REVIEW ARTICLE

Newborn Screening and Diagnosis of Infants with Congenital Adrenal Hyperplasia

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Congenital adrenal hyperplasia (CAH) is an autosomal recessive endocrine disorder which can manifest after birth with ambiguous genitalia and salt-wasting crisis. However, genital ambiguity is not seen in male babies and may be mild in female babies, leading to a missed diagnosis of classical CAH at birth. In this review, we provide a standard operating protocol for routine newborn screening for CAH in Indian settings. A standardization of first tier screening tests with a single consistent set of cut-off values stratified by gestational age is also suggested. The protocol also recommends a two-tier protocol of initial immunoassay/time resolved fluoroimmunoassay followed by liquid chromatography tandem mass spectrometry for confirmation of screen positive babies, wherever feasible. Routine molecular and genetic testing is not essential for establishing the diagnosis in all screen positive babies, but has significant utility in prenatal diagnosis and genetic counseling for future pregnancy.

Keywords: *17OHP, Cortisol, Fluoroimmunoassay, Tandem mass spectrometry*.

Congenital Adrenal Hyperplasia (CAH) is an autosomal recessive disorder with an incidence ranging from 1:10,000 to 1:20,000 births [1].The screen positive rate of CAH among a cohort of 104,066 babies screened at birth in India was 1 in 5762 as per a recent report [2].The most common defect in CAH is deficiency of enzyme 21-hydroxylase caused by mutation in *CYP21A2* gene, which comprises about 95% of all forms of CAH. Inadequate cortisol leads to an increase of ACTH which further stimulates adrenals, resulting in hyperplasia. A defective corticosteroid and mineralocorticoid enzymatic pathway shunts the steroid precursors to alternate derivatives like androgens and sex hormones.

The classical variety of CAH presents early as genital ambiguity in newborn females (due to excess sex hormones and their derivatives) or as adrenal crisis in both boys and girls. Adrenal crisis is characterized by insufficient corticosteroid and aldosterone production which causes hyponatremic dehydration and shock (salt wasting type). Patients with adequate aldosterone production without salt wasting who have signs of prenatal virilization are termed as simple virilizers classified under classical CAH. A mild non-classical form (NCCAH) of the disorder is also recognized in which presentation is in adolescence or later.

We herein provide guidance, in the Indian context, for diagnosis and referral of babies in early infancy with classical forms of CAH with 21-hydroxylase deficiency.

METHODS

A 2017 group of experts in the field of pediatric endocrinology and newborn screening (Delhi Pediatric Endocrinology Newborn Screening group- DePENS) used a semi-structured search strategy for preparing this review. The primary database used to search information was Medline through PubMed. The search was performed in September 2017 and updated till January 2019. Both MeSH and keyword based inputs were searched for articles pertaining to diagnosis and management of classical CAH with 21-hydroxylase deficiency in childhood. Systematic reviews, meta-analysis and randomized controlled trials were given priority. Articles pertaining to the management of CAH in adulthood were not included.

NEWBORN SCREENING

Incorporation of screening for 21-hydroxylase deficiency in to all newborn screening programs is recommended, wherever feasible.

Neonatal CAH is a disease which satisfies all the criteria

under newborn screening (NBS) checklist proposed by Wilson and Jungner [3]. NBS can help in early diagnosis, timely treatment and correct gender assignment of babies with classicalCAH [1,4]. Male babies with classical CAH may go undetected in the absence of genital ambiguity. Institution of specific steroid therapy can be life-saving in babies with salt-losing CAH where adrenal crisis may be misdiagnosed as sepsis. In addition, NBS can recognize simple virilizing forms in male newborn who would otherwise present later in childhood with features of precocious puberty. The final height of affected boys may be significantly compromised by that time due to advanced epiphyseal maturation [5]. However, NBS may not detect non-classical forms consistently when performed at birth [4].

The prevalence rate of CAH has shown an increase in the post-screening era. Sweden reported an increase in prevalence of salt-wasting CAH from 1 in 18,600 (1969-1986) to 1 in 12,800 (from 1989-1994) after introduction of NBS [6]. The incidence of CAH reported from Australia and Italy is variable from 1 in 15,488 or 18,105 births (in screened population) to 1 in 18,034 or 25,462 births (in unscreened population) [7,8].The incidence of screen positive CAH among cohort of 104,066 babies screened at birth in India was 1 in 5762 as per a recent report. There were marked regional differences with highest from Chennai (1:2036) to lowest from Mumbai (1:9983). The incidence of salt-wasting CAH was higher (1 in 6934) than simple virilizing type (1 in 20,801) [2]. Another study done on a cohort of 18,300 newborn in Andhra Pradesh showed an incidence of 1 in 2600.The screen positives were confirmed on recall in this study [9]. A prospective study on 11,200 newborns from Bangalore from 2007 to 2013showed a similar incidence of 1:2800 (confirmed cases) [10].

