1. Abstract

Fetomaternal hemorrhage (FMH) occurs normally throughout pregnancy with an increment occurring during the birthing process. In cases with a significant difference in red blood cell antigenicity between the fetus and mother, allosensitization of the maternal immune system can occur, which causes significant morbidity and mortality in that or subsequent pregnancies. Flow cytometric methods for detection and enumeration of fetal red blood cells (RBCs) in pregnant women using anti-HbF-FITC (Fetal Hemoglobin) have been validated for reliability and are often used to detect fetomaternal hemorrhage. We evaluated new methods for allosensitization and fetal RBC detection.

2. Study Aims

2.1. Develop a Probability State Model (PSM) for analysis of flow cytometric listmode data files using anti-HbF methods for the detection of fetal red blood cells (RBCs) in maternal blood samples as used in diagnostic assessment of fetomaternal hemorrhage.

2.2. Compare PSM methods used in this study to manual analysis achieved in other clinical laboratories using an expert system (GemStone software) to manual data analysis with WinList™ software (Verify Software House) as performed by blinded analysts by an expert developer of the anti-HbF method in > 100 data files from analysis of diagnostic clinical samples. This includes the use of the gating regions developed by experts and the gating approach used for fetal cell exclusion.

2.3. Examine data from above comparative analysis to determine the correlation between the methods and determine if any bias is identified between PSM and expert manual analysis.

2.4. Determine potential for PSM data analysis to provide automated analysis of flow cytometric data for clinical fetomaternal hemorrhage testing and provide a more robust alternative to the current practice of subjective gating and subpopulation region definition that contribute to the documented imprecision or variation between various diagnostic laboratories performing flow cytometric fetal RBC detection for prenatal women.

3. Methods

3.1. Materials:

- Clinical blood samples submitted for analysis for fetomaternal hemorrhage as EDTA anti-coagulated blood
- Artificial mixtures of ABD and FITC blood type matched adult and cord (maternal) blood samples
- PSM cell populations: three-level control material for fetomaternal hemorrhage assays (Triplum Diagnostics, Bangor, Maine, USA)
- PBS buffer (0.9% buffered saline solution) with 0.5% bovine albumin, pH 7.4
- FMH QuikQuant assay for flow cytometry detection of fetomaternal hemorrhage (Triplum Diagnostics, Bangor, Maine, USA)
- Gates: Adult RBCs defined by experience or auto-fluorescence signal
- Adult F Cell region defined by high level FETALTrol control
- FETALTrol, stabilized three level control material for fetomaternal hemorrhage assays (Trillium Diagnostics, Bangor, Maine, USA)

3.2. Sample Processing and Preparation: All samples were processed as detailed in the instructions for use for the FMH QuikQuant assay including:

- Centrifuge sample and decant supernatant
- Add 10 microliters of FMH QuikQuant reagent A (anti-HbF-FITC antibody and propidium iodide) and 40 microliters PBS/BSA, incubate 15 minutes
- Analyze on flow cytometer, collecting at least 50,000 events for parameters of FLS, SS (log), FL1 (anti-HbF-FITC), FL2 (auto-fluorescence), and FL3 (propidium iodide).

3.3. Manual "Expert" Data Analysis with WinList software:

- Data files are analyzed independently by each of the authors (BD) using the approach outlined previously described (Davis, Brit, Davis YT. Enumeration of Fetal Red Blood Cells, Cells, and F-Integrin Cells in Human Blood. In: Current Protocols in Cytometry, Unit 617, Editors Robinson Jr., Ofer A., John Wiley and Sons, Inc., 2004)
- Specific gating and analysis approach shown in Figure 1.

3.4. Probability State Model with GemStone software:

- Method developed by author (BD) based upon principles used by expert analyst
- Data files analyzed independently of "expert" with minimal adjustments to sub-population regions

4. Results

4.1. Probability State Modeling (PSM) uses a set of expression profiles for one or more listmode parameters to assign events to states and cell types probabilistically. The PSM can be designed to mimic subjective sub-division of cells in flow analysis. The operator visually examines intensity and flow spread of anti-HbF for a FITC high control cell (A ~ 5% fetal cells) and makes a sample intensity estimate for the Adult RBC population (SS, HbF negative cells, blue population). The model automatically adjusts for the RBC intensities of the F Cells (C, green population) and Adult RBCs (red population) to reach a consensus that can occur in an FMH sample. The expression profiles in the models used in these studies are designed to allow, allowing the modeling process to assign events to the most probable cell type. This provides for a more reproducible analysis from operator to operator, and sets the stage for future total automation of the data analysis.

4.2. A Probability State Model (PSM) uses a set of expression profiles for one or more listmode parameters to assign events to states and cell types probabilistically. A PSM can be designed to mimic subjective sub-division of cells in flow analysis. The operator visually examines intensity and flow spread of anti-HbF for a FITC high control cell (A ~ 5% fetal cells) and makes a sample intensity estimate for the Adult RBC population (SS, HbF negative cells, blue population). The model automatically adjusts for the RBC intensities of the F Cells (C, green population) and Adult RBCs (red population) to reach a consensus that can occur in an FMH sample. The expression profiles in the models used in these studies are designed to allow, allowing the modeling process to assign events to the most probable cell type. This provides for a more reproducible analysis from operator to operator, and sets the stage for future total automation of the data analysis.

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4.4. Model development by author (BD) based upon principles used by expert analyst

4.5. Flow cytometric data analysis is performed using WinList. The principal of analysis are to gate on red cells, excluding aggregates, and to include any nucleated cells (lymphocytes and nucleated red cells, identified by F- staining), then identify fetal RBCs by high hemoglobin (Hb) content, Adult RBCs by the absence of Hb, and fetal cell by the intermediate level of Hb.

5. Benefits of Probability State Modeling

5.1. Improved algorithms for the analysis and classification of HBF-containing Red Blood Cells

6. Conclusions

6.1. Probability State Modeling provides equivalent results to manual data analysis with standard listmode analysis software by highly experienced individuals for fetal RBC analysis, a form of rare event analysis.

6.2. The GemStone PSM protocol requires no or minimal adjustment of gate or regions, providing a high likelihood that PSM analysis could be done in an automated format, thereby removing an important source of inter-individual variability in the current method of data analysis.

6.3. PSM analysis by GemStone and the improved, no-wash method of FMH QuikQuant together promise to further improve the accuracy, precision, and technical simplicity of fetal RBC detection in clinical flow cytometry.

6.4. Further study is warranted to further improve the GemStone protocol for data analysis of clinical samples and document the degree of improved performance over current manual methods of flow cytometric data analysis.