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GUIDELINES FOR LABORATORY ASSESSMENT OF FETOMATERNAL HAEMORRHAGE

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Laboratory Assessment of Fetomaternal Haemorrhage

1. Background

In 1997 the National Health and Medical Research Council (NHMRC) of Australia appointed a working party to develop "best practice" guidelines for the use of Rh D immunoglobulin. In March 1999 "Guidelines on the prophylactic use of Rh D immunoglobulin (Anti-D) in obstetrics" were endorsed and published by the NHMRC.

Among other issues, the document produced a number of recommendations concerning testing for fetomaternal haemorrhage (FMH) and the methods currently available and commented that "….the accuracy and practicality of the routine use of these tests is variable and they are not used uniformly in all centres". Further recommendations were made concerning the value of the Kleihauer test and the suitability of flow cytometry in the assessment of fetomaternal haemorrhage.

Members of the Scientific Subcommittee (SSC) of the Australian & New Zealand Society of Blood Transfusion (ANZSBT) were included in the consultative process that produced the guidelines. It was generally thought that the ANZSBT was the appropriate organisation to take the initiative and provide direction to laboratories for tests used in assessment of fetomaternal haemorrhage.

Contingent to this task the ANZSBT SSC has assessed the available literature, quality assurance program results, and consulted with the wider scientific community with expertise in the area of FMH assessment to produce recommendations for laboratory testing for FMH.

2. Introduction

One of the complications of pregnancy is transplacental transfer of fetal erythrocytes, or FMH, into the maternal circulation. Some studies have shown that small bleeds of less than 1 ml of blood occur in 96% of all pregnancies, and that larger losses of approximately 30 ml occur in up to 0.3% of all pregnancies. Although situations are recognised when it is more likely that a large FMH may occur, these often occur without significant signs or symptoms in either the mother or fetus. In many cases the cause of significant FMH remains obscure and is not predictable.

It is well known that FMH with blood group incompatibility between the fetus and the mother may induce the production of maternal red blood cell antibodies. These antibodies can then cross the placenta and elicit an immune mediated haemolytic anaemia in the fetus, or Haemolytic Disease of the Newborn (HDN). Whilst this is known to occur with many blood group antigens historically HDN has most commonly occurred with a Rh D negative mother and a Rh D positive fetus, with the fetal red cells inducing production of maternal anti-D. This subsequently destroys the fetal red cells.

The widespread adoption of post partum immunoprophylaxis with a single dose of Rh D immunoglobulin dramatically reduced the incidence of Rh D immunisation, and HDN. However despite this the incidence of Rh D immunisation during pregnancy remains at approximately 1-2%. One of the causes of this can be a FMH of a volume larger than the protection offered by a single dose of Rh D Immunoglobulin.

The NHMRC guidelines aim to overcome this by requiring a quantitative assessment of FMH in all Rh D negative pregnant women at delivery and/or following events or procedures that are known to increase the risk of FMH occurring after the first trimester. In the event that a large FMH has occurred further doses of Rh D immunoglobulin can then be administered.

Detection of fetal cells in the maternal circulation during pregnancy is also valuable in the evaluation of fetal welfare following maternal trauma, investigating fetal hydrops or near term fetal death and severe fetal anaemia.

The most commonly used tests rely on the detection of red cells containing fetal haemoglobin (HbF) or the detection of Rh D positive fetal red cells in the maternal (Rh D negative) circulation. If laboratory testing indicates that a FMH larger than that covered by the standard dose of Rh D Immunoglobulin has occurred, further doses of Rh D Immunoglobulin should then be given.

Rh D Immunoglobulin currently available is formulated to provide protection at a rate of approximately 20 µg (100 IU) of Rh D Immunoglobulin for each one (1) ml of Rh D positive red cells. CSL manufacture Rh D Immunoglobulin in both a 50 µg/250 IU dose for use in the first trimester, and a 125 µg/625 IU dose for use in all other indications. This 125 µg/625 IU dose is known to prevent sensitisation in FMH of 6 ml of red cells or approximately 12 ml of whole blood.

It is important to note that other manufacturer's product may enter the Australasian market in the future [see further note p 11].

