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Comparison of two quantitative methods of microscopic feto-maternal haemorrhage evaluation**

Porównanie dwóch ilościowych metod mikroskopowej oceny krwawienia płodowo-matczynego

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Summary

Introduction. Quantification of feto-maternal haemorrhage (FMH) is essential for determination of an accurate dose of anti-RhD Ig in immunoprophylaxis of haemolytic disease of foetus and newborn in RhD negative mothers. The Kleihauer-Betke (K-B) test is one of some methods introduced to our laboratory for FMH investigation. In some countries it has been used routinely for many years, but in Poland only occasionally. K-B test is based on the different properties of haemoglobin F (HbF) in fetal red blood cells (RBCs) and haemoglobin A (HbA) in maternal RBCs. HbF is more resistant than HbA to both alkali denaturation and acid elution.

Material and methods. Dried and stained blood films were examined using two quantitative methods: in the 1st of them we counted twice (2 slides) foetal RBCs between 1000 maternal "ghost" cells, in the 2nd we evaluated at least 10 000 cells, using a Miller square. These methods were performed 10 times for each prepared mixtures with 0.1%, 0.25%, 0.5%, 1% and 2% of foetal RBCs in adult blood, imitating FMH.

Results. Coefficients of variation (CV) were appropriate in the 2nd method (< 20%) and estimated volumes of FMH were accurate. These values were not proper using the 1st method (CV about 30% to 64%).

Conclusions. K-B test can be the sensitive method for quantification of FMH if we assess at least 10 000 RBCs.

Key words: feto-maternal haemorrhage, Kleihauer-Betke test, immunoglobulin anti-RhD, haemolytic disease of foetus/newborn

Streszczenie

Wprowadzenie. Ilościowa ocena krwawienia płodowo-matczynego (FMH) ma zasadnicze znaczenie dla ustalenia odpowiedniej dawki Ig anty-RhD w immunoprofilaktyce choroby hemolitycznej płodu i noworodka u RhD ujemnych matek. Test Kleihauera-Betke (K-B) jest jedną z metod wprowadzoną do naszego laboratorium w celu badania FMH. W niektórych krajach jest on stosowany rutynowo od wielu lat, a w Polsce tylko wyjątkowo. Oparty jest na różnych własnościach hemoglobiny F (HbF) w krwinkach czerwonych płodu i hemoglobiny A (HbA) w krwinkach matki. HbF jest bardziej odporna na alkaliczną denaturację i kwaśną elucję niż HbA.

Materiał i metody. Wysuszone i zabarwione rozmazy krwinek czerwonych po denaturacji i kwaśnej elucji oceniano ilościowo dwiema metodami. W pierwszej liczono 2 x krwinki płodowe wśród 1000 „cieni" krwinek matki (dwa szkiełka), w drugiej oceniano co najmniej 10 000 krwinek stosując siatkę Millera. Postępowanie przeprowadzono po 10 razy dla mieszanin: 0,1%, 0,25%, 0,5%, 1%, 2%, krwinek płodowych w krwi osób dorosłych, które imitowały FMH.

Wyniki. Współczynniki zmienności (WZ) były odpowiednie w drugiej metodzie odczytu (< 20%) i obliczone objętości FMH odpowiadały rzeczywistym objętościom w dopuszczalnym zakresie. Te wartości nie były prawidłowe w pierwszej metodzie odczytu (WZ od ok. 30% do 100%).

Wnioski. K-B może być czułą metodą ilościową badania FMH, jeśli w odczycie ocenia się co najmniej 10 000 krwinek czerwonych.

Słowa kluczowe: przeciek płodowo-matczyny, test Kleihauera-Betke, immunoglobulina anty-RhD, choroba hemolityczna płodu/noworodka

INTRODUCTION

The pathogenesis of hemolytic disease of newborn (HDN) was discovered in the fourties of 20th century. HDN has usually affected RhD-(negative) women who gave birth to RHD+(positive) child. D antigen is strongly immunogenic and its distribution in Cauca-

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sian population [about 20% RhD-(negative) and 80% RhD+(positive) people] cause higher risk of HDN than any other antigen among several hundred known today. Parturition, last trimester and different irregularities of pregnancy and parturition can lead to increased feto-maternal leakage, especially that blood vessels of mother and child are very close to each other in placenta. If red blood cells carrying foreign antigens on their surfaces enter the women's circulation, her immunologic system may start producing IgG antibodies, which can pass through the placenta and destroy blood cells of foetus in subsequent pregnancy.

