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A prospective observational study to evaluate effect of heat inactivation on ABO titers performed by column agglutination technology and conventional tube technique

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Abstract:

BACKGROUND AND AIMS: When determining ABO antibody titers, immunoglobulin G (IgG) antibodies can be masked by immunoglobulin M (IgM) antibodies. Hence, the measurement of actual concentration of IgG requires methods like heat inactivation (HI) of plasma. This study was aimed at determining the effects of HI on IgM and IgG titers performed by conventional tube technique (CTT) and column agglutination technique (CAT).

MATERIALS AND METHODS: This was a prospective, observational study conducted from October 2019 to March 2020. All consecutive A, B, and O group donors who gave consent for participation were included. All samples were consecutively tested by CTT and CAT, before and after HI (pCTT, pCAT).

RESULTS: A total of 300 donors were included. IgG titers were found to be more than IgM titers. For group O, IgG titer results were higher for both anti-A and anti-B compared to group A and B. For group A, B, and O, pretreatment results were higher than posttreatment IgG titer results. Median anti-A titers were similar to median anti-B titers across all categories. Median IgM and IgG titers were higher for group O individuals than nongroup O individuals. There was reduction in IgG and IgM titers after HI of plasma. One log reduction in median titers was observed when ABO titers were performed by CAT and CTT.

CONCLUSION: There is one log difference between median antibody titers estimated using heat inactivated and nonheat inactivated plasma. The use of HI for ABO isoagglutinin titer estimation can be considered in low resource settings.

Keywords:

ABO, column agglutination technology, conventional tube technique, heat inactivation, titration

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Introduction

The two most important immunoglobulin types in transfusion science are immunoglobulin G (IgG) and immunoglobulin M (IgM). Anti-A and anti-B of blood groups A and B are predominantly IgM type while those of blood group O are predominantly of IgG type.[1,2] ABO antibodies are clinically significant and play a pivotal role during solid organ or hematopoietic stem cell transplant.[2-9] Concentration of these antibodies regulate immune reactions related to transfusion and transplantation; and hence, their measurement is important for such patients. Since IgG antibodies are believed to play a crucial role in graft outcome, measurement of IgG antibodies alone becomes important. The concentration of IgG can be masked by IgM antibodies

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which can lead to overestimation of IgG titers.[9] This results in increased cost of treatment costs due to vigorous use of immunosuppressants, more number of therapeutic apheresis sessions, and increased hospital stay. In order to determine the actual concentration of IgG antibodies, IgM antibodies need to be inactivated. Several methods have been described in the past to inactivate IgM such as heat inactivation at 63°C, and use of sulfhydryl reagents such as 2-mercaptoethanol (ME) and dithiothreitol (DTT).[10,11] The use of heat has several advantages over DTT such as less time consumption, no dilution of the sample and avoidance of the use of chemical agents. Since the effects of heat are relatively less on IgG, they are minimally affected.[12] However, the effects of heat are less specific than DTT as only heat sensitive proteins are denatured.

Quantitation of ABO antibodies is routinely performed by titration. Titration can be performed by different techniques. Conventional test tube technique (CTT) has been used widely but due to certain limitations of this procedure such as being labor-intensive and requiring more time along with low reproducibility, inter-observer and inter-laboratory variability, other semi and fully automated methods of titration such as column agglutination technology (CAT) and solid phase red cell adherence/hemagglutination (HA) are available.[13-16] Many studies have compared CAT with CTT and have concluded that CAT has more advantages over CTT because of high reproducibility and objectivity as well as stable well-defined end points of agglutination reactions.[14,17-20] The aim of the present study was to study the effect of HI on ABO isohemagglutinin titers performed by CTT and CAT and to compare the results of ABO isohemagglutinin titers obtained by HI of plasma performed by CTT and CAT.

Materials and Methods

Settings and design

This was a prospective, observational study conducted in the Department of Transfusion Medicine at a tertiary healthcare center from October 2019 to March 2020. All samples were simultaneously tested by CTT and CAT for anti-A and anti-B titration. For both CTT as well as CAT, plasma from each donor was heat inactivated and tests were performed before and after treatment. All results were recorded for comparison.

