

Original Research Article

Comparative evaluation of effect of DTT treatment and heat inactivation on ABO isoagglutinin titers

ABSTRACT:

Background and aims: In order to determine actual concentration of clinically significant IgG antibodies, there is a need to inactivate IgM antibodies by, which can be performed by DTT or heat. The aim of the present study was to compare the effect of DTT treatment and heat inactivation on ABO isoagglutinin titers performed by column agglutination technology (CAT) and conventional tube technique (CTT).

Materials and methods: This was a prospective, observational study conducted from October 2019 to March 2020. All consecutive A, B and O group donors were included. Serum from each donor was treated with DTT and heat and tests were performed by CTT and CAT before and after treatment.

Results: A total of 300 whole blood donors participated in this study; 100 each for group A, B and O. Anti-A and anti-B IgG titers of group O individuals were higher than corresponding titers of non-O individuals. Anti-A titers in group O individuals were slightly higher than anti-B titers, whereas anti-A and anti-B titers from non-O individuals were found to be similar. Heat treated titers were higher than DTT treated titers. Median IgG titers were higher than median IgM titers. Group O titers were higher than non-O titers.

Conclusion: There is overestimation of ABO IgG antibody titers and hence, estimation of ABO IgG titers after inactivation of IgM antibodies is strongly recommended. DTT treatment was found to be superior to heat inactivation in terms of efficiency in elimination of IgM activity.

KEYWORDS: Isohemagglutinin titer, ABO, Conventional tube technique, Column agglutination technique, DTT, heat inactivation

INTRODUCTION:

The most important component of pre-transfusion testing is ABO blood group. [1] ABO antibodies are capable of causing intravascular hemolysis, renal failure and even death if not considered in pre-transfusion testing. [2] They are also known to play a major role in outcome of ABO incompatible grafts in case of solid organ as well as hematopoietic stem cell transplant. [3-6] Immune reactions like hyperacute graft rejection after ABO incompatible solid organ transplant as well as complications of hematopoietic stem cell transplant like pure red cell aplasia and delayed engraftment are some other scenarios where ABO antibodies play an important role. [7,8] Measurement of the concentration of these isohemagglutinins is crucial because it is responsible for immune reactions related to transfusion and transplantation. Titration is a semi-quantitative method of antibody quantitation. Determination and monitoring of isoagglutinin titres play an important role in the outcome of solid organ and hematopoietic stem cell transplant.

IgM and IgG antibodies are the immunoglobulin types which are important to any transfusion service. Anti-A and anti-B antibodies belonging to individuals of A and B blood group are predominantly IgM type while those of blood group O are predominantly IgG type. While testing for IgG antibody titers, their concentration can be masked by IgM antibodies. [1] In order to determine the actual concentration of the clinically significant IgG antibodies, there is a need to

inactivate these IgM antibodies. Literature provides methods to inactivate IgM antibodies such as heat inactivation and use of sulfhydryl reagents such as 2-mercaptoethanol (ME) and dithiothreitol (DTT; also called Cleland's reagent). [9,10] IgG antibodies are less susceptible to these treatments because the disulphide bonds between chains are not as labile as those of IgM subunits. [11]But they may get slightly affected. [12] Inactivation of IgM antibodies as a part of routine testing in clinical laboratories has been recommended where IgM interference is suspected. [13,14] DTT being an expensive reagent is not easily accessible to all transfusion services. Heat inactivation provides a cheaper and easy to perform alternative to DTT in resource constraint settings. However, there is scarcity of data with respect to comparison of the effect of DTT and heat inactivation on ABO antibody titers.

Conventional tube technique (CTT) has been widely used as a method of antibody titration. However, CTT being a manual method, has certain disadvantages; it is time consuming, labor-intensive, has inter-observer as well as inter-laboratory variations. Like other immunohematological investigations, titration is being offered on various automated immunohematology analyzers with the advantage of high throughput, less inter-observer and inter-laboratory variation and easy for laboratory personnel. These analyzers are based on different techniques like column agglutination technology (CAT) and solid phase red cell adherence (SPRCA)/ hemagglutination (HA). There are studies which compare results obtained using different methods of titration. [15-21] Many of these techniques conclude that the results of the age old CTT do not correlate with the results obtained by newer techniques. [1,22-24]

The aim of the present study was to compare the effect of DTT treatment and heat inactivation on ABO isoagglutinin titers performed by CAT and CTT.

MATERIALS AND METHODS:

1.1 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 consecutive A, B and O blood group donors were included samples were simultaneously tested by CTT and CAT for anti-A and anti-B titration. For studying the effect of DTT and heat inactivation, serum from each donor was treated with DTT and heat and tests were performed by CTT and CAT before and after treatment. All results were recorded for comparison.

1.2 Study population

All consecutive 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. [25,26] Pilot tubes collected at the time of donation were used for titration. No additional samples were drawn. After performing the routine donor 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°C. 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.

1.3 Methods of titration:

1.3.1 CONVENTIONAL TEST TUBE TECHNIQUE (CTT): Titration was performed by CTT according to the method described in AABB technical manual. [4] 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.

- 1.3.2 **COLUMN AGGLUTINATION TECHNIQUE (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 test sample were prepared as for CTT and testing was performed as per manufacturer's instructions. The reactions were then read and recorded on a case reporting form. The titer end point was the highest dilution yielding 1+ agglutination visible to the naked eye.
- 1.3.3 **DTT PREPARATION:** 0.01M DTT was prepared by dissolving 0.154g of DTT in 100ml of PBS (pH 7.3) as described in the steps detailed in AABB technical manual. [4]
- 1.3.4 **DTT TREATMENT OF SERUM:** Serum was treated with 0.01M DTT as per the method described in AABB technical manual. [4] One volume of the prepared DTT was combined with one volume of serum. The mixture was incubated at 37°C for 30 to 45 minutes mixing every 5 minutes. From this mixture serial dilutions were prepared and antibody titration was performed using column agglutination technique for both IgM and IgG. For dilution control, one volume of patient serum was mixed with one volume of PBS and that mixture was used for serial dilutions and titration. This was done to show that reactivity was not reduced simply by dilution of serum.
- 1.3.5 **HEAT INACTIVATION OF PLASMA:** Heat inactivation of plasma was performed by the method described in American Society for Histocompatibility and Immunogenetics (ASHI) Laboratory Manual. [27] Desired amount of plasma was taken in a test tube and was placed in pre-heated 63° C heat block for exactly 13 minutes using a stop watch.

