Review Article

# Congenital Adrenal Hyperplasia – A Comprehensive Review of Genetic Studies on 21-Hydroxylase Deficiency from India

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## Abstract

Congenital adrenal hyperplasia (CAH) comprises a heterogeneous group of autosomal recessive disorders impairing adrenal steroidogenesis. Most cases are caused by mutations in the *CYP21A2* gene resulting in 21-hydroxylase (21-OH) deficiency (21-OHD). The genetics of 21-OH CAH is complexed by a highly homologous pseudogene *CYP21A1P* imposing several limitations in the molecular analysis. Therefore, genetic testing is still not a part of routine CAH diagnosis and is mainly dependent on 17-hydroxy progesterone (OHP) measurements. There are very few reports of *CYP21A2* gene analysis from India and there is no comprehensive review available on genetic testing and the spectrum of *CYP21A2* mutations from the country. This review focuses on the molecular aspects of 21-OHD and the genetic studies on *CYP21A2* gene reported from India. The results of these studies insist the compelling need for large-scale *CYP21A2* genetic testing and newborn screening (NBS) in India. With a high disease prevalence and consanguinity rates, robust and cost-effective genetic testing for 21-OH CAH would enable an accurate diagnosis in routine clinical practice. Whereas establishing affordable genotyping assays even in secondary care or resource-poor settings of the country can identify 90% of the mutations that are pseudogene derived, initiatives on reference laboratories for CAH across the nation with comprehensive genetic testing facilities will be beneficial in those requiring extended analysis of *CYP21A2* gene. Further to this, incorporating genetic testing in NBS and carrier screening programmes will enable early diagnosis, better risk assessment and community-based management.

**Keywords:** 21-hydroxylase deficiency, congenital adrenal hyperplasia, *CYP21A2*, genetic testing, India

## Introduction

Adrenal corticosteroids and androgens are involved in several metabolic and direct cellular functions under the strenuous regulation of hypothalamic–pituitary adrenal (HPA) axis. Faulty steroidogenesis may result in several disorders, out of which congenital adrenal hyperplasia (CAH) is the major cause of primary adrenal insufficiency.[1] These enzyme deficiencies result in a clinical spectrum of abnormalities that ranges from atypical genitalia and infertility to severe life-threatening conditions.[2] The enzyme 21-hydroxylase (21-OH), critical for cortisol synthesis when impaired, accounts for 90% of patients with CAH.[3]

Although CAH is a monogenic disorder, the phenotypic manifestations are highly heterogeneous. Based on clinical severity, CAH is classified into the classical form: salt wasting (SW), simple virilizing (SV) and the non-classical forms. The classical SW is the most severe form, which expresses itself early in life with severe electrolyte imbalance and shock.[4] Followed by this is the SV type with atypical genitalia and a markedly increased secretion of adrenal androgens.[5] The non-classical type (NCCAH), which has been referred to as late-onset CAH (LOCAH), bears similarity to the SV type manifesting peri-pubertally with hirsutism, oligomenorrhoea or virilization in girls and, at times, diagnosed during evaluation for infertility later in life.

## Clinical Diagnosis

In newborns presenting with SW crisis or atypical genitalia and adolescents or adults presenting with oligomenorrhoea,

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amenorrhoea, hirsutism, or infertility, hormonal testing is usually carried out for diagnosis. Elevated 17-hydroxy progesterone (17-OHP), the major substrate for 21-hydroxylase (21-OH), is the hallmark for diagnosis of CAH due to 21-hydroxylase deficiency (21-OHD). Measurement of basal 17-OHP early in the morning is usually done for CAH screening, and a value of >10 ng/ml is diagnostic of 21-OHD, while a value less than 2ng/ml excludes 21-OHD.[6] An intermediate value of 2–10 ng/ml requires cosyntropin stimulation. First-tier newborn screening (NBS) is done by measuring 17-OHP in dried blood spots by heel prick after 24–74 hours of life. Preterm newborns should preferably be screened 2–4 weeks after birth before discharge. The diagnostic cut-offs in neonates born preterm weighing <2.5 kg are 53.5 ng/ml (≤32 weeks) and 27.7 ng/ml (32–36 weeks) and in those weighing ≥2.5 kg, the cut-offs are 33.7 ng/ml (≤32 weeks) and 24.7 ng/ml (32–36 weeks). The cut-off is ≥24.7 ng/ml for those born at term.[7] Those whose mothers received glucocorticoids during the antenatal period have to be tested again after 2 weeks. However, 17-OHP measured by ELISA yields high false-positive results due to assay interference by other steroid intermediates.[8] Therefore, in most cases, second-tier confirmation with liquid chromatography-mass spectrometry (LC-MS/MS) is carried out.[6,9] Adrenocortical steroid profiling post cosyntropin stimulation is recommended when 17-OHP values are borderline to biochemically confirm 21-OHD and to differentiate from other enzyme defects.[9,10] Commercially available adrenal steroid profiling in India includes cortisol (µg/dl), 17-OHP (ng/dl), testosterone (ng/dl), androstenedione (ng/dl), Dehydroepiandrosterone sulfate (DHEAS) (µg/dl), DHEA (ng/ml), progesterone (ng/ml), 11-deoxycortisol (ng/dl), corticosterone (ng/dl), estradiol (pg/ml) and aldosterone (ng/dl).[11] Also, several studies have evaluated the utility of 21-deoxycortisol in the diagnosis of CAH, suggesting it as the key biochemical marker in replacement of 17-OHP measurements.[12-14] Table 1 summarizes the biochemical profiling in 21-OHD and other forms of CAH.[15]

