

Special Report

Report of a joint WHO/WFH meeting on the control of haemophilia: carrier detection and prenatal diagnosis

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Introduction

In 1977 WHO published in its *Bulletin* a Memorandum on Methods for the Detection of Haemophilia Carriers. This was produced following a WHO/WFH Meeting of Investigators held in Geneva in November 1976, and has served as a valuable reference article to all interested in the genetics of haemophilia. The analyses discussed were based on phenotypic assessment, which, at that time, was the only procedure available.

The molecular biology revolution in genetics has, during the 1980s, made enormous contributions to our understanding of the molecular basis of the haemophilias and allowed for precise carrier detection and prenatal diagnosis. WHO and WFH held a joint meeting in March 1990 to consider the Prevention and Control of Haemophilia, and recommended that WHO should organize, in collaboration with WFH, a meeting of experts to update the 1977 WHO/WFH Memorandum 'in view of the recent explosive development of DNA technology and their continuing simplification'.

Assessment of the problem

The carrier state in haemophilia

(i) Clinical and genetic considerations

Carriers (females) of haemophilia usually inherit their abnormal factor VIII or factor IX gene from either of their parents. Since they have a second normal X-chromosomal gene from the other parent the clotting factor level is around 50% of normal which is sufficient for normal haemostasis.

Bleeding symptoms do occur, however, in carriers if their clotting factor level is in the range of mild haemophilia, below 40%. This may be due to homozygosity, Turner's syndrome, other chromosomal abnormalities, extreme lyonization, or the coinheritance of a variant von Willebrand factor allele (i.e. von Willebrand's disease Normandy). With the increasing success of patient associations, the chances of carriers marrying patients might be expected to rise, but until now, homozygosity has been distinctly rare, just as the unlikely coincidence of Turner's syndrome and carriership. Occasional true heterozygous carriers with low clotting factor levels due to extreme lyonization, however, are known in all major haemophilia centres. Within the perspective of this document, this is relevant for invasive procedures for prenatal diagnosis and for the management of delivery.

Worries about the risk of haemophilia affecting their offspring are the motive for possible carriers to seek advice. The first step in this genetic counselling procedure is to find out why the consultand thinks that she might carry the gene. This may lead to any of three conclusions: she is not a carrier, she is an obligatory carrier, she is a possible carrier.

Carriership is excluded if haemophilia occurs in the paternal family without her father himself being affected. *Carriership is obligatory* if her father has haemophilia or if she has maternal relatives with haemophilia as well as an affected child. In these situations each newborn son has a 50% chance of being affected. If the consultand has more than one son with haemophilia without other affected relatives, she may be either a true heterozygote, or a mosaic.¹⁻⁴ In the case of somatic or germ line mosaicism the recurrence risk of the disease in subsequent newborn sons depends on the proportion of ova carrying the abnormal gene, which is difficult to establish. *Carriership is possible* if the consultand has affected relatives on the maternal side and no affected children, or if she has one affected son and no other affected relatives. In the last case, there are four possibilities: the consultand may have inherited the gene through the silent maternal line, which makes her a true heterozygote; she may be a mosaic; the affected son may have received the abnormal gene from a mutation in the single ovum that he originated from; or, the affected son may himself be a somatic mosaic with a large proportion of factor VIII- or IX-producing cells carrying the mutation.⁵

After this first step, the genetic counsellor proceeds with rigorous pedigree analysis, clotting factor assays and DNA studies in order to minimize uncertainty and to set the stage for decision-making about prenatal diagnosis. These phases will be dealt with in detail below.

(ii) Psychological considerations

Every woman who considers carrier testing for haemophilia, has a multitude of sociological and psychological influences which might affect her perceptions of the personal implications of possible carrier status. Some influences may be created by the ethnic and/or religious background of the individual. A particular society's perception of a woman who carries the gene for haemophilia may certainly influence whether or not a woman at risk chooses to be tested. Decisions to undergo carrier testing may also be dependent upon the degree of anxiety about haemophilia and its complications, as well as the availability and safety of treatment. Women who live in countries where treatment for haemophilia is inadequate, may be very interested in carrier detection and prenatal diagnosis, and there-

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fore a more aggressive approach to testing may be successful.

Preconceived notions about the clinical aspects of the disease will be formed as a result of the degree of, and content of contact with male haemophilic relatives. Women who have male relatives with more clinically severe disease, with inhibitors, or AIDS, may be more inclined to seek out carrier testing. This is especially true if an individual has been able to closely observe the effects of the disease on themselves and their family over time. Women who are related to those with milder clinical disease, or who have more distantly related haemophilic relatives, may have different perceptions regarding carrier testing. In the case of mild clinical disease in the family, women may see no need for carrier testing because of perceptions that haemophilia will not have a large personal impact. Those with more distantly related haemophilic relatives may be eager to pursue testing because of a perceived low risk, but may be unprepared if positive results are obtained.

It is important that formal counselling be done before laboratory tests are even considered, in order to resolve conflicts that may exist between a woman's desire to learn of her carrier status and the implications of possible results. It should be emphasized that the results of carrier testing may also be that the woman is not a carrier of haemophilia. The anxieties a woman might have regarding genetic testing, may also be complicated by the type of testing available and the requirements of such, as well as the reliability of the tests involved. The possibility of inconclusive test results, or results that are not highly accurate may deter some women from choosing to be tested.

The psychological impact of other family members must also be considered when counselling. Some sisters, daughters, and mothers of haemophilic males may have ambiguous feelings about their decisions regarding carrier testing. They often consider the effects of their own decisions regarding genetic testing on their male relative(s), and may not always make decisions based on their own needs. Family members will become an important part of counselling if they are called in for blood sampling. In this case a family counselling session should be offered. Involvement of spouses or long term partners in counselling sessions is also important, so that the couple can make mutually beneficial decisions regarding testing. The partner's perceptions of haemophilia, and the degree of reproductive risks he is willing to take, will play an important role in this process. Some women wait to undergo carrier testing until they are pregnant. They may be referred by their obstetricians, or they may elect to undergo testing because of extreme anxiety. Carrier testing under these conditions is very stressful for the

patients and medical personnel. The women seek rapid, accurate diagnosis of the carrier state so that decisions can be made about prenatal testing at the appropriate time. Therefore carrier testing during pregnancy is not recommended, rather it should be done prior to this time.

Many women who choose to undergo carrier testing may perceive themselves as either carriers or non-carriers prior to actual laboratory testing. This perception may influence how an individual assimilates the results of her laboratory testing, therefore it is an important issue to discuss during counselling. Those who assume that they are non-carriers will be supported by negative results, but may feel shock and surprise if given positive results. Those who assume that they are carriers may be relieved when given negative results, but they may also feel guilty for 'escaping' their family's genetic burden, and they may have difficulty coping with their new status if life decisions have been made based on their assumptions of carrier status. Any woman who receives positive results will need a great deal of support, as they then must deal with the reality of their carrier status within the context of their family and their society.

Prenatal diagnosis

(i) Clinical and genetic considerations

In families at risk of having a child with haemophilia, assessment of carrier status and counselling regarding prenatal diagnosis should ideally be carried out before conception. Pregnant carriers requesting prenatal diagnosis should be counselled as to the available options, including the techniques for foetal tissue sampling, their limitations and potential complications. If the foetus is affected, the options of (i) continuing with the pregnancy and either keeping or adopting their child, and (ii) terminating the pregnancy, are reviewed.

Diagnostic centres. Prenatal diagnosis should be undertaken in centres with full genetic, haematological and obstetric expertise.

Diagnostic tests. In the early days of prenatal diagnosis of the haemophilias (until the mid-1980s) the policy was to perform amniocentesis at 16 weeks followed by foetal blood sampling at 20 weeks for phenotypic diagnosis in male foetuses. Subsequently, with the application of recombinant DNA techniques to the analysis of placental biopsy material, parents were offered the advantage of first trimester diagnosis, with the additional benefit that in the presence of a male foetus only one invasive test was required. However, foetal

blood sampling for phenotypic diagnosis is still the preferred method in patients presenting in the second trimester of pregnancy, and it is necessary for those patients who are not informative for any of the available DNA probes, for those requiring confirmation where normality is based on a linked probe, and those who have sporadic haemophilia or lack key relatives. Second trimester diagnosis may also be the preferred option for those patients wishing to avoid invasive testing for female foetuses because foetal sexing can now be performed by ultrasonography at 16–20 weeks.

Termination of pregnancy. Traditionally, termination of pregnancy in the first trimester was performed under general anaesthesia by dilatation of the cervix and evacuation of the uterine contents. In the second trimester termination involved induction of labour and delivery of the foetus. Recently with the more widespread uptake of second trimester dilatation and evacuation, one of the potential advantages of first trimester diagnosis (less traumatic termination), may not be valid.

(ii) Psychological considerations

Prenatal diagnosis of the haemophilias holds a multitude of psychological considerations for the woman and her partner as well as the wider family and the community as a whole. Parental anxieties arise from (i) the risks of having an affected child with lifelong morbidity, (ii) attending a hospital and having a potentially painful invasive test, (iii) miscarrying as a result of invasive testing, (iv) receiving an abnormal result, (v) making a decision on whether to continue or terminate an affected pregnancy, (vi) undergoing a termination, with its potential short-term and long-term complications. The parents are also subjected to further, either real or perceived, pressures. The wider family, especially affected members, close friends, and even their doctors, may hold strong views concerning quality of life and attitudes to termination. Prevailing, cultural, religious and moral values within a society may impose additional stresses on the parents.

Awareness of these problems and psychological support at each step of prenatal diagnosis, constitute the basis of a good diagnostic centre.

Present status of genetic diagnosis for haemophilia

Assessment of carrier status

(i) Family data—risk assessment

A pedigree of the family has to be carefully drawn with accurate information on the males being affected with

haemophilia or not. If more than one haemophiliac exists or has existed in the family, the case is familial. If the haemophiliac is the only known case in the family it is considered as isolated. These two types of families have to be discussed separately.

Familial cases. Haemophilia A and B are X-linked, recessive disorders. The segregation probabilities are thus 50% for a carrier female to transmit the X-linked gene to each child, male or female, while the haemophilic male will have only normal sons and carrier daughters. Study of the pedigree alone will identify some females as *obligate carriers*. An obligate carrier is defined as a woman who:

- has a father who is a haemophiliac (with the rare exception of him being a somatic mosaic);
- has more than one haemophilic son (identical twins excluded) or one haemophilic son and a daughter who has given birth to a haemophilic son;
- has a haemophilic son and a well documented haemophiliac on the maternal side of the family.

Obligate carriers have a probability of 1.0 for carrier-ship and need no further investigation.

Females in the pedigree who are not obligate carriers are to be considered as possible carriers. If a consultand is a possible carrier her probability of carriership calculated from pedigree data should be done in two steps. In the first step only information from the pedigree anterior to her is used, and the pedigree from the consultand to the nearest maternal relative who is a haemophiliac or an obligate female carrier is examined. For each step vertical or horizontal in the pedigree from this person, a probability of 0.5 is taken and these factors are multiplied together to arrive at the probability of the consultand being a carrier.

This is illustrated in the pedigree in Figure 1. I:2 is an obligate carrier since she has given birth to a son with haemophilia and a daughter who has a son with haemophilia. II:4 is one step horizontal to the haemophiliac (or one step vertical to the obligate carrier) and thus has a probability of 0.5 for carriership. III:2 is one step horizontal and one step vertical to the haemophiliac (or two steps vertical to the obligate carrier) and thus has a probability for carriership $0.5 \times 0.5 = 0.25$.

If the consultand has male descendants her probability for carriership has to be modified by taking these into consideration. If the consultand has healthy male descendants her probability of being a carrier is diminished.

The probability for III:2 being a carrier according to the anterior family history is 0.25. She has given birth to two healthy sons. The next step is to calculate the likelihood that III:2 would have two normal sons, sep-

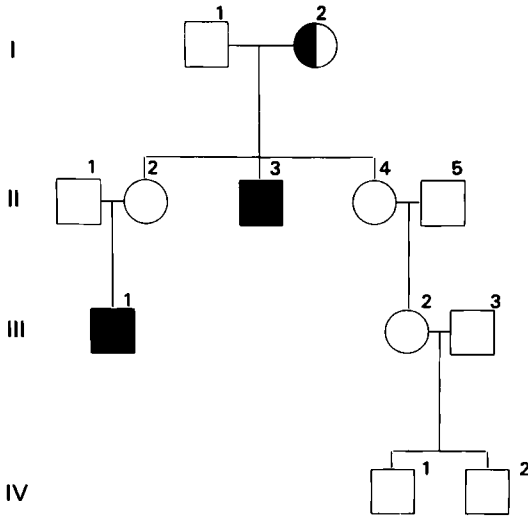


Figure 1. Example of a pedigree for calculating anterior probabilities of carriership.

arately for the two possible cases that she is or is not a carrier. If she is a carrier the likelihood that her two sons would be healthy is $(0.5)^2 = 0.25$, since each son has a probability of 0.5 of not receiving the abnormal gene. If she is not a carrier the likelihood that her two sons would be normal is $1^2 = 1$. These two likelihoods expressed as odds for carriership give $(0.5)^2:1^2$, i.e. 0.25:1.

In order to modify the probability of 0.25 obtained from the anterior pedigree by taking into consideration the odds obtained from the pedigree of the descendants, the former probability (P) must be expressed as odds; $0.25:0.75 = 1:3$. The odds in the anterior and descendant pedigree is multiplied to arrive at the final odds for carriership obtained from pedigree data; $0.25 \times 1:1 \times 3 = 0.25:3$. The odds for carriership can then be transformed again into a probability according to the formula; odds a:b corresponds to the probability according to the formula; $P = a/(a + b)$; $0.25/(0.25 + 3) = 0.08$. Expressed in words, the fact that III:3 has given birth to two healthy sons reduces her probability of carriership from 0.25 to 0.08.

These calculations are illustrated in Table 1 with the general formula applying to a consultant having n sons. With one normal son ($n = 1$), the final probability is reduced from 0.25 to 0.14, with two normal ($n = 2$) sons it is reduced to 0.08, and so on.

