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ORIGINAL ARTICLE

Short Prewarming at 41°C to Correct the Interference in Samples with the Presence of Cold Agglutination

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SUMMARY

Background: **Cold agglutinins (CAs) in blood samples can cause a reversible agglutination of red blood cell (RBC) which result in an incorrect complete blood count (CBC). So, it is important to explore new simple and feasible treatment conditions for clinical work.**
Methods: **The CAs group included 32 samples with CAs. The parameters of CBC at room temperature or after prewarming at 37°C or 41°C for different time periods were compared. The consistency and correlation of those parameters were analyzed. The morphology of erythrocytes in the CAs group was observed manually. The control group included 45 samples without CAs and prewarmed at 37°C or 41°C for different time periods. The differences were also analyzed.**
Results: **CAs have a significant effect on CBC. After prewarming at 37°C or 41°C the interferences are all corrected. Consider prewarming at 37°C for 120 minutes as the standard procedure. The consistency and correlation analysis showed there was no statistical difference between the results of each subgroup and standard group, except the MCHC of group 41°C 10 minutes. The correlation of parameters between all subgroups and the standard group is satisfied. Microscopic examination showed no RBC aggregation or fragmentation after prewarming at 41°C or 37°C. According to the maximum bias requirements for expert performance in Validation, Verification, and Quality Assurance of Automated Hematology Analyzers, 2nd Edition (CLSI H26-A2), the differences in overall results in control group are negligible.**
Conclusions: **The 41°C 2 minutes prewarming method is a rapid and effective way for treating samples with CAs. It is an efficient way to obtain more reliable CBC results, without specific instruments.**
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KEYWORDS

cold agglutinins, cold agglutinin disease, complete blood count, interference

INTRODUCTION

Cold agglutinin disease (CAD) is a type of autoimmune hemolytic anemia [1]. The autoantibodies that cause hemolysis are called cold agglutinins (CAs), which most often is the immunoglobulin M (IgM) class that reacts with the antigen on the erythrocyte surface depending on the ambient temperature. Phlebotomy, even cooling of blood during passage through acral parts of the circulation allows CAs to bind to erythrocytes and cause agglutination. However, when the ambient temperature returns to 37°C, IgM-CA detaches from the cell surface, allowing agglutinated erythrocytes to separate from each other [2]. Thus, early studies have found that the effects of CAs on red blood cells (CBC) can be gradually corrected as the temperature increases [3]. Due to the lack of clear guidelines to regulate this, it is common for laboratories to use 37°C for 120 minutes prewarming to eliminate interference by CAs which is time-consuming and laborious [4]. So, it is particularly important to explore new simple and feasible treatment conditions for clinical work. Complete blood count (CBC) is one of the most commonly used tests in clinical practice and the interference of CAs is particularly significant. A study has shown that the interference of CAs with CBC can be eliminated by using a short water bath at 41°C. Therefore, our study compared the effects of 30 minutes and 120 minutes prewarming at 37°C to investigate the feasibility of prewarming samples at 41°C for 2 minutes or 10 minutes.

MATERIALS AND METHODS

Subjects

All enrolled samples were obtained from outpatients of Zhongshan Hospital, Fudan University between January 2022 and May 2022, and all peripheral blood samples were collected in K_2EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA).

CAs group: 32 specimens were enrolled, and all specimens were confirmed to have erythrocyte cold agglutinins, confirmed by the optical microscope. There was no jaundice, lipemia, or hemolysis.

Control group: 45 specimens were randomly selected. All specimens were confirmed without CAs by the optical microscope and free of jaundice, lipemia, and hemolysis.

Instrument

All tests were performed using Sysmex XN series (Sysmex, Kobe, Japan) and supporting reagents.

Specimen testing

Specimens from the CAs group were divided into 5 aliquots (250 μL) and treated as follows:

1) Mix upside down at room temperature.
2) Prewarm at 37°C for 30 minutes.
3) Prewarm at 37°C for 120 minutes.
4) Prewarm at 41°C for 2 minutes.
5) Prewarm at 41°C for 10 minutes.

After the samples were processed as described above, tests were performed using the CBC+RET channel (groups 1, 3, 4, 5) or the CBC channel (group 2), and the following parameters were recorded: red blood cell (RBC), mean cellular hemoglobin (MCHC), hemoglobin (HGB), hematocrit (HCT), reticulocyte% (RET%), reticulocyte #(RET#). Consider the 37°C for 120 minutes group as the standard group.

