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Mini Review

Newborn screening for congenital adrenal hyperplasia: Utility of liquid chromatography with tandem mass spectrometry as a secondary test

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Highlights

- Liquid chromatography with tandem mass spectrometry for second-tier testing is useful for congenital adrenal hyperplasia screening in Japan.

Abstract. In Japan, newborn screening (NBS) for congenital adrenal hyperplasia (CAH) began in 1989. NBS is useful for early diagnosis and preventing gender misidentification, however, it has a higher false positive rate for CAH compared to other diseases detected by neonatal screening. Recently, it has become clear that using liquid chromatography with tandem mass spectrometry (LC-MS/MS) for second-tier testing reduces false positive rates and repeat blood sampling. LC-MS/MS commonly measures cortisol (F), androstenedione (A4), 11-deoxycortsiol (11DOF), 21-deoxycortisol (21DOF), and 17-hydroxyprogesterone (17OHP) levels. The ratios for (21DOF+17OHP)/F and (17OHP+A4)/F have been used to establish cut-off values for the second-tier test. In Japan, the recall rate is reduced using the 11DOF/17OHP ratio as well as the ratios for (21DOF+17OHP)/F and (17OHP+A4)/F for the second-tier test. Currently, second-tier testing using LC-MS/MS for CAH neonatal screening is unfeasible in all regions of Japan due to equipment costs, however, it will hopefully be available nationwide in the future.

Key words: congenital adrenal hyperplasia, false positive, recall rate, liquid chromatography with tandem mass spectrometry, 11-deoxycortisol

Introduction

In Japan, newborn screening (NBS) for congenital adrenal hyperplasia (CAH) began in 1989. This screening type allowed for CAH detection, especially 21-hydroxylase deficiency (21OHD), early treatment, and avoiding gender misidentification (1–3). However, NBS has a higher false positive rate for CAH compared to other newborn screening diseases, especially in low birth weight and preterm infants (1–3). Recently, various countries have attempted to reduce the false positive rate through steroid analyses using liquid chromatography with tandem mass spectrometry (LC-MS/MS) as a second-tier test for CAH (4–17). Certain regions in Japan have also begun conducting steroid analyses using LC-MS/MS (18–22). This review describes the current status, problems, and challenges of NBS for CAH.

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Challenges of CAH screening in newborns

21OHD accounts for most CAH cases (1–3). This disease causes virilization of the external genitalia in girls and early adrenal insufficiency post-birth. In the severe salt-wasting type, adrenal insufficiency occurs 7–10 d after birth (23). In these cases, early detection and steroid supplementation is likely to improve prognosis. In Japan, NBS for CAH was introduced nationwide in 1989 (1–3). Owing to 17-hydroxyprogesterone (17OHP) accumulation in 21OHD, NBS for CAH includes 17OHP level measurement in blood spots using an enzyme-linked immunosorbent assay (ELISA). However, CAH screening has a low positive predictive value (PPV) and high false positive rates. In certain developed countries, CAH is subject to NBS, however, in the United Kingdom, CAH is not included in the NBS.

The low PPV and high false positive rates in CAH screening is explained by several reasons (1–3). First, 17OHP levels are high in umbilical cord blood and decrease within 1–2 d after birth. Thus, these levels are likely to be high in early NBS. Second, 17OHP levels increase in neonates due to various types of stress and diseases in the absence of 21OHD. Third, the fetal adrenal glands produce 17OHP, leading to false positive results, especially in preterm infants. Finally, the antibodies used for 17OHP ELISA assessment exhibits cross-reactivity with other fetal adrenal glands-secreted steroids.

Before LC-MS/MS, a study in Sapporo city reported a PPV of 1.8% (2). A report from Tokyo used 17OHP cut-off values based on gestational age, and the PPVs were 33.3% for term infants and 2% for gestational ages < 37 wk (3). In a study from Sweden (24), cut-off values were set per gestational age, which is similar to the Tokyo report. However, the PPVs were 22% for full-term infants and 2% for 35–36 wk of gestation, the latter of which is consistent with the Tokyo report results.

Global findings for second-tier test using LC-MS/MS

To increase the PPV and reduce false negatives, the Endocrine Society guidelines from 2018 and Japanese Society for Pediatric Endocrinology guidelines from 2021 recommend the use of LC-MS/MS for second-tier analysis (25, 26). **Figure 1** displays the adrenal steroidogenic pathways in 21OHD. LC-MS/MS commonly measures levels of cortisol (F), androstenedione (A4), 11-deoxycortsiol (11DOF), 21-deocycortisol (21DOF), and 17OHP. In a report by Lacey *et al.* (4), among 101 false-positive samples, 86 were below the cut-off value of LC-MS/MS-measured 17OHP. Janzen *et al.* (5) demonstrated the combined usefulness of the (21DOF+17OHP)/F ratio and 17OHP levels in the second-tier test. The (17OHP+A4)/F ratio also effectively reduced false positive rates (7, 8). Since then, screening efficiency was improved by measuring the levels of several steroid metabolites and using their ratios. Recent reports from overseas are summarized in **Table 1** (6–10, 11, 13–17). As shown in **Table 1**, 17OHP and (17OHP+A4)/F were frequently used as parameters for the second-tier test, and the PPV ranged from 7.0 to 100%. However, the protocol for using LC-MS/MS as the second-tier test was not consistent in every region. For instance, in Wisconsin (10), the cut-off value for 17OHP in the first-tier test was

