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Detection by capillary electrophoresis of restriction fragment length polymorphism

Analysis of a polymerase chain reaction-amplified product of the DXS 164 locus in the dystrophin gene

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ABSTRACT

Capillary electrophoresis (CE) was used to characterize restriction fragment length polymorphism (RFLP) in a polymerase chain reaction (PCR)-amplified product of a 740-base pairs DNA fragment from the DXS 164 locus of the dystrophin gene. The polymorphic alleles of 740 and 520/220 base pairs revealed by *Xmn*I digestion were analysed from homozygous and heterozygous individuals by CE. Our studies show that extraction in phenol–chloroform may be useful in PCR-amplified product purification. Excellent separation was obtained in a short time. The data indicate that CE is suitable for genomic analysis such as carrier detection and prenatal diagnosis of X-linked recessive disorders after purification of PCR-amplified products.

INTRODUCTION

Specific endonucleases digest DNA, producing DNA fragments that differ in size among individuals. By identifying the association between different DNA fragments (known as restriction fragment length polymorphisms or RFLPs) and the appearance of a particular inheritable trait or disorder within a family tree, inheritance patterns in families have been studied and numerous genetic diseases have been detected and mapped to specific chromosomes [1].

Traditionally, DNA polymorphisms have been detected by analysing point mutations in restriction-enzyme sites that give rise to altered length fragments. These are detected using DNA transfer to a solid support and its hybridization with labelled molecular probes [2]. Differences in the banding pattern between individuals whose DNA has been digested with the same enzyme

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will reveal an RFLP. The polymorphism can be detected quicker and more economically by polymerase chain reaction (PCR) amplification of a stretch of DNA followed by detection of the presence or absence of the restriction-enzyme cutting sites on ethidium-stained agarose gels. PCR coupled to (multiple) restriction digests allows dramatically faster analysis of RFLPs than the Southern-blotting technique [3].

The availability of genetic markers, which reveal DNA polymorphism by restriction-endonuclease analysis, and/or variable number of sequence length repeats on the X chromosome, has improved the prenatal genotype prediction and carrier detection of several X-linked diseases, such as Duchenne muscular dystrophy, ornithine transcarbamyl transferase deficiency, retinitis pigmentosa, adrenal hypoplasia, chronic granulomatous disease and McLeod phenotype [4,5]. Because slab gel electrophoresis is time-consuming, labour-intensive, and difficult to quantify, capillary electrophoresis (CE) may become an attractive alternative to agarose or polyacrylamide gel electrophoresis. In this paper we describe the application of CE in the detection of DXS164/*Xmn*I polymorphism after PCR amplification of genomic DNA.

EXPERIMENTAL

Subjects

Venous blood samples were obtained from unrelated adult donors at the National Blood Transfusion Center, Italian Red Cross, Rome, Italy. Subjects were of both sexes and from different parts of Italy. The blood samples were collected after informed consent.

DNA sample preparation: agarose gel electrophoresis

Circulating leucocytes were separated from venous blood anticoagulated with 0.5% EDTA. DNA was extracted from the cells by urea lysis, phenol–chloroform extraction, ethanol precipitation and Tris–EDTA resuspension. Human genomic DNA was amplified by PCR under the following conditions: a 100-μl volume containing 0.5–1 μg of genomic DNA, with 1 μM of each primer (5′-GACTGGAGCAAGGGTCGCC-3′ and 5′-ACAATTTCCCTTTCATTCCAG-3′), 200 μM of each deoxynucleotidetriphosphate (dNTP), 0.3 U of Amplitaq (Perkin-Elmer Cetus) and 1 × Perkin-Elmer PCR buffer was subjected to 29 cycles of amplification. PCR was performed on a DNA thermal cycler (PE Cetus Instruments, Norwalk, CT, USA). Cycle temperature conditions were 40 s at 94°C, 1 min at 57°C and 1 min at 72°C.

Amplified DNA was then sequentially digested with *Xmn*I at 37°C overnight. DNA fragments were analysed by agarose gel electrophoresis using 2% agarose gels and TBE buffer (0.089 *M* Tris, 0.089 *M* boric acid, 2 m*M* EDTA, pH 8.5). Run conditions were 60 mA, 70 V, for 5–6 h. DNA was visualized by ethidium bromide staining at a concentration of 10 μg/ml. For analysis by CE, the digested samples were subjected to phenol–chloroform (3:1, v/v) extraction. DNA size standard low range [72–1350 base pairs (bp)] was used. DNA molecular weight markers (*Hae*III digest of ϕX 174 DNA, New England Biolabs, Beverly, MA, USA) were reconstituted in distilled water to a final concentration of 10 μg/ml.

