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Stability of MERS-CoV RNA on spin columns of RNA extraction kit at room temperature

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ABSTRACT

Middle East respiratory syndrome coronavirus (MERS-CoV) is an emerging virus causing a highly fatal respiratory disease in humans. Confirmation of MERS-CoV infection and molecular study on the virus may require transportation of samples to specialized laboratories. While freezing at −80 °C is the gold standard method for RNA preservation, maintaining the integrity of viral RNA during transport will require additional precautions and, as a result, increase transport costs. We aimed at testing the stability of MERS-CoV RNA on spin columns of RNA extraction kit at room temperature for 16 weeks. Respiratory samples spiked with stock culture of MERS-CoV were extracted and loaded on QIAamp Viral RNA Mini Kit spin columns and preserved at room temperature. Amount of viral RNA was evaluated periodically by real-time quantitative reverse-transcription polymerase chain reaction. Minimal changes in cycle threshold values over the study period were noted, suggesting stability of viral RNA by this preservation method.

1. Introduction

Coronaviruses are a large group of enveloped single-stranded, positive sense RNA viruses. Five of the 7 human coronaviruses belong to the genera *Betacoronavirus*, with severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV2 significant human pathogens (Sahin et al., 2020).

MERS-CoV is the causative virus of a highly lethal respiratory disease in humans, the Middle East respiratory syndrome. It was identified for the first time in the Kingdom of Saudi Arabia in 2012 with a documented fatality rate of 37% (WHO, 2020; Zaki et al., 2012).

Confirmation of suspected cases of MERS-CoV infection necessitates detection of unique sequences of virus RNA including real-time reverse-transcription polymerase chain reaction (rRT-PCR), with confirmation by nucleic acid sequencing when necessary according to the WHO algorithm for testing patients under investigation for MERS-CoV (WHO, 2018). For this reason, the transport of samples across experienced or more equipped laboratories, especially in developing and low-resource countries, may be essential for complete diagnosis and further study (WHO, 2018).

Infectious materials transported across countries or within the same country can present risks to both people and the environment. In order to minimize those risks, the transport and shipment of specimens should be subjected to certain regulations. These regulations are defined by the United Nations Committee of Experts on the Transport of Dangerous Goods and WHO guidelines concerning infectious substances transport, which aim to provide minimum requirements for the safe handling, packaging, and transport of infectious material (WHO, 2019).

Specimens should be stored at −80 °C and shipped on dry ice to preserve the virus for further testing. For long distance transportation, the combined use of dry ice and frozen gel icepacks may be required. Meeting such conditions would surely increase the cost of shipping samples (CDC, 2015).

Numerous studies have investigated the preservation of clinical samples for extended periods of time, but the quality of RNA is influenced by temperature changes, tissue processing protocols, and storage conditions. Physical or chemical methods are used for the preservation of RNA, and the selection of the appropriate method is mainly based on availability and cost (Mathay et al., 2012; Salehi and Najafi, 2014; Stevenson et al., 2015).

MERS-CoV (isolate HCoV-EMC/2012) showed environmental stability at relatively low temperature and humidity conditions (20 °C and 40% relative humidity), and a marked decline in viability was noticed at higher temperatures and humidity (Van Doremalen et al., 2013).

While RNA quality is influenced by storage conditions, we adapted the spin column technique used to extract and purify MERS-CoV RNA to preserve the nucleic acid in situ at ambient temperature, which will allow the storage or transport of extracted viral RNA for longer periods of time, up to several weeks if cold storage cannot be guaranteed, or will add too much to the cost, which is important especially-in low resource countries.

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Table 1
Sequence of primers and probe used in rRT-qPCR.

	Sequence
Primer_1_F	5′ AAT TTg ggg TCT ggg CTA gT 3′
Primer_1_R	5′ TAT ggg TgC TAA Tgg CAA CA 3′
Probe	FAM 5′ TTg ACA TAC AAA TCA AAC TgC ATg 3′ BBQ

F = forward; R = reverse; FAM = 6-carboxyfluorescein; BBQ = blackberry quencher.

2. Methods

2.1. Preparation of samples

Sputum and nasopharyngeal aspirate mix was spiked with stock culture of inactivated MERS-CoV of a known titer of 1×10^8 copies/mL that was kept in −80 °C and supplied by Prof. Ali Zaki (Ain Shams University, Cairo, Egypt). It was added stepwise, and real-time PCR was performed until obtaining a cycle threshold (Ct) value of 20–25. This concentration is based on an average expected for clinical samples (Min et al., 2016).

2.2. Viral RNA extraction

Samples were extracted using QIAamp Viral RNA Mini Kit (52904: Qiagen, Hilden, Germany) following manufacturer's guidelines (spin protocol) in which the samples were lysed and then loaded onto the QIAamp Mini spin columns. Total volume of the sample (stock virus spiked sample) used in extraction was 140 μL per column according to the kit instructions and the same for all spin columns used in the study. The RNA binds to the membrane, and contaminants were washed away efficiently in 2 steps using wash buffer AW1 followed by wash buffer AW2. The last elution step was postponed, and spin columns were stored in closed containers at room temperature (22–25 °C).

