

Introduction

Fetomaternal hemorrhage (FMH) refers to the passage of fetal blood into the maternal circulation before or during delivery. Besides the deleterious effects of anemia in the fetus, allosensitization of the mother to red cell (particularly Rh) antigens may result in significant mortality/morbidity in the current or a future fetus. Another major indication for the testing of maternal blood for the presence of fetal cells is assessment of placental injury following acute trauma in a pregnant woman. As FMH may be asymptomatic in the mother, the diagnosis requires a high index of clinical suspicion augmented by laboratory testing for the presence fetal red cells in maternal blood. Quantitation of fetal red cells in maternal blood is used to estimate the size of the FMH and calculate the dose of RhIG needed to prevent Rh-sensitization in a woman who is Rh-negative and delivers an Rh-positive infant, or who has lost a pregnancy. Calculation of RhIG dose requires an assay of sufficient precision and accuracy near the clinically important 30 mL threshold (which translates to a fetal red cell frequency of 0.6% or 12 fetal cells/2000 adult cells in the Kleihauer-Betke assay), above which additional vials of RhIG are required. In the lowest ranges (0.01-0.1%), detection of fetal red cells becomes a problem in rare event analysis – the forte of flow cytometry (FCM). Therefore, in addition to the time-honored, semi-quantitative Kleihauer-Betke (KB) test, Allina Health Laboratory is currently developing a flow cytometric assay to complement the assessment of fetal red cells in maternal blood.
Embryonic, Fetal, and Adult Hemoglobins

Three embryonic hemoglobins (Gower1, Gower2, Portland) are present during the first trimester, but not in cord blood. Near the end of the first trimester a switch leads to production of fetal hemoglobin (Hb F), comprised of two alpha chains and two gamma chains (fetal precursor of adult beta chains). Hemoglobin A (alpha2/beta2) comprises approximately 10% of total hemoglobin in the second and third trimesters, the remainder being Hb F. Fetal hemoglobin (alpha2/gamma2), and the adult hemoglobins, Hb A (alpha2/beta2) and A2 (alpha2/delta2) are all present in cord blood. At the time of birth, B-globin synthesis has begun replacing gamma chains synthesis, such that at term, Hb F comprises 60-80% of total Hb, the remainder being Hb A. By 16-20 weeks old Hb F comprises approximately 3% of total Hb, and only about 1% (normal adult level) by 1-2 years.

F-cells

In the second trimester fetus the red cells contain predominantly Hb F. In the adult, Hb F is restricted to a few red cells called F-cells. F-cells contain 20%–25% Hb F and make up 0.5%–7.0% of circulating adult RBCs. The bone marrow responds to stress by increasing the production of F-cells, notably in patients with hemoglobinopathies, including sickle cell disease (with and without hydroxyurea treatment intended to increase F-cells), beta-thalassemia, as well as in hereditary persistence of fetal hemoglobin (HPFH) and in normal pregnancy. In 25% of pregnant women, F-cell production in the bone marrow begins to increase at 8 weeks gestation and peaks at 18–22 weeks, reaching levels in the maternal blood as high as 7% by 32 weeks. Hence, the presence of F-cells may give either a false-positive KB test result or may overestimate the magnitude of a true FMH. See Images 1, 2, and 3.

Rh Immunization - Basics Facts

RhIG prophylaxis in D-negative mothers within 72 hours of birth of a D+ or weak D+ baby reduces the risk of Rh immunization from approximately 16% to 1-2%. Antenatal RhIG administered at 28 weeks gestation to all unsensitized Rh-negative women AND within 72 hours of birth further reduces the anti-D immunization rate to 0.1%. In addition, RhIG is indicated for Rh-negative women under the following circumstances in which the baby’s Rh status is unknown: miscarriage/abortion; ectopic pregnancy; vaginal bleeding anytime during pregnancy, fetal demise, amniocentesis, chorionic villus sampling, cordocentesis, external cephalic version of a breech fetus, and blunt trauma to the abdomen. In the US, standard practice is that all Rh- women who have not been alloimmunized to the RhD blood group receive one vial of RhIG at 26-28 weeks, followed by rosette test screening following delivery if the infant is Rh(D) positive or Rh(D)variant. One vial (300 lg) of RhIG is sufficient to protect against 30 mL of fetal whole blood or 15 mL of fetal RBCs. FMH during term delivery can, on rare occasion (3/1,000 births), exceed 30 mL of fetal whole blood, necessitating accurate quantification of the FMH and more than one vial of RhIG.
Laboratory Tests Used in the Assessment of Fetal Maternal Hemorrhage

Essential Points

Rosette Test (RT)
- Qualitative test detecting a FMH of >10cc
- A positive RT means an Rh - mother delivering an Rh+ fetus may not be covered by a standard single dose of RhIG; if positive, KB or FCM is required.
- Indirectly detects D+ fetal cells in maternal blood using indicator D+ RBCs
- Only applicable to Rh (D) - mothers