3. Techniques Available to Assess FMH

Acid Elution Test

The acid-elution technique has been used for many years as the principal method for assessment of FMH. The original test described by Kleihauer *et al* differentiates red cells containing fetal haemoglobin (HbF) from those containing adult haemoglobin by the relative resistance of HbF to elution at a low pH. After counter-staining, the fetal red cells stand out as brightly stained cells in a field of "ghost" red blood cells. A number of variations to the original method have been described. Commercially available tests for the procedure are also available.

The advantages of these tests are that they are:

- not dependent on the presence or absence of the Rh D antigen;
- they require only basic laboratory equipment, and;
- are inexpensive to perform.

There are however significant disadvantages to these tests. This is highlighted by recent Quality Assurance Programs that show a wide variation in results. This variation is due to a combination of factors including:

- Technique sensitivity. The Haemoglobin elution step is sensitive to pH, time and temperature;
- Subjective interpretation of the stained blood film;
- Experience of the scientist/technician performing the test;
- Assumptions in the calculation of results;
- Increased levels of HbF in maternal red cells during pregnancy. It is known that in about 25% of pregnant women, the level of maternal HbF rises above the upper limit of normal (0.9%) at about 8 weeks gestation and may persist until the 32nd week.
- Hereditary persistence of fetal haemoglobin. This has been reported at levels of 1-2% in specific populations

The success of the test is highly dependent on a combination of factors. Experienced staff, the use of thin blood films, and use of an ocular grid all contribute to more consistent results.

In addition the use of control slides and participation in a quality control program are essential. Further details and recommendations are provided in Appendix 3.

Flow Cytometric Testing

Flow cytometers are designed to quantitate small numbers of cells present in a larger cell population. Differentiation and quantitation of the populations is most often achieved using fluorescent labelled antibodies.

Flow cytometry allows large numbers of cells (eg. 50,000 – 100,000) to be readily counted. The test should then be more sensitive and accurate; and produce objective, quantitative results. However flow cytometers are expensive and generally available only in major centres and require staff with specific expertise to operate the analyser.

Assessment of FMH can be achieved with flow cytometry using commercially available antibodies to the Rh D antigen or to Haemoglobin F [HbF].

In situations where the mother is known to be Rh D negative and the fetus is known to be Rh D positive the use of anti-D provides sensitive and accurate assessment of FMH, and determination of Rh D Immunoglobulin requirements.

However where the mother and the fetus are of the same Rh D type or unknown anti-D cannot be used to determine whether FMH has occurred. Similarly it is of no value when the mother is Rh D positive. In these circumstances determination of FMH by flow cytometry should use anti-HbF.

With the introduction of antenatal Rh D Immunoglobulin prophylaxis programs anti-HbF will also be the reagent of choice for FMH determination.

Further details and recommendations relating to the use of flow cytometry and testing using anti-D and anti-HbF are provided at Appendix 4.

As mentioned above flow cytometric tests using fluorescent labelled anti-HbF are also available. In common with the Kleihauer test, use of HbF as a marker of FMH can result in false positive results due to hereditary persistence of fetal haemoglobin, increased levels of HbF in maternal red cells during pregnancy and in some disease states such as thalassaemia.

Further, since all adult red cells contain small amounts of HbF (resulting in a 'background' level), discrimination of fetal and adult cells can be difficult and at times uncertain. Commercial manufacturers have recently combined antibody tests for other unique properties of fetal red cells (eg. quantitation of carbonic anhydrase, Ii antigens) with HbF testing in an attempt to increase test specificity.

Other Techniques

Other techniques are available and have been used but are known to produce a significant false negative rate. They are included for completeness only and are NOT recommended.

a) Rosette Test

The rosette test utilises an Indirect Antiglobulin Test with increased sensitivity achieved by the addition of Rh D positive "indicator" red cells. These indicator cells adhere to the anti-D coating the minor population of Rh D positive fetal red cells. This results in clusters or rosettes that can be counted microscopically.

Commercial kits are available for screening samples to detect FMH, using the rosetting technique.

The rosette test is only applicable in situations where the fetus is Rh D positive and mother is Rh D negative. It is not therefore not a replacement for the Kleihauer test.

b) Enzyme linked antiglobulin test (ELAT)

The enzyme linked antiglobulin test is a quantitative test for FMH based on the indirect antiglobulin test.

c) Surrogate Tests

Elevation of maternal serum alpha-fetoprotein (AFP) has been reported as a consequence of FMH.

