

# Application of three intragenic DNA polymorphisms for carrier detection in haemophilia B

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**SUMMARY** In the west of Scotland use of a single intragenic restriction fragment length polymorphism (*F9(VIII)/TaqI*) allowed definitive genetic counselling for 45% of females at risk of being carriers for haemophilia B. Two further intragenic RFLPs, *F9(VIII)/XmnI* and *F9(XIII)/DdeI*, have been applied to this population and by using all three polymorphisms the carrier status could be determined in 68% of females at risk. Linkage disequilibrium was apparent between these three RFLPs, and in the west of Scotland the single most clinically useful polymorphism was *F9(VIII)/TaqI* followed by *F9(XIII)/DdeI* and then *F9(VIII)/XmnI*. Overall, prenatal diagnosis by DNA analysis could be offered to 31 of 37 (84%) carriers (obligate and detected) in these families.

DNA analysis using intragenic or closely linked restriction fragment length polymorphisms (RFLPs) is rapidly gaining acceptance as a reliable and practical approach to carrier detection in both haemophilia B<sup>1-4</sup> and haemophilia A.<sup>5,6</sup> A single factor IX intragenic RFLP (*F9(VIII)/TaqI*) was informative for 42% of obligate carriers and permitted definitive counselling of 14 of 31 (45%) females at risk who were tested in the west of Scotland.<sup>4</sup> We wish to report our experience with the two other known factor IX RFLPs in these previously reported families and in one further family from the west of Scotland.

## Methods

The methods for DNA extraction, digestion, and the identification of specific fragments have been described previously<sup>4</sup> and only modifications are outlined below. Probe VIII is a 2.5 kb segment of the factor IX gene comprising 2.4 kb of introns and the 'd' exon.<sup>7</sup> Fragment polymorphisms can be identified with this probe using the restriction enzymes *TaqI*<sup>1</sup> and *XmnI*.<sup>2</sup> Probe XIII identifies a fragment polymorphism using *DdeI*.<sup>2</sup> In this latter RFLP the fragments differ in size by only 50 bp and, in order to demonstrate this, electrophoresis was performed in 17 cm 1.2% agarose gels after ethanol precipitation of the digests. Hybond N (Amersham) was used in

place of nitrocellulose for Southern blotting in these experiments.

## Results

Probe VIII identifies two bands in *TaqI* digested DNA from males and two or three bands in *TaqI* digested DNA from female subjects. The 5.3 kb band occurred in all subjects and the polymorphic fragments were 1.8 kb or 1.3 kb. When DNA is digested with *XmnI* and probed with VIII only a single band is apparent in males (11.5 kb or 6.5 kb), whereas females may be homozygous for either fragment or heterozygous (11.5 kb/6.5 kb). Probe XIII similarly only identifies a single band in *DdeI* digested male DNA (1.75 kb or 1.7 kb) whereas females may be homozygous for either fragment or heterozygous.

Fig 1 illustrates these RFLPs and the frequency of each fragment for unrelated Scottish subjects is indicated in table 1. From the Hardy-Weinberg equilibrium the expected heterozygote frequencies for each RFLP are *F9(VIII)/TaqI* 46%, *F9(VIII)/XmnI* 43%, and *F9(XIII)/DdeI* 35%.

Ten families from the west of Scotland segregating for haemophilia B have been studied to date. The pedigrees of nine of these have already been described<sup>4</sup> and are presented with results of carrier detection and outcomes of recent pregnancies in figs 2 to 11. In family 1756 (fig 2), III.9 underwent prenatal diagnosis after amniocentesis and a male fetus without haemophilia B was predicted by DNA analysis. Unfortunately, this pregnancy was also at a

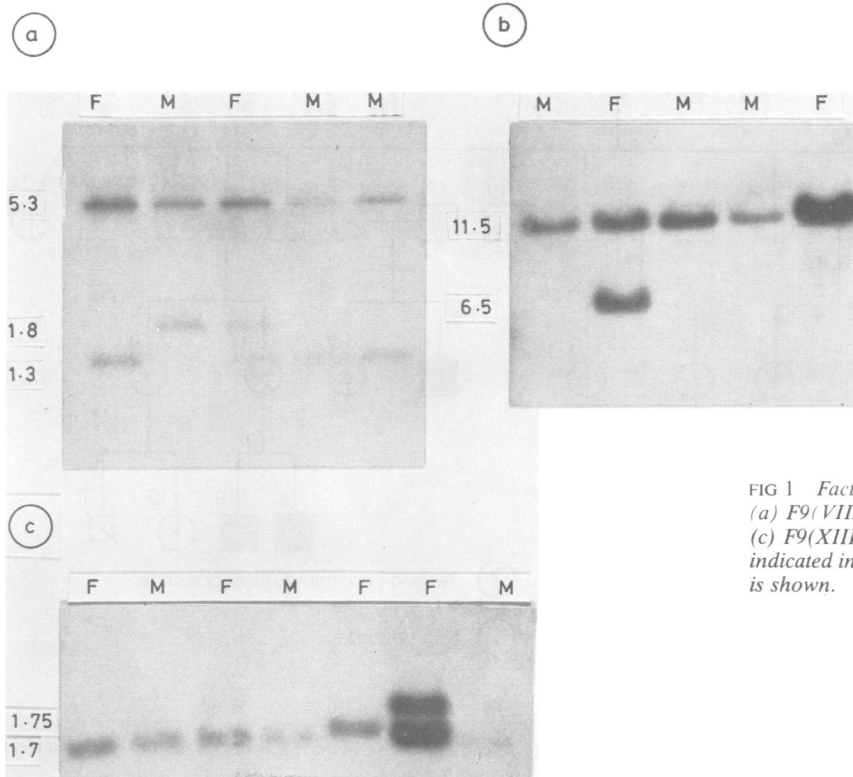


FIG 1 Factor IX intragenic RFLPs. (a) F9(VIII)/TaqI, (b) F9(VIII)/XmnI, (c) F9(XIII)/DdeI. Fragment sizes are indicated in kb and the sex of each subject is shown.

