Fekadu Alemu, Getachew Tesfaye Beyene, Ethiop Med J, 2022, Vol. 60, No. 2

ORIGINAL ARTICLE

EFFECT OF THERMAL INACTIVATION OF BIOLOGICAL SPECIMENS ON THE LIMIT OF DETECTION OF RT-PCR IN THE DIAGNOSIS OF SARS COV-2

Fekadu Alemu, MSc.[1$], Getachew Tesfaye Beyene, PhD.[1$*], Eyerus Selomon, BSc[1], Zewudu Selomon, BSc[1], Sinknesh Wolde, MSc[1], Desalegn Abeje Tefera, BSc[1], Dawit Hailu Alemayehu, MSc[1], Adane Mihret, PhD[1], Alemseged Abdissa, PhD[1], Andargachew Mulu, PhD[1]

ABSTRACT

Background: *Coronavirus disease 2019 (COVID-19) is characterized as a global pandemic by World Health Organization. For the safety of medical laboratory personnel and the environment, thermal inactivation of clinical samples is a common practice performed by most laboratories before nucleic acid extraction. However, there are conflicting reports in the literature regarding the effect of thermal inactivation on the analytical sensitivity of molecular assays.*

Objective*: To test the impact of thermal inactivation using alternative methods on the analytical sensitivity of respiratory samples.*

Method: *We compared the impact of thermal inactivation of biological samples after adding lysis buffer at 72^0C for 10 minutes by dry heat block and water bath on analytical sensitivity of severe acute respiratory syndrome coronavirus 2 (SARS-CoV2). Furthermore, we tested the effect of the thermal inactivation method at 56^0C using a water bath on the detection rate of SARS-CoV2.*

Result: *All samples tested positive in dry heat block was also tested positive in the water bath. We observed a similar viral detection rate of viral RNA at 56^0C for 15 min and 30 min, whereas inactivating samples at 56^0C on the water bath for 45 minutes drastically reduces the virus detection rate by 20%.*

Conclusion*: Water bath is not inferior to dry heat block to treat samples with lysis buffer, and can be used instead of dry heat block in district laboratories. However, the inactivation of samples at 56^0C over 30 minutes drastically reduces the virus detection rate. Hence, samples shall not be heat-treated before nucleic acid extraction.*

Keywords: *SARS-CoV2, COVID-19, heat inactivation, heat block, water bath*

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) is the virus responsible for the coronavirus disease 2019 (COVID-19) pandemic [1]. The World Health Organization (WHO) on March 11, 2020, has declared the COVID-19 outbreak a global pandemic [2], and then the demand for laboratory equipment, chemicals, and personal protective equipment has soared. In resources limited countries like Ethiopia, the availability of the right and properly functioning laboratory equipment is scarce. Given that the expansion of the pandemic to different regional states in the country is rapid, it is very difficult for district laboratories to avail all the necessary and standard equipment used for SARS-CoV2 diagnosis in this emergency time. Hence, the shortage of laboratory equipment and consumables needed for the extraction and detection of SARS-CoV-2 RNA in respiratory samples has forced many laboratories to find alternative approaches for sample preparation [3]. To protect medical laboratory personnel from infection, most laboratories inactivate the virus, causing COVID-19 in clinical samples before nucleic acid extraction and testing by applying high temperatures [4]. However, the outcome of applying different methods of heat inactivation on the detection of the viral RNA is not well explored and findings are controversial.

Therefore, this study aims to explore the impact of heat inactivation of the nasopharyngeal swab on the water bath and compare with heat treatment of samples after adding lysis buffer using dry heat [heat block] and moist heat [water bath] on reverse transcription-polymerase chain reaction (RT-PCR).

METHODS

We performed two experimental designs to show the impact of temperature on the test result of nasopharyngeal swab (NPS) samples that were known positive for SARS-CoV2 RNA. In the first experiment, we compared the impact of dry heat block with water bath heat inactivation of samples after lysis buffer is applied to the samples. Briefly, in a standard Da An Gene Nucleic Acid extraction protocol next to the addition of proteinase K, we apply lysis buffer into the samples followed by a dry heat block at 72^0C for 10 minutes. However, in most of our district laboratories, there is a huge shortage of dry heat blocks, hence we wanted to test if water bath inactivation (moist heating) at 72^0C for 10 minutes can be used instead of dry heat block to facilitate viral RNA extraction. For this purpose, randomly 20 NPS samples sent to AHRI's SARS-CoV2

[1]Armauer Hansen Research institute, Jumma Road ALERT Compound, P.O. Box address 1005, Addis Ababa, Ethiopia

$These authors contributed equally

*Corresponding Author: E-mail: getachew.tesfaye@ahri.gov.et

laboratory for routine diagnosis that were confirmed positive for SARS-CoV-2 RNA were included.