Study population

All consecutive A, B and O blood group donors who were eligible to donate blood as per the guidelines laid down by the Drugs and Cosmetics Act, 1940 and the standards for blood banks and blood transfusion services were included in the study with a planned sample size of 150, 50 for each group.[21,22] Samples collected in pilot tubes at the time of donation were used. After performing the routine testing, antibody titration was performed from the residual sample either on same day or on the next day of collection. If tested on the next day, the sample was stored at 4□. All donors who did not give consent to participate in the study, donors reactive for transfusion transmitted infections, samples with positive direct antiglobulin test or positive antibody screen were excluded from the study.

Methods of titration

CTT

Titration was performed by CTT according to the method described in AABB technical manual.[1] The titer end point was the reciprocal of the highest dilution yielding 1+ agglutination with naked eye. The reactions were recorded for IgM and IgG on a case reporting form.

Heat inactivation of plasma

Heat inactivation of plasma was performed by the method described in ASHI Laboratory Manual.[23] Desired amount of plasma was taken in a test tube and was placed in pre-heated 63°C heat block (MK-10, Bioexon, India) for exactly 13 min using a stop watch. After completion of 13 min, the tube was removed from heat block and was centrifuged. The supernatant was removed into another labeled test tube and was used for titration. The heat-treated sample was used on the same day.

CAT

For IgM titer, Neutral Ortho BioVue System cassettes (Ortho Clinical Diagnostics, Raritan, New Jersey, USA) were used while for IgG, Anti-IgG Monospecific Ortho BioVue System cassettes (Ortho Clinical Diagnostics, Raritan, New Jersey, USA) were used. Dilutions of heat inactivated test sample were prepared as for CTT and testing was performed as per manufacturer's instructions. The reactions were then read and recorded on the case reporting form. The titer end point was the highest dilution yielding 1+, 2+, and 3+ agglutination visible to the naked eye.

Statistical analysis

Data were entered into an MS excel sheet; numerical values, percentages, mean and standard deviation were calculated. Statistical analysis was performed using SPSS software (Version 25.0.0.0, 2017, Chicago, Illinois, USA). Median IgM and IgG titers were calculated for anti-A and anti-B obtained by CTT and pre HI CAT (CAT) as well as post HI CAT (pCAT). Correlation between CTT and CAT and between CTT and pCAT were tested using Spearman's rho for all samples. The strength of the correlation was calculated using a guide for the absolute value of r_s.[24] The guide is as follows:

- 0.0–0.19-very weak

- 0.20–0.39-weak
- 0.40–0.69– moderate
- 0.7–0.89-strong
- 0.9–1.0-very strong.

Nonparametric Wilcoxon signed-rank paired test was used to test for significance comparing IgG results between CTT and pCTT, CTT and CAT, pCTT and CAT and pCTT and pCAT used for a given sample. For this purpose, a total of 10 samples (every 10th sample) were included.

Results

A total of 300 whole blood donors participated in this study; 100 each for group A, B and O. For group O, the mean age of participants was 31.91 ± 7.8 years and 5% (5 of 100) were female. For group A, the mean age of participants was 31.25 ± 6.12 years and 6% (6 of 100) were females. For group B, the mean age of participants was 32.59 ± 7.62 years and 10% (10 of 100) were females.

Figure 1 illustrates the distribution of IgG titer results obtained by CTT pre- and post-heat inactivation using a box and whisker plot. For group O, IgG titer results were higher for both anti-A and anti-B compared to group A and B. Anti-A IgG titer results were higher for group O individuals as compared to group B individuals. Similarly, anti-B IgG titer results were higher for group O individuals as compared to group A individuals. For group A, B, and O, pretreatment results were higher than posttreatment IgG titer results. For group O, while distribution of anti-A and anti-B pretreatment titer results was similar, post-treatment titer results were higher for anti-B. Non-O anti-A titer results were lower than anti-B results.