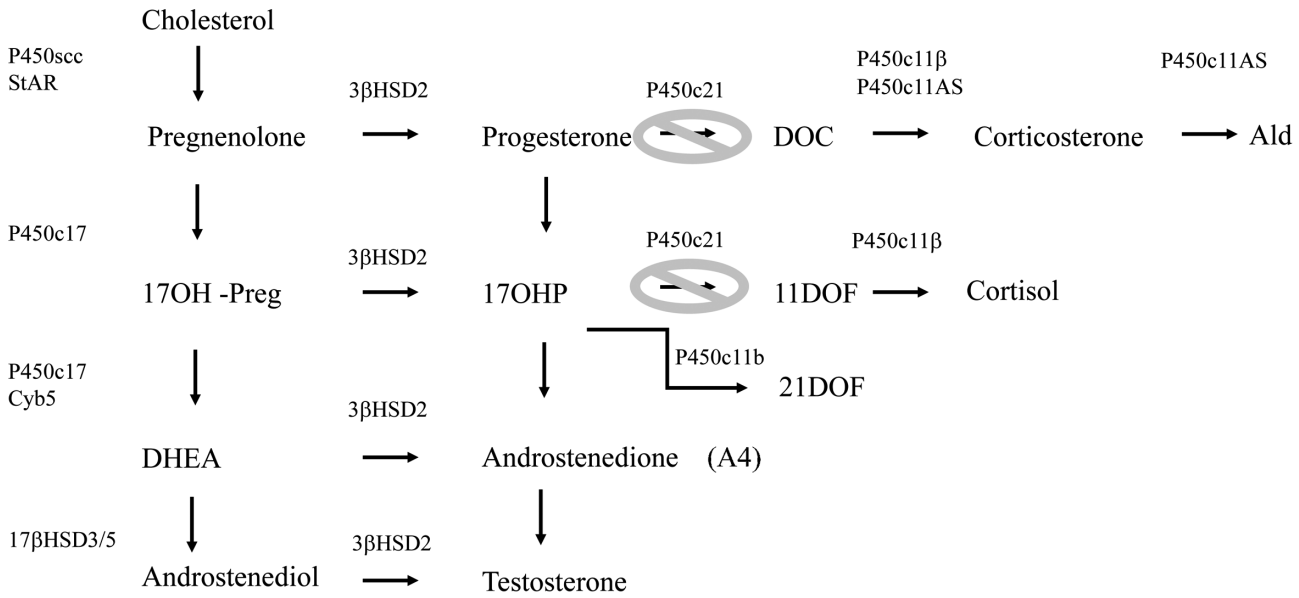

Fig. 1. Steroidgenesis pathway. P450scc, cholesterol side chain cleavage enzyme; StAR, steroidogenic acute regulatory protein; 3βHSD2, 3β-hydroxysterod dehydrogenase type 2; P450c21, 21-hydroxylase; P450c11β, 11β–hydorxylase; P450c11AS, aldosterone synthase; P450c17, 17α-hydroxylase/17, 20-lyase; Cyb5, cytochrome b5; 17bHSD3/5, 17β-hydroxysteriod dehydrogenase type 3 or 5; DOC, deoxycorticosterone; Ald, aldosterone; DHEA, dehydroepiandrosterone; 11DOF, 1-deoxycortisol; 21DOF, 21-deoxycortisol.

