Rapid and safe determination of human apolipoprotein E genotypes by miniaturised SDS-PAGE in non-insulin dependent diabetes mellitus

C Clavel, A Durlach, V Durlach, P Birembaut

Abstract

**Aims**—To present a non-isotopic procedure for the analysis of the different apolipoprotein E genotypes in normal subjects and patients with non-insulin dependent diabetes mellitus.

**Methods**—Apolipoprotein E genotypes were detected following polymerase chain reaction and miniaturised sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (PhastSystem Pharmacia).

**Results**—The time taken from extraction of DNA from 6 μl whole blood to the final result was eight hours. The allele frequencies in patients with diabetes mellitus in our series were similar to those found in the control subjects.

**Conclusions**—SDS-PAGE is rapid, reliable and safe with few drawbacks, can be used as a routine procedure and may easily replace apolipoprotein E phenotyping.


Keywords: Apolipoprotein E, SDS-PAGE, non-insulin dependent diabetes mellitus.

Of the different constituents of lipoprotein, apolipoprotein E is essential for the catabolism of lipoproteins by receptor mediated endocytosis. Apolipoprotein E is a structural component of several classes of plasma lipoproteins such as chylomicrons and their remnants, very low density lipoprotein (VLDL) and its intermediate density lipoprotein (IDL) remnants, and a high density lipoprotein (HDL) subpopulation. Apolipoprotein E is also involved in the conversion of VLDL to low density lipoprotein (LDL) by lipoprotein lipase and is a ligand for two types of high affinity receptors on most cell plasma membranes: the apolipoprotein E receptor and the LDL (B/E) receptors. Thus, apolipoprotein E mediates hepatic elimination of potentially atherogenic remnants particles generated by the lipolysis of triglycerides rich in lipoproteins (chylomicrons, VLDL). Apart from its role in lipoprotein metabolism, apolipoprotein E is synthesised in most cells and organs. Apolipoprotein E synthesis is increased following injury and is implicated in immunoregulation, repair and modulation of cell growth. A recent paper even suggested a functional pathogenic role for a particular apolipoprotein E isomorph in Alzheimer’s disease.

The human apolipoprotein E gene spans 3-7 kilobases including four exons and three introns and is located on chromosome 19. Structural variants of apolipoprotein E were first identified using isoelectric focusing (IEF). Now, either cloned DNA fragments, acting as probes, or synthesised oligonucleotides can be used to detect DNA mutations and/or polymorphisms which are associated, genetically linked or causally related to lipoprotein disorders. Three co-dominant alleles ε2, ε3 and ε4 encode three common major isoforms of apolipoprotein E: from acidic to basic, E2, E3 and E4, respectively. Rare variants have been identified using IEF and sequencing. Three alleles determine six different apolipoprotein Es: three homozygous phenotypes E2/E2, E3/E3, E4/E4 and three heterozygous phenotypes: E2/E3, E2/E4, E3/E4, all relatively common in the population. These variations result from Arg-Cys interchanges at residues 112 and 158 of apolipoprotein E and are caused by a single nucleotide alteration at two loci in the fourth exon of the apolipoprotein E gene. These allelic variations affect receptor binding and influence total, LDL cholesterol and triglyceride concentrations.

Apolipoprotein E3 is the ancestral protein and the most common of these isoforms. It has a Cys residue at position 112 and an Arg residue at position 158 in the receptor binding region of apolipoprotein E. The mutant E2 isoform (112 Cys, 158 Cys) binds with reduced affinity to cellular receptors. It is associated with decreased cholesterol concentrations and increased triglyceride concentrations. E2/E2 phenotypes are detected in more than 90% of patients with type III hyperlipoproteinemia. The E4 isoform (112 Arg, 158 Arg) is associated with increased concentrations of total and LDL cholesterol, and decreased triglyceride concentrations. Thus, because of the high apolipoprotein E polymorphism in the general population and its relation with a potentially atherogenic lipid profile, there is growing interest in developing a method for the rapid analysis of apolipoprotein E polymorphism.

Given the higher risk of cardiovascular disease in patients with non-insulin dependent diabetes mellitus (NIDDM), which is partially related to their lipid disorders, the forms of apolipoprotein E occurring frequently in this population are of particular interest. In previous studies, Snowden et al and Wenham et al...
emphasised discrepancies between the results of genotyping and phenotyping of apolipoprotein E in normal control and diabetic populations (13% to 17%). IEF can lead to the erroneous assignment of an apolipoprotein E phenotype even after pretreatment with neuraminidase. Thus, genotyping by DNA analysis is the preferred method for determining apolipoprotein E status.