We aimed the second experimental design to assess the impact of water bath heat inactivation at 56^0C directly on NPS samples over time before initiation of nucleic acid extraction. Here we took 15 NPS samples randomly that were confirmed positive for SARS-CoV-2 RNA from the samples AHRI received for routine COVID-19 diagnosis. The samples were heat-treated by a water bath at 56^0C for 15 minutes, 30 minutes, and 45 minutes.

RNA extraction
Viral nucleic acid (NA) was extracted from 200 µL respiratory samples (NPS and oropharyngeal swabs in viral transport medium using the NA extraction and purification reagent, Dn An Gene Co., Ltd, as recommended by the manufacturer (Da An Gene Co., Ltd, of Sun Yat-sen University, China). Briefly, 50 µL proteinase K and 200 µL lysis buffer were mixed with 200 µL NP and/or Nasal swab samples. Then, the lysed samples were heat-inactivated parallelly on a dry heat block and in a water bath at 72^0C for 10 minutes, followed by the addition of inhibitor remover and subsequent washing. Finally, the NA was eluted in 50 µL molecular grade water preheated at 72^0C.

RT-PCR for the detection of viral RNA
In the experiments, the careGENETM COVID-19 RT-PCR kit was used to detect SARS-CoV2 RNA. The kit has the following combination of detection targets and fluorescent reporters; nucleocapsid (N) gene reported by FAM, RNA-dependent RNA polymerase (RdRP) gene by CY5, and the internal control (IC) by ROX. Regardless of the RT-PCR system, the kit has the limit of detection10copies/ µL of viral RNA. The analytical sensitivity (cut-off value) of the careGENETM for positive tests is a cycle threshold of ≤ 43, and any reading above 43 is a negative test. Finally, the amplification reaction mixes for both experiments were run on Agilent Technologies Stratagene, Max3005P RT-PCR system according to the protocol provided by the manufacturers.

Ethics: The study is approved by the Armauer Hansen Research Institute/ALERT Ethics Review Committee.

RESULT

The first experiment where we have compared the use of dry heat block and water bath at a temperature of 72^0C for 10 minutes after the addition of lysis buffer during RNA extraction shows that all the 20 samples that were positive in dry heat block turned out to be all positive in the water bath. The Ct values of the samples tested by dry heat block and water bath were nearly similar (P-value = 0.00023). Interestingly, the overall average of the change in the Ct value (ΔCt) for the N gene (in the FAM Chanelle) of the samples processed by water bath demonstrated a 2.79 lower Ct value than the sample treated by heat block. We observed a similar result for RdRP gene (in the CY5 channel) that is, samples inactivated by water bath had a 2.63 lower Ct value than the sample treated by dry heat block (Table 1).

In the experiment where we tested the impact of heat inactivation by the use of water bath directly on NPS samples at 56^0C over time, we observed that the N-gene which is reported by HEX channel showed similar analytical sensitivity (detection level) when samples were treated at 56^0C for 15 min and 30 min. That is, out of the 15 samples that were confirmed positive before heat treatment, 6.67% of the specimens were detected negative for the N-gene; whereas 13.3% of the samples were turned negative for the RdRP gene when they were heated 56^0C for 15 min and 20 min. When the time of inactivation increased to 45 min, 20% of the samples turned negative for both N gene and RdRP gene (Table 2).

DISCUSSION

Heat treatment of virus inactivation rate depends on ways of applying heat [5]. Concerning the modes of transmission of SARS-CoV-2 inactivation rate under heat treatment at 70^0C can vary by almost two orders of magnitude depending on the treatment procedure [6]. Here, we tested the impact of different sources of heat on the analytical sensitivity or the limit of detection of SARS-CoV-2 RNA. The study shows that all the samples that were positive when treated by dry heat block during lysis nearly all turned positive when heat treated by a water bath. This suggests that the water bath is not inferior in its use for virus inactivation during lysis for RNA extraction, and can be used in place of a dry heat block in district laboratories where there is a shortage. The United States Centre for Disease Control and Prevention advised moist heat as the method for virus inactivation [7]. Interestingly, in line with this, our data shows samples heat treated with water bath during lysis have a higher viral load, on average a difference of Ct value greater than 2.5 for both N and RdRP genes, showing that SARS-CoV2 inactivation by water bath during lysis is advantageous over dry heat block because it lowers the limit of detection of the viral RNA.

