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Predicting respiratory distress syndrome at birth using fast test based on spectroscopy of gastric aspirates. 1. Biochemical part

Peter Schousboe[1], Henrik Verder[1], Torben E. Jessen[2], Christian Heiring[3], Lars Bender[4], Finn Ebbesen[4], Marianne Dahl[5], Christian Eschen[1], Jesper Fenger-Grøn[6], Agnar Höskuldsson[1], Jes Reinholdt[7], Nikolaos Scoutaris[1], Heidi Smedegaard[8]

1. Department of Paediatrics Holbaek University Hospital, Holbaek, Denmark

2. Department of Clinical Biochemistry, Holbaek University Hospital, Holbaek, Denmark

3. Department of Neonatology Rigshospitalet, University of Copenhagen, Denmark

4. Department of Paediatrics Aalborg Hospital, University of Aalborg, Denmark

5. Department of Paediatrics Odense Hospital, University of Southern Denmark

6. Department of Paediatrics Kolding Hospital, University of Southern Denmark

7. Department of Paediatrics Herlev Hospital, University of Copenhagen, Denmark

8. Department of Paediatrics Hvidovre Hospital, University of Copenhagen, Denmark

Short title: Bedside Lung Maturity Method

Corresponding author: C. Heiring, Department of Neonatology Rigshospitalet, University of Copenhagen. Blegdamsvej 9, 2100 Copenhagen Ø, Denmark.

Tel +45 2217 4087. Fax: +45 3545 5021. Email: christian.heiring@regionh.dk

Abstract

Aim: To develop a fast bedside lung maturity test.

Methods: Gastric aspirates obtained from premature infants, contain lamellar bodies, carrying lung surfactant. To estimate lung maturity we isolated lamellar bodies from fresh gastric aspirates by centrifugation. Erythrocytes and other cells were lysed by adding water and discarded subsequently with the supernatant. Mid-infrared spectroscopy was then performed to measure the lung maturity as lecithin-sphingomyelin ratio. Lecithin was determined as dipalmitoylphosphatidylcholine, the most surface-active-phospholipid. Algorithms to measure lecithin and sphingomyelin concentrations in fresh gastric aspirates were developed on aspirates from 140 premature infants. Each gastric aspirate sample was divided in two samples: one for mass spectrometry as reference and one for spectroscopy. Development of the algorithm is described in details in Appendix S1.

Results: Gastric aspirates stored at 4-5°C avoid flocculation of proteins and phospholipids in contrast to when the aspirates were frozen and thawed. Omission of freezing and concentration of the lung surfactant by centrifugation combined with diminished influence of proteins improves the spectroscopic measurement of lecithin-sphingomyelin ratio. Measurement of lecithin-sphingomyelin ratio by the new method was performed within 10-15 minutes.

Conclusion: We present a new fast bedside lung maturity test on fresh gastric aspirate for early targeted surfactant treatment.

Key notes

- Respiratory distress syndrome is a major cause of mortality and morbidity in premature infants and a fast biochemical method to measure lung maturity at birth is needed for early targeted surfactant treatment
- A spectroscopic bedside method to measure lecithin-sphingomyelin ratio (L/S) on gastric aspirate was developed
- The new method can be performed in 10-15 min and a prototype of a point-of-care instrument has been built and clinical trials planned

Keywords

Gastric aspirate, Mid-infrared spectroscopy, Lamellar bodies, Prematurity, Respiratory distress syndrome, Lecithin-sphingomyelin ratio (L/S)

Introduction

A fast bedside diagnostic test for lung maturity is needed for optimal targeted surfactant treatment of respiratory distress syndrome (RDS). Although the etiology of RDS has been known for more than half a century, a fast method with high sensitivity and specificity has only now been developed. We previously developed a laboratory lung maturity test on gastric aspirates (GAS) and analysis by mid-infrared spectroscopy of lecithin-sphingomyelin ratio (L/S) on frozen and thawed GAS (1). Lecithin was measured as dipalmitoylphosphatidylcholine (DPPC), the dominant lecithin and the lung surfactant phospholipid with the highest surface activity.

