

The Assessment of Feto-Maternal Hemorrhage in an Artificial Model Using Anti-D and Anti-Fetal Hemoglobin Antibody by Flow Cytometry

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ABSTRACT

Background: When fetal red cells enter the maternal circulation from placenta, an event would be happened that is described as feto-maternal hemorrhage (FMH). This life-threatening condition could be detected by using RBC antigens (surface antigens and intracellular antigens). Therefore, the measurement of fetal RBC in an artificial model would be useful to calculate FMH and consequently the dosage of Rhogam for prophylaxis. The aim of the present study was to evaluate FMH in an artificial mixture model. **Methods:** A series of 40 artificial specimens were prepared consisting of Rh(D) negative adult blood (non-immunized) spiked with varying amounts of Rh(D) positive cord blood from mothers between 20-30 years old in Shahid Beheshti Hospital, Tehran, Iran. Monoclonal anti-D and anti-HbF (fetal hemoglobin) were used for detection of fetal RBC in artificial mixture sample modeling. **Results:** This study showed that the percentage of fetal cells in artificial sample for anti-D antigen is in ranges of 0.28%-0.32% for a 0.25% dilution mixture, and 1.3%-2.05% for the mixture with dilution 2%. In addition, the ranges of data for anti-HbF staining was obtained 0.2%-0.34% for the 0.25% dilution sample, and the ranges of 1.04-1.8% for the 2% dilution. The regression analysis indicated that the correlation of anti-D assessment with expected standard method was $r^2 = 0.9672$ and anti-HbF assessment was $r^2 = 0.8842$. **Conclusion:** Although both molecule targets could be used for detection of fetal RBC, in this model, anti-D staining was more accurate than anti-HbF staining. However, since anti-D can not be utilized for low-density or weak phenotype and other incompatibility, the anti-HbF labeling could be used for all FMH. *Iran. Biomed. J.* 12 (1): 43-48, 2008

Keywords: Feto-maternal hemorrhage (FMH), Fetal hemoglobin (HbF), Anti-D

INTRODUCTION

Hemolytic disease of the newborn (HDN) is a condition in which the life span of the infants' red cells is shortened by the action of specific antibodies derived from the mother via placental transfer [1, 2]. When fetal red cells enter the maternal circulation from placenta, an event would be happened that is described as feto-maternal hemorrhage (FMH) [3, 4]. FMH can lead to life-threatening anemia in the fetus and the newborn child. The pathogenic backgrounds for the hemorrhage are for example, placental malformation, trauma or choriocarcinoma and so on [5, 6]. Once a volume of fetal blood with at least 0.1 ml enters the mother circulation, the FMH would be occur [7-9]. The most frequent cause of sever HDN is D antigen that

is due to the high immunogenicity of D antigen [5, 6]. Fetal hemoglobin (HbF) is a predominant form of hemoglobin expressed in the developing fetus, appearing a few weeks post-conception and persisting for a few months post-birth [10].

The production of maternal anti-D antibodies against fetal RBC because of Rh incompatibility may be prevented by the administration of prophylactic anti-D antibodies within 72 hours of FMH [11]. A dose of 100 μ g (500 IU) anti-D antibody is sufficient to prevent an antibody response to 5 ml fetal RBC [12-14]. If we quantify the size of FMH correctly, we are able to calculate the dose of anti-D antibody and reduce the rate of disorders.

The Kleihauer-Betke acid-elution technique is a standard for detection of fetal RBC, but it is not very

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accurate and is very time consuming. Recently, flow cytometry has been used for detection of fetal RBC in maternal peripheral blood that is based on antigenic differences. It can provide a clearly pattern between maternal and fetal RBC, and different antigens must be examined, because mother and fetus might share several antigens [15, 16].

The method for fetal cell detection has been reported using RBC surface antigens, particularly with anti-D polyclonal or monoclonal reagents [7, 17]. Fetal RBC can be accurately identified and quantified using intracellular detection of HbF, which is a new approach for isolating RBC [18, 19].

The flow cytometry assay is used to measure FMH by using anti-D or anti-HbF antibody in maternal blood. However, the accuracy of the test needs to be justified for set up. We established an artificial model, which fetal RBC or cord blood RBC spiked in known blood serial dilutions for make an expected FMH by standard protocol and compared with the volume of FMH that flow cytometer reads. The result could be used for the measurement of the Rhogam dosage.

