Original Article

Efficiency of *BclI* Restriction Fragment Length Polymorphism for Detection of Hemophilia A Carriers in Sistan and Baluchestan Province, Southeast of Iran

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Abstract

Background: Indirect genetic diagnosis using polymorphic DNA markers can detect carriers of hemophilia A. This technique is preferable in developing countries because of its simplicity and cost effectiveness compared to direct mutation analysis. In the present study, we examined usefulness of intragenic marker *BclI* restriction fragment length polymorphism (RFLP) at intron 18, for carrier detection. How this marker is informative was tested in 102 members of 16 hemophiliac families from Sistan and Baluchestan province, Southeast of Iran.

Methods: Blood samples were obtained from 29 hemophilia A patients and 73 of their relatives, after taking informed consents. DNA was extracted using proteinase K digestion followed by DNA precipitation. Factor VIII gene polymorphism was identified by the polymerase chain reaction/RFLP which is both sensitive and economical.

Results: Our results showed that almost 69.8% of X-chromosomes had restriction site for *BclI* enzyme. The heterozygosity rate for *BclI* polymorphism in tested women was 61.4%, signifying the usefulness of this marker in carrier detection. The informative rate respecting this polymorphism was 43.7% meaning that a remarkable percent of families from the target population could be diagnosed using this marker alone.

Conclusion: In Sistan and Baluchestan province where there is limited access to sophisticated facilities of molecular diagnosis, use of PCR-based analysis of DNA polymorphism in the *BclI* locus can be used to identify a remarkable percentage of the carriers and even for prenatal diagnosis. Meanwhile, it is necessary to evaluate the effectiveness of other polymorphic DNA markers to enhance the informative rate.
Iran J Med Sci 2008; 33(1): 33-36.

Keywords ● Hemophilia A ● RFLP ● carrier detection ● Iran

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Introduction

Hemophilia A, an X-linked recessive disorder, is characterized by a deficiency in the activity of coagulation factor VIII. Generally, substitution therapy with factor

VIII is satisfactory, but cost constraint is a major problem in developing countries. Therefore, carrier detection and prenatal diagnosis is useful in these countries and play an important role in limiting the propagation of the disease.

Genetic diagnosis of hemophilia A using polymorphic DNA markers can detect carriers of hemophilia A. This is preferable in developing countries because of its simplicity and cost effectiveness compared to direct mutation analysis. Many polymorphic markers within and near the factor VIII gene have been reported as being useful for the carrier detection and prenatal diagnosis. These markers include *BcI*/intron 18 polymorphism,[1] *XbaI*/intron 22 polymorphism,[2,3] intron 13/22 dinucleotide repeat polymorphism,[4] and extragenic St14 variable number tandem repeat (VNTR).[5] Of these, *BcI* is the most widely used marker.[1,3,6-9]

The allele frequencies in a given factor VIII restriction fragment length polymorphism (RFLP) differ significantly in various ethnic groups.[3,9,10] Therefore, the clinical usefulness of a DNA polymorphism should be determined according to ethnicity of the population under study.

"Sistan and Baluchestan" is a province located in southeastern Iran, where the prevalence of hemophilia A is about eight per 100,000 among male population and the socioeconomic condition of the local population is well below the average for the country.

In this study, we examined whether polymerase chain reaction (PCR)-based analysis of DNA polymorphism in the *BcI* locus was efficient enough for carrier detection in the population of Sistan and Baluchestan.

Materials and Methods

After taking informed concents, blood samples (10 mL) collected in EDTA tubes were obtained from 29 patients with hemophilia A and 73 of their relatives including parents and sisters who wished to be tested. DNA was extracted using proteinase K digestion followed by DNA precipitation.

The *BcI* polymorphism was analyzed by restriction enzyme-PCR. PCR was performed by adding 0.1–0.5 μg of DNA to 50 μL PCR reaction mixture consisting of 50 mM KCl, 10 mM Tris HCl [pH 8.3], 1.5 mM $MgCl_2$, 50 μM of each dNTP, and 0.5μM of each primer and one U of Taq polymerase. The sequence of primers used is given in table 1.

After five min at 95 °C for initial denaturation, 30 cycles of PCR were performed (denaturation: one min at 95 °C; annealing: one min at 50 °C; extension: one min at 72 °C) with an automated thermal cycler. Then, 26 μL of amplified product was digested with *BcI* restriction enzyme at 55 °C for 2 h. Amplicons (before and after treatment with restriction endonuclease) were submitted to agarose gel electrophoresis and stained with ethidium bromide. For characterization of molecular size in electrophoresis, φX174 DNA digested with *Hae*III was used as marker. The sizes of the fragments generated are given in table 1.