After completion of 13 minutes, the tube was removed from heat block and was centrifuge. The supernatant was removed into another labelled test tube and was used for titration. The heat-treated sample was used on the same day.

1.4 ETHICAL APPROVAL: All donors who consented to participate in the present study were included. The study was approved by the institutional review board (IRB) and the institutional ethics committee (IEC).

1.5 STATISTICAL ANALYSIS: Data was entered in an MS excel sheet (version 16.0, 2016, Microsoft Corporation, Washington, USA); numerical values, percentages, mean and standard deviation was calculated. Statistical analysis was performed using SPSS software (Version 25.0.0.0, Chicago, USA). Box and whisker plot for distribution of post treatment anti-A and anti-B IgG titers performed by CAT and CTT was plotted. Median IgM and IgG titres were calculated for anti-A and anti-B obtained by CTT and CAT; both pre and post DTT and heat treatment. Correlation between the treatments was tested using Spearman's rho for all samples. The strength of the correlation was calculated using the following guide for the absolute value of r_s :

0.0-0.19 - very weak

0.20-0.39 - weak

0.40-0.59 - moderate

0.60-0.79 - strong

0.80-1.0 - very strong

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 females. 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 anti-A and anti-B IgG titers post DTT treatment and post HI treatment performed by CTT in the box and whisker plot. Anti-A and anti-B IgG titers of group O individuals were higher than corresponding titers of non-O individuals. Anti-A titers in group O individuals were slightly higher than anti-B titers, whereas anti-A and anti-B titers from non-O individuals were found to be similar. Heat treated titers were higher than DTT treated titers.

Figure 2 illustrates a comparison of median anti-A and anti-B titers after DTT and HI treatment performed by CTT and CAT. Median heat treated IgM titers were higher than median DTT treated IgM titers when performed by CTT and CAT. Median IgG titers were higher than median IgM titers. Group O titers were higher than non-O titers. Median titers performed by CAT were higher than median IgG titers. For non-O individuals, IgG and IgM titers post HI treatment were higher than IgG and IgM titers post DTT treatment. While group O IgM heat treated titers were found to be higher than group O IgM DTT treated titers, group O IgG heat treated titers were mostly similar to group O IgG DTT treated titers.

Figure 3 illustrates a comparison of the frequency of distribution of IgG titers performed by CTT and CAT, both post DTT and HI treatment for A, B and O blood groups. In general, results of anti-A and anti-B IgG titers performed by CAT are higher as compared to titers performed by CTT. Also, distribution of Anti-B titers show a shift to right as compared to anti-A titers, which

indicated that anti-B titers were higher as compared to anti-A titers. Post treatment IgG titers show a shift to left for both anti-A and anti-B. This shift is more evident for post DTT treatment results.

Table 1 lists the Spearman's rho (r_s) for correlation between CTT and CAT, pre and post DTT treatment. The statistical analysis was performed for IgG titers for both anti-A and anti-B antibodies individually. The results show that IgG measurement of anti-A and anti-B of group O individuals showed strong to very strong correlation 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 to moderate correlation between CTT and pCTT, very strong correlation between CTT and CAT, moderate to strong correlation between pCAT and pCTT. However, correlation between CTT and pCAT, CAT and pCAT was very weak to weak for anti-A and anti-B IgG titers.

Table 2 lists the Spearman's rho (r_s) for correlation between CTT and CAT, pre and post HI treatment. The statistical analysis was performed for IgG titers for both anti-A and anti-B antibodies individually. The results show that IgG measurement of anti-A and anti-B of group O individuals showed strong correlation between CTT and CAT, before and after HI treatment. In contrast, correlation calculation performed for anti-A and anti-B titer estimation by CTT and CAT showed weak to moderate correlation between CTT and pCTT, strong correlation between CTT and CAT, moderate to strong correlation between pCAT and pCTT. However, correlation between CTT and pCAT, CAT and pCAT was weak for anti-A and anti-B IgG titers.

DISCUSSION:

ABO isoagglutinins play a major role in outcome of ABO incompatible grafts in case of solid organ as well as hematopoietic stem cell transplant. [28-30] Since the concentration of these antibodies regulate the immune reactions related to transfusion and transplantation, their measurement and preoperative as well as postoperative monitoring is very important. O blood group individuals are known to possess more IgG ABO isoagglutinins as compared to A and B blood groups. [1] In the present study, IgG titers measured by CAT and CTT both were higher than IgM titers. This indicates that presence of IgM antibodies in samples leads to overestimation of IgG titers which can directly impact management of ABO incompatible transplant recipients. Without the use of DTT or heat inactivation, there is estimation of total antibody titer. When comparing 1+ reaction strength, both IgM and IgG titers were found to be more when measured by CAT as compared to CTT. IgG antibodies are believed to play a crucial role in graft outcome and hence IgG titre estimation with use of a method to inactivate IgM antibodies like DTT treatment of plasma or heat inactivation has been recommended. [13,14] Both these methods have been known to inactivates IgM antibodies with minimal effect on IgG antibodies. ^[11,14] The technique of heating the plasma in order to remove IgM antibodies activity was first mentioned in 1981 by Steinberg and Cook. [31] A few studies have been done in cases of solid organ transplant in which the presence of IgM antibodies caused false positive CDC crossmatch and the use of heat inactivation technique led to negative crossmatch results by eliminating the effect of IgM. [32,33] However, the effect of heat on the ABO isohemagglutinin titers have not been studied as compared to DTT or 2-ME.

