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GUIDELINES

The estimation of fetomaternal haemorrhage*

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1. INTRODUCTION

Transplacental haemorrhage (TPH) may occur following a sensitizing event during pregnancy or at delivery and can lead to RhD immunization. Assessment of TPH is an important element in determining the amount of anti-D to be administered to a RhD-negative mother following a sensitizing event or after delivery of an RhD-positive infant. The joint Consensus Conference of the Royal College of Physicians of Edinburgh/Royal College of Obstetricians and Gynaecologists on anti-D prophylaxis stated that 1–2% of RhD-negative pregnant women at risk still become sensitized.

The guidelines for the use of anti-D immunoglobulin for Rh prophylaxis (UK Blood Transfusion Services, Immunoglobulin Working Party, 1991) stated that at least 500 iu of anti-D must be given to every RhD-negative woman with no preformed anti-D within 72 h of delivery of an RhD-positive infant. This dose will be sufficient to prevent sensitization from a 4-mL fetal red blood cell bleed. 0·4% of women have a TPH of greater than 4 mL and up to 0·3% greater than 15 mL and will not be protected by the standard 500-iu dose of anti-D. It is therefore important that the size of any fetomaternal bleed is accurately estimated so that if necessary a supplementary dose of anti-D can be administered and maternal alloimmunization prevented.

2. PURPOSE OF THE GUIDELINES

Despite earlier publications describing methods for assessing fetomaternal haemorrhage (UK National Blood Transfusion Services, Immunoglobulin Working Party, 1991; Wagstaff, 1978) it is recognized that there is little standardization of techniques (Milkins *et al*., 1997), a fact which can lead to inaccuracies in determining the size of any fetomaternal haemorrhage (FMH).

These guidelines set out recommendations for best practice and include: (i) quality assurance, (ii) sample requirements, (iii) serological testing, (iv) criteria for the assessment of FMH, (v) techniques for assessing FMH, (vi) examination of the blood film, (vii) calculation of the FMH and (viii) follow up and confirmation of the result.

3. QUALITY ASSURANCE

It is recommended that: (i) all laboratories carrying out FMH assessment participate in an External Quality Assurance scheme, e.g. UK NEQAS; (ii) Standard Operating Procedures are available for the technique in use; (iii) training protocols are available; (iv) there is a mechanism in place for ongoing assessment of staff proficiency for the technique in use.

4. SAMPLE REQUIREMENTS

4.1. *Women from whom blood samples are required*. All RhD-negative women at delivery and RhD-negative women following a potentially sensitizing event after 20 weeks gestation. A sensitizing event may be defined as therapeutic termination of pregnancy, spontaneous complete or incomplete abortion, amniocentesis, chorionic villus sampling, fetal blood sampling, insertion of shunts, embryo reduction, antepartum haemorrhage, external cephalic version, closed abdominal injury, ectopic pregnancy, intrauterine death and still birth (Lee *et al.*, 1999). Sensitizing events before 20 weeks do not require a fetomaternal haemorrhage assessment.

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4.2. *Type of samples*

At delivery: (i) mother – an EDTA sample for fetomaternal haemorrhage assessment and a clotted or EDTA sample for blood grouping and antibody screening; (ii) from the cord – an EDTA or clotted sample as locally required taken with a syringe and needle from a cord vessel for blood grouping.

Following a sensitizing event between 20 weeks and delivery: (i) an EDTA sample from the mother.

4.3. *Sample labelling*. The samples should be labelled in accordance with the BCSH guidelines on pretransfusion compatibility testing (BCSH, 1996). The samples should also be clearly marked 'Cord' and 'Maternal' as appropriate. The request form should contain the full patient's details including location and relevant clinical details including the date and time of delivery or sensitizing event.

4.4. *Timing of samples*. The maternal sample should be taken within 2 h of a normal delivery, manual removal of placenta or sensitizing event. The sample should be processed and results reported in sufficient time to ensure that if necessary a supplementary dose of anti-D can be given within 72 h of the delivery or sensitizing event.

