AMPLIFICATION OF Bcl I REGION OF THE FACTOR VIII GENE BY PCR

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Abstract. A study was initiated to amplify by polymerase chain reaction (PCR), a short factor VIII gene fragment containing the Bcl I restriction site from hemophilia patients using published primer sequences. Preliminary findings indicated that the resulting fragment is 142 bp long. This fragment, when digested with Bcl I restriction enzyme, produced two fragments, 99 bp and 43 bp in length. Polymorphism in the Bcl I region can be used to detect carrier state in the family members of the hemophiliacs.

INTRODUCTION

Hemophilia A is the most common inherited bleeding disorder characterized by the loss of clotting factor VIII, a component in the blood coagulation pathway. The evaluation of restriction fragment length polymorphism (RFLP) by Southern blotting has greatly improved prevention of the disease by carrier detection and prenatal diagnosis.

A rapid non-radioactive technique based on enzymatic amplification of a short factor VIII gene segment containing the polymorphism Bcl I site of intron 18 has been published by several investigators (Kogan *et al*, 1987; Gitschier *et al*, 1985).

In this paper we report the preliminary findings of the application of polymerase chain reaction (PCR) technique in the amplification of the factor VIII gene fragment containing the Bcl I restriction site in hemophilia patients.

MATERIALS AND METHODS

DNA from blood samples collected in EDTA from hemophilia patients from the National Blood Transfusion Centre Hospital Kuala Lumpur and Hospital Malacca was extracted by proteinase K digestion followed by phenol-chloroform extraction (Kunkel *et al*, 1977). Amplification of the short factor VIII gene fragment containing the Bcl I site was performed according to a modification of the standard procedure (Saiki *et al*, 1985). Target sequences were amplified in a 100 µl reaction volume containing 1 µl of genomic DNA; 1.25 mM each of deoxyadenosine triphosphate, deoxycytosine triphosphate, deoxyguanine triphosphate and deoxythymidine triphosphate, 50 pmol of each oligonucleotide primers; all in 10 µl of 10 X PCR reaction buffer (100 mM Tris HCl, pH 8.3, 0.5 M KCl, 15 mM $MgCl_2$ and 0.1% gelatin). The above mixture was covered with 60 µl mineral oil to avoid evaporation. The mixture was heated at 94°C for 7 minutes to denature the DNA, allowed to cool at room temperature for 2 minutes. Then 2.5 units of *Taq* polymerase was added to each sample which was then heated at 72°C for 2 minutes. The samples were then transferred to the thermal cycler programmed with the following steps; denaturation temperature 94°C for 1 minute, annealing temperature 57°C for 1 minute and extension temperature 72°C for 1 minute. The above steps were repeated for 32 cycles, followed by a 7 minutes elongation at 72°C after the last cycle.

The PCR product was then electrophoresed on 2% agarose gel at 100 V for 2 hours and stained with ethidium bromide before being exposed under the ultraviolet light for assessment and photography.

These PCR products were then incubated at 50°C for 2 hours with the Bcl I restriction enzyme to identify the presence or absence of digestion site. The digested products were then electrophoresed under the same condition as mentioned above.

RESULTS

Using published specific primers for Bcl 1 region (Kogan *et al*, 1987) we were able to obtained a 142 bp DNA fragment, amplified through PCR for the hemophilia patients analysed. Fig 1 shows the fragments obtained for some of the hemophilia patients studied. The size of the fragment ampli-

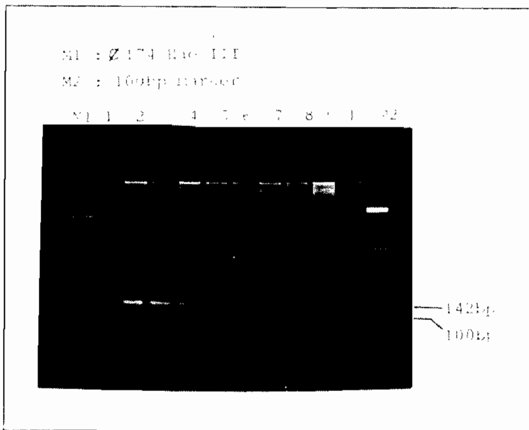

Fig 1–DNA samples from 10 hemophilia patients showing the 142bp fragment of factor VIII gene containing the Bcl I restriction site.

fied was determine by 100 bp reference marker and ϕ174/Hae III marker run concurrently with the PCR samples.

