection of SARS-CoV2 in self-collected saliva and dry oral swabs

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### ABSTRACT

**Background:** Ongoing need of alternative strategies for SARS-CoV-2 detection is undeniable. Self-collected samples without viral transport media (VTM), coupled with simple nucleic acid extraction methods for SARS-CoV-2 PCR are beneficial.

**Objectives:** To evaluate results of SARS-CoV-2 PCR using simple nucleic acid extraction methods from self-collected saliva and oral swabs without VTM.

**Methods:** A cross-sectional single-centre study was conducted on 125 participants (101 SARS-CoV-2 positive cases and 24 controls). PCR was performed following five simple nucleic acid extraction methods on self-collect saliva and oral swabs without VTM and results were compared with gold standard PCR. For saliva, kit-based extraction (SKE), Proteinase K and Heat extraction (SPHE), only Heat extraction (SHE) methods and for dry oral swabs, Proteinase K and Heat extraction (DPHE) and only Heat extraction (DHE) was performed.

**Results:** SARS-CoV-2 was detected in self-collected saliva and oral swabs. 93.07% were correctly classified as positive by SKE, 69.31% by SHE, 67.33% by SPHE, 67.33% by DPHE and 55.45% by DHE. Discriminant power of SKE was significantly higher than other methods (p-value < 0.001) with good-fair agreement of alternate extraction methods against gold standard.

**Conclusion:** Combination of self-collected saliva/ oral-swab without VTM and alternative RNA extraction methods offer a simplified, economical substitute strategy for SARS-CoV-2 detection.

### Introduction

The world has witnessed major waves of the COVID-19 pandemic caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV2) and is still enduring the same with a lot of morbidity and mortality (Chih-Cheng Lai et al 2020). This unprecedented pandemic challenged the healthcare system like never before and pressed the need for transformation in diagnostics, treatment, and prevention to a great extent. Early laboratory diagnosis of COVID-19 is necessary for confirmation and initiation of treatment, as well as to break the chain of transmission (Sharma et al 2021).

With evolving modalities, diagnostic scenario of COVID-19 has changed drastically.

Currently, Real Time reverse transcription Polymerase Chain Reaction (rRT-PCR) using nasopharyngeal / oropharyngeal (NP/OP) swab is the gold standard for detection of SARS-CoV-2 infection (Liu et al 2020). However, sample collection, transportation and nucleic acid extraction have certain limitations as far as this gold standard is concerned, which can be overcome by alternative options (Hwang et al 2018, Ambrosi et al 2021).

In view of risk associated (while collecting NP/OP samples) to the patient as well as to the health care worker (HCW), self-collected, non-invasive samples such as saliva/ dry oral swabs are valuable alternatives (Wyllie et al 2020), especially in children and non-co-operative patients. Also, performance of the gold standard rRT-PCR can be affected by the viral RNA extraction procedure, a crucial variable that ascertains the

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sample positivity. Though quite a few laboratories deploy automated nucleic acid extraction systems, many smaller labs still use manual extraction methods and reliable protocols for RNA extraction are necessary (S Sahajpal et al 2020). The COVID-19 pandemic also challenged the reagent supply chain leading to a shortage of viral transport media (VTM), nucleic acid extraction kits etc during the pandemic (Smith et al 2020, Wyllie et al 2020). As a response, newer sample collection protocols (saliva/ dry oral swabs) that can be transported without VTM and alternative methods for RNA extraction such as prior heat-processing of samples and use of Proteinase K have evolved as economical methods, enabling an extremely fast down streaming at much lesser cost thereby easing the logistics (Srivatsan et al 2021, Guan et al 2021, Azmi et al 2021). Also, Multi-step RNA extraction is a bottleneck that impedes mass testing for COVID-19. Alternative, simpler RNA extraction protocols are more suitable and have a tremendous potential to minimize the need for laboratory set-up and trained professionals, when integrated with a similar simplified method for detection (Nique et al 2021).

The present study attempted to analyse alternate, low-cost RNA extraction methods for detection of SARS-CoV2 in saliva and dry oral swabs without VTM.

