Original article

Comparative study of ABO antibody titers using conventional tube technique and automated column agglutination technique

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Abstract:

Background: *ABO antibody titration is an integral part for management of ABO incompatible organ transplantation, especially ABO-incompatible kidney transplants. Due to the precision of an automation, we planned to apply a fully automated antibody titration method using the column agglutination technique (auto-CAT) instead of a conventional tube technique (CTT).* ***Objective:*** *To compare and correlate ABO antibody titers using CTT and auto-CAT.* ***Materials and Methods:*** *A total of 180 donor serum samples consisted of 60 samples for each A, B and O blood groups, antibody titrations were performed using CTT and auto-CAT simultaneously. Spearman's correlation and Bland-Altman plot were used to determine the correlation of ABO antibody titers between the two methods and to compare the methods agreement, respectively.* ***Results:*** *In all samples, the concordance rate of CTT and auto-CAT for IgM and IgG ABO antibody titers were 81.67% to 98.30% and correlated well with spearman rho correlation coefficient of 0.73-0.94 ($p < 0.001$). The Bland-Altman plot showed that the mean differences between the two methods were -0.39 to 0.53.* ***Conclusion:*** *This study demonstrated that ABO antibody titers measured by auto-CAT and CTT had a highly significant correlation, and good agreement indicated reliable results. Thus auto-CAT can be implemented to perform antibody titration in routine laboratory testing.*

Keywords : ● *Titer of ABO antibodies* ● *Column agglutination technique*

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Introduction

The immunoglobulin class of antibody in the ABO system are IgM and IgG and even traces of IgA. Anti-A and anti-B in peoples of group B and A are mainly IgM with small amount of IgG, whereas most group O peoples have mainly IgG anti-A and anti-B. It was found that both IgM and IgG in ABO system can react best at 20-24°C or lower and they can also react up to 37°C. In addition, they can bind to complement leading to hemolysis. Antigens of ABO system found on red blood cell surface and besides, they can be found on white blood cells, platelets, epithelial cells and endothelial cells of blood vessels and various organs such as kidney, liver, heart as well as in body secretions. Therefore, antibody in ABO system is the main cause of acute hemolytic transfusion reaction, hemolytic disease of the fetus and newborn and hemolysis in major-ABO incompatible allogeneic hematopoietic stem cell transplantation. In addition, it can cause hyperacute rejection in case of ABO incompatible liver, cardiac and kidney transplantations.[1-4]

ABO incompatible kidney transplantation had long been considered as absolute contraindication. At present, due to increasing organ shortage, rendering the development of strategies to overcome the ABO antibodies barrier. Kidney transplantation across ABO blood group can now be successfully performed by desensitization to ABO antigen prior to transplantation. This procedure is able to reduce anti-A and anti-B titers to the optimal level so that the transplantation can be safely performed. In addition, the monitoring to maintain continual low anti-A and anti-B titers is crucial to the graft survival. The increase in titer of anti-A and anti-B is one of the indicators for antibody-mediated rejection (AMR).[5-7]

King Chulalongkorn Memorial Hospital had performed the first ABO incompatible kidney transplantation in Thailand in 2008.[8] The number of patients to be transplanted has increased each year. Since the target anti-A and anti-B titer for transplantation is ≤ 16[9], the method for antibody titration should yield the accurate and stable result. The anti-A and anti-B titers are the major-key decision for transplantation. The wrong result on anti-A and anti-B titers may lead to AMR and graft loss. In addition, anti-A and anti-B titration is also important to other medication processes such as ABO non-identical platelet transfusion, stem cell transplantation including liver and cardiac transplantations.[10-13] King Chulalongkorn Memorial Hospital blood bank performs anti-A and anti-B titration by conventional tube technique (CTT) which is the low-cost standard technique. IgM and IgG anti-A and anti-B could be determined at the same test. However, the disadvantage of this technique is its too many steps and all of the procedures are performed manually which was time consuming. There are many steps which could be sources of error, such as dilution of plasma, washing of red cells, especially, reading of the test result which is subjective. All of these factors make it difficult to control the quality of the test.[7,14-16] At present, the development of column agglutination technique (CAT) enables it possible to perform fully automated antibody titration (auto-CAT). The automation goes all the way through from entering the samples into the equipment to the determination and recording the results. It is considered as objective and reproducible technique yet less time consuming. The only disadvantage of this technique is its high cost. There were many comparative studies between CTT and CAT for anti-A and anti-B titration. Among these, majority recommendations were to use CAT as the standard method for ABO antibody titration.[17-23] However, most of them used manual CAT (gel test) whereas quite a few performed fully automated antibody titration.[20]

This study was aimed to compare the anti-A and anti-B titers obtained from CTT and auto-CAT by correlation and agreement testing. The finding will be used as guideline for the development of the test for our routine anti-A and anti-B titration in place of CTT. In addition, the clinicians may consider to adjust the target ABO-antibody titers for pre and post-kidney transplantation.

