Allele-Specific PCR and Next Generation Sequencing based genetic screening for Congenital Adrenal Hyperplasia in India

Lavanya Ravichandran
Christian Medical College Vellore
Sophy Korula
Christian Medical College Vellore
Asha H.S.
Christian Medical College Vellore
Deny Varghese
Christian Medical College Vellore
Parthiban R
Christian Medical College Vellore
Jabasteen Johnson
Christian Medical College Vellore
Janani Ishwarya
Christian Medical College Vellore
Sahana Shetty
Christian Medical College Vellore
Kripa Elizabeth Cherian
Christian Medical College Vellore
Felix Jebasingh
Christian Medical College Vellore
Nitin Kapoor
Christian Medical College Vellore
Divya Pachat
Christian Medical College Vellore
Sarah Mathai
Christian Medical College Vellore
Anna Simon
Christian Medical College Vellore
Simon Rajaratnam
Christian Medical College Vellore
Thomas V Paul
Christian Medical College Vellore
Nihal Thomas
Christian Medical College Vellore
Aaron Chapla (aaronchapla@gmail.com)
Christian Medical College Vellore https://orcid.org/0000-0003-4157-6041

Research Article

Keywords: Congenital Adrenal Hyperplasia, Allele-Specific PCR for CYP21A2 gene, Targeted Next Generation Sequencing, Parallel multigene testing, India

Posted Date: May 26th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-535031/v1

Abstract

Purpose

Genotyping CYP21A2 gene is known to be extremely challenging and is not utilized as a first tier diagnostic tool in routine clinical practice. Also, with the advent of massive parallel sequencing technology, there is a need for investigating extended panel of genes implicated in CAH. This study aims to establish a comprehensive genetic screening strategy for five genes in CAH.

Methods

Allele-Specific Polymerase Chain Reaction (ASPCR) for hotspot mutations in CYP21A2 gene followed by targeted Next-Generation Sequencing (NGS) of CYP21A2, CYP11B1, CYP17A1, POR, and CYP19A1 genes were carried out to screen 72 clinically diagnosed CAH subjects from India.

Results

Utilizing ASPCR, 88.7% (n = 55/62) of the subjects suspected with 21 hydroxylase deficiency were positive for CYP21A2 hotspot mutations. Utilizing NGS, the ASPCR assay was found to highly sensitive and specific for screening these hotspot mutations. Additionally, through targeted NGS, six study subjects were positive for other CYP21A2 variants: one with a novel c.1274G > T, three with c.1451G > C and one with c.143A > G variant. One subject was compound heterozygous for c.955C > T/c.1042G > A variants identified using ASPCR and NGS. One subject suspected for a simple virilizing 21-hydroxylase deficiency was positive for a CYP19A1:c.1142A > T variant. CYP11B1 variants (c.1201-1G > A, c.1200 + 1delG, c.412C > T, c.1024C > T, c.1012dup, c.623G > A) were identified in all six subjects suspected for 11 beta-hydroxylase deficiency.

Conclusion

With an overall mutation-positivity rate of 97.2% (140/144 alleles), ASPCR followed by a multigene targeted NGS assay has shown to be highly sensitive and specific as a cost-effective and comprehensive diagnostic tool for CAH in a clinical setting.

Introduction

Congenital Adrenal Hyperplasia (CAH) includes a heterogeneous group of autosomal recessive disorders resulting from molecular defects in any one of the enzymes involved in adrenal steroidogenesis. Deficiency of 21-hydroxylase, an enzyme that is crucial for the synthesis of aldosterone and cortisol, accounts for over 90% of patients with CAH [1]. The unequal crossing over and gene conversion events between the functional CYP21A2 gene and non-functional CYP21A1P pseudogene contribute to 95% of mutations in 21-OH deficiency [2].

The general incidence of classical CAH in Caucasians is around 1 in 15000, whereas in India, the cumulative prevalence is as high as 1 in 5762 [3][4]. Biochemical investigations in CAH based on 17 hydroxyprogesterone (17OHP) measurements have shown to yield false-positive results due to assay interference with other steroid intermediates [5]. Further, these enzymatic assays cannot explain disease severity or distinguish heterozygotes. Despite these drawbacks in biochemical testing, genetic screening in 21-0H CAH is still not used as a first-tier diagnostic tool [6] due to pseudogene imposed complexities in genotyping the CYP21A2 gene. However, with a high prevalence and carrier frequency, there is a need for cost-effective, sensitive, and specific genetic screening strategies to confirm CAH diagnosis, understand the phenotypic severity, rule out carrier state and provide genetic counseling.

The other enzyme defects in steroidogenesis include 11 hydroxylase (CYP11A1, CYP11B1), 3β-hydroxy steroid dehydrogenase (HSD3B2), 17α hydroxylase (CYP17A1), and cytochrome P450 oxidoreductase (POR). Though these gene defects contribute to relatively rare forms of CAH, there is a need for molecular investigations to analyze the mutation spectrum in these genes. Also, with the advent of NGS-based screening, the clinical utility of these strategies in CAH needs to be evaluated.

Materials And Methods

Participants-Baseline Characteristics

Sixty-five subjects with biochemical confirmation of CAH were recruited from the Departments of Paediatric Endocrinology and Endocrinology, Diabetes & Metabolism, Christian Medical College, Vellore. Additionally, seven subjects were recruited from Aster MIMS, Kerala. Patients presenting with salt-wasting crisis in the neonatal period or infancy, or ambiguous genitalia noted early in life, or with oligomenorrhoea, amenorrhoea, or infertility as adolescents or adults were suspected for 21-OH deficiency. Virilized females or males with hyperandrogenism and systemic hypertension with or without hypokalemia were suspected of having 11β -OH deficiency. In vitro determination of 17-OHP was performed using a radioimmunoassay kit from Immunotec®. The functional sensitivity of this assay was 0.064ng/ml, and the inter-assay coefficient of variation was < 11.5% with duplicates of 11 different series. Those with equivocal results (2 to 10 ng/ml) were confirmed by ACTH stimulated 17-OHP testing. Informed consent for genetic testing from the adult subjects and assent from the parents of the paediatric subjects were obtained. The study was approved by the Institutional Review Board (IRB Min Nos: 10814/ 2017 and 10903/2017), CMCH, Vellore, India.

