Original Article

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Quick Response Code:

Website: https://journals.lww.com/ijhm

DOI: 10.4103/ijh.ijh_8_24

Validity of the current anti-D immunoglobulin doses for the prevention of hemolytic disease of the newborn in Duhok/Iraq

Hazheen Hisham Saifullah, Adil Abozaid Eissa[1]

Abstract:

BACKGROUND: The current study was initiated to evaluate the comparability of fetomaternal hemorrhage (FMH) measurement using flow cytometry (FCM) and Kleihauer–Betke test (KBT) and also to evaluate the validity of the current anti-D immunoglobulin doses for the prevention of hemolytic disease of the newborn in Duhok/Iraq.

MATERIALS AND METHODS: The current study included 101 pregnant women with Rh(D)-negative blood group who had Rh (D)-positive husbands. Their blood was tested for blood groping, FMH by FCM and KBT, and indirect Coombs test, 1 h following sensitizing events and 72 h after giving anti-D. Furthermore, blood from newborns was examined for blood group and direct Coombs test.

RESULTS: The main sensitizing event was parturition (62.4%) followed by cesarean section (32.7%). The indirect Coombs was positive in 32 cases while the direct Coombs test was positive in 19 cases. In 63.4% of cases, the ABO blood groups were incompatible between mothers and their babies. When FMH was checked by KBT method, it was found that 16 (15.8%) participants had FMH ranging 1.2–51 mL (median 4.35 mL), while FMH was positive in 27 (26.7%) participants by FCM method ranging 1.2–54.4 ml (median 9.5 mL). About 4–5 patients had FMH (measured by KBT and FCM, respectively) of >12 mL and only 1% had a volume of >30 mL. The difference between KBT and FCM for FMH measurement was statistically significant with $P < 0.001$ when assessed by paired *t*-test and has a highly significant positive correlation with each other. The correlation of FMH was statistically significant with maternal gravidity, number of anti-D received before (moderate positive correlation), gestational age, and newborn hemoglobin (negative correlation). However, the correlation was not significant between FMH and the following factors: maternal parity, maternal hematological parameters, and ABO compatibility of mother and their babies with $P > 0.05$.

CONCLUSION: Inadequate doses of anti-D had been given previously that resulted in sensitization in at least one-quarter of the cases, and this necessitated proper measurement of FMH in all Rh (D) mothers following sensitizing events.

Keywords:

Anti-D immunoglobulin, fetomaternal hemorrhage, flow cytometry, Kleihauer–Betke test, sensitizing events

Department of Laboratory, Azadi Teaching Hospital, Kirkuk, [1]Department of Pathology, College of Medicine, University of Duhok, Duhok, Iraq

Address for correspondence:
Dr. Adil Abozaid Eissa, Department of Pathology, College of Medicine, University of Duhok, Duhok, Iraq.
E-mail: adilkhr77@uod.ac

Submission: 31-01-2024
Revised: 18-03-2024
Accepted: 19-03-2024
Published: 16-04-2024

Introduction

Fetomaternal hemorrhage (FMH) as a result of the transplacental transfer of fetal red blood cells (RBCs) into the maternal circulation and the subsequent sensitization is a recognized complication of pregnancy.[1]

Typically, FMH occurs in small quantities during pregnancy and experiences an increase during parturition.[2,3] Several studies have indicated that minor hemorrhages, measuring <1 mL of blood,

How to cite this article: Saifullah HH, Eissa AA. Validity of the current anti-D immunoglobulin doses for the prevention of hemolytic disease of the newborn in Duhok/Iraq. Iraqi J Hematol 2024;13:79-84.

are present in 96% of all pregnancies. Additionally, larger hemorrhages of approximately 30 mL are observed in up to 0.3% of all pregnancies. It is common for such occurrences to happen without notable signs or symptoms in either the mother or fetus. In numerous instances, the etiology of substantial FMH remains uncertain and lacks predictability.[1-3] FMH can lead to alloimmunization of the mother. This occurs as a result of the mother's exposure to antigens present on the surface of fetal RBCs, which are inherited from the father. The antigens that exhibit the highest immunogenicity are RhD, c, Kell, E, Kidd, and Duffy, in descending order. Alloimmunization has the potential to result in hemolytic disease of the newborn (HDN) when maternal immunoglobulin G (IgG)-type antibodies cross the placenta.[4-6]

