mented fecal sample is stored in moist form, rather than as a dry smear on a test card, and sampling is delayed, the positivity score falls rapidly. Apparently, the degradation of heme through the removal of the iron atom \([5, 7]\) is more rapid in moist samples than in the thin, relatively dry smears on the sample cards. Thus, erroneous results (false negatives) will tend to occur when smearing of stools onto the sample collection device is delayed.

This finding is particularly relevant to two areas. First, pathology laboratories that receive stools in an unaltered moist form are likely to miss pathological amounts of blood because of bacterial degradation of the heme. Second, studies in which occult blood testing is performed on moist samples sent to the laboratory, with a resulting delay in smearing of the test card, may not give reliable estimates of the clinical performance of these tests, e.g., in screening for colorectal neoplasia.

We conclude that the test cards for fecal occult blood tests based on the pseudoperoxidase activity of heme should be prepared immediately after defecation. Transport of moist samples to the laboratory should be avoided; that procedure results in reduced sensitivity.

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References


Nonisotopic Detection of Point Mutations in CYP21B Gene in Steroid 21-Hydroxylase Deficiency, Bogotá Esquiera, José M. Varela, Carlos Jarielo, Antonio Oliver, and Ricardo Gracia (Depts. of 1 Biochem. and 2 Pediatr. Endocrinol., Hosp. La Paz, Paseo Castellana 261, 28046 Madrid, Spain; *author for correspondence: fax 34 1 3582733)

Classical steroid 21-hydroxylase (21-OH; EC 1.14.99.10) deficiency is the enzymatic defect underlying 90–95% of congenital adrenal hyperplasias. The affected enzyme, cytochrome P450c21, is encoded by the gene CYP21B, which is found in the human leukocyte antigen (HLA) class III gene region on chromosome 6p21.3, together with a nonfunctional pseudogene [1]. HLA genotyping has been used to detect 21-OH deficiency carriers and perform prenatal diagnosis in families with a previously haploinsufficient affected child, but de novo mutations or intra-HLA recombination has sometimes invalidated the approach [2]. Frequent homozygosity and poor expression of some of these markers in amniocytes have made prenatal diagnosis difficult [3]. Molecular identification of these markers after easy PCR techniques has only been developed for the class II genes. Prenatal diagnosis and carrier detection have also been performed through biochemical evaluation of 17-OH progesterone (17OHP), either in amniotic fluid or after corticotropic (adrenocorticotropic hormone; ACTH) stimulation, respectively. Unfortunately, dexamethasone treatment to the mother, to prevent prenatal virilization, suppresses 17OHP in amniotic fluid [4], and the results for post-ACTH 17OHP carriers overlap those of controls [1, 3, 5].

Direct analysis of the gene is now feasible and offers a more accurate identification of patients and carriers in affected families [6–9]. Moreover, the strong genotype–phenotype relationship found in this disease [7–9] reinforces interest in finding the mutations underlying this deficiency. The severity of the disorder is related to the degree of impairment of the enzymatic activity, which depends, in turn, on the severity of the mutation. In addition, we must consider that patients with mild forms of the deficiency may carry severe mutations [7–9], that will not be detected by methods other than direct gene analysis (see below, Fig. 1A).

Point mutations in the CYP21B gene are the molecular defect in 70% of the alleles for severely affected patients and in almost all of the alleles associated with the late-onset forms of 21-OH deficiency [5–9]. These point mutations are probably products of gene conversion events between the pseudogene and the CYP21B gene, and screening for a limited number of mutations allows the characterization of most of the mutated alleles, including de novo mutations [6]. We have recently reported the distribution and frequency of CYP21B mutations in the 21-OH-deficient Spanish population [9].

Allele-specific oligonucleotide (ASO) hybridization methods, based on isotopic labeling, are used to detect these mutations [2, 4, 7–11]. Highly sensitive isotopic methods have the disadvantage of the short half-life of labeled oligonucleotides and are not feasible in many clinical laboratories, given that special installations are needed. We have designed a protocol for the nonradioactive detection of point mutations in the 21-OH gene. This nonisotopic protocol was applied to 15 families with 21-OH-deficient family members—5 with classical (CL) and 10 with late-onset (LO) forms. We also used a protocol involving radiolabeling to study 38 21-OH-deficient families (25 CL and 13 LO, some reported previously [9]). Five families were studied with both methods. Genomic DNA was isolated from peripheral blood leukocytes. Oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) 391 DNA synthesizer. PCR amplifications were carried out with a Pharmacia-LKB (Uppsala, Sweden) Gene ATAQ thermal controller. The CYP21B gene was amplified in three overlapping fragments: exon 1 to exon 3 (fragment a), exon 3 to the noncoding 3′ termini (b), and exon 2 to exon 6 (c) as described previously [9]. Exons 3 and 6 are the regions selected for specific amplification of the gene [7–11]. The denatured PCR-amplified fragments
were applied to positively charged nylon membranes and hybridized to the ASOs. The ASOs (100 pmol) were 3'end-labeled for 15 min at 37 °C with digoxigenin (DIG)-11-ddUTP and terminal transferase (Boehringer Mannheim, Mannheim, Germany), allowing the incorporation of a single labeled group per molecule.