## Genetics of 21-Hydroxylase Deficiency

Steroid 21-OH, a member of the cytochrome p450 family, is encoded by the 3.2 kb long *CYP21A2* (cytochrome P450 family 21 subfamily A member 2) gene with 10 exons on chromosome 6 with a complex tandem arrangement in the MHC class III region. Located 30 kb away from its non-processed pseudogene *CYP21A1P* (cytochrome P450 family 21 subfamily A member 1 pseudogene), it alternates with *C4B* and *C4A*, *RP1 (STK19)* and *RP2 (STK19B),* and *TNXA* and *TNXB* genes.[16] This region comprising the RCCX module can be monomodular, bimodular, or trimodular in genomic arrangement.[17] A non-functional pseudogene (98% homologous to the functional gene) in the bimodular variation of the RCCX module forms the ground for unequal crossing over and gene conversion events resulting in disease-causing mutations.[18] The pseudogene itself is functionally inactivated by several pathogenic mutations within its sequence.

Upto 95% of mutations in 21 OHD are transferred from pseudogene to functional gene through non-allelic homologous recombination.[19,20] These are broadly grouped into two: (1) structural rearrangement from unequal meiotic crossing over; this facilitates large deletions, duplications and chimeras in up to 30% of the cases.[21,22] The 30 kb deletion is the most frequent and leads to chimeric genes. Nine different chimeras (CH1–CH9) have been identified so far, seven of which are classical chimeras associated with classical forms and the other two are attenuated resulting in non-classical forms. Followed by large deletions are gene conversions mediating comparatively shorter sequence transfers while other rearrangements are less prevalent.[23] (2) Point mutations by micro conversion; several point mutations are passed on from pseudogene to functional gene during micro conversion in meiosis and is reported in 70–75% of the cases.[24] Such recurrent micro conversion mutations include I2G splice mutation, P30L, 8BPDEL, I172N, E6CLUS (I235N, V236E, and M238K), V281L, Q318X, and R356W. Figure 1 depicts the molecular mechanisms and the most common pseudogene-derived mutations in 21-OHD.

Besides this, around 240 variants have been identified housing several Single-nucleotide variants (SNVs), small deletions, insertions and complex rearrangements.[25] *De novo* mutations are usually rare and reported in less than 5% of the cases.[21] Founder effect in non-pseudogene-derived mutations has also been observed.[26,27]

**Table 1: Summary of biochemical profiling in different forms of CAH, adapted from Yau M, 2000**

| Type of CAH | Biochemical profile |
|---|---|
| Classical 21-OH deficiency, salt wasting | Elevated 17-OHP, DHEA and androstenedione, elevated K, low Na, $CO_2$ |
| Classical 21-OH deficiency, simple virilizing | Elevated 17-OHP, DHEA and androstenedione, normal electrolytes |
| Non-classical 21-OH deficiency | Elevated 17-OHP, DHEA and androstenedione on ACTH stimulation |
| Classical CAH 11β-deficiency | Elevated DOC, 11-deoxycortisol (S); androgens, low K, elevated Na, $CO_2$ |
| Non-classical CAH 11β-deficiency | Elevated 11-deoxycortisol±DOC, elevated androgens |
| 17α-OH deficiency | Normal or decreased androgens and oestrogen, elevated DOC, corticosterone |
| 17,20-Lyase deficiency | Decreased androgens and oestrogens |
| 3β-HSD deficiency, classical | Elevated DHEA, 17-pregnenolone, low androstenedione, testosterone, elevated K, low Na, $CO_2$ |
| 3β-HSD deficiency, non-classical | Elevated DHEA, 17-pregnenolone, low androstenedione, testosterone |
| P450 oxidoreductase deficiency | Partial, combined and variable defects of P450c21, P450c17 and P450 aromatase activity |
| Lipoid CAH | All steroid products low |

