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Rapid assessment of haemophilia A carrier state by non-invasive techniques using the polymerase chain reaction

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Abstract

The polymerase chain reaction (PCR) was used to amplify two polymorphic regions in the factor VIII gene. In vitro synthesis of DNA was achieved using samples obtained from buccal cells, urine, and hair follicles in addition to purified genomic and crude DNA samples prepared from whole blood. Female members of two kindreds affected with haemophilia A were assessed for carrier state using direct restriction fragment length polymorphism analysis of amplified gene products in the *Bc1I* and *XbaI* regions. It is concluded that this is a non-invasive, rapid, and inexpensive technique for carrier detection.

Haemophilia A is an inheritable haemorrhagic disorder caused by a heterogeneous array of genetic lesions that result in a deficiency of the blood clotting protein factor VIII.[1] Because the gene for factor VIII is located on the distal portion of the long arm of the X chromosome, phenotypically normal females within an affected kindred may be carriers of the disorder. Detection of haemophilia carriers is typically performed by gene tracking of affected X chromosomes using restriction fragment length polymorphism (RFLP) analysis.[2] Two frequently occurring RFLPs have been described within the factor VIII gene.[3,4] The first, a *Bc1I* polymorphism, is situated 3′ of exon 18 and secondly, an *XbaI* polymorphism is present within intron 22. Recently, Kogan *et al* described the use of PCR amplified gene products for the direct assessment of RFLPs within the factor VIII gene.[5] We examined the feasibility of using DNA obtained by non-invasive techniques as target material for rapid direct RFLP analyses in haemophilia A.

Methods

Genomic DNA was obtained from 10–20 ml blood collected into 3·8% trisodium citrate using the method of Kunkel *et al*.[6] Crude DNA was obtained from 200 μl anticoagulated blood, 10 ml water mouthwash, or 50 ml urine samples by centrifugation at 2000 ×*g*. Cell pellets were then resuspended and boiled in 200 μl of water for five minutes followed by centrifugation at 13000 ×*g* for two minutes; 30 μl of the supernatant was used in each reaction. Ten hair roots boiled in 200 μl of water were similarly used to produce a crude DNA sample. Amplifications using heat stable *Taq* polymerase were performed according to a modification of the procedure of Kogan *et al*.[5] Target sequences were amplified in 100 μl reaction volume containing 1 μg of genomic DNA or 30 μl of crude preparation, 1·5 mM of deoxycytosine triphosphate, deoxyadenosine triphosphate, deoxythymidine triphosphate, and deoxyguanosine triphosphate, and 50 pmol of each oligonucleotide primer all in a reaction buffer of 10 mM TRIS-HCl (pH 8·3), 50 mM KCl, 15 mM $MgCl_2$ and 0·05% gelatin. Oligonucelotide primers were as described by Kogan *et al*[5]; primers 7:1 and 7:10 were used for the *XbaI* region and primers 8:1 and 8:2 for the *Bc1I* region. After five minutes of initial denaturation at 95°C 30 to 40 cycles were performed comprising annealing for one minute at 55°C (37°C for *Bc1I* primers), extension at 73°C for two minutes, and denaturation at 93°C for one minute. One unit of *Taq* polymerase was added after initial denaturation. An additional 1 unit of enzyme was added at cycle 20. Ten μl of amplified product was digested using 10 units of restriction endonuclease incubated in a suitable buffer for two hours.

The resultant fragments were viewed under ultraviolet light following electrophoresis using a 4% submarine low gelling temperature agarose stained with ethidium bromide.

Results

AMPLIFICATION OF POLYMORPHIC REGIONS WITHIN THE FACTOR VIII GENE

When using the *Bc1I* primers a single fragment

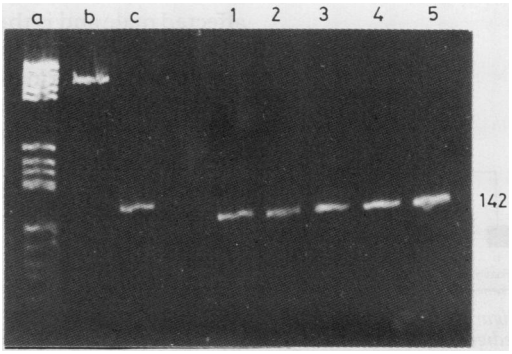

Figure 1 Bc1I amplified products. Lane (a) Hae III digest of pBR322; lane (b) 500 base pair marker; lane (c) 150 base pair marker; lanes 1–5 are PCR amplified products using clean DNA, whole blood, mouthwash, urine and hair, respectively. Size in base pairs is shown on right hand side.

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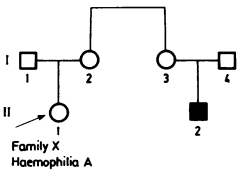

Figure 2 Family X pedigree information using Bc1 PCR. Boxes depict males, circles females. II2 is a severe haemophiliac. III is the propositus case.