In this study we present a sensitive, easy, rapid, non-isotopic procedure for the analysis of the different apolipoprotein E genotypes which can be used in routine practice.

Methods

The study population comprised 104 unrelated French patients with NIDDM (58 men and 46 women) aged between 31 and 79 years with a mean (SD) age of 57 (11) years, all attending the Reims Diabetes Centre. The control group comprised 85 non-diabetic subjects (50 men and 35 women with a mean (SD) age of 55.4 (9.3) years. The study was approved by the local ethics committee. Statistical analysis of genotype distributions was performed by testing the difference in allele frequencies between the NIDDM and control populations. We could not use the χ² test because of the small size of our study population and therefore applied Fisher’s exact test. Significance was set at the 5% level.

DNA was extracted from 189 peripheral blood samples using the InstaGene DNA purification matrix (732-6030; Bio-Rad, Ivry-sur-Seine, France). The InstaGene matrix permits fast and easy preparation of polymerase chain reaction (PCR) amplifiable DNA by eliminating the labour intensive phenol/chloroform extraction steps. A simple cell lysis step achieved by boiling in the presence of matrix is sufficient. This is possible because the matrix efficiently absorbs cell lysis products that interfere with the PCR amplification process. Moreover, the InstaGene bottle is cheap (£40 for 100 extractions from 6 µl whole blood).

Extraction takes one hour.

Briefly, 3 to 6 µl whole blood were mixed with 1 ml water, incubated for 20 minutes at room temperature and centrifuged at 10,000 rpm for three minutes. The InstaGene matrix (200 µl) was added to the pellet and incubated for 20 minutes at 56°C. The sample was then vortexed, incubated for eight minutes at 100°C, and spun for two minutes; 20 µl of the resulting supernatant fluid were used per 50 µl PCR reaction. The remainder was stored at −20°C.

Apolipoprotein E PCR products 244 base-pairs long, containing amino acid positions 112 to 158, were generated. Non-labelled primers F4 (5′-ACAGAATTCGCCGCCGCGTCGGTA C A C C-3′) and F6 (5′-TAAAGCTTGGCACGGCGTGTC ACCAAGGA-3′), proposed by Emi et al., in standard conditions in a final volume of 30 µl were used to amplify the DNA. The PCR was performed on approximately 0.2 µg genomic DNA from peripheral blood samples.

The PCR mixture contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin (w/v), 200 µM each of the four deoxyribonucleotides, and 10 pM each of the non-labelled primers. The DNA samples were first denatured at 95°C for six minutes and then 1 unit Taq DNA polymerase (BioTaq, Bioprobe Systems, Montravel-sous-Bois, France) was added. Then these samples were denatured again at 95°C for one minute, annealed with PCR primers at 60°C for one minute, extended at 70°C for two minutes in a thermocycler (Trioblock, Eurogentec, Seraing, Belgium) for 30 cycles, with a final extension of seven minutes.

PCR products were digested with 5 units of HhaI (Eurogentec) for three hours or overnight at 37°C. Electrophoresis was carried out using PhastSystem PAGE, on preformed polyacrylamide gels (33 × 50 × 0.45 mm). These gels have a 13 mm stacking zone, at a concentration of 7.5% polyacrylamide and cross-linking of 3%, and a 32 mm homogenous zone of 20% with 2% cross-linking (Pharmacia 17-0624-01). Moreover, we chose a discontinuous buffer system (Native at the anode and SDS buffer at the cathode) rather than a standard continuous buffer (Native buffer at both the anode and cathode). Like the buffer system in the gels, we used 0.112 M acetate (leading ion) (Pharmacia 17-0517-01) and 0.112 M Tris (pH 6.4) (anode) as the Native buffer and SDS-PAGE buffer (0.20 M tricine (Pharmacia 17-0516-01), 0.20 M Tris and 0.55% SDS, pH 7.5) (cathode). The discontinuous buffer system gave the same results as the continuous buffer system, but was faster. Three microlitres of restriction enzyme digestion products were mixed with 0.05% Bromophenol Blue and 0.05% Xylene Cyanol. This mixture was loaded on to the 20% gel at the cathode.

Environmental conditions (table 1), with a constant gel temperature during electrophoresis (30 minutes), are critical and were optimal at 15°C for a total of 98 volts/hours (AVh) (maximum total of 100 AVh). PCR products were detected using the silver staining method, with a slightly modified Development Technique (file no. 210 in the PhastSystem) (table 2).