1 in 4 risk for cystic fibrosis as IV.10 is so affected. Amniotic fluid biochemistry and ultrasonography suggested a recurrence of cystic fibrosis which was confirmed at necropsy after termination of pregnancy. Family 5215 (fig 11) were referred for genetic counselling of the consultand II.4. The obligate carrier I.2 is informative for both F9(VIII)/TaqI and F9(VIII)/XmnI. Paternal absence prevents interpretation for the second of these polymorphisms, but the consultand II.4 is homozygous 1.3 kb/1.3 kb for the

first polymorphism and is thus not a carrier, as in this family haemophilia B is linked to the 1.8 kb fragment.

Table 2 shows the RFLP results for the obligate carriers in these 10 families. Among the haemophilia B obligate carriers, 46% (13/28) were informative for F9(VIII)/TaqI, 30% (8/27) were informative for F9(VIII)/XmnI, and 44% (12/27) were informative for F9(XIII)/DdeI. Six of the 28 obligate carriers were not informative for any RFLP, 12 were informative for a single RFLP, nine for two, and one carrier was informative for all three RFLPs.

Thirty-one females at risk have been traced and tested. DNA analysis indicated that six females were carriers and that 15 were not; no conclusion was possible from DNA analysis for 10 females at risk (table 3, figs 2 to 11). Three females at risk who could not be counselled on the basis of DNA analysis had levels of factor IX coagulant activity well outside our normal range, as determined by the one stage clotting assay based on partial thromboplastin time (pedigree 4549, III.5: factor IX level 52%; pedigree 4565, IV.2: level 42%; and pedigree 4555, IV.4: level 50%). Although normal coagulant studies do not exclude

TABLE 1 Fragment frequencies for factor IX intragenic RFLPs in Scottish subjects.

RFLP	Polymorphic fragments (kb)	Frequency	No of unrelated X chromosomes analysed
F9(VIII)/TaqI	1.8	0.63	46
	1.3	0.37	
F9(VIII)/XmnI	11.5	0.68	60
	6.5	0.32	
F9(XIII)/DdeI	1.75	0.23	30
	1.7	0.77	

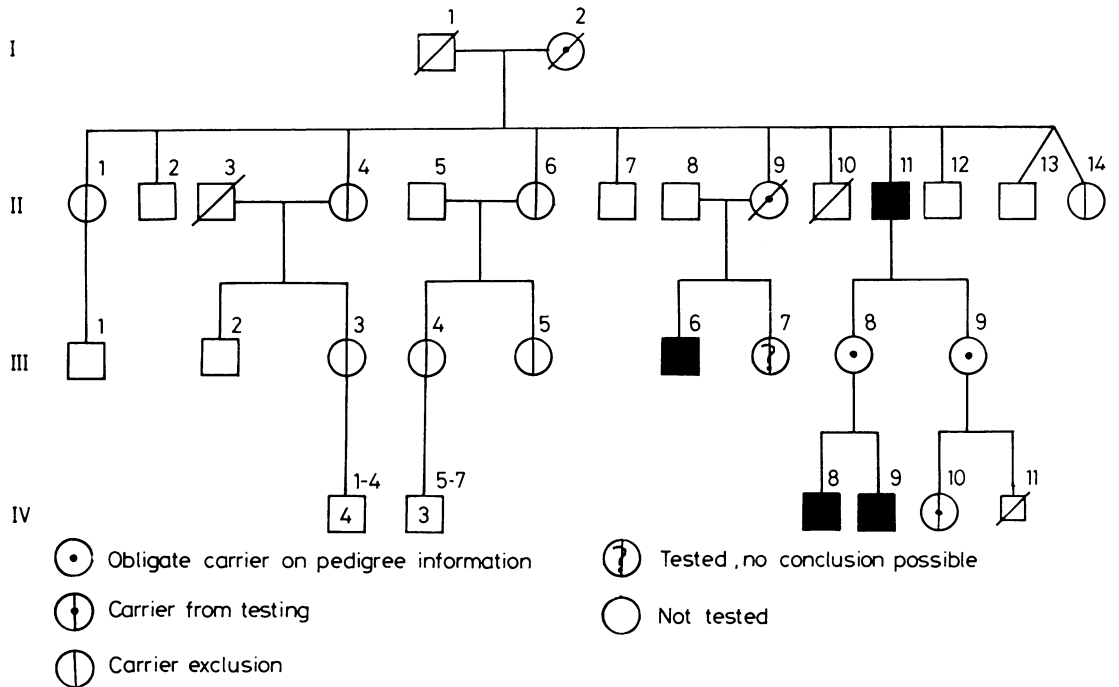


FIG 2 Pedigree 1756.