Materials and Methods

Materials

1. EDTA donor blood samples were obtained from The National Blood Centre, Thai Red Cross Society during December 2008 to March 2009. They all were tested as normal ABO group and revealed no unexpected red cell alloantibody. A total of 180 blood samples were from 60 of each A, B and O blood groups. After separation from EDTA blood, the plasma samples were kept frozen at -20 C until tested. Both IgM and IgG anti-A and anti-B titers were determined by CTT and auto-CAT simultaneously.

2. Standard cells and reagents

a) 3% A_1 cells and B cells and antihuman globulin serum (AHG) for CTT, manufactured by NBC.

b) 0.8% Affirmagen (A_1, B), reverse diluent cassette and Anti-IgG, -C3d; polyspecific cassette for auto-CAT, manufactured by Ortho Clinical Diagnostics (UK).

3. Equipment

3.1. Serologic centrifuge, manufactured by Kokuson corporation, Japan

3.2 Automatic cell washer, manufactured by Helmer scientific, USA.

3.3 Incubator 37°C

3.4 ORTHO VISION Analyzer, manufactured by Ortho Clinical Diagnostics, UK.

Methods

1. Anti-A and anti-B titration using CTT[24]

Serial two-fold dilutions of serum samples: 500 µL of saline is first added to each tube for 12 tubes. 500 µL of serum is then added to tube 1 and mix well. Transfer 500 µL of diluted serum from tube 1 into tube 2 and so on until tube 12 which is 1:2048 dilution. To perform the test, transfer 200 µL of diluted serum from tube 1 to 12 into 2 new sets of 12 tubes (12 x 75 mm.). The first and the second sets are for the determination of IgM and IgG antibodies, respectively. Then add 100 µL of 3% A cell or B cells into all tubes. Mix all the tubes well. Incubate one set at room temperature for 15 min. At the same time incubate the second set at 37°C for 30 min. After the end of the incubation time, all tubes of the first set are centrifuged at 3,400 rpm for 15 sec. Then examine for the agglutination. The last dilution tube that give weak agglutination examined with the naked eye[15] indicates the titer of the sample. After the incubation time of the second set of test, wash the cells in each tube 3 times using automated cell washing equipment. Then 2 drops of AHG are added to each tube. All the tubes are centrifuged at 3,400 rpm for 15 sec. Then examine for the agglutination and determine for IgG anti-A and anti-B titers the same way as above mentioned.

2. Determinations of anti-A and anti-B titers using auto-CAT equipment and CTT are performed simultaneously.

Each plasma sample is divided into two portions. One portion is subjected to be tested by auto-CAT. The equipment will automatically perform all steps for anti-A and anti-B titrations. Starting from serial two-fold dilution from 1:2 to 1:1024. Then 40 µL of diluted plasma will be tested with 50 µL of 0.8% Affirmagen (A_1 and B) in each microcolumn. One cassette consisted of 6 microcolumns. In case of titration for IgM anti-A and anti-B, the equipment will perform the test on the reverse diluent cassette and immediately after adding A_1 and B cell, centrifuge for result reading. In case of titration for IgG anti-A and anti-B, the equipment will perform the test on anti-IgG,-C3d; polyspecific cassette. The test are incubated at 37°C for 15 min. Then centrifuge for the result reading for 5 min. The equipment will show the result in form of figure for the titers of both IgM and IgG anti-A and anti-B. The last column containing diluted plasma that gives 0.5+ (weak) is considered as the titer of the test sample.[20]

Statistical analysis

The titers of IgM and IgG anti-A and anti-B obtained from CTT and auto-CAT were compared by inference statistic using Wilcoxon signed rank test and analysis for correlation between the two techniques. Spearman

correlation coefficient was applied to analyze the agreement of IgM and IgG anti-A and anti-B titers obtained from both techniques. The natural log to transform titers by Bland-Altman plot and Lin's Concordance. STATA Version 15 (StataCorp. 2017. Stata Statistical Software: release 15. College Station, TX: StataCorp LLC.) was used for statistical analysis. The statistically significant difference was $p < 0.05$.

This research project was approved from The National Blood Centre, Thai Red Cross Society IRB: code 16/2561.

