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Original Research Article

Effect of delay in processing and storage temperature on diagnosis of SARS-CoV-2 by RTPCR testing

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ABSTRACT

Purpose: A large number of new molecular or virology laboratories have been established to increase the testing capacity for SARS-CoV-2. Due to heavy workload, there is delay in testing of samples. In order to avoid the negative effect of delayed testing on RTPCR results guidelines are issued from WHO and CDC to transport samples in cold chain. However, in pandemic situations the recommended guidelines for transport and storage conditions are often compromised. This study was conducted to evaluate the effect of sample storage conditions at different temperatures on the results of RT PCR test.

Methods: Total 275 samples were included in this study, among these 126 samples tested positive and 149 samples tested negative. All samples were aliquoted into two and the aliquotes stored in duplicate at 4 °C and room temperature. All aliquots stored at both the temperatures were tested by RTPCR every 24 hours up to 5 days.

Results: Diagnostic accuracy decreased from day1 to day 5 at both the storage temperatures i,e 4 °C and room temperature in comparison to the initial day results. Positivity decreased on an average of 9.02% at 4 °C and at 9.27% at room temperature per day. Among total 126 positive samples on an average false negative and failure of internal control at 4 °C and room temperature was 8.86%, 8.22% and 3.64%, and 4.12%, respectively. All the samples with CT value < 30 remained positive at both temperatures up to 5 days. Few samples with >30 CT value showed variable results i.e. positive, negative or internal control failure from day 1 (2nd day after sample collection) onwards.

Conclusions: There was no significant difference between RT PCR results of samples stored at 4 °C and room temperature up to 5 days of collection. However internal control failure was more in samples stored at room temperature. Therefore, samples received without cold chain also may be processed by RTPCR and should not be rejected.

1. Introduction

The current pandemic of severe acute respiratory disease is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The virus was first reported from Wuhan, China, in late 2019 which later spread worldwide [1]. With increasing number of infected people in a short period of time, the pandemic caused an enormous burden on the healthcare system including the diagnostic laboratories. Molecular test methods are considered the only reliable means of diagnosing an active case of COVID−19, particularly early in the course of infection, and are the only means of determining whether a patient is contagious to others or not. Real-time reverse transcription–quantitative polymerase chain reaction (RT-qPCR) helps not only to diagnose patients but also to determine the plan of treatment and to assess disease progression.

Abbreviations: RT-PCR, Real Time Polymerase Chain reaction; WHO, World health organization; CDC, Central disease control; CT Value, Cyclic Threshold; COVID-19, Corona virus infectious disease; SARS-CoV-2, severe acute respiratory syndrome virus-Corona Virus-2; VTM, Viral Transport Medium; RNA, Ribonucleic acid; NPV, Negative predictive value; PPV, Positive predictive value.

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Various techniques, media types, transport conditions have been developed to avoid false test results for SARS-CoV-2 by RTPCR [2]. Due to heavy load sometimes there may be delay in processing wherein samples need to be stored for long duration for further processing.

The recommended storage temperature for nasopharyngeal swabs is 4 °C during transport and it is recommended that RT-PCR be performed as soon as possible. It is stated that the sample can be stored at 4 °C or between 2 °C and 8 °C for up to 4 or 5 days if the swab is in viral transport medium (VTM) [3,4]. VTMs are used to preserve the viruses for PCR and other molecular techniques or viral culture techniques [5,6]. Many studies previously assessed the effect of environmental factors like temperature and humidity on the survival of SARS CoV-2 [7–9]. But limited information is available regarding effect of temperature on RTPCR testing.

This study was conducted to compare RTPCR results of Nasopharyngeal swabs collected for SARS-CoV-2 detection in Viral transport medium and stored at 4 °C and Room temperature conditions up to 5 days and to observe the effect of temperature and delay in processing on RT PCR results.

2. Materials and methods

This is a prospective case control observational study, conducted in department of clinical virology, SVIMS, hospital from 01 to 06–2021 to 01-09-2021. The study was approved by institutional ethical committee with IEC No. 1196. A total of 300 nasopharyngeal samples were prospectively collected and processed for RTPCR testing within 3 hours of sample receiving.

