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# On fetomaternal hemorrhage

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# Quantification of fetomaternal hemorrhage: a comparative study of the manual and automated microscopic Kleihauer-Betke tests and flow cytometry in clinical samples

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## Abstract

***Objective:*** Quantification of fetomaternal hemorrhage (FMH) in 44 patients at risk by the manual and automated microscopic analysis of Kleihauer-Betke stained slides and by flow cytometry.

***Methods:*** Blood smears were stained and evaluated manually according to the Kleihauer-Betke test (KBT). The same slides were used for automated microscopy. In addition, flow cytometry using anti-HbF immunostaining was performed.

***Results:*** FMH>0.1% was detected in 4 patients by manual and automated KBT and by flow cytometry. FMH was absent according to all three methods in 13 patients, whereas in 27 patients FMH<0.1% was detected either by manual or automated KBT or both. Moderate agreement was observed between the manual and automated KBT (weighted $\kappa$=0.56, 95% CI 0.33–0.78). Agreement between the manual KBT and flow cytometry was fair (weighted $\kappa$=0.40, 95% CI 0.15–0.66).

***Conclusions:*** Automated microscopic detection of fetal cells in clinical samples provided accurate quantification comparable to the manual KBT, both in small and large FMH. Flow cytometry was only capable of quantifying FMH>0.1%.

## Introduction

After exposure to fetal Rh D positive red cells during pregnancy and delivery, Rh D negative women are at risk of alloimmunization. In subsequent pregnancies, red cell alloimmunization may lead to severe fetal anemia, hydrops fetalis, heart failure and even fetal death. The routine administration of anti-D immunoglobulin to Rh D negative women after delivery of a Rh D positive infant, has decreased the risk of alloimmunization to approximately 2%.[1] Antenatal prophylaxis has reduced the risk even further to 0.2%.[2] However, despite well-organized prophylaxis programs, Rh D alloimmunization continues to occur as a serious complication of pregnancy. Reliable detection and quantification of fetal red cells in maternal blood, therefore, is important for the assessment of fetomaternal hemorrhage (FMH) in Rh D negative patients. In addition, a reliable diagnostic test is needed for Rh D positive patients suspected of FMH. Both in normal and pathological conditions, such as placental abruption or abdominal trauma, significant FMH may occur.[3,4] Also, invasive diagnostic tests and other obstetrical interventions are known for their potential risk of FMH.[5] Even in uncomplicated pregnancy and delivery, small amounts of fetal cells cross the placental barrier and can be detected in maternal blood.[6]

To prevent alloimmunization in Rh D negative patients, an appropriate amount of anti-D immunoglobulin has to be administered.[5] The frequently used standard dose of 300 μg anti-D immunoglobulin is sufficient to clear 15 ml of Rh D positive cells (30 ml of whole blood).[7] When the FMH volume is larger than 15 ml Rh D positive cells, additional doses of anti-D immunoglobulin are required.

The Kleihauer-Betke test (KBT), based on resistance of fetal hemoglobin (HbF) to acid elution, is widely used to determine the FMH volume.[8] Although this method has proven to be clinically useful in the detection of fetal red cells in maternal blood, some studies showed an unacceptable high inter-observer and inter-laboratory variability.[9-12] Over the last ten years several studies reported the use of flow cytometric assays for the quantification of fetal red cells in adult blood using polyclonal antibodies to human D surface antigen and monoclonal antibody to HbF. Flow cytometry (FCM) demonstrated high sensitivity and statistical accuracy in the detection and quantification of substantial FMH.[9,10,12-15] Recently, we developed an automated microscopic approach for the quantification of fetal red cells in artificially spiked adult samples of Kleihauer-Betke stained slides, to improve the accuracy and objectivity of the standard KBT.[16]

In the present study we compared the manual and automated microscopic analysis of Kleihauer-Betke stained slides and FCM using monoclonal anti-HbF in unselected clinical samples of patients at risk of FMH. Our main target was to investigate the quantitative performance of all three methods for FMH ranging from

very small to large volumes. Secondly, our objective was to provide arguments for a more reliable strategy for the administration of anti-D immunoglobulin, based on an accurate technique for the quantification of especially small FMH. For patients with <0.1% FMH, the dose of anti-D immunoglobulin could be adjusted taking a margin of safety into account, thereby reducing both costs and risk of viral transmission.