In the present paper we describe a new bedside method for lung maturity measurement. The analyses were performed on lamellar bodies (LB) isolated from fresh GAS by a single centrifugation step and measured by mid-infrared Fourier Transform Infrared Spectroscopy (FTIR). Surfactant is produced in the lung alveoli as LB and secreted with lung fluid into the

amniotic fluid and GAS. To estimate L/S, we developed an algorithm on fresh GAS. The samples were divided into two aliquots: one for FTIR analysis and one for mass spectroscopy (MS) measuring the concentrations of DPPC and sphingomyelin (SM).

The new method was evaluated in an observational study comparing L/S with development of RDS and the results are published in an accompanying article in this issue of Acta (2).

Methods

Gastric aspirates

GAS obtained immediately after birth were stored at 4-5^{o}C and analysed by FTIR spectroscopy within ten days.

Spectroscopic improvements

The FTIR spectroscopy was performed by dry transmission (3) and the spectroscopic signal was enhanced by concentrating the surfactant thus avoiding the interference of proteins, salts or flocculent protein clots previously referred to as mucus (1).

Standard procedure for sample analysis

A reference method for FTIR spectroscopy based on mass spectrometry (MS) was prepared from a standard procedure, which included: GAS (200 µL) diluted 4-fold with water and centrifuged at 4,000 x *g* for 4 minutes. After removal of the supernatant, the samples were suspended in 100 µL of water and split into 2 x 50 µL aliquots: 50 µL was measured by FTIR and 50 µL was analysed by MS to estimate the content of DPPC and SM was performed at Southampton University Hospital as described earlier (1). Internal standards comprised DPPC (16:0), the isotopic labelled 40^{13}C-DPPC, SM (16:0) and SM (6:0), the latter SM metabolite is not detected in biological material. FTIR analyses were performed by dry transmission on CaF_2 windows (1 mm thick and 13 mm diameter, Chrystran.com). The samples (50 µL) were

applied onto the CaF_2 and dried on a hotplate (90°C). The FTIR spectra were measured by a Bruker Tensor 27, equipped with a DTGS detector (60 scans and a resolution of 4 cm^{-1}).

The L/S algorithm

The L/S algorithm was built on samples from infants with gestational ages of 24-36 weeks. The calibration model was established using partial least squares regression analysis (4,5), which quantitatively correlated spectral values from 55 samples with known DPPC concentration and 85 samples with known SM concentration. Calculation of the L/S ratio based on the FTIR spectra was performed by the algorithm described in Appendix S1 in the online supplement section.

Electron microscopy

Electron microscopy (EM) was used to verify that the GAS precipitates contain LB. GAS samples were treated by the standard method and the precipitated LB were fixated in 4% paraformaldehyde.

Results

Comparison of frozen and fresh GAS

Fresh and frozen GAS was compared in 30 cases. A flocculent, insoluble and flowing substance appeared to be generated by freezing and thawing GAS in all cases. Fresh unfrozen GAS was homogeneous and easily diluted in all cases. A representative example of a frozen and thawed GAS compared with a fresh GAS is presented in Figures 1A and B.

Mass spectroscopy

MS analyses of the flocculent material from frozen samples revealed both a wide range of proteins (data not shown) and a high content of DPPC and SM. Freezing and thawing of a GAS may change the balance between DPPC and SM in the liquid part of the thawed

sample. Therefore, we used the more homogeneous fresh GAS in our analyses of DPPC and SM.

Purification of the LB fraction

Surfactant as LB was isolated from GAS by a single centrifugation step. The precipitate contains the LB fraction (Fig. 2), while the discarded supernatant contains the soluble proteins and salts (substances known to interfere with spectroscopic measurement). The analysis time was 10-15 minutes.

Stability of phospholipids during storage

The stability of DPPC in four fresh GAS with different gestational ages was investigated. The phospholipid concentrations were measured by MS at birth and again after storing for four weeks at 4-5° C. The DPPC concentrations were stable and unchanged during the period (Fig. 3).

Transmission electron microscopy

Electron microscopic analysis of LB precipitate isolated from GAS by the standard procedure showed the well-known characteristic structure of LB (Fig. 4).

Samples containing blood

The method was tested in GAS samples contaminated with blood. When exposed to hypotonic conditions, erythrocytes burst and most of the cellular material is removed along with the supernatant (Fig. 5).The low centrifugation force used in our standard procedure to precipitate LB left most of the ruptured membranes of erythrocytes and other lysed cells in the supernatant after centrifugation.

