An Infant with Persistent Jaundice and a Normal Newborn Direct Bilirubin Measurement

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CASE DESCRIPTION

A 54-day-old infant of Asian descent presented with jaundice. He first started appearing yellow a few weeks after birth. His pediatrician initially recommended increasing sunlight exposure. At subsequent visits, the pediatrician recommended stopping breastfeeding. Despite these interventions, the infant's jaundice persisted and his stools became pale. At 52 days of life (DoL),3 he had a serum bilirubin measured, and the reported “Bilirubin, Direct” concentration of 5.54 mg/dL (reference interval, 0.0–0.4 mg/dL) prompted an immediate referral (see Table 1 for a summary of laboratory results).

The infant's physical examination and evaluation results were most consistent with biliary atresia (BA). He had marked jaundice, with a reported “Bili Conjugated” of 4.7 mg/dL (reference interval, 0.0–0.2 mg/dL), as well as increased aspartate aminotransferase, alanine aminotransferase, and γ-glutamyltransferase activities. He otherwise appeared well and had 2 newborn screens with results within reference intervals, making infectious or metabolic etiologies unlikely. Furthermore, protease inhibitor typing, chest radiograph, and abdominal ultrasound revealed no abnormalities, arguing against other liver-associated causes such as α1-antitrypsin disease, Alagille syndrome, and choledochal cyst.

There was one laboratory result, however, that was inconsistent with BA: his newborn conjugated bilirubin concentration, reported as “Neonatal Dbil.” In our experience, infants with BA have newborn direct or conjugated bilirubin concentrations that exceed their birth hospital’s derived reference interval (1). In contrast, this infant had a reported “Neonatal Dbil” concentration of 0.5 mg/dL on DoL 1, which was within the birth hospital’s reported reference interval of 0.0–0.6 mg/dL. The bilirubin was measured using a Vitros analyzer, and the reference interval was derived by the manufacturer based on “40 apparently healthy neonates” (2).

Because infants with BA treated earlier have the best outcomes, we continued the evaluation despite the discrepant newborn bilirubin concentrations. He promptly underwent liver biopsy, which showed fibrosis and bile duct proliferation characteristic of BA. Subsequent intraoperative cholangiogram confirmed the BA diagnosis. However, one important question still remained: how could the infant’s reportedly normal “Neonatal Dbil” concentration at birth be explained?

DISCUSSION

As many as 15% of infants may present to their pediatricians for evaluation of jaundice (3). Most have increased unconjugated bilirubin concentrations, which can usually be treated supportively with increasing sunlight exposure or switching from breast milk to formula. Some infants, on the other hand, have high conjugated bilirubin concentrations. These infants may have more serious diseases that require prompt intervention, because increased conjugated bilirubin concentrations are a marker for a variety of infectious, metabolic, and/or liver conditions.

For infants with increased conjugated bilirubin concentrations, practitioners should review the bilirubin measurements in the newborn period to help make the diagnosis. Newborn total bilirubin concentrations are often measured to determine need for phototherapy and, as in this case, total as well as conjugated (commonly referred to as “Dbil,” “direct,” or “conjugated”) concentrations are reported. If the newborn conjugated concentration is high, the practitioner can assume the infant was born with disease and should suspect metabolic or liver-related causes. If the newborn conjugated con-

QUESTIONS TO CONSIDER

1. What is the difference between “Neonatal Dbil,” “Bilirubin, Direct,” and “Bili Conjugated”?
2. How should reference intervals be established?
3. Why are the reference intervals for the 3 tests in Table 1 different?
centrations across the hepatocyte’s membrane correctly. However, if bilirubin is not transported across the liver’s membrane correctly (as in viral infections) or if bilirubin is not transported across the hepatocyte’s membrane correctly (as in Dubin-Johnson syndrome). They can also increase in diseases such as BA where bile ducts are obstructed. Normal bile flow ceases, preventing all components of bile, including conjugated bilirubin, from passing appropriately. Instead, bile backs up into the liver and eventually into the bloodstream. Delta bilirubin is a third form of conjugated bilirubin, which is only present in chronic liver diseases. Delta bilirubin forms when serum concentrations of bilirubin mono- and diglucuronide are so high that some covalently bind with albumin. Delta bilirubin’s bond with albumin is essentially irreversible, and delta bilirubin clearance follows the slow kinetics of albumin clearance. As a result, delta bilirubin concentrations can be present even after bilirubin mono- and diglucuronide have been cleared and a patient’s primary liver problem has resolved.

