Rapid Second-Tier Molecular Genetic Analysis for Congenital Adrenal Hyperplasia Attributable to Steroid 21-Hydroxylase Deficiency

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Background: Neonatal screening for steroid 21-hydroxylase (CYP21) deficiency is performed to identify congenital adrenal hyperplasia (CAH). The immunologic assay for 17α-hydroxyprogesterone (17-OHP) has a high rate of false positives. We assessed the potential for increasing the specificity for CAH by use of a second step involving analysis of the CYP21 gene.

Methods: Between January 1999 and December 2003, a total of 810,000 newborns were screened. Of these, 7920 had to be retested because their 17-OHP values were above the cutoff of the assay. Sixty-one had positive 17-OHP values in their recall samples and were diagnosed as having CAH. We used a rapid assay for common mutations of the CYP21 gene to analyze these 61 samples. In a prospective study, 198 consecutive samples that had increased 17-OHP and 100 samples that had normal 17-OHP concentrations were genotyped.

Results: Fifty-nine of 61 cases diagnosed as having CAH were confirmed genetically as CYP21 deficiencies. One patient had a 3β-hydroxysteroid dehydrogenase deficiency, and one patient carried no CYP21 mutations. The 198 increased 17-OHP results were designated as false positives after immunologic testing of recall samples. None of these samples exhibited the genetic pattern consistent with CYP21 deficiency.

Conclusions: If samples with increased 17-OHP values were screened genetically, the number of retests would decrease by ~90%, but the overall sensitivity of CAH screening would remain the same. Adding a second-tier genetic step would require a modest increase in costs, but is counterbalanced by fewer recalls, less clinical follow-up, and a reduction in unnecessary worry for families.

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Congenital adrenal hyperplasia (CAH) attributable to a deficiency of steroid-21-hydroxylase (CYP21) is among the most common inborn errors of metabolism. Clinical manifestations of CAH include ambiguous genitalia in girls and precocious pseudopuberty in both sexes resulting from high concentrations of adrenal androgens (simple virilizing form), with additional excessive renal sodium excretion in the salt-wasting form. The worldwide incidence of these two classic forms of CAH is estimated to be ~1 in 16,000 (1). In addition to rare exceptions such as 11β-hydroxylase deficiency, CYP21 deficiency is responsible for almost all CAH cases detected in screening of dried blood spots by 17α-hydroxyprogesterone (17-OHP) immunoassays (2). The primary goal of CAH screening, which is now included in many newborn-screening programs worldwide, is the detection of the severe form of CYP21 deficiency to prevent life-threatening salt-wasting crises in the first weeks of life. The immunologic method, however, has a high number of false-positive results attributable to cross-reactivity with other steroids, and early sampling and neonatal stress induce high concentrations of 17-OHP in premature babies (3–6). The false-positive rate has been reduced by adjustment of 17-OHP cutoff values for birth weight, gestational age, and recently, sampling age (5–7); however, ~1% of newborns still must be retested and in most

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4 Nonstandard abbreviations: CAH, congenital adrenal hyperplasia; CYP21, steroid 21-hydroxylase; and 17-OHP, 17α-hydroxyprogesterone.
cases turn out negative. Those false positives require clinical follow-up and, most importantly, cause unnecessary worry for many families. In some regions, these drawbacks are considered to outweigh the benefits of CAH screening.

To improve CAH neonatal screening, we applied a new strategy in which the conventional immunologic method is followed by rapid CYP21 genetic testing of 17-OHP-positive samples.

**Materials and Methods**

**PARTICIPANTS AND GENETIC ANALYSIS**

As part of a comprehensive population screening program in Bavaria and North-Rhine Westfalia, Germany, 810,000 babies were screened for CAH between January 1999 and December 2003 by use of the AutoDelfia Neonatal 17-OHP test and the Wallac model 1235 automatic immunoassay system. The recommended age for sampling was set at the third day of life. Of those tested, 7,920 babies had to be retested because of a positive 17-OHP screening value. Sixty-one showed clinical and biochemical signs of CAH. DNA for these 61 babies was extracted from Guthrie card punches and retrospectively screened as described previously (8). In addition, 100 newborns with normal 17-OHP were analyzed with Guthrie card punches and retrospectively screened for 11 point mutations and an 8-bp deletion of the CYP21 gene. Written consent for genetic testing was obtained from the parents or guardians before the analysis. The screening method involved PCR amplification and minisequencing via a protocol similar to that published by Krone et al. (8). If PCR failed, the DNA was analyzed with a novel LightCycler real-time PCR assay to exclude large deletions of the CYP21 gene. Homozygosity or compound heterozygosity for the mutations detected in the assay would account for ~95% of symptomatic CYP21 deficiency in our area (9). According to this study, the sensitivity of the assay, i.e., its ability to detect at least one mutated allele in confirmed CAH patients, is >99% (9).

