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Impact of heat-inactivation on the detection of SARS-CoV-2 IgM and IgG antibody by ELISA

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ABSTRACT

Background: To establish a safe and accurate method for detecting SARS-CoV-2 IgM and IgG, we assessed the impact of sera after heat-inactivation on the SARS-CoV-2 IgM and IgG levels measured by ELISA-immunoassay.

Methods: The serum samples of 62 patients with COVID-19 and 18 healthy controls were collected in Hankou’s Hospital of Wuhan from February 27 to March 6, 2020. Before and after the samples were inactivated, the levels of IgM and IgG antibodies were measured.

Results: The indexes of antibodies after inactivated were significantly higher than those in fresh sera, while the positive rates in all participants or in patients with COVID-19 did not change. The positive coincidence rate, negative coincidence rate and total coincidence rate of IgM antibodies before and after inactivation were 100.00% (55/55), 96.00% (24/25) and 98.75% (79/80), respectively (κ = 0.971, P < 0.001), while those for IgG antibodies were 98.21% (55/56), 91.67% (22/24) and 98.75% (79/80) respectively (κ = 0.910, P < 0.001). These results showed a good consistency.

Conclusions: Heating-activation does not decrease the diagnostic efficacy of SARS-CoV-2 IgM or IgG antibodies. Sera inactivated by heating at 56 °C for 30 min should be recommended to minimize the risk of virus contamination of laboratory staff.

1. Introduction

As the largest known RNA viruses, coronaviruses (CoVs) are further divided into four genera: α-CoVs, β-CoVs, γ-CoVs, and δ-CoVs, among which α- and β-CoVs are able to infect mammals [1,2], whereas the other two genera can infect birds and could also infect mammals. According to the previous researches, COVID-19 was caused by SARS-CoV-2 [3], a new member of β-CoVs that was recently isolated from human airway epithelial cells, characterized by next-generation sequencing in January 2020 [4].

This public health concern intense attention not only within China but internationally. Although the COVID-19 outbreak has led to implementation extraordinary public health measures to reduce further spread of the virus within China and elsewhere. On March 11, 2020, the coronavirus outbreak was declared a global pandemic by the WHO. The spread and potential lasting existence of the COVID-19 pose severe threats to the world [5]. Consequently, how to increase the detection of the virus and find more potential therapeutic strategies become of vital importance for scientists and clinicians globally.

The nucleic acid of SARS-CoV-2 RT-PCR test has become the standard method of diagnosis of COVID-19 [6], although these real-time PCR test kits have many limitations such as high false negative rates and long turnaround times [7]. Some companies have developed specific antibodies of SARS-CoV-2 testing kit to quickly identify infected patients to prevent virus transmission and to assure timely treatment. It is widely accepted that IgM provides the first line of defense during viral infections, prior to the generation of adaptive, high affinity IgG responses that are important for long term immunity and
immunological memory [8]. According to Chinese Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia (Trial Version 7), SARS-CoV-2 specific IgM becomes detectable around 3–5 days after onset and IgG reaches a titration of at least 4-fold increase during convalescence compared with the acute [9]. Therefore the detection of IgM and IgG antiviral antibodies in the serum samples from patients can be additional evidence to confirm COVID-19. To reduce the risk of accidental exposure of laboratory workers, sera of patients will be heat-inactivated. Previous study showed that coronaviruses are vulnerable to heat and heating at 56 °C for 30 min could reduce more than 4 log10 TCID50/ml of MERS virus (the sixth human coronavirus identified) [10]. Furthermore, a recent study showed that exposure to 56 °C for 30 min could decrease the viral titer of SARS-CoV-2 significantly although the number of detectable RNA copies of virus was not affected [11]. However, the potential impact of heat-inactivation on the levels of SARS-CoV-2 IgM or IgG antibodies is not published. In order to establish a safe and accurate method for detecting SARS-CoV-2 specific antibodies, we retrospectively assessed the impact of sera heat-inactivation at 56 °C for 30 min on the levels of SARS-CoV-2 IgM or IgG antibodies.