Results

The F4/F6 primers gave amplified DNA fragments 244 base pairs long with six possible HhaI restriction sites and two polymorphic sites (fig 1). Figures 2A and 2B illustrate the char-

Table 1 Electrophoresis protocol

<table>
<thead>
<tr>
<th>Step</th>
<th>Pre-run</th>
<th>Step 2 Sample application</th>
<th>Step 3 Run</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>250V 10 mA 3 W 1 Vh</td>
<td>250V 10 mA 3 W 1 Vh</td>
<td>250V 10 mA 3 W 98 Vh</td>
</tr>
</tbody>
</table>

Table 2 Silver staining protocol

<table>
<thead>
<tr>
<th>Step</th>
<th>Time (minutes)</th>
<th>Temperature (°C)</th>
<th>Staining reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>50</td>
<td>Trichloroacetic acid 20% Glutaraldehyde 5%</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>50</td>
<td>H₂O</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>50</td>
<td>H₂O</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>50</td>
<td>H₂O</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>40</td>
<td>AgNO₃ (0.5%)</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>30</td>
<td>H₂O</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>30</td>
<td>H₂O</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>30</td>
<td>Developer</td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>30</td>
<td>Developer</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>30</td>
<td>Reductor</td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>30</td>
<td>Glycerol (20%)</td>
</tr>
</tbody>
</table>
Determination of apolipoprotein E genotypes using SDS-PAGE

![Diagram](image)

**Figure 1** Partial restriction map of the polymorphic region of the apolipoprotein E (apo E) gene and locations of constant HhaI cleavage sites (small arrows) and polymorphic sites (large arrows). The distances between polymorphic HhaI site are in base pairs.

**Table 3** Genotype counts and frequencies in French patients with NIDDM

<table>
<thead>
<tr>
<th>Types</th>
<th>e2/e2</th>
<th>e2/e3</th>
<th>e2/e4</th>
<th>e3/e3</th>
<th>e3/e4</th>
<th>e4/e4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIDDM</td>
<td>1</td>
<td>13</td>
<td>0</td>
<td>69</td>
<td>19</td>
<td>2</td>
<td>104</td>
</tr>
<tr>
<td>NIDDM (%)</td>
<td>1</td>
<td>12-5</td>
<td>0</td>
<td>66-3</td>
<td>18-2</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>Controls (%)</td>
<td>1-2</td>
<td>11-8</td>
<td>1-2</td>
<td>67-0</td>
<td>17-6</td>
<td>1-2</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 2 A: Diagrammatic representation of the major banding patterns of six apolipoprotein E genotypes obtained on 20% polyacrylamide Phast-Gel; B: electrophoretic pattern of the free NIDDM apolipoprotein genotypes. A, e2/e2; B, e2/e3; C, e3/e3; D, e4/e4; E, e3/e3; F, pBR 322 ladder digested by Hae III.

Table 4 Allele frequencies in the two French groups

<table>
<thead>
<tr>
<th>Alleles</th>
<th>e2</th>
<th>e3</th>
<th>e4</th>
</tr>
</thead>
<tbody>
<tr>
<td>French patients with NIDDM (n=208)</td>
<td>7-2</td>
<td>81-7</td>
<td>11-1</td>
</tr>
<tr>
<td>French control population (n=170)</td>
<td>7-6</td>
<td>81-8</td>
<td>10-6</td>
</tr>
</tbody>
</table>

As expected, the e3/e3 genotype was the most common and >81% of our samples had at least one e3 allele. The next most common allele was e4. Heterozygotes for e2/e4 were not detected in our series. The allele frequencies in patients with NIDDM were similar to those in the control subjects.

**Discussion**

Apolipoprotein E single gene locus polymorphisms appear to have an important effect on lipid profile. Data on the status of apolipoprotein E in several populations are estimated differently. Most authors have analysed the frequencies of apolipoprotein E phenotypes in patients with NIDDM.14-15 Snowden et al11 and Shriver et al12 compared the genotypes of patients with NIDDM with the phenotypes of normal control subjects. These various studies did not reveal any differences in the isoform frequencies in patients with NIDDM compared with a non-diabetic population, with the exception of the study by Snowden et al.11 However, when the genotypes of patients with NIDDM were compared with the phenotypes of the control population, the apolipoprotein E status was similar in the two populations.11 13

Nevertheless, the discrepancies observed between genotypes and phenotypes in normal or diabetic subjects11 12 may lead to the erroneous estimation of the apolipoprotein E status. Moreover, phenotypic analysis may lack discriminative power and perhaps reliability.16 IEF cannot distinguish rare variants of apolipoprotein E that have the same net charge as the three common isoforms. Electrophoretically silent structural variations may also escape detection.7 Minor glycosylated isoforms complicate the interpretation of phenotypes on IEF gels, especially with the superimposition of glycosylated and non-glycosylated forms.11 12 All of these drawbacks make phenotyping cumbersome, difficult to interpret and time-consuming.

