



Gargle lavage & saliva: Feasible & cheaper alternatives to nasal & throat swabs for diagnosis of COVID-19

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Background & objectives: In the present scenario, the most common sample for diagnosis of COVID-19 by reverse transcription polymerase chain reaction (RT-PCR) is nasal and throat swab (NTS). Other sampling options such as gargle lavage have found limited application in clinical use mostly because of unavailability of an appropriate gargling liquid. This study was conducted to assess the stability of SARS-CoV-2 RNA in normal saline at 4°C that can serve as a gargling liquid as well as a transport medium. The study also looked at the agreement between NTS and gargle lavage/saliva for the detection of SARS-CoV-2.

Methods: In 29 consecutive real-time RT-PCR (rRT-PCR) positive COVID-19 patients, paired NTS, gargle and saliva samples were taken. Samples were processed by rRT-PCR for the detection of SARS-CoV-2 RNA. To assess the SARS-CoV-2 RNA stability in normal saline, gargle lavage specimens were divided into two aliquots; one subset of the specimen was run within 4-6 h along with the routine samples (NTS and saliva) and the other subset was stored at 4°C and processed after 24-30 h. Agreement between cycle threshold (Ct) values from both the runs was compared using Bland-Altman (BA) analysis.

Results: The positivity rates of rRT-PCR in NTS, saliva and gargle lavage samples were 82.7 (24/29), 79.3 (23/29) and 86.2 per cent (25/29), respectively. BA plot showed a good agreement between the Ct values of fresh and stored gargle samples, stipulating that there were no significant differences in the approximate viral load levels between the fresh and stored gargle lavage samples (bias: *E* gene -0.64, *N* gene -0.51, *ORF* gene -0.19).

Interpretation & conclusions: Our study results show stability of SARS-CoV-2 RNA in the gargle samples collected using normal saline up to 24-30 h. Gargle lavage and saliva specimen collection are cost-effective and acceptable methods of sampling for the detection of SARS-CoV-2 RNA by rRT-PCR. These simplified, inexpensive and acceptable methods of specimen collection would reduce the cost and workload on healthcare workers for sample collection.

Key words COVID-19 - gargle lavage - nasal swab - throat swab - RNA stability - saliva - SARS-CoV-2

COVID-19 caused by SARS-CoV-2 was declared a pandemic by the WHO in March 2020¹. The outbreak has involved over 150 countries all over the globe, with approximately 171 million cases, and currently, India is the second most affected country². It is through a multipronged approach that this pandemic can be brought under control and adequate testing is one of the most important measures. Effective testing strategy does not only depend on the availability of a reliable test but also on an acceptable method of sample collection. The most common method for sample collection for the reverse transcription is the testing of (RT)-PCR combined nasal and throat swab (NTS). Proper NTS sampling requires not only deputation of trained individuals but also requires protective gears, flocked swabs and viral transport medium (VTM). Alternative sampling methods such as collection of gargle lavage and saliva can be much more advantageous compared to NTS³. Although there are multiple reports of gargle and saliva being an acceptable alternative method, there is little clarity on the stability of viral RNA in these clinical specimens^{4,5}. Stability of clinical specimens can significantly affect the acceptability of these methods, keeping in mind the possibility of delay in transport and processing of samples in the real-world scenario. Therefore, this study was undertaken to evaluate the stability of viral RNA in the gargle lavage when stored at 4°C for one day. The agreement between NTS and gargle lavage/saliva samples was also studied for the detection of SARS-CoV-2.

Material & Methods

A cross-sectional, single-centre study was conducted at the All India Institute of Medical Sciences (AIIMS), New Delhi, India. NTS, saliva and gargle lavage samples were collected from 29 consecutive rRT-PCR-confirmed COVID-19 patients, admitted to the isolation wards (within 48 h of diagnosis). All the participants provided written informed consent. Children (age <18 yr) and patients who were not able to perform gargling or unable to follow instructions were excluded from the study. The study was approved by the Institutional Ethics Committee of AIIMS, New Delhi (IECPG-193/20.05.2020).

Sample collection was done in the following order: NTS followed by saliva followed by gargle lavage. Nasal swab was collected at the level of middle turbinate from both nostrils. The throat swab sample was collected from the posterior pharyngeal wall and the tonsillar area by trained healthcare workers (HCWs) using nylon-flocked swab. The swabs were

placed immediately into a sterile tube containing 2-3 ml of viral transport medium (VTM) and sealed properly. Saliva specimens were self-collected by the participants. They were asked to spit saliva multiple times in a sterile container. Sampling container was sealed securely after obtaining about two ml of saliva.

