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Original Article

Clinical Utility of Second-tier Testing in Newborn Screening for Congenital Adrenal Hyperplasia: The Hong Kong Experience

MCW ***Yeung***, TCH ***Chan***, CM ***Mak***

Abstract ***Objective:*** The present study aimed to evaluate the utility of steroid profiling using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as a second-tier test for congenital adrenal hyperplasia (CAH) newborn screening. ***Methods:*** The newborn screening results of 40,754 newborns who were screened for CAH from April 2016 to March 2019 were included in this study. First tier test involved measurement of 17-hydroxyprogesterone (17-OHP) in dried blood spot using dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA). Cases with positive first-tier screen were subjected to second-tier test utilizing LC-MS/MS to measure 17-OHP, androstenedione and cortisol in the same dried blood spot samples. ***Results:*** Of the 40,754 newborns screened, 422 (1.04%) were screen positive by first-tier test and required second-tier test. Among them, 4 (0.01%) were screen positive by second-tier test and were recalled for further workup. Two of whom were diagnosed with CAH. Also one neonate with negative screen was subsequently diagnosed with CAH during work up for fever and hyponatraemia and hyperkalaemia. The 2 true positive and 1 false negative cases are all salt-wasting 21-hydroxylase deficiency. The estimated incidence of classical CAH in the screened population was 1:13,585. ***Conclusions:*** Second-tier steroid profiling by LC-MS/MS can significantly reduce false positive rate and avoid unnecessary recalls in newborn screening for CAH. However, false negative screen still occurs and any patients with clinical features of CAH should receive diagnostic testing regardless of newborn screening results.

Key words Congenital adrenal hyperplasia; False positive; Newborn screening; Second-tier

Introduction

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders caused by defect in adrenal steroidogenesis. Among this group, 21-hydroxylase deficiency is the most common form and it accounts for around 95% of all CAH cases.[1] In this article, CAH refers to 21 hydroxylase deficiency unless otherwise specified. 21-hydroxylase is responsible for converting 17-hydroxyprogesterone (17-OHP) to 11-deoxycortisol and progesterone to 11-deoxycorticosterone. A defect of this enzyme caused by mutations in the *CYP21A2* gene can lead to varying degree of mineralocorticoid deficiency and hyperandrogenism, presenting clinically as salt wasting and virilising phenotype respectively. The incidence of CAH was reported to be 1 in 6,084 in Chinese.[2] In view of the potentially fatal salt wasting crises in severe CAH and the morbidity of hyperandrogenism such as precocious puberty and shortened height in milder form of CAH, newborn screening for this disorder has been introduced in many countries.[3]

Department of Pathology, Hong Kong Children's Hospital, 1 Shing Cheong Road, Kowloon Bay, Kowloon, Hong Kong SAR

MCW **Yeung** (楊雋永) *MBBS, FRCPA, FHKAM(Pathology)*
TCH **Chan** (陳進希) *MBBS*
CM **Mak** (麥苗) *MD, FRCPA, FHKAM(Pathology)*

Correspondence to: Dr MCW **Yeung**
Email: ycw186@ha.org.hk

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Currently, first-tier screening methodology for CAH is based on measuring 17-OHP levels in dried blood spots by immunoassays, in particular dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA). This screening method, although being fast, simple and easily automated, suffers from poor specificity or high false positive rate due to several reasons: First, premature and low birth-weight neonates have elevated 17-OHP levels due to functional deficiency in steroidogenic enzymes and perinatal stress.[4] Second, immunoassays are susceptible to cross-reactivity of antibodies with other steroids present in blood, which include steroid intermediates such as 17-hydroxyprenenolone and its monosulfate metabolite.[5] Strategies to improve positive predictive value of CAH screening include improvement in analytical specificity of antibodies employed in immunoassay provided by assay manufacturers and adoption of gestational-age or birth weight adjusted cutoff values for 17-OHP,[6,7] all with limited success. Another strategy is second-tier screening tests, which are tests performed on the same dried blood spot specimen used for first-tier screen. The most widely adopted second-tier test for CAH is steroid profiling by liquid chromatography-tandem mass spectrometry (LC-MS/MS). It was initially described by Lacey et al who simultaneously measured 17-OHP, androstenedione and cortisol by LC-MS/MS[8] and subsequent report from their centre showed a nearly 90% reduction in false positive rate and an improvement of the positive predictive value from the reported 0.5 to 4.7%.[9] Similar reports from other centers using this strategy further supported its clinical value.[10-12] For this reason, our laboratory provided LC-MS/MS-based second-tier test when newborn screening for CAH was started in Hong Kong in April 2016. Drawing on data in the last 3 years, we aim to report our experience on first- and second-tier screening for CAH.