The ASO protocols performed in this study are based on the temperature dependence shown by the hybrids, which consist of the denatured PCR-amplified fragments and the ASOs. The discrimination between normal and mutated oligonucleotide sequences is based in the presence of mismatched pairs. Oligonucleotides for ASO hybridization were 17 or 18 nucleotides long (see sequences in Table 1) with the mutation located in the middle of the sequence because the instability is greatest when the mismatch is in the middle of the duplex [12]. The stability of these hybrids also depends on ionic strength, which was kept constant at 1 mol/L NaCl throughout the hybridization and washing processes. An empirical formula based on the G, C, A, or T base composition

dissociation temperature, \( T_d = 4 \) (number of G+C) + 2 (number of A+T)
valid for duplexes from 11 to 20 bases in 1 mol/L NaCl, allows calculation of the \( T_d \). A sensitive, specific isotopic protocol was established in which each set of oligonucleotide probe pairs was hybridized, based on length and base composition, at temperatures 4–10 °C below \( T_d \), and washed at \( T_d \) [9]. However, the use of these temperatures in the nonradioactive protocol affected the detection limit of the assay, making it necessary to lower the hybridization and washing temperatures. Considering that every mismatched base in the duplexes reduces \( T_d \) 5–10 °C [12], we performed the stringent wash at the temperatures indicated in Table 1. To improve the specificity, which was lower under the new conditions, we added an excess of unlabeled probe corresponding to the other allele as a competitor to the hybridization mixture. Sensitive, specific results were obtained when using a 16-fold excess (2–3 nmol/L labeled oligonucleotide vs 30–60 nmol/L unlabeled oligonucleotide) in all systems except for the insertion of T at position 306 (which required 80 nmol/L unlabeled oligonucleotide). Each PCR-amplified fragment included several mutations, which we studied after stripping and reprobing the membranes.

DIG-labeled oligonucleotides were detected, after incubation with an alkaline phosphatase-conjugated antibody, by using a chemiluminescent substrate—dissodium 3-(4-methoxyspiro-1,2-dioxetane-3-2'5' chloro)tricyclo [3.3.1.1'2']decane -4-yl) phenyl phosphate (CSPD; Boehringer Mannheim)—essentially as described by the manufacturer with the following modification: Incubating the substrate in the hybridization bottles with a small volume (5 mL) of the diluted substrate (0.05 instead of 0.25 mmol/L CSPD) improved background readings and reproducibility of the method.

Figure 1 shows our results for two heterozygous, mildly 21-OH-deficient patients. The most frequent genotype in LO forms of 21-OH deficiency is homozygosity for the mild Val281Leu mutation [1, 5, 7, 9]; a nonclassic form may also result from heterozygosity of point mutations [7, 9], although few such examples have been documented. Fig. 1A illustrates a mild form of the deficiency carrying a severe mutation. One-third of LO 21-OH-deficient patients in a Spanish sample (3 of 10 in this DIG series and 8 of 20 in the whole sample (this study and the one preceding [9]) were carriers of severe mutations. Fig. 1B shows an interesting finding that resulted from the molecular analysis of this family. The patient's brother was found to be an undiagnosed LO 21-OH-deficient-patient, genotypically identical to the proband, his sister (Val281Leu/Pro453Ser). Later biochemical evaluation confirmed the molecular diagnosis (see Fig. 1 legend). Two additional patients in our whole series were diagnosed after molecular 21-OH gene analysis.

The hybridization signals obtained with the nonisotopic method were easily interpretable and allowed unequivocal identification of mutants, as Fig. 1 illustrates for several point mutations. The investigators were blinded to the clinical histories during the interpretation of results and in those from families studied by both methods, but no false-positive or false-negative results were obtained. All 30 positive PCR products with the mutant ASOs in the DIG protocol had given positive hybridization signals with 32P-labeled oligonucleotide. None of the negative samples gave positive dots. Three repetitions of the nonradioactive protocol for the positive and
Table 1. Optimization of ASO hybridization for nonisotopic detection of point mutations in 21-OH deficiency.

