

DETECTION OF HEMOPHILIA A CARRIERS BY DNA ANALYSIS. COMPARISON WITH COAGULATION TESTS

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ABSTRACT

We employed DNA analysis for the detection of hemophilia A carriers in 23 possible carriers from eight unrelated Brazilian families. Two factor VIII (FVIII) gene defects were identified and used for family studies: a large deletion involving the exons 16-26, and a TaqI site mutation in the exon 24. For all remaining families, carrier detection was successfully performed by the analysis of six polymorphic sites associated with the FVIII gene: BclI, HindIII, XbaI, BglI, MspI, and the extragenic TaqI/St14 system. Results obtained by coagulation assays combined with pedigree analysis showed an inaccuracy of 38% when compared to DNA studies. In addition, allele frequencies for each of the six polymorphic sites and the derived haplotypes were determined in a sample of 32 unrelated Brazilian subjects. The allele frequencies of the intragenic sites were similar to those observed for other Caucasian populations thus far studied.

INTRODUCTION

Hemophilia A (HA) is a common X-linked disorder caused by the deficiency of the coagulant factor VIII (FVIII). It affects about 20 per 100,000 males and its clinical picture is characterized by recurrent bleedings in multiple sites (Levine, 1987). The frequency and severity of bleeding episodes depend on the functional levels of FVIII, which vary from < 1% to 49% (normal range 50-200%). Due to the severity of the disease, worsened by the high incidence of transfusion transmitted hepatitis and acquired immunodeficiency syndrome, genetic counseling is often requested by affected families. Until recently, the detection of hemophilia A carriers was based on pedigree analysis and

the combined measurements of coagulant FVIII and von Willebrand factor. Although easily carried out, this method is always probabilistic and presents an estimated error of 10 to 25% (Barrow *et al.*, 1982; Green *et al.*, 1986; White and Shoemaker, 1989).

The cloning and characterization of the FVIII gene (Gitschier *et al.*, 1984; Toole *et al.*, 1984) has allowed the use of DNA methodology for carrier detection and prenatal diagnosis of hemophilia A. DNA methods have significantly improved the accuracy of genetic counseling of hemophilia A when compared to classical biochemical methods (Antonarakis, 1988; White and Shoemaker, 1989). Two approaches may be used: a direct method, which requires the recognition of the FVIII gene defect in each family under study, and an indirect method, which is based on the analysis of DNA polymorphisms associated with the FVIII gene. Since the use of classical DNA methods allows the identification of the gene defect in only 5-15% of the patients, genetic counseling is usually performed by the analysis of DNA polymorphisms. Several polymorphic sites have been identified in association with the FVIII gene (White and Shoemaker, 1989; Tuddenham *et al.*, 1991). Allele frequencies for each site vary considerably in distinct populations (Antonarakis, 1988; La Salle *et al.*, 1990; Graham *et al.*, 1990). Although numerous studies have been carried out to determine the most informative polymorphic sites in different ethnic groups, no previous study has been conducted in a Brazilian population.

We report the results of carrier detection of hemophilia A by the identification of the gene defect and the analysis of six polymorphic sites associated with the FVIII gene.

SUBJECTS AND METHODS

Subjects

Carrier status was investigated in 23 daughters of obligatory carriers from eight unrelated families. At least two patients from each family had a confirmed diagnosis of severe (six families) or moderate (two families) hemophilia A. Carrier status was determined by both coagulation assays and DNA methods in 21 females; in two cases (from family B, Table I) only DNA methods were performed. A total of 69 subjects, including 13 hemophiliac patients, 17 obligatory and 23 possible carriers, and 16 not at-risk relatives, were studied by DNA methods. All patients included in this study are under regular follow-up in the Hemostasis Outpatient Clinics of the University Hospital in Ribeirão Preto.

The polymorphic sites linked to the FVIII gene were also investigated in a sample of 32 unrelated Brazilian subjects (16 females and 16 males), composed of members of the families under study and voluntary blood donors. Two subjects were

classified as Black, two as Mulatto, and 28 as Caucasian (22 of these reported European ancestry).

Table I - Identification of FVIII gene defects and analysis of DNA polymorphisms in eight Brazilian families affected by hemophilia A.

Family	Gene Defect	Informative polymorphism					
		BclI	XbaI	MspI	HindIII	BglI	St14
A	Deletion exons 16-26	No	No	No	No	No	Yes
B	TaqI site mutation at exon 24	-	-	-	-	-	-
C	Not identified	Yes	Yes	Yes	Yes	No	Yes
D	Not identified	Yes	Yes	Yes	Yes	No	Yes
E	Not identified	Yes	Yes	Yes	Yes	No	Yes
F	Not identified	No	Yes	No	No	No	Yes
G ^{2gn*}	Not identified	Yes	No	Yes	Yes	Yes	No
G ^{3gn*}	-	No	Yes	No	No	No	Yes
H	Not identified	No	Yes**	No	No	No	Yes

* The second and third generations of family G are listed separately because of the differences in the informative polymorphisms.