5. SEROLOGICAL TECHNIQUES

ABO and RhD typing must be carried out on both the maternal and the cord samples. The maternal sample should also be screened for clinically significant red-cell antibodies. Techniques used should be in accordance with the BCSH guidelines on pretransfusion compatibility testing (BCSH, 1996).

Anti-D detected in the maternal plasma/serum may be immune in origin or due to the administration of prophylactic anti-D. Where it is not certain that the anti-D present is immune in origin a standard dose of anti-D should be administered.

6. CRITERIA FOR PERFORMING AN FMH ESTIMATION

An FMH estimation should be performed if a RhD-negative woman has delivered an RhD-positive baby or if a fetal blood group is not available. This will allow an appropriate supplementary dose of anti D immunoglobulin to be given to the woman within the correct time period when necessary.

FMH estimation should also be performed following a potentially sensitizing event after 20 weeks gestation in an RhD-negative woman (see 4.2).

Fetomaternal haemorrhage assessment is not indicated if preformed immune anti-D is present in the mother's plasma/serum.

7. METHODS OF ASSESSMENT FOR FMH

Techniques used for fetomaternal assessment include acid elution (Mollison, 1972), rosetting (Jones & Silver, 1958) and flow cytometry (Johnson *et al*., 1995). The techniques recommended for the screening and quantification of FMH in this document are: (a) acid elution and (b) flow cytometry. Flow cytometry for the estimation of FMH is an evolving technique and offers the advantage that if an anti-D reagent is used to relabel the fetal cells, RhD-positive cells are detected. It is recognized that not all laboratories have access to a flow cytometer and that in many laboratories only the acid elution technique is available.

8. ACID ELUTION TECHNIQUE

It is very important that particular attention is paid to detail. Two recommended methods can be found in Appendix 1.

All other methods (including commercial kits) should be standardized against one of these two recommended methods.

Care must be taken to exclude false positive results. Increased levels of HbF are seen in various genetic disorders including β and β thalassaemias and hereditary persistence of fetal haemoglobin.

8.1. *Slide preparation*. Thin blood films are freshly made on clean dry slides, previously degreased if necessary. The thickness of the blood film is important for accurate results and it is recommended that the maternal whole blood sample is diluted 1:2 or 1:3 with saline before making the film. It is important to ensure that the diluted sample is well mixed immediately before the film is made. Each slide must be spread evenly and when examined under the microscope should show red cells touching but not overlapping.

8.2. *Controls*. Controls must be performed with each batch of slides stained and should be treated in exactly the same way as the maternal sample. Positive control: fresh EDTA cord blood diluted 1:100 to adult EDTA male blood. The sample should be well mixed before film preparation. Negative control: adult male blood.

8.3. *Staining*. The effectiveness of the staining is dependent upon a number of factors including; temperature, age, quality and pH of the stain. All these factors must be standardized.

8.4. *Examination of the stained films*. It is recommended that this is divided into screening and quantification. Controls must be examined first to ensure that the staining and preparation are satisfactory. If the controls are not to the required standard the whole process must be repeated.

8.4.1. *Screening*. A minimum of 25 fields should be examined using a ×10 objective. If no fetal red cells are seen the FMH can be reported as <4 mL fetal red cells and no further anti-D is needed. If any fetal red cells are seen a full quantification must be performed.

8.4.2. *Quantification*. Slides giving a positive screening result must be examined further to estimate the number of fetal cells present. It is recommended that fetal cells are expressed as a proportion of adult cells with a minimum of 6000 cells being counted using a ×40 objective. Lowering the condensor may make it easier to count ghosts. This method is aided by the use of a Miller Square disc or an Index Square. Their use is described in Appendix 2.

8.5. *Calculation of the FMH*. This is calculated using the formula described by Mollison (1972). This assumes that the maternal red cell volume is 1800 mL, fetal cells are 22% larger than maternal cells and only 92% of fetal cells stain darkly. The fetal bleed should be calculated thus:

Uncorrected volume of bleed = $1800 \times$ fetal cells counted (F)/Adult cells counted (A)
Corrected for fetal volume (1.22) = $(1800 \times F/A) \times 1.22 = J$ and corrected for staining efficiency (1·09) = $J \times 1.09$ = fetal bleed

An example is given in Appendix 2. When the fetomaternal bleed has been determined a supplementary dose of anti-D is given in accordance with recommendations, i.e. 125 iu mL fetal red cells. A minimum of 500 iu must be given. When there is an FMH of more than 15 mL it is preferable to use the larger anti-D Ig IM preparation (2500 iu or 5000 iu) (Lee *et al*., 1999).

