Original Article

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Does Sample Collected in Plain Vial Provides Better Results Than Anticoagulated Blood in the Antiglobulin Test?

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Abstract

Context: The antiglobulin test is an important test in the detection of immunoglobulin or complement bound on the surface of red blood cells (RBC) and serum antibodies. It is possible that anticoagulants used for collection of blood samples may affect the end result of this test. *Aim:* To study the variation in degree of agglutination between the antiglobulin test performed on plain blood and anticoagulated blood. *Settings and Design:* Antiglobulin Test was performed on blood samples collected from umbilical cord of 100 new-born babies during delivery in plain vials and vials with anti-coagulant like ethylenediaminetetraacetic acid (EDTA) and citrate. *Methods and Materials:* 6 ml of blood samples were collected from umbilical cord immediately after delivery and 2 ml each transferred into: EDTA, Citrate and Plain vials. The sample thus collected was used for performing the direct antiglobulin test. 10% red cell suspension was sensitized with Anti D IgG (as supplied by Tulip diagnostics) at different dilutions of 1/10, 1/20, 1/30 and 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. The picture is taken of microscopic field using MIPS (micro image processing system) and saved. *Statistical Analysis used:* Friedman test and Wilcoxon signed-rank test. *Results:* The degree of agglutination at various dilutions obtained by using plain blood samples was found to be higher than that obtained by using anticoagulated blood. The citrated blood samples gave intermediate agglutination results between blood samples collected in plain and EDTA vials.

Keywords: Antiglobulin Test; Agglutination; EDTA; Citrate.

Introduction

The antiglobulin test was first described in 1945 by Robert Royston Amos Coombs and colleagues [1,2]. The indirect antiglobulin test was reinforced by Grove-Rasmussen in 1964 as a screening method for detection of serum antibodies [3]. Direct antiglobulin test (DAT) was first described in 1908 by Moreschi [4] and is performed to detect surface bound IgG or complement to erythrocyte antigens. Its major utility is in differentiating immune-mediated hemolysis from immune-independent causes. However end result of the test may be affected by the anticoagulants like EDTA and citrate used in the collection of blood samples. This study focuses on comparison of the end result obtained on performing antiglobulin test using plain, EDTA and citrate blood samples. This study is first of its kind. Extensive search in archives and internet has not revealed any such studies.

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Subjects and Methods

Sources of Data: Blood samples were collected from umbilical cord of 100 cases during delivery.

Method of Sample Collection

6 ml. of blood samples were collected from Umbilical

cord immediately after delivery and 2 ml. each transferred into:

1. EDTA vial
2. Citrate vial
3. Plain vial

The samples thus collected were used for performing the antiglobulin test as follows:

Procedure

A. Washing the Red Blood Cells

- The blood samples were centrifuged and the plasma/serum was discarded.
- 0.5 ml of the Red Blood Cells sediment was mixed with 4 ml of normal saline by vigorous shaking.
- Centrifuge at 2000 rpm for 5 minutes. The supernatant was removed and the sediment was resuspended in the saline. This procedure was repeated 3 times.

B. 5% suspension of washed Red Blood Cells is prepared.

C. Sensitization with anti-D IgG at different dilutions as follows:-

- *Undiluted (UD):* 100 μL of Red Blood Cells suspension taken and 200 μL of undiluted anti-D IgG added.
- *1/10 Dilution:* 100 μL of Red Blood Cells suspension taken and 200 μL of 1/10 diluted anti-D IgG added.
- *1/20 Dilution:* 100 μL of Red Blood Cells suspension taken and 200 μL of 1/20 diluted anti-D IgG added.
- *1/30 Dilution*: 100 μL of Red Blood Cells suspension taken and 200 μL of 1/30 diluted anti-D IgG added.
- *1/40 Dilution:* 100 μL of Red Blood Cells suspension taken and 200 μL of 1/40 diluted anti-D IgG added.

D. Mixed well and incubated for 45 minutes.

E. The incubated preparation was washed for 3 times in saline.

F. The supernatant was decanted completely and a 5% saline suspension was prepared.

G. 100 μL of Coombs (anti human globulin) reagent was added to 50 μL of 5% saline suspension. Mixed well and immediately centrifuged at 1000 rpm for 10 seconds.

H. The sediment was agitated and transferred to a glass slide.

I. The agglutination was observed under a microscope at 5X objective. The picture was taken of microscopic field using MIPS (micro image processing system) and saved.