Long-Range PCR and restriction digestion

DNA extraction from whole blood was carried out using the QIAGEN® kit (Hilden, Germany). Long-range PCR amplification of the CYP21A2 (6.2 kbp) and CYP21A1P (6.1 kbp) genes was carried out as per the published protocols [7]. Specificity of the amplification was further validated by restriction digestion with TaqI enzyme at 65ºC for two hours [7].

Allele-Specific PCR for hotspot screening

The CYP21A2 long-range PCR product was used as a template for novel in-house designed allele-specific PCR (ASPCR) primers to genotype eight known hotspot mutation in 21-OH deficiency – P30L, I2G, 8BPdel, I172N, E6CLUS (I235N, V236E, M238K) V281L, Q318X, and R356W. The assay was standardized using different primer sets for the wild type and mutant alleles. The ASPCR was carried out with EmeraldAmp® Max PCR master mix (Takara Bio Inc, Japan) (Table 1). All the samples were genotyped along with appropriate positive and negative controls, and the results were validated with both Sanger and Next Generation Sequencing (Fig 1).

Next-Generation Sequencing strategy

A multiplex PCR was utilized for target enrichment followed by NGS for five genes which include CYP21A2, CYP11B1, CYP17A1, and POR along with CYP19A1 gene that causes aromatase deficiency mimicking CAH. The PCR assay was standardized with Qiagen® Multiplex PCR mix to amplify the coding and splice site regions of the above genes with the in-house designed primers. These PCR products were sheered separately and pooled along with the long-range PCR product of the CYP21A2 gene. Library preparation and NGS using Ion Torrent PGM™ was carried out as per previously published protocols [8]. Data analysis was carried out using the Ion torrent suit software (Version 5.0.4.0) and DNA Star software v13. The classification of the identified variants was based on ACMG 2015 guidelines [9]. Varsome and other widely available online tools were used for data interpretation [10, 11]. All the clinically relevant variants were validated using Sanger sequencing.

Multiplex Ligation-dependent Probe Amplification (MLPA)

MLPA was carried out for the samples suspected of large deletions and rearrangements based on long-range PCR and restriction digestion results. The assay was standardized with SALSA MLPA Probemix P050 from MRC-Holland® [12] using reference samples as per the manufacturer's protocol. The results were analyzed using coffalyser software [13].

Results

A total of 72 subjects (49 paediatric and 23 adults) were included in the study, of whom 66 subjects were clinically suspected of having 21-OH deficiency while six were suspected for 11β -OH deficiency. Sixty-seven were from the southern part of the country, and five were from northern India, with 32 males and 40 females.

Among the subjects with 21-OH deficiency, 60.6% (n=40) were of Salt-Wasting (SW) phenotype, 31.8% (n=21) with Simple Virilizing (SV) phenotype and 7.6% (n=5) with Non-Classical (NC) CAH. The age of diagnosis varied from 1 to 95 days in SW type, two weeks to 8 years in SV type, and 3.5 to 26 years in NC CAH. The consanguinity proportions were 42.5%(SW), 30%(SV), and 50%(NC). The mean basal 17-OHP values were 127.9, 36.1, and 21 ng/ml in SW, SV, and NC phenotype respectively. Subjects with SW phenotype presented with mean sodium of 126.4 meq/l and mean potassium of 7.1 meq/l. A short synacthen test confirmed poor cortisol response among 17 patients with SW phenotype. In four males and two female subjects suspected of 11β -OH deficiency (5 Paediatric and 1 adult), the age of diagnosis varied from 2.5 to 14 years with a mean basal 17-OHP of 10.7 ng/ml, and 40% of the patients were born of consanguineous marriages.

Long range PCR and MLPA

The long-range PCR yielded specific amplification of the functional and pseudogene in 62/66 samples with appropriate restriction digestion patterns. Subjects C15, C62, and C71 had only the functional gene amplified with a restriction digestion pattern of the pseudogene. On the other hand, subject C29 with only the pseudogene amplification gave a restriction digestion pattern of the functional gene. Based on these results, MLPA was carried out, which showed a homozygous large deletion involving 5' of CYP21A1P and 3' of CYP21A2 in subjects C15, C62, and C71. These three subjects were homozygous positive for all eight hotspot mutations in ASPCR. MLPA results also confirmed a large gene conversion involving 5' of CYP21A2 and 3' of CYP21A1P in subject C29.

Allele Specific PCR for hotspots screening

Utilizing the in-house designed ASPCR approach, CYP21A2 hotspot mutations were identified in 55/62 subjects - 33 SW, 19 SV, and 3 NC CAH. Out of 33 subjects with SW phenotype, 25 (75.8%) had biallelic mutations, seven (21.2%) had multiple heterozygous mutations, while one subject (C3) was positive only for a heterozygous Q318X mutation. Among the 19 subjects with SV phenotype, 10 (52.6%) had biallelic mutations, eight (42.1%) had multiple heterozygous mutations, and one subject (C33) was heterozygous for the 8BP deletion in exon 3. Out of five non-classical subjects, two were positive for biallelic mutations (40%) and one subject (C65) had a single heterozygous V281L hotspot mutation. The remaining seven subjects were negative for ASPCR.

Next Generation Sequencing for a targeted panel of 5 genes in CAH

The NGS assay for a single gene CYP21A2 was carried out for all the samples with 21-OH deficiency - both positive and negative for ASPCR. Five gene NGS panel was utilized for those subjects negative for ASPCR and those with suspected 11β -OH deficiency. The Multiplex PCR - NGS assay covered the complete coding and splice site regions of five genes included in the panel. The mean base coverage for five genes was 700X and >99 % of the target had a minimum

coverage of 20X. Further, with NGS, no additional samples were positive for the eight hotspot mutations corroborating the sensitivity and specificity of the ASPCR assay.