Methods

From April 2016 to March 2019, a total of 40,754 newborns were screened for CAH in Hong Kong. Demographic data and screening and diagnostic information of screened neonates were collected and analysed. Dried blood spot samples were collected on whatman 903 filter paper between 24 and 72 hours of life. For neonates of less than 34 weeks gestation or birth weight less than 2,000 gram or neonates admitted to neonatal intensive care unit, a second dried blood spot sample will be collected on day 28 of life or at discharge whichever comes first. The first-tier test for CAH is based on 17-OHP measurements by automated, time-resolved fluoroimmunoassay (DELFIA, Perkin Elmer Life Sciences, Turku, Finland). For term neonates, a cutoff of 20 nmol/L was initially used to define elevated 17-OHP in first tier test and was later revised to 25 nmol/L in September 2018 after review of our reference data. For preterm neonates, cutoff is based on gestational age and age at sampling according to recommendations of the International Society for Neonatal Screening.[13] Samples with elevated 17-OHP by this primary screen were submitted for second tier test as described by Lacey et al,[8] in which LC-MS/MS is used to simultaneously measures 17-OHP, cortisol and androstenedione (4-AD). The ratio (17-OHP + 4-AD)/cortisol is then calculated. This ratio is based on the fact that the metabolic block at 21-hydroxylase causes reduced cortisol production and accumulation of precursors including 17-OHP, and shunting of precursors into androgen production, causing elevation in 4-AD. In sick neonates, whose elevation in 17-OHP is due to stress-related adrenal stimulation, cortisol is expected to be elevated, thus normalising the ratio. With the analytes 17-OHP, 4-AD and cortisol expressed in units of ng/mL serum, the cutoff for the ratio (17-OHP + 4-AD)/cortisol was set at 2 after review of our own population data and taking reference to cutoff used by other participants in the Newborn Screening Quality Assurance Program (NSQAP) CAH second tier proficiency testing program.[14]

Results

During the 3-year study period, a total of 40,754 infants were screened by first-tier test for CAH. There were 1.04% of all screened infants (n=422) found to have elevated 17-OHP above cutoff. Their newborn screening dried blood spot samples were subjected to LC-MS/MS second-tier test and 4 were found to have elevated (17-OHP + 4-AD)/cortisol ratio and determined to be screened positive for CAH, giving the overall recall rate of 0.01%. We deduced that if second-tier test was not available and all elevated 17-OHP cases from first-tier immunoassay were recalled, the recall rate would be dramatically raised to 1.04%. But in reality the cutoff would need to be increased to avoid such high recall rate in newborn screening program for CAH without second-tier test.

Among the 40,754 infants, 5448 of them were preterm (defined as birth before 37 weeks of gestation). A total of

51 preterm infants were screened positive by first-tier test and all of them were screened negative by second-tier test. Regarding the timing of sample collection, 82 dried blood spot samples were collected before 24 hours of life and 9 of them were screened positive by first-tier test and all 9 samples were normal on second-tier test. The higher screened positive rate (11%) on first-tier test in sample collected before 24 hours of life can be attributed to the fact that those are sick infants that required transfusion and neonatal intensive care unit admission and the dried blood spot collected before 24 hours of life was a pre-transfusion sample.

All screening-positive infants were assessed clinically by paediatricians and underwent further biochemical investigations, including serum 17-OHP, plasma electrolytes, short synacthen test, and urine steroid profiling. Consequently, among the 4 screened positive infants, 2 were diagnosed with CAH. First case (TP1) was a male full term neonate who was asymptomatic and was called back on day 7 of life for the abnormal newborn screening result. Physical examination showed normal blood pressure and normal external genitalia. Blood test on day 7 showed borderline low sodium of 134 mmol/L and mildly elevated potassium of 6.2 mmol/L. Second case (TP2) was a full term male neonate who was delivered by emergency cesarean section due to failed induction. He had transient tachypnea of the newborn and early onset neonatal jaundice due to glucose-6-phosphate dehydrogenase deficiency. His electrolyte was normal on day 2 of life but started to develop hyponatraemia (plasma sodium 136 mmol/L) and hyperkalaemia (plasma potassium 6.3 mmol/L) on day 5 of life, which was the time when the abnormal newborn screening result was available. Both positive cases had elevated plasma ACTH and renin and urine steroid profile of pattern compatible with 21-hydroxylase deficiency and both cases responded well to fludrocortisone and hydrocortisone. The clinical information and screening and subsequent follow up test results for these 2 true positive cases (TP1 and TP2) are summarised in Table 1. Regarding the 2 false positive cases, both had borderline 17-OHP results on first-tier test (21 nmol/L and 23 nmol/L) and also borderline elevated (17-OHP + 4AD)/cortisol ratio (2.2 and 2.4). Subsequent investigations showed both cases had normal plasma 17-OHP and normal urine or blood steroid profile.

