Effect of Blood Contamination on Delta 450 Bilirubin Measurement: An in Vitro Comparison of Two Corrective Methods, Kirk Foster, Jennifer Moore, Kelly Hankins, Curtis A. Parvin, and Ann M. Gronowski

Amniotic fluid bilirubin peaks (1.6–1.8 mg/L) at ~19–22 weeks of gestation (1). An increase in amniotic fluid bilirubin concentration after 25 weeks indicates extensive hemolysis of fetal blood and potential erythroblastosis fetalis in which amniotic fluid bilirubin can reach ~10 mg/L (2, 3).

Bilirubin in amniotic fluid can be measured by direct spectrophotometry, most commonly by the “delta 450 bilirubin” method (4). Contaminating pigments, such as hemoglobin, can interfere in the method. Chloroform extraction has been used to extract bilirubin, leaving water-soluble pigments in the aqueous layer. The delta 450 bilirubin is then measured in the chloroform fraction and interpreted by use of the Liley plot (5, 6).

A delta 410 correction formula also exists that theoretically accounts for hemoglobin interference. The delta 410 absorbance is measured and used to correct the delta 450 bilirubin (4, 6, 7).

Our objective was to examine the effect of hemolyzed-whole-blood contamination on the delta 450 bilirubin measurement and to compare corrective methods.

After receiving Institutional Review Board approval, we pooled amniotic fluid specimens collected for physician-ordered fetal lung maturity assessment (stored at ~20 °C). Visibly bloody or meconium-contaminated samples were not used. The pool was centrifuged at 1100g for 5 min, and the supernatant was filtered through a 22 μm filter (Millipore Corp.) and a 1.2 μm RA filter (Millipore). Bilirubin was undetectable in the pool by delta 450 measurement.

We prepared a 600 mg/L stock solution of unconjugated bilirubin (ICN Biomedicals Inc.) by dissolving 6.0 mg of bilirubin in 0.1 mL of dimethyl sulfoxide (Sigma Chemical Co.) and 0.2 mL of sodium carbonate (100 mmol/L). The solution was vortex-mixed until the bilirubin was dissolved. We then added 9.5 mL of pooled amniotic fluid, followed by 0.2 mL of HCl (100 mmol/L) to bring the pH to neutral. This solution was protected from light, poured into a foil-wrapped container, and stored at 4 °C.

Blood was collected from a volunteer donor into an EDTA tube. To lyse the erythrocytes, we subjected the blood to at least one freeze-thaw cycle at ~20 °C. The total hemoglobin concentration and oxyhemoglobin and met-hemoglobin fractions, as measured on a blood gas analyzer, were 185 g/L, 89%, and 10% in the first experiment and 180 g/L, 79%, and 19% in the second.

The bilirubin solution was added to the pooled amniotic fluid to produce final bilirubin concentrations of 1.5, 4.5, 9, and 18 mg/L (corresponding to delta 450 bilirubin measurements of 0.076, 0.22, 0.43, and 0.82, respectively). To each of these groups, we added whole blood to produce hemoglobin concentrations of 0, 0.04, 0.2, and 0.4 g/L. Tubes were protected from light by use of aluminum foil. Samples were analyzed immediately on a Spectronic Genesys spectrophotometer (Spectronic Instruments, Inc.) in quartz cuvettes with water as the reference unless otherwise indicated.

Amniotic fluid samples were scanned from 365 to 550 nm. The absorbances at 365, 450, and 550 nm were plotted vs wavelength on semilog paper. The baseline was drawn between the absorbances at 365 and 550 nm. The delta 450 was calculated as the difference between the absorbance and baseline at 450 nm (4).

After spectrophotometric scanning, the samples were mixed with an equal volume of spectrophotometry-grade chloroform (Sigma Chemical Co.) and vortex-mixed for 30 s. After centrifugation (1600g for 10 min at 2–4 °C), the chloroform layer was carefully removed and scanned in quartz cuvettes with chloroform as the reference (5).

For the recovery experiment, we used leftover amniotic fluid samples with no visible blood contamination that had been sent to the laboratory for physician-ordered delta 450 bilirubin measurements and had been wrapped in aluminum foil and frozen at ~70 °C. Samples were thawed and mixed, and the delta 450 was measured immediately. After chloroform extraction, the delta 450 was measured immediately again.

The effects of bilirubin and hemoglobin on delta 450 measurements were estimated by ANOVA using the SAS Proc Mixed procedure (SAS Institute).

Addition of bilirubin up to 18 mg/L to amniotic fluid produced a linear increase in delta 450 bilirubin measurements (not shown). The effect of blood contamination on the delta 450 bilirubin measurement (Fig. 1A) was more pronounced (P = 0.0012) at lower bilirubin concentrations (1.5 mg/L; []). Hemoglobin decreased the absolute delta 450 bilirubin measurement from a baseline of 0.076 to 0.039 at 0.4 g/L hemoglobin. It was not until very high, pathologic concentrations of bilirubin (>9 mg/L; delta 450 >0.43; ■ and ○) that the delta 450 bilirubin measurement, after the addition of blood, was within 10% of baseline.

Chloroform extraction reduced the effect of hemoglobin contamination (Fig. 1B), but decreased (P <0.0001) the measured concentrations of bilirubin, even in samples with no hemoglobin added. At higher concentrations of
bilirubin, the protein layer that formed between the organic and aqueous layers was yellow, suggesting that some of the bilirubin was trapped there.

The significant decrease in the delta 450 measurement as a result of chloroform extraction, even in the absence of added hemoglobin, was unexpected. To confirm this, we examined the effect of chloroform extraction on 10 random amniotic fluid samples with delta 450 measurements ranging from 0.024 to 0.157. Chloroform extraction decreased the delta 450 measurement in 9 of 10 samples with a mean change of −54% (range, −92 to 25%).