Isolated cases. An isolated case of haemophilia may result from transmission of the haemophilia gene through asymptomatic females in whom the gene has remained undetected; from a new mutation in the mother, resulting in her being a carrier, or a new mutation in the haemophiliac (= true *de novo* mutation). The existence of somatic mosaicism and germ line mosaicism has also to be taken into consideration.^{2,3}

The true proportion of *de novo* mutations will depend upon the mutation rate in males *vs* females (v/u). If it is higher in males, a high proportion of mothers of isolated cases will be carriers. If it is higher in females, many isolated haemophiliacs will be the result of true *de novo* mutations. The sex ratio of mutation frequencies in haemophilia has not been definitely established. Most studies show a higher mutation frequency in the male than in the female. In haemophilia A v/u has been estimated as 5.0⁶ and 9.6^{7,8} in two recent studies. In haemophilia B it has been estimated as 11.⁹ Even if these figures are cautiously interpreted, most mothers of isolated haemophiliacs are carriers. For practical purposes one can approximate the genetic probability to be 0.85 for carriership in mothers of an isolated case.

(ii) Phenotypic assessment of haemophilia A and B

Glossary of terms. Factor VIII:C (factor VIII coagulant activity)—the coagulant activity of factor VIII as assessed from the normalizing effect on the activated partial thromboplastin time (APTT) of plasma containing less than the 1% of the normal factor VIII:C concentration.

Factor VIII:Ag (factor VIII antigen)—the factor VIII protein as assessed by immunoassays.

vWF:Ag (von Willebrand factor antigen)—the von Willebrand factor protein as assessed by immunoassays.

Factor IX:C (factor IX coagulant activity)—the

Table 1.

Information	Probability or odds		
	Carriership	Noncarriership	Carrier:noncarrier
Anterior to III:2	0.25	0.75	1:3
Descendants of III:2			$(0.5)^n:1$
Anterior and Descendants	$(0.5)^n/(0.5)^n + 3$	$3/(0.5)^n + 3$	$(0.5)^n:3$

coagulant activity of factor IX as assessed from the normalizing effect on the APTT of plasma containing less than 1% of the normal factor IX:C concentration.

Factor IX:Ag (factor IX antigen)—the factor IX protein as assessed by immunoassays.

Substrate plasma is plasma devoid of factor VIII:C or IX:C, respectively, and used in the coagulation assay.

Test plasma is the plasma sample taken from an individual to be tested.

Local working standard plasma is a pool of plasma used as a 'control' plasma. It should be calibrated in international units (IU).

Laboratory standards. Factor VIII:C: All factor VIII:C estimations must be assayed by comparison with an international standard and expressed as international units (IU) of factor VIII coagulant activity. One IU of VIII:C is by definition the factor VIII coagulant activity in 1 ml of 'fresh normal human plasma'. The IU is defined by the 'International Standard for Blood Coagulation Factor VIII Plasma Human' (at present 90/550), which is available from the National Institute for Biological Standards and Control, London, UK.

In the local laboratory, pooled citrated plasma from at least 20 healthy donors, having an age and blood group distribution comparable to those of the local population from which test subjects are drawn, may be used as the working standard. Every new batch of this local standard plasma has to be calibrated against the international standard plasma or an 'intermediate' standard which has been calibrated against the international standard. When calibrating, the standard plasma should be tested three times in triplicate and a conversion factor be calculated for local U VIII:C/ml to IU VIII:C/ml. A new working standard should be prepared every 2–3 months to minimize inaccuracy due to deterioration in storage. The standard must be stored at -70°C .

Factor VIII:Ag: The local working standard calibrated against the international standard as described above is to be used as standard.

vWF:Ag: The local working standard should be prepared according to the guidelines above and calibrated against the international standard for vWF (at present 5th British Standard for Blood Coagulation Factors, plasma human 91/516).

Factor IX:C: The local working standard should be prepared according to the guidelines above and calibrated against the 'International Standard for Blood Coagulation Factor IX Human Plasma' (at present the 5th British Standard for Blood Coagulation Factors, plasma human 91/516). This standard may also be used for Factor IX:Ag measurements.

Sampling of blood. Both carriers and controls have to be in good health at the time of sampling since inflammatory states, liver and other diseases and certain drugs may influence the concentrations of the coagulation factors.

The syringes and tubes used should be of plastic or siliconized glass. Vacuum tubes may be used. The anti-coagulant should be 0.11 or 0.13 M sodium citrate, 1 vol. to 9 vol. of blood. The tubes should be turned upside down two to three times immediately after sampling. Centrifugation for at least 20 min at $2\,000 \times g$ should be performed as soon as possible. The plasma should not be haemolysed. If plasma is not analysed immediately it should be frozen at -70°C and not stored more than a few months.

The results of VIII:C and vWF:Ag determinations will not be confounded if carriers are pregnant until the 22nd week of gestation or taking oral contraceptives at the time of blood sampling.^{10–12} The age (haemophilia A and B) and blood group (haemophilia A) has to be considered in both carriers and controls.^{13,14} The data on the effect of age on IX:C concentrations are ambiguous; no effect was found by Graham *et al.*¹⁵ whereas Orstavik *et al.*¹⁶ in a population of twins found a significantly higher value in the senior twins. Oestrogen-containing drugs, like oral contraceptives, result in higher concentrations of both IX:C and IX:Ag.¹⁷

Coagulant assays. VIII:C should be measured by a one-stage clotting assay or chromogenic substrate method.

The one-stage method is based on the test sample's ability to correct the APTT in plasma which has a $<1\%$ VIII:C.¹⁸ The one-stage assay uses a partial thromboplastin, such as cephalin, as a substitute for platelets in the test plasma (phospholipids). Activation of the contact phase can be accomplished by substances such as celite. Phospholipid/activator reagents differ with respect to phospholipid content, the presence of oxidation products, their procoagulant potency and precision.¹⁹

The chromogenic substrate assay for measuring VIII:C has been shown to have a correlation of 0.92–0.98 to one-stage clotting assays.²⁰ In a comparison between different VIII:C assays, the chromogenic substrate assay was found to have the highest precision.²¹

VIII:Ag can be measured by various immunoradiometric (IRMA) or enzyme-linked immunosorbent (ELISA) assays.²² Most of these assays are two-site solid phase assays using polyclonal or monoclonal antibodies as the solid phase and a radio- or enzyme-labelled tracer. Some reports have favoured VIII:Ag measurement over VIII:C for carrier detection, while other reports have come to the opposite result.^{23,24} The local experience with the different methods may favour

one over the other, but there is no universal advantage of using VIII:Ag instead of VIII:C or vice versa.

vWF:Ag can be measured quantitatively with two different methods; electroimmunoassay (EIA)²⁵ and IRMA or ELISA.²⁶ EIA is performed by the 'rocket technique'.²⁷ IRMA and ELISA techniques are usually performed as two-site solid phase assays and several modifications exist. In a first step, tubes or microtitre wells are incubated with polyclonal or monoclonal antibody against vWF. In a second step the plasma to be tested is incubated in various dilutions. In the third step radiolabelled or enzyme-linked antibody is added as a tracer and measured after incubation and washing procedures. The dose-response curves of the working standard and the test samples must be parallel in order to calculate the vWF:Ag concentration in the sample tested. The standard working plasma should be calibrated against the international standard or an 'intermediate' standard. EIA is as satisfactory a method as IRMA or ELISA in detection of carriers of haemophilia A and the experience at the local laboratory should make the choice.²⁴

IX:C can be measured by one-stage or two-stage assays based on the same principles as VIII:C assays. The same precautions concerning sampling, working standard plasma, international standard plasma and test plasma are applicable to IX:C assays. A chromogenic substrate method which uses a factor Xa substrate, has been described based on the conversion of factor IX in plasma to IXa by addition of a semi-pure factor XIa in the presence of calcium.²⁸

IX:Ag can be measured by various immunological assays, the most reliable being IRMA and ELISA.^{29,30} When these techniques were introduced it was found that haemophilia B could be classified into many subgroups according to the amount of IX:Ag present. CRM+ (cross reacting material positive) had normal IX:Ag, CRM^R reduced amounts and CRM- undetectable IX:Ag. In the CRM^R group two subgroups can be identified; one where the factor IX activity and antigen are reduced to the same degree (IX:C/IX:Ag approx. = 1.0), and one group where IX:C is significantly more reduced than the IX:Ag (ratio IX:C/IX:Ag < 0.5). Haemophilia B- is a designation of CRM- patients and CRM^R with IX:C/IX:Ag ratio of approximately 1.0. Haemophilia B+ are CRM+ patients and CRM^R with IX:C/IX:Ag ratio < 0.5. Haemophilia Bm are CRM+ or CRM^R patients who have a prolonged one-stage prothrombin time when using ox-brain thromboplastin in the assay.³¹ Haemophilia B Leyden refers to CRM- and CRM^R patients where the deficiency of IX:C and IX:Ag is only transient. After puberty the levels increase with an average rate of 0.04–0.05 IU/ml each year.³²

The average concentrations of IX:C or IX:Ag are lower in haemophilia B carriers than in non-carrier women. Both concentrations are influenced by the random inactivation of one of the X-chromosomes (Lyon-phenomenon).³³ Haemophilia B+ carriers also have lower concentrations of IX:C, though their IX:Ag may vary widely as a result of the Lyon-phenomenon.³⁴ No consensus exists on the most effective way of classifying carriers of different types of haemophilia B. Measurement of IX:Ag offers only limited predictive improvement, mainly in haemophilia B+ families according to Kasper *et al.*³⁵ and Pechet *et al.*³⁶ Orstavik *et al.*³⁴ on the other hand, found that IX:Ag was of discriminant value in both haemophilia B- and B+ families. In another study the most efficient way of classifying haemophilia B- carriers was univariate discrimination based on IX:Ag. For haemophilia B+ carriers bivariate linear discriminant analysis using both IX:C and IX:Ag gave the best results.¹⁴ In this paper univariate linear discriminant analysis is advocated for haemophilia B, using IX:C measurements (see below).

Odds ratios based on laboratory data. On average, carriers of haemophilia A or B have about 50% of the normal levels of factor VIII or IX. Due to considerable overlap between the levels in carriers and normal women it is usually not possible to establish carrier status on these laboratory data in an unambiguous way. Therefore, the laboratory data are used to calculate an odds ratio favouring carriership: an odds ratio 'X' means that the laboratory findings in the consultand are X-times more likely to be found in a carrier than in a non-carrier. The subsequent use of Bayes' rule allows one to combine this odds ratio with the probability of carriership derived from the pedigree analysis and to obtain a 'final' probability of carriership. In the 1977 WHO memorandum³⁷ four ways were described to calculate odds from laboratory data. Currently, however, the preferred method for haemophilia A is bivariate linear discriminant analysis based on factor VIII:C and vWF:Ag measurements accommodating the effects of age and ABO blood group.²⁴ For haemophilia B, univariate linear discriminant analysis is advocated using factor IX:C measurements and applying a correction for the use of oral contraceptives.^{16,38} Although the estimation of FIX:Ag levels may be advantageous in some cases,³⁴ the routine application of this assay does not seem to be justified.^{35,36,38}

Both in haemophilia A and B, laboratory data are obtained in reference groups of carriers and non-carriers using exactly the same methods, reagents and standards as used for prospective consultands. Also the subjects used for reference purposes should be as similar as possible in all respects to the consultands. This

used to require the recruitment of about 30 obligatory carriers and an equal number of non-related but very similar women. Since DNA technology now allows one to definitely prove or disprove carriership one may use the laboratory data on previously diagnosed consultands for reference purposes. At all times, however, one should be aware that age, blood type, severity of haemophilia, pregnancy, use of oral contraceptives, and probably other factors may exert an influence on the outcome of the laboratory tests. Consequently it is important to note whether the reference subjects and prospective consultands are similar in these respects. Furthermore, the laboratory data usually need to be transformed such that the distribution of the data for the reference groups are normal or at least non-skewed.

In Tables 2 and 3 we have provided one approach for haemophilia A using the universal discriminant²⁴ which obviates the need to study a reference group of

carriers, and one approach for haemophilia B applicable to both CRM positive and CRM negative forms.¹⁶ Considering the primary position of DNA analysis in carrier diagnosis we feel that these straightforward approaches should be sufficient in most cases. The calculations can be easily carried out in a spreadsheet-type computer program.

Combining pedigree and laboratory data. The pedigree probability of carriership (P) has to be transformed to the corresponding odds ($a:b$) according to the formula $a:b = P:(1-P)$. The probability $P = a/(a + b)$. In familial haemophilia the anterior pedigree itself supplies a probability of the consultand being a carrier. If descendants exist one can modify the anterior probability with the odds of being a carrier derived from the descendant pedigree. In this method, the anterior probability is first transformed to odds before combining the two (Table 4).

The odds of being a carrier from the laboratory data, as calculated above, is multiplied with the pedigree

Table 2. Haemophilia A, bivariate universal discriminant analysis. This method requires factor VIII:C and von Willebrand factor antigen reference data non-carriers only. Data are transformed using the natural logarithm and the effects of age and ABO blood type are accommodated²⁴

Enter for consultand:

α = age in years
 β = ABO-bloodtype: 0 for 0, 1 for non-0
 y = vWF:Ag in IU/ml
 δ = VIII:C in IU/ml
 π = Genetic probability of carriership (fraction of 1)

Enter for normal reference group:

μ_c = mean of Ln transformed vWF:Ag levels in IU/ml
 μ_x = mean of Ln transformed VIII:C levels in IU/ml

Compute for consultand:

$x = \ln(y) - \mu_c$
 $y = \ln(\delta) - \mu_x$

Compute the coefficients for the modified discriminants:

$a = -0.0955 - 0.0156\alpha + 0.000196\alpha^2 + 0.0298\beta$
 $b = 0.649 - 0.00184\alpha + 0.0000314\alpha^2 + 0.117\beta$

Compute the predicted means of the discriminants for carriers and normals:

$c = -0.391 - 0.00571\alpha + 0.0001\alpha^2 - 0.0648\beta$
 $d = -0.0347 - 0.00171\alpha + 0.0000473\alpha^2 + 0.0754\beta$

and compute the odds ratio:

$e = ax + by$
 $f = 4.28(e - c)$
 $g = 7.97(e - d)$
 $h = 0.623 + 0.5(f + g)(f - g)$
 $LR = \exp(-h)$, the odds ratio favouring carriership*

Compute the final probability of carriership:

$P_c = \pi LR / (\pi LR + 1 - \pi)$

*Note: $\exp(-h)$ denotes 'e', the base of natural logarithm, raised to the power of $-h$.