MATERIALS AND METHODS

A series of 40 artificial specimens were prepared consisting of Rh(D)-negative adult blood (non-immunized) spiked with varying amounts of Rh(D)-positive cord blood (from mothers between 20-30 years old in Shahid Beheshti Hospital, Tehran, Iran). Each sample was tested by flow cytometry using anti-D and anti-HbF antibodies staining.

Blood samples and preparation of artificial FMH mixtures. D+ cord blood samples (n = 10) with O blood group (from mothers between 20-30 years old in Shahid Beheshti Hospital) were collected in EDTA tubes and stored at 4°C. D- negative adult blood with O blood group was obtained from non-immunized healthy male (prepared from Blood Bank Center, Isfahan, Iran). RBC was counted with an automated cell counter (Coulter counter, H1 Teknicon, Seyed-Alshohada Hospital, Isfahan, Iran). An artificial FMH was established according to the standard protocol pointed by Greiss *et al.* [5]. The expected FMH for preparing standard spiked sample is presented in Table 1.

Positive control was prepared from D-positive of O blood group and negative control prepared from D-negative adult male of O blood group (n = 10).

Table 1. The expected FMH for preparing standard spiked sample [5].

FMH (ml) RBC	Percentage of D+ cells	Volume of D-negative cells (μ l)	Volume of D+ Cells (μ l)
6	0.25	1000	2.5
11	0.50	1000	5.0
22	1.00	1000	10.0
44	2.00	1000	20.0

Then, the mixtures of D positive cord blood and D negative adult blood were washed three times in phosphate buffered saline, (pH 7.4) and prepared for two staining methods.

Reagents:

The fetal cell count kit II which consists of eight reagents quantities human fetal red blood cells in maternal blood. The test is based on the sensitive and accurate flow cytometric detection of HbF and carbonic anhydrase (CA) present in different red blood cells populations. Monoclonal anti-D was purchased and used in the assay according to the manufacture instruction. Anti-HbF staining has been performed as follows:

Fixation and permeabilization. Fixative solution A (100 μ l) containing <0.1% sodium azide was added to a 5-ml conically-bottomed tube, then 10 μ l EDTA-anticoagulated whole blood (2.5×10^7 Cell/ μ l), was added, mixed and vortexed and finally 100 μ l of fixative solution B, buffer formaldehyde was added and vortexed. The mixed cell suspension was incubated at room temperature for 30 minutes. After washing, 100 μ l of permeabilization solution containing SDS was added and resuspended the cells by vortexing. The cell suspension was incubated for 3-4 minutes, washed and resuspended in 1 ml reagent D.

Immunofluorescent staining. In a new conical-bottomed tube, 10 μ l reagent E-Rabbit anti-human CA, 10 μ l reagent F-Mouse anti-human HbF and 25-50 μ l erythrocytes suspension (cell suspension previous step) were added together and mixed well. The suspension was incubated at room temperature in dark for 15 minutes, the cell suspension was resuspended in 100 μ l PBS and then 10 μ l reagent G (Goat anti-Rabbit IgG (H+L) FITC) was added to the cell suspension. Reagent H (10 μ l, goat fab anti-mouse IgG (H+L) R-PE) was added to the cell suspension, the mixture was incubated at room temperature in dark for 15 minutes, the supernatant was discarded and the cell suspension was

resuspended in 100 - 200 μ l PBS to perform the flow cytometry analysis.

Anti-D staining. A volume of 20 μ l of mixed artificial whole blood (2.5×10^7 cell/ μ l) was added to 2 μ l of monoclonal anti-D with directly conjugated R-Phycoerythrin and the mixture was incubated at room temperature for 30 minutes. Then, the cells were washed three times in PBS azide and were resuspended in 200 μ l of PBS for subsequent flow cytometric analysis.