J. The microscopic pictures were divided into 6 groups:-

- *4+:* Almost 100% of RBC's clumped with large single or only few clumps.
- *3+:* About 75% of RBC's clumped with several large clumps which are visible even macroscopically.
- *2+:* About 50% of RBC's clumped. The clumps are many and small even visible macroscopically.
- *1+:* About 25% of RBC's clumped. The clumps are tiny and numerous. Macroscopically just visible.
- *+/-:* Less than 25% of RBC's clumped. Tiny clumps visible microscopically only.
- *-ve:* No clumps, evenly distributed RBC's, No clumps visible microscopically.

Inclusion Criteria

All types of deliveries (normal, assisted or caesarean). Both gender newborns. No previous haematological investigation of the mother. No specific ABO blood group, but only RH+ blood is included for the study.

Exclusion Criteria

RH -ve blood samples and those blood sample which contained cold auto agglutinins.

Stastical Analysis

Friedman test and Wilcoxon signed-rank test were performed. A p value less than or equal to 0.05 was considered significant.

Results

Figure 1 showing degree of agglutination seen with plain blood and anticoagulated blood. 60% of the cases showed 4+ agglutination with undiluted samples used (Table 1). This percentage dropped down to 40% with undiluted samples when citrate blood samples were used (Table 2) and 34% with undiluted (UD) blood samples when EDTA blood samples were used (Table 3).

Whereas when samples with maximum dilution (1/40) were used negative results (no agglutination) were obtained with 33% of plain blood samples, 56% with citrate blood samples and 67% with EDTA blood samples.

The details of 25th percentiles, 50th median and 75th percentiles along with Friedman test value and p value at various dilutions of plain blood samples, citrate blood samples and of EDTA blood samples are presented respectively in Table 4, Table 5 and Table 6. From these tables it was noted that the degree of agglutination was maximum with undiluted blood samples and minimum or nil with 1/40 dilutions and there was a steady decline as the dilutions increased which was evident from the Friedman test value which is between 380 to 400 in all 3 types of samples. Similarly the p value was consistently less than 0.001 in all 3 samples which shows highly significant difference in the end results of undiluted and various dilution samples. Table 7 shows the Wilcoxon signed-rank test Z and p values obtained on comparing EDTA with plain blood, citrate with plain and citrate with EDTA of undiluted and different dilution samples. It is evident from the table that there was considerable difference between EDTA and plain blood with Wilcoxon signed-ranks test Z being very high ranging between 3.5 and 6.5 in all the dilutions. The values were not so high when plain blood samples were compared with citrate with Wilcoxon signed-ranks test Z ranging between 2.0 and 4.5 except in the dilution (1/40) where it was 1.02. When citrate was compared with EDTA the Wilcoxon signed-ranks test Z values obtained do not show any consistent pattern at different dilutions.

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

		- ve		+/-		1+		2+		3+		4+		Total	
	Sample	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%
CORD BLOOD SAMPLE	Plain Blood UD	0	.0%	0	.0%	0	.0%	5	5.0%	35	35.0%	60	60.0%	100	100.0%
	Plain Blood 1/10.	0	.0%	0	.0%	5	5.0%	37	37.0%	58	58.0%	0	.0%	100	100.0%
	Plain Blood 1/20.	0	.0%	3	3.0%	39	39.0%	58	58.0%	0	.0%	0	.0%	100	100.0%
	Plain Blood 1/30.	0	.0%	33	33%	67	67.0%	0	.0%	0	.0%	0	.0%	100	100.0%
	Plain Blood 1/40.	33%	33.0%	65	65%	2	2.0%	0	.0%	0	.0%	0	.0%	100	100.0%

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

Sample	- ve		+/-		1		2		3		4		Total	
	No. of Samples	%	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%	4	4.0%	56	56.0%	40	40.0%	100	100.0%
Citrate 1/10.	0	.0%	0	.0%	4	4.0%	54	54.0%	42	42.0%	0	.0%	100	100.0%
Citrate 1/20.	0	.0%	4	4.0%	59	59.0%	37	37.0%	0	.0%	0	.0%	100	100.0%
Citrate 1/30.	3	3.0%	60	60.0%	37	37.0%	0	.0%	0	.0%	0	.0%	100	100.0%
Citrate 1/40.	56	56.0%	44	44.0%	0	.0%	0	.0%	0	.0%	0	.0%	100	100.0%

