Documenta Haematologica -
Revista Romana de Hematologie | 2024, Vol. 2, Nr. 4

https://doi.org/10.59854/dhrrh.2024.2.4.167

– ORIGINAL PAPERS –

Study of Haemolytic Disease of the Foetus and Newborn with Positive Direct Coomb's Test in Single Centre

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Abstract

Objectives: *HDFN is caused by maternal alloantibodies against foetal erythrocytes that might cause morbidity and mortality in the foetus and newborn. Our study aims to investigate the incidence of Haemolytic disease of foetus and newborn (HDFN), Direct Coomb's test (DCT), cord blood and to identify the eluted antibodies in those patients. Besides, to evaluate the adapted protocol regarding Rosette and Kleihauer Betke test.*

Methods/Materials: *A retrospective study of all newborns with positive DCT using cord blood samples. Descriptive data were analyzed as frequency and percentage or mean and range. Bivariate analysis for categorical variables was done using chi square or fisher exact test. The p value was at 95% confidence interval. All statistical analysis was done using SPSS version 29.*

Outcomes: *The commonest cause of positive cord blood DCT in newborn babies without evidence of haemolysis was due to ABO, RH and Kell blood groups' mismatch between mothers and their babies. Correlation analysis of positive DCT with hemolysis parameters in the babies was unremarkable with insignificant p. value. We found no significant correlation between HDFN and hemoglobin level, Reticulocyte count, and serum bilirubin. The only significant correlation was between the positivity of DCT and anti RhD eluted antibodies with p=0.02.*

Conclusion*: The commonest cause of positive cord blood DCT in newborns without evidence of hemolysis was due to ABO, Rh and Kell blood groups' mismatch. DCT positivity has significant correlation with anti RhD eluted alloantibodies. We suggest considering RhD alloantibodies a predictive tool to initiate prophylactic management for preventing susceptible haemolytic events in newborns.*

Keywords*: HDFN; DCT; Eluted antibodies; ABO; Other blood groups.*

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Introduction

Haemolytic disease of the foetus and newborn (HDFN) is a rare immune-mediated blood disorder that affects foetuses and neonates.[1]

In 1932, Louis Diamond, Kenneth Blackfan, and James Baty published the first clear description of HDFN based on peripheral blood smears obtained from infants with the condition.[2]

It has taken over 3 centuries for the medical community to understand the aetiology underlying this condition and develop prophylactic treatments to prevent HDFN-related complications.

HDFN is a disease caused by maternal alloantibodies of the IgG class against fetal red blood cell (RBC) antigens. there are two mechanisms causing hemolytic disease of the fetus and newborn. First, the fetomaternal pair can

have inherent ABO incompatibility, which occurs in 15 to 25% of pregnancies.3 Only about 1% of those pairs with high IgG titres will develop HDFN due to ABO incompatibility.3 In ABO incompatibility, naturally occurring antigens against A or B blood types are present in mothers with O blood type.[4]

The second mechanism most commonly causing HDFN is through fetomaternal haemorrhage (FMH), where maternal antibodies develop after exposure to fetal blood. When fetal RBCs enter the maternal blood circulation, maternal antibodies can develop to an antigen presented on the fetal RBC surface. The most common antigen involved in this mechanism is the Rhesus D (RhD) antigen.[4]

Most of the severe cases of HDFN are caused by alloimmunization against the D antigen, a part of the Rh blood group system. Other contributors are antibodies targeting the K antigen (of the Kell blood group system) and c and other Rh antigens.4 Worldwide, anti-D mediated HDFN still accounts for 160,000 perinatal deaths and 10,000 patients with disabilities every year.5 By implementing sustainable prevention, screening and disease treatment measures in all countries this will systemically reduce unnecessary perinatal deaths.5 Rhesus D-negative (RhD) immunoprophylaxis was first introduced in 1968, which dropped the incidence of HDFN from 1% of all newborns worldwide (with 50% mortality) to 0.5%.4 The incidence of HDFN decreased even further to 0.1% with the administration of antepartum RhD immune-prophylaxis. However, despite adequate RhD immunoprophylaxis, an estimated 1 to 3 in 1000 Rh-negative women still develop alloimmunization today.[5]

The rosette screen test is highly sensitive in qualitatively detecting 10 mL of fetal whole blood in the maternal circulation. The Kleihauer-Betke acid-elution test, the most widely used confirmatory test for quantifying FMH, relies on the principle that fetal RBCs contain mostly fetal hemoglobin (HbF), which is resistant to acid-elution whereas adult hemoglobin is acid-sensitive.[3]

As appropriate dosing is calculated based on the volume of FMH, the prompt and accurate laboratory assessment of FMH is highly desirable.[5]

Although the Kleihauer-Betke test is inexpensive and requires no special equipment, it lacks standardization and precision.3 Besides, it may not be accurate in conditions with elevated fetal erythrocytes.[3]

The objective of the current study is to illustrate the incidence of HDFN with positive direct Coomb's test (DCT). In addition to, identifying the eluted antibodies in these cases of HDFN. This study was done in King Fahd Hospital of the University (KFHU), KSA, for 2 years (2022-2023). We compared our results with different results from Saudi Arabia, Sudan and Uganda, within different timelines.

