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Lamellar Body Count and Stable Microbubble Test on Gastric Aspirates from Preterm Infants for the Diagnosis of Respiratory Distress Syndrome

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Key Words
Respiratory distress syndrome · Pulmonary surfactant · Preterm newborn · Lamellar body count · Stable microbubble test

Abstract
Background: Lamellar body count (LBC) in amniotic fluid is being used to identify infants at risk of respiratory distress syndrome (RDS) who would benefit from surfactant prophylaxis or very early therapy. The test in gastric aspirates of newborns has not been properly explored. ***Objective:*** The main objective of this research was to evaluate the performance of LBC alone or in combination with the stable microbubble test (SMT), done on gastric aspirates from preterm babies to predict RDS. ***Methods:*** A total of 34 preterm infants with RDS and 29 without RDS, with a gestational age between 24 and 34 weeks, were included in the study. Gastric fluid was collected in the delivery room. A diluent (dithiothreitol) allowed all samples to be processed, even the thickest and non-homogeneous ones, without centrifugation. The SMT was done for comparison. ***Results:*** The best cut-off value was <42,000 lamellar bodies/μl to predict RDS, with a sensitivity of 92% (95% CI 73–100%) and specificity of 86% (95% CI 77–95%). The area under the receiver-operating characteristic curve was 0.928 (95% CI 0.86–0.99). SMT showed similar results. LBC and SMT together in series (positive result if both tests were positive) showed a sensitivity of 100% and a specificity of 86%. ***Conclusion:*** LBC on gastric aspirates diluted in a solution of dithiothreitol can be rapidly and easily performed, and may be used alone or in combination with SMT as a predictor of RDS, allowing selective prophylaxis or very early treatment only in surfactant-deficient newborns.
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Introduction

The use of some rapid tests on amniotic fluid [1–4], on tracheal aspirates [5–7] and on gastric aspirates [8–12] has been suggested for the estimation of lung maturity in preterm infants and for helping in the decision to provide prophylactic or very early surfactant therapy. One of the tests used to assess lung maturity is the lamellar body count (LBC), described in amniotic fluid by Dubin [13] in 1989, with the use of a cell counter. The similarity of lamellar body size to platelet size allows the use of the automated blood cell counter for the quantification of lamellar bodies. This test is currently widespread in ob-

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stetric clinical practice for the assessment of fetal lung maturity, as it is accurate and performed quickly at low cost.

Quite recently, attempts have been made to assess LBC in the gastric aspirate of newborn infants [14, 15]. The advantage of LBC over other physical tests (click test, stable microbubble test (SMT)) is that it is not operator-dependent and it can be done in a few minutes in a regular laboratory. The use of gastric fluid instead of amniotic fluid to count lamellar bodies may have additional advantages. Gastric fluid is easily collected after birth and it may be free of blood even when amniotic fluid is grossly contaminated. It can also be obtained when amniotic fluid cannot be collected. The performance of LBC in gastric fluid has not yet been consistently determined.

Another simple and rapid test for the assessment of lung maturity is the SMT developed by Pattle et al. [16] in 1979. The test is done in a few minutes at the bedside, and can be performed on amniotic fluid, tracheal or gastric aspirate specimens. The performance of the test in gastric aspirates to diagnose respiratory distress syndrome (RDS) has already been determined [8–12, 17].

We hypothesized that LBC in gastric aspirates, done with a modified technique that allows to do the test in nearly all samples, may prove to be very useful in neonatal clinical practice. The main objective of the present study was to assess LBC in the gastric aspirate of newborn infants with and without RDS in order to evaluate the performance of the test to predict the syndrome. We also performed SMT in gastric aspirate of the same infants for comparison with the LBC and also to verify if the combined tests would improve their performance.

Patients and Methods

This prospective study was conducted at the Neonatal Intensive Care Unit of the Hospital São Lucas, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil, between October 2007 and June 2008. The study protocol was approved by the local Research Ethics Committee.

Patients born at 24–34 weeks of gestation were included. The infants were enrolled consecutively until the desired sample of each group (with and without RDS) was reached. Gestational age at birth was determined according to the date of the last menstrual period and confirmed by early fetal ultrasound (before 20 weeks) or by the New Ballard Score [18]. It was planned not to include patients with pneumonia, congenital heart disease, meconium-stained amniotic fluid, pneumothorax, diaphragmatic hernia and/or genetic syndromes that compromised the respiratory function. The diagnosis of RDS was established according to clinical criteria (tachypnea, costal and external retractions, grunting, necessity of $FiO_2 > 0.40$) associated with a compatible radiological pattern (diffuse reticulogranular pattern and presence of air bronchograms).