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classified according to birth weight, whereas 17OHP, (17OHP+A4)/F, and (17OHP+21DOF)/F were used in the second-tier test. Accordingly, the PPV was 1% pre-LC-MS/MS introduction but increased to 17% post-LC-MS/MS introduction. In a report from the Netherlands (13), the first-tier test was stratified by gestation weeks, and the second-tier test was by stratified by 21DOF measurement. Herein, the PPV increased to 53% post-LC-MS/MS introduction. Five out of thirty-seven patients exhibiting elevated 21DOF levels in the second-tier test were not referred to the hospital. Therefore, whether these cases were true or false positives could not be determined. de Hora *et al*. (12) and Fujikura *et al*. (18) reported that the 21DOF levels were reduced in several patients with 21OHD. Using 21DOF-based indicators may result in delayed 21OHD diagnosis. de Hora *et al*. (17) employed cut-off values for 17OHP that were adjusted for birth weight in the first- and second-tier tests. Then, 21DOF and (17OHP+A4)/F were used in the second-tier test. Thus, the PPV increased from 10.1% pre-LC-MS/MS introduction to 45.8% post-LC-MS/MS introduction. In the Korean study (9), over 2-yr, 5,852 blood spots were analyzed, and 2.2% of samples exceeded the cut-off value for 17OHP in the first-tier test [0.8% (37/4474) for term infants and 6.4% (88/1378) for preterm neonates. The second-tier test, using LC-MS/MS based on 17OHP and (17OHP+A4)/F ratio showed positive results in two patients, both of whom had 21OHD. Pre-LC-MS/MS introduction, the average follow-up period until baseline 17OHP was 75 d. However, post-LC-MS/MS introduction, the follow-up period was shortened to an average of 7 d.

Status of LC-MS/MS as the second-tier test in Japan

Reports from Japan on the application of LC-MS/MS for secondary testing are summarized in **Table 2** (18–22). Fujikura *et al*. (18) were the first in Japan document the use of LC-MS/MS in second-tier testing for CAH. Accordingly, the cut-off values were as follows: ≥ 4 ng/mL of 17OHP or ≥ 3 ng/mL of 17OHP and ≥ 0.2 of (17OHP+4A/F) in neonates born post-37 wk of gestation; ≥ 6 ng/mL of 17OHP or ≥ 5 ng/mL of 17OHP and ≥ 0.2 of (17OHP+4A/F) in neonates born pre-36 wk of gestation. Thus, the overall recall rate decreased from 0.65% to 0.32%. Specifically, it decreased from 0.09% to 0.02% for neonates born post-37 wk of gestation, and from 7.9% to 4.0% for neonates born pre-36 wk of gestation. Furthermore, Yamagishi *et al*. (20) demonstrated the utility of the 11DOF/17OHP ratio. From 2013 onwards, the criteria for second-tier testing did not include gestational age (**Table 2**). Therefore, the overall recall rate decreased to 0.061%. Specifically, it decreased to 0.0061% for neonates born after 37 wk of gestation and 0.055% for neonates born before 36 wk of gestation. Additionally, the PPV improved to 75%. The PPV of the second-tier test in Sapporo using LC-MS/MS and Tokyo Metropolitan simulation was better than that reported in other countries. Kanda *et al*. (19) and Isobe *et al*. (21) incorporated 11DOF/17OHP into the cut-off value (**Table 2**). In the Miyazaki prefecture, the overall recall rate decreased from 1.54% to 0.38% (≥ 36 wk, decreased from 0.72% to 0.02%; < 36 wk, decreased from 0.83% to 0.36%). In Saitama city, the recall rate decreased from 1.71% to 0.81%. Furthermore, Watanabe *et al*. (22) documented the results of an algorithm-based simulation for second-tier testing using LC-MS/MS. This algorithm combines the 17-OHP, 21-DOF, 11DOF/17OHP, and

Table 1. Use of liquid chromatography with tandem mass spectrometry (LC-MS/MS) for the second-tier test in NBS for CAH

State (USA) or country	Parameter of second-tier test	PPV[1] before LC-MS/MS (%)	PPV after LC-MS/MS (%)	Improvement rate after LC-MS/MS (%)
Minnesota (6)	17OHP[2], (17OHP+A4[3])/F[4]	0.8	7.3	812
Utah (7)	17OHP, (17OHP+A4)/F	0.4	9.4	2250
California (8)	17OHP, (17OHP+A4)/F	NA	7.0	NA
Wisconsin (10)	17OHP, (17OHP+A4)/F (17OHP+21DOF[5])/F	1	17	1600
Denmark (15)	17OHP, (17OHP+A4)/F	NA	55	NA
Netherland (13)	21DOF	24.7	53	114
China (16)	17OHP, (17OHP+A4)/F	1.4	26.0	1757
New Zealand (17)	17OHP, (17OHP+A4)/F 21DOF	10.1	45.8	353
Australia (11)	17OHP, (17OHP+A4)/F		71.4	NA
Italy (14)	17OHP, (17OHP+A4)/F 21DOF 11DOF[6]	0.24	2.54	958
Korea (9)	17OHP, (17OHP+A4)/F	1.9	100	5163

[1] PPV, positive predictive value; [2] 17OHP, 17-hydroxyprogesterone; [3] A4, androstenedione; [4] F, cortisol; [5] 21DOF, 21-deoxycortisol; [6] 11DOF, 11-deoxycortisol; NA, not available.