** An extragenic XbaI polymorphism was detected; the XbaI intragenic site was uninformative.

Coagulation assays

Blood samples were mixed 9:1 in the anticoagulant 3.8% trisodium citrate and centrifuged at high speed. The platelet-poor plasma was processed immediately or stored at -80°C and the pellet used for DNA analysis. Factor VIII coagulant activity (FVIII:C) was measured by one-stage assay (Rizza and Rhymes, 1982), using commercial FVIII-deficient plasma (Baxter, USA). von Willebrand factor antigen (vWF:Ag) was measured by rocket immunoelectrophoresis (Zimmerman *et al.*, 1982), using commercial plates (Helena, USA). Calibration curves for both determinations were prepared with commercial international standards ("Assayed Reference Plasma", Helena, USA). All measurements were performed in duplicate or triplicate.

Probability calculations

"Pedigree probability" of carriership (πc) was determined as previously described (WHO Working Group, 1977). The "likelihood ratio" favoring carriership (LR)

was calculated by linear discriminant analysis (Elston *et al.*, 1976; WHO Working Group, 1977). The final probability ($P(C)$) of carriership was determined after the combination of πc and LR values (Graham *et al.*, 1982). The discriminant used in this study was determined as described by Elston *et al.* (1976), based on the results of the coagulation assays (FVIII:C and vWF:Ag) and the ages in two additional samples: (1) 27 unrelated control females (voluntary females without family history or clinical picture of clotting disorders) and (2) 22 unrelated obligatory carriers of hemophilia A.

DNA analysis

Molecular defects of the FVIII gene were investigated by the Southern blotting method in all families.

Six polymorphic sites linked to the FVIII gene were studied: BclI (intron 18), HindIII (intron 19), XbaI (intron 22), BglI (intron 25), MspI (at 5' of exon 26), and the extragenic taqI/St14 system (Figure 1). The BclI and HindIII sites were analyzed by the Polymerase Chain Reaction method as described by Kogan *et al.* (1987) and Graham *et al.* (1990) respectively. The XbaI, BglI, and MspI sites, and TaqI/St14 system were analyzed by the Southern blotting method as described by Wion *et al.* (1986), Antonarakis *et al.* (1985), Yossoufian *et al.* (1987), and Oberlé *et al.* (1985b) respectively. The nomenclature used for the description of the alleles of the TaqI/St14 system was that proposed by Oberlé *et al.* (1985a,b).

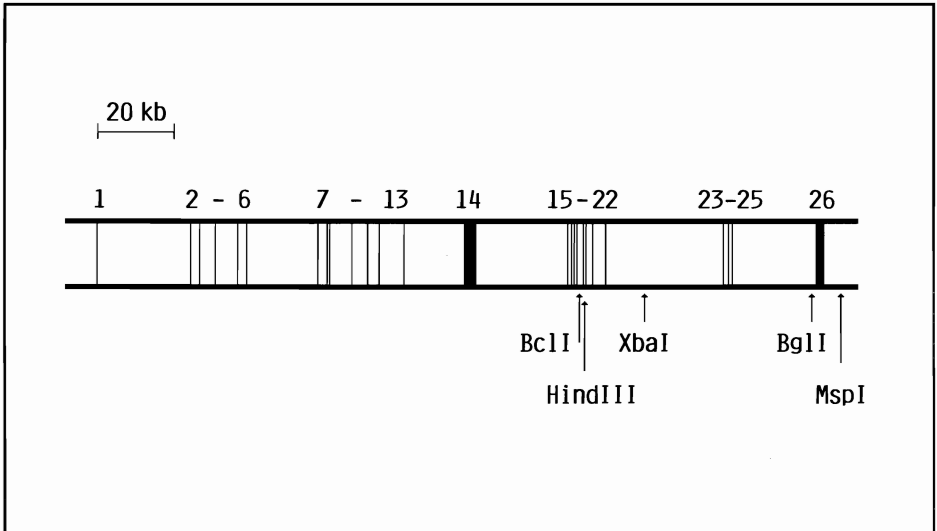


Figure 1 - Structure of the FVIII gene showing its 26 exons and the location of the five polymorphic sites investigated in this study.