2. Materials and Methods

2.1. Study design and participants

For this retrospective, single-center study, we recruited 62 patients at Hankou Hospital in Wuhan, China from February 26 to March 6, 2020. All patients were diagnosed as having COVID-19 according to WHO interim guidance were enrolled in this study [12]. And 18 healthy controls in hospital were added to help the assessment. The study was approved by Hankou Hospital Ethics Committee (No. HKYY-2020-028).

2.2. Specimen

Throat-swab specimens from the upper respiratory tract that were obtained from all the cases and controls were maintained in viral-transport medium. And serum samples taken from peripheral venous were maintained in 5 ml vacuum blood collection tubes with separation gel.

2.3. Laboratory measurements

Laboratory measurements were conducted on collected specimens. SARS-CoV-2 infection was confirmed by viral nucleic acid detection with real-time RT-PCR using the same protocol described previously [13]. The IgM and IgG antibodies of SARS-CoV-2 were measured by ELISA-imunoassay using SARS-CoV-2 antibody detection kit (Guangzhou Darui Biotechnology Co, Ltd). The assay is based on a double-antigen sandwich principle that detects antibodies binding SARS-CoV-2 spike protein receptor binding domain (RBD) in sera. Serum samples were centrifuged at 3000RPM for 15 min to obtained serum samples. Then 500 μl fresh serum was dispensed in B2 biosafety cabinet. The rest serum was incubated at 56 °C for 30 min in a water bath and cooled to room temperature by UV irradiation in the biosafety cabinet for 30 min afterwards.

2.4. Antibody detection protocol

Serum was diluted in dilution buffer by 1:100. Then the diluted serum and 100 μl negative or positive controls were added to the wells coated with recombinant SARS-CoV-2 antigen and incubated for 40 min at 37 °C. Wells were washed by washing solution in the kit for 5 times followed by the addition of HRP-conjugated SARS-CoV-2 antigen and subsequent incubation for 20 min at 37 °C. Wells were washed five times and urea peroxide and tetramethylbenzidine were added for colorimetric reaction. Following 10 min of incubation at 37 °C, the reaction was stopped by 2 mol/L H2SO4 solution and the resultant absorbance was read on a microplate reader at 450 nm.

2.5. Serological interpretation

The cut-off value of IgM antibody was defined as the mean index of negative control duplicate wells plus 0.25, while that of IgG antibody was defined as the mean index of negative control duplicate wells plus 20% of the mean index of positive control duplicate wells. And the results determined by the manufacture are; negative < cut-off value; cut-off value ≤ weak positive < 0.5; greater than 0.5 positive. All testing was done in accordance with the manufacturer’s guidelines [14].

2.6. Statistical analysis

We present continuous measurements as mean (SD) if they are normally distributed or median (IQR) if they are not, and categorical variables as count (%). For laboratory results, we also assessed whether the measurements were outside the normal range. We used SPSS (version 24.0) for all analyses. Wilcoxon test was used to compare non-parametric variables before and after heating. Categorical variables was compared with Kruskal-Wallis test. Kappa test was used to evaluate the consistency of detection between fresh sera and heated sera. Statistical significance was defined as \( P < 0.05 \).

3. Results

3.1. Baseline characteristics of all participants

The cut-off values of different dates for the assay were shown in Table 1. Baseline characteristics of 80 participants are shown in Table 2, stratified by cases/healthy controls. 77.50% of participants were patients. 60% of participants were male. The median age was 65 ranged from 26 to 94 in all participants. The optical density (OD) at 450 nm of antibodies measured after heating were significantly higher than those in fresh sera (Wilcoxon test: \( Z = -3.729 \) for IgM, \( P < 0.001; Z = -7.609 \) for IgG, \( P < 0.001 \)). The specific data were shown in Fig. 1A and Fig. 1B.

3.2. The indexes of antibodies and detection results in healthy controls

Among the 18 healthy controls, the indexes of IgG antibody increased when the sera were heat-inactivated (Wilcoxon test: \( Z = -3.224, P = 0.001 \)), while there were no statistical differences for IgM in both conditions (Wilcoxon test: \( Z = -1.136, P = 0.256 \)). However, the detection results of both of IgM and IgG antibodies remained negative whether the sera heated or not.