FIGS 2-10 Pedigrees of families with haemophilia B from the west of Scotland. The carrier status of females in these pedigrees is indicated.

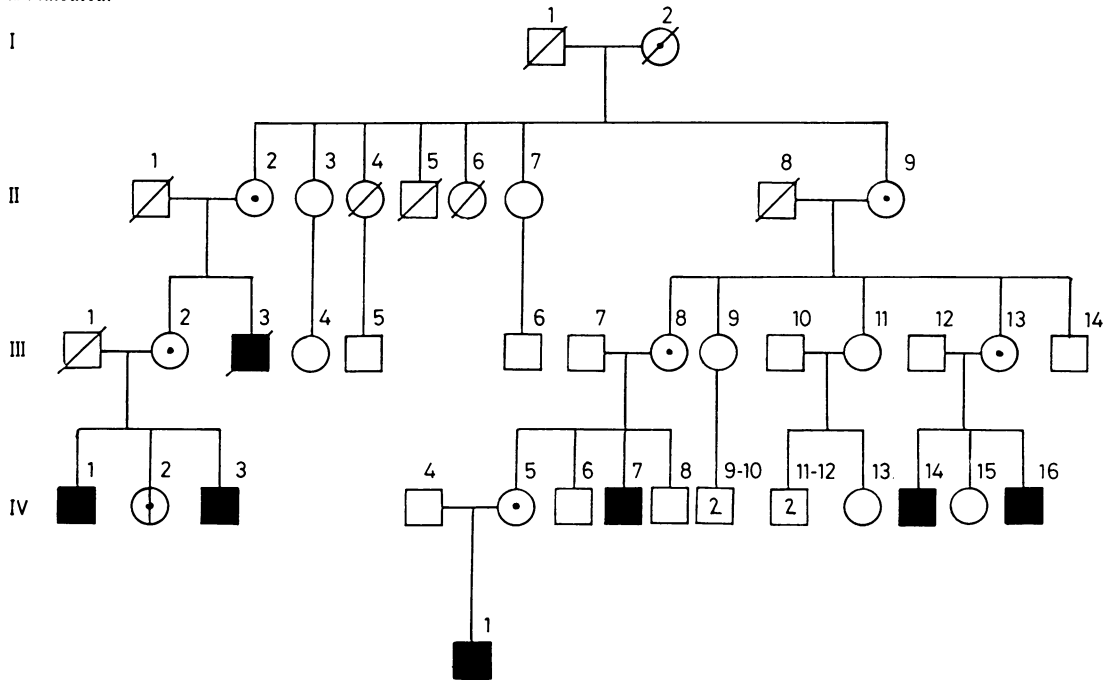


FIG 3 Pedigree 2377.

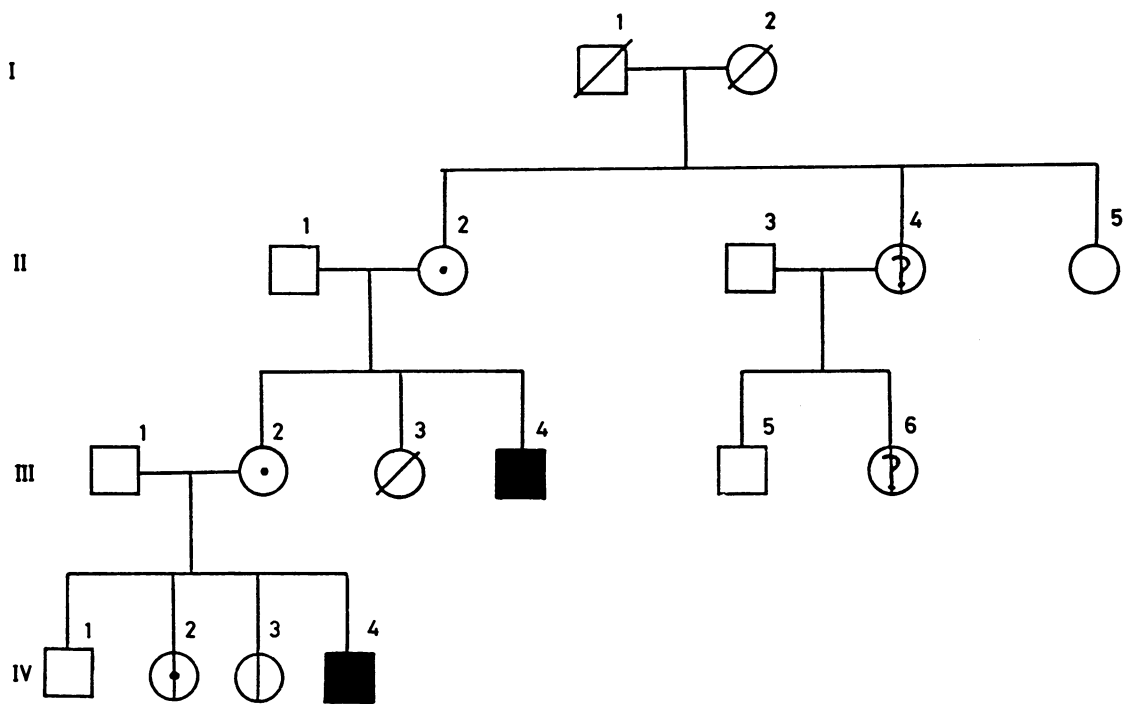


FIG 4 Pedigree 3569.