Studies suggest that heat-inactivated biological

Table 1: A comparison of the Ct values of the targets, N and RdRP genes reported by FAM and CY5, respectively of the NP samples treated at 72^0C for 10 minutes using dry heat block and water bath for RNA extraction. ROX channel is the reporter for internal control.

Samples ID	***Dry heat block***			***Water bath***			***Change in Ct values (†ΔCt)***	
	ROX	FAM	CY5	ROX	FAM	CY5	‡FAM_{Dry}-FAM_{Wet}	§$CY5_{Dry}$-$CY5_{Wet}$
AHRI-16584	18.64	13.77	16.50	17.65	12.13	15.29	1.64	1.20
AHRI-16063	17.04	14.48	16.60	16.51	14.13	16.14	0.35	0.47
AHRI-16055	16.93	13.86	17.10	16.76	12.83	14.98	1.03	2.09
AHRI-16283	16.48	18.11	20.80	14.94	16.46	19.01	1.65	1.77
AHRI-16307	23.03	23.17	24.20	21.78	21.97	23.31	1.20	0.89
AHRI-16442	16.25	29.49	29.60	16.18	28.71	29.17	0.78	0.43
AHRI-16281	15.16	30.65	29.70	15.45	29.49	28.85	1.16	0.81
AHRI-16448	16.86	28.98	29.10	16.02	24.93	24.80	4.05	4.30
AHRI-16392	14.82	33.72	32.80	14.29	29.23	29.12	4.49	3.67
AHRI-16441	16.84	30.17	30.50	16.58	29.15	30.58	1.02	-0.08
AHRI-16417	17.91	32.73	32.30	17.50	31.05	30.71	1.68	1.55
AHRI-16447	16.05	31.51	31.90	16.20	29.96	30.31	1.55	1.56
AHRI-16404	16.82	33.27	33.30	15.78	31.07	31.83	2.20	1.47
AHRI-16437	16.55	35.6	34.10	15.14	31.40	32.46	4.20	1.60
AHRI-16447	16.05	31.51	31.90	16.20	29.96	30.31	1.55	1.56
AHRI-16439	19.37	43	29.60	16.66	33.07	NCt	11.40	NCt
AHRI-16478	14.61	31.75	32.8	15.46	29.99	31.43	1.76	1.36
AHRI-16403	19.42	29.2	41.4	17.44	NCt	28.9	NCt	12.46
AHRI-16435	19.65	36.54	41.7	19.67	36.56	31.58	0.2	10.14
AHRI-16466	15.36	44.63	NCt	15.65	33.38	32.46	11.3	NCt

† Refers to change in Ct value
‡ Refers to subtraction of Ct value in FAM channel reading of the water bath from a FAM channel reading in the dry heat block
§ Refers to subtraction of Ct value in Cy5 channel reading of the water bath from a Cy5 channel reading of dry heat block
NCt: No cycle threshold

Table 2: Positive detection rates of SARS-CoV2 NP samples for N- and RdRP-genes after direct heat inactivation using water bath at 56^0C over time.

Total number of samples	No treatment (Zero time)	Heat treatment at 56^0C					
		15 min		30 min		45 min	
		N-gene	RdRP gene	N-gene	RdRP gene	N-gene	RdRP gene
15	100%	93.3%	86.7%	93.3%	86.7%	80%	80%

samples may not be suitable for proper detection of viral RNA as there is a possible reduction in the sensitivity and increase in the limit of detection of viral RNA resulting in a significant number of false-negative results [6,8]. In this context, in our second experiment, we tested the impact of water bath heat inactivation methods over time on the detection rate of SARS-CoV2. Our data show a similar detection rate of the viral RNA when applying heat of 56^0C for 15 min and 30 min while heating the NPS samples for 45 minutes [which is the standard temperature-time combination to inactivate viruses] drastically reduces the virus detection rate that is, 20% (3/15) positive samples turned to negative. This implies that the standard temperature-time combination to inactivate viruses has a negative impact on the RT-PCR-based diagnosis. This agrees with a study that reported after heat inactivation treatment 13% (6/46) of positive samples turned negative [4] and is in line with WHO recommendation. We have also observed that there is a difference in the detection rate of the virus regarding the two target genes, N gene has a higher detection rate where 6.67% of positive samples turned in to negative whereas 13.3% of positive samples turned to negative for RdRP gene. An earlier study also reported that the Ct value for RdRP gene was 1.2 times larger than that of the N gene [9], suggesting that primer sets for detecting the SARS-CoV2 N gene might be the choice for highly sensitive detection of the virus.