Materials and Methods:

We started this retrospective study upon the approval of Imam Abdulrahman bin Faisal University (IAU) Institutional Review Board (NCBE registration No.: HAP-05-D-003) IRB number PGS-2023-01-283, dated December 14th, 2023, and in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). All patients' consents were obtained according to the IAU research ethics. Besides, newborns' data were retrieved after their parents/guardian consents were obtained. We confirm that there were no physical, psychological, social, legal, financial, or other risks to participants, as the study involves data that has already been collected previously for clinical purposes. In addition, the current study was designed with strict measures to protect participants' confidentiality. This study data was collected retrospectively from (QuadraMed electronic system) using patients' hospital medical record numbers.

We reviewed all newborn babies with positive DCT of cord blood samples at KFHU, KSA. We retrieved retrospective data starting from January 1st, 2022, to November 30, 2023, and included 106 newborn babies who had positive DCT of cord blood samples. The antibodies were eluted from infants' erythrocytes and were identified. The Data collected from mothers' files included ABO blood group typing, Rosette test, Kleihauer-Betke test and antibodies' titration.

For patients with red blood cell sensitization, the antibody specificity was determined. Certain blood group antibodies such as anti-I, -P1, -Lea, and -Leb, may be ignored because the corresponding (cognate) antigens are incompletely developed at birth.

Data collected from baby files are ABO blood group typing, DCT, hemoglobin level, reticulocyte percentage, and serum bilirubin level. If the DAT was positive with the anti-IgG reagent, an antibody elution test should be performed.

Laboratory Investigations:

Blood groups and Direct coombs test:

ID-Card DiaClon ABO/Rh (Diahem AG, Diagnostic Products, Switzerland, 8180 Bülach, Schlosserstrasse 4 Commercial Registry of Canton Zürich) for newborns DVI+ uses monoclonal antisera and red cell suspense diluent, without incubation.

Elution test:

Done by gamma elu-kit 11 (Lemminkaeisenkatu 62 FIN-20520 Turku, Finland) is intended for use in the rapid acid elution of antibodies from intact red blood cells. Elution is a procedure that allows for the uncoupling (elution) of bound antibodies from sensitized RBCs. Once the eluate is obtained it should be run against an extended antibody panel to determine the specificity of the antibodies.5

Antibodies screening: Indirect Coomb's test (ICT) and antibody identification were performed for mothers of babies who had positive DCT.

ID-DiaCell I-II-III (Bio-Rad Company, 1000 Alfred Nobel Drive Hercules, California 94547 USA) is a 3-cell screening for patients. It contains a set of 3 vials for IAT and NaCl testing, including two RhD positive cells (CCDee, Cw+, ccDEE) and one cell RhD negative (cc.ee). Besides, a double dose for Fya, Fyb, Jka, Jkb, M, S and s, and at least one cell positive for K, Lea, Leb, P1 and N. It has been tested by serology for HLA Class I antigens.

Antibodies identification: ID-DiaPanel-P papainized (Bio-Rad Company, 1000 Alfred Nobel Drive Hercules, California 94547 USA) test cells for antibody identification which is a set of 11 vials for enzymatic testing.

Fetomaternal hemorrhage screen test (The Rosette Test):

For detection of D-Positive Red Cells in D-negative Mothers. It was done by Immucor FMH Rapid screen test (Immucor Products; 5 Isnor Drive Dartmouth Nova Scotia B3B 1M1, Canada). The result is positive if five or more agglutinates of red blood cells are observed. Positive test indicates the presence of D-positive fetal red blood cells in possibly significant numbers in the maternal circulation. If four or lesser erythrocytes' agglutinates were observed, it indicates that a large feto-maternal haemorrhage is less likely present.

Kleihauer-Betke Test:

It was done using sigma-aldrich fetal hemoglobin kit (SIMMLER, INC., 4564 NORTH SQUARE DRIVE HIGH RIDGE, MO 63049, USA). Used for estimation of fetal hemoglobin containing erythrocytes in the maternal circulation.