Kleihauer-Betke (KB)
- Slide-based acid elution differentially stains fetal and maternal red cells
- Semi-quantitative
- Reliability depends on technical skill and stain quality
- Susceptible to errors resulting in underestimation or overestimation of a FMH

Flow Cytometry (FCM)
- Detects fetal red cells by fluorescence intensity of monoclonal antibody bound to Hb F
- Highly quantitative, automated, high precision and accuracy
- Objectively distinguishes maternal F-cells from fetal RBCs.
- May have clinical utility in recognizing increased maternal Hb F

Details of Tests

ROSETTE TEST (RT)

The RT is a highly sensitive qualitative method for the detection of a FMH of >10cc. The test is appropriate on post-partum maternal blood when the mother is D- and the baby (cord blood) D+. A D- mother who delivers a D- or D variant baby requires a quantitative fetal red cell test (KB or FCM) (the lab reflexes the test for the physician).

Maternal blood is mixed with anti-D reagent, which binds to any D+ fetal cells that may be present. Unbound anti-D is removed by washing. Next, D+ indicator cells are added which form clumps (rosettes) of D+ indicator cells around any sensitized D+ fetal cells. If the rosette test is negative, a single vial of RhIG is administered to a D- mother. A positive test indicates a significant (>10 cc) FMH and may require more than one vial of RhIG, depending on the results of a quantitative test for fetal red cells, either the KB or FCM. The RT may be falsely positive if the mother is D variant (see below). As the RT relies on the presence of the D antigen to distinguish fetal from maternal cells, it cannot be used to detect FMH in D+ mothers, a D- mother carrying a D- fetus, or in the case of a known variant D fetus/neonate. In the latter situations, a quantitative test based on detection of fetal Hb (not D antigen) is required.
The Kleihauer-Betke (KB) stain acid-elution technique is based on the principle that fetal hemoglobin is relatively insoluble in an acid citrate-phosphate buffer, whereas Hb A and its variants are soluble and readily elute out of the cell. After staining with eosin the bright pink cells fetal cells can be distinguished from adult erythrocytes, which appear as pale pink or colorless “ghosts.” Leukocytes retain the eosin and will be an intermediate color (see Images 1, 2, and 3). The quantitation of fetal cells in the maternal blood sample is a simple two cell differential count. Generally, 2000 cells are counted, and the ratio of fetal: adult cells calculated. The method of Kleihauer (1996) is used to calculate the amount of fetal blood in the maternal circulation as derived from the fetal: maternal red cell ratio:

- Assumes maternal blood volume = 5000 cc
- All fetal cells stains positively
- MCV fetal cells = MCV adult cells

\[ \text{cc whole fetal blood in material circulation} = \% \text{ fetal cells} \times 50 \]

30cc is the threshold for one 300 ug vial of RhoGAM (RhIG)

Note: by this method, 30cc is equivalent to 0.6% = 12 fetal RBCs/2000 adult RBCs

Number of vials of RhIG required = \( \frac{\text{cc whole fetal blood}}{30} \)

Although the above formula is widely used for the calculation of vials of RhIG (RhoGam) required, at Allina Health Laboratory, the method of Dr. Polesky (endorsed by Dr. John Jones) is used in which 22.5 cc rather than 30 cc is the divisor, resulting in a “margin of safety,” in terms of RhIG vials. This approach is warranted because published studies have found that giving too little Rh antibody can actually cause immune enhancement. If the calculations place the dose at the upper limit for a certain number of RhIG vials, an additional vial can be safely added to the calculated dose. Reports in the literature indicate that even large doses (10-12 vials) can be given without adverse reactions.

**Advantages of KB**

The KB is relatively easy and cheap with faster turnaround time than FCM. The simplicity of the KB and 24 hour availability make it ideal for stat testing.

**Disadvantages of KB**

The KB is only semi-quantitative, although this is only a significant objection when calculating RhIG dose, an indication for which the major imperative is to avoid under-dosing with RhIG. In practice, the major disadvantages of the KB test are the subjective nature of the interpretation and the test’s inherent variability based on staining quality. Both over- and underestimation of FMH have been reported, but most studies report a tendency of the KB test to overestimate FMH. As noted above, overestimation is less of a problem for RhIG than underestimation, as the latter could result in inadequate RhIG dosing and Rh sensitization. In the prenatal
assessments of FMH as a marker of placental injury, however, overestimation (or a false positive) might lead to an erroneous diagnosis of FMH and placental injury, resulting in unnecessary interventions, some of which may carry risk for the fetus. The major factor leading to overestimation in the KB test is the presence of F-cells that may appear dark pink, and possibly failure to adjust for larger maternal circulating volume (for women weighing > 70 kg). If mother and fetus are ABO incompatible, the fetal cells may disappear from the mother's circulation quickly, and an underestimate of the size of a FMH will be made. Incomplete staining of fetal cells in the KB test also likely contributes to underestimation.

