IJPRP

Indian Journal of Pathology: Research and Practice
Volume 9 Number 1 (Part II), January - April 2020
DOI: http://dx.doi.org/10.21088/ijprp.2278.148X.9120.37

Original Research Article

Effects of Anticoagulants on Clarity of the Agglutination in Coombs Test

Siddhartha Shanker Sinha[1], Yogesh Kumar Yadav[2], Sonal Saxena[3], Pooja Jaiswal[4], Juhi Chauhan[5]

[1]Associate Professor, [3]Assistant Professor, [4]Associate Professor, [5]Senior Resident, Department of Pathology, Integral Institute of Medical Sciences and Research, Lucknow, Uttar Pradesh 226026, India. [2]Associate Professor, Department of Pathology, Autonomous State Medical College, Ayodhya, Uttar Pradesh 224001, India.

Corresponding Author:
Yogesh Kumar Yadav, Associate Professor, Department of Pathology, Autonomous State Medical College, Ayodhya, Uttar Pradesh 224001, India.

E-mail: dr.yogi007@gmail.com

Received on 14.11.2019

Accepted on 29.01.2020

Abstract

Context: Antiglobulin (Coombs) test is very important test in the detection of incomplete (IgG) antibodies in the blood, either in free form (Indirect coombs test) or bound to the red blood cell (Direct coombs test). There is a possibility that anticoagulants like Ethylenediaminetetraacetic acid (EDTA), Citrate, etc used for the collection of blood sample, may effect the end result of the test. *Aims:* The present study is aimed at comparison of the end result (the clarity of agglutination) between the coombs test performed on plain blood and anti- coagulated blood (EDTA and Citrate). *Settings and Design:* Blood samples collected from blood donors at the time of blood donation in plain and anti- coagulated blood (EDTA and Citrate). Samples were subjected to coombs test at various dilutions and agglutination was observed under the microscope. *Methods and Material:* 6 ml. of blood samples will be collected from the 100 blood donors at the time of blood donation in and 2 ml each transferred into; EDTA bottle, Citrate bottle and Plain bottle. The sample thus collected was used for performing the Anti-globulin test. 10% red cell suspension was sensitized with Anti D IgG (as supplied by Tulip Diagnostics) at different dilutions (1/10, 1/20, 1/30, 1/40). The washed saline suspension of RBC's is treated with Anti- human globulin serum and agglutination is observed under a microscope at 5X objective. *Statistical analysis used:* Friedman test. *Results:* The degree of agglutination obtained by using plain blood samples at various dilutions, tend to be higher compared to the same obtained by using citrate and EDTA blood samples. The citrate blood samples gives intermediate pattern between plain blood samples and EDTA blood samples. *Conclusions:* The use of plain blood was found to give better results compared to citrate and EDTA blood in performing the antiglobulin test. The chances of missing the detection of low titres of anti-D IgG is more with EDTA blood samples when compared to citrate blood samples and least with plain blood samples.

Keywords: Antiglobulin; Coombs; Anticoagulants, Ethylenediaminetetraacetic acid (EDTA), Citrate

How to cite this article:
Siddhartha Shanker Sinha, Yogesh Kumar Yadav, Sonal Saxena, et al. Effects of Anticoagulants on Clarity of the Agglutination in Coombs Test. Indian J Pathol Res Pract. 2020;9(1 Part II):241–247.

Introduction

Coombs test (also known as antiglobulin test or AGT) refers to two clinical blood tests used in immunohematology and immunology. The two types of Coombs tests are the direct Coombs test (also known as direct antiglobulin test or DAT), and the indirect Coombs test (also known as indirect antiglobulin test or IAT).

The more commonly used test, the Direct Coombs test, is used to test for autoimmune hemolytic anemia.

Antiglobulin (Coombs test) is very sensitive and is an important test in the detection of incomplete (IgG) antibodies in the blood either in free form or bound to RBC. There is possibility that anticoagulants like ethylene diamine tetra acetic acid (EDTA) and citrate, etc used in the collection of blood samples may affect the end result of the test.