However, many other fetal conditions are associated with raised levels of AFP. Hence this technique is not recommended as a sole indicator of FMH.

Other tests have also been used as surrogate markers of FMH. These include placental alkaline phosphatase (PLAP), polymerase chain reaction [PCR], fluorescence in situ hybridisation [FISH].

Summary and Recommendation

It is apparent that there is little standardisation of techniques to assess FMH. This increases the potential for inaccuracy in reporting and subsequent over or under utilisation of Rh D immunoglobulin with the consequential potential for an adverse patient outcome in either the current or future pregnancies. The recent introduction of the RCPA Quality Assurance Program (QAP) for assessment of FMH has highlighted the technical difficulties associated with techniques routinely utilised in the quantitation of FMH.

It is generally accepted that flow cytometry is the method of choice to quantitate FMH. Despite the lower number of users, unpublished figures from the RCPA QAP surveys and educational exercises demonstrate that flow cytometry produces results within a narrower range, smaller standard deviation and a reduced coefficient of variation [CV] compared to the Kleihauer test.

Whilst Kleihauer tests are more commonly available, the technique generally suffers from increased method and operator variability, low level of reproducibility, poor preparation technique and lack of standardisation between laboratories and individuals. Despite these inadequacies, with a standardised approach to test variables and a very prescriptive method, the Kleihauer test may be used.

The Scientific Sub-Committee of the ANZSBT therefore recommends that:

1. All Rh D negative pregnant women who do not have 'pre-formed' anti-D detected in their plasma and undergo a potentially sensitising event **during** the first trimester of pregnancy receive one vial of CSL Rh D immunoglobulin (50 µg/250 IU). In twin or multiple pregnancies reference should be made to the product PID.

2. All Rh D negative pregnant women who do not have 'pre-formed' anti-D detected in their plasma and undergo a potentially sensitising event **after** the first trimester of pregnancy receive one vial of CSL Rh D immunoglobulin (125 µg/625 IU).

   In addition quantitative measurement of FMH should be performed and results made available within 72 hours. At no time should a single dose of Rh D immunoglobulin be withheld based upon, or pending, the results of a test to quantitate FMH.

3. Flow cytometry is accepted as the most accurate quantative test for FMH, but, it is not widely available, requires costly equipment and scientists experienced in this methodology. The SSC believes that this is the method of choice for quantation if readily available.
   However until flow cytometry becomes more widely available recommendation 4 below is of paramount importance.

4. Laboratories undertaking quantitative assessment of FMH by any method must show acceptable performance in internal and external quality assurance programs and have clearly defined test methods, continuing assessment protocols and documented staff training programs to ensure accuracy and reproducibility of results.

   Results should be reported in a format that allows easy correlation with product inserts of locally available Rh D immunoglobulin.

5. Where FMH quantitation shows that fetomaternal haemorrhage greater than that covered by the dose already administered has occurred, administration of an additional dose/s of Rh D immunoglobulin sufficient to provide immunoprophylaxis must be administered and preferably within 72 hrs. Recommended results calculations and dosage administration tables are provided in Appendices 3, 4 and 5.

6. For large bleeds follow up testing should be performed on a sample collected 48 hrs post Rh D immunoglobulin administration to determine if further dosing is required.

   Supplemental Rh D immunoglobulin should be administered if:

   1] FMH is still positive, **and**

   2] Rh D immunoglobulin is not detected in maternal plasma by IAT.

   Further recommendations regarding sensitising events, specimen requirements, testing protocols and dosage are provided in the appendices.

Appendix 1: Patients Requiring Testing for FMH

A maternal sample should be taken from all Rh D negative women following a potentially sensitising event occurring after the first trimester of pregnancy to determine the extent of the fetomaternal haemorrhage.

Sensitising events include (but are not limited to):

- delivery
- termination of pregnancy,
- spontaneous abortion,
- amniocentesis,
- chorionic villus sampling,
- fetal blood sampling,
- insertion of shunts,
- embryo reduction,
- antepartum haemorrhage,
- external cephalic version,
- abdominal trauma,
- ectopic pregnancy,
- intrauterine death, and
- stillbirth.

The sample should be processed and results reported within 72 hours so that, if necessary, a supplementary dose/s of Rh D Immunoglobulin can be given within 72 hours of delivery or the sensitising event.