Specimens from the control group were divided into 4 aliquots (250 μL) and the above operations were repeated for subgroups 1, 3, 4, and 5. After the specimens were processed, tests were performed using the CBC channel. Then the following parameters were recorded: RBC, WBC, PLT (denoted by $RBC_{control}$, $WBC_{control}$, and $PLT_{control}$).

Data analysis

All statistical analyses were completed using GraphPad Prism (Ver8.0.2.263).

The verification of the normal distribution of data was performed with the Shapiro-Wilk test. Normally distributed data are expressed using mean ± SD, while non-normally distributed data are expressed as the median with quartiles (P25 and P75). Consistency analysis was performed with a paired *t*-test and Wilcoxon test. The agreement between different subgroups was assessed with Pearson's correlation coefficient (r2). Percent difference analysis was performed using the formula: (pre-treatment result - post-treatment result)/pre-treatment result * 100%.

RESULTS

Cold agglutination group

The comparison between the CBC and RET parameters performed at room temperature and after different prewarming conditions.

The main results of the CBC and RET parameters performed at room temperature and after prewarming under different conditions in the CAs group as well as the consistency analysis and correlation analysis are shown in Table 1 and 2. CAS has a significant effect on RBC, HCT, MCHC, and RET#, with a decrease in the RBC, HCT, and RET# and a significant false high value of MCHC but seems to have no effect on HGB (all subgroup $p > 0.05$, compared with RT group). The consistency analysis and correlation analysis between the results of each subgroup and the standard group showed there was no statistical difference between the results of each subgroup and those of the standard group, except

Table 1. CAs group: comparison between the CBC parameters performed at room temperature and after prewarming under different conditions.

Parameter	Mean ± SD	p-value vs. group$_{RT}$	p-value vs. group$_{37°C\ 120\ minutes}$	r^2 vs. group$_{37°C\ 120\ minutes}$
RBC$_{RT}$	2.19 ± 0.15	-	-	-
RBC$_{37°C\ 120\ minutes}$	3.34 ± 0.16	< 0.0001	-	-
RBC$_{37°C\ 30\ minutes}$	3.30 ± 0.19	< 0.0001	0.9627	0.9614
RBC$_{41°C\ 2\ minutes}$	3.33 ± 0.17	< 0.0001	0.0872	0.9814
RBC$_{41°C\ 10\ minutes}$	3.31 ± 0.16	< 0.0001	0.0961	0.9796
HCT$_{RT}$	21.7 ± 1.42	-	-	-
HCT$_{37°C\ 120\ minutes}$	28.9 ± 1.53	< 0.0001	-	-
HCT$_{37°C\ 30\ minutes}$	29.3 ± 1.76	< 0.0001	0.9	0.9516
HCT$_{41°C\ 2\ minutes}$	29.2 ± 1.56	< 0.0001	0.5217	0.9749
HCT$_{41°C\ 10\ minutes}$	28.9 ± 1.52	< 0.0001	0.9277	0.9723
MCHC$_{RT}$	449 (399; 549)	-	-	-
MCHC$_{37°C\ 120\ minutes}$	343 ± 3.66	< 0.0001	-	-
MCHC$_{37°C\ 30\ minutes}$	348 ± 5.10	< 0.0001	0.1313	0.8092
MCHC$_{41°C\ 2\ minutes}$	345 ± 3.38	< 0.0001	0.3401	0.8516
MCHC$_{41°C\ 10\ minutes}$	347 ± 3.18	< 0.0001	<u>0.0383</u>	0.8243
HGB$_{RT}$	100 ± 5.25	-	-	-
HGB $_{37°C\ 120\ minutes}$	100 ± 5.17	0.1512	-	-
HGB $_{37°C\ 30\ minutes}$	103 ± 5.25	0.4045	-	-
HGB $_{41°C\ 2\ minutes}$	101 ± 5.47	0.2756	-	-
HGB $_{41°C\ 10\ minutes}$	101 ± 5.39	0.1501	-	-

RBC - red blood cell (*10^{12}/L), HCT - hematocrit (%), MCHC - mean cellular hemoglobin concentration (g/L), HGB - hemoglobin (g/L), RT - room temperature.
Statistically significant variation p-value < 0.05. Statistically significant differences underlined.
*** - shown as median (lower/upper quartile).**

Table 2. CAs group: comparison of reticulocyte assay results performed at room temperature and after prewarming under different conditions.