Additional homozygous CYP21A2 variants were identified in five out of seven subjects who were negative for ASPCR. These variants include c.1451G>C(p.Arg484Pro) in three subjects, c.143A>G(p.Tyr48Cys) in one subject and a novel c.1274G>T(p.Gly425Val) variant in one subject

Interestingly, subjects C31 and C32 with SW phenotype and subject C47 with SV phenotype were positive for homozygous CYP21A2:c.1451G>C variant. The younger sibling of subject C31 with a similar clinical presentation of SW phenotype was also homozygous positive for this recurrent variant. This variant has been previously reported in patients with both SW and SV phenotype [14, 15]. Subject C3, one year old female with SW phenotype, was heterozygous positive for Q318X mutation identified through ASPCR and heterozygous positive for CYP21A2:c.1042G>A(p.Ala348Thr) variant identified through NGS. This subject was compound heterozygous for the above variants and inherited the Q318X mutation from the mother and A348T variant from the father. In subjects C33 and C65, who only had single heterozygous hotspot mutation identified in ASPCR, no additional CYP21A2 variants were identified through NGS. Subject C63 with a non-classical phenotype was negative for variants in all the five genes screened.

Subject C46, who was initially suspected of having a 21- OH SV phenotype, was negative for mutations in the CYP21A2 gene but was found to be positive for an aromatase gene CYP19A1:c.1142T>A(p.Asp381Val) variant. Born to second-degree consanguineous parents, this subject had ambiguous genitalia and clitoromegaly at birth with an elevated 17-OHP of 15.4 ng/ml. Deficiency in the aromatase enzyme due to CYP19A1 mutations has been reported to cause ambiguous genitalia in 46XX females [16]. A recent report has also shown that aromatase deficiency can mimic SV CAH [17]. The majority of the in silico tools support a pathogenic prediction, and only two heterozygous alleles have been reported in South Asians so far (MAF - 0.0000653, GnomAD exomes). However, there is a need for further investigations and family screening to confirm its pathogenicity, and based on ACMG 2015 guidelines, this variant has been classified as a Variant of Uncertain Significance (VUS).

Out of six subjects with suspected 11β -OH deficiency, four had homozygous, and two had compound heterozygous variants; four novel and two reported variants were identified in total (CYP11B1:c.1201-1G>A, c.1200+1delG, c.412C>T, c.623G>A, c.1024C>T, and c.1012dupC). Subject C56, an eight year old female born of second degree consanguineous marriage, presenting with hyperpigmentation, clitoromegaly, and hypertension, was homozygous positive for CYP11B1:c.412C>T (p.Arg138Cys). In vitro studies of this variant had demonstrated partially impaired CYP11B1 activity [18]. Subject C53 is a 13-year-old male, presenting with hypertension and hypokalemia from 2.5 years of age and was found to be compound heterozygous for two reported missense variants CYP11B1:c.412C>T (p.Arg138Cys) and CYP11B1:c.623G>A (p.Arg208Gln). Subject C54, a 12-year-old male, with hypertension and hyperpigmentation from five years of age, was compound heterozygous for a novel nonsense mutation CYP11B1:c.1024C>T (p.Gln342Ter) and a novel duplication CYP11B1:c.1012dupC (Gln338ProfsTer16) resulting in a frameshift at codon 338 followed by premature termination at codon 353 instead of 504.

Two unrelated subjects born to second degree consanguineous parents (C51 and C55) were homozygous positive for a novel splice variant CYP11B1:c.1201-1G>A. Subject C51, a 19-year-old male, presented with precocious puberty at two years of age, followed by recurrent hypokalemic paralysis and hypokalemic cardiomyopathy. Subject C55 had enlarged clitoris from birth and hypokalemic paralysis with hypertension at four years of age. Subject C52, presenting with hypertension, true puberty, and aggressive behavior, was positive for a novel homozygous splice variant CYP11B1:c.1200+1delG. The in silico analysis for these splice variants predict aberrant splicing and requires functional assays to confirm its pathogenicity.

No mutations were identified in the CYP17A1 and POR genes. The details of the individual variants identified through NGS assay are mentioned in Table 2a and 2b, and the complete workflow with the results is depicted in Fig 2.

Parental Screening

Family screening was carried out using Sanger sequencing and the carrier status was confirmed in 18 out of 21 available paediatric parental samples. De novo homozygous mutations were identified in three probands as their parents were negative for those mutations. Family screening is incomplete in the other study subjects due to the unavailability of one or both parental samples.

Genotype-phenotype correlation

Majority of the subjects with SW phenotype in this study had null or group A mutations which are known to result in <1% of the enzymatic activity, and I2G hotspot was the predominant genotype identified. The SV phenotype had most of its genotype falling in group B (1-2 % enzyme activity), with the predominant genotype being I172N. Two out of five NCCAH subjects were positive for I2G mutation in the homozygous state under Group A. All the subjects with null mutations presented with SW phenotype. 9% of the classical subjects were positive for P30L, which usually is predicted to result in NCCAH. I2G, a SW genotype was identified in 28% of the subjects with SV phenotype. I172N, usually reported in SV phenotype was seen in 16 % of the subjects with SW phenotype. Eleven subjects had multiple homozygous and heterozygous mutations, indicating smaller gene conversions involving multiple exons that are frequently observed in 21-OH deficiency [19]. The frequency of the mutated alleles and their associated phenotype is depicted in Fig 3. Table 3 explains the different groups of genotypes identified and their correlation with clinical phenotypes.

Discussion

The genes implicated in CAH are well recognized for over decades, but genotyping in 21-OH deficiency is not routinely used as a first-line diagnostic tool. The underlying reasons include challenges in primer specificity to avoid pseudogene interference, assay standardization, cost-effectiveness, and analysis of complex rearrangements. In the present study, allele-specific PCR and NGS-based strategies have been established and utilized to study the spectrum of mutations in 72 Indian subjects.

ASPCR is highly preferred for genotyping as it is much simpler, cost-effective and does not require radioactive probes or restriction enzymes in comparison to southern blotting and RFLP. Our initial efforts to replicate published ASPCR protocols did not achieve expected specificity and sensitivity. Therefore, novel primer sets for the CYP21A2 hotspot mutations were designed in-house, and standardization was performed using specific template concentrations, primer dilutions, and annealing temperatures. Following standardization with appropriate controls, the study subjects were genotyped, and the results were validated with NGS and Sanger sequencing. Through these efforts, we have established an ASPCR assay that is 100 % sensitive and specific, with a diagnostic yield of 88.7% (55/62). These results suggest that ASPCR is the most inexpensive tool for mass screening of the hotspot mutations in 21-OH deficiency.

