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Screening and identification of familial defective apolipoprotein B-100 in clinical samples by capillary gel electrophoresis

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Abstract

Familial defective apolipoprotein B-100 (FDB) is a dominantly inherited disorder. It is characterized by a decreased affinity of low density lipoprotein (LDL) for the LDL receptor, as a consequence of a substitution of adenine by guanine in exon 26 of the apolipoprotein B-100 gene, coding for the putative LDL receptor-binding domain of the mature protein. This disorder is associated with a strikingly high incidence of arteriosclerosis and tends to cause disease and premature death. In this communication we describe a rapid capillary gel electrophoretic method in combination with molecular biology techniques to facilitate the diagnosis of FDB. Mutation screening for FDB is performed by an allele-specific amplification followed by capillary gel electrophoresis (CGE). For the combined polymerase chain reaction (PCR)–CGE method, a total analysis time of only 3 h is needed, a period that is normally necessary for the run and for staining of the gel only, not including the time for PCR, gel casting, etc. In our pilot study 4 of 43 hypercholesterolemic patients were found to have the predominant apoB 3500 codon mutation. The verification is demonstrated by DNA-sequencing. This pilot study will be followed by a large cohort analysis of the south-west German population to determine the frequency of FDB in this area. The PCR–CGE method on the Dionex capillary electrophoresis system (CES I) allows rapid, fully automated detection of the mutation resulting in the unequivocal diagnosis of FDB.

Keywords: Apolipoproteins; Lipoproteins; DNA, molecular diagnostics

1. Introduction

Human apolipoprotein B-100 (apoB-100) is a glycoprotein with a mass of circa $550 \cdot 10^3$ (512 932, calculated on the basis of its amino acid sequence), consisting of 4536 amino acid residues and appears to be the only protein component of plasma low density lipoprotein (LDL), carrying most of the plasma cholesterol. The key role of LDL is the binding and uptake of these particles mainly by liver, but also by peripheral cells. About 80% of this uptake is accomplished by the cell surface LDL receptor, and the remainder is removed by a non-specific low-affinity process [1]. The high-affinity interaction between LDL and the LDL receptor occurs via apoB-100, which is the physiological ligand and is therefore largely responsible for regulating plasma levels of LDL. Mutations, resulting in structural changes in either the LDL receptor or the apoB-100, decrease the efficient receptor-mediated

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uptake of LDL. While mutations in the LDL receptor gene have long been known to cause familial hypercholesterolemia, the existence of a mutation in an apoB-100 has been recognized more recently and is under intensive investigation [1–5].

Familial defective apolipoprotein B-100 (FDB) is one of the major causes of monogenic hypercholesterolemia, a dominantly inherited genetic disease, characterized by high LDL levels, tendon xanthomata, arcus lipoides, carotid arteriosclerosis and premature coronary heart disease [6,7]. Base changes in the apoB-100 gene, from CGG to CAG [3], from CGG to TGG [5], both at nucleotide 10 708 (codon 3500), and the probably rare CGC to TGC transition at nucleotide 10 800 (codon 3531) [1,8] were found to cause FDB. These transitions in exon 26 of the apoB gene, resulting in amino acid substitutions (Arg_{3500}→Gln, Arg_{3500}→Trp and Arg_{3531}→Cys), disrupt the conformation of the receptor-binding domain of apoB-100 and reduce the affinity of LDL to the LDL receptor down to 3–9% of the normal amount [5,9–11]. The putative receptor-binding domain approximately spans from codon 3000 to 4000 [12]. Although the apoB-100 Arg_{3500}→Gln exchange appears to be the most common mutation leading to FDB, other mutations could also cause binding abnormalities, but perhaps less severe ones [1,13]. The frequency of the Arg_{3500}→Gln mutation is in the range of 1:500 to 1:700 in North America and Europe [6,13,14], and in the group with hyperlipidemia about 3% have FDB [6,11,13]. Most patients are heterozygous for the defect, but recently homozygous individuals have been described also [15]. The purpose of this communication is to present a fast, exact and reliable analytical method for the diagnosis of FDB, suitable for mutation screening.

