

Development of the acid elution method (Kleihauer test) in the assessment of fetomaternal hemorrhage

Dezvoltarea metodei elutiei acide (testul Kleihauer) pentru evaluarea hemoragiei feto-materne

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Abstract

Fetomaternal hemorrhage (FMH) is the physiopathological mechanism involved in the hemolytic disease of the newborn (HDN). Its assessment is necessary in order to adjust the dose of anti-D immunoglobulin administered to Rh-negative pregnant women with incompatible pregnancy. Since no assessment test is currently employed in Romania, we propose the acid elution test. Material and method. The paper presents the stages involved in this method's implementation. After establishing the method and the research batches, the test was performed on samples from healthy adults and Rh D positive newborns. An ANOVA variance test was subsequently performed. Results. The percent of fetal erythrocytes in negative controls allows the use of this sample in order to apply the proposed method. The statistically insignificant difference in variance between the samples in the positive control batch shows variance equivalence between the numbers of detected fetal erythrocytes. Conclusion. The analyzed batches allow initiating the procedures in order to apply the proposed method and assessing its parameters (sensitivity, specificity).

Keywords: hemolytic disease of the newborn, fetomaternal hemorrhage, acid elution, F hemoglobin

Rezumat

Hemoragia feto-maternă (FMH) constituie mecanismul fiziopatologic în boala hemolitică a noului născut (BHNN), iar evaluarea ei este necesară pentru adaptarea dozelor de imunoglobulină anti D administrate gravidei Rh negativ cu sarcină incompatibilă. Deoarece în România nu se practică în prezent nici un test de evaluare, propunem testul eluției acide. Material și metodă. Lucrarea de față prezintă etapele standardizării acestei metode. După stabilirea metodei și a loturilor de cercetat s-a efectuat testul pe un număr de eşantioane provenind de la adulți sănătoși și nou născuți Rh D pozitiv. Testarea s-a realizat prin aplicarea testului de varianță ANOVA. Rezultate. Procentul de hematii fetale din probele de control negativ permite utilizarea acestui eşantion

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pentru aplicarea metodei. Diferența ne semnificativă statistic între probele recoltate pentru lotul de control pozitiv indică echivalența de varianță între valorile numărului de hematii fetale detectate. Concluzie. Loturile analizate permit inițierea procedurilor de aplicarea a metodei propuse și de evaluare a parametrilor săi (sensibilitate, specificitate).

Cuvinte cheie : boala hemolitică a noului născut, hemoragia feto-maternă, eluția acidă, hemoglobina F

Introduction

The first technique of tracking fetal cells in maternal circulation was developed by Kleihauer, Braun and Betke in 1958 and it continues to be the reference method in assessing FMH (1). This test helped understand the mechanisms that lead to alloimmunization in pregnancy and provides useful data for prophylaxis and treatment strategies in HDN. Several variants of this method are used in current practice, including a rapid technique which uses commercially available kits and an automated cell counting technique (2 – 6).

After studying the specialty literature and international regulations and taking into account our laboratory's expertise and technical base, we chose the classic method proposed by Mollison in 1972, which in turn is based upon both his personal experience and the original method elaborated by Kleihauer and Betke in 1960 (7).

The aim of this paper is to present the stages of the proposed method's standardization: establishing the method, the working samples, the control batches and the method of statistical analysis that allows limiting the method's error in order to attain a valid technique.

This method will be validated in our immunohematology laboratory and compared to the gel agglutination assay (as a "golden standard") by using ID-FMH Screening –Test (8) reagents.

Material and method

The test makes use of the fact that F hemoglobin (HbF) is resistant to acid elution while A hemoglobin (HbA) is not. If a fixated

thin layer blood smear is immersed in an acid buffer solution, adult erythrocytes will lose their hemoglobin in the buffer and keep only their stroma, while fetal erythrocytes will remain intact. The percent of fetal red blood cells in maternal blood is used to calculate the volume of the FMH.

Sampling

Samples will be harvested on EDTA or ACD through the usual phlebotomy technique and following the laboratory's standard operating procedure.