8.6. *Women with high levels of HbF*. A standard dose of anti-D should be given. If free anti-D is present in the plasma no further anti-D immunoglobulin is necessary; however, if no free anti-D is present a supplementary dose should be given and the original and a new sample should be sent to an appropriate centre for flow cytometric analysis.

9. FLOW CYTOMETRY

The flow cytometry method is evolving and therefore only general guidance is given and a detailed method is not included.

9.1. *Sample*

9.1.1. Anticoagulated blood must be used.

9.1.2. The washing procedure must be validated to ensure that: (a) all cells are sedimented by the centrifugation procedure and, (b) all cells are thoroughly resuspended before staining.

9.2. *Staining*. Indirect and direct methods of staining are available for identifying RhD-positive fetal red cells.

9.2.1. *Indirect staining*. (i) A polyclonal antiserum or monoclonal antibody which gives clear discrimination between RhD-positive and RhD-negative cells must be used. (ii) It is recommended that a fluorescent isothiocyanate (FITC) or phycoerythrin (PE)-labelled Fab anti-human IgG reagent is used to prevent agglutination of fetal RhD-positive red cells. (iii) The anti-D and fluorescent anti-IgG reagent should be tested in a checkerboard assay to determine the optimum combination that gives maximum discrimination between negative and positive populations.

9.2.2. *Direct staining*. FITC- or PE-labelled monoclonal anti-D antibodies are commercially available. The antibodies must be validated against mixtures of D-positive and D-negative cells to ensure that a clear discrimination between negative and positive populations can be made. The direct test should always be done on a maternal sample prior to the administration of anti-D.

Where the mother has received a dose of prophylactic anti-D it is possible that the RhD-positive fetal cells will be sensitized to such an extent that binding of the fluorescent-labelled anti-D will be inhibited. This may falsely indicate that no RhD-positive cells are present. It is recommended that where it is known that the mother has received anti-D, an indirect and direct flow cytometric test should be undertaken.

9.3. *Quantification*. RBCs should be selected for analysis by using forward and side scatter parameters. A gate setting should be selected such that >99% of red cells will be analysed for fluorescent staining. Validation of the gate settings may be accomplished by back gating with RBCs stained for example with an antiglycophorin–FITC reagent. The use of the following in-house standards is recommended which use ABO-compatible RhD-negative and RhD-positive donors:

- 100% D-negative cells
- 99% D-negative and 1% D-positive (approximating an 22-mL bleed)
- 99.75% D-negative and 0.25% D-positive (approximating a 5.5-mL bleed)

It is recommended that, as a minimum, duplicate test samples be analysed. To minimize the coefficient of variation obtainable, no less than 500 000 events should be collected. Where the flow cytometer does not allow this, samples should be continuously reanalysed until an analysis of a sufficient number of cells (through summation by simple addition of the results from several individual counts) has been obtained.

The gain setting on FL1 or FL2 should be set so that <5% of negative events fall below the first log decade.

Regions must be set on FL1 or FL2 to separate negative and positive cells; less than 0·05% of negative events should fall in the positive region. Background positive events obtained with 100% D-negative cells must be subtracted from the test positive events.