Majority of the earlier reports have utilized a combination of techniques to detect pseudogene-derived point mutations, large rearrangements, and Sanger sequencing was employed to rule out other mutations in the CYP21A2 gene [19]. One of the studies from India had reported mutations in 96.4% of the alleles, including four novel variants utilizing RFLP, SSCP, and nested PCR [20]. In other studies, exclusively screening the hotspot mutations, *Asanuma et al* has reported 82% [21], *Bas et al* with 84.6% [22] *Mathur, R. et al* with 78.6% [23] and *Marumudi et al* with a 74.2% [24] positivity. The varying yield under these studies could be due to the difference in the sample size and the number of hotspot mutations screened. Table 4 gives a comparison of these study results with previous reports on 21OH deficiency from India.

In line with previous reports, we identified the I2G splice variant as the single most common mutation in the overall study population and in the SW phenotype. Studies from the USA, UK, Cuba, Vietnam, China, Sweden have reported the I2G splice variant as the most common pseudogene-derived mutation in their study cohorts [25–30]. In a mixed population study, *New Maria I. et al* reported I2G and V281Lmutations to be the prominent mutations identified in the largest cohort of 1507 subjects [31]. Even though 30kb deletion is one of the most common mutation observed in Classical 21-OH CAH [32], we have identified this deletion in only three subjects with salt-wasting CAH.

In general, the genotype-phenotype correlation is strong in SW and NCCAH, as reported previously [15, 31, 33]. In our cohort, the correlation between null and group A genotypes with their corresponding SW phenotype was high. The concordance was poor among NCCAH subjects. The discrepancies in genotype – phenotype concordance of P30L, I2G, and I172N mutations could be attributed to the phenotypic heterogeneity that is often reported in CAH [34][35].

To further screen those subjects negative for the hotspot mutations and other forms of CAH, a five gene panel for targeted Next-generation sequencing was established. The NGS-based assay achieved complete coverage of coding and splice site regions across the targeted panel of genes and picked up all the hotspot mutations identified earlier with ASPCR. However, the NGS data analysis for CYP21A2 gene is challenging and requires specific bed files (defining the target region) along with the need to allow increased mismatches in case of suspected rearrangements to achieve alignment of the majority of NGS reads. Interestingly, utilizing NGS based strategy, we have also identified a recurrent CYP21A2 mutation c.1451G>C in three (4.5%) different families. Further, studies in large cohorts are required to confirm this finding to include this recurrent mutation in ASPCR based screening in India. 2 subjects (3%) carried a single hotspot mutation only on single allele. Nonetheless, there are earlier reports in CAH patients with only one affected allele [36, 37]. One subject with non-classical phenotype (1.5%) was negative for all the five genes screened requiring additional investigations to confirm the genetic diagnosis.

Recent reports by *Gangodkar et al* from India [38] and *Karaoğlan et al* from Turkey [39] have utilized NGS-based screening for the CYP21A2 gene and identified variants in 82.6% and 79.5% of CAH subjects. In this study, we have screened an extended NGS panel of genes in CAH, which is beneficial in patients with overlapping phenotypes of milder forms of CAH. One of the subjects in this cohort suspected of having SV- 21 hydroxylase deficiency was positive for a CYP19A1 variant. Although NGS-based multigene testing provides a robust strategy, it is still expensive and requires complex computational infrastructure and expertise for its clinical utility in a developing country like India. Therefore, we recommend ASPCR as a first-tier screening tool for the CYP21A2 hotspots, followed by targeted NGS only for those subjects who are negative for ASPCR and with other forms of CAH.

Study Limitations

Due to the small number of subjects under the category of rare forms of CAH, the NGS assay for CYP11B1, CYP17A1, POR and CYP19A1 genes requires further validation. The NGS assay needs the inclusion of other genes (HSD3B2, StAR, and CYP11A1) implicated in CAH and requires future studies in large cohorts to investigate these rarer forms of CAH.

Conclusion

To conclude, ASPCR followed by a multigene targeted NGS assay has shown to be a robust strategy for comprehensive and cost-effective genetic screening of CAH in India. With this strategy, the genotype of 140 out of 144 (97.2%) alleles in a total of 72 subjects was characterized. These protocols could pave a way forward for its utility as first-tier testing in a clinical setting for CAH diagnosis, carrier screening, newborn screening, and prenatal testing.

Declarations

Acknowledgements: Nil

Funding: The study was supported by independent FLUID research grants and Molecular Endocrinology Laboratory funds from Christian Medical College, Vellore, India.

Data availability: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability: Not applicable

Authors' contributions

All the authors have made substantive contributions to the article. Aaron Chapla, Sophy Korula, Asha H.S., and Sahana Shetty contributed to the study conception and design. Clinical evaluation and follow up of the participants were carried out by Sophy Korula, Asha. H.S., Sahana Shetty, Kripa Elizabeth Cherian, Felix Jebasingh, Nitin Kapoor, Divya Pachat, Sarah Mathai, Anna Simon, Simon Rajaratnam, Thomas V Paul and Nihal Thomas. Biochemical investigations were performed by Janani Ishwarya. Data collection and Genetic testing was carried out by Aaron Chapla, Lavanya Ravichandran, Deny Varghese, Parthiban and Jabasteen Johnson. Data analysis and interpretation was carried out by Lavanya Ravichandran and Aaron Chapla. The first draft of the manuscript was written by Lavanya Ravichandran and Aaron Chapla and all authors commented on previous versions of the manuscript. Sarah Mathai, Anna Simon, Simon Rajaratnam, Thomas V Paul and Nihal Thomas provided critical revision for important intellectual content. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflicts of interest.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the Christian Medical College, Vellore and with the 1964 Helsinki Declaration and its later amendments. This study was approved by the Institutional review board of the Christian Medical College, Vellore (IRB numbers: 10814/ 2017 and 10903/2017).