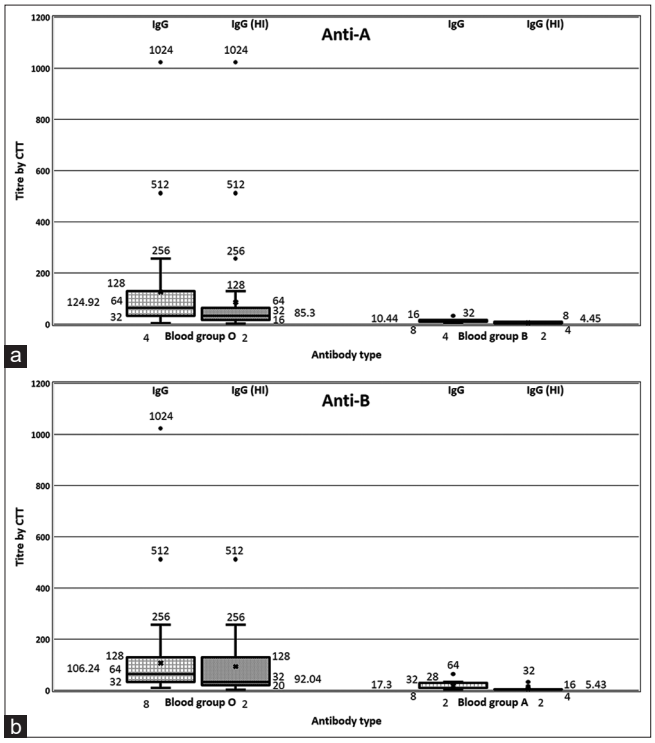

Figure 1: Distribution of anti-A and anti-B titers performed by CTT (a) Anti-A immunoglobulin G titer before and after HI treatment (b) Anti-B immunoglobulin G titer before and after heat inactivation

Figure 2 shows the comparison between median IgG and IgM titers for anti-A and anti-B performed by CAT and CTT before and after DTT treatment. Median titers obtained by CAT were higher than median titers obtained by CTT. Median blood group O titers were higher than median titers from A and B blood group. Median IgM titers were higher for group O individuals than nongroup O individuals. Median IgG titers were higher for group O individuals than nongroup O individuals when measured by both methods. Median anti-A titers were similar to median anti-B titers across all categories. With the use of heat, one log reduction of group O IgG titers was observed. Similarly, with the use of heat, one log reduction of group A/B IgG titers was observed. For group A and B individuals, median IgM titer results were similar or higher than IgG titer results. However, for group O individuals, median IgG titer results were similar or higher than median IgM titer results.

Figure 3 illustrates the IgG results obtained for every 10th sample by all three methods. A comparison between CTT and CAT, CTT and pCTT, CTT and pCAT, pCTT and pCAT was performed using Wilcoxon signed-rank paired test for significance. When comparing anti-A and anti-B IgG results for group O individuals, results of CTT and SPRCA were not found to be significant ($P > 0.05$), whereas, results of CTT and CAT were found to be significant ($P < 0.05$). However, when comparing anti-A and anti-B IgG results for nongroup O individuals, results of CTT and CAT, results of CTT and SPRCA were found to be significant ($P < 0.05$).

Tables 1 and 2 list the Spearman's rho (r_s) for correlation between CTT and CAT, pre- and post DTT treatment. The statistical analysis was performed for IgM and IgG titers for both anti-A and anti-B antibodies individually. The results show that IgM and IgG measurement of anti-A and anti-B of group O individuals showed strong correlation (r_s: 0.7–0.89) between CTT and CAT, before and after DTT treatment. In contrast, correlation calculation performed for anti-A and anti-B titer estimation by CTT and CAT showed weak (r_s: 0.20–0.39) to moderate (r_s: 0.40–0.69) correlation between CTT and pCTT, strong (r_s: 0.7–0.89) correlation between CTT and CAT, moderate (r_s: 0.40–0.69) to strong (r_s: 0.7–0.89)

