Protocol Article

Allele-specific and multiplex PCR based tools for cost-effective and comprehensive genetic testing in Congenital Adrenal Hyperplasia

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A B S T R A C T

Congenital Adrenal Hyperplasia (CAH) is an autosomal recessive disorder due to enzyme defects in adrenal steroidogenesis. Several genes code for these enzymes, out of which mutations in the CYP21A2 gene resulting in 21 hydroxylase deficiency, contribute to the most common form of CAH. However, pseudogene imposed challenges complicate genotyping CYP21A2 gene, and there is also a lack of comprehensive molecular investigations in other genetic forms of CAH in India. Here, we describe a cost-effective, highly specific, and sensitive Allele Specific PCR (ASPCR) assay designed and optimized in-house to screen eight common pathogenic mutations in the CYP21A2 gene. We have also established and utilized a multiplex PCR assay for target enrichment and Next-generation sequencing (NGS) of CYP11B1, CYP17A1, POR, and CYP19A1 genes. Following preliminary amplification of the functional gene CYP21A2, ASPCR based genotyping of eight common mutations - P30L, I2G, 8BPdel, I172N, E6CLUS (I235N, V236E, M238K) V281L, Q318X, and R356W was carried out. These results were further validated using Sanger and Next-generation sequencing. Once optimized to be specific and sensitive, the advantage of ASPCR in CYP21A2 genotyping extends to provide genetic screening for both adult and paediatric subjects and carrier testing at a low cost and less time. Furthermore, multiplex PCR coupled NGS has shown to be cost-effective and robust for parallel multigene sequencing in CAH.

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Specifications table

| Subject Area; | Biochemistry, Genetics and Molecular Biology |
|----------------|-----------------------------------------------|
| More specific subject area; | Genotyping with Allele Specific PCR and target enrichment with multiplex PCR |
| Protocol name; | Allele Specific and Multiplex PCR for genetic testing in CAH |
| Reagents/tools; | Gentra Puregene DNA extraction kit from QIAGEN®  
TaKaRa LA PCR™ Kit (ver.2.1)  
EmeraldAmp® Max PCR master mix  
QIAGEN® Multiplex PCR Kit |
| Experimental design; | 1. Long-range PCR for CYP21A2 gene amplification followed by Allele Specific PCR for screening eight hotspot mutations.  
2. Multiplex PCR for target enrichment of CYP11B1, CYP17A1, CYP19A1 and POR genes |
| Trial registration; | N/A |
| Ethics; | N/A |
| Value of the Protocol; | • ASPCR - highly specific and sensitive to identify eight pseudogene derived mutations in the CYP21A2 gene.  
• Multiplex PCR – cost-effective and robust for target enrichment of CAH related genes  
• Together aids in comprehensive genetic screening for CAH in clinical settings |

Description of the protocol

DNA extraction and long-range PCR

DNA extraction was carried out with 2 ml EDTA whole blood using Gentra Puregene kit from QIAGEN® (Hilden, Germany) and quantified using NanoDrop™ spectrophotometer. Long-range PCRs were utilized for locus-specific amplification of the functional gene CYP21A2 (6.2 kbp) and pseudogene CYP21A1P (6.1 kbp) with TaKaRa LA PCR™ Kit (ver.2.1) using previously published protocols [1]. In addition, the results were validated with TaqI restriction digestion [1]. Based on these results, samples suspected for large 30 kbp deletion and large gene conversion were validated with MLPA and additional long-range PCRs with specific primers for these rearrangements described previously by Greene et al [2]. Figs. 1 and 2 show results of locus-specific amplification and restriction digestion of the above genes and their interpretation in identifying rearrangements.

Allele Specific PCR (ASPCR) for screening eight hotspot mutations in CYP21A2 gene

The long-range PCR product of the CYP21A2 gene was utilized as a template for Allele Specific PCR (ASPCR) to genotype eight common hotspot mutations in the CYP21A2 gene. ASPCR, a modified application of conventional PCR technique, is a strategy to detect point mutations and small deletions by deliberately introducing mismatches in the primers. Primer designing is crucial in ASPCR to generate detectable amplicons from the mutation target while minimizing false priming at the non-target allele. A wild-type (WT) primer complementary to the normal sequence is designed for each target sequence harboring the hotspot mutation. A mutant (MT) primer complementary to the 3’ terminal base of the mutation under study is also designed for the same target. A reverse primer common for both the WT and MT is designed to maintain the same size for both WT and MT products. The wild type primer will provide amplification only with the wild type allele and there is no amplification when the allele is mutated.