To provide effective prophylaxis of HDN all RhD-(negative) women, who gave birth to RhD+(positive) child should receive anti-RhD IgG immunoglobulin which neutralizes foetal cells and reduces the risk of sensitization. This prophylaxis was applied in the sixties, in Poland at the beginning of the seventies. However standard dose of anti-RhD IgG still has not been established and in different countries various standard dose are suggested in pregnancy and after complicated or non-complicated delivery. Also, there are several approaches to the evaluation of the size of FMH.

The first method of quantification of FMH was described in the fifties and is called the Kleihauer-Betke (K-B) test. In Poland this method is not in routine use, only one laboratory rarely perform it in the suspicion of large FMH. The procedure of the test is placed in Polish guidelines for blood banks.

Because we undertake investigation for quantification of FMH using different methods like microscopic, serological and flow cytometric, the aim of our study was to evaluate two ways of examination of stained films in K-B test: first way recommended by Polish and American procedures and second ordered by UK guidelines.

MATERIAL AND METHODS

Blood Samples

Adult donor's blood collected into EDTA was mixed with umbilical cord blood: blood count was done for each sample and then the dilution of foetal cells among adult cells was made to get artificial dilutions: 0.1, 0.25, 0.5, 1, 2%. For each dilution 4-5 solutions were performed and then 10 K-B tests were carried out.

The principle of the test

K-B test is used to measure foetal cells with haemoglobin F (HbF) among adult's cells with hemoglobin A (HbA). HbF is resistant for alkali denaturation and acid elution while HbA is removed from cells. After staining, adult cells without haemoglobin are seen as "ghost" cells, while foetal cells appear as rose-pink in color.

Performance of the test

Tests were performed using reagents ready for use (Fetal Red Cell Kit, Guest Medical UK) with the following procedure: 1) 150 μl of whole blood from the sample was mixed with the same amount of phosphate buffered saline; 2) blood smears (two for every examination) were prepared on glass slides and air-dried for 20 minutes in room temperature; 3) slides were fixed in ethanol (Fixing Reagent) for 2 minutes; 4) after being rinsed with tap water, slides were flooded with Eluting Reagent for 30 seconds and rinsed again; 5) slides were counterstained with erytrosin (Counterstain) for 3 minutes; 6) each slide was microscopically examined (10x eyepiece, 40x objective) using two methods of counting:

Method 1:

Foetal and adult cells were counted on two slides (1000 cells on each slide) and then the percentage of foetal cells was determined. From any foetal cell or one per 1000 (0,1%) to 20 (2%) was detected.

Method 2:

Foetal and adult cells were counted on two slides, but at least 5000 cells on each slide. For accurate quantification Miller square was used (fig. 1). This special microscopic eyepiece graticule divides power field into two squares: the small one is 9x smaller than the large one. Foetal cells were counted in the large square and adult cells were counted in the small one. Then the amount of adult cells was multiplied by 9 and it was the amount of adult cells in the large square – usually it was about 200. Screening of about 25 high power fields was enough to count 5000 cells on the slide (10 000 in one examination).

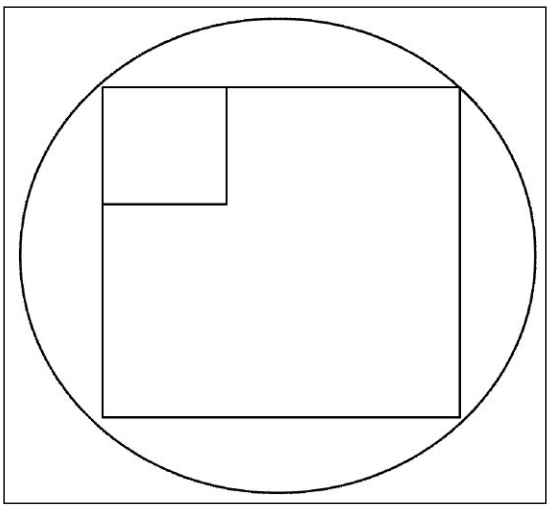

Fig. 1. The Miller square for the Kleihauer-Betke test.