In a 5-week routine screening period, the same genetic testing method was anonymously used on 198 consecutive samples (of 16,748) that were positive for 17-OHP in the primary screening. Of these, 195 had normal 17-OHP values in the recall sample. 17-OHP cutoff values were adjusted for birth weight and sampling age as described previously (7). In addition, 100 newborns with normal 17-OHP values were analyzed by this method.

**PRIMERS AND PROBES**

Primers for minisequencing were as described previously (8). An additional primer (5'-TCG GTG GGA GGG TAC CTG AA-3') was used for seminested PCR. Primers and probes of the LightCycler real-time PCR assay to detect large deletions of the CYP21 gene were designed with LightCycler Probe Design Software, Ver. 1.0 (Roche Molecular Biochemicals). The sequences of these PCR primers were 5'-AAG AAG GTC AGG CCC TC-3' and 5'-AGA ACT CCT GGG TCA GC-3'. The primers recognize 215- and 207-bp regions of the functional CYP21 gene and the CYP21 pseudogene, respectively. The anchor probe (5'-ACA AGC TGG TGT CTA RGA ACT ACC CGG A-3') was labeled with fluorescein at the 3' end. The detection probe for the functional CYP21 gene was 3'-phosphorylated and labeled at the 5' end with LightCycler Red 640 and had the sequence 5'-TGT CCT TGG GAG ACT ACT CCC-3'. The sequence of the detection probe specific for the pseudogene and an 8-bp deletion was 5'-TGT CTT TGG CTC TCT TG-3'; the probe was 3'-phosphorylated and labeled at the 5' end with LightCycler Red 705. All oligonucleotides were synthesized by Metabion.

**DNA EXTRACTION**

DNA was extracted from three Guthrie card punches (diameter of 3.2 mm each) with the QIAamp DNA Blood Mini Kit (Qiagen). We added 180 μL of buffer ATL (Qiagen) to the punches and incubated them at 85 °C for 10 min. After addition of 20 μL of proteinase K (Qiagen), incubation was continued for 30 min at 56 °C. All further steps of DNA extraction were performed according to the manufacturer's instructions. DNA was eluted in a volume of 50 μL.

**AMPLIFICATION OF THE CYP21 GENE AND MINISEQUENCING**

The functional CYP21 gene was amplified by seminested PCR. PCR and minisequencing were performed as described previously (8).

**LIGHTCYCLER REAL-TIME PCR ASSAY TO DETECT LARGE DELETIONS OF THE CYP21 GENE**

Rapid-cycling PCR was performed in a LightCycler (Roche Molecular Biochemicals). PCR was done in glass capillaries (Roche Molecular Biochemicals) that contained 10 μL of reaction mixture. Each 10 μL of reaction mixture contained 6 pmol of the forward primer, 10 pmol of the reverse primer, 2 pmol each of anchor and the two detection probes, 2 μL of LightCycler-FastStart DNA Master Hybridization ProbesPLUS (which includes reaction buffer, nucleotides, and Taq polymerase; Roche Molecular Biochemicals), and 2.5 μL of DNA. The thermocycling conditions for amplification were as follows: 95 °C for 10 min for initial denaturation and activation of Taq polymerase, followed by 35 thermal cycles of 95 °C for 0 s, 60 °C for 10 s, and 72 °C for 20 s, with a ramping rate of 20 °C/s. Fluorescence was measured during each 60 °C stage.

If a functional CYP21 gene was present, a fluorescent signal was produced in the channel for detection of the LightCycler Red 640-labeled probe (functional CYP21 gene) and in the channel for detection of a LightCycler Red 705-labeled probe (CYP21 pseudogene). In the case of a homozygous large deletion of the CYP21 gene or a homozygous 8-bp deletion, only a signal from the pseudogene-specific probe was detected. If there was no DNA amplification in the PCR for minisequencing and no LightCycler Red 640 signal, this was highly indicative of a homozygous large deletion within the CYP21 gene and...
not of PCR failure, e.g., attributable to inhibition or low DNA extraction efficiency (Fig. 1).

