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Thermal inactivation of COVID-19 specimens improves RNA quality and quantity

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Abstract

The rapid spread of coronavirus disease 2019 (COVID-19), a disease caused by severe acute respiratory syndrome coronavirus 2, poses a huge demand for immediate diagnosis. Real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) of nasopharyngeal (NP) and oropharyngeal (OP) swabs have been used to confirm the clinical diagnosis. To avoid the risk of viral-exposure of laboratory workers, thermal inactivation is currently recommended but has unknown effects on the accuracy of the rRT-PCR results. Thirty-six NP/OP specimens were collected from COVID-19 patients and subjected to thermal inactivation (60°C for 30 min) or the RNA extraction processes to activate the form. Here, our data showed that the concentration of extracted-RNA increases upon thermal inactivation compared to the active form ($p = .028$). Significantly higher levels of RNA copy number were obtained in inactivated compared to the active samples for both N and ORF1ab genes ($p = .009$, $p = .032$, respectively). Thermal inactivation elevated concentration and copy number of extracted-RNA, possibly through viral-capsid degradation and/or nucleoprotein denaturation.

KEYWORDS

COVID-19, nucleic acids, nucleoproteins, real-time polymerase chain reaction, virus inactivation

1 | INTRODUCTION

The ongoing outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is responsible for coronavirus disease 2019 (COVID-19) is another pandemic after the influenza pandemic in 1918. In December 2019, a cluster of unexplained pneumonia was reported in Wuhan China and the infection has spread rapidly to over 213 countries and territories. Clinically, manifestations of COVID-19 include fever, cough, dyspnea, diarrhea, multiple organ failure, and even death. Lymphocytopenia is also common laboratory evidence in these patients. However, the risk of death will increase with age and the presence of comorbid illnesses (Cheng et al., 2020). The SARS-CoV-2 is an enveloped non-segmented positive-sense RNA virus, a new member of β-coronavirus genera with 79.5% amino acid similarity to SARS-CoV (Cascella et al., 2020). It has been shown that the SARS-CoV-2 genome encodes 14 open reading frames (ORFs) that translates to two polyproteins (pp1a and pp1b), are auto-proteolytically processed into 16 non-structural proteins (Nsp1-16) and subsequently form the replicase or transcriptase complex (Gordon et al., 2020). The 3′ end of the SARS-CoV-2 genome also encodes four structural proteins that are important for virion assembly, including envelope (E) protein,

nucleocapsid (N) protein, spike (S) glycoprotein, and matrix (M) protein. Among these, N protein as a multifunctional protein forms a ribonucleoprotein complex in combination with genomic RNA, which is inserted into the viral envelope carrying other structural proteins. It also displays pleiotropic effects, such as cell cycle arrest and apoptosis induction (McBride et al., 2014). This ability of N protein to RNA-binding is critical for encapsidation, discontinuous transcription of the viral genome, and polymerase template switching (Mateos-Gomez et al., 2011). Indeed, Coronavirus-N protein with a conserved two-domain (NTD and CTD) which is linked by a poorly structured linkage region domain (Zhang & Zhao, 2020) acts as a RNA-chaperone that promotes the correct folding of the hammerhead ribozyme, in vitro (Zuniga et al., 2007). SARS-CoV-2 requires angiotensin-converting enzyme 2 (ACE2) as a receptor for entrance to the host cells that is a key determinant for the pathogenesis of infection. The S-glycoprotein, which contains S1 and S2 subunits, can bind to the ACE2 that highly expresses on the alveolar epithelial type 2 cells and enterocytes of the small intestine (Guo et al., 2020). The SARS-CoV-2 probably enters into the cells either via endocytosis or via plasma membrane fusion and the viral RNA is released to the cytoplasm (Hoffmann et al., 2020).

Qualitative detection of SARS-CoV-2-nucleic acid by real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) of upper respiratory specimens, such as nasopharyngeal swabs (NP), oropharyngeal swabs (OP), BAL, sputum, and tracheal aspirates has been typically used to confirm the clinical diagnosis (W. Wang et al., 2020). Detection of viral RNA is not only helpful in the diagnosis of an active infection but also provides significant epidemiological information. However, there is a risk of exposure to the virus in clinical specimens for laboratory workers. Tremendous attempts have been made to mitigate the outbreak of infection in laboratories. There are several methods for virus inactivation, including using chemical agents, such as Triton X-100, which is recommended by the Center for Disease Control and Prevention (CDC). Moreover, heating the sample at 60°C for 1 h for viral hemorrhagic fever agent is another possibility (Pastorino et al., 2020). Other efforts for viral-inactivation are conducted through ultraviolet light (UV), gamma irradiation, formaldehyde and glutaraldehyde, and heat treatment of virus (Darnell et al., 2004). Although inactivation of coronaviruses can be achieved through several techniques, obtaining sufficient amounts of viral-RNA templates in specimens for subsequent steps is critical. In the present study, we have examined the effect of heat on the extracted-RNA concentration and quantities of total RNA which is based on rRT-PCR results on SARS-CoV-2 samples.