2.3. RNA elution and quantification

Baseline testing of 5 spin columns was performed, and viral RNA stability was evaluated periodically for 16 weeks. Every week, 5 spin columns that were preserved at room temperature were eluted with RNase-free buffer according to manufacturer's guidelines and analyzed by real-time quantitative RT-PCR (rRT-qPCR) to determine the Ct value of preserved RNA. Real-time RT-PCR was performed in Real-Time PCR machine StepOne (Applied Biosystems, Foster City, CA) using Verso 1-Step qRT-PCR Kit Plus ROX Vial (AB4100A: Thermo Fisher Scientific, South San Francisco, CA) according to the manufacturer's guidelines with 5 μg of RNA input per reaction. Primers and probe sets (Table 1) were designed to target the RNA-dependent RNA polymerase gene region using Applied Biosystems® Primer Express Software.

The following cycling parameters were used: at 50 °C for 30 min, then 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. Each run included a negative and positive control. Each sample was run in triplicate, and mean Ct value for each sample was recorded. Standard curve was constructed by plotting Ct values versus the logarithm of the MERS-CoV RNA amount, which showed a linear relationship with an R^2 of 0.995 and an efficiency of 91%.

3. Results

Real-time PCR results as a Ct value could be considered as a semi-quantitative viral load estimator in clinical samples. Fig. 1 shows the average Ct values recorded during the study period. Extracted MERS-CoV RNA on silica membrane of spin columns remained stable for 16 weeks when stored at room temperature. The average starting Ct value at week 0 was 18.524±0.84, reaching 20.25±1.82 at week 16 (Fig. 2), with a difference of approximately 1.73 between baseline Ct record and 16 weeks later at the end of the experiment.

4. Discussion

RNA viruses including coronaviruses exhibit higher mutation rates noted recently by the emergence of new viruses, including MERS-CoV in the Kingdom of Saudi Arabia and currently SARS-CoV-2 that was first detected in China and evolved into a global pandemic (Salata et al., 2019).

These emerging viruses are the focus of much research, and it is critical that transporting these potentially hazardous agents between reference laboratories for research purposes should be a careful balance between biosafety precautions and maintaining the quality of transported materials. This will add more to the effort and cost of ensuring safe delivery to the processing area.

Preservation of RNA has been widely reviewed; many methods are available, either preservation of the clinical specimen itself or pure extracted RNA, the gold standard method for preservation of RNA is freezing at −80 °C (Daum et al., 2011; Foss et al., 2016; Liu et al., 2015; Mathay et al., 2012).

Other methods for preserving RNA at room temperature are investigated: The Flinders Technology Associates (FTA) card and the RNASound card. Both are filter papers containing chemicals designed to preserve nucleic acids in clinical samples (Cardona-Ospina et al., 2019).

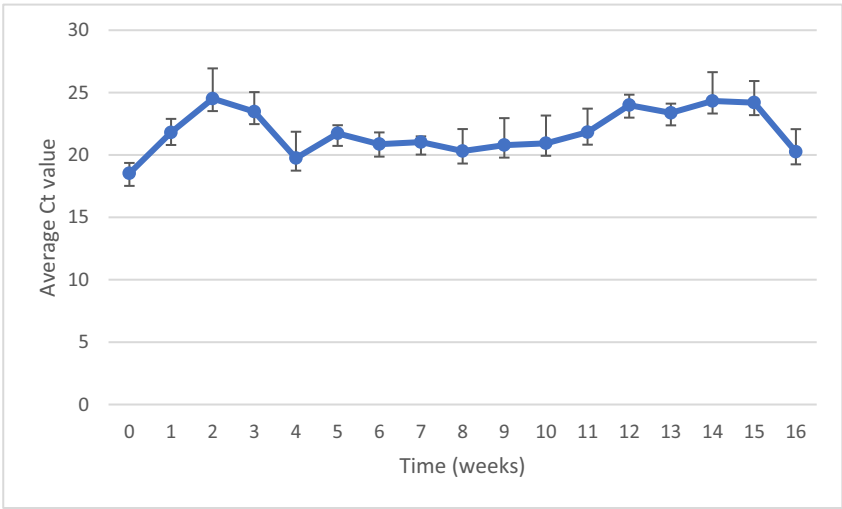

Fig. 1. Average Ct values recorded along study period.