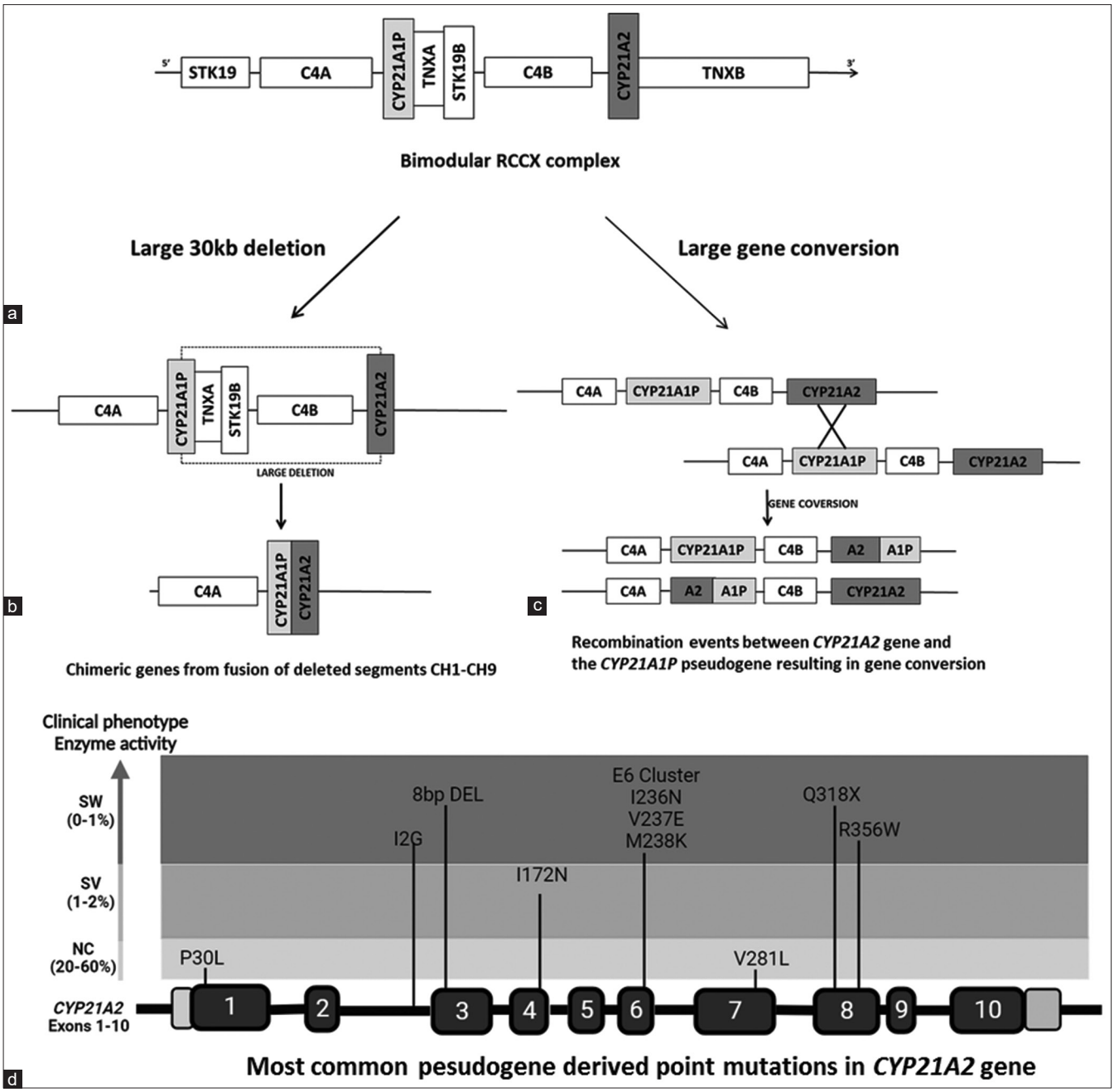



**Figure 1:** Schematic representation of molecular mechanisms in 21-OH deficiency. a) Bimodular RCCX complex with *CYP21A2, C4B, TNXB* and *STK19B* genes. b) Large 30 kb deletion encompassing 5′ of *CYP21A1P* and 3 ′ of *CYP21A2* genes. The remaining segment of these two genes fuse together to form different chimeric genes. c) Large gene conversion replacing functional gene sequence partly or completely with that of pseudogene. d) The most common pseudogene derived hotspot mutations in *CYP21A2* gene

## Genotype–Phenotype Correlation

CAH mutations inherited in an autosomal recessive manner are mostly compound heterozygous.[28] These mutations are grouped into four based on clinical severity and functional activity. The 'null' mutations result in absent *in vitro* activity, group A with 1% activity, group B with 2% activity and group C retaining 10–20% activity.[29] The mutation spectrum includes Q318X, R356W, E6CLUS and 8BPDEL of the Null group (SW), I2G of group A, I172N and I177T of group B (SV) and V281L, P30L and P453S of group C (NC).[30] Null and group A mutations principally promote a SW phenotype with high clinical severity. Following this, group B mutations comprise the SV phenotype and group C produces the mildly affected non-classical type.[10] SW mutations induce adverse effects on membrane anchoring and enzyme stability with the loss of 21-OH function. On the other hand, SV mutations negatively impact the transmembrane region and hydrophobic residues with 2% enzyme activity, whereas those that cause a mild NC type interfere with the oxidoreductase function.[31] Direct genotype–phenotype concordance has been observed in

90% of the cases.[32] Phenotypic variability has been observed in I2G, I172N and P30L mutations.[28,29]

## Molecular Analysis of *CYP21A2* Gene

Molecular analysis of *CYP21A2* gene is highly advantageous in overcoming the pitfalls with biochemical testing of 17-OHP to confirm the diagnosis; enable carrier screening, prenatal diagnosis (PND), and NBS; and offer genetic counselling. However, genotyping *CYP21A2* gene is complicated by pseudogene-derived complex rearrangements and deletions.[33] Also, parental screening is often required to differentiate compound heterozygote variants in these rearrangements.[34] There are very few reports on molecular analysis of *CYP21A2* gene from India. Most of the studies have focused on genotyping the common pseudogene-derived mutations[30,35-38] whereas few others have focused on complete gene analysis.[39-41] Table 2a explains the various molecular methods adapted and the most common mutations reported from Indian subjects. There is a wide range of mutations noticed across the studies that could be due to differences in sample size and the various techniques adapted. Conducting CAH genetic studies in large cohorts in the country will thus validate the results and identify the actual spread of these common mutations. Table 2b is a comparison of different techniques utilized in India and their diagnostic yield in CAH to other reported studies across the world.

The techniques adapted by the Indian authors and their pros and cons are discussed next.

