BAL fluid cytology in the assessment of infectious lung disease

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The need for rapid laboratory results has marked the application of bronchoalveolar lavage (BAL) fluid cytology as a valuable tool in the assessment of infectious lung conditions. The techniques of BAL fluid processing presented here can be performed in a microbiological laboratory. The diagnostic value of BAL fluid cytology for the prediction of ventilator-associated pneumonia is discussed.

The quantitative culture of bronchoalveolar lavage (BAL) fluid is recommended as a reference method in the diagnosis of ventilator-associated pneumonia (VAP) (Baselski et al, 1992). Prospective studies demonstrated that the early administration of antimicrobial therapy reduces the mortality rate of VAP. However, because of a culture delay of 24–72 hours, the information becomes available too late to influence patient survival (Limia et al, 1997).

We became engaged in BAL fluid cytology 3 years ago, when we looked at microscopy as a tool for earlier diagnosis of VAP. We implemented a standardized protocol for BAL fluid microscopy as a routine diagnostic service. In this paper, we describe this protocol and report on the BAL fluid cytological findings in patients suspected of having VAP. Emphasis will be placed on practical considerations of specimen processing and the clinical relevance of microscopic findings will be discussed.

PROCESSING OF THE BAL FLUID

During bronchoscopy, four 50 ml aliquots of sterile 0.9% sodium chloride (NaCl) are instilled in the wedged segment and immediately aspirated. The aliquots are separately recovered in non-adhesive polypropylene containers which are consecutively numbered. They are promptly transported and hand-delivered to the laboratory, thereby bypassing the central transport facility. This policy precludes excessive transport delays and circumvents the need for refrigeration of the BAL fluid, which may hamper the growth of environmentally sensitive bacteria.

BAL fluid samples are considered of urgent priority and are processed immediately upon arrival in the laboratory. Designed for application in a diagnostic setting, the goals of our protocol are a short turnaround time and the facility to perform the analysis during weekends and out of hours. Processing steps that are common in the research setting, such as filtration, centrifugation and resuspension of the BAL fluid, are therefore deliberately omitted. For safety reasons, all operations are carried out in a biological safety cabinet.

The volume of the recovered BAL fluid and its macroscopic appearance are recorded. The first aliquot, which represents the bronchial fraction, is separated, and the remaining fractions are pooled for further analysis. The total cell count is performed in a Fuchs–Rosenthal haemocytometer chamber. All nucleated cells are counted, and the average of two successive counts is reported as the number of cells per ml.

Figure 1. Cytospin chambers are loaded in the cytospin head which is placed within a biosafety cabinet.

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TABLE 1.
Cytocentrifugation of bronchoalveolar lavage (BAL) fluid samples: number of drops per cytospin chamber and Cytospin operating conditions

BAL fluid total cell count/ml	Number of drops per chamber	Shandon Cytospin 3 operating conditions
<50 000	7	Low acceleration, 650 rpm, 20 min
50 000–100 000	4–5	Low acceleration, 650 rpm, 10 min.
100 000–200 000	3–4	Low acceleration, 650 rpm, 10 min
200 000–300 000	3	Low acceleration, 650 rpm, 10 min
300 000–400 000	2–3	Low acceleration, 650 rpm, 10 min
>500 000	Dilute in balanced electrolyte solution to 4–5 drops final volume	Low acceleration, 650 rpm, 10 min
Haemorrhagic BAL fluid	Add an additional drop to the number of drops listed above	Low acceleration, 650 rpm, 10 min

Specimen drops are those from sterile disposable bulb pipettes (Transfer pipettes, Sartsted, Germany). As the drop size is dependent on the type of pipette, it is recommended to rely on a single pipette type for standardization of this procedure. Modified from Capron et al (1990)

CYTOCENTRIFUGATION

Slides are prepared by cytocentrifugation. This process involves a low-speed centrifugation of the BAL fluid in a sample chamber that is fixed onto a microscope slide (*Figure 1*). Cells are deposited in a 6 mm diameter monolayer (the cytocentrifuge spot) and the fluid is absorbed into a filter pad. At present, we use the Shandon Cytospin 3 apparatus (Shandon Scientific Ltd, Astmoor, England).

For obtaining easy-to-read monolayer preparations, it is imperative to adjust the number of drops per chamber to the total cell count, and to chose cytocentrifugation parameters that result in both a preserved cell morphology and an optimal recovery of organisms (*Table 1*). An elevated speed increases the recovery of tiny organisms but damages larger and vulnerable cells. Because we perform stains on air dried specimens, we prefer a long centrifugation time as it allows complete absorption of the BAL fluid into the filter pad. The slides are allowed to air dry and are fixed for 10 minutes by an overlay of absolute methanol.

CHOICE OF STAINS

The Gram and the May–Grünwald Giemsa (MGG) stains are routinely performed on all submitted BAL fluid samples. Optional stains such as the methenamine silver nitrate stain and immunofluorescent stains are performed when clinically indicated. Romanovsky stains such as the MGG stain render nuclear features in less detail as compared to Papanicolaou stains and are less suitable for the detection of malignancy and viral inclusion bodies. However, they are excellent for identification of leukocytes and clearly stain extracellular substances such as mucus.

In our laboratory, MGG working solutions are made freshly each day. For uniform staining, the jars are gently rotated onto the platform of a benchtop orbital shaker. After drying, the stained preparations are sealed with a cover glass by means of a xylene-free mountant (Histomount, Shandon).

CELLULAR COMPONENTS OF THE BAL FLUID

Cytological evaluation of BAL fluid requires an understanding of the cell population of the distal airways. Alveolar macrophages are the main cell population, accounting for 90–95% of the cells in normal BAL fluid samples (*Figure 2*). Alveolar macrophages are mononucleated cells that range from 10 μm to 40 μm in diameter. Multinucleated macrophages are occasionally seen but they do not imply infection or foreign body reaction.

Squamous epithelial cells (SECs) originate from the oral cavity. SECs are large, flat cells which occur singly or in sheets. Their nuclei are comparatively small and uniformly round. The cytoplasm appears dark or pale blue, and the cell borders are distinctly sharp. Bronchial epithelial cells (BECs) are ciliated cells and mucus-secreting goblet cells, the former are most frequently observed (*Figures 3* and *4*). Type II pneumocytes (alveolar lining cells) are round or cuboidal cells with a vacuolated cytoplasm. Contrary to macrophages, they shed as cohesive cellular clusters. Reactive type II pneumocytes may resemble malignant cells (*Figure 2*) (Grotte et al, 1990).

Basophils or mast cells have an oval nucleus which is often partially covered by numerous small, reddish-purple granules. The other cell types commonly found in BAL fluid specimens include lymphocytes, neutrophils and eosinophils. Plasma cells occasionally occur in BAL fluid specimens (*Figure 5*). The morphology of these