## Materials and methods

### Patients

A total of 101 COVID-19 rRT-PCR positive patients presenting to designated COVID hospital attached to our Medical College in Bengaluru, India between February to November 2021, were included in the study. The control group included 24 COVID-19 negative health care workers (HCWs). Institutional ethical clearance (IEC no. BMCRI/ PS/ 09/20-21) and informed consent from the study subjects was taken. As the study was being conducted at dedicated COVID-19 hospital with confirmed and potentially infectious patients, a convenient sample size was taken. Patients who did not consent to be a part of the study, patients with severe COVID-19 on ventilator, and patients where sample could not be collected (e.g. – unconscious patients) were excluded from the study.

### Data Collection

Demographic data were recorded using a proforma. Details were entered into an Excel sheet.

### Sample Collection

Saliva and dry oral swab were collected at the time of patient's admission (within 24-48 hours of positive gold standard rRT-PCR) to COVID-19 hospital. Patients were asked to collect unstimulated whole saliva, by repeatedly spitting into a sterile, container (without any transport media) until roughly a third full (excluding bubbles), before securely closing it (M Ott et al 2020). Dry oral swabs were collected using nylon flocked swabs, patients were asked to roll the swab in the oral cavity, on the tongue and cheeks and put the swab in a sterile container, without any VTM<sup>9</sup>. Samples were transported at room temperature to the laboratory for further processing, samples with delay in transportation were stored at 4 ° c for a maximum of up to 24 hours.

### Processing of saliva samples

Upon receipt at laboratory, all the specimens were handled and processed under Bio Safety Level 2 as per standard guidelines.

### RNA Extraction Methods

Five methods of RNA extraction were carried out.

For the saliva samples: (Figure 1 and 2)

- 1 Kit based extraction (SKE) using commercially available kit (Qiagen)

- 2 Proteinase K and Heat extraction (SPHE)
- 3 Heat extraction (SHE).

For dry oral swab samples: (Figure 3)

- 1 Proteinase K and Heat extraction (DPHE)
- 2 Heat extraction (DHE).

Methods for extraction have been described in detail in Figure 1, 2 and 3.

### Detection of SARS-CoV-2

RNA extracted by all five methods was subjected to RT-PCR for SARS CoV2 detection, using a commercially available, ICMR approved kit (Q-line) following manufacturer's protocol for E and RdRp genes.

### Data analysis

Data was analysed using R software version 4.1.1 and Excel. Categorical variables are given in the form of a frequency table. Continuous variables are given in Mean  $\pm$  SD/ Median (Min, Max) form. Receiver operating characteristic curve (ROC) analysis was conducted to compare results from different methods with gold standard (actual COVID status) and pairwise comparisons of the ROC curves were performed. Kappa agreement of different methods with gold standard and with each other were checked. One-way ANOVA was used to compare the positive sample's CT values of E and RdRp genes between different methods. Bland Altman plot was used for the comparison of positive sample's cycling threshold (CT) values of E gene and RdRp gene for different methods. P-value less than or equal to 0.05 indicates statistical significance.

## Results

### Overall summary of SARS-CoV2 Detection through 5 different extraction methods

Data contains measurements on 125 samples (101 were COVID-19 positive and 24 were COVID-19 negative as per rRT-PCR (gold standard method)). Five different methods of RNA extraction were analysed for rRT-PCR with E and RdRp gene targets.

