Improved Fluorescence Polarization Assay for Use in Evaluating Fetal Lung Maturity. III. Retrospective Clinical Evaluation and Comparison with the Lecithin/Sphingomyelin Ratio

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We clinically evaluated, retrospectively, our improved fluorescence polarization assay for fetal lung maturity. The procedure requires 0.5 mL of amniotic fluid and a standard clinical laboratory fluorescence polarimeter (TDx Analyzer, Abbott Laboratories). We measured the L/S ratios for 93 freshly collected amniotic fluids, uncontaminated with blood or meconium, collected within three days of delivery. The fluids were stored frozen for eight to 32 months, then thawed and assayed for net fluorescence polarization. Fourteen of the infants developed respiratory distress syndrome; five, transient tachypnea of the newborn; and 74, no respiratory distress. The polarization assay and lecithin/sphingomyelin ratio had equivalent receiver operating characteristic curves, indicating no difference in their clinical performance. Although a prospective study with fresh amniotic fluid specimens will be necessary to establish a definitive reference range, the present study shows that this assay can be used to rapidly predict fetal lung maturity.

Additional Keyphrases: amniotic fluid • receiver operating characteristic curves • sensitivity, specificity compared • cutoff (threshold) values • diabetes • reference interval

The neonatal respiratory distress syndrome (RDS) occurs in infants that lack adequate amounts of pulmonary surfactant at birth (1). Laboratory assays that are used to predict fetal lung maturity by measuring surfactant in amniotic fluid are therefore important in the management of many pregnancies (2). Most laboratories measure the lecithin/sphingomyelin ratio (L/S ratio) of amniotic fluid to assess fetal lung maturity. Unfortunately, the value obtained for this ratio is highly method-dependent (2, 3) and the determination is imprecise and has a slow turn-around time (4–5 h in our laboratory). Other types of assays of amniotic fluid (4–10) also have drawbacks, both for the clinical laboratory and for the clinician. The ideal assay for evaluating fetal lung maturity would be rapid, clinically reliable, and available in most laboratories at any time.

Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) is a promising assay technique: rapid, simple, and precise (10). Many clinical studies have shown that the DPH assay predicts fetal lung maturity as well as the L/S ratio (9, 11–21) does. However, the DPH assay as originally described has several shortcomings that limit its applicability in the clinical laboratory. We therefore developed an improved procedure (22) in which a different fluorophore, NBD-PC, is used and which can be performed with a commercial clinical laboratory instrument (TDx Analyzer, Abbott Laboratories). Results correlate well with the L/S ratio (r = −0.85) and the assay is easily performed as a routine clinical test (23).

Here we describe a retrospective clinical evaluation of this improved polarization assay. Polarization measurements were performed on a collection of amniotic fluid specimens that had been stored frozen after being obtained within three days of delivery. The L/S ratios had already been measured on these specimens at the time of amniocentesis. We show that this polarization assay has clinical utility equivalent to the L/S ratio.

Materials and Methods

Specimen collection and assay procedures. During two years we obtained 102 specimens of amniotic fluid from patients at University Hospital; the transabdominal amniocentesis was done within three days before delivery. These specimens were selected without conscious bias from all amniotic fluids submitted for assay during this period. Specimens were kept at 4°C until the L/S ratio was evaluated (usually within 24 h of collection) as described elsewhere (23). Uncentrifuged specimens were stored at −20°C for eight to 32 months. They were then allowed to thaw at room temperature and centrifuged (400 x g, 2 min, 4°C). The supernatants were used in the fluorescence polarization assay as described elsewhere (23), which included an assay temperature of 35°C.

