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Liquid chromatography–mass spectroscopy as a tool in the rapid diagnosis of biliary atresia: a pilot study

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ABSTRACT

Introduction: Biliary atresia (BA) is a neonatal obstructive cholangiopathy requiring rapid intervention to prevent end-stage liver failure and death. Low bile acid levels in stool, detectable with high-performance liquid chromatography–mass spectroscopy, may reflect extrahepatic biliary obstruction in cholestasis.
Hypothesis: Stool bile acid content can differentiate BA from non-BA forms of cholestasis.
Methods: Stool samples from four healthy and nine cholestatic patients were collected following internal review board approval. Bile acids were extracted and separated on a 4000-Q-Trap HPLC-MS system.
Results: Total bile acid content was highest in samples from healthy relative to cholestatic patients: 3354.01 ± 2102.56, 1476.27 ± 1361.07, and 34.29 ± 10.30 μM/mg of stool in healthy, total parenteral nutrition-associated cholestasis, and BA samples, respectively. Mean cholic acid and chenodeoxycholic acid concentrations in healthy samples (2017.5 ± 1413.6 and 876.83 ± 660.60 μM/mg) were higher than in TPN cholestatic samples (93.99 ± 131.55 and 232.34 ± 293.41 μM/mg). The most dramatic reduction in cholic acid and chenodeoxycholic acid was observed in BA samples (0.65 ± 0.47 and 1.22 ± 0.80 μM/mg).
Conclusion: Bile acid content in stool is reduced in cholestatic patients relative to healthy patients with the most dramatic reduction observed in BA-patients.

Biliary atresia (BA) is a fatal neonatal disease characterized by progressive fibro-inflammatory destruction of the bile ducts, leading to end-stage liver disease and death by 2 years if left untreated [1]. Definitive diagnosis requires direct evidence of extrahepatic biliary obstruction, typically with intraoperative cholangiogram to visualize the biliary tract. Delayed diagnosis is considered a missed opportunity for treatment. Currently, the only known treatment is to restore biliary drainage via a surgical portoenterostomy (Kasai procedure), which involves excision of the entire extrahepatic biliary tree with drainage of the residual biliary ductules into a jejunal Roux-en-Y limb. Success of surgery is defined by the achievement of normal serum bilirubin concentration within 6 months and theoretically depends on the patency of these microscopic biliary ductules at the time of operation [2]. Age is strongly associated with successful outcomes, with multiple case series advocating surgery prior to 60 days of life – at which point every 10 days of delay decreases success rates by 50% [3]. While the notion of age cutoffs for surgery is controversial, the tenet of early surgery since the introduction of the Kasai procedure continues to underscore BA management today. Until the etiology of BA is better understood, early and accurate diagnosis will continue to be the mainstay of treatment, necessitating better strategies to meet a limited window of therapy.

The current gold standard for diagnosis of BA is liver biopsy and intraoperative cholangiogram. A high level of suspicion is warranted before subjecting cholestatic infants to the morbidity of this invasive procedure. Conjugated jaundice and pale stools comprise a clinical presentation that is compelling for the diagnosis of BA, yet frequently prompts initial workup for the wide range of more common infective, genetic, and metabolic etiologies for cholestasis. Screening algorithms for BA that incorporate laboratory and radiographic data have been useful in stratifying risk but lack diagnostic power due to the clinical heterogeneity of BA and overlap with other causes of neonatal cholestasis [4,5]. These same parameters have been used to generate predictive scores once the diagnosis of BA is confirmed. One recent scoring methodology cited high diagnostic accuracy (100% sensitivity and 97.6% specificity) by including 12 parameters, with higher scores largely attributable to histopathology findings obtained from liver biopsies [6]. However, no test stands alone for the diagnosis of BA nor can they altogether exclude BA outside of cholangiogram. Ultimately, the cost of additional time and false-negative tests must be balanced against the possibility of a negative laparotomy that holds diagnostic accuracy.

Other diagnostic tests have been suggested as less-invasive methods of diagnosing BA. Endoscopic retrograde cholangiopancreatography has

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been shown to be feasible in the differential diagnosis of some pediatric hepatobiliary diseases and has variable visualization rates of the pancreaticobiliary system in BA. Furthermore, endoscopic retrograde cholangiopancreatography has less success in infants relative to older children [7]. Since biliary obstruction results in the lack of bile flow into the intestine, tests such as the hepatobiliary iminodiacetic acid scan, stool color cards, and duodenal tube tests may be useful. Radioisotope uptake by the liver on hepatobiliary iminodiacetic acid scan results in reduced or absent excretion into the intestine within 24 h in severe intrahepatic cholestasis, but absent excretion from severe cholestasis is by no means exclusive to BA [8]. Population-based screening using stool color cards has proven to be cost-effective in countries outside the US where the incidence of BA is higher, although incorrect judgment of stool color continues to hinder diagnosis and still requires invasive confirmation of disease. Similarly, duodenal tube testing, which involves bile sampling at the distal duodenum, may qualitatively exclude BA when yellow biliary fluid is observed within 24 h. However, equivocal cases still arise, and invasive surgical cholangiograms are still mandated [9].

A validated, noninvasive method by which to discriminate between BA and non-BA patients rapidly and accurately could change the treatment paradigm in BA. Measuring the bile acid content of stool may provide such a noninvasive test to confirm BA without the need for intraoperative cholangiogram and minimize the risk of a negative laparotomy. We hypothesize that stool bile acid content, detectable using high-performance liquid chromatography–mass spectrometry (HPLC-MS), can effectively differentiate BA from non-BA forms of cholestasis.