**Results**

**GENETIC ANALYSIS OF CAH PATIENTS DETECTED IN NEONATAL SCREENING**

Of 7920 newborns positive in 17-OHP screening, 61 showed clinical and biochemical signs of CAH. CYP21 deficiency was confirmed genetically in 59 cases, i.e., a mutation was found on both alleles (homozygosity or compound heterozygosity). A comparison of the observed frequencies of genotypes in this screened group with those in a retrospective study on confirmed CAH patients with CYP21 deficiency from southern Germany is shown in Table 1. Genotypes causing a salt-wasting phenotype were statistically more frequent among CAH patients

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**Fig. 1. LightCycler real-time PCR assay to detect the 8-bp deletion (del) in exon 3 and large deletions in the CYP21 gene.**

(A), schematic diagram of primers and probes; (B), interpretation.
detected in neonatal screening ($P = 0.001$, $\chi^2$ test). In one patient, CAH was caused by 3β-hydroxysteroid dehydrogenase deficiency. One female newborn without CYP21 mutations was reported to have ambiguous genitalia at birth and increased 17-OHP in the corticotropin stimulation test. The 17-OHP values for this patient were 340 nmol/L in the initial screening and 545 nmol/L in the recall sample (reference interval 60 nmol/L).

### GENETIC ANALYSIS OF ROUTINE SAMPLES WITH INCREASED 17-OHP

None of the 198 routine samples that showed increased 17-OHP values in the primary screening had a genetic pattern consistent with CYP21 deficiency. In 85 cases, no mutation could be detected. In 13 samples, a mutation was found on only one allele (Q318X, n = 3; E6 cluster, n = 1; V281L, n = 6; P30L, n = 1; P453S, n = 2). Consecutive retests for 17-OHP were below the threshold value in 195 of these infants and were not indicative for CYP21 deficiency. In two cases, no recall samples were sent. One female newborn who presented signs of virilization at birth was reported to suffer from CAH attributable to 11β-hydroxylase deficiency. The 17-OHP values of this child were 50 nmol/L in the initial screening and 69 nmol/L in the recall sample (reference interval <30 nmol/L).

### GENETIC ANALYSIS OF ROUTINE SAMPLES WITH NORMAL 17-OHP

Ten of 100 samples with normal 17-OHP values carried a mutation on only one allele (Q318X, n = 4; large gene deletion, n = 1; I2 G, n = 1; P30L, n = 1; P453S, n = 3). The frequency of heterozygosity was not significantly different between the group that had increased 17-OHP values in the primary screen but normal values in the recall sample (13 of 195) and the group with normal 17-OHP values in the primary screen (10 of 100; $P = 0.413$, $\chi^2$ test).

### Discussion

Newborn screening can minimize delays in the diagnosis of CAH attributable to CYP21 deficiency. In particular, screening has been shown to reduce morbidity and mortality from adrenal salt-wasting crises (1). Because of this immediate benefit, CAH screening aimed at detecting CYP21 deficiency has been introduced in newborn-screening programs in many countries around the world. Despite several adjustments that increase the specificity of the immunologic 17-OHP assay, a high number of false positives still occurs (5–7). In 5 years of screening by our laboratory, 7920 of 810 000 newborns had increased 17-OHP values in the primary screening. Of these, 7856 were negative in the recall samples.

