Original Article

Evaluation of FMH QuikQuant for the Detection and Quantification of Fetomaternal Hemorrhage

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Background: The Kleihauer–Betke test (KBT) is the most widely used assay for fetomaternal hemorrhage (FMH) detection in rhesus D negative women. In the current study, we sought to evaluate the performance of a flow cytometry (FCM) kit (FMH QuikQuant) using an anti-HbF antibody.

Methods: Eighty-three pregnant women, 58 umbilical cord blood (UCB) dilutions in adult blood, and 6 control samples were tested in parallel with FCM and KBT.

Results: Firstly, we compared for each assay, on the 58 UCB preparations, results obtained to dilutions prepared. FCM results showed an excellent correlation (r = 0.97), a high reproducibility with a coefficient of variation lower than 20% for values reaching 10 fetal RBCs. KBT values were correlated (r = 0.94) but exhibited a poor reproducibility. Then, we compared both techniques on all samples. FCM showed a good correlation with KBT (r = 0.87) but the KBT exhibited a systematic overestimation of the FMH. For 8 out of 83 pregnant women, KBT was positive. Five were concordant with FCM results (KBT+/FCM+). On the three discordant (KBT+/FCM−), 2 were finally classified as false positive of the KBT because a second control sample was negative and additionally, FCM identified an increased rate of F cells. One discordant case (KBT+/FCM−) remained unexplained. By receiver operating characteristic analysis, we found a threshold at 4.5 RBCs for FCM with a sensitivity of 89.8% and a specificity of 93.2%.

Conclusions: The FMH QuikQuant kit is a reliable and highly reproducible FCM method for FMH quantification. © 2012 International Clinical Cytometry Society

Key terms: flow cytometry; fetomaternal hemorrhage; F cells; anti-HbF antibody

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Correct detection and quantification of fetomaternal hemorrhage (FMH) is critical for the obstetrical management of Rhesus D (RhD) negative pregnant women. The amount of fetal red blood cells (RBCs) in the maternal circulation determines the therapeutic dose of anti-RhD immunoglobulin necessary to prevent alloimmunization and FMH occurrence (1). Besides, FMH testing contributes to the management of severe situations such as decreased fetal activity, neonatal anemia, or unexplained stillbirth (2).

The Kleihauer–Betke test (KBT) is the most widely used approach for FMH detection. This test is based on the visual microscopic counting of fetal RBCs on a maternal blood film. In acid conditions, fetal and adult hemoglobin have differences in solubility properties. Hemoglobin F (HbF) resists to acid elution and fetal RBCs are stained in bright pink, while hemoglobin is eluted from adult RBCs that appear as ghost cells (3). Nevertheless, the KBT is labor-intensive and inaccurate, theoretically leading to inappropriate dose of anti-RhD

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immunoglobulin (I). This lack of precision is in part due to the subjective identification of adult cells with increased content of HbF, also called F cells, physiologically increased during pregnancy (5). F cells appear as light pink RBCs and it remains challenging to distinguish them from fetal RBCs, leading to false-positive KBT results (6). Automatic detection of fetal cells on blood films stained with K8 method was reported to be more precise than KBT decreasing especially the interobserver variation (7).

Flow cytometry (FCM) is a candidate method for FMH quantification. Indeed, FCM assays exhibit a better reproducibility and a more reliable quantification of fetal RBCs than KBT (8). Initially, this FCM method used surface RBC antigens, particularly with anti-RhD antibodies (9–11). However, such strategy is limited to the determination of anti-RhD immunoglobulin dose, as it cannot distinguish fetal RBCs from adult cells in cases of RhD compatibility. In the last decade, FCM approaches using intracellular detection of HbF have been developed (4,12–17). Monoclonal anti-HbF antibody allows discrimination of three distinct populations: fetal RBCs, F cells, and adult RBCs (12,14). The quantification of F cells provided by FCM eliminates a major drawback of KBT. In this context, FMH QuikQuant is a new CE marked kit including a monoclonal anti-HbF antibody and propidium iodoide (PI) as a specific marker of nucleated cells (18,19).