Parameter	Median (lower/upper quartile)	p-value vs. agglutinin	p-value vs. group$_{37°C\ 120\ minutes}$
RET%$_{RT}$	3.3 (1.7; 6.1)	-	-
RET% $_{37°C\ 120\ minutes}$	3.4 (1.8; 6.2)	<u>0.0028</u>	-
RET% $_{41°C\ 2\ minutes}$	3.5 (1.9; 6.3)	<u>0.0012</u>	0.1011
RET% $_{41°C\ 10\ minutes}$	3.5 (1.8; 6.2)	<u>0.0023</u>	0.75
RET#$_{RT}$	77.9 (31.9; 124.5)	-	-
RET# $_{37°C\ 120\ minutes}$	109.2 (65.8; 187.5)	<u>< 0.0001</u>	-
RET# $_{41°C\ 2\ minutes}$	113.4 (72.0; 184.4)	<u>< 0.0001</u>	0.0941
RET# $_{41°C\ 10\ minutes}$	103.9 (69.2; 188.7)	<u>< 0.0001</u>	0.148

RET% - reticulocyte (%), RET# - reticulocyte (*10^9/L), RT - room temperature.
Statistically significant variation p-value < 0.05. Statistically significant differences underlined.

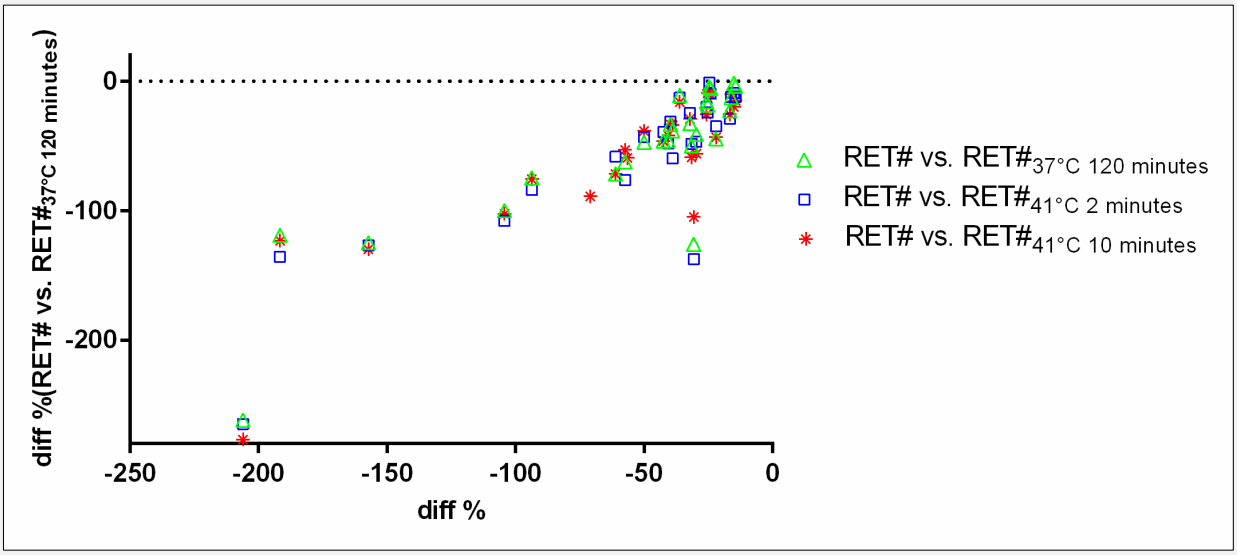

Figure 1. CAs group: Relationship between the percentage difference of RET# in different prewarming subgroups and the percentage difference of RBC in the standard group.

RBC - red blood cell, RET# - reticulocyte (*10^9/L), diff% - percentage difference.

