

ORIGINAL ARTICLE

The Estimation of Foetomaternal Haemorrhage by Flowcytometry

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ABSTRAK

Pendarahan sel darah bayi jenis rhesus (Rh) positif ke dalam aliran darah ibu berdarah Rh negatif boleh berlaku semasa mengandung dan semasa proses kelahiran. Perbezaan jenis darah ini menyebabkan ibu menghasilkan Rh antibody dan menyebabkan pemusnahan sel pramatang pada darah bayi. Ujian Kleihauer adalah kaedah biasa digunakan untuk mengukur pendarahan sel darah bayi. Walaubagaimanapun tiada penyelarasan khusus antara makmal bagi ujian ini dan keputusannya kurang tepat. Teknik sitometri aliran adalah kaedah baru menggunakan anti HbF monoclonal. Ia boleh mengkaji lebih banyak sel, lebih sensitif dan tepat. Tujuan kajian ini adalah untuk mengukur pendarahan sel darah bayi di dalam aliran darah ibu menggunakan dua teknik tersebut dan menentukan tahap korelasi antara keduanya. 98 sampel darah ibu mengandung lebih dari 20 minggu dan selepas bersalin telah dianalisa. Kajian mendapati tahap korelasi yang sederhana antara dua kaedah ujian, $r = 0.655$ ($p < 0.05$) dan tahap keharmonian adalah 66.3%. Walau bagaimanapun, kaedah sitometri aliran member bacaan yang lebih tinggi untuk 70.4% kes berbanding kaedah Kleihauer. Pemerhatian ini mungkin disebabkan oleh cahaya otoflores yang dihasilkan oleh kontaminasi sel darah putih atau penggunaan anti HbF yang kurang spesifik berbanding anti-D monoclonal. Ia boleh juga berpunca dari jenis fluorokrom yang digunakan. Oleh itu, untuk meningkatkan ketepatan keputusan ujian, analisis dua label glycophorin A yang spesifik untuk sel darah merah dan perbandingan antara anti HbF dan anti-D adalah disyorkan.

Kata kunci: pendarahan sel darah bayi, Kleihauer test, sitometri aliran, anti HbF

ABSTRACT

Foetomaternal haemorrhage (FMH) may occur following a sensitizing event, during pregnancy or at delivery. In cases of rhesus (Rh) incompatibility between mother

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and the fetus, it can thus subject to the haemolytic disease of the newborn. The Kleihauer test for quantification of FMH lacks standardization and results are less accurate. Furthermore, it cannot differentiate the foetal cell from the adult HbF. Flowcytometry analysis using monoclonal antibodies, is a new technique for the quantification of FMH and it allows larger number of cells to be analysed. It is also able to differentiate the foetal cell from maternal HbF, and thus is more sensitive and accurate. The objective of our study was to determine the FMH using the flowcytometric analysis of anti-HbF antibody and to correlate the FMH using flow cytometry and the standard Kleihauer test. Ninety eight peripheral blood samples from pregnant women at more than 20 weeks of pregnancy and post delivery were analyzed by both methods. The percentage of the foetal cells were recorded and the FMH were calculated. We found a fair correlation between the two methods with the correlation coefficient $r = 0.633$ ($p < 0.05$). The concordance rate was 66.3%. Flow cytometric method, however, gave higher values in 70.4% of cases as compared to the Kleihauer test. These could be due to the autofluorescence of contaminated white blood cell or using anti HbF which is less specific than anti-D. Other possible factors could be due to the fluorochrome used. Therefore, in order to increase the accuracy, we recommend the use of dual labeling of red cells with glycophorin A which is a marker for red cells and compare the use of monoclonal anti HbF and anti-D for analysis.