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

Sample	-ve		+ve / -ve		1+		2+		3+		4+		Total	
	No. of samples	%	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%	4	4.0%	62	62.0%	34	34.0	100	100%
EDTA 1/10	0	.0%	0	.0%	7	7.0%	65	65.0%	28	28.0%	0	0%	100	100%
EDTA 1/20	0	.0%	7	7.0%	65	65.0%	28	28.0%	0	0%	0	0%	100	100%
EDTA 1/30	7	7.0%	61	61.0%	32	32.0%	0	0%	0	0%	0	0%	100	100%
EDTA 1/40	67	67.0%	33	33.0%	0	0%	0	0%	0	0%	0	0%	100	100%

Table 4: Cord blood samples of plain blood in various dilutions

	Sample	N	25th Percentiles	50th (Median)	75th Percentiles	Friedman Test Value	p value
Sample Card Blood	Plain Blood UD	100	4.00	5.00	5.00	396.603	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		

Table 5: Cord blood samples of citrate blood in various dilutions

	Sample	N	25th Percentiles	50th (Median)	75th Percentiles	Friedman Test Value	p value
Card Blood Sample	Citrate UD	100	4.00	4.00	5.00	397.574	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		

Table 6: Cord blood samples of EDTA blood in various dilutions

	Sample	N	25th Percentiles	50th (Median)	75th Percentiles	Friedman Test Value	p value
Card Blood Sample	EDTA UD	100	4.00	4.00	5.00	396.093	p<0.001
	EDTA 1/10.	100	3.00	3.00	4.00		HS
	EDTA 1/20.	100	2.00	2.00	3.00		
	EDTA 1/30.	100	1.00	1.00	2.00		
	EDTA 1/40.	100	.00	.00	1.00		

Table 7: Comparison between plain, citrate and EDTA blood samples at various dilutions

Sample	Wilcoxon Signed Ranks Test Z	p value	
CORD BLOOD SAMPLE			
EDTA UD - Plain Blood UD	4.11	p<0.001	HS
CITRATE UD - Plain Blood UD	2.61	0.009	HS
CITRATE UD - EDTA UD	0.82	0.415	NS
EDTA 1/10. - Plain Blood 1/10.	4.93	p<0.001	HS
CITRATE 1/10. - Plain Blood 1/10.	2.10	0.036	sig
CITRATE 1/10. - EDTA 1/10.	2.29	0.022	sig
EDTA 1/20. - Plain Blood 1/20.	5.24	p<0.001	HS
CITRATE 1/20. - Plain Blood 1/20.	3.18	0.001	HS
CITRATE 1/20. - EDTA 1/20.	1.66	0.096	NS
EDTA 1/30. - Plain Blood 1/30.	5.60	p<0.001	HS
CITRATE 1/30. - Plain Blood 1/30.	4.53	p<0.001	HS
CITRATE 1/30. - EDTA 1/30.	1.21	0.226	NS
EDTA 1/40. - Plain Blood 1/40.	4.01	p<0.001	HS
CITRATE 1/40. - Plain Blood 1/40.	3.50	p<0.001	HS
CITRATE 1/40. - EDTA 1/40.	0.14	0.886	NS

