



Correspondence

A preliminary evaluation of normal saline as an alternative to viral transport medium for COVID-19 diagnosis

Sir,

The current pandemic of coronavirus disease-2019 (COVID-19) across the globe has exhausted the healthcare facilities including the diagnostic and sample collection centres across the world. Real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is regarded as the gold standard for the diagnosis of COVID-19 from nasopharyngeal (NP) and/or oropharyngeal (OP) swab specimens, collected in viral transport medium (VTM)¹. The preferred and most commonly collected specimen for COVID-19 diagnosis is a NP swab placed in VTM^{2,3}. During the COVID-19 pandemic, every major country across the world conducted millions of tests, and there was a huge requirement of VTM, which is rather costly. The cost of diagnosis of COVID-19 including the consumables required for sample collection is putting the healthcare facilities under huge economic stress. VTMs are used for transport of viruses solely to keep them viable for isolation in tissue culture facilities and for diagnosis by nucleic acid test, *i.e.* RT-qPCR test^{4,5}. However, the requirement of viable viruses for diagnosis by RT-qPCR is not mandatory as observed in other studies where dry swabs kept at 37°C detected several respiratory viruses including influenza A, influenza B, respiratory syncytial virus and human metapneumovirus even after two weeks^{5,6}. Moreover, another recent comparative study on transport media has documented that even ethanol which is commonly available in hospitals are as good as VTM upto three days at 37°C for SARS-CoV-2 diagnosis by RT-PCR⁷. VTM is made up of two balanced salt solutions: Hanks A (20X containing 16.0 g NaCl, 0.80g KCl, 0.40 g MgSO₄.7H₂O, 0.28 g CaCl₂; dissolved in 80 ml distilled water, CaCl₂ is dissolved separately in 10 ml of double distilled water) and Hanks B (20X containing 0.12 g Na₂HPO₄.2H₂O, 0.12 g KH₂PO₄

and 2 g glucose in 100 ml distilled water) with sterile heat inactivated foetal bovine serum (FBS) 10 per cent, NaHCO₃ (3.5%), antibiotics (gentamycin sulphate 50 mg/ml and amphotericin B 250 µg/ml) and phenol red (0.5%) as pH indicator adjusting pH to 7.2 with 1N NaOH⁸. Usually, 3 ml of this solution is dispensed in sterile 15 ml conical centrifuge tubes or 1.5 ml in small 5 ml tubes. To circumvent the use of costly VTMs and easy availability of sterile normal saline (NS), the equivalence of NS as a medium for sample collection was evaluated in comparison to VTMs for the purpose of diagnosis of COVID-19 by real-time RT-PCR at Regional viral research and diagnostics laboratory, ICMR-RMRC, Dibrugarh, Assam.

In this preliminary study conducted with approval of the Institutional Ethical Committee of ICMR-RMRC, Dibrugarh, 35 known COVID-19 cases diagnosed by RT-qPCR admitted in Mohendra Mohan Choudhury Hospital, Guwahati and Assam Medical College and Hospital, Dibrugarh, Assam, and 100 COVID-19- negative cases (by RT-qPCR) in May-June 2020 were enrolled (after written informed consent) for the comparison of NS and VTM for equivalence (*i.e.* qualitative results as well as cycle threshold or Ct value variations) as well as stability of storage up to 10 days. NP samples were collected using a nylon flocked NP swab with breakpoint as per the protocol for COVID-19 diagnosis. Sterile NS (3 ml, used in intravenous infusion) was dispensed in 15 ml sterile conical centrifuge tubes for use in sample collection and no antibiotics were put in the NS solution. Commercial VTM [HiViral Transport Kit (A) MS2760A-50NO, HiMedia, Mumbai] was used for the comparison. After collection, both VTM and NS samples were divided into two aliquots with one kept at 4°C and the other at room temperature (RT ~25 to 32°C) and processed for RT-qPCR after 48 h (two days), 120 h (five days)

and 240 h (10 days). Overall, there were 510 numbers of measurements each for NS and VTM (three genes measured at 48, 120 and 240 h at 4°C and 48 and 120 h at RT). Measurements at 240 h at RT were not conducted due to non-availability of the assay with the same kit.