Out of 300 samples 25 samples were excluded because of internal control failure (on day zero) and insufficient quantity for five days of testing. Total 275 samples were aliquoted into 2 two vials containing equal volume of VTM solution and stored at 4 °C and room temperature (20–22 °C). RNA from samples was extracted by automated RNA extraction system (Hi-Media insta96) according to manufacturer instruction. All samples (samples from 4 °C and Room temperature) were tested every 24 hours for 5 days by RTPCR with the SARS- CoV-2 detection kit (Genes 2 viral detect kit) according to the Manufacturer's instructions. Primers of the kit targeted three viral genes: E gene, RdRp gene and N gene of SARS-CoV-2 and one housekeeping gene (RNase P). All RTPCR testing were done by using Applied Biosystems AutoQuant studio-5 (96) platform. As per kit protocol CT value of 37 was considered as cut off for differentiating between positive and negative results.

Statistical analysis: All data were arranged in excel spread sheets; percentages and histogram were generated by excel software. Mean, standard deviation, paired t-test and Sensitivity, Specificity, Positive predictive value (PPV), Negative predictive value (NPV), Diagnostic accuracy were calculated by open Epi online statistical tool.

3. Results

Total of 275 samples were tested by RTPCR, among these 126 tested positive (45.82%) and 149 (54.18%) tested negative. Out of 126 positive samples, 43 (34.13%) samples had CT value < 20, 37 samples had CT value between 21 to 25, 31 samples had CT value between 26 to 30 and 15 samples had Ct value > 30.

Taking the initial day (Day 0) results as reference, the performance of the test was evaluated for 4°C and room temperature storage. As shown in Table 1 sensitivity, NPV, and diagnostic accuracy decreased at both the temperatures however the specificity and PPV were well maintained on throughout 5 days and were comparable to the initial day results (Table 1).

Of the 126 positive samples, 84 (66.66%) samples stored at 4 °C and 80 samples stored at room temperature tested positive on all five days. Percentage positivity decreased each day However there was no significant difference in positivity of samples at two temperatures with decrease in positivity at an average of 9.02% per day as shown in Fig. 1.

Table 1
Comparison between 4 °C and Room temperature in all five days of testing.

Days & Temp	Sensitivity	Specificity	PPV	NPV	Diagnostics accuracy
Day 1					
4 °C	98.36%	100%	100%	99.26%	99.26%
RT	98.39%	100%	100%	98.66%	99.26%
Day 2					
4 °C	90.83%	98.65%	98.20%	92.99%	95.15%
RT	83.19%	91.72%	89.19%	86.93%	87.88%
Day 3					
4 °C	82.64%	95.92%	94.34%	87.04%	89.93%
RT	91.58%	91.67%	87.88%	94.29%	91.63%
Day 4					
4 °C	79.20%	100%	100%	90.51%	90.51%
RT	79.67%	99.33%	98.99%	85.55%	90.44%
Day 5					
4 °C	75.63%	100%	100%	83.71%	89.18%
RT	75.83%	93.92%	91%	82.74%	85.82%

Note: RT: Room Temperature, 4 °C: 4 degree temperature.

Table 2
Types of Errors among 4 °C and Room Temperature with initial results. Very major error: Positives become negative, Major error: Negatives become positive, minor error: positive/negative to repeat sample due to internal control failure and/or single target gene amplified.

4 °C Temperature					
Type of error	**DAY-1**	**DAY-2**	**DAY-3**	**DAY-4**	**DAY-5**
Very major error	2	11	21	26	29
Major error	0	2	6	0	0
Minor error	4	7	7	1	7
Room temparature					
Type of error	**DAY-1**	**DAY-2**	**DAY-3**	**DAY-4**	**DAY-5**
Very major error	2	20	17	25	29
Major error	0	12	12	1	9
Minor error	4	11	13	3	7

Similarly, the false negative and internal control failure rate per day was similar at both the temperatures with an average of 8.5 and 3.8% respectively. Overall discordant results (initially negative testing positive and initially positive testing negative) percentage was about 12.4% (Data not shown) (see Fig. 3).

CT values were analyzed on each day for both temperatures. The mean CT values of all positives increased at both temperatures each day. All the samples with CT value < 30 remained positive at both temperatures up to 5 days. The PCR results of the samples with original CT value more than 30 changed on storage at both the temperatures. Among negative samples, average RNase P gene CT value in initial sample was 24.4 (Day 0). The difference in the internal control CT value was only of 1–2 cycle from day 1 to day 5 between 4 °C and room temperature storage. The failure of internal control was more at room temperature with 40 (includes all 5 days) samples in comparison to failure of only 25 samples at 4 °C. Internal control failure started from day 3 onwards.