Traditionally, heat inactivation of sera has been done at 56°C for 30 minutes to inhibit the complement activity. [34,35] Hasekura et al heated sera at 70°C for 10 minutes and observed a significant decline in both IgM and IgG titers of anti-A and anti-B. [36] On the other hand, Riley

et al heated plasma at 63°C for 10 minutes to ameliorate the effect of IgM in false positive CDC crossmatch. [33] In the present study heat inactivation was performed at 63°C for 13 minutes in accordance with ASHI. [27]

CAT has been recommended for various immunohematological investigations due to its sensitivity. However, for antibody level determination, it is not explored well. [20,37] There was difference between the antibody titer results obtained by CTT and CAT, both pre and post treatment. CAT results were found to be higher in general compared to CTT results. Median anti-A and anti-B IgG titer results showed a one to two fold difference between CTT and CAT with titers reported by CAT being higher. We compared antibody titer obtained using DTT and heat inactivated and untreated samples. A reduction in anti-A and anti-B titers was observed by DTT treatment as well as by heat inactivation, both by CTT as well as CAT. Median IgG and IgM titers showed a decrease with treatment; both DTT and heat. However, this reduction was more with DTT treatment. DTT treatment was found to be more effective in reducing the titers across all categories as compared to HI. Median IgM titers post DTT treatment showed a two to three fold decrease whereas with heat inactivation, one fold decrease was observed.

Nayak et al compared results of 50 samples and concluded that there was poor agreement between IgG titers performed by CAT and CTT. [38] Matsuura et al enrolled 10 individuals with blood group O and performed antibody titration simultaneously using CTT and automated CAT. They used DTT treated plasma for automated titer estimation by CAT to define the cut-off value in antibody titration and found 45% concordance and a significant positive correlation between CTT and automated CAT with weak strength of reaction. They recommended use of DTT for titer estimation by automated CAT. [39] In the present study, when correlation was calculated, in the present study, IgG titer results of anti-A and anti-B showed strong correlation between CTT

and CAT for DTT and HI treatment. The results show that IgG measurement of anti-A and anti-B of group O individuals showed strong to very strong correlation between CTT and CAT, before and after DTT treatment. The results show that IgG measurement of anti-A and anti-B of group O individuals showed strong correlation between CTT and CAT, before and after HI treatment.

Park et al tested 60 individuals with blood group O for titer estimation by CTT and CAT, with and without DTT treatment. They observed that higher median titers of anti-A and anti-B were obtained by CAT than CTT. Median titers were found to be higher in CAT as compared to post DTT treatment CAT titers. They concluded from their study that results obtained by CAT with or without DTT treatment were more sensitive than CTT for group O individuals. [40] Shim et al compared three methods of antibody titration using 40 samples and found that the median IgM and IgG titres were higher by CAT with the agreement being better for IgM compared to IgG. [41] In the present study, median titers were determined separately for IgM and IgG for anti-A and anti-B as illustrated in figure 2. Median titers observed by post DTT treatment performed by CAT were mostly higher than those obtained by CTT. The distribution of titers illustrated in figure 3 can be used to deduce that IgG antibody titer results are higher when performed by CAT as compared to CTT, DTT or heat treatment of plasma can lead to decrease in titers by eliminating the effect of IgM and anti-B titers were higher than anti-A titers.

While heat inactivation was found to be less efficient in inactivating IgM antibodies, as compared to DTT it is a relatively simple and cost-effective method. It consumes less time making the tedious process of titration easier as compared to DTT treatment. Also, equipment needed for heat inactivation is accessible to most laboratories in comparison to DTT treatment which is an expensive reagent and hence, not readily available to all transfusion services

especially in resource constraint settings such as ours. Strengths of this study include a robust sample size. 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.

CONCLUSION:

The findings of the present study suggest that without DTT or heat inactivation of plasma, there is definite overestimation of ABO antibody titers and hence, authors strongly recommend estimation of ABO IgG titers after inactivation of IgM antibodies. DTT treatment was found to be superior to heat inactivation in terms of efficiency in elimination of IgM activity. However, for resource constraint settings, where DTT is not accessible, heat inactivation of plasma offers a satisfactory alternative. CAT results were found to be more sensitive than CTT titer results. However, clinical impact of these titer results need to be performed to find an appropriate method for titer estimation.

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TABLES

Table 1: The correlation of pre DTT treatment (CTT, CAT) and post DTT treatment (pCTT, pCAT) results obtained by CTT and CAT measuring IgG antibodies for anti-A and anti-B (Blood group A,B,O)

Antibody	Blood group	Comparing methods	Spearman's rho	P-value	Strength of correlation	Association	Direction of correlation
IgG							
Anti-A	B	CTT – pCTT	0.33	<0.05	Weak	Significant	Negative
Anti-A	B	CTT – CAT	0.89	<0.05	Very strong	Significant	Positive