## *CYP21A2* Gene Amplification

The key challenge in the molecular diagnosis of 21-OHD is specific amplification of the functional gene *CYP21A2*. The functional and the pseudogene differ by only 65 nucleotides in the genomic sequence.[48] This complicates the primer designing to achieve specific amplification of the functional gene. The most routinely used primers for *CYP21A2* amplification include a specific sequence in exon 3 and exon 6.[49] However, a large deletion or a mutation in the primer binding site often results in failure of polymerase chain reaction (PCR) amplification. Alternatively, long-range PCRs are utilized to specifically amplify the functional and pseudogene separately.[50] Following the PCR amplification, restriction digestion can also give clues for large gene conversion and gene deletion.[41] Another strategy is to use four primer sets for locus-specific amplification of *CYP21A2*, *CYP21A1P*, 30 kb deletion and gene conversion.[51]

## Methods for Identification of Pseudogene-Derived Point Mutations

### *Allele-specific PCR (ASPCR)*

By designing primers with wild-type and mutant-specific 3′-terminal mismatches, this technique enables the identification of known mutations in two separate PCR reactions. For the I2G splice mutation, possessing two wild-type and one mutant allele, three PCR reactions are required. It is simple, cost effective and less time-consuming and does not require digestive enzymes or radioactive probes. On the other hand, it involves challenges in primer designing and requires careful standardization to avoid false-positive and negative results. Two studies from India have adapted ASPCR to identify the most common pseudogene-derived pathogenic mutations.[37,41]

### *Restriction digestion*

For genotyping the common mutations, the amplified *CYP21A2* gene product is treated with specific restriction enzymes for each mutation. Many pseudogene-derived hotspot mutations may create or remove restriction sites. However, incomplete digestion can limit the sensitivity.[34] The restriction enzymes for *CYP21A2* hotspot mutations reported in Indian studies include Hha 1 for P30L and IG, Taq 1 for I172N, ApaL 1 for V281L and R339H, Pst 1 for Q318X, Fnu4 H1 for R356W and Hha1 for P450Ser.[36]

### *Single-stranded conformational polymorphisms (SSCP)*

This technique enables the identification of unknown mutations by assessing changes in DNA conformation. The target gene is amplified with conventional PCR. The amplified fragments are denatured and allowed to re-anneal. When run on agarose gels, even a single base change can cause changes in the conformational pattern and a subsequent shift in the electrophoretic mobility. SSCP has been used to identify non-pseudogene-derived novel mutations in the *CYP21A2* gene.[40] Nevertheless, it is less sensitive for larger fragments and is largely replaced by newer techniques.

### *Large deletion and duplication analysis*

Primary amplification of *CYP21A2* and *CYP21A1P* with long-range PCR, followed by restriction digestion, could yield differential PCR product sizes or digested patterns that provide initial clues for large rearrangements and gene conversion. Multiplex ligation-dependent probe amplification (MLPA), being the gold standard technique for analysing copy number variations, can be utilized for validation of these large deletions and rearrangements in 21-OHD. These assays collectively have replaced Southern blotting for genetic diagnosis of CAH.[21] However, results acquired with the combination of techniques require careful interpretation to identify the actual extent of rearrangement and the resulting chimeric genes. Also, differentiating these chimeras into classic and attenuated is essential for genotype–phenotype correlation.[49]

### *Direct sequencing*

Following preliminary amplification of *CYP21A2* gene, direct sequencing has been mostly employed to detect known hotspot mutations and novel variants and to identify the extent of rearrangements. However, with the advent of next-generation sequencing (NGS) technology, targeted gene/panel sequencing in CAH with these strategies is being evaluated. In terms of clinical utility, there is a need for establishing robust computational pipelines to overcome challenges associated with the data analysis of *CYP21A2* gene.[39,41] The application, pros and cons of these different techniques are given in Table 3.

**Table 2a: Molecular methods adapted by Indian authors and the results on the most common *CYP21A2* mutations from India**

| Study | Mathur *et al.* | Marumudi *et al.* | Yadav *et al.* | Ganesh *et al.* | Khajuria *et al.* | Nageshwari *et al.* | Gangodkar *et al.* | Ravichandran *et al.* | Monteiro *et al.* |
|---|---|---|---|---|---|---|---|---|---|
| Year of publication | 2001 | 2012 | 2015 | 2015 | 2017 | 2017 | 2020 | 2021 | 2023 |
| Sample size | 28 | 62 | 13 | 10 | 55 | 11 | 310 | 72 | 46 |
| Techniques | PCR-based method to identify gene deletion and ASPCR for 5 common mutations | Restriction digestion for 9 common mutations | Restriction digestion for 8 common mutations | Not described | PCR-based method to identify gene deletion, nested PCR for 7 common mutations and SSCP for novel mutations | Restriction digestion for 9 common mutations | PCR-based method to identify 30 kb deletion and NGS for common and novel mutations | ASPCR for common mutations and NGS for extended screening | MLPA and NGS |
| % mutations identified | 78.6 | 74.2 | 100 | 60 | 96.4 | 100 | 86.8 | 97.2 | 100 |
| P30l | 2.2 | 46 | 23.1 | | | 100 | | 12.2 | |
| I2G | 27.2 | 48 | 15.4 | 33.3 | 20 | 36.3 | 29 | 67.6 | 35.8 |
| 8BPDEL | NA | 26 | 7.6 | 66.7 | 12.7 | 9 | 3.9 | 38.5 | 7.7 |
| I172N | 31.8 | 26 | | | 12.7 | 72.7 | 14.1 | 52.7 | |
| E6CLUS | NA | NA | NA | | 5.4 | NA | | 10.7 | |
| V281L | NA | 0 | | | 3.6 | 0 | | 7.6 | |
| Q318X | 22.7 | 35 | | | 9 | 90 | 12.5 | 6.1 | |
| R339H | NA | 0 | NA | | | 0 | | | |
| R356W | 0 | 20 | | | 14.5 | 18.2 | | 4.6 | 6.6 |
| P453S | NA | 0 | | | | 81.8 | | | |
| P267L | | | | | 1.8 | NA | | | |
| L308fs/F306+T | | | | | 5.4 | | | | |
| Gene deletion/30 kb deletion | 15.9 | NA | NA | | 3.6 | | 18.2 | 16.7 | 35.8 |
| Compound heterozygous for different mutations | | | 53.8 | | | | | | |
| Other rearrangements | | NA | NA | | | NA | 6.5 | | |
| Frequent genotype | I172N | I2G | p30l | 8bp del | I2G | p30l | I2G | I2G | 30 del/ I2G |

