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Original Research Article

EVALUATION OF STABILITY OF RNA IN VIRAL TRANSPORT MEDIA FOR THE DETECTION OF SARS-COV-2 BY RT-PCR UNDER DIFFERENT TEMPERATURE CONDITIONS

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ABSTRACT

Background: Severe acute respiratory syndrome coronavirus-2 (SARS CoV-2) was first detected and described in December 2019 in the Wuhan City of China, as the causative agent of COVID 19 and has led to global spread causing universal pandemic. For diagnosis of COVID 19, collected sample in the viral transport media (VTM) need to be transported and stored maintaining cold chain. Sometimes it is difficult to maintain the transport and storing conditions of the collected samples for covid19 testing according to the guidelines in pandemics. The objective of this study was to determine stability of SARS-CoV-2 RNA in VTM at different temperatures.**Methods:** A total 92 remnant SARS CoV-2 positive and 8 SARS CoV-2 negative samples by RT PCR were selected for the study. Results of all samples which were stored at 3 different temperatures (4^0C, ambient temp & 37^0C) were analyzed. All samples were put in PCR on Day 1 (24 hrs), Day 3 (72 hrs) and Day 7 (168 hrs) .**Result:** Till day 7, all the SARS CoV-2 positive samples showed very good sensitivity at all three different (4^0C, ambient temp & 37^0C) temperatures. There was no loss of stability of the SARS CoV-2 RNA in positive samples except one sample on day 7 at 4^0C and 2 samples on day 3 & day 7 at ambient temp gave inconclusive results. **Conclusion:** In resource-limited situations such as pandemics, transportation and storage of nasopharyngeal / oropharyngeal swab samples in VTM at ambient temperature may be possible can be considered an option.

Keywords: Nasopharyngeal, Oropharyngeal Swab, Viral Transport Medium, SARS CoV-2, RT-PCR, Temperature

INTRODUCTION

The In Dec,2019, a novel coronavirus-Severe acute respiratory syndrome coronavirus (SARS CoV-2) was first identified in Wuhan, China as the causative agent of Covid-19 (coronavirus disease-19). Covid - 19 is a clinical syndrome that varies from asymptomatic cases to influenza like illness that can progress even to acute lung injury /acute respiratory distress syndrome with substantial mortality. (1)

Collection of respiratory samples in a viral transport media followed by RNA extraction and RTPCR is the recommended diagnostic line for testing the presence of SARS CoV-2 in patients. (2) It is known to be the most sensitive test for laboratory detection of COVID 19 disease.(3) Oropharyngeal swabs (OP), Nasal swabs, Nasopharyngeal swabs, lower respiratory tract aspirates, Sputum, Saliva, Bronchio alveolar lavage (BAL), Nasopharyngeal wash/aspirate, or Nasal aspirate are all considered important specimens for laboratory detection of COVID 19.(4) Oropharyngeal and Nasopharyngeal swabs collected in VTM are the most widely used specimens. VTM is made up of balanced salt solution, foetal bovine serum, antibiotics, and antifungals (5), is used to transport viruses while keeping them alive.

Any media used for sample collection and transportation for SARS CoV-2 detection by RT PCR should fulfill following requirements i.e. it

should be free from PCR inhibitors, no degradation of nucleic acid, compatibility with molecular diagnostics, can be easily used in field settings, and it should be cheaper and easily available.(6)

Furthermore, it has been recommended by various health organizations like the World Health Organization, the Centers for Disease Control and Prevention, the European Center for Disease Prevention and Control, that the accuracy of RT-PCR results depends largely upon proper specimen collection and storage.(5,7,8) Proper specimen storage means keeping the collected sample at the desired temperature of 4-8 degrees Celsius (maintaining cold chain) before processing for real time PCR).

Sometimes it is difficult to maintain transport, shipment, and storage conditions in line with the guidelines in a developing economy like India, particularly when there is a large number of sample collected during a pandemic.(9)

Packaging and transporting specimens to laboratories, which are sometimes hundreds of kilometres away, can be time-consuming and resource-intensive as well.(10) All of these problems result in diagnostic delays and diagnostic testing rationing.(11)

For PCR testing of SARS CoV2 replication capable viruses are not required but preserved nucleic acid is a must.(10) However for culture and further testing with the isolates, live and intact viruses are necessary for which we need to transport and store samples maintaining the required temperature cold chain.

Many environmental studies have quoted the effect of temperature on virus' viability, (12) but little is known about the impact of temperature on nucleic acid detection by PCR.

This study was aimed, to assess the effect of temperature on detection of SARS CoV-2 RNA by RT PCR by using respiratory specimens collected in VTM and stored at 4ºC, ambient and at higher temperature (37ºC). Latter two temperatures simulated field conditions in which specimens remain for hours.