3.3. The indexes of antibodies and detection results in cases

When 62 sera of patients analyzed, as seen in Table 3, the indexes of IgM or IgG antibody increased when the sera were heat-inactivated (Wilcoxon test: \( Z = -3.390 \) for IgM, \( P = 0.001; Z = -6.797 \) for IgG, \( P < 0.001 \)). Additionally, the positive detection rate in patients did not
change when sera were heat-inactivated. ($\chi^2$ test: $\chi^2 = 0.086, P = 0.769$ for IgM before and after inactivation; $\chi^2 = 0.100, P = 0.752$ for IgG before and after inactivation).

### 3.4. Biological interpretation discrepancy and consistency test

Then we analyzed the biological interpretation of IgM and IgG before and after heating. There was one sample changed from negative to positive after heating in IgM detection. One positive sample became negative while two negative samples became positive after heating in IgG detection. There was 1 sample changed from negative to positive both in IgM and IgG detection. The data were shown in Fig. 1C and D and the specific changed samples were plotted in red color.

Furthermore, Table 4 showed that the positive coincident rates, negative coincident rates and total coincident rates of SARS-CoV-2 antibodies in patients before and after inactivation. The Kappa value of the test for IgM was 0.971, and the positive coincidence rate, negative coincidence rate and total coincidence rate of IgM antibodies before and after inactivation were 100.00% (55/55), 96.00% (24/25) and 98.75% (79/80), respectively. In addition, The Kappa value of the test for IgG was 0.910, and the positive coincidence rate, negative coincidence rate and total coincidence rate of IgG antibodies before and after inactivation were 98.21% (55/56), 91.67% (22/24) and 98.75% (79/80).

The results above showed that the consistency of the antibodies detection before and after heating was high although the specific indexes of antibodies changed slightly.

### 4. Discussion

As the outbreak and spread of COVID-19, scientists and clinicians globally are working swiftly to combat this disease. The diagnostic assays have been developed rapidly in China and other countries, and have played significant roles in diagnosis, monitoring, surveillance and infection control [15].

The nucleic acid of SARS-CoV-2 RT-PCR test has become the standard method of diagnosis of COVID-19 [6]. However, there are many limitations for the real-time PCR test kits: (1) The PCR tests require certified laboratories, expensive equipment and trained technicians to operate. (2) The high false negative rate of PCR makes it difficult to discover all of the COVID-19 patients and the asymptomatic infection [7]. The process of blood sample collection is more controllable making the detection of antibody more reliable. Furthermore, ELISA has a high sensitivity of IgM detection, which is beneficial to early diagnosis of COVID-19 patients. Thus some companies have developed specific antibodies of SARS-CoV-2 testing kit to quickly identify infected patients to prevent virus transmission and to assure timely treatment. Rapid detection of both IgM and IgG antibodies will play vital role in diagnosis and treatment of COVID-19.

Evidence showed that SARS-CoV-2 could be detected in blood, raising the possibilities of blood transmission [16]. According to previous data, SARS-CoV RNA was detected in 50% of plasma and 78% of serum samples during the first week of illness [17]. Heating treatment at 56 °C for 30 min is widely used to inactivate the virus for further detection or research. It has been demonstrated that during detection, inactivation of virus could reduce the risk of accidental exposure of laboratory workers [18,19]. However, the potential impact of heat-inactivation of sera for SARS-CoV-2 IgM or IgG is not validated.

In our study, the indexes of sera IgM and IgG increased after heating-activation using ELISA. The positive detection rate in patients did not change when sera were heat-inactivated. The positive coincidence rate, negative coincidence rate and total coincidence rate of IgM antibodies before and after inactivation were 100.00% (55/55),

### Table 2

Baseline characteristics of participants.

|                | All | Cases | Healthy controls |
|----------------|-----|-------|------------------|
| N              | 80  | 62    | 18               |
| Age (years)    | 65(26–94) | 65(28–94) | 66 (26–94) |
| Sex (male)     | 48(60.0%) | 37(59.7%) | 11(61.1%) |