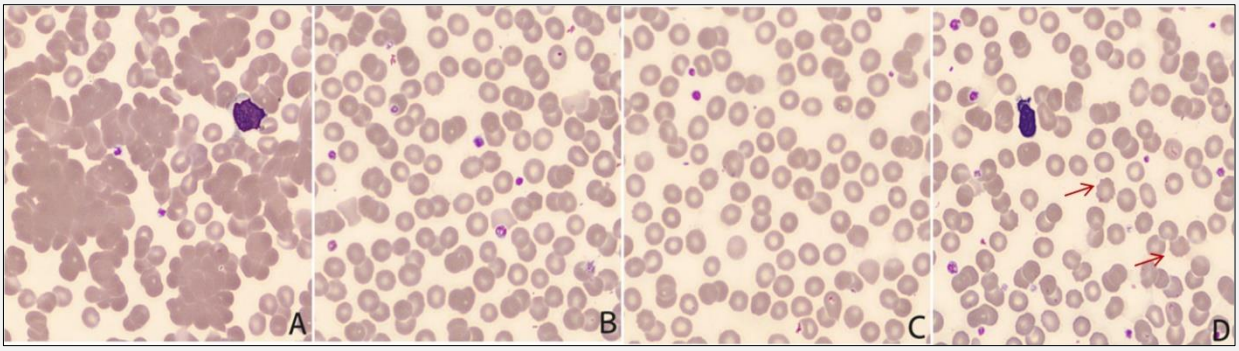

Figure 2. CAs group: Microscopic examination of peripheral blood smears after different prewarming treatments.

(A - room temperature, B - 37°C 120 minutes, C - 42°C 2 minutes, D - 41°C 10 minutes, 1,000 × objective - Wright-Giemsa stain). Few red blood cells present with mildly wrinkling (arrows shown).

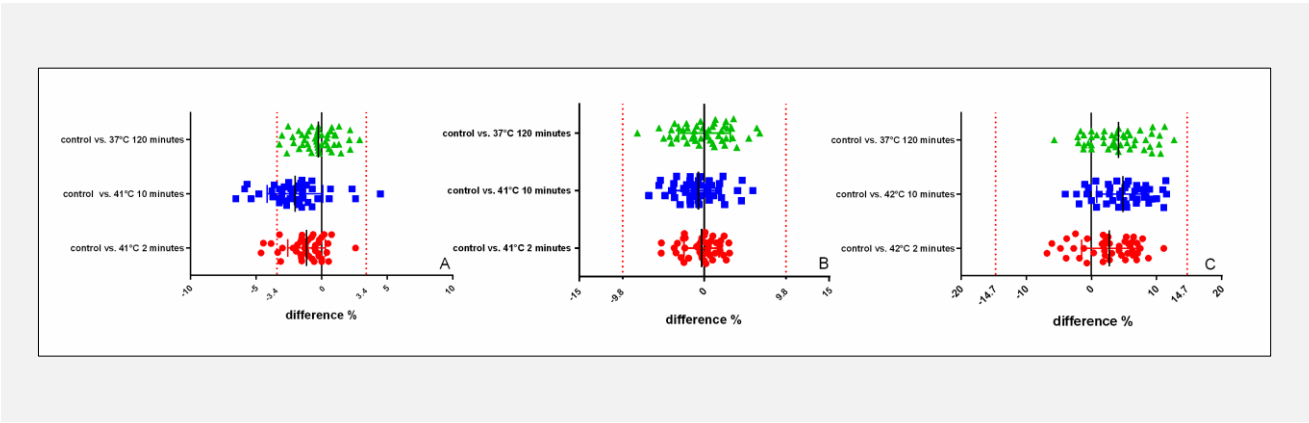

Figure 3. Control group: Differences in CBC results after warming under different conditions. RBC (A), WBC (B), and PLT (C) results of the control specimens after warming under different conditions

The dashed lines show maximum bias requirements for expert performance of each parameter in the analytical quality specifications for routine tests in clinical hematology (CLSI H26-A2) (RBC ≤ 3.4%, WBC ≤ 9.8%, PLT ≤ 14.7%).

the MCHC results of the group$_{41°C10\ minutes}$. The correlation in those parameters between all subgroups and the standard group is good.

The relationship between the percentage difference of RET# in different prewarming subgroups and the percentage difference of RBC in the standard group are shown in Figure 1. Data showed that the size of the percentage difference of RET# results in different subgroups was proportional to that of RBC results in the standard group.

CAs group

Microscopic examination of peripheral blood smears after prewarming of samples under different conditions. The microscopic examination of blood smear at room temperature as well as after warming at 37°C for 120 minutes, 41°C for 2 minutes, and 41°C for 10 minutes is shown in Figure 2.

Control group

Differences in CBC results after prewarming under different conditions

The percentage differences of RBC, WBC, and PLT results after prewarming under different conditions are compared in Figure 3. The data showed that the overall differences in the WBC results of samples in the control group after prewarming at 37°C for 120 minutes, 41°C for 2 minutes, and 10 minutes were slight (mean percent differences: -0.01%, -0.34%, -0.74%). In contrast, there was a mild increase in RBC and a decrease in PLT results after prewarming (mean percent difference RBC: -0.24%, -1.14%, -2.03%; PLT: 4.13%, 2.74%, 4.83%).