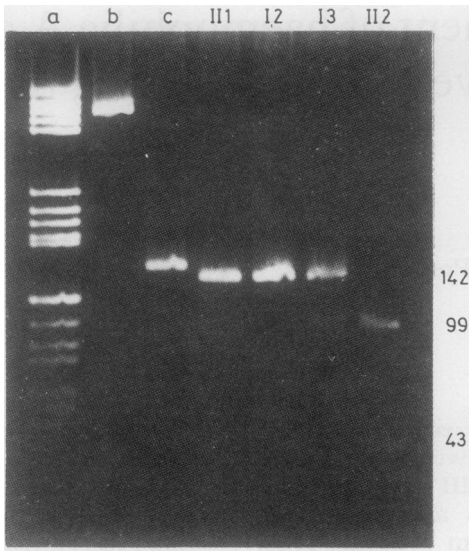

Figure 3 Lane (a) Hae III digest of pBR322; lane (b) 500 base pair marker; lane (c) 150 base pair marker. Lanes II1 to II2 depict BclI PCR products from family X.

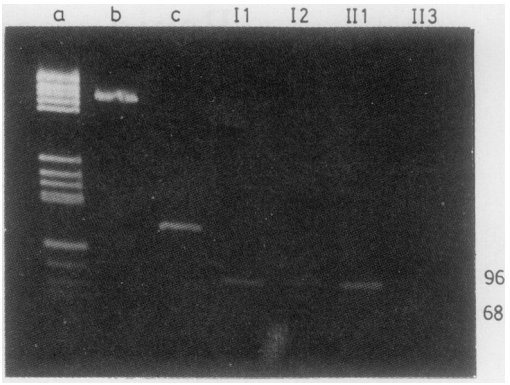

Figure 5 Lane (a) Hae III digest of pBR322; lane (b) 500 base pair marker; lane (c) 150 base pair marker. Lanes I1 to II3 depict XbaI PCR products from family Y. (lanes I2 and II3 exhibit pale staining bands at 68 base pairs).

was amplified from purified DNA or any of the crude preparations (fig 1). The resultant band was 142 base pairs in size. Similarly, an *XbaI* amplified region was obtained using all the sources of DNA and a band of 96 base pairs was observed. The *BclI* region, when exposed to *BclI* restriction endonuclease, was cleaved asymmetrically to give two products of 99 and 43 base pairs. The *XbaI* region was cleaved to produce a pale staining band at 68 base pairs, the smaller band was not observed. Much of the *XbaI* region amplified DNA from a male with a known positive site for the enzyme would not digest. No difference in digestion was observed in the amplified fragments from each source material.

ASSESSMENT FOR CARRIER STATE

Mouthwash samples were used to diagnose carrier state in two families. The first family were informative using the *BclI* amplified region (fig 2). The propositus case was shown to be homozygous negative for the cleavage site, as was her mother. The mother of the haemophilic patient was heterozygous and the affected boy had a positive cleavage site within his factor VIII gene. The propositus case had not inherited the same X chromosome as the affected male and is therefore, by exclusion, not a carrier of haemophilia A (fig 3). The second family were informative for the *XbaI* region (fig 4). The propositus case inherited a positive allele from her mother and a negative allele from her father. The negative site inherited by the affected boy from his mother indicated that the propositus case is not a carrier of the disorder (fig 5).

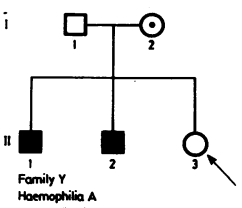

Figure 4 Family Y pedigree information using XbaI PCR. Boxes depict males, circles females. I2 is an obligate carrier by virtue of having two affected sons II1 and II2. II3 is the propositus case.

Discussion

The method of choice for gene analysis of carriers of haemophilia A is typically RFLP assessment by Southern blotting.[2] Such techniques are lengthy, technically involved, and require the use of substantial quantitites of radioisotopes. Rapid analyses by the PCR have been reported for prenatal diagnosis and carrier testing in a variety of disorders including sickle cell anaemia, phenylketonuria, and cystic fibrosis in addition to haemophilia. Furthermore, fetal sexing using the PCR has been reported using amiocytes, chorionic villi, and single cells taken from developing blastocysts.[7-12] PCR has also been applied to the study of residual leukaemia.[13] As far as we know this is the first report of rapid gene analysis in haemophilia achieved by the PCR on DNA obtained from buccal cells, hair follicles, and urine samples. We found that all the crude material amplified well using thermostable DNA polymerase and cycling was completed within a few hours. Analysis using the method described here can be performed within one day: conventional procedures normally take up to 10 days.

Our findings that the amplified *XbaI* region will only partially cleave with *XbaI* restriction endonuclease supports the work of others who prospose that a good proportion of the product is not factor VIII gene in origin and therefore does not possess a restriction site for this enzyme.[6] This is probably due to a lack of specificity of the primers and consequently reduces the diagnostic capacity of this particular reaction. The use of further *XbaI* primers should improve this.

The use of non-invasive techniques is a principal attraction of the procedure with regard to ease of collection of samples, particularly relating to younger family members. Diagnosis can be made quickly and cheaply with minimum inconvenience to patients under investigation. PCR procedures are technically undemanding, obviate the need for radioisotope handling, and lend themselves easily to automation. Despite this the risk of false results due to contaminating material or by the lack of fidelity of the reaction is a potential problem. Furthermore, the stability of stored crude DNA samples is as yet unknown.

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