Chart review. Without knowledge of the assay results the medical records of all 102 mothers and 105 infants were reviewed for the following information: indication for amniocentesis, clinically estimated gestational age, use of steroids or tocolytics, route of delivery, labor interval before delivery, premature rupture of membranes, birthdate, gestational age by pediatric examination (Dubowitz), Apgar scores at 1 and 5 min, birth weight, neonatal RDS, neonatal death, and presence of congenital anomaly. The diagnosis of RDS required a compatible chest radiograph, positive clinical signs (tachypnea, cyanosis, grunting, and/or retractions), and progressive increases in therapeutic oxygen requirements. Severity was graded according to therapeutic criteria: severe RDS, >40% O2 atmosphere and intubation; moderate RDS, >40% O2 atmosphere and increased air pressure; and mild RDS, <40% O2 atmosphere and normal air pressure. Infants requiring mild, non-progressive oxygen therapy for less than 36 h were classified as having trans-
Transient tachypnea of the newborn rather than RDS. Transient tachypnea is sometimes diagnosed as mild RDS but has low risk of mortality or residual neurological deficits. These cases were excluded from analysis rather than being arbitrarily classified as RDS or no RDS.

Results

Seven specimens (7%) were excluded because they were contaminated with blood (more than 10 mL/L) or meconium, both of which are known to interfere with this assay (22); two additional infants with respiratory distress were excluded from the evaluation because the pulmonary disease was judged to be caused by sepsis. Five infants developed transient tachypnea of the newborn; these cases were not used in calculating the sensitivities or specificities of each assay.

Of the 88 remaining cases, 14 developed RDS and 74 had no respiratory distress. Although there were three twin births, each set of twins was counted as only one case, because a single amniotic fluid was tested and the clinical outcomes of the twins were concordant. Table 1 lists the net fluorescence polarization and L/S ratio for the infants who developed RDS.

As can be seen in Figure 1, there is a strong inverse correlation between fluorescence polarization and L/S ratio ($r = -0.77$, $P_{rel} = -0.0257$ L/S $+$ 0.3367, samples with L/S $>$ 4.9 excluded, n = 84). The regression slope of polarization onto L/S ratio for these frozen samples, $-0.0257$ per L/S unit, is significantly less ($p = 0.0272$, F test) than the regression slope found with fresh samples, $-0.0320$ per L/S unit (23).

The 74 specimens from infants without RDS had a mean value for fluorescence polarization of 0.2563 (SEM = 0.00346) and mean L/S ratio of 3.11 (SEM = 0.127). The 14 specimens from infants with RDS had a mean polarization of 0.3213 (SEM = 0.00571) and a mean L/S ratio of 1.07 (SEM = 0.170). Although the differences between these means are highly significant ($p < 0.00001$), the clinical usefulness of either of these two assays is more properly assessed by determining the degree of overlap between RDS and non-RDS cases.

Figure 2 shows cumulative frequency plots of both assays for infants with and without RDS. Clinical sensitivity—the true positive rate—is the fraction of cases with mature lungs having a test result indicating fetal lung immaturity. Clinical specificity—1 minus the false positive rate—is the fraction of cases with mature lungs having a test result indicating maturity. Sensitivity and specificity can be determined for any threshold from the frequency plots, as in the dashed lines in the figure demonstrate. If an L/S ratio of $<2.0$ is assumed to indicate fetal lung immaturity, then the sensitivity of the L/S ratio is 84% and its specificity is 87%. If one selects a threshold of 0.300 for net fluorescence polarization, then the sensitivity of the polarization assay is 87% and its specificity is 92%. The mother of the infant with the lowest polarization result ($P_{rel} = 0.259$) was an insulin-dependent diabetic. Of the two cases of RDS with an L/S ratio of 2.0 or greater, one mother had diabetes.

The clinical utility of both assays can be compared by use of a receiver operating characteristic curve, as in Figure 3 (24). Receiver operating curves permit the simultaneous display of the sensitivity and specificity for more than one

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**Table 1. Assay Results and Clinical Outcomes for 14 Infants Developing RDS**