All samples were extracted using a commercial spin-column-based extraction kit (QIAamp Viral RNA Mini Kit, Qiagen, Hilden, Germany). RT-qPCR (TaqMan assay) was performed using an *in vitro* diagnostic (IVD) FDA emergency use authorization commercial COVID-19 RT-PCR diagnosis kit (TaqPath COVID-19 Combo Kit, ThermoFisher Scientific, Waltham, USA) in a real-time PCR machine (ABI 7500, ThermoFisher Scientific, Waltham, USA). The Ct-values of all three genes (*N*, *S* and *Orf1 ab*) used for the detection for SARS-CoV-2 were entered in Microsoft (MS) office Excel software (Microsoft office professional plus 2019, Microsoft Corporation, Redmond, USA). A Ct-value of up to 40 was set as the cut-off for analysis as mentioned in the kit.

The mean, median, standard deviation (SD) and standard error of mean (SEM) were calculated for all Ct-values across all parameters. Significance was measured using paired *t* test in MS office Excel for Ct-values for samples collected in VTM and NS. The sensitivity, specificity, positive-predictive value (PPV) and negative-predictive value (NPV) were calculated both for samples collected in NS and VTMs using a 2 × 2 table. To test the equivalence or proportional bias between NS versus VTM collected samples, a Bland–Altman scatter plot analysis was performed in SPSS statistical software version 26 (SPSS version 26, SPSS Inc., Chicago, USA). Analysis was done on Ct-value difference between NS collected and VTM collected samples at 4°C after 48 h storage. A one sample *t* test was performed before performing the Bland–Altman analysis, and further a linear regression analysis was done to assess proportional bias and any significance of mean difference between the tests.

A total of 135 samples were subjected to RT-qPCR for the diagnosis of SARS-CoV-2, of which 35 samples were from known positives by RT-qPCR done within three days before the current test. The Ct-values ranged between 18.2 and 39.0. Among the 35 known positives, 34 samples were SARS-CoV-2 positive in both in VTM and NS collected samples. One different sample each was not detected by both assays. One hundred RT-qPCR negative samples for SARS-CoV-2 were

also retested with samples collected in NS and VTM and all of these were found negative for SARS-CoV-2 in both VTM and NS collected samples.

The mean Ct-values for all three genes (cumulative Ct-values) of samples collected in NS was compared to samples collected in VTM. The mean cumulative Ct-values in NS and VTM samples were 27.76 and 28.46 at 48 h (4°C), 27.6 and 27.66 at 120 h (4°C), 27.75 and 28.29 at 240 h (4°C), 28.93 and 30.02 at 48 h (RT) and 27.58 and 27.53 at 120 h (RT), respectively which was significant in paired *t* test ($P < 0.05$). Furthermore, it was seen that samples stored at RT in NS after 48 h had lower Ct-value (*i.e.* 28.29 in NS compared to 30.02 in VTM), and after five days, the Ct-values variations were < 0.1 cycle difference between NS and VTM stored samples. The overall mean Ct-values in NS and VTM across all parameters were 27.92 for NS and 28.38 for VTM and mean Ct-value difference was 0.46 cycle. Fig. 1A and B displays the median Ct-values and SEM across different parameters at 4°C and at room temperature, respectively. A Bland–Altman scatter plot analysis was performed for the Ct-values obtained from NS versus VTM collected samples stored at 4°C after 48 h for 81 measurements (Ct-values of *N*, *S* and *Orf1ab* genes). Samples (either NS or VTM) with missing Ct-values for any genes were excluded from the analysis. The mean Ct-value difference between NS and VTM collected samples was first analyzed by a one-sample *t* test, which showed a significantly lower Ct-values for NS collected samples compared to VTM collected samples ($P = 0.016$, mean: -1.256 , 95% confidence interval: -2.27 to -0.236). Fig. 2 shows a Bland–Altman scatter plot which failed to reveal any proportional bias between the two tests on linear regression analysis ($P = 0.863$; mean = 0.018 , SE = 0.104).