**Table 2b: Comparison of molecular techniques and the diagnostic yield reported in Indian studies to other reports across the world**

| Genetic study | Year of publication | Country | Genetic testing strategy | Diagnostic yield (%) | Sample size (*n*) | Reference |
|---|---|---|---|---|---|---|
| *Mathur, et al*. | 2001 | India | ASPCR | 78 | 28 | [37] |
| *Marumudi et al*. | 2012 | India | Restriction digestion | 74.2 | 62 | [36] |
| *Yadav, et al*. | 2015 | India | Restriction digestion | 100 | 13 | [38] |
| *Khajuria et al*. | 2017 | India | Nested PCR, SSCP, RFLP | 96.4 | 55 | [40] |
| *Gangodkar et al*. | 2020 | India | NGS | 82.6 | 310 | [39] |
| *Ravichandran et al*. | 2021 | India | ASPCR, NGS | 96.4 | 72 | [41] |
| *Monteiro et al.* | 2023 | India | MLPA, NGS | 100 | 46 | [42] |
| *De Carvalho et al*. | 2016 | Brazil | MLPA, Sanger sequencing, ASPCR | 91.75 | 480 | [26] |
| *Somasundaram et al*. | 2020 | Sri Lanka | ASPCR | 100 | 30 | [43] |
| *Marino et al*. | 2011 | Argentina | ASPCR, RFLP, Sanger sequencing, Southern blotting | 95.3 | 454 | [44] |
| *Krone et al*. | 2000 | South Germany | Sanger sequencing, Southern blotting | 98.7 | 155 | [29] |
| *Espinosa Reyes et al*. | 2020 | Cuba | 2-phase PCR | 56 | 55 | [45] |
| *Xia et al*. | 2022 | China | MLPA, Sanger sequencing | 100 | 155 | [46] |
| *Concolino et al*. | 2023 | Italy | MLPA, Sanger sequencing | 100 | 245 | [47] |

NGS=Next-generation sequencing; MLPA=Multiplex ligation-dependent probe amplification; ASPCR=Allele-specific polymerase chain reaction; RFLP=Restriction fragment length polymorphism; SSCP=Single-stranded conformational polymorphisms; PCR=Polymerase chain reaction

**Table 3: The utility, pros and cons of different molecular techniques in the analysis of *CYP21A2* gene defects**

| Technique | Targeted mutations | Pros | Cons |
|---|---|---|---|
| ASPCR | Point mutations/ small INDELS | 1. Cost effective<br>2. Rapid<br>3. Less laborious<br>4. Does not require enzymes, radioactive or fluorescent probes<br>5. Feasible in small labs<br>6. Can differentiate chimeric genes in combination with long-range PCR results | 1. Crucial standardization to achieve optimal results<br>2. Can detect only known mutations |
| Restriction digestion | Point mutations | 1. Cost effective<br>2. Less laborious | 1. Requirement of suitable enzymes that create or destroy restriction sites<br>2. Can detect only known mutations |
| Southern blotting | Copy number variations | 1. Can detect CNVs to the whole extent of RCCX module | 1. Potential risk from radioactive biohazard<br>2. Large amounts of DNA<br>3. Tedious<br>4. Cannot determine junction sites of chimeras |
| MLPA | Copy number variations | 1. Does not require primary amplification<br>2. Less time consuming<br>3. High throughput | 1. Duplication masks deletion<br>2. Highly sensitive<br>3. Requires extensive knowledge of RCCX module for interpretation<br>4. Challenges to determine phase of deletion/duplication<br>5. Validation with other methods |
| Sanger sequencing | Point mutations | 1. Cost effective<br>2. Identifies junction sites of chimeras<br>3. Identifies novel mutations | 1. Challenges in interpretation of chromatogram if heterozygous rearrangements present |
| Next-generation sequencing | Point mutations/ small INDELS | 1. Targeted panel of multiple genes sequenced and analysed at the same time<br>2. Identifying novel mutations<br>3. Advantageous in mild and overlapping phenotypes of CAH | 1. Pseudogene-imposed challenges in read alignments<br>2. Knowledge on complex bioinformatics pipelines<br>3. Cannot detect large deletions and duplications<br>4. Requires adequate coverage of target regions |

## Genotype–Phenotype Correlation in Indian studies

The I2G mutation is the most prevalent genotype reported by *Marumudi et al*.[36], *Khajuria et al*.[40], *Ravichandran et al*.[41] and *Gangodkar et al*.[39] in their cohorts. *Yadav et al*.[38] have reported p30L to be predominant in their study population whereas *Mathur et al*.[37] have reported I172N. *Marumdi et al*.[36] have also reported p30L in a relatively high frequency in comparison to other studies (46%).

Although genotype–phenotype correlation is high in SW and NCCAH,[28,52,53] there exists heterogeneity in the genotype and phenotype concordance of 21-OHD. Phenotypic variability is commonly observed in P30L, I2G and I172N mutations. Though P30L is predominantly associated with NCCAH, 30% of classical CAH harbour P30L mutation. I2G, a SW mutation, is seen in 20% of SV patients and I172N of SV group is reported in 25% of SW phenotype.[2] Phenotypic variability has been observed by *Mathur et al*.,[43] *Ravichandran et al*.,[43] *Gangotkar et al*. *and Khajuria et al*.[40] in I2G and I172N mutations. *Khajuria et al*.[40] have reported an NCCAH subject with 8 bp deletion.