Flow cytometric analyses. Flow cytometric analyses were performed on a fluorescence-activated cell sorter (FACS calibur, BD company, USA), and sample acquisition was performed on 50,000 to 100,000 cells at a flow rate of 5,000 events per second. Side-scatter and forward scatter thresholds were set to exclude small particles such as buffer contaminates and platelets. Gates were set to include RBC but to exclude auto fluorescent nucleated cells. Data analysis was performed with the cell quest software (Becton Dickinson, USA) the region of analysis for fetal RBC and was determined by using the positive control samples containing fetal RBC from cord blood.

RESULTS

Flow cytometric results indicate fluorescent signals of artificial mixtures at different fetal RBC concentrations clearly. These analyses were done according to the spiked sample with 0.25, 0.5, 1, 2% of fetal RBC Rh(D) positive stained with anti-D IgG-PE, the RBC gated by FSC/SSC dot-plot diagram. In (SSC/FL2) diagram, RBC Rh(D) positive was selected. The cytometric analysis of a spiked sample with 0.25, 0.5, 1, 2% of fetal RBC Rh(D) positive stained with anti-HbF by Fetal Cell Count Kit showed 3 cell populations: A1, no CA and high HbF content (Fetal RBC's); A2, CA and low HbF content (F-Cells); A4, CA and no HbF content (adult RBC's). The results of the quantitative studies are shown in Table 2.

Following formula was used to calculate the FMH for anti-D labeling [7]:

$$\text{FMH} = 1800 \times 1.22 \frac{(\text{number of positive events} - \text{number of events in negative control})}{\text{total number of events counted}}$$

Or

$$= 1800 \times 22 \frac{(\% \text{positive events} - \% \text{events in negative control})}{100}$$

i.e. $\text{FMH} = 22 (\% \text{positive events} - \% \text{events in negative control})$

Table 2. Percentage of fetal cells in range of 0.25% and 0.5% (a) and 1% and 2% (b) estimated FMH.

(a)	Sample	FC with anti-D		FC with anti-HbF		0.25% FMH	0.5% FMH
		0.25% FMH	0.5% FMH	0.25% FMH	0.5% FMH		
	1	0.30	5.05	0.57	10.90	0.24	6.1
	2	0.32	5.40	0.56	10.70	NA	NA
	3	0.28	4.60	0.58	11.70	0.28	6.9
	4	0.30	5.05	0.53	10.12	0.20	5.0
	5	0.30	5.10	0.47	9.24	0.20	5.0
	6	0.31	5.20	0.59	11.40	0.20	4.4
	7	0.31	5.20	0.50	9.50	0.34	5.5
	8	0.32	5.20	0.53	10.12	0.31	4.8
	9	NA	NA	0.49	7.90	NA	NA
	Mean \pm SD	0.305 ± 1.30		0.5356 ± 1.24		0.261 ± 7.081	0.496 ± 0.119

(b)	Sample	FC with anti-D		FC with anti-HbF		1% FMH	2% FMH
		1% FMH	2% FMH	1% FMH	2% FMH		
	1	1.05	21.70	2.05	43.5	0.80	21.70
	2	0.98	19.80	1.90	41.5	NA	NA
	3	NA	NA	1.30	28.0	NA	NA
	4	1.02	20.90	1.87	39.5	0.82	21.90
	5	0.95	20.24	1.90	40.2	0.73	20.10
	6	0.94	19.15	1.82	38.5	0.93	18.48
	7	0.92	18.70	1.74	36.7	0.96	19.14
	8	1.03	19.80	1.88	39.8	0.94	18.70
	9	1.00	20.40	1.78	37.6	0.97	19.36
	Mean \pm SD	0.98 ± 4.6		1.804 ± 0.20		0.878 ± 9.4	1.48 ± 0.281

For anti-HbF staining. Using the fetal cell count kit II (according to the fetal cell count):

$$\text{Degree of FMT} = \frac{\% fRBC}{(\% mRBs + \% F - \text{cells})} \times 2400$$

The data obtained in the present study show that the percentage of fetal cells in artificial mixtures for anti-D staining, for 0.25% dilution mixtures, the measured ranges that acquired were 0.28%-0.32% with ranges of 4.6 ml-5.4 ml FMH (Table 2a), and for mixtures with dilution 2%, were 1.3%-2.05% with ranges of 28 ml-43 ml FMH (Table 2b). In the other protocol, for anti-HbF staining, the ranges of data were 0.2%-0.34% for 0.25% equivalent to 4.4-6.9 ml FMH (Table 2a) and 1.04%-1.8% for mixture dilution 2% with 24.4-48 ml FMH (Table 2b).