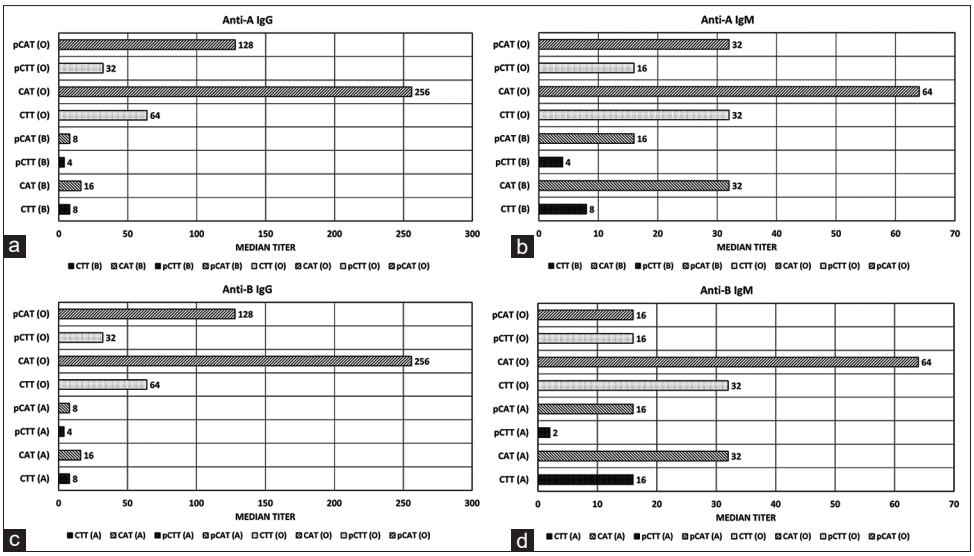

Figure 2: Comparison of median anti-A and anti-B titers before and after heat inactivation performed by CTT and CAT (a) Median anti-A immunoglobulin G titers (b) Median anti-A immunoglobulin M titers (c) Median anti-B immunoglobulin G titers (d) Median anti-B immunoglobulin M titers (CAT, CTT- Pre DTT treatment; pCAT, pCTT – Post heat inactivation)

Table 1: The correlation of pre- and post-heat inactivation results obtained by conventional test tube and column agglutination technique measuring IgG antibodies for anti-A and anti-B (blood group A, B, O)

Antibody IgG	Blood group	Comparing methods	Spearman's rho	*P*	Strength of correlation	Association	Direction of correlation
Anti-A	B	CTT-pCTT	0.31	<0.05	Weak	Significant	Negative
Anti-A	B	CTT-CAT	0.68	<0.05	Moderate	Significant	Positive
Anti-A	B	CTT-pCAT	0.17	<0.05	Very weak	Significant	Positive
Anti-A	B	CAT-pCAT	0.30	<0.05	Weak	Significant	Positive
Anti-A	B	CAT-pCTT	0.49	<0.05	Moderate	Significant	Positive
Anti-A	B	pCAT-pCTT	0.45	<0.05	Moderate	Significant	Positive
Anti-A	O	CTT-pCTT	0.77	<0.05	Strong	Significant	Positive
Anti-A	O	CTT-CAT	0.69	<0.05	Moderate	Significant	Positive
Anti-A	O	CTT-pCAT	0.60	<0.05	Moderate	Significant	Positive
Anti-A	O	CAT-pCAT	0.75	<0.05	Strong	Significant	Positive
Anti-A	O	CAT-pCTT	0.76	<0.05	Strong	Significant	Positive
Anti-A	O	pCAT-pCTT	0.79	<0.05	Strong	Significant	Positive
Anti-B	A	CTT-pCTT	0.51	<0.05	Moderate	Significant	Positive
Anti-B	A	CTT-CAT	0.74	<0.05	Strong	Significant	Positive
Anti-B	A	CTT-pCAT	0.28	<0.05	Weak	Significant	Positive
Anti-B	A	CAT-pCAT	0.31	<0.05	Weak	Significant	Positive
Anti-B	A	CAT-pCTT	0.62	<0.05	Moderate	Significant	Positive
Anti-B	A	pCAT-pCTT	0.73	<0.05	Strong	Significant	Positive
Anti-B	O	CTT-pCTT	0.70	<0.05	Strong	Significant	Positive
Anti-B	O	CTT-CAT	0.71	<0.05	Strong	Significant	Positive
Anti-B	O	CTT-pCAT	0.76	<0.05	Strong	Significant	Positive
Anti-B	O	CAT-pCAT	0.65	<0.05	Moderate	Significant	Positive
Anti-B	O	CAT-pCTT	0.62	<0.05	Moderate	Significant	Positive
Anti-B	O	pCAT-pCTT	0.77	<0.05	Strong	Significant	Positive

