Rapid detection of 21-hydroxylase deficiency mutations by allele-specific in vitro amplification and capillary zone electrophoresis

Paola Carrera, Anna Maria Barbieri, Maurizio Ferrari, Pier Giorgio Righetti, Marilena Perego, and Cecilia Gelfi

A quick diagnosis of the classic form of 21-hydroxylase deficiency (simple virilizing and salt wasting) is of great importance, especially for prenatal diagnosis and treatment in pregnancies at risk. A method for simultaneous detection of common point mutations in the P450c21 B gene is here proposed by combining a nested PCR amplification refractory mutation system (ARMS) with capillary zone electrophoresis (CZE) in sieving liquid polymers. In the first PCR, B genes are selectively amplified. In the nested reaction, ARMS-detected wild-type and mutated alleles are separately pooled and resolved by CZE. CZE is performed in coated capillaries in the presence of 30 g/L hydroxyethyl cellulose in the background electrolyte for size separation of the DNA analytes. For high-sensitivity detection the electrophoresis buffer contains the fluorescent dye SYBR Green I. Laser-induced fluorescence detection is obtained by excitation at 488 nm and signal collection at 520 nm. Specificity and reproducibility of the protocols were established by using samples from 75 Italian families with 21-hydroxylase deficiency already genotyped by allele-specific oligonucleotide hybridization or direct sequencing. Whereas dot-blot is time consuming because of the high number of hybridizations with radioactive probes, this present protocol is more rapid, giving sufficient separation on CZE after PCR reactions without preconcentration or desalting of samples.

Steroid 21-hydroxylase deficiency is a recessive inherited disease accounting for ~90% of congenital adrenal hyperplasia (CAH). Lack of 21-hydroxylation results in the accumulation of 17-hydroxyprogesterone, and stimulates excessive androgen production [11]. The severe form of this disease occurs with a frequency of 1:14 000 live births [2]. In this form prenatal virilization in females is observed (simple virilizing, SV) and in 70% of cases it also causes salt wasting (SW). In the classical form, prevention of virilization in females is feasible by prenatal diagnosis and treatment with dexamethasone starting at early stages of pregnancy [3]. The milder nonclassical form (late onset, LO), with postnatal virilization, is more common, with variable frequencies in different ethnic groups (1:27–1:2000). The LO form may also be asymptomatic, called cryptic form [4]. The 21-hydroxylase enzyme is encoded by the P450c21-B gene, located within the HLA complex, on chromosome 6p21.3. The B gene and a 98% homologous pseudogene form a tandem repeat adjacent to C4B and C4A genes, respectively. A certain heterogeneity of mutations in the active gene has been described, such as whole gene deletion, gene conversion [5, 6], and, more frequently, several common point mutations. The majority of small rearrangements, which are also present in the pseudogene, are probably the result of small-scale gene conversions [7]. We recently described the distribution of different classes of mutations in the Italian population [8, 9]. In particular, the distribution of gene rearrangements has been compared with that found in other ethnic groups and the rearrangements have been correlated with clinical phenotypes, including classic, nonclassic, and cryptic forms of the disease.

Molecular diagnosis of the disease can be performed with direct methods for identifying the different classes of

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6 Nonstandard abbreviations: CAH, congenital adrenal hyperplasia; SV, simple virilizing; SW, salt wasting; LO, late onset; ARMS, amplification-refractory mutation system; CZE, capillary zone electrophoresis; ASO, allele-specific oligonucleotide; LIF, laser-induced fluorescence; HEC, hydroxyethyl cellulose; and TBE, Tris–borate–EDTA buffer.
mutations. Conventional Southern transfer hybridization allows detection of gene deletion and large-scale gene conversion, whereas point gene conversions can be scored by a variety of PCR-based methods. Several protocols in which selective amplification of the active gene is performed before screening for different pathological mutations [10–14] have been proposed. The development of rapid methods for direct detection of mutations is of great importance, especially for prenatal diagnosis of 21-hydroxylase deficiency, because prenatal treatment can reduce or avoid virilization in affected female fetuses.

In this work we describe a new protocol for the simultaneous detection of the most common point mutations in the 21-hydroxylase active gene, by combining the PCR-based amplification-refractory mutation system (ARMS) with capillary zone electrophoresis (CZE).

Materials and Methods

A protocol was established by using samples from 75 Italian families with 21-hydroxylase deficiency already genotyped for gross rearrangements by restriction mapping analysis and for point mutations by PCR and either hybridization with allele-specific oligonucleotides (ASOs) or direct sequencing [8, 9].