1. Materials and methods

This study was approved by the Internal Review Board at Children's Hospital Los Angeles (CHLA-14-00225 and CCI-10-00148). A prospective study was performed on all infants between July 2014 and July 2015 who were <100 days when workup was initiated for conjugated jaundice. A comparison cohort consisted of healthy patients in the same age range. Stool samples were collected and stored at −80 °C. The patient's age and laboratory data were recorded at time of collection. Final diagnoses were confirmed upon subsequent review of laboratory and imaging data, in addition to operative and pathology reports. Processing was performed by lyophilizing 10-mg samples of stool overnight and bile acid extraction carried out with solid phase extraction as previously described [10]. Bile acid content was analyzed using the 4000-Q-Trap HPLC-MS system (Applied Biosystems, Waltham, MA).

2. Results

2.1. Patient characteristics

Stool analysis was performed on patients who underwent workup for conjugated jaundice. Final diagnoses were confirmed to be BA in 3 patients, and total parenteral nutrition (TPN)-related cholestasis in 3 patients. Additional diagnoses following cholestatic workup included choledochal cyst, Alagille syndrome, and ornithine transcarbamylase deficiency. As a comparison cohort, four stool samples were obtained from healthy controls with average age spanning 32 ± 9 days of life at the time of collection. Patient characteristics are listed in Table 1.

2.1.1. Bile acid concentrations in stool

We analyzed stool extracts via HPLC-MS and determined total bile acid concentrations in addition to levels of cholic acid (CA) and chenodeoxycholic acid (CDCA), the primary bile acids synthesized de novo in the liver. The mean total bile acid concentrations were 3354.01 ± 2102.56, 1476.27 ± 1361.07, and 34.29 ± 10.30 μM/mg of stool in healthy, TPN cholestasis, and BA samples, respectively. Notably, an over 40-fold difference between TPN cholestasis and BA samples was observed in stool bile acid content whereas a less than 1.4-fold difference was observed based on serum bilirubin level, the standard marker of cholestasis. No definitive conclusion can be drawn based on wide standard deviations within each sample group.

Table 1
Demographics, laboratory values, and final pathology associated with stool samples at time of collection.

Sample	Age (d)	Serum bilirubin (mg/dL) total (*Direct)	Diagnosis
1	26	4.2 (2)	BA
2	73	6.8 (2.7)	BA
3	67	8.2 (3.9)	BA
4	71	9.2 (5.1)	TPN cholestasis
5	27	11.4 (6.1)	TPN cholestasis
6	93	6 (2.0)	TPN cholestasis
7	53	7.1 (3.1)	Choledochal cyst
8	70	11.8 (7.4)	Alagille syndrome
9	146	0.3	OTC
10+	27	—	Control
11+	29	—	Control
12+	25	—	Control
13+	46	—	Control

+ = healthy control; OTC, ornithine transcarbamylase.

In healthy stool samples, CA made up more than half of total bile acid content, compared to CDCA (57.88 ± 5.08% and 24.76 ± 4.84%, respectively). Mean CA and CDCA concentrations in healthy stools were 2017.5 ± 1413.6 μM/mg and 876.83 ± 660.60 μM/mg, respectively. The disproportionate ratio of CA to CDCA was reversed in cholestatic stool samples with more contribution from CDCA than CA, although this trend was not statistically significant. In TPN cholestatic stool samples, 14.61 ± 6.38% of total bile acids were attributable to CDCA compared to 4.12 ± 4.03% comprising CA, corresponding to concentrations of 232.34 ± 293.41 μM/mg and 93.99 ± 131.55 μM/mg. Bile acid content was lowest in BA stool samples, with 3.95 ± 3.14% of total bile acids comprising CDCA compared to 2.26 ± 1.85% comprising CA. Mean values of CDCA and CA in BA stool were 1.22 ± 0.80 μM/mg and 0.65 ± 0.47 μM/mg, respectively. Patients with Alagille syndrome, ornithine transcarbamylase deficiency, and choledochal cyst had very low total bile acid levels. The patient with Alagille syndrome had a >5-fold higher level of both CA and CDCA compared to BA. However, since there was only 1 patient in each of these 3 diagnoses, we cannot draw any conclusions (Table 2).

Measurements of total bile acid, CA, and CDCA concentrations in control, TPN cholestasis, and BA patients are compared in Fig. 1.

3. Discussion

This pilot study aims to demonstrate the rationale that threshold levels of bile acids in stool can be used to accurately differentiate BA from non-BA patients. In addition to total bile acids, specific quantities or ratios of primary bile acid concentrations may be valuable using efficient stool extraction methods and HPLC. The capacity to identify and quantify an entire spectrum of conjugated and nonconjugated bile acids, thereby detecting even minute bile acid metabolites in the stools of cholestatic patients, may potentially lead to an even more reliable, noninvasive test for the exclusion of BA without the need for operative diagnosis.

Bile acids are synthesized in the liver and secreted into the small intestine as free acids or as glycine or taurine conjugates. In addition to the 2 primary bile acids, CA and CDCA, there are secondary bile acids, including

Table 2
Concentrations of bile acids are shown in μM/mg stool.

Sample	n	Total bile acid	CA (%)	CDCA (%)
Control	4	3354.01	2017.50 (57.88)	876.83 (24.76)
TPN cholestasis	3	1476.27	93.99 (4.13)	232.34 (14.61)
BA	3	34.29	0.65 (2.26)	1.22 (3.95)
Alagille syndrome	1	17.78	3.59 (20.17)	6.53 (36.71)
OTC	1	101.81	0.29 (0.28)	0.76 (0.75)
Choledochal cyst	1	34.34	3.36 (9.78)	2.45 (7.12)