MATERIAL AND METHODS

Sample collection and storage:

The present study was an observational analytical study conducted in Department of Microbiology, SMS Medical College Jaipur. We analyzed the data of Covid-19 positive patients from our records and selected 92 patient's nasopharyngeal swab and oropharyngeal swabs which were stored in VTM (Vitromed healthcare, Biotech park, Jaipur, Rajasthan) for this study. Patients' samples showing a wide range of different Ct values were selected. The patient samples showing CT value > 35, were excluded from the study. Patient's verbal consent was taken at the time of sample collection and rest the study was done on the collected samples, patients' details were not disclosed. This study was conducted in December 2020.

VTM samples were aliquotted in three sets of eppendorf tubes (1 ml volume in each) after thorough vortexing. Each set of VTM samples aliquots were kept at 4ºC, ambient temperature (23º-26ºC) and 37ºC (Incubator) respectively. Refrigerator and Incubator temperatures were monitored by thermometer continuously and regularly.

In addition to these samples, we took eight negative samples also, which were stored under same conditions as a control group.

First RT PCR test of these samples was already done on the day sample were received in the lab i.e. within 24 hrs of collection. After that, all the samples (stored at 4ºC, ambient temperature 23ºC to 26ºC) and 37 ºC and were tested on day 1, day 3, and day 7).We used automated extraction system (Perkins Elmer chemgic 360) for RNA extraction followed by RT PCR by TruPCR SARS–COV-2 detection kit as per manufacturer's instructions. We noted the Ct values of all the samples and compared them.

Extraction:

After thorough vortexing , followed by brief centrifugation of the VTM tubes , 300 µl of the sample was transferred to a 96 deep well processing plate to which 4 µl Poly (A) RNA , 10 µl of proteinase K, 300 µl lysis buffer along with 150µl magnetic beads and 900 µl of RNA binding buffer were already been added.

The beads/ RNA mixture was washed with washing buffer and eluates were obtained in elution buffer in the automated system (PerkinsElmerchemagic 360).

Real time PCR (TruPCR master mix)

The primers used in TruPCR RT PCR kit are designed to target E gene, N/RdRp and RnasePgenes. For PCR, 10 ul RNA and 15 ul PCR master mix solution containing 10 ul master mix

reagent, 0.35 ul Enzyme mix and 4.65 ul of primer probe mix. Cyclic conditions used as per the manufacturer's instructions were 50° C for 15 mins, 95°C for 5 mins, then 38 repeat cycles of 95°C for 5 secs, 60°C for 40 secs and 72°C for 15 secs, using BioradCFx platform.

Statistical analysis

The statistical software -SPSS version 20 was used for the analysis of the data and Microsoft word and Excel have been used to generate tables. Cohens' kappa coefficient was used to estimate agreement between RTPCR detection using VTM stored at different temperatures.

RESULTS

A total 92 remnant SARS CoV-2 positive and 8 SARS CoV-2 negative samples were processed for PCR. Results of all samples which were stored at 3 different temperatures (4^0C, ambient temp & 37^0C) were analyzed. Table:1

Table:1 Results of SARS CoV -2 positive specimens stored at 3 Different temperature conditions

Day		**+4^0 C**				**Ambient Temp. 23°- 26° C**				**+37^{0}C**			
	Available positive Sample	P*	N$	IR@	IN#	P*	N$	IR@	IN#	P*	N$	IR@	IN#
Day-1	92	92	0	0	0	92	0	0	0	92	0	0	0
Day-3	92	92	0	0	0	91	0	1	0	92	0	0	0
Day-7	92	91	0	1	0	91	0	1	0	92	0	0	0

** Novel coronavirus SARS CoV-2 RNA detected,* $ *Novel coronavirus SARS CoV-2 RNA NOT detected, @ (Inconclusive result) IR, # Invalid*

Table2: Result Interpretation

Result Interpretation	N/RDRP	E gene	RNaseP (Internal control)
Novel coronavirus SARS CoV-2 RNA detected	+	+	+/-
Novel coronavirus SARS CoV-2 RNA NOT detected	_	--	+
(Inconclusive result) IR	--	+	+/-
Invalid	_	_	_

All samples were put in PCR on Day 1 (24 hrs), Day 3 (72 hrs) and Day 7 (168 hrs) .One positive and one negative control were put in each PCR run and their results came within established QC ranges.

All 8 negative samples gave negative results on PCR for SARS CoV-2 with valid graph of RNAase P, demonstrating that the swabs were free of any kind of SARS Cov-2 contamination.

PCR results of the SARS CoV-2 positive specimens stored at 3 different temperature are shown in table 1.