Table 1. Primers used for PCR and nested ARMS. a

<table>
<thead>
<tr>
<th>Product (bp)</th>
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<tr>
<td>1590</td>
</tr>
<tr>
<td>1683</td>
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</tbody>
</table>

SELECTIVE AMPLIFICATION OF P450C21B GENES

PCR was carried out on genomic DNAs isolated from peripheral blood lymphocytes by standard procedures. To minimize allele drop-out, B genes were selectively amplified in two overlapping fragments by using the forward B-specific primer 606 with the reverse primer 2197 for the first fragment and the forward primer –280 with the reverse B-specific primer 1394R for the second one (Table 1). Overlapping fragments were designed to share a quite large region (606–1394) containing the mutations in intron 2 and in exon 4. The PCR reaction was performed in the presence of 20 pmol of each oligonucleotide, either 250 ng of genomic DNA or 5 ng of constructs obtained by cloning into TA vectors (Invitrogen) patients’ amplified B gene sequences, and 2.5 U of Taq polymerase in a final volume of 50 µL containing 10 mmol/L Tris-HCl pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, and 0.2 mmol/L each deoxynucleotide.

A first round of amplification was carried out for 30 cycles (denaturation, 30 s at 94 °C; annealing, 30 s at 60 °C; extension, 2 min at 72 °C). The PCR reaction was ended by incubating for 5 min at 72 °C. A Gene Amp PCR system 9600 thermal cycler was used (Perkin-Elmer). The amplified products were analyzed by 1% agarose gel electrophoresis.

Table 1. Primers used for PCR and nested ARMS. a

<table>
<thead>
<tr>
<th>Primers for first-round PCR</th>
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<tbody>
<tr>
<td>606: aggtcaggccctagtctctcaa</td>
</tr>
<tr>
<td>2197R: ctcgggctttcactcatc</td>
</tr>
<tr>
<td>–280: cctgcacaggtggacacc</td>
</tr>
<tr>
<td>1394R: gctgcacaggtggacacc</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Primers for second-round PCR ARMS</th>
</tr>
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<tbody>
<tr>
<td>655C-R: cttagacaccctgtctcaggagga</td>
</tr>
<tr>
<td>441: taatgccccaggcttgtaaatctca</td>
</tr>
<tr>
<td>655AR: cttagacaccctgtctcaggagga</td>
</tr>
<tr>
<td>655GR: cttagacaccctgtctcaggagga</td>
</tr>
<tr>
<td>433: gtcctcgttggccacaggtggacacc</td>
</tr>
<tr>
<td>5w1685: aacactccggctctctgcaactac</td>
</tr>
<tr>
<td>3del679: tccaagactccggctctctgca</td>
</tr>
<tr>
<td>979R: agagactttctcttctcaggagaggg</td>
</tr>
<tr>
<td>999T: ggaatcttctctctctctctctctcag</td>
</tr>
<tr>
<td>999A: ggaatcttctctctctctctctctcag</td>
</tr>
<tr>
<td>1141R: taggctggctaattctctctcaggagaggg</td>
</tr>
<tr>
<td>1683G-R: gtcctcgcagctgtgacac</td>
</tr>
<tr>
<td>1557: tccacccctctgaggagac</td>
</tr>
<tr>
<td>1683T: gtcctgcagctgtgacac</td>
</tr>
<tr>
<td>1560: cgtctctctctcaggagagaggg</td>
</tr>
<tr>
<td>1993C: cgatctgccccctcggctctcaggagaga</td>
</tr>
<tr>
<td>1993T: cgatctgccccctcggctctcaggagaga</td>
</tr>
<tr>
<td>2144R: tgccttggctgagctgtgacagaggacaa</td>
</tr>
</tbody>
</table>

a The allele-specific primers are named with the nucleotide position and with the base present in the corresponding allele. For the mutation in intron 2 and the missense at codon 281, allele-specific primers were designed on the reverse strand and each was used with a different forward primer. R suffix denotes the reverse primers.

b B-gene specificities are bold-faced.

c Mismatched positions are bold-faced.
d The region of the 8-bp deletion is underlined. Mutated allele.

e Temperature for annealing/extension.
phoresis, and specificity of the system was confirmed in patients with P450c21B homozygous deletion.

**ARMS SYSTEM**

In the second round of PCR, 0.5 μL of the B gene-amplified fragments, diluted 1:20, were amplified with primers specific for each allele. Amplification reactions were identical to that described above, except for use of 1 U of Taq polymerase. Amplifications were performed by using a two-step protocol with 30 cycles of denaturation at 94 °C for 30 s, followed by annealing/extension for 1 min at temperatures indicated in Table 1 plus a 1-s extension/cycle. The final annealing/extension step was 5 min followed by 15 min at 37 °C to facilitate reannealing of complementary chains. In Table 1 the primer sequences, the mutations detected, the annealing temperatures, and the length of PCR products are listed. As positive and negative controls in each set of reactions, DNA samples from subjects previously genotyped as homozygous normal or homozygous mutant or heterozygous were included.

Amplified products were analyzed in 2% agarose gels and by CZE. Before CZE, PCR products with primers specific for the normal and mutated sequences were pooled into the wild-type and mutant set, respectively (see Table 1).