The participants were provided with a sterile screw-capped container pre-filled with normal saline (5 ml) for gargle lavage. The containers were prepared outside the COVID-19 facility to avoid any contamination. Participants were asked to perform the gargle for 15-20 sec and spit back lavage into the same container. Samples were sealed and transported as per the institutional protocol maintaining cold chain.

Assessment of RNA stability: To assess the RNA stability in normal saline, gargle specimens were divided into two aliquots; one subset of specimen was run within 4-6 h along with the routine samples and the other subset was processed after 24-30 h (stored at 4°C). Thereafter, the cycle threshold (Ct) values between both the results were compared to measure the difference between the two samples.

Sample processing and rRT-PCR: Samples were received in negative pressure BSL-2 facility for processing and centrifuged at 1500 g for 15 min. One aliquot of gargle lavage was stored at 4°C for testing on subsequent day. Saliva samples were incubated with lysis buffer for extended incubation to completely liquefy the sample. RNA was extracted with the MagMAX automated extraction system using MagMAX Viral/Pathogen II nucleic acid isolation kit following 200 µl sample input volume protocol (Thermo Fisher Scientific Inc., USA). rRT-PCR testing for SARS-CoV-2 was performed by Fosun COVID-19 RT-PCR detection kit (Shanghai, China) using Agilent AriaMx real-time PCR system (Agilent Technologies Inc., USA). The PCR assay detected *ORF1ab*, *N* and *E* genes of SARS CoV-2. An internal reference, added during extraction, was also simultaneously identified in the assay to assess extraction efficiency and possible inhibition. In samples with discrepancy, an additional PCR testing for ribonuclease P (RNP), a housekeeping gene, was also performed to assess adequacy of clinical material in the submitted sample.

Statistical analysis: The categorical variables were represented by counts and percentages, whereas the quantitative variables were represented by mean±standard deviation (SD). The test positivity

rate was calculated by ratio of positive test result to the total number of participants. Agreement between the fresh and stored gargle samples was assessed by Bland–Altman (BA) analysis. A scatter plot was constructed in which the differences between the paired measurements (Ct values) were plotted on Y-axis and fresh sample was plotted on X-axis. The mean difference (bias) in values obtained with the two methods was represented by a central horizontal line on the plot. The SD of differences between paired measurements was used to construct horizontal lines above and below the central horizontal line to represent the upper and lower limits of agreement (mean bias \pm 1.96 SD). The Chi-square test was used for comparison of test positivity rates between NTS, gargle lavage and saliva specimens. The data were analyzed using STATA 15 (Stata Statistical Software: Release 15. StataCorp LLC, TX, USA).

Results & Discussion

Twenty nine rRT-PCR-confirmed COVID-19 patients were enrolled in the study and 87 paired samples (NTS, saliva and gargle lavage; three specimens from each participant) from these 29 participants were analysed. The mean (\pm SD) age of the participants was 40.2 (\pm 16) yr, and 20 (69.0%) were male. Of the 29 participants, 20 (69%) participants were symptomatic for COVID-19, and the median duration of illness was three days (1-6 days) (Table I).

The positivity rates of SARS-CoV-2 in NTS, saliva and fresh gargle lavage samples were

82.7 (24/29), 79.3 (23/29) and 86.2 per cent (25/29), respectively (Tables II-IV). In four asymptomatic patients, all three samples (fresh and stored gargle and saliva sample) were negative for SARS-CoV-2 within 48 h of baseline testing (Table IV). In one patient, gargle and saliva samples were positive, but NTS was negative because of improper sample collection, as the clinical material was found inadequate (confirmed using the internal control *RNP* gene in the rRT-PCR assay).

SARS-CoV-2 RNA in the fresh and stored gargle samples was compared using the Ct values of *E*, *N* and *ORF* genes, to understand the stability of the genetic material in normal saline kept at 4°C over 24-30 h. BA analysis showed excellent agreement between the two, indicating that there were no significant differences in the approximate viral load levels between the fresh and stored samples (bias: *E* gene -0.64, *N* gene -0.51, *ORF* gene -0.19) (Figs 1-3). However, the positive test rate in the fresh gargle was higher than the stored gargle, 86.2 per cent (25/29) versus 82.7 per cent (24/29), respectively (Table IV). The initial Ct value for *E* gene was 32.2 and for *ORF* gene was 32.1 in the fresh gargle sample which became negative after 24 h of storage.

SARS-CoV-2 can survive on surfaces such as plastic and stainless steel for up to 72 h and on papers and cardboard for up to 24 h⁶.

