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Quantification of Fetal Red Blood Cells in Adult Blood Using Classical Kleihauer-Betke Method and a Dual Colour Flow Cytometry Technique.

Objective: To assess the sensitivity of flow cytometry (FCM) in the detection and quantification of fetal red blood cells (RBC) in comparison with the traditional Kleihauer-Betke test (KBT).Fetomaternal-haemorrhage (FMH) is a serious complication of pregnancy. Substantial FMH may lead to life-threatening anaemia in the fetus or newborn. In addition, exposure of Rhesus (Rh) D negative women to small amounts of fetal Rh D positive red cells during pregnancy or delivery may result in sensitization. Accurate detection and quantification is important as FMH is related to many obstetrical disorders and invasive procedures and may lead to critical complications.
Material and Methods: A series of 32 artificial samples were prepared consisting of volunteer's adult blood spiked with varying amounts of cord blood from maternity ward of the Hospital Kepala Batas. The flow cytometric method and the KBT were used for detection of fetal RBCs in artificial mixture sample modeling.
Results: This study showed a significant difference between the results by the traditional KBT and the expected values. And the results were higher than expected, while there were no significant differences between the results of the FCM and the expected values, but the results were generally on the lower side.
Conclusion: We concluded that the KBT could be used in the routine diagnostic work to calculate FMH volume; the reagents are cheaper and require less expensive device for detection of fetal cells. However, KBT cannot be utilized for discriminating between fetal red cells and adult red cells with high fetal haemoglobin (F cells).
The flow cytometry with dual staining technique could be used for the required purpose. However, the technique demands an expert technician because the kit is expensive and takes longer preparation time.

Keywords: Red Blood Cell, Kleihauer-Betke Method, Flow cytometry (FCM), Fetus.

INTRODUCTION

The transfer of fetal red blood cells into maternal circulation, or fetomaternal- haemorrhage, may occur during pregnancy or at delivery due to spontaneous or induced disruption of placental barrier, usually without any antepartum maternal or fetal signs {1, 2}. However, a number of pathological conditions, such as abdominal trauma {3, 4}, placental abruption {2, 5, 6}, or choriocarcinoma {7, 8} are identified as risk factors for the occurrence of significant FMH. In addition, a number of prenatal invasive procedures and obstetrical interventions during pregnancy and delivery can cause breakdown of the placental barrier place patients at risk. However, in most of the cases only small volume of blood, usually less than 1 mL transfers from fetus to the mother {1}. Conversely, massive FMH, greater than 30 mL of whole blood, occurs in only about 3 out of 1000 pregnancies and might lead to serious complications of pregnancy, including life-threatening anaemia in the fetus or a newborn {9}. In addition, exposure of Rhesus (Rh) D negative women to small amounts of fetal Rh D positive red cells during pregnancy or delivery may result in sensitization to D-antigen {10}. Therefore, it is of clinical importance to develop new sensitive techniques to detect fetal cells in the adult blood and to quantify the FMH.In 1957 Kleihauer demonstrated the presence of fetal cells in the maternal circulation by application of the acid elution principle to identify fetal erythrocytes. This method is based on the fact that fetal haemoglobin (HbF) is more resistant to acid elution than adult haemoglobin (HbA) {10}.Accurate detection and quantification is important as FMH is related to many obstetrical disorders and invasive procedures and may lead to critical complications such as fetal exsanguination and red cell immunization {2}. In addition, estimation of FMH is an important dose-determining measure for the administration of RhIg in D–negative pregnant or newly delivered women to prevent anti-D immunization and haemolytic disease of the newborn (HDN) {11}.While the Kleihauer test is sensitive, its accuracy in quantifying FMH is open to question because of numerous sources of error including variations in the thickness of blood films, the number of red cells in a low power microscope field, the fact that some fetal cells do not stain, and the difficulty of classifying cells of intermediate staining. Furthermore, the Kleihauer test is unable to differentiate between fetal red cells and F cells which may be present in women with sickle cell and thalassaemic syndromes, hereditary persistence of fetal haemoglobin (HPFH), and in up to 25% of normal women in the second trimester of pregnancy {12, 13, 14}.Several investigators focused on the development of more reliable methods for FMH quantification using flow cytometry {15. 16}. Most of these alternative tests are based principally on flow cytometry quantitation of fetal cells identified by a specific blood group antigen that serves as a marker, for example, RhD-positive RBCs in the blood of an RhD–negative mother {16, 17}.Some groups have proposed the use of antibodies to HbF for the specific identification of fetal cells {18, 19}. A major advantage of this method is that it is not dependent on the blood group of the mother or the fetus. The discrimination between maternal F cells and fetal RBCs is

