Comparison of Quantitative Acid-elution Technique and Flow Cytometry for Detecting Fetomaternal Hemorrhage*

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ABSTRACT

A study of the Kleihauer-Betke acid-elution technique for quantitating fetomaternal hemorrhage was performed to assess intra- and inter-technologist accuracy and precision as well as to delineate the statistically valid domain of the test as usually performed. The results were then compared to a parallel study quantitating fetomaternal hemorrhage by flow cytometry. Additionally, a statistical model for estimating efficacy of treatment with Rh immune globulin in the prevention of pregnancy-associated Rh(D) isoimmunization was developed. The results indicate that the acid-elution technique can be performed in a reproducible manner with acceptable accuracy and precision with whole blood fetomaternal hemorrhages 25 ml and higher if a background correction for false positive identification of fetal cells is included. Flow cytometric determination reveals significantly increased accuracy in comparison to the corresponding Kleihauer-Betke results.

Introduction

Without maternal prophylaxis with Rh immune globulin (abbreviated RhIG), the overall risk of maternal Rh(D) isoimmunization is 15 percent with an Rh(D)-positive ABO compatible fetus (1).

Immunization results from fetomaternal hemorrhage (FMH). Routine postpartum maternal prophylaxis within three days of delivery reduces the rate of immunization to approximately two percent of those women at risk.1 Routine use of both antenatal and postpartum RhIG prophylaxis further reduces the incidence of Rh immunization.2

In the United States, the routine prophylactic postpartum dose of RhIG is 300 micrograms. Experiments performed on Rh negative adult volunteers injected
with Rh positive adult red cells suggest that this standard dose of RhIG provides adequate protection against isoimmunization from a volume of approximately 30 ml of Rh positive whole blood.11 This corresponds to 0.6 percent Rh positive cells in an adult with a five liter blood volume.

The overall incidence of Rh immunization with the routine use of both antenatal and postpartum prophylaxis is approximately one in 400.3 This figure is at least in part attributable to the fact that approximately one in 300 to one in 4301,3 women have a greater than 30 ml FMH. Thus, a reduction in the rate of Rh immunization can be effected if a sensitive and reliable method for the recognition of FMHs in excess of 30 ml was employed.

There are a variety of techniques in general use for the quantitation of the magnitude of FMH.12 In the USA, the Kleihauer-Betke (KB) test or some variant of it is in wide use. Often the KB test is combined with another technique to increase reliability. However, there are few studies that have evaluated the reliability of the KB technique.5,6,7

In this paper, a study of the KB test was undertaken so that the statistically valid domain of the test could be delineated and intra- and inter-technologist reproducibility and bias assessed for the test as typically performed. The results were then compared with those of a parallel study quantitating FMH by flow cytometry.4,8 The study was carried out by determining the percent fetal cells in artificially prepared mixtures of fetal/adult erythrocytes.

Material and Methods

Fetal-Adult Erythrocyte Mixtures

The Rh negative red cells were collected by venipuncture from a consenting adult volunteer. Umbilical cord Rh positive red cells, collected with parental informed consent, were used as a source of fetal cells. The red cells were stored at 4°C in ACD (Trisodium citrate, 22 g per l; citric acid, 8 g per l; dextrose, 24.5 g per l, adjusted to pH 7.1 with 1M NaOH). Fetal/adult erythrocyte mixtures with the following percentages of fetal cells were prepared: 1.0, 0.5, 0.1, 0.01 and 0.001.

"Kleihauer-Betke" Technique

In these determinations, commercial reagents* were used with eluting solution consisting of alcoholic ferric chloride/hematoxylin at pH 1.3 to 1.6 and staining solution of erythrosin. It should be noted that this is the Nierhaus10 modification of the standard KB technique which allows simultaneous elution and hematoxylin staining in contrast to the standard technique where these are separate steps. Additionally, the eluting solutions are different (viz, citrate-phosphate buffer at pH 3.5 in the standard technique). With these techniques, cells with hemoglobin F stain dark pink and appear refractile while cells with hemoglobin A are recognized only as pale "ghosts" as with the standard technique.

In this paper, the modified test will be henceforth referred to as the "KB test".

The protocol was as follows: standard manual blood smears were prepared and air dried 20 minutes, fixed in 80 percent ethanol for five minutes, rinsed immediately in running tap water, and allowed to dry. The slides were then placed in eluting solution for 20 seconds, rinsed with running tap water, and then air dried.