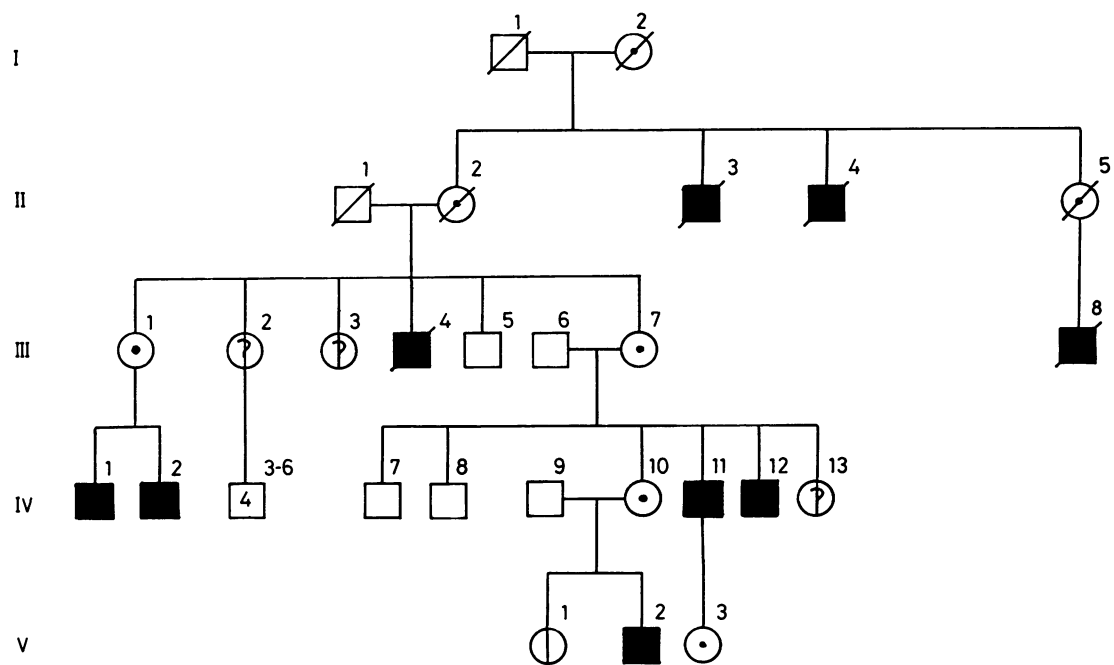


FIG 5 Pedigree 4542.

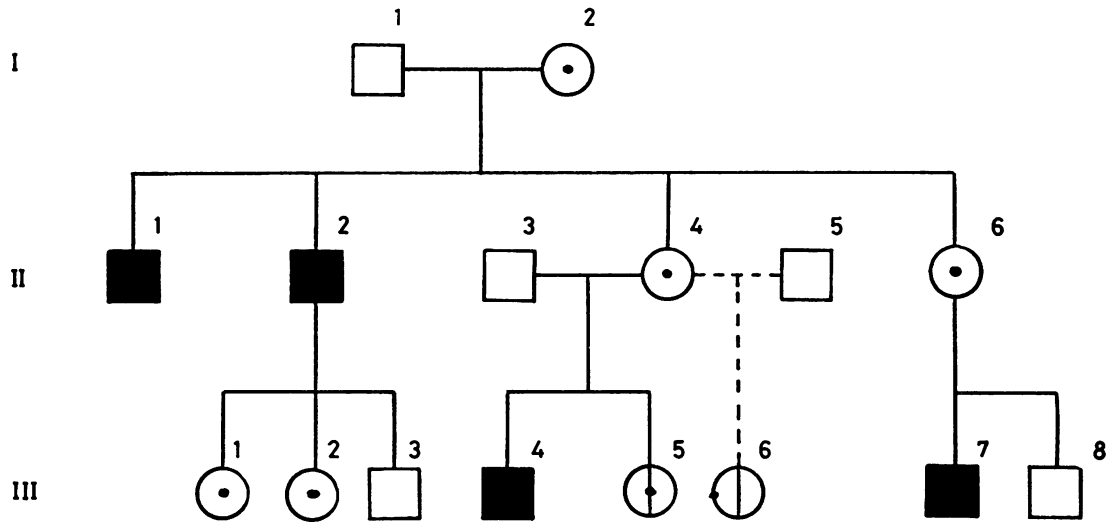


FIG 6 Pedigree 4549.

carrier status of haemophilia B, these abnormal results indicate a high probability that these three females are carriers. Thus, DNA analysis permitted definitive genetic counselling in 68% of females at risk and, if combined with the above coagulation results, 77% of females at risk could be counselled.