The mortality rate in CAH varies from 0-4% in unscreened cohort [6]. NBSwill reduce time to diagnosis, duration of hospitalization, severity of clinical manifestations, diagnostic uncertainty and reduce mortality in cases of salt wasting crisis. The importance of NBS to save lives in ethnic populations with high prevalence where timely clinical diagnosis is infrequent and CAH related deaths occur frequently, is undoubted. Thus, incorporation of screening for CAH should be considered as a component of NBS programme.

Standardization of first-tier screening tests with a single consistent set of cut-off values stratified by gestational age is recommended

It is recommended that first-tier screens for CAH employ fluoroimmunoassay to measure 17- hydroxy progesterone (17-OHP) in dried blood spots by heel prick methodon the same filter paper cards as are used for other tests in NBS [1].The use of cord blood is not recommended as the level of 17-OHP is significantly high immediately after birth [11]. Fluoroimmunoassay has supplanted radioimmunoassay and ELISA in most NBS programs [5,12]. It is recommended that the sample should be obtained between 24 to 72 hours of life as 17-OHP levels are normally high at birth and decrease rapidly in the first few postnatal days. Though in order to decrease the false positive rate it would be ideal to collect samples after 72 hours of birth, high birth rates necessitate screening after 24 hours or day 2 of life. Accessible births in the rural setting as collected by paramedical health workers maybe as delayed till 7 days. Hence it may be practical to collect samples between 24 hours and 7 days of life. In contrast, 17-OHP levels increase with time in affected neonates [4]. This makes diagnostic accuracy questionable in the first couple of days which can be an issue if newborns are discharged early. 17-OHP levels are higher in preterm, sick and stressed babies [4,12]. The cutoff values used should be adjusted for these factors to reduce recall rate. The combined use of gestational age and birth weight significantly improved predictive value in NBS for CAH [13]. However, 17-OHP, values correlate better with the gestational age rather than birthweight. Newborn screening programs in Switzerland and Netherlands have adopted the gestational age cut-offs which have improved the positive predictive value of screening [14,15]. The authors recommend the use of the gestation specific cut-offs (whole blood units in nmol/L) for Indian newborns as shown in ***Table* I,** which are based on data collected from a large multicentric study from Delhi

TABLE I GESTATIOAL AGE AND BIRTHWEIGHT-BASED CUT-OFFS FOR BLOOD LEVELS OF 17-HYDROXY PROGESTERONE FOR NEWBORN SCREENING FOR CONGENITAL ADRENAL HYPERPLASIA

Gestational age (completed wk)	*Birthweight < 2500 g*	*Birthweight ≥2500 g*
≤32 wk	81	51
33-36 wk	42	37.5
≥37 wk	37.5	37.5
Birthweight	*Preterm (<37 wk)*	*Term (≥37 wk)*
<1000 g	189	153
1000-1499 g	82	71
1500-2499 g	42	37.5
≥2500 g	37.5	37.5

Blood values when performed between 2-7th day of life; all values in nmoL/L (convert nmol/L to ng/mL by multiplying by 0.66).

[8].The use of birthweight-based cut-offs should be done only when accurate gestational age assessment by first trimester ultrasonography or record of last menstrual period is not available.

Blood 17-OHP values are considered borderline between 37.5-90 nmol/L and positive beyond 90 nmol/L, as per the fluoroimmunoassay kit-cut off values [2].The newborn screening programs in every country adopts its own cut-off value based on their population study. Cut-offs based on weight and gestational age are given in ***Table* I**. A multiplication factor of 0.66 with whole blood units (in nmol/L) may be used to obtain serum units (in ng/mL) of 17-OHP cut-offs [2].