2 | MATERIALS AND METHODS

2.1 | Preparation of patients samples

A total of 36 hospitalized-suspected COVID-19 (21 male, 15 female; mean ± SD of age was 64.56 ± 21.52 years) were enrolled in this study after written informed consent. The study was approved by the Semnan University of Medical Sciences (IR. SEMUMS. REC.1399.197). According to the CDC instructions, specimens were collected from the upper respiratory tract through collecting OP and NP swabs from 36 hospitalized-suspected COVID-19 patients using Dacron swabs (CMC Medical Devices & Drugs). Immediately, swabs were placed into sterile 15 ml conical centrifuge tubes (Thermo Fisher Scientific) containing 2 ml of Dulbecco's modified Eagle's medium culture medium and 1% streptomycin (Gibco) and transferred to the laboratory in a container with ice or frozen gel packs.

2.2 | Thermal inactivation

To determine the effect of thermal inactivation on the integrity of RNA, collected specimens from suspected-patients were divided into two groups (inactivated and active). Subsequently, inactivated groups were incubated at 60°C for 30 min (Kampf et al., 2020).

2.3 | RNA extraction procedure

For RNA extraction, 400 μl of culture medium was treated by 10 μl carrier RNA(1 mg/ml) and 20 μl proteinase k(10 mg/ml) provided by a MagCore® 202 Low PCR Inhibition kit (MagCore® Viral Nucleic Acid Extraction Kit). Total RNA extracted using the manufacturer's instructions were followed without modification by the automated Viral Nucleic Acid Extractor using magnetic-particle technology (MagCore®). RNA eluted into 60 μl of elution buffer to obtain high concentration viral RNA and was used as the template for rRT-PCR. The concentration of extracted-RNAs was measured and the ratio of the absorbance at 260 and 280 nm (A260/280) was determined using a NanoDrop OneC Spectrophotometer (Thermo Scientific NanoDrop). The extracted RNA samples were stored at −70°C for later analyses.

2.4 | Real-time reverse-transcriptase-polymerase chain reaction

For quantitative detection of SARS-CoV-2 N- and ORF1ab-gene, rRT-PCR was performed on both OP and NP swabs from suspected people with symptoms of COVID-19 using a detection kit for 2019 novel coronavirus (2019-nCoV) RNA (PCR-Fluorescence Probing; DaAn Gene Co., Ltd. Sun Yat-Sen University) according to the manufacturer's protocol. All RNA concentrations were determined by a nanodrop Spectrophotometer (Thermo Scientific NanoDrop). A final volume of 25 μl PCR reaction mixture for each sample, containing 17 μl of solution A (contains specific primers, probes, Tris (hydroxymethyl) aminomethane-hydrochloric acid buffer), 3 μl of solution B (contains hot start Taq DNA polymerase and c-MMLV reverse transcriptase), 5 μl of extracted-RNA sample, and positive/negative control was incubated in the 8-tube (Applied Biosystems). The negative control RNA template containing Pseudo virus with internal

control fragment (Provided by DaAn Gene rRT-PCR detection kit) was extracted along with patients' samples with the same extraction method. The positive control vial (Provided by DaAn Gene rRT-PCR detection kit) contains Pseudo virus target fragments and internal control fragments. Subsequently, the strips were centrifuged for 8 s at 6000 RPM (Hettich). The reaction was carried out on a StepOne™ Real-Time PCR System (Applied Biosystems), according to the manufacturer's instructions. According to the detection kit recommendation, the PCR cycling program was started with 50°C for 15 min, 95°C for 15 min for initial denaturation of the templates, and followed by 45 cycles of 94°C for 15 s and 55°C for 45 s. Both N- and ORF1ab-genes of each sample were measured in the FAM and VIC detecting channels respectively; and cycle threshold (C_t) values ≤40 were considered positive (Zhou et al., 2020).

2.5 | Statistical analysis

All statistical calculations were conducted with Prism 8.0.2 software (GraphPad Software). Statistical analysis between the paired samples was performed using a paired two-tailed *t-test*. $p < .05$ were considered statistically significant.