Similarly, a mutant primer can amplify only the DNA sequence that carries the mutation. This enables the identification of the hotspot mutation under simple PCR conditions. Mismatches at the penultimate bases are often intentionally added to increase the specificity of the ASPCR [3]. If the terminal destabilization is weak, a strong destabilizing mismatch is added at the penultimate base and vice versa with a strong destabilization at the terminal base. Two WT forward primers were designed for 12G splice site mutation, including two WT alleles A and C. For all the eight hotspot mutations, common internal control primers were designed in such a way, it is amplified with both WT and MT alleles.
Fig. 1. a) 1% Agarose gel image of locus-specific amplification of CYP21A2 and CYP21A1P genes. M-1 Kbp ladder, lane 1: functional gene product CYP21A2 (6.2 kbp) amplified with primers CYP779f/Tena36F and lane 2: pseudogene product CYP21A1P (6.1 kbp) amplified with primers CYP779f/XA-36F adapted from Lee et al. [1]. b) Agarose gel image (1%) - restriction digestion of CYP21A2 and CYP21A1P genes with TaqI. The product sizes of digested products from the functional gene were 3.7 kbp & 2.4 kbp shown in lane 1 and the digested products from the pseudogene were 3.2 kbp & 2.3 kbp shown in lane 2.

Fig. 2. a) Restriction digestion [1] results with TaqI in large gene conversion on 1% agarose gel electrophoresis – Lane 1 and 2 shows normal restriction digested fragments of functional and pseudogene amplified with long range PCR in the negative control. In one subject, there was no amplification with functional gene primers CYP779f/Tena36F2, and so there were no digested products as seen in lane 3. However, there was amplification with the pseudogene primers (CYP779f/XA-36F) with a restriction digestion pattern similar to the functional gene, as shown in lane 4. This suggests a homozygous large gene conversion involving the proximal end of CYP21A2 and the distal end of CYP21A1P genes. b) Restriction digestion results in large 30 kbp deletion on 1% agarose gel electrophoresis: Lane 4 and 5 show normal restriction digested fragments of the functional and pseudogene in the negative control. Lane 1 shows a restriction digestion pattern of a sample with homozygous 30 kbp deletion. Since the deletion involves forming a chimeric (fusion) gene with the proximal end of CYP21A1P and the distal end of CYP21A2 genes, there is no amplification with pseudogene primers (CYP779f/XA-36F). However, the product amplified with functional gene primers (CYP779f/Tena36F2) gives a restriction digestion pattern similar to that of the pseudogene. A heterozygous 30 kbp deletion on one allele results in amplification with both the primer sets, but the product from functional gene primers produces three restriction digestion bands resulting in a combination of functional and pseudogene, as seen in lane 2.
Pre-clean up

The long-range PCR product of the CYP21A2 gene is purified using Agencourt AMPure XP (Beckman Coulter Life Sciences, USA) magnetic beads with the following protocol. This cleaned up product is used as a template for ASPCR.

Mix long-range PCR product of CYP21A2 gene (6.2 kbp) with 1.8x volume of Agencourt AMPure XP magnetic beads

↓

Vortex to mix the contents and incubate for 5 minutes at room temperature

↓

On a magnetic stand, separate the beads with the template from the clear supernatant and wash with 200μl of 70% ethanol [× 2 times]

↓

Spin and remove the excess ethanol

↓

Allow complete evaporation of ethanol

↓

Elute with 20μl of 0.1x TE buffer

Standardization of ASPCR conditions

The ASPCR was in house standardized with Emerald Amp® Max PCR master mix (Takara Bio Inc, Japan) in 15 μl reaction volume. The primer sequences are given below in table 1.

- WF- Wildtype Forward, MF- Mutant Forward, F-Forward, R-Reverse, WR-Wildtype Reverse, MR-Mutant Reverse.
- *The underlined sequences were adapted from Lee et al. [1]

Optimal annealing temperature and template concentration were utilized with appropriate positive and negative controls, and the below conditions were finalized to achieve optimal results. P30L hotspot mutation required primer redesigning to overcome false-positive results. Change in DNA extraction techniques can also affect the specificity of ASPCR and might require further standardization of the template concentration used. Details of the ASPCR reaction mix and program are mentioned in Tables 2a and 2b.

Following this, samples were screened for all the hotspot mutations with mutant primers, and the results were also validated with Sanger and NGS sequencing (Fig. 3).

MLPA and ASPCR in identifying chimeric genes

Large 30 kbp deletion in 21-hydroxylase deficiency results in the formation of chimeric genes involving the proximal end of CYP21A1P and the distal end of CYP21A2 genes. MLPA (Multiplex Ligation-dependent Probe Amplification) is the most common technique employed in molecular analysis of large deletions and duplications in routine clinical practice. In this study, we utilized MLPA to validate large 30 kbp deletion suspected from the results of long-range PCR and restriction digestion using SALSA MLPA CAH Probemix P050 C1 from MRC-Holland [4]. Simultaneously allele-specific PCR was also carried out. Results of some of these samples are discussed below in Fig. 4.