As the standard slab gel electrophoresis method for the analysis of polymerase chain reaction (PCR) products (agarose or polyacrylamide) is time-consuming, labour-intensive and difficult to quantify, there is a need to introduce new reliable analytical techniques. A progressive impulse towards clinical routine analysis of PCR products is the combination of PCR and capillary gel electrophoresis (CGE) [16]. The high degree of automation, rapid performance of electropherograms with the highest resolution, minute sample amounts required, on-line evaluation of the results, etc., make this combination an attractive tool for large cohort analyses to determine the frequency of mutations in the population or for special clinical molecular diagnosis. In the past, CGE proved to be successful for genetic typing and diagnosis of neoplastic disorders [17–20] or inherited diseases like Duchenne and Becker muscular dystrophies [21,22], cystic fibrosis [23,24], medium-chain acyl-coenzyme A dehydrogenase deficiency [25], congenital adrenal hyperplasia [26] or von Willebrand factor gene [27].

Examples of the power of CGE analysis of PCR products in the field of detection of arteriosclerotic risk factors are: (1) the determination of the insertion/deletion polymorphism of the angiotensin I-converting enzyme gene, which is thought to be an arteriosclerotic risk factor, completed in less than 10 min with high accuracy, resolution and speed [28]; (2) the analysis of the variable number of tandem repeats (VNTR) in the apolipoprotein B gene, detectable within 17 min in a range of between 600 and 1000 bp [29] or (3) the polymorphisms in the apolipoprotein E gene by restriction fragment-length polymorphism (RFLP), where CGE resolves fragments differing in only 6 bp with high reproducibility [30].

In this communication, preliminary results on capillary gel electrophoretic separations of PCR products and screening of apoB-100 Arg_{3500}→Gln mutations of codon 3500 in the south-west German population are described. In a pilot study, investigating the DNA of 43 selected patients, the efficiency of capillary electrophoresis for large cohort analyses in a fully automated way, in comparison with the conventional slab gel electrophoresis technique, is demonstrated.

2. Experimental

2.1. Materials

DNA isolation: DNA was isolated and purified from 200 μl of whole blood (EDTA) by QIAamp-spin columns according to the protocol given by the manufacturer (QIAamp Blood Kit, QIAGEN, Hilden, Germany). A 10-μl volume (200–500 ng) of the eluate was used for amplification by PCR.

2.2. Capillary electrophoresis

Coated capillaries (47 cm×50 μm I.D.) from Bio-Rad (Munich, Germany) were used for all electrophoretic runs. The separations were performed on a Dionex (Idstein, Germany) capillary electrophoresis system (CES I) equipped with an automatic constant volume sample injection system, a high sensitivity UV–VIS detector with wavelength programming and a dedicated computer system with a Microsoft Windows interface. The separations were performed with 100 m*M* Tris–borate–EDTA, pH 8.3, containing 10 μ*M* ethidium bromide and 0.7% hydroxyethyl cellulose at 12 kV (all from Sigma, Deisenhofen, Germany). The capillary was rinsed for 180 s with water, followed by 240 s with buffer after each run. PCR products were directly introduced by electrokinetic injection (60 s at 4 kV) and detected at λ=260 nm.

2.3. Polyacrylamide gel electrophoresis procedure

Following the PCR, 1 μl of each reaction mixture was loaded onto a homogeneous polyacrylamide gel (0.5×260×125 mm), containing 5% acrylamide and 1.5% crosslinkers (Serva, Heidelberg, Germany). The electrode buffer was 20 m*M* 3-[N-morpholino]-propanesulfonic acid (MOPS) and 1 m*M* EDTA (Sigma), pH 8.0. The DNA electrophoresis was performed on a Multiphor II horizontal electrophoresis system (LKB Pharmacia, Freiburg, Germany) at a maximum of 100 V at 10°C for 1 h, continued for another hour with a maximum setting of 200 V, until the marker front had reached the end of the gel. Upon completion of electrophoresis, the DNA was fixed in ethanol–acetic acid–water (1:0.5:8.5, v/v/v) for 6 min and was then stained with the silver staining kit according to the QIAGEN protocol (QIAGEN). After drying the gel overnight at room temperature, each sample lane of the silver-stained gel was analyzed visually and the results were written in a report.