Results

One-hundred and two individuals from 16 families, comprised of 29 hemophiliacs, 16 unaffected males, 16 obligate carriers and 41 possible carriers, were sampled for the present study. According to the pedigree analysis, 14 out of these 16 families had a history of hemophilia A. The results of restriction enzyme-PCR analysis for one of these families with two affected sons, are shown in figure 1. Summary of the results related to the analysis of *BcI* polymorphic site is given in table 2. Of the women tested for the above-mentioned polymorphism, 61.4% were found to be heterozygous. As obligate carriers with X-linked alleles are useful for pedigree analysis, the above percentage justifies the usefulness of *BcI* marker in detection of the carrier state. Indeed, 43.7% of families could be diagnosed using this marker alone. Of 41 possible carriers, seven women

Table 1: Primers used for the analysis of factor VIII gene *BcI* polymorphism.[7]

| Primers	Sequence 5ȼ–3ȼ	PCR product size (bp)	Absence of site (bp)	presence of site (bp)
BcI forward	TAA AAG CTT TAA ATG GTC TAG GC	142	142 (−)	99 and 43 (+)
BcI reverse	TTC GAA TTC TGA AAT TAT CTT GTT C			

Table 2: Allele frequencies of *BcI* RFLP

Number of X Chromosomes	Allele frequencies (%)[a]		Heterozygosity rate (%)[b]	Informative families (%)
	+	−		
159	69.8	30.2	61.4	43.7%

[a] frequency refers to the number of alleles detected divided by the total number of alleles analyzed.
[b] heterozygosity refers to the percentage of women found to be heterozygous for the polymorphism among the total number of women analyzed.

were diagnosed as carrier and 10 as non-carriers. The prevalence of a '+' allele for *Bcl*I restriction (allele digested by *Bcl*I) in the studied population was found to be 69.8%.
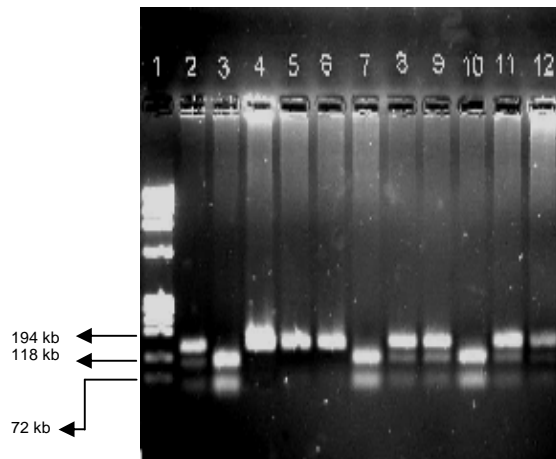
Figure 1: Alleles of intragenic *Bcl*I RFLP in a hemophilic family.
Lane 1: *Hae*III digested φX174 DNA size marker. Lanes 2–4: −/+ ,+/+ and −/− controls, respectively. Lanes 5–6: affected sons. Lane 7: unaffected father. Lane 8: mother (obligate carrier). Lanes 9–12: sisters. Three out of four sisters (lanes 9, 11 and 12) are carriers and one of them (lane 10) is diagnosed as non-carrier.

Discussion

In developing countries like Iran, carrier detection and prenatal diagnosis of hemophilia will be a great step forward in reducing the burden of hemophilia care in the society. However, direct diagnosis of the molecular defects in hemophilia is often difficult, due to the high heterogeneity of mutations and the size and structural complexity of the factor VIII gene, so linkage analysis with RFLP is the most useful approach.[11,12]

Since Iran is a country with a population composed of a wide variety of ethnic groups, acquiring data about genetic variations in different regions of the country should be of great help for assuming a suitable strategy for diagnosing the carriers, and also for prenatal diagnosis. In this context a few studies have been performed in the country and their results indicated the usefulness of RFLP analyses in detection of hemophilia A carriers.[13,14]

The preliminary study to estimate the heterozygosity for *Bcl*I locus in the target population, showed a rate of 61.4% which is significantly higher than the similar rates in other populations; the rate is 42% in whites, 21% in Koreans, and 26.5% in Japanese.[1,3,15] The observed rate also differs significantly from what has been reported from other parts of the country, i.e., 48% and 28.57%.[13,14] On the other hand it is almost the same as the rates obtained from some parts of India, especially

the northern areas of that country which have had rates as high as 58%–60% in various studies.[8,9,16] It should be mentioned that based on our previous experiments, the studied population, also showed notable resemblance with Indian population in some other mutations and haplotypes including the more common mutations for β-thalassemia. The informative rate respecting this polymorphism was 43.7% which confirmed the efficiency of *Bcl*I RFLP in detecting carriers in the population studied. This finding is in accordance with the reported findings from an identical study on a sample population composed of families who were referred from different parts of Iran.[13] The efficiency of *Bcl*I RFLP in detecting carriers varies in different ethnic groups— 25.9% in Koreans, 41% in American whites and 64% in Indians.[3,8,17]

Although the informative rate of 43.7% achieved in our study is quite helpful in detecting the carrier state, other studies have shown that by using 3–4 markers simultaneously, the carrier state can be determined in about 80% of the affected families.[8,18] Therefore, the next step to assign the strategy of detecting carriers and prenatal diagnosis would be to investigate the informative rate of other polymorphic markers. Among the available markers, the intragenic markers are preferable in view of their high accuracy in diagnosis and also because their risk of error due to miotic recombination is less than 1%.[19]

In conclusion, analysis of *Bcl*I polymorphism is effective and feasible in carrier detection and we suggest it for carrier screening and prenatal diagnosis in hemophilic families. To be capable of detecting carriers in more families, utilizing other RFLP sites would be helpful.

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