## Material and methods

### *Samples*

Blood samples from 44 patients, admitted to our obstetrical department and at risk of FMH, were collected in EDTA vacutainer tubes (Becton Dickinson, Ruthford, NJ). FMH detection was indicated either because of Rh D incompatibility or used as a diagnostic tool for the assessment of FMH. Samples were stored at 4 °C and processed within 48 hours of collection. ABO and Rh type matched umbilical cord blood and adult blood from healthy non-pregnant volunteers was collected in EDTA vacutainer tubes for the preparation of control samples. After washing with phosphate-buffered saline (PBS), artificial dilutions consisting of 0%, 0.0001%, 0.001%, 0.01%, 0.1%, 1%, 5% and 10% fetal red cells in adult blood were prepared and analyzed by manual KBT, automated microscopy and FCM.

### *Kleihauer-Betke test*

#### Preparation of the slides

Hundred µl of each sample was mixed with 100 µl PBS. Conventional blood smears were prepared on glass slides using 2.5 µl of this diluted solution. Fixation of the cells and elution of HbA were performed as described previously.[8,17] The slides were air-dried for 30 minutes at room temperature and fixed in 80% ethanol for 5 minutes. After rinsing with tap water, they were placed in citrate buffer at 37 °C for exactly 5 minutes and rinsed again. The citrate buffer consisted of 750 ml 0.1 M $C_6H_8O_7$. $H_2O$ and 250 ml 0.2 M $Na_2HPO_4.2H_2O$; pH 3.20 to 3.30 at 37 °C. The slides were counterstained with Papanicolaou dye (hematoxylin solution, Merck Diagnostica, Darmstadt, Germany) for 3 minutes at room temperature and rinsed again in tap water. Finally, the slides were placed in Sørensen buffer for 5 minutes and stained with erythrosin B (Merck Diagnostica, Darmstadt, Germany), for another 5 minutes at room temperature. The Sørensen buffer consisted of 400 ml 0.067 M $Na_2HPO_4.2H_2O$, 340 ml 0.067 M $KH_2PO_4$ and 260 ml distilled water. The pH of this solution was 6.9 at room temperature. After rinsing with tap water, the slides were air-dried at room temperature.

Manual evaluation of Kleihauer-Betke stained slides

Fetal red cells were counted in 400 subsequent microscopic fields using a 40x objective.[17] Adult red blood cells, containing small amounts of HbF, were distinguished from fetal red cells by intensity and intracellular distribution of the pink staining. Only bright pink stained cells were classified as fetal. The fetal red cell percentage was calculated from the proportion of the detected number of fetal red cells and the estimated total number of background red cells in 400 fields.

Automated microscopic analysis of Kleihauer-Betke stained slides [16]

After manual counting, the same slides were embedded in Fluoromount embedding medium (Gurr, BDH Limited Poole, UK) and sealed with a cover glass. An automated microscope (Olympus BX-60), equipped with a scanning stage (Maerzhauser Co, Wetzlar, Germany) and a MDS 1 image analysis system (Applied Imaging Corporation, Santa Clara, CA) was used. At least 500 low power fields (10x objective) were scanned with a two-step filter-switch method to identify fetal red cells by bright field analysis. A green filter was applied to select all pink stained cells and a red filter to identify and exclude the hematoxylin counterstained white blood cells. Optimal software settings for detection and analysis were defined on a set of test samples.

Automatically detected cells were stored in a database and relocated for direct microscopic verification. Only bright pink cells were selected as fetal. The fetal red cell percentage was calculated from the proportion of the detected number of fetal red cells to the estimated total number of background red cells (at least 1,000,000).

