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Effects of Different Temperature and Time Durations of Virus Inactivation on Results of Real-time Fluorescence PCR Testing of COVID-19 Viruses*

Ze-gang WU†, Hong-yun ZHENG†, Jian GU†, Feng LI, Rui-long LV, Ya-yun DENG, Wan-zhou XU, Yong-qing TONG#
Department of Clinical Laboratory, Renmin Hospital of Wuhan University, Wuhan 430060, China

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Summary: The novel coronavirus SARS-CoV-2 caused an outbreak of pneumonia in Wuhan, Hubei province of China in January 2020. This study aims to investigate the effects of different temperature and time durations of virus inactivation on the results of PCR testing for SARS-CoV-2. Twelve patients at the Renmin Hospital of Wuhan University suspected of being infected with SARS-CoV-2 were selected on February 13, 2020 and throat swabs were taken. The swabs were stored at room temperature (20–25°C), then divided into aliquots and subjected to different temperature for different periods in order to inactivate the viruses (56°C for 30, 45, 60 min; 65, 70, 80°C for 10, 15, 20 min). Control aliquots were stored at room temperature for 60 min. Then all aliquots were tested in a real-time fluorescence PCR using primers against SARS-CoV-2. Regardless of inactivation temperature and time, 7 of 12 cases (58.3%) tested were positive for SARS-CoV-2 by PCR, and cycle threshold values were similar. These results suggest that virus inactivation parameters exert minimal influence on PCR test results. Inactivation at 65°C for 10 min may be sufficient to ensure safe, reliable testing.
Key words: SARS-CoV-2; COVID-19; throat swabs; real-time fluorescence PCR

Numerous infections of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) have occurred in Wuhan, Hubei since December 2019, and the number of infections has rapidly increased[1]. New infections have since occurred in other regions of China and the other countries[2–4]. The SARS-CoV-2 has been added to the Chinese National Health Committee's list of Class A infectious diseases requiring prevention and control under the Infectious Disease Control Law. As of April 10, 2020, a total of 81 953 confirmed cases and 3339 deaths (4.07%) have been reported nationwide[5]. Since the beginning of the epidemic in late December 2019, SARS-CoV-2 has now spread to all continents, and up to April 10, 2020, the WHO reported 1 521 252 confirmed cases and 92 798 deaths globally (Situation Report-81).

Real-time fluorescence PCR detection of SARS-CoV-2 nucleic acids is one of the methods for confirming infection[6]. Due to the highly infectious nature of the virus, staff who perform nucleic acid detection are at great risk of infection and therefore require protective equipment and other measures to minimize this risk. According to the current COVID-19 diagnosis and treatment guidelines, the virus can be inactivated at 56°C for 30 min[7]. Whether this inactivation process affects the PCR results is currently unclear.

Therefore, this study compared the results of PCR testing of throat swabs following sample inactivation at different temperature for different periods. We also intended to identify the best inactivation temperature and time duration that can minimize infection risk to laboratory staff without affecting test results.

1 MATERIALS AND METHODS

1.1 Ethical Approval

This study was conducted in accordance with the Declaration of Helsinki and approved by the Research Ethics Committee of Renmin Hospital of Wuhan University. The requirement for written informed consent was waived given the context of emerging infectious diseases.

Ze-gang WU, E-mail: wuzegang1234@163.com; Hong-yun ZHENG, E-mail: shenzheng2008@163.com; Jian GU, E-mail: tekjian@gmail.com
†These authors contributed equally to this work.
#Corresponding author, E-mail: tytsing@163.com
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1.2 Selection of Patients and Samples

Throat swabs from a convenience sample of 12 patients suspected of being infected with SARS-CoV-2, aged 32–75 years (average, 52.57 years) in Renmin Hospital of Wuhan University were collected on February 13, 2020. All 12 patients were living in Wuhan and had fever, fatigue, and dry cough. Swabs were placed immediately in vials containing 3 mL of preservation solution containing antibiotics, protein protection agent, buffer, glycerin, and nuclease inhibitors (Health Gene Technologies, China) and stored at room temperature (20–25°C) until inactivation (see below). The diagnostic criteria for suspected cases of COVID-19 were consistent with the New coronavirus pneumonia diagnosis and treatment, a clinical guideline and database (trial version 6)[7].