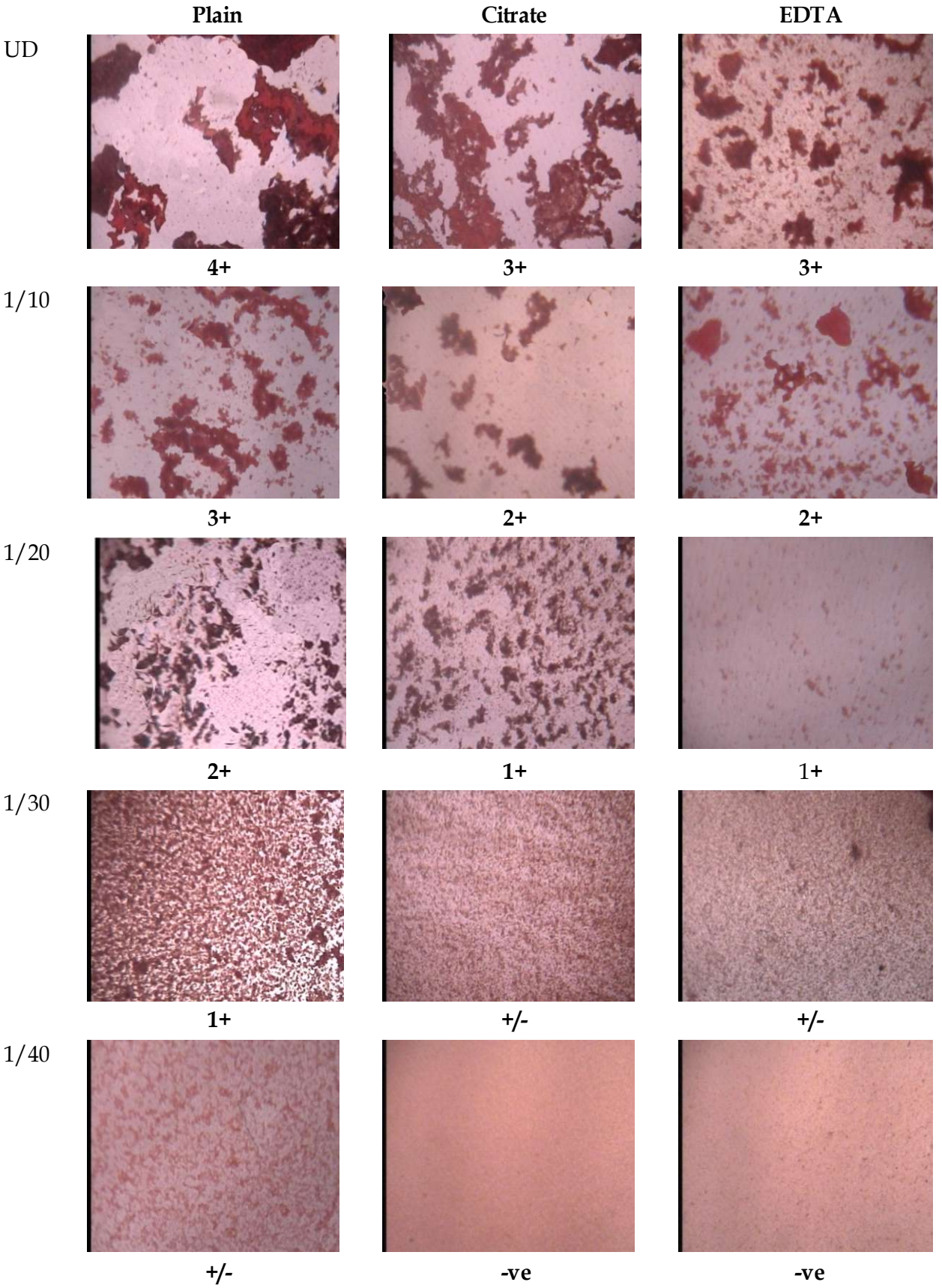

Fig. 1: Antiglobulin test: Degree of agglutination seen with plain sample and sample with anticoagulant (x50X)

Discussion

The indirect antiglobulin test is used by the blood banks in detecting serum alloantibodies. The DAT helps in detection of immunoglobulins or complement, particularly IgG and C3d, attached to the surface of red blood cell [5]. The DAT is therefore used to diagnose haemolytic disease of newborn, autoimmune haemolytic anaemia, drug-induced hemolysis and haemolytic transfusion reactions [5].

The serum has theoretical advantage over plasma for antibody detection as anticoagulants inhibit complement activation and therefore probably hinder the detection of few complement activating antibodies. On the contrary, plasma is being extensively used in the automated systems of grouping and screening of antibodies. Scott et al. have reported comparison of plasma and serum for detection of antibody using gel system [6].

The general practice is to send umbilical cord blood samples from labour room to laboratory in EDTA vials for performing DAT. In the present study, it was noted that degree of agglutination is maximum with undiluted blood samples and reduces as the dilution increases indicating that degree of agglutination is proportionate to the titre of anti-D IgG. Moreover, the degree of agglutination obtained by using plain blood samples at various dilutions, was higher as compared to those using citrate and EDTA blood samples as indicated by the Wilcoxon signed rank test Z and p values. These findings were in accordance with Baker F. J. et al who mentioned that the use of clotted blood (defibrinated) is preferable to anticoagulated blood for the detection of coated antibodies [7]. T

his point is supported by Dacie and Lewis who felt serum (clotted blood) is preferable to plasma for the detection of free red cell antibodies but also mentioned that plasma is increasingly being used for convenience purposes [8].

However, McKenzie preferred EDTA samples for Direct Antiglobulin test procedure because EDTA chelates Ca^{++} and Mg^{++}, preventing the in vitro binding of complement to red cells that can be mediated by naturally occurring cold reactive antibodies [9].

Conclusion

From the results obtained and analyzed in this study we concluded that plain blood gives better results compared to anticoagulated blood in the antiglobulin test. The detection of low titres of anti-D IgG is most likely to be missed with EDTA blood and least with plain blood samples.

Further studies are recommended to develop a consensus on the use of plain blood samples instead of anticoagulated blood samples in performing Antiglobulin test.

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