The samples collected in NS and VTM both missed detection of one positive sample out of 35 known positive but both samples were different. This discrepancy may be due to the error in sample collection or may be due to not using the same nasal cavity of the patient for collecting both the samples. The sensitivity, specificity, PPV and NPV were 97.1, 100, 100 and 99 per cent, respectively, for both NS and VTM collected samples compared with earlier known values performed by RT-qPCR test for COVID-19 diagnosis (Table). Further, there was no significant change in Ct-values in both NS and VTM stored samples at 4°C and RT over time up to 10 and five

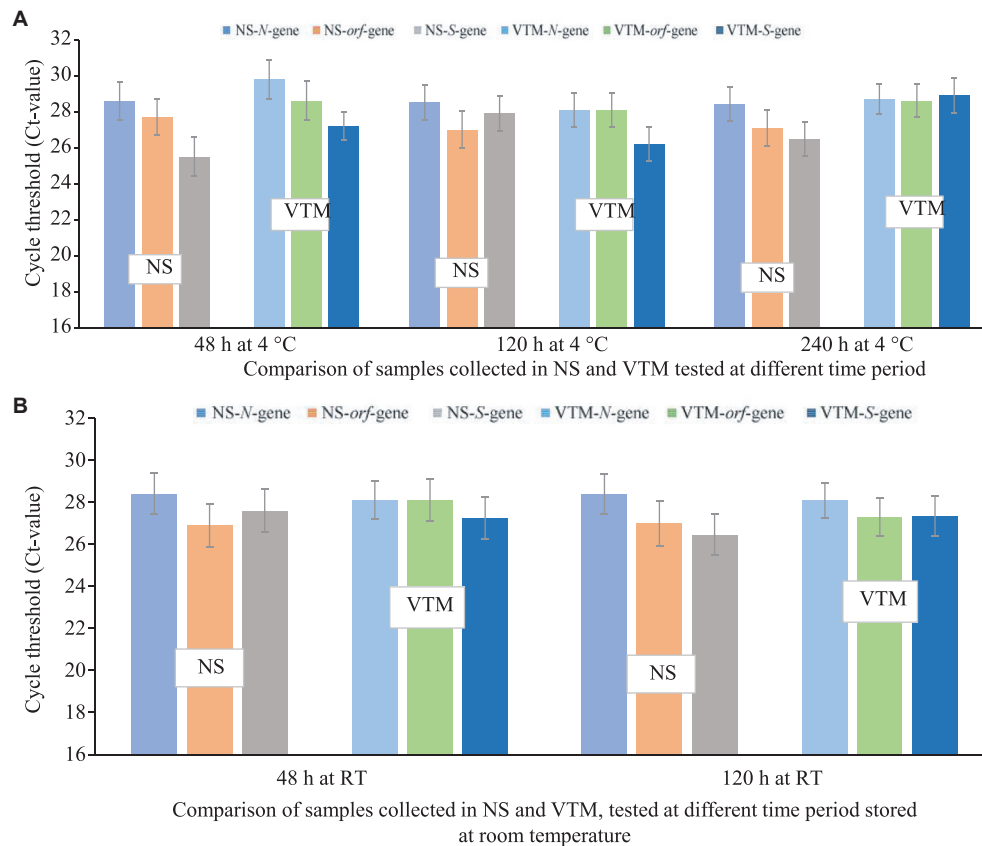


Fig. 1. (A) The median Ct-value of reverse transcription-quantitative polymerase chain reaction for SARS-CoV-2 genes (*N*, *Orf* and *S* genes) for samples collected in normal saline (NS) compared with viral transport medium (VTM) tested at different time intervals and stored at 4°C, while, **(B)** displays the median Ct-value of samples stored at room temperature (RT ~ 25°C) at two different time intervals (n=34).

Table. Sensitivity, specificity, positive-predictive value (PPV) and negative-predictive value (NPV) for samples collected in normal saline (NS) tested by real-time reverse transcription-polymerase chain reaction for SARS-CoV-2 (n=135)*

Conditions	True positive	True negative	Totals
Samples detected positive in NS	34 (a)	0 (b)	34 (a+b)
Samples detected negative in NS	1 (c)	100 (d)	101 (c+d)
Total	35 (a+c)	100 (b+d)	135 (a+b+c+d)
Sensitivity		97.1% (a/a+c)	
Specificity		100% (d/b+d)	
PPV		100% (a/a+b)	
NPV		99% (d/c+d)	

*VTM collected samples were taken as the gold standard for calculation of sensitivity, specificity, PPV and NPV of NS collected samples. Samples collected in VTM and NS both failed to detect one positive sample out of 35 known positive samples and both VTM and NS missed detection of a different positive sample. As such sensitivity of VTM was 97.1 per cent in comparison to NS collected samples.

days, respectively. Thus, with the data from limited numbers of samples, it may be guardedly stated that samples collected in NS are equally good as those collected in VTM for diagnosis of COVID-19 across different parameters for up to 10 days at 4°C or up to five days at RT.