The difference in results acquired on 5 days in comparison to initial day (Day zero) were considered as errors and were defined as: Very major error- Positives becoming negative, Major error- Negatives becoming positive, Minor error: positive/negative sample had internal control failure and/or single target gene amplified (Table 2).

Very major error results increased with each day at both temperatures however there was no significant difference in errors at samples stored at the two temperatures (Fig. 2). Very major errors were common in samples with CT value more than 30 and very few in samples with CT value less than 20. In major error results, observed 28 and above CT values only. Up to 5 days the difference in CT value of the specific SARSCOV−2 genes was not significant for samples stored at two different temperatures, however average internal control failure was significantly more at room temperature as compared to 4 °C (p value < 0.00001).

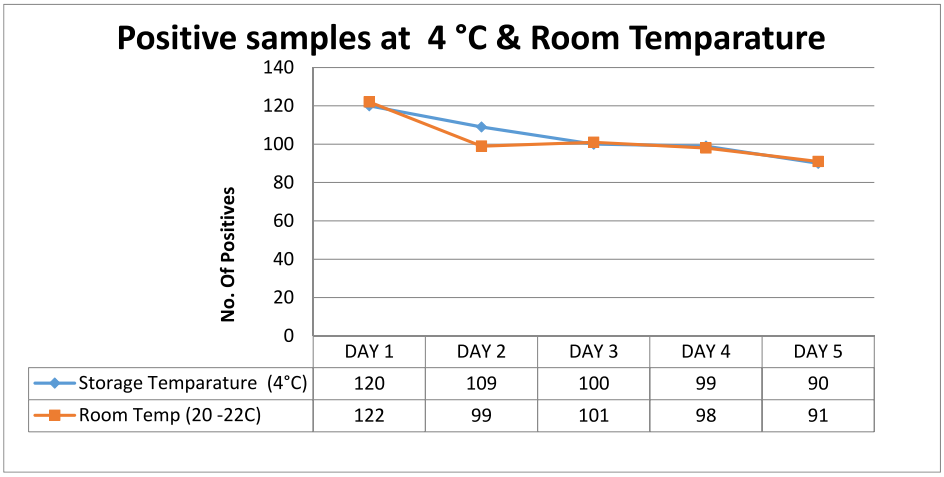

Fig. 1. Diagrammatic representation of decreasing no. of positive results among subsequent days at both temperatures compare to initial true results.