Taking into account 10 examinations of each RBC mixture dilution the mean percentage of foetal cells, standard deviation (SD) and coefficient of variation (CV) were calculated using formulas:

$$SD = \pm\sqrt{\frac{\Sigma(xi - \bar{x})^2}{n - 1}}$$

$$CV = \frac{SD}{\bar{x}} \times 100$$

The following Mollison's formula was used to convert hypothetical percentage into milliliters of FMH where

1800 is average volume of RBC of adult women; coefficient 1.08 means that 92% of foetal cells stain darkly and 1.22 means that foetal cells are 22% larger than adult cells.

$$\text{FMH ml} = \frac{\text{\% blood cells x 1800 x 1.22 x 1.08}}{100}$$

RESULTS

All calculation are placed in table 1. The coefficient of variation in first method (2000 cells count) was high: from 64% and 50% for two lowest dilution (0.1 and 0.25%) of foetal cells to about 30% for other dilutions. In second method (10 000 count) CV was under 20%, except the 0.1 concentration (CV about 30%). The volumes of FMH calculated for K-B test results obtained by second method were more adequate to expected values and the range between maximum and minimum values were narrower in comparison to the first method.

DISCUSSION

Quantification of foetal red blood cells in maternal blood sample gives an information on the amount of foetal blood in maternal blood stream. This is very helpful to administer the appropriate amount of anti-RhD immunoglobulin to RhD-(negative) mother after delivery RhD+(positive) child, knowing that 20 μg of IgG neutralize 1 ml of RhD+(positive) cells. In Poland RhD-(negative) women get standard dose 150 μg anti-RhD IgG, which is enough to protect against 7,5 ml of RhD+(positive) cells. Double dose (300 μg) is recommended in clinical condition associated with potential placental trauma. Implementation of K-B test or other method for evaluation of FMH for all women would allow to adjust the dose of anti-RhD IgG to individual needs, which improve accuracy and safety of immunoporphylaxis. In UK all Rh-(negative) women after delivery are tested for FMH. This strategy allowed to decrease standard dose of anti-RhD IgG to 100 μg. In the case of volume of FMH higher than 4 ml, the K-B result is checked by flow cytometry and, if necessary the accurate dose of immunoglobulin is given. Such optimization of use of anti-D IgG gives benefits including saving this medicine (reduced dose in comparison to Poland) and providing better protection against immunization for women with large FMH.

The K-B test detects cells with haemoglobin F so can be used to assess FMH not only for RhD-(negative) women. It could be applied in the population-based study or to confirm the necessity of intrauterine transfusion when fetus is losing blood into mother's circulation. Some investigators contest the sensitivity of the K-B test and recommend the use of the method based on flow cytometry. Others claim that these tests are comparable, what our experiments (unpublished data) prove as well. Differences between K-B and FC could arise from various amount of cells taking under consideration. Foetal cells are searched usually between 50 000 cells in FC method while in K-B test between 2000 (first method of screening). Our experiment shows that if the second method of examination is used, and 10 000 cells are counted, the results are very close to the expected values. Application of Miller square enable to estimate the number of cells in microscopic field with satisfactory accuracy, although only 1/9 of the each field is analyzed. Calculated CV for second method of counting is less than 20% for nearly all of the dilutions, what is required for most of analytical methods. In the case of the lowest FMH, this factor was around 30%. This result can be concerned as adequate, since the differences of calculated foetus blood volumes (1.94, 2.77 and 3.6 ml) in the total volume of mother's blood (5000 ml) are clinically irrelevant. In all three cases the risk assessment as well as the amount of anti-RhD Ig dosage will be equal and the lowest of applied. In the case of the first method of counting, CV values were high, resulting in percentage spread of foetus RBCs in the total volume of mother's blood. Thereby, the difference of defined FMH was relevant.

Basing on our results, it can be concluded that the Kleihauer-Betke test with the number of 10 000 RBCs analyzed is sensitive and reliable for quantitative assessment of FMH. Low cost of reagents and availability of laboratory equipment are reasons for implementation of K-B test in most diagnostic laboratories.

Table 1. Results obtained from Kleihauer-Betke test, reading with two methods in blood mixtures imitating feto-maternal haemorrhage.

FMH mixtures		FMH evaluation			
		Method 1		Method 2	
dilution (%)	volume (ml)	volume (ml)	CV (%)	volume (ml)	CV (%)
0.1	2.4	3.50 ± 2.32	64.20	2.77 ± 0.83	28.83
0.25	6	6.29 ± 2.98	47.76	6.29 ± 0.97	15.48
0.5	12	10.08 ± 3.34	32.90	12.84 ± 1.53	11.80
1.0	24	25.18 ± 7.09	28.14	22.66 ± 3.39	17.23
2.0	48	39.70 ± 10.78	27.21	44.81 ± 48	10.70

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