2.4. Polymerase chain reaction

The amplification was performed in an Eppendorf Mastercycler 5330 (Eppendorf, Hamburg, Germany). Two 100 μl samples were used for the analysis of each individual: 200 μ*M* dNTP's, 1 μ*M* of primers apob2: 5′-gag aac ata caa gca aag cca ccc tg-3′ and primer apobg: 5′-ctt act tga att cca aga gca cac g-3′, yielding a 227 bp product, or primer apoba: 5′-gaa ttc caa gag cac aca-3′ and apobpi 5′-aga agc cac act cca acg ca-3′, yielding a 113 bp product, 1.5 m*M* $MgCl_2$ and 0.5 units of Taq polymerase (Appligene, Heidelberg, Germany) were mixed in PCR buffer (50 m*M* KCl, 10 m*M* Tris–HCl, 0.001% gelatine, pH 8.3). Cycling was performed with an incubation time of 5 min at 95°C, followed by 35 cycles at 95°C for 45 s, 60°C for 45 s, 72°C for 30 s and finally at 72°C for 5 min.

The primer apoba contains an adenine residue that corresponds to the apolipoprotein B mutation in codon 3500. In the presence of the mutant allele, amplification with the primers apoba and apobpi results in a PCR product of 113 base pairs. The PCR reaction with primers apobg and apob2 yields a 227 bp product in the presence of the normal allele.

2.5. DNA sequencing

The DNA was sequenced to confirm the mutations identified by the allele-specific PCR. Those samples, showing the 113 bp fragment were reamplified with a different set of primers, whereas one of them was biotinylated for isolating single-strands by the binding of biotin-labelled PCR products to streptavidin-coated magnetic beads (Dynabeads, Deutsche Dynal, Hamburg, Germany). Single-strand DNA sequencing yields much better results than double-strand sequencing [31]. The procedure was performed with a γ-^{33}P-ATP-end-labelled sequencing primer following a cycle-sequencing protocol from Stratagene (CyclistTM DNA Sequencing Kit, Stratagene, Heidelberg, Germany). The products were analyzed on a Flow Gen SE 1350 electrophoresis apparatus (Froebel, Lindau, Germany) in a 6% polyacrylamide gel (0.4×400×360 mm) at 1800 V for 2 h. After the run, the gel was dried and mounted on Kodak X Omat X-ray film (Sigma) for 24 h.

2.6. Subjects

43 patients with combined hyperlipidemia or familial hypercholesterolemia were investigated, recruited from the lipid outpatient clinic of the Medical

Center Hospital, Department of Medicine IV, University of Tübingen. The selection criteria for all patients were cholesterol levels (chol.) in serum of ≥7.0 mmol/l.

Patient J.M. (64 years of age) had a history of peripheral and cerebrovascular disease, occlusion of the arteria carotis interna and hypercholesterolemia (chol. 10 mmol/l, LDL–chol. 7 mmol/l). The family history in patient A.S. (24 years of age) revealed recurrent myocardial infarction of the father and her mother died at the age of 63 with acute myocardial infarction. Patient A.S. had hypercholesterolemia (12 mmol/l) and until now showed no signs of atherosclerotic disease. Patient E.S. (67 years of age) had myocardial infarction at the age of 51, tendon xanthomata and arcus lipoides, and the cholesterol level was 10.9 mmol/l before treatment. Patient R.S. (44 years of age), the son of E.S., had tendon xanthomata, arcus lipoides, no signs of arteriosclerosis and the level of cholesterol was 10 mmol/l before treatment. To lower the high cholesterol levels, all patients were treated with individual doses of hydroxy-methyl-glutaryl-coenzyme A reductase inhibitor (simvastatin), and their cholesterol levels returned to levels in the upper reference range of cholesterol during therapy. In this preliminary study, we preselected the collective by analysing only patients with familial hypercholesterolemia with the intention of definitely finding a FDB mutation.