Keywords: foetomaternal haemorrhage, Kleihauer test, flow cytometry, anti HbF

INTRODUCTION

FOETOMATERNAL HAEMORRHAGE AND THE USE OF ANTI-D

Foetomaternal haemorrhage (FMH) may occur following a sensitizing event during pregnancy or at delivery and can lead to RhD immunization. If this occurs in a RhD-negative woman with a RhD-positive infant, there is a risk of HDN and can be fatal (Augustson et al. 2006). Assessment of FMH is an important element in determining the amount of anti-D to be administered to a RhD-negative mother following a sensitizing event or after delivery of an RhD-positive infant.

The guidelines for the use of anti-D

immunoglobulin for Rh prophylaxis (National Blood Transfusion Services, Immunoglobulin Working Party, 1991) stated that at least 500 IU of anti-D must be given to every RhD-negative woman with no preformed anti-D within 72hrs of delivery of an RhD-positive infant. This dose will be sufficient to prevent sensitization from a 4ml foetal red blood cell bleed (BCSH Blood Transfusion and General Haematology Task Force 1999). Statistics have shown that 0.4% of women have a FMH of greater than 4 ml and up to 0.3% greater than 15 ml thus will not be protected by the standard 500 IU dose of anti-D (BCSH Blood Transfusion and General Haematology Task Force 2011; Bayliss et al. 1991). It is therefore important

that the volume of any foetomaternal bleed is accurately estimated so that if necessary a supplementary dose of anti-D can be administered and maternal alloimmunization prevented.

Despite the policy, 1-2% of RhD-negative pregnant women at risk still become sensitized (BCSH Blood Transfusion and General Haematology Task Force 1999). A Kleihauer screening test is usually performed in all cases to detect the 1% of women who have a bleed larger than 4ml. In these cases the Kleihauer test is used to quantify the haemorrhage and determine the additional dose of anti-D immunoglobulin. Cases with large bleeds are monitored for the disappearance of haemoglobin F containing cells using the Kleihauer test and for the presence of free anti-D in maternal serum.

Despite earlier publications describing methods for assessing foetomaternal haemorrhage (Duguid & Bromilow 1999) it is recognized that there is little standardization of techniques (BCSH Blood Transfusion and General Haematology Task Force 1999; Johnson et al. 1993), a fact which can lead to inaccuracies in determining the size of any foetomaternal haemorrhage.

The Kleihauer acid elution test is the standard method for the detection and quantitation of foetomaternal haemorrhage in the UK. This test identifies foetal cells in the maternal circulation by the relative resistance of cells containing haemoglobin F to acid elution (Kleihauer et al. 1957). While the Kleihauer test is sensitive, its accuracy in quantifying FMH is open to

question because of increased method and operator variability, low level of reproducibility, poor preparation technique and lack of standardization between laboratories and individuals.

Numerous sources of error includes variations in the thickness of blood films, the number of red cells in a low power microscope field, the fact that some foetal cells do not stain, and the difficulty of classifying cells of intermediate staining.

Furthermore, the Kleihauer test is unable to differentiate between foetal red cells and adult haemoglobin F containing cells which may lead to false positive diagnoses due to increased levels of maternal HbF cells as a result of physiological variations during pregnancy (25% in second trimester) or traits such as thalassaemia, sickle cell anaemia or hereditary persistence of foetal Hb (HPFH) (Duckett & Constantine 1997; Corsetti et al. 1987). Descriptions of the Kleihauer test method vary between sources and therefore it is not surprising that wide variations between laboratories occur. In view of the problems with Kleihauer test, a new technology using flow cytometer for detection of HbF has been introduced.

Flow cytometry technique uses monoclonal or polyclonal antibodies to HbF, either directly conjugated with a fluorochrome or used with a secondary conjugated antibody to identify foetal cells in the maternal circulation (Davis et al. 1998; Brown & Wittwer 2000). The red cells are fixed and permeabilized to allow the antibody access to the haemoglobin in the cytosol, and the intensity of the

staining of the cells distinguishes foetal cells from adult 'F' cells. It allows large numbers of cells e.g. 50 000 - 100 000 to be readily counted. The test should then be more sensitive and accurate; and produce objective, quantitative results. However, flow cytometers are expensive and generally available only in major centers and require staff with specific expertise to operate the analyzer.