CTT=Conventional test tube, CAT=Column agglutination technique, pCTT=post heat inactivation CTT, pCAT=post heat inactivation CAT

correlation between pCAT and pCTT. However, correlation between CTT and pCAT, CAT, and pCAT was moderate (r_s: 0.40–0.69) to strong (r_s: 0.7–0.89) for anti-A and anti-B IgM titers and correlation between CTT and pCAT, CAT and pCAT was weak (r_s: 0.20–0.39) for anti-A and anti-B IgG titers.

Discussion

The technique of heating plasma in order to inactivate IgM antibodies was first described in 1981 by Steinberg and Cook.[25] Studies performed previously for pretransplant work-up of solid organ transplant recipients have

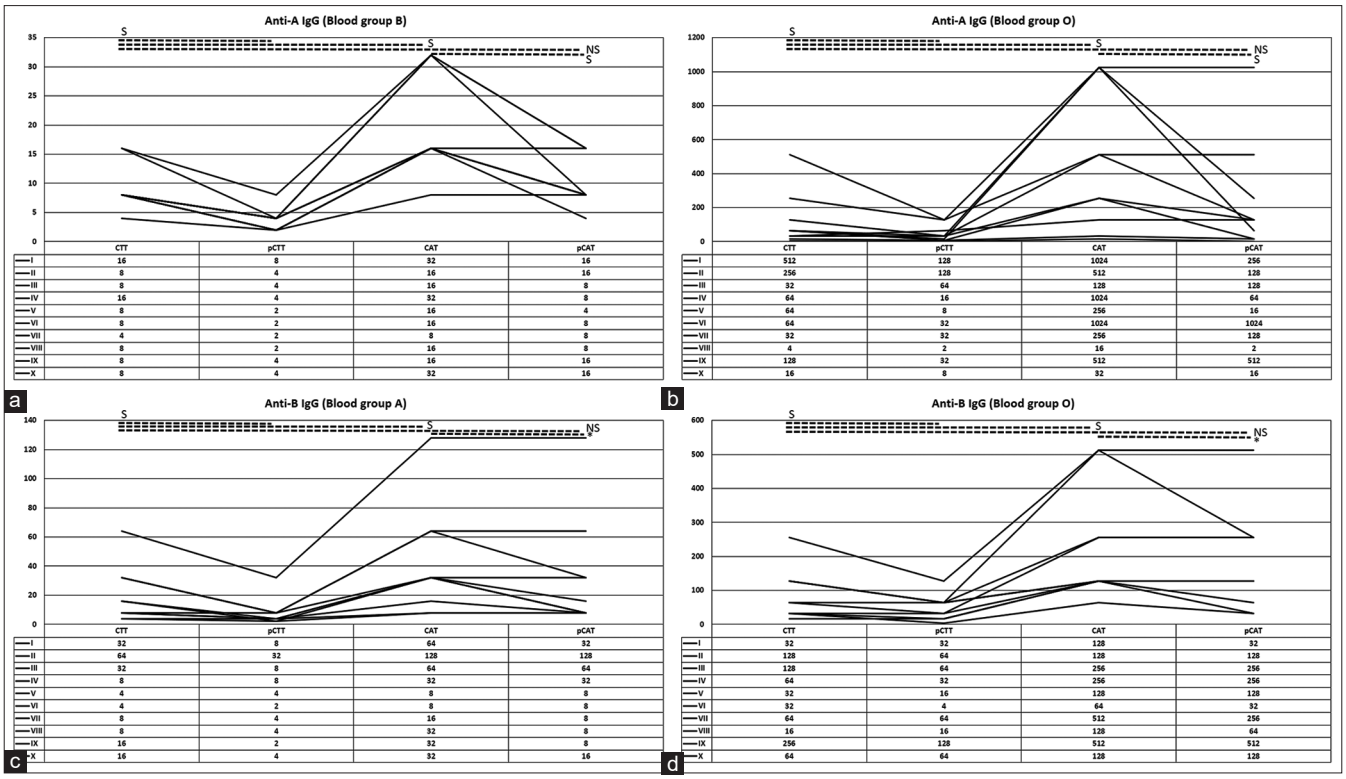

Figure 3: Comparison of immunoglobulin G titers performed by CTT and CAT for 10 samples pre and post heat inactivation (a) Anti-A titer (blood group B) (b) Anti-A titer (blood group O) (c) Anti-B titer (blood group A) (d) Anti-B titer (blood group O)/ Wilcoxon signed rank test was used for calculating significance using $P < 0.05$. S indicates significant, NS indicates not significant and * indicates could not be calculated

Table 2: The correlation of pre- and post-heat inactivation results obtained by conventional test tube and column agglutination technique measuring IgM antibodies for anti-A and anti-B (blood group A, B, O)

Antibody IgM	Blood group	Comparing methods	Spearman's rho	*P*	Strength of correlation	Association	Direction of correlation
Anti-A	B	CTT-pCTT	0.32	<0.05	Weak	Significant	Positive
Anti-A	B	CTT-CAT	0.69	<0.05	Moderate	Significant	Positive
Anti-A	B	CTT-pCAT	0.51	<0.05	Moderate	Significant	Positive
Anti-A	B	CAT-pCAT	0.57	<0.05	Moderate	Significant	Positive
Anti-A	B	CAT-pCTT	0.19	<0.05	Very weak	Significant	Positive
Anti-A	B	pCAT-pCTT	0.46	<0.05	Moderate	Significant	Positive