Southern blotting. Genomic DNA was isolated from blood leucocytes by phenol/chloroform extractions (Sambrook *et al.*, 1989). Five to ten μg of DNA were digested with the appropriate enzymes, separated on 0.8-1% agarose gel, and transferred to nylon membranes (Sambrook *et al.*, 1989). The filters were hybridized to specific probes at 42°C for 16-48 hs, washed at 65°C for 120 min, and exposed to X-ray film (X-Omat AR, Kodak), with intensifying screens, at -70°C from 1 to 7 days. Three probes were used for the investigation of molecular defects: Kpn/Sac - a 1.8-kb SacI/KpnI cDNA fragment containing exons 1-12; p51-61 - a 4.7-kb EcoRI cDNA fragment containing from exon 14 to the beginning of exon 26; and p1.8-a 1.8 EcoRI cDNA fragment containing the remainder of exon 26 (Toole *et al.*, 1984). For analyses of DNA polymorphisms, four probes were employed: p1.8 for BglII polymorphism, p625.3 (a 5.4 EcoRI genomic fragment encoding the 3' flanking region of the FVIII gene) for MspI polymorphism, a 1.1-kb EcoRI/SstI fragment from the genomic probe p482.6 for XbaI polymorphism, and St14 (3.0 EcoRI genomic fragment) for TaqI/St14 polymorphic system (Wion *et al.*, 1986; Youssoufian *et al.*, 1987; La Salle *et al.*, 1989). For the study of the XbaI polymorphic site DNA samples were double digested with XbaI and KpnI. All probes were labelled with [α -32P]dCTP, using the "Random Primed DNA Labelling Kit" (Boehringer, Mannheim, FRG). The cDNA probes were a gift from "Genetics Institute" and probe p625.3 was a gift from J. Gitschier.

Polymerase Chain Reaction (PCR). The BclII polymorphic site was investigated using the primers described by Kogan *et al.* (1987). For analysis of the polymorphic HindIII site, intron 19 was amplified using partial segments of the primers described by Graham *et al.* (1990): 5'-CATCTACATGCTGGGATGAGC-3' and 5'-AAGCCATTCCCAGGGAGTCT-3'. The primers were synthesized on an Applied Biosystem oligonucleotide synthesizer. PCR was performed in 25 μl total reaction volume containing 100 ng (HindIII) or 200 ng (BclII) of genomic DNA, 10 mM tris-HCl pH 8.5, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 200 μM of each dNTP, 0.13 μM (HindIII) or 0.25 μM (BclII) of each primer, and 1U of Taq DNA polymerase; 35 cycles of amplification were carried out (94°C for 30s, 55°C for 20s, and 72°C for 60s). After PCR, the amplified samples were digested with the appropriate enzyme in excess and the fragments were separated on 6% polyacrilamide gel (BclII site) or 3% agarose gel (HindIII site).

RESULTS

Molecular defects of the FVIII gene

A gross deletion of the FVIII gene (at least 95 kb in extension) was identified in family A. The DNA sample from patient II-1, digested with TaqI and hybridized to

probe p51-61, showed the absence of the 2.8-kb, 2.4-kb, 2.2-kb, and 1.4-kb bands, and the presence of an abnormal 1.1-kb band (Figure 2). The deletion was confirmed and mapped by the digestion of the DNA sample with EcoRI and BamHI, and hybridization with cDNA and genomic probes (data not shown). Detailed characterization of the gene defect has been reported elsewhere (Figueiredo *et al.*, 1992). The deletion involves the exons 16-26 and is associated with FVIII inhibitors in affected members. The abnormal 1.1-kb TaqI fragment generated by the gene defect was used as a marker for carrier detection (Figure 2).