While the Kleihauer test is a simple, sensitive and cost effective technique for detecting patients with a FMH below 4 ml, flow cytometry offers several advantages in patients with larger FMH; 50 000 – 100 000 cells are assessed individually using the flow cytometer and several sources of inherent errors in the Kleihauer test are eliminated (Bromilow & Duguid 1997; Duckett & Constantine 1997; Corsetti et al. 1987). Raised maternal HbF concentrations render the Kleihauer test inconclusive, whereas the flow cytometer is able to distinguish foetal cells from adult cells. Furthermore, it can detect potentially immunising foetal cells even in cases where the Rh status of the fetus is not known. This method can be used to test mothers of any blood group and is not Rhesus dependent (Mollison 1972; Johnson et al. 1993; Nance et al. 1989).

The main objectives of the present study were to determine the FMH using the flow cytometric analysis of anti-HbF antibody and to correlate the FMH using flow cytometric with anti-HbF method and the standard Kleihauer test.

MATERIALS & METHODS

STUDY DESIGN

This was a comparative study to measure FMH in pregnant women using flow cytometry in comparison with conventional Kleihauer test. The study was performed using fresh whole blood collected in EDTA container from pregnant women attended the O&G clinic or admitted in UKMMC between 2005-2006 with prior consent from respective patients before samples were withdrawn. All the samples were run in the flow cytometry laboratory, Blood Bank Unit, Universiti Kebangsaan Malaysia Medical Centre (UKMMC). Demographic and clinical data of these patients were retrieved from the Record Department of UKMMC. A total of 98 pregnant women were recruited for this study. They were uneventful pregnant women from 28 weeks to 32 weeks period of gestation attending Antenatal Clinic (n=62), or pregnant women with sensitizing events after 20 weeks of gestation (n=15) or pregnant women after spontaneous vaginal delivery. Women who were known to have Thalassaemia, were excluded. Three ml of maternal whole blood were collected into anticoagulated EDTA tube with prior consent. The samples were taken within two hours after spontaneous vaginal delivery, at any time during uneventful pregnancy between 28-32 weeks or whenever patient admitted to hospital after post-sensitizing event. The samples were mixed thoroughly and checked for haemolysis or blood clots. They were then sent to the laboratory and processed within four hours after

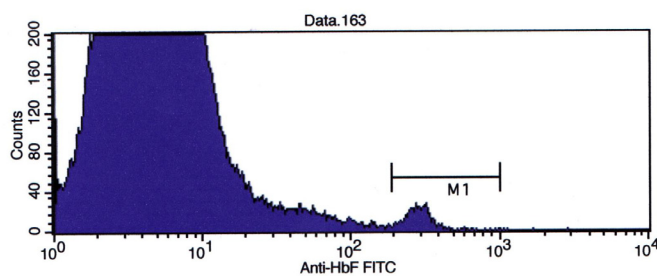


Figure 1: A representative histogram plot of an estimated 1% artificial mixture showing a population of foetal cells in adult cells.

collection. Haemolysed or clotted sample were rejected. The positive control sample was prepared in-house according to a documented protocol. The method described by the Members of the Scientific Subcommittee (SSC) of the Australian & New Zealand Society of Blood Transfusion (ANZSBT).