3 | RESULTS

3.1 | The effect of thermal inactivation on the integrity of extracted-RNA

According to the results, the median RNA concentration was significantly higher in the inactivated group compared to the active group ($p = .028$). However, no significant differences were observed in A260/280 ratios between the inactivated and active group ($p = .421$; Table 1, Figure 1).

3.2 | Impact of thermal inactivation on real-time PCR results of extracted-RNA

To investigate the impact of thermal inactivation on the amount of extracted-RNA, both inactivated and active samples were analyzed for expression of SARS-Cov-2 N- and ORF1ab-gene using rRT-PCR assay after complementary DNA synthesis. The C_t as the number of cycles required for the fluorescent signal to cross the threshold was determined to provide the impact of heat treatment on extracted-RNA expression. According to the obtained results, inactivated samples have significantly lower C_t values than active samples for expression of N- and ORF1ab-gene ($p = .009$, $p = .032$, respectively; Table 2, Figure 2). Moreover, a comparison of C_t values between a thermal-inactivated and active form of samples could reveal a false-negative result. The rRT-PCR results of one patient showed high C_t values for both N- and ORF1ab-genes in the active form (C_t value ≥40), whereas C_t values equal to 35.92 and 35.16 for N- and ORF1ab-gene were obtained in the inactivated form of a sample, respectively.

4 | DISCUSSION

There is heavy exposure to infectious-viruses in laboratories that increases the risk of infection. However, as SARS-CoV-2 is an emerging virus, there is little information about the impacts of viral-inactivation and infectivity to reduce the risk of exposure for laboratory personnel. Generally, in the case of most viruses, there are two mechanisms for viral-inactivation including the degradation of the viral capsid (protein denaturation), and the degradation of the viral-RNA (RNA inactivation) (Dimmiock, 1967; Y. Wang et al., 2004). Based on the experiences of other enveloped-RNA viruses, it is supposed that the inactivation of SARS-CoV-2 can be achieved through several techniques, such as chemical, UV, and heat treatment procedures. Among these, it has been shown that heat treatment can inactivate viruses through denaturation of the secondary structure of proteins, disturbance of cell-attachment, replication of genomic RNA, and transcription processes of the subgenomic messenger RNA (mRNA; Schlegel et al., 2001). As reported by Lee et al., heat treatment can affect the infectivity of viruses by thermal aggregation of the SARS-CoV membrane protein due to a conformational change and protein degradation (Lee et al., 2005). Furthermore, the current reference standard test for COVID-19 diagnosis is the real-time PCR assay (Cheng et al., 2020). To achieve accurate and precise real-time PCR results, a high-quality nucleic acid template is needed. According to "The Laboratory Technical Guidelines for Detection 2019-nCoV" collected specimens can be incubated at 56°C or higher temperature before RNA extraction to inactivate the virus (Zhang & Zhao, 2020). In this study, we investigate the effect of thermal inactivation (60°C for 30 min) on the integrity and quality of extracted-viral RNA, based on the Real-Time PCR results of inactivated samples compared to active (control) samples. Our data showed that upon heat treatment, the concentration of extracted-RNA increase compared to the active forms of samples. Besides the suitable quality and integrity of extracted-RNA in heat-inactivated samples, the real-time PCR results were significantly better in the inactivated group than the active form of viruses. These results demonstrate that C_t values decreased when viral-samples were thermally inactivated. In other words, in the thermal-inactivated samples, there were higher copy numbers of RNA templates than the active form of samples. Consistent with these results, Pastorino et al. reported that the 56°C for 30 min and 60°C for 60 min protocols do not affect the integrity and copy number of SARS-CoV-2 RNA (Pastorino et al., 2020). Also, two types of research studies by Darnell and Chen on SARS-CoV and SARS-CoV-2 have found that virus incubation at 56°C for 20–30 min can inactivate the viruses, without a significant effect on qRT-PCR results (Darnell et al., 2004; Zhang & Zhao, 2020). The studies on structural properties of heat-treated virions imply that

TABLE 1 Comparison of extracted-RNA quantity and quality between inactivated and active groups ($n = 36$)