Discussion

In this study we describe the development of a new fast, reliable, non-invasive bedside test using simple FTIR technology to measure L/S on GAS. Our former L/S method was a laboratory test performed on frozen and thawed GAS (1). We are now working with fresh GAS, and thereby avoiding flocculation of proteins and phospholipids which otherwise would make it difficult to solubilize the samples for spectroscopy. Furthermore, by concentrating LB, we reduce the influence of substances irrelevant for analysis of lung surfactant.

Previously, Liu et al. 1998 (6) performed mid-infrared spectroscopy to measure lung maturity on amniotic fluid. This set-up, quite different from ours, was hindered by difficulties with the spectroscopy due to high contents of compounds interfering with the L/S measurement. Furthermore, difficulties with the reference method meant that the method was never used clinically.

Erythrocytes and other cells are often present in GAS. To reduce the contamination of GAS from these sources in order to improve the phospholipid measurements, it has earlier been common practice to centrifuge amniotic fluid or GAS and subsequently discard the precipitate prior to measurement of L/S. However, this procedure reduces the amount of surfactant, resulting in less accurate measurements of lung maturity.

The first step of our new L/S method was dilution of GAS samples in pure water, lysing erythrocytes and other cells in the hypotonic environment. In the subsequent centrifugation step, the lysed erythrocyte content and cell membranes remained in the supernatant and the LB precipitate was used for FTIR analysis. This new dilution procedure also reduced salt and protein concentrations, resulting in stable and reliable FTIR measurements. Furthermore, the viscosity was lowered, allowing LB to precipitate by centrifugation at low *g*-force.

SM is sparsely present in the outer membranes of erythrocytes (7). Therefore, the effective removal of erythrocytes with the new method may have slightly increased the L/S values and the L/S cut-off value, as seen when comparing the observational study (2) with our earlier study (1).

Development of the algorithm for L/S estimation was based on reference samples selected to cover as large a concentration range of DPPC and SM as possible. Wavenumbers correlated to the FTIR signals and the concentrations of the reference samples, DPPC and SM, were included. FTIR is unable to distinguish small differences in the length of the fatty acid chains in long chain lipids. However, our approach is to feed the algorithm with MS measured reference concentrations of DPPC and SM and in this way develop the algorithm to select FTIR wavenumbers that correlate optimally with the reference concentration of DPPC and SM. Whether the algorithm includes spectral values from phospholipids and sphingolipids that differ marginally from DPPC and SM is of less importance as long as it finds the wave numbers that correlate optimally with the concentration of DPPC and SM. A more detailed overview of the stepwise calibration procedure has previously been published (1,7) and is described in more details in Appendix S1.

Along with the development of a new method for early diagnosis of RDS and targeted early surfactant treatment we have finished developing the first automated prototype of a point-of-care instrument. Using this instrument and the new method an L/S result can be obtained within 10-15 min after birth.

Conclusions

We have developed a point-of care method suitable for fast analysis of lung maturity in preterm neonates where the influence of blood-contamination is negligible. The L/S values obtained by this method have been compared with development of RDS in 72 premature infants in a Danish multicentre study presented in an accompanying article in this issue of Acta Paediatrica (2).

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Abbreviations

DPPC, dipalmitoylphosphatidylcholine; EM, Electron microscopy; FTIR, Fourier transform infrared spectroscopy; GAS, gastric aspirates;

LB, lamellar bodies; L/S, lecithin-sphingomyelin ratio; MS, mass spectroscopy;

RDS, respiratory distress syndrome; SM, sphingomyelin

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Conflict of interest

Agnar Höskuldsson and Henrik Verder hold part of a patent for spectroscopic analysis of biological samples and Peter Schousboe and Henrik Verder hold part of a patent for a foetal lung maturity test based on lamellar body purification. The authors have no other conflicts of interest.

Figure 1 A and B.