**Normal Range for KB**

There is no consensus among reference laboratories or published literature. Currently, at Allina Health Laboratory 1/1000 (2/2000) is the threshold for a positive KB result.

![Image 1. Negative KB stain with pink to pale ghost-like unstained adult RBCs](image-url)
Image 2. Positive control slide with dark red fetal RBCs and background negative adult RBCs

Image 3. Maternal blood sample with increased F-cells (single arrows) and weakly stained neutrophil (double arrows). Negative KB stain.
FLOW CYTOMETRY (FCM)

CAP proficiency surveys have repeatedly shown that FCM using anti-HbF antibodies is more precise (CV’s <20%) than the KB (CV’s 32%–80%), and is more accurate. FCM using monoclonal antibodies to Hb F represents the state-of-the-art for the detection of FMH when quantitative accuracy is important. In published literature reports, FCM is more objective, quantitative, reproducible, sensitive, and specific than the KB method. As a side benefit, the percentage of F-cells in the mother can be directly quantitated and may correlate with clinical conditions in which the Hb F level is raised. The FCM method utilizes monoclonal antibodies to Hb F incubated with permeabilized suspensions of red cells in whole blood to achieve intracellular staining. The fluorescence intensity of 50,000 red cells stained with fluorochrome-tagged anti-Hb F monoclonal antibody can be obtained in the cytomter in a few minutes. The intensity of fluorescence of each stained red cell is proportional to the concentration of Hb F. The data is presented as a single parameter histogram. Control blood spiked with cord blood is used to define the region of fetal red cell fluorescence intensity. The assay objectively distinguishes F-cells from fetal red cells. See Figures 1, 2, and 3.

Reference intervals for fetal red cells in maternal blood samples

Surprisingly little published reference range data is available regarding fetal red cells in maternal blood specimens analyzed by FCM. Equally surprising is the lack of consensus as to whether or not the percentage of red cells in normal pregnant women varies with gestational age.

As a generalization, published studies suggest pregnant women beyond 20 weeks gestation should have <0.50% fetal red cells. Non-pregnant adults should have <0.10%. The Allina flow cytometry lab is currently developing reference intervals for enumeration of fetal RBCs in maternal blood by FCM.

Some comparative published data based on various FCM methods:

De Wit et al, 2011:
- 236 pregnant women: mean: 0.047% Range: 0.00% to 0.50%
- Non-pregnant (male/female) adults: Range: 0.00 - 0.125% (all <0.125%)

Davis et al, 1998:
- 150 non-pregnant (male/female) adults: Range: 0.00 - 0.09% mean: 0.02% (all <0.1%)

Pora, et al, 2007:
- 74 non-pregnant females: mean: 0.007% Range: 0.00% - 0.03% (all <0.03%)
Figure 1

Flow histogram of normal (non-pregnant) adult female blood. Normal level of F-cells (1.12%) with 0.00% fetal RBCs.

Figure 2

Flow histogram of normal adult female blood spiked with fetal RBCs to 3.70%. Normal level of F-cells (2.40%), clearly separated from fetal RBCs.
D Variant Blood Issues

The Rh blood group system is a highly polymorphic red cell antigen system, among which the D antigen is the most immunogenic (Connie M. Wethoff, PhD, MT (ASCP)SBB, 2008) and the major cause of hemolytic disease of the fetus and newborn (HDFN). Anti-D is readily formed in D-negative (D-) women exposed to D-positive (D+) fetal RBCs. The use of Rh immune globulin (RhIG) to prevent primary immunization has dramatically decreased HDFN due to anti-D. Therefore, appropriate assignment of D antigen status is a critical blood bank issue for obstetric patients as well as patients undergoing chronic transfusion therapy. However, the serologic distinction between D-positive and D-negative RBCs is not always straightforward.

In recent years, the simple algorithm in which a D- mother giving birth to a D+ infant receives appropriately-dosed anti-D prophylaxis has been challenged by recognition that 0.2-1.0% of whites may inherit a variant of the D antigen typically reported as “weak” or “variable” when typed by standard blood bank techniques. Different ethnic groups have different rates and percentages of these variant D antigens.