The analysis based on the artificial mixture dilution shows that the mean \pm SD of D percentage in D staining method was 0.3050 ± 1.309 ($n = 8$) and HbF method was 0.2614 ± 7.081 for the 0.25% standard dilution preparation.

The data obtained for 0.5% dilution for D percentage method was 0.5356 ± 1.246 and for HbF method was determined 0.4967 ± 0.119 , respectively. For standard dilution 1%, the mean of D staining method was 0.9863 ± 4.658 and for HbF method was 0.8786 ± 9.40 and, finally in 2% dilution of mixtures, this mean of D method was 1.804 ± 0.20 and with HbF staining was 1.48 ± 0.281 . So, the mean of HbF staining in dilution 0.25% and 0.5% was much more close to the artificial mixtures (Table 2a). In contrast, in the dilution of 1.00% and 2.00% in artificial mixture, the mean of D method was much more close to the mean of expected method.

So, the data indicate that the D labeling method is much more accurate than HbF labeling. Therefore, in these cases we quantified FMH, and measured what dose of Rhogam is used. The plot shows that, although the population of F-cells is considerable, the population of real fetal RBC's can be distinguished accurately. The results of comparison in two flow cytometric methods have a good correlation ($r^2 = 0.9672$, $r^2 = 0.8842$). As shown in Figures 1 and 2, there was a correlation between expected and measured percentage and FMH.

DISCUSSION

New methods for FMH quantification such as flow cytometry are now available. This method enables rapid cell analysis with high sensitivity and detect very small bleeds (i.e. 1 fetal cell in 100,000

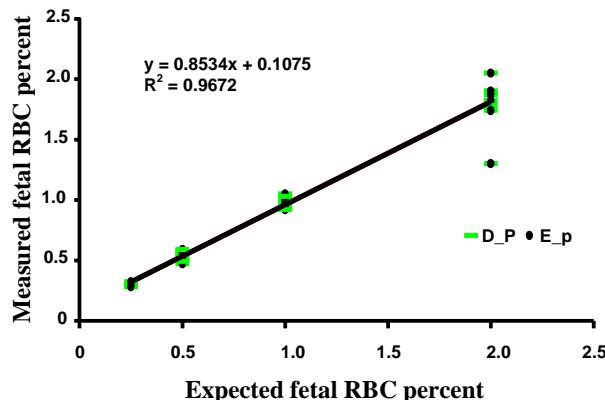


Fig. 1. Percentage of expected cells versus the percentage of fetal cells deleted by anti-Hb labeling for all spiked samples labeling ($n = 36$), which resulted in a linear regression of $y = 0.6845x + 0.1389$, $R^2 = 0.8842$.

maternal cells) has been applied recently by several groups [6, 20, 21].

There are two methods of flow cytometric approaches using monoclonal antibodies to HbF or antigen D. This quantification of fetal RBC's is most commonly used to estimate the degree of FMH: - In case of trauma with suspected placental injury, or, in the situation of Rhesus-D incompatibility between the fetus and the mother for prevention of HDN 22.

The result of the present study using flow cytometry described the percentage of fetal cells in maternal blood and measured FMH that we determined dose of RhIg should be given based on measured FMH or percentage of fetal RBC.

We do the cytometric technique included a direct technique based on staining of RhD antigen and an indirect technique based on hemoglobin F staining.