FLOW CYTOMETRY

In the flow cytometry method, the foetal red cells were identified using the monoclonal antibody against the HbF. The monoclonal anti-HbF used in this study was acquired commercially of Isotype IgG1, clone: WBAC HbF1 (Chemicon, USA). It was designed for the detection and quantitation of HbF positive RBC arising in the circulation of mothers as a consequence of foetomaternal haemorrhage (FMH). The format in the study used the purified immunoglobulin conjugated to FITC. SILENIUS@FITC conjugated anti-HbF was supplied in phosphate buffered saline, pH 7.2, containing 0.2% BSA and 0.1% Sodium Azide. To maintain its applicability, the monoclonal antibody was stored at 2-8°C in undiluted aliquots and is protected from sunlight. The methods for the cell suspension

and reagent preparations were performed and prepared according to the manufacturer's instruction. The reagents included the wash buffer, prefixation solution, permeabilization solution and fixing solution. All the reagents were freshly prepared for every batch of test run. If not analyzed immediately, the cell suspension preparation can be stored at 4°C in the dark for a maximum period of 24 hrs prior to analysis.

The cell suspension were then analyzed on a FAC Scan flow cytometer. For each test, 50,000 events gating were collected on the RBC population. A linear forward scatter versus linear side scatter gate was selected. The vertical scale of fluorescence intensity histograms were adjusted so that the small foetal-HbF region, the adult 'F' cells and the adult negative cells could be visualized. The foetal-HbF region was set using the positive control and marked as 'M'. The statistic analysis for the selected region was acquired and it showed the percentage foetal cells under the area marked as 'M' (Figure 1). The FMH was then calculated using the formula according to BCSH guidelines on the estimation of FMH (BCSH Blood

Transfusion and General Haematology Task Force 1999);

FMH (ml)=

$$\frac{\text{Area under the curve (M)} \times 2400}{100 - \text{Area under the curve (M)}}$$

To optimize the flow cytometer settings, two following standards were used; Mouse anti-IgG antibody was used and run in parallel with each sample tested to identify any non-specific antibody binding. A positive control sample which contained 1% of HbF (10ml of cord blood added to 990ml blood of a male person) was run in parallel with each batch of samples tested.

KELIHAUER'S TEST (ACID ELUTION TEST)

The thin blood films were prepared from the EDTA-anticoagulated samples from pregnant women. It was done by an experienced senior laboratory technician in accordance with the laboratories standard method and according to the local haematology laboratory standard operating procedure. The positive control slide was made from the smear of fresh cord blood to differentiate between adult and foetal cells. The control slides were performed with each batch of slides and treated in exactly the same way as the samples. Prior to the quantification of the FMH, screening of the control slides was performed to ensure that the staining were satisfactory. If not, the entire process was repeated and fresh new slides were obtained for the same sample.

The quantification of FMH was carried out using a light microscope under 400 x magnification. An area of the film where cells were touching but not overlapping, was selected. Any foetal cell was counted in the area. The slide was moved across in sequence so that the next counting was continued for a minimum of 6000 adult cells ie. 15 fields examined under x400 magnification, and the number of foetal cells were recorded. The FMH was calculated using the formula:

FMH (ml) =

$$\frac{\text{Number of Foetal cell (F)} \times 2400}{\text{Number of Adult cell (A)}}$$

LINEARITY STUDY

Spiked samples were prepared by adding a known percentage of foetal cells to adult male cells (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 1%, 5%). They were subjected to the FMH quantification by both methods (Figure 2-4). This test represented the linearity study experiment on both methods.

RESULTS

A total of 98 samples from pregnant women attended to Antenatal Clinic or admitted to UKMMC had quantification of FMH by flow cytometry with anti-HbF and Kleihauer test. The age of the patients are between 20 and 42 (median=30). None of them had any background medical problems. The following patients were included: women with uneventful pregnancy attending Antenatal Clinic (n=62), post spontaneous vaginal delivery (n=21)

and women admitted for antepartum haemorrhage or other sensitizing events (n=15).