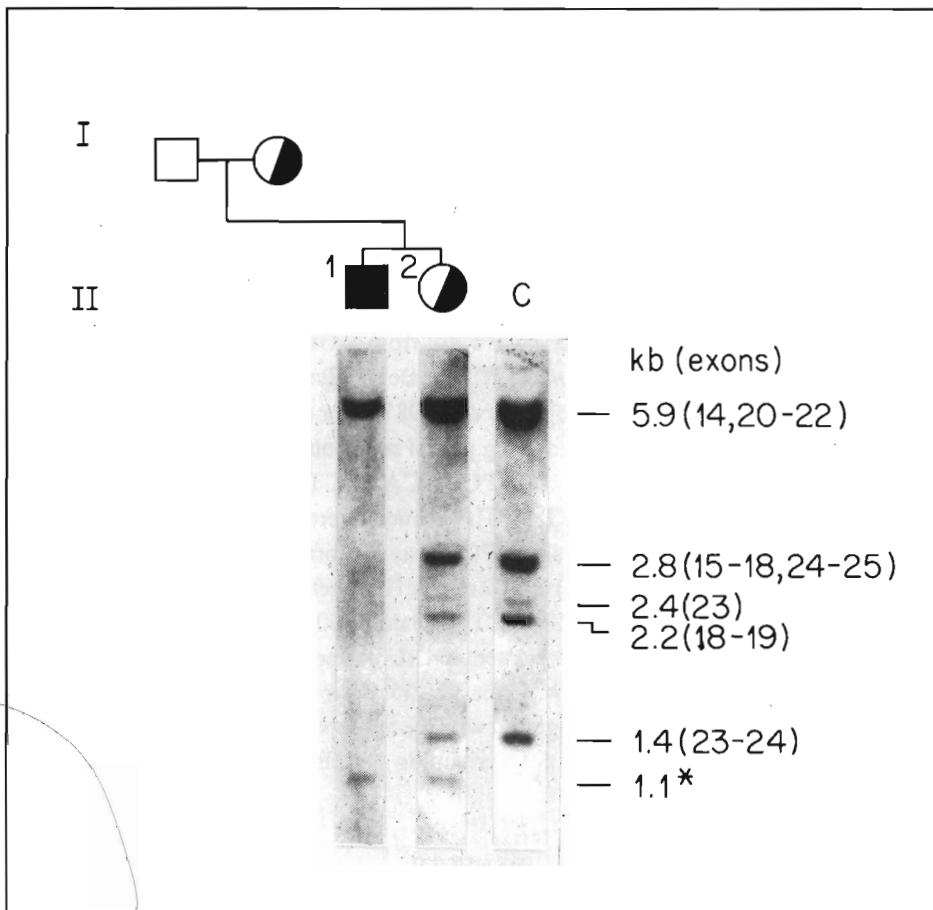


Figure 2 - Identification of the FVIII gene defect in the family A. The DNA samples were digested with TaqI and hybridized to probe p51-61. Patient II-1 shows the absence of bands which correspond to exons 15-25 and an abnormal 1.1-kb band (indicated by an asterisk). Female II-2 was diagnosed as a carrier by the presence of the abnormal band and a reduced intensity of the normal bands containing exons 15-25. C - normal female control.

In family B, a TaqI site mutation in exon 24 was identified. The DNA sample from patient II-2, digested with TaqI and hybridized to probe p51-61, revealed the absence of the 1.4-kb band, a reduced intensity of the 2.8-kb band, and an abnormal 4.2-kb band (Figure 3). A normal hybridization pattern was observed when the DNA sample was digested with BamHI or EcoRI and hybridized to the same probe (data not shown). These results indicate a mutation involving the TaqI site of exon 24 of the FVIII gene. The patient presented severe hemophilia A without FVIII inhibitors. We performed the carrier investigation in the possible carriers from the family based on the abnormal restriction pattern generated by the TaqI enzyme (Figure 3).

No other abnormalities were detected in the remaining families (C-H) by restriction-endonuclease analysis.

Carrier detection by DNA polymorphisms

In families C through H the analysis of FVIII-associated polymorphisms allowed the definition of the carrier status in all possible carriers (18 cases) (Table I). The