To our knowledge, no work has been carried out on the comparison between genotypes of apolipoprotein E in patients with NIDDM and those of non-diabetic control subjects. In the present study, we chose this homogeneous approach, but did not find any significant difference in the distribution of apolipoprotein E polymorphisms between these two populations.

PCR procedures are largely applied for genotyping. PCR methods amplify the sequences containing e2 and e4 allelic substitution sites at positions 112 and 158.120 The close proximity of the variable DNA sites of the apolipoprotein E gene permits their co-amplification within a single DNA fragment. Some authors have extended their PCR re-

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action by using other primers which cover other domains (receptor binding domain)\(^1\), and others amplify with biotin labelled primers.\(^2\) PCR amplified DNA products can be subjected to dot blot analysis with allele specific oligonucleotide (ASO) probes.\(^1\) Most frequently, separate hybridisations are necessary with each of the four radiolabelled oligonucleotides to distinguish 112 Cys, 112 Arg, 158 Cys, and 158 Arg codons. A nested primer approach to PCR is also possible.\(^3\)\(^\text{11}\) ASO probes and dot blots are accurate, but expensive, methods, are time-consuming and involve several steps. They are also particularly sensitive to non-specific binding and background radioactivity, so that a particular sample often requires multiple testing to obtain an unambiguous hybridisation pattern.

Two more rapid analyses of known mutations can be performed by direct electrophoresis of PCR mixtures: the ARMS (amplified refractory mutation system) method, which requires some non-labelled oligonucleotides with mismatches close to the 3' end.\(^3\)\(^\text{12}\) Hybridisation with labelled ASO probes is not necessary and the analysis is carried out by agarose gel electrophoresis. The second technique incorporates restriction isotyping. Accurate genotyping is achieved by examination of the \(H\)hal or the \(C\)fj restriction products generated from PCR amplified DNA using classic PAGE.\(^3\)\(^\text{13}\)\(^\text{14}\)

We improved the SDS-PAGE technique by using the PhastSystem (Pharmacia). A microprocessor automatically regulates all of the parameters during the separation and development runs. This system provides preformed polycrylamide gels with their appropriate buffer strips. Gels are processed with an optimised silver staining method. The method is safe and designed to give reproducible, highly sensitive staining without background. Therefore, each genotype can be directly visualised by silver staining of a unique and reliable combination of restriction fragments for homozygotes and heterozygotes. The silver staining technique is 10 times more sensitive than traditional electrophoresis with ethidium bromide. This system enables detection of quantities as small as 75 pg of DNA. Moreover, even the smallest DNA fragments (16, 18 and 19 base pairs) can be visualised on the preformed gels. This system avoids the use of toxic ethidium bromide and acrylamide powder. Moreover, analysis of the single stranded conformation polymorphisms of the PCR products can be performed using the PhastSystem.\(^3\)\(^\text{15}\)

Miniaturised SDS–PAGE, including separation and silver staining, can be completed within 60 minutes. The time taken from extraction of DNA from 6 μl whole blood to the final result is eight hours. SDS-PAGE incorporating the PhastSystem is more expensive than classic PAGE, but is less time-consuming and therefore is more profitable (table 5).

The method presented in this paper for apolipoprotein E genotyping for routine purposes in many fields of pathology. This technique using the PhastSystem may easily replace apolipoprotein E phenotyping. In the future, sequencing of the apolipoprotein E gene may permit the analysis of new variants with linkage to polymorphic restriction sites. Our methodology could be easily used to detect other nucleotide substitutions.\(^3\)

We thank Dr Morel for the statistical analysis. Dr A Durall is supported by a grant from the Fondation pour la Recherche Médicale.

<table>
<thead>
<tr>
<th>Methodology</th>
<th>PCR and dot blot with radiolabelled ASO</th>
<th>PCR and restriction isotyping. Analysis on classic PAGE</th>
<th>PCR and restriction isotyping. Analysis on the PhastSystem</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Sensitivity</td>
<td>0–1–1 pg Duration from blood sampling to the final result Effective handling Cost of reagents (excluding the PCR set-up) for one assay Toxicity</td>
<td>1–5 ng (ethidium bromide) 48 hours two hours (two gels) Use of radiolabelled probes Use of formamide in the hybridisation mixtures</td>
<td>75 pg (AgNO(_3)) eight hours one hour (two gels) Use of ethidium bromide Handling of acrylamide powder</td>
</tr>
</tbody>
</table>

15. Eto M, Watanabe K, Iwashima Y, Morikawa A, Oshima...
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