based on the lower expression of HbF in the former population. An adequate setting to differentiate these cells carrying HbF, however, is difficult to achieve, particularly in samples with large amounts of F cells {18}. Other studies used a combination of a murine monoclonal antibody directed to HbF and a polyclonal antibody to carbonic anhydrase (CA).The CA isoenzymes that are mainly represented by CAI and CAII in RBCs are fully expressed only after birth {20-22}.In this study we established an artificial model, by spiking fetal RBCs into adult blood at serial dilutions, to compare the traditional method, (KBT), with a flow cytometric technique. Finally, by using a combination of anti-HbF and anti-CAII, the discrimination between true fetal cells and possible false positive F cells can be achieved, by staining the small population of contaminating F cells with anti-CAII, thereby moving it away from the fetal cell population.Since both the KBT and a dual-color flow cytometric method using Fetal Cell Count Kit are used in some of the European countries for detection and quantification of fetal cells in maternal blood, we feel the importance to introduce this new technique in Malaysia, optimization of this techniques, and evaluation of the superiority between the two techniques for diagnostic purposes.The general objective of the study was to assess the sensitivity of the flow cytometric technique in the detection and quantification of fetal RBCs in comparison with the traditional KBT. The specific objectives were to discriminate fetal RBCs from adult RBCs by using both techniques, and to evaluate the sensitivity of a dual-color flow cytometric method for detecting fetal RBCs by using Fetal Cell Count kit in comparison with KBT.

MATERIAL AND METHODS

Blood samples: Adult blood from healthy volunteers and umbilical cord blood was collected in EDTA blood collection tubes and stored at 4°C for no longer than 72hours. Red cell counts of adult blood and cord blood samples were determined using a cell counter (Sysmex KS21) and dilution with phosphate-buffered saline (PBS) was applied to equalize red cell counts in samples. Eight different dilutions were made on each cord blood and ABO compatible adult blood samples. Of each dilution, four replicate measurements were performed on different occasions. Each sample was analyzed by both KBT and FCM.Dilutions for each sample mixture ranged from 0.025% to 4% (0.025%; 0.05%; 0.1%; 0.2%; 0.5%; 1%; 2%; and 4%), fetal red cells to adult blood. Two controls (5% and 50%) cord blood in adult blood for the KBT and the FMC method were prepared, respectively.

Methods:The flow cytometric method, by using the Fetal Cell Count kit (IQP-379 from IQ Products), and the traditional Kleihauer-Betke test (285D, Sigma Aldrich, Germany) were used for detection of fetal Red Blood Cells (RBCs) in artificial mixture sample modeling. The manufacturer procedures were followed for both methods.