3. Results and discussion

Previously, FDB patients were identified by the use of either monoclonal antibodies binding with higher affinity to the abnormal LDL particle [2] or based on the hybridisation of genomic or PCR-amplified DNA to radiolabelled allele-specific oligonucleotide probes, complementary to either the normal or the mutant apoB-100 gene [3,4,32]. These tedious methods require the use of radioisotopes, nowadays mostly replaced by non-isotopic allele-specific oligonucleotides [13], mutagenic PCR (introducing restriction enzyme cutting sites [33,34]), allele-specific PCR [14] or mutagenically separated PCR [35]. Analyses of PCR products is mainly performed by agarose gel electrophoresis combined with ethidium bromide staining, or the more sensitive polyacrylamide gel electrophoresis (PAGE) followed by silver staining procedures. These analyses are less time-consuming, but still labour-intensive and require operator proficiency. Gel casting and especially sample preparation are the most difficult operation steps, as the sample volumes are in the 1–5 μl range and precise transfer into the small, close sample slots on the supporting medium is essential. Fig. 1 illustrates the silver-stained routine PAGE separation of apoB-100 PCR fragments from various individuals showing an apoB-100 Arg_{3500}→Gln mutation in lane 5 (two lanes per individual).

The high speed, automatic and unequivocal separation of apoB-100 PCR fragments by CGE, as presented in the following, does not suffer from such drawbacks. Using a 113 bp (=FDB mutation) and a 227 bp fragment (non-FDB mutation), amplified in two different allele-specific PCRs, reliable results are

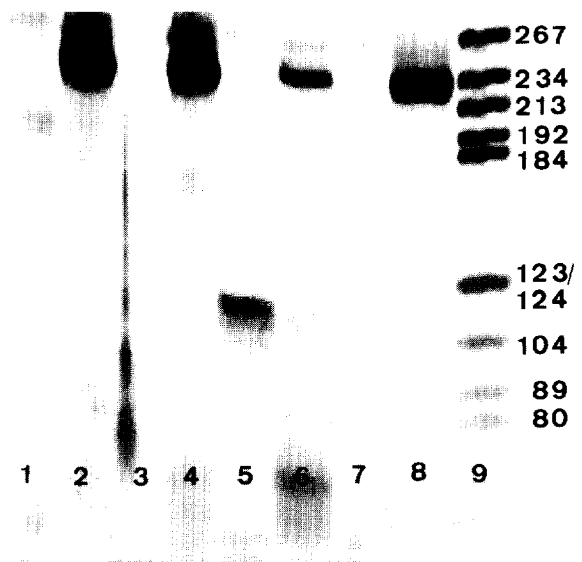


Fig. 1. Horizontal polyacrylamide gel of PCR fragments of DNA, from normal individuals and from a FDB_{3500} (Arg→Gln) heterozygote, on a 5% gel (0.5×260×125 mm), stained with silver. Running conditions: 100 V for 1 h followed by 200 V for 1 h; 20 m*M* MOPS buffer (pH 8.0). Lanes 1 and 2=non-FDB patient; lanes 3 and 4=non-FDB patient; lanes 5 and 6=FDB patient (lane 5 shows a 113 bp fragment, i.e. the product of the mutant allele; lane 6 with the 227 bp fragment corresponds to the normal allele, indicating the heterozygous status of this subject); lanes 7 and 8=non-FDB patient; lane 9=molecular mass marker (DNA fragment lengths in base pairs).

obtained, under faster separation conditions. According to the literature [17,36–41], different running conditions and hydroxyethyl cellulose concentrations are compared with regard to speed and a sufficient separation profile in the area of interest. Fig. 2 shows the optimised CGE separation of the molecular mass marker pBR 322, cut with restriction endonuclease *Hae III*. Under the conditions used, rapid analysis in less than 11 min is possible. The expected fragments, amplified for the analysis of the apoB-100 mutation Arg_{3500}→Gln, can be separated in an extremely short time. In Fig. 3, the patterns for the different geno-

