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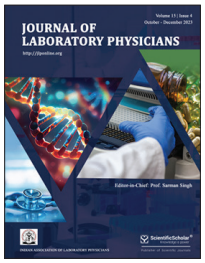

Original Article

Effects of storage temperature on throat swabs preserved in different transport media for detection of SARS-CoV-2 by reverse transcriptase polymerase chain reaction

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ABSTRACT

Objectives: The coronavirus disease 2019 (COVID-19) pandemic resulted in an increased need for molecular diagnostic testing. Delay in the specimen processing and storage of samples in laboratories leads to degradation of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral RNA. Inactivation transport medium (ITM) contains chaotropic agents that inactivate the virus and stabilize SARS-CoV-2 RNA for a longer duration, even at room temperature. The effect of different temperatures and duration of storage of samples in viral transport media (VTM) and ITM for detection of SARS-CoV-2 RNA was assessed.

Materials and Methods: Samples from COVID-19 patients were aliquoted in ITM and VTM and kept at ambient temperature, 37°C and 45°C. SARS-CoV-2 viral RNA was extracted. Multiplex real-time polymerase chain reaction was done on days 0, 1, 3, and 5, and cycle threshold (Ct) values were noted.

Statistical Analysis: Data were analyzed using the Statistical Package for the Social Sciences version 26.0. Linear variables were summarized as mean and standard deviations. One-way analysis of variance test with *post hoc* Tukey honestly significant difference was used to compare mean value between different loops and for pair-wise comparison. $P < 0.05$ was taken as significant.

Results: The mean Ct values of both the Orf and E genes of the samples in VTM and ITM were stable across all temperature conditions on day 1. On day 5, the increase in Ct values for both E and Orf genes were significantly higher for VTM than ITM at ambient temperature, 37°C and 45°C. Ribonuclease P failure was significantly higher for VTM than ITM at ambient temperature and 37°C on day 3 and at all temperatures on day 5.

Conclusions: ITM is a valuable transport media that can preserve SARS-CoV-2 for up to 5 days at ambient temperature and 37°C. As it renders the samples non-infectious, thus reducing the potential of biohazard events, this transport medium can be used effectively for the collection and transportation of SARS-CoV-2 samples, especially from remote or isolated healthcare facilities.

Keywords: ITM, Ribonucleic acid (RNA), severe acute respiratory syndrome coronavirus 2, Viral transport media

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by a newly identified virus – severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The novel disease, which began in Wuhan,

China, in December 2019, was declared a pandemic by the World Health Organization on March 11, 2020.[1] In India, the first case was reported in the state of Kerala on January 30, 2020.[2] Subsequently, the country witnessed a drastic rise in the number of cases across all states and union territories.

Various methods have been developed for the diagnosis of COVID-19, including the detection of viral nucleic acids and antigens. Detection of viral nucleic acids based on reverse transcription - quantitative polymerase chain reaction (RT-qPCR) is the most popular, sensitive, and specific method.[3]

The current guidelines of the Centers for Disease Control and Prevention recommend the collection of respiratory samples in a viral transport media (VTM) or saline followed by RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR) for testing the presence of SARS-CoV-2 in patients.[4,5] It is known to be the gold standard for laboratory detection of COVID-19 disease.[6] Oropharyngeal and nasopharyngeal swabs collected in VTM are the most widely used specimens. VTM is made up of a balanced salt solution, fetal bovine serum, antibiotics, and antifungals.

The virus remains in an infectious state in VTM, thus posing a significant biohazard both during transport and in the laboratory, and requires special safety procedures of packaging, transport, and treatment under Biosafety Level 2 conditions in the laboratory, which causes a considerable bottleneck in the processing workflow.[7] Therefore, alternative transport media that are safe and suitable for molecular diagnosis are needed.

In a resource-poor country like India, samples have to be collected from remote places, and their transportation takes several hours before they reach the testing laboratory. The major challenge is to maintain the cold chain and proper packing to prevent the risk of infection as the virus remains viable in VTM. VTM is a suitable medium for virus culture, but in situations where only molecular diagnosis is required, a need is felt for a transport media that does not require a cold chain, inactivates the virus, and keeps the nucleic acid intact, making it safe to handle.