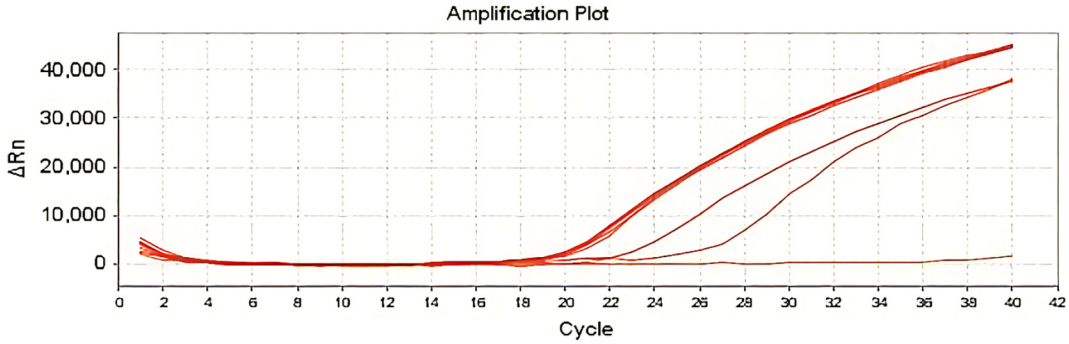

Fig. 2. Amplification plot of preserved MERS-CoV RNA.

FTA cards have been used mainly for the preservation of DNA; however, their use has been extended for the preservation of RNA, but according to the manufacturer's recommendations, they should be used immediately or kept frozen. Cardona-Ospina et al. (2019) reviewed the use of FTA cards for the preservation of viral RNA at different temperatures and different processing methods for a variety of RNA viruses (although not including MERS-CoV) and showed a wide variation in the length of time the viral RNA could be maintained.

Avian influenza virus RNA may be stored for up to 5 months at room temperature using FTA cards, while some arboviruses' (zika virus, chikungunya virus, dengue virus) RNA may be successfully recovered from FTA cards after 28 days. Similarly, the RNASound card effectively maintained influenza virus RNA for about 28 days at room temperature (Hall-Mendelin et al., 2017; Lau and Hurt, 2016).

Both FTA cards and RNASound cards carry the advantage of preserving clinical samples immediately, hence their appeal in field studies, but RNASound cards are sometimes preferred over FTA cards because viral RNA is easily eluted (Lau and Hurt, 2016).

Recently developed cellulose disks (UNEXP) saturated with a nucleic acid extraction buffer (UNEX) have successfully maintained norovirus at room temperature for up to 2 weeks (Cromeans et al., 2019).

In this study, silica membrane (of spin column extraction kit) was used to preserve MERS-CoV extracted RNA for up to 16 weeks at room temperature. The level of viral RNA remained stable during the preservation period, denoted by the almost stable Ct value recorded during real-time PCR (1.73 difference in mean Ct value over study period).

We used Ct value as an indicator of viral stability. Ct value was used to represent viral load of some respiratory viruses including MERS-CoV (Feikin et al., 2015; Fuller et al., 2013; Memish et al., 2014). The reaction conditions were fixed to minimize errors in Ct values not related to the template. It is estimated that a 1-unit difference in Ct value would be equivalent to approximately 2-fold change of concentration of virus copies. This is provided that RT-PCR is 100% efficient (Fuller et al., 2013).

For this study, experiment clinical samples were not available; however, spiked sputum and nasopharyngeal aspirate mixture was used to mimic the natural conditions from which the virus is extracted. We tested the stability of 1 concentration of RNA on silica membrane. Although stability of various RNA loads on different matrices is not related to concentration tested (Relova et al., 2018), we recommend future testing of stability of various viral RNA concentrations on spin columns. Carrier RNA added during the RNA extraction protocol improves binding of viral nucleic acids to the silica membrane even with few target molecules in the sample (Shaw et al., 2009).

Accumulated data in our laboratory indicate that viral RNA is stable in the silica membrane of Qiagen RNA extraction kit for variable lengths of time. We shipped norovirus RNA extracted from stool to the United States, and subsequent amplification and phylogenic analysis was successful. We also shipped RNA extracted from the sputum for the metapneumovirus diagnosis and successfully tested it in the Netherlands. Lastly, RNA of 2012 MERS-CoV from a patient's sputum was shipped to the Erasmus University Medical Center in the Netherlands. It was tested by RT-PCR and successfully sequenced for phylogenetic analysis (Zaki, 2012). The average time from extraction to final processing was approximately 3 weeks, and RNA was shipped at ambient temperature.

There are no documented reports on preservation of MERS-CoV RNA for extended periods of time; however Moscoso et al. and Manswr et al. preserved infectious bronchitis virus which is a member of the *Gammacoronavirus* genus for 36 days and 21 days, respectively, at room temperature using FTA cards (Manswr et al., 2018; Moscoso et al., 2005).

5. Conclusion

We are the first group to demonstrate the use of spin columns to preserve MERS-CoV RNA and show stability of the preserved RNA for up to 4 months. The spin column method for viral RNA preservation has many advantages over other methods; there are no need for special equipment other than that used to extract RNA and no need for special chemicals or storage conditions. It could be considered cost effective as some of the other methods of preserving viral RNA are usually followed by the use of an RNA extraction kit to ensure RNA purification (Lau and Hurt, 2016).

Further research is needed to investigate the use of this method for longer durations and applications on different types of viruses and even for higher temperature storage.

Declarations of interest

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Author contributions

A.Z. supervised and carried out experimental work. N.A. analyzed the data and wrote the first draft of the manuscript. All authors were involved in conception, design of the study, resources, manuscript revision, and approval of the final version of the manuscript.

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