Of the 21 females whose carrier status was determined by DNA analysis, 17 could be advised on

the basis of the  $F9(VIII)/TaqI$  RFLP result. A further three were dependent upon the  $F9(VIII)/XmnI$  result and one upon the  $F9(XIII)/DdeI$  result. This sequence reflects the historical order of RFLP discovery and not all females at risk were tested for each RFLP.  $F9(VIII)/TaqI$  alone was informative for 17 of 31 (55%) females at risk,  $F9(VIII)/XmnI$  alone was informative for four of 15 (27%) females at risk

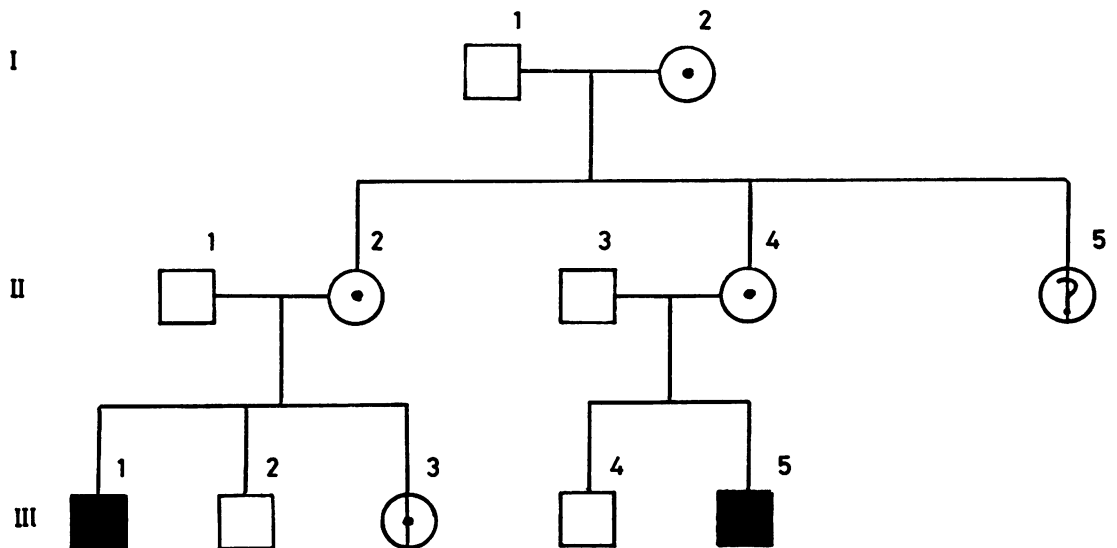


FIG 7 Pedigree 4551.

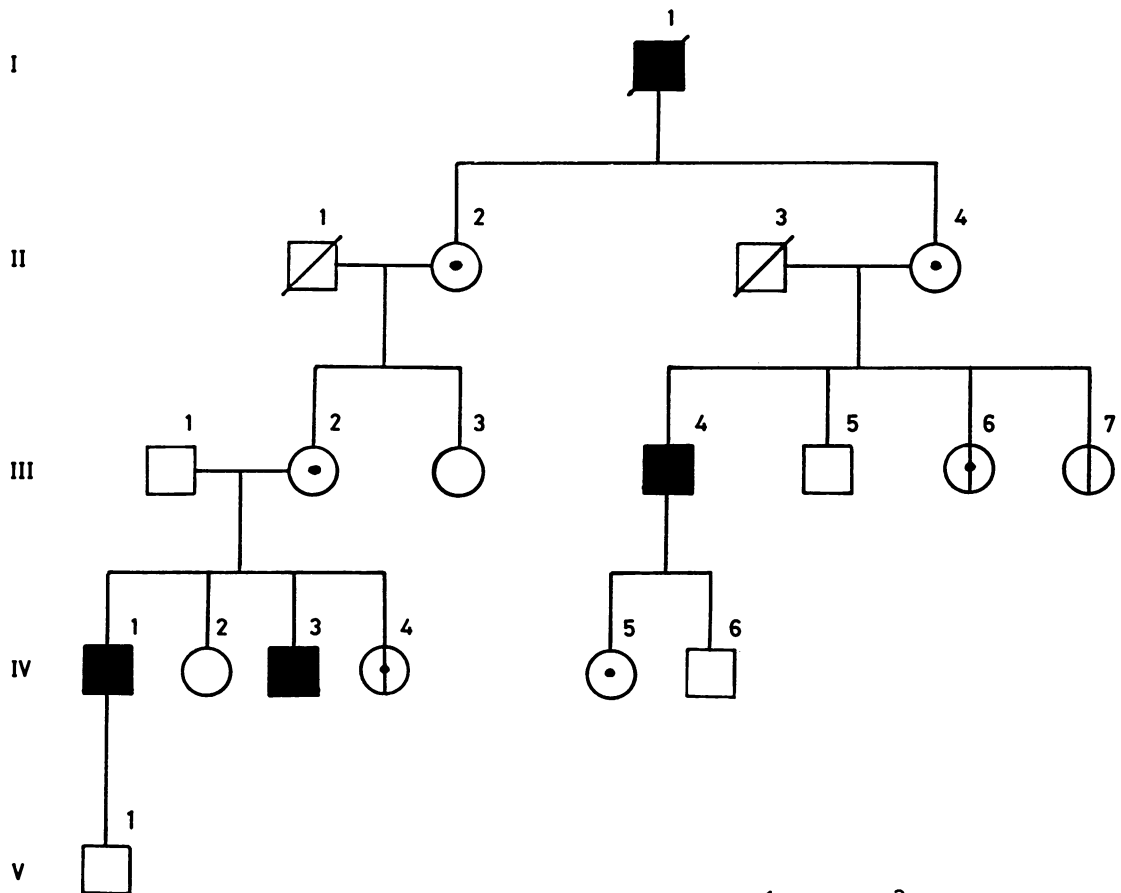


FIG 8 Pedigree 4555.

tested, and *F9(XIII)/DdeI* alone was informative for five of 15 (33%) females at risk tested. The DNA results on carriers detected in the course of this study are indicated in table 3. All nine could be offered prenatal diagnosis by DNA analysis and four were informative for all three intragenic RFLPs. Overall, prenatal diagnosis by DNA analysis could be offered to 31 of 37 (84%) carriers (obligate and detected) in these families.