The present study is aimed at the comparison of the end result (clarity of agglutination) obtained on performing Coombs test using plain, EDTA and citrate blood samples.

Materials and Methods

Blood samples were collected from 100 blood donors at the time of blood donation.

Method of collection of data

6 ml. of blood samples are collected from the blood donors at the time of bleeding and 2 ml. each transferred into; EDTA bottle, Citrate bottle and Plain bottle. Blood samples are collected from the blood donors at the time of bleeding at blood bank. The samples thus collected were used for performing the Anti-globulin test. The Anti-globulin test was done as follows:

A. Centrifuge the blood sample and discard the plasma/serum.
   a) 0.5 ml of the Red Blood Cells sediment is mixed with 4 ml of normal saline by vigorous shaking.
   b) Centrifuge at 2000 rpm for 5 minutes. The supernatant is removed and the sediment is resuspended in the saline. This procedure is repeated 3 times.
B. 5% suspension of washed Red Blood Cells was prepared.
C. The Red Blood Cells are sensitized with anti-D IgG as follows:-
   a) *Undiluted*: Take 100 μL of Red Blood Cells suspension and add 200 μL of undiluted anti-D IgG.
   b) *1/10 Dilution:* Take 100 μL of Red Blood Cells suspension and 200 μL of 1/10 diluted anti-D IgG.
   c) *1/20 Dilution:* Take 100 μL of Red Blood Cells suspension and 200 μL of 1/20 diluted anti-D IgG.
   d) *1/30 Dilution:* Take 100 μL of Red Blood Cells suspension and 200 μL of 1/30 diluted anti-D IgG.
   e) *1/40 Dilution:* Take 100 μL of Red Blood Cells suspension and 200 μL of 1/40 diluted anti-D IgG.
D. Mix well and incubate for 45 minutes.
E. Wash the incubated preparation for 3 times in saline.
F. Decant the supernatant completely. Prepare a 5% saline suspension.
G. Add 100 μL of Coombs (anti human globulin) reagent to 50 μL of 5% saline suspension. Mix well and immediately centrifuge at 1000 rpm for 10 seconds.
H. The sediment is agitated and transferred to a glass slide.
I. The agglutination is observed under a microscope at 5X objective.
J. The microscopic pictures are divided into 6 groups:
   a) *4+:* Almost 100% of RBC's are clumped with large single or only few clumps.
   b) *3+:* About 75% of RBC's are clumped with several large clumps which are visible even macroscopically.
   c) *2+:* About 50% of RBC's are clumped. The clumps are many and small even visible macroscopically.
   d) *1+:* About 25% of RBC's are clumped. The clumps are tiny and numerous. Macroscopically just visible.
   e) *+/-:* Less then 25% of RBC's are clumped. Tiny clumps visible microscopically only.
   f) *-ve:* No clumps, evenly distributed RBC's, No clumps visible microscopically.

Inclusion criteria

Donor selection as per drug control act. No prior

investigation of donor expect haemoglobin. Sample wad collected only from the tubing of the blood bag.

Exclusion criteria

RH -ve blood samples and those blood samples which contains cold auto agglutinins (those clump in bovine albumin).

Results

The application of Coombs test was done on Donor's Blood and the results obtained are presented in the form of charts and table.

The results are presented in the Fig. 1 (donor's plain blood samples), Fig. 2 (donor's citrate blood samples) and Fig. 3 (donor's EDTA blood samples). The results obtained from donor's blood samples are presented in Table 1 (plain blood), Table 2 (citrate clood) and Table 3 (EDTA blood).