Table 3. Haemophilia B, univariate linear discriminant analysis. Factor IX:C levels are required for both the carrier and non-carrier reference groups assuming that the reference subjects do not use oral contraceptives. The factor IX levels of the consultand need to be corrected by a factor of 0.75 if she does. In this example the data are transformed by the square root to obtain symmetrical distributions

Enter for consultand:

α = Factor IX:C in IU/ml
 β = Oral contraceptive use: 0 for no, 1 for yes
 π = Genetic probability of carriership (fraction of 1)

Enter for the carrier and non-carrier reference groups:

μ_c = mean of square-root-transformed factor IX:C levels of the carriers in IU/ml
 σ_c = standard deviation of square-root-transformed factor IX:C levels of the carriers in IU/ml
 μ_n = mean of square-root-transformed factor IX:C levels of the non-carriers in IU/ml
 σ_n = standard deviation of square-root-transformed factor IX:C levels of the non-carriers in IU/ml

Compute transformed and corrected factor IX:C for consultand:

$a = [(1 - 0.25\beta)\alpha]^{1/2}$

Compute the odds ratio:

$b = 1/(2\sigma_c^2)$
 $c = 1/(2\sigma_n^2)$
 $d = (a - \mu_c)^2$
 $e = (a - \mu_n)^2$
 $f = bd - ce$
 $g = \exp(f)^*$
 $LR = \sigma_n / (g\sigma_c)$, the odds ratio favouring carriership

Compute the final probability of carriership:

$P_c = \pi LR / (\pi LR + 1 - \pi)$

*Note: $\exp(f)$ denotes 'e', the base of natural logarithm, raised to the power of f .

Table 4. Combining pedigree and laboratory data

Odds from pedigree	1:3
Odds from the laboratory data	10:1
Combined odds	$1 \times 10:3 \times 1 = 10:3$
Final probability	$10/(10 + 3) = 0.77$

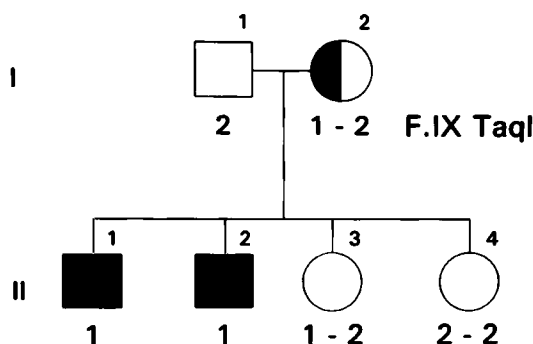


Figure 2. Example of the use of the intragenic factor IX *TaqI* polymorphism in determining carrier status in two potential carriers of the disorder, II.3 and II.4. The haemophilia B mutation is segregating with the '1' allele in this kindred. Potential carrier II.3 has inherited the *TaqI* '1' allele from her obligate carrier mother and can thus be identified as a carrier of haemophilia B with a greater than 99% probability. In contrast, her sister II.4 has inherited the *TaqI* '2' allele which is associated with the normal factor IX gene and can therefore be told that she is not a carrier.

odds to arrive at a combined odds. This is then again transformed into a probability by the formula $P = a/(a + b)$. The example in Table 4 illustrates the calculations.

(iii) The genotypic assessment of haemophilia A and B: genetic linkage using intragenic polymorphism analysis

The genes encoding factor VIII and factor IX. The factor VIII gene is situated at the telomeric end of the long arm of the X chromosome at band Xq28. The gene encompasses 186 kbp of genomic DNA (approximately 0.1% of the DNA sequence on the X chromosome) and comprises 26 exons ranging in size from 69 bp to 3.1 kbp.³⁹ Recent studies indicate that three different transcripts result from the processing of this gene. The normal factor VIII mRNA of 9 kbp is accompanied by two smaller transcripts of 1.8 kbp and 2.5 kbp, both of which appear to originate from intron 22 of the gene. The significance of these two alternative products is currently unclear. The factor IX gene is situated at band Xq27, approximately 40 megabases

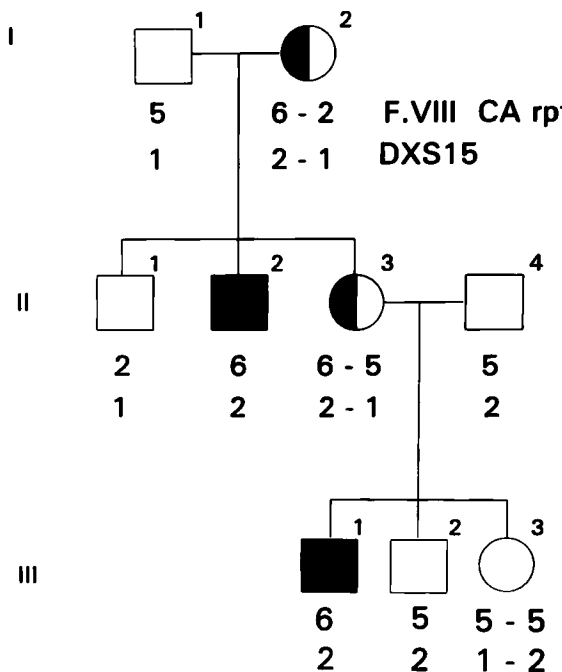


Figure 3. The use of two polymorphisms in segregation analysis of a haemophilia A family. The two markers shown are the multi-allelic intron 13 CA repeat and the extragenic DXS15 polymorphism which is located approximately five recombination units distant from the factor VIII gene. The haemophilia A mutation is segregating with the intron 13 '6' allele in this family. With this information in mind, the potential carrier female III.3 is identified as a non-carrier. The coincident analysis of the DXS15 marker shows that a recombination event has occurred between this locus and the factor VIII gene in the unaffected male III.2. In this individual, analysis with this extragenic marker alone would have incorrectly predicted that he had inherited the haemophilia A mutation.

centromeric of the factor VIII locus on the X chromosome. The gene is approximately 33.5 kbp long, comprises 8 exons and encodes an mRNA of 1.4 kbp.⁴⁰

Introduction to genetic linkage. The mutations that result in haemophilia A and B are heterogeneous and in most instances involve changes of single nucleotides.^{41,42} These two factors in two large genes have made the direct detection of haemophilic mutations a difficult challenge. As a result, many laboratories continue to assess the genetic status of potential carriers of haemophilic mutations through the use of indirect genetic markers of the factor VIII and factor IX genes. These genetic polymorphisms represent natural variations of the genome sequence which occur in the general population and which can be used as con-

venient landmarks to track mutant genes through families (Figures 2 and 3).

When the polymorphisms occur within the gene of interest (intragenic polymorphisms), the likelihood of the polymorphic marker becoming unlinked from the mutation through genetic recombination is related to the size of the gene. To date, there are no reports of intragenic recombination events in either haemophilia A or B and thus one can assume that genetic diagnosis based on the analysis of an intragenic polymorphism in these conditions is extremely accurate.

This discussion will summarize information concerning the types of polymorphisms occurring within the factor VIII and factor IX genes and their utility in genetic diagnostic studies.

Types of sequence variation within the factor VIII and factor IX genes. In order to be able to track individual copies of the factor VIII and factor IX genes, it is essential to have available polymorphic sequences which will differentiate between the two gene copies present in females. The presence of heterozygosity or informativeness for a polymorphism is a prerequisite which must be satisfied if genotypic studies are to be effective. In addition, as alluded to above, the polymorphic sequence should either be within the gene with which the disease is segregating or close enough to ensure that the possibility for genetic recombination between the polymorphism and the disease mutation is minimal. The final point to re-emphasize is that although some polymorphisms may be in allelic association (linkage disequilibrium) with particular mutations, the polymorphisms themselves represent phenotypically neutral sequence variation that is found in the general population.

Two types of polymorphic sequence exist within the haemophilia genes (Figure 4 and Tables 5–8). The most frequent and simple examples are the bi-allelic polymorphisms resulting from single nucleotide substitutions which either create or abolish restriction endonuclease sites (restriction fragment length polymorphisms; RFLPs). The limitation of these bi-allelic systems is that the maximum level of heterozygosity or informativeness is 50%. One bi-allelic insertion/deletion polymorphism also exists in intron 1 of the factor IX gene.

Two multi-allelic intragenic polymorphisms have been identified to date in these genes. These CA dinucleotide repeat sequences occur within introns 13 and 22 of the factor VIII gene. The intron 13 repeat with eight alleles reported has a heterozygosity rating of approximately 80%.⁴³ In light of the fact that the overall frequency of sequence polymorphism in the human genome is probably in the order of 1 nucleotide in

every kilobase the scarcity of polymorphisms in both of the haemophilic genes and in factor VIII in particular is noteworthy.

The extraction of DNA. High molecular weight DNA is most often obtained from blood leukocytes by treatment with proteinase K followed by phenol:chloroform extraction. The preferred anticoagulant for blood collection is either EDTA or sodium citrate. In light of its interference with subsequent test procedures heparin is not recommended as an anticoagulant for these studies. The transport of samples from one centre to another can be accomplished in a number of ways. Ideally, DNA should be extracted at source and then sent through the regular postal system, either in aqueous solution (10 mM Tris/1 mM EDTA), precipitated in ethanol or following lyophilization. If DNA extraction at source is not feasible, the anticoagulated whole blood should be decanted into polypropylene tubes, individually packaged in plastic or polystyrene containers and sent on dry ice via courier service to the testing laboratory. Whole blood samples can either be stored at -70°C without additional manipulations or leukocyte pellets can be prepared prior to storage. In most instances using standard extraction protocols, between 200 and 500 μg of DNA will be obtained from a 10 ml blood sample. Abbreviated DNA extraction protocols have been proposed in the preparation of material for the polymerase chain reaction and these methods will result in material of adequate quality in most instances.

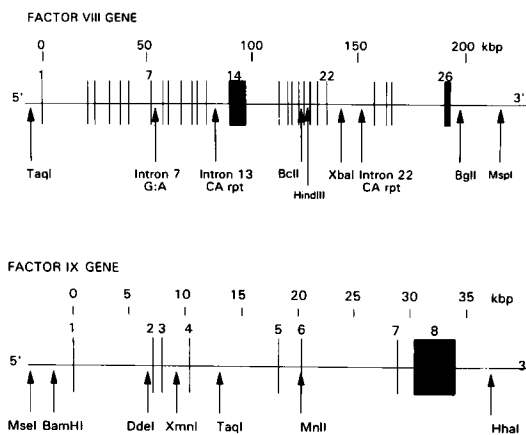


Figure 4. Line diagrams of the factor VIII and factor IX genes with the sites of common polymorphic sequences identified.

Table 5. Factor VIII intragenic DNA polymorphisms

Site	Restriction enzyme	Testing methods (PCR or Southern blot)	Heterozygosity	
			(%)	Ref.
5' flanking region	<i>TaqI</i>	Blot Probe: p701.1	40	102
Intron 7	-	PCR (G or A) Primers: 5' TGCAGAACATGAGCCAATTC 3' 5' TAATGTACCCAAGTTTTAGG 3' ASO Probes: 5' GCAAGACACTCTGACATTG 3' 5' GCAAGACACTCTAACATTG 3'	32	103
Intron 13	-	PCR (CA repeat) Primers: 5' TGCATCACTGTACATATGTATCTT 3' 5' CCAAATTACATATGAATAAGCC 3'	80	43
Intron 18	<i>BclI</i>	PCR Primers: 5' TAAAAGCTTTAAATGGTCTAGGC 3' 5' TTCGAATTCTGAAATTATCTTGTTT 3'	42	46
Intron 19	<i>HindIII</i>	PCR Primers: 5' GGCGAGCATCTACATGCTGGGATGAGC 3' 5' GTCCAGAAGCCATCCCAGGGGAGTCT 3'	42	63, 104
Intron 22	<i>XbaI</i>	PCR and blot Primers: 5' CACGAGCTCTCCATCTGAACATG 3' 5' GGGCTGCAGGGGGGGGGGACAACAG 3' Probe: p482.6	48	46, 48
Intron 22		PCR (CA Repeat) Primers: 5' TTCTAAGAATGTAGTGTGTG 3' 5' TAATGCCACATTATAGA 3'	44	105
3' of exon 26	<i>BglI</i>	Blot Probe: Factor VIII cDNA-C	18	106
3' of exon 26	<i>MspI</i>	Blot Probe: p625.3	44	107

Analysis of the polymorphic sequences. The two molecular genetic techniques used to identify DNA polymorphisms are those of Southern blotting⁴⁴ and the polymerase chain reaction (PCR).⁴⁵ The former method has been in use for more than a decade and involves a relatively labour-intensive schedule comprising the capillary transfer of endonuclease-digested DNA fragments from an agarose gel to a membrane support and the subsequent probing of the membrane with a radiolabelled DNA fragment representing the sequence of interest. Studies utilizing Southern analysis require a minimum of 5 µg of high molecular weight DNA for testing and take at least 5–7 days to produce results. The polymerase chain reaction has now

replaced Southern blotting in many instances. This technique utilizes synthetic oligonucleotide primers to select for specific sequences of interest which are then amplified *in vitro* to produce a targeted product which is present in more than a million-fold its original concentration. The power of this method has resulted in several advantages including the ability to work with very small starting quantities of DNA (less than 1 µg), increased simplicity and biosafety (non-radioisotopic methods) and the completion of tests within 48 h.