The junction sites to classify classical and attenuated chimeras depend on the series of deleterious pseudogene mutations present in the extent of rearrangement. However, CYP21A2 probes in the
Table 1
Primer sequences for in house designed ASPCR to genotype eight common pseudogene derived mutations in CYP21A2 gene.

| S.NO | PRIMER NAME | 5’ PRIMER SEQUENCE 3’ |
|------|-------------|-----------------------|
| 1    | CAH ARMS INTERNAL CONTROL F | TGTGGCGGTGTAGTTGGTGTGG |
| 2    | CAH ARMS INTERNAL CONTROL R | GGGGACTTGTTCAGGGTGAGGA |
| 3    | CAH P30L WF | CTCCGGAGCTCCACCTCCCT |
| 4    | CAH P30L MF | CTCCGGAGCTCCACCTCCCT |
| 5    | CAH P30L R | TCAGTCCAGAAGAGAGGGCT |
| 6    | CAH I2G WF [C allele] | TCCCCACCTCCAGCCCCCC |
| 7    | CAH I2G WF [A allele] | TCCCCACCTCCAGCCCCCTA |
| 8    | CAH I2G MF [G allele] | TCCCCACCTCCAGCCCCGG |
| 9    | CAH I2G R | TCAGTCCAGAAGAGAGGGCT |
| 10   | CAH 8BPDEL WF | CCGGACCTGTCCTTGGGAGACTAC |
| 11   | CAH 8BPDEL MF | TACCCGGACCTGTCCTTGGTC |
| 12   | CAH 8BPDEL R | *AGCCCCACGCAGACAGTCTCA |
| 13   | CAH I172N WF | TCTCCCTCACCTGCAGCATCAT |
| 14   | CAH I172N MF | TCTCCCTCACCTGCAGCATCAA |
| 15   | CAH I172N R | GAGGGTGTTTGCTGTGGTCTCA |
| 16   | CAH EX 6 CLUS WF | ATCACATCGTGGAGATGCAGCT |
| 17   | CAH EX 6 CLUS MF | GAGGGATCACAACGAGGAGAA |
| 18   | CAH EX 6 CLUS R | *AGCCCCACGCAGACAGTCTCA |
| 19   | CAH V281L WF | GACAGCTCCTGGAAGGGCACG |
| 20   | CAH V281L MF | GACAGCTCCTGGAAGGGCACT |
| 21   | CAH V281L R | TCTCCCTCACCTGCAGCATCA |
| 22   | CAH Q318X WF | CCAGATTCAGCAGCGACTGC |
| 23   | CAH Q318X MF | CCAGATTCAGCAGCGACTGT |
| 24   | CAH Q318X R | CTCGGACCCACAGATGACT |
| 25   | CAH R356W F | *CTGGAGCCACTGTCCTACCA |
| 26   | CAH R356W WR | GCCAAGCCACAACCGGCCC |
| 27   | CAH R356W MR | GCCAAGCCACAACCGGCCC |

Table 2a
ASPCR reaction Mix.

| Contents                                      | Volume |
|-----------------------------------------------|--------|
| EmeraldAmp® Max PCR master mix                | 7.5 μl |
| Forward primer                                | 1 μl   |
| Reverse primer                                | 1 μl   |
| Internal forward primer                       | 1 μl   |
| Internal reverse primer                       | 1 μl   |
| Template*                                     | 1 μl   |
| Sterile water                                 | 2.5 μl |
| Total                                         | 15 μl  |

*Template - cleaned up PCR product of CYP21A2 gene (diluted concentration: 5-8 ng/μl).
Primer concentration used: 10 pmol/μl.

Table 2b
ASPCR - Thermal cycler conditions.

| Stage 1×1                                      | Initial denaturation | 95 °C | 5 minutes |
|------------------------------------------------|----------------------|-------|-----------|
| Denaturation                                   |                      | 98 °C | 10 seconds|
| Stage 2×20                                     | Annealing            | 68 °C | 30 seconds|
|                                                 | 70 °C (for I2G only) |       |           |
| Extension                                      |                      | 72 °C | 1 minute  |
| Stage 3×1                                      | Final extension       | 72 °C | 5 minutes |
P30L – a) Gel image with ASPCR product of 1027 bp; Samples 1, 4, 7, 9, and 10 were positive. NS amplification seen at the top of the gel in the positive samples did not interfere with the interpretation of the results. b) and c) - corresponding NGS and Sanger sequencing images of CYP21A2:c.92C>T

I2G- a) Gel image with ASPCR product of 453 bp; Samples 2, 4, 5, 7, and 8 were positive. NS amplification seen at ~800bp did not interfere with the interpretation of the results. b) and c) - corresponding NGS and Sanger sequencing images of CYP21A2:c.293-13C>G

8BPdel- a) Gel image with ASPCR product of 806 bp; Samples 1, 4, 7, 8, 9, and 10 were positive. NS amplifications seen at ~450 and 600bp did not interfere with the interpretation of the results. b) and c) - corresponding NGS and Sanger sequencing images of CYP21A2:c.332_339delGAGACTAC