From the Tables and Charts it is also observed that:-

- The degree of agglutination is maximum in undiluted samples and decline as the dilution increases.
- Degree of agglutination is more in plain blood samples compare to those of citrate blood samples and EDTA blood samples.
- Between citrate and EDTA blood samples the degree of agglutination is comparatively

Table 1: Number and percentage of agglutination in plain blood samples

Donor's Sample	-ve		+/-		1+		2+		3+		4+	
	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples		%	No. of samples
Plain Blood UD	0	0	0	0	0	0	0	0	35	35.0	65	65
Plain Blood 1/10	0	0	0	0	0	0	36	36	64	64.0	0	0
Plain Blood 1/20	0	0	0	0	32	32	68	68	0	0	0	0
Plain Blood 1/30	0	0	33	33	60	60	7	7.0	0	0	0	0
Plain Blood 1/40	47	47	53	53	0	0	0	0	0	0	0	0

Abbreviations: UD (undiluted)

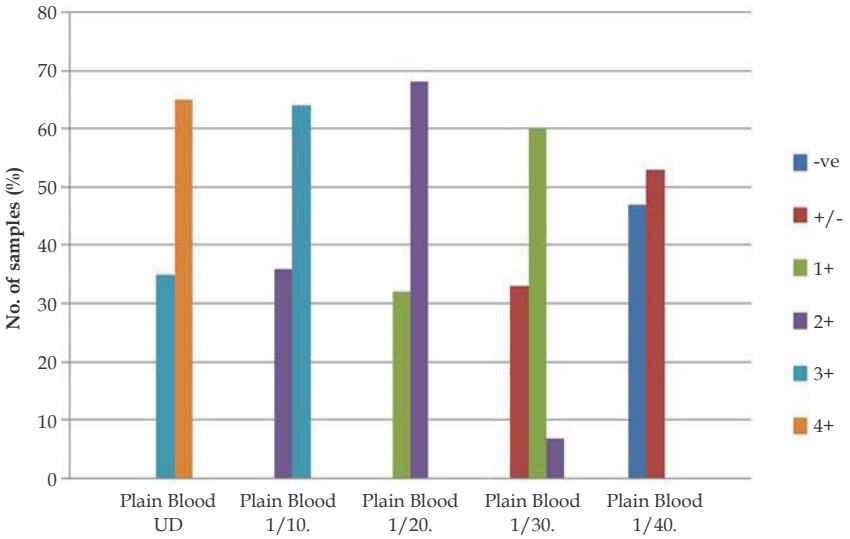

Fig. 1: Percentage and results of donor's blood samples in plain blood.

Table 2: Number and percentage of agglutination in citrate blood samples

Donor's Sample	-ve		+/-		1+		2+		3+		4+	
	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%
Citrate UD	0	0	0	0	0	0	0	0	51	51	49	49
Citrate 1/1	0	0	0	0	6	6	50	50	44	44	0	0
Citrate 1/2	0	0	6	6	48	48	46	46	0	0	0	0
Citrate 1/30	6	6	50	50	44	44	0	0	0	0	0	0
Citrate 1/4	55	55	45	45	0	0	0	0	0	0	0	0

Abbreviations: UD (undiluted)

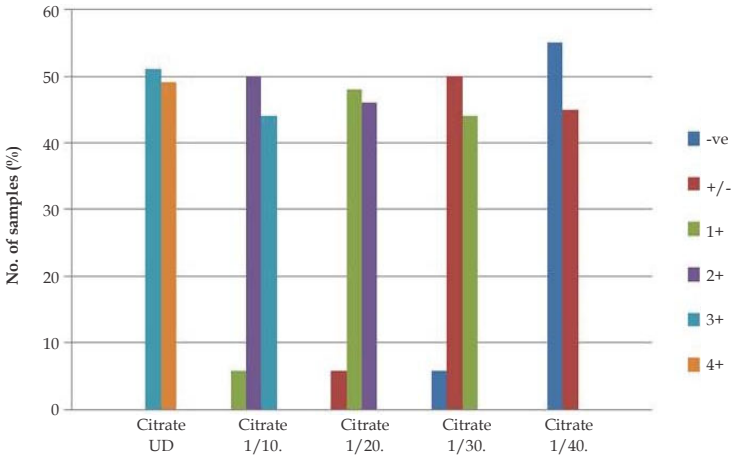

Fig. 2: Percentage and results of donor's blood samples in citrate blood.

