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TITLE: Fetal Maternal Bleed Testing

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Hello, my name is Eapen Jacob. I am an Assistant Professor of Laboratory Medicine at the Mayo Clinic in Rochester MN. Welcome to this Pearl of Laboratory Medicine on "**Fetal Maternal Bleed Testing."**

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Hemolytic Disease of the Fetus and Newborn or HDFN is a clinically significant disorder which historically has been a leading cause of death in the fetus and newborns. In it fetal red blood cells are destroyed by maternal antibodies which target paternal antigens expressed on the fetal cells. For me it is one of the success stories of modern medicine in that the original theory as to its cause (developed in large part by a pathologist from Chicago Dr. Ruth Darrow in the 1940's) to successful treatment and prevention occurred over the space of approximately 30 years.

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In general, the main immunizing event is thought to occur during labor as fetal cells enter the maternal circulation. Unlike neonatal alloimmune thrombocytopenia, the immunization occurs during one pregnancy and the destruction of the cells (in this case RBC's) typically occurs in a subsequent pregnancy. Other events such as trauma, interventional procedures, and spontaneous/induced abortions can also lead to immunization earlier in the pregnancy although most occur at labor and delivery.
In the figure to the right, rbc in the fetal circulation are separated from the maternal circulation although oxygen and other substances can cross. One such substance is IgG using the neonatal FcGamma receptor. After this occurs binding to fetal rbcs may

occur (if a cognate antigen is present) and destruction results. Historically the major antigen in question was D (Rh antigen).
With destruction of rbc there are multiple consequences. The fetus becomes anemic. Compensatory mechanisms lead to increased cardiac output, potentially failure and extramedullary hematopoiesis. Eventually hydrops. Interestingly during in utero HDF the breakdown products are metabolized by the mother's liver. After birth however, the child's liver is not able to fully convert the bilirubin which results in jaundice and kernicterus (neurologic complications)

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Treatment developed in the last 50 years has dramatically decreased infant mortality. This includes bili-lights which help to convert insoluble unconjugated bilirubin into a more soluble form which can then be excreted. More invasive methods such as exchange transfusion are possible including in utero transfusion. These among other efforts have significantly brought down morbidity and mortality but have not eliminated them.

In the 1960s various groups in North America and Europe realized that anti-D formation might be prevented by infusing anti-D antibody. Somewhat surprisingly, this has been shown to work very well. The key to its use is recognition of potential immunizing events. At this point the standard of care dictates a single dose of RhIg be given at 28 weeks gestation in Rh negative mothers (to prevent immunization events during pregnancy) and then a second dose after labor and delivery in RhNeg mothers with Rh Positive infants. The key to the second dose is that it be given within 3 days of delivery and it be of the proper amount to cover the fetal maternal bleed.

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One vial of RhIg will be sufficient to prevent immunization with 30 ml of fetal blood (15 ml of fetal rbc's) from historical studies. Luckily less than 0.5% of deliveries lead to such bleeds.

To determine when additional RhIg is needed first a qualitative test is used. The Rosette test is a serologic test which is designed to be positive when more than one dose of RhIg will be needed. Then a quantitative test is used. Historically a KB is preformed which makes use of the fact that fetal hgb is more resistant to acid pH than adult hgb. Due to this, adult cells have a ghost-like appearance compared to the more darkly staining fetal cells. This is a highly manual test which requires hand counting and has been shown in PT testing to under report bleeds in some instances.

Flow cytometric measurement allows for various advantages not the least of which is a larger number of cells being counted. This adds to the accuracy and precision of the testing especially at low level bleeds.

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Flow cytometric testing is based on being able to distinguish fetal from adult cells using antigenic targets such as fetal hemoglobin. The cells have been permeabilized allowing for the labeled antibody targeting fetal hgb to enter the cells. In this example a small population of fetal cells are identified in third decade. The majority of the cells are adult cells with primarily adult hemoglobin. One drawback of this method is that a typical adult has a small amount of fetal hemoglobin containing cells; labeled here as adult F cells. In our hands these typically stain less intensely than the true fetal cells. Daily controls are necessary to determine the proper placement of gates. For instance, low titer antibodies, particularly in homebrew systems can make distinguishing fetal cells from typical adult and adult f cells difficult.

Although designed and FDA approved for the detection of fetal cells in the mother's circulation, such assays can also be theoretically used to measure increases in fetal hemoglobin containing cells; eg in therapeutic interventions to treat sickle cell anemia. This may be complicated however, depending on how the fetal hemoglobin is distributed in the rbc population.