Gastric aspiration of these patients was performed in the delivery room using a short gastric aspiration tube size 6 or 8 french coupled to a 10- or 20-ml syringe. SMT was done in fresh samples and LBC in frozen samples at –20°C for 72 h. LBC was performed by the laboratory technician who was blinded to the condition of the infants. SMT was done by the neonatologist present at birth and repeated by two other staff members who were not aware of the patients' condition. All the staff of the unit is trained in the technique as SMT has been routinely used for many years. The good inter- and intra-observer agreement in the SMT performed in gastric aspirates has already been reported [19].

To perform LBC, diluted dithiothreitol (DTT; Invitrogen, Calif., USA) in distilled water (10 mg/ml) was prepared in advance. It was kept frozen at –20°C in Eppendorf tubes until its use. DTT solution and gastric fluid thawing occurred at room temperature until it was visually confirmed. The amount of gastric aspirate used for each LBC ranged from 50 to 100 μl. The sample was placed in a test tube containing the diluted DTT in a proportion of 1 part of gastric aspirate to 6 parts of DTT. This diluted sample was vortexed for 10 s using a Vortex Ap 56 shaker (Phoenix, Araraquara, Brazil), and finally the material was aspirated by the automated Sysmex XT-1800i cell counter (Sysmex Corp., Japan). LBC was performed using the platelet channel results.

In order to verify the adequacy of LBC in frozen samples in the first 22 patients, with or without RDS, the test was done in the same samples, fresh and frozen, for 72 h, both diluted in the same way in DTT solution. In the first 10 patients in whom gastric aspirates diluted in saline at the same concentration of the samples diluted in DTT solution were considered homogeneously liquefied to be processed without centrifugation, LBC was done to verify the correlation with the fresh and frozen samples diluted in DTT. All results obtained were multiplied by 7 to correct for the dilution.

The SMT was carried out immediately after collection, within the first 30 min of life, at the newborn intensive care unit, according to the method described by Pattle et al. [16] with slight modifications. An aliquot of 40 μl of gastric aspirate was suctioned into a Pasteur pipette (Brand GmbH & Co., Wertheimer, Germany) with 11.5 cm stem and 1 mm diameter and a 2-ml rubber cap, and placed on a count chamber (Neubauer Improved Bright-Line, Loptik Labor, Germany), without the slide cover. Holding the pipette vertically, with the tip almost touching the count chamber, the aliquot was quickly suctioned in and expelled out 20 times. It was then expelled over the count chamber, which was immediately inverted and placed under a binocular microscope, forming a hanging drop. After 4 min, the count area was examined with a magnification of 10 × 10, and the microbubbles (bubbles <15 μm) were counted. Five of the 25 squares forming 1 mm^2 were counted (one square in each quadrant and the central square), and the number was multiplied by 5. If <100 stable microbubbles were present per mm^2, the whole mm^2 was considered.

It was calculated that around 30 patients would be required in each group to estimate sensitivity and specificity with a maximum error margin of 15 for a 95% confidence interval (95% CI). Descriptive statistics were used for the quantitative variables. The unpaired tests were used for comparison between the two groups, and Mann-Whitney test for LBC and SMT.

Table 1. Correlation of LBCs done in the same samples, fresh and frozen, diluted in saline and in DTT solution

n	Type of samples	Median	IQR	CC	p
22	LBC-DTT fresh vs. LBC-DTT frozen	45,500 38,500	14,000–77,000 28,000–98,000	0.95	<0.001
10	LBC-saline fresh vs. LBC-saline frozen	31,500 35,000	14,000–77,000 14,000–77,000	0.99	<0.001
10	LBC-saline fresh vs. LBC-DTT fresh	31,500 31,500	14,000–77,000 14,000–63,000	0.99	<0.001
10	LBC-saline frozen vs. LBC-DTT frozen	35,000 35,000	14,000–77,000 21,000–98,000	0.93	<0.001

n = Number of samples; IQR = interquartile range; CC = Spearman correlation coefficient; LBC = lamellar body count; DTT = diluted in dithiothreitol.

Table 2. LBC and SMT on gastric aspirates of patients with and without RDS

	n	Median	IQR	Minimum	Maximum	p
LBC						
RDS	34	21,000	14,000–35,000	0	70,000	<0.001
Non-RDS	29	98,000	63,000–231,000	14,000	490,000	
SMT (SMB)						
RDS	34	7	3–10	0	21	<0.001
Non-RDS	29	50	25–150	4	280	

n = Number of patients; IQR = interquartile range; LBC = lamellar body count/μl; SMT = stable microbubble test; RDS = respiratory distress syndrome; SMB = stable microbubbles/mm^2.

