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Reverse-hybridization assay for rapid detection of common *CYP21A2* mutations in dried blood spots from newborns with elevated 17-OH progesterone

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ABSTRACT

Background: Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder most commonly caused by defects in the *CYP21A2* gene. Neonatal CAH-screening based on 17-hydroxyprogesterone (17-OHP) measurements prevents life-threatening salt wasting conditions in newborns, but results in a considerable false-positive rate. Therefore, efficient second tier tests are required.
Methods: We developed a reverse-hybridization test strip-based assay (CAH StripAssay) covering the most prevalent *CYP21A2* point mutations/small insertions/deletions occurring in Middle European populations. Assay specificity was validated using plasmid clones, and wild-type and mutant reference DNAs. Its practicability was evaluated in 271 samples from patients with CAH, suspected CAH, and dried blood spots from screening-positive newborns.
Results: All eleven point mutations and 51% of large deletions/conversions could be unambiguously identified when compared to reference methods (DNA sequencing, MLPA). After exclusion of rare mutations (6.4%) not covered by the StripAssay, the overall detection rate was 85%. Undetected heterozygous deletions/conversions caused a lack of information, but did not result in an incorrect prediction of phenotypes.
Conclusions: Our novel CAH StripAssay proved to be a fast (7 h) and reliable method for detection of common *CYP21A2* mutations. Implemented as a second-tier test in CAH newborn screening, it has the potential to significantly reduce recall rates.

1. Introduction

Congenital adrenal hyperplasia (CAH; OMIM ID: 201910) is an autosomal recessive disorder of the adrenal cortex (incidence 1:10,000–15,000) caused in about 95% of cases by genetic defects in the steroid 21-hydroxylase gene *CYP21A2*. The resulting disorder of adrenal steroid metabolism is characterized by a lack of cortisol and aldosterone biosynthesis. As a consequence, steroid precursors upstream of the enzymatic block are shunted into the androgen pathway, leading to androgen excess starting from early intrauterine life. Depending on severity of the different *CYP21A2* mutations, CAH results in a wide range of clinical manifestations including classic salt-wasting (SW) CAH, classic simple-virilizing (SV) CAH as well as mild non-classic (NC) forms of the disease [1]. Various degrees of genital virilization

Abbreviations: CAH, congenital adrenal hyperplasia; CYP21A2, cytochrome P450, family 21, subfamily A, polypeptide 2; 17-OHP, 17-hydroxyprogesterone; MLPA, multiplex ligation-dependent probe amplification; SW, salt-wasting; SV, simple virilizing; NC, nonclassic; fs, frameshift; CYP21P1, cytochrome P450, family 21, subfamily A, polypeptide 1 pseudogene.

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in females (46,XX) are present at birth, whereas males (46,XY) may appear inconspicuous. Since SW predominates in classic CAH (70%), and clinical signs may be overlooked at birth, particularly in boys, a life-threatening condition due to a salt-wasting crisis during the first weeks of life may occur. For this reason, newborn screening programs based on 17-hydroxyprogesterone (17-OHP) levels have been introduced in various countries. However, neonatal 17-OHP testing has a considerable false-positive rate of around 0.7%, mainly due to low birth weight, stress and assay cross-reactivity. Hence, recall rates are high despite adjustments to confounding variables [2,3] and result in a substantial economical burden and emotional stress in parents. To overcome this shortcoming we developed a test strip-based reverse-hybridization assay (CAH StripAssay) for rapid simultaneous detection of 11 *CYP21A2* mutations most commonly encountered in Middle European populations and evaluated its practicability.

2. Material and methods

2.1. DNA samples and recombinant plasmid clones

Anticoagulated or dried blood samples from patients, who had been previously typed positive for one of the 11 most frequent mutations [p.P30L (c.89C>T), I2 splice (c.290-13A/C>G), p.G110fs (c.329_336del) or "Del 8 bp E3", p.I172N (c.515T>A), [p.I236N (c.707T>A), p.V237E (c.710T>A), p.M239K (c.716T>A)] or "cluster E6", p.V281L (c.841G>T), p.L307fs (c.920_921insT), p.Q318X (c.952C>T), p.R356W (c.1066C>T), p.P453S (c.1357C>T) and p.R483P (c.1448G>C)] [4] were collected and anonymized prior to processing. DNA was extracted using the GEN^{X}TRACT Blood DNA Extraction System (ViennaLab Diagnostics). Subsequently, these genomic DNA samples were used to generate recombinant plasmid clones for each mutant allele (TOPO TA Cloning Kit, Invitrogen). The presence of a mutation was confirmed by dideoxy DNA sequencing. These plasmid clones served as homozygous templates to design and optimize oligonucleotide capture probes.