Our study sought to evaluate the performance of the FMH QuikQuant kit in combination with the FCM analyzer FC500 (Beckman Coulter). The analysis of artificial mixtures containing from 1 to 100 fetal RBCs per 10,000 adult RBCs together with the investigation of pregnant women samples allowed us to validate this FCM kit as a reliable and efficient method to screen for FMH disease.

DESIGN AND METHODS
Patient Samples and Study Design

The KBT is routinely performed in our laboratory to detect fetal RBCs after explicit request from the obstetric unit. Residual blood samples following a KBT analysis were prospectively included according to institutional review board and national guidelines. All specimens anticoagulated with K2 Ethylenediaminetetraacetic acid (EDTA), were stored at 4°C and tested with the FMH QuikQuant kit (Trillium Diagnostic, Bangor, ME) within 48 h after blood collection. The age of the patient and clinical conditions were collected from each case.

Otherwise, positive control samples were prepared with dilutions of fetal RBCs in adult RBCs (1/10,000, 1/2,000, 1/1,000, 1/500, and 1/100) and tested in duplicate from 1 to 100 fetal RBCs per 10,000 adult RBCs. Two slides were immediately prepared for KBT and 10 µL were used for FCM analysis.

In parallel, samples from healthy RhD negative donors from the National Blood Bank (Etablissement Français du Sang, Rennes, France) were included to establish a reference value of F cells in the non-pregnant population. Finally, FETALLrol (Trillium Diagnostic), a stabilized three-level control set for FMH assay was used.

Kleihauer–Betke Test

Two blood films from each pregnant woman or umbilical cord blood (UCB) samples were fixed in 80% of ethanol for 5 min. Fixed newborn blood films served as positive control for this technique. Maternal hemoglobin A was removed by an acid elution in hematoxylin solution during 1 min 30 sec while HbF resisted to the elution. Then, slides were stained with eosin 0.5% for 2 min before microscopic (×500) reading by two independent microscopists. Adult cells appeared as faint ghosts whereas fetal cells were colored in dark pink. The number of fetal RBCs was counted on 25 fields (10,000 total RBCs). Dim pink cells were considered as adult F cells. KBT were reported as positive when the count of fetal RBCs were higher than 8 per 10,000 adult cells (2,20).

Flow Cytometry Analysis

We used the FMH QuikQuant kit following the recommendations of the manufacturer. Briefly, 10 µL of diluted EDTA anticoagulated blood (1/20 in QuikQuant buffer) was added to 0.75 mL of glutaraldehyde (0.04% in phosphate buffered saline (PBS)) and incubated for 5 min at room temperature (RT). Then, 1.5 mL of permeabilization solution Trillium Intra-Cell was added and incubated for 10 min at RT before a 5 min centrifugation (600g at 4°C). After decanting the supernantant, 25 µL of QuikQuan antibody reagent was added and incubated for 10 min in the dark. This reagent contains fluorescein isothiocyanate labeled monoclonal anti-HbF and PI. Finally, after two washing steps with the QuikQuant buffer, stained cells were analyzed on a flow cytometer FC500 (Beckman Coulter, Miami, FL). About 100,000 RBCs were analyzed with Kaluza software (Beckman Coulter) as described in Figure 1. Firstly, nucleated cells were excluded by PI. Then fetal RBCs were detected as expressing bright anti-HbF fluorescence, as guided by the FETALtrol high control with 1.00% of fetal RBC. Further samples were analyzed without modification of this positive gate. Lastly, after exclusion of fetal RBCs with the boolean (RBCs) AND NOT [fetal RBCs], F cells were discriminated from adult RBCs on a monoparametric anti-HbF histogram (Fig. 1). The following equation was used to report the results: fetal RBCs = [fetal RBCs]/([RBCs] – [fetal RBCs]) × 10,000 and F cells = [F cells]/[RBCs] × 100 (in %).