Some studies have shown that lysis buffers of nucleic acid extraction kits have the potential to be used as RNA stabilization agents at adverse temperatures and over an extended period and have the potential to inactivate viruses completely.[8,9] There are studies on guanidine thiocyanate-based media that are suitable for virus inactivation and RT-qPCR detection assays.[10-12]

Inactivation transport medium (ITM) is an Indian Council of Medical Research (ICMR) approved medium that contains chaotropic agents, such as guanidine thiocyanate, ethanol and chelating agent, and ethylenediamine tetraacetic acid that allows transport of specimens without the need for cooling and improves the biosafety profile of the entire diagnostic workflow. ITM is a licensed specimen collection medium and swab validated by ICMR supplied by Promea Therapeutics, Hyderabad, Telangana, India (Licence Number: MFG/IVD/2020/000122). The present study aimed to evaluate ITM for molecular detection of SARS-CoV-2 and not for culture isolation of the virus.

MATERIALS AND METHODS

Sample collection and storage

This comparative observational study was conducted in the Department of Microbiology, SMS Medical College, Jaipur, over 2 months (September–October 2022). The study was approved by the Institutional Ethical Committee (560MC/EC/2022). A total of 100 samples were collected prospectively. Out of these, 80 samples were randomly collected from adult patients who were found positive for SARS-CoV-2 by RT-qPCR by routine testing in our laboratory. These patients were either hospitalized or quarantined after being diagnosed positive for SARS-CoV-2. In addition, 20 healthy volunteers were recruited as negative controls.

Two oropharyngeal samples were collected, one for ITM and the other for VTM (Vitromed Healthcare, Biotech Park, Jaipur, Rajasthan, India) for this study. Consent was taken at the time of sample collection. Samples were tested before storage (day 0) to obtain an initial cycle threshold (Ct) value. The RNA viral load was estimated based on Ct values.

Sample from each VTM and ITM was aliquoted in three sets of Eppendorf tubes (0.8 mL volume in each) and, after thorough vortexing incubated at ambient temperature, 37°C and 45°C. The samples were incubated at 45°C in an incubator. The temperature of 45°C was chosen as it is the average temperature in summer in this part of the country, and samples collected from various health facilities have to be transported from the sampling site to a COVID-19 RT-PCR laboratory. The average travel time for transporting these samples may take 6–8 hours.

The temperatures of incubators were monitored continuously by thermometers and recorded using data loggers. Samples for each temperature were processed by different researchers, and the findings were shared with the analyzing person separately without disclosing the duration and storage conditions of the aliquots using codes.

RNA extraction was done by an automated RNA extraction system (Perkins Elmer Chemagic 360) according to the manufacturer's instructions. All samples were tested on days 0, 1, 3, and 5 by COVID sure Multiplex Real-time RT-PCR detection kit as per the manufacturer's instructions. Primers of the kit were the E gene, Orf-1ab of SARS CoV-2, and one housekeeping gene (RNase P). Real-time PCR was performed on the Quantstudio5 (Applied Biosystems, Foster City, USA).

Ct value of 36 was considered as a cutoff for differentiating between positive and negative results as per kit protocol. Ct values of all the samples were noted and compared.

Statistical analysis

Data were entered into a Microsoft Excel sheet and were subjected to statistical analysis. Linear variables were summarized as mean and standard deviations and nominal/categorical variations as percentages (%).

One-way analysis of variance test with *post hoc* Tukey honestly significant difference was used to compare mean value between different loops and for pair-wise comparison. $P < 0.05$ was taken as significant. The Statistical Package for the Social Sciences 26.0 software was used for all statistical calculations.

RESULTS

A total of 100 samples were tested; 20 were from healthy volunteers recruited as negative controls, and 80 were positive samples. Out of 80 positive samples, 37 (46.25%) samples had a Ct value <25, 33 (41.25%) samples had a Ct value between 25 and 30, and 10 (12.5%) samples had a Ct value >30. The recovery of SARS-CoV-2 RNA from swabs stored in VTM and ITM on day 1, day 3, and day 5 at ambient temperature, 37°C, and 45°C were evaluated. All controls in both VTM and ITM tested negative across all temperature conditions on all days. Eighty samples that tested positive for SARS-CoV-2 were analyzed.