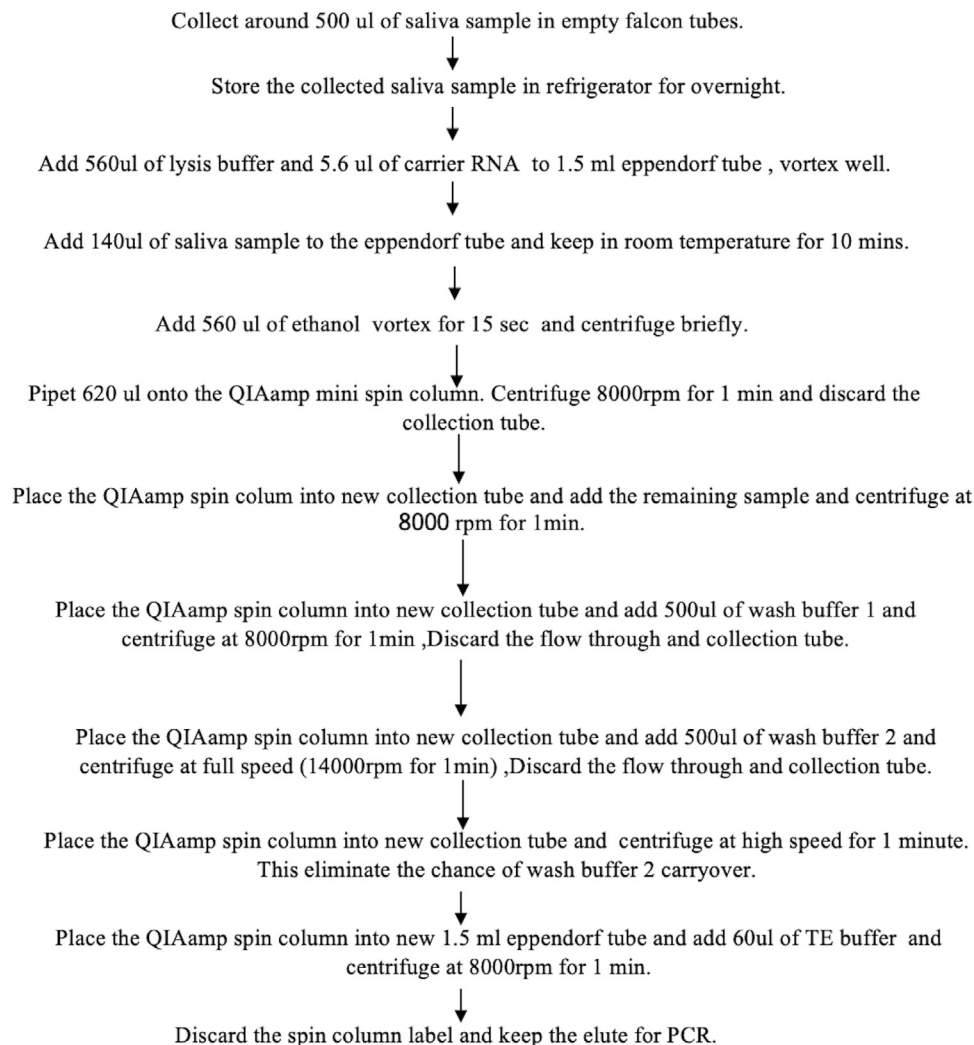
Out of 101 subjects with positive COVID status, 69.31% were males and 30.69% were females. A majority of subjects belonged to 41-60 years age group. 03 samples were excluded due to processing errors and results of these were ignored for the further analysis. Of the remaining 98 samples, 93.07% were correctly classified as positive by SKE, 69.31% by SHE, 67.33% by SPHE, 67.33% were correctly classified as positive by DPHE and 55.45% using DHE. Table 1 gives the agreement of different methods with COVID-19 rRT-PCR status (Gold standard).

### Accuracy assessment of all 5 methods with respect to RT-PCR status of samples

From Kappa tests, we observed that there is significant (almost perfect) agreement of the results based on SKE with Gold standard (p-value < 0.001). There is significant- moderate agreement between the results of SPHE, SHE and DPHE with Gold standard (p-value < 0.001). There is significant- fair agreement between the results of DHE and Gold standard (p-value < 0.001). Table 2 shows the diagnostic analysis of different methods with COVID-19 rRT-PCR status (gold standard).

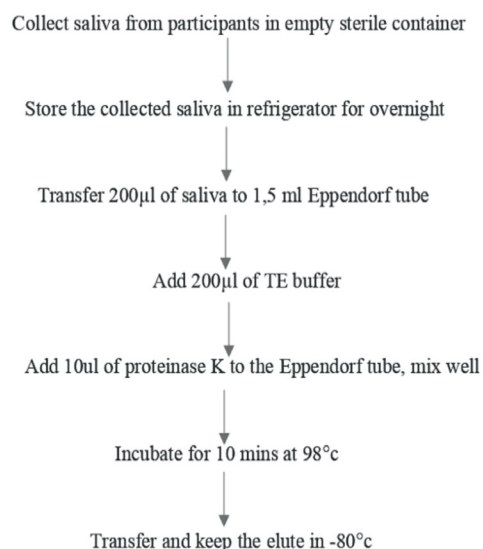
From pairwise comparison of the ROC curves (Figure 4), the discriminant power of the SKE method is significantly higher than all other methods (all p-values < 0.001). The discriminant power of the SPHE method is not significantly lower than the SHE method (p-value = 0.8612). The discriminant power of the SPHE method is the same as that of DPHE (p-value = 1). The discriminant power of the SPHE method is not significantly lower than DHE (p-value = 0.3402). The