Test tubes are visually inspected on arrival at the laboratory. Samples with blood clots or hemolysis are not accepted for processing since they yield false positive or negative results. Similarly, if the test tubes are faulty new samples will be needed. It is recommended that samples reach the laboratory in less than 2 hours since harvesting. Test tubes will be labeled according to the standard operating procedures of the laboratory. The time allotted to performing the technique and conveying the results has to be adequate in order to allow the administration of supplementary anti-D IgG in case this should prove necessary (less than 72 hours after birth or any risk event).

1. Test samples: 3 ml of whole blood sampled from Rh D negative or positive pregnant women or women lately confined, at no more than 2 hours after giving birth or after an event that could induce FMH

2. Control samples

- 1-2 ml of cord blood from a Rh D positive newborn
- 3 ml of blood from an adult male ABO identical with the cord blood, Rh negative.

Materials and reagents

Distilled water, isotonic saline solution, dibasic sodium phosphate, 0.1 M citric acid, 80% ethyl alcohol, DiaQuick Panoptic reagents (Reagent Ltd., Budapest, Hungary), laboratory glassware and consumables.

Apparatus

Microscope with cell counting device, centrifuge, automated pipettes with adjustable volume, analytic scales, 37° C thermostat.

Phosphate buffer preparation

36 ml of 0.1 M citric acid and 14 ml of dibasic sodium phosphate are mixed in a volumetric flask. pH is measured and adjusted to the critical value of 3,2-3,3 by adding citric acid, if it is too elevated, or dibasic sodium phosphate if it is too low. The buffer will be kept at 4° C.

Blood smear preparation

Positive (artificial mixes of adult male blood and cord blood of the same type) and negative (blood from repeat donors) control smears are prepared. All smears will follow identical processing.

Elution and staining

- smears are fixated by immersion in an Erlenmeyer flask which contains 80% ethyl alcohol for 5 minutes and are subsequently air-dried
- fixated smears are immersed in another Erlenmeyer flask containing phosphate buffer at 37° C for 5 minutes
- smears are washed with distilled water and are left to dry
- the staining procedure is as follows:
 - smears are immersed in an Erlenmeyer flask containing DiaQuick Panoptic red solution for one second; this step is repeated 10 times
 - smears are immersed in an Erlenmeyer flask containing DiaQuick Panoptic blue solution for one second; this step is repeated 5 times
 - smears are washed with distilled water and left to dry for further examination.

If the smears have been properly processed, they should meet the following criteria:

- complete elution of HbA makes adult erythrocytes easy to identify
- fetal erythrocytes are intensely stained
- leucocytes are easy to differentiate and cannot be mistaken for fetal erythrocytes
- presence of artifacts is minimal.

Screening test

- 25 fields are examined at 400x magnification;
- if no fetal cells are found the result is negative;
- if a single fetal erythrocyte is encountered quantitative assessment will follow.

Quantitative assessment

In order to enhance the precision of the calculations, at least 2000 cells will be counted using 200x or 400x magnifications.

Calculation

Before calculating the absolute count of fetal erythrocytes the following must be taken into account (15):

- fetal red blood cells are 22% larger than adult ones, thus the volume will be higher than indicated by the erythrocyte count;
- only 92% of fetal erythrocytes stain;
- some adult erythrocytes have a certain degree of HbF preservation;
- a total volume of 5000 ml of maternal blood is arbitrarily considered;
- maternal total erythrocyte mass (in a 5000 ml volume) is 1800 ml.

The fetal bleed (VFEM) should be calculated as follows:

- Uncorrected volume of bleed =
= 1800 x FE / AE
- Corrected for fetal volume =
= (1800 x FE/AE) x 1,22 = J
- Corrected for staining efficiency =
= J x 1,09 = fetal bleed,

where VFEM = Volume of fetal erythrocytes in maternal circulation (ml), AE = Adult erythrocytes count, FE = Fetal erythrocytes count.

Limitations

It is recommended that the samples are kept at +4° C during transportation in order to preserve their quality. The following technical aspects are extremely important:

- temperature, pH of the buffer solution and concentration of ethyl alcohol must be carefully observed as they all are critical points
- blood may be diluted 1:3 with saline to ensure thin blood smears
- samples will be very well mixed for at least 10 minutes before processing
- samples can be preserved at most for a week at +4° C prior to smear preparation
- glassware must be clean and grease-free
- glass slides will be dried in a vertical position, with the thin side up.