Orf gene

The mean Ct values of the Orf gene of the samples in VTM and ITM were stable across all temperature conditions on day 1. On day 3, Ct values significantly increased at 45°C as compared to ambient temperature and 37°C in VTM ($P = 0.012$). In ITM, Ct values were found to be significantly higher at 45°C as compared to 37°C ($P = 0.008$). However, in both the media on day 5, significantly higher Ct values were observed at 45°C as compared to ambient temperature and 37°C. (VTM; $P \leq 0.001$, ITM; $P \leq 0.001$) [Table 1].

E gene

The mean Ct values of the samples in VTM and ITM were stable across all temperature conditions on day 1. On day 3, Ct values significantly increased at 45°C as compared to ambient temperature and 37°C in both VTM and ITM (VTM; $P \leq 0.001$, ITM; $P \leq 0.001$).

On day 5, significantly higher Ct values were observed at 37°C and 45°C as compared to ambient temperature in VTM. ($P \leq 0.001$) However, in ITM, significantly higher Ct values were observed at 45°C as compared to ambient temperature and 37°C ($P \leq 0.001$) [Table 2].

Comparison of change in Ct values as an effect on storage temperature

To determine the stability of RNA in VTM and ITM at different storage temperatures, the change in Ct value was compared.

For the Orf gene on day 3, it was observed that the increase in Ct value for VTM was significantly higher than the increase in Ct values for ITM at ambient temperature ($P = 0.012$) but not found significant at 37°C ($P = 0.071$) and 45°C ($P = 0.109$).

On day 5, the increase in Ct values was significantly higher for VTM than ITM at ambient temperature ($P \leq 0.001$), 37°C ($P = 0.005$), and 45°C ($P = 0.038$).

For E gene on day 3, it was observed that the increase in Ct value for VTM was significantly higher than the increase in Ct values for ITM at ambient temperature ($P = 0.015$) and 37°C ($P = 0.049$) but not found significant at 45°C. ($P = 0.118$).On

Table 1: Variations in Ct values of Orf gene in VTM and ITM at different temperatures and storage duration.

Day	Temperature	*n*	VTM		*n*	ITM	
			Mean Ct value (±SD)	*P*<0.05 between*		Mean Ct value (±SD)	*P*<0.05 between*
0		80	23.68 (4.38)		80	23.66 (4.17)	
1	AT#		24.43 (4.14)	-		24.31 (4.07)	-
	37°C	80	24.49 (3.56)		80	23.81 (3.37)	
	45°C		25.40 (3.92)			24.95 (3.68)	
3	AT#		26.43 (4.04)	AT and 45°C 37°C and 45°C		25.69 (3.75)	37°C and 45°C
	37°C	80	26.39 (3.39)		80	25.29 (3.38)	
	45°C		28.05 (4.43)			27.11 (4.37)	
5	AT#		28.89 (3.51)	AT and 45°C		27.04 (3.56)	AT and 45°C
	37°C	80	29.70 (3.64)	37°C and 45°C	80	27.81 (3.39)	37°C and 45°C
	45°C		31.33 (4.35)			30.16 (4.17)	

*ANOVA: Analysis of variance *post hoc* Tukey honestly significant difference, #AT: Ambient temperature, VTM: Viral transport medium, Ct: Cycle threshold, SD: Standard deviation, ITM: Inactivation transport medium

Table 2: Variations in Ct values of the E gene in VTM and ITM in different temperatures and storage duration.