A logistic regression model with LBC or SMT as explanatory variables was used for the prediction of RDS. Sensitivity and specificity of the tests were determined. Receiver-operating characteristic (ROC) curves were obtained, estimating the areas under the curves (AUCs), and the best cut-off point was defined for each test. AUCs were compared using the method of DeLong et al. [20].

In case of association of tests in series (test derived from individual tests, being considered positive if both tests were positive), the characteristics relative to the quality of the combined test in terms of sensitivity (s) and specificity (sp) were obtained by the following formulas: $s_s = s_{LBC} x s_{SMT}$; $sp_s = sp_{LBC} + sp_{SMT} - sp_{LBC} x sp_{SMT}$. Youden's index ($YI = s_s + sp_s - 1$) values, obtained considering the combination of tests in series, were used to fit a quadratic response surface regression model with LBC and SMT values and to plot the response surface [21]. $p < 0.05$ was regarded as statistically significant.

Results

Gastric aspirates were collected from 63 patients: 34 with RDS and 29 without RDS. Mean gestational age and mean birth weight and their standard deviations were 30 ± 3 weeks and 1,167 ± 436 g for the RDS group and 32 ± 2 weeks and 1,790 ± 458 g for the non-RDS group. Comparisons of LBC in the same fresh and frozen samples and diluted in saline and DTT showed a very good correlation (table 1). Once this very good correlation was established, it was decided to follow the study performing the test only in frozen samples diluted in DTT solution, which were available in all patients. Thus the analysis shown in this article refers only to LBC in this type of sample. LBC and SMT were significantly lower in the RDS group ($p < 0.001$) (table 2).

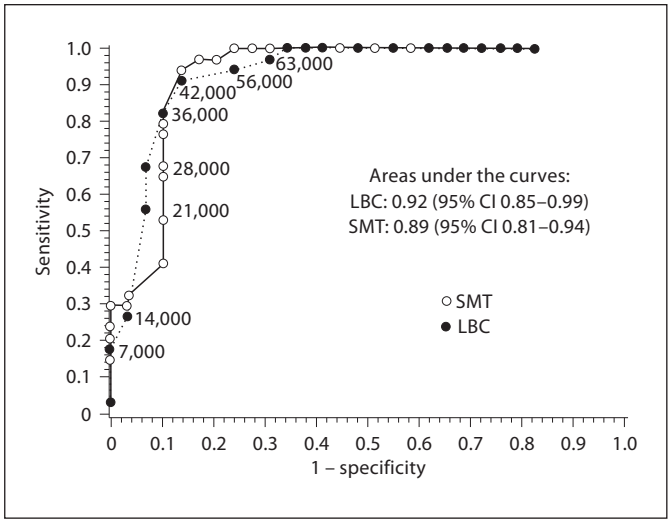

Fig. 1. ROC curves for LBC and SMT to diagnose RDS considering different cut-off points.

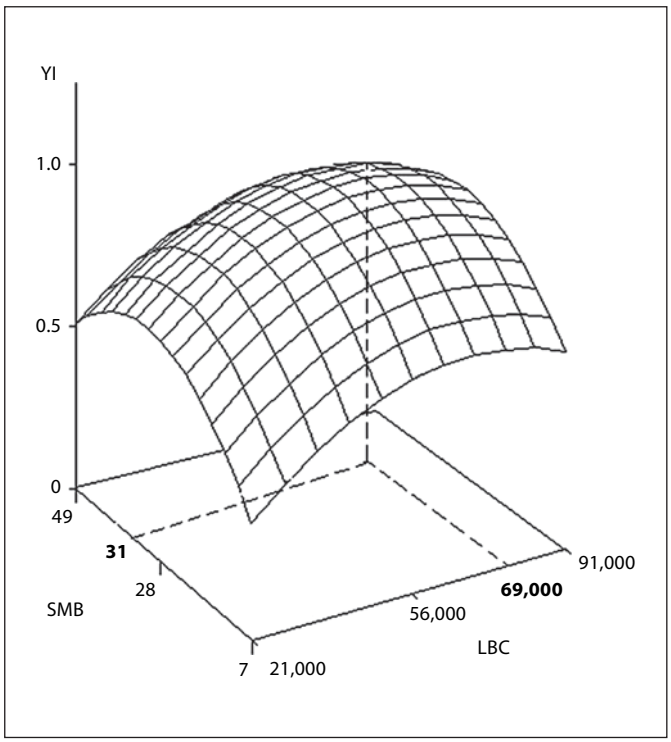

Fig. 2. Graphic representation of YI according to LBC and SMT in series. The vertical dashed line indicates the estimated highest value for the index and the best combination of both tests for the diagnosis of RDS of the newborn.