Despite considerable discussion in the literature about the effect of whole-blood contamination on delta 450 bilirubin measurements (4–6), including Liley’s original report (4), we have found no published report that carefully quantifies the effect of such contamination. This study confirms that hemolyzed whole blood does interfere with the measurement of bilirubin absorbance at 450 nm. The negative interference seen in Fig. 1A could change interpretation of an individual’s Liley curve from the indeterminate range to the unaffected range, depending on gestational age. This observation contradicts reports that hemoglobin causes an increase in the delta 450 bilirubin (4). To address this contradiction, we carefully examined the spectrophotometric scans. We found that as hemoglobin increases there is a sharp absorbance peak, close to 410 nm, which produces a baseline with a more negative slope. We hypothesize that this causes the difference between the peak at 450 and baseline to decrease, hence giving a smaller delta 450 measurement. At bilirubin concentrations >9 mg/L, blood contamination appeared to have no effect; results were within 10% of baseline values. At no time did we see a false-positive increase in delta 450 bilirubin. These experiments were done with hemolyzed blood containing >10% methemoglobin. We have also examined fresh hemolyzed blood samples with <1.1% methemoglobin and found the same trend.

Because hemolyzed blood contamination was believed to cause a positive, not negative, interference on delta 450 bilirubin measurement (4), the delta 410 correction method was established. However, it is clear that a delta 410 correction cannot correct for hemolyzed blood contamination because it subtracts an additional 5% from an absorbance measurement that is not falsely increased.

The data in Fig. 1B clearly show that chloroform extraction separates blood (aqueous fraction) from bilirubin (chloroform fraction). However, after chloroform extraction, bilirubin measurements were reduced by >60% compared with measurements before extraction. Original reports of the chloroform extraction method recommend that post-extraction measurements be plotted by use of the Liley plot. However, these data would suggest that if the Liley plot is to be used, new cutoffs need to be derived because all delta 450 results are decreased after extraction.

Chloroform extraction does eliminate interference from hemoglobin; therefore, this method would appear to be effective if all samples (not just visibly bloody samples) were extracted and either new cutoff values were derived or trends in serial delta 450 bilirubin measurements were followed rather than absolute delta 450 measurements.

One study has examined whether chloroform-extracted samples correlate better with fetal outcome than unextracted samples (6). The authors determined that chloro-
form extraction was better than both the uncorrected delta 450 and the delta 410 correction (6). Interestingly, they also reported that chloroform extraction caused a decrease (mean of 20%) in the delta 450 measurement in 90% (93 of 103) of specimens. However, they attributed this to the presence of blood that is not detected by visual inspection in the majority of amniotic fluid samples. Hochberg et al. (5) also reported that results for 11 of 21 (52%) samples were significantly different after chloroform extraction. Of these 11 samples, the results for 8 were decreased with a mean difference of −43%. Regardless of whether the cause of the decrease is bilirubin trapped in the protein layer, as we speculate, or visually undetectable hemoglobin, it is clear that the post-chloroform extraction delta 450 measurement is lower than the pre-chloroform extraction measurement in the majority of samples. However, the Liley plot was created using unextracted delta 450 bilirubin measurements. Therefore, if chloroform is used, a new so-called “Liley curve” needs to be derived.

Recently, Egberts et al. (8) reported an iterative spectrophotometric method for the determination of bilirubin in amniotic fluid. This method corrects for contamination with oxy- and methemoglobin. Furthermore, the authors were able to extrapolate their data to a Liley chart and showed excellent correlation. Further studies correlating outcomes are needed, but this method looks promising for assessing erythroblastosis fetalis.

Our data were generated from in vitro experiments that may not duplicate all in vivo conditions. The amount of mel- and oxyhemoglobin may be different in vivo. Clinical outcomes studies are required to determine the effect of hemolyzed blood contamination on patient management. These data suggest that the delta 450 bilirubin measurement, delta 410 correction, and chloroform extraction can all lead to false interpretations of fetal hemolytic status if the amniotic fluid sample is contaminated with hemolyzed blood. The clinical impact of these findings is not clear, but on the basis of these data, we recommend that amniotic fluid samples containing hemolyzed blood not be used to assess fetal hemolysis.

References

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Inflammatory bowel disease (IBD) represents a spectrum of disorders that affect the gastrointestinal tract (1). IBD includes two major entities, Crohn disease and ulcerative colitis (UC). Although the etiology of IBD is unknown at present, it is believed to be an immunologically mediated disease (2). Over the last 40 years, various (auto)antibodies have been described in IBD (3). Anti-Saccharomyces cerevisiae antibodies (4) and perinuclear anti-neutrophil cytoplasmic antibodies (pANCAs) (5) have relatively high prevalence in patients with Crohn disease and UC, respectively. Unlike ANCs present in vasculitis (6) and in Wegener granulomatosis (7), the exact target antigen of UC-associated pANCAs has not been identified (5, 8).

As a consequence, immunofluorescence microscopy is the only widely available technique for screening for these antibodies. Commercially available substrates, however, are not standardized, and part of the discrepancy in results could be attributable to differences among the substrates/assays used, as reported recently (9). Moreover, because specific microscopic criteria to distinguish UC-associated pANCAs from pANCAs seen in vasculitis vary among laboratories, discrepant results could also be attributable to an investigator’s interpretation of the fluorescence pattern.

Despite these methodologic problems, it has been suggested that the determination of pANCAs in UC could serve as an adjunct to conventional tools in the diagnosis of IBD and could be used for better phenotypic classification of the disease. Therefore, pANCA analysis is