2.2. Test strip preparation, PCR, and reverse-hybridization

A series of candidate 15–25-mer oligonucleotides, encoding all wild-type and mutant alleles, were defined using the NCBI Reference Sequence databank (NCBI RefSeq ID: NG007941.2). Probes were chemically synthesized and immobilized as an array of parallel lines on a nitrocellulose membrane, which was then sliced into 3 mm test strips [5]. In order to analyze reference plasmid clones, as well as genomic DNA samples, the entire *CYP21A2* gene was amplified in three overlapping fragments using biotinylated PCR primer pairs that do not co-amplify the highly homologous pseudogene *CYP21A1P* [6]. Fragment A enfolds the region from exons 1 to 6, while fragments B and C from exons 3 to 10 and exons 5 to 10, respectively. PCR (25 μl) contained 1–200 ng DNA template and comprised an initial denaturation step (95 °C for 2 min), followed by 40 cycles of 95 °C (30 s)–62 °C (30 s)–72 °C (2 min 30 s) and a final extension step (72 °C for 7 min). Biotinylated amplification products were hybridized to the preliminary probe arrays under controlled stringency (45 ± 0.5 °C), and specifically bound fragments were visualized at room temperature using streptavidin–alkaline phosphatase conjugate and enzymatic color reaction [5]. Our final array design was based on a set of capture probes that discriminated best between wild-type and mutant alleles, and included a 5′-biotinylated control oligonucleotide to verify the functionality of the detection system.

2.3. Assay validation and clinical evaluation

The specificity of the CAH StripAssay was validated by hybridizing test strips against PCR products obtained from mutant plasmid clones, as well as wild-type, heterozygous and homozygous mutant reference DNA samples. In a second step, we evaluated the CAH StripAssay for routine diagnostic purposes on 271 DNA samples, randomly chosen from (a) CAH patients, (b) individuals suspected to carry *CYP21A2* mutations, and (c) newborns with elevated 17-OHP levels, in comparison with DNA sequencing [7,8] and multiplex ligation-dependent probe amplification (MLPA) [9]. The study was approved by the Institutional Ethics Review Boards and informed consent signed by patients or caregivers.

3. Results

3.1. Validation studies

All 11 point mutations covered by the StripAssay could be unambiguously identified. Examples of homozygous (mutant signal present and corresponding wild-type signal absent), heterozygous (wild-type and corresponding mutant signal present) and wild-type (mutant signal absent) DNA samples and plasmid clones are shown in Fig. 1. In addition to the designated point mutations, the CAH StripAssay was capable of indicating a defined fraction of large gene deletions and conversions that produce chimeric alleles with 5′- and 3′-ends corresponding to *CYP21A1P* and *CYP21A2*, respectively [10,11]. If the junction site of the *CYP21A1P*/*CYP21A2* chimera was located in the region upstream of cluster E6, a series of mutant probes ranging from p.P30L to p.I172N stained positive (Fig. 1, strip 23). Homozygous chimeras with junction sites downstream of cluster E6, as well as homozygous deletions of the complete *CYP21A2* gene, resulted in allelic dropout due to missing primer-binding sites, with test strips showing neither wild-type nor mutant signals (data not shown). If the second chromosome was *CYP21A2* wild-type or carried a point mutation, the heterozygous state of such chimeras could not be identified. Essentially, these types of *CYP21A1P*/*CYP21A2* chimeras, as well as complete *CYP21A2* deletions, caused a partial PCR dropout, and only the allele carrying a wild-type sequence or a point mutation was amplified. In the latter case, mutations appeared to be homozygous instead of hemizygous (Fig. 1, strip 22).