Table 3: Number and percentage of agglutination in EDTA blood samples

Donor's Sample	-ve		+/-		1+		2+		3+		4+	
	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%
EDTA UD	0	0	0	0	0	0	0	0	60	60	40	40
EDTA 1/10	0	0	0	0	0	0	65	65	35	35	0	0
EDTA 1/20	0	0	3	3	69	69	28	28	0	0	0	0
EDTA 1/30	24	24	53	53	23	23	0	0	0	0	0	0
EDTA 1/40	81	81	19	19	0	0	0	0	0	0	0	0

Abbreviations: EDTA (Ethylenediaminetetraacetic acid), UD (undiluted)

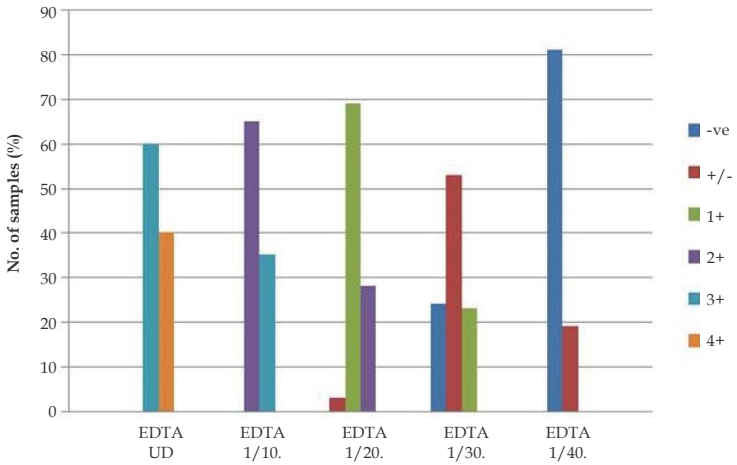

Fig. 3: Percentage and results of donor's blood samples in Ethylenediaminetetraacetic acid blood

more with citrate than EDTA blood samples.

Sixty-five percent of donor's blood sample showed 4+ agglutination with undiluted samples when plain blood samples are used. This percentage drops down to 49% with donor's blood undiluted samples when citrate blood samples are used; and 40% donor's undiluted blood samples when EDTA blood samples are used.

Whereas when samples with maximum dilution (1/40) are used negative results (no agglutination) are obtained with 47% donor's blood samples when plain blood sample is used. 55% of donor's blood samples when citrate blood samples is used. 81% donor's blood samples with EDTA blood samples.

The details of 25th percentiles, 50th median and 75th percentiles along with Friedman test value and p value of donor's blood samples at various dilutions of plain blood samples are presented in Table 4, of citrate blood samples in Table 5 and of EDTA blood samples in Table 6.

From the Tables 4, 5 and 6 it is noted that the degree of agglutination is maximum with undiluted blood samples and minimum or nil with 1/40 dilutions and there is a study decline as the dilutions increases which is evident from the Friedman test value which is between 380 to 400 in all 3 types of samples of both categories. Similarly the p value is consistently less than 0.001 in all 3 samples which shows highly significant deference

Table 4: Donor's blood samples of plain blood in various dilutions - Friedman test and *P*-value

Donor's Sample	*N*	25th Percentiles	50th (Median)	75th Percentiles	Friedman test value	*p*-value
Plain Blood UD	100	4.00	5.00	5.00	392.353	$p < 0.001$
Plain Blood 1/10	100	3.00	4.00	4.00		HS
Plain Blood 1/20	100	2.00	3.00	3.00		
Plain Blood 1/30	100	1.00	2.00	2.00		
Plain Blood 1/40	100	.00	1.00	1.00		

Abbreviations: UD (undiluted)

Table 5: Donor's blood samples of citrate blood in various dilutions-Friedman test and *P*-value

Donor's Sample	*N*	25th Percentiles	50th (Median)	75th Percentiles	Friedman test value	*p*-value
Citrate UD	100	4.00	4.00	5.00	369.494	p<0.001
Citrate 1/10	100	3.00	3.00	4.00		HS
Citrate 1/20	100	2.00	2.00	3.00		
Citrate 1/30	100	1.00	1.00	2.00		
Citrate 1/40	100	00	00	1.00		