In these families, seven females at risk cannot be counselled despite DNA analysis and coagulation studies. For two of these females the problem is maternal non-information for all three intragenic RFLPs (pedigree 4542, IV.13; pedigree 4551, II.5) and for the other five unavailability of one or both parents for testing prevented interpretation of the consultand's DNA result (pedigree 1756, III.7; pedigree 3569, II.4 and III.6; pedigree 4542, III.2 and

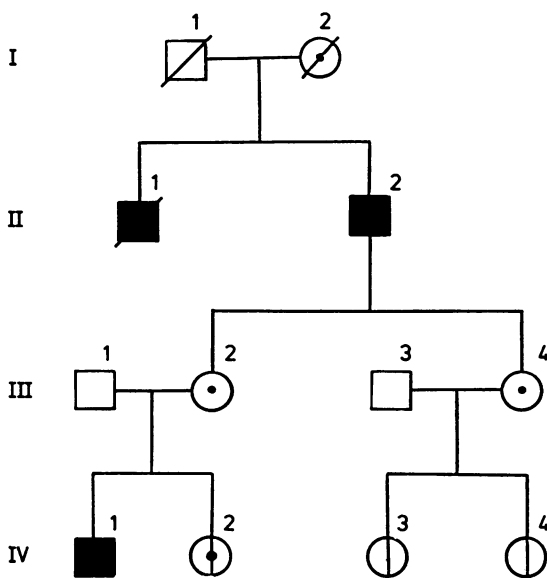


FIG 9 Pedigree 4559.

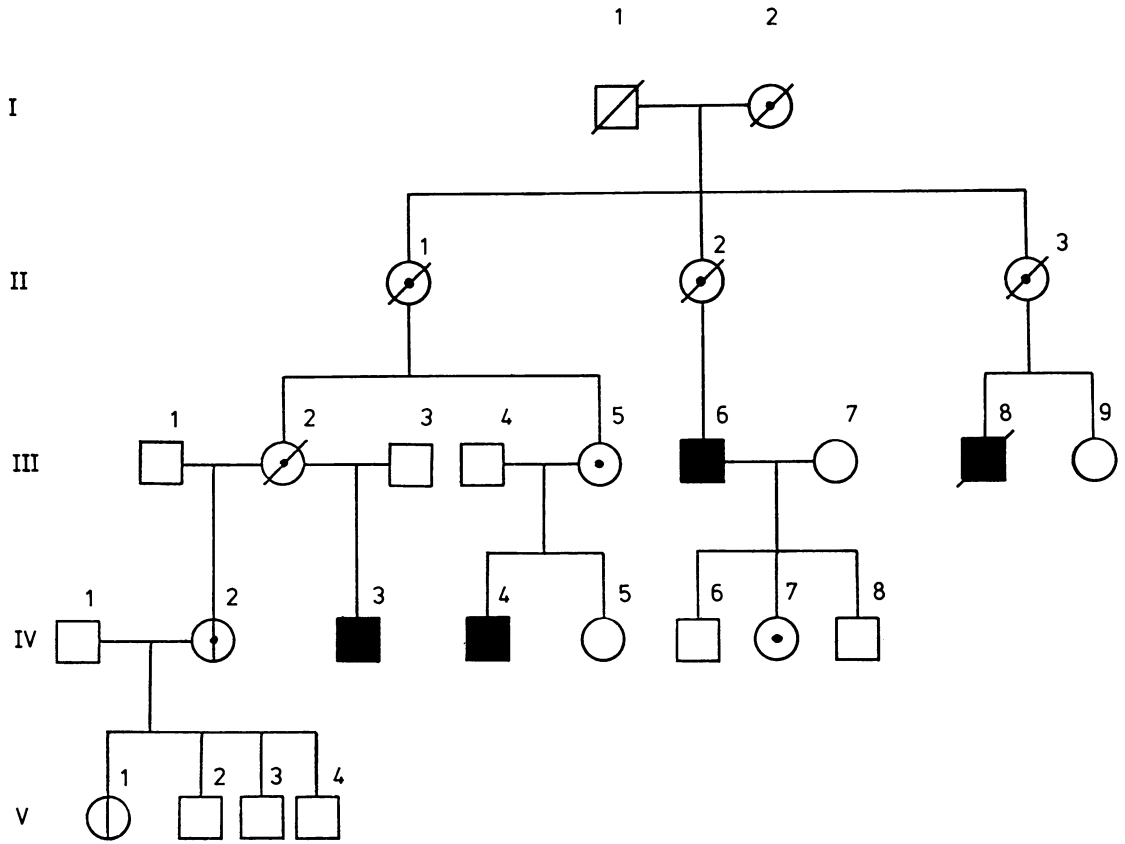


FIG 10 Pedigree 4565.