Informed Consent: Informed consent for genetic testing from all the adult subjects and assent from the parents of all the paediatric subjects were obtained.

References

1. P.C. White, P.W. Speiser (2000) Congenital Adrenal Hyperplasia due to 21-Hydroxylase Deficiency. 21:47
2. A. Massimi, M. Malaponti, L. Federici et al., Functional and structural analysis of four novel mutations of CYP21A2 gene in Italian patients with 21-hydroxylase deficiency. Horm Metab Res **46**, 515–520 (2014). https://doi.org/10.1055/s-0034-1371864
3. ICMR Task Force on Inherited Metabolic Disorders, Newborn Screening for Congenital Hypothyroidism and Congenital Adrenal Hyperplasia. Indian J. Pediatr. **85**, 935–940 (2018). https://doi.org/10.1007/s12098-018-2645-9
4. P. Vats, A. Dabas, V. Jain et al., Newborn Screening and Diagnosis of Infants with Congenital Adrenal Hyperplasia. Indian Pediatr. **57**, 49–55 (2020)
5. J.-H. Choi, G.-H. Kim, H.-W. Yoo, Recent advances in biochemical and molecular analysis of congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Ann Pediatr Endocrinol Metab **21**, 1 (2016). https://doi.org/10.6065/apem.2016.21.1.1
6. D.P. Merke, R.J. Auchus, Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. N. Engl. J. Med. **383**, 1248–1261 (2020). https://doi.org/10.1056/NEJMra1909786
7. H.-H. Lee, Mutational analysis of CYP21A2 gene and CYP21A1P pseudogene: long-range PCR on genomic DNA. Methods Mol. Biol. **1167**, 275–287 (2014). https://doi.org/10.1007/978-1-4939-0835-6_19
8. A. Chapla, M.D. Mruthyunjaya, H.S. Asha et al., Maturity onset diabetes of the young in India - a distinctive mutation pattern identified through targeted next-generation sequencing. Clin. Endocrinol. (Oxf) **82**, 533–542 (2015). https://doi.org/10.1111/cen.12541
9. S. Richards, N. Aziz, S. Bale et al., Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet. Sci. **17**, 405–423 (2015). https://doi.org/10.1038/gim.2015.30
10. C. Kopanos, V. Tsiolkas, A. Kouris et al., VarSome: the human genomic variant search engine. Bioinformatics **35**, 1978–1980 (2019). https://doi.org/10.1093/bioinformatics/bty897
11. J.M. Schwarz, D.N. Cooper, M. Schuelke, D. Seelow, MutationTaster2: mutation prediction for the deep-sequencing age. Nat. Methods **11**, 361–362 (2014). https://doi.org/10.1038/nmeth.2890
12. J.P. Schouten, C.J. McElgunn, R. Waaijer et al., Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res **30**, e57 (2002)
13. C. Jordy (2011) Analysis of MLPA Data Using Novel Software Coffalyser.NET by MRC-Holland. Joost van den Berg ED1 - Ahmed Badr Eldin (ed) Modern Approaches To Quality Control. IntechOpen, Rijeka, p Ch. 7
14. S. Baumgartner-Parzer, M. Witsch-Baumgartner, W. Hoeppner, EMQN best practice guidelines for molecular genetic testing and reporting of 21-hydroxylase deficiency. Eur J Hum Genet **28**, 1341–1367 (2020). https://doi.org/10.1038/s41431-020-0653-5
15. G.P. Finkielstain, W. Chen, S.P. Mehta et al., Comprehensive genetic analysis of 182 unrelated families with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. J Clin Endocrinol Metab **96**, E161–E172 (2011). https://doi.org/10.1210/jc.2010-0319
16. S.E. Bulun, AROMATASE DEFICIENCY. Fertil. Steril. **101**, 323–329 (2014). https://doi.org/10.1016/j.fertnstert.2013.12.022
17. F. Dursun, S. Ceylaner, A Novel Homozygous CYP19A1 Gene Mutation: Aromatase Deficiency Mimicking Congenital Adrenal Hyperplasia in an Infant without Obvious Maternal Virilisation. J Clin Res Pediatr Endocrinol **11**, 196–201 (2019). https://doi.org/10.4274/jcrpe.galenos.2018.2018.0140
18. N. Reisch, W. Högler, S. Parajes et al., A Diagnosis Not to Be Missed: Nonclassic Steroid 11β-Hydroxylase Deficiency Presenting With Premature Adrenarche and Hirsutism. The Journal of Clinical Endocrinology & Metabolism **98**, E1620–E1625 (2013). https://doi.org/10.1210/jc.2013-1306
19. D. Pignatelli, B.L. Carvalho, A. Palmeiro et al. (2019) The Complexities in Genotyping of Congenital Adrenal Hyperplasia: 21-Hydroxylase Deficiency. Front Endocrinol (Lausanne) 10:. https://doi.org/10.3389/fendo.2019.00432