To reduce the number of recalls, we have designed a

### Table 1. Frequencies of genotypes in newborns with genetically confirmed CYP21 deficiency.

<table>
<thead>
<tr>
<th>Mutation group</th>
<th>Predicted clinical phenotype of CAH</th>
<th>Genotype</th>
<th>No. of patients in our study</th>
<th>No. of patients in a retrospective study (9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null</td>
<td>Salt wasting</td>
<td>Del/Del</td>
<td>9</td>
<td>16 (27%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q318X/(Q318X, Del)</td>
<td>4</td>
<td>32 (21%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R356W/(R356W, Del)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>E6 cluster/(E6 cluster, Del)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R356W/Del 8 bp</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>16</td>
<td>32 (21%)</td>
</tr>
<tr>
<td>A</td>
<td>Salt wasting</td>
<td>I2 G/(I2 G, Del)</td>
<td>26</td>
<td>30 (51%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I2 G/R356W</td>
<td>2</td>
<td>50 (34%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I2 G/E6 cluster</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>I2 G/Del 8 bp</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>30</td>
<td>50 (34%)</td>
</tr>
<tr>
<td>B</td>
<td>Simple virilizing</td>
<td>I172N/I2 G</td>
<td>5</td>
<td>9 (15%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I172N/(I172N, Del)</td>
<td>4</td>
<td>50 (34%)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>9</td>
<td>50 (34%)</td>
</tr>
<tr>
<td>C</td>
<td>Nonclassical</td>
<td>P30L/I2 G</td>
<td>1</td>
<td>4 (7%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P30L/(P30L, Del)</td>
<td>2</td>
<td>17 (11%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P453S/I2 G</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>4</td>
<td>17 (11%)</td>
</tr>
</tbody>
</table>

a The patients are grouped according to the predicted severity of CYP21 mutations (18, 19). Null, no CYP21 activity. Group A, the I2 G mutation has only minimal residual CYP21 activity. This group includes patients homozygous for the I2 G mutation or compound heterozygous for I2 G with null mutations. Group B, the I172N mutation retains ~2% of CYP21 activity compared with healthy controls. This group includes patients homozygous for the I172N mutation or compound heterozygous for I172N with either a null or the I2 G mutation. Group C, partial decrease in CYP21 activity.

b Del, CYP21 gene deletion or large gene conversion; E6 cluster = I235N, V236E, and M238K; I2 G = intron 2 splice site mutation. Genotypes shown in parentheses cannot be differentiated by the technique used (8, 20, 21).

c Values in parentheses are the percentages of the total number of patients in the respective studies.
two-tier strategy (Fig. 2). If the 17-OHP value in the primary screening is higher than the cutoff, DNA is extracted from the blood spot and analyzed for mutations of the CYP21 gene. Cutoff values for “increased” (low likelihood for CAH) and “markedly increased” 17-OHP (high likelihood for CAH) were determined with respect to birth weight and sampling age (7). If a newborn has a markedly increased 17-OHP, a recall sample is immediately requested, regardless of the genetic result, to avoid any delay in diagnosis. Samples with increased 17-OHP but no mutations are considered negative for CAH, whereas newborns with one mutation are very likely carriers of CYP21 deficiency. We propose that only those with one mutation be retested biochemically; this would catch cases of CYP21 deficiency that involve a rare or de novo mutation that this genetic assay cannot detect. Genetic analysis is completed within 16–30 h and therefore causes no relevant delay in the screening process.

The two-tier strategy was prospectively evaluated on 16,748 samples. Of these, 198 had 17-OHP values above the assay cutoff. Thirteen of the 198 carried a mutation on one allele. These, along with 11 patients who had markedly increased 17-OHP values in the primary screen, would have to be retested (Fig. 2). The proposed two-tier strategy therefore would have eliminated the need for retesting in 88% of the cases. On the basis of 7920 recalls in 5 years, this strategy would have made at least 6969 recalls unnecessary.

A higher frequency of CYP21 mutations might be expected among children with increased 17-OHP values. Although based on a small sample size, our data showed no significant difference between the frequency of CYP21 heterozygosity in this group and the frequency in samples with normal 17-OHP. Equally, the relative frequencies of specific mutations associated with nonclassical CAH (V281L, P30L, and P453S) did not differ between these two groups.

In the context of newborn screening, identification of CYP21 heterozygotes represents unwanted surplus information that requires follow-up and/or genetic counseling (depending on the strategy of the program). However, in the proposed two-tier procedure, the detection rate for heterozygotes can be estimated to be only 0.07%, based on a recall rate of 0.7% and a prediction that 10% from the recall group will be heterozygotes (7). We propose that a second sample should be obtained from these patients and tested for 17-OHP to catch any CAH attributable to compound heterozygosity of the CYP21 gene with a rare or de novo mutation.

This two-tier strategy would miss individuals with CYP21 deficiencies who have mildly increased 17-OHP values and are homozygous or compound heterozygous

<table>
<thead>
<tr>
<th>Immunologic assay</th>
<th>Genetic analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-OHP normal&lt;sup&gt;+&lt;/sup&gt;</td>
<td>No evidence for CAH</td>
</tr>
<tr>
<td>17-OHP markedly increased&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Mutations on both alleles</td>
</tr>
<tr>
<td>17-OHP increased&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Mutation on one allele</td>
</tr>
</tbody>
</table>

<sup>+</sup> 17-OHP cutoff values adjusted for age and birth weight (7).