Only the three most concentrated artificially prepared fetal/adult erythrocyte

* Fetal hemoglobin elution method, Boehringer Mannheim Diagnostics, catalog number 124249.
mixtures were counted with the KB technique because of statistical limitations on the lower level of sensitivity of the method as usually performed. Four of the five dilutions were tested by flow cytometry.

Each of four technologists, experienced in performing the KB test, received nine coded blood specimens consisting of three different aliquots of the three most concentrated fetal/adult mixtures. Each technologist made a KB slide as previously described for each of the nine samples, and counted the fetal and adult cells in five high-power fields (dry 40 x objective, 10 x ocular). Additionally, three coded aliquots of pure adult cells were distributed and the same counts performed to serve as controls for determining background counts. The experience of the technologists ranged from three to eight years with a mean of six years experience in the performance of the test at least once a month.

Calculation of theoretical sensitivity limits of the KB technique was carried out using the Poisson distribution. The theoretically appropriate statistical description of the KB technique is actually the hypergeometric distribution, but because the number of cells counted is so much smaller than the number of cells present in the actual preparation, the binomial distribution is a good approximation. Since the fetal/adult mixtures are so dilute, the binomial distribution is well-approximated by the Poisson distribution in this region.

Flow Cytometry Immunoassay

The immunoassay is a double antibody technique in which the artificial fetal/maternal erythrocyte mixture is first incubated with anti-D antiserum* and then allowed to react with 0.76 micrometer fluorescent polymer immunospheres† to which goat anti-human IgG‡ was covalently linked. The sample was then analyzed by flow cytometry.§ Data analysis to determine the percentage of fetal cells was performed using locally developed software.‖ Complete experimental details of this procedure will be presented elsewhere.¶

Results

Background Counts

Background count refers to the number of erythrocytes perceived as fetal in a KB smear prepared exclusively from adult cells. These "false positives" occur in part, because the appearance of cells represents a continuum from the ideal adult "ghost" to the ideal dark pink, refractile fetal cell. In the usual smear prepared from adult cells, the background count is very low. This background will also be present in fetal/adult mixtures and thus could play an important role in determining the lower level of sensitivity as well as the accuracy of the method. The best background control would be late prepartum maternal blood free of fetal cells; however, the frequent occurrence of prepartum FMH makes this specimen generally unavailable. Pregravid maternal blood could be considered, but there are obvious difficulties with this approach. Because of the previously-cited constraints, pure adult red cells were used as a background control.

The background rates for the four technologists were 0.041, 0.014, 0.174, 0.016.

* Special preparation from Ortho Pharmaceuticals, Raritan, NJ.
† "MX" type, "green", Covalent Technology, Ann Arbor, MI.
‡ Cappel Laboratories, Cochranville, PA.
§ EPICS V multiparameter cell sorter, Coulter Electronics, Hialeah, FL.
‖ "ROMP"—Rochester Multiparameter Analysis System.
and 0.045 percent fetal cells, respectively. There is an order of magnitude difference between the extremes.

The use of the background correction was indeed found to improve the accuracy of the KB technique. This will be described in further detail in the section entitled “Flow Cytometry Results and Comparison with KB Results”.

**ACCURACY, BIAS AND PRECISION**

The number of maternal and fetal cells detected in five high power fields for each of the three slides at each of the three dilutions for each technologist is presented in table I. The mean percentages of fetal cells (averaged over the 15 high power fields) for each of the three concentrations for each of the four technologists were calculated and background corrected by subtraction of the appropriate background rate from the observed rates. These data are presented in table II and reveal that the corrected rate of fetal cell identification of technologist three at the 0.1 percent concentration is actually negative. This is a result of his relatively high background correction. Additionally, the entries in parentheses represent an estimate of technologist bias which is defined as the difference between the corrected rate and true rate expressed as a percentage of the true rate. Bias thus refers to the systematic counting error relative to the true rate.

In table II it is shown that the magnitude of bias generally decreases with increasing fetal cell concentration (except for technologist 3); so that, bias at the 0.1 percent level ranges from approximately —10 to 60 percent, at the 0.5 percent level 10 to 20 percent, and at the 1.0 percent level 5 to 10 percent. Technologist 3 generally had the most bias, and consistently undercounts, at least in part, because of his high background rate. Technologists 1, 2, and 4 appear to be similar in regard to accuracy.