There was one false negative case detected during this 3-year period. This false negative case (FN1) is a term female infant with borderline elevated 17-OHP of 25.4 nmol/L (cutoff >25 nmol/L). Subsequent second-tier LC-MS/MS test showed normal 17-OHP and normal (17-OHP + 4-AD)/cortisol ratio of 0.37 (see Table 1 case FN1 for summary). She presented at 1 month of age with fever and was noted to have clitoromegaly, hyponatremia and mild hyperkalaemia. Serum 17-OHP was 718 nmol/L (reference interval <8 nmol/L) at presentation and urine steroid profile detected significant elevation of metabolites of 17-OHP. She was treated with hydrocortisone and fludrocortisone with good response. With both true positive and false negative cases included, the overall incidence is estimated to be 1:13,585 for classical CAH.

Discussion

The purpose of this study was to explore the clinical utility of second-tier testing in newborn screening for CAH. Our data showed that the addition of LC-MS/MS-based second-tier steroid profile to immunoassay-based first-tier CAH newborn screening, the recall rate for CAH can be lowered from 1.04% to 0.01%, with an improvement in positive predictive value from 0.47% to 50%. This directly translates to less unnecessary hospitalisation and investigations of healthy infants, which can generate significant parental anxiety and potentially leads to

Table 1 Summary of the clinical and biochemical findings in the confirmed cases of CAH

Case	Gender	Gestation (weeks)	Age of diagnosis	First tier 17-OHP (nmol/L)	Second tier 17-OHP (nmol/L)	Second tier (17-OHP + 4-AD) / cortisol ratio	CAH subtype	Serum 17-OHP at diagnosis (nmol/L)
TP1	Male	40	7 days	127	89	2.0	SW	114
TP2	Male	38	5 days	207	120	4.1	SW	281
FN1	Female	41	1 month	25.4	6.8	0.37	SW	718

CAH: congenital adrenal hyperplasia; 17-OHP: 17-hydroxyprogesterone; 4-AD: androstenedione; SW: salt-wasting form of 21-droxylase deficiency

dysfunctional parent-child relationships.[15] To further improve the positive predictive value of second tier test for CAH, inclusion of additional analytes in the LC-MS/MS assay has been suggested. Janzen et al improved on second-tier test method of Lacey et al,[8] which is the method used by our laboratory, by adding 21-deoxycortisol and 11-deoxycortisol into the steroid panel.[16] 21-deoxycortisol, being the product of 11β-hydroxylation of 17-OHP, is not elevated in preterm infants and is highly specific for 21-hydroxylase deficiency. In their study, the ratio of (21-deoxycortisol + 17-OHP)/cortisol is calculated and was shown to be significantly elevated in cases of 21-hydroxylase deficiency with no false positive result, resulting in a positive predictive value of 100%. Also, 21-deoxycortisol and 11-deoxycortisol allows differentiation of 21-hydroxylase deficiency and 11 β-hydroxylase deficiency, hence this second-tier test can perform simultaneous confirmation and subtyping of CAH.[17] Also, since this second tier test do not require additional equipment and the cost of acquiring the standards of these two analytes is limited, adding 21-deocycortisol and 11-deoxycortisol into the second-tier steroid panel incurs minimal additional cost.

In this study, we have one cases of false negative case (FN1), who actually had mildly elevated 17-OHP on first-tier test but normal 17-OHP level and normal (17-OHP + 4-AD)/cortisol ratio by our LC-MS/MS second-tier test. This case is similar to the experience reported by the Minnesota newborn screening program. Using a two-tier newborn screening protocol, they reported 11 false negative cases with four of them actually had an abnormal first-tier screen which was subsequently overridden by a normal second-tier result.[18] Also, the false negative rate of one-tier and two-tier protocols had no statistically significant difference. Therefore, it is clear that a normal second-tier steroid profile result should not be regarded as ruling out classic CAH totally and a high level of suspicion should be maintained in patients presenting with suspected CAH.

