

EDUCATIONAL COMMENTARY – FETAL CELL DETECTION AND QUANTIFICATION

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LEARNING OUTCOMES

On completion of this exercise, the participant should be able to

- understand the difference between fetal cell detection methods and quantitative methods.
- appreciate the clinical importance of fetal cell detection and quantification.

Introduction

The D antigen is the most immunogenic of the blood group antigens. Exposure to as little as 0.1 to 1.0 mL of D-positive red blood cells (RBCs) in a D-negative individual can elicit an immune response. Despite the potent immunogenicity of the D antigen, without prophylaxis, only 16% of D-negative mothers with D-positive infants will develop anti-D. Prior to the advent of Rh immune globulin (RhIG), anti-D was the most common cause of hemolytic disease of the fetus and newborn (HDFN) which leads to a high fetal mortality rate in subsequent pregnancies of immunized mothers.

Unless trauma is involved during the pregnancy, the largest volume of fetomaternal hemorrhage (FMH) usually occurs during delivery. If the mother becomes sensitized at the time of delivery, HDFN will not be seen until subsequent pregnancies. The use of RhIG is important to prevent alloimmunization; when used correctly, RhIG can prevent anti-D formation in 99.9% of cases. The College of Obstetricians and Gynecologists recommends that an antepartum dose of RhIG be given to D-negative mothers at approximately 28 weeks' gestation and a postpartum dose be given within 72 hours after delivery of a D-positive newborn. RhIG should also be given to D-negative women after miscarriage, abortion, ectopic pregnancy, amniocentesis, or chorionic villus sampling. The typical dose used in the United States, 300 µg, protects a D-negative person from immunization to the D antigen when exposed to up to 30 mL of D-positive whole blood (15 mL RBCs). The 1% to 2% of cases where RhIG treatment fails may be a result of too small of a dose or delay in injecting RhIG.¹ To prevent alloimmunization, an FMH must be properly detected and quantified.

Fetal Cell Detection Methods

Historically, the microscopic weak D test and an enzyme-linked antiglobulin test (ELAT) have been used to detect the presence of D-positive fetal cells in D-negative maternal circulation.² However, there are more sensitive and standardized tests available now, and these tests are no longer used. The only FDA-approved screening test is the rosette test. In this screening test, RBCs from a D-negative patient are

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incubated with anti-D, washed, and then microscopically examined following the addition of enzyme-treated, D-positive RBCs. If D-positive fetal cells are present, the indicator cells will form aggregates (rosettes) around the coated fetal cells (**Figure 1**). This test can detect a fetal whole-blood bleed of 10 mL or more in the maternal circulation. FDA-approved commercial kits are available for the rosette test. This easy-to-perform and cost-effective screening test does not require highly specialized equipment. It can be performed at any time and has a turnaround time of 1 to 2 hours.

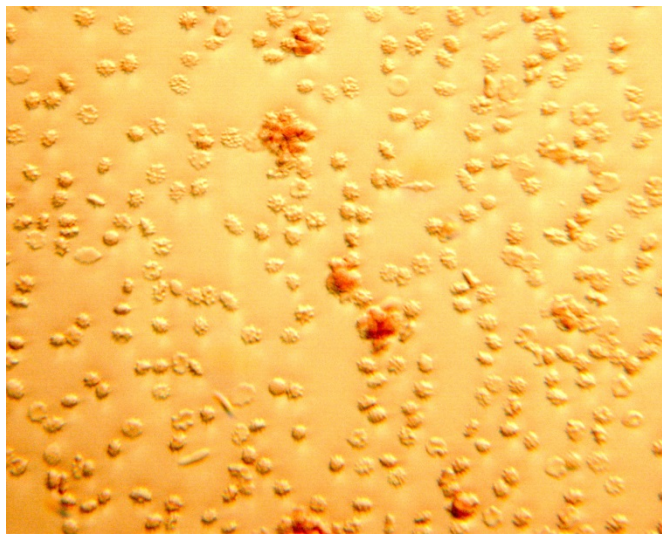


Figure 1: Positive rosette test. Photo compliments of Mount Sinai Hospital Blood Transfusion Laboratory, Toronto, Ontario.

Despite the ease of use, the rosette screening test has limitations. Because the test works by detecting D-positive fetal cells in D-negative circulation, false-positive results can occur if the mother is a weak D, and false-negative results can occur if the fetal cells are weak D. If the maternal blood has a positive DAT, a false-positive result on the rosette test may occur because the autoantibody can cross react with the indicator cells.³ In the case of ABO incompatibility between the mother and fetus, the fetal cells may be destroyed before the testing is performed, causing a false-negative result. A rosette screening test cannot detect an FMH in D-positive women or when the fetus is D-negative.