In order to study the precision of the determination, the standard deviations of the corrected percentage of fetal cells were calculated based on the data from the 15 high power fields for each technologist at each dilution and are presented in table III. Additionally, the entries in parentheses are the corresponding standard deviations as a percentage of the actual frequency of fetal cells present. This is similar to a coeffi-

### TABLE I

<table>
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TABLE III
Technologist Precision Expressed as the Standard Deviation of the Background-Corrected Percentage of Fetal Cells Calculated Based on the Data from the 15 High Power Fields for Each Technologist at Each Dilution

<table>
<thead>
<tr>
<th>Technologist</th>
<th>Percent Fetal Cells</th>
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<tr>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
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</table>

The numbers in parentheses are the standard deviations as a percentage of the actual frequency of fetal cells present.

The numbers in parentheses are the standard deviations as a percentage of the actual frequency of fetal cells present.

icient of variation. All of the technologists appear similar with respect to precision.

These data show that precision generally increases with increasing fetal cell concentration. However, there appears to be a threshold phenomenon occurring somewhere between the 0.1 percent and the 0.5 percent fetal cell concentrations above which there is little improvement in precision. The variability at the 0.1 percent level is two to three times that at the threshold level.

It should be noted that the experimentally observed coefficients of variation are consistently three to four times greater than those predicted by simple Poisson analysis. This is most likely a consequence of the actual, non-ideal Poisson nature of the KB technique with its associated laboratory and perceptual uncertainties.

VARIATION IN MICROSCOPIC FIELD CELL DENSITY

To assess further the intra- and inter-technologist variation in performance of the test, a three-way analysis of variance was carried out on the total number of maternal cells in each high-power field with regard to technologist, dilution, and field number. All two-way interactions were also included and, as is customary for analysis of such data, a square root transformation was used to stabilize the variance. The analysis resulted in a value of 78.4 percent for R-square (coefficient of determination). The analysis revealed a systematic variation in the number of maternal cells per field among the four individual technologists (F-ratio 111.42, p-value 0.0001) and also a smaller but possibly more interesting, systematic variation among the three dilutions (F-ratio 15.52, p-value 0.0001).

The nature of these variations can be more clearly seen in figure 1 which is a plot of the mean maternal cell count for each dilution versus technologist. It suggests a striking consistency in the number of cells per field counted by each individual technologist and to a lesser extent a smaller systematic dilutional effect in the number of cells per field chosen for counting. The consistency of an individual technologist's counting is not surprising owing to the individual nature of interpretation of the criteria for choice of fields for counting. There does not appear to be a well defined trend in the systematic variation in the number of maternal cells per field with regard to each individual technologist's dilutions; however, a slight tendency for all four technologists to count more densely packed fields as dilution increases seems apparent.

LOWER LIMIT OF SENSITIVITY

In order to estimate the lower limit of sensitivity of the KB method, the probability of detecting a difference between a relatively low nominal rate of, for example, 0.09 percent fetal cells and a representative background rate of 0.04 percent was calculated.

Poisson analysis indicates that counting five high power fields yields a proba-
Corsetti, Cox, Leary, Cox, Blumberg, and Doherty

Flow Cytometry and Comparison with KB Results

In Table IV are shown the KB and flow cytometry results for the percentage of fetal cells in the mixtures along with the experimental error. Also included (column 4) is the average magnitude of error for the KB technique. This is the average over the four technologists of the absolute value of each technologist’s corrected percent error (from Table II) for each dilution and is a reasonable measure of the percentage error in the results of any single technologist. These are the values that should be used in making comparisons between the KB and flow results since the other reported percentage errors of the KB technique listed in Table IV (columns 2 and 3) were based on data from all four technologists and as such are not representative of the results of any single individual technologist. They are included only to demonstrate the significant improvement in the KB results using the background correction.

At the 1.0 percent level, the flow results have an experimental error of 2.2 percent which is approximately a fifth of the average magnitude of error of the KB technique at the same level. At the 0.1 percent level the experimental error of the flow technique is still only 4.3 percent, whereas that of the KB is 54.6 percent. It is only at the 0.001 percent level that the flow technique exhibits significant experimental error of 46.3.

