Acid elution test for fetal hemoglobin (Kleihauer-Betke test, fetal cells test)

**METHOD**

Blood smears or smears of body fluid, after air drying and alcohol fixation, are immersed in a citrate phosphate buffer (pH 3.3) at 37°C or an alcoholic ferric chloride solution (pH 1.3-1.6) at 20-25°C. The preparations are then stained with hematoxylin and eosin and examined microscopically. The number of fetal cells (erythrocytes containing hemoglobin F), which appear deeply stained, are counted. The percentage of fetal cells is calculated based on the total number of red blood cells present. On specimens of maternal blood, the results are reported as the volume of fetal red blood cells. A formula for the conversion to volume is as follows:

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\text{Volume (ml) of fetal red cells} = \frac{2400 \times \# \text{of adult}}{\# \text{of fetal red cells in maternal blood}}
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**CLINICAL INDICATIONS**

The acid elution test for fetal hemoglobin (AET) was first described by Kleihauer et al. in 1957. The test is used to detect the presence of fetal red blood cells in the maternal circulation. This information is considered in the decision to administer Rho(D) immune globulin (RhIGG) in cases of suspected maternal Rh sensitization (positive micro D\(^u\) test on maternal blood). It is also used to assess the magnitude of fetal-maternal hemorrhage to determine the amount of RhIGG to administer. It is generally advised that one vial of RhIGG is sufficient for 15 ml of fetal red cells (30 ml whole blood).

The AET may be employed on cerebrospinal fluid to assist in the diagnosis of intracranial hemorrhage in the neonate. In those infants who have been transfused with adult blood prior to lumbar puncture, the percentage of fetal cells in the cerebrospinal fluid and peripheral blood may be compared. If the percentage of fetal cells is much higher in the cerebrospinal fluid, this suggests an intracranial hemorrhage. If the percentage of fetal cells in the cerebrospinal fluid is similar to that in the peripheral blood, it is more likely a traumatic tap.

Finally, the AET may be useful in distinguishing those forms of hereditary persistence of fetal hemoglobin (HPFH) in which all red blood cells show an increase in fetal hemoglobin, from beta thalassemia minor, where only a proportion of the cells are so affected.

**CLINICAL AND TECHNICAL LIMITATIONS**

The AET relies on the difference in resistance to acid elution of fetal and adult hemoglobin. Fetal hemoglobin (HbF) resists such treatment, remains intracellular, and erythrocytes containing HbF will take up a stain which is applied after acid
elution of a smear of blood or body fluid containing blood. In contrast, adult hemoglobin (HbA) is susceptible to acid elution and will dissolve out of cells, which are seen as pale "ghosts" after staining.

The percentage of HbF in blood varies normally with age. At birth, HbF constitutes 53 to 95% of total hemoglobin. It decreases after birth by approximately 3 to 4% per week. After age 2, the reference range for the percentage of HbF in peripheral blood is 0 to 2 percent. The fetus is able to synthesize adult hemoglobin to some degree prior to birth, but this factor is taken into account in the conversion formula noted earlier.

The presence of fetal erythrocytes in the maternal circulation has been reported at 9 weeks' gestation. The chance of finding fetal cells in the mother's blood increases as pregnancy progresses and with the number of examinations. An incidence of 28.9 to 50% by the third trimester of pregnancy has been found. Thus, transplacental passage of small amounts of fetal blood is a common event during normal pregnancy. Larger volumes of blood may enter the maternal circulation at the time of delivery and as a result of obstetrical manipulations, amniocentesis, or abortion. Some data indicates that transplacental hemorrhage of even small amounts of blood (less than 0.1 ml) can cause maternal Rh sensitization.

Demonstration of HbF-containing cells in maternal blood as direct evidence of fetal-maternal bleeds has been challenged by some investigators, who note that HbF may be of maternal origin. Mollison cites several studies that have shown that there is an increased production of HbF during pregnancy in normal subjects. The rise in the level of HbF begins at about the tenth week of pregnancy and continues until approximately week 32, with peak production occurring between the twenty-third and thirty-first weeks.

Increased HbF can also be seen in healthy subjects with HPFH. Several types of this condition have been described. In some forms, HbF can be detected in only a proportion of the circulating red blood cells, while in other forms there is an equal distribution of HbF in all cells. Rarely, an anemic patient may receive blood shortly before delivery from a donor with HPFH. As a result, the results of the AET could be misinterpreted as positive in the absence of significant fetal-maternal hemorrhage.

Elevated values for the percentage of HbF may be observed in a variety of anemias and hematologic conditions. These include aplastic anemia, sickle cell anemia, hereditary spherocytic anemia, hemoglobin H disease, and thalassemia major and minor. The increase in percentage of HbF may be particularly marked in cases of thalassemia major, where a range of 40 to 90% has been reported.

Other sources of false positives with the use of the AET include confusion of reticulocytes with fetal cells. Some caution is advised in the use of the AET in the diagnosis of neonatal intracranial hemorrhage. If the infant has been transfused with adult blood prior to the occurrence of the CNS event, the percentage of fetal cells would be expected to be similar in both the infant's cerebrospinal fluid and peripheral blood. This would yield misleading results, which suggest a traumatic tap in the presence of intracranial hemorrhage. In addition, mixing a hemorrhagic cerebrospinal fluid and systemic blood during the lumbar puncture procedure might
reduce the difference in the percentage of fetal cells in the blood and spinal fluid, also giving a false negative test.

The technique used in the AET is capable of detecting as little as .05-0.1 ml of fetal blood in the maternal circulation. The precision of the B-K test has not been addressed satisfactorily in the literature. One manufacturer claims that results of day to day repetition are within 10%. On a theoretical basis, assuming a smear with .5% fetal cells, the number of cells observed could range from 0 to 10. Actual precision achieved in the laboratory is typically less than that calculated theoretically. Good precision would be especially difficult to attain with low concentrations of fetal cells, and would require the counting of large numbers of cells.

The B-K test has been criticized because of its poor accuracy. This may reflect differences in the thickness of smears and in the magnification at which the smears are inspected. A 1972 survey of 11 laboratories in England performing the B-K test showed that 92% of the results fell within a range of 50% to 200% of the correct answer. The rest exceeded this range. A 1980 College of American Pathologists survey of over 250 laboratories found a false positive rate of 40% and a false negative rate of 12% using Kleihauer technics. In view of these discrepancies, some authors feel that the AET results should be regarded as an estimate. They recommend administering a dose of RhlgG that is twice the amount calculated on the basis of the fetal cell count. However, this may result in overtreatment.

Most investigators agree that other methods for the detection and quantification of HbF are more sensitive and accurate than the B-K test. These alternative technics include immunofluorescent methods, enzyme multiplied immunoassays, and enzyme-linked antiglobulin assays. Hess compared an enzyme-linked antiglobulin test with B-K methods and found the former to be more reproducible. However, these other tests are also subject to the errors of interpretation described earlier regarding HbF production during pregnancy and with certain hematologic conditions. The microscopic Du test, a commonly used screening test for the detection of fetal maternal hemorrhage in RhlgG candidates, has a false negative rate similar to that of the AET. Its false positive rate is less, 8.5 percent.

A great deal of work is needed to determine the precision and accuracy of the AET and its alternatives.

REFERENCES