Statistical Analysis

Standard statistical methods, included Spearman correlation test and Bland–Altman test were used for data analysis using GraphPad 5.0 (Prism Software). Non-parametric continuous variables were compared with Mann and Whitney test. Receiver operating characteristic (ROC) curve was built using a positive threshold for KBT of eight fetal RBCs in order to define the optimal threshold for the FCM method. For all the hypotheses, P value < 0.05 was considered as statistically significant.
RESULTS

Samples Characteristics

Fifty-eight artificial dilutions containing from 1 to 100 fetal RBCs in 10,000 adult cells were prepared from seven UCB samples. Eighty-three pregnant women were included in this study from June 30 to August 29, 2011. The average age was 29.5 years (17.2–45.2 years). The KBT was requested for delivery of rhesus-negative women (55 cases), diminution of active fetal movements (10 cases), antepartum hemorrhage (8 cases), abdominal traumas during the third trimester (4 cases), intracranial deaths (4 cases), and placental abnormalities (2 cases).

Validation of FMH QuikQuant Kit

Firstly, we sought to evaluate the performance of FMH QuikQuant kit on prepared UCB dilutions. We compared the results obtained by KBT and FCM with the expected number of fetal RBCs (Figs. 2A and 2B). FCM and KBT showed an excellent correlation with expected results from UCB dilutions with a Spearman \( r \) of 0.97 (95% confidence interval (CI) 0.95–0.98) and of 0.94 (95% CI 0.90–0.96), respectively (Fig. 2B). The level of fetal RBCs detected by FCM matched perfectly the prepared dilutions whereas KBT overestimated systematically the amount of fetal RBCs (Table 1). Moreover, FCM exhibited a better reproducibility than KBT with a coefficient of variation respectively lower than 20% (for value higher than 10 fetal cells) and higher than 29% (Table 1).

We then compared the FCM to the KBT on 141 samples (58 UCB dilutions and 83 pregnant women) and 6 FETAltrol samples. Firstly, we confirmed the systematic overestimation of the KBT as demonstrated by the Bland–Altman test with a bias of 22.3 (Fig. 2C). Furthermore, FCM showed a good correlation with KBT with a Spearman \( r \) of 0.87 (95% CI 0.82–0.91) (Fig. 2D).

Finally, we defined the positive threshold of FCM using a ROC curve accounting of (i) 86 negative samples, i.e., KBT < 8 (11 UCB dilution and 75 pregnant women) and (ii) 53 positive samples, i.e., KBT ≥8 (47 UCB and 8 pregnant women) (Fig. 3). The area under the curve (AUC) was at 0.975 (95% CI 0.955–0.995). The threshold of FCM was defined at 4.3 fetal RBCs per 10,000 adult RBCs, with a sensitivity of 89.8% (95% CI 79.2–96.18%) and a specificity of 95.2% (95% CI 85.8–97.5%).

Pregnant Women Samples

In 8 out of 83 (9.6%), the KBT was positive (KBT+) with an amount from 9 to 40 fetal RBCs per 10,000 adult RBCs. The KBT was negative (KBT−) for 75

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Figs. 2. Comparison between Kleihauer-Betke test and FCM. A: Examples of cord blood dilutions samples (from 1/100 to 1/10,000) analyzed in FCM in parallel with KBT. The number of fetal RBC per 10,000 adult RBC obtained with FCM are reported in red on the dot-plots. B: Comparison of FCM (gray triangles) and KBT (clear circles) results with expected results from each dilution (N = 58). Spearman ρ of 0.97 for the correlation between FCM data and expected results and ρ of 0.94 between KBT and expected results. C: 83 pregnant women blood samples (black circles), 58 dilutions (gray triangles) and the three control levels (FetalStar) (clear square) in duplicate were analyzed both by FCM and KBT. FCM and KBT showed a good correlation (ρ of 0.87). D: Bland-Altman dot-plot of the same 147 samples showed a systematic overestimation by KBT. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

women. Fetal RBCs were undetectable for 73 out of 75 cases whereas 2 cases showed an amount of respectively 2 and 4 fetal RBCs (Fig. 2C).