Most of the polymorphisms in the factor VIII and factor IX genes can now be studied through the analysis of DNA which has been amplified *in vitro* by PCR.^{46,47} The only reason to establish Southern Blot-

Table 6. Factor VIII intragenic DNA polymorphisms

Site	Restriction enzyme	Southern blot alleles	Allelic frequencies	PCR alleles
5' flanking region	<i>TaqI</i>	9.5 kb 4.0 kb	72% 28%	- -
Intron 7	-	-	79% 21%	G A
Intron 13	-	-	CA Repeat 8 alleles	
Intron 18	<i>BclI</i>	1.1 kb 0.88 kb	29% 71%	142 bp 99 + 43 bp
Intron 19	<i>HindIII</i>	2.7 kb 2.6 kb	70% 30%	469 + 248 bp 469 + 167 + 81 bp
Intron 22	<i>XbaI</i>	6.2 kb 4.8 + 1.4 kb	41% 59%	96 bp 68 + 28 bp
Intron 22	-	25 Repeats 26 Repeats	33% 67%	CA Repeat
3' of exon 26	<i>BglI</i>	20 kb 5 kb	10% 90%	- -
3' of exon 26	<i>MspI</i>	7.5 kb 4.3 + 3.2 kb	68% 32%	- -

Table 7. Factor IX intragenic DNA polymorphisms

Site	Restriction enzyme	Testing method (PCR or Southern blot)	Heterozygosity	
			(%)	Ref.
5' flanking region	<i>MseI</i>	PCR	44	108
5' flanking region	<i>BamHI</i>	PCR 5' GATAGAGAACTGGAAGTAGACCC 3' 5' TTAGGTCITTCACAGAGTAGATT 3'	11	51, 109
Intron 1	<i>DdeI</i>	Primers: Sequences not reported PCR	36	47, 60
Intron 3	<i>XmnI</i>	Primers: 5' GGGACCACTGGTATAATGTGG 3' 5' CTGGAGGATAGATGTCTCTATCTG 5' PCR	41	47, 60
Intron 4	<i>TaqI</i>	Primers: 5' AATCAGAGACTGCTGATTGACTT 3' 5' AAACAGCCAGATAAAGCCTCCA 3' PCR	45	47, 110
Codon 148	<i>MnII</i>	Primers: 5' CTGGAGTATGACTGGCCAATTATCC 3' 5' GGTACACAAGGATTCTAAGGTTG 3' PCR	45	111
3' flanking region	<i>HbaI</i>	Primers: 5' GATTTGAAAACGTCCATGAAAATAAC 3' 5' AAGTACCTGCCAAGGGAATTGACCTGG 3' PCR	48	57
		Primers: 5' ACAGGCACCTGCCATCACTT 3' 5' AGATTTCAAGCTACCAACAT 3'		

Table 8. Factor IX intragenic DNA polymorphisms

Site	Restriction enzyme	Southern blot alleles	Allelic frequencies	PCR alleles
5' flanking region	<i>MseI</i>	–	33% 67%	83 bp 57 + 26 bp
5' flanking region	<i>BamHI</i>	15 kb 13 kb	52% 48%	356 bp 216 + 140 bp
Intron 1	<i>DdeI</i>	1.75 kb 1.70 kb	24% 76%	369 bp 317 bp
Intron 3	<i>XmnI</i>	11.5 kb 6.5 kb	71% 29%	222 bp 154 + 68 bp
Intron 4	<i>MspI</i>	5.8 kb 3.4 kb	22% 78%	– –
Intron 4	<i>TaqI</i>	1.8 kb 1.3 kb	65% 35%	163 bp 124 + 39 bp
Codon 148	<i>MnII</i>	–	33% 67%	126 + 279 bp 126 + 120 + 159 bp
3' flanking region	<i>HbaI</i>	–	61% 39%	230 bp 150 + 80 bp

ting for haemophilia genetic diagnosis is for the analysis of the *XbaI* and *BglI* polymorphisms in the factor VIII gene. Assessment of the *XbaI* polymorphic genotype by PCR is complicated by the co-amplification of homologous sequences adjacent to, but outside of, the factor VIII gene. This complexity can be resolved by Southern analysis.⁴⁸

The principal requirements for analysis of these intragenic polymorphisms are as follows: a reliable source of amplification primers, a programmable temperature cycling apparatus (Thermal Cycler) and suitable vertical electrophoresis equipment. The only polymorphisms which can be studied by PCR but which require radioisotopic detection are the highly informative factor VIII intron 13 and intron 22 CA repeats. The analysis of these variable sequences requires end-labelling of one of the amplification primers with either gamma ³²P or gamma ³⁵S, ATP with subsequent autoradiography.

Following DNA extraction, the PCR amplification of these various sequences takes approximately 3 h, after which the amplified products are digested with the appropriate restriction enzyme and analysed by polyacrylamide gel electrophoresis. As with all PCR studies, the complication of sample contamination with extraneous DNA must be guarded against. Most laboratories perform their PCR studies in a designated clean area and use a dedicated set of equipment (including positive displacement pipettes) and supplies to minimize the risk of DNA carry-over. In addition, the inclusion of a 'no DNA template' blank tube in each experiment provides a further safeguard against this

problem. Finally, although the endonuclease digestion of most PCR products will proceed to completion uneventfully, the inclusion of previously genotyped PCR amplified samples in each test run ensures that all components of the endonuclease reaction have been added and are functional. In addition, the inclusion of an invariant endonuclease site within the amplified fragment further assists in evaluating the digestion process.

Haemophilia A—testing strategy. The strategy for polymorphism analysis in any particular family must take into account factors including the site of the polymorphism, the heterozygosity rating of the marker and the ethnic origin of the family. This latter consideration will be addressed in detail later in this section of the text.

The recently described intron 13 CA repeat polymorphism appears to be informative in approximately 80% of females and thus represents the logical starting point for analysis of factor VIII polymorphisms (Figure 5).

This sequence can be amplified in a 'multiplex' PCR with the other, intron 22, CA repeat and the two sequences analysed simultaneously. The fact that these sequences require the use of radiolabelled amplification primer and electrophoretic separation of the products on a DNA sequencing gel may, however, result in some laboratories reserving the analysis of this marker for those cases in which the two other frequent *BclI* and *XbaI* polymorphisms are uninformative. Fortunately, these four markers are not in allelic associ-

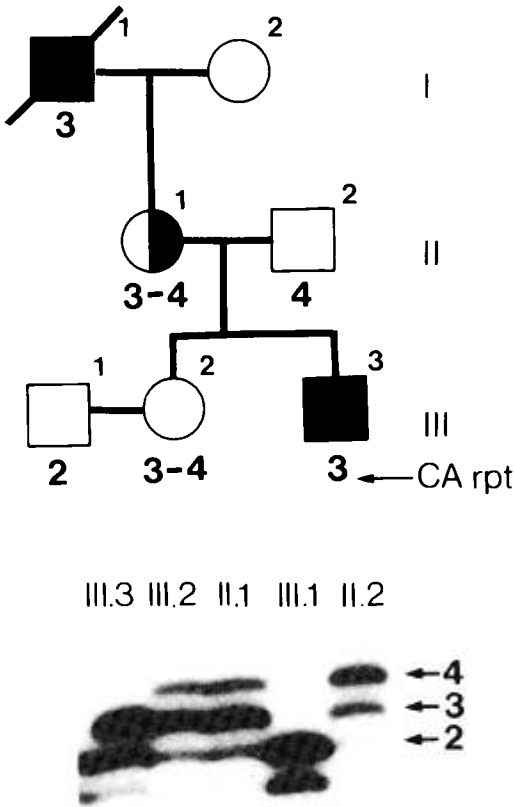


Figure 5. Analysis of the CA dinucleotide repeat sequence from intron 13 of the factor VIII gene. The electrophoretic analysis of CA repeat sequences is routinely accompanied by the presence of fainter autoradiographic bands below the CA repeat allele. The genotype is read as the fragment(s) of greatest autoradiographic intensity. The haemophilic mutation in this family is segregating with the '3' allele. The potential carrier, III.2, has inherited the mutant factor VIII gene from her mother and can be offered prenatal diagnosis using this marker with an error rate of less than 1%.

ation (linkage disequilibrium) and more than 95% of females will be informative for one or more of these polymorphisms. The remaining families should be tested with the *Bgl*I marker and with the intron 7 polymorphism which will be informative in approximately 10% of females who are homozygous for the absence of the *Bcl*I polymorphic site.

Haemophilia B. The combined use of the *Taq*I, *Xmn*I, *Dde*I, *Hba*I and *Mnl*I polymorphisms will provide informative results in approximately 85% of females in haemophilia B genotype testing. Unfortunately, however, all these bi-allelic polymorphisms are in some

degree of allelic association and there is no hyper-variable sequence within or close to factor IX. Therefore, there will still be between 10% and 15% of families with haemophilia B in whom intragenic polymorphism analysis is uninformative and where either linked extragenic markers will have to be investigated or direct mutation detection will be necessary.

Advantages of genotype assignment with polymorphism testing. Where a prior family history of haemophilia exists, and an intragenic polymorphism is informative, diagnostic results with an error rate of less than 1% are attainable. The methods are straightforward, rapid and inexpensive to perform. Thus, in many families requesting genetic diagnosis of haemophilia, the use of intragenic polymorphism analysis represents the diagnostic strategy of choice.

Limitations to the use of polymorphism testing in haemophilia. Although in many instances, the use of an informative intragenic polymorphism will provide highly accurate genetic diagnosis of haemophilia, there are, nonetheless, some limitations to this diagnostic strategy.⁴⁹ All of these drawbacks relate to the fact that the haemophilic mutation itself is not identified by these methods.

The requirement for family sampling. Polymorphism linkage analysis requires the participation of a minimum number of key individuals from a haemophilic family. At least one affected male should be available for testing to identify the polymorphic allele which is associated with the mutation in the family requesting diagnosis. With the recent catastrophe of HIV infection in the haemophilia population, this initial requirement may be compromised by early deaths of haemophilic males. However, even in the instances where all affected males in the family are deceased, the recovery of DNA from stored pathological samples for analysis by PCR still makes polymorphism testing feasible.

In addition to getting all appropriate family members to agree to participate in genetic testing, it is also vital that all stated family relationships (particularly paternity) are correct.

Families with an isolated affected haemophiliac. Sporadic cases of haemophilia comprise approximately 30–50% of the total haemophilic population. In these families, because polymorphism analysis does not identify the haemophilic mutation directly, it is not possible to ascertain at which level of the pedigree the mutation arose. In fact, past studies have indicated that approximately 85% of mothers of isolated haemo-

philiacs are carriers but in individual diagnostic cases, unless the coagulation studies of the mother are strongly suggestive of her being a carrier, it is probably unwise to attempt the diagnosis of a haemophilic allele by polymorphism testing. Therefore, in these families, polymorphism studies can only be used to exclude transmission of the haemophilic mutation.

The requirement for heterozygosity and possibility of genetic recombination. As detailed above, for polymorphism linkage studies to yield useful information, it must be possible to differentiate between the two X chromosomes in key females through the presence of polymorphic heterozygosity. In the study of haemophilia A with intragenic markers, this requirement is now achieved in more than 95% of families due in large part to the intron 13 CA repeat polymorphism. In the analysis of the factor IX gene where a similarly multi-allelic sequence does not exist, some 10–15% of families will still require diagnostic studies with linked extragenic markers to achieve informative results. In these latter studies, the possibility of genetic recombination between the polymorphic site and the haemophilic mutation adds an additional uncertainty to the precision of genetic diagnosis.

(iv) Ethnic variation

Factor IX polymorphisms. In Caucasians, the use of six intragenic Factor IX gene RFLP sites allows linkage of the gene in approximately 80% of families.⁵⁰ These sites are *TaqI*, *XmnI*, *DdeI*, *MspI*, *BamHI* as well as the residue 148 (Thr/Ala) base change (*MnII*). However, there is marked ethnic variation in the incidence of heterozygosity for these sites. In Blacks, both the 5' *BamHI* and the intron 3 *BamHI* sites are useful, with heterozygosity rates of 0.46 and 0.22 respectively.⁵¹ In comparison, informativity at these sites is rare in the Caucasian populations.^{51,52} The incidence of heterozygosity for RFLP sites of different ethnic groups are given in Table 9. Orientals, such as Chinese⁵³ and Japanese,⁵⁴ Filipinos⁵⁵ as well as Malays⁵⁶ were found to have a low incidence or absence of these common polymorphisms. Thus prenatal or carrier testing by this approach is virtually impossible in these people. An *MseI* RFLP in the 5' flanking region of the Factor IX gene has been recently described, however, and shown to be informative in South-east Asian populations.

Extragenic polymorphisms. The recently described extragenic *HhaI* polymorphic site, located 8 kb 3' to the factor IX gene⁵⁷ is however more polymorphic. The heterozygosity rate in Caucasians was 0.48 and that in Chinese was 0.28. For the Chinese, this appeared to be the only useful RFLP for linkage of the haemophilia B

gene.⁵⁵ Since this site is still relatively close to the gene, it is less likely to be susceptible to recombinant events. To date, no case of recombination with factor IX has been reported using this site.

Linked intergenic polymorphisms. An *SstI* RFLP at locus DXS99 can be detected by probe pX58dIIIc.⁵⁸ This polymorphism gives rise to two alleles of 5.9 kb and 8.8 kb respectively, with a frequency of 0.43 for the former. The polymorphic locus DXS99 is mapped to Xq26–q27 and tightly linked to the factor IX gene. The precise genetic distance between this locus and the factor IX gene has yet to be determined, but thus far, no recombination had been detected in 39 informative meioses, giving a LOD score of 9.79 at $\theta = 0.0$, with 95% confidence limit of $\theta = 0-0.06$. Since this marker and the FIX loci do not appear to be in linkage disequilibrium,⁵⁸ the analysis of this *SstI* RFLP at DXS99 in conjunction with the six intragenic sites in the factor IX gene should increase the diagnostic efficiency to 90% of females at risk.

Linkage disequilibrium of factor IX polymorphisms. In Caucasians, the *TaqI*, *XmnI*, *MspI* and *MnII* RFLPs show marked linkage disequilibrium (allelic association), thus the use of all four sites would only increase the diagnostic efficiency to 55% as opposed to 45% when using the *TaqI* site alone.^{56,59,60} However, *DdeI* and 3' *HhaI* polymorphisms showed much less disequilibrium and the combined use of these two sites and the *TaqI* site increases the heterozygosity rate to almost 76%.^{57,59}

In the American Blacks, the linkage disequilibrium between *TaqI* and *MspI* is less marked. While the intragenic *BamHI*+ alleles and *MspI*- alleles showed disequilibrium, the 5' *BamHI*, *DdeI* and *XmnI* sites appeared to be in equilibrium. The combined use of these latter sites showed an observed frequency of heterozygosity of 87% for Black females (*vs* ~60% in Caucasians).⁵²

No linkage disequilibrium was observed between the *HhaI* locus at the 3' end of the factor IX gene and the other intragenic loci,⁵⁷ thus it is likely that this polymorphic marker will be extremely useful for factor IX carrier testing. This has certainly been the case for Orientals,⁵³ who lack heterozygosity for the common intragenic RFLP sites.