In cases of antenatal bleeding due to continual antepartum haemorrhage [APH] weekly testing for FMH and antibody screening by IAT should be performed while either the test for FMH is positive and/or the antibody screen is negative. At this stage a further dose of Rh D Immunoglobulin should be given and the testing cycle restarted.

Appendix 2: Sample Handling

Sample collection

EDTA or ACD anticoagulated blood samples are required as a cell source for the investigation of FMH. Clotted samples are unreliable.

Ideally, samples should be collected within 2 hours of the sensitising event and prior to administration of Rh D immunoglobulin.

Sample Transport

It is desirable that samples for investigation of FMH be maintained between $2^{o}C$ and $8^{o}C$ during transit. Temperature extremes may compromise the quality of the sample and should be avoided.

Packaging, labelling and transport of specimens should comply with all local, state, national and international regulations for the regions through which the specimens will pass.

Sample Integrity

The specimen should be visually inspected for haemolysis or container defects that may lead to sample leakage. If the specimen shows any visual signs of deterioration, recollection, if possible, is the preferred option.

Samples, that have been collected inappropriately, may be processed by the laboratory according to a local documented and approved policy. The deficiencies in the sample should be recorded and the final report should reflect the effect that these deficiencies may have had on the results. Results from clotted, leaking or haemolysed samples may produce a false negative result or underestimate the size of a FMH.

Appendix 3: Acid Elution Testing

Commercial Kits

Laboratories utilising commercial kits must follow the manufacturers recommendations and test method.

In-house Methods

Laboratories using locally developed test methods must follow a documented, validated and approved method.

Blood Films

Thin blood films should be prepared in accordance with the laboratories standard method.

Controls

Control slides must be performed to ensure the staining protocol adequately differentiates between adult and fetal cells, and to standardise the counting of fetal cells.

The following control protocol is recommended.

- Laboratories performing less than 20 tests per week should run a calculated control in parallel with each run.
- Where laboratories regularly perform greater than 20 Kleihauer tests per week a weekly calculated control is acceptable.

Controls should be set to detect a bleed greater than that covered by the standard dose of prophylactic anti-D. CSL Rh D immunoglobulin (125 µg/625 IU) should protect against 6 ml fetal red cells (12 ml whole blood). This is equivalent to 0.25% fetal cells in the maternal circulation.

Controls may be prepared in-house according to a documented protocol. One method is shown below for information.

1. Collect 10 ml of adult blood from a known Rh D Negative individual.
2. Collect 1-2 ml of Rh D Positive cord blood. This must be ABO blood group identical to the adult sample.
3. Perform a Red Blood Cell (RBC) count on each sample.
4. Calculate the volume of cord cells to add to the adult cells using the following formula.

   A = Adult RBC count

   B = No. of adult cells in 10 ml = $\frac{A \times 10}{1000}$

   C = % fetal cells required

   Hence: *Number of fetal cells required to be added* = $\frac{C \times B}{100}$

   Convert the number of cells to a volume:

   Volume of fetal cells to be added (ml) = $\frac{No.\ of\ \ foetal\ cells (as\ above) \times 1000}{Foetal\ RBC\ count}$

5. Add the calculated volume of fetal cells to the adult sample, mix well and make thin blood films as per the laboratories standard method.

Examination of blood films

Examination of the film and counting of fetal and adult red cells requires sufficient cells to be counted to ensure statistical validity at the low end of desired sensitivity. This may be achieved by:

- Using an eye-piece graticule to systematically count fetal and adult red cells, or
- Counting the fetal cells in a minimum of 50 low power fields, and estimating the number of maternal cells by counting the number of maternal cells present in 2–3 low power fields.

Calculation of FMH

From bibliography references 2, 11 and 14, the calculation of fetomaternal haemorrhage using an acid elution technique is based on the following assumptions:

- Fetal red cells are approximately 22% larger than maternal cells.
- Only 92% of fetal red cells stain darkly
- Maternal red cell volume is approximately 1800 ml.

The fetal bleed should be calculated thus:

Uncorrected volume of bleed = 1800 x fetal cells counted [F] / adult cells counted [A]

Corrected for fetal volume (1.22) = (1800 x F / A) x 1.22 = J.

Corrected for staining efficiency (1.09) = J x 1.09 = Fetal bleed.