***Flow cytometry***

Staining protocol

The red blood cell (RBC) count of each patient sample was determined on a Sysmex K-1000 cell counter (Baxter Scientific, Chicago, IL) and adjusted to a final concentration of 2.5 x $10^6$/μl with PBS. Approximately 2.5 x $10^7$ red cells (10 μl) were fixed in 1 ml freshly prepared, cold 0.05% glutaraldehyde in PBS, thoroughly mixed, and incubated at room temperature for 10 minutes. The samples were washed 3 times using 2 ml PBS supplemented with 1% bovine serum albumin (PBS/1% BSA). The cells were resuspended and permeabilized in 0.5 ml 0.1% Triton X-100 (Sigma, St Louis, MO) in PBS/1% BSA at room temperature for exactly 4 minutes, and then washed with 2 ml PBS/1% BSA. The cell pellet was resuspended in 0.25 ml PBS/1% BSA. Five μl of this suspension was incubated with 5 μl of fluorescein isothiocyanate (FITC)-conjugated anti-HbF antibody (Caltag, Burlingame, CA) and 35 μl PBS/1% BSA in the dark for 15 minutes at room temperature. The cells were washed twice in PBS/1% BSA and resuspended in 0.25 ml PBS/1% BSA. Flow cytometric analysis was performed within 4 hours. Control samples were included with each run.

Flow cytometric analysis
A FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) was used for the flow cytometric analysis. Sample acquisition was performed on a minimum of 100,000 cells, with collection of measures for forward scatter (FSC) and side scatter (SSC), log green fluorescence (525-nm band pass filter), and autofluorescence using log orange fluorescence (575-nm band pass filter). A light scatter threshold was set to exclude nonspecific signals from buffer contaminants and platelets. Data analysis was performed with CellQuest (Becton Dickinson, San Jose, CA). The region for analysis of fetal red cells was determined using a positive control sample. The artificial dilutions of cord blood in adult blood were used to fine-tune the flow cytometric assay with respect to gate setting and amplification.

***Statistical analysis***
Correlation between results from patient and control samples, measured by the manual KBT, automated microscopy and FCM was performed using linear regression and Pearson's correlation coefficient (r) both after log transformation (SPSS 10.0 for windows, SPSS Inc., Chicago, IL). A coefficient of variation for each method was calculated by the performance of 5 replicate determinations of the control samples (Excel2000, Microsoft Inc., Redmond, WA). To assess agreement between methods in patient samples a weighted kappa ($\kappa$) was calculated (SAS, SAS Inc, Cary, NC). The value of $\kappa$ was assigned a degree of agreement as defined by cited literature.[18]

## Results

***Patient samples***
Blood samples from 44 patients between 25 and 42 weeks of gestation, admitted to the Department of Obstetrics of the Leiden University Medical Center, were analyzed. The medical records of all patients and, when available, pathological reports were reviewed. Patients' characteristics are given in table 1. Substantial FMH, as detected by all three methods, occurred in 4 patients. One patient with obstructed labor underwent a ventouse-assisted delivery and fundal pressure. Another patient had a caesarean section, complicated by a difficult removal of the placenta. The third patient presented with an antepartum fetal death at term. Pathological investigation of the placenta revealed an intra-placental choriocarcinoma. The fourth patient had a placental abruption. A caesarean section was performed immediately. The infant was anemic (hemoglobin 3.0 g/dl) at birth, but recovered after a blood transfusion.

**Table 1 -** FMH grouped by obstetrical diagnosis in 44 patients.

| | | FMH | | |
|---|---|---|---|---|
| Diagnosis | n | 0%* | <0.1%** | >0.1%* |
| abdominal trauma | 9 | 3 | 6 | 0 |
| vaginal bleeding: | | | | |
| marginal placenta previa | 2 | 1 | 1 | 0 |
| preterm labor | 2 | 1 | 1 | 0 |
| unknown | 6 | 4 | 2 | 0 |
| antepartum fetal death: | | | | |
| choriocarcinoma | 1 | 0 | 0 | 1 |
| placental infarct | 1 | 0 | 1 | 0 |
| chorionic vein thrombosis | 1 | 0 | 1 | 0 |
| unknown | 2 | 1 | 1 | 0 |
| complicated delivery (fundal pressure) | 2 | 0 | 1 | 1 |
| manual removal of the placenta | 2 | 0 | 2 | 0 |
| placental abruption | 3 | 0 | 2 | 1 |
| caesarean section: | | | | |
| breech presentation | 3 | 1 | 2 | 0 |
| placenta previa | 2 | 1 | 1 | 0 |
| monochorionic twin pregnancy | 2 | 0 | 1 | 1[#] |
| dichorionic twin pregnancy | 1 | 0 | 1 | 0 |
| intra-uterine growth restriction | 1 | 0 | 1 | 0 |
| neonatal anemia: | | | | |
| monochorionic twin pregnancy | 1 | 0 | 1 | 0 |
| ventouse assisted delivery | 1 | 0 | 1 | 0 |
| unknown | 2 | 1 | 1 | 0 |
| Total | 44 | 13 | 27 | 4 |