Factor VIII polymorphisms. As with factor IX, the incidence of factor VIII RFLPs differ significantly in various racial groups,^{61,62} thus before a prenatal diagnosis programme can be instituted in a particular region, the RFLPs for that population should be studied, to decide the most suitable sites for use.

Table 9. Frequencies of heterozygosity for polymorphisms associated with factor IX gene in different ethnic groups

Polymorphic sites	Alleles (kbp)	Caucasian ⁵⁹		Japanese ⁵⁴		Chinese ^{61,64}		Malays ⁵⁶		American Blacks ⁶²	
		Allele freq.	Hetero	Allele freq.	Hetero	Allele freq.	Hetero	Allele freq.	Hetero	Allele freq.	Hetero
5' <i>Bam</i> HI	25 (-)	0.98	0.0421 ⁵¹	-	-	1.0	0	1.0	0	0.64	0.46
	23 (+)	0.02				0.0		0.0		0.36	
<i>Taq</i> I	1.8 (-)	0.65	0.45	1.0	0	0.99	0.02	0.99	0.02	0.86	0.24
	1.3 (+)	0.35		0.0		0.01		0.01		0.14	
<i>Xmn</i> I	11.5 (-)	0.71	0.41	1.0	0	0.99	0.02	1.0	0	0.88	0.21
	6.5 (+)	0.29		0.0		0.01		0.0		0.12	
<i>Dde</i> I	1.75	0.24	0.36	0.0	0	1.0	0	-	-	0.36	0.46
	1.7	0.76		1.0		0.0		-		0.64	
<i>Msp</i> I	5.8 (-)	0.20	0.32	0.0	0	-	-	-	-	0.39	0.47
	3.4 (+)	0.80		1.0		-		-		0.61	
<i>Bam</i> HI (2)	25 (-)	0.94	0.11	-	-	0.98	0.04	-	-	0.87	0.22
	16 (+)	0.06		-		0.02		-		0.13	
Residue 148 (<i>Mnl</i> I)	Thre (ACT)	0.67	0.44	-	-	0.97	0.06	0.97	0.06	0.89	0.1956
	Ala (GCT)	0.33		-		0.03		0.03		0.11	
3' <i>Hba</i> I	0.23 (-)	0.39	0.48	-	-	0.83	0.28	0.91	0.11	0.43	0.49(2934)
	0.15 (+)	0.61		-		0.17		0.06		0.57	
5' <i>Mse</i> I	(-)	0.67	0.44	-	-	-	-	-	-	-	-
	(+)	0.33		-		-		-		-	

Asians ⁵⁶		Maori ¹¹²		Polynesian ¹¹²		Thai ¹⁰⁸	
Allele freq.	Hetero	Allele freq.	Hetero	Allele freq.	Hetero	Allele freq.	Hetero
0.64	0.46	-	-	-	-	-	-
0.36		-		-		-	
0.96	0.07	0.93	0.13	0.99	0.02	-	-
0.04		0.07		0.01		-	
0.96	0.07	0.94	0.11	-	-	-	-
0.04		0.06		-		-	
-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-
0.93	0.07	-	-	-	-	-	-
0.04		-		-		-	
0.32	0.43	-	-	-	-	-	-
0.68		-		-		-	
-	-	-	-	-	-	0.79	0.33
-		-		-		0.21	

Hetero = Heterozygosity

Table 10 summarizes the incidence of *Bcl*I, *Xba*I, *Bgl*II and *Hind*III polymorphisms in the factor VIII gene in various ethnic groups. The positive incidence of *Bgl*II polymorphism is higher in Chinese than in other races. Of particular interest is that in American Blacks, the rates of the (+) site for *Bcl*I and *Hind*III polymorphisms are the reverse of what is observed in other ethnic groups.⁶³ Table 10 also shows the female heterozygosity rate. The *Bcl*I dimorphism is more informative in Mediterraneans, Indians and Japanese (42-47%) compared to Caucasian, American Blacks, Chinese and Malays (31-39%). The *Hind*III and *Xba*I

RFLPs showed similar heterozygosity in the various groups tested, whilst the *Bgl*II RFLP is most useful in American Blacks (38%), but useless in Chinese (0%).

No data is currently available concerning the ethnic variability of allele frequencies for the intragenic CA repeat polymorphisms.

Linked intergenic polymorphisms. The physical mapping of the q28 region of the X chromosome has revealed that the loci DXS52 and DXS15 are 1-2 Mb centromeric to the factor VIII gene. The highly polymorphic St 14 probe detects a polymorphism within

Table 10. Frequencies of heterozygosity for polymorphisms associated with factor VIII gene in different ethnic groups

Polymorphic sites	Alleles (kbp)	Caucasian		Japanese		Chinese		Malays		American Blacks		Asians		Maori		Polynesian	
		Allele freq.	Hetero	Allele freq.	Hetero	Allele freq.	Hetero	Allele freq.	Hetero	Allele freq.	Hetero	Allele freq.	Hetero	Allele freq.	Hetero	Allele freq.	Hetero
<i>BclI</i>	1.1 (-)	0.27	0.39	0.30	0.42	0.21	0.33	0.21	0.33	0.81	0.31	0.31	0.43	0.33	0.44	0.43	0.49
	0.88 (+)	0.73		0.70		0.79		0.79		0.19		0.69		0.67		0.57	
<i>HindIII</i>	2.7 (-)	0.74	0.38	-	-	0.76	0.37	0.77	0.35	0.22	0.34	0.71	0.41	-	-	-	-
	2.6 (+)	0.26		-	-	0.24		0.23		0.78		0.29		-	-	-	-
<i>XbaI</i>	6.2 (-)	0.44	0.49	0.41	0.48	0.42	0.49	-	-	-	-	-	-	-	0.49	-	0.5
	4.8 + 1.4 (+)	0.56		0.54		0.58		-		-		-		-		-	
<i>BglII</i>	20 (-)	0.15	0.25	0.09	0.16	0.0	0	-	-	0.26	0.38	0.06	0.11	-	0.11	-	0.02
	5 (+)	0.85		0.91		1.0		-		0.74		0.94		-		-	
<i>MspI</i> (1)	7.5 (-)	0.68	0.43	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4.3 + 3.2 (+)	0.32		-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>MspI</i> (2)	4.0 (-)	-	0.00	-	0.45	-	-	-	-	-	0.00	-	0.13	-	-	-	-
	3.8 (+)	-		-		-		-		-		-		-		-	

Hetero = heterozygosity

the cluster MN12, cpX67, and DX13.⁶⁴ The two extragenic RFLPs, *BglII*/DX13⁶⁵ and *TaqI*/St 14⁶⁶ are both closely linked to the haemophilia A locus. The *BglII* RFLP detected by the DX13 probe shows two alleles, 5.8 kb (allele 1) and 2.8 kb (allele 2) respectively. In an initial study in Caucasians, 50% of the females were found to be heterozygous and no cross-over was observed between the DX13 and haemophilia A locus amongst 42 X-chromosomes studied.⁶⁵ In an Italian population, 60% of obligate carriers were heterozygous, with an equal incidence of alleles 1 and 2. One recombination was observed amongst 31 meioses, giving a recombination fraction of 0.045 and LOD score of 2.1.⁶⁷ Ethnic variation was observed with this RFLP. In the Japanese, of 108 chromosomes analysed, the frequencies of alleles 1 and 2 were 0.16 and 0.84 respectively, with a 27% heterozygosity rate.⁶⁸

The *TaqI* RFLP detectable with the St 14.1 probe gives two independent systems of alleles.⁶⁶ System I has eight alleles (1 to 8) ranging from 6.6 to 3.4 kb in length, and system II has two alleles, 'A' (5.5 kb) and 'B' (4.1 and 1.4 kb respectively). A summary of the data relating to the frequency of the different *TaqI* alleles shows that the incidence of alleles 7 and 8 were higher, whilst those for alleles 3 and 4 were lower in Chinese than Caucasians. No alleles 1 and 2 were detected in the Chinese⁶² and Japanese.⁶⁸ Other than this, the Japanese appeared to be similar to the Caucasians as far as other allelic frequencies in the *TaqI*/St 14 polymorphisms are concerned.⁶⁸ In all three ethnic groups, the heterozygous rate for alleles 1 to 8 were similar (0.712 to 0.798). Whilst in Chinese, the incidence of A and B alleles were 55% and 45% respectively (see Table 11).

Caution should be exercised when using intergenic linkage probes for diagnosis because of the possibility of meiotic recombination.^{69,70} With the St 14.1 probe,

reports from world literature suggested a genetic disease of 3 cM, and this should be taken into account during genetic counselling. The use of the DX13 probe would be even more prone to error, as the cross-over rate with the factor VIII gene is thought to be about 4.5%.⁷¹

Linkage disequilibrium of factor VIII polymorphisms.

There is a strong linkage disequilibrium between the intron 18 *BclI*, intron 19 *HindIII* and intron 25 *BglII* sites.^{63,70} Thus little additional information will be gained in using more than one of these three sites. In contrast, the *XbaI* site in intron 22 is often informative in females who are homozygous for the *BclI* site.⁷² Even though the *BclI* and *XbaI* sites are not in complete linkage equilibrium, with a disequilibrium coefficient of 0.0722–0.1627 in various ethnic groups reported, the combined use of these two sites would significantly increase the informativeness of 79% in Japanese, 64–69% in Caucasians and 52% in Chinese females respectively.^{62,68,72} In certain populations, multiple *XbaI* polymorphisms have been described, e.g. Chinese⁶⁸ and Canadian.⁷³ Although these other polymorphisms may well be non-factor VIII sequences which are detected by the factor VIII intron 22 probe (p482.6), they are closely linked to the factor VIII gene and the combined use of all the *XbaI* and *BclI* RFLPs would increase the detection rate to 67% in Chinese.

Due to the highly polymorphic nature of the St 14.1 probe, the combined use of the two intragenic (*BclI* and *XbaI*) and one extragenic (*TaqI*/St 14.1) polymorphisms should allow carrier detection or prenatal diagnosis in 96–100% of females at risk.^{62,67,68}

(v) *Direct mutation detection in the haemophilias*

Linkage analysis for carrier detection and prenatal diagnosis of haemophilia has widely appreciated ben-

Table 11. *TaqI* system I alleles detected by probe St14.1 at locus DXS52 on the X chromosome in various ethnic groups

System I alleles	kbp	Frequency (%)			
		Chinese ⁶²	Caucasians ⁶⁶	Italian ⁶⁷	Japanese ⁶⁸
1	6.6	0	0.5	0.6	0
2	5.3	0	4.5	4.6	0
3	4.8	9	12	15	13
4	4.5	2	36	32	33
5	4.1	4	20	15	16
6	4.0	18	20	15	16
7	3.9	23	11	18	9
8	3.4	44	15.5	14.5	29

efits including, rapidity, relative technical simplicity, wide availability and definitive diagnostic results in a high proportion of cases. However it has certain inherent drawbacks and limitations which have been previously described.

In principle, all these defects of linkage analysis may be circumvented by identifying the specific mutation in a given kindred. It is then sufficient merely to check the putative carrier or foetus at risk for the relevant defect. In addition, identification of mutations provides scientifically interesting information that may give clues on structure and function of factor VIII and IX, on mechanisms of mutation or on phenotype/genotype aspects such as the risk of inhibitor formation.

The obvious drawbacks of mutation analysis in haemophilia A and B are that the two genes involved are large and complex and the mutations therein extremely heterogeneous, as expected for X-linked sub-lethal disorders. However methods for rapid screening of large regions of DNA for small lesions have been developed in the past 5 years which now make this approach feasible in expert laboratories. These methods were first applied to the smaller gene, factor IX, with impressive results in several centres. For example, a project to identify all the mutations causing haemophilia B in the UK population is now well advanced with successful identification of a causative gene lesion in 160 out of 161 patient DNAs analysed so far (F. Giannelli, personal communication). The methods used for haemophilia B analysis comprise Southern blotting with a full length cDNA probe which detects deletions down to about 1.5 kb. This yields diagnostic information in about 1% of cases. For more detailed analysis, enzymatic amplification of each exon is performed using the polymerase chain reaction (PCR) followed by chemical cleavage mismatch detection (CCD). A positive signal from CCD is confirmed by direct sequencing.

Mutation analysis in haemophilia A has to cope with the fact that the 26 exons of the factor VIII gene are distributed over 186 kbp of genomic DNA and exon 14

is over 3 kbp in length. Southern blotting after digestion of genomic DNA with the enzyme *TaqI* yields diagnostic information in ~5% of cases due to the somewhat higher incidence of large deletions in haemophilia A and the occurrence of mutation hotspots in five *TaqI* sites. Currently three screening methods are being used to screen enzymatically amplified exons and flanking regions of the factor VIII gene. Higuchi and colleagues^{74,75} have applied denaturing gradient gel electrophoresis and reported on their results in 29 mild or moderate cases and 30 severe cases. Forty-five oligonucleotide primer sets were required to amplify 99% of the coding region and 41 of 50 splice junctions. The disease producing mutation was found in 25 out of 29 mild or moderate cases (85%) but in only 16 of 30 severe cases (53%). Even allowing for incomplete coverage of splice junctions there is a clear implication that a high proportion of mutations causing severe haemophilia A lie outwith the previously accepted essential regions of the factor VIII gene. This highly interesting finding has been confirmed using an alternative approach (see 'Developments in direct defect detection' below). Consequently until these unidentified mutations are located there is going to be a limitation on the effectiveness of even direct defect detection, underlining the continuing importance of phenotype assays and polymorphism analysis in carrier and foetal diagnosis.