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Although typical bleeds only require 1-2 vials of RhIg, occasionally large bleeds such as this one in the top figure are detected.

More problematic is the second example in which a large population of presumed adult f cells are present. The percentage of fetal hemoglobin in the mother's circulation can be increased during pregnancy complicating enumeration of fetal maternal bleeding. Proper gating is essential here to allow for distinguishing between the true fetal cells which stain/fluoresce more intensely.

Most troublesome are large amounts of such cells seen in presumed hereditary persistence of fetal hemoglobin (HPFH) cases. In these cases, the shoulder of HPFH cells starts to encroach on the true fetal cell gate. This prevents an accurate determination of the fetal cells as in all likelihood some of the cells in the gate are not truly fetal in origin. Due to the consequences of missing a fetal bleed that can result in an immunizing event, it is safer to count these cells and cover with the appropriate amount of RhIg. In a mother with known HPFH, this can make it difficult or impossible to determine a FMB accurately.

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Dual antibody platforms can be helpful in such situations. Again, the single antibody set up is shown with a moderate number of adult f cells and no significant fetal bleed. In the bottom left blue histogram, anti-fHgb is used on the X axis and anti-D on the Y. Thus, relevant true fetal bleeds would be in the RUQ.

Similarly, in the lower right a second antibody such as this one to CA can be used to distinguish fetal cells (which should be negative) from adult cells. Combine with the fetal HGB antibody the true fetal cells are in the LUQ. Theoretically adult cells with higher amount of fetal hemoglobin can then be distinguished from true fetal cells.

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Some potential pitfalls can occur. E.g. if the mother of child is a partial D phenotype, distinguishing the cells with an anti-D antibody may be problematic. In addition if a significant amount of anti-D has been given to mother prior to the lab draw, it may mask the D epitope from the diagnostic antibody. Use of antibodies distinguishing fetal from adult cells by antigen maturation could potentially be an issue if this does not occur as expected in the mother/infant pair.

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The dosing of RhIg is based on knowing the amount of fetal cells in the maternal circulation using one of the quantitative tests. Then using the conversion for vials of RhIg is performed. Rounding is up if 0.5 or above then a safety margin is added of 1 additional vial. Therefore, even if the calculation of RhiG suggests 0; 1 vial of RhIg is always given if the mother is RhNeg and the child is RhPos.

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Typically, the blood bank does not know the weight of the mother. If it is known, it can be substituted into the equation. If not, the standard is to use 70kg. In this example of a relatively large bleed, the maternal circulation is approximately 4900 ml of whole blood; with a 1% content of fetal cells a bleed of 49 ml results. This converts to 1.6 vials of RhIg which rounds up to 2. Adding the safety margin of 1 additional vial gives 3 vials of RhIg. Some test prep books advocate a short cut in these calculations however I think it is useful to know what the standard calculation is. This allows for personalization of the dose if the mother is not 70kg. In addition, it's important to note I use whole blood in my calculation which leads to the 30 ml/vial conversion. Some use the 15 ml of fetal rbc/vial. One just needs to remember to calculate the volume of fetal bleed appropriately.

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One of the major causes of immunization is not recognizing when RhIg should be given. Which scenario does not require RhIg in a Rh-negative mother?

The essential issue is to recognize the mother is Rh Negative. If the fetus is Rh positive or the Rh is not known, then RhIg should be given.

In scenario 1 the Rh of the fetus is likely not known. RhIg is therefore required.
In scenario 2 the Rh is also likely not known so RhIg is recommended. In the third trimester, a qualitative or quantitative test for amount of fetal bleed should also performed.
In scenario 3 the fetus is known to be Rh negative so RhIg is not required. It is possible that the fetal bleed detected could cause an immunizing event but RhIg would not prevent it since the fetal cells do not have the target antigen (the cells do not have D on their surface)
In scenario 4 the fetus is Rh positive with a negative Rosette test on mom. That tells us that additional doses of RhIg are not required. However, the baseline dose of 1 vial would still be given since the test is designed to tell us if additional RhIg is needed only.
Finally, scenario 5. Even if the mother is planning to have no future children at risk for HDFN, she may very well need a transfusion some time in her life. Preventing the potential formation of anti-D antibody complicating future transfusions justifies the use of RhIg here.

Slide 13: References

Slide 14: Disclosures

Slide 15: Thank You from www.TraineeCouncil.org

Thank you for joining me on this Pearl of Laboratory Medicine on Fetal Maternal Bleed Testing Using Flow Cytometry.