Statistical analysis was done with SPSS (10.00)

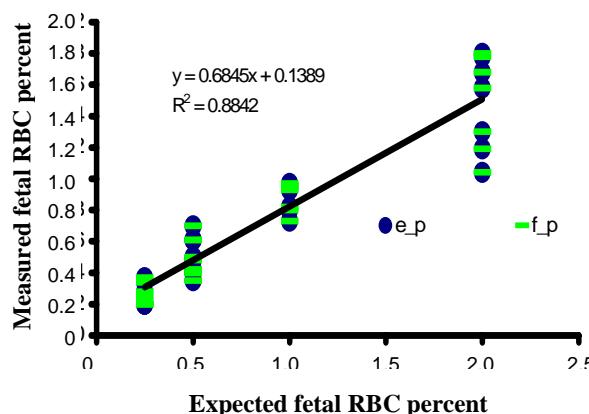


Fig. 2. Percentage of expected cells versus the percentage of fetal cells deleted by anti-Hb labeling for all spiked samples labeling ($n = 36$), which resulted in a linear regression of $y = 0.6845x + 0.1389$; $R^2 = 0.8842$.

and showed that both techniques are sensitive and accurate in the range studied (0.25-2%) but anti-D staining much more accurate than another. ($r^2 = 0.9672$, $r^2 = 0.8842$).

Nance *et al.* [22] and Johnson *et al.* [15] indication showed these results are in agreement with our representation. They pointed out that hemoglobin F staining can be used for detection of fetal RBC in different clinical situations and is not restricted to RhD negative mothers delivering RhD positive.

The result of Nelson *et al.* [8] have suggested the use of directly conjugated monoclonal anti-HbF method for the quantification FMH is comparable to the flow cytometry method using anti-D.

In addition, Kennedy [7] reported that the estimation of FMH by flow cytometry using directly conjugated anti-HbF may significantly underestimate the number of fetal red cells present in the samples containing $\geq 1\%$ fetal cells in comparison to labeling with anti-D staining.

Davis *et al.* [23] described an approach to flow cytometric detection of fetal cell using antibodies to HbF, that allows broad application of flow cytometric to all clinical situation and they suggested anti-HbF labeling method is highly accurate.

Ochesbein-Imhof *et al.* [24] recommended that the use of monoclonal anti-D labeling for quantification of FMH for the prevention of RhD alloimmunization is high accurate.

Otherwise recommended doses vary between different countries that ranges are from 100-300 μg . In our country, Iran we did not quantify FMH but till now, we administrate one dose 300 μg to any patient. So, we decided to quantify the rate of FMH and doses of Rhogam.

Quantification of FMH using flow cytometry allows to measurement of precise dosage of RhD immunoglobulin for protection against anti-D alloimmunization.

In conclusion, our data show that anti-D labeling significantly is much more accurate than HbF labeling. Although, the flow cytometric approach using anti-D is applicable only to the clinical situations with RhD antigen incompatibility (anti-HbF labeling detect all incompatibility) and cannot be utilized in all cases of maternal trauma and suspected FMH, or samples involving low-density D antigen or the weak D phenotype, however, to allow adequate immunoprophylaxis in D-negative mothers with massive FMH, we recommended that anti-D labeling should be used in the routine flow cytometric estimation of FMH.