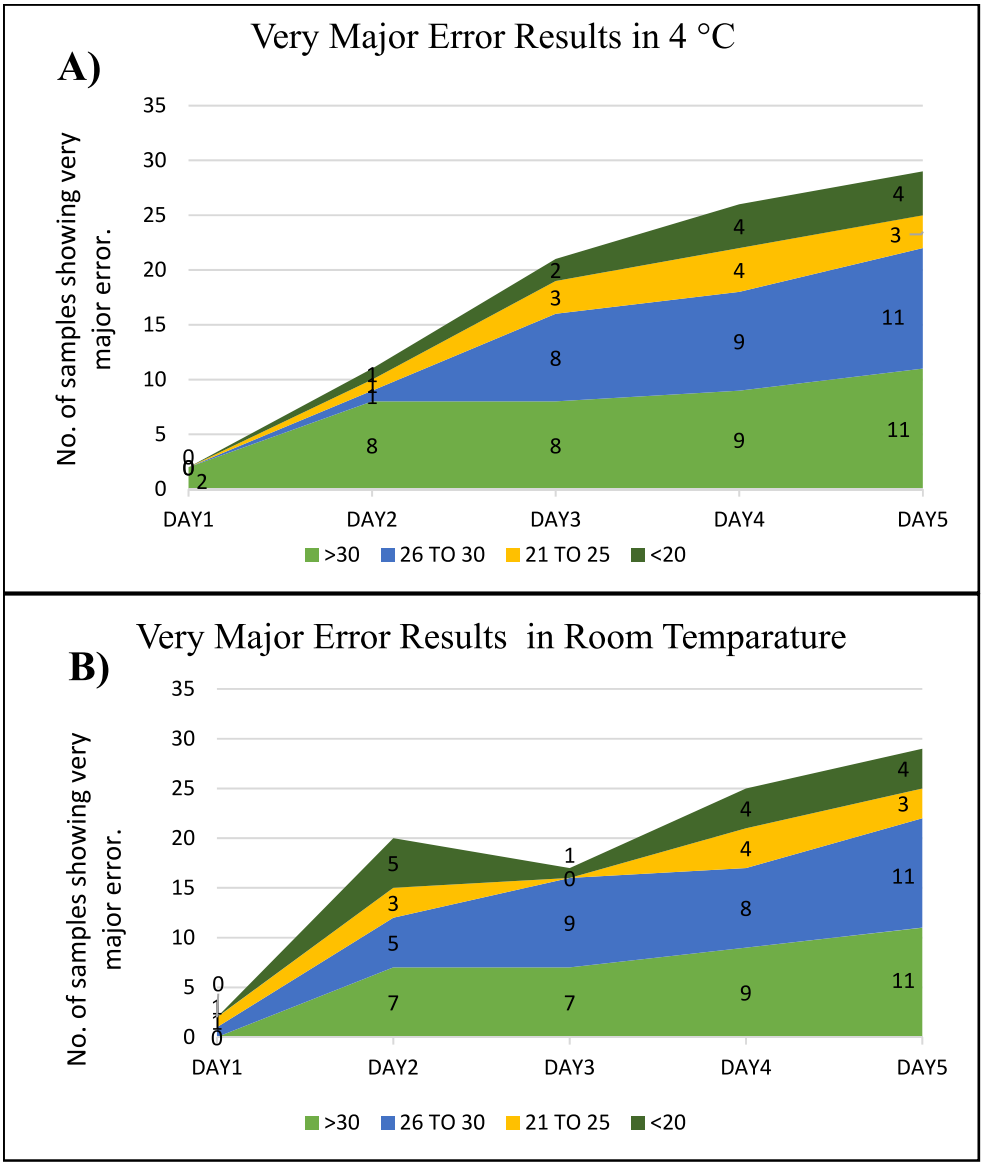

Fig. 2. A & B representing Ct Value differences in Very major error samples (False negative results) from Initial true positive results in subsequent days with both storage temperatures.