3.2. Assay evaluation in a clinical setting

Analysis of 271 samples from patients/newborns with CAH or suspected CAH yielded complete concordance between StripAssay results and reference methods for all covered point mutations, with p.V281L (19.3%), I2 splice (18.8%), p.I172N (9.1%), p.Q318X (5.5%), p.P30L (4.1%) and p.R356W (2.8%) being the most prevalent (Table 1). In addition, 55 (15.2%) out of 107 (29.6%) large deletions and conversions could be identified. The remaining 52 (14.4%) undetected chromosomal rearrangements were either heterozygous *CYP21A1P*/*CYP21A2* chimeras with junction sites downstream of cluster E6, or heterozygous *CYP21A2* deletions. Rare mutations not covered by the StripAssay were present in 23 (6.4%) alleles. In summary, the CAH StripAssay detected 287 (79.3%) out of 362 alleles carrying a *CYP21A2* mutation. After exclusion of rare mutations, the overall detection rate was 85%.

4. Discussion

Several different methods that can identify *CYP21A2* mutations, such as the amplification-created restriction site approach [12], multiplex minisequencing [13], and direct gene sequencing [7,8], have been applied to CAH genotyping. Chromosomal rearrangements have been investigated by Southern blotting [14,15], quantitative real-time PCR [16,17] and multiplex ligation-dependent probe amplification (MLPA) [9]. Direct sequencing in combination with MLPA offers the highest diagnostic information, however, being very time-consuming and costly, does not represent an attractive second-tier test for newborn screening programs at this stage [18]. We have therefore developed a novel reverse-hybridization assay for detection of the 11 most prevalent