Complete digestion with Bcl I restriction enzyme resulting in only fragments 99 bp and 43 bp were observed in half of the cases investigated, however, the other half showed incomplete digestion of PCR products which resulted in the 99 bp fragment and remains of undigested 142 bp fragment as demonstrated in Fig 2.

The presence or absence of the site can be followed in much the same way as it is in the Southern blot analysis of Bcl I-digested genomic DNA sample and polymorphisms determined by hybridization with allele-specific oligonucleotide probes.

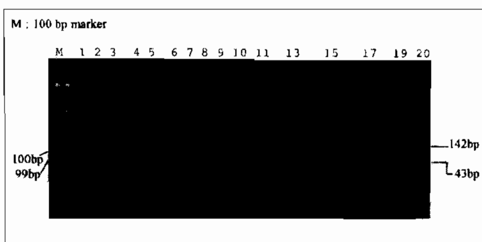

Fig 2–Bcl I digestion of amplified samples showing complete digestion resulting in a 99 bp and 43 bp fragments in lanes 2, 4, 6, 10, 16, 18 and 20. Incomplete digestion is observed in lanes 14, 17 and 19.

DISCUSSION

Specific amplification of target sequences by Taq polymerase allows rapid prediction by different approaches. Digestion of amplified samples known as restriction-site polymorphism as in our study is applicable to disease that are diagnosed by the analysis of associated restriction-site polymorphism and to diseases in which mutation alters a restriction-site. However, sequence polymorphism including that do not alter the restriction-site can be analysed by hybridization of amplified sample to allele-specific oligonucleotide probes (Orkin *et al*, 1984). In disorders caused by gene deletions diagnosis can be made by observing the presence or absence of the sequences in the amplified samples (Nakahori *et al*, 1986). These different applications of PCR reflects the flexibility of the amplification with Taq polymerase for genetic diagnosis.

In the case of hemophilia diagnosis, the presence of polymorphism in the Bcl I region of the factor VIII gene are currently being used for the accurate identification of carriers and for prenatal diagnosis by chorionic villus sampling in the first trimester (Kogan *et al*, 1987). These polymorphism involves the recognition site for the restriction enzymes Bcl I, Bgl I and Xba I; the presence or absence of the sites provides a tag for the hemophilia mutation in a pedigree analysis.

Our preliminary findings showed that the PCR can be use successfully to amplify the short factor VIII fragment of interest such as Bcl I region with the use of specific primers and appropriate PCR conditions. Digestion with restriction enzyme can detect the presence or absence of the restriction site, however, approprite amount and suitable condition for incubation has to be determined in order not to end up with incomplete digestion as in our cases. Hybridization with specific oligonucleotide probe will aid in confirming the presence or absence of mutation at the restriction site of interest.

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REFERENCES

Gitschier J, Dragna D, Edward CD, *et al.* Genetic mapping and diagnosis of haemophilia A achieved through a Bcl I polymorphism in the factor VIII gene. *Nature* 1985; 314 : 738-40.

Kogan SC, Doherty M, Gitschier J. An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequence. *N Engl J Med* 1987; 317 : 985-90.

Kunkel LM, Smith KD, Bayer SH, *et al.* Analysis of human Y-chromosome specific reiterated DNA in chromosome variant. *Proc Natl Acad Sci USA* 1977; 74 : 1245-9.

Nakahori Y, Mitani K, Yamada M, *et al.* A human Y-chromosome specific repeated DNA family (DYZ1) consists of a tandem array of pentanucleotides. *Nucleic Acids Res* 1986; 14 : 7569-80.

Orkin SH, Kazazian HH JR. The mutation and polymorphism of the human β-globin gene and its surrounding DNA. *Ann Res Genet* 1984; 18 : 131-71.

Saikai RK, Scharf S, Faloona F, *et al.* Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 1985; 230 : 1350-4.