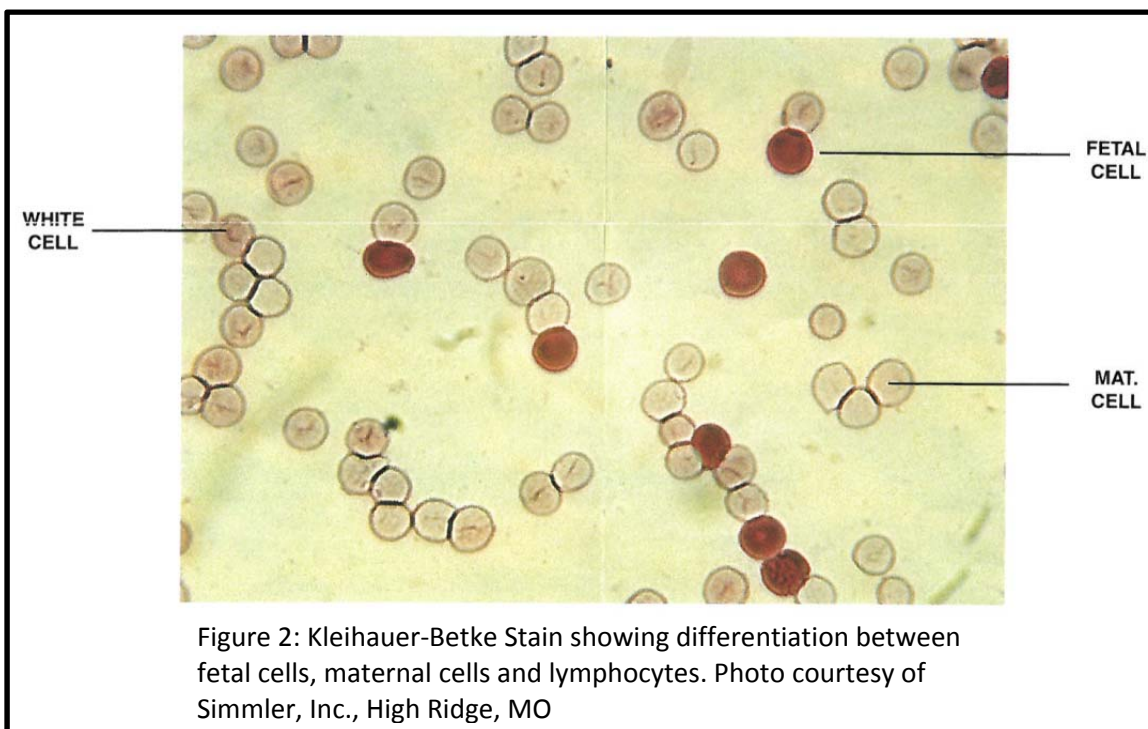
Gel microcolumns have also been used as a screening method to detect an FMH. However, this method is not accurate when there is a large FMH and should not be used for large-volume quantification.⁴

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When the result of the screening test is negative, 1 dose of RhIG is still given to the D-negative mother. When the screening test is positive, it is necessary to quantify the volume of fetal cells in maternal circulation and calculate an adequate dose of RhIG. Estimates suggest that an FMH of greater than 30 mL occurs in only approximately 0.3% of pregnancies.⁵

FMH Quantification Using Kleihauer-Betke Stain

The Kleihauer-Betke (K-B) acid-elution assay is the most widely used quantitative method for measuring fetal RBCs in maternal circulation. The principle of the K-B assay is that fetal hemoglobin (HbF) resists denaturation from acid treatment, and therefore retains a dark stain. Because adult hemoglobin is denatured by acid, these cells will not retain the stain and will appear white or lighter in comparison to fetal cells (**Figure 2**).