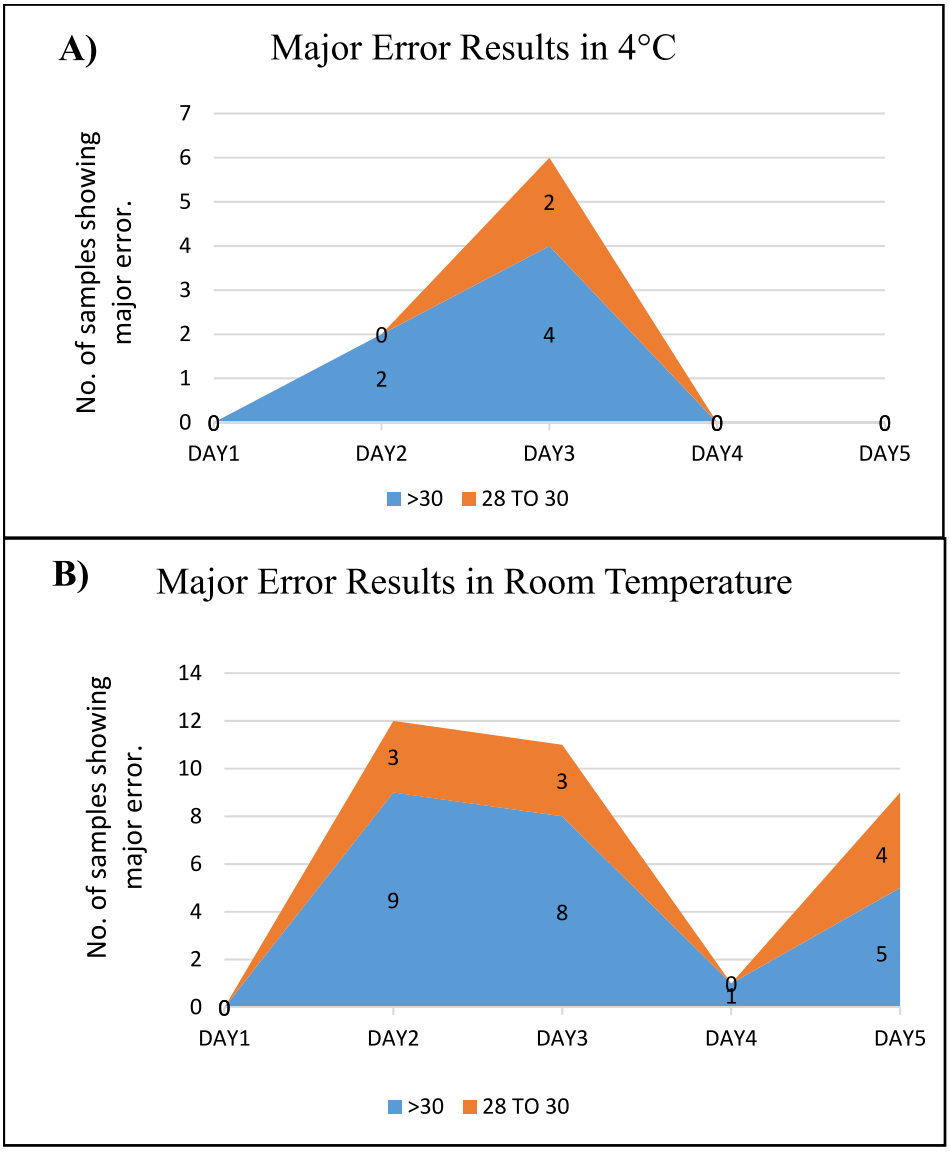

Fig. 3. A & B Representing Ct Values of False Positive Samples compared to initial true negatives results at both storage Temperatures.

4. Discussion

The World Health Organization (WHO) and CDC recommend cooled storage (2–8 °C) and transport of respiratory specimens in a specific viral transport medium (VTM) up to 5 days for accurate results by molecular testing.

SARS COV-2 RNA has been successfully amplified from viral transport media stored at room temperature for up to 14 days. Amy A. Rogers et al. (3) reported >2 CT value increase over 14 days at room temperature and under refrigerated storage conditions. They also reported that the increasing linear trend of Ct values in refrigerated samples over time had no effect on the interpretation of the results of the positive samples. In our study we have noticed that there was change in interpretation of results for some of the samples. This change in result was more for samples stored at room temperature. In few samples with >30 CT value samples stored at refrigerated conditions also there was change of results from positive to negative. This is in contradiction to the study by Amy A. Rogers et al. who reported that the increasing linear trend of CT values in refrigerated samples over time had no effect on the interpretation of the results of the positive samples [2]. The quality of the transport media or technical errors may be the reason for these contradictory results.

In a study by Yilmaz et al. [10], storage of samples at room temperature or refrigerated conditions did not affect significantly the CT value for the first 3 days, however fourth day onwards in samples stored at room temperature the CT values started to increase. They reported that all positives had increase in their mean CT values at both the temperatures [10]. In our study also the mean CT value of all the samples increased at both the temperatures however there was no significant difference in the mean increase of the CT values at the two storage temperatures.

Some of the samples initially positive, turned negative on subsequent testing or internal control could not be detected at both temperatures. Presence of amplification inhibitors, organisms in quantity below the detection level of the assay and inappropriate collection, transportation, and improper handling or processing, variability in virus shedding, sample collection too early and low analytic sensitivity of the kit can be various reasons for a false negative sample. Delay in sample processing can sometimes lead to overgrowth of the bacteria or fungal contaminants thus inhibiting the PCR. False negative result due to delay in processing can have major implications on the spread of infection and thus can pose great challenge to control of the current pandemic. A similar study conducted on four common viruses with different properties

investigated, different types of swabs and transport media and different storage conditions. In that study all the four viruses namely Influenza virus, enterovirus, herpes simplex virus and adenovirus studied could be detected at 4 °C as well as 22 °C up to 7 days by PCR [6]. In corroboration to that study our results matched at both the temperatures however there was rise of CT value, false negative results and failure to detect internal control with longer periods of storage.