It is noteworthy that all three CAH cases (2 true positive and 1 false negative) are all salt wasting type of CAH. It is possible that there are other false negative cases that are not identified, especially milder forms of CAH including classical simple virilising and non-classical CAH. To minimise the risk of missing clinically important cases of CAH, some states in the United States mandated that a second screen be performed on all newborns at 8-14 days of age.[19,20] A study comparing detection rates of first and second screen reported that majority of simple virilising and non-classical CAH cases were detected on the second screen that would have been missed by first screen.[21] Also, the second screen allowed detection of an additional 6.5% of classical salt wasting cases.[21] However, the practice of collecting a second specimen for all infants after discharge may not be practical in all newborn screen programs and its cost-effectiveness is still controversial.[20,22]

Molecular genetic second-tier screening has been suggested as an alternative to biochemical second tier test in several small scale studies. The genotyping approach ranged from detection of a targeted panel of mutations using real-time PCR.[23] or minisequencing[24] to whole *CYP21A2* gene sequencing with multiplex ligation-dependent probe amplification (MLPA).[25] Molecular second-tier test has been well established in newborn screening programs for cystic fibrosis, however its application in newborn screening for CAH has several caveats: First, the functional *CYP21A2* gene is in close proximity with its nonfunctional pseudogene *CYP21P* and they share 98% homology in exons and about 96% in introns.[26] Hence, any PCR-based methods must utilise primers specific to sequence of *CYP21A2* to avoid amplification of the pseudogene. Second, up to 30% of pathogenic variants causing CAH are gene deletions/gene conversions.[27] Techniques to detect such copy number change, such as MLPA, needed to be incorporated, which complicates the genetic second tier testing protocols. Third, a single *CYP21A2* allele may carry more than one pathogenic mutations. When two heterozygous mutations are detected, genotyping of the parents is needed to determine whether the mutations are in cis or in trans configuration. This approach is not possible in the setting of second tier newborn screen when the only sample available to screening laboratory is the dried blood spot card of the neonate. Overall, given the above technical difficulties, molecular genetic second screen for CAH is still considered to be tedious and costly compared with LC-MS/MS based approach. In 2018, the Endocrine Society stated that LC-MS/MS, in preference to all other methods (including genotyping), is the test of choice in second-tier screen for CAH.[28]

Conclusion

In conclusion, this study suggests that LC-MS/MS based steroid panel as second-tier test for CAH reduces false positive rate and reduce unnecessary follow up testing,

healthcare expenditures and worry of parents. However, false negative result can occur in both first- and second-tier test and a negative newborn screening result does not rule out all possibility of CAH, even for severe classical salt wasting type. Therefore, any patient with clinical features of CAH should receive diagnostic testing.

Conflict of Interest

The authors have no conflicts of interest to disclose.