Antibodies Titration:

Serial antibody titres were used to detect ongoing sensitization, presumably due to the presence of fetal red blood cells' antigens. If there was a previously affected pregnancy, trending of the antibody titre will not be a reliable measure of increasing sensitization. In addition, transfusion laboratories establish critical antibody titres at which the antibody strength has reached a level that may lead to significant fetal anemia (titres of 1:16-32 are commonly used). However, because the Kell blood group antigens are present on early red blood cell precursors, a maternal anti-K of relatively low titre, such as 8, may lead to severe hypoproliferative anemia.[4]

Data analysis:

Descriptive data such as the total population (Isoimmunization, Rh related, and ABO) were analyzed as frequency and percentage or mean and range. Bivariate analysis for categorical variables was done using chi square or fisher exact test. This analysis was done to assess relation between DCT positive results (Yes, No), and patient blood group, Antibodies, Haemoglobin level (g/dl) (Low <15, Normal range 15-21, High >21), Reticulocyte % (Very low <1.8, Low < 3, Normal 3-6 and High >6), Total Serum bilirubin (µmol/I) (Normal <8 and High >8). The p value was at 95% confidence interval. All statistical analysis was done using SPSS version 29.

Results:

In the present study, we included a total of 106 mothers. Out of them we studied 90 mothers with blood group "O" (O positive and O negative) and their newborn babies. 90.22% of mothers studied were in the O positive blood group, and the remaining 7.22% were O negative (Table 1).

Blood groups	Maternal number (%)
O+	83 (92.22)
O-	7 (7.77)

ABO incompatibility	**Frequencies (%)**
O-A incompatibility	36 (34)
O-B incompatibility	54 (50)

Table 1: *Maternal ABO blood groups and mothers/babies' incompatibility*

58.88% of newborn babies have B+ blood group, and 36.66% have A+ blood group. All those newborn babies had positive DCT. As regards positive DCT cases with ABO incompatibility, 40% had O-A blood group incompatibility, and 60% had O-B blood group incompatibility. The positive DCT cases were 31.11%

having low Hb (<15 g/dl) and 51.11% having normal Hb (15-21 g/dl).

Furthermore, 26.66% of DCT positive newborns had increased reticulocyte count (>6%), which clearly indicated that those infants had hemolysis. In addition, 4.44% of DCT positive newborns had high bilirubin (>8 µmol/l). All the studied variables showed non-significant association with positive DCT (p>0.01) (Table 2).

Blood group	Positive DCT	p-value
A+	33 (36.66)	0.72
B+	53 (58.88)	
B-	1 (1.11)	
A-	3 (3.33)	
ABO incompatibility		
O-A	36 (40)	0.71
O-B	54 (60)	
Haemoglobin level (g/dl)		
Low <15	28 (31.11)	0.25
Normal range 15-21	46 (51.11)	
High >24	0 (0)	
Reticulocyte level (%)		
Low<3	1 (1.11)	0.73
Normal 3-6	49 (54.44)	
High >6	24 (26.66)	
Total serum bilirubin (µmol/l)		
Normal< 8	70 (77.77)	0.47
High >8	4 (4.44)	

Table 2: *Association of positive DCT with lab tests of hemolysis (n=90)*

Total 106 newborns' eluates were collected and evaluated. 16 newborn babies who had hemolysis revealed antibodies against Rh and Kell blood group antigens (Table 3).

Eluted infants' blood antibodies	N	%
Anti D	7	7
Anti c	2	1
Anti e	1	1
Anti E	1	1
Anti D and C	1	1
Anti E and c	1	1
Anti D, E and c	1	1
Other (K)	2	2
ABO	90	85%
Total	106	100%

Table 3: *Eluted antibodies in infants' blood due to alloimmunization against ABO, Rh and other blood groups (n=106)*

Infants with positive DCT had normal hemoglobin levels (mean 15.3 gm/dl), normal or high reticulocyte percentages (from 3.6 to 16.1%), and normal serum bilirubin level <8 µmol/l. Significant correlation with positivity of DCT and anti RhD recovered from infant erythrocytes with p =0.02. Most antibodies identified were against Rh system (Table 4).

Blood type	Rh D negative	Rh D negative combined with any of (C, E, c, e, k) negatively
A-	2 (30)	1 (25)
AB-	1 (10)	0
B-	2 (30)	1(25)
B+	0	0
O-	2 (30)	2 (50)
O+	0	0
Total	7	4

Table 4: *Number of mothers with negative Rh (n=11)*

There were no statistically significant differences between infants with positive DCT results as regards hemoglobin level, reticulocyte percentages, and total serum bilirubin levels (Table 5).