I172N- a) Gel image with ASPCR product of 765 bp; Sample 4 was positive. b) and c) - corresponding NGS and Sanger sequencing images of CYP21A2:c.518T>A

Fig. 3. Gel images of ASPCR results, NGS alignments and chromatogram of Sanger sequencing results for the eight CYP21A2 hotspot mutations screened. a) Agarose gel image (2%) showing ASPCR results for P30L, I2G, BBPDEL, I172N, E6 CLUS, V281L, Q318X, and R356W mutation screening of different samples with appropriate Positive Control (PC), Negative Control (NC) and a No Template Control (NTC - To detect reagent contamination) run with mutant primers. IC indicates internal control at 180bp. 1 to n represent samples from different subjects, M indicates 100 bp marker. NS (Non-specific) may indicate non-specific amplification from different combinations of the allele-specific and internal control primers. However, these non-specific products did not interfere with the identification of samples positive and negative for ASPCR. Utilizing these Allele-specific PCR the positive and negative control were compared with the test samples for genotyping. b) and c) NGS results and chromatogram of Sanger validation of the eight hotspot mutations showing the same hotspot mutation corresponding to ASPCR. The chromosome coordinates of the NGS results indicate the alignment of the reads to the CYP21A2 gene and not to the pseudogene CYP21A1P.
E6CLUS - a) Gel image with ASPCR product of 502 bp; Sample 1 was positive. NS amplification seen at ~350 bp in the positive samples did not interfere with the interpretation of the results. b) and c) - corresponding NGS and Sanger sequencing images of CYP21A2:c.710T>A, c.713T>A and c.719T>A

V281L - a) Gel image with ASPCR product of 662 bp; Sample 2 and 4 were positive. NS amplification seen at the top of the gel in the positive samples did not interfere with the interpretation of the results. b) and c) - corresponding NGS and Sanger sequencing images of CYP21A2:c.844G>T

Q318X - a) Gel image with ASPCR product of 931 bp; Sample 5 was positive. b) and c) - corresponding NGS and Sanger sequencing images of CYP21A2:c.955C>T

R356W - a) Gel image with ASPCR product of 955 bp; Sample 7 was positive. NS amplification at ~650 bp and did not interfere with the interpretation of the results. b) and c) - corresponding NGS and Sanger sequencing images of CYP21A2:c.1069C>T

Fig. 3. Continued
Table 3
In house designed primer sequences for amplifying CYP17A1, POR and CYP19A1 genes.