It is recommended that infants whose mothers received antenatal corticosteroid treatment be retested after 2 weeks or at discharge, whichever is later

Antenatal corticosteroids used in preterm deliveries to facilitate fetal pulmonary maturation carry higher chances of interfering with CAH screening results as corticosteroids can cross the placenta and suppress the fetal hypothalamic pituitary axis. This may reduce the blood spot 17-OHP level thus leading to false negative result when performed at discharge or within 72 hours of birth. A reduction in serum 17OHP to upto 30% was seen after multiple courses of steroids [16], while inconsistent results have been obtained across otherstudies with a single course of steroids [17]. Thus, history of all institutional deliveries should be reviewed specially of the preterm babies for history of antenatal steroids. It is recommended that such infants (term or preterm) should be retested after two weeks of life, provided the baby is monitored carefully between the two screenings for salt losses and has easy access to health care services.For preterm babies, the cut-off should correspond to the cut-off for the corrected gestational age at two weeks, while for term babies the cut-off remains the same.

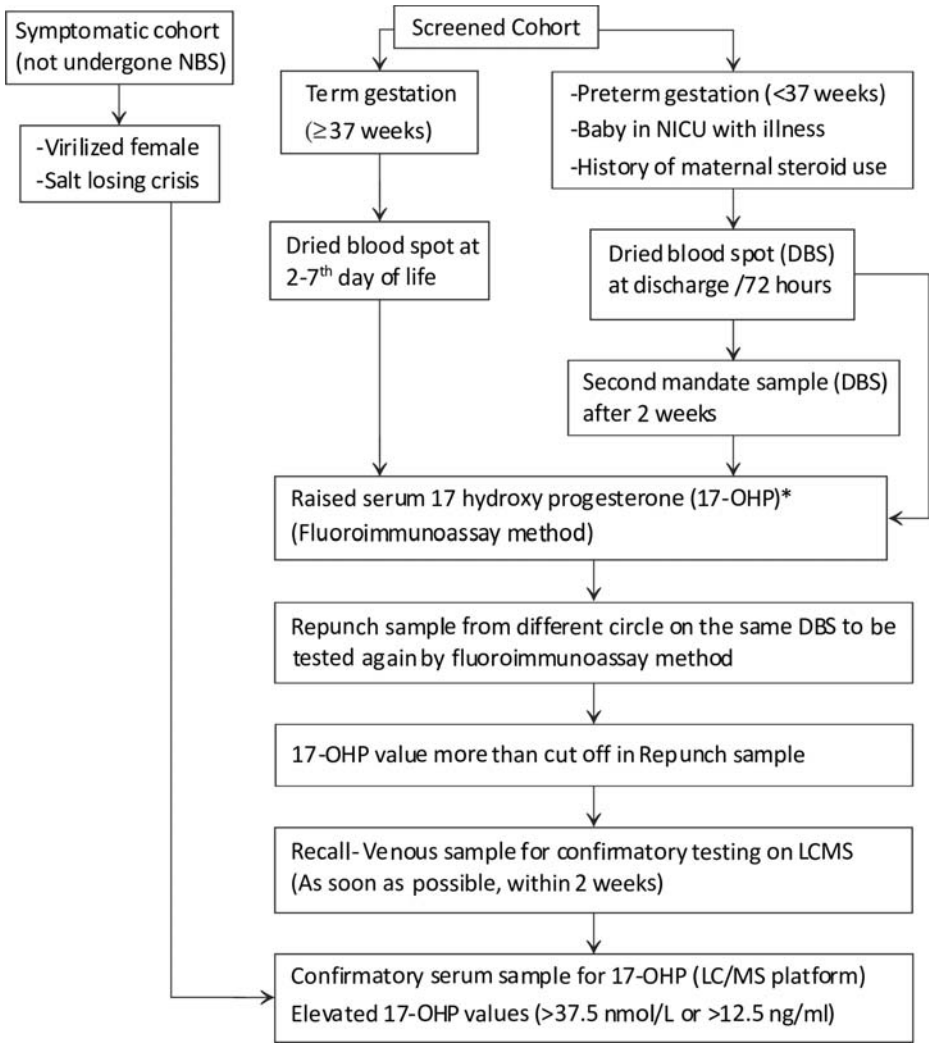
A two tier protocol (initial time resolved fluoroimmuno-assay with evaluation of positive tests by liquid chroma-tography-tandem mass spectrometry) is recommended for confirmation of all babies tested screen positive

The first sample for NBS should be collected on DBS after 24 hours till 72 hours of life to be processed by an initial fluoroimmunoassay. Babies admitted in intensive care, preterms and those whose mothers have received antenatal steroids should also have a mandatory repeat DBS tested after 2 weeks. In borderline and positive cases, a second repunch sample from a different circle on the same DBS is analyzed by fluoroimmunoassay (Repunch). In case the repunch spot also tests positive, the baby should be recalled immediately for a repeat venous blood sample for the second tier confirmatory testing, which is done by liquid chromatography-tandem mass spectrometry (LC-MS) (***Fig.* 1).** LC/MS is a diagnostic test for confirming the screen positives which is recommended by all the newborn screening programs. LCMS can profile the steroids separately into cortisol, 17-OH progesterone and 17-deoxycorticosterone and can thus differentiate the peak obtained in fluoro-immunoassay into its different components. Thus, it is both used as a diagnostic test and for confirming the screen positive cases.