Accurate identification and measurement of FMH is of utmost importance in the obstetric care of pregnant women who are Rh(D) negative. The quantity of fetal RBCs present in the maternal circulation is a determining factor in establishing the appropriate therapeutic dosage of anti-Rh(D) Ig. This dosage is crucial in preventing alloimmunization and the occurrence of HDN.[7,8]

The presence of anti-D alloantibodies can have varying clinical outcomes. While some cases may not exhibit any noticeable symptoms, others can result in severe complications such as hemolytic disease in the fetus and newborn, fetal anemia, hydrops fetalis, or even fatality for both the current and future pregnancies.[9,10]

The administration of Rh(D) immune globulin (RhDIg) can effectively mask the antigenic sites present on fetal RBCs, thereby preventing any subsequent immune responses from the maternal immune system. The assessment of FMH is a crucial consideration when determining the appropriate dosage of RhDIg. The standard protocol for RhDIg administration and prescription in Iraq dictates that Rh(D)-negative women carrying Rh(D)-positive fetuses should be given a dosage of 1500 international units (IU) (300 μg) of RhDIg. This dosage is sufficient for concealing up to 12 mL of fetal RBCs.[11,12]

Multiple techniques are employed for the quantitation of FMH. One such method is the Kleihauer–Betke test (KBT), which is conducted by evaluating the resistance of fetal hemoglobin (Hb) to acid elution. The KBT method is cost-effective and can be conducted using basic laboratory equipment. However, it is important to note that the sensitivity of the test can be influenced by various factors throughout the entire process, including film preparation, staining, elution, and interpretation of the stained blood films.[13,14]

The flow cytometric technique is a contemporary method used to assess fetal Rh(D) RBCs in maternal blood. This is achieved through the utilization of monoclonal antibodies targeting fetal markers such as HbF or surface Rh(D) antigens.[15]

Through this methodology, our objective was to assess the comparability of FMH measurement using flow cytometry (FCM) and Kleihauer testing. Additionally, we aimed to evaluate the practicality of these methods in ensuring the accurate administration of appropriate doses of anti-D Ig to Rh(D)-negative women.

Materials and Methods

Samples

This cross-sectional study included 101 pregnant women who were Rh(D) negative at their most recent prenatal obstetric visit at Duhok Maternity and Obstetric Hospital, Iraq. The study protocol received approval from the Ethical and Research Committees of the Department of Medical Laboratory Science at Duhok College of Health Sciences. The patient's permission was obtained prior to enrollment, after a comprehensive discussion about the study. The demographic information, including the age of the patient, as well as the clinical conditions, were obtained from each individual.

All women recruited were Rh(D) negative married to Rh(D)-positive husbands and giving birth to Rh(D)-positive babies. Four milliliters of peripheral blood from women who gave birth to Rh(D)-positive newborns or who had sensitizing events was collected within 1 h after sensitizing event in an ethylenediaminetetraacetic acid (EDTA) and gel tubes for FMH estimation and indirect Coombs test. Another sample of peripheral blood in EDTA tube was collected after 72 h from administration of anti-D Ig from all enrolled women for FMH estimation to confirm the efficacy of the given anti-D doses.

Half a millimeter of blood was taken from each newborn at the time of delivery for blood grouping, and in case of positive Rh(D), the remaining blood was used for direct antiglobulin test otherwise the mother was excluded.