Flow cytometric method*:** The Fetal Cell Count kit is intended for the discrimination and quantitative detection of the human fetal red blood cells in maternal blood. The Fetal Cell Count methodology is based on the combination of two antibodies. One is directed against fetal haemoglobin (HbF), which is present in fetal RBCs and normally in a trivial amount in adult RBCs (called F-cells). The second antibody is directed against Carbonic Anhydrase (CA), an enzyme only present in adult RBCs and start to appear in the RBCs after childbirth. The dual-color flow cytometric method allows simultaneous detection of these intracellular antigens, while the use of formaldehyde as fixative and sodium dodecyl sulfate (SDS) for permeabilization of fixed RBCs results in low background staining, negligible HbF leakage and minimal clumping. Each dilution mixture underwent several steps: fixation, permeabilization, and then fluorescent staining by the two types of antibodies. Antibody directed towards HbF is labeled with phycoerythrin (PE) and the antibody directed towards CA is labeled with fluorescein isothiocyanate (FITC). A negative control was used from the unstained fixed and permeablized sample. Two single positive controls were for detecting HbF (fetal RBCs), or positive CA (adult RBCs), and a third positive control was prepared by mixing (50/50) (fetal and adult RBCs) stained sample used to set up the quadrants to calculate the percentage of fetal cells and adult cells separately, and to find if there are adult RBCs with high HbF (F-cells) ***(Figure 1).

Kleihauer-Betke test: This method is based on the fact that fetal haemoglobin is more resistant to acid elution than adult haemoglobin. When blood smears are immersed in acid buffer for 3 minutes, rinsed thoroughly with distilled water, the adult haemoglobin is eluted leaving ghost cells, whereas fetal haemoglobin is not. Blood smears were made from the eight different dilution mixtures plus the 5% positive control, dried and stained. Ten thousand red cells were screened under the microscope using x40 objective, and the total fetal cells were recorded ***(Figures 2 & 3)***.

Statistical analysis:We used Statistical Package of Social Science (SPSS for window, version12.0.1; SPSS) to create and analyze the data. Data are presented as mean ± standard deviation. Comparison between KBT and FCM method was done by paired sample t-test. Difference was considered statistically significant for two sided P value≤ 0.05. We also measured the correlation between the KBT and the FCM through linear regression and Pearson's correlation coefficient (r).

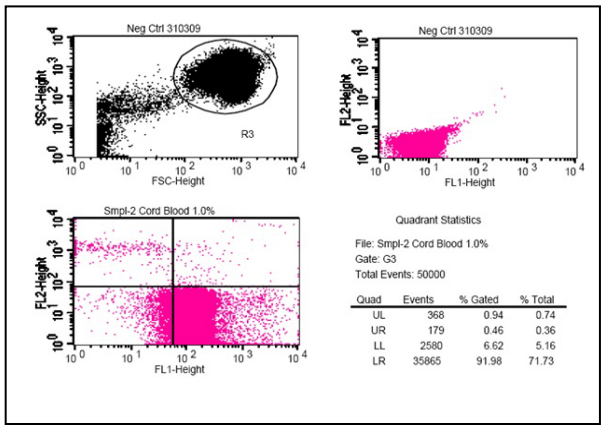

FIG. 1: Flow cytometric figure showing gating RBCs on FSC/SSC plot, negative control on FL1/FL2 plot, cord RBCs on the upper left quadrant, and adult RBCs on the lower right quadrant with its statistics (under Quadrant Statistics).

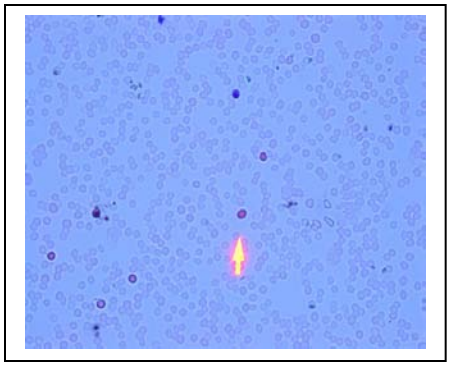

FIG. 2: KBT: microscopic field after staining showing seven fetal RBCs among ghost adult RBCs (40X objective)