**CONJUGATED BILIRUBIN IS INCREASED IN LIVER DISEASE**

Serum generally contains 2 types of bilirubin. The first type, unconjugated bilirubin, forms when old red blood cells are cleared and heme is degraded. Unconjugated bilirubin can present a problem in neonates, because increased concentrations can accumulate in the developing brain and cause the devastating neurological disease kernicterus. As a result, high unconjugated concentrations in newborns are treated with phototherapy, which lowers unconjugated bilirubin by converting it to dozens of different isomers that are more efficiently cleared from the circulation (4).

The second type, conjugated bilirubin, is formed when hepatocytes process unconjugated bilirubin for excretion. Hepatocytes collect unconjugated bilirubin from the circulation and make it more water soluble by attaching—or conjugating—1 or 2 glucuronide moieties to bilirubin through a well-characterized esterification reaction. Hepatocytes then secrete the bilirubin mono- and diglucuronide into the canalicular space, where it dissolves in bile and ultimately passes out of the body with stools (4).

Conjugated isoforms accumulate in serum in a variety of liver diseases. For example, bilirubin mono- and diglucuronide concentrations can increase if hepatocytes lyse (as in viral infections) or if bilirubin is not transported across the hepatocyte’s membrane correctly (as in

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**Table 1. Summary of fractionated bilirubin results.**

<table>
<thead>
<tr>
<th>Day of life</th>
<th>Test name</th>
<th>Assay</th>
<th>Instrument</th>
<th>Result, mg/dL</th>
<th>Reference interval, mg/dL</th>
<th>Reference interval source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>“Neonatal Dbil”</td>
<td>Direct spectrophotometry</td>
<td>Vitros</td>
<td>0.5</td>
<td>0.0–0.6</td>
<td>Manufacturer</td>
</tr>
<tr>
<td>52</td>
<td>“Bilirubin, Direct”</td>
<td>Chemical reaction (Diazo)</td>
<td>Roche</td>
<td>5.54</td>
<td>0.0–0.4</td>
<td>Laboratory derived</td>
</tr>
<tr>
<td>54</td>
<td>“Bili Conjugated”</td>
<td>Direct spectrophotometry</td>
<td>Vitros</td>
<td>4.7</td>
<td>0.0–0.2</td>
<td>Laboratory derived</td>
</tr>
</tbody>
</table>

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Unfortunately, as highlighted by this case, practitioners face a number of challenges in interpreting newborn conjugated bilirubin concentrations correctly. Our infant did indeed have high conjugated bilirubin concentrations at birth, consistent with his diagnosis of BA. However, his newborn concentration was overlooked because of 2 subtle yet critical details: (a) the result was reported as “Dbil,” when in fact “conjugated” bilirubin was assayed; and (b) the reference interval was too broad for newborn “conjugated” bilirubin assays. How these errors occurred—and continue to occur—can be understood by examining the nuances of conjugated bilirubin measurements.

**DIRECT AND CONJUGATED ASSAYS ARE NOT EQUIVALENT**

The first issue in this case was an issue in reporting. The laboratory reported a “Dbil” result when in fact “conjugated” bilirubin was assayed. “Direct” and “conjugated” bilirubin assays are widely available, and, as in this patient, are often performed in the same patient at different times. As a result, the 2 are often confused and used interchangeably. However, the 2 are very different assays, measuring different bilirubin fractions using unrelated technologies.

“Direct” bilirubin assays measure all conjugated bilirubin (bilirubin monoglucuronide, bilirubin diglucuronide, and delta bilirubin) as well as some unconjugated bilirubin. “Direct” assays involve a chemical reaction with diazo dyes, followed by quantification of azobilirubin produced over a specified time. All conjugated bilirubin forms react quickly, whereas unconjugated bilirubin forms react more slowly (unconjugated bilirubin forms can react quickly if an accelerant is added, as is done for total bilirubin measurements). Hence, the “direct” assays always include delta bilirubin and a small amount of unconjugated bilirubin (4).

The “conjugated” bilirubin assay, on the other hand, measures bilirubin mono- and diglucuronide alone. This assay is based on direct spectrophotometry, using the BuBc slide on the Vitros analyzer. The BuBc slide shifts the absorbance spectrum of bilirubin mono-/diglucuronide by...
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30–40 nm, thereby allowing these forms to be quantified separately from unconjugated and delta forms (5). As a result, “conjugated” measurements are usually less than “direct” measurements, because they do not include delta bilirubin or any unconjugated bilirubin [compare DoL 54 and 52 concentrations in this case (Table 1)].