9.4. *Calculation of FMH*. The percentage of RhD-positive events (i.e. presumed red cells) is used to calculate the volume of fetal blood from the presumed maternal blood volume. The maternal blood volume is assumed to be 1800 mL of packed red cells; no account is taken of maternal haematocrit, maternal body mass or the occurrence of maternal haemodilution just prior to delivery. Cord red cell volume is assumed to be 22% greater than that of adult red cells; hence the volume of fetal cells calculated as a percentage of the maternal red cell number found to be RhD-positive should be proportionately increased in order to take this difference into account. Thus, if 0·5% of the maternal blood is D positive, then the fetal bleed will be calculated to be:

uncorrected for fetal RBC volume:

$$1800 \times 5/1000 = 9$$

corrected for fetal RBC volume:

$$= 9 + (9 \times 22/100) = 10{\cdot}98.$$

10. CONFIRMATION

For any FMH greater than 4 mL an appropriate supplementary dose of anti-D immunoglobulin must be given immediately. A repeat estimation of the FMH should be carried out 48 h following the initial anti D injection. The serum plasma should be screened for anti-D.

The protocol for follow up and confirmation that must be followed is shown in Table 1.

It is recommended that wherever possible a transplacental haemorrhage of greater than 4 mL should be confirmed by flow cytometric analysis. If this method is not available then a separate operator should confirm the FMH by the Kleihauer technique or if this is not possible, e.g. out of routine working hours a new film should be examined.

11. FOLLOW UP

Where a large fetal maternal haemorrhage (>4 mL) has been identified a sample should be taken from the mother 6 months after the sensitizing event and the serum or plasma tested for the presence of anti-D.

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Table 1

Acid elution/flow cytometry	Serum/plasma	Action
No fetal cells present	No free anti-D	Give further dose of anti-D Re-test the serum/plasma for presence of free anti-D in 48 h
No fetal cells present	Free anti-D	No further action
Fetal cells present	No free anti-D	Quantify and give appropriate further dose of anti-D Repeat FMH assessment in 48 h
Fetal cells present	Free anti-D	Repeat FMH assessment in 48 h

APPENDIX 1

Demonstration of fetal haemoglobin in individual red cells using acid elution technique

Principle. Fetal haemoglobin (HbF) is more resistant than adult haemoglobin (HbA) to both alkali denaturation and acid elution. When dry blood films are fixed and then immersed in an acid buffer solution, HbA is denatured and eluted, leaving red-cell ghosts. Red cells containing HbF are resistant and the haemoglobin can be stained; these cells stand out in a sea of ghost maternal cells. There are many factors which influence the quality of the results.

Two techniques will be described. The first is based on the classical Kleihauer method, and the second is a shorter modification on which commercially available kits are based. For both *strict timing* is essential.

Method 1

Reagents:

Fixative

Solution A: citric acid monohydrate 0·1 M ($C_6H_8O_7$ H_2O MW 210.14), 21·01 g to 1 litre distilled water.

Solution B: disodium hydrogen phosphate 0·2 M (Na_2HPO_4. $2H_2O$ MW 177.99) 35·60 g to 1 litre distilled water.

Working buffer: pH 3·3
Solution A: 73·4 mL
Solution B: 26·6 mL

Check pH and adjust if necessary; pH is critical.

Stains

Ehrlich's acid haematoxylin
Eosin Y 10 g L^{-1} in distilled water

Method

1 Make the films as described above.
2 Fill a Coplin jar with the working citrate-phosphate buffer (pH 3·3) and allow to warm to 37°C. Allow air bubbles to escape.
3 Fix slides in 80% ethanol for 5 min. Rinse in tap water and air dry.
4 Immerse slides in citrate-phosphate buffer at 37 °C for 5 min. Agitate slides at 1 and 3 min to ensure even exposure to the buffer.
5 Rinse slides in tap water and air dry.
6 Stain slides in Ehrlich's acid haematoxylin for 3 min. Rinse slides in tap water.
7 Counterstain with eosin for 3 min. Rinse in tap water and dry.

Results: Haemoglobin F-containing cells are stained densely red and are refractile. The cells that contained HbA are ghosts. White cells stain grey/blue.

Method 2—rapid technique

Reagents:

Solution A:
Haematoxylin 7·5 g in 100% ethanol to 1 litre

Solution B:
Ferric chloride 24 g
Hydrochloric acid (2·5 mol L^{-1}) 20 mL
Distilled water to 1 litre

Eluting solution (pH 1·5)
Solution A: 2 parts
Solution B: 1 part
80% Ethanol 1 part

Counterstain: Eosin Y 10 g L^{-1} in distilled water

Solutions A and B can be stored at room temperature. If stored in a refrigerator, the eluting solution must be allowed to reach room temperature before use. The eluting solution should be made up fresh each time.