Infants' characteristics	DCT positive		Chi^2	P-value
	No	Yes		
	number (%)	number (%)		
Infant Blood group				
A+ (n=3)	0	3 (100)	4.148	0.246
AB+ (n=1)	0	1 (100)		
B+ (n=3)	0	3 (100)		
O+ (n=9)	4 (44)	5 (56)		
Haemoglobin level				
Low <14	2 (25)	6 (75)	0.873	1
Normal range 14-24	2 (25)	6 (75)		
High >24	0	0		
Reticulocyte level (%)				
Very low <1.8	0	0	0.762	0.585
Low< 3	0	0		
Normal 3-6	3 (33)	6 (67)		
High >6	1 (14)	6 (86)		
Total Serum bilirubin (µmol/I)				
Normal <8	4 (27)	11 (73)	0.356	1
High >8	0	1 (100)		
Antibodies were identified				
Anti D	0	7 (100)	13.333	0.02
Anti c	2 (100)	0		
Anti e	0	1 (100)		
Anti E	1 (100)	0		
Anti D and C	0	2 (100)		
Anti E and c	1 (100)	0		
Anti D, E, K, C	0	2 (100)		

Table 5: *Infants' characteristics and DCT positive results (n=16)*

On the other hand, Table 6 shows results of infants' antibody titration for Anti D, C, c, K, E and e.

Infant and mother's readings		*Titration*		
		Mean (SD)	*Minimum*	*Maximum*
Antibodies	***Anti c***	-	-	-
	Anti C	*1.41 (0.22)*	*1.20*	*1.64*
	Anti D	*1.33 (0.17)*	*1.13*	*1.64*
	Anti e	*1.20*	*1.20*	*1.20*
	Anti E	*1.21 (0.16)*	*1.10*	*1.32*
	Anti K	*1.36 (0.06)*	*1.32*	*1.40*
DCT positive	***No***	-	-	-
	Yes	*1.32 (0.16)*	*1.10*	*1.64*

Table 6: *infants' antibody titration for Anti D, C, c, K, E and e.*

Discussion:

A total 106 newborns with positive cord blood DCT were enrolled in this study. We found the commonest cause of positive DCT without evidence of haemolysis is due to ABO incompatibility in 85% of our studied cases. Besides, the other 15% of positive DCT cases without haemolysis were caused by Rh and other blood group (K) antigens incompatibility. These results agree with other published papers.6-10

All studied newborn babies with positive cord blood DCT showed no statistically significant differences as regards parameters of hemolysis which are reticulocyte %, serum bilirubin, and hemoglobin levels. The only statistically significant finding was found between positive DCT and the prevalence of anti- RhD antibody (p value= 0.02). 92.22% of mothers (n=83) have blood group O positive, while 7.77% of cases are O negative (n=7).

50% of our positive DCT newborns have blood group B (n=54), and (34%) have blood group O or A (n=36). These findings agree with previously published local study.6 This is in contrast with one study that concluded the most common cause of HDFN is caused by other non-ABO blood groups.11 In our study the antibodies recovered by elution tests and identified were against Rh (D, E, e, C, and c) and against Kell blood group systems. The highest frequencies of maternal antibodies were anti- RhD (52%), anti- c (14%), anti- E (14%), anti- e (5%), and anti- K (10%). In our hospital screen tests are done to all newborns on umbilical cord blood samples. The newborn's screening tests are direct antiglobulin test (DCT), and ABO blood group typing. DCT is one of the most performed investigations in HDFN, as a part of diagnostic workup.4 DCT is simple, quick, and inexpensive test. A positive DCT does not necessarily indicate that haemolysis is occurring and a diagnostic workup for haemolysis should be done before a diagnosis of HDFN can be made. On the other hand, negative DCT does not rule out HDFN, and does not rule out non-immune haemolytic aetiology underlying clinical hyperbilirubinaemia.12,13 However, positive DCT might be an early predictor for development of HDFN. Therefore, close follow up is recommended for these cases to avoid possible late-onset severe anemia, particularly for infants born to Rh-negative mothers.14,15 In our study DCT positivity on neonatal cord blood samples does not display evidence of HDFN. Yet, neither rules out a potentiality of late onset hemolysis due to sensitization of mothers' immune system. This was suggested as we identified elevated mothers' IgG antibodies which can cross the placenta and target neonatal erythrocytes at or near body temperature.