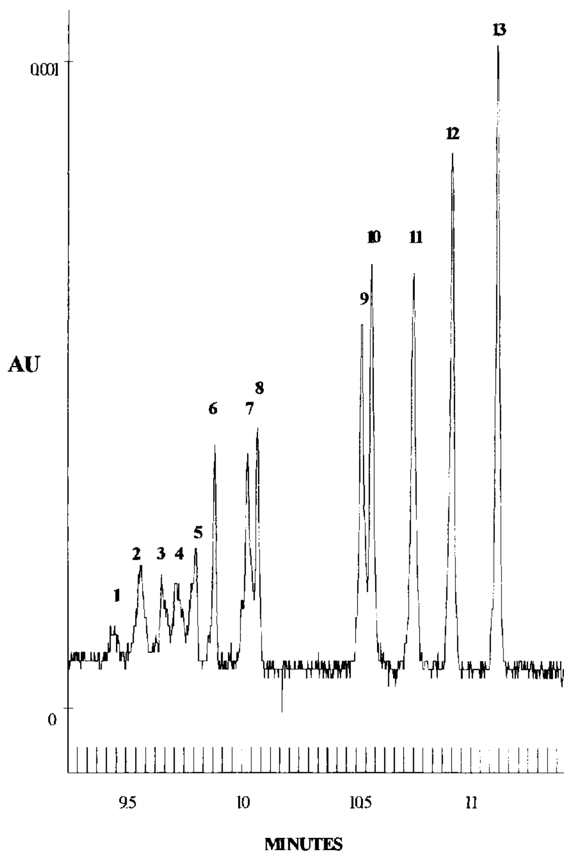
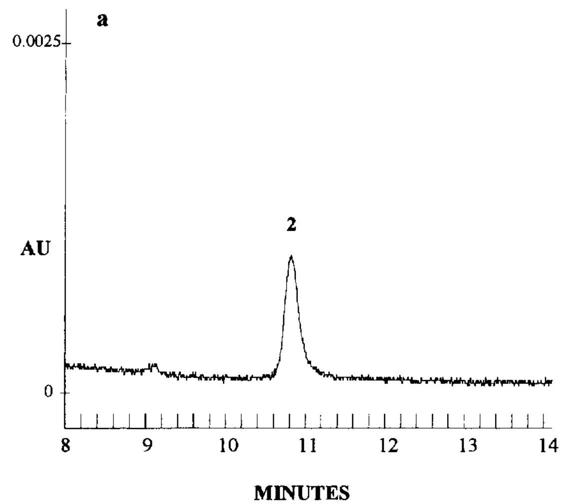


Fig. 2. Capillary electropherogram of molecular mass marker pBR 322 cut with restriction endonuclease *Hae* III. Electrophoretic conditions: Capillary, coated fused-silica (47 cm×50 μm); loading, 15 s at 4 kV; running conditions, 12 kV; buffer, 100 m*M* Tris–borate–EDTA, 10 μ*M* ethidium bromide and 0.7% hydroxyethyl cellulose, pH 8.3; detection, UV at λ 260 nm; equipment, Dionex CES I; peaks (fragment lengths in base pairs): 1=51; 2=57; 3=64; 4=80; 5=89; 6=104; 7=123; 8=124; 9=184; 10=192; 11=213; 12=234 and 13=267.



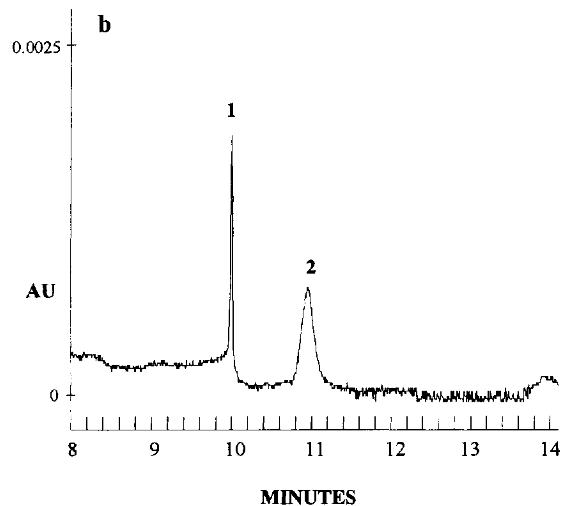


Fig. 3. Capillary electropherogram of PCR products from patients with different apoB-100 genotypes. For separation conditions see legend to Fig. 2; except that loading conditions used were 60 s at 4 kV. (a) non-familial defective apolipoprotein B-100; (b) familial defective apolipoprotein B-100. Peaks: 1=113 bp and 2=227 bp.