Kleihauer–Betke acid-elution test

The KBT test was conducted in accordance with the methodology outlined by Kleihauer, Braun, and Betke in 1957, with minor adjustments.[16] Fresh, air-dried blood films were prepared. After drying, the slides were fixed in 80% ethanol for 5 min. Next, the slides were placed in the elution solution for 20 s. Finally, slides were placed on the counter for 2 min. The slides were washed and dried between each step.[17] HbA undergoes denaturation

and subsequent elution, resulting in the formation of red cell ghosts. Erythrocytes that include fetal HbF exhibit resistance to acidic conditions, allowing for effective staining of the Hb. Consequently, these cells become easily distinguishable among a multitude of maternal cells with a pale appearance. The newborn cells were quantified using microscopic observation at a high magnification objective lens (×40). The volume of FMH was calculated using Mollison formula which assumes that the maternal red cell volume is 1800 mL, fetal cells are 22% larger than maternal cells, and only 92% of fetal cells stain darkly.[18]

$$\frac{\text{Number of fetal cells per HPF}}{\text{Number of maternal cells per HPF}} \times 1800 \times \frac{122}{100} \times \frac{100}{92}$$

Flow cytometry protocol

The techniques used for the identification of fetal cells specifically focus on Rh(D) antigen detection, using fluorochrome-conjugated anti-D antibodies (BRAD3) obtained from the International Blood Group Laboratory (Bristol, UK). Patients' samples were washed in PBS two times and 5% suspension of washed cells was incubated with FITC-BRAD3 for 30 min at 37°C. After washing in PBS once, the cells were suspended in 0.5 mL of PBS and vortexed prior to introduction into flow cytometer. In each experiment, 100,000 events were analyzed. The calculation method of the test is as follows (assuming that the maternal red cell volume is 1800 mL and the fetal cells are 22% larger than maternal cells).[18]

$$\frac{\text{Percentage fetal cells cells}}{100} \times 1800 \times \frac{122}{100}$$

Statistical analysis

The Statistical Package for the Social Sciences software, version 22 (SPSS Inc., Chicago, IL, USA), was used for statistical and data analysis. The ranges (median) were used to present the results. The Mann–Whitney and Spearman correlations were utilized as applicable to correlate different parameters with the amount of FMH, and the paired *t*-test was used to compare FMH by the two methods; $P < 0.05$ was deemed statistically significant.

Results

One hundred and one Rh(D)-negative pregnant ladies were enrolled in the current study. Their mean age was 29 (4.2) years. The obstetrics and clinical characteristics of the study population are shown in Table 1. The mean maternal Hb was 10.26 (0.99) g/dL, while the mean newborn Hb was 15.8 (4.54) g/dL. The indirect Coombs was positive in 31.7% of cases while the direct Coombs test was positive in 18.8%. In 63.4% of cases, the ABO blood groups were incompatible between the mother and her baby. These findings are shown in Table 2.

Table 1: Obstetrics and clinical characteristics of the study population

Variable	*n* (%)
Gravidity	
1	19 (18.8)
2–4	73 (72.3)
>4	9 (8.9)
Maternal parity	
0	19 (18.8)
1–2	60 (59.4)
3–4	22 (21.8)
Gestational age (weeks)	
<37	5 (5)
37	6 (5.9)
38	21 (20.7)
39	5 (5)
40	64 (63.4)
History of neonatal jaundice	
Yes	46 (45.5)
No	55 (54.5)
History of blood transfusion	
0	93 (92.1)
1	7 (6.9)
2	1 (1.0)
History of receiving anti-D	
0	48 (47.5)
1	18 (17.8)
2	19 (18.8)
>3	16 (15.9)
Sensitizing event	
Vaginal delivery	63 (62.4)
C-section	33 (32.7)
Miscarriage	2 (2.0)
Stillbirth	2 (2.0)
Abdominal trauma	1 (1.0)

Table 2: Maternal and newborn laboratory measurements

Measurement	Range (median)
Maternal Hb (g/dL)	7.8–12.1 (9.8)
Maternal WBC count (10^9/L)	4.8–13.4 (6.7)
Maternal platelets count (10^9/L)	285–312 (178)
Newborn Hb (g/dL)	3.9–22 (12.3)
Variables	***n* (%)**
Indirect Coombs test (maternal blood sample)	
Positive	32 (31.7)
Negative	69 (68.3)
Direct Coombs test (neonatal blood sample)	
Positive	19 (18.8)
Negative	82 (81.2)
ABO compatibility	
Compatible	37 (36.6)
Incompatible	64 (63.4)

WBC=White blood cell, Hb=Hemoglobin

When FMH was checked by KBT method, it was found that 16 (15.8%) participants had FMH ranging 1.2–51 mL (median 4.35 mL). On the other hand, FMH was positive in 27 (26.7%) participants by FCM method ranging 1.2–54.4 mL (median 9.5 mL). In both methods, most of those with FMH had a volume <4 mL; 4–5 patients had FMH of >12.0 mL, and only 1% in each method had a volume of >30 mL [Table 3].