**Extraction using Commercially available kits (Qiagen)**

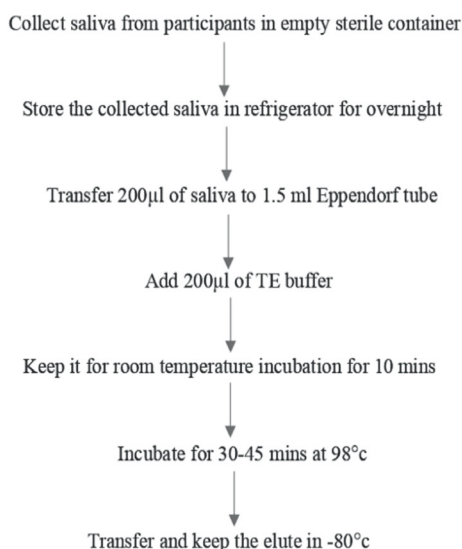


**Figure 1.** Procedure for Saliva Kit based extraction (SKE).

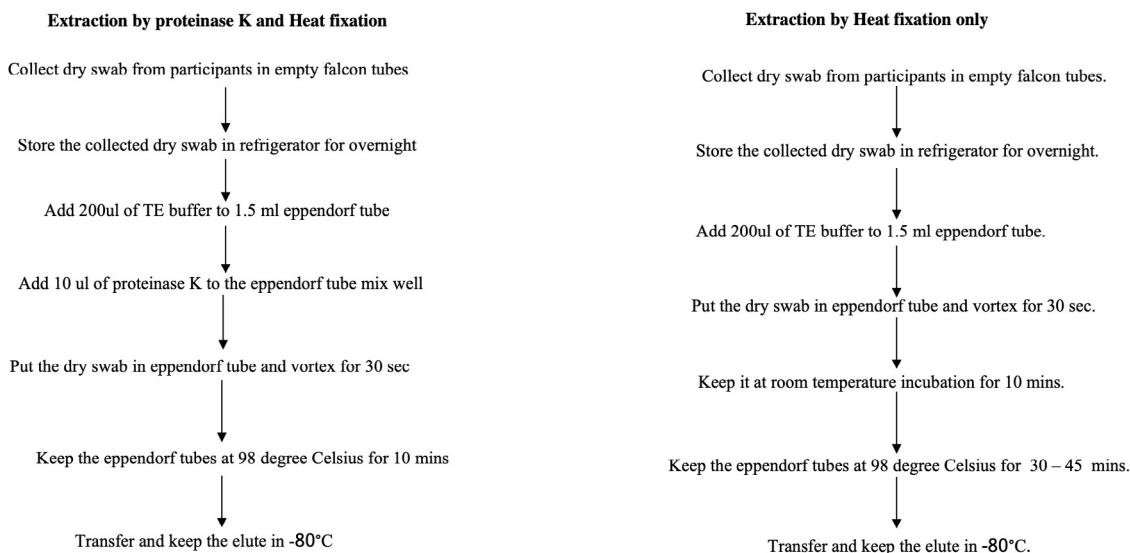
**Extraction by proteinase K and Heat fixation**



**Extraction by Heat fixation only**



**Figure 2.** Procedure for saliva- extraction by Proteinase K and heath fixation (SPHE) (Left flow chart) and by heat fixation only (SHE) (Right flow chart)