Another powerful and technically simpler approach to mutation screening is based on the property of single-stranded DNA to form self-associated loop structures that are highly sequence dependent. The conformation adopted strongly influences rate of migration of single-stranded DNA in non-denaturing polyacrylamide gel electrophoresis, enabling detection of sequence polymorphism (or mutation). The screening method exploiting these phenomena is called Single Stranded Conformational Polymorphism (SSCP) analysis. A comparison of SSCP with CCD for mutation detection in factor VIII exons 1 to 14 has yielded essentially identical sensitivity (E. G. D. Tuddenham, unpublished observations), so this method may become more widely utilized. Finally direct sequencing of amplified DNA is used to identify any specific mutation. Methods based on the use of streptavidin-coated magnetic microbeads with biotinylated primers yield very clear and reproducible sequencing results from PCR amplified DNA.

Data in haemophilia A. A compilation of mutations in the factor VIII gene updated to August 1991 has been published in *Nucleic Acids Research*.⁴¹ Eighty-one different point mutations, six insertions, seven small deletions and 60 large deletions are catalogued.

Information where available is also provided for factor VIII coagulant and antigen level, clinical severity and inhibitor status. A unique number has been assigned to each patient for future identification. Thirty-eight per cent of point mutations are located in CpG dinucleotides. This frequency is biased by screening with *TaqI* which identifies five such sites, but an unbiased estimate from Higuchi's data²⁴ is similar at 32%. Recurrent mutation in CpG dinucleotides has occurred in at least 16 sites, where identity by descent can be excluded on RFLP haplotype or extreme geographical separation. Recurrence at non-CpG sites also occurs, for example at Arg 2307 Leu, found in American, German and Japanese patients.

This international database will be updated annually and published in *Nucleic Acids Research*. New cases for inclusion should be submitted to Dr S. Antonarakis, The Johns Hopkins University, Baltimore, USA or Dr E. Tuddenham, MRC Harrow, UK. The haemophilia A database has been submitted to the Genome Database (Welch Medical Library, 1830 East Monument Street, Baltimore, MD 21205, USA) and will be accessible there by electronic means. In future as the number of patients successfully analysed increases the only practical means of updating and retrieval will be *via* computerized databases, organized nationally and internationally. Appropriate security of information access will be built into these systems.

Data in haemophilia B. A compilation of point mutations, short deletions and insertions in the factor IX gene has been produced by Giannelli *et al.*⁴²

The data tabulated includes levels of factor IX clotting activity and antigen, inhibitor status but not clinical severity. Three hundred and eighty-eight mutants are listed, representing 206 different molecular alterations. These are distributed across the entire coding region from the signal peptide to codon 411, only five residues before the stop codon. Eight examples of mutations in the promoter region giving rise to the Leyden phenotype are recorded. Twenty-nine examples of mutations affecting RNA processing have been observed. One hundred and seventeen examples of mutations associated with normal levels of a non-functional factor IX protein have now been identified providing a wealth of potential information on structure and function of factor IX. This information has now been incorporated into the EMBL database, providing on-line access to updates.

The UK national register of mutations in haemophilia B is being maintained by Dr Giannelli at Guy's Hospital, allowing carrier and antenatal diagnosis to be performed by mutation specific analysis. This may be a model for other national mutation databases.

Dr Ljung (Malmo) has established a Swedish national database for haemophilia B. Out of 44 mutations analysed to date, 14 deletion or nonsense genotypes were found, amongst which five patients have inhibitors. Thirty missense mutations were characterized, amongst which were no patients with inhibitors. This exemplifies the potential for important clinical correlations arising from mutation analysis.

(vi) The application of polymorphism analysis and direct defect detection in developed and developing countries

The aim of molecular genetics in haemophilia studies is to identify the genetic lesion which causes the disease in a particular affected individual. The identification of this defect within family members then represents the ideal way to assess carrier status in females or to perform prenatal diagnosis in the unborn foetus.

Developments in molecular genetics mean that it is now possible to identify practically all the functional mutations in the factor IX gene in patients with haemophilia B and a significant number within the factor VIII gene in patients with haemophilia A (see above). The techniques used are specialized and require staff with molecular biology experience, and several groups within Europe and North America have established laboratories where these analyses are performed upon request. They also provide a follow-up service for subsequent carrier status assessment and prenatal diagnosis.

In these countries the identification of the specific defect will increasingly replace family studies based on gene tracking of polymorphisms (see above). Although polymorphism analysis, particularly when based on PCR technology is extremely simple to perform, the problems related to non-informative family members, non-paternity and sporadic disease mean that where the technology is available defect detection techniques are preferable. The recently reported cases of germline mosaicism in haemophilia present problems for family studies based on either approach. It is also expected that increased technological advances will make defect detection easier and more applicable to the routine laboratory. However without these advances it is probable that polymorphism based gene tracking will still remain important in those laboratories where molecular biology experience for defect detection is not available.

It is unlikely that direct defect detection will be available in the foreseeable future in many countries where the identification and treatment of the disease itself is only in its infancy. However, it should be pointed out that genetic analysis of families with patients with hae-

mophilia, even though the treatment of the haemophilic may not be optimal, can still be of assistance to the family by identifying carriers and preventing the birth of more affected individuals. Where resources are scarce it is probable that the least expensive techniques of gene tracking which will involve, at the moment, polymorphism analysis will be the only ones applicable. It is, therefore, important that such techniques should be made as simple as possible and their application in developing countries is something that may well advance quite rapidly in the next decade under the guidance of the World Federation of Haemophilia.

Prenatal diagnosis

(i) Obstetric techniques

Ultrasonography. All pregnant woman should be offered a detailed ultrasound examination for (i) confirmation of foetal viability and gestational age, (ii) the diagnosis of multiple pregnancies, and (iii) exclusion of both major malformations but also smaller defects, that may lead to the diagnosis of an underlying chromosomal abnormality or genetic haemophilia, ultrasonography at 16–20 weeks will reliably diagnose the foetal sex. In the presence of a female foetus, invasive foetal testing can be avoided.

Foetal blood sampling. In the 1970s and early 1980s the method of foetal blood sampling was fetoscopy, which involved the introduction into the amniotic cavity of an endoscope (3 mm in diameter). Blood vessels in the chorionic plate or umbilical cord were visualized and punctured to provide pure foetal blood.⁷⁶ The patients were usually hospitalized and the procedure was carried out under heavy sedation. The technique was confined to very few centres and in the best hands the procedure related risk of foetal death was 2–5%. More recently, improvements in imaging by ultrasonography have made fetoscopic guidance unnecessary and foetal blood can be obtained by ultrasound guided puncture of an umbilical cord vessel (cordocentesis) or the foetal heart (cardiocentesis). The preferred method is cordocentesis, which involves the ultrasound guided insertion of a 20- or 22-gauge needle through the maternal abdomen and into an umbilical cord vessel.^{77,78} The procedure is carried out on an outpatient basis and no maternal sedation or anaesthesia are required. Several centres throughout the world have now developed considerable expertise in this technique. Maternal complications are negligible. The risk of foetal death following cordocentesis is approximately 1–2%. The risks are higher when the mother is obese, the placenta is posterior and the gestation at sampling 16–19 weeks rather than 20–21 weeks.

Chorion villus sampling (CVS). Placenta tissue can be successfully obtained from as early as 6 weeks' gestation by the transabdominal or transcervical entry of aspiration cannulas, biopsy forceps, or needles of variable sizes.^{79,80} More than 200 000 procedures have now been performed throughout the world and the technique is carried out in many centres. However, the recent report on the possible association between CVS at less than 10 weeks and foetal limb abnormalities is likely to confine its application to pregnancies beyond this gestation.⁸¹ Furthermore, the European MRC trial has demonstrated a significantly higher risk of foetal death after CVS than after second trimester amniocentesis.⁸² Despite these limitations, CVS has the advantage of providing prenatal diagnosis in the first trimester rather than at 18–20 weeks as with traditional amniocentesis.

Amniocentesis. In the context of prenatal diagnosis of haemophilia, amniocentesis is used for foetal sexing and DNA analysis. When amniocentesis was first performed, it was limited to 16 weeks onwards, because at earlier gestations there was a high failure rate in obtaining amniotic fluid. However, during the past 5 years several studies have established the feasibility of early amniocentesis at 10–14 weeks.⁸³ Furthermore, the original apprehension about the smaller cellular content of amniotic fluid has not been substantiated; although the number of amniotic fluid cells at 10 weeks is smaller than at 16 weeks, the number of viable cells is the same. At amniocentesis, a 20- or 22-gauge needle is guided by ultrasound into the amniotic cavity and 10 ml of fluid is aspirated. Cell culture and cytogenetic analysis are successful in 98% of the cases and results can be available within 2–3 weeks.⁸³ For diagnosis of haemophilia, DNA can be extracted from the cells either directly after separation or after culture.

Summary of possible strategies for prenatal diagnosis of haemophilia.

1. Ultrasound scanning at 16–20 weeks for foetal sexing, followed by cordocentesis in male foetuses at 20 weeks for phenotypic diagnosis. The advantage is avoidance of invasive testing in 50% of cases. The disadvantage is late diagnosis.
2. Either CVS or amniocentesis at 10–14 weeks. The extent to which CVS or early amniocentesis will be the preferred technique depends on the results of a large prospective randomized trial comparing the two for diagnostic accuracy, and both short-term and long-term safety. The advantage is early diagnosis and the disadvantage is unnecessary risk of invasive testing in 50% of the cases. Furthermore, the risks of early CVS or amnio may be higher than those of cordocentesis.

Foetal blood. Meticulous care, consistency and speed are critical in collecting the diagnostic blood samples. All the necessary tubes are prepared before cordocentesis. Three successive foetal blood samples are aspirated into 1 ml plastic syringes. The first sample is collected into a heparin tube for (i) immediate cell size analysis and (ii) subsequent Kleihauer-Betke testing, to confirm its foetal origin.

The subsequent two samples are delivered into six polystyrene precipitin tubes, exactly to the 500 μ l mark without airlocks or bubbling. The polystyrene tubes contain accurate volumes of buffered-citrate anticoagulant; in two tubes the citrate to foetal blood ratio is 1:1 and in three it is 1:9. The tubes are held sideways between the forefinger and thumb and jerked vigorously two to three times to mix. The rest of each sample is collected into heparin tubes for (i) cell size analysis and determination of haematocrit, which are compared to the results of the first sample, to ensure that all samples are foetal and not contaminated by amniotic fluid, and (ii) immunoradiometric assay of VIII:Ag or IX:Ag in the supernatant plasma. All tubes containing foetal samples are capped, placed in ice, and taken to the laboratory for assay, where they are left undisturbed for 1 h.

Amniotic fluid. Amniotic fluid (10 ml) is collected into a sterile container, and kept at 4°C until further analysis. The sample is divided into two, one half to be used for direct DNA analysis and the other for cell culture to confirm the initial result. The amniocytes are separated by centrifugation in Eppendorf tubes and PCR is performed on the DNA, which is extracted using Proteinase K and phenol, followed by ethanol precipitation.

Chorionic villi. Samples are aspirated into culture medium and examined under a dissecting microscope for any contamination with decidua, in which case the tissues must be separated immediately after collection. The chorionic villi are noted for a central vascular core and budding cytotrophoblast, unlike decidua which are more sheet-like and less vascular. The chorionic villi are weighed in a pre-weighed Eppendorf tube; the minimum required is 6 mg, although 60–80 mg of wet tissue is usually obtained.

Measurements on foetal blood. Both factors VIII and IX are measured in all cases to provide an added check on the validity of the samples. In addition, factor V is measured as an indicator of possible consumption or activation of VIII.⁸⁴ When the foetus is at risk of haemophilia B, factor X is also measured. A modified one-stage, dose-response bioassay is used.⁸⁴

1. The tubes are examined to exclude any clots, platelet clumps indicating possible activation of the sample.
2. The plasma is separated after spinning in a refrigerated centrifuge (2 000 \times g for 15 min), collected into small polypropylene tubes and kept on ice until tested.
3. Standard assay curves: serial plasma dilutions of the standard (20-donor fresh plasma pool) stored in liquid nitrogen are made in barbitone buffer from 1/10 to 1/80, and duplicates tested in one-stage dose-response bioassay, for factor VIII and factor IX coagulant activities. A buffer blank is included in both assays, and the results are plotted separately on double-log paper to give a standard graph for each assay.
4. Screening assay: 100 μ l of foetal 1:1 plasma is diluted 1/10 and 1/20 in buffer. The coagulometer clotting times are plotted against concentration on double-log paper, and the values read from standard curves.
5. Definitive assay: if the results of the screening assay are in the normal foetal range, the assays are confirmed on a 1:9 plasma sample diluted 1/10 and 1/20. However, if the screening assay indicates severe deficiency, the 1:9 plasma sample is diluted only 1/5 and 1/10 before testing. The clotting times of both dilutions will approximate the buffer blank if the plasma contains <1 unit/dl factor VIIIc or IX activity.

(ii) Prenatal diagnosis of haemophilia A and B by DNA analysis

The effective use of DNA analysis for prenatal diagnosis of haemophilia involves prior planning and coordination between the testing laboratory and an obstetrician specialist in foetal medicine.

The use of PCR-based genetic testing has greatly simplified the prenatal diagnostic strategies used in screening for haemophilia.⁴⁶ The ability to perform PCR studies on less than 1 μ g of DNA makes this technique especially useful in prenatal analysis where the yield of foetal tissue for testing may be limiting. There has now been considerable experience with the use of PCR-based prenatal testing for both polymorphism linkage analysis and more recently for direct mutation detection in the haemophilias.

Analysis of chorionic villus tissue. The extraction of DNA from both CVS material and amniocytes is best carried out by alternative, 'gentler' methods to those used for blood extractions. All routine precautions to avoid sample contamination with extraneous DNA must be strictly adhered to and the possibility of maternal contamination of the CVS tissue must also be kept in mind. Most laboratories will perform two sets

of tests on the CVS DNA. The first involves sexing of the foetus through the use of amplification primers corresponding to the regions ZFX and ZFY on the X and Y chromosome respectively. The DNA is then amplified for haemophilia genotyping using either one of the previously detailed intragenic polymorphisms or one of the methods used for direct mutation analysis. Diagnostic studies are achievable within 48 h of receipt of the CVS tissue.