![Fig. 2. Two-tier strategy in newborn screening for CAH attributable to CYP21 deficiency.](image-url)
for rare/de novo mutations or a rare/de novo mutation and a deletion of the CYP21 gene. Both are not detected by minisequencing and the LightCycler assay, and no recall samples would be requested. However, based on the estimated allelic frequencies of those mutations (9) and an incidence of 1 in 16 000 (11), this pattern might occur in 1 of 400 patients, i.e., 1 of only 6 000 000 screened babies. In our test group, a CAH diagnosis based on clinical criteria and a biochemical CYP21 defect was confirmed by genetic testing in all but one case. However, this child was reported to have shown signs of virilization and increased 17-OHP in the corticotropin stimulation test. The 17-OHP values of this patient were markedly increased in the initial screening as well as in the recall sample. The infant was therefore referred to a pediatric endocrinologist. According to our two-tier strategy (Fig. 2), this infant would have been referred even if no genetic defect were detected. In one infant from our prospective study group, CAH was caused by a deficiency of 11β-hydroxylase, and one case of 3β-hydroxysteroid dehydrogenase deficiency was confirmed among the 61 CAH patients we analyzed retrospectively. CAH screening programs cannot be aimed at detecting causes of CAH other than CYP21 deficiency because the sensitivity of the 17-OHP assay for those defects is unclear. They certainly would not be detected by a second-tier genetic assay directed at increasing the specificity of 17-OHP screening. Moreover, 11β-hydroxylase and 3β-hydroxysteroid dehydrogenase deficiencies are extremely rare, but both can cause salt wasting (10–12). Interestingly, in the sample from the infant suffering from 3β-hydroxysteroid dehydrogenase deficiency, we detected 17-OHP values that were also markedly increased (255 and 599 nmol/L; reference interval <40 nmol/L), and the patient was referred to an endocrinologist.

The low specificity of the initial 17-OHP assay is well documented (5–7). Recently, another two-tier strategy for CAH screening was described that combines immunologic determination of 17-OHP with liquid chromatographic–tandem mass spectrometric measurement of a steroid profile as the confirmation step (13). Interestingly, second-tier steroid profiling as well as our genetic strategy will reduce immunologic retesting in ~90% of the cases (14). Steroid profiling could be advantageous for screening laboratories serving populations in which the very small share of carrier detection concomitant to second-tier genetic testing might be considered a potential risk. The liquid chromatography–tandem mass spectrometry method, however, requires greater technical sophistication and expertise, which might not be readily available and applicable in many regional screening programs that use tandem mass spectrometry for screening of metabolic disorders (15, 16). Our method for CYP21 genetic analysis can be done with much less expensive instrumentation that is already present in many laboratories doing molecular biology. The cost for the assay is also reasonable: the €40 (€10 for reagents) per second-tier genetic assay (in <1% of 17-OHP-positive samples) would add only an additional 30 cents/sample to the first-tier immunologic CAH screening costs. An additional advantage of the genetic approach as the second-tier test is that genetic confirmation strongly supports the diagnosis of CAH.

In conclusion, rapid second-tier genetic confirmation or exclusion of CAH attributable to CYP21 deficiency can be highly valuable whenever increased 17-OHP concentrations in recall samples could be the result of prematurity and/or neonatal stress. The overall increase in specificity of the screening process may minimize the risk of stigmatization, unnecessary treatment with its attendant side effects, and the costs for clinical follow-up. In addition, immediate genetic confirmation of steroid CYP21 deficiency allows early treatment, which eliminates the risk of the salt-wasting syndrome. A two-tier strategy combining an immunologic technique with genetic confirmation has already been successfully introduced for neonatal screening for cystic fibrosis (17). In the majority of the screening programs, the nonspecificity of the 17-OHP assay accounts for >80% of all recalls. Because only ~0.7% of all samples have to be analyzed genetically, the two-tier strategy can easily be integrated into the screening procedure. Genetic second-tier testing for CYP21 mutations could dramatically reduce the need for recall samples in CAH screening and consequently in the entire screening program, thus avoiding unnecessary worry for many families.

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