## Novel *CYP21A2* Variants Reported from India

Though most of the reports from India focus on the common pathogenic variants, there are few reports identifying novel variants. *Khajuria et al*.[40] have reported two novel missense variants in heterozygous state with other mutations in a cohort of 55 subjects screened using SSCP. *Gangodkar et al*.[39] have identified 9 novel variants in 310 subjects using NGS: 6 missense, 2 frameshift and 1 nonsense variant (4 homozygous and 5 heterozygous variants with known variant on the other allele). *Ravichandran et al*.[41] have reported two novel missense variants using NGS: one homozygous and one compound heterozygous variant. It is interesting to note that 5 out of these 13 novel variants were located in exon 8. The details of these variants are listed in Table 4. Identification of these novel variants is essential to expand the knowledge on the spectrum of *CYP21A2* mutation in the Indian population.

## Genotyping in Pregnancy and Prenatal Diagnosis

Genotyping pregnant women at risk of delivering offspring with CAH is of high importance to enable early diagnosis and may allow medical intervention to avoid virilization of female foetuses. This will also aid in appropriate and timely genetic counselling. The risk of a classical CAH mother having a CAH affected child when the partner's genotype is unknown is 120. Conversely, If the mother is affected with non classical CAH, the risk of the child being affected is 1 in 360 [Table 5].[54] These probabilities are based on the carrier frequency data available from the west. There is no report from India on the carrier frequency of classical and non-classical mutations in healthy individuals. The risk can be much higher due to the high rates of consanguinity in the country.[55] Thus, genetic diagnosis in affected probands and carrier testing in their partners before planning pregnancy will be extremely beneficial. Continuous follow-up of both the partners being carriers or CAH-affected individuals marrying a carrier will benefit in better management of CAH-associated pregnancies and drastically reduce the incidence of CAH in India.

Following genotyping of the parents, PND is carried out with chorionic villi sampling (CVS) or amniocentesis sampling in the growing foetus. Since 17-OHP measurement is generally unreliable if the mother is already on dexamethasone,[56] genotyping is highly advantageous in not only making an accurate diagnosis but also distinguishing between foetus affected with SW, SV, and NCCAH mutations. The PND sample is usually sent for karyotyping and variable number of tandem repeats (VNTR) analysis to check for maternal contamination followed by genotyping of CAH mutations. If the foetus is of 46 XX karyotype and with a positive genotype, prenatal therapy with dexamethasone is possible to prevent virilization.[57] Simultaneously, if the foetus is unaffected, unnecessary administration of dexamethasone can be avoided. *Dubey et al*. in 2017[56] have performed genetic screening in 15 foetuses at risk for CAH using MLPA and Sanger sequencing and identified six foetuses with biallelic mutations, six heterozygote carriers and three healthy foetuses with homozygous wild-type alleles that allowed informed decisions on further management of pregnancy. The use of non-invasive prenatal testing with cell-free DNA that can be done in the fourth week of pregnancy may allow for early clinical intervention.[58,59]

## Newborn Screening in CAH

CAH satisfies the NBS criteria (Wilson and Junger) and is recommended with 17-OHP measurements for first-tier

**Table 4: Novel *CYP21A2* variants reported from India (Transcript ID: NM_000500.9)**

| Study | Codon change | Protein change | Type | Genotype | Clinical phenotype | Functional impact* |
|---|---|---|---|---|---|---|
| *Gangodkar et al*., 2021 | c. 158_159delCT | Gln54GlufsTer25 | Frameshift | Homozygous | SW CAH | Likely pathogenic |
| | c. 274A>G | Arg92Gly | Missense | Homozygous | SW CAH | Likely pathogenic |
| | c. 1118G>A | Ser373Asn | Missense | Heterozygous | SW CAH | Likely pathogenic |
| | c. 1136T>A | Ile379Asn | Missense | Heterozygous | SW CAH | Likely pathogenic |
| | c. 1005_1006delCC | Pro336LeufsTer45 | Frameshift | Homozygous | SW CAH | Likely pathogenic |
| | c. 1112C>A | Pro371His | Missense | Heterozygous | SW CAH | Variant of unknown significance |
| | c. 339C>A | Tyr113Ter | Stop gained | Heterozygous | SW CAH | Likely pathogenic |
| | c. 169G>A | Gly57Arg | Missense | Heterozygous | SV CAH | Likely pathogenic |
| | c. 725T>C | Leu242Pro | Missense | Homozygous | SW CAH | Likely pathogenic |
| *Ravichandran et al*. | c. 1274G>T | Gly425Val | Missense | Homozygous | SW CAH | Likely pathogenic |
| | c. 1042G>A | Ala348Thr | Missense | Heterozygous | SW CAH | Likely pathogenic |
| *Khajuria et al*. | c. 1095C>A | His365Asn | Missense | Heterozygous | NCCAH | Medium |
| | c. 916T>G | Phe306Val | Missense | Heterozygous | NCCAH | Low |