Discussion

To reduce the incidence of pregnancy-associated maternal Rh isoimmunization to its lowest possible value, a quick, dependable test to determine FMH in excess of that effectively treatable with the standard 300 microgram dose of RhIG is necessary. The KB test has been commonly used for this purpose, but there has been surprisingly little experimental data to support its use. This study is such an attempt and, in general, the following may be concluded. First, there is significant increase in accuracy with the use of the background correction. Second, the test can be performed in a reproducible manner with acceptable accuracy and precision at the level of approximately 0.50, and counting ten high power fields a probability of approximately 0.70, of being able to detect a difference between 0.09 percent and 0.04 percent fetal cell mixtures. Thus, counting reliability below 0.1 percent is very poor.
ACID-ELUTION VS. FLOW CYTOMETRY FOR FMH

TABLE IV

Comparison of the Percentage of Fetal Red Cells in Artificially Prepared Fetal/Adult Red-Cell Mixtures and Percent Error as Determined by the KB Technique and Flow Cytometry

<table>
<thead>
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</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.077 ±7.7</td>
<td>1.011 ±1.1</td>
<td>11.2</td>
<td>1.022 ±2.2</td>
</tr>
<tr>
<td>0.5</td>
<td>0.629 ±25.8</td>
<td>0.561 ±12.2</td>
<td>12.6</td>
<td>ND</td>
</tr>
<tr>
<td>0.1</td>
<td>0.160 ±60.0</td>
<td>0.092 ±8.0</td>
<td>54.6</td>
<td>0.104 ±4.3</td>
</tr>
<tr>
<td>0.01</td>
<td>ND</td>
<td>--</td>
<td>--</td>
<td>0.0113 ±12.7</td>
</tr>
<tr>
<td>0.001</td>
<td>ND</td>
<td>--</td>
<td>--</td>
<td>0.000537 ±46.3</td>
</tr>
</tbody>
</table>

The KB results were averages over the four technologists without and with background correction. The average magnitude of error was calculated by taking the average of the absolute value of each technologist's corrected percent error over the four technologists (from Table II) for each dilution.

of FMH requiring more than the standard dose of RhIG. Third, the accuracy and precision of the test is unacceptable at the 0.1 percent level as demonstrated by both theoretical and empirical results.

Inspection of table IV reveals that at the level of FMHs of interest (greater than 0.1 percent fetal cells), the flow cytometric results statistically approach the ideal test. However, the adequacy and relative ease of performance of the KB test along with the relatively complex sample preparation and sophisticated instrumentation necessary for flow cytometry make its use for this application somewhat impractical at the present time. However, the technique is ideally suited for a number of research applications, some of which include the quantitation of FMH throughout pregnancy, survival studies of fetal red cells in postpartum mothers treated with RhIG to determine mechanism of action, and quantitation of Rh antigen on red cells.

Appendix

A Statistical Model to Assess Efficacy of Treatment

In order to relate the previous results to the clinical prevention of pregnancy-related Rh isoimmunization using the KB test, estimates of several parameters were made. The first was an estimate of the proportion of FMHs greater than 0.6 percent fetal cells (those hemorrhages not adequately treatable with one standard dose of RhIG). Fitting and extrapolation of the data of Ness9 indicate that approximately 0.6 percent of women at risk for pregnancy associated Rh isoimmunization have FMHs greater than 0.6 percent. In spite of the absence of any FMH greater than 0.6 percent fetal cells in the series of Ness, the extrapolated value of 0.6 percent is well within the range of previous studies on large populations as reviewed by Zipursky14 where 0.2 to 0.7 percent of women at risk have FMHs greater than 0.6 percent fetal cells.

The second and third parameters estimated were the proportion of women who need a second dose of RhIG and receive it (N-R); and the proportion of women who do not need a second dose of RhIG but receive it (NN-R). These two determinations were made as a function of the percentage of fetal cells used as a cutoff for determining therapy with a second dose of RhIG. The results are given in figure 2. The behavior of these parameters is due to the size distribution of FMHs and the Poisson nature of the measurements. A description of the sta-
Efficacy of treatment with a second standard dose of RhIG as a function of the level of fetal cells in the maternal circulation used as a cutoff for treatment. The abscissa is given as the percentage of fetal cells in the maternal circulation used as cutoff. (A) Ordinate is the percentage of women who need a second dose and receive it—(N-R). (B) Ordinate is the percentage of women who do not need a second dose and receive it—(NN-R).

**Figure 2.**

The statistical methodology used to generate these results may be found in the section entitled “The Model”.