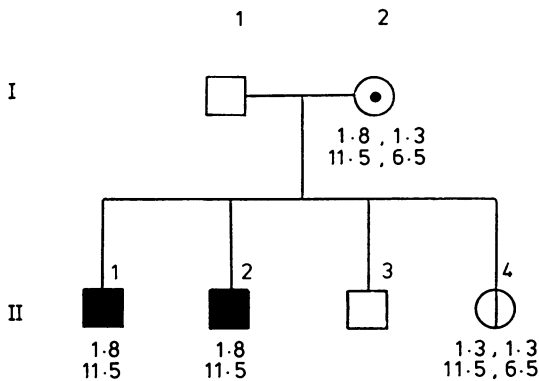


FIG 11 Partial pedigree (5215) of a family with haemophilia B. In this family the average level of factor IX activity is 1% in the affected males. The RFLP results for F9(VIII)/TaqI and F9(VIII)/XmnI are indicated for each subject.

III.3). Three of this later group are, however, beyond reproductive age.

The usefulness of each RFLP is less than predicted due to linkage disequilibrium between these three polymorphisms within the factor IX gene. Table 4 compares the observed intragenic RFLP haplotypes in 31 normal X chromosomes with the numbers expected assuming random association on the basis of population fragment frequencies. Thus, two-thirds of normal factor IX genes studied had 1.3-11.5-1.7, 1.3-11.5-1.75, or 1.8-11.5-1.7 haplotypes. A goodness of fit analysis shows that the difference from observed frequency is statistically significant ( $p < 0.05$ ). Three different haplotypes are apparent in the haemophilia B families, two of which are common among the normal population.

**Discussion**

Winship *et al*<sup>2</sup> characterised the two new factor IX intragenic polymorphisms. The F9(VIII)/XmnI

TABLE 2 DNA analysis on obligate carriers for haemophilia B (fragment sizes are in kb and fragment heterozygotes are underlined).

Pedigree No	Subject	RFLP		
		<i>F9(VIII)/TaqI</i>	<i>F9(VIII)/XmnI</i>	<i>F9(XIII)/DdeI</i>
1756	III.8	<u>1.8,1.3</u>	11.5,11.5	<u>1.75,1.7</u>
	III.9	<u>1.8,1.3</u>	11.5,11.5	<u>1.75,1.7</u>
2377	II.2	<u>1.8,1.3</u>	11.5,11.5	1.7, 1.7
	III.2	<u>1.8,1.3</u>	<u>11.5, 6.5</u>	1.7, 1.7
	II.9	<u>1.8,1.3</u>	<u>11.5, 6.5</u>	<u>1.75,1.7</u>
	III.8	1.3,1.3	11.5,11.5	1.7, 1.7
	III.13	1.3,1.3	11.5,11.5	1.7, 1.7
3569	IV.5	1.3,1.3	11.5,11.5	<u>1.75,1.7</u>
	II.2	1.3,1.3	<u>11.5, 6.5</u>	1.7, 1.7
4542	III.2	<u>1.8,1.3</u>	11.5,11.5	<u>1.75,1.7</u>
	III.1	1.3,1.3	11.5,11.5	<u>1.75,1.7</u>
	III.7	1.3,1.3	11.5,11.5	1.7, 1.7
	IV.10	1.3,1.3	11.5,11.5	<u>1.75,1.7</u>
4549	V.3	1.3,1.3	11.5,11.5	1.7, 1.7
	I.2	1.3,1.3	<u>11.5, 6.5</u>	<u>1.75,1.7</u>
	II.4	1.3,1.3	<u>11.5, 6.5</u>	<u>1.75,1.7</u>
	II.6	1.3,1.3	6.5, 6.5	<u>1.75,1.7</u>
	III.1	<u>1.8,1.3</u>	<u>11.5, 6.5</u>	1.7, 1.7
4551	III.2	1.3,1.3	NT	NT
	I.2	1.8,1.8	11.5,11.5	1.7, 1.7
	II.2	<u>1.8,1.3</u>	11.5,11.5	1.7, 1.7
4555	II.4	<u>1.8,1.3</u>	11.5,11.5	1.7, 1.7
	II.4	1.3,1.3	<u>11.5, 6.5</u>	<u>1.75,1.7</u>
4559	IV.5	1.3,1.3	11.5,11.5	<u>1.75,1.7</u>
	III.2	<u>1.8,1.3</u>	11.5,11.5	<u>1.75,1.7</u>
4565	III.4	<u>1.8,1.3</u>	11.5,11.5	1.7, 1.7
5215	IV.7	<u>1.8,1.3</u>	11.5,11.5	1.7, 1.7
	I.2	<u>1.8,1.3</u>	<u>11.5, 6.5</u>	1.7, 1.7
Totals for obligate carriers		1.8,1.8	11.5,11.5	1.75,1.75
		1/28	18/27	0/27
		<u>1.8,1.3</u>	<u>11.5, 6.5</u>	<u>1.75,1.7</u>
	13/28	8/27	12/27	
	1.3,1.3	6.5, 6.5	1.7, 1.7	
	14/28	1/27	15/27	

NT = not tested.

The intragenic factor IX haplotypes associated with the disease mutation in each family are: pedigree 1756, 1.3/11.5/1.7; 2377, 1.3/11.5/1.7; 3569, 1.3/11.5/1.7; 4542, 1.3/11.5/1.7; 4549, 1.3/6.5/1.7; 4551, 1.8/11.5/1.7; 4555, 1.3/11.5/1.7; 4559, 1.8/11.5/1.7; 4565, 1.8/11.5/1.7; and 5215, 1.8/11.5/1.7. The RFLP order is as in the table.