<table>
<thead>
<tr>
<th>Indication for amniocentesis</th>
<th>Route of delivery</th>
<th>Gestation, weeks</th>
<th>Fetal weight, g</th>
<th>$P_{rel}$</th>
<th>L/S ratio</th>
<th>Degree of RDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premature labor</td>
<td>CS</td>
<td>32</td>
<td>2030</td>
<td>0.346</td>
<td>0.8</td>
<td>Severe</td>
</tr>
<tr>
<td>Premature labor</td>
<td>CS</td>
<td>28</td>
<td>1580</td>
<td>0.342</td>
<td>0.5</td>
<td>Severe</td>
</tr>
<tr>
<td>Premature labor</td>
<td>Vag</td>
<td>23</td>
<td>1370</td>
<td>0.338</td>
<td>0.5</td>
<td>Severe</td>
</tr>
<tr>
<td>Previous CS</td>
<td>CS</td>
<td>34</td>
<td>2070</td>
<td>0.335</td>
<td>1.2</td>
<td>Severe</td>
</tr>
<tr>
<td>Premature labor</td>
<td>CS</td>
<td>31</td>
<td>1330</td>
<td>0.332</td>
<td>0.4</td>
<td>Severe</td>
</tr>
<tr>
<td>Placenta previa</td>
<td>CS</td>
<td>34</td>
<td>1880</td>
<td>0.326</td>
<td>0.6</td>
<td>Mild</td>
</tr>
<tr>
<td>Premature labor</td>
<td>Vag</td>
<td>30</td>
<td>1380</td>
<td>0.324</td>
<td>0.9</td>
<td>Severe</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>Vag</td>
<td>35</td>
<td>2450</td>
<td>0.324</td>
<td>1.1</td>
<td>Severe</td>
</tr>
<tr>
<td>Pre-eclampsia, mild</td>
<td>CS</td>
<td>27</td>
<td>1030</td>
<td>0.323</td>
<td>1.0</td>
<td>Severe</td>
</tr>
<tr>
<td>Pre-eclampsia, severe</td>
<td>CS</td>
<td>29</td>
<td>790</td>
<td>0.319</td>
<td>0.5</td>
<td>Severe</td>
</tr>
<tr>
<td>Premature labor</td>
<td>CS</td>
<td>32</td>
<td>2200</td>
<td>0.316</td>
<td>1.4</td>
<td>Severe</td>
</tr>
<tr>
<td>Placenta previa</td>
<td>CS</td>
<td>31</td>
<td>1865</td>
<td>0.310</td>
<td>1.4</td>
<td>Mild</td>
</tr>
<tr>
<td>Fetal distress, twins</td>
<td>Vag</td>
<td>31</td>
<td>740</td>
<td>0.304</td>
<td>2.3</td>
<td>Severe</td>
</tr>
<tr>
<td>Chronic hypertension</td>
<td>CS</td>
<td>32</td>
<td>2660</td>
<td>0.259</td>
<td>2.5</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

*Abbreviations used are CS, cesarean section; Vag, vaginal delivery. *Mother treated with steroids before birth. *Mother has diabetes mellitus. *Neonatal death.
Tests for fetal lung maturity are important in several clinical situations. Lung maturity should be established before elective cesarean section or induction of labor for a patient with an imprecise clinical estimate of gestational age. These tests are also useful in managing pregnant patients with pre-eclampsia, erythroblastosis fetalis, or placenta previa. Premature birth may be helpful in each of these medical conditions. The clinician must decide when the risk of RDS is low enough to warrant therapeutic, albeit premature, delivery of the infant. Fetal lung maturity testing also affects the management of premature labor and premature rupture of membranes, because tocolytic therapy can successfully postpone birth. This therapy, however, has a number of significant side effects on the mother (25). If a rapid laboratory test indicated lung maturity, many clinicians would have the option to withhold tocolytics and allow labor to progress.

Clinical Performance of the NBD-PC Assay

The net fluorescence polarization of NBD-PC predicts fetal lung maturity as well as the more time-consuming L/S
ratio does (see Figure 3). Clinical studies in which a similar methodology was used to determine the L/S ratio \( (12, 15, 26-30) \) have not utilized receiver operating characteristic curves. When plotted, the reported sensitivity and specificity pairs for these methods are close to the L/S ratio curve in Figure 3. Thus the L/S ratio method we used as a comparison in this report is representative of similar published methods.