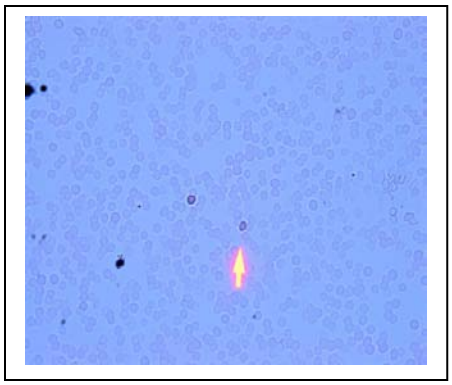

FIG. 3: KBT: microscopic field after staining showing two fetal RBCs among ghost adult RBCs (40X objective)

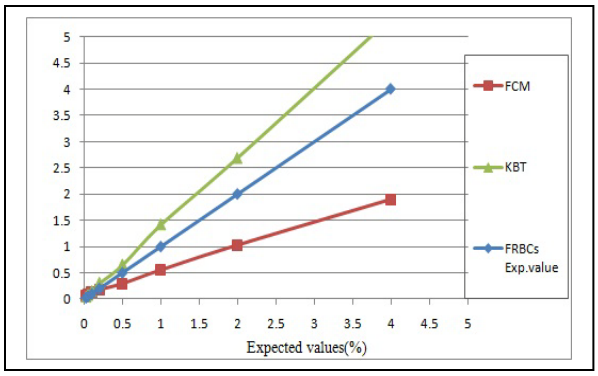

FIG.4: Plot of expected (experimental) and measured values for both KBT & FCM.

RESULTS

All samples of different mixture dilutions of adult blood and cord blood were analyzed. For each dilution a mean and a standard deviation (SD) were calculated from the number of fetal red cells detected by FCM and KBT. ***Tables (I, II, III, IV, and V)***, summarize the main results of the study for both KBT and FCM techniques. Comparison between KBT and FCM results was performed in a total of 32 dilutions of the spiked samples at four different occasions. The data obtained from the present study ***(Table III)*** showed no significant difference between FCM and KBT on dilutions: 0.025%, 0.05%, and 0.1% (p value = 0.283, 0.116, and 0.332, respectively). However, in artificial mixtures for 0.2% dilution there was a significant difference between the two methods (p value = 0.009), this is also shown in dilutions: 1%, 2%, and 4% (p value = 0.049, 0.026, and 0.023 respectively).The differences between KBT results and the expected values ***(Table IV)*** at dilution 0.025% and 0.05%, were insignificant (p value = 0.080, 0.063, respectively), while in dilutions: 0.1%, 0.2%, 1%, 2%, and 4% showed significant differences (p value = 0.032, 0.031, 0.026, 0.014, and 0.011, respectively). The insignificant difference seen in dilution 0.5% could be considered incorrect and due to a technical fault as it is located between two significant results. The differences between FCM results and expected values (table V) at dilution 0.025% appeared insignificant (p value = 0.263), but this result was affected by unexpectedly very high value (outlier result) in one of the 4 replicate results at this dilution (0.74) which falsely raised the mean of this replicate. A significant difference (p value = 0.034) was seen in 0.05% dilution. From dilution 0.1% up to 1% difference was insignificant (p value = 0.083 - 0.095). However, in the last two dilutions, 2% and 4%, the p value indicated significant differences, but if we look at them closely, they were nearly 0.05 (for 2% the p value was 0.045, and for 4% the p value = 0.041) and probably one can consider them nearly insignificant.The mean percentages of fetal red cells detected by flow cytometry and the manual KBT compared to experimental values are given in ***Figure 4***. In the ranges from 0.025% to 4.0%, the standard manual KBT results were higher than the expected values, whereas the flow cytometry showed lower results than the expected values in the ranges from 0.5% to 4.0%. However, the results of both techniques were comparable. Both methods correlated well (r=0.870, p value < 0.05). As shown in ***Figure 4***, the experimental values obtained with the eight diluted mixtures started from 0.025% to 4.0% of fetal RBCs were in correlation with the values for FCM (r=0.912, $p < 0.05$) and KBT (r=0.994, $p < 0.05$). The linearity was also confirmed with the data obtained from artificial mixtures (0.025% to 4.0%) prepared during the evaluation process with a high correlation coefficient.