References

1. White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Endocr Rev 2000;21:245-91.
2. Zhong K, Wang W, He F, Wang Z. The status of neonatal screening in China, 2013. J Med Screen 2016;23:59-61.
3. Therrell BL, Padilla CD, Loeber JG, et al. Current status of newborn screening worldwide: 2015. Semin Perinatol 2015;39: 171-87.
4. Nomura S. Immature adrenal steroidogenesis in preterm infants. Early Hum Dev 1997;49:225-33.
5. Wong T, Shackleton CH, Covey TR, Ellis G. Identification of the steroids in neonatal plasma that interfere with 17 alpha-hydroxyprogesterone radioimmunoassays. Clin Chem 1992;38: 1830-7.
6. Nordenstrom A, Wedell A, Hagenfeldt L, Marcus C, Larsson A. Neonatal screening for congenital adrenal hyperplasia: 17-hydroxyprogesterone levels and CYP21 genotypes in preterm infants. Pediatrics 2001;108:E68.
7. Allen DB, Hoffman GL, Fitzpatrick P, Laessig R, Maby S, Slyper A. Improved precision of newborn screening for congenital adrenal hyperplasia using weight-adjusted criteria for 17-hydroxyprogesterone levels. J Pediatr 1997;130:128-33.
8. Lacey JM, Minutti CZ, Magera MJ, et al. Improved specificity of newborn screening for congenital adrenal hyperplasia by second-tier steroid profiling using tandem mass spectrometry. Clin Chem 2004;50:621-5.
9. Minutti CZ, Lacey JM, Magera MJ, et al. Steroid profiling by tandem mass spectrometry improves the positive predictive value of newborn screening for congenital adrenal hyperplasia. J Clin Endocrinol Metab 2004;89:3687-93.
10. Schwarz E, Liu A, Randall H, et al. Use of steroid profiling by UPLC-MS/MS as a second tier test in newborn screening for congenital adrenal hyperplasia: the Utah experience. Pediatr Res 2009;66:230-5.
11. Votava F, Novotna D, Kracmar P, et al. Lessons learned from 5 years of newborn screening for congenital adrenal hyperplasia in the Czech Republic: 17-hydroxyprogesterone, genotypes, and screening performance. Eur J Pediatr 2012;171:935-40.
12. Seo JY, Park HD, Kim JW, et al. Steroid profiling for congenital adrenal hyperplasia by tandem mass spectrometry as a second-tier test reduces follow-up burdens in a tertiary care hospital: a retrospective and prospective evaluation. J Perinat Med 2014;42: 121-7.
13. Blankenstein O, Stopsack M, Fingerhut R, Loeber G, Torresani T. The ISNS 17OHP initiative: Establishing of 17OHP cut-off levels by international collaboration. Ces-slov Pediatr 2009;64:192-3.
14. De Jesus VR, Simms DA, Schiffer J, Kennedy M, Mei JV, Hannon WH. Pilot proficiency testing study for second tier congenital adrenal hyperplasia newborn screening. Clin Chim Acta 2010;411: 1684-7.
15. Gurian EA, Kinnamon DD, Henry JJ, Waisbren SE. Expanded newborn screening for biochemical disorders: the effect of a false-positive result. Pediatrics 2006;117:1915-21.
16. Janzen N, Peter M, Sander S, et al. Newborn screening for congenital adrenal hyperplasia: additional steroid profile using liquid chromatography-tandem mass spectrometry. J Clin Endocrinol Metab 2007;92:2581-9.
17. Monostori P, Szabo P, Marginean O, Bereczki C, Karg E. Concurrent Confirmation and Differential Diagnosis of Congenital Adrenal Hyperplasia from Dried Blood Spots: Application of a Second-Tier LC-MS/MS Assay in a Cross-Border Cooperation for Newborn Screening. Horm Res Paediatr 2015;84:311-8.
18. Sarafoglou K, Banks K, Gaviglio A, Hietala A, McCann M, Thomas W. Comparison of one-tier and two-tier newborn screening metrics for congenital adrenal hyperplasia. Pediatrics 2012;130:e1261-8.
19. Chan CL, McFann K, Taylor L, Wright D, Zeitler PS, Barker JM. Congenital adrenal hyperplasia and the second newborn screen. J Pediatr 2013;163:109-13.e1.
20. Brosnan CA, Brosnan P, Therrell BL, et al. A comparative cost analysis of newborn screening for classic congenital adrenal hyperplasia in Texas. Public Health Rep 1998;113:170-8.
21. Held PK, Shapira SK, Hinton CF, Jones E, Hannon WH, Ojodu J. Congenital adrenal hyperplasia cases identified by newborn screening in one- and two-screen states. Mol Genet Metab 2015; 116:133-8.
22. Yoo BK, Grosse SD. The cost effectiveness of screening newborns for congenital adrenal hyperplasia. Public Health Genomics 2009; 12:67-72.
23. Kosel S, Burggraf S, Fingerhut R, Dorr HG, Roscher AA, Olgemoller B. Rapid second-tier molecular genetic analysis for congenital adrenal hyperplasia attributable to steroid 21-hydroxylase deficiency. Clin Chem 2005;51:298-304.
24. Krone N, Braun A, Weinert S, et al. Multiplex minisequencing of the 21-hydroxylase gene as a rapid strategy to confirm congenital adrenal hyperplasia. Clin Chem 2002;48:818-25.
25. Silveira EL, Elnecave RH, dos Santos EP, et al. Molecular analysis of CYP21A2 can optimize the follow-up of positive results in newborn screening for congenital adrenal hyperplasia. Clin Genet 2009;76:503-10.
26. Wedell A. Molecular genetics of 21-hydroxylase deficiency. Endocr Dev 2011;20:80-7.
27. Baumgartner-Parzer SM, Schulze E, Waldhausl W, et al. Mutational spectrum of the steroid 21-hydroxylase gene in Austria: identification of a novel missense mutation. J Clin Endocrinol Metab 2001;86: 4771-5.
28. Speiser PW, Arlt W, Auchus RJ, et al. Congenital Adrenal Hyperplasia Due to Steroid 21-Hydroxylase Deficiency: An Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab 2018;103:4043-88.