Control samples will be examined first to ensure that preparation and staining were well performed; if the controls are not satisfactory the whole process will be repeated.

Precautions

- during the process ethyl alcohol will be kept away from any source of heat since it is flammable
- phosphate buffer is toxic upon inhalation, contact with integument or mucosae or accidental ingestion, and as such it is to be treated considering all safety regulations of the laboratory
- ethyl alcohol is irritating for the eyes
- DiaQuick Panoptic reagents are toxic upon accidental ingestion or eye contact.

Establishing research groups

Acid elution test was performed on two batches:

a) Negative control batch consisting of male and female blood donors; this batch was used in order to assess normal HbF values in adults in the geographical area in which the study was performed. Samples were selected from repeat donors who meet the legal criteria for donating blood.

The negative control batch was divided

into two categories:

A. Women – 50 samples

B. Men – 50 samples

3 blood smears were prepared out of each sample, processed and examined at 400x magnification to observe possible persistence of HbF. Only those erythrocytes with a homogenous pink color were interpreted as F erythrocytes, while the intermediary ones were interpreted as A erythrocytes.

b) Positive control batches

Artificial samples were prepared by mixing cord blood with the same type - Rh D positive - adult male blood. Prior to mixing, blood samples were washed two times with saline solution.

Hematocrit (Ht) was determined for each sample in the first stage. Ht values were equalized to 33.5 through dilution with saline solution. Several mixes were subsequently prepared:

a) 0.2% = 10 µl cord blood + 5000 µl adult blood – 6 samples

b) 0.3% = 15 µl cord blood + 5000 µl adult blood – 6 samples

c) 1% = 10 µl cord blood + 1000 µl adult blood – 6 samples.

10 smears were prepared out of each sample and followed identical processing.

Results and discussions

Examination of blood smears (Figure 1)

- it is recommended that smears are examined in the regions where they are thinner and blood cells are uniformly spread, with small spaces between them and without overlapping
- fetal erythrocytes appear stained in red or dark pink, with a more conspicuous center
- only the membrane of adult erythrocytes is stained
- leukocytes appear with a blue-gray nucleus and a pale pink cytoplasm
- intermediary cells with partially stained

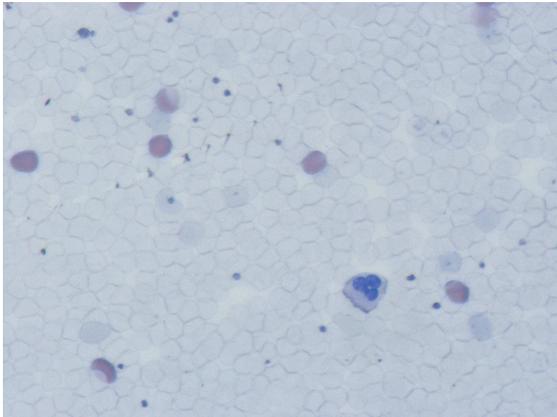


Figure 1. Blood smear obtained by Kleihauer method

hemoglobin (persistence of HbF) are considered adult cells.

The test is divided in two stages: screening and quantitative assessment.

In the negative control batch, no fetal erythrocytes were detected.

Results of positive control batches with known dilutions are presented in *Figures 2, 3, and 4*. Results assessed by using repeated measurements and ANOVA statistical test indicate the homogeneity of the measurements ($p = 0.9651$, $p = 0.9294$, $p = 0.9427$).

Although the proposed method is the oldest among FMH testing techniques, it has a very important contribution to HDN prevention.