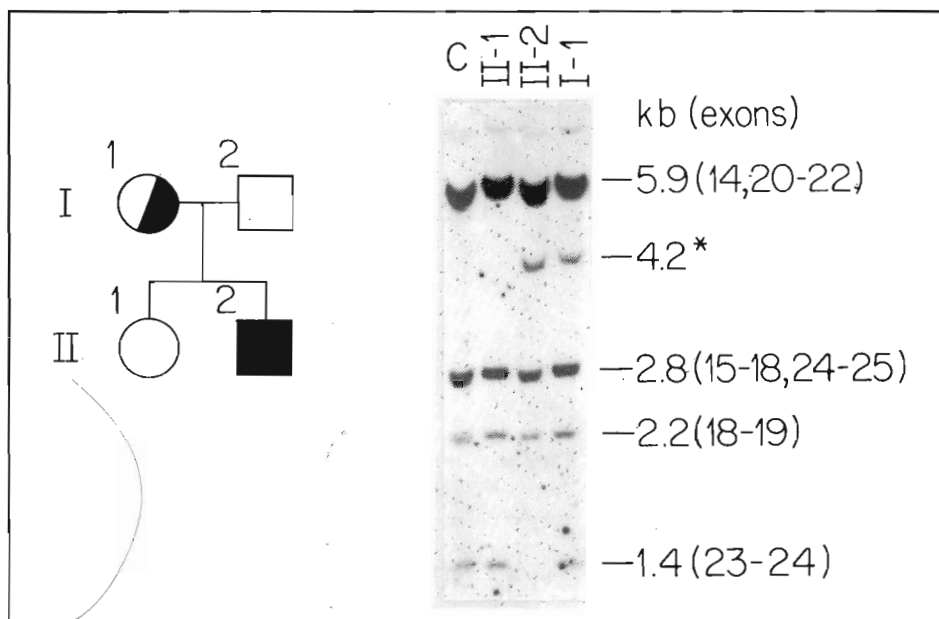


Figure 3 - Identification of a mutation involving the TaqI site of exon 24 of the FVIII gene in family B. DNA samples were digested with TaqI and hybridized to probe p51-61. Patient II-2 shows the absence of the 1.4-kb band, a reduced intensity of the 2.8-kb band, and an abnormal 4.2-kb band (indicated by an asterisk). As expected, the restriction pattern of the DNA sample from his mother (I-1) reveals the abnormal 4.2-kb band in addition to the normal bands. As female II-1 did not inherit the 4.2-kb band, a diagnosis of non carrier was established. C - normal control.

BclII polymorphism was informative in 10/18 cases (4/6 families), HindIII in 10/18 cases (4/6 families), XbaI in 16/18 cases (6/6 families), BglII in 2/18 cases (1/6 families), MspI in 10/18 cases (4/6 families), and TaqI/St14 system in 16/18 cases (6/6 families). In 8/18 cases the BclII, HindIII, XbaI, and MspI sites, and TaqI/St14 system were all informative. The combination of BclII and XbaI sites provided information in all cases. Using the combination of BclII, XbaI, BglII, and St14, all cases were informative for at least two polymorphic sites. No recombination was detected between the St14 locus and the intragenic sites.

Figure 4 illustrates the pedigree of family E with the results of 4 polymorphisms, which allowed the investigation of the carrier status in 3 females.

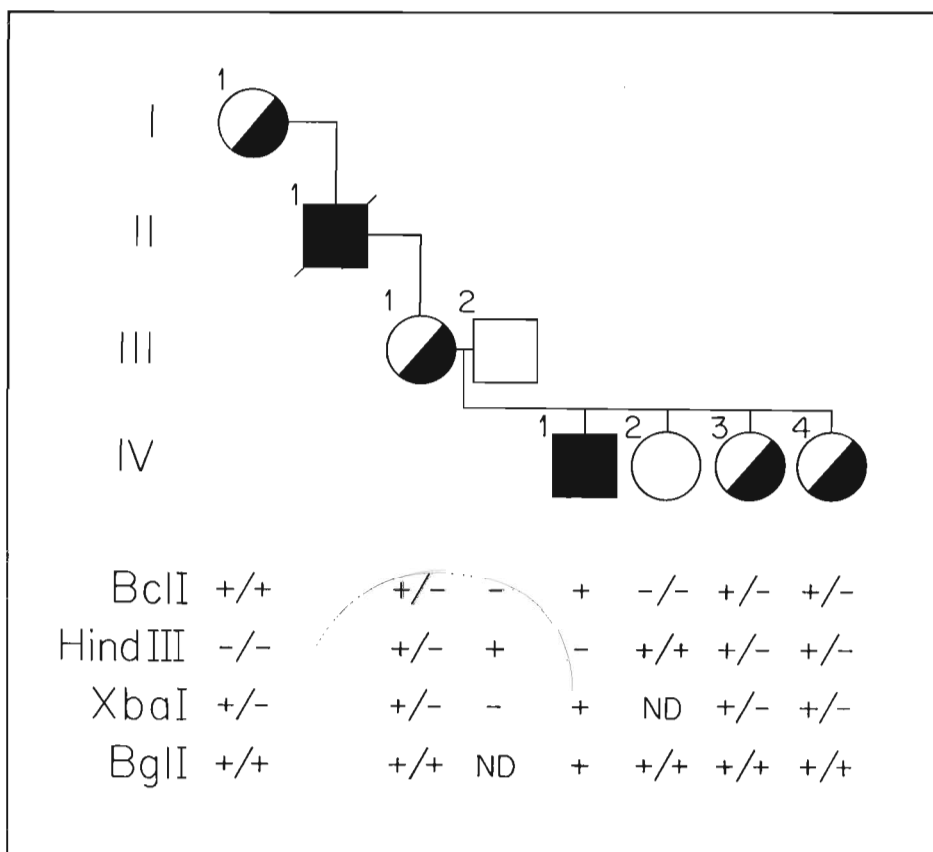


Figure 4 - Partial pedigree of family E with the results of four intragenic polymorphisms. Females I-1 and III-1 are obligatory carriers (determined by a complete pedigree analysis). The diagnosis of females IV-2, IV-3, and IV-4 was performed by analysis of the BclII, HindIII, and XbaI sites (since female III-1 is heterozygous for all these sites). Females IV-3 and IV-4 were diagnosed as carriers and female IV-2 as a non-carrier.