DISCUSSION

The results of the present study using flow cytometric technique and Kleihauer-Betke test describe the percentage of fetal cells in adult blood in artificial mixtures of eight dilutions from 0.025% to 4.0%. For each dilution, four replicate measurements were performed on four different occasions. We compared the flow cytometric technique by using two antibodies to intracellular antigen, (anti-F) and (anti-CA), and acid elution technique, the traditional Kliehauer test, and we found that there is no significant difference between KBT and the expected value (the experimental dilution) at lower two dilutions (0.025% and 0.05%; *p* value = 0.080 and 0.063 respectively). However, there was a significant difference between FCM and expected value at these two dilutions. This might be false because in our methodology we acquired 50,000 events which might not be enough to detect very low positive fetal cells and what is measured probably considered as an auto-fluorescent signals. The reason for this assumption is due to some previous studies that suggested acquiring 100,000 or

Table I: Descriptive statistics shows the minimum and maximum results and means with standard deviation (SD) for the KBT done at 4 replicate measurements.

Test No.	Dilutions %	Minimum	Maximum	Mean ± Std. Deviation
1	0.025	0.03	0.05	0.04 ± 0.01
2	0.05	0.06	0.09	0.08 ± 0.02
3	0.1	0.12	0.17	0.15 ± 0.02
4	0.2	0.25	0.36	0.31 ± 0.06
5	0.5	0.55	0.76	0.66 ± 0.11
6	1.0	1.20	1.60	1.43 ± 0.21
7	2.0	2.45	3.05	2.70 ± 0.27
8	4.0	4.95	6.01	5.37 ± 0.48

Table II: Descriptive statistics shows the minimum and maximum results and means with standard deviation (SD) for the FCM done at 4 replicate measurements.

Test No.	Dilutions %	Minimum	Maximum	Mean ± Std. Deviation
1	0.025	0.07	0.74	0.25 ± 0.33
2	0.05	0.08	0.15	0.12 ± 0.04
3	0.1	0.09	0.20	0.17 ± 0.05
4	0.2	0.16	0.24	0.22 ± 0.04
5	0.5	0.26	0.48	0.37 ± 0.12
6	1.0	0.44	0.94	0.69 ± 0.26
7	2.0	0.90	1.69	1.28 ± 0.43
8	4.0	1.66	3.26	2.44 ± 0.90

Table III: Mean difference between the KBT and FCM at different dilution with *p* value.

Dilution %	Mean difference	t statistics	*P* value
0.025	-0.21250	-1.305	0.283
0.05	-0.04000	-2.191	0.116
0.1	-0.02000	-1.155	0.332
0.2	0.09250	5.976	0.009
0.5	0.28500	2.467	0.090
1.0	0.73750	3.208	0.049
2.0	1.42250	4.099	0.026
4.0	2.92750	4.340	0.023

Table IV: Mean difference between the KBT and expected value at different dilution with *p* value

Dilutions %	Mean difference	t statistics	*p* value
0.025	0.01250	2.611	0.080
0.05	0.02500	2.887	0.063
0.1	0.04500	3.781	0.032
0.2	0.10750	3.867	0.031
0.5	0.15500	2.810	0.067
1.0	0.42500	4.123	0.026
2.0	0.70250	5.125	0.014
4.0	1.36750	5.642	0.011

Table V: Mean difference between the FCM and expected value at different dilution with *p* value