No significant differences were observed in the Δ Cq-values over time which is Consistent with a study from Austria wherein viral stability was tested through long, non-cooled transports or sample storages. Even the initial viral load did not have any significant effect on the ΔCq and RNA stability either which is in corroboration with their study. No differences could be observed in the detection of the two target genes E and Orf in specimens with different viral loads at both the temperatures. The Austria study reported that non-cooled samples and compromised storage conditions did not affect the viral stability and can be used for increasing the testing capacity and mass screening of samples for SARS COV-2 [11]. Another study from turkey in 2020 with limited sample size, concluded that whenever sample transportation or storage at cold chain conditions becomes a limiting factor for the pandemic laboratory, keeping samples at ambient temperature enables much more testing [12].

Basso D et al [12], reported a slight CT increase only within the first 48 h, suggesting a potential decay of the viral molecular signal. They also suggested high false negative rates in asymptomatic patients, and fluctuating rRT-PCR results in COVID-19 hospitalized patients could be due to technical issues of sample processing. They also reported that temperature and time of storage of nasopharyngeal swabs for SARS-CoV-2 testing did not affect the reproducibility of the RTPCR results significantly and emphasized proper nasopharyngeal swab sampling for avoiding false negative results particularly the low viral load samples. In our study it was observed that the various types of errors were common in samples which had higher initial CT value (>30) and as the CT value decreased the errors also decreased. Similarly, it was also observed that although the performance of the kit decreased from initial to the day 5, however it did not vary significantly at the two storage temperatures.

It was observed that some of the samples had negative on initial day tested positive on subsequent testing. This could be due to technical errors, therefore, retesting of samples whenever there is strong clinical suspicion may give better results.

Basso D et al, [12] concluded that prior nucleic acid extraction before storage maximizes the RNA preservation of the sample. They also observed that samples with low viral load and CT values above 33 may yield unreliable results in repeated tests during storage [12]. In our study samples with CT values more than 30 became negative or inconclusive after 1st day of testing. However, we stored samples without nucleic acid extraction similar to routine situations in Indian settings.

In a study by Yilmaz et al. [10] majority of the samples stored at 4 °C had consistent CT values over 5 days, however CT values increased by 1–4 amplification cycles for most of the samples stored at room temperature over 5 days. In our study though the CT values increased at regular intervals over 5 days there was no significant difference between the increase of the CT values at room temperature or refrigerated conditions. Yilmaz et al. reported that positive samples with CT value less than 25 can be stored for at least five days at both 4 °C and room temperature without affecting the positivity [10]. Different studies suggested that, transport and storage of nasopharyngeal swabs or oropharyngeal swabs in viral transport media at room temperature upto 48 hours after collection for RTPCR is reliable. However, storage for longer period refrigeration of sample is preferable upto 5 days without affecting the reproducibility of RTPCR results [10,12]. In our study there was no significant difference in results of samples stored at the two temperatures for the SARSCOV-2 specific genes for 5 days, however internal failures do occur in samples on longer storage particularly if stored at room temperature. In our study diagnostics accuracy in comparison to initial day results of the test decreased by 10% on day1 to day 5 at 4 °C. Whereas at room temperature it decreased by 13.44% from day 1 to day 5.

The false-negative (FN) rates of SARS-CoV-2 from respiratory tract samples vary from 1 to 30% [13,14]. The World Health Organization (WHO) recommends repeat testing (including sampling of the lower respiratory tract) in symptomatic individuals of COVID-19. Several studies suggested repeat testing after an interval of 1–6 days following the first negative test [13–18]. In our study some samples negative on day zero tested positive on subsequent testing, however repeat testing of all the samples is not possible in a pandemic situation, therefore it is recommended that whenever possible repeat testing with the initial sample in symptomatic patients may be very useful and the overall performance of the RTPCR test can be improved. Kanji JM recommended to interpret the COVID-19 molecular test in the overall context of clinical scenario [15,19], and repeat testing to be done wherever there is high index of clinical suspicion.

5. Conclusion

The storage temperature does not significantly affect the sample results for SARSCOV-2 specific genes at 4 °C and Room temperature up to 5 days of collection. However internal control failure occurs more frequently in samples stored at room temperature than refrigerated samples. Despite of all technical expertise discordant results do occur so repeat testing in clinically suspected patients is recommended. The performance of RTPCR is best when the samples are tested at the earliest possible.

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Author contributions

Conception and design of study: Srikar A.
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Revising the manuscript critically for important intellectual content: Usha K.
Approval of the version of the manuscript to be published (the names of all authors must be listed): Usha K.

Declaration of competing interest

We declare that they are no known conflict of interest.

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