Druce *et al*⁷ evaluated the use of sterile normal saline for the transport of specimen for detection of influenza A pdmH1N1 by PCR. They showed

Table I. Baseline demographic and clinical characteristics of the study participants (n=29)

| Characteristic | Total, n (%) | Symptomatic, n (%) | Asymptomatic, n (%) |
|-------------------------------|-----------------|--------------------|---------------------|
| Participants | 29 (100) | 20 (68.9) | 9 (31.0) |
| Age (yr, mean \pm SD) | 40.2 \pm 16.2 | 44.2 \pm 16.4 | 31.3 \pm 11.6 |
| Sex | | | |
| Male | 18 (62.0) | 10 (50.0) | 8 (88.8) |
| Female | 11 (38.0) | 10 (50.0) | 1 (11.1) |
| Duration of illness (days) | | | |
| Median | - | 3 | - |
| Range | - | 1-6 | - |
| Comorbidities | 5 (17.2) | 5 (25) | 0 |
| NTS positive | 24 (82.7) | 17 (85) | 7 (77.7) |
| Saliva positive | 23 (79.3) | 16 (80) | 7 (77.7) |
| Gargle lavage (immediate) | 25 (86.2) | 18 (90) | 7 (77.7) |
| Gargle lavage (after 24-30 h) | 24 (82.7) | 17 (85) | 7 (77.7) |

SD, standard deviation; NTS, nasal-throat swab

that saline and VTM had comparable efficacy in conserving viral RNA, in iced specimens over seven days⁷. Further, the WHO guidelines on sample collection mention that sterile normal saline can be used if VTM is not available⁸. Our study showed stability of viral RNA in the normal saline stored in an optimal environment for up to 24-30 h, indicating that the preservation of replication compatibility is not essential for the identification of viral RNA. Further, a major advantage of normal saline (over conventional VTM) is widespread availability and cost-effectiveness.

Mittal *et al*³ showed gargle lavage using saline as a viable alternative to conventional swab collection with excellent agreement between both the methods. However, these samples were processed immediately after collection and did not evaluate RNA stability in saline. Saito *et al*⁹ reported higher viral load of SARS-CoV-2 in the gargle sample as compared to

swab. Similarly, Guo *et al*⁵ identified that the positive testing rate of throat washing was higher than the nasopharyngeal swabs. In this study, the positive testing rates were similar between NTS and gargle lavage. A meta-analysis showed that significant proportion of SARS-CoV RNA (88.8-99.9%) was detected in the gargle lavage and saliva in a cell-free form¹⁰. For SARS-CoV-2, Ehre¹¹ has shown that airway epithelial cells release the virus in high quantity. Therefore, it is reasonable to believe that sufficient quantity of RNA can be present in the saliva and gargle lavage.

Obtaining gargle lavage and saliva samples was easy, quick, safe and acceptable. Only 1-2 ml of throat saliva or a gargled specimen with 5-7 ml of sterile normal saline needs to be collected into a sterile container. Since it can be self-collected, saliva or gargled samples circumvent the need for skilled health workers at collection centres.

Table II. Comparison of nasal-throat swab and saliva specimens for detection of SARS-CoV-2

| | NTS | | |
|------------------------|-----------------|-----------------|--------------|
| | Positive, n (%) | Negative, n (%) | Total, n (%) |
| Saliva | | | |
| Positive | 22 (75.8) | 1 (3.4) | 23 (79.3) |
| Negative | 2 (6.8) | 4 (13.7) | 6 (20.6) |
| Total | 24 (82.7) | 5 (17.2) | 29 |
| NTS, nasal-throat swab | | | |

Table III. Comparison of nasal-throat swab and gargle lavage specimens for detection of SARS-CoV-2

| | NTS | | |
|------------------------------|-----------------|-----------------|--------------|
| | Positive, n (%) | Negative, n (%) | Total, n (%) |
| Gargle lavage (fresh sample) | | | |
| Positive | 24 (82.7) | 1 (3.4) | 25 (86.2) |
| Negative | 0 | 4 (13.7) | 4 (13.7) |
| Total | 24 (82.7) | 5 (17.2) | 29 |
| NTS, nasal-throat swab | | | |

Table IV. Comparison of positivity rate between fresh and stored gargle lavage specimens to detect stability of SARS-CoV-2

| | Fresh gargle lavage sample (run within 4-6 h of sampling) | | |
|--|---|-----------------|--------------|
| | Positive, n (%) | Negative, n (%) | Total, n (%) |
| Stored gargle sample (run after 24-30 h of sampling) | | | |
| Positive | 24 (82.7) | 0 | 24 (82.7) |
| Negative | 1 | 4 | 5 (17.2) |
| Total | 25 (86.2) | 4 (13.7) | 29 |