Neither NBD-PC fluorescence polarization nor L/S ratio determination perfectly distinguishes RDS from non-RDS cases. The clinician may choose different thresholds for predicting pulmonary maturity, depending upon the condition of the mother and fetus. If the mother has had a normal pregnancy and an elective cesarean section is indicated, then a fetal lung maturity assay should be highly predictive of pulmonary maturity (non-RDS), i.e., the sensitivity should be near 100%. In this situation, the threshold for L/S ratio should be 2.4 and for fluorescence polarization, 0.280. This strategy minimizes the chance of delivering an infant with RDS. For these thresholds, the specificities of the assays are 66% and 51%, respectively. For a patient with severe pre-eclampsia, however, the thresholds should be highly predictive of pulmonary immaturity (RDS); therefore, the specificity should be near 100%. The threshold for L/S ratio would be 1.4; for fluorescence polarization, 0.310. This strategy minimizes the chance of not delivering an infant with mature lungs. The respective sensitivities would be 81% and 81%.

Pulmonary surfactant production in the fetus begins at 30 weeks of gestation and usually reaches mature levels by 37 weeks (4). If fluorescence polarization indicates the degree of surfactant production then a decrease should be observed during this gestational time period. Figure 4 clearly demonstrates this decrease. At any given gestational age, patients with diabetes mellitus appear to have lower fluorescence polarization values (Figure 4, dotted line). Simon et al. (31) found similar changes for the DPH fluorescence polarization assay in Classes D, F, and R diabetes but not in Classes A, B, or C. We did not have enough cases of maternal diabetes to test whether there were differences between classes for the NBD-PC assay as well. This difference indicates that patients with diabetes may be a subpopulation and therefore require a separate reference interval (tentatively, 0.020–0.040 lower).

Advantages of the NBD-PC Assay

The present assay has several clinical advantages over L/S ratio assays for the prediction of fetal lung maturity. (The analytical ones are discussed elsewhere \( (22, 23) \).) Through the use of a standard such as Triton X-100 \( (22, 23) \), interlaboratory variation can be minimized. Therefore, many laboratories can jointly establish a reference interval. The assay requires less than 30 min and can be made available on an emergency basis at any time of day. Timely testing will allow the obstetrician additional choices for the management of many pregnancies. For example, the decision to use tocolytic therapy for premature labor may be postponed until after the assay results are known. The fluorescence polarization assay requires only ¼ as much amniotic fluid as does the L/S ratio assay (0.5 mL vs 2.0 mL). This smaller sample requirement will facilitate repeated testing for questionable results and will decrease the frequency of assay failure owing to an insufficient quantity of sample.

Although the actual polarization values for this assay cannot be compared directly with values obtained by the DPH-based assay \( (22) \), the reported sensitivity and specificity pairs for the DPH assays \( (9, 11-21, 32) \) fall close to the receiver operating characteristic curve for the NBD-PC assay in Figure 3. Thus, the NBD-PC assay appears to be clinically equivalent to the DPH assay.

Limitations of This Study

The specimens used in this study were stored frozen for up to 32 months. Freezing for up to one month did not significantly alter the NBD-PC polarization values of 30 paired samples \( (23) \). The frozen specimens analyzed retrospectively in this study appear to differ, however, from the fresh specimens analyzed prospectively in a separate study \( (23) \). The slope of the regression of polarization onto L/S ratio was \(-0.0587\) for the frozen specimens in this study, whereas it was \(-0.0320\) for fresh specimens. The reference interval could therefore differ for fresh amniotic fluids.

It may also be preferable to establish zones of risk for RDS based on this assay, rather than a single threshold for predicting RDS. Although polarization values exceeding 0.300 clearly carry a high risk of RDS, lower values may also carry a high risk of RDS in certain subpopulations of patients, such as diabetics.

We conclude that our clinical evaluation of the present assay indicates that it would improve clinical care. At all thresholds the sensitivities and specificities are equivalent to the L/S ratio, but the present procedure is much more rapid and precise. The NBD-PC assay also represents a technical improvement over the DPH assay, while promising a similar clinical predictive value. These results must, however, be confirmed by a prospective clinical study with fresh amniotic fluid. The reference interval for amniotic

![Figure 4. Effect of gestational age on net fluorescence polarization of NBD-PC](image-url)
fluids analyzed before freezing may well differ from that for frozen specimens. The reference interval for patients with diabetes mellitus is also likely to differ.

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References