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 x [F / A]

Rh D Immunoglobulin Dosage

One vial of Rh D Immunoglobulin (125 µg/625 IU) is sufficient for 6 ml of red cells. A dosage table is provided at Appendix 5 for information.

Limitations

- It is known that in about 25% of pregnant women, the level of maternal HbF rises above the upper limit of normal (0.9%) at about 8-10 weeks gestation and may persist until the 32nd week.
- Hereditary persistence of fetal haemoglobin has been reported at levels of 1-2% in specific populations.

Reporting

If 0 - 0.05% of fetal red cells detected, report as less than 1ml bleed. For levels above this quantitate and report as '%' and 'volume' of fetal red cells detected.

If the calculated bleed is greater than that covered by a single dose of Rh D immunoglobulin, then the required number of vials should be calculated and administered. Reporting should include both an assessment of the size of the FMH and the number of vials of Rh D Immunoglobulin (125 µg/625 IU) required for immunoprophalyxis.

Appendix 4: Flow Cytometry

NOTE: This protocol is provided for the assessment of FMH by the use of anti-D if the mother is RhD negative and the fetus is RhD positive and for the use of anti-HbF in other circumstances.

Reagents

Commercially manufactured reagents are to be stored and used in accordance with the manufacturer's instructions.

In-house reagents are to be manufactured according to documented, validated and approved methods.

Standards and Controls

Control specimens should be run in parallel with each assay. The following controls are recommended:

- 100% Rh D Negative adult cells
- 0.25% Rh D Positive fetal cells in Rh D Negative adult cells
- With post delivery specimens it is recommended that the matching fetal/cord blood sample be run as a positive 'gating' control.

Control specimens may be manufactured in accordance with the method specified in Appendix 3.

Test Method

Test protocols are to be documented, validated and approved.

Calculation of FMH

From bibliography references 2 and 11, the calculation of fetomaternal haemorrhage using flow cytometry technique is based on the following assumptions:

The volume of fetal packed red cells (rather than fetal whole blood) in the maternal sample is calculated by multiplying an assumed maternal red cell volume of 1800ml by the number of positive events [fraction of cells] against anti-D or anti-HbF. Again fetal cells are assumed to be 22% larger than maternal cells.

Hence, for a maternal sample containing 0.5% fetal red cells, this would be calculated as:

Uncorrected for fetal RBC volume: **1800 x 5 / 1000 = 9**

Corrected for fetal RBC volume: **9 + (9 x 22/100) = 10.98 mls.**

A simpler approach is to use the % fetal red cells and simply use the conversion chart in Appendix 5 [ie 0.5% = 12mls].

Fetal cells that possess partial Rh D antigens or variant Rh D antigens may not be detected by this method. If using commercially available Anti-D refer to manufacturer's product insert for details.

Reporting

If 0 - 0.05% of fetal red cells detected, report as less than 1ml bleed. For levels above this quantitate and report as '%' and 'volume' of fetal red cells detected.

If the calculated bleed is greater than that covered by a single dose of Rh D immunoglobulin, then the required number of vials should be calculated and administered. Reporting should include both an assessment of the size of the FMH and the number of vials of Rh D Immunoglobulin (125 µg/625 IU) required for immunoprophalyxis.

Appendix 5: Rh D Immunoglobulin Dosage.

The following table for the administration of Rh(D) Immunoglobulin has been drawn up for the following RhD Immunoglobulin products

CSL Australia - 625 iu/ml

WinRho 600 iu/ml

and has been calculated for the following FMH:-

%Rh(D) Positive fetal cells	Estimated FMH (mls)	No of vials of CSL Anti-D (625IU/ml)	No of vials of WinRho (600IU/ml)
0.125%	3 mls	1	1
0.25%	6 mls	1	1
0.5%	12 mls	2	2
0.75%	18 mls	3	3
1.0%	24 mls	4	4

For management of bleeds >24ml consultation with the referring MO or specialist should be undertaken.

Whilst CSL Rh D immunoglobulin is currently [September 2002] the only available product, at various times the listed other manufacturers products may be available. These will have different formulation and potency. Hence users will need to consult the manufacturers product information to check the correct dosage.

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Addendum

A manual fluorescence microscopy method to quantitate fetal cells carrying the D antigen has recently been published. This method has not been evaluated in the preparation of these guidelines.

[Oshsenbein-Imhof N, et al. Transfusion, 2002; 42: 947-953.]

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