REFERENCES

1. Vengelen-Tyler, V., Brecher, M.E. and Butch, S.H. (1999) Technical manual. 13th ed. Maryland: American Association Blood Banks AABB, United State, pp. 495-512.
2. Mollison, P.L., Engelfriet, C.P. and Contreras, M. (1997) Blood Transfusion Clinical. 10th ed. Oxford: Blackwell Science, UK, pp. 390-424.
3. Urbaniak, S.J. and Greiss, M.A. (2000) Rh haemolytic disease of the fetus and the newborn. *Blood Rev.* 14: 44-61.
4. Stoll, B.J. and Kliegman, R.M. (2004) Blood Disorders. In: *Nelson text book of pediatrics*. (Behman, R.E., Kliegman, R.M. and Jenson, H.B. eds.), 17th ed., Philadelphia, Saunders Co, pp. 601-606.
5. Greiss, M.A., Armstrong-Isher, S.S., Perera, W.S., Brown, P.M. and Urbaniak, S.J. (2002) Semiautomated data analysis of flow cytometric estimation of fetomaternal hemorrhage in D-women. *Transfusion* 42 (8): 1067-1078.
6. Bayliss, K.M., Kueck, B.D., Johnson, S.T., Fueger, J.T., McFadden, P.W., Mikulski, D. and Gottschall, J.L. (1991) Detecting fetomaternal hemorrhage: a comparison of five methods. *Transfusion* 31 (4): 303-307.
7. Kennedy, G.A., Show, R., Just, S., Bryson, G., Battistutta, F., Rowell, J. and Williams, B. (2003) Quantification of feto-maternal haemorrhage (FMH) by flow cytometry: anti-fetal haemoglobin labeling potentially underestimates massive FMH in comparison to labeling with anti-D. *Transfus. Med.* 13 (1): 25-33.
8. Kumpel, B.M. (2002) On the mechanism of tolerance to the Rh D antigen mediated by passive anti-D (Rh D prophylaxis). *Immunol. Lett.* 82 (1-2): 67-73.
9. Cunningham, F.G., Gant, N.F. and Leveno, K.J. (2001) *Williams Obstetrics*, 21th ed., New York, McGraw-Hill Co., pp. 1056-1067.
10. Sebring, E.S. and Polesky, H.F. (1990) Fetomaternal haemorrhage: incidence, risk factors, time of occurrence, and clinical effects. *Transfusion* 30 (4): 344-356.
11. Boucher, M., Rinfret, D. and Varin, J. (1996) Fetomaternal hemorrhage after external cephalic version: an overestimated risk. *Am. J. Obstet. Gynecol.* 174: 360-362.
12. Ness, P.M., Baldwin, M.L. and Niebyl, J.R. (1987) Clinical high-risk designation does not predict excess fetal-maternal hemorrhage. *Am. J. Obstet. Gynecol.* 156 (1): 154-158.
13. Kumpel, B.M., Wiener, E., Urbaniak, S.J. and Bradley, B.A. (1989) Human monoclonal anti-D antibodies II: the relationship between IgG subclass, Gm allotype and Fc mediated function. *Br. J. Haematol.* 71 (3): 415-420.
14. Liod-Evans, P., Guest, A.R., Austin, E.B. and Scott,

M.L. (1999) Use of a phycoerythrin-conjugated anti-glycophorin: a monoclonal antibody as a double label to improve the accuracy of FMH quantification by flow cytometry. *Transfuse. Med.* 9 (2): 155-160.

15. Janssen, W.C.M. and Hoffman, J.J.M.L. (2002) Evaluation of flow cytometric enumeration of fetal erythrocytes in maternal blood. *Clinical. Laboratory. Haematol.* 24 (2): 89-92.

16. Bowman, J.M. (1988) The prevention of Rh immunization. *Transfus. Med. Rev.* 2 (3): 129-150.

17. Morrison, J. (2000) Audit of anti-D immunoglobulin administration to pregnant Rhesus D negative women following sensitising events. *J. Obstet. Gynecol.* 20 (4): 371-373.

18. Nelson, M., Zarkos Popp, H. and Gibson, J.A. (1998) Flow-cytometric equivalent of the Kleihauer test. *Vox. Sanguinis.* 75 (3): 234-241.

19. Lloyd-Evans, P., Guest, A.R., Voak, D. and Scott, M.L. (1999) Detection of weak D and D^{vi} red cells in D-negative mixtures by flow cytometry: implications for feto-maternal haemorrhage quantification and D typing policies for newborns. *Br. J. Haematol.* 104 (3): 621-625.

20. Duguid, J.K.M. and Bromilow, I. (1999) Laboratory measurement of feto-maternal haemorrhage and its clinical relevance. *Transfuse. Med. Rev.* 13 (1): 43-48.

21. Polesky, H. and Sebring, E. (1981) Evaluation of methods for detection and quantification of fetal cells and their effects on RhIgG usage. *Am. J. Clin. Pathol.* 76 (suppl): 525-529.

22. Nance, S.J. and Garratty, G. (1987) Application of flow cytometry to immunohematology. *J. Immunol. Methods* 101 (1): 127-131.

23. Davis, B.H., Olsen, S., Bigelow, N.C. and Chen, J.C. (1998) Detection of fetal red cells in fetomaternal haemorrhage using a fetal haemoglobin monoclonal antibody by flow cytometry. *Transfusion* 38: 749-756.

24. Ochsenbein, N., Ochsenbein, A.F., Seifert, B., Huch, A., Huch, R. and Zimmermann, R. (2002) Quantification of fetomaternal haemorrhage by fluorescence microscopy is equivalent to flow cytometry. *Transfusion* 42: 947-953.