Day	Temperature	*n*	VTM		*n*	ITM	
			Mean Ct value (±SD)	*P*<0.05 between *		Mean Ct value (±SD)	*P*<0.05 between *
0		80	24.44 (4.15)		80	24.04 (4.44)	
1	AT#		25.45 (3.88)	-		25.11 (3.80)	-
	37°C	80	24.99 (3.71)		80	24.51 (3.36)	
	45°C		26.31 (3.92)			25.50 (3.86)	
3	AT#		27.09 (4.00)	AT and 45°C		25.95 (3.65)	AT and 45°C
	37°C	80	27.14 (3.19)	37°C and 45°C	80	25.46 (3.58)	37°C and 45°C
	45°C		29.18 (4.15)			27.95 (4.19)	
5	AT#		28.69 (3.40)	AT and 37°C		27.04 (3.80)	AT and 45°C
	37°C	80	30.16 (3.32)	AT and 45°C	80	28.23 (3.30)	37°C and 45°C
	45°C		33.30 (4.14)	37°C and 45°C		31.38 (4.10)	

*ANOVA: Analysis of variance *post hoc* Tukey honestly significant differenc. #AT: Ambient temperature, VTM: Viral transport medium, Ct: Cycle threshold, SD: Standard deviation, ITM: Inactivation transport medium

Table 3: Comparison of change in mean Ct values as an effect of storage temperature in two media for different genes.

Gene	Day	Temperature	*n*	VTM Mean (±SD)	ITM Mean (±SD)	*P*-value
ORF gene	1	AT#		0.75 (0.93)	0.65 (1.16)	0.55
		37°C	80	0.81 (4.14)	0.15 (4.18)	0.32
		45°C		1.73 (3.47)	1.29 (2.94)	0.39
	3	AT#		2.75 (1.93)	2.03 (1.69)	0.01
		37°C	80	2.71 (3.57)	1.63 (3.99)	0.07
		45°C		4.38 (3.60)	3.45 (3.66)	0.11
	5	AT#		5.21 (2.76)	3.38 (2.60)	<0.001
		37°C	80	6.03 (3.87)	4.15 (4.46)	0.005
		45°C		7.65 (3.58)	6.50 (3.36)	0.04
E gene	1	AT#		1.01 (1.59)	1.08 (1.89)	0.82
		37°C	80	0.55 (4.04)	0.48 (4.18)	0.91
		45°C		1.88 (3.00)	1.46 (3.19)	0.40
	3	AT#		2.65 (1.87)	1.91 (1.90)	0.01
		37°C	80	2.70 (3.71)	1.43 (4.40)	0.05
	5	AT#		4.25 (2.75)	3.00 (3.30)	0.01
		37°C	80	5.73 (3.60)	4.19 (4.41)	0.02
		45°C		8.86 (3.87)	7.34 (3.71)	0.01

#AT: Ambient temperature, VTM: Viral transport medium, Ct: Cycle threshold, SD: Standard deviation, ITM: Inactivation transport medium

day 5, the increase in Ct values was significantly higher for VTM than ITM at ambient temperature (P = 0.010), 37°C (P = 0.017), and 45°C (P = 0.012).

On comparing the two media in significantly more samples, viral RNA was not detected in VTM at 37°C and 45°C at day 5 for both Orf and E genes, respectively, as compared to ITM. (P = 0.036, P = 0.003 for Orf and P = 0.020, and P = 0.002 for E gene) [Table 3].

On day 3, internal control (RNaseP) failure was significantly higher for VTM than ITM at ambient temperature (P = 0.001) and 37°C (P = 0.006). On day 5, the internal control (RNaseP) failure was significantly higher for VTM than ITM at ambient temperature (<0.001), 37°C (<0.001), and 45°C (<0.001).

DISCUSSION

During the COVID-19 pandemic, to contain the fast-spreading disease, public health authorities across the globe resorted to a mass testing strategy.[13] The accuracy of a test result is dependent on the pre-analytical step, which involves sample collection, packaging, storage, and transportation. Transportation of samples is a challenge for health facilities that are located at significant distances away from RT-PCR laboratories. Other factors include failures in the different analytic stages or data analysis.[14-16]

In this research, ITM, a guanidine-based inactivation transport media that maintains the RNA quality of SARS-CoV-2 during transportation without cold chain, was evaluated. Guanidinium thiocyanate or guanidinium

hydrochloride has been shown to inactivate SARS-CoV-2 and can be used for RT-PCR applications.[17-19]

Here, we analyzed the real-time PCR diagnostic performance for SARS-CoV-2 on oropharyngeal swab samples stored in VTM and ITM at ambient temperature, 37°C and 45°C. The Ct values obtained for each sample were analyzed to assess the impact of the two media types and storage conditions.