Dilutions %	Mean difference	t statistics	*p* value
0.025	0.22500	1.376	0.263
0.05	0.06500	3.702	0.034
0.1	0.06500	2.566	0.083
0.2	0.01500	0.792	0.486
0.5	-0.13000	-2.142	0.122
1.0	-0.31250	-2.142	0.095
2.0	-0.72000	-3.318	0.045
4.0	-1.56000	-3.462	0.041

even 200,000 events to be sensitive enough to detect such low number of fetal cells and compared to KBT {23}. Another important finding in our results is that by using the KBT there was a significant difference between the measured values and the expected values at dilutions from 0.1% and above, while by using the FCM technique, by using the dual stain (anti-F and anti-CA), the results showed no significant difference from the expected values from dilution 0.1% until dilution 4.0%. This suggests that using the FCM method with this new dual staining technique gives more accurate and reasonable measurements in comparison with expected (experimental) dilutions than using the KBT traditional method. The significant difference between the measured value and the experimental dilutions seen with the KBT method were due to the higher results in the latter. Our data obtained from the present study showed a good correlation between the two method, a finding reported by other investigators {17, 23}, and no significant difference at lower dilutions (table III), but at dilution 0.2% and above there was a significant difference between the two techniques, except at dilution 0.5% which might be due to a random error rather than a true result as it is located in-between significant difference results. The higher values seen with the KBT compared to the experimental dilutions seen in our study is also noted by other researchers {15, 23}. This is probably caused by lack of consistency in the methods used by various laboratories and subjectivity of the manual KBT resulting in large inter-observer and inter-laboratory variability {24, 25}.The overestimation of fetal cell count by the KBT may also be explained by an erroneous low count of maternal cells favored by the ghost appearance of adult RBCs after acid elution. Another reason for high values could be due to a high adult F cell percentage that resists acid elution and could not be discriminated from the fetal cell population, and this can lead to an overestimation by using KBT. Adult RBCs containing HbF (F cells) are evidenced in patients with abnormal Hb, as in thalassaemia, sickle cell anaemia and in hereditary persistence of HbF, as well as in some normal situations {26, 27}. Although there was no significant difference between measured values using the FCM method and the expected values at dilutions 0.1% up to 4.0%, figure 4 shows that the curve is clearly below that of the curve for the expected values. The lower values obtained by the flow cytometric method in our experiment could be due to several variables including the improper staining of intracellular antigen (HbF & CA) which causes false low results. Another variable is technical cause from inaccurate delineation between fetal population and adult population using quadrant stat technique. A third possible variable could be the number of photons after exciting the fluorescence material, as the measurement depends on the number of signals transmitted from the photons to electronic signals which are amplified and converted to digital signals. Last variable could be that, acquiring of 50,000 is below the threshold for obtaining more accurate and sensitive results at very low dilutions.

DISCUSSION

The results of the present study using flow cytometric technique and Kleihauer-Betke test describe the percentage of fetal cells in adult blood in artificial mixtures of eight dilutions from 0.025% to 4.0%. For each dilution, four replicate measurements were performed on four different occasions. We compared the flow cytometric technique by using two antibodies to intracellular antigen, (anti-F) and (anti-CA), and acid elution technique, the traditional Kliehauer test, and we found that there is no significant difference between KBT and the expected value (the experimental dilution) at lower two dilutions (0.025% and 0.05%; *p* value = 0.080 and 0.063 respectively). However, there was a significant difference between FCM and expected value at these two dilutions. This might be false because in our methodology we acquired 50,000 events which might not be enough to detect very low positive fetal cells and what is measured probably considered as an auto-fluorescent signals. The reason for this assumption is due to some previous studies that suggested acquiring 100,000 or even 200,000 events to be sensitive enough to detect such low number of fetal cells and compared to KBT {23}. Another important finding in our results is that by using the KBT there was a significant difference between the measured values and the expected values at dilutions from 0.1% and above, while by using the FCM technique, by using the dual stain (anti-F and anti-CA), the results showed no significant difference from the expected values from dilution 0.1% until dilution 4.0%. This suggests that using the FCM method with this new dual staining technique gives more accurate and reasonable measurements in comparison with expected (experimental) dilutions than using the KBT traditional method. The significant difference between the measured value and the experimental dilutions seen with the KBT method were due to the higher results in the latter. Our data obtained from the present study showed a good correlation between the two method, a finding reported by other investigators {17, 23}, and no significant difference at lower dilutions (table III), but at dilution 0.2% and above