Amniocyte DNA analysis. Two options are available in testing cells obtained at amniocentesis. Direct analysis of DNA extracted from amniocytes spun down from 10 ml of amniotic fluid is now feasible and avoids the 2 week hiatus before sufficient quantities of cultured amniocytes are available for analysis. Once again, in light of the small quantities of foetal cells available for amplification, PCR contamination must be guarded against using techniques previously alluded to in this document. The strategy for DNA testing is identical to that outlined for CVS-based diagnoses.

General considerations

All laboratories offering DNA testing for prenatal diagnosis of haemophilia should be familiar with the processing of both chorionic villus and amniocyte samples. In almost all cases, the investigations required will involve PCR amplification as the first step in the manipulation of the DNA. With both CVS and amniocentesis, direct DNA testing is available and results can be produced within 48 h of receiving the foetal samples.

(i) Genetic counselling services for haemophilia

The individual or individuals involved in genetic counselling for haemophilia should have proficiency in the following areas: haemophilia and haemophilia care, human genetics, prenatal diagnosis, molecular genetics, and interpersonal communication and counselling. Proficiency may be gained through specific medical/clinical genetics or genetic counselling training programmes, coursework or laboratory experience. If all of the aforementioned aspects cannot be covered in the training of one individual, then a team approach to counselling is recommended so that expertise may be available in all areas. When a team approach is utilized, one individual should coordinate activities of the team, and act as an interface between the patient and team members. Liaison and communication between professional colleagues is highly recommended, especially between those who perform and interpret laboratory tests and those who provide clinical information to the patients and their families.

The aims of genetic counselling services should be to

(1) provide patients with sufficient information to make informed choices regarding carrier testing and prenatal diagnosis, and (2) to provide psychosocial support to the patient throughout the process of testing. Information should be given in a non-directive manner prior to the time of actual testing, so that patients and their families can make choices regarding their medical care.

All potential carriers should be tested early in life to determine factor VIII or IX activity levels. This should be done for safety reasons alone, to detect individuals who may have bleeding problems in certain situations. Then, at the age of informed consent, carrier testing should be offered to all female relatives of haemophilic males. The individual undergoing testing should be aware of the purposes of the test and its implications. It is preferable that carrier testing be completed before a potential carrier becomes pregnant. Contact can be made with females at risk directly, or through their male relatives. An accurate family history should be obtained, and the specific clinical diagnosis of haemophilic males within the family should be ascertained. Knowledge of severity of disease is extremely important when counselling family members regarding reproductive risks. If a potential carrier is not familiar with the clinical presentation of haemophilia, then various methods of introduction to the disease should be considered, such as audiovisual materials, selected reading, and/or personal interviews with affected individuals and their families.

Technical aspects of both phenotypic and genotypic carrier testing should be discussed with each potential carrier, as well as accuracy and limitations of the tests. Individuals at risk should be informed that the most accurate type of testing available at this time is genotypic testing, and that direct defect detection is preferred if it is available. If genotypic testing is to be done, family counselling should be considered so that all family members may be informed of the nature of the test, and the possible knowledge that will be gained upon completion of testing. All females with negative carrier test results should carefully be advised of the accuracy of the diagnosis, especially in the case of phenotypic testing.

Women who have positive carrier test results should be advised of all of their reproductive options, including adoption and *in vitro* fertilization where applicable. They should also be aware of the current treatment options and prognosis for an individual with haemophilia in their area. If they consider having a child of their own, all prenatal testing options should be discussed, as well as the appropriate methodology for termination of an affected pregnancy. Risks for each testing procedure should be given as accurately as

possible, taking into consideration the testing facilities which would be available to the patient. Counselling and psychological support should be available to the patient during all aspects of prenatal testing.

(ii) *The present situation in developed and developing countries and the role of the World Federation of Haemophilia*

Only recently have methods for prenatal diagnosis of haemophilia moved from the research laboratory to the clinical setting. Because of this, they are at present only available in developed countries, usually in academic settings.

Developed countries. There is very little information internationally on the availability, organization and proficiency of centres that provide these diagnostic services. The information available stems from publications or communications at meetings. As examples, the situation in Italy and the USA, two countries where the provision of prenatal diagnosis of haemophilia appears to be arranged with different strategies will be described. Italy, a country with a population of 56 million, has registered 2 036 patients with severe and moderate haemophilia A and B, i.e. those that are most likely to be concerned with prenatal diagnosis. The distribution of the diagnostic services is regional, with units established in Milan and Genova (Northern Italy), Rome (Central Italy), Naples (Southern Italy) and Genoa (located on the large island of Sardinia). With the exception of the latter, these diagnostic units are established within comprehensive haemophilia centres and enlist the integrated cooperation of a coagulation laboratory that provides phenotypic diagnosis, an obstetric service that performs techniques such as ultrasound, amniocentesis and chorionic villus sampling, and a laboratory of molecular biology that performs DNA analysis. Delivery to the patient of diagnostic information and genetic counselling are provided by genetic counsellors belonging to the staff of the haemophilia centres. This situation is particularly advantageous for the families that are regularly followed at such centres, because the stressful situation of prenatal diagnosis is dealt with by the same staff who attend to haemophiliacs in their day-to-day care. This regional organization has developed spontaneously in Italy, around haemophilia centres which chose to develop DNA techniques for research purposes. There is no state or regional recognition of the diagnostic services, which are funded with research money provided by the hospitals or universities where the haemophilia centres are established.

The regional organization of Italy, based on con-

veniently sized and located units providing the coordinated services needed for prenatal diagnosis, contrasts with the organization of other countries such as the USA. In this country with a population of 250 million, and approximately 20 000 haemophiliacs, very few haemophilia centres (for instance, in San Francisco and Chapel Hill, NC) have established their own services of prenatal diagnosis, spanning from counselling and obstetric procedures to DNA diagnosis. Most centres enlist the help of obstetric units (not necessarily located in the same hospital) to perform chorionic villus sampling or amniocentesis and then send DNA samples to institutions (such as the Mayo Clinic, Johns Hopkins University and the University of North Carolina at Chapel Hill) that have a large experience in molecular biology of congenital coagulation disorders. This system has some appeal, because these large units acquire considerable proficiency by analysing a large number of DNA samples. On the other hand, it has the disadvantage of being somewhat fragmented, in that the consultands are referred from a comprehensive haemophilia centre (not always the centre where regular care is provided) to an obstetric unit for chorionic villus sampling or amniocentesis (not always in the same State), with the actual DNA diagnosis eventually carried out in a totally different institution.

Developing countries. As recent initiatives in primary health care reduce infant mortality, congenital disorders are inevitably beginning to be recognized in developing countries. The burden of handicapping genetic diseases such as haemophilia is heavier in developing countries, because the infrastructure and services needed to assist the handicapped are usually primitive or non-existent. Because of the lack or deficiency of the social support system, the heavy burden of chronic disease for the individual and the family adds emphasis on the desirability of prevention. This is particularly evident for conditions such as haemophilia, which requires expensive therapeutic measures. Hence, there is a great demand for prenatal diagnosis of haemophilia and allied disorders in developing countries. As a result of this demand, there are many families at risk that seek genetic diagnostic testing in developed countries, but obviously these services can only be afforded by the richest. Local diagnostic facilities are practically non-existent, with the exception of a few academic centres where research interest in molecular biology of coagulation defects has prompted the applications of DNA techniques to carrier detection and prenatal diagnosis of haemophilia. The current availability of comprehensive haemophilia centres in developing countries is too scanty to envisage the rapid development of a network of genetic services as fea-

ible and reasonable. On the other hand, it must be realized that knowledge spreads faster than technology, which is expensive. This will create significant problems in developing countries, because knowledge about haemophilia and the corresponding expectations are fast out-distancing the ability to provide modern therapy.

The role of the World Federation of Haemophilia. Despite the fact that WFH is the only international organization that has the institutional goal of striving for the improvement of the well-being of patients of haemophilia, the Federation has as yet no information on the availability and organization of units for prenatal diagnosis in member countries (National Member Organization, NMO). It is, therefore, recommended that the Medical Advisory Board or the Medical Secretaries of the WFH, in collaboration with WHO, take action to gather this important information, by questionnaires or other methods of survey. This input is essential to prepare a registry of diagnostic units and plan further action. The ongoing designation of a few International Haemophilia Training Centres of the WFH as WHO Collaborating Centres on Haemophilia, should provide a network where staff from developing countries that choose to develop genetic service for prenatal diagnosis of haemophilia can be trained.

Future considerations for the genetic diagnosis and management of haemophilia

The post-HIV situation

Although the impact of HIV-I infection on the haemophilic population differs widely from country to country, this threatening infection is an important cause of death among haemophiliacs. The death of a large number of haemophiliacs from AIDS has important implications on the feasibility of carrier detection or prenatal diagnosis in haemophilic families at risk. The presently available diagnostic methods based on recombinant DNA technology rely heavily on the availability of the DNA from at least one affected member of the haemophilic families. This need materializes not only when RFLPs are employed to track the abnormal allele, as occurs most often in the diagnosis of haemophilia A, but also when the disease causing DNA defect is searched for directly, as occurs more and more frequently in haemophilia B.

Unfortunately, because of HIV infection, these studies cannot be carried out in some families at risk because critical affected family members have died, so

that their DNA is not available for examination. On the other hand, blood for DNA extraction, and the DNA itself, are stable if stored under proper conditions and may be used for studies many years later. To avoid the aforementioned pitfalls that make carrier detection and prenatal diagnosis often impossible, the World Federation of Haemophilia has recently recommended that samples of blood suitable for DNA extraction be obtained from all persons with haemophilia A and B, particularly from those affected by HIV infection or other potentially lethal conditions. A technique suitable for processing and storing DNA samples has also been recommended.⁸⁵ These samples stored at the haemophilia centres locally or nationally will provide valuable material for future studies and represent a resource that will be increasingly important as DNA analysis provides a better understanding of the molecular basis of haemophilia.

It is therefore recommended that WHO should also suggest the organization of the storage of DNA samples from patients with haemophilia and allied congenital coagulation disorders to all member countries.

Developments of RFLP/VNTR detection techniques: applications for developing countries

Genetic diagnosis of haemophilia A and B can be performed most effectively by direct detection of the mutation itself. However, with the size of the factor VIII gene and the limited number of mutations identified, it is often not possible for the clinical laboratory to perform this type of diagnosis routinely. Even for the factor IX gene, although much smaller in size compared to the factor VIII gene, but the mutations causing haemophilia B are highly heterogenous and unrelated patients are expected to carry independent mutations. Thus diagnosis at the DNA level often relies on the familial segregation of linked restriction fragment length polymorphisms (RFLPs). The classical approach to RFLP analysis involved the enzyme digestion of genomic DNA and Southern hybridization with radiolabelled DNA probes. This is often laborious and time-consuming. With the development of *in vitro* DNA amplification using polymerase chain reaction (PCR), it is possible to selectively increase the number of copies of a particular DNA fragment a million-fold. The amplification of target DNA allows for more convenient, non-radioactive detection methods. After amplification of a DNA fragment encompassing the polymorphic site, the presence or absence of the polymorphism can be determined by direct restriction enzyme analysis and visualization of the digested product under UV after polyacrylamide gel electrophoresis.⁴⁶ This technique has been applied to various factor VIII and factor IX intragenic and extragenic

RFLPs.^{46,56,57,63} Another approach which would obviate the need for restriction enzyme digestion is PCR followed by differential hybridization with sequence-specific oligonucleotide probes. These probes can be linked to horseradish peroxidase and a colour detection system used instead of radioisotopes.⁸⁶ When hybridized under appropriate conditions, the synthetic DNA probes will anneal to their complementary target sequence in the test sample, and form a stable duplex, only if they are perfectly matched. The use of shorter probes (15 bases in length) will further magnify the mismatch, so that it can be easily detected. This method is therefore applicable for detection of the absence or presence of the polymorphic site, if two probes, complementary to the different sequences are used. The main requirement is that the sequences around the RFLP sites should be known. The horseradish peroxidase-conjugated oligonucleotide probes are convenient to use, since they can be hybridized at the same temperature and stringently washed at $\pm 2^\circ\text{C}$ difference, so that only the stable DNA-oligonucleotide probe duplex remains. The probe also has the advantage of being stable for more than 1 year after preparation when stored at 4°C . This is particularly attractive for many developing countries, where availability of radioisotope poses a problem. The conjugation of horseradish peroxidase to the oligonucleotide probe requires the incorporation of a thiol-modifier at the 5' end during oligonucleotide synthesis and subsequent coupling of the horseradish peroxidase to the oligonucleotide at the 5' end.

A more recent development to this technique is the use of immobilized sequence-specific oligonucleotide probes for genetic analysis of PCR-amplified DNA.⁸⁷ Unlike the former method of immobilized DNA, where each probe requires a separate hybridization, this method would enable simultaneous screening of a sample for a number of RFLPs at an amplified locus. In this format, the oligonucleotides are given homopolymer tails with terminal deoxyribonucleotidyl-transferase, spotted onto a nylon membrane and covalently bound by UV irradiation. Due to their long lengths, the tails are preferentially bound to the nylon, leaving the oligonucleotide probe free to hybridize. The target DNA sample to be tested is PCR-amplified with biotinylated primers and then hybridized to the nylon membrane containing the immobilized probes under stringent conditions. The detection of hybridized material is made via binding of streptavidin-horseradish peroxidase to the biotinylated PCR-DNA, followed by a colour development in red leuco dye (Eastman Kodak). Since a number of nylon strips of immobilized probes can be prepared at one time and stored for future use, this 'reverse dot-blot' method

appears a simple and robust technique that may even lend itself to semi-automation. Even if the various RFLPs are located on too long a stretch of DNA for a single PCR reaction, as in the case of the intragenic factor VIII RFLPs, it may be possible to devise a 'multiplex PCR' reaction for a simultaneous amplification of various DNA segments, followed by hybridization with immobilized probes. One of the prerequisites of this technique is that all the immobilized probes need to be sequence specific under the same hybridization conditions. If necessary, this requirement can be met by adjusting the length, position and strand specificity of the probe or by varying the amounts of each probe that are applied onto the nylon membrane.⁸⁷ Once the conditions are established for a particular set of RFLPs, the method would allow quick and easy haplotype analysis for any given gene locus.