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Table 2. Use of liquid chromatography with tandem mass spectrometry (LC-MS/MS) for the second-tier test in NBS for CAH in Japan

Area	Parameters of second-tier test	Cut-off value for second-tier test	PPV[1] before LC-MS/MS (%)	PPV after LC-MS/MS (%)	Recall rate before LC-MS/MS (%)	Recall rate after LC-MS/MS (%)	PPV before LC-MS/MS (%)	PPV after LC-MS/MS (%)
Sapporo city (20)	17OHP[2] (17OHP+A4[3])/F[4] 11DOF[5] /17OHP	Immediate referral ① 17OHP ≥ 50 ng/mL or ② ≥ 37 wk of gestation and 17OHP ≥ 20 ng/mL or ③ 21DOF ≥ 2 ng/mL Resampling 17OHP ≥ 2.5 ng/ml and 11DOF/17OHP ≤ 0.2 and (17OHP+A4)/F ≥ 0.2	25	75	0.65	0.061 ≥ 37 wk[6] 0.0061 < 37 wk 0.055	NA	75
Saitama city (21)	17OHP 11DOF/17OHP	Immediate referral ① 21DOF ≥1.0 ng/mL or ② 17OHP ≥ 30 ng/mL (< 37 wk) 17OHP ≥ 20 ng/mL (≥ 37 wk) Resampling 17OHP ≥ 1.0 ng/mL and (17OHP+A4)/F ≥ 0.10 and 11DOF/17OHP ≤0.3	NA	NA	1.71	0.18	NA	NA
Miyazaki prefecture (19)	17OHP (17OHP+A4)/F 11DOF/17OHP	Resampling ① 17OHP ≥ 5 ng/mL or ② 17OHP ≥ 2 ng/mL and 11DOF/17OHP ≤ 0.2 or 17OHP ≥ 2 ng/mL and (17OHP+A4)/F ≥ 0.1	NA	NA	1.54 ≥ 36 wk 0.72 < 36 wk 0.83	0.38 ≥ 36 wk 0.02 < 36 wk 0.36	NA	NA
Tokyo Metropolitan (22)[7]	17OHP (17OHP+A4)/F 11DOF/17OHP	Immediate referral ① 17OHP > 5.0 ng/mL and 21DOF > 1.0 ng/mL or ② 17OHP > 5.0 ng/mL and 11DOF/17OHP < 0.1 and (17OHP+A4)/F > 2.0 Resampling 17OHP > 1.5 ng/mL and 11DOF/17OHP < 0.3 and (17OHP+A4)/F > 0.3	41	92	0.45	0.0259	41	91

[1] PPV, positive predictive value; [2] 17OHP, 17-hydroxyprogesterone; [3] A4, androstenedione; [4] F, cortisol.; [5] 11DOF, 11-deoxycortisol; [6] Gestational age; [7] Simulation results, not actual data; NA, not available.

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17OHP+4A/F levels for any gestational age. The PPV increased from 41% to 91%, and the recall rate decreased from 0.45% to 0.0259%. However, the actual simulation results in the Tokyo Metropolitan area remain to be observed.

Should LC-MS/MS be used as a first-tier test, it will improve PPV. However, the PPV improvement rate is likely to be similar to that obtained when LC-MS/MS is used as a second-tier test. Testing cost depends on the number of tests. However, if > 30,000 neonates are tested annually, a backup LC-MS/MS device would be required, whose cost for one test is higher than that of ELISA. Therefore, the total cost of LC-MS/MS as a first-tier test is expected to be about twice as much per sample as second-tier LC-MS/MS. The testing duration will also be longer than that of a first-tier test using the ELISA method.

High rates of false-negative results lead to unnecessary parental concerns and an increased resampling burden for neonates, inspectors, and consultant doctors. Reduced false-negative results improve clinical practice by minimizing the need for subspecialty endocrine evaluations. Conversely, a true-positive can be achieved via endocrine consultation and timely confirmatory testing.

In developed countries where blood spot NBS services are provided, LC-MS/MS equipment is used to screen patients for inherited metabolic diseases. For experienced scientists, using LC-MS/MS to simultaneously measure the levels of multiple steroids is not technically difficult, however, the biggest challenge is cost. Watanabe *et al*. (22) estimated that the cost per sample is approximately 300 yen, which is similar to the cost of ELISA. Annually, a cost reduction of 120,000 yen is achieved by reducing the number of tests and minimizing repeated blood sampling. Furthermore, a reduction in the need for confirmatory tests is likely to result in further cost reductions. However, the LC-MS/MS equipment itself costs 80 million yen, with estimated maintenance costs of 4 million yen per annum, although this price will hopefully decrease in the future.

Conclusion

Second-tier testing using LC-MS/MS for CAH screening reduces the burden of follow-up care and improves clinical practice by minimizing the necessity of subspecialty care, such as endocrine evaluation. The benefits and disadvantages of this test will need to be further examined by government agencies, testing facilities, and the various professions involved in NBS.

Conflict of interests: The author have nothing to declare.

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