The analysis of the XbaI polymorphism in family H revealed the presence of a 5.1-kb band, in addition to the expected 6.6-kb constant band and the 6.2-kb and 4.8-kb polymorphic bands (Figure 5). This unusual allele, which has been previously reported by Wehnert *et al.* (1990), is generated by a XbaI polymorphic site located in the intergenic region at DXS 115 (Patterson *et al.*, 1989). This extragenic polymorphism was used for carrier investigation in two females (Figure 5). The family was also informative for the TaqI/St14 system (which produced concordant results), but was not informative for all intragenic polymorphic sites (including XbaI).

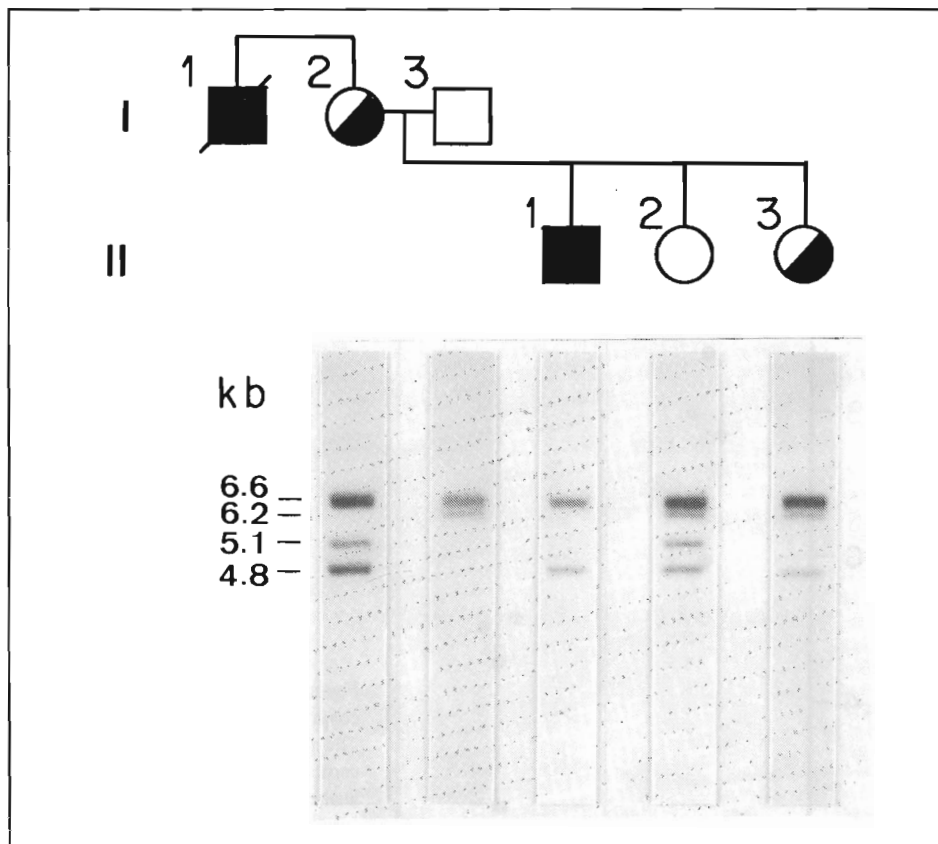


Figure 5 - Detection of carriers in Family II by the analysis of an extragenic XbaI polymorphism. The obligatory carrier I-2 is homozygous for the 4.8-kb XbaI intragenic allele and presents an unusual 5.1-kb XbaI extragenic allele. Since the hemophiliac patient (II-1) did not inherit the 5.1-kb allele his mother is heterozygous for this polymorphism, and the 5.1-kb allele is linked to the normal chromosome. By the analysis of its segregation, the female II-2 was diagnosed as a non-carrier and female II-3 as a carrier.

Comparison of coagulation assays and DNA methods

The probability of carriership ($P(C)$), calculated on the basis of pedigree data and the results of coagulation assays (see methods), was compared with the results of the DNA-based methods in 21 possible carriers from 7 families (Figure 6). In 13/21 cases the coagulation assays indicated a small ($< 25\%$) or high ($> 75\%$) $P(C)$ in accordance with results of the DNA methods. In 6/21 cases an inconclusive $P(C)$ (25%-75%) was obtained, and in two cases the results of the coagulation assays clearly differed from the DNA analysis (indicated by arrows in Figure 6). One of these cases is shown in Figure 4 (subject IV-4). The female presented a $P(C)$ of 0.121 (FVIII:C - 69%, vWF:Ag - 69%) whereas the analysis of five informative polymorphic sites indicated a carrier diagnosis.