In the present study, in samples stored in VTM and ITM at various temperatures, it was observed that mean Ct values for Orf and E genes were stable on day 1 for both media. On comparing the change of Ct values as an effect on storage temperature, it was observed that there was a significant increase in Ct values for the Orf gene at day 3 in VTM at ambient temperature compared to ITM. For the E gene at day 3, a significant increase of Ct values in samples stored in VTM was found at ambient temperature and 37°C as compared to samples stored in ITM. This suggests that viral RNA degradation by day 3 was more pronounced in VTM than in ITM. On day 5 of incubation, the Ct values significantly increased at all temperatures for both Orf and E genes in samples stored in VTM as compared to ITM, and this difference was statistically significant.

On day 3, RNase P failure was observed in a significantly higher number of samples stored in VTM at ambient temperature and at 37°C as compared to ITM, and this was more pronounced on day 5. On day 5 at 45^{0}C, RNase P failure was detected in 37/80 (46.25%) samples stored in VTM as compared to 15/80 (18.75%) samples stored in ITM. This indicates that the virus remained more stable in ITM even at this high temperature.

Perumal *et al*., in their study, reported samples to be stable for up to 48 h but there was a slight decline in viral RNA quantity at day 3 in samples stored in lysis buffer at ambient temperatures.[8] However, Wiraswati *et al*. reported no significant variation in Ct values up to 18 days in samples stored in a guanidine based inactivation transport medium at ambient temperature as well as in samples heated for 3 h at 40°C.[20] Yilmaz Gulec *et al*. observed that among positive samples stored in VTM, there was no change in Ct values up to 3 days in samples stored at ambient temperatures; however, from the 4th day onward in samples stored at room temperature, the Ct values started to increase.[21] Agaoglu *et al*. reported that samples collected in VTM and stored at ambient temperature remained positive for 6 days without any false-negative result. However, they observed that Ct values of the Orf gene significantly decreased over time which is contrary to the present study.[22] Anagoni *et al*. and Li *et al*., in positive samples stored in VTM, reported an increase in the mean Ct values each day.[23,24] A variable stability has been reported by different authors in both VTM and guanidine-based formulations. Specific buffer formulations or technical errors may be the reason for these variable results.

The degradation of nucleic acids can potentially compromise the precision of molecular detection techniques. As a result, it is crucial to prevent nucleic acid degradation by maintaining a cold chain for sample preservation or by incorporating viral stabilizing agents into the transport medium. Here, we observed that direct sampling into ITM resulted in a lower Ct value of SARS-CoV-2 by RT-qPCR compared to sampling into VTM over an extended period. This could be due to the beneficial effect of nucleic acid stabilization by ITM. VTM is the recommended medium for sample collection and transport as it can also help in isolating the virus, but ITM appears to be a good alternative when large numbers of samples have to be tested by molecular tests. It also helps in the easy and safe transport of samples and saves time for lysis of samples.

Limitations

As it was primarily a laboratory-based study, clinical data could not be included.

Ideally, samples should have been tested in duplicate/triplicate to get more accurate results, but we were unable to do so due to insufficient sample volume.

Viral cultures could not be done to assess the viral inactivation in ITM.

The samples were collected from patients admitted to a single center, so the possibility of selection bias may not be ruled out.

CONCLUSIONS

The present study concludes that ITM is compatible with the diagnostic molecular testing platform but not for the culture of the virus. It is a valuable transport media that can preserve SARS-CoV-2 RNA for 5 days at ambient temperature and 37°C. ITM renders the sample non- infectious, thus reducing the potential of biohazard events occurring, as most of the samples are in the form of RNA. Therefore, this transport medium can effectively facilitate the transportation and collection of SARS-CoV-2 specimens, particularly from remote or isolated areas in healthcare centers.

Ethical approval

The study was approved by Institutional Ethical Committee, approval number (560MC/EC/2022).

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Use of artificial intelligence (AI)-assisted technology for manuscript preparation

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript, and no images were manipulated using AI.

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