| S.NO | PRIMER NAME | 5’ PRIMER SEQUENCE | 3’ PRIMER SEQUENCE |
|------|-------------|---------------------|--------------------|
| 1    | CYP17A1 EX1 F  | TCCAAGCCTTGACTCCTGAG |               |
| 2    | CYP17A1 EX1 R  | ACAGTGAAGTCCTTGAGAGC |               |
| 3    | CYP17A1 EX2-3 F | AAGGAAGAGTAGGGGAGAGAG |               |
| 4    | CYP17A1 EX2-3 R | AAAAGATGGGATCTGGCCGG |               |
| 5    | CYP17A1 EX4 F  | CTCTCTCTGTTAGAATGTTG |               |
| 6    | CYP17A1 EX4 R  | CGCCCCAGCTCTGTAAGTCA |               |
| 7    | CYP17A1 EX5-6 F | CTGCCCCAGACTTGCTCTACT |                |
| 8    | CYP17A1 EX5-6 R | AGTAGTTGATGGTTGACTGACTT |             |
| 9    | CYP17A1 EX7-8 F | AAACGCACACCCACATACAC |               |
| 10   | CYP17A1 EX7-8 R | GAGCTCGAGTGTCCTGAGAA |               |
| 11   | POR EX1 F     | CATTTCCTGCAGCCCCAG  |               |
| 12   | POR EX1 R     | TTCTCCGAGTGCTCCTGTG  |               |
| 13   | POR EX2 F     | GGATACTTCCCCCTCTTGTG |               |
| 14   | POR EX2 R     | CGGAGAGAAAAATTGGGAGTG |               |
| 15   | POR EX3 F     | GTGACCTTTGCGCTCTTTTG |               |
| 16   | POR EX3 R     | GCAGGGATGGCAATGACC  |               |
| 17   | POR EX4 F     | GGCTTCCCCATCTTGTG    |               |
| 18   | POR EX4 R     | GCCACTGCGACGCTCTAA  |               |
| 19   | POR EX5-6 F   | GTCAACCAGATGAAGCTCTC |             |
| 20   | POR EX5-6 R   | GCAGGGATGGCAATGACC  |               |
| 21   | POR EX7 F     | TATGCAACCTTCCCCCTCCT |             |
| 22   | POR EX7 R     | TCGAGAGTAAAGCCGCTAGT |             |
| 23   | POR EX8-9 F   | GCCCTTTGAGTGAAACGGA |               |
| 24   | POR EX8-9 R   | GCCTAAGCAGAGCCTACC  |               |
| 25   | POR EX10-11 F | CAGGGAGGACATGAGAGAG |               |
| 26   | POR EX10-11 R | GAGAATTGCTCACAAGCC |               |
| 27   | POR EX12-13 F | CTGCGACAAATGTAGGGGAT |             |
| 28   | POR EX12-13 R | AAGGGTGGTGCTGTGGAGG  |             |
| 29   | POR EX14-15 F | ACGAAGTGCGCATGAGG  |               |
| 30   | POR EX14-15 R | AAGGTGAATGCGGAGGTAGG |             |
| 31   | CYP19A1 EX 1 F | CTTGGCTCCTCCCCATCACC |             |
| 32   | CYP19A1 EX 1 R | TCGAACCACATGGGAGG |               |
| 33   | CYP19A1 EX 2 F | GTCTGCTCAAATGGTGATCAA |            |
| 34   | CYP19A1 EX 2 R | TTTCTCCACAGTCTCTGTG  |               |
| 35   | CYP19A1 EX 3 F | ATGGGAGAATGGAGCGCTCAT |          |
| 36   | CYP19A1 EX 3 R | TCAGGaAAACCAATATATGCTT |          |
| 37   | CYP19A1 EX 4 F | AGAAGATGCTTATATCAGGCG |          |
| 38   | CYP19A1 EX 4 R | CGAGGTCAGGACCAGCTGCT |          |
| 39   | CYP19A1 EX 5 F | CCTATCTCCCTCCCTTCTTCT |         |
| 40   | CYP19A1 EX 5 R | GTGAGCTCCTCTCTCTGAGG  |             |
| 41   | CYP19A1 EX 6 F | GTGAGGCAAGACGACAAATC |            |
| 42   | CYP19A1 EX 6 R | TCGACCAGTTCCTCAACTCAA |          |
| 43   | CYP19A1 EX 7 F | AGCTAATCTGCGGACCTAAA |            |
| 44   | CYP19A1 EX 7 R | GTGGTCTTTGAGGTGGGATT |            |
| 45   | CYP19A1 EX 8 F | GTCCAGCTCAGATCGAGAC |               |
| 46   | CYP19A1 EX 8 R | AGAGGAGGACGGAAAAGATG |            |
| 47   | CYP19A1 EX 9 F | GCTAATAATATAGGAGTCCC |            |
| 48   | CYP19A1 EX 9 R | GAAGGCTTTGAGATGAAATC |            |
| 49   | CYP19A1 EX 10 F | CATAAAAGGTCATAGTCC |            |
| 50   | CYP19A1 EX 10 R | CCTTTGGTTAGGAGCAGAGA |            |
| 51   | CYP19B1 EX 1-2 F | TGCAAGGCAAGCAGCAG |            |
| 52   | CYP19B1 EX 1-2 R | TGCTCCAGCTCTCAGGCT |            |
| 53   | CYP19B1 EX 3-5 F | AGAAATTCTCCTGCCCTTA |            |
| 54   | CYP19B1 EX 3-5 R | GACACCTGGGCGCGCCTGTA |            |
| 55   | CYP19B1 EX 6-9 F | TGACCTCGACTGCTGCTT |            |
| 56   | CYP19B1 EX6-9 R | GAGACCTGACTGCTGCTT |            |

* Primers for the CYP11B1 gene were adapted from white et al. [6].
Fig. 4. MLPA images of a) reference sample with copy number 1 for all the probes b) a sample positive for 30 kbp homozygous deletion with loss of eight probes in CYP21A2 gene. This sample was homozygous positive for all the eight common mutations screened with ASPCR, indicating the formation of classic chimera CH8 [5]. c) A sample positive for heterozygous 30 kbp deletion with a copy number of 0.5 in several CYP21A2 probes. The black arrowheads indicate the copy number of MLPA probes for Intron 2 splice site – each for wild type allele C and A to be zero with DQ of 0 and 0.09, respectively. With ASPCR, this subject was heterozygous for P30L, 8BPdel, I172N, E6CLUS and V281L and homozygous for I2G mutations. The parental screening revealed that the mother was a carrier for 30 kbp deletion and the father for the I2G splice variant. These results indicate that the subject is heterozygous for 30 kbp deletion with chimeric gene CH5 [5] on one allele and I2G splice mutation on the other allele.

utilized MLPA assay span only till exon 7 out of 10 (probe: CYP21A2-7(WT) wt F306+T). But Q318X and R356W probes are also required to identify chimeras CH3 and CH8. Therefore, the ASPCR, including these mutations, is advantageous to identify the above chimeras.