types are shown: The normal allele exhibits one 227 bp peak with a mean migration time of 10.91 min (Fig. 3a), while the mutation pattern shows two peaks, one with a mean migration time of 10.0 min (113 bp) and a second one with coincident migration time as observed for the non-FDB patient, indicating a heterozygous mutation (Fig. 3b), thus allowing unequivocal differentiation between FDB and non-

FDB types. The high stability of the separation profile, with a coefficient of variation for the migration time of 0.82% (113 bp fragment) and 0.52% (227 bp fragment), and thus the diagnostic safety to detect a mutation, is demonstrated by six consecutive runs (Fig. 4).

As described in other communications, it is recommended that the salt, primer and nucleotide content in the PCR product samples is reduced prior to injection, because high amounts of salt will strongly diminish the total amount of electrokinetically introduced DNA entering the capillary and excess primers and nucleotides may overlay the separation profile [23,37,42–45]. We successfully tried to eliminate this additional time-consuming step by direct injection of the PCR products (diluted 1:5 with water) without sample clean-up, obtaining well-resolved signals for detection with no disturbance by the PCR components (Fig. 4). As the salt, nucleotide and primer concentration in every apoB-100 PCR reaction is identical, we could not recognize any interfering effects. This procedure allows 44 samples to be analysed by a fully automatic method overnight via the sequential analytical mode (see Table 1). In parallel, e.g. with ten capillaries, an increased sample throughput (one order of magnitude greater) could be achieved. Another remarkable aspect of PCR product analysis by CGE is that the samples can be run immediately without incurring additional costs, in contrast to routine gel electrophoresis DNA analysis where normally samples are collected until all slots on the gel can be used, for economical reasons.

Out of the 43 patients with familial hypercholesterolemia, screened in this preliminary study, four with classic FDB_{3500} mutation were found. DNA sequencing showed the known G→A mutation in all four cases (Fig. 5). No mutations were found in the other patients. The hypercholesterolemia in these patients is possibly due to mutations in the LDL receptor gene or to idiopathic hypercholesterolemia [46].

These results demonstrate that capillary electrophoresis has several advantages over slab gel electrophoresis (Table 1). As Joule heating is dissipated very efficiently, caused by the high surface-to-volume ratio of the capillary, high voltage in the kilovolt range is applicable, reducing separation