1.3 Virus Inactivation

We used sterile EP tubes to divide each sample into 12 tubes, 300 μL per tube. The aliquots were incubated in water baths at room temperature for 60 min (control); at 56°C for 30, 45, or 60 min; or at 65, 70, or 80°C for 10, 15, or 20 min. Nucleic acid was extracted using a Smart Labassist-32 type nucleic acid extraction/purification analyzer (TANBead, Taiwan, China). Then levels of mRNA were determined in all samples using real-time fluorescence PCR on a LightCycler 480 fluorescence PCR system (Roche, Switzerland). Reagents and primers were purchased from the SARS-CoV-2 ORF1ab/N Gene Dual Nucleic Acid Detection Kit (Huirui Biotechnology, Shanghai, China), which contained forward primer 5′-TCAGA-ATGCCAATCTCCCCAAC-3′, reverse primer 5-AA-AGGTCCACCCGATACATTGA-3′ and probe 5′-CY5-CTAGTTACACTAGCCATCCTTACTGC-3′ BHQ1). Each PCR reaction contained 7.5 μL of PCR reaction solution, 5 μL of ORF1ab/N gene reaction solution, 1.5 μL of enzyme mixture, and 11 μL of nucleic acid extract. Reactions were subjected to 50°C for 15 min, 95°C for 5 min, followed by 45 cycles of 95°C for 10 s, and 55°C for 45 s. According to the manufacturer's instructions, the criterion for judging the results is cycle threshold (Ct) <40.

1.4 Statistical Analysis

Data were analyzed using SPSS 19.0 (IBM, USA). Data were expressed as mean±SD. Data distribution was assessed using the Kolmogorov-Smirnov test. Analysis of variance was used to compare Ct values between groups. Differences were considered significant when $P<0.05$.

2 RESULTS

2.1 Levels of SARS-CoV-2 RNA in Non-inactivated Control Samples

Based on analysis of the control samples, 7 of 12 (58.3%) patients were tested positive for SARS-CoV-2 nucleic acid (table 1). One patient was classified as having a high concentration of nucleic acid (Ct <30), 3 as having a medium concentration (Ct, 30–35), and 3 as having a low concentration (Ct, 36–40).

Table 1 Cycle threshold values and overall results of real-time fluorescence PCR testing for SARS-CoV-2 RNA in non-inactivated control samples

Samples	Cycle threshold	Result
1	34.83	Positive
2	33.58	Positive
3	Unknown	Negative
4	35.67	Positive
5	38.64	Positive
6	27.59	Positive
7	Unknown	Negative
8	Unknown	Negative
9	33.79	Positive
10	Unknown	Negative
11	38.24	Positive
12	Unknown	Negative

2.2 Levels of SARS-CoV-2 RNA in Inactivated Samples

Inactivated samples showed similar Ct values and similar overall positive/negative results as the control samples. The SARS-CoV-2 RNA was processed with 4 temperature values and 3 time durations were set at each temperature value [56°C (30, 45, and 60 min); 65°C/70°C/80°C (10, 15, and 20 min). The Ct values of the test results were similar, and there were no statistically significant differences among the time points ($P>0.05$) (table 2). Similarly, when the processing time was uniform but the processing temperature was different, the test results were not statistically different ($P>0.05$) (table 3).

In addition, the 7 positive samples included

Table 2 Comparison of SARS-CoV-2 nucleic acid concentration at different treatment time durations

Processing temperature (°C)	Time (min)/cycle threshold			*F*	*P*
56°C	30	45	60	0.177	0.839
	34.33±1.31	33.77±1.16	34.90±1.51		
65°C	10	15	20	0.006	0.994
	33.68±1.57	33.49±1.24	33.49±1.20		
70°C	10	15	20	0.248	0.783
	34.04±1.31	33.44±1.16	34.7±1.39		
80°C	10	15	20	0.128	0.881
	34.26±1.28	35.18±1.46	34.98±1.32		

Table 3 Comparison of SARS-CoV-2 nucleic acid concentration at different treatment temperature

Inactivation time (min)	Cycle threshold after inactivation at			*F*	*P*
	65°C	70°C	80°C		
10	33.68±1.57	34.04±1.31	34.26±1.28	0.045	0.956
15	33.49±1.24	33.44±1.16	35.18±1.46	0.589	0.565
20	33.49±1.20	34.72±1.39	34.98±0.73	0.372	0.695

high-concentration, medium-concentration, and low-concentration samples, and the results showed that each concentration curve showed a typical fluorescence quantitative amplification curve (fig. 1). These results suggest minimal influence of inactivation temperature or duration on levels of viral RNA prior to PCR testing.