20. R. Khajuria, R. Walia, A. Bhansali, R. Prasad, The spectrum of CYP21A2 mutations in Congenital Adrenal Hyperplasia in an Indian cohort. Clin. Chim. Acta. **464**, 189–194 (2017). https://doi.org/10.1016/j.cca.2016.11.037
21. A. Asanuma, T. Ohura, E. Ogawa et al., Molecular analysis of Japanese patients with steroid 21-hydroxylase deficiency. J. Hum. Genet. **44**, 312–317 (1999). https://doi.org/10.1007/s100380050167
22. F. Baş, H. Kayserili, F. Darendeliler et al., CYP21A2 Gene Mutations in Congenital Adrenal Hyperplasia: Genotype – phenotype correlation in Turkish children. J Clin Res Pediatr Endocrinol **1**, 116–128 (2009). https://doi.org/10.4008/jcrpe.v1i3.49
23. R. Mathur, P.S. Menon, M. Kabra et al., Molecular characterization of mutations in Indian children with congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency. J. Pediatr. Endocrinol. Metab. **14**, 27–35 (2001)
24. E. Marumudi, A. Sharma, B. Kulshreshtha et al., Molecular genetic analysis of CYP21A2 gene in patients with congenital adrenal hyperplasia. Indian J Endocrinol Metab **16**, 384–388 (2012). https://doi.org/10.4103/2230-8210.95679
25. D.V. Chi, T.H. Tran, D.H. Nguyen et al., Novel variants of CYP21A2 in Vietnamese patients with congenital adrenal hyperplasia. Mol Genet Genomic Med **7**, e623 (2019). https://doi.org/10.1002/mgg3.623
26. T.M. Espinosa Reyes, T. Collazo Mesa, P.A. Lantigua Cruz et al., Molecular diagnosis of patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. BMC Endocr Disord **20**, 165 (2020). https://doi.org/10.1186/s12902-020-00643-z
27. M. Lako, S. Ramsden, R.D. Campbell, T. Strachan, Mutation screening in British 21-hydroxylase deficiency families and development of novel microsatellite based approaches to prenatal diagnosis. J. Med. Genet. **36**, 119–124 (1999)
28. P.W. Speiser, J. Dupont, D. Zhu et al., Disease expression and molecular genotype in congenital adrenal hyperplasia due to 21-hydroxylase deficiency. J. Clin. Invest. **90**, 584–595 (1992). https://doi.org/10.1172/JCI115897
29. A. Wedell, A. Thilén, E.M. Ritzén et al., Mutational spectrum of the steroid 21-hydroxylase gene in Sweden: implications for genetic diagnosis and association with disease manifestation. J Clin Endocrinol Metab **78**, 1145–1152 (1994). https://doi.org/10.1210/jcem.78.5.8175971
30. C. Xu, W. Jia, X. Cheng et al., Genotype-phenotype correlation study and mutational and hormonal analysis in a Chinese cohort with 21-hydroxylase deficiency. Mol Genet Genomic Med **7**, e671 (2019). https://doi.org/10.1002/mgg3.671
31. M.I. New, M. Abraham, B. Gonzalez et al., Genotype–phenotype correlation in 1,507 families with congenital adrenal hyperplasia owing to 21-hydroxylase deficiency. Proc Natl Acad Sci U S A **110**, 2611–2616 (2013). https://doi.org/10.1073/pnas.1300057110
32. C.N. Greene, S.K. Cordovado, D.P. Turner et al., Novel method to characterize CYP21A2 in Florida patients with congenital adrenal hyperplasia and commercially available cell lines. Molecular Genetics and Metabolism Reports **1**, 312–323 (2014). https://doi.org/10.1016/j.ymgmr.2014.07.002
33. M. New, M. Yau, O. Lekarev et al., Congenital Adrenal Hyperplasia, in *Endotext*, ed. by K.R. Feingold, B. Anawalt, A. Boyce et al. (MDText.com, Inc., South Dartmouth (MA), 2000)
34. M.I. New, O. Lekarev, D. Mancenido et al. (2014) Congenital Adrenal Hyperplasia Owing to 21-Hydroxylase Deficiency. Genetic Steroid Disorders. Elsevier, pp. 29–51
35. F. Hannah-Shmouni, W. Chen, D.P. Merke, Genetics of Congenital Adrenal Hyperplasia. Best Pract Res Clin Endocrinol Metab **23**, 181–192 (2009). https://doi.org/10.1016/j.beem.2008.10.014
36. N. Krone, A. Braun, A.A. Roscher et al., Predicting phenotype in steroid 21-hydroxylase deficiency? Comprehensive genotyping in 155 unrelated, well defined patients from southern Germany. J Clin Endocrinol Metab **85**, 1059–1065 (2000). https://doi.org/10.1210/jcem.85.3.6441
37. R.C. Wilson, A.B. Mercado, K.C. Cheng, M.I. New, Steroid 21-hydroxylase deficiency: genotype may not predict phenotype. J Clin Endocrinol Metab **80**, 2322–2329 (1995). https://doi.org/10.1210/jcem.80.8.7629224
38. P. Gangodkar, V. Khadilkar, P. Raghupathy et al. (2020) Clinical application of a novel next generation sequencing assay for CYP21A2 gene in 310 cases of 21- hydroxylase congenital adrenal hyperplasia from India. Endocrine. https://doi.org/10.1007/s12020-020-02494-z
39. M. Karaoğlan, G. Nacarkahya, E.H. Aytaç, M. Keskin, Challenges of CYP21A2 genotyping in children with 21-hydroxylase deficiency: determination of genotype-phenotype correlation using next generation sequencing in Southeastern Anatolia. J. Endocrinol. Invest. (2021). https://doi.org/10.1007/s40618-021-01546-z
40. N. Krone, I.T. Rose, D.S. Willis et al., Genotype-Phenotype Correlation in 153 Adult Patients With Congenital Adrenal Hyperplasia due to 21-Hydroxylase Deficiency: Analysis of the United Kingdom Congenital Adrenal Hyperplasia Adult Study Executive (CaHASE) Cohort. J Clin Endocrinol Metab **98**, E346–E354 (2013). https://doi.org/10.1210/jc.2012-3343
41. S. Yadav, S. Birla, E. Marumudi et al., Clinical profile and inheritance pattern of CYP21A2 gene mutations in patients with classical congenital adrenal hyperplasia from 10 families. Indian J Endocrinol Metab **19**, 644–648 (2015). https://doi.org/10.4103/2230-8210.163191

Tables

Table 1: Details of the Allele Specific PCR for screening eight hotspot mutations in CYP21A2 gene

Hotspot mutation	P30L	I2G	8bp Deletion	I172N	E6 cluster	V281L	Q318X	R356W
Product size (Bp)	1027	453	806	765	502	662	931	955

PCR conditions

Stage 1 × 1	Initial denaturation	95□C	5 minutes
Stage 2 × 20	Denaturation	98□C	10 seconds
	Annealing	68□C 70□C (for I2G only)	30 seconds
	Extension	72□C	1 minute
Stage 3 × 1	Final extension	72□C	5 minutes