Multiplex PCR based target enrichment for NGS testing in CAH

A multiplex PCR program was designed to comprehensively screen for CYP21A2, CYP11B1, CYP17A1 and POR genes in CAH along with the CYP19A1 gene that causes aromatase deficiency mimicking CAH.
The coding and splice site regions of four genes - CYP11B1, CYP17A1, POR and CYP19A1 were amplified in 28 amplicons in 6 groups. Primers for the CYP11B1 gene were adapted from white et al. [6]. The primers were pooled into six groups based on the amplicon sizes (Table 3 and 4). The multiplex PCR was carried out using QIAGEN® Multiplex PCR kit. The PCR reaction mix and the conditions are described in table 5 and 6 respectively. The concentration of primers used was 10 pmol/μl. The PCR products were visualized on 2% agarose gel electrophoresis (Fig. 5.). Multiplex PCR products were
Table 4
Grouping details of primers for multiplex PCR.

| Group No. | Product | volume (μl) |
|-----------|---------|-------------|
| GROUP 1   |         |             |
| 1         | POR EX7 F | 387          | 10          |
|           | POR EX7 R | 485          | 10          |
| 2         | POR EX3 F | 545          | 10          |
|           | POR EX3 R | 10           |             |
| 3         | POR EX1 F | 589          | 10          |
|           | POR EX1 R | 10           |             |
| 4         | CYP19A1 EX 9F | 1X TE | 70 |
|           | CYP19A1 EX 9R | 10 |
| GROUP 2   |         |             |
| 1         | CYP19A1 EX 4F | 470          | 15          |
|           | CYP19A1 EX 4R | 10           |             |
| 2         | CYP19A1 EX 1F | 506          | 10          |
|           | CYP19A1 EX 1R | 10           |             |
| 3         | POR EX4 F  | 581          | 10          |
|           | POR EX4 R  | 10           |             |
| 4         | CYP19A1 EX 6F | 778          | 10          |
|           | CYP19A1 EX 6R | 10           |             |
| 5         | POR EX10-11 F  | 1448         | 10          |
|           | POR EX10-11 R | 10           |             |
| 6         | CYP11B1 EX1-2 F | 1X TE | 50 |
|           | CYP11B1 EX1-2R | 10 |
| GROUP 3   |         |             |
| 1         | CYP19A1 EX 7F | 468          | 10          |
|           | CYP19A1 EX 7R | 10           |             |
| 2         | CYP19A1 EX 3F | 534          | 10          |
|           | CYP19A1 EX 3R | 10           |             |
| 3         | CYP19A1 EX 5F | 600          | 10          |
|           | CYP19A1 EX 5R | 10           |             |
| 4         | POR EX10-11 F | 768          | 10          |
|           | POR EX10-11 R | 10           |             |
| 5         | POR EX5-6 F  | 850          | 10          |
|           | POR EX5-6 R  | 10           |             |
| 6         | CYP11B1 EX 3-5F | 1409         | 10          |
|           | CYP11B1 EX3-5R | 10 |
| GROUP 4   |         |             |
| 1         | CYP19A1 EX 2F | 471          | 10          |
|           | CYP19A1 EX 2R | 10           |             |
| 2         | POR EX2 F   | 527          | 10          |
|           | POR EX2 R   | 10           |             |
| 3         | CYP19A1 EX 8F | 585          | 10          |
|           | CYP19A1 EX 8R | 10           |             |
| 4         | POR EX12-13 F | 649          | 20          |
|           | POR EX12-13 R | 20           |             |
| 5         | CYP17A1 EX2-3 F | 795          | 10          |
|           | CYP17A1 EX2-3 R | 10           |             |
| 6         | CYP17A1 EX5-6 F | 950          | 10          |
|           | CYP17A1 EX5-6 R | 10 |
| 7         | CYP11B1 EX 6-9F | 1541         | 10          |
|           | CYP11B1 EX6-9R | 10 |

(continued on next page)
Table 4 (continued)

| Group No. | Product | Size (bp) | volume (μl) |
|-----------|---------|-----------|-------------|
| GROUP 5   |         |           |             |
| 1         | CYP17A1 EX4 F | 443       | 2           |
|           | CYP17A1 EX4 R |          | 2           |
| 2         | POR EX14-15 F | 818       | 3           |
|           | POR EX14-15 R |          | 3           |
|           | TOTAL      |           | 10          |
| GROUP 6   |         |           |             |
| 1         | POR EX8-9 F | 566       | 1           |
|           | POR EX8-9 R |          | 1           |
| 2         | CYP17A1 EX1 F | 972       | 1           |
|           | CYP17A1 EX1 R |          | 1           |
|           | 1X TE      | 6         |             |
|           | TOTAL      | 10        |             |

Table 5
Multiplex PCR reaction mix.

| Contents                     | Volume |
|------------------------------|--------|
| 2x QIAGEN Multiplex PCR Master Mix | 7.5 μl |
| Q solution                   | 1.5 μl |
| DNA                          | 1 μl   |
| Primer pool                  | 3 μl   |
| Sterile water                | 2 μl   |
| Total                        | 15 μl  |

Primer concentration used: 10 pmol/μl.