*As reported by the authors

**Table 5: The risk probability of a CAH mother having a CAH child**

| Mother's phenotype | Father's genotype | Probability of child having CAH |
|---|---|---|
| Classical CAH | Unknown | 1 in 120 |
| NCCAH | Unknown | 1 in 360 |

testing.[60] This NBS recommendation is to provide early diagnosis to reduce mortality in severe cases with adrenal crisis, proper gender assignment and prevent the missing of detection of CAH males.[61] First-tier biochemical screening of 17-OHP is usually done by immunoassays like radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA).[62] NBS for CAH has been implemented in all 50 states of America[63] and in most other developing countries. In a screening study of 6.5 million newborns worldwide, the overall incidence of CAH was reported to be 1 in 15,000 live births.[64] The incidence of classical 21-OHD in the general population varies from 1 in 10,000 to 20,000[65] whereas the non-classical incidence is 1 in 1000.[54] The reported prevalence is 1:10,000–16,000 in the United States and Europe, 1:21,000 in Japan and 1:23,000 in New Zealand.[66]

The infant mortality rate (IMR) in India is 30.6 as per recent report from UNICEF.[67] Even with a high IMR, there is no national policy for CAH-NBS in India. There is a pressing need for implementing a uniform nationwide NBS programme for CAH. Several small-scale NBS studies have been carried out using 17-OHP screening in India. The first expanded NBS study was carried out in Hyderabad on 18,300 babies, which showed a prevalence of 1 in 2575.[68] Since then, several studies have been conducted across the country by ICMR task force in which 104,066 newborns were screened.[69] In comparison to the west, the incidence of CAH in India is high – 1 in 5762 – with remarkable regional differences. This study has also reported an incidence of 1 in 6934 in SW CAH and 1 in 20,801 in SV CAH. The highest incidence has been observed in Chennai (1:2036) and the least in Mumbai (1:9983). Another single-centre study has reported an incidence of 1 in 2800 ($n$ = 11,200 newborns screened) in south India.[70] The increased rate of incidence reported in Indian studies in comparison to the west can be attributed to high rate of consanguineous marriages in the country. Following the ICMR pilot study, three states in India – Kerala, Goa and Chandigarh – have initiated government-funded NBS for CAH. These states exhibit an IMR well below the Indian average of 33.[71]

NBS with 17-OHP measurements provide the necessary data suggesting a high incidence of CAH in the country; however, several factors limit the sensitivity of 17-OHP assay, so this assay cannot be considered as a confirmed diagnostic test. Reference range varies based on age, birth weight and sex. Though cord blood for measuring 17-OHP in newborns is non-invasive and easily available, it is not the ideal source as 17-OHP values are significantly higher in the first few days after birth.[72] The recommended sample collection is usually after 24 hours of birth to 7 days. The other factors that limit the sensitivity of 17-OHP results being used as a diagnostic marker are mode of delivery, gestational age and gender.[73] Immature adrenal function in preterm babies, conditions that induce stress and differences in methodologies adapted by the laboratories may lead to false-positive results whereas high exposure of the foetus to maternal cortisol and glucocorticoid treatments before delivery leads to false-negative results.[74] High false-positive rates ranging from 9% to 100% in first-tier screening have been reported in NBS for CAH in Indian studies.[9] This decreases the positive predictive value of first-tier testing,[75] insisting the need for better testing strategies.

## Overcoming the Challenges in CAH-NBS

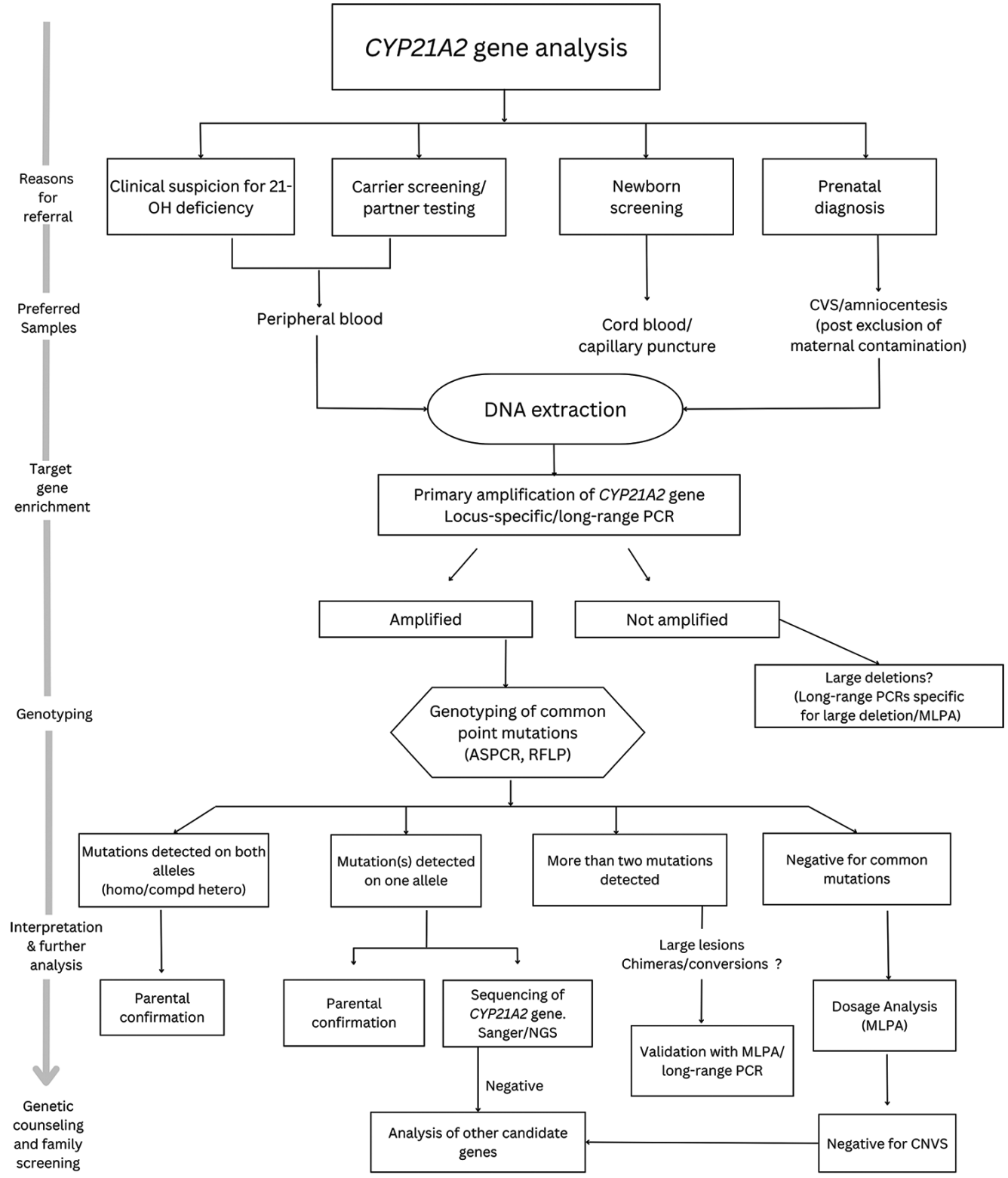
Major concerns associated with undiagnosed early infant deaths and wrong gender assignment in virilized females drive the need for immediate implementation of CAH-NBS in India. However, several logistics like resources, cost effectiveness, and widespread availability of reliable second-tier screening should also be addressed.