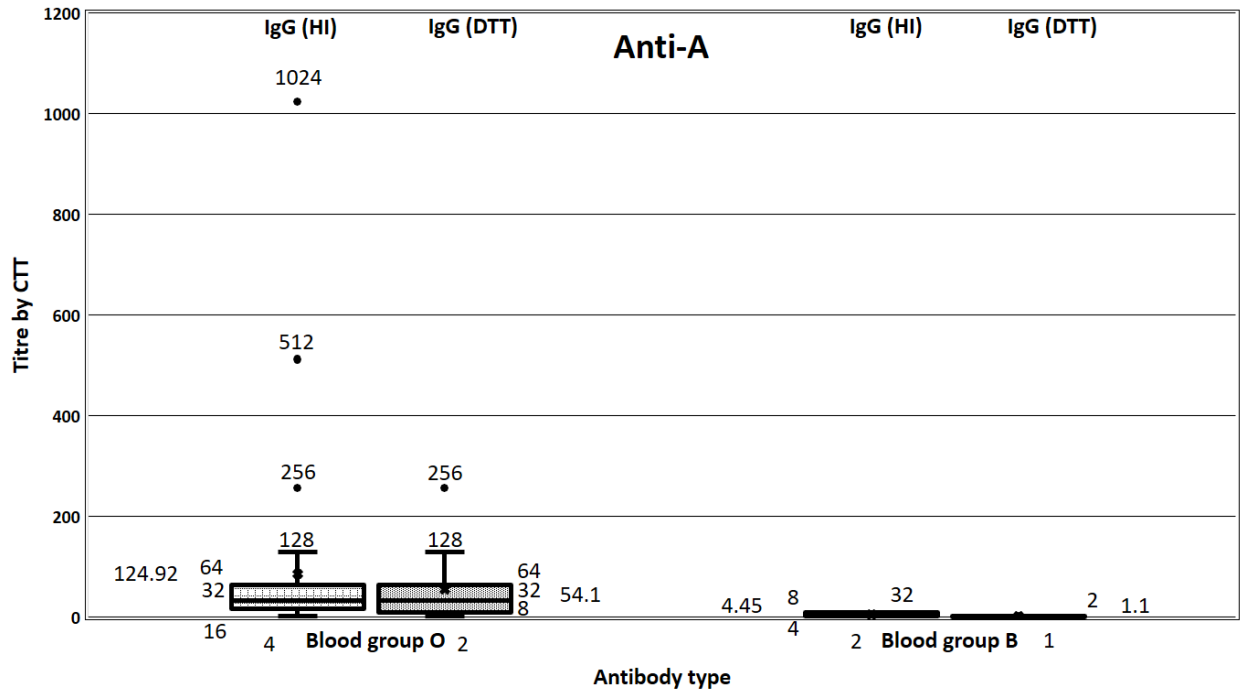
Anti-A	B	CTT – pCAT	0.09	>0.05	Very weak	Not significant	Positive
Anti-A	B	CAT – pCAT	0.03	>0.05	Very weak	Not significant	Positive
Anti-A	B	CAT - pCTT	0.55	<0.05	Moderate	Significant	Positive
Anti-A	B	pCAT - pCTT	0.48	<0.05	Moderate	Significant	Positive
Anti-A	O	CTT – pCTT	0.78	<0.05	Strong	Significant	Positive
Anti-A	O	CTT – CAT	0.77	<0.05	Strong	Significant	Positive
Anti-A	O	CTT – pCAT	0.62	<0.05	Strong	Significant	Positive
Anti-A	O	CAT – pCAT	0.77	<0.05	Strong	Significant	Positive
Anti-A	O	CAT - pCTT	0.78	<0.05	Strong	Significant	Positive
Anti-A	O	pCAT - pCTT	0.85	<0.05	Very strong	Significant	Positive
Anti-B	A	CTT – pCTT	0.59	<0.05	Moderate	Significant	Positive
Anti-B	A	CTT – CAT	0.96	<0.05	Very strong	Significant	Positive
Anti-B	A	CTT – pCAT	0.30	<0.05	Weak	Significant	Positive
Anti-B	A	CAT – pCAT	0.34	<0.05	Weak	Significant	Positive
Anti-B	A	CAT - pCTT	0.63	<0.05	Strong	Significant	Positive
Anti-B	A	pCAT - pCTT	0.75	<0.05	Strong	Significant	Positive
Anti-B	O	CTT – pCTT	0.73	<0.05	Strong	Significant	Positive
Anti-B	O	CTT – CAT	0.78	<0.05	Strong	Significant	Positive
Anti-B	O	CTT – pCAT	0.73	<0.05	Strong	Significant	Positive
Anti-B	O	CAT – pCAT	0.69	<0.05	Strong	Significant	Positive
Anti-B	O	CAT - pCTT	0.69	<0.05	Strong	Significant	Positive
Anti-B	O	pCAT - pCTT	0.81	<0.05	Very strong	Significant	Positive

Table 2: The correlation of pre heat inactivation (CTT, CAT) and post heat inactivation (pCTT, pCAT) results obtained by CTT and CAT measuring IgG antibodies for anti-A and anti-B (Blood group A,B,O)

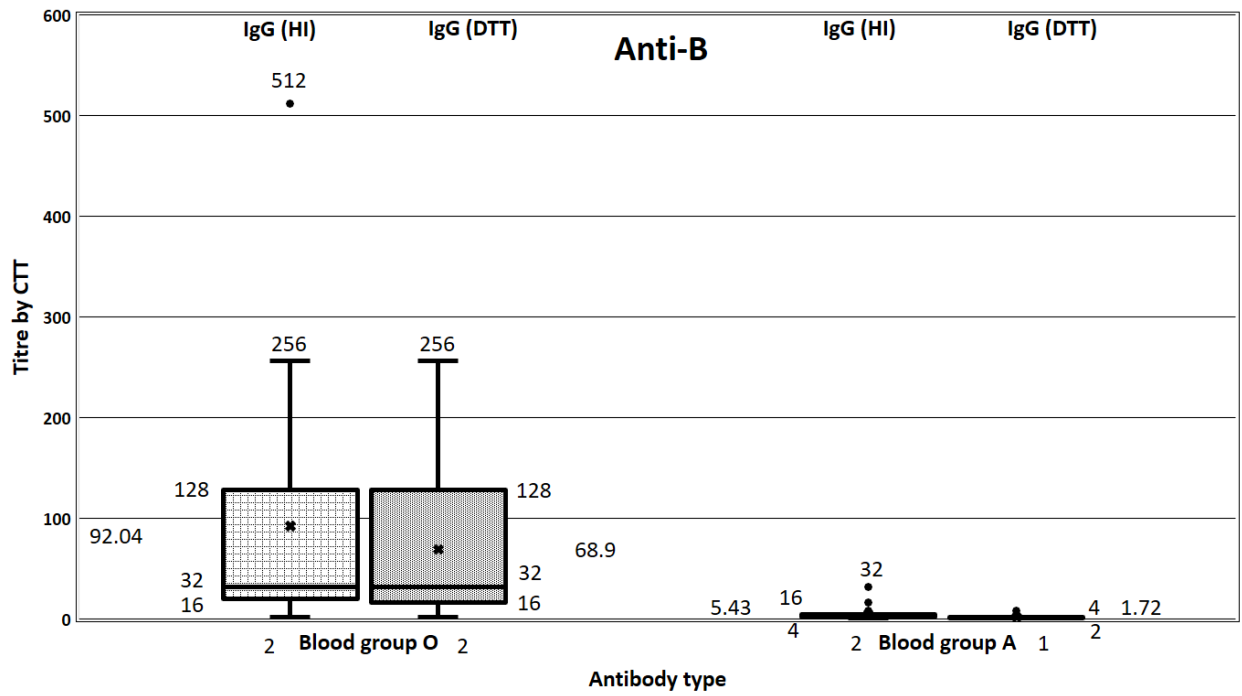
Antibody	Blood group	Comparing methods	Spearman's rho	P-value	Strength of correlation	Association	Direction of correlation
IgG							
Anti-A	B	CTT – pCTT	0.31	<0.05	Weak	Significant	Negative
Anti-A	B	CTT – CAT	0.68	<0.05	Moderate	Significant	Positive
Anti-A	B	CTT – pCAT	0.17	<0.05	Very weak	Significant	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