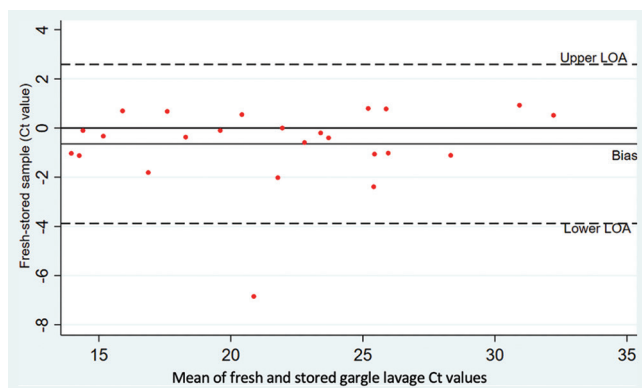


Fig. 1. Bland–Altman plot between fresh and stored gargle samples for *E* gene. Difference plot of cycle threshold (Ct) values of fresh gargle versus stored gargle samples in sample pairs for *E* gene target. Stability of SARS-CoV-2 RNA in fresh gargle samples was comparable to the stored gargle samples. However, Ct values of the stored gargle samples were higher than that of the fresh sample, as indicated by the line of mean difference (bias of -0.64 for *E* gene). The dotted lines represent upper and lower limits of agreement (LOA).

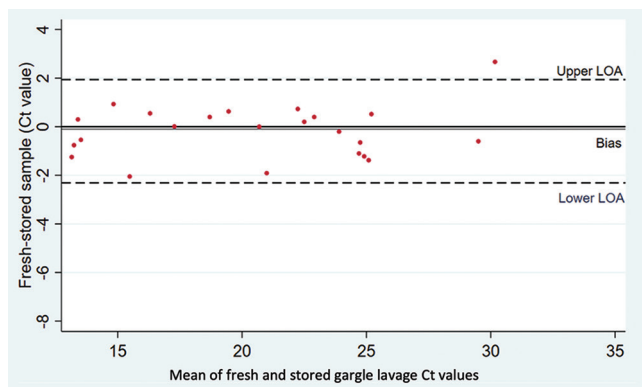


Fig. 3. Bland–Altman plot between fresh and stored gargle samples for *ORF* gene. Difference plot of cycle threshold (Ct) values of fresh gargle versus stored gargle samples in sample pairs for *ORF* gene targets. Stability of SARS-CoV-2 RNA in fresh gargle samples was comparable to the stored gargle samples. However, Ct values of the stored gargle samples were higher than that of the fresh sample, as indicated by the line of mean difference (bias of -0.19 for *ORF* gene). The dotted lines represent upper and lower limits of agreement (LOA).

The major limitation was the limited number of participants and a cross-sectional single-centre study.

To conclude, the stability of viral RNA in normal saline was found to be good and may be utilized as an appropriate medium for collection and transport of gargle lavage specimen for the detection SARS-CoV-2. Gargle and saliva specimen can provide a reasonable alternative to NTS collection. These methods would further reduce financial burden and workload of HCWs for sample collection and pave the way for simplified, inexpensive, effective and acceptable collection

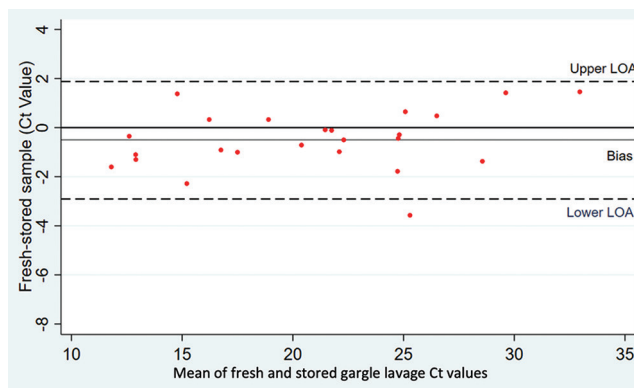


Fig. 2. Bland–Altman plot between fresh and stored gargle samples for *N* gene. Difference plot of cycle threshold (Ct) values of fresh gargle versus stored gargle samples in sample pairs for *N* gene targets. Stability of SARS-CoV-2 RNA in fresh gargle samples was comparable to the stored gargle samples. However, Ct values of the stored gargle samples were higher than that of the fresh sample, as indicated by the line of mean difference (bias of -0.51 for *N* gene). The dotted lines represent upper and lower limits of agreement (LOA).

procedures. Studies with longitudinally collected gargle lavage and saliva specimens from a larger number of SARS-CoV-2 suspects would help determine the more precise extent of association and selection of preferred sample for testing, duration of infectiveness and viral stability during storage.

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Conflicts of Interest: None.

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