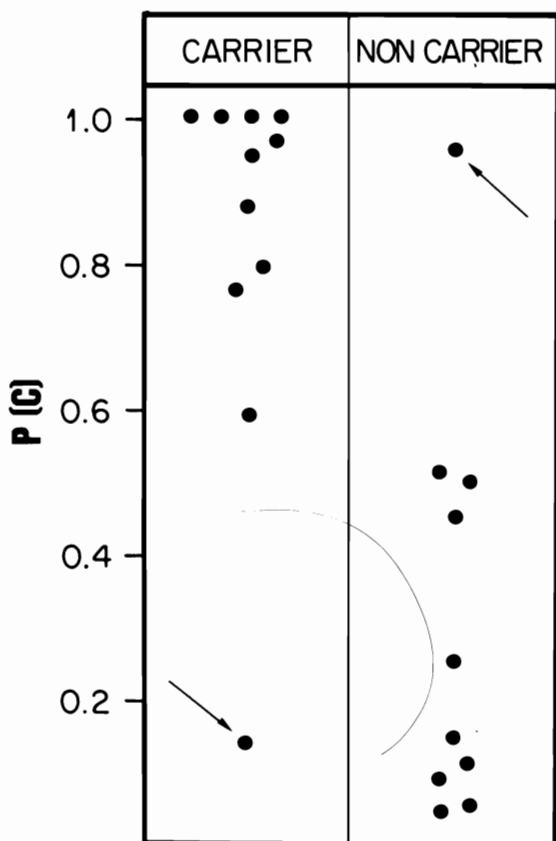


Figure 6 - Comparison of results using coagulation assays and DNA methods. The possible carriers were divided into carriers and non-carriers based on DNA analysis and compared with probability of carriership ($P(C)$), which has calculated using pedigree data and results of coagulation methods. In two cases (arrows), the two methods presented discrepant results, and the phenotype method gave uninformative results in six additional cases.

Allele frequencies of the FVIII gene-associated polymorphisms and haplotypes in unrelated Brazilian subjects

The results of the analysis of six polymorphic sites associated with the FVIII gene in 48 chromosomes from 32 unrelated Brazilian subjects are summarized in Tables II and III. The BclI, HindIII, XbaI, and MspI sites showed similarly high values of heterozygosity rates (0.45-0.55), whereas the BglI site was almost monomorphic, with an observed heterozygosity rate of 0.14. No differences were detected between the observed and the expected heterozygosity rates (Tables II). The analysis of the TaqI/St14 multiallelic system revealed a prevalence of alleles 3 (49%) and 4 (23%) (Table III). The observed heterozygosity rate for this system was 0.82. In the biallelic system, the α allele was much more prevalent (0.92) than the β allele (0.08) (Table III).

Table II - Allele frequencies of five polymorphic sites linked to the FVIII gene in Brazilian subjects.

Polymorphic site	No. of chromosomes	Allele frequencies (+/-) ^a	Heterozygosity rate (Observed/Expected), p ^b
BclI	46	0.67/0.33	0.50/0.44, p > 0.5
HindIII	43	0.30/0.70	0.50/0.42, p > 0.5
XbaI	40	0.40/0.60	0.55/0.48, p > 0.5
BglI	41	0.95/0.05	0.14/0.10, p > 0.5
MspI	35	0.66/0.34	0.45/0.45, p > 0.5

^a+ and - indicate the presence and the absence of the restriction site respectively. ^bThe statistical significance of the difference between observed and expected values was calculated by Chi-square distribution.

For the analysis of FVIII gene haplotypes, only the BclI, HindIII, XbaI, BglI and MspI sites were considered. The haplotypes could be determined in 26 chromosomes; multiple heterozygosity precluded haplotype determination in the remaining chromosomes. Only four haplotypes were observed: +++++ (38%), +++-+ (35%), -+-+ (23%), and -+--- (4%).

DISCUSSION

We demonstrated that the carrier status of all Brazilian females under study could be determined by DNA analysis. The identification of the FVIII gene defect was

Table III - Allele frequencies of the multiallelic and biallelic TaqI/St14 polymorphic systems in Brazilian subjects.

System	Allele (kb)	No. of chromosomes (frequency)
Multiallelic	1 (6.6)	0 (0)
	2 (5.4)	2 (0.05)
	2A (5.2)	3 (0.08)
	3 (4.8)	19 (0.49)
	4 (4.5)	9 (0.23)
	5 (4.1)	2 (0.05)
	6 (4.0)	1 (0.03)
	7 (3.9)	0 (0)
	7A (3.6)	1 (0.03)
8 (3.4)	2 (0.05)	
Biallelic	α	36 (0.92)
	β	3 (0.08)

accomplished in two (out of eight) families, and the restriction patterns of the abnormal genes were used for carrier detection (Figures 2 and 3). The deletion found in family A had not been previously identified in any other population, which corroborates the results of previous studies which demonstrate that the FVIII deletions are very heterogeneous (Tuddenham *et al.*, 1991). Conversely, the TaqI site mutation identified in family B is a recurrent mutation (Gitschier *et al.*, 1985; Bernardi *et al.*, 1989; Casula *et al.*, 1990; Tuddenham *et al.*, 1991), probably involving the CpG dinucleotide.