Anti-A	O	CTT-pCTT	0.60	<0.05	Moderate	Significant	Positive
Anti-A	O	CTT-CAT	0.74	<0.05	Strong	Significant	Positive
Anti-A	O	CTT-pCAT	0.63	<0.05	Moderate	Significant	Positive
Anti-A	O	CAT-pCAT	0.71	<0.05	Strong	Significant	Positive
Anti-A	O	CAT-pCTT	0.65	<0.05	Moderate	Significant	Positive
Anti-A	O	pCAT-pCTT	0.78	<0.05	Strong	Significant	Positive
Anti-B	A	CTT-pCTT	0.27	<0.05	Weak	Significant	Positive
Anti-B	A	CTT-CAT	0.78	<0.05	Strong	Significant	Positive
Anti-B	A	CTT-pCAT	0.63	<0.05	Moderate	Significant	Positive
Anti-B	A	CAT-pCAT	0.65	<0.05	Moderate	Significant	Positive
Anti-B	A	CAT-pCTT	0.29	<0.05	Weak	Significant	Positive
Anti-B	A	pCAT-pCTT	0.60	<0.05	Moderate	Significant	Positive
Anti-B	O	CTT-pCTT	0.69	<0.05	Moderate	Significant	Positive
Anti-B	O	CTT-CAT	0.75	<0.05	Strong	Significant	Positive
Anti-B	O	CTT-pCAT	0.62	<0.05	Moderate	Significant	Positive
Anti-B	O	CAT-pCAT	0.71	<0.05	Strong	Significant	Positive
Anti-B	O	CAT-pCTT	0.77	<0.05	Strong	Significant	Positive
Anti-B	O	pCAT-pCTT	0.74	<0.05	Strong	Significant	Positive

CTT=Conventional test tube, CAT=Column agglutination technique, pCTT=post heat inactivation CTT, pCAT=post heat inactivation CAT

concluded that presence of IgM antibodies can cause false positive Centers for Disease Control and Prevention (CDC) crossmatch and subsequent use of heat inactivation technique can eliminate this false positivity by decreasing interference of IgM antibodies.[26,27] However, the effect of heat inactivation on the ABO isohemagglutinin titers have not been studied in detail when compared to study of the effect of DTT or 2-ME on ABO titers. Hasekura and Ishimori studied the effects of heat on IgG, IgM, and IgA titers in 300 females with ABO incompatible fetus or newborns.[28] The present study was performed for 300 healthy whole blood donors which included 21 females.

