Heat inactivation decreases the qualitative real-time RT-PCR detection rates of clinical samples with high cycle threshold values in COVID-19

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ABSTRACT

SARS-CoV-2 has caused COVID-19 pandemic globally in the beginning of 2020, and qualitative real-time RT-PCR has become the gold standard in diagnosis. As SARSCoV-2 with strong transmissibility and pathogenicity, it has become a professional consensus that clinical samples from suspected patients should be heat inactivated at 56℃ for 30 min before further processing. However, previous studies on the effect of inactivation on qualitative real-time RT-PCR were conducted with diluted samples rather than clinical samples. The aim of this study was to investigate whether heat inactivation on clinical samples before detection will affect the accuracy of qualitative real-time RT-PCR detection. All 46 throat swab samples from 46 confirmed inpatients were detected by qualitative real-time RT-PCR directly, as well as after heat inactivation. Heat-Inactivation has significantly influenced the qualitative detection results on clinical samples, especially weakly positive samples. The results indicate the urgency to establish a more suitable protocol for COVID-19 clinical sample's inactivation.

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1. Introduction

SARS-CoV-2 had firstly caused an outbreak of COVID-19 in Wuhan City, China, in December 2019 (Wu et al., 2020a) (https://www.who.int/csr/don/05-january-2020-pneumonia-of-unkown-cause-china/en/). Then, the virus spread rapidly through person to person (Chan et al., 2020). According to Coronavirus disease (COVID-19) situation reports released by WHO, by May 31, 2020, about 5.9 million cases were confirmed globally, including 367, 166 deaths (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports).

Development of accurate and prompt diagnosis is critically important in COVID-19 control and prevention (Yip et al., 2020). Qualitative real-time reverse transcription-polymerase chain reaction (qualitative real-time RT-PCR) has become the gold standard in COVID-19 diagnosis. Till now, hundreds of RNA detection products have been rapidly developed for use in epidemic control. However, a great number of false negative results were generated by qualitative real-time RT-PCR in patients who were identified as positive by clinical diagnosis. Such false negative results have been attributed to improper sampling time, method and location, nuclease degradation (inappropriate sample preservation or sample inactivation), and insensitivity of diagnosis reagents (lack of adequate clinical verification and optimization) (Wang et al., 2020). With relatively high false negative rates, feces, blood, urine and anal swabs were not appropriate for qualitative real-time RT-PCR detection of SARS-CoV-2 (Wang et al., 2020; Wu et al., 2020b). Based on the virus' high transmissibility and pathogenicity, the Chinese Society of Laboratory Medicine (Chinese Society of Laboratory Medicine, 2020) recommended heat inactivation of clinical samples at 56 ℃ for 30 min before processing for detection. Therefore, heat-inactivation was considered as one of the possible causes of false negative. Previous studies have different conclusions on whether sample inactivation reduces the detection rate of SARS-CoV-2, and only from using diluted samples. One study reported that samples diluted to low concentrations appeared false negative after treatment with different methods of inactivation (Duan et al., 2020). By contraries,

another study concluded that inactivation with high temperature or 75% ethanol had no effect on qualitative real-time RT-PCR detection in 2 diluted samples (Chen et al., 2020). As there was no published investigation on clinical samples without dilution, the influence caused by inactivation on detection rate of real-clinical samples was still unknown. This study is first to report on the effect of heat inactivation and reduced detection by qualitative real-time RT-PCR using 46 COVID-19 clinical samples.

The aim of this study was to investigate the influence of heat inactivation on COVID-19 samples at 56 °C for 30 min on the detection sensitivity of SARS-CoV-2 by qualitative real-time RT-PCR.

2. Materials and methods

2.1. Clinical samples

Between February 18, 2020, to February 23, 2020, 46 throat swab samples were collected from 46 confirmed inpatients in Chongqing University Central Hospital. The patients were diagnosed with COVID-19 based on the Handbook of COVID-19 Diagnosis and Treatment released by National Health Commission of the People's Republic of China (http://www.nhc. gov.cn/yzygj/s7653p/202003/46c9294a7dfe4cef80dc7f5912eb1989/ files/ce3e6945832a438eaae415350a8ce964.pdf) (The handbook provides a summary of epidemiological characteristics, clinical manifestations and pathologic changes of COVID-19 and etiological characteristics of SARS-CoV-2, and formulated the diagnostic criteria, clinical classification criteria and treatment plans for COVID-19. COVID-19 cases should be confirmed by nucleic acid tests or antibody tests). Samples were preserved in 1 mL of sterile viral preservation medium and stored at -80 °C (Disposable Virus Sampling Tubes, Chongqing Lingjun Medical Equipment Co., Ltd., Chongqing, China).

2.2. Detection of SARS-CoV-2 RNA by qualitative real-time RT-PCR

The samples of the treated group were heat inactivated at 56 °C water bath for 30 minutes, while matched samples of the untreated group (non-heat inactivated) were maintained at 4 °C for the same time. A mixture of pseudovirus containing target fragments and internal control fragments was used as positive control and pseudovirus containing internal control fragments was used as negative control. The viral RNA was extracted from 200 μL of each sample, and was eluted with 50 μL of elution buffer using the Viral Nucleic Acid Isolation Kit (Magnetic Beads) (Bioperfectus, China), according to the manufacturer's instructions.

Qualitative real-time RT-PCR was performed on a 25 μL reaction mixture containing 5 μL RNA template extracted from treated and untreated samples or controls using the COVID-19 Qualitative Real Time PCR Kit (Da An Gene, China) which targeted internal control, ORF1ab and N gene and, with the following conditions: reverse transcription at 50 °C for 15 minutes, initial denaturation at 95 °C for 15 seconds, followed by 45 cycles at 94 °C for 15 seconds and 55 °C for 45 seconds. PCR was performed in Bio-Rad CFX96 Real-time PCR system.