DISCUSSION

Although cold agglutinin disease may be rare, cold agglutinins exist commonly and interfere with laboratory testing significantly. According to retrospective data statistics, the positive rate of cold agglutinins in our lab was about 0.1% under normal conditions. The interference of CAs on laboratory assays is multifaceted, it has been suggested that blood samples for a certain test such as electrophoresis and immunofixation should keep at 37 - 38°C during sampling and separation of serum or plasma to avoid false low values and low sensitivity [5]. This interference with CBC and flow cytometry is even more significant, and erythrocyte aggregation may make the results of cell count less accurate. Whereas CBC is one of the most commonly used clinical tests. CA can cause a false reduction in RBC and an increase in MCHC. Since the decrease in RBCs can be seen in a variety of pathophysiologic conditions, it is less specific for the identification of CAs. However, MCHC, as a ratio of hemoglobin to hematocrit, is relatively stable and has less intra- and inter-individual variability than RBC [6] and is more likely to attract clinical attention if it is significantly abnormal.

For most labs, it is possible to take the blood sample directly on-site and measure it immediately without cooling down. We would add a note “Erythrocytes agglutination can be seen in the blood smear” in the report to give clinicians a hint and remind these patients to inform us before blood collection so that the test can be performed in time.

But the interference of CAs is still difficult to avoid entirely in the whole process of laboratory testing. Although there are several methods to eliminate the effect of CAs on the assay, such as pre-dilution and multiple plasma exchange, the most commonly used method is to

prewarm the specimen at 37°C for 120 minutes which is time-consuming. Therefore, this experiment was conducted to determine whether a shorter prewarming time (30 minutes) at 37°C could achieve the same corrective effect. Recently, it has been reported that the Sysmex XN series analyzer can use an optical method to count RBCs in the RET channel by fluorescence flow cytometry after warming at 41°C in the incubation chamber for 1 minute [7]. That can help us eliminate the interference of CAs on the assay to obtain corrected RBC results without pretreatment. However, this method requires specific instruments and cannot be applied in all laboratories. Other studies have shown that the interference caused by CAs can be effectively corrected with a short time high-temperature water bath by bringing the specimen temperature to 41°C, while the effect of high titer CAs can be corrected by the longer 41°C water bath (more than 30 minutes) [8]. Therefore, this study attempted to analyze whether a shorter period (2 minutes or 10 minutes) of 41°C prewarming can effectively correct the interference caused by CAs.

Data showed that CAs have a significant effect on RBC, HCT, and MCHC, with a decrease in RBC and HCT and a significant increase in MCHC. Of the MCHC results, 96.9% (31/32) are greater than 375 g/L, which is significantly higher than the upper limit of the reference range. After prewarming at 37°C for 30 minutes/120 minutes and 41°C for 2 minutes/10 minutes, the interferences mentioned above are corrected.

The results obtained after prewarming at 37°C for 120 minutes were set as the standard, and the results of other prewarming subgroups were compared with the standard group for consistency analysis and correlation analysis. There was no statistical difference between the results of each parameter in all subgroups and the standard group except for the MCHC results in $group_{41°C\ 10\ minutes}$ ($p = 0.0383$). The correlations between the results of each parameter in all subgroups and the standard group are reasonable, with each correlation coefficient (r^2) greater than 0.80 (Table 1). The data showed that sample prewarming at 37°C for 30 minutes basically eliminate the interference of CAs, but for an individual sample with high titer CAs (RBC $0.47*10^9$/L; MCHC 1,891 g/L), the MCHC results were still at high values (392 g/L) after prewarming for 30 minutes and the RBC results were still reduced ($2.74*10^9$/L). But after prewarming at 37°C for 120 minutes, the MCHC and RBC results could return to normal (356 g/L; $3.74*10^9$/L), and after prewarming at 41°C for 2 minutes and 10 minutes, the MCHC and RBC results also returned to the same level (2 minutes: 364 g/L, $3.42*10^9$/L; 10 minutes: 365 g/L, $3.43*10^9$/L).