Inspection of figure 2A reveals the intuitively expected increase in women needing and receiving RhIG (N-R) with decreasing cutoff value. Moreover, it is demonstrated in figure 2A that as the cutoff value decreases there is an almost linear increase in the value of N-R to a plateau level occurring at a cutoff of approximately 0.46 percent fetal cells. Likewise, inspection of figure 2B demonstrates the expected increase in women not needing and receiving RhIG (NN-R) with decreasing cutoff. That is, figure 2B reveals low values of NN-R down to a threshold value of approximately 0.71 percent fetal cells below which there is a rapid increase in the value of NN-R.

The following is an example of the use of this model in determining the efficacy of treatment with a second dose of RhIG as a function of FMH cutoff level that could prove useful in the optimization of treatment. That is, from figure 2, the commonly used 0.6 percent FMH cutoff yields an estimate of 86 percent for the proportion of women who need and receive a second dose of RhIG (N-R), and 0.15 percent for the proportion of women who do not need a second dose of RhIG but receive it (NN-R). These values of N-R and NN-R may not be optimum with regard to broad treatment goals. In light of the apparent relative lack of deleterious effects associated with the use of RhIG, a decrease in the treatment cutoff level to increase the proportion of women who need RhIG and receive it might be more desirable. Inspection of figure 2 reveals a better treatment cutoff level might well be 0.46 percent giving N-R of 99 percent and NN-R of 0.68 percent.

The results are based on the assumption of a technique for FMH quantitation possessing perfect accuracy and precision. This is clearly not the case for the KB test. In tables II and III it is shown that at the optimum cutoff concentration of 0.46 percent fetal cells, the KB technique overcounts by about 15 percent and has a variability of approximately 33 percent. The tendency for overcounting can be considered a built-in safety feature. However, this level of variability implies that a proportion of hemorrhages
determined by the KB technique as less than the cutoff value, and therefore not requiring extra RhIG, will correspond to hemorrhages greater than the cutoff value. So as not to miss these cases with the KB technique, the cutoff value for therapy with extra RhIG would be lowered. Previous workers recognized this problem and attempted to deal with it by simply doubling the dose of RhIG calculated from the KB results. As the following calculation demonstrates, this turns out to be reasonable procedure. Doubling the dose of RhIG is approximately equivalent to using one-half the idealized optimum value of the cutoff value which in this case would result in a cutoff of 0.23 percent. Assuming a normal distribution and a variability of about 50 percent at this level, the probability of a greater than 0.46 percent FMH is

\[ P_N = 1 - \left( \frac{\mu}{\mu + 1} \right)^n \exp \left( -\frac{m}{\mu} \right) \sum_{k=0}^{m-1} P(k,m) \left[ 1 - \left( \frac{\mu}{\mu + 1} \right)^m - k \right] \]

where \( P(k,m) = \frac{m^k k!}{e^m} \) are Poisson probabilities and \( m = 39.0 \). Similarly, the probability that an at risk woman needs but does not receive therapy is

\[ 1 - \left( \frac{\mu}{\mu + 1} \right)^m - P_N. \]

From these two, the remaining probabilities can be calculated by subtraction.

The next step in this analysis is to calculate the probability that a woman at risk who does not need therapy does not receive it. The decision was assumed to be based on a Poisson random variable with mean \( x \), determined by the mother and sampled from the mixed exponential distribution fitted previously. For a given decision point of \( m \), therapy was given if the observed Poisson variate exceeded \( m \). Using these assumptions the above probability can be shown to be equal to

Using this model, the probability that a woman at risk does not need therapy, assuming a cutoff of \( m = 39.0 \), is

\[ 0.892 + 0.108 \times \int_0^{m_0} \frac{1}{\mu} \exp \left( -\frac{x}{\mu} \right) dx = 0.994 \]

The Model

Data were available from Ness et al\(^{12}\) on the distribution of FMHs for at risk mothers (mothers who delivered Rh-positive infants). The first step was to model this frequency distribution as a mixed probability distribution, having both a discrete and continuous component. It was assumed that \( \Pr(0 \text{ ml}) = 0.892 \) and the remaining probability was assumed exponentially distributed having density, \( 0.108/\mu \exp(-x/\mu) \), where the value of \( \mu \) was chosen to provide the best fit to the observed data. The value of \( \mu = 13.5 \) gives 6.7 percent and 2.55 percent for the intervals (0,13) and (14,26) as compared to 7.0 percent and 2.7 percent, respectively, and was judged adequate for the amount of data.

Acknowledgments

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References