Figure 1: Distribution of anti-A and anti-B IgG titers post DTT and post HI treatment performed by CTT [a] Anti-A titers [b] Anti-B

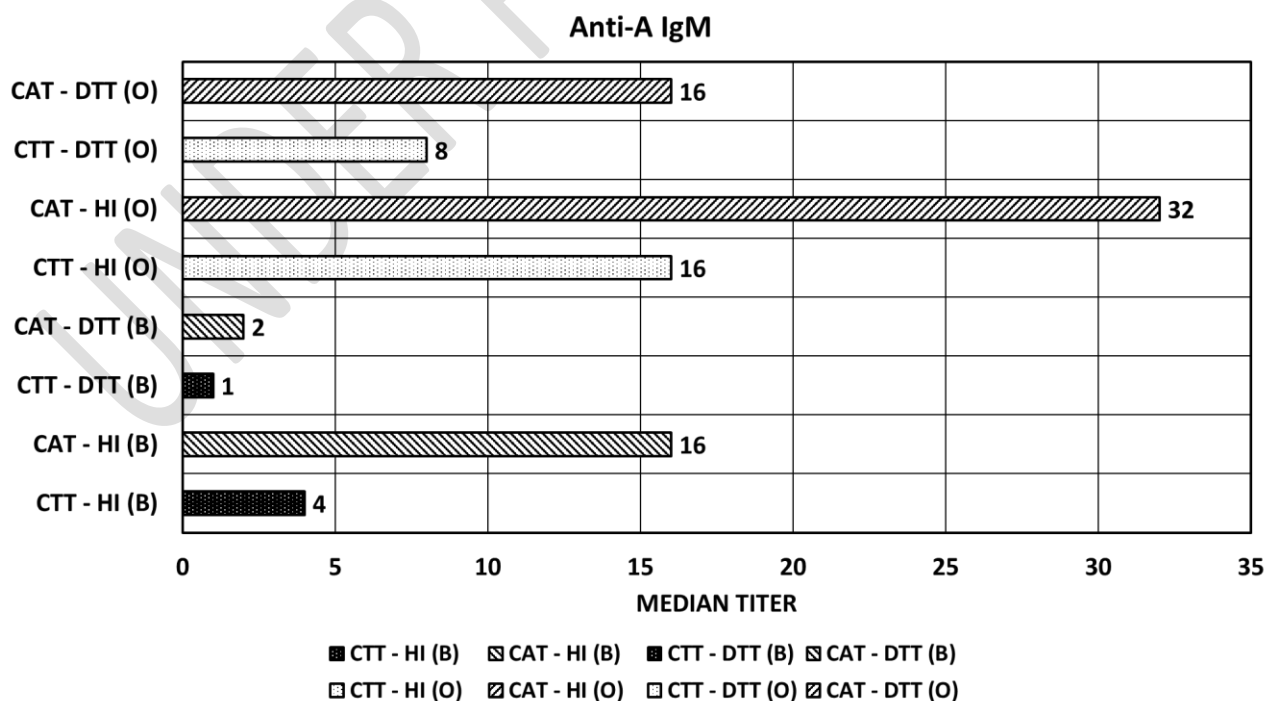
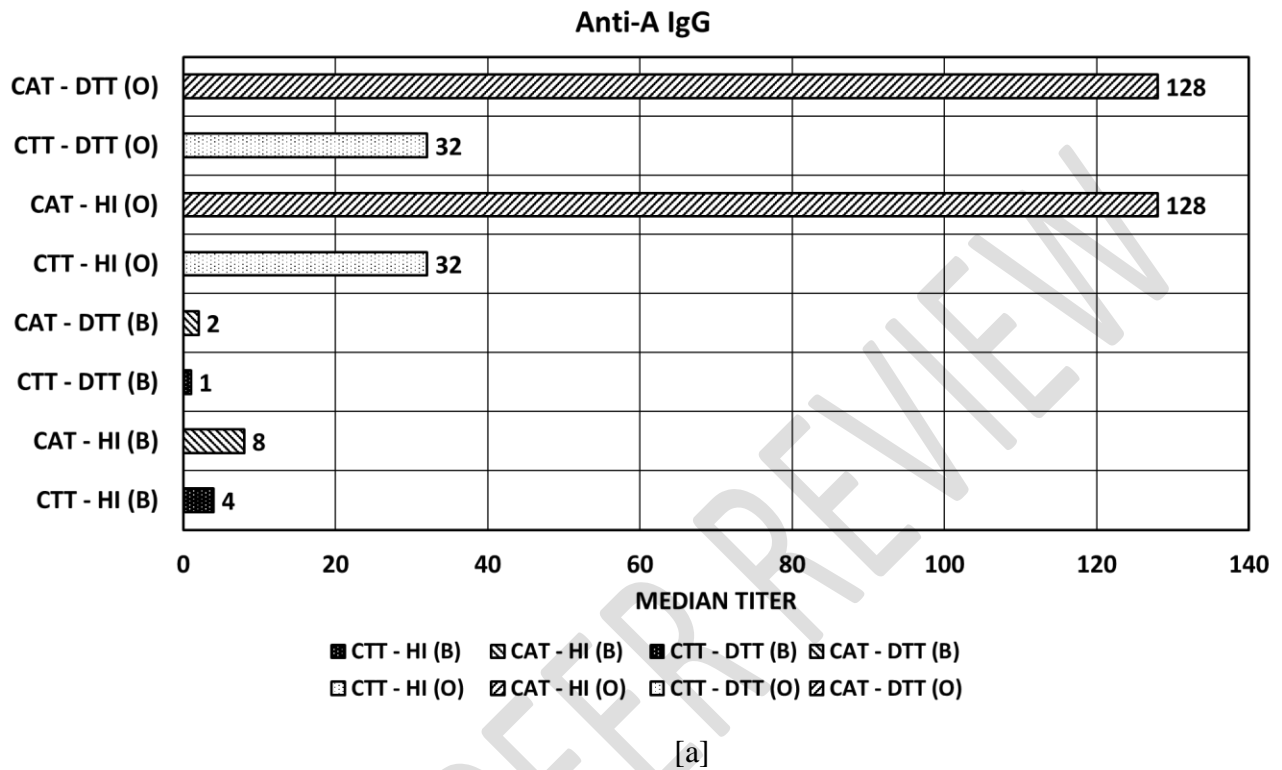