LINEARITY STUDY

In the linearity experiment, the FMH estimated by the flow cytometer were plotted against the theoretical (known) estimated FMH (Figure 2). The results showed a correlation coefficient of $r = 0.992$. The FMH estimated by Kleihauer test plotted against the theoretical estimated FMH (Figure 3) showed a correlation coefficient of $r = 0.87$. The correlation study between the flow cytometer and the Kleihauer test for the FMH are shown in Figure 4. All the results of % foetal cells and the FMH were shown to be higher in the Kleihauer test compared to the theoretical estimation and the flow cytometry. The results of the linearity study showed a good correlation between the flow cytometry and the kleihauer test in estimating the FMH with the correlation coefficient of $r = 0.896$ respectively. All tests showed statistically significant results with p value less than 0.05 ($p < 0.05$)

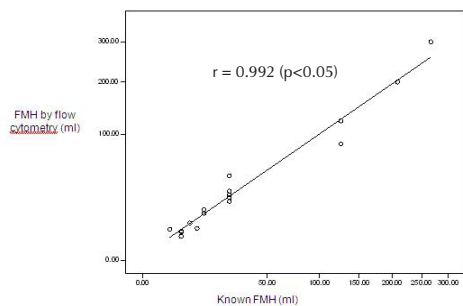


Figure 2: Linearity study showing the correlation graph between the FMH estimated by the flow cytometry (y-axis) and the theoretically estimated FMH (x-axis).

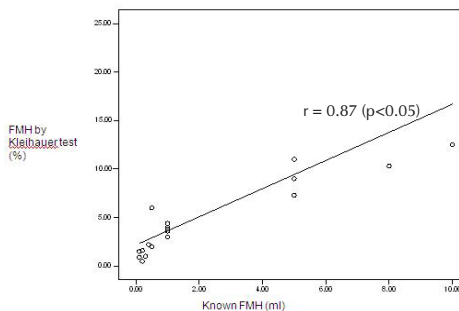


Figure 3: Linearity study showing the correlation graph between the FMH estimated by the Kleihauer's test (y-axis) and the theoretically estimated FMH (x-axis).

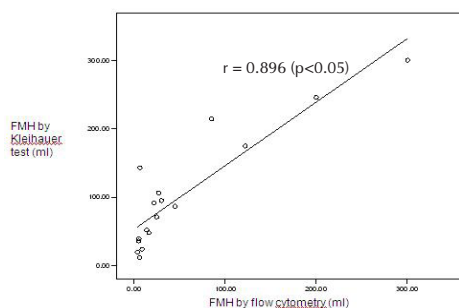


Figure 4: Linearity study showing the correlation graph between the FMH estimated by the flow cytometry (x-axis) and the Kleihauer's test (y-axis).

QUANTIFICATION OF FMH IN PREGNANT WOMEN

In this study, the results of the % foetal cells and the FMH from the patients' samples showed fair correlation between flow cytometric method and Kleihauer test with correlation coefficient of $r = 0.476$ and $r = 0.633$ ($p < 0.05$), respectively (Figure 5 and 6). Overall, the results from the patients' sample analysis of the FMH were shown to be higher by the flowcytometry in comparison to Kleihauer test. In 70 cases (71.4%), the size of FMH quantitated by flow cytometry were

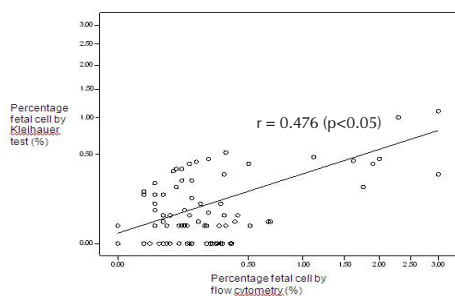


Figure 5: The correlation graph between the % foetal cells estimated by flow cytometry (x-axis) and the Kleihauer test (y-axis).