CE instrumentation and buffer system

CE was performed using the HPE 100 high-performance capillary electrophoresis system from Bio-Rad Labs. (Richmond, CA, USA), with data collection by a Hewlett-Packard 3394A integrator. A 50 cm × 50 μm I.D. preassembled coated capillary (Bio-Rad) was conditioned with separation buffer and pre-electropheresed before each analysis. Samples and standards were loaded electrophoretically at a negative polarity by applying 180 V/cm for 8 and 15 s, respectively. Separations were performed at negative polarity under constant voltage at 160 V/cm. A typical run lasted 25 min. The separation buffer consisted of a 0.5% hydroxypropyl methylcellulose (HPMC; Sigma, St. Louis, MO, USA) in TBE. The buffer was filtered by a Millipore filter (0.45 μm) to remove particulates and degassed at 0.01 atm (1 atm = 101 325 Pa) by a standard vacuum pump for 30 min. DNA size standard low range (88–1746 bp) was used (*Eco*RI digest of pBR322 from Bio-Rad). DNA molecular markers were reconstituted in distilled water to a

final concentration of 10 μg/ml. Ultraviolet absorbance was monitored at 260 nm. By serial dilution of the size standard up to 0.0025 μg/ml, a diminution of the peak area was detected. This may be useful when quantification is needed. In any case, no variation in the migration time was observed and the resolution was maintained. The identification of the DNA fragment size was determined by plotting log bp *vs.* the migration time. A good correlation was obtained ($r = 0.977$). In preliminary experiments, the same sample was injected sequentially. The migration time was reduced from run to run by 15 ± 1 s (mean ± S.D., $n = 7$), while the electropherograms show the same profile. Since the drift was constant and predictable, we calculated the DNA fragment sizes taking into account this factor.

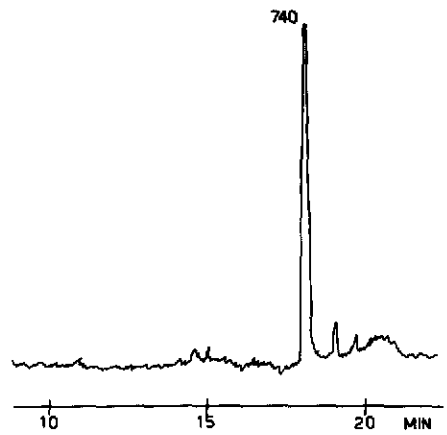

Fig. 2. CE of the PCR-amplified fragment. Detection was by absorbance at 260 nm (0.005 full scale). Migration times (min) are indicated on the *x*-axis.

RESULTS

Fig. 1 shows the separation of samples from various individuals demonstrating *Xmn*I polymorphism on agarose gel. The amplification product is a fragment of 740 bp, and *Xmn*I digestion distinguished a two-allele polymorphism of 520 and 220 bp. Lane M shows DNA size markers. Lane U shows the amplified fragment, lane d the digested amplified fragment. PCR amplification of the 740-bp DNA fragment from an intron in the DXS 164 locus and subsequent digestion by *Xmn*I yields polymorphic fragments of 520 and 220 bp, corresponding to alleles A1 and A2, respectively. Individuals homozygous for A1 reveal only the 740-bp fragment. Those homozygous for A2 reveal the 520- and 220-bp fragments. Heterozygous individuals (A1–A2) yield three fragments upon electrophoretic separation.

Fig. 2 shows the electropherogram (CE) of the PCR-amplified fragment. The size (740 bp) was determined by comparing the mobility with a calibration run of standard low-range DNA fragments from Bio Rad. On the basis of this calibration, the sizes of the fragments (740, 520, 220 bp) were determined. This result corresponds with that estimated by agarose gel electrophoresis. Digestion of the fragment in an A2

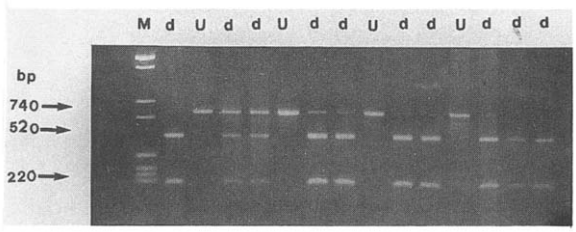

Fig. 1. Separation on agarose gel of samples from various individuals demonstrating *Xmn*I polymorphism. PCR amplification of a 740-bp DNA fragment from an intron in the DXS 164 locus and subsequent digestion by *Xmn*I. For details see text.