[a]

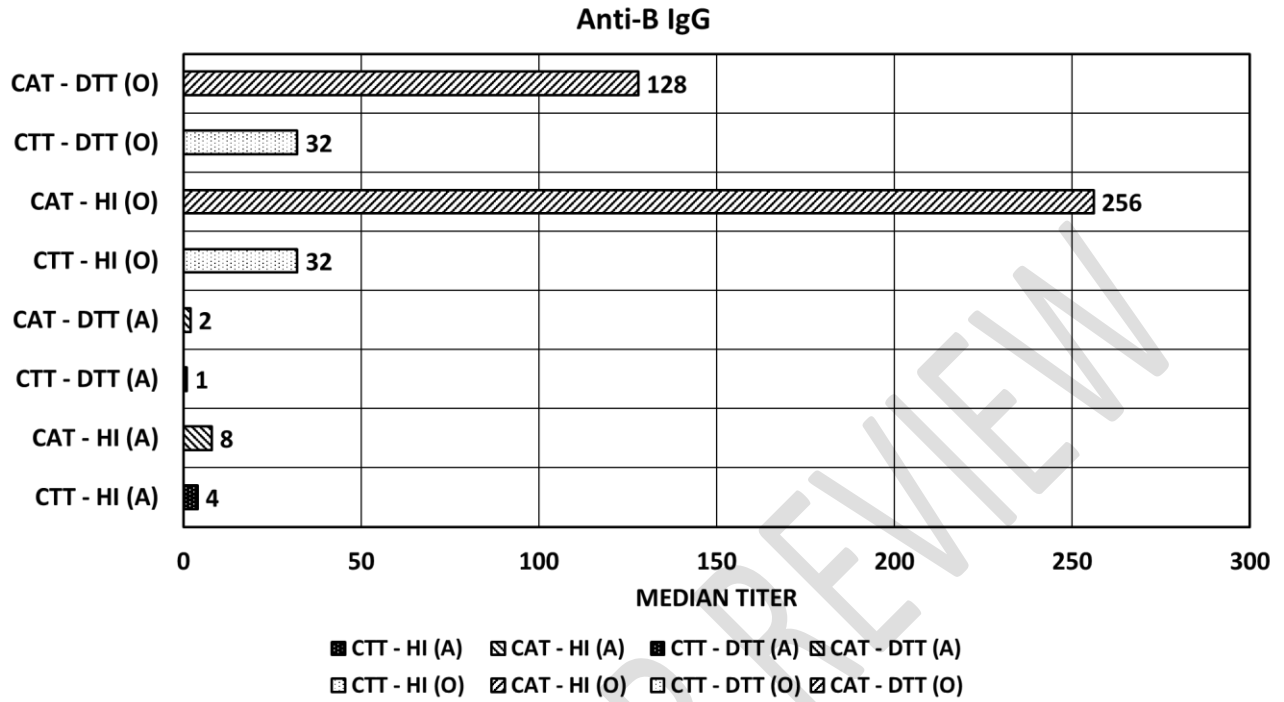


[b]

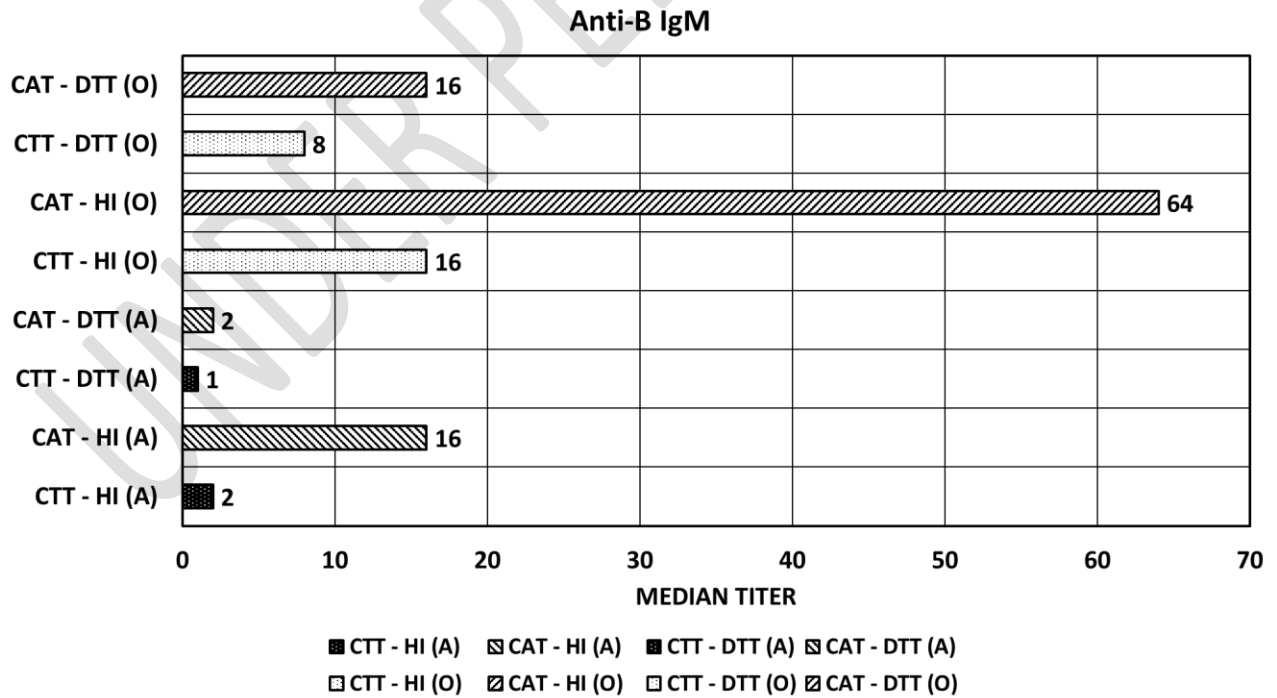
Figure 2: Comparison of median anti-A and anti-B titers before and after DTT and HI treatment performed by CTT and CAT with 1+ strength as end point [a] Median anti-A IgG titers [b] Median anti-A IgM titers [c] Median anti-B IgG titers [d] Median anti-B IgM titers