Though “direct” and “conjugated” assays are the most commonly available, they are not the only ways to measure conjugated bilirubin concentrations. For example, the bilirubin oxidase and vanadate oxidation methods are enzymatic and chemical assays, respectively. They involve converting conjugated bilirubin forms into biliverdin and, unlike the diazo method, are unaffected by coexisting substances such as hemoglobin or vitamin C (6). HPLC, currently used mainly for research purposes (4), can also be used. HPLC offers the advantage of detecting minor bilirubin fractions such as those produced with phototherapy.

**“DIRECT” AND “CONJUGATED” REFERENCE INTERVALS SHOULD BE VERIFIED**

The second issue in this case was an issue of borrowing reference intervals. Many laboratories face challenges with pediatric reference intervals. Few have the resources to derive their own ranges of values for every test and age. Instead they borrow reference intervals from manufacturers, and now, more recently, from large initiatives such as the CALIPER (Canadian Laboratory Initiative on Pediatric Reference Intervals) database (7). In many scenarios, this is appropriate; however, “direct” and “conjugated” bilirubin assays deserve special consideration.

For example, “direct” assay methods differ from laboratory to laboratory, complicating the use of a single reference interval. “Direct” measurements using the diazoo method vary depending on a number of site-specific factors, including how long the chemical reaction is allowed to proceed, the pH of the reaction, the strength of the diazo reagents, and the instrument used (4). As result, reference intervals that combine data from many laboratories, such as a published range of approximately 0.0–1.0 mg/dL from 2898 infants age 0–14 days, are too broad to be of practical use (8). Instead, derived reference intervals similar to the range from the DoL 52 measurement in this case are clinically more meaningful.

For the “conjugated” assay, borrowing reference intervals poses a different problem, as demonstrated in this case. The “conjugated” assay should vary less from laboratory to laboratory because it always uses the same reagent (the BuBc slide) and is performed on the same instrument (the Vitros analyzer). However, the manufacturer’s reference interval of 0.0–0.6 mg/dL does not match that derived in clinical practice. For example, a much narrower range of 0.0–0.3 mg/dL was calculated from 64095 newborns ages 0–14 days who had a clinical reason to have bilirubin measured (8). Similarly, our hospital and others with the Vitros analyzer independently derived a reference interval of 0.0–0.2 mg/dL by using concentrations from cohorts of healthy newborns.

We surmise 2 reasons for why such a broad reference interval was used by the manufacturer. First, the manufacturer may have used too small of a sample size for their reference interval calculations. The manufacturer reports using measurements from 40 newborns for its reference interval, whereas the standard is to calculate reference ranges using samples from at least 120 individuals (2, 9). Second, widening the reference interval could reduce false positives and increase specificity. The upper limit of the reference interval is traditionally defined as the highest 2.5% of concentrations, resulting in increased concentrations in as many as 1 in 40 cases. By broadening the reference interval beyond the standard limits, the high positive rate would certainly decrease; however, it does so at the expense of missing cases with serious disease, such as the infant in this case.

**CLINICAL IMPLICATIONS**

The newborn in this case was overlooked because of 2 subtle but clinically important problems, which we were able to uncover only after considerable investigation. The most important clue was realizing that the laboratory was actually measuring “conjugated” bilirubin concentrations. Whereas a “direct” bilirubin concentration of 0.5 mg/dL could be within the reference interval because of measurement variations, a “conjugated” bilirubin concentration of 0.5 mg/dL is well above all published and independently derived reference intervals. This discrepancy prompted us to further question how the laboratory obtained its reference intervals.

Importantly, if only 1 of the 2 problems had occurred, this infant could have been recognized in the newborn period. For example, had the test been labeled correctly, some providers would have recognized the high “conjugated” bilirubin concentration despite the reference interval provided. Similarly, had a narrower reference interval been used, all providers would have identified the bilirubin concentration as abnormal regardless of how the test was labeled. Unfortunately, when both problems are combined, the test result becomes impossible to interpret correctly without more information.