The analysis of the segregation of an abnormal FVIII gene constitutes the most accurate method for the identification of hemophilia A carriers. Unfortunately, the Southern blotting method allows the characterization of the gene lesion in a small percentage of cases only. So, for the majority of families carrier detection should be performed by the analysis of polymorphisms linked to the FVIII gene. Using this approach, we could determine the carrier status of all the possible carriers from the remaining families. The combination of BclI and XbaI sites provided information in all cases. The other intragenic sites (HindIII and BglII) and the MspI site did not give any significant additional information. These results are similar to those reported by Wion *et al.* (1986), Janco *et al.* (1987), and Pecorara *et al.*, (1987). The extragenic polymorphic

system identified by the St 14 probe was informative in most cases. Although the isolated use of this polymorphism has been associated with a misdiagnosis risk of 3-5% due to a possible recombination between the extragenic site and the abnormal FVIII gene (Antonarakis, 1988), no recombination was detected between the St14 locus and the intragenic sites in our study. Thus, the combined analysis of the BclI and XbaI sites and the TaqI/St14 system seems to be the best approach for the detection of hemophilia A carriers in the population under study.

The coagulation assays revealed a high level of uncertainty in 6/21 females (29%), and a discrepant diagnosis (when compared to the DNA-based methods) in two cases (10%). Similar results were observed by Brocker-Vriends *et al.* (1987) and la Salle *et al.* (1989). Therefore, the inaccuracy and error observed with the coagulation assays was much higher than the estimated error for the DNA methods based on intragenic (< 0.2%) or extragenic (3-5%) polymorphisms (Brocker-Vriends *et al.*, 1987; Antonarakis, 1988).

The allele frequencies for the intragenic FVIII polymorphic sites in unrelated Brazilian subjects are very similar to those observed for Caucasian populations thus far studied (White and Shoemaker, 1989; Graham *et al.*, 1990; la Salle *et al.*, 1990). This is not surprising since the great majority of the subjects included in this study are White of European descent. The allele frequencies observed for the MspI site and the TaqI/St14 system differed slightly from the previous studies on Caucasian populations (Oberlé *et al.*, 1985b; Youssoufian *et al.*, 1987; la Salle *et al.*, 1990). The small number of haplotypes found in this Brazilian population may reflect the reported linkage disequilibrium between the BcII site and most other intragenic sites (Antonarakis, 1988). The scarcity of studies reporting FVIII gene polymorphisms and haplotypes does not allow a more appropriate comparison with other populations.

In conclusion, the results presented here are useful for the development of a strategy for the detection of hemophilia A carriers in the Brazilian population. However, these results should not be extrapolated to all regions of Brazil, especially in Northern and Northeastern areas where the contribution of the Black population is preponderant. Further studies are necessary to determine the best combination of polymorphic sites to be used in those regions.

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RESUMO

Utilizamos métodos de análise de DNA para detecção de portadoras de hemofilia A em 23 possíveis portadoras oriundas de 8 famílias brasileiras não-relacionadas. Em duas famílias, a investigação de portadoras foi realizada através da identificação prévia das anormalidades do gene do fator VIII (FVIII). Foram caracterizadas uma grande deleção envolvendo os exons 16-26 e uma mutação do sítio TaqI do exon 24. Nos casos restantes a análise familiar foi realizada através do estudo de seis polimorfismos associados ao gene do FVIII: BclI, HindIII, XbaI, BglII, MspI e o sistema extragênico TaqI-St14. Os resultados obtidos por métodos bioquímicos clássicos (análise do pedigree associada a resultados de dosagens de fatores de coagulação) mostraram-se imprecisos em 38% dos casos, quando comparados com os resultados obtidos pelos métodos de análise gênica. Adicionalmente, foram determinadas as freqüências alélicas de cada um dos sítios polimórficos em uma amostra de 32 indivíduos brasileiros não-relacionados. As freqüências dos alelos dos sítios intragênicos não diferiram significativamente das freqüências observadas em outras populações caucasóides anteriormente estudadas. Os resultados aqui apresentados são úteis para a definição da melhor estratégia para a detecção de portadoras de hemofilia A na população brasileira.

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