Figure 3

*Flow histogram showing increased F-cells (18.35%) in a pregnant female. Fetal RBCs (spiked from cord blood) are clearly separated from maternal F-cells.*
Two genes, RHD and RHCE, lie in close proximity on chromosome 1 and encode two Rh proteins: one, the D antigen, and the other, CE antigens in various combinations (ce, cE, Ce, or CE). The resulting RHD/RHCE recombinant gene encodes the final red cell Rh membrane protein. Because of the large number of potential RHD/RHCE combinations, the theoretical number of antigenically different Rh proteins is enormous. Strong ethnic linkage limits the likelihood of Rh variants that may be encountered in an individual patient. Most D+ individuals have an RhD protein on their red cells that consists of a full complement of antigens. However, numerous RHD alleles are known that may result in diminished or altered expression of the RhD protein. The term “weak D” is applied to the red cells of individuals whose D antigen appears weak or negative in serologic blood bank testing, even though they are not truly Rh negative (in true Rh-negative people the RHD gene is deleted and RhD protein absent). A more appropriate term is “D variant” to describe this D antigen with diminished numbers of antigens. The ability of the laboratory to detect the D antigen is dependent on the reagents and typing methods used. Hence, Rh typing results may vary between different labs, and a person with D variant red cells may be told they are Rh+ by one lab and Rh- by another.

There are three types of clinically important D variant phenotypes. “Partial D” variants have mutations that eliminate one or more protein epitopes, but do not reduce the amount of membrane Rh protein. People who are partial D variants type as D+ but may develop anti-D when exposed by transfusion or during pregnancy to the complete D antigen. Women who are partial D variants and have developed anti-D are at risk for HDFN. There are around 60 known partial D variants. To avoid anti-D alloimmunization, pregnant women and transfusion recipients who type as partial D variants should be regarded as D-negative.

“Weak D” variants have a reduced number of qualitatively normal D antigens. There are more than 50 different mutations that cause weak D expression. As weak D variants contain a full complement of epitopes, they usually do not form anti-D on exposure to D+ RBCs. A third type of D variant of clinical importance is the DEL variant. DEL RBCs express very low quantities of D antigen that cannot be detected on routine serologic testing. They are infrequent in whites but often found in Asian ethnic populations. RBC units with DEL phenotype can cause alloimmunization and usually type serologically as D-negative.

**Some general guidelines with respect to D variant blood typing**

**For Transfusion Recipients:** Serologically determined weak D individuals are considered D- for transfusion purposes.

**For Pregnancy:** Mothers who are partial D variants should be considered D- and receive standard RhIg prophylaxis and have a KB performed to detect any fetal RBCs that may be present. Pregnant women previously shown to be weak D variants should receive RhIg in their current pregnancy.

**For Blood Donors (and cord blood testing):** Both “weak D” and “partial D” people are considered D+ as donors. The law requires blood centers to label blood as D+ if it contains any amount of the D antigen. “Partial D” people are treated as D- when receiving a blood transfusion. But when donating, their blood is labeled as D+ because some parts of the D
antigen are present. So, whether “weak D” or “partial D,” the donor’s blood will only be given to D+ (Rh positive) recipients.

According to the American Association of Blood Banks, weak D testing (antiglobulin phase of anti-D testing) is not required in pregnant women but is mandatory in blood donor and cord blood testing.

**Current practice guidelines at Allina Health Laboratory**

Without molecular testing, it is impossible to know with certainty if a patient is susceptible to Rh alloimmunization when receiving D+ positive blood. In the Allina Health Laboratory, both “partial D” and “weak D” are regarded as “D variants.”

Current laboratory practice methods interpret D variant typing as D- when the person is a pregnant woman or a patient, but as D+ when that person is a blood donor or a newborn. This strategy ensures that all pregnant women, particularly those with weak or partial D phenotype, receive RhIG after delivering a D+ or D variant infant.

Note that Allina Health Laboratory practice recommendations will change as of February, 2014. The following changes in result reporting will take place in Excellian:

Rh testing (called “D Antigen testing”) will be reported as:

- Positive
- Negative
- D Variant

For D variant results, the following *COMMENT* will be added to the report:

“Patient is identified as a D Variant which represents weak D or partial D. Differential determination between these cannot be made using routine blood bank testing methods. For the safety of the patient, D variants are considered D antigen negative (Rh negative) for transfusion and maternal Rh immune globulin administration. Rh negative mothers who deliver a D variant infant are a candidate for Rh immune globulin and should undergo testing with a Fetal Maternal Hemorrhage Screen.”

An Excellian notification will be published when the above recommendations become active. Further recommendations from the American Congress of Obstetricians and Gynecologists (ACOG) and the American Association of Blood Banks (AABB) are anticipated in 2014.
References:

Fetomaternal Hemorrhage


Fetal Red Cell Enumeration by Flow Cytometry


Rh Blood Group System and D Variant Practice Issues


For questions, comments, or suggestions about this newsletter or other laboratory issues, please contact Lauren Anthony, MD, Medical Director of Allina Health Laboratory, (612) 863-0409 or Lauren.Anthony@allina.com