Among those measured by both methods, the difference between the mean volumes of them was statistically significant with $P < 0.001$ when assessed by paired *t*-test. The measures in both methods have a highly significant positive correlation with each other, as shown in Table 4.

Table 5 illustrates the correlation between FMH measured by KBT with different maternal and newborn factors. The correlation with maternal gravidity was a moderate positive correlation, and this correlation was statistically significant. The correlation was highly significant statistically but in a negative direction with gestational age. A significant negative correlation was with newborn Hb and a positive correlation seen with the number of anti-D received before. However, the correlation was not significant between FMH and the following factors: maternal parity, maternal Hb, WBC count, and platelet count.

The association between FMH in relation to ABO compatibility of mother with baby was statistically not significant using both FMH and KB, as shown in Table 6.

Table 3: Fetomaternal hemorrhage calculated by Kleihauer–Betke test and flow cytometry

Method	Amount of FMH	Frequency (%)
KBT: 1.2–51 mL (median 4.35 mL)	Negative	85 (84.2)
	<4 mL	8 (7.9)
	4–12 mL	4 (4.0)
	12.1–30 mL	3 (3.0)
	>30 mL	1 (1.0)
FCM: 1.2–54.4 mL (median 9.5 mL)	Negative	74 (73.3)
	<4 mL	12 (11.9)
	4–12 mL	10 (9.9)
	12.1–30 mL	4 (4)
	>30 mL	1 (1.0)

FMH=Fetomaternal hemorrhage, KBT=Kleihauer–Betke test, FCM=Flow cytometry

Table 4: Correlation between Kleihauer–Betke test and flow cytometry in detecting fetomaternal hemorrhage

Method	Range (median) (mL)	Paired *t*-test	Correlation	*P*
KBT	1.2–51 (4.35)	6.13	0.980	<0.01
FCM	1.2–54.4 (9.5)			

KBT=Kleihauer–Betke test, FCM=Flow cytometry

Discussion

Globally, measurement of FMH using a KBT is regularly executed on all women with Rh(D)-negative blood group after any sensitizing event that might be associated with the risk of FMH to guarantee that an enough anti-D Ig dose is given.[19,20] In our region, the test is not routinely done, thus the current study is initiated mainly to evaluate the efficacy of the routinely given anti-D dose and the need of the test in the region.

Previous studies from the region had demonstrated that the frequency of Rh(D) positivity in the community is 91.1%[21] and this means that 8.1% of all women *"only 8.9% of females are of Rh(D) negative and 91.1% of their newborn are of Rh(D) positive blood so multiplying 8.9 × 91.1% = 8.09% of pregnant ladies will have incompatible babies"* in the region are at risk of giving birth to a child with Rh(D)-positive blood and are at risk of sensitization in case no appropriate dose of anti-D is provided.

Neonatal hyperbilirubinemia is a common manifestation of blood group incompatibilities between the mother and the newborn, in addition to various other causes like intrauterine transfusion, FMH or fetofetal transfusion, hematological diseases with increased hemolysis like G6PD deficiency, and neonatal polycythemia as a result of intrauterine hypoxia, and can be prevented efficiently with appropriate measurement of FMH and administration of appropriate doses of anti-D Ig.[22]

Parity and gravida were as expected from the region to be high as compared to other developed countries, and

Table 5: Correlation between fetomaternal hemorrhage calculated by Kleihauer–Betke test and different maternal and newborn variables