Abbreviations: UD (undiluted)

Table 6: Donor's blood samples of EDTA blood in various dilutions-Friedman test and *P*-value

Donor's Sample	N	25th Percentiles	50th (Median)	75th Percentiles	Friedman test value	*p*-value
EDTA UD	100	4.00	4.00	5.00	387.893	$p < 0.001$
EDTA 1/10	100	3.00	3.00	4.00		
EDTA 1/20	100	2.00	2.00	3.00		
EDTA 1/30	100	1.00	1.00	1.00		
EDTA 1/40	100	00	00	00		

Abbreviations: EDTA (Ethylenediaminetetraacetic acid), UD (undiluted)

in the end results of undiluted and various dilution samples.

Discussion

The antiglobulin test was first introduced by Dr Robin Coombs in 1945, since than this test is referred as the Coombs' test.[1] This immunohematologic technique has become a important tool in detecting immune-mediated hemolytic anemia, non immune hemolytic anemia, hemolysis of the newborn and transfusion reactions.[1,2] The direct antiglobulin test (DAT) is a method of demonstrating the presence of immunoglobulin (Ig) and/or complement, bound to the surface of red blood cells (RBCs) by the use

of anti-human globulin to form a agglutination reaction which can be visibly detected. The test is very specific and sensitive in the detection of erythrocytic auto and alloantibodies.[3,4] Although an immune mechanism for hemolysis, the DAT may have certain pitfalls it can be negative for certain reasons. Autoantibodies which cause hemolysis perticularly IgA or IgM have a negative DAT because in this test we can only detect RBC that are coated with IgG or C3d.[5] When lower titer of potent antibodies present, the DAT may fail to detect them.[6] when adequate titer of low affinity antibodies are present which are clinically relevant may get removed at the time of washing in DAT testing resulting in negative test.[7] However, the conventional method of Coomb's test is stilll highly effective and reliable, even after the development of newer methods like microtiter plates, gel microcolumns, capillary tubes and flow cytometry.[8]

Gel and solid phase methods offer some advantages over the tube method such as automation and technically easy to perform.[9,10] However, the essential principle remains the same.

The indirect coomb's test can be performed by serum or plasma while direct by red blood cells of the blood samples. However, Lewis S.M. et al. preferred serum to plasma for indirect coombs test, but also quoted that plasma is being used increasingly for convenience purpose.[8] When plasma is used, complement is inhibited by the EDTA anticoagulant, which effects the detection of certain antibody (e.g. Kidd) or gives weak reaction with anti-D IgG.[8] Baker et al. for direct coombs test mentioned that Red Blood Cells preferably from clot should be used for performing antiglobulin test.[11,12] The reagent to be used to modulate antibody binding in coombs test can be better Understood by studying anti-Rh(D) antibodies on a molecular level.[13] In the present work it is noticed that the degree of clumping in antiglobulin test done with various dilutions of anti-D IgG is more with plain blood samples compared with those to EDTA blood samples. An attempt is also made to compare the results of plain blood samples and EDTA blood samples with Citrate blood samples. It is noticed that the result with citrate blood samples are in between those obtained from plain blood and EDTA blood. The above finding are consistent with the view of both Lewis et al. and Baker et al., that plain blood is preferred in performing the antiglobulin test.

In the blood bank the donor's blood is collected in ACD/CPD bags. After disconnecting the needle from the donors vein the blood in the tubing is made to go into the bag, then a small portion of this blood is squeezed into a glass tube for performing various tests. The Red Blood Cells from the citrated blood sample is used for doing a Coombs cross-match and for the detection of irregular antibodies. In the present study the blood samples were collected from the tubings directly in plain bottle, EDTA bottle and Citrate bottle (without prior mixing ACD/CPD).