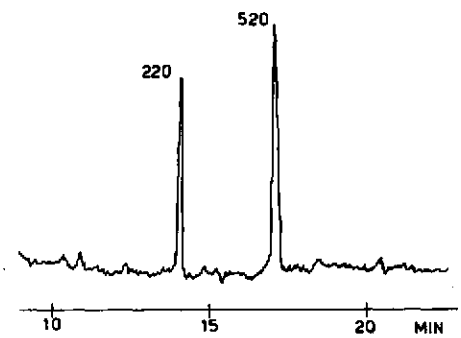

Fig. 3. CE that indicates homozygous alleles (A2–A2). Detection was by absorbance at 260 nm (0.005 full scale). Migration times (min) are indicated on the *x*-axis.

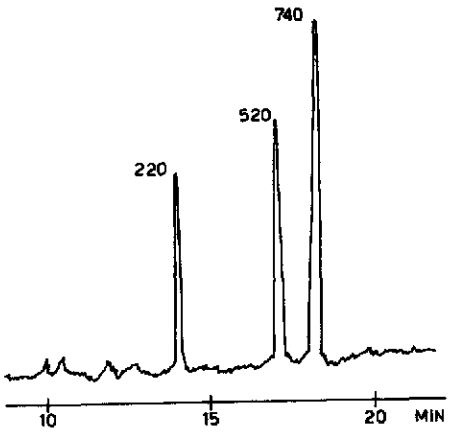

Fig. 4. CE of a sample taken from a woman. The presence of three fragments (744, 520 and 220 bp) demonstrates the heterozygosity of the subject (A1–A2). Detection was by absorbance at 260 nm (0.005 full scale). Migration times (min) are indicated on the *x*-axis.

homozygous sample yields two fragments (520 and 220 bp). The fragments are well separated (Fig. 3). Fig. 4 shows the electropherogram (CE) of a sample taken from a woman. The presence of three fragments (740, 520, 220 bp) demonstrates the heterozygosity of the subject (A1–A2) [6].

DISCUSSION

Our results demonstrate that CE can be applied to genomic diagnosis using RFLP restriction enzyme-digested PCR samples. Excellent separation is achieved for the DNA restriction fragments. In our study it may be seen that it is possible to determine unambiguously homo- and heterozygous patterns. To improve the detectability in cases in which the samples do not contain enough DNA, it is possible to increase the injection time or the voltage. One solution to the sample detectability problem is to employ more powerful detection schemes, *e.g.*, laser-induced fluorescence or electrochemical detectors [7]. Recently, a method by which more samples can be injected and simultaneously focused into a narrow zone has been introduced, taking advantage of injecting a small water plug [8].

Linear polymers have been used as buffer additives to separate the DNA fragments by acting as molecular sieves [9,10]. In our experiments the use of methylcellulose derivative was essential to adequately separate RLFPs. Good resolution may be obtained only after purification of the PCR products, which we achieved by extraction in phenol–chloroform. Recently, an ultrafiltration method has been introduced [11].

In our experiments, a shift in the migration times was observed. The observed drift is due to the change in temperature of the capillary and its environment. For this reason, the size determination of the DNA fragments based on mobility *vs.* bp number plot of calibration standards can only be a good approximation. However, since the drift was constant and predictable, this method was more than sufficient for our purpose, owing to the large difference in the size of the analysed fragments. It is conceivable that this problem may be overcome by using the instruments, now commercially available, that allow adequate temperature control and heat removal from the capillary.

Determination of DNA restriction fragments with a sieving buffer containing ethidium bromide and a coated capillary has recently been achieved [12]. The ethidium bromide induces significant shifts in mobility of the DNA fragments, depending on their size. Its addition has demonstrated the usefulness of CE in detecting PCR-amplified retroviral DNA sequences, and RFLP of an oncogene [12,13].

CE may be useful for detecting RFLP because it allows a reduction in analysis time compared with the standard method. Samples can be loaded automatically and run overnight unattended, and the same capillary can be used for hundreds of runs. Further optimization of CE conditions would allow a reduction of CE run time (*e.g.*, when larger differences in DNA fragments are analysed) [12]. In future, it will be possible to estimate the size of unknown DNA fragments based on calibration data using special software. Further improvements in quantitation should gain CE wide acceptance in diagnostic and biomedical research applications.

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