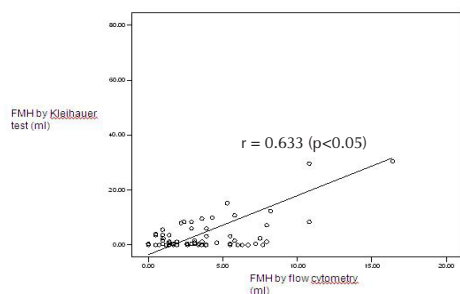


Figure 6: The correlation graph between the estimated FMH by flow cytometry (x-axis) and Kleihauer test (y-axis).

higher than that estimated by Kleihauer test. Only 27 cases (27.6%) showed that the FMH quantification by Kleihauer test were higher compared to flow cytometry. One case showed no foetal cell detected by both methods (1%). Twenty eight out of all cases (28.6%) were detected positive for fetal cells by flow cytometry method (0.06 to 5.5%) but was negative by the Kleihauer test. None of the results which were detected positive by the Kleihauer test were negative by the flow cytometer. These results, suggested that flow cytometer was more sensitive than the Kleihauer test in detecting the foetal cell. There were 23 (23.7%) cases in which flow

cytometry methods and the Kleihauer methods indicated a FMH of more than 4ml and 45 (46.4%) cases indicated FMH of less than 4ml, thus giving the concordance rate of 70.1%. A further 53 (54.6%) cases were associated with at least one method giving a result to suggest a bleed of more than 4ml. Twenty one (21.6%) cases showed FMH more than 4 ml when estimated by flow cytometer method but less than 4ml by Kleihauer test. Only 9 (9.3%) cases with FMH more than 4ml estimated by Kleihauer test were estimated less than 4ml by the flow cytometer method. Analysis of all the results showed a fair correlation between the two methods for assessing the size of FMH and indicated that flow cytometry testing generally showed larger amount of FMH compared to Kleihauer test. However, flow cytometer seemed to be more sensitive in detecting the significant FMH of >4ml.

DISCUSSION

FMH estimation by flowcytometric technique can be done using monoclonal anti-HbF or monoclonal anti-D. The quantification by monoclonal anti-HbF has the advantage that it can be achieved irrespective of the blood groups of the mother and infants therefore enabling a flow cytometric method independently of the Rh antigens. Furthermore, the presence of maternal F cells did not interfere with the estimation since the intensity of staining is usually less than that of foetal cells. In the Kleihauer technique, there was no difference between an adult F cell and a true foetal cell. The

addition of a calculated percentage of foetal cells from cord blood cells is used to help in identifying the cut-off level. Nevertheless, it is known that only 95% of foetal cells stain with Kleihauer and a small percentage do not. Flowcytometric method on the other hand differentiates the adult F cell and a true foetal cells by virtue of their intensity in staining of the HbF.

The current local policy for anti-D prophylaxis dosage is 500 IU (200 mg) of anti-D which can theoretically neutralize 4 ml of foetal cells. Additional dosage of anti-D is given according to the FMH estimated by the Kleihauer test. It is very crucial to estimate FMH accurately because underestimation of the FMH can be dangerous as a lower dosage of anti-D would be given to such cases therefore, would not be sufficient to clear the foetal cells from the maternal circulation. On the other hand, overestimation of the FMH may result in giving a higher dosage of anti-D to such cases and would not be cost-effective.

In the present study, the linearity study results showed very good correlation between the known FMH and the results obtained by flowcytometric method and the Kleihauer's test. There was also good correlation with the % foetal cells and FMH results obtained by flow cytometry and Kleihauer's test. However, all the results of the % foetal cells and the FMH from the Kleihauer test showed higher values than the flow cytometer and the estimated theoretical % foetal cells. The results of flow cytometric method on the other hand showed, concordance results to the estimated theoretical values. Hence,

we believe that the flow cytometer is a more accurate method to estimate the FMH than the Kleihauer test.

Duckett & Constantine in (1997) had shown that a large inter-observer and inter-hospital variation in interpreting Kleihauer test slides where there was agreement in only 46% of cases. Janssen & Hoffmann (2002) suggested that the quantification of foetal red cells by flow cytometry with monoclonal anti-HbF to be highly accurate based on their linearity study.