Table 2a: Details of NGS variants identified in CYP21A2 and CYP19A1 genes

ject D	Phenotype	Gene Symbol	HGVS Nomenclature	HGVS_ Predicted_Protein change	Genotype	Exon/ Intron position	Type of variant	No. of pathogenic predictions available on Varsome	Novel/reported	MAF in South Asians	No of heterozygotes reported	ACMG classification
30	SW CAH	CYP21A2	NM_000500.7:c.1274G>T	NP_000491.4:p.(Gly425Val)	homozygous	Exon 10	missense	17 out of 18	Novel			Likely Pathogenic PM2, PM5, PI PP3
31, 32	SW CAH	CYP21A2	NM_000500.7:c.1451G>C	NP_000491.4:p.(Arg484Pro)	homozygous	Exon 10	missense	12 out of 17	rs200005406	0.000101	3	Likely Pathogenic PM1, PM2, PP2,PP3
47	SV CAH											
50	NCCAH	CYP21A2	NM_000500.7:c.143A>G	NP_000491.4:p.(Tyr48Cys)	homozygous	Exon 1	missense	8 out of 20	rs566306310	0.0000363	1	Variant of uncertain significanc PM1, PM2, PI
3	SW CAH	CYP21A2	NM_000500.7:c.1042G>A	NP_000491.4:p.(Ala348Thr)	heterozygous	Exon 8	missense	17 out of 18	Novel			Likely Pathogenic PM1, PM2, PI PP3
46	SV CAH	CYP19A1	NM_000103.3:c.1142A>T	NP_000094.2:p.(Asp381Val)	homozygous	Exon 9	missense	16 out of 21	rs773015145	0.0000653	2	Variant of uncertain significanc PM2,PP3,BP

Table 2b: Details of NGS variants identified in CYP11B1 gene of subjects with 11 β – OH deficiency

ject D	HGVS Nomenclature	HGVS_ Predicted_Protein change	Genotype	Exon/ Intron position	Type of variant	No. of pathogenic predictions available on Varsome	Novel/reported	MAF in South Asians	No of heterozygotes reported	ACMG classification (9)
51 ıd 55	NM_000497.3:c.1201-1G>A	-	homozygous	Intron 7	splice variant	6 out of 7	novel			Likely Pathogenic PVS1, PM2 , PP3
52	NM_000497.3:c.1200+1del	-	homozygous	Intron 7	splice variant	not available	novel			Likely Pathogenic PVS1, PM2 , PP3
53	NM_000497.3:c.623G>A	NP_000488.3:p.(Arg208Gln)	heterozygous	Exon 4	missense	14 out of 21	rs200559974	not reported in South Asians		Variant of uncertain significance PM2, PP2, PP3
53	NM_000497.3:c.412C>T	NP_000488.3:p.(Arg138Cys)	heterozygous	Exon 3	missense	10 out of 21	rs764251434	0.0000989	3	Likely Pathogenic PM1, PM2, PP2, PP3
56			homozygous							
54	NM_000497.3:c.1024C>T	NP_000488.3:p.(Gln342Ter)	heterozygous	Exon6	nonsense	4 out of 9	novel			Pathogenic PVS1, PM2 , PP3
	NM_000497.3:c.1012dup	NP_000488.3:p.(Gln338ProfsTer16)	heterozygous	Exon6	duplication	not available	novel			Pathogenic PVS1, PM2 , PP3

Table 3: Genotype - phenotype correlation in subjects with 21-hydroxylase deficiency

HENOTYPE	Total	GENOTYPE*						
		Null	Group A	Group B	Group C	Unclassified	Incomplete	Inconclusive
alt Wasting	40	16	12	2	1	4	5	
ıple Virilizing	20		4	9	1	1	4	1
on classical	4		2			1		1
Total	64	16	18	11	2	6	9	2

Subject C46 with CYP19A1 variant and subject C65 negative for 5 genes were not included in the above table.

****Description of alleles*** [35, 40]

Null mutations (0% enzyme activity) - loss of function mutations - 30kb deletion, large rearrangement, 8BP DEL, E6 CLUS and R356W

GROUP A (less than 1 % enzyme activity) - I2G in homozygous state, compound heterozygous with null group mutations

GROUP B (1-2% enzyme activity) - I172N in homozygous/ compound heterozygous with group A mutation

GROUP C (20-60% enzyme activity) - P30L or V281L in compound heterozygous with group A/group B mutations

UNCLASSIFIED- Q318X/NGS, NGS variants

INCOMPLETE - Parental screening is pending

INCONCLUSIVE - positive only for a single heterozygous mutation

Table 4: Comparison of the present study to previously reported results from India

Study	Molecular techniques	Genes analyzed	Diagnostic yield% (sample size)	Predominant mutation in CYP21A2 gene
Mathur, R., et al ,2001	ASPCR for 6 hotspots	CYP21A2	78 (n=28)	I172N
Marumudi et al,2012	Genotyping with restriction digestion	CYP21A2	74.2 (n=62)	I2G
khajuria et al, 2017	Nested PCR, SSCP, RFLP	CYP21A2	96.4 (n=55)	I2G
Yadav, Sarita, et al, 2015	Genotyping with restriction digestion	CYP21A2	100 (n=13)	P30L
Gangodkar et al, 2020	NGS	CYP21A2	82.6 (n=310)	I2G
Present study	ASPCR, NGS	CYP21A2, CYP11B1, CYP17A1, CYP19A1, POR	97.2 (n=72)	I2G

References: [20, 23, 24, 38, 41]