Result

It was found that concordance rate of total samples tested for ABO antibodies by the two techniques were 81.67% to 98.30% (when compared the titers that were not exceeded 1 dilution difference). Among these samples, IgG ABO antibody titers was in agreement more than IgM. The highest concordance rate of IgG anti-A was 98.30% (59 cases) in group O. Whereas concordance rate of IgG anti-A was 96.67% (58 cases) in group B and the least concordance rate of IgM anti-B was 81.67% (49 cases) in group A (Table 1).

The comparison of medians of IgM and IgG ant-A and anti-B titers in A, B and O blood groups by CTT and auto-CAT showed statistically significant difference ($p < 0.001$). Accept median of IgM anti-A in B and IgG anti-A and anti-B in O blood group by the two techniques were not statistically significant difference (Table 2).

It was observed that IgM and IgG of anti-A and anti-B titers of the total samples by CTT and auto-CAT showed high correlation in the same direction with significant difference. Spearman correlation coefficient; rho of IgG anti-A and anti-B was 0.94 and 0.90, respectively and IgM anti-A and anti-B rho was 0.86 and 0.73, respectively with $p < 0.001$. For agreement by Lin's Concordance, it was found that anti-A and anti-B titers by the two techniques were in good agreement)[27] with $p < 0.001$. Lin's Concordance correlation coefficient (CCC) of IgM anti-A and anti-B was 0.83 and 0.72, respectively. While IgG anti-A and anti-B titers were 0.93 and 0.88, respectively (Table 3).

Table 1 Concordance of ABO antibody titer results by CAT and CTT

Blood group antibody (no. samples)	IgM				IgG			
	Concordance*	CAT Identical to CTT titer	CAT > CTT titer	CAT < CTT titer	Concordance*	CAT Identical to CTT titer	CAT > CTT titer	CAT < CTT titer
Anti-B in blood group A (60)	49 (81.67%)	24 (40.00%)	5 (8.33%)	31 (51.67%)	54 (90.00%)	28 (46.67%)	2 (3.33%)	30 (50.00%)
Anti-A in blood group B (60)	56 (93.30%)	30 (50.00%)	13 (21.67%)	17 (28.33%)	58 (96.67%)	27 (45.00%)	10 (16.67%)	23 (38.33%)
Anti-A in blood group O (60)	54 (90.00%)	23 (38.33%)	35 (58.33%)	2 (3.33%)	59 (98.30%)	34 (56.67%)	13 (21.67%)	13 (21.67%)
Anti-B in blood group O (60)	51 (85.00%)	20 (33.33%)	37 (61.67%)	3 (5.00%)	57 (95.00%)	26 (43.33%)	23 (38.33%)	11 (18.33%)

CTT= conventional tube test; CAT= column agglutination technique; *concordance within one dilution difference

Table 2 Median comparison of ABO antibody titers between CTT and CAT

Blood group antibodies (no. samples)	Median (min-max) of antibody titer in each method					
	CTT IgM	CAT IgM	*p*-value*	CTT IgG	CAT IgG	*p*-value*
Anti-B in blood group A (60)	64 (8-512)	32 (4-512)	< 0.001	64 (8-1024)	64 (8-512)	< 0.001
Anti-A in blood group B (60)	32 (4-256)	32 (4-128)	0.58	64 (4-256)	32 (2-128)	0.04
Anti-A in blood group O (60)	128 (8-512)	192 (16-1024)	< 0.001	256 (64-4096)	256 (64-1024)	0.56
Anti-B in blood group O (60)	128 (8-512)	128 (16-1024)	< 0.001	512 (32-2048)	512 (16-1024)	0.06

CTT= conventional tube test; CAT= column agglutination technique; **p*-value from Wilcoxon signed rank test

Table 3 Correlation and agreement of ABO antibody titers between CTT and CAT among all samples

Blood group antibodies (no. samples)	Spearman correlation coefficient (rho)		Lin's Concordance correlation coefficient (CCC)*	
	IgM	IgG	IgM	IgG
Anti-A (120)	0.86 ($p < 0.001$)	0.94 ($p < 0.001$)	0.83 ($p < 0.001$)	0.93 ($p < 0.001$)
Anti-B (120)	0.73 ($p < 0.001$)	0.90 ($p < 0.001$)	0.72 ($p < 0.001$)	0.88 ($p < 0.001$)

*based on natural log-transformation of titer's values

Table 4 Agreement of ABO antibody titers between CTT and CAT using Bland-Altman plot*