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CONCLUSION

This study showed that by using the standard KBT one is able to enumerate fetal red cells varying from very small to large numbers and therefore is suitable for routine clinical use in the assessment of FMH in the majority of cases and it would be preferred because the reagent is cheaper, it does not need an expensive device and can be done in any routine diagnostic laboratory. Also we found the staining procedure for the KBT is easier than the staining procedure using Fetal Cell Count kit by flow cytometry.Flow cytometric technique, using the dual staining reagents is sensitive in measuring the fetal red cells and the adult red cells and clearly separates the two populations. From this study the FCM appeared to be more accurate and sensitive in quantifying fetal and adult red cells. Also the FCM technique using the dual staining is able to differentiate between fetal red cells and adult F cells, while the traditional KBT technique is unable to discriminate between the two types of red cells. However, FCM reagents are more expensive. There are special cases when the number of F cells is high as HPFH, thalassaemia, and sickle cell anaemia. In these cases, using KBT can give false high result, but use of the FCM with this new staining technique is able to differentiate between fetal cells and adult F cells and so can avoid inappropriate clinical approach, as e.g., injecting high dose of anti-D to prevent RBCs sensitization, and also to accurately measure FMH post trauma.

REFERENCES

1- *Bowman J M, Pollock J M, Penston L E. Fetomaternal transplacental hemorrhage during pregnancy and after delivery. Vox Sang. 1986; 5: pp.117–121.*

2- *Sebring E S, Polesky H F. Fetomaternal hemorrhage: incidence, risk factors, time of occurrence, and clinical effects. Transfusion. 1990; 30: pp.344-357.*

3- *Pearlman M D, Tintinalli J E, Lorenz R P. Blunt trauma during pregnancy. N Engl J Med. 1990; 323: pp.1609-1613.*

4- *Muench M V, Baschat A A, Reddy U M, Mighty H E, Weiner C P, et al. Kleihauer- Betke testing is important in all cases of maternal trauma. J Trauma. 2004; 57: pp. 1094-1098.*

5- *Cardwell M S. Ultrasound diagnosis of abruptio placentae with fetomaternal. Am J Obstet Gynecol. 1987; 157: pp. 358-359.*

6- *Kelsey P, Reilly J T. BCSH Blood Transfusion and General Haematology Task Forces. The estimation of fetomaternal haemorrhage. Transfusion Med. 1999**;** 9: pp.87-92.*

7- *Moya F R, Perez A, Reece E A. Severe fetomaternal hemorrhage. A report of four cases. J Reprod Med. 1987; 32: pp. 243-246.*

8- *Santamaria M, Benirschke K, Carpenter P M, Baldwin V J , Pritchard J A. Transplacental hemorrhage associated with placental neoplasms. Pediatr Pathol. 1987; 7: pp. 601-615.*

9- *American College of Obstetricians and Gynecologists (ACOG), Practice bulletin. Prevention of Rh D alloimmunization. Clinical management guidelines for obstetrician -gynecologists. American College of Obstetrics and Gynecology. Int J Gynaecol Obstet. 1999; 66:pp. 63–70.*

10- *Kleihauer E, Braun H, Betke K. Demonstration of fetal hemoglobulin in erythrocytes by elution. Klin Wochenschr. 1957; 35: pp. 637-638.*

11- *Parker J, Wray J, Gooch A, Robson S, Qureshi H. Guidelines for the use of prophylactic anti-D immunoglobulin. London (UK): British Committee for Standards in Haematology (BCSH); 2006.*