**CAPILLARY ELECTROPHORESIS**

CZE analyses were performed with a Beckman P/ACE System 5000 equipped with a laser-induced fluorescence (LIF) detector providing 488 nm excitation with a 520 nm bandpass filter. We used a 27 cm (20 cm to the detector window) × 100 (i.d.) capillary coated by a slight modification of Hjerten’s protocol [15] with a novel monomer (N-acryloyl amino propanol) that combines high hydrolytic stability with high hydrophilicity [15–17]. The capillary was filled with a sonicated solution of 30 g/L hydroxyethyl cellulose (HEC; 27 000 M_r) from Polysciences dissolved in 89 mmol/L Tris–borate buffer with 1 mmol/L EDTA (TBE), pH 8.3, and a 1:30 000-diluted solution of SYBR Green I (FMC Bio Products) as fluorescent dye. After 3 min of purging, the samples were injected either by pressure or electrokinetically, depending on whether they were previously desalted by a Centricon 30 (Amicon) or not. After each run the capillary was washed for 5 min in water and then in TBE buffer. The separation was performed at 100 V/cm at 25 °C external temperature.

**Results**

The ARMS is a rapid and reliable method for detecting any mutation involving single base changes or small deletions. Initially, 20-mer sequence-specific primers were designed for all mutations. Only the ARMS for the V281L substitution gave specific results by amplifying target alleles. To increase the specificity of the other mutants, we designed longer primers (30-mer), and a mismatched residue at the penultimate position of primers was introduced. Mismatches were selected as previously described [18]. The calculated melting temperature (T_m) of the oligonucleotides in the nested ARMS was ~90–95 °C, allowing us to use a two-temperature amplification profile. With those primers we observed a good reproducibility by testing this system in CAH patients and the previously genotyped parents. In all the samples analyzed by this protocol, we have excellent agreement with the results obtained by ASO or sequencing. In Fig. 1 are shown all the possible peaks that we can obtain in the wild-type and mutant sets, after resolution in a 27-cm coated capillary in the presence of 30 g/L HEC as sieving liquid polymer in TBE buffer. We can see that also the polymorphism A/C in the same 655 position of intron 2 mutation is well resolved. Easy identification of the two systems of fragments can be achieved with acceptable and reproducible separations (though not to baseline) of similar-size fragments (the couples 172/180 bp and 243/252 bp).

We next report a series of family profilings, showing how CZE can identify carrier parents and affected children in families with steroid 21-hydroxylase deficiency. In Figs. 2 through 5 the results of CZE of nested ARMS with the wild-type-specific and with the mutant-specific prim-
ers are shown in parallel for each subject in these families. In Figs. 2 and 3, two families with the classic form of the disease in the offspring and the cryptic form in the parents are analyzed. In Figs. 4 and 5, two additional families with the SW form of the disease are shown. In each family study, the differential analysis of the spectrum of bands produced by each member of the family when amplifying the wild-type and mutant regions allows a precise diagnosis.

Discussion
We had previously reported the use of CZE in sieving liquid polymers for the analysis of 21-hydroxylase deficiency [19]. In that paper detection of a single mutation,
namely, an 8-bp deletion in exon 3 of the B gene, was reported. The two products (normal, 135-bp, and disease-linked, 127-bp, fragments) were easily separated in 6% T liquid linear polyacrylamide by exploiting the natural UV absorbance of DNA fragments at 254 nm.

In the present report we developed a protocol that presents the advantage of rapidly and specifically detecting five common mutations in steroid 21-hydroxylase deficiency by using a nested ARMS coupled with detection of amplified products by CZE. In comparison with dot-blot technique, which is time consuming because of the high number of hybridizations with specific oligonucleotides, this protocol is more rapid, nonradioactive, and does not require ethidium bromide staining.
The major problem of this method is to differentiate between failed PCR and homozygosity. In our protocol, we included as external controls DNA samples from subjects who had been previously genotyped, either homozygous or heterozygous. The control PCR reactions were set up in parallel with the DNA samples to be tested and amplifications were run simultaneously. In addition, by amplifying DNA samples in 11 different reactions we had indirect evidence about the quality of templates. Finally, a very important point is family genotyping, which is advisable to confirm transmission of mutations. Moreover, direct genotyping is crucial in families in which a cryptic parent is present; in this case, indirect methods do not allow identification of cryptogenic alleles. Therefore, the development of a rapid method for direct detection of mutations in the P450c21-B gene is an important goal, especially for the prenatal diagnosis of the disease when considering the possibility of performing, in pregnancies at risk, prenatal treatment.

The introduction in CZE of fluorescent labels (the SYBR Green I, recently reported in CZE by Skeidsvoll and Ueland [20]), with detection at 520 nm, allows an extraordinary increment of sensitivity (by at least $10^3$ to $10^5$) in sample analysis. Additionally, concentration and desalting steps are not required when using LIF detection [21–23]. As the sieving liquid polymer we used a commercially available HEC, which has low viscosity and can be pumped automatically into the capillary, giving maximum run-to-run reproducibility. Finally, all the electrophoretic runs can be manipulated with computer algorithms, which allows the overlay of several runs for easy comparison among different samples and consequent easy assessment of correct genotypes in families.

In conclusion, CZE in sieving liquid polymers of PCR-amplified DNA fragments is rapidly becoming the technique of choice in genetic analysis, viral and microbial infection detection, molecular biology, and forensic medicine. Several reviews have already appeared, highlighting the technical aspects of this methodology and the achievements in these various fields [24–28].

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References