In conclusion, the water bath is not inferior to dry heat block for virus inactivation for detecting SARS-CoV2 RNA and can substitute dry heat block in district laboratories where there is a shortage. However, the inactivation of NPS samples at 56^0C over 30 minutes drastically reduces the virus detection rate and increases false negativity, and thus sample should not be heat-treated before nucleic acid extraction.

Conflict of interest

The authors have declared that no competing interests exist

Acknowledgment

We would like to thank all the brave men and women staff members working in the AHRI's SARS-CoV2 laboratory and those who participated in sample reception and laboratory cleaning.

Authors' contribution

FA: Formal analysis, Methodology, Writing - review & editing
GTB: Conceptualization, Formal analysis, Methodology, Writing - review & editing
ES: Investigation, Methodology, review & editing
ZS: Investigation, Methodology, review & editing
SW: Investigation, Methodology, review & editing
DAT: Investigation, Methodology, review & editing
DHA: Formal analysis, Methodology, Writing - review & editing
AM: Investigation, Methodology, review & editing
AA: Investigation, Methodology, Supervision, Writing - review & editing
AnM: Conceptualization, Formal analysis, Methodology, Writing - review & editing

REFERENCES

1. Guo Y-R, Cao Q-D, Hong Z-S, Tan Y-Y, Chen S-D, Jin H-J, et al. The origin, transmission and clinical therapies on coronavirus disease 2019 (COVID-19) outbreak – an update on the status. Mil Med Res. 2020;7: 1–10. doi:10.1186/s40779-020-00240-0
2. Cucinotta D, Vanelli M. WHO declares COVID-19 a pandemic. Acta Biomed. 2020;91: 157–160. doi:10.23750/abm.v91i1.9397
3. Calvez RM, Taylor A, Calvo-Bado L, Fink CG. Molecular detection of SARS-CoV-2 using a reagent-free approach. medRxiv. 2020; 2020.04.28.20083626.

4. Zou J, Zhi S, Chen M, Su XX, Kang L, Li C, et al. Heat inactivation decreases the qualitative real-time RT-PCR detection rates of clinical samples with high cycle threshold values in COVID-19. Diagn Microbiol Infect Dis. 2020;98: 115109. doi:10.1016/j.diagmicrobio.2020.115109
5. Gamble A, Fischer RJ, Morris DH, Yinda KC, Munster VJ, Lloyd-Smith JO. Heat-treated virus inactivation rate depends strongly on treatment procedure. bioRxiv. 2020; 2020.08.10.242206. Available: https://www.biorxiv.org/content/10.1101/2020.08.10.242206v1%0Ahttps://www.biorxiv.org/content/10.1101/2020.08.10.242206v1.abstract
6. Pastorino B, Touret F, Gilles M, de Lamballerie X, Charrel RN. Heat inactivation of different types of SARS-CoV-2 samples: What protocols for biosafety, molecular detection and serological diagnostics? Viruses. 2020;12: 6–13. doi:10.3390/v12070735
7. Rutala WA, Weber DJ. Disinfection and sterilization in healthcare facilities. Bennett Brachman's Hosp Infect Sixth Ed. 2013. doi:10.1017/9781107153165.009
8. Pan Y, Long L, Zhang D, Yuan T, Cui S, Yang P, et al. Potential False-Negative Nucleic Acid Testing Results for Severe Acute Respiratory Syndrome Coronavirus 2 from Thermal Inactivation of Samples with Low Viral Loads. Clin Chem. 2020;66: 794–801. doi:10.1093/clinchem/hvaa091
9. Syndrome R, Rna C, Number C, Chen H, Wu R, Xing Y, et al. Influence of Different Inactivation Methods on Severe Acute Respiratory Syndrome Coronavirus 2 RNA Copy Number. J Clin Microbiol. 2020;58: 1–8.