* FMH detected by manual KBT, automated microscopy and flow cytometry

** FMH detected by manual KBT or automated microscopy only

[#] complicated by difficult removal of the placenta

### *Distribution of fetal cell percentages between methods*

A comparison of the results from manual KBT, automated microscopy and FCM, divided into the categories 0%, <0.1% and >0.1% FMH, is given in table 2. All three methods were negative in 13 patients. FMH varying from 0.0001% to 0.1% was found in 27 patients either by automated microscopy (n=10) or manual KBT (n=3) or both methods (n=14). In these patients, FCM was unable to distinguish the signal of fetal cells from the background of HbF containing adult cells. In 4 patients FCM and both manual KBT and automated microscopy detected a FMH >0.1%, as shown in table 3. The FMH volume was calculated assuming a red cell volume of 1800 ml in pregnant women.[19] FCM tended to detect slightly higher fetal red cell percentages than the

manual KBT and automated microscopy. In one patient diagnosed with a placental abruption, FCM detected a substantially higher fetal red cell percentage than the manual and automated KBT.

***Agreement and correlation between methods***

The correlation between the expected and detected fetal red cell percentage measured in control samples by manual KBT, automated microscopy and FCM was good (r= 0.99, 0.99 and 0.96). The coefficient of variation for each method studied in 5 replicate determinations was less than 5% in the majority of control samples, except for the 0.0001% dilution the coefficient of variation was 14%. Calculations of agreement between methods in patient samples were based on the results given in table 2. Moderate agreement was observed between automated microscopy and the manual KBT (weighted $\kappa$=0.56, 95% CI 0.33–0.78). A comparison of the fetal red cell percentage measured by the manual KBT and automated microscopy after log transformation is illustrated in figure 1 (r=0.77). Agreement between FCM and the manual KBT was fair (weighted $\kappa$=0.40, 95% CI 0.15–0.66) and correlation (r) was 0.69.

**Table 2 -** A comparison of the results from manual KBT, automated microscopy and flow cytometry in 44 patient samples.

| | automated microscopy | | | flow cytometry | |
|---|---|---|---|---|---|
| manual KBT | 0% | <0.1% | >0.1% | 0%* | >0.1% |
| 0% | 13 | 10 | 0 | 23 | 0 |
| <0.1% | 3 | 14 | 0 | 17 | 0 |
| >0.1% | 0 | 0 | 4 | 0 | 4 |
| total | 16 | 24 | 4 | 40 | 4 |

* the cut-off value for a positive flow cytometric test result was ≥0.1%

**Table 3 -** A comparison of the fetal red cell percentage (%) and the FMH volume (ml) measured by manual KBT, automated microscopy and flow cytometry in 4 patients with substantial FMH.

| | manual KBT | | aut. microscopy | | flow cytometry | |
|---|---|---|---|---|---|---|
| sample | % | (ml)* | % | (ml)* | % | (ml)* |
| No. 1 (complicated delivery) | 0.27 | (4.9) | 0.25 | (4.5) | 0.3 | (5.4) |
| No. 2 (caesarean section) | 4.2 | (76) | 4.0 | (72) | 5.0 | (90) |
| No. 3 (choriocarcinoma) | 7.3 | (131) | 7.7 | (139) | 8.3 | (149) |
| No. 4 (placental abruption) | 7.0 | (126) | 8.0 | (144) | 13.0 | (234) |

* The volume of FMH (ml) was calculated, assuming a maternal red cell volume of 1800 ml