Developments in direct defect detection

Analysis of large multiexonic genes on an exon by exon basis becomes increasingly laborious and time consuming as the number of exons increases. Although the factor IX gene with 8 exons is tractable, by present screening methods, the 26 exons of factor VIII stretch resources of time, personnel and laboratory equipment to the limits such that only a few laboratories have taken up the challenge of mutation analysis in haemophilia A. The recent discovery that small amounts of processed mRNA for many if not all genes are present in tissues that do not normally express those genes promises to greatly speed up analysis of genes whose normal tissue of specific expression is inaccessible.

'Ectopically transcribed' mRNA can be isolated from peripheral blood lymphocytes, reverse transcribed to produce cDNA, then enzymatically amplified with specific primers. The PCR products representing regions of processed mRNA can then be screened with chemical cleavage mismatch detection, and sequenced to identify mutations.⁸⁸ Preliminary experience with this approach suggests that mutations, including those affecting mRNA processing can be successfully identified in most cases of moderate or mild haemophilia A and in up to 60% of cases of severe haemophilia A (F. Giannelli, personal communication).

The speed and power of this method are not in doubt but the degree to which the technology can be transferred from its originator (highly expert) laboratory to other groups remains to be established. Also the problem of unidentified mutations in severe cases of haemophilia A remains.

Other developments which may impact the speed and facility of direct defect detection include those centred on the automation of sequencing and screening

methods. DNA sequencing equipment with laser scanning devices for detection of fluorescent labels incorporated in oligonucleotide primers or terminators can greatly increase the rate of data acquisition. If such equipment becomes more widely available, direct sequencing of entire coding regions of factor IX or factor VIII might be feasible. The same equipment is highly suited to rapid multiplexed analysis of VNTR—CA repeat type polymorphic markers. This could greatly facilitate linkage analysis—perhaps in a centralized facility. The present cost of automated DNA scanning equipment will limit its use to large expert laboratories and national or regional DNA diagnostic centres.

Advances in prenatal diagnostic techniques

Advances in molecular genetics, such as DNA amplification of a single cell by PCR, have stimulated research into the feasibility of genetic diagnosis in the pre-implantation embryo and in foetal cells in the maternal circulation.

Pre-implantation genetic diagnosis

The potential advantages of pre-implantation diagnosis are (i) avoidance of repeated terminations in couples at high risk for affected offspring, and (ii) correction of the disease by such measures as gene therapy. Possible approaches for pre-implantation diagnosis include biopsy from the (i) polar body, (ii) six to eight cell embryo on day 3, (iii) blastocyst at days 5–6.

Polar body biopsy

Demonstration of the affected allele in the sample implies that the primary oocyte carries the normal allele. However, the possibility of recombination should be considered, which is up to 50% for genes near the telomeres. Furthermore, PCR on single cells can fail to produce sufficient DNA for diagnosis in approximately 20% of cases. Of the 83 cases in which this method was attempted (including 28 at risk of haemophilia) only one successful pregnancy was achieved, and in this case diagnosis by CVS demonstrated the foetus to be affected.^{89,90}

Early cell embryo biopsy

This procedure which involves either the aspiration or herniation of one cell from the six to eight cell embryo does not result in obvious abnormal development of foetuses in cattle and mice, although, in mice, where like humans, the pre-implantation period is relatively short, the viability of the embryos is reduced. In one human study, 38 embryos underwent biopsy; 30 had two pronuclei (normal), and eight had either three or no pronuclei. In 27 of the 30 normal embryos, morpho-

logical development was assessed and ten (37%) developed into blastocysts; this percentage is similar to that of unmanipulated embryos.^{91,92}

Therefore, it appears that removal of one cell from an eight cell embryo does not affect embryo development or success rate of establishing a viable pregnancy. However, as with polar body biopsy, material from one cell is not always sufficient for successful PCR amplification. As an alternative to PCR, fluorescence *in situ* hybridization may be applied. However the latter technique is less suitable for diagnosis of unique sequence DNA mutations.

Blastocyst biopsy

This technique involves slitting the zona and allowing 10–30 cells to extrude. The herniated cells are cut off and can be used for replicate assays. Although the frequency of hatching is apparently not affected by the biopsy, no blastocysts have yet been transferred back to the mother.⁹³

Recovery of foetal cells from maternal blood

The possibility of recovering foetal cells from maternal blood was first raised by the finding of male metaphases in the blood of pregnant women. More recently, the presence of foetal cells in the maternal circulation has been confirmed by the combined use of (i) monoclonal antibodies against foetal specific antigens on syncytiotrophoblasts or HLA-A2 antigen positive lymphocytes from HLA-A2 antigen negative women or transferrin receptor and glycophorin-A on erythroblasts, (ii) flow cytometry, to isolate or enrich foetal cells, and (iii) PCR amplification of Y chromosome specific sequences.^{94–97}

Gene therapy—a reality?

Haemophilias A and B are considered suitable targets for development of gene therapy on the following grounds:

- (i) The relevant genes have been cloned.
- (ii) Tight regulation of expression is not essential.
- (iii) A modest increase in circulating levels of factor VIII or IX would greatly improve the clinical bleeding tendency, e.g. 0% to 10% converts severe to mild bleeding.
- (iv) Haemophilia is a lifelong condition with severe effects on the sufferer.
- (v) Treatment by replacement of deficient factor is not entirely satisfactory, since it involves frequent injections of highly expensive products that are not free of all risks.

However, haemophilia is not amongst the very first genetic disorders to be targeted for gene therapy as it is

not invariably fatal and the best present treatment is successful in controlling the major clinical symptoms, and in achieving a relatively normal life for sufferers.

The basic concept of gene therapy is straightforward. It is proposed that a normally functioning gene be transferred into somatic cells of the recipient, such that those cells make and continue to make sufficient gene product to correct the inherited defect. In the case of haemophilia A or B, either factor VIII or IX must find its way into the circulation for whichever cell type is chosen for gene transfer, in a correctly modified form, at a rate sufficient to raise the circulating level of clotting factor to 10% of normal. The problem may therefore be conveniently considered under several headings.

Somatic cell type targeted for gene transfer. *In vivo* factor VIII is synthesized by hepatocytes and by some as yet unidentified cell type in the lymphoid system, particularly the spleen. However, ectopic production might be satisfactory, for example in haematopoietic cells. Thus target cells under consideration include, endothelial cells, hepatocytes, fibroblasts, myoblasts and bone marrow stem cells.

Vector for DNA transfer. Since a highly efficient transfer system is essential to deliver DNA to a large number of cells into which the DNA should be stably integrated, retroviruses have attracted most attention. The retrovirus is modified so that essential coding sequences required for packing and therefore infectivity are replaced by cDNA inserts representing a therapeutic gene.

Packaged virus is obtained using a cell line which contains defective viral DNA producing the deleted packaging proteins but no infectious viral RNA. Vector design is undergoing continuous development at the present time, but a limitation of retrovirus is that only about 7 kb of inserted DNA can be effectively packaged. Therefore a modified factor VIII gene must be used, lacking the B domain, which is dispensable for normal function. The retroviruses life cycle leads to stable integration of viral DNA but only into actively replicating cells, which precludes the use of hepatocytes as target host cells. Therefore, alternative vectors have been developed including adenoviruses. The maximum size of foreign gene insert in these viruses is 5 kb. Non-virus based transfer methods are also being developed.

Animal models. Relevant animal models for stable DNA transfer, integration and expression are needed. As regards haemophilia, dog models of haemophilia A and B are available,^{98,99} and at least one colony of haemophilic cats is being maintained. No mouse model of

haemophilia exists but it is now feasible to produce mouse lines defective in any specified gene sequence by targeted homologous recombination in embryonic stem cells. Subsequent recovery of fertile chimeric adults with the modified genotype present amongst their gametes is obtained by injecting targeted ES cells into morula stage embryos. Further breeding produces mice with the targeted gene disrupted in all their cells, provided that the phenotype is compatible with embryonic development. By this means many new strains of mice representing human hereditary defects have been produced.

Physical acquisition of cells, genetic modification and reintroduction, long-term expression of clotting factor and safety

These aspects can only adequately be studied in suitable and relevant animal models, such as those mentioned above. Thus, to be considered successful, a gene transfer experiment would have to be conducted on safely acquired cells from the intended haemophilic recipient, that were efficiently transfected *in vitro*, reintroduced and shown to continue functioning for years or the lifetime of the animal. There must be no detectable adverse side effects, such as malignant replication of genetically modified cells or local adverse effects of ectopic coagulation factor production, over many animal life times.

Gene transfer experiments involving factors VIII or IX

Factor IX. Modified retrovirus containing human factor IX coding sequence has been transferred into hepatoma cell lines, murine rat and human fibroblasts. The majority of the factor IX protein expressed by these cells was correctly gamma-carboxylated and functional *in vitro*. Canine factor IX has been expressed in dog fibroblasts and bovine endothelial cells. When fibroblasts secreting human factor IX were transplanted into syngeneic mice, human factor IX could be detected in their circulation for 2 weeks until an immunological response occurred. However, the vector was inactivated by an epigenetic mechanism regardless of the immune response to foreign protein.¹⁰⁰

Factor VIII. Functional B-domainless human factor VIII has been secreted from human fibroblasts transfected with a retroviral vector containing B-domain deleted factor VIII cDNA. When transferred to normal mice these cells could be recovered after 2 months and shown to still synthesize factor VIII. However, no factor VIII was detected in the recipient mice, probably due to the short half-life of human factor VIII in this animal.¹⁰¹

General conclusions

Although considerable progress has been made towards developing gene therapy, no satisfactory experiments in animal models have yet been described. Clearly there is a need to use conspecific factor in a relevant animal model before the success or otherwise of gene therapy can be judged.

The nearest approach has been reached with dog factor IX in dog fibroblasts *in vitro*. Presumably these will be tested in haemophilic dogs in the near future. On this basis it has been predicted that clinical trials will start before 1995. This prediction seems over-optimistic to the present group who doubt that such trials could ethically be started before the millennium, in view of the caveats expressed above about demonstration of efficacy and safety in extensive animal trials. Nevertheless it is certain that extensive efforts to validate gene therapy in haemophilia will be undertaken over the next 10 to 20 years with some considerable chance of ultimate success. Should such procedures be shown to be safe, efficacious and reasonably cheap then gene therapy might become the therapy of choice—especially in developing countries where other means of support are too expensive to be widely available. Conversely, if the price of recombinant factor VIII were to fall very considerably and especially if the oral route became feasible then gene therapy would be less attractive.

Conclusions and recommendations

Recent advances in molecular genetic procedures have resulted in a realization that accurate DNA-based carrier and prenatal diagnosis in haemophilia can be successfully achieved in many countries. The provision of these accurate methods combined with the availability of professional counselling and obstetric procedures performed by specialists in foetal medicine is seen as the ultimate goal for the effective genetic control of haemophilia. The role of WHO and WFH in encouraging the provision of such facilities was recognized.

Techniques for specific mutation detection are now available, particularly in relation to haemophilia B and developments in this area should be actively encouraged in order to make such procedures simpler and more applicable to routine analysis. It was however accepted that at present in most cases diagnoses will be based on DNA polymorphism analysis.

Differences between the cultural, social and economic situations in different countries will significantly affect the application of genetic procedures and the ethnic variation in frequency in many of the polymorphic markers will also result in varying efficiency of

such procedures. Services for carrier detection and prenatal diagnosis of haemophilia should be established in the locale where they will be used. This will take into account local ethnic and other influences.

The participants agreed that the present situation with regard to pre-implantation diagnosis of haemophilia and gene therapy, whilst offering exciting possibilities, was unlikely to affect significantly the requirements for genetic analysis procedures in the foreseeable future.

Future research into the feasibility of non-invasive foetal testing and gene therapy for haemophilia should be encouraged.

The following recommendations were adopted:

1. There should be a multidisciplinary approach to the prenatal diagnosis and carrier detection of haemophilia involving experts in the fields of genetic counselling, haemophilia care, molecular genetics and foetal medicine.
2. Potential carriers of haemophilia should be tested for clotting factor deficiencies early in childhood to assess the potential for clinical bleeding problems. These same potential haemophilia carriers should subsequently be offered carrier diagnosis after the age of consent and preferably prior to pregnancy.
3. Initial risk assessment for carriership should be based on pedigree analysis. Carrier testing by phenotypic analysis should include, where practical, the calibration of all reference material used in assays against approved international standards, and the final probability of carriership should be based on the combined likelihoods obtained from pedigree and laboratory analysis.
4. Genotype assessment offers a more accurate method for carrier detection. The genotypic analysis of haemophilia will most often involve the use of linked polymorphic markers to follow the inheritance of a haemophilic gene within a pedigree. This assessment should initially involve the study of the most informative intragenic markers with reference to the ethnic origin of the family being tested. Where circumstances permit, the genetic diagnosis of haemophilia should be based on the direct identification of the disease-causing mutation in the factor VIII or factor IX gene.
5. Family members undergoing counselling should be made familiar with the limitations of laboratory testing and thus be able to provide informed consent. The counselling process should be coordinated by a single well-trained individual, who should be familiar with the concepts of haemophilia care and clinical/molecular genetics. The genetic counselling process must also include a critical evaluation of the

type of haemophilia in male relatives of the consultant (haemophilia A or B, its severity, etc.).

6. DNA should be acquired and stored locally or nationally from all haemophiliacs to facilitate future genetic diagnoses in families. National Haemophilia Societies should be actively involved in this process.
7. All professionals involved in providing genetic services to haemophiliacs should attain appropriate levels of training. In this regard an international registry of potential training centres should be established and maintained by WFH. Some of these centres may be considered, after due process, as WHO Collaborating Centres.
8. WFH should conduct an international survey with the assistance of WHO to assess the facilities available for prenatal diagnosis of haemophilia, and subsequently an international registry of such facilities should be established.
9. Existing international data bases for specific haemophilia mutations and the development of new technologies to identify such mutations should be supported by the WFH and WHO. Where possible, all genetic information should be added to National Haemophilia Registries.

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