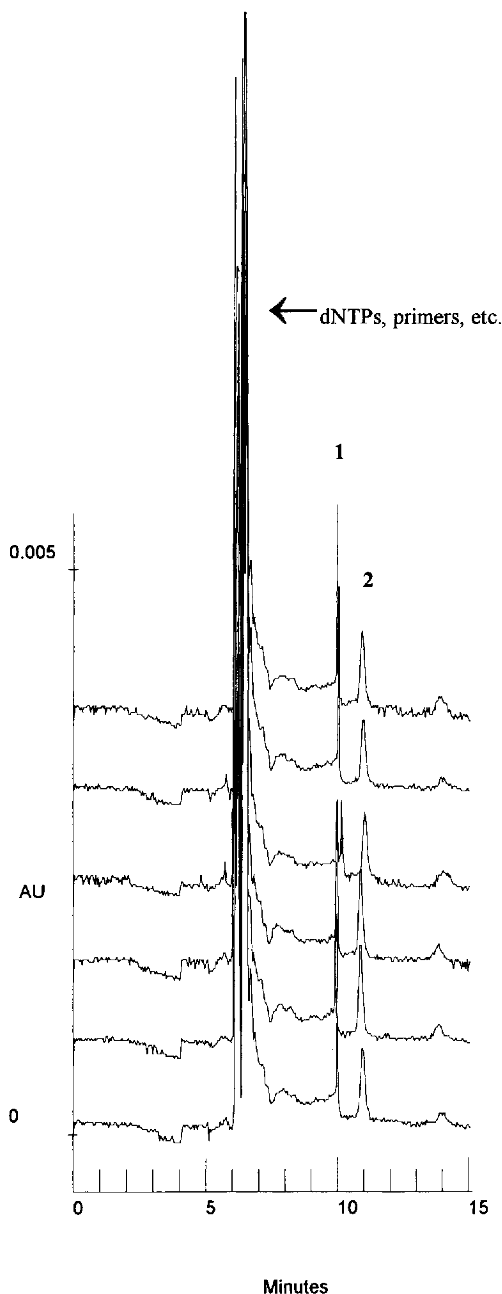


Fig. 4. Six consecutive injections (overlaid) of an apoB-100 mutation (patient E.S.). For separation conditions see legend to Fig. 2; except that the loading conditions used were 60 s at 4 kV. Peaks: 1=113 bp and 2=227 bp.

Table 1
Comparison of the time consumption required for the different steps involved in capillary gel electrophoresis and the routinely used polyacrylamide gel electrophoresis for the screening of apolipoprotein B-100

	Horizontal polyacrylamide gel electrophoresis[a]	Capillary gel electrophoresis
Gel casting and polymerization	90 min	none
Electrophoresis preparation	10 min	10 min (including sample vial loading)
Dilution of samples	none	30 s per sample
Sample application time	10 min	60 s per sample
Total run time	120 min	19 min (44 samples overnight)
Staining	60 min	on-column detection
Analysis and documentation of the results	30 min	directly after detection via PC

[a] 40 parellel lanes.

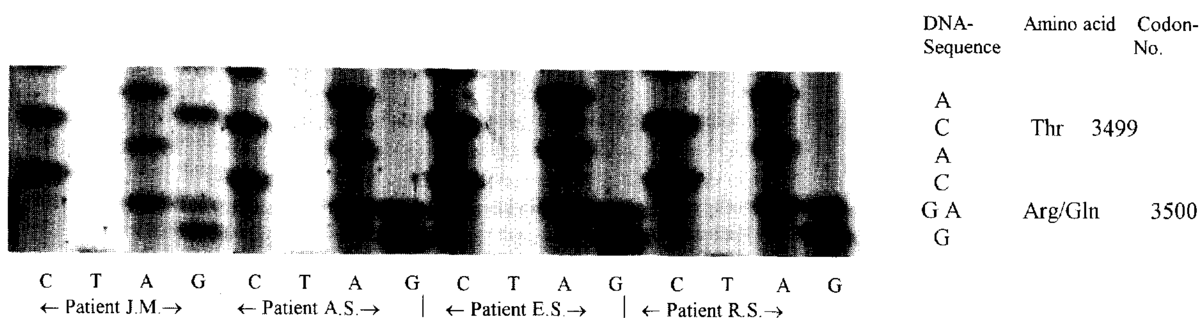


Fig. 5. Autoradiograph of a 6% polyacrylamide gel (0.4×400×360 mm) showing the DNA sequences of FDB patients with the Arg→Gln mutation in codon 3500. Running conditions: 1800 V for 2 h; 89 m*M* TBE buffer (pH 8.3). The derived DNA sequences, the corresponding amino acids and the codon numbers are indicated on the right side of the figure. The DNA sequence of every FDB patient is shown in four lanes indicating the four different bases (C=cytosine; T=thymidine; A=adenine and G=guanine).

times to minutes (Fig. 3). No gel casting and staining is needed. Samples can be loaded automatically from a carousel. Minute amount of sample volume (in the nanoliter range) is required, as detection through the capillary is performed. Evaluating the specific separation parameters, this method allows fully automated analyses of DNA mixtures over a vast range of molecular sizes including, data reporting and storage. It may well be that a multiplex array of capillaries [47] or capillary electrophoresis chips [48] will further revolutionize DNA analysis in clinical laboratories in the future.

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