Till day 7, all the SARS CoV-2 positive samples showed very good sensitivity at all three different (4^0C, ambient temp & 37^0C) temperatures. There was no loss of stability of the SARS CoV-2 positive

samples (only ±2 Ct value variation was noted) except one sample on day 7 at 4^0C and 2 samples on

day 3 & day 7 at ambient temp gave inconclusive result. To our surprise, all the samples which were stored in incubator at 37^0C give positive result without any false negativity.

After day 7 the available test materials was insufficient for further PCR assay.

Table 3 depicts a strong agreement between the positivity in RT PCR results of VTM stored at all temperatures.

Table:3

Tests	Cohen's kappa(k)	Association
Gold standard vs D1at -4˚C	1	Almost perfect agreement
Gold standard vs D3 at -4˚C	1	Almost perfect agreement
Gold standard vs D7 at -4˚C	0.936	Almost perfect agreement
Gold standard vs D1 at Ambient temperature	1	Almost perfect agreement
Gold standard vs D3 at Ambient temperature	0.936	Almost perfect agreement
Gold standard vs D7 at Ambient temperature	0.936	Almost perfect agreement
Gold standard vs D1 at 37˚C	1	Almost perfect agreement
Gold standard vs D3 at 37˚C	1	Almost perfect agreement
Gold standard vs D7 at 37˚C	1	Almost perfect agreement

DISCUSSION

Limited- stability studies are available in the literature for SARS-CoV-2 RNA. A study of SARS-CoV-2 in aerosols and on surfaces demonstrated that culturable SARS-CoV-2 was detectable in aerosols for upto 3h, upto 4h on copper, upto 24 h n cardboard, and upto 2 to 3 days on plastic and stainless steel (13) Given the persistence of SARS-CoV-2 in the environment, it is not surprising that RNA can be reliably amplified from vtm after relatively long storage times, even at room temperature.

In the present study, we analyzed the RT-PCR diagnostic performances for SARS-CoV-2 on nasopharyngeal and oropharyngeal swabs samples stored at three different temperatures (4^0C, ambient temp & 37^0C). These samples at three different temperatures were compared for detection of SARS-CoV-2 virus by RT-PCR. While many studies have investigated the effect of environmental factors such as temperature and humidity on virus survival, little is known about the effect of temperature on SARS-CoV-2 RNA detection by RT-PCR.(12)

In a few studies in this area related to other viruses (Adenovirus, HSV-2, HHV-8, Enterovirus), it has been reported that the storage of samples at ambient temperature did not affect the positive results.(**14)** In present study it was clearly shown that nasopharyngeal and oropharyngeal swabs samples stored at three different temperatures (4^0C,ambient temp & 37^0C) remain positive for SARS-CoV-2 virus by RT-PCR for at least 7 days .

It is recommended by CDC, WHO and many other health authorities that the samples should be stored at 2ºC -8ºC for up to 3-5 days and -70ºC if more than 5 days' storage is required (6,8,9). Maintaining such temperature during shipment and transportation, especially in pandemics /epidemics time is cumbersome and involves extra cost and many logistic issues. Many laboratories tend to reject the samples, if these guidelines are not followed.

In this study, it was noted that SARS CoV2 RNA was consistently detected in all three storage conditions for up to 7 days. This is in concordance with studies done by Agaoglu NB (9) and Julian Druce,(14) Former has shown that nasopharyngeal and oropharyngeal NP/OP samples in VTM kept at ambient temperature remain positive in SARS CoV-2 PCR test for 5 days and latter has reported that amplifiable results are obtained even if samples are kept at 25ºC and 37 ºC. Though he has also reported that after 3 days at 37ºC,there was gradual loss of nucleic acid integrity in four viruses (Influenza A, HSV-2, enterovirus and adenovirus 7), but in our study no such loss at 37ºC for SARS CoV-2 was observed.

Samples with wide range of Ct values were taken in the current study but all the samples even those with higher Ct values showed almost same Ct values despite incubating them at 37º C for 7 days.

Researchers have studied the temperature effect on SARS CoV-2 RNA for up to 25ºC but in tropical areas like Rajasthan, most of the times in a year the temperature remains more than 30ºC. This is the reason we kept one set of samples in the incubator 37ºC and analyzed the effect.

Taking into consideration the data of our study, we recommend that even if temperature conditions are not fulfilled, the samples can be analyzed and reported .

Limitations: Further studies are required to access the reproducibility of SARS-CoV2 RNA detection by RT-PCR in VTM samples in tropical climates with higher ambient temperature and humidity.

Recommendations: It is recommended that even if temperature conditions are not fulfilled during shipment, the samples for covid19 testing can be analyzed and reported with special remark regarding temperature at which samples were transported and/or stored.

CONCLUSION

During epidemics/pandemics, when sample storage/transportation at cold chain conditions becomes a limiting factor in enhancing testing, keeping samples at higher (ambient 25ºC/37ºC) temperature can enable much more testing

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