According to the kit's instruction, a positive result was identified with a Ct<40. Only if results of internal control were normal, N+ ORF1ab+ double positive samples were confirmed as positive, N+ ORF1ab- or N- ORF1ab+ single positive samples as suspected and double negative samples as negative.

In this study, virus amount >10×LOD were regarded as higher virus amount. Since no quantitative RT-PCR assays have been validated, the Ct value of 10×LOD could not be exactly given. Consequently, the Ct value of 37, an integer, was chosen.

2.3. Statistical analysis

Statistical analysis on positive rates of the inactivation group and the untreated group was performed using Wilcoxon signed rank test. All statistical analyses were performed with SPSS software.

3. Results

In order to investigate the effect of heat-inactivation on SARS-CoV-2 detection accuracy in clinical samples, 46 throat swab samples were collected from inpatients diagnosed as COVID-19. The cases had an average age of 52.35 ± 5.77 years, with male-female ratio at 1.19: 1. The average days from confirmation to sample collection was 17 ± 2.00 days; 10.87%, 76.09%, and 13.04% were clinically classified as mild, normal and critically severe type, respectively.

In the inactivation group, 69.57% cases were N gene positive and 52.17% were ORF1ab gene positive. 24, 8 and 14 cases were confirmed as positive, suspected and negative cases respectively. In the untreated group, 89.13% cases were N gene positive and 78.26% were ORF1ab gene positive; 36, 5 and 5 cases were confirmed as positive, suspected and negative cases respectively. The 5 cases which tested negative might had eliminated the viruses after therapy. There was a significant difference in positive rates of double targets detection among two groups ($P = 0.000$) (Table 1). After heat inactivation treatment, 13.04% (6/46) positive samples turned to negative, 13.04% (6/46) positive samples tuned to suspected and 6.52% (3/46) suspected samples turned to negative (Table 1).

According to Ct value of untreated group, samples were divided into 2 sub-groups of higher amount of virus (Ct<37) and lower amount of virus (Ct≥37) respectively. In higher amount of virus sub-group, the detection results of both gene of the inactivation group completely matched the outcomes of the untreated group, but some of them showed higher Ct values than their corresponding untreated samples. However, in lower amount of virus sub-groups, the N gene positive rates in inactivation and untreated group were 39.13% and 78.26% (P value was 0.003) separately, while 24.14% and 65.52% in ORF1ab gene (with P value of 0.001), means that inactivation lead to a large number of false negatives (Table 2).

4. Discussion

In early 2020, SARS-CoV-2 has caused global pandemics (Bommer et al., 2017). Various measures were implemented to prevent the outbreak, and accurate diagnosis was one of the forceful measures. Among pathogen diagnosis technologies, qualitative real-time RT-PCR was used as the golden standard for patient confirmation. However, false negatives were common in newly infected patients and discharged patients who remained infectious (Suo et al., 2020), and this brought tremendous threaten to disease control and prevention. More efforts should be done to improve the accuracy of nucleic acid detection.

Besides the reagents themselves and sampling sites, appropriate viral preservation mediums and inactivation methods played important roles in clinical nucleic acid testing. Two research teams had performed studies on diluted samples which were very different from clinical samples and drew opposite conclusions (Chen et al., 2020; Duan et al., 2020).

Table 1
Positive detection rates of COVID-19 samples by single targets and double targets.

	N		ORF1ab		Double targets		
	Positive rate	P value	Positive rate	P value	Positive rate	Suspective rate	P value
Inactivation group	69.57% (32/46)	.003	52.17% (24/46)	.001	52.17% (24/46)	17.39% (8/46)	.000
Untreated group	89.13% (41/46)		78.26% (36/46)		78.26% (36/46)	10.87% (5/46)	

Table 2
Positive detection rates of COVID-19 samples by single target in groups with different virus amount.

	N		P value	ORF1ab		P value
	Positive rate			Positive rate		
	Inactivation group	Untreated group		Inactivation group	Untreated group	
Higher amount of virus (Ct<37)	100% (23/23)	100% (23/23)	1.000	100% (17/17)	100% (17/17)	1.000
Lower amount of virus (Ct≥37)	39.13% (9/23)	78.26% (18/23)	0.003	24.14% (7/29)	65.52% (19/29)	0.001

This was the first study to compare the detection rate of SARS-CoV-2 in undiluted clinical samples with heat inactivation or not, especially those with lower amount of virus. The research demonstrated that heat inactivation largely decreased the detection rates. Furthermore, this study showed that missed detections were all identified in samples with higher Ct values (lower amount of virus). There might be 3 reasons for this situation. Firstly, the present viral preservation medium was designed for protection of virus in low temperatures for later virus isolation, but not in a high temperature for virus inactivation, thus when heat-inactivated, virus nucleic acids might easily degraded. Secondly, treating at 56 °C for 30 min might be overly restrictive for SARS-CoV-2 RNA. Thirdly, prolonged heating might result to the production of PCR inhibitors.

False negatives in nucleic acid detections of SARS-CoV-2 should draw sufficient attentions. More proper virus preservation medium for virus nucleic acid detection and sampling strategies for both virus isolation and detection should be developed. More studies on both effective and mild inactivation conditions for SARS-CoV-2 should be conducted as this was a newly identified virus.

5. Conclusions

To our knowledge, this is the first study performing such research with many clinical samples. The study indicated that heat inactivation treatment before detection would reduce detection rates of SARS-CoV-2 in weakly positive clinical samples by qualitative real-time RT-PCR. All in all, this study provided important clues for increasing detection rates of SARS-CoV-2 RNA.

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Declarations of competing interest

None.

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