Figures

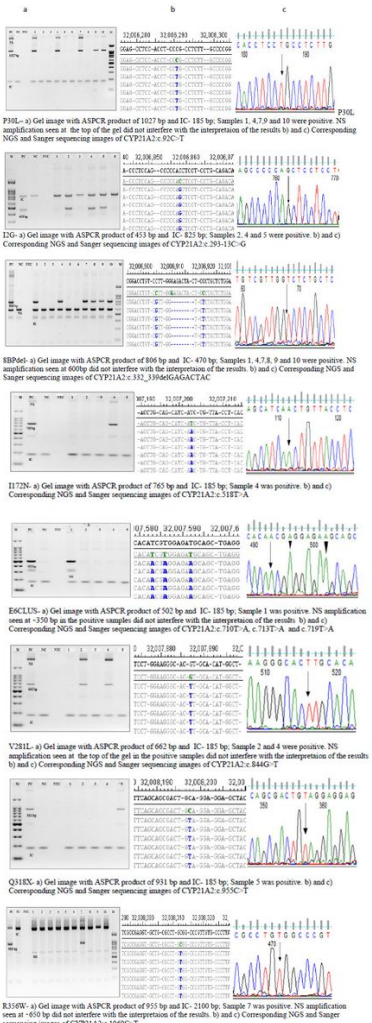


Figure 1

Gel images of ASPCR results, NGS alignments and chromatogram of Sanger sequencing results for the 8 CYP21A2 hotspot mutations screened a) 2% agarose gel showing ASPCR results for P30L, I2G, 8BPDEL, I172N, E6 CLUS, V281L, Q318X and R356W hotspot screening of different samples with appropriate Positive Control (PC), Negative Control (NC) and a No Template control (NTC - To rule out reagent contamination) run with mutant primers. IC indicates internal control, 1 to n represents 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 bands did not interfere with the identification of samples positive and negative for ASPCR. Each Allele specific product was clearly differentiated in positive and negative control to which the test samples were compared. b) and c) NGS results and Chromatogram of Sanger validation of 8 hotspot mutations showing the same hotspot mutation corresponding to ASPCR. The chromosome coordinates of the NGS results indicate alignment of the reads to CYP21A2 gene and not to the pseudogene CYP21A1P.

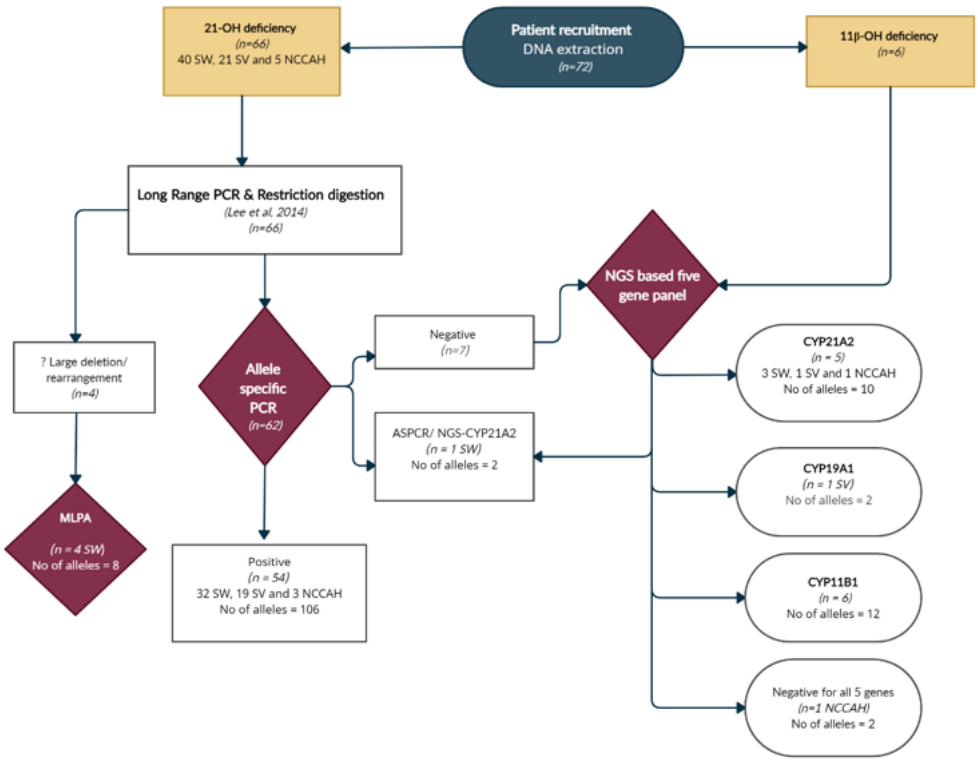
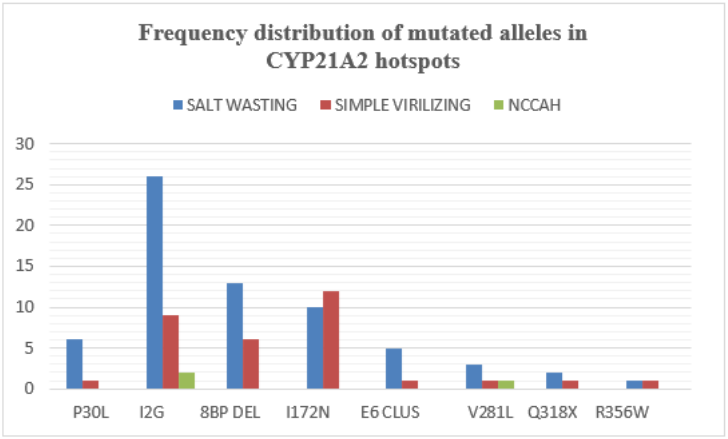
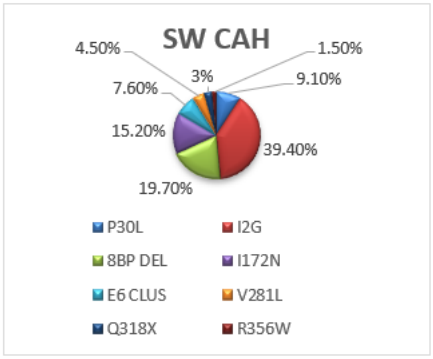


Figure 2

Methodology and results of comprehensive genetic testing for 5 genes in 72 subjects with Congenital Adrenal Hyperplasia





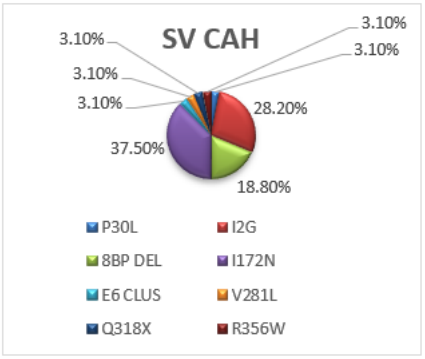


Figure 3

Frequency distribution of mutated alleles with CYP21A2 hotspots