12- *Popat N, Wood W G, Weatherall D J , Turnbull A C. Pattern of maternal F-cell production during pregnancy. Lancet. 1977; 2: pp. 377-379.*

13- *Duckett J R A, Constantine G. The Kleihauer technique: an accurate method of quantifying fetomaternal hemorrhage? Br J Obstet Gynaecol.1997; 104: pp. 845-846.*

14- *Conley C L, Weatherall D J, Richadson S N, Shepard M K, Charache S. Hereditary Persistence of Fetal Hemoglobin: A Study of 79 Affected Persons in 15 Negro Families in Baltimor. Blood, 1963; 21: pp. 261-281.*

15- *Bayliss K M, Kueck B D, Johnson S T, Fueger J T, McFadden P W & Mikulski D. Detecting fetomaternal hemorrhage: comparison of five methods. Transfusion; 1991; 31: pp.303-307.*

16- *Lloyd-Evans P, Kumpel B M, Bromelow I, Austin E, Taylo E. Use of directly conjugated monoclonal anti-D (BRAD-3) for quantification of fetomaternal hemorrhage by flow cytometry. Transfusion. 1996; 36: pp. 432-437.*

17- *Cupp J E, Leary J F, Cernichiari E, Wood J C S, Doherty R A. Rare-event analysis methods for detection of fetal red blood cells in maternal blood. Cytometry. 1984; 5: pp.138-144.*

18- *Davis B H, Olsen S, Bigelow N C, Chen J C. Detection of fetal red cells in fetomaternal hemorrhage using a fetal hemoglobin monoclonal antibody by flow cytometry. Transfusion.1998; 38: pp. 749-756.*

19- *Dziegiel M H, Nielsen L K, Berkowicz A. Detecting fetomaternal hemorrhage by flow cytometry. Curr Opin Hematol. 2006; 13: pp.490-495.*

20- *Tashian R E. The carbonic anhydrases: widening perspectives on their evolution, expression and function. Bioessays. 1989; 10: pp.186-192.*

21- *Brady H J M, Edwards M, Linch D C, Knott L, Barlow J H, et al. Expression of the human Carbonic anhydrase I gene is activated late in fetal erythroid development and regulated by stage specific trans-acting factors. Br J Haematol. 1990; 76: pp. 135-142.*

22- *Ali Akbar S, Brown E R. Measurement of human erythrocyte CAI and CAII in adult, newborn, and fetal blood. Clin Biochem. 1996; 29: pp.157-164.*

23- *Valerie P, Janine B, Pierre G, Pascaline B, Dominique R, et al. Identification and quantification of fetal red blood cells in maternal blood by a dual-color flow cytometric method: evaluation of the fetal cell count kit. Transfusion. 2007; 47: pp. 1281-1289.*

24- *Corsetti J P, Cox C, Leary J F, Cox M T, Blumberg N, et al. Comparison of quantitative acid-elution technique and flow cytometry for detecting fetomaternal hemorrhage. Ann Clin Lab Sci. 1987; 17: pp.197-206.*

25- *Johnson P R, Tait R C, Austin E B, Lee D. Flow cytometry in diagnosis and management of large fetomaternal haemorrhage. J Clin Pathol. 1995; 48: pp. 1005-1008.*

26- *Pembrey M E, Wood W G, Weatherall D J, Perrine R P. Fetal haemoglobin production and sickle gene in the oases of Eastern Saudia Arabia. Br J Haematol. 1978; 40: pp.415- 429.*

27- *Hoyer J D, Penz C S, Fairbanks V F, Hanson C A, Katzmann J A. Flow cytometric measurement of hemoglobin F in RBCs: diagnostic usefulness in the distinction of hereditary persistence of fetal haemoglobin (HPFH) and hemoglobin S-HPFH from other conditions with elevated levels of hemoglobin F. Am J Clin Pathol. 2002; 117: pp.857-863.*