Fig. 1. The indexes of IgM or IgG in sera. A: IgM indexes in all sera before and after inactivation; B: IgG indexes in all sera before and after heating; C: The consistency of IgM indexes before and after heating (the biological interpretation sample was plotted in red color and the CV was 13.12% versus serum after inactivation); D: The consistency of IgG indexes before and after heating (the biological interpretation samples were plotted in red color and the CVs were 68.73%, 37.61% and 31.68% versus sera after inactivation).
Previous study indicated that heating inactivation of sera may bring to false negative results in anti-Toxoplasma IgM testing [20]. The main reason for this may be the production of aggregates composed of IgA, IgM, IgG an albumin resulting from heat-induced aggregation of serum proteins [21]. However, some other studies showed that heating sera may lead to false positive results or eliminate heat-labile factors which could give false-positive results. For instance, using the Abbott HIV EIA assay with heating sera may cause false-positive results or eliminate heat-labile factors which could give false-positive results. For instance, using the Abbott HIV EIA assay with heating sera may cause false-positive results or eliminate heat-labile factors which could give false-positive results. For instance, using the Abbott HIV EIA assay with heating sera may cause false-positive results or eliminate heat-labile factors which could give false-positive results. For instance, using the Abbott HIV EIA assay with heating sera may cause false-positive results.

In summary, sera inactivated by heating at 56 °C for 30 min could minimize the risk of virus contamination and did not impair the positive detection rate using SARS-CoV-2 antibody detection kit (ELISA-immunoassay) and eventually represents a valuable contribution to a safer COVID-19 serological diagnosis. However, as the small samples in our study, more experiments and samples in other laboratories are needed to validate the results.

Table 3
The indexes of antibodies and detection results in cases.

| Antibody | Fresh ELISA | Inactivated ELISA | Z | P | Positive rates | χ² | P |
|----------|-------------|-------------------|---|---|----------------|----|---|
| IgM      | 0.868       | 1.229             | −3.390 | 0.001 | 55/62 | 56/62 | 0.086 | 0.769 |
|          | (0.148–3.469) | (0.175–3.358) |        |    |      |      |    |    |
| IgG      | 0.438       | 0.633             | −6.797 | <0.001 | 56/62 | 57/62 | 0.100 | 0.752 |
|          | (0.044–0.996) | (0.071–1.282) |        |    |      |      |    |    |

* P = 0.001 vs. the fresh IgM group for Wilcoxon test.

Table 4
The serological results based on IgM or IgG before and after heating.

| Antibody | Results | Inactivation sera | Total | k | P |
|----------|---------|-------------------|-------|---|---|
| Fresh sera | IgM | Positive | 55 | 0 | 55 | 0.971 | 0.000<sup>a</sup> |
|           | Negative | 1 | 24 | 25 | 0.996 | 0.000<sup>a</sup> |
|           | Total | 56 | 24 | 80 |       |       |       |
| IgG | Positive | 55 | 1 | 56 | 0.910 | 0.000<sup>d</sup> |
|        | Negative | 2 | 22 | 24 |       |       |       |
|        | Total | 57 | 23 | 80 |       |       |       |

<sup>a</sup> P < 0.001 vs. the fresh IgM group for kappa test.
<sup>d</sup> P < 0.001 vs. the fresh IgG group for kappa test.

96.00% (24/25) and 98.75% (79/80), respectively. In addition, The positive coincidence rate, negative coincidence rate and total coincidence rate of IgG antibodies before and after inactivation were 98.21% (55/56), 91.67% (22/24) and 98.75% (79/80). The Kappa test result showed that the consistency of the antibodies detection before and after heating was high using SARS-CoV-2 antibody detection kit (ELISA-immunoassay).

5. Conclusion
In summary, sera inactivated by heating at 56 °C for 30 min could minimize the risk of virus contamination and did not impair the positive detection rate using SARS-CoV-2 antibody detection kit (ELISA-immunoassay) and eventually represents a valuable contribution to a safer COVID-19 serological diagnosis. However, as the small samples in our study, more experiments and samples in other laboratories are needed to validate the results.

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Author contributions
All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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Declaration of Competing Interest
The authors declare that they have no competing interests.

Ethical approval
The study was approved by Hankou Hospital Ethics Committee (No. HKYY-2020-028).

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