Table 6
Multiplex PCR program.

| Stage | PCR Program     | Temperature | Time    |
|-------|-----------------|-------------|---------|
| 1     | Initial denaturation | 95 °C       | 10 minutes |
|       |                  | 98 °C       | 5 minutes  |
| 2     | Denaturation     | 98 °C       | 30 seconds |
|       | Annealing        | 60 °C       | 90 seconds |
| 3     | Extension        | 72 °C       | 90 seconds |
|       | Final extension  | 72 °C       | 10 minutes |

The above comprehensive strategy, clinically significant variants were identified in CYP21A2, CYP11B1 and CYP19A1 genes in 97.2% of the study subjects (n=72) suspected for 21 hydroxylase and 11 beta hydroxylase deficiency. No disease-causing variants were identified in CYP17A1 and POR genes. However, several polymorphisms were identified in the above two genes (table 8) indicating effective use of this CAH - NGS panel in clinical settings.
| Amplicon No | Contig_start | Contig_end | Region ID | ave_base reads | fwd_base reads | rev_base reads | Cov 20x | Cov 100x | Cov 500x |
|------------|--------------|------------|-----------|---------------|--------------|--------------|---------|---------|---------|
| 1          | 104590181    | 104591627  | chr10:104590181-104591627 | 517.914 | 415.200 | 334222 | 1447 | 1420 | 824 |
| 2          | 104596499    | 104597469  | chr10:104596499-104597469 | 1166.65 | 605.352 | 527465 | 971 | 971 | 960 |
| 3          | 104592145    | 104593093  | chr10:104592145-104593093 | 1713.268 | 952.225 | 673666 | 949 | 949 | 949 |
| 4          | 104594438    | 104595231  | chr10:104594438-104595231 | 2463.586 | 980.654 | 975433 | 794 | 794 | 794 |
| 5          | 104593641    | 104594084  | chr10:104593641-104594084 | 3555.056 | 879.897 | 698548 | 442 | 442 | 442 |
| 6          | 51528876     | 51529408   | chr15:51528876-51529408 | 2727.535 | 895.187 | 558589 | 533 | 533 | 533 |
| 7          | 51510437     | 51511062   | chr15:51510437-51511062 | 2763.912 | 657.054 | 1073155 | 626 | 626 | 626 |
| 8          | 51502646     | 51503422   | chr15:51502646-51503422 | 2783.369 | 1274.280 | 888398 | 777 | 777 | 777 |
| 9          | 51514346     | 51514944   | chr15:51514346-51514944 | 3165.851 | 5837.330 | 1059012 | 599 | 599 | 599 |
| 10         | 51506984     | 51507567   | chr15:51506984-51507567 | 3259.106 | 964.143 | 939175 | 584 | 584 | 584 |
| 11         | 51630548     | 51631052   | chr15:51630548-51631052 | 3544.501 | 918.710 | 871263 | 505 | 505 | 505 |
| 12         | 51504333     | 51504920   | chr15:51504333-51504920 | 3768.374 | 1191.552 | 1024252 | 588 | 588 | 588 |
| 13         | 51519816     | 51520284   | chr15:51519816-51520284 | 4440.981 | 1244.142 | 838678 | 469 | 469 | 469 |
| 14         | 51507754     | 51508220   | chr15:51507754-51508220 | 4909.906 | 1122.681 | 1170245 | 467 | 467 | 467 |
| 15         | 51534813     | 51535282   | chr15:51534813-51535282 | 6990.323 | 1714.667 | 1570785 | 470 | 470 | 470 |
| 16         | 32005398     | 32011605   | chr8:32005398-32011605 | 808.908 | 2537.725 | 2478950 | 6208 | 6208 | 4977 |
| 17 & 18    | 75614821     | 75615956   | chr7:75614821-75615956 & chr:75615407-75615956 | 591.27 | 2855.556 | 386127 | 1136 | 1136 | 872 |
| 19 & 20    | 75609528     | 75611028   | chr7:75609528-75611028 & chr:75610180-75611028 | 1317.141 | 985.186 | 998142 | 1431 | 1431 | 1339 |
| 21         | 75613940     | 75614706   | chr7:75613940-75614706 | 1458.856 | 613.624 | 526031 | 767 | 767 | 767 |
| 22         | 75583197     | 75583740   | chr7:75583197-75583740 | 1953.465 | 603.651 | 459304 | 544 | 544 | 544 |
| 23         | 75612700     | 75613264   | chr7:75612700-75613264 | 2065.257 | 6418.78 | 524992 | 565 | 565 | 565 |
| 24         | 75601606     | 75602131   | chr7:75601606-75602131 | 3238.409 | 8863.636 | 817040 | 526 | 526 | 526 |
| 25         | 75608573     | 7560956    | chr7:75608573-7560956 | 3296.955 | 830.463 | 765263 | 484 | 484 | 484 |
| 26         | 75611472     | 75611857   | chr7:75611472-75611857 | 3574.839 | 6898.546 | 690342 | 386 | 386 | 386 |