Figure 1 shows the ROC curves for the LBC and SMT. Note that there is a large area under the LBC curve (0.92, 95% CI 0.85–0.99) and SMT curve (0.89, 95% CI 0.81–0.96) suggesting good sensitivity/specificity for both tests. There is no statistically significant difference between the AUCs (p = 0.901). At a cut-off point of <42,000 lamellar bodies/μl (the highest point in YI), a sensitivity of 92% (95% CI 73–100%) and a specificity of 86% (95% CI 77–95%) were obtained. The SMT yielded a sensitivity of 94% (95% CI 77–100%) and a specificity of 86% (95% CI 76–95%) with a cut-off point of <14 SMB/mm^2. With cut-off points of <15 and <18 SMB/mm^2, the sensitivity of the SMT amounted to 97 and 98%, whereas specificity corresponded to 83 and 78%, respectively. We were unable to calculate predictive values and accuracy due to the study design.

At the best observed combination of LBC and SMT in series, sensitivity was 100% and specificity was 86%. The number of incorrect diagnoses fell from 7 to 4 with the association of the tests. The graphic representation of the results of the tests in series is shown in figure 2. In this association the highest estimate for the YI was 0.96 (95% CI 0.94–0.98) corresponding to a LBC of 69,000/μl and a SMT of 31 SMB/mm^2. It should be observed that the best cut-off points for the association of the tests are not the same as for the tests done separately.

Discussion

In this study it was shown that LBC in gastric aspirates of preterm infants has both high sensitivity and specificity for predicting RDS. A way was also presented to overcome technical difficulties to perform the test in gastric fluid. Gastric aspirate is a thick and non-homogeneous fluid, which hinders its processing by the cell counter without centrifugation even when diluted in saline. Many liquefying agents were tried in order to reduce the consistency of the sample, but they substantially changed the number of lamellar bodies or were not efficient enough to make the samples acceptable to be passed through the counter. The investigation into LBC in gastric aspirate could only be resumed more recently after the use of DTT, which turned out to be quite efficient to liquefy the samples without changing the LBC. This diluent has already been mentioned in other studies, but not with the same purpose [22–24].

In the present study, the LBC showed good sensitivity and specificity for the diagnosis of RDS (both >85%), and the best cut-off point was established as <42,000 lamellar

bodies/μl. With a cut-off point of 50,000 lamellar bodies/μl, sensitivity slightly increased to 93%, but specificity fell to 81%. This cut-off point of <42,000 lamellar bodies/μl in the LBC on gastric aspirate of newborn infants is similar to the cut-off points recommended for the determination of fetal lung maturity in amniotic fluid [25–27], but it was much higher than that obtained in another study [14]. In this latter study, the authors performed the LBC on gastric aspirates submitted to centrifugation. Centrifugation has been considered in several studies as it can reduce the LBC. Dubin [13] reported that the centrifugation of amniotic fluid samples at 500 *g* for 5 min could reduce the LBC by 10–40%. Using the same rate and length of centrifugation in amniotic fluid, Roiz-Hernández et al. [28] state that up to one third of the lamellar bodies may be lost to sedimentation. The use of DTT allowed carrying out all LBC without centrifugation, while dilution in saline did not permit the analysis of many samples as they were thick and clumpy. The DTT for this test is of very low cost, as hundreds of samples can be processed using only one bottle of the product.

The SMT on gastric aspirate yielded a remarkably lower value in RDS patients, with good sensitivity and specificity, confirming previously reported results [8–12, 17]. The best cut-off point in this study was <14 SMB/mm^2.

The advantage of the LBC over SMT in gastric aspirates is that the count is made by an equipment available at any hospital laboratory and that it does not rely on the visual observation of an operator. The advantage of the SMT over the LBC test on gastric aspirates is that for this test there is no need for dilution or centrifugation and it can be done at the delivery room or at the newborn intensive care unit with a simple microscope. Both tests can be performed in less than 10 min and are of very low cost compared with biochemical tests.

The LBC and SMT in gastric aspirates of newborn infants are comparable tests for the diagnosis of lung immaturity. The choice between one test or the other depends on the staff's experience with each of these tests. It is possible that the association improves the accuracy of the tests. This study has not enough power to answer this question, however if the difference found in this study is real, the number of incorrect tests would be reduced to nearly half. The good sensitivity and specificity of the tests, associated to their simplicity and low cost, clearly indicate that they may play a role in helping the neonatologist to decide for the administration of prophylactic surfactant, in a more selective and judicious manner, to very immature infants, as well as for its very early administration to not so immature infants who start with respiratory symptoms soon after birth, suspicious of RDS. We speculate that the tests may also help to indicate which preterm infants will do well on early nasal continuous positive airway pressure.

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