[b]

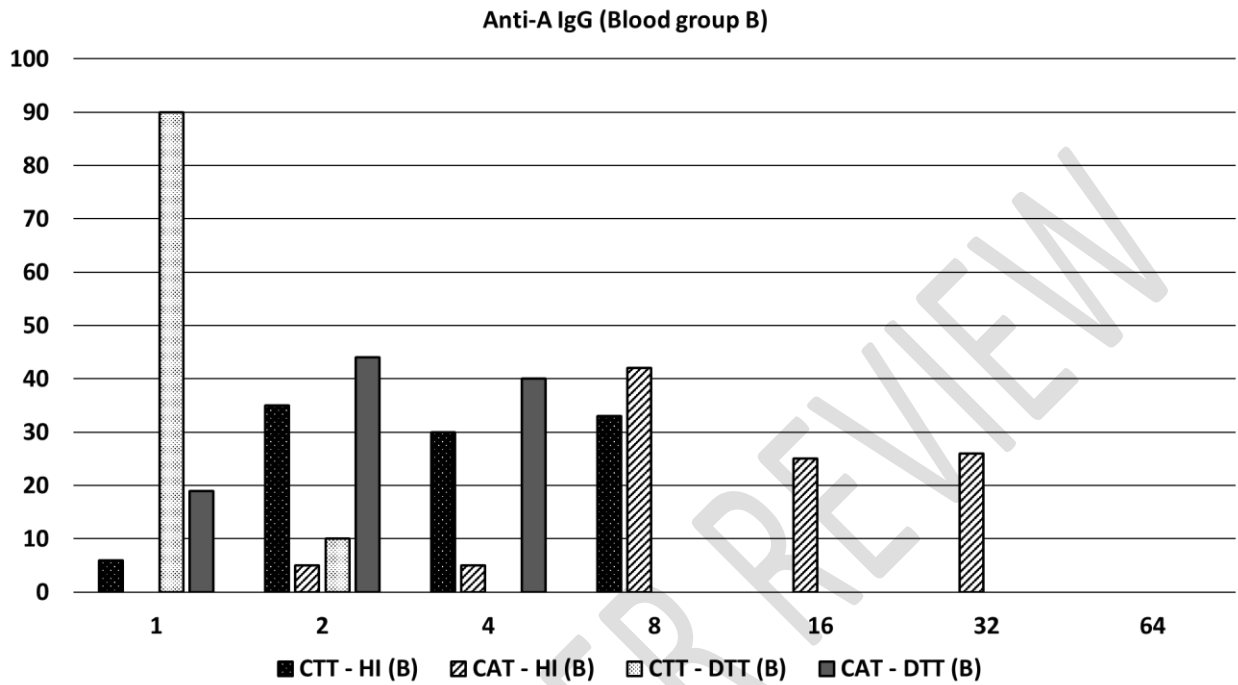


[c]

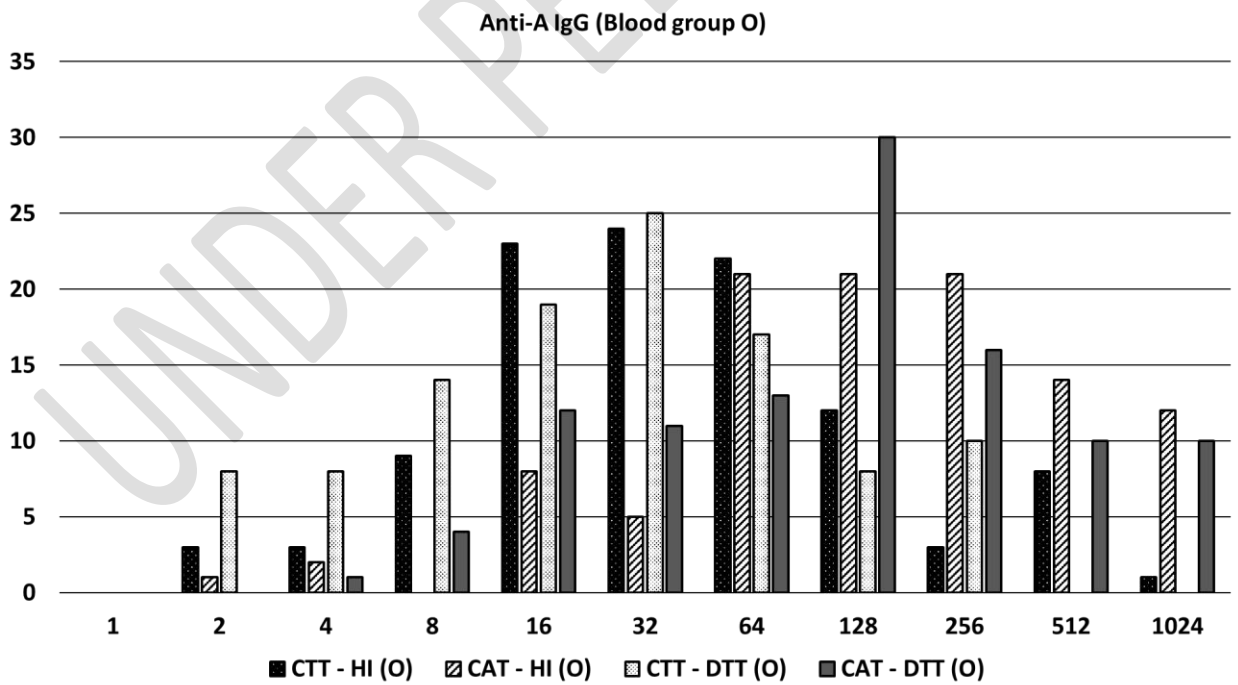


[d]