The effect of CAs on RET results owns limited reports which were found mainly in RET# in this study. Although there was a statistical difference in RET% results before and after prewarming treatment, the values showed almost no difference. But the results of RET# show a clear difference in the values (see Table 2). In addition, data analysis also showed that the percentage of the difference of RET# results in different subgroups after prewarming treatment was proportional to the percentage of the difference of RBC results in the standard group after prewarming treatment (see Figure 1). This may be because the RET% is counted in the RET channel by fluorescence flow cytometry, after stabilization and warming at 41°C in the incubation chamber for 1 minute, therefore having less effect on CAs. While RET# is the product of RET% results times the RBC results counted with the traditional impedance method, so the interference degree of RET# is consistent with RBC. There was no statistical difference between the results of RET% and RET# after 41°C with 2 minutes or 10 minutes prewarming and the results after 37°C with 120 minutes prewarming. This may further indicate that short prewarming at 41°C can replace 37°C with 120 minutes prewarming and eliminate detection interference caused by CAs.

We also randomly selected some specimens with CAs and stained blood smears immediately after prewarming under different conditions. Microscopic examination showed no aggregation of erythrocytes after short prewarming at 41°C or 120 minutes prewarming at 37°C, both without erythrocyte fragmentation. Only a small number of erythrocytes were found to be mildly crinkled in the smear of the sample prewarming at 41°C for 10 minutes (see Figure 2D). This indicates that a short period of 41°C incubation does not cause damage to the erythrocytes and ensures the reliability of the results.

To verify whether prewarming at different conditions effected on the CBC results, we also selected some normal samples for the control test. The analysis of the results showed that there was no overall difference in the WBC results of the control group after prewarming at 37°C for 120 minutes, 41°C for 2 minutes, and 10 minutes (mean percent difference: -0.01%, -0.34%, -0.74%). Meanwhile, there was a slight increase in RBC and a decrease in PLT results after prewarming at three different conditions (mean percent difference RBC: -0.24%, -1.14%, -2.03%; PLT: 4.13%, 2.74%, 4.83%). If the maximum bias requirements for expert performance in Validation, Verification, and Quality Assurance of Automated Hematology Analyzers, 2nd Edition (CLSI H26-A2) [9] (RBC ≤ 3.4%, WBC ≤ 9.8, PLT ≤ 14.7%) were used as the standard, the percentage differences of RBC, WBC, and PLT results after prewarming in the $group_{37°C\ 120\ minutes}$ were all within the permissible range. While in the $group_{41°C\ 2\ minutes}$ and $group_{41°C\ 10\ minutes}$ the percentage differences of WBC and PLT results after prewarming were also all within the permissible range, and the percentage differences in RBC results exceeded the permissible range of 3.4% in 6.7% and 20%, respectively, whose maximum percentage differences were -4.62% and -6.54%, respectively. Although it is not clear what the specific cause is of this difference in results, normal samples are not subjected to prewarming. For samples with CAs, this deviation is almost negligible compared to the large percentage difference in results after prewarming caused by erythrocyte

agglutination (mean percentage difference after prewarming at 37°C for 120 minutes: -79.5%, maximum percentage difference: -555.3%). So short-time prewarming at 41°C is still an efficient and acceptable method to eliminate interference of CAs.

Compared to our work, the method based on Antonio LG et al. is to prewarm samples to 41°C, which is done by rapidly warming up the samples at 45°C to reach a core temperature of 41°C. Although the prewarming time was only 1 minute, significant erythrocyte fragments and hemolysis could occur when the specimen reached 45°C in our experiments. Another study reported that heating affected RBC membrane stability, and the deformability of RBCs significantly worsened at higher temperatures and longer period [10]. In contrast, 41°C prewarming did not destroy erythrocytes. The results of the consistency analysis and the different analysis of the control group showed that 30 minutes prewarming at 37°C could resolve the interference by CAs in most samples, but could not completely remove the RBC agglutination of the higher titer CAs samples. The 41°C 2 minutes prewarming method is a rapid and effective method for the treatment of samples with CAs. Xie et al. found the influence of cold agglutinins can't be eliminated by 37°C 120 minutes prewarming in individual specimens containing high-titer CAs [8], We also found the similar phenomena in our past work. It is still necessary to explore effective methods to eliminate the interference of high-titer CA in such specimens anyway, short prewarming at 41°C can replace the traditional 37°C 120 minutes prewarming in daily work and does not require specific instrumentation. With the global epidemic of COVID-19, CAD is becoming increasingly common in clinical settings [11-13]. We believe that the rapid and effective interference elimination methods we report can be helpful to more clinical laboratories.

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Declaration of Interest:

Authors state no conflict of interest.

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