Blood group antibodies (no. samples)	IgM		IgG	
	Bias (mean difference)	95% Limit of Agreement	Bias (mean difference)	95% Limit of Agreement (lower, upper)
Anti-B in blood group A (60)	-0.39	-1.70, 0.92	-0.39	-1.37, 0.59
Anti-A in blood group B (60)	-0.03	-1.25, 1.18	-0.17	-1.22, 0.88
Anti-A in blood group O (60)	0.46	-0.53, 1.45	-0.07	-1.06, 0.92
Anti-B in blood group O (60)	0.53	-0.68, 1.74	0.15	-0.97, 1.27

*based on natural log-transformation of titer's values

The agreement analysis for the two techniques by Bland-Altman plot using mean difference of test results. The natural log that transformed from the titer values were used for calculation. It was found that the mean difference between log titer of IgM anti-A and anti-B in A, B and O blood groups by auto-CAT and CTT was in the range of -0.39 to 0.53. The mean difference between log titer of IgG anti-A and anti-B in A, B and O blood groups by auto- CAT and CTT was in the range of -0.39 to 0.15 (Table 4).

Discussion

This study indicated that ABO antibody titration using auto-CAT was reliable. The results were in good agreement and correlated well with CTT at the high level. It consumed less operation time than CTT. The mean time for the titration of IgM and IgG ABO antibodies in A and B blood groups by auto-CAT and CTT were 44 and 54 min., respectively. While IgM and IgG anti-A and anti-B in blood group O by auto-CAT and CTT were 62 and 75 min., respectively.

Although, median of IgM and IgG ABO antibody titers by CTT were similar to auto-CAT, the high variation of the medians resulting in statistically significant difference was found in almost all of blood groups by CTT and auto-CAT (Table 2), except the median titers of IgM anti-A in B, and IgG anti-A and anti-B titer in O blood groups showed no significant difference. This observation was different from the study of Park ES et al.[18] Their study revealed the median of IgG anti-A and anti-B in B and A blood groups by CTT and auto-CAT showed no significant difference. Whereas the median of IgG anti-A and anti-B in O blood group by auto- CAT was statistically significant higher than CTT (anti-A, $p < 0.001$; anti-B, $p < 0.001$). The discrepancy may be due to the different technique such as they used manual-CAT and LISS/Coombs card from Diamed, Switzerland, which was difference in details of the technique. This may lead to the difference of antibody titers.[16,28-30]

According to the American Association of Blood Banks (AABB),[24] antibody titration is the measurement of antibody concentration by serial two-fold dilution which is semi-quantitative method and may result in high variation of the test. On the other hand, there were many variations in the substances in plasma itself which resulting in the acceptance of one dilution plus or minus as no difference. Therefore, the significant difference in titers should exceed 3 dilutions. This study revealed that concordance rate of the test samples which their titers were not exceed one dilution

by auto-CAT and CTT were very high as 81.67% to 98.30%. The concordance rate IgG antibody titers in O and B were as high as 98.3% and 96.67%, respectively (Table 1). This finding was in agreement with Shirey RS et al.[30] They found that concordance rate of IgG antibody titers in O blood group by CTT and auto-CAT was 100%. In addition, the number of test samples with the same titer had 86% concordance rate, while those with the different titers not exceed 1 dilution was 14%. The similarity of the two studies may be due to the similar use of material and method to perform the IgG titration which included the separation of the test set of plasma in the step of IAT by CTT from IgM detection. Starting from incubation at 37°C without RT phase, including considered the last weak positive as the end point. AuBuchon JP, et al.[15] had showed that the reading of weak reaction from the tube with weak positive could reduce the difference in the result between the examiners. In addition, Shirey RS, et al. also used Ortho Clinical Diagnostics cassette for IgG detection. If compared the number of samples with the same titer, the concordance rate of IgG anti-A and anti-B in O blood group was lower than their study which were 56.67% (34 cases) and 43.33% (26 cases), respectively. In addition, 25 cases (41.67%) and 31 cases (51.67%) had IgG anti-A and anti-B titers difference not exceed 1 dilution, respectively. The rest of samples, 1 case (1.67%) and 3 cases (5.00%) had 2 dilutions difference in titer for anti-A and anti-B, respectively (Table 1). The difference in the result may be due to the difference of gel manual-CAT from auto-CAT.

The correlation study of IgM and IgG ABO antibodies by auto-CAT and CTT showed that Spearman correlation coefficient; rho of IgG and IgM antibody titer were very high as rho > 0.90 and > 0.73, respectively (Table 3), indicating that both techniques had high correlation which were in agreement with many reports.[19-20] However, good correlation only may not be sufficient to clearly indicate agreement of the two techniques. Other test for agreement should be considered.