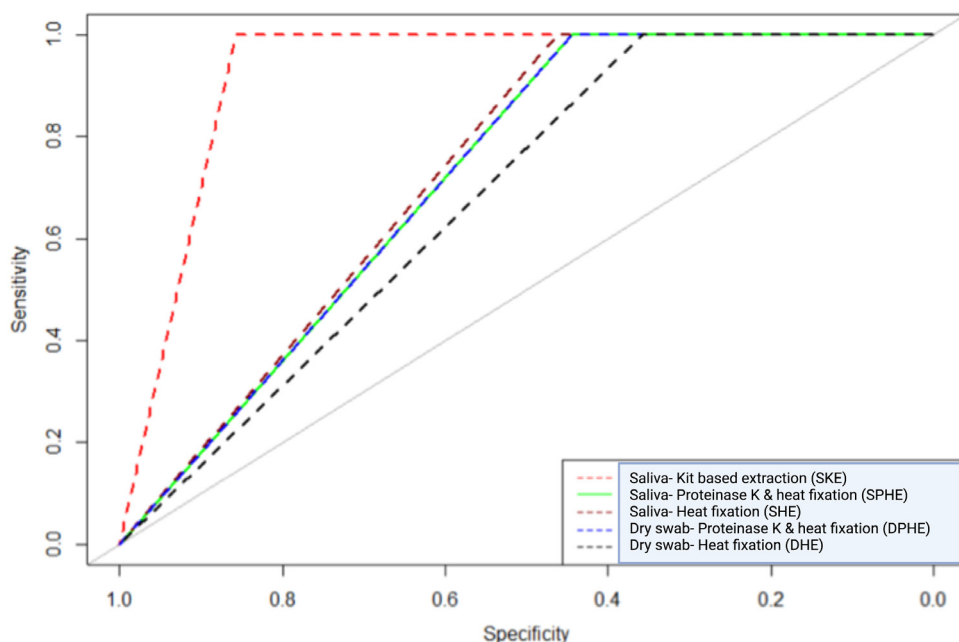


**Figure 3.** Procedure for dry oral swab- extraction by Proteinase K and heath fixation (DPHE) (Left flow chart) and by heat fixation only (DHE) (Right flow chart)

**Table 1**  
Agreement of different methods with COVID-19 rRT-PCR status.

Methods	Result	Positive	Negative	Kappa coefficient (95% CI)	p-value
Saliva- Kit extraction (SKE)	Positive	94	0	0.90 (0.81 – 0.996)	< 0.001 <sup>K*</sup>
	Negative	4	24		
Saliva- Proteinase K & heat fixation (SPHE)	Positive	68	0	0.48 (0.31 – 0.64)	< 0.001 <sup>K*</sup>
	Negative	30	24		
Saliva- heat fixation (SHE)	Positive	70	0	0.49 (0.33 – 0.66)	< 0.001 <sup>K*</sup>
	Negative	28	24		
Dry swab - Proteinase K and heat fixation (DPHE)	Positive	68	0	0.48 (0.31 – 0.64)	< 0.001 <sup>K*</sup>
	Negative	30	24		
Dry swab - heat fixation (DHE)	Positive	55	0	0.33 (0.17 – 0.49)	< 0.001 <sup>K*</sup>
	Negative	43	24		

(K\* = kappa)



**Figure 4.** ROC curve of different methods in predicting true COVID-19 status.

**Table 2**  
Diagnostic analysis of different methods with COVID-19 rRT-PCR status

Methods	Sensitivity(95% CI)	Specificity(95% CI)	PPV (95% CI)	NPV(95% CI)	Accuracy(95% CI)	AU-ROC(95% CI)
Saliva- Kit based extraction (SKE)	95.92(89.88 - 98.88)	100(85.75 - 100)	100(96.15 - 100)	85.71(67.33 - 95.97)	96.72(91.82 - 99.10)	0.93(0.86 - 0.99)
Saliva- Proteinase K & heat fixation (SPHE)	69.39(59.26 - 78.3)	100(85.75 - 100)	100(94.72 - 100)	44.44(30.92 - 58.6)	75.41(66.79 - 82.75)	0.72(0.66 - 0.79)
Saliva- heat fixation(SHE)	71.43(61.42 - 80.1)	100(85.75 - 100)	100(94.87 - 100)	46.15(32.23 - 60.53)	77.05(68.57 - 84.18)	0.73(0.66 - 0.80)
Dry swab - Proteinase K and heat fixation (DPHE)	69.39(59.26 - 78.3)	100(85.75 - 100)	100(94.72 - 100)	44.44(30.92 - 58.6)	75.41(66.79 - 82.75)	0.72(0.66 - 0.79)
Dry swab - heat fixation (DHE)	56.12(45.73 - 66.13)	100(85.75 - 100)	100(93.51 - 100)	35.82(24.47 - 48.47)	64.75(55.59 - 73.18)	0.68(0.62 - 0.74)

discriminant power of the SHE method is not significantly higher than the DPHE (p-value = 0.8612) and DHE methods (p-value = 0.2595). Discriminant power of DPHE is not significantly higher than DHE (p-value = 0.3402).