The K-B assay can be used to determine the volume of fetal RBCs regardless of the mother's D-antigen status; however, it is most commonly used for RhIG prophylaxis. By counting the number of fetal cells in the total number of adult cells, the number of doses of RhIG needed can be calculated. The AABB *Technical Manual* provides a common calculation to determine the FMH (mL):

$$\frac{(\text{Fetal cells} \times \text{maternal blood volume})}{\text{Total RBCs counted (should be at least 2000)}} = \text{FMH (mL)}$$

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The FMH can also be resulted as the % fetal cells:

$$[\text{Fetal cells counted} / \text{total RBCs counted}] \times 100 = \% \text{ fetal cells}$$

After calculating the total FMH or the % fetal cells, the number of vials to inject can be determined. The AABB Technical Manual and some manufacturer package inserts provide tables for RhIG dosage based on % fetal cells seen. The number of vials can also be calculated by dividing the total FMH by 30 mL. To prevent sensitization, 1 dose of RhIG is needed for every 30 mL of FMH. The calculated number of vials is typically rounded up or down, and then 1 vial is added to the total to protect from inaccuracy in the KB testing method:

$$\text{FMH (mL)} / 30 \text{ mL} = \# \text{ of vials (round up/down)} + 1 \text{ vial} = \text{total \# of vials to inject}$$

Here is an example of a calculation:

Fetal Cells counted = 11 Total RBCs counted = 2610 Estimated Maternal Blood Volume = 5000 mL

$$\text{FMH} = [11 \times 5000] / 2610 = 21.1 \text{ mL}$$

$$\% \text{ fetal cells} = [11 / 2610] \times 100 = 0.42\%$$

$$\text{Total number of vials calculated} = 21.1 \text{ mL} / 30 \text{ mL} = 0.70 \text{ (round up)} + 1 \text{ vial} = 2 \text{ vials total}$$

The commonly used maternal blood volume is 5000 mL, which is only an estimate. The College of American Pathologists (CAP) has published an “RhIG Dose Calculator,” which allows the user to input the mother’s weight and height to more accurately calculate total blood volume. The subjectivity, poor reproducibility, and time-consuming nature of the K-B assay are significant limitations. Other limitations of this test are listed in the **Box** below.

Box. Sources of Error in the K-B Stain Assay

- Hematologic disorders producing increased levels of fetal-type cells can cause false-positive results
- 0.5%-7.0% of normal adult RBCs may contain 20% to 25% HbF
- Lymphocytes may retain a darker stain, which may be misinterpreted as fetal cells

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FMH Quantification Using Flow Cytometry

Another quantitative test method that is used in lieu of the Kleihauer-Betke assay is flow cytometry. In the 2013 HBF-04 CAP survey, 5% of the 929 participants used flow cytometry to quantify fetal cells, a slight increase from 4.2% in the 2009 survey.^{6,7} There are several FDA-approved flow cytometry methods that use either anti-HbF or anti-D or a combination of both. These methods have an advantage over the K-B assay. Flow cytometry is automated and more accurate; it can also distinguish between adult HbF and fetal cells. However, because this method is much more expensive than the K-B assay and requires special equipment and specialized personnel to operate, it is unsuitable as a STAT test.

HbF antibodies are bound to a fluorescein or to a dye and, following special treatment of the maternal blood, bind to fetal cells in the specimen. The anti-D flow cytometry method targets the D antigen on the fetal cells in D-negative maternal blood. Because this method cannot detect weak D or partial D, it may produce false-negative results. Although flow cytometry methods are more accurate than the K-B assay, the subjectivity of traditional manual gating when analyzing cell populations can reduce precision. Some studies have implemented the patented approach of Probability State Modeling (PSM), which allows for fully automated analysis.⁸ The greater accuracy and reproducibility of flow cytometry are an advantage over the K-B assay method.

Hematology analyzers with immunofluorescence capabilities can offer an analogous antibody-based method to flow cytometry that requires no washing steps and minimal technical expertise.⁹ The same FITC-conjugated antibodies used in the flow cytometer kits have been used in published studies on hematology analyzers and have shown comparable performance to the flow cytometers. It may be necessary to process the data using a third party software system when using the hematology analyzers. In most laboratory settings, these types of hematology analyzers are available throughout the day to allow for STAT testing capabilities. Validation of all flow cytometry testing methods is required to prove the assay performs as expected.

Summary

Proper detection and quantification of D-positive fetal cells in D-negative mothers is necessary to provide proper prophylaxis against immunization. Despite the success of RhIG, there remains a failure rate among recipients. This may be a result of failure to quantify and calculate the correct dose required. Proper calculation of FMH must be validated in each laboratory. Clearly there is an opportunity for an inexpensive, more accurate, and less subjective quantitative method to detect fetal cells in maternal circulation. This is clear from studies and PT reports that have been done. The AABB Technical Manual states in the Methods section that “The accuracy and precision of this procedure are poor...”. Flow cytometry is more accurate and precise, but expensive.

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