This method is easy to perform, but it however does raise some difficulties related to

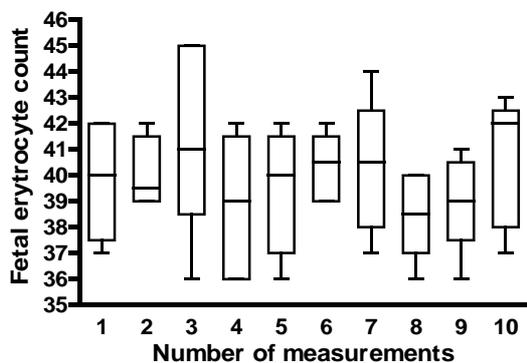


Figure 2. Number of detected fetal erythrocytes in 2:1000 dilution

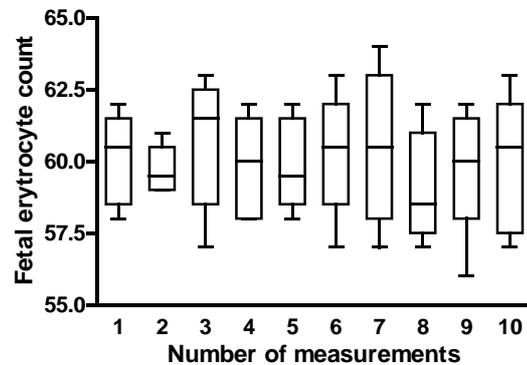


Figure 3. Number of detected fetal erythrocytes in 3:1000 dilution

the possibility of subjective smear interpretation, hereditary persistence of HbF in certain population and in diseases like thalassemia or drepanocytosis or sharp elevation of HbF levels in the last 2 months of pregnancy (9, 10). These difficulties can be easily overcome through the expertise of the laboratory technician and by participating in external quality control programs (11).

The absence of F erythrocyte-like red blood cells in the studied negative control batches indicates that these cannot represent a source of errors in the acid elution test in Rh negative pregnant women with incompatible pregnancies.

As far as the positive control batches are concerned, ANOVA statistical analysis shows a variance equivalence of the total F erythrocytes count in all 3 dilutions. In all three

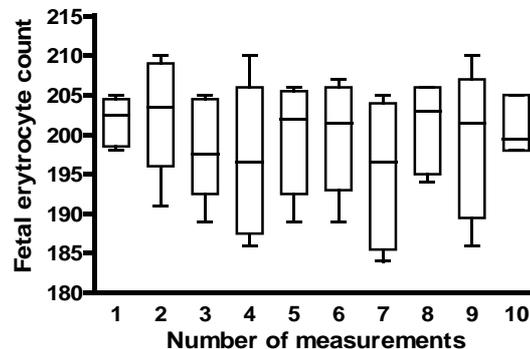


Figure 4. Number of detected fetal erythrocytes in 1:100 dilution

cases the values returned by the statistical test are greater than 0.05 (threshold significance value), a fact that indicates the lack of a statistically significant difference with regards to variance between studied batches.

Other than the diagnosis of FMH in pregnant or recently confined women, the acid elution method can be used:

- in newborn if a FMH is suspected (maternal erythrocytes in fetal circulation)
- in mildly positive Rh D recently confined women (who were Rh D negative prior to giving birth), because massive FMH may be suspected in this case
- if the newborn develops an unexpected anemia or intrauterine death
- in cord blood that was intrauterinely harvested through cordocentesis or from the amniotic fluid (2, 4, 6, 12).
- in monitoring the clearance of fetal RhD-positive red cells in FMH following RhD immunoglobulin administration (13).

The acid elution test has several major advantages:

- it is a simple and easy to implement method
- it is not limited by the presence or absence of D antigen, therefore it is useful in all forms of HDN
- it requires only usual laboratory hardware and it does not imply large costs, and as such it is affordable for any type of laboratory (3, 6).

No test for assessing FMH is currently in use in Romania, and the control of anti-D IgG efficiency through assessing passive anti-D is sporadic. In addition, since anti-D IgG is administered through a national program only after birth, most of the cases of FMH (or other risk event) during pregnancy remain undiagnosed and thus increase the risk of apparition of antibodies involved in HDN.

Conclusions

In conclusion, testing the values obtained by assessing the negative control batch

allows the use of such a selection in order to subsequently apply the test. The same conclusion can be formulated with regards to the positive control batch, on the background of establishing the parameters of the Kleihauer test (internal and external validity level).

In our opinion, the proposed test is precise and sensitive enough to track even a minute amount of fetal erythrocytes. Since its clinical usefulness is undeniable, this method can be used in Romanian medical practice.

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