RFLP is a site polymorphism which lies 4 to 4.5 kb 5' to the *F9(VIII)/TaqI* site polymorphism. The *F9(XIII)/DdeI* RFLP is 1.5 kb 5' to the *F9(VIII)/XmnI* RFLP and is a length polymorphism due to a 50 bp insertion of Z type DNA sequence. Thus, these three RFLPs are within 6 kb of each other within the factor IX gene. Edwards<sup>8</sup> has estimated that a DNA segment 100 kb long has only a 50% chance of rearrangement in 700 generations or about 20 000 years. Hence, any haemophilia B family, even if extensive, would be expected to show non-random association (linkage disequilibrium) with a particular intragenic RFLP haplotype. Similarly, linkage disequilibrium would be expected between each of the RFLPs. This was apparent in the present study and also in that of Winship *et al.*,<sup>2</sup> although only pairs of RFLPs were presented in the latter study.

If a disease mutation is very rare and is selectively

neutral or advantageous (to the heterozygote), then linkage disequilibrium may be apparent at a population level. This has been observed for haemoglobin S,<sup>9</sup> several  $\beta$  thalassaemia mutations,<sup>10</sup> and the Z allele of  $\alpha_1$  antitrypsin deficiency.<sup>11</sup> It was not apparent in the present study and this would suggest that most of the haemophilia B mutations are of relatively recent origin, as might be expected for a deleterious X linked trait.

Linkage disequilibrium has an important practical consequence. In the present study, although each newly discovered RFLP increased the proportion of females at risk who could be definitively counselled, the yield with each new RFLP was successively less. Despite this limitation, the combination of three intragenic RFLPs permitted definitive carrier detection in 68% of females at risk. This is a significant improvement in comparison with the use of only a



TABLE 3 DNA analysis on carriers detected by DNA analysis or haematological testing (fragment sizes are in kb and fragment heterozygotes are underlined).

Pedigree No	Subject	RFLP		
		F9(VIII)/TaqI	F9(VIII)/XmnI	F9(XIII)/DdeI
1756	IV.10	1.3,1.3	11.5,11.5	1.75,1.7
2377	IV.2	<u>1.8,1.3</u>	11.5,11.5	<u>1.75,1.7</u>
3569	IV.2	1.3,1.3	11.5,11.5	<u>1.75,1.7</u>
4549	III.5	<u>1.8,1.3</u>	11.5, 6.5	<u>1.75,1.7</u>
4551	III.3	<u>1.8,1.3</u>	11.5, 6.5	<u>1.75,1.7</u>
4555	III.6	<u>1.8,1.3</u>	11.5,11.5	1.7, 1.7
4559	IV.4	<u>1.8,1.3</u>	11.5, 6.5	<u>1.75,1.7</u>
4559	IV.2	<u>1.8,1.3</u>	11.5, 6.5	<u>1.75,1.7</u>
4565	IV.2	<u>1.8,1.3</u>	11.5, 6.5	1.7, 1.7

TABLE 4 Intragenic factor IX haplotypes in 31 normal X chromosomes.

Haplotype F9(VIII)/TaqI	F9(VIII)/XmnI	F9(XIII)/DdeI	No observed	No expected
1.8	11.5	1.75	3	3
1.8	11.5	1.7	5	10
1.8	6.5	1.75	2	2
1.8	6.5	1.7	2	5
1.3	11.5	1.75	5	1
1.3	11.5	1.7	10	6
1.3	6.5	1.75	2	1
1.3	6.5	1.7	2	3

single intragenic RFLP, which in turn is better than haematological testing alone for carrier detection in haemophilia B. The delineation of further intragenic or flanking tightly linked RFLPs should improve carrier detection by DNA analysis still further, but for most (five of seven) of our females at risk, who cannot yet be counselled using this technology, the problem is non-availability of parental DNA. This renders DNA interpretation impossible except by the demonstration of homozygosity for the non-disease linked RFLP fragment. In this situation, direct detection of a mutation within a family, perhaps via an oligonucleotide probe, might be of clinical value.

The clinical and haematological variation among families with haemophilia B no doubt reflects a diverse heterogeneity of molecular pathology. Complete factor IX gene deletions appear to be rare but have been a consistent finding in patients who develop antifactor IX antibodies.<sup>12-14</sup> Chen *et al*<sup>15</sup> have described a family with a partial factor IX deletion of 10 ± 0.3 kb which included the two exons coding for amino acids 85 to 195. Despite this large deletion, there was evidence of transcription and translation of the gene and no antifactor IX antibodies were produced. A point mutation is suspected for factor IX Chapel Hill on the basis of an altered amino acid composition with substitution of

histidine for arginine at position 145.<sup>16</sup> More recently, a donor splice site mutation at the 3' end of exon f with GT replaced by TT has been demonstrated in a patient with severe CRM negative haemophilia B, and oligonucleotide probes were constructed which could be used for carrier detection in this family.<sup>17</sup> However, this involved sequence analysis of the entire factor IX gene in order to define the disease mutation and screening of 14 further severe CRM negative patients with haemophilia B using this allele specific oligonucleotide probe did not detect further examples of this mutation. Thus, for practical purposes, carrier detection by intragenic RFLPs is likely to remain the first line of approach for carrier detection in haemophilia B.

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