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 BclI 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. 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. Two frequently occurring RFLPs have been described within the factor VIII gene. The first, a BclI 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. 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. 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. 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, deoxyguanosine triphosphate, and deoxythymidine 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 MgCl₂ and 0.05% gelatin. Oligonucleotide primers were as described by Kogan et al.; primers 7:1 and 7:10 were used for the XbaI region and primers 8:1 and 8:2 for the BclI 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 BclI 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 visualised under ultraviolet light following electrophoresis using 4% submarine low gelling temperature agarose stained with ethidium bromide.

Results
AMPLIFICATION OF POLYMORPHIC REGIONS WITHIN THE FACTOR VIII GENE
When using the BclI primers a single fragment

Figure 1 BclI 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.
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).

Discussion

The method of choice for gene analysis of carriers of haemophilia A is typically RFLP assessment by Southern blotting. Such techniques are lengthy, technically involved, and require the use of substantial quantities 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 amniocytes, chorionic villi, and single cells taken from developing blastocysts. PCR has also been applied to the study of residual leukaemia. 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 propose 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. 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, obviating 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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