Evaluation of buffered saline and NS for use in COVID-19 diagnosis has been made earlier and a 100 per cent qualitative agreement as well as Ct-value variation of <2 cycles for samples stored in NS, phosphate-buffered saline (PBS), minimal essential medium (MEM) and VTM over a seven-day

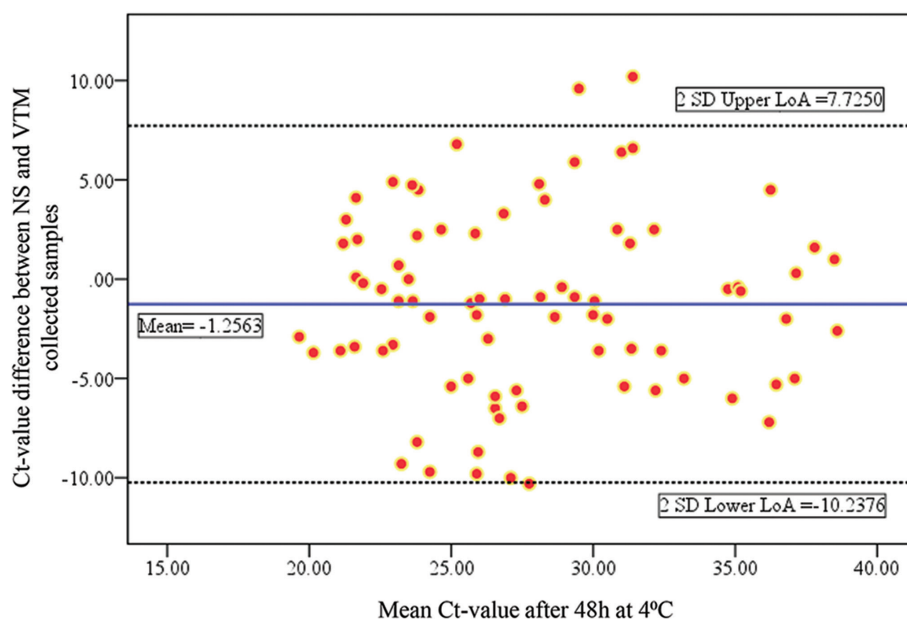


Fig. 2. Displays a scatter plot (Bland-Altman plot) to show the mean Ct-value of reverse transcription-quantitative polymerase chain reaction test and its difference between normal saline (NS) versus viral transport medium (VTM) collected samples tested after 48 h stored at 4°C to discern the equivalence bias between the two techniques. The two-standard deviation of mean Ct-values for upper and lower limits of agreement (LoA) are shown in dotted lines. The scatter plot displays the differences between the NS and VTM collected samples plotted against the average of both the techniques.

period was seen². A study from India has reported that NS gargle lavage may be a viable alternative to swabs for the collection of samples for COVID-19 diagnosis⁹. This study showed that gargle lavage samples collected in NS were comparable to swabs collected in VTM⁹. Radbel *et al*¹⁰ evaluated VTMs with PBS and found that PBS was also a suitable alternative as transport medium for SARS-CoV-2 diagnosis by RT-qPCR. The results of our study supported the fact that samples collected in NS were equally good as VTM for sample transport and storage for the diagnosis of SARS-CoV-2 by RT-qPCR. Sterile NS does not contain any known PCR inhibitors, while VTMs containing CaCl₂ have a potential to inhibit the PCR reaction as calcium ions, a known inhibitor of PCR can compete with co-factors of DNA Taq polymerase¹¹.

This preliminary study provides a basis for further studies on the use of NS as an alternative to the VTM for sample collection for the diagnosis of COVID-19 by RT-qPCR test including in remote regions where NS may be easily available, but VTMs may not be available. However, the limitation of using samples collected in NS is that such samples may not be suitable for virus isolation. Although the limited sample size of the study makes it difficult to provide significant advantage of using NS rather than VTM,

this preliminary study indicates that sterile NS may be equally good as VTM for the collection of samples for diagnosis of COVID-19 by RT-qPCR test and serves as basis for further research into this aspect.

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