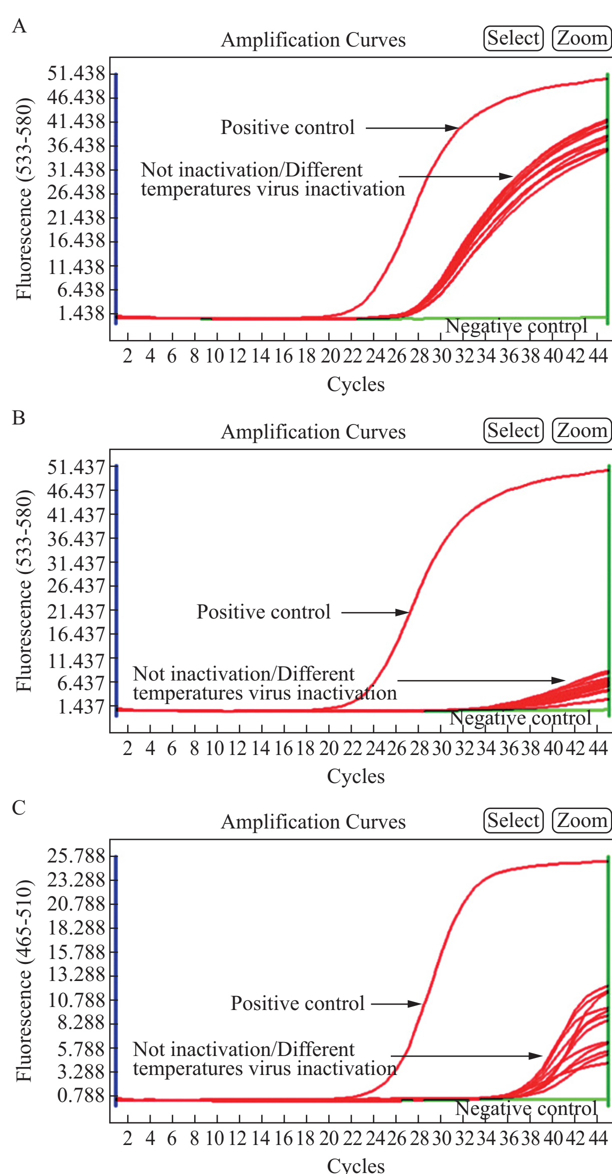

Fig. 1 Typical PCR amplification curves of patients whose throat swabs showed (A) high concentration of SARS-CoV-2 RNA (cycle threshold <30), (B) medium concentration (cycle threshold of 30–35), or (C) low concentration (cycle threshold >35). Each panel shows the results of a single patient at different temperatures for different periods of virus inactivation.

3 DISCUSSION

With the sudden outbreak of the new SARS-CoV-2 and its rapid spread, the health of the general public is seriously threatened[8]. The focus of COVID-19 prevention and control is mainly on early detection, diagnosis, isolation, and treatment. Real-time fluorescence PCR to detect viral nucleic acid is one of the major diagnostic methods[9]. Given the highly infectious nature of the SARS-CoV-2, it is of great importance to find ways to reduce risk of virus spread among laboratory personnel. This will have important implications in carrying out SARS-CoV-2 testing worldwide.

Previous studies have shown that SARS-CoV was completely inactivated by heating at 56°C for 60 min or longer[10]. Similarly, SARS-CoV-2 is sensitive to heat and can be effectively inactivated at 56°C for 30 min[7]. In this study, we found that different inactivation temperatures and different inactivation time durations had no significant effect on PCR detection of SARS-CoV-2 RNA. This may be related to the protective effects of the preservation solution, which contains antibiotics, protein protection agent, buffer, glycerin, and nuclease inhibitors. The inactivation temperature should be high enough to eliminate virus but not high enough to damage viral RNA or the PCR tube. In order to improve the laboratory testing efficiency as much as possible, the inactivation time duration should not be too long. Based on our results, we suggest virus inactivation at 65°C for 10 min. This recommendation should be verified using larger number of samples and different reagent test kits.

Conflict of Interest Statement

I declare on behalf of my co-authors and myself that we do not have any conflict of interest to declare.

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