<table>
<thead>
<tr>
<th>Mutation*</th>
<th>Sequence (5'-3')b</th>
<th>T. *c</th>
<th>Fragment*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro 30 (N)</td>
<td>TCC ACC TCC GCC CTC TTG</td>
<td>51</td>
<td>a (ex 1)</td>
</tr>
<tr>
<td>Leu 30 (M)</td>
<td>TCC ACC TCC TGC CTC TTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 A (N)*</td>
<td>AGC CCC CA G CTC CTC CT</td>
<td>51</td>
<td>a, b (in 2)</td>
</tr>
<tr>
<td>2 C (N)*</td>
<td>AGC CCC CA G CTC CTC CT</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>2 G (M)*</td>
<td>AGC CCC CA G CTC CTC CT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile 172 (N)</td>
<td>CAG CAT CAT CT G TTA CC</td>
<td>46</td>
<td>b, c (ex 4)</td>
</tr>
<tr>
<td>Asn 172 (M)</td>
<td>CAG CAT CA G TTA CC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 (N)</td>
<td>CAC ATC GT C GAG ATG CAG</td>
<td>45</td>
<td>c (ex 6)</td>
</tr>
<tr>
<td>6 (M)</td>
<td>CAC AAC GAC GAG AAG CAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val 281 (N)</td>
<td>AAG GCC AGC TGC ACA TG</td>
<td>49</td>
<td>c (ex 7)</td>
</tr>
<tr>
<td>Leu 281 (M)</td>
<td>AAG GCC AT C TGC ACA TG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>306 (N)</td>
<td>AGC AAA AAA ACC AGC GCC</td>
<td>49</td>
<td>c (ex 7)</td>
</tr>
<tr>
<td>306 insT (M)</td>
<td>AGC AA A AAC CAC GGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gin 318 (N)</td>
<td>AGC GAC TGC AGG AGG AG</td>
<td>51</td>
<td>c (ex 8)</td>
</tr>
<tr>
<td>Stop 318 (M)</td>
<td>AGC GAC TGT AGG AGG AG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg 356 (N)</td>
<td>TGC GCC GT G GCC CGC TT</td>
<td>57</td>
<td>c (ex 8)</td>
</tr>
<tr>
<td>Trp 356 (M)</td>
<td>TGC GTG GCC CGC TT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro 453 (N)</td>
<td>CCC GGA GGG CAG CAG CTT</td>
<td>59</td>
<td>c (ex 10)</td>
</tr>
<tr>
<td>Ser 453 (M)</td>
<td>CCC GGA GGA CAG CAG CTT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mutations in the coding region are given as amino acid in the normal (N) or mutated (M) sequence and its position in the protein, except for the splicing mutation at position 655 in intron 2, called "2", followed by the base that is changed; the triple codon in exon 6, called "6"; and the frameshift mutation in exon 7 at nucleotide position 306, which is an insertion of a T (insT).

b Sequences (5' to 3') of the oligonucleotide pairs specific for the normal and mutated sequences. The bases that differ are underlined.

c Stringent washing temperature. Prehybridization, hybridization, and the non-stringent washing were performed at 42°C, for 2 h, 16 h, and 90 min, respectively. Two aliquots (15 and 5 ml) of 5 x SSC (20 x SSC: 3 mol/L NaCl, 0.3 mol/L sodium citrate) containing 10 g/L blocking reagent (Boehringer Mannheim), 1 g/L lauryl sarcosine, and 0.2 g/L sodium dodecyl sulfate were used in the pre- and hybridization steps, respectively. Stringent and nonstringent washes were made with 30 ml of 5 x SSC.

d PCR-amplified fragment/s in which the mutation is analyzed. In parentheses is given the number of the exon (ex) or intron (in) in which the mutation is located.

Position 655 in intron 2 (see Fig. 1) has three alleles, two found in the normal population (A or C), and another (G) that is the splicing mutation in the mutated alleles. See text for identification of a, b, and c.

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negative controls of each mutation (different PCR amplification, membrane, solutions, days, technicians) gave identical results.

Besides point mutations, the PCR-ASO protocol also allowed the characterization of some large conversions and deletions. Those conversion and deletions with one of the CYP21B-specific regions in their sequence are PCR-amplified and react positively to several mutated oligonucleotides [6-9].

The nonisotopic protocol has been as successful as the isotopic one for detection of point mutations, and the concordance of results has been total. Although slightly longer than the radioactive protocol, because additional incubations are required to develop the signal, the DIG protocol with the chemiluminescent substrate is safe, rapid, and sensitive and can be performed in all laboratories without special installations. Use of the chemiluminescent substrate permits reprobing of the membranes, which is important when several mutations or polymorphisms must be tested on the same amplified DNA fragment. In addition, the DIG 3'end-labeling has been performed on unmodified, previously synthesized oligonucleotides. Stock oligonucleotides synthesized for other purposes are suitable, and, more importantly, the labeled oligonucleotide is stable for months and ready for use when required.

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Quantification of prostate-specific antigen (PSA) is widely used for early detection and monitoring of prostate cancer [1], even though numerous recent publications have unambiguously