Maternal alloantibodies remain in the neonatal circulation for up to six months after birth and continue to trigger hemolysis.2 These alloantibodies, including alloantibodies against Kell antigen, can also suppress the erythropoiesis causing prolonged neonatal anemia or late onset anemia up to three months after birth.2 It was reported that positive DCT on neonatal cord blood erythrocytes close to delivery time may alert the clinician to consider prophylactic management to avoid HDFN.16 However, a negative DCT result may allow clinicians to adopt a more conservative approach and spare the neonate from many laboratory investigations or invasive investigation procedures. Risk stratification of HDFN using DCT obtained early after delivery, can be one of the many tools available to clinicians in identifying and efficiently managing newborns at risk for severe hyperbilirubinemia.16 It was reported that blood type incompatibility increases the frequency of neonatal

hyperbilirubinemia only in the DAT positive infants.17 This later study contradicts to other two studies conducted in our hospital that negate the DCT as a valuable screening tool which is overused in our centre, and questioned its role for selective neonatal screening testing.[6,18]

Some investigators reported that DCT is not recommended as a screening test for HDFN owing to its poor positive predictive value.19 However, in critical situations that require evaluating DCT are when there is suspicion of HDFN due to clinically evident hemolysis or hyperbilirubinemia in the neonate.19 In such critical cases, even with a negative maternal antibody screening tests, the DCT should be checked for the foetus owing to the possibility of a low frequency antigen that might not be present on the screening cells.19 Clinical monitoring of infants for jaundice in the first week of life rather than reliance on DCT screening is likely more effective management approach.[19]

Another practice undertaken by some centres is selective cord blood testing (ABO typing and DAT) for infants born to blood group O positive mothers rather than routine testing of all newborn infants. This has been shown by several studies to reduce costs without increasing the risk of clinically significant hyperbilirubinaemia.[12-14]

We believe that the neonatal testing protocol applied in our hospital to screen for RH negative mothers is valid. This screening protocol is constantly reviewed and checked by College of American Pathologist (CAP) proficiency testing twice annually.

Although the manual Kleihauer-Betke test is the most widely used test to quantify the volume of FMH, it has many limitations. Aside from being laborious to perform, the accuracy and precision of the test might be suboptimal because of lack of standardization leading to slight variations in test characteristics.[3,20] These variations include thickness of screened blood smears, pH variations of the test buffer solutions, interobserver and interhospital variations of results' interpretation, and statistical imprecision associated with rare event analysis. These variations may result in overestimation or underestimation of FMH.20 It was reported that tendency of the Kleihauer-Betke test to overestimate FMH is more than underestimation of FMH.20 We think that overestimating FMH is preferred than underestimating it, as the latter can result in inadequate Rh immune globulin antibody dosing with subsequent immune sensitization.[3,20]

A recent study suggested assessing feta haemoglobin (Hb F) by using haemoglobin electrophoresis21 This study concluded the usefulness of hemoglobin electrophoresis in investigating suspected FMH, as it is accessible procedure in most hospitals, less time consuming, and cost effective.21 Still hemoglobin electrophoresis procedure has some limitations regarding the inability to distinguish maternal from infant Hb F.[21]

Conclusion:

Our current study concluded that the most common cause of positive cord blood DCT test in newborns without evidence of haemolysis was due to ABO, Rh and Kell blood groups' mismatch between the mother and the baby. The frequent eluted antibodies identified because of ABO incompatibility were anti B (58.88%), anti A (36.66%), anti Rh {anti D (52%), anti c (14%), anti E (14%), anti e (5%)} and anti- Kell blood group (10%). The highest Maternal antibody titration noticed were anti D, C, E, K, e.

Advances in maternal-fetal medicine, including the non-invasive feto-maternal testing, will result in significant improvements in HDFN outcomes and the avoidance of maternal allo-sensitization. Future advancements in blood typing and non-invasive testing will help women and their children with blood group incompatibility.

No funding for this study

Conflicts of interest

I undersign, certificate that I do not have any financial or personal relationships that might bias the content of this work.

The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law. Informed consent was obtained from all the patients included in the study.

Acknowledgments:

The authors would like to thank The Deanship for scientific research, Imam Abdulrahman Bin Faisal University, for their continuous support. In addition, the authors would like to thank the information technology (IT) Department in King Fahd Hospital of The University for their valuable help to collect the electronic data of studied Patients throughout the research duration.

Authorship and contribution:

Both authors made substantial contributions to all the following: (1) the conception and design of the study, acquisition of data, analysis, and interpretation of data, (2) drafting the article and revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Funding:
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data Availability statement:
The data that support the findings of this study are available on request from the corresponding author, [Y.O.].

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