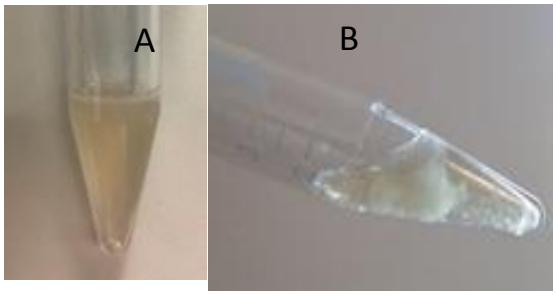


Figure 2.

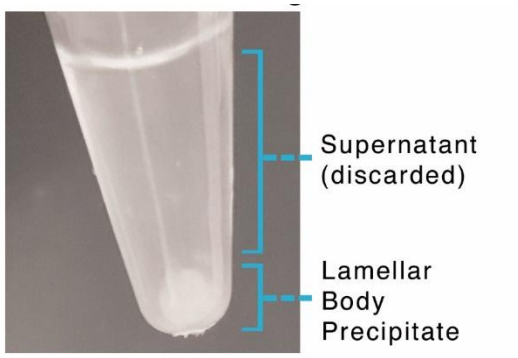


Figure 3.

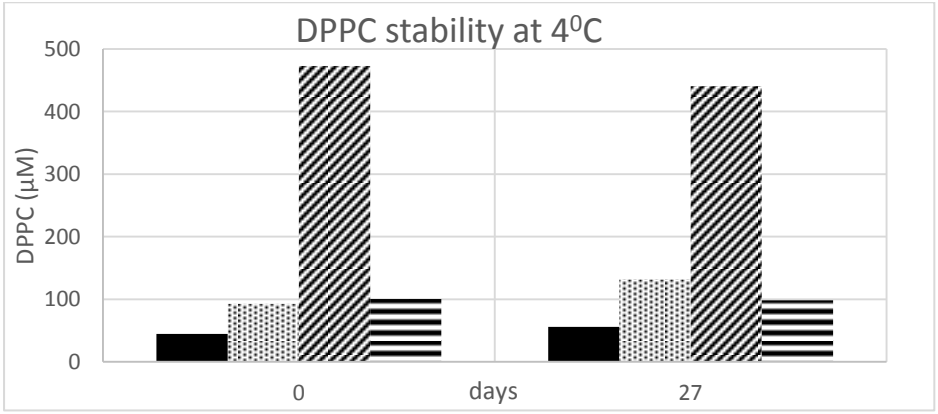
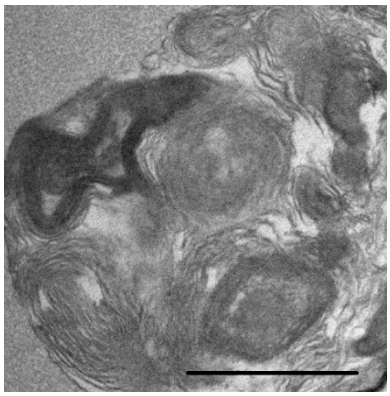
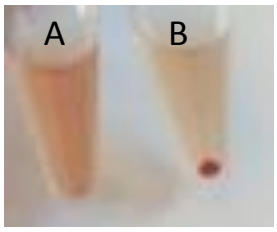


Figure 4.



Figs. 5A and B.



Legends to Figures

Figure 1A and B. Fresh gastric aspirate (A) and frozen and thawed gastric aspirate (B). The thawed sample is heterogeneous and composed of a liquid phase and an insoluble flocculent phase. The aggregated material is mainly consisting of proteins and phospholipids. The fresh sample is homogeneous in its composition.

Figure 2. Gastric aspirate after centrifugation at 4,000 x *g* for 4 minutes.

Figure 3. Four gastric aspirates were stored at 4-5°C for a period of 27 days. From each sample, 100 µL was withdrawn for lipid extraction at several time points followed by mass

spectroscopy of DPPC. The graph shows the initiate and the final measurement of DPPC for each sample. The corresponding samples are shown by the same pattern.

Figure 4. Transmission electron microscopy of lamellar bodies from gastric aspirate in precipitate (Gauge =1 µm).

Figure 5A and B. A bloody sample of gastric aspirate was split into two. The samples were either diluted with water (A) or saline (B) and centrifuged at 4,000 x *g* for 4 minutes. The coloured supernatant in the water sample indicate burst erythrocytes where the pellet in saline clearly shows a precipitate of erythrocytes.