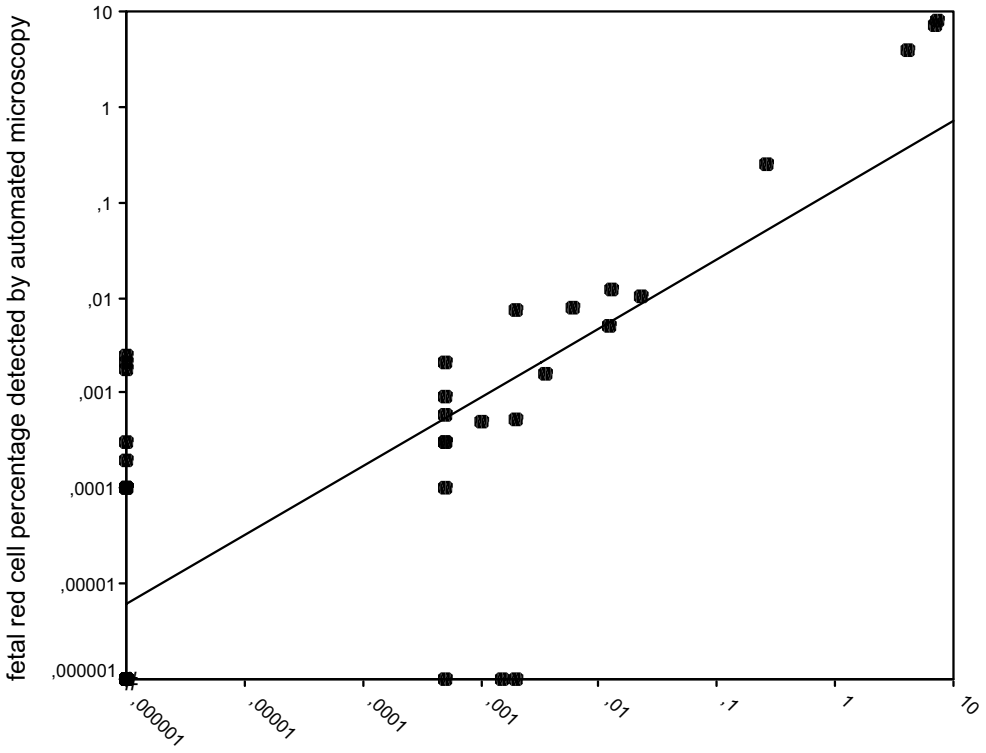


**Figure 1 -** A linearity study of the fetal cell percentage measured by manual KBT and automated microscopy in 44 patient samples (log y= -0.878+0.723log(x), r=0.77). The zero values were transformed to 0.00001% for the purpose of a logarithmic expression (#).

## Discussion

In the present study we quantified FMH in a high-risk patient group by manual and automated microscopic analyses of Kleihauer-Betke stained slides and by FCM. Moderate agreement was found between the manual and automated KBT, due to the differences between these methods in the smaller ranges of FMH. Fair agreement was observed between the manual KBT and FCM, mainly because of the insensitivity of FCM to detect FMH <0.1%.

In the majority of patients a FMH between 0% and 0.1% was detected. Large FMH was diagnosed in 4 high-risk obstetrical cases. In three of these patients with FMH ranging from 0.3 to 8.3% quantification was almost identical with the three methods. In one patient FCM detected a FMH of 13%, while both the manual and

automated KBT resulted in 7%. The implausible high level of FMH, as estimated by FCM, may be explained by a high maternal F cell percentage, which could not be discriminated from the fetal cell population, leading to an overestimation by FCM.

In a recent publication on the relation between third trimester bleeding and the occurrence of FMH >0.01% measured by the KBT, no significant increase of FMH was found compared to non-complicated pregnancies.[20] A FMH >0.01% was found in 4 out of 91 cases with vaginal bleeding. In our study, we detected a FMH between 0.001% and 0.002% by manual and automated KBT in 3 out of 10 pregnancies complicated by vaginal bleeding without signs of placental abruption. Vaginal bleeding due to placental abruption was diagnosed in 3 patients. One of these patients had a substantial FMH and in the other two patients a very small FMH was detected.