*Agreement of various extraction methodologies with each other: A Sub-group analysis*

- Agreement between results of Saliva sample by all three methods: *SKE, SPHE and SHE.*

The Kappa Test for the agreement of results between all the three combinations shows that there is significant moderate agreement between results. The Kappa coefficient between SKE and SPHE at 95% Confidence Interval (CI) is 0.51(0.36 - 0.67), between SHE and SPHE at 95% CI is 0.44(0.29 - 0.59) and between SHE and SKE at 95% CI is 0.51(0.36 - 0.67). All are significant with p<0.001.

- Agreement between results of Dry Swab samples by two methods: *DPHE and DHE*

Agreement between the results for DHE and DPHE fixation shows that there is significant fair agreement between results. The Kappa coefficient at 95% CI is 0.36(0.20 - 0.52) and is significant at p<0.001.

- Agreement between results of Saliva samples and Dry swab samples by various extraction methods

Agreements were assessed between extraction methods for different samples as follows. The Kappa coefficient between DPHE and SKE at 95% CI is 0.51(0.36 - 0.67), between DPHE and SPHE at 95% CI is 0.35(0.19 - 0.51), between DPHE and SHE at 95% CI is 0.47(0.32 - 0.62), between DHE and SKE at 95% CI is 0.41(0.26 - 0.56), between DHE and SPHE at 95% CI is 0.24(0.07 - 0.40) and between DHE and SHE at 95% CI is 0.42(0.27-0.58).

**Discussion**

Sustained need for SARS-CoV-2 detection during the current pandemic is foreseeable. There is a dire need to have matching volume of hassle-free SARS-CoV-2 testing capacity, that can overcome the limitations of the present gold standard method. This can be achieved by validation and optimization of alternative protocols to implement simpler diagnostics and ensure continuity of testing (Nique et al 2021). It is important to disseminate data on alternative testing protocols in the current global scenario, so that they can provide inputs for diagnostic algorithms and thereby inform strategies.

During this unparalleled pandemic with many unknowns, where research can be challenging and outcomes can be rewarding, the present study has merit in deploying a simple substitute strategy to simplify diagnostics by a combination of unconventional samples (self-collected saliva and oral swab without VTM) with alternative, simpler, cost-effective RNA extraction methods.

The present gold standard, RT-PCR with nasopharyngeal/oropharyngeal (throat and nasal swabs) NP/OP samples for the detection of SARS-CoV-2, requires a visit to a health care facility, resulting in crowding at sample collection centres, putting the HCW at a substantial risk (Chih-Cheng Lai et al 2020). Additionally, supply chain issues in procurement of VTM, reagents, kits and other critical materials to perform and sustain gold standard PCR has been a great challenge and had resulted in delay in testing and reporting and of course heavy pricing of tests in the beginning of the pandemic (Wyllie et al 2020).

Published studies have shown the use of saliva and oral swab, which can be self-collected, as alternative samples for diagnosis of SARS-CoV2 (Chu et al 2020, Ku et al 2021). Further, transportation of these samples without VTM reduces the cost and eases logistics. Though saliva has been approved as alternative sample, to the best of our knowledge, there are no published studies from India on utility of dry oral swabs



for sample collection. While many articles, reviews and reports explain the numbers behind efficiency of alternative samples (Chu et al 2020, Ku et al 2021, de Paula et al 2022) few studies directly shed light on the alternative methods of nucleic acid extraction of these untraditional samples collected without VTM (Jayaprakasam et al 2021). Alternative, low-cost nucleic acid extraction methods applied to these unorthodox samples further provides benefits in terms of ease, less dependence on reagents/ equipment, time taken and cost. This article curates the analysis of the various extraction methods for saliva and dry oral swab samples.