| 27         | 143957567    | 143958974  | chr8:143957567-143958974 | 391.872 | 291466 | 260290 | 1408 | 1360 | 233 |
| 28         | 143955781    | 143957320  | chr8:143955781-143957320 | 1041.747 | 699797 | 904493 | 1540 | 1540 | 1509 |
| 29         | 143960421    | 143961293  | chr8:143960421-143961293 | 1608.318 | 725207 | 678555 | 873 | 873 | 873 |
**Fig. 6.** a. Coverage analysis report of a representative sample sequenced for CYP21A2 gene with 100% of the target having a minimum coverage of 20X reads. b. Coverage analysis report of a representative sample sequenced for five genes CAH panel in 29 amplicons with 99.72% of the target having a minimum coverage of 20X reads and 99.44% of the target with 100X reads.
| Subject ID | Gene | Ref Base | Called Base | Codon change | Protein change | Genotype | Effect | dbsNP ID | MAF in South Asians |
|------------|------|----------|-------------|--------------|---------------|----------|--------|----------|---------------------|
| C3         | CYP17A1 | G       | A           | c.138C>T     | p.His46=     | Homozygous | Synonymous | 6162 | 0.476               |
| C3         | POR  | C       | A|C | c.195G>T     | p.Ser65=     | Heterozygous | Synonymous | 663 | 0.359               |
| C3          | POR | G | A|G | c.1716G>A     | p.Ser572=    | Heterozygous | Synonymous | 1057870 | 0.278           |
| C3          | POR | A | G|A | c.387A>G      | p.Pro129=    | Heterozygous | Synonymous | 1135612 | 0.206           |
| C3          | POR | T | C   | c.1455T>C    | p.Ala485=    | Heterozygous | Synonymous | 2228104 | 0.932           |
| C3          | POR | G | A       | c.138C>T     | p.His46= | Heterozygous | Synonymous | 6162 | 0.476               |
| C3          | POR | C       | A|C | c.195G>T     | p.Ser65=     | Heterozygous | Synonymous | 6162 | 0.359               |
| C3          | POR | T | C   | c.1455T>C    | p.Ala485=    | Heterozygous | Synonymous | 1057868 | 0.354           |
| C3          | POR | G       | A|G | c.138C>T     | p.His46=     | Heterozygous | Synonymous | 6162 | 0.476               |
| C3          | POR | C       | T|C | c.1508C>T    | p.Ala503Val  | Heterozygous | Non-synonymous | 2228104 | 0.932           |
| C3          | POR | A | G|A | c.387A>G      | p.Pro129=    | Heterozygous | Synonymous | 1135612 | 0.206           |
| C3          | POR | T | C   | c.1455T>C    | p.Ala485=    | Heterozygous | Synonymous | 2228104 | 0.932           |
| C3          | POR | G | A       | c.138C>T     | p.His46=     | Heterozygous | Synonymous | 6162 | 0.476               |
| C3          | POR | C       | A|C | c.195G>T     | p.Ser65=     | Heterozygous | Synonymous | 6162 | 0.359               |
| C3          | POR | A | G|A | c.387A>G      | p.Pro129=    | Heterozygous | Synonymous | 1135612 | 0.206           |
| C3          | POR | T | C   | c.1455T>C    | p.Ala485=    | Heterozygous | Synonymous | 2228104 | 0.932           |
| C3          | POR | G | A       | c.138C>T     | p.His46=     | Heterozygous | Synonymous | 6162 | 0.476               |
| C3          | POR | C       | T|C | c.1508C>T    | p.Ala503Val  | Heterozygous | Non-synonymous | 2228104 | 0.932           |

**Conclusion**

The ASPCR assay was found to be highly specific and sensitive to detect all eight hotspot mutations in CYP21A2 gene that were also identified by NGS and Sanger sequencing, validating its sensitivity and specificity. This assay is a simple cost-effective technique to genotype point mutations in CYP21A2 gene and to identify junction sites in chimeric genes of CYP21A2 - CYP21A1P rearrangement that contributes to more than 90% of mutations in 21 - hydroxylase deficiency. Careful standardization
enabled accurate and precise results that can provide a genetic diagnosis to a significant proportion of the CAH cohort in a clinical setting. The multiplex PCR assay enables a cost-effective step in NGS processing of CAH genes achieving uniform coverage matrices across the genes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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