Bland-Altman plot[31] is the tool for agreement study of the two techniques by considering from the mean difference of the two techniques. The mean difference of the results from the new and the standard techniques of each test sample is considered. The best mean difference is equal to zero which is impossible in fact. Therefore, in practice, the acceptable mean difference at the range of ± 1.96 SD or assigned as limit of agreement (LOA) at 95% confident interval is used. Due to non-normality distribution of this study, the titers had to be transformed into natural log before applying Bland-Altman plot Altman plot. This study revealed that mean difference of log titer of ABO antibodies in A, B and O blood groups by auto-CAT and CTT were closed to zero (-0.39 to 0.53) for each blood group (Table 4). This finding indicated that the titers tested by auto-CAT and CTT were similar or in good agreement. After converting the log titer (antilog value) to actual titers revealed that mean difference between titers obtained by auto-CAT and CTT not exceed 1.70 (Table 5) which was very little difference. This should not have impact on the patient actual titer.

The agreement study between titers was obtained from auto-CAT and CTT using Lin's Concordance (Lin's CCC).[27] It was found that Lin's CCC of IgM and IgG were ≥ 0.72 and 0.8, respectively (Table 3), indicating that the results of IgM and IgG titers by both techniques were moderately to highly agreeable, respectively. This was different from the study of Bhangale, et al.[19] They found that IgG ABO antibody titers by CAT and CTT showed Lin's CCC equal to 0.58. The disagreement may be due to the difference in materials and methods used. They used manual CAT (gel test) Diamed, Switzerlan, including the use of plasma which were destroyed for IgM and consider end point at 1+. However, Bhangale A, et al. found that Spearman correlation coefficient value of the two techniques was 0.94 which was closed to the result of this study. In addition, they concluded that the antibody titration by the two techniques were of high correlation.

Table 5 Mean difference of ABO antibody titers using antilog value between CTT and CAT

Blood group antibodies (no. samples)	Bias (mean difference)	
	IgM	IgG
Anti-B in blood group A (60)	0.68	0.68
Anti-A in blood group B (60)	0.97	0.84
Anti-A in blood group O (60)	1.58	0.93
Anti-B in blood group O (60)	1.70	1.16
Anti-A in all subjects (120)	1.23	0.33
Anti-B in all subjects (120)	1.07	0.89

Antibody in ABO system is mainly IgM and IgG. The IgM antibody in this system is capable to react in the range of 4-37°C, the same as IgG antibody. Therefore, to perform the titration of IgG antibody, IgM antibody in plasma should be destroyed before testing for IAT. Dithiothreitol (DTT) is mostly used for this purpose. It can mitigate the interference of IgM from IgG.[32] There were many studies reported that the average titers of IgG antibody which did not use DTT treatment were higher than titers obtained from DTT treated plasma samples.[18,20,28] Due to the difficulty in process of DTT treatment and its time consuming,[33] most of blood banks including us do not use DTT in the routine work and accept the result in IAT as the titer of IgG antibody. Actually, this should be the titer of total antibody according to the College of American Pathologists.[16,28]

To perform IgG antibody titration, many studies used monospecific anti-IgG for CTT and used anti-IgG cassette for CAT in order to avoid the effect of complement which may cause higher result than actual result. However, Vanamalar A, et al.[34] concluded that monospecific AHG gave more sensitive reaction than polyospecific AHG. In this study, antihuman globulin (AHG), polyspecific (IgG & C3d) was used for CTT and polyspecific cassette was used for auto-CAT.

King Chulalongkorn Memorial Hospital Blood Bank uses these reagents in the routine work. To compare polyspecific cassette and anti-IgG cassette, 45% of IgG antibody gave the same titer while 55% of IgG antibody from anti-IgG cassette gave 1 dilution higher which was not significant difference.[24]

The limitation of this study: only apparently healthy blood donors were included. Most of them possessed high titer of ABO antibodies. Further study in the future should include the comparison among the patients, especially ABO incompatible kidney transplant candidates. These patients are subjected to maintain low level of ABO antibodies prior to transplantation and throughout their life. So that the information can be effectively applied for the patient therapeutic use.

Conclusion

This study showed that ABO antibody titration by ORTHO VISION Analyzer CAT and the standard CTT gave high correlation and good agreement which reflexed the reliability of auto-CAT. In addition to other advantages such as fast result report, objective and reproducibility of the technique will enable clinicians to successfully provide good care to the patients, especially the ABO incompatible kidney transplant patients. The result of this study renders us to use fully automation (ORTHO VISION Analyzer) which is already use for routine work to perform the antibody titration as well in order to prevent AMR in ABO incompatible kidney transplant patients.

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