Our results show that self-collected saliva and oral swab samples without VTM can detect SARS-CoV-2 in COVID-19 patients. Sensitivity was higher for saliva (93.07% by SKE, 69.31% by SHE, 67.33% by SPHE) than oral swab (67.33% DPHE and 55.45% using DHE). Similar findings were seen in another study (de Paula et al 2022) where saliva showed a sensitivity of 87.3% and 65.9% using oral swab. Another study also documented that saline gargle was a comparable alternative to a nasopharyngeal swab, but the lower sensitivity of the oral swab makes them less useful (Kandel et al 2021). Self-collected saliva without VTM is thus advantageous over oral swabs in the present pandemic to fulfil the requirements of mass testing, as sterile containers for saliva are easily available in comparison to swabs and also cost less. Also, a higher percentage of saliva samples is known to remain positive for a longer duration after the COVID-19 diagnosis compared to nasopharyngeal swab (NPS) (Ku et al 2021), offering an advantage for large scale community testing.

The foremost findings in our study elaborate upon the differences in the use of various extraction methods for the processing of these samples. From pairwise comparison of the ROC curves, the discriminant power of the SKE method was significantly higher than all other alternative methods (all p-values < 0.001). Kit based extraction from saliva had the best result as corroborated by another study (Azmi et al 2021) with close agreement of saliva samples following kit-based RNA-extraction, with 98.7% positive agreement. One more study found that the sensitivity of SARS-CoV-2 detection from saliva is comparable, if not superior to nasopharyngeal swabs (Wyllie et al 2020). Our results reiterate that kit-based extraction from saliva is reliable for SARS-CoV-2 detection.

We evaluated alternate extraction free protocol for saliva and oral swab and found good- fair agreement of various alternate low-cost RNA extraction methods with COVID-19 rRT-PCR status (Gold standard). The discriminant power of the SPHE method was same as that of DPHE (p-value = 1). From Kappa tests, we observed that there was significant-moderate agreement between the results of SPHE, SHE and DPHE with Gold standard (p-value < 0.001) and significant- fair agreement between the results of DHE and Gold standard (p-value < 0.001). Chu et al observed that the RT-PCR positive rate was significantly higher for proteinase K and heat (PKH group) PKH (80 % [40/50]) than those of heat only (58 % [29/50]; P = 0.001). The difference in our study may be related to the rRT-PCR assay used, as the type of RT-PCR assay can affect the results (Chu et al 2020). Sally A. Mahmoud et al performed analysis of saliva samples by nucleic-acid-extraction-free, RT-qPCR method for SARS-CoV-2 detection where salivary samples were treated with proteinase K and heat inactivation followed by a direct input in the RT-PCR machine, and reported 85.34% sensitivity as compared to our study which showed 69.39% sensitivity. The drawback of their study was that they did not analyse different extraction methods for processing, which has been covered in this novel study (Mahmoud et al 2021). Iqbal Azmi et al also showed 95.7% agreement for positive test samples and 100% agreement for the negative samples by optimized RNA extraction-free protocol, suggesting that saliva can be directly used for the detection of SARS-CoV-2 without the need for costly and time-consuming extraction methods (Azmi et al 2021).

In alternative extraction protocols, proteinase K offers many advantages: it homogenizes saliva, prevents the degradation of SAR-CoV-2 RNA by digesting RNase and improves the efficiency of RT-PCR by digesting the proteins in clinical samples like saliva. In view of easy avail-

ability, lack of supply issues, low cost and simplicity of extraction procedure, proteinase K combined with heat treatment fares well over kit-based extraction for massive screening using self- collected samples like saliva in developing counties, in-spite of lesser sensitivity.

To conclude, our results show that self-collected saliva and oral swab samples without VTM can detect SARS-CoV-2 in COVID-19 patients, however self-collected saliva without VTM is advantageous over oral swabs. Though saliva has been approved as alternative sample, to the best of our knowledge, there are no published studies from India on utility of dry oral swabs for sample collection. Alternate extraction free protocols for saliva and oral swab in our study showed good- fair agreement with COVID-19 rRT-PCR status (Gold standard). Deployment of combination of alternative samples that can be self-collected (saliva and oral swab) and simple, low-cost RNA extraction methods would ensure safe sample collection, reduced processing time and lesser cost for detection of SARS-CoV-2 during the ongoing pandemic.

### 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.

### Conflict of Interest

None.

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### Ethical Approval statement

Institutional ethical approval taken (IEC no. BMCRI/ PS/ 09/20-21).

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