	Concentration (ng/μl)		A260/280	
	Inactivated	Active	Inactivated	Active
Sample 1	6.3	1.0	1.10	1.82
Sample 2	2.5	2.0	1.49	1.75
Sample 3	4.7	2.4	1.82	1.58
Sample 4	1.2	1.4	1.29	0.98
Sample 5	22.7	2.5	1.06	0.99
Sample 6	5.1	4.1	1.39	1.07
Sample 7	4.9	3.0	1.00	1.11
Sample 8	1.7	8.0	0.84	1.00
Sample 9	3.1	1.5	0.96	0.82
Sample 10	3.6	4.6	1.25	1.05
Sample 11	1.4	1.6	1.20	0.95
Sample 12	15.2	10.4	1.39	1.44
Sample 13	2.9	1.5	1.32	0.91
Sample 14	1.2	1.5	0.77	0.99
Sample 15	7.3	11.1	1.38	1.18
Sample 16	1.7	6.6	1.06	1.77
Sample 17	21.4	9.4	1.40	1.57
Sample 18	36.4	3.5	0.48	1.28
Sample 19	55.2	13.6	1.13	1.28
Sample 20	1.3	3.7	1.12	1.46
Sample 21	5.3	11.4	1.45	0.28
Sample 22	2.9	2.2	1.12	0.65
Sample 23	112	19.1	1.83	1.77
Sample 24	5.3	4.4	1.40	1.40
Sample 25	7.5	7.8	1.34	12.03
Sample 26	18.8	28.3	1.63	0.90
Sample 27	2.1	1.3	1.10	0.94
Sample 28	0.6	0.9	0.35	1.13
Sample 29	4.8	6.3	1.19	1.13
Sample 30	1.3	1.3	0.91	0.68
Sample 31	4.8	2.3	1.25	1.02
Sample 32	58.2	28.1	1.77	1.72
Sample 33	1.7	0.7	0.96	0.56
Sample 34	74.8	36.0	1.81	1.69
Sample 35	6.1	3.5	1.58	1.14
Sample 36	7.7	4.3	1.25	1.30
Mean ± *SEM*	14.27 ± 4.04	6.98 ± 1.41	1.23 ± 0.05	1.48 ± 0.30
Median (range)	4.850 (0.6–112.0)	3.60 (0.7–36.0)	1.25 (0.35–1.83)	1.13 (0.28–12.03)
p Value[a]	.028		.421	

[a]Paired *t* test.

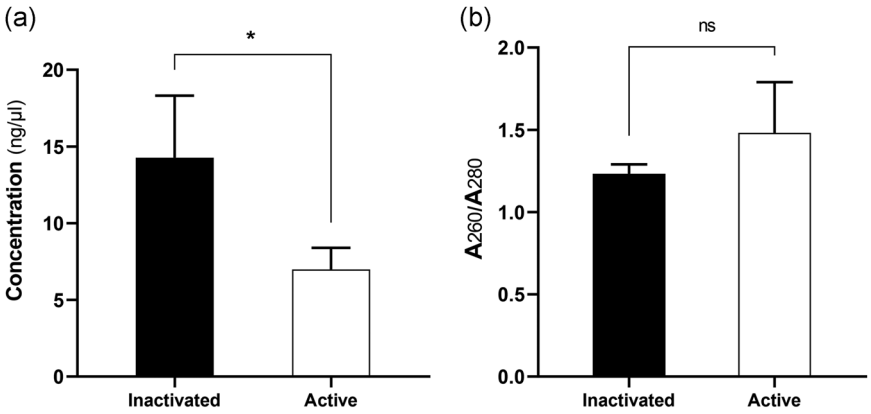

FIGURE 1 The effect of thermal inactivation on the integrity of extracted-RNA. To evaluate the effect of SARS-CoV-2 inactivation through heat treatment, suspected specimens of the upper respiratory tract from 36 hospitalized patients were divided into two (inactivated and active) group. Inactivated samples were incubated at 60°C for 30 min and subsequently, RNA extraction was performed for both inactivated and active samples. The concentration and purity of extracted-RNAs were determined using a NanoDrop Spectrophotometer. (a) The concentration of extracted-RNA was higher in inactivated samples than active samples ($p = .028$). (b) No significant differences were found between A260/280 in inactivated and active samples ($p = .421$). Significant p values were calculated using the paired t test after passing the normality test (Kolmogorov–Smirnov). Values are the mean ± *SEM* (error bars). ns, nonsignificant; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2. $^{*}p < .05$