In order to have high sensitivity, the cut-off levels for 17- OHP are typically set low enough to detect a positive proportion of approximately 0.3-0.5%. A study published from the New York screening program for CAH from 2007-14 reported a recall rate of 13,050 samples out of 1,96,2433 samples screened (0.66%). Out of this only 105 cases were confirmed positive (0.8% of the recalls) [18]. As the actual prevalence of CAH is approximately 0.01 to 0.02%, this effectively means that approximately 98% of screen positives would be false positive [19]. This in turn means that specificity of initial immunoassay is quite low and majority of screen positive cases are false positive. The cost of following up each false positive case could be avoided with use of a more specific second tier test. The specific test that should be ordered for confirmation of positive result on fluoroimmunoassay/immunoassay should be a biochemical assay on venous sample collected after immediate recall.

A similar two-tier protocol is being made feasible in resource-limited settings. Positive tested samples on immunoassay can be sent to the dedicated laboratory equipped to perform LC-MS/MS method with a priority label from centres that do not have similar facilities. It is recommended that LC-MS/MS be carried out on venous samples; however, in cases where venous sample is not available, DBS samples (whole blood) can also be used for the second tier testing.

Liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) is an alternate option where steroid ratios are measured. This is a good confirmatory test which can be performed easily on serum samples. The principle of organic solvent extraction increases its specificity over an immunoassay [20,21]. Many CAH screening programs have reported an increase in positive predictive value, from 0.8 to 7.6% in Minnesota and 0.4 to 9.3% in Utah after implementing this approach [22,23]. A German program tested 1609 screen positive samples(out of 242,500 samples screened) using a modified LC-MS/MS protocol which used a ratio of the sum of 17-OHP and 21-deoxycortisol levels, divided by the cortisol level. They



Gestational age cut offs preferred instead of birth weight wherever available.

FIG. 1 *Suggested algorithm for screening of infant for congenital adrenal hyperplasia.*

concluded that a cut-off ratio of 0.53 had a positive predictive value of 100% [24].

The downstream cost of high recall rates with false-positive screen is difficult to estimate. The cost-effectiveness of NBS for CAH has not been well analyzed. A false positive screen may also be a cause of significant undue parental stress. However, justification for NBS in CAH scores over these minor issues. Use of better diagnostic tests will help to avert these logistic issues [5].

The use of molecular and genetic tests should be reserved for research settings where resources permit.They are recommended strongly for prenatal diagnosis and genetic counselling for future pregnancies

Molecular testing can be offered to babies who test positive on biochemical screening. The most common mutations detected are the *CYP21A2* gene mutations. One of the 10 common mutations is present in 90% of the affected patients, thus absence of these mutations would deem the diagnosis of CAH unlikely while presence of at least one mutation would warrant further workup [12]. Hotspots from both the northern and southern Indian cohort of patients with CAH have identified a panel of 9 to 10 common mutations which can be offered to screen positive patients in laboratories where molecular facilities are available [2]. Genotyping of screening samples has been proposed as a useful second tier test in lieu of LCMS in several studies [25]. Kosel, *et al.* [27] in their study

reported a decrease in number of retests by 90% when the screen positive 17-OHP values were screened by molecular means [26]. However, no large scale study has demonstrated cost wise efficacy of this strategy. It is currently more expensive than LC-MS/MS on a per sample basis and is not in use in any nationwide newborn screening program.

The genotype also helps in determining the degree of enzyme impairment, which in turn determines the severity of hormonal abnormalities. Studies have demonstrated genotypic-phenotypic correlation in 50% of the cases [12,27]. A deletion or intronic splice mutation in I2G is commonly associated with salt wasting CAH, I172N mutations and V281L were commonly associated with simple virilizing and non-classical CAH, respectively [28]. However, genotype may not always differentiate between salt wasting and non-salt wasting forms. For example, patients with V281L or P30L mutations, which have been traditionally associated with non-classical CAH may present with virilization [29].