To overcome the challenges with 17-OHP testing by LC-MS/MS, genetic testing for the most common mutations (screening for hotspot mutations) can be considered in those with elevated 17-OHP on first-line NBS with a turnaround time of 5–7 days.[55] This can be a suitable alternative for the second-tier LC-MS for accurate and cost-effective diagnosis of 21-OHD.

## Cost-Effective Genetic Testing in CAH

In a developing country like India, cost effectiveness is one of the key factors determining the availability of genetic testing to a larger extent. High CAH prevalence in the country, high consanguinity rates and the importance for genetic testing in diagnosis, carrier screening, PND and NBS drive the need for affordable genetic testing with highly sensitive and specific molecular strategies. Although the cost of whole genome/exome sequencing (WGS/WES) has drastically come down over the years[76] and the current pricing ranges from 20,000 to 25,000 INR,[77] it is still expensive for developing countries like India. Also WGS/WES cannot be directly beneficial in many cases with CAH because of the low target coverage and complicated data analysis. Since several rearrangements and chimeras are involved, often a combination of techniques is required to accurately identify mutations on both alleles of *CYP21A2* gene. The reported cost of CAH genetic testing in India varies from 5000 to 36,000 INR.[78] The cost largely depends on the techniques involved and the extent of mutations covered. Since identifying pseudogene-derived common point mutations can also cover micro conversions and give clues on chimeras, genotyping assays can be an effective first-stage assay in CAH genetic testing. A model algorithm of step-wise and comprehensive genetic screening in 21-OHD is shown in Figure 2.

At the author's centre, a cost-effective genotyping assay with ASPCR (cost 2600 INR) for the common hotspot




NGS=Next-generation Sequencing ; CNVS=Copy Number Variations; MLPA=Multiplex Ligation-dependent Probe Amplification; ASPCR=Allele-specific Polymerase Chain Reaction; CVS=Chorionic Villi Sampling

**Figure 2:** Flow chart on step-wise approach for comprehensive genetic testing in 21-OH deficiency

mutations and chimeras has been developed that is beneficial in >85% of the subjects with CAH.[79] This simple PCR-based analysis does not require radioactive probes or enzymes like other genotyping assays. ASPCR does not also require

instrumentation facility for fragment separation as in the case of MLPA. Therefore, this can be easily established even in resource-poor settings, enabling affordable genetic testing and identifying disease-causing CAH mutations in 85–90% of the cases. This can also provide a means for efficient second-tier testing to validate the positive results of 17-OHP-based NBS, carrier testing and community-based screening in CAH.

### Treatment and Management

Treatment usually involves lifelong supplementation with mineralocorticoids and glucocorticoids to replace adrenal steroid hormone deficiency, ensure adequate growth in children, suppress excessive androgen secretion in children and adolescents and manage infertility and other long-term consequences. With the recent advances in technology, several studies are in progress to develop alternative therapeutic approaches. They include trials with administration of corticotrophin-releasing hormone (CRH) receptor and melanocortin-2 receptor (MC2R) antagonists, adrenolytic agents and corticotrophin antibodies.[65] However, results from on-going research on gene therapy in animal models include adenoviral-*CYP21A1* vectors[80] and fibroblasts expressing 21-OH in mice,[81] which can temporarily enhance glucocorticoid and mineralocorticoid hormone synthesis. Nevertheless, there is no report on clinical trials to witness translational success of these approaches in patient care.

## Conclusion

Diagnosing and managing CAH presents formidable challenges when not promptly identified and treated. This condition not only carries the risk of life-threatening consequences but also subjects individuals to social stigmatization as they progress through life. Given the high prevalence of CAH and elevated rates of consanguinity in India, the development of widely available, comprehensive and cost effective genetic testing approaches become paramount in achieving a definitive diagnosis for this disorder.

Furthermore, a holistic strategy encompassing genetic screening for carrier status, identification of at-risk communities, genetic counselling for affected families, and advocacy for government policies promoting nationwide NBS programmes is imperative. This multi-faceted approach is essential for early diagnosis, enhanced patient care and implementation of community-based management strategies.

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### Conflicts of interest

There are no conflicts of interest.

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