The present study as revealed that

1. The degree of agglutination is maximum with undiluted blood samples and reduces as the dilution increases. This indicates the degree of agglutination is proportionate to titre of anti-D IgG.
2. The degree of agglutination obtained by using plain blood samples at various dilutions, tend to be higher compared to the same obtained by using citrate and EDTA blood samples shows greater difference as indicated by the Wilicoxon Signed Ranks Test Z and *p*-value as shown in Table 10. The difference is more so with higher dilution samples which indicates that there is a possibility that a low titre of anti-D IgG (both by direct antiglobulin test and indirect antiglobulin test) may be missed if EDTA blood samples are used compare to plain blood samples.

The citrate blood samples gives intermediate pattern between plain blood samples and EDTA blood samples.

Microscopy and grading of agglutination is highly subjective. The present study is a first of its kind. Extensive search in archives and internet has not revealed any such studies. This is the reason why very minimal references are available.

Conclusion

From the results obtained and analyzed in the present study the following conclusions are made:-

1. The use of plain blood is found to give better results compared to citrate and EDTA blood in performing the antiglobulin test.
2. The chances of missing the detection of low titres of anti-D IgG is more with EDTA blood samples when compared to citrate blood samples and least with plain blood samples.
3. Due to these reasons it is recommended to carry forward and have further studies and evolve a consensus on the use of plain or

anticoagulated blood samples in reporting Coombs test.

Acknowledgement: none

Conflict of Interest: none

Key Messages: For performing coombs test, use of plain blood instead of anticoagulated blood gives better results. There is better probability detection of low titres of anti-D IgG with plain blood samples as compared to anticoagulated blood (Ethylenediaminetetraacetic acid (EDTA) or Citrate).

References

1. Coombs RR, Mourant AE, Race RR. A new test for the detection of weak and incomplete Rh agglutinins. Br J Exp Pathol 1945;26:255–66.
2. Rosse WF. The antiglobulin test in autoimmune hemolytic anemia. Annu Rev Med 1975;26:331–6.
3. Rumsey DH, Ciesielski DJ. New protocols in serologic testing: A review of techniques to meet today's challenges. Immunohematology 2000;16:131–7.
4. Widmann FK, Barnes A, Case J, et al. Technical Manual, 8th ed. Washington DC: AABB; 1981:422–516.
5. McGann PT, McDade J, Mortier NA, et al. IgA-mediated autoimmune hemolytic anemia in an infant. Pediatr Blood Cancer 2011;56:837–9.
6. Merry AH, Thomson EE, Rawlinson VI, et al. Quantitation of IgG on erythrocytes: correlation of number of IgG molecules per cell with the strength of the direct and indirect antiglobulin tests. Vox Sang 1984;47:73–81.
7. Garratty G. Immune hemolytic anemia associated with negative routine serology. Semin Hematol 2005;42:156–64.
8. Bain BJ, Bates I, Laffan MA, et al. Dacie and Lewis Practical Haematology, 11th ed. Edinburgh: Elsevier Churchill Livingstone 2012.pp.229–44.
9. Alwar V, Shantahla Devi AM, Sitalakshmi S, et al. Evaluation of the use of gel card system for assessment of direct Coombs test: Weighing the pros and cons. Indian J Hematol Blood Transfus 2012;28(1):15–8.
10. Moulds JM, Diekman L, Wells TD. Evaluation of column technology for direct antiglobulin testing. Immunohematology 1998;14(4):146–8.
11. F. J. Baker. Blood group technique. In: Introduction to medical laboratory technology 5th ed F. J. Baker and R.E. Silverton, London: The English Language Book Society and Butterworths publishers 1978. pp.673–4.
12. Linda A. Smith. Hemolytic Anemia: Immune anemias. In: Clinical laboratory hematology, 2nd ed. Shirlyn B. Mckenzie and J. Lynne Williams, New Jersey: Pearson clinical laboratory science series 2010.pp.345–7.
13. Chang T Y, Siegel D L. Genetic and immunological properties of phage-displayed human anti-Rh(D) antibodies: Implications for Rh(D) epitope topology. Amer J Hematol 1998;91:3066–78.