Thus, genotyping carries implications for prenatal diagnosis and genetic counseling for next pregnancy.It is not required before initiation of treatment in index case of classical CAH. It is recommended for prenatal diagnosis or where diagnosis is questionable.

Urinary steroid profiling by Gas chromatography Mass Spectrometry (GCMS) is not recommended as a routine confirmatory test

Urinary steroid profiling (USP) is a biochemical analytical technique for the diagnosis of various types of steroidogenesis defects including those leading to CAH as it can identify and quantify a series of steroid metabolites both above and below the enzymatic block simultaneously in a single analysis [30]. Urine steroid profiling by GCMS requires time consuming preanalytical sample preparation. Apart from the technical challenges, an important limitation is the inability to perform these tests in a high through put format where large number of samples need to be processed in a short span of time. This has limited the use of GCMS to a few experienced research laboratories and is yet to be adapted to large scale commercial assays [31]. It can provide a rapid simplified differential diagnosis for CAH, where available [32].

DIAGNOSIS IN BABIES WHO HAVE NOT UNDERGONE NEWBORN SCREENING

A morning baseline serum 17 OHP is recommended in symptomatic individuals

A newborn who has not undergone genetic screening at birth should be offered screening for CAH anytime he/she presents during the neonatal period irrespective of whether symptomatic for CAH or not. Alternately, any neonate with ambiguous genitalia or suspicion of CAH on metabolic work-up should also be offered confirmatory testing on venous sample for CAH on priority basis. In these babies, a single measurement of serum 17-OHP prior to steroid administration must be sent and interpreted.

A complete adrenocortical profile is recommended to differentiate 21-OH deficiency from other enzyme defects and to diagnose borderline cases. Alternatively the steroid profiling done on tandem mass may help identify a subset of these disorders

The possibility of an alternative diagnosis other than 21-hydroxylase deficiency may be considered in neonates/infants with clinical or lab markers pointing to defects other than 21- OH deficiency. The other causes of CAH include 11β-hydroxylase deficiency, 17 a hydroxylase deficiency, 3b hydroxysteroid dehydrogenase deficiency and lipoid CAH. Only 21-OH deficiency and 11 β-hydroxylase deficiency are predominantly virilizing diseases. Patients with other causes of CAH have impaired production of cortisol by the adrenals as well as gonadal steroids. Male patients will have undervirilization, while female patients may or may not exhibit virilization. Clinical features may appear similar and basal serum 17-OHP may not be fully discriminatory in all such cases. Precursors to product ratios on LC-MS/MS are important in differentiating the various enzyme defects. In order to differentiate the various enzyme defects, serum 17-OHP, cortisol, 11-deoxycortisol, 17-OH pregnenalone, dihydroepiandrostenedione and andro-

TABLE II SUBTYPES OF CONGENITAL ADRENAL HYPERPLASIA

Subtypes	*Phenotype*	*Elevated metabolites*
11β-hydroxylase deficiency	Female virilization	Deoxycorticosterone, 11-deoxycotisol
17 α-hydroxylase deficiency	Male undervirilization Female virilization +/-	Deoxycorticosterone, corticosterone
3β-hydroxysteroid dehydrogenase deficiency	Male undervirilization Female virilization +/-	Dihydroepiandrostenedione, 17-OH pregnenalone

stenedione should be measured [12,27]. The metabolites elevated in the various subtypes are shown in ***Table* II**. Apart from 17-OH pregnenalone, the rest of tests are available on the LC-MS/MS platform. These tests can be performed on a venous sample collected as soon as possible but preferably within first two weeks of life. These disorders are less common and such children should be immediately referred to the endocrinologist for further workup. It should be noted that thepurpose of newborn screening is identification of babies with 21-hyroxylase deficiency, which is the commonest.

CONCLUSIONS

CAH is a disease associated with significant morbidity, mortality and long-term complications. The timely diagnosis and treatment is challenging in the absence of newborn screening. Screening for CAH with DBS using fluoroimmunoassays is recommended for all babies. The confirmation of diagnosis must be made using LC-MS method, which is getting widely available. Genetic diagnosis should be used for diagnostic confirmation where resources permit but definitely – for prenatal testing and counselling.

Contributors: SK,AS,VJ,MK,SY: conceived the idea; AD,PV,PS,PB,RS,NG,RK: drafted and designed the manuscript. The group led to the development of the manuscript and share the primary responsibility for the final content. All authors have read and approved the final manuscript.

Funding: None; *Competing interest*: None stated.

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