Correlated factors FMH (mL)	Range (median): 8.26 (10.54)	Correlation coefficient (*r*)	*P*
Gravidity	1–7 (2.5)	0.622	0.007
Maternal parity	0–4 (1.6)	0.286	0.237
Gestational age (week)	24–40 (39.6)	–0.699	0.0025
History of anti-D receiving	0–5 (0.9)	0.405	0.019
Newborns Hb (g/dL)	3.9–22 (12.3)	–0.405	0.041
Maternal Hb (g/dL)	7.8–12.1 (9.8)	–0.102	0.356 (NS)
WBC count (10^9/L)	4.8–13.4 (6.7)	0.098	0.123 (NS)
Platelet count (10^9/L)	285–312 (178)	0.3217	0.078 (NS)

WBC=White blood cell, Hb=Hemoglobin, FMH=Fetomaternal hemorrhage

Table 6: Association between fetomaternal hemorrhage and ABO compatibility

Method	Compatible ABO range (median) (mL)	Incompatible ABO range (median) (mL)	*P**
KBT (mL)	0–51.0 (4.3)	0–18 (3.12)	0.145 (NS)
FCM (mL)	0–54.4 (13.4)	0–13.6 (4.12)	0.131 (NS)

*By Mann–Whitney test. KBT=Kleihauer–Betke test, NS=Not significant, FCM=Flow cytometry

this might have a negative impact on the rate of perinatal complication including the amount of FMH and the incidence of sensitization of Rh(D)-negative women.[23] So that accurate measurement of FMH volume and then giving adequate doses of anti-D are essential in such risky patients for prevention of HDN.

Detection of positive indirect Coombs test higher than positive direct Coombs test might indicate the presence of nonspecific antibodies or incompatible antibodies as majorities of enrolled cases showed incompatible ABO blood group and might indicate that incompatible antibodies not passed the placenta or were not associated with HDN. Hence, only certain antibodies that pass the placenta are of clinical significance and associated with HDN like anti-Rh (D, c) and anti-Kell antibodies as the corresponding antibodies of IgG that can pass the placenta and are of high immunity.[24] The current study showed a very high prevalence of sensitization (31.7%) in contrast to the previous study by Alhaidari and Abdul-Razza from Bagdad/Iraq (10.4%) and also to the global meta-analysis study that was conducted by the Cochrane Collaboration in 2000 (1%), and this is mainly related to the failure of FMH measurement.[25,26]

The current study revealed a highly significant positive correlation of both methods utilized for the measurement of FMH and showed also that the amount of FMH measured by flow cytometry (mean 16.05 mL) is significantly higher than that measured by KBT (mean 13.1 mL), and this is consistent with a previous study by Bromilow and Duguid who contributed the reasons to the difficulties in standardization of KBT and the problem in the interpretation.[19]

The routinely recommend doses of anti-D given in the postpartum period is 1500 IU and usually enough to cover 12 ml of FMH.[27] This indicates that 4–5/100 of our patients with FMH of more than 12.0 mL are receiving inadequate doses of anti-D and makes them susceptible to develop sensitization with the risk of HDN in subsequent pregnancies.

Studied factors that showed to have significant correlation with the rate and the amount of FMH include maternal gravidity (multigravida is the major risk factor in both placenta previa and abruptio placentae that contribute to antepartum hemorrhage and increased risk of FMH), gestational age at the time of sensitizing events (placenta abnormalities commonly lead to preterm and FMH), previous history of receiving anti-D (cannot be explained), and the newborn Hb level (reflects the amount of blood that passed into maternal circulation), while maternal hematological parameters did not show a significant correlation with the rate and the amount of the FMH, and this may explain the annotation that the clinical manifestations of FMH are usually subtle, nonspecific, and difficult to identify at the time of the event.[28-30]

Conclusion

A higher than average percentage of sensitization among the enrolled women was primarily caused by inadequate anti-D Ig doses due to a lack of successful FMH measurement. Additionally, a significant portion of cases had FMH larger than 12 mL, meaning they were not covered by the recommended 1500 µ dose. For this reason, it is important to include FMC in the assessment process, particularly after administering the recommended dose, to determine whether additional anti-D doses are required. When compared to FMH measurements made by KBT, flow cytometry was substantially linked to increased FMH volume.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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