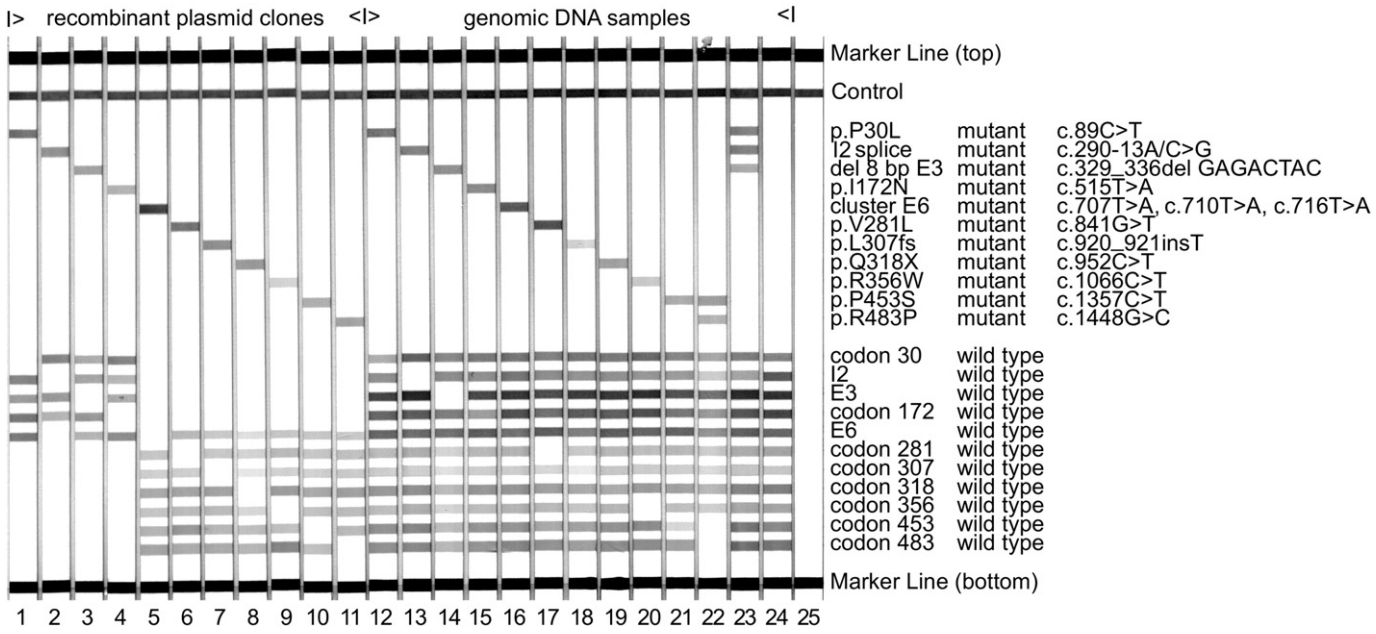

Fig. 1. Reverse-hybridization test strips after hybridization to various PCR products and enzymatic color development. Strips on the left show recombinant plasmid clones encoding mutations p.P30L (1), I2 splice (2), Del 8 bp E3 (3), p.I172N (4), cluster E6 (5), p.V281L (6), p.L307fs (7), p.Q318X (8), p.R356W (9), p.P453S (10) and p.R483P (11). Strips on the right show genomic DNA samples heterozygous for p.P30L (12), p.I172N (15), cluster E6 (16), p.L307fs (18), p.Q318X (19), p.P453S (21), homozygous for I2 splice (13), Del 8 bp E3 (14), p.V281L (17), p.R356W (20), hemizygous for p.P453S and p.R483P (22), heterozygous for a large deletion or conversion (23), a wild-type DNA (24) and a negative PCR control with water in place of DNA (25).

CYP21A2 mutations in Middle European populations. Its reliability could be demonstrated by complete concordance between StripAssay results and reference methods for all covered mutations. Additionally, in our cohort the StripAssay identified 51% of large deletions and conversions, which account for 27–39% of mutant alleles in Middle Europe [4,19]. Overall, 79.3% of mutant *CYP21A2* alleles were detected. Apart from rare mutations (6.4%) not covered by the test design, the remainder comprised undetected deletions/conversions, since the StripAssay approach is limited by its inability to identify chimeric *CYP21A1P*/*CYP21A2* genes with junction sites downstream of cluster E6 as well as complete *CYP21A2* deletions in samples which are heterozygous for these lesions.

Table 1
Number of mutated *CYP21A2* alleles identified in 271 DNA samples using the CAH StripAssay and reference methods.

Mutation	No. of alleles (%)	No. of alleles (%)
	CAH StripAssay	Reference methods[a]
p.P30L	15 (4.1)	15 (4.1)
I2 splice	68 (18.8)	68 (18.8)
Del 8 bp E3	6 (1.7)	6 (1.7)
p.I172N	33 (9.1)	33 (9.1)
Cluster E6	1 (0.3)	1 (0.3)
p.V281L	70 (19.3)	70 (19.3)
p.L307fs	2 (0.6)	2 (0.6)
p.Q318X	20 (5.5)	20 (5.5)
p.R356W	10 (2.8)	10 (2.8)
p.P453S	6 (1.7)	6 (1.7)
p.R483P	1 (0.3)	1 (0.3)
Deletions/conversions[b]	55 (15.2)	107 (29.6)
Rare mutations	0 (0.0)	23 (6.4)
Undetected mutations[c]	75 (20.7)	0 (0.0)
Total	362 (100.0)	362 (100.0)

[a] DNA sequencing and MLPA.
[b] Large deletions or conversions leading to chimeric *CYP21A1P*/*CYP21A2* genes with junction sites upstream of cluster E6; homozygous *CYP21A1P*/*CYP21A2* genes with junction sites downstream of cluster E6; and homozygous *CYP21A2* deletions.
[c] Rare mutations; heterozygous *CYP21A1P*/*CYP21A2* genes with junction sites downstream of cluster E6; and heterozygous *CYP21A2* deletions.

Consequently, hemizygosity for a point mutation cannot be discriminated from homozygosity whenever such a large rearrangement is present on the other chromosome. This deficit, however, is alleviated by the fact that the clinical phenotype of CAH is typically linked to the less severe mutation, and thus such situations will ultimately lead to the same diagnosis. This also holds true for wild-type alleles combined with these chimeras or deletions, leading to a wild-type pattern that conceals the carrier status, but suggests the correct unaffected phenotype. Ultimately, the uncertainty that may arise due to undetected deletions/conversions can be overcome easily by using the reverse-hybridization technique in combination with methods determining gene copy numbers, such as the quantitative real-time PCR approach of Parajes et al. [16]. When adding this method to StripAssay analysis, the detection rate of large deletions and conversions could be increased from 51% to 100% in our cohort (data not shown).

In conclusion, the CAH StripAssay provides a fast (7 h) and reliable protocol to identify common *CYP21A2* mutations. Only small amounts of DNA (≥1 ng) are required, allowing analysis from dried blood spot punches. If implemented as a second-tier test in CAH newborn screening programs, a significant reduction of recall rates should be achievable.

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