Theoretically, the error prevented what could have been an earlier diagnosis and treatment, which in turn correlates with delaying or even preventing need for liver transplantation (10). With an abnormal newborn concentration, the infant’s pediatrician would have been advised to repeat the test at the 2-week well-child visit. Although this method introduces a small delay, in our experience it effectively excludes many of the newborns who test high but do not have liver disease. This infant would have retested high at 2 weeks and would have then been referred to us urgently. We would have performed
Points to Remember

- Newborn “direct” and “conjugated” bilirubin concentrations can help identify infants with serious liver diseases such as BA.
- “Direct” assays use a chemical reaction and measure bilirubin monoglucuronide, bilirubin diglucuronide, delta bilirubin, and a small amount of unconjugated bilirubin.
- The “conjugated” assay uses spectrophotometry and measures bilirubin monoglucuronide and bilirubin diglucuronide.
- Reference intervals for newborn “direct” or “conjugated” bilirubin concentrations should be verified independently by each laboratory.

References


Commentary

Allah B. Haafiz

BA is the most common cause of liver transplantation in children. The success of the Kasai procedure progressively diminishes with older age at the time of surgery. Early identification of conjugated hyperbilirubinemia remains the first step toward the timely diagnosis of BA. In this context, Harpavat et al. present an illustrative case that underscores the subtle yet clinically relevant difference between conjugated and direct hyperbilirubinemia. An elegant discussion follows the case presentation showing how the synonymous use of “direct” and “conjugated” bilirubin is incorrect, misleading, and can delay the diagnosis of BA. This discussion is timely because most clinicians are not familiar with the highlighted biochemical and methodological nuances. However, the discussion does not assertively link this case with, and thus draw the attention of the reader to, the wider implications of the issues so elegantly presented. For example, the main message of this report—the incorrect interpretation of “Neonatal Dbil” delaying the diagnosis of BA—reinforces the conclusions of a recent landmark study published by the same group, which is casually referenced in the context of the case presentation. This notable study (1) documented that direct-reacting or conjugated bilirubin is increased as early as 24–48 h after birth in most children with the perinatal form of BA, which accounts for 70%–85% of BA in infants. These findings raise the possibility that measurements of conjugated or direct bilirubin could be used as a screening tool for early detection...
of BA. Documentation of cases such as the one presented here by Harpavat et al., and additional studies addressing sensitivity and cost issues, will help to shift the focus to screening all children rather than the current standard of care of screening only those children having persistent jaundice beyond 2 weeks of life. Such a shift could have a dramatic positive impact on the care and outcome of BA, which remains a formidable cause of morbidity in children.

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Reference


Commentary

Dennis J. Dietzen*

Not all neonatal jaundice is created equal. Unconjugated hyperbilirubinemia and its associated risk of kernicterus get lots of publicity but conjugated hyperbilirubinemia presents a formidable diagnostic challenge. In addition to BA, the differential diagnosis includes infection, Alagille syndrome, hypothyroidism, galactosemia, bile acid metabolic defects, cystic fibrosis, α1-antitrypsin deficiency, and genetic syndromes like PFIC (progressive familial intrahepatic cholestasis). In the case of BA, early recognition and treatment is essential to spare liver function. The first clues to cholestasis are often biochemical because affected infants remain asymptomatic or only mildly symptomatic for many days or weeks after birth. The laboratory tools at our disposal, however, are not designed to detect small increases of conjugated bilirubin. The conjugated bilirubin assay relies on the intrinsic absorptivity of conjugated bilirubin between 420–430 nm and may be compromised by chromophores that absorb in the same region. Direct diazo methods compare poorly across platforms and are variably sensitive to the often substantial amounts of unconjugated bilirubin in neonatal blood. Neither measure of bilirubin glucuronides provides exceptional precision near the upper limit of the reference interval. Given truncation of direct or conjugated bilirubin to the nearest 0.1 mg/dL (1.7 μmol/L), the smallest detectable change at 0.2 mg/dL (3.4 μmol/L) is 50%. Better tools are clearly needed.

Finally, this case illustrates the importance of clinical context when applying reference intervals. When analyte values in unaffected and affected patients overlap, a clinical cutoff must account for the downside of missing disease as well as the consequences of overdiagnosis. In the case of pediatric cholestasis, optimizing sensitivity spares livers at the potential cost of a repeat bilirubin measurement. Optimizing specificity may lead to irreversible hepatic failure and liver transplant, or death. As the authors of this case point out, optimizing sensitivity in this context is preferable.

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