at low temperatures random degradation of the nucleic acid can be accomplished, whereas the major conformational changes can occur in virus structure upon treatment with high temperature (Laude, 1981). It is reported that UV and heat treatments can cause irreparable damage of the RNA that leads to loss of viral-RNA replication and following processes. Heat treatment also resulted in envelope disruption and sensitization of the viral-capsid to proteolytic digestion (Pfaender et al., 2015). Thus, one of the possible mechanisms for more quality and quantity of extracted-RNA upon this thermal inactivation protocol is the capsid degradation and aggregation of the SARS-CoV membrane protein that leads to the viral-RNA release and more RNA template. Moreover, among coronaviruses proteins, the N protein is the major virion structural proteins with an essential role in assembling the RNA of coronavirus, RNA replication, and mRNA transcription/translation may be more sensitive to inactivation treatments than other structural proteins due to the highest hydrophilicity and pI (isoelectric point) without any cysteine residues (Compton et al., 1987; Y. Wang et al., 2004). Indeed, the thermal-induced unfolding of N protein at 55°C leads to complete denaturation (Duan et al., 2003). Based on a study by Wang and his colleagues, another possibility is the low stability of N protein that causes ribonucleoprotein complex destruction and release of the viral-RNA.

Taken together, in the present study we demonstrated that in addition to reducing the risk of viral contamination, the quality and quantity of extracted-RNA can increase upon accurate thermal inactivation (60°C for 30 min). However, there is an urgent need to

TABLE 2 Comparison of C_t values between inactivated and active groups ($n = 7$)

	SARS-CoV-2 N gene		SARS-CoV-2 ORF1ab gene	
	Inactivated	Active	Inactivated	Active
Sample 1	33.50	33.15	33.92	35.40
Sample 2	30.55	32.01	31.59	31.04
Sample 3	29.97	31.73	30.25	31.47
Sample 4	27.39	32.53	25.95	40.00
Sample 5	17.32	20.71	19.45	27.67
Sample 6	35.92	40.00	35.16	40.00
Sample 7	28.13	30.85	28.51	30.21
Mean ± *SEM*	28.97 ± 2.24	31.57 ± 5.67	29.26 ± 2.02	32.54 ± 1.51
p Value[a]	.009	.032		

Abbreviation: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.
[a]Paired t test.

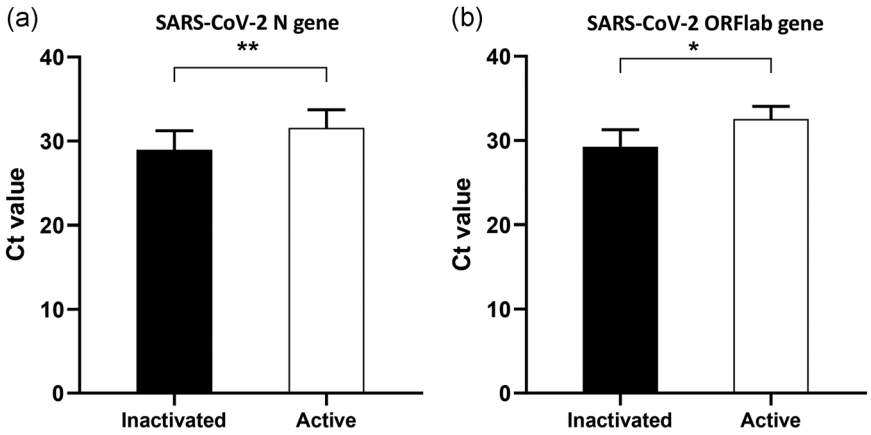

FIGURE 2 Impact of thermal inactivation on real-time PCR results of extracted-RNA. To determine the effect of thermal inactivation protocol (60°C, 30 min) on SARS-CoV-2 quantity, the N- and ORF1ab-gene expression was assessed in both inactivated and active samples using real-time PCR assay ($n = 7$). (a) Results showed that the expression of N-gene with C_t value mean 28.97 in inactivated samples is lower than its expression in active samples with C_t value mean 31.57 ($p = .009$). (b) Similarly, the expression of ORF1ab-gene in inactivated samples had a lower C_t value with a mean of 29.26 than its expression in active samples with a mean of C_t value 32.54 ($p = .032$). Significant p values were calculated using the paired *t-test* after passing the normality test (Kolmogorov–Smirnov). Values are the mean ± *SEM* (error bars). PCR, polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2. $^{*}p < .05$; $^{**}p < .01$

identify the mechanisms of action of thermal inactivation in the SARS-CoV-2 case. These results can be used to achieve more accurate and safe protocols for virus laboratory diagnosis.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Parviz Kokhaei: conceptualization and design of the study, project administration, writing-review, and editing. Maral Hemati: formal analysis and writing-original draft. Mohsen Soosanabadi, Tahereh Ghorashi, Hadi Ghaffari, Azadeh Vahedi, Elaheh Sabbaghian, Zahra Rasouli Nejad, Amir Salati, and Navid Danaei: acquisition of data and methodology.

DATA AVAILABILITY STATEMENT

The authors confirm that their article contains a data availability statement.

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