With FCM, the 95th percentile of the KBT population was at 4.5 fetal RBCs (0.045%). Interestingly, this value was concordant with the positive threshold established with the ROC curve (4.3 fetal RBCs, 0.043%). Using this threshold, only five out of eight KBT + were diagnosed by FCM. However, two samples were reviewed as negative on control sample addressed by the physician, and these KBT were finally classified as false positive. Indeed, these slides showed a high rate of F cells, colored in pale pink, and counted as fetal RBCs by the slide readers (Fig. 4). FCM confirmed the negativity of these samples with a count respectively of 1.9 and 3.2 fetal RBCs per 10,000 and a high level of F cells was
Table 1

<table>
<thead>
<tr>
<th>Dilution of cord blood in fetal blood</th>
<th>N</th>
<th>Expected results</th>
<th>FCM Results (mean)</th>
<th>CV (%)</th>
<th>KBT Results (mean)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/100 (1.00%)</td>
<td>10</td>
<td>100</td>
<td>97.4</td>
<td>10.0</td>
<td>273.0</td>
<td>29.0</td>
</tr>
<tr>
<td>1/500 (0.2%)</td>
<td>10</td>
<td>20</td>
<td>22.1</td>
<td>16.5</td>
<td>62.2</td>
<td>42.2</td>
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<tr>
<td>1/1,000 (0.10%)</td>
<td>14</td>
<td>10</td>
<td>10.7</td>
<td>17.7</td>
<td>35.6</td>
<td>39.1</td>
</tr>
<tr>
<td>1/2,000 (0.05%)</td>
<td>14</td>
<td>5</td>
<td>5.7</td>
<td>34.6</td>
<td>17.7</td>
<td>38.6</td>
</tr>
<tr>
<td>1/10,000 (0.01%)</td>
<td>10</td>
<td>1</td>
<td>1.9</td>
<td>29.8</td>
<td>3.3</td>
<td>43.0</td>
</tr>
</tbody>
</table>

Fifty-eight control samples containing 100, 20, 10, 5, or 1 fetal per 10,000 total RBCs were prepared from seven cord bloods. These samples were analyzed either by flow cytometry with the FMH QuikQuant kit (FCM) and by the Kleihauer-Betke test (KBT). Results are expressed as number of fetal RBCs per 10,000 RBCs, except CV (%). N: number of samples. CV: coefficient of variation.

detected, respectively of 5.4% and 8.9%. The last discordant (KBT+/FCM−) case remained unexplained. Otherwise, we found only one discordance KBT+/FCM+ among the 75 women without FMH. In this case, the fetal RBCs count was at 4 per 10,000 (0.04%) by KBT and 10.3 (0.103%) by FCM.

F Cells Distribution

Finally, to define a reference range, we measured the F cells percentage in the 83 pregnant women and in 10 RhD-negative healthy donors. The rate of F cells in pregnant women population was 2.2% (from 0.5 to 11.3%), with a 95th percentile of 5.4%. F cell distribution was comparable to healthy samples with a mean of 3.4% F cells (from 0.9 to 13.6%) (data not shown).

DISCUSSION

All immunization prevention potentially concerns all the RhD negative pregnant women. An accurate quantification of FMH is important and defines the right dose of anti-D therapy. The KBT is an efficient screening test, when performed with correct attention in an experienced center (8,15). However, this assay lacks specificity and exhibits a large inter-observer variation resulting in potential overtreatment risks related to plasma-derived products and unnecessary treatment cost (20–22). In accordance with previous studies, we reported an overestimation of FMH by the KBT (12,15,23). The KBT presents stained maternal F cells that may be counted as fetal RBCs, leading to these false positive results (20,23,24). Indeed, in this study, we identified two discordant cases with a false positive KBT due to a high F cells level identified with FCM method. As recommended in case of positive KBT, a control sample has been performed, which concluded of the absence of detectable fetal RBCs by two observers.

Anti-RhD antibodies allow an accurate detection and quantification of RhD+ fetal RBCs in RhD− maternal circulation (1,25). This strategy allows the positive identification of RhD+ cells, while the use of HbF content does not discriminate negative from positive RhD cells. On the other hand, the use of anti-RhD strategy alone is unable to detect FMH in other conditions lacking RhD fetomaternal incompatibility such as abdominal trauma stillbirth, or decreased fetal activity. The FMH Quik-Quant kit shows two major advantages: (i) it accurately quantifies fetal RBCs independently of RhD status, (ii) F cells are easily identified as intermediate anti-HbF fluorescence cells.