Over the past years a large proportion of the hospitals have abandoned the traditional KBT and now use flow cytometric assays for the quantification of FMH. This change is due to presumed unreliability of the KBT, mainly as a consequence of modifications of the test and analysis of an insufficient number of microscopic fields, leading to large inter-observer and inter-laboratory variability.[11,12] Publications on the performance of FCM using anti-HbF or anti-D have shown that FCM is capable of quantifying only FMH ≥0.1% with accuracy.[9,10,12,13,15] The relation between the manual KBT and flow cytometric assays strongly depends on the KBT method used. Both underestimation[9] and overestimation[10,15] of the fetal cell percentage by the KBT are reported. A study on the quantitative performance of the KBT, fluorescence microscopy and FCM with anti-D immunostaining in artificially spiked samples, demonstrated that the KBT is inappropriate in quantifying FMH in the range of 0 to 1% fetal cells, while fluorescence microscopy and FCM were accurate.[21] These findings are in contrast to our study of clinical samples, where FMH <0.1% was detected by the manual and automated KBT and not by FCM.

The reported differences may partially be explained by the fact that statistical precision is related to the number of fields evaluated and the varying thickness of the blood smear. If recommendations are followed and a sufficient number of microscopic fields is counted using a 40x objective, then, in our opinion even inexperienced laboratory staff can perform the KBT.[17]

Recently, we have shown that automated microscopic analysis of Kleihauer-Betke slides demonstrates high reproducibility, very small inter-observer and intra-observer variability and good correlation with the manual KBT.[16] The automated microscopic procedure, as performed in the present study, is capable of detecting both small and substantial FMH. In the very small range of FMH we found fetal red cells in the maternal circulation varying from 0.0001% to 0.1% in 27 patients either by automated microscopy or manual KBT or both.

There is consensus on the importance of diagnosing large FMH in clinically indicated cases such as severe fetal anemia and for the appropriate dosing of anti-D immunoglobulin. Accurate quantification of small FMH is particularly important, considering the fact that there is a dose-dependent relation between the volume of Rh D positive red blood cells to which a Rh D negative person is exposed and the incidence of Rh D alloimmunization, with volumes as small as 0.1 ml or 0.006% red cells resulting in antibody formation.[22] In addition, very small amounts of FMH in pregnancy may evoke sensibilization, which might result in detectable antibody formation in a subsequent pregnancy.[19] Consequently, the FMH volume is an important consideration in the risk of sensitization of Rh D negative women. Therefore, an accurate and standardized method capable of detecting <0.1% fetal red cells in the maternal circulation is needed. To overcome laboratory and observer variability, we have studied the application of an automated readout of manually stained Kleihauer-Betke slides. The software used for the automated analysis was primarily developed to show “proof of principle”.[16] After an automated, highly standardized Kleihauer-Betke staining procedure, algorithms for further fine-tuning of the analysis software may ultimately result in an accurate and highly sensitive procedure.

Despite all efforts, the estimation of the total maternal blood volume, will remain an unreliable factor in calculating the FMH volume. As recommended, a maternal red cell volume of 1800 ml is used for the calculation of the FMH volume.[3,19] However, the true red cell volume in pregnant women depends on biological and pathophysiological factors such as gestational age and the individual hematocrite level.

Although our study contains a small number of cases, the results indicate that the dosage of anti-D immunoglobulin might be further fine-tuned, especially for patients with FMH <0.1%. In our opinion, anti-D immunoglobulin should be restricted to those patients who need it. The administration of a relatively large dose of 300 μg to all Rh D negative patients who are at risk of alloimmunization after sensitizing events, may eventually lead to a future shortage of anti-D immunoglobulin, obtained from volunteers with high circulating antibody levels. In addition, considering cost-effectiveness and the fact that anti-D immunoglobulin is a blood product with a small potential risk of viral transmission (e.g. prion disease), one could discuss another strategy. With the application of a method more reliable in quantifying smaller volumes of FMH, the anti-D immunoglobulin dose could be adjusted to the detected FMH percentage including a margin of safety. The automated analysis of Kleihauer-Betke stained slides, which is not influenced by individual observers, is the preferable procedure for the quantification of FMH.

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