In our study, the results showed that there was a fair correlation between the % foetal cells and FMH results obtained by flow cytometry and Kleihauer test, $r = 0.633$ ($p < 0.05$). Previous studies done by different authors in observing the correlation between the two methods in quantitating the FMH have shown that there is good correlation between the two methods with the majority obtained a correlation coefficient $r > 0.8$ ($p < 0.05$). Furthermore, our results, showed that in general, the flow cytometer gave higher results than the Kleihauer test. Other previous studies (Kennedy et al. 2003) on the other hand, showed that the Kleihauer test overestimates the FMH compared to the flow cytometry by using both anti-HbF and anti-D. However, they also found that in samples containing $< 0.6\%$ foetal cells, no significant difference in the detection of foetal cells between anti-HbF and anti-D ($P = 0.11$) but in samples containing $> 1\%$ foetal cells, anti HbF significantly underestimates the percentage of foetal cells ($P = 0.0001$). However, we were unable to determine from our findings whether the flow cytometry overestimate the

FMH or as opposed whether the Kleihauer test underestimates the FMH. The conclusion may be determined by the use of another third outcome or a 'gold standard' for the estimation of FMH. This 'gold standard' has not been established yet. We also found in our study that some of the cases which were positive for foetal cells detected by flow cytometry were detected as negative by the Kleihauer test. None of the cases which were positive for the Kleihauer test were detected as negative by the flow cytometry method. These findings suggest that the flow cytometry is more sensitive in detecting the foetal cells.

The causes of the difference in the results from previous studies using flow cytometry have been suggested by some authors. The flow cytometry methodology with different reagents, the use of PE-conjugated anti-HbF instead of FITC-conjugated anti Hb-F, the use of PBS azide for washes and resuspension instead of PBS with bovine serum albumin, and a longer incubation time with the Triton-X reagent (8 mins instead of 3-5 mins). Another factor to be considered is the contamination by the white cell which can exhibit autofluorescence and may cause an overestimation of the FMH as demonstrated by Janssen & Hoffman (2002). Several authors have suggested the use of monoclonal anti-CD45 or propidium iodide, a fluorescent dye specific for DNA to check for the contamination. Previous studies have mentioned various differences in the methodology that may cause the different results by flow cytometry. Johnson et al. (1995) and Llyod-Evans et al. (1999) have shown that monoclonal

anti-D is more accurate compared with anti-HbF in detecting foetal cells and quantification of FMH. The use of phycoerythrin (PE) or fluorescein (FITC) as fluorescence dye may also cause different results. Few authors have suggested that the use of PBS azide rather than PBS with bovine serum albumin and longer incubation with Triton-X could contribute to the difference in the results. However, so far, there has been no individual study to look specifically into this matter. White cell autofluorescence can give false positive results. The use of double labeling with phycoerythrin conjugated anti-glycophorin A monoclonal antibody rather than single labeling with either anti-HbF or anti-D can improve the accuracy of FMH quantification (Llyod-Evans et al. 1999). The use of monoclonal anti-CD45 and propidium iodide to check for contamination of nucleated cells have also been suggested and proved to improve the accuracy (Janssen & Hoffman 2002).

In our study, we used single labeling technique with FITC conjugated anti Hb-F, PBS azide during washing procedure and adopted the 3-5 minutes incubation time with Triton-X (permeabilizing solution) as recommended by the manufacturer. We did not employ double labeling to exclude white cell contamination. These different in the methodology may have contributed to our findings. We therefore, recommend to use a double labeling rather than single labeling of the monoclonal antibody in order to improve the accuracy in the quantification of FMH. We also would like to further evaluate and compare

the use of monoclonal anti-HbF and monoclonal anti-D in the evaluation of FMH.

CONCLUSION

In conclusion, our study showed a fair correlation between the FMH quantification by flow cytometric method and the Kleihauer test. In addition, the linearity study showed that the flow cytometer is very accurate in detecting the foetal cells thus estimating the FMH.

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