Method

1 Make films as described above.
2 Fix in 80% ethanol for 5 min. Air dry.
3 Flood slides with the eluting solution at room temperature for 20 s. Rinse in distilled water and air dry.
4 Stain with 1% eosin for 2 min.
5 Rinse in tap water and air dry.

The staining characteristics are identical to those described above. Examination and reporting is described elsewhere in the document.

APPENDIX 2

Quantification of FMH using visual counting

Using a miller square (Fig. 1)

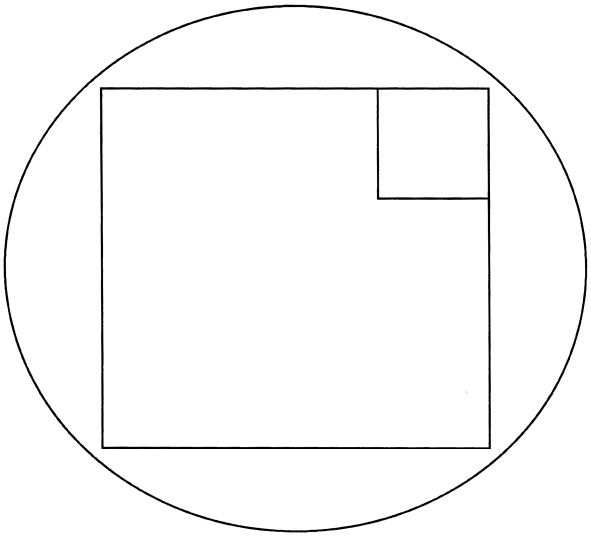

Fig. 1. Miller square.

1 Using a ×40 objective, select an area of the film where cells are touching but not overlapping appreciably.
2 Count adult cells (*A*) in the small square, including all cells which overlap the left hand or upper edges but not those overlapping the right hand or lower edges.
3 Count fetal cells (*F*) in the large square, treating cells overlapping the edges as above.
4 Move across the slide so that the next area to fall within the counting grid is contiguous with the preceding one and repeat the procedure.
5 Continue until the proportion of fetal cells among a minimum of 6000 adult cells has been counted.
6 Assume the total adult cells scanned $(A \times 9) = 6003$ and total fetal cells counted = 18.
7 Calculate the FMH using the formula of Mollison:

Uncorrected volume of bleed	$1800 \times 18/6003$	$= 5{\cdot}4$
Corrected for fetal volume	$5{\cdot}44 \times 1{\cdot}22$	$= 6{\cdot}6$
Corrected for staining efficiency	$6{\cdot}6 \times 1{\cdot}09$	$= 7{\cdot}2$

A shortcut method of achieving this same calculation is to multiply the ratio of adult to fetal cells by 2400 as described by Mollison:
Corrected bleed = $2400 \times 18/6003 = 7{\cdot}2$ mL.

Note: it is essential to survey a minimum of 6000 cells to achieve reasonable precision. In the above example the 95% confidence limits of an estimate of 7·2 mL would be 6·36–8·03 mL, allowing for the fact that only 667 adult cells are actually counted (this number being multiplied by 9 to estimate the number of adult cells actually scanned). If 6000 cells were actually counted the 95% confidence limits would be narrower, 6·91–7·49 mL.

Using an indexed square

The procedure is as above except that adult cells are counted in 10 of 100 squares in a 10×10 grid and the number of adult cells is then $A \times 10$.

Suppliers of both graticules:
Graticules Ltd., Morley Road, Tonbridge, Kent TN9 1RN, UK
Miller Square E57 16 mm Order no. 01A16032, 19 mm–01A19032, 21 mm 01A21032
Indexed Square E35 16 mm Order no. 01B16221, 19 mm 01B19221, 21 mm 01B121221, price on 1.11.97 £33
Use of either graticule requires a Kellner × 10 (F10) eyepiece, Order no. 5E 02081