Recently, the FMH QuikQuant kit has been tested on the CELL-DYN Sapphire hematology analyzer (Abbott Diagnostics) (26). A positive threshold of 12.5 fetal RBCs per 10,000 maternal RBCs has been defined on a panel of pregnant women without FMH risk factors (26). However, in this study, the KBT was not used to identify women with FMH. Our results based on both negative and positive samples proposed a positive threshold of 4.3 fetal RBCs per 10,000 maternal RBCs. We used cord blood dilutions containing a known number of fetal RBCs. FCM exhibited a high reproducibility and a good correlation with KBT, which is the most widely used assay in clinical laboratory. We found a coefficient of variation lower than 20% for values higher than 10 fetal RBCs per 10,000 maternal RBCs (0.1%) which is better than the KBT and in accordance with previous studies (3,13). Additionally, the kit design includes a positive marker of nucleated cells, thus

![area 0.975](image)

**Fig. 3.** Threshold determination for the flow cytometric method. Fifty-five positive KBT (i.e., 8 pregnant women and 47 cord blood dilutions) and 86 negative KBT (i.e., 75 pregnant women and 11 cord blood dilutions) were used to build this ROC curve. The black arrow corresponded to a value of 0.3 fetal RBCs per 10,000 maternal RBCs, defined as the best threshold for FMH QuikQuant. It exhibited a sensitivity of 89.8% and a specificity of 93.2% for this threshold.
facilitating exclusion of these interfering cells with auto-
fluorescence levels similar in fluorescein isothiocyanate
(FITC) fluorescence intensity as anti-HBF labeled fetal
RBC (12,27). We adapted the gating strategy to enhance
the fetal RBCs discrimination. Specifically, after exclusion
of nucleated cells, fetal RBCs were visualized on a bi-parametric
dot-plot SSC vs anti-HBF fluorescence, rather than
on a single parameter histogram of HBF fluorescence.
The placement of fetal RBCs gate on this bi-parametric
dot-plot, although still driven by a high FetalRBC control,
was much easier than the proposed single anti-HBF histo-
gram where the gate was more subjective to place.

Although KBT is inexpensive, it does not meet the
quality standard required for a quantitative assay due to
a lack of standardization and a large inter-observer vari-
ation. FCM methods are standardized, reproducible
and the gating protocol established for this study lead to
a quick and reliable interpretation of the histograms with-
out modification of the gate.

In addition, FCM assay allows to accurately quantify F
cells as well as adult RBCs with an increased amount of
HBF. The accurate quantification of F cells could be useful
in hemoglobin diseases, hereditary persistence of HBF,
and myelodysplastic syndromes (28–30). Using
FMH QuikQuant kit, we found exactly the same distribu-
tion of F cells values in pregnant women than in healthy
donors matched in aged suggesting that pregnancy is
not the unique physiological condition with an
increased level of HBF containing erythrocytes.

The major limitation of FCM is the lack of availability
24 h/day. However, urgent indications of FMH testing
are becoming a subject of discussion. Although the ab-
dominal trauma remained an emergency indication in
some centers, the performance of KBT is not consistent
with this context (21,31). In fact, no difference in the
rate of positive KBT between the trauma group and a
low risk control were reported by Emery and coll. (21).
In accordance with FCM integration in routine practice,
a recent study proposed that FCM was a better diagnosis
assay than KBT for the diagnostic evaluation of fetal ane-
mia of unknown cause (32).

Other FCM strategies have been employed combining
specific anti-HBF antibodies with a second antibody en-
abling a second parameter to differentiate fetal from
maternal RBCs. Anti-human carbonic anhydrase has
been used (15). This strategy is based upon the observa-
tion that adult RBCs express more carbonic anhydrase
than fetal cells, allowing a good separation even if adult
cells contained high values of HBF (35). Another stra-
 egy consisted in a double staining with anti-HBF and
with a monoclonal anti-D antibody (34).

In conclusion, FMH QuikQuant is a reproducible and
accurate CE marked flow cytometric method to detect
and quantify FMH and F cells. This technique was per-
formed and analyzed in one hour and a half for a 10
samples series, including controls, which is compatible
with increasing laboratory efficiency demands.

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