Figure 3: Comparison of frequency of distribution of IgG titers (pre and post DTT and HI treatment) performed by CTT and CAT [a] Anti-A (blood group B) [b] Anti-A (blood group O) [c] Anti-B (blood group A) [d] Anti-B (blood group O)

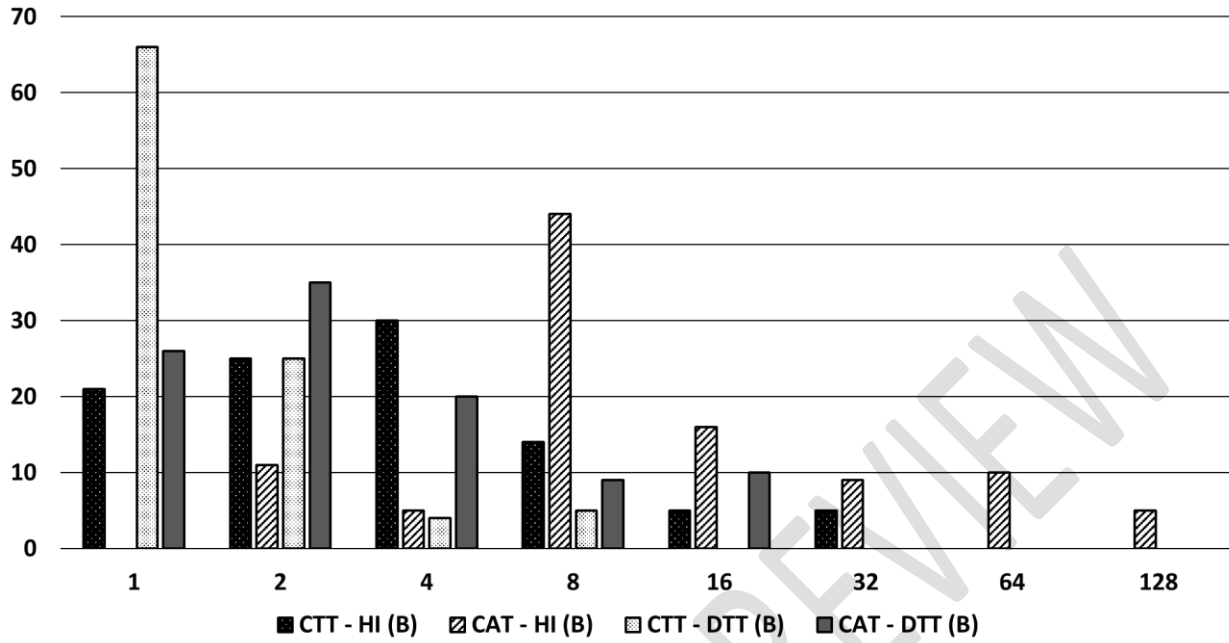


[a]



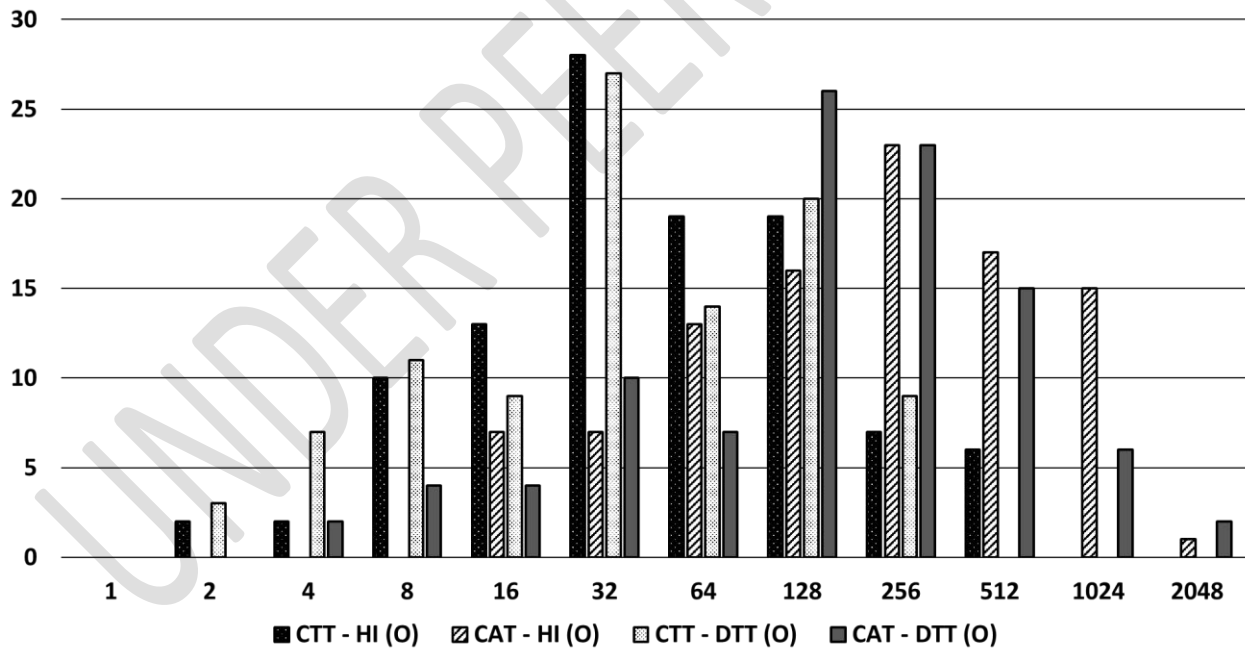
[b]

Anti-B IgG (Blood group A)



[c]

Anti-B IgG (Blood group O)



[d]