O blood group individuals are known to possess more IgG ABO isohemagglutinins as compared to A and B blood groups.[29] A cross-sectional analytical study including 560 donors conducted over 20 months found that the prevalence of high titer anti-A and anti-B IgM antibodies was 36% and 32%, respectively while the prevalence of high titer anti-A and anti-B IgG antibodies was 9.6% and 5.5%, respectively.[2] Titration was performed by the double dilution tube agglutination technique. In the present study, IgG titers for anti-A and anti-B measured by both CAT and CTT were higher than IgM titers. When comparing results, both IgM and IgG titers were found to be more when performed by CAT as compared to CTT. For group O, while distribution of anti-A and anti-B pretreatment titer results was similar, posttreatment titer results were found to be higher for anti-B. This could be because of higher anti-B IgG antibodies than anti-A IgG in these individuals.

Historically, heat inactivation of sera has been performed at 56°C for 30 min to inhibit the complement activity.[30,31] Hasekura *et al*. heated sera at 70°C for 10 min and observed a significant decline in both IgM and IgG titers of anti-A and anti-B titers.[28] Riley *et al*. heated plasma at 63°C for 10 min to ameliorate the effect of IgM in false-positive CDC crossmatch.[27] In the present study, HI was performed at 63°C for 13 min as per ASHI laboratory manual.[22] Similar to Hasekura *et al*., who expected more difference in titers according to the temperature, the authors of the present study did not observe a significant difference in anti-A and anti-B titers for both IgM and IgG types after HI of plasma.[28] In general, one-fold decrease in median titers was observed with HI of plasma. While reduction in titers does indicate that the presence of IgM antibodies in samples leads to overestimation of IgG titers, this effect was quite evident when titers were performed by CTT whereas CAT results showed a modest decline only.

CTT has been the conventional method for all immunohematology investigations including ABO antibody titration, it is being replaced at various centers with other semi and fully automated techniques which do reduce the inter-observer variation and require much less expertise. However, these techniques are expensive and despite various advantages are not available to transfusion services in resource constraint settings. In the present study, the median IgG titers for both anti-A and anti-B were higher when performed by CAT in comparison to CTT. Park *et al*. compared only IgG titers of CTT with CAT and found that for blood group O, the titers were more in CAT than CTT.[32] Median anti-A and anti-B titers by CTT for were found to be 32 in their study while in the present study, it was 8.[32] Nayak *et al*. compared results of 50 samples and concluded that there was poor agreement between IgG titers performed by CAT and CTT.[33]

While HI helps in reducing the interference of IgM antibodies, it is not a very effective method for determination of IgG titers. This effect is much more evident when performed by CTT. The use of HI is a simple, less time consuming, low cost method requiring simple equipment like a heat block or water bath which is available in most laboratories for IgM inactivation when compared to use of DTT. Strengths of this study include a robust sample size. To the best of our knowledge, this is the first study which assesses the effect of heat on anti-A and anti-B titers in 300 individuals, 100 each for group A, B, and O with use of two different methods; CAT and CTT. Limitations of this study include inability to assess clinical impact of titration performed before and after heat inactivation of plasma and inability to assess effect of on samples preserved overnight for titration.

Conclusion

There is overestimation of IgG titers when performed by CTT as well as CAT which can increase the cost of treatment for a patient undergoing transplant. Hence, the authors strongly recommend inactivation of IgM antibodies before estimation of IgG titers. CAT was found to be a more sensitive method when compared to C7T. However, despite the various drawbacks of manual method, the authors use CTT with inactivation of IgM for reporting IgG titers. There is a satisfactory effect of heat inactivation on titers of ABO isohemagglutinins, which is modest when performed by CAT as compared to CTT. While a clinical impact of ABO titers with HI treatment needs to be studied, HI treatment of plasma for ABO titer determination provides a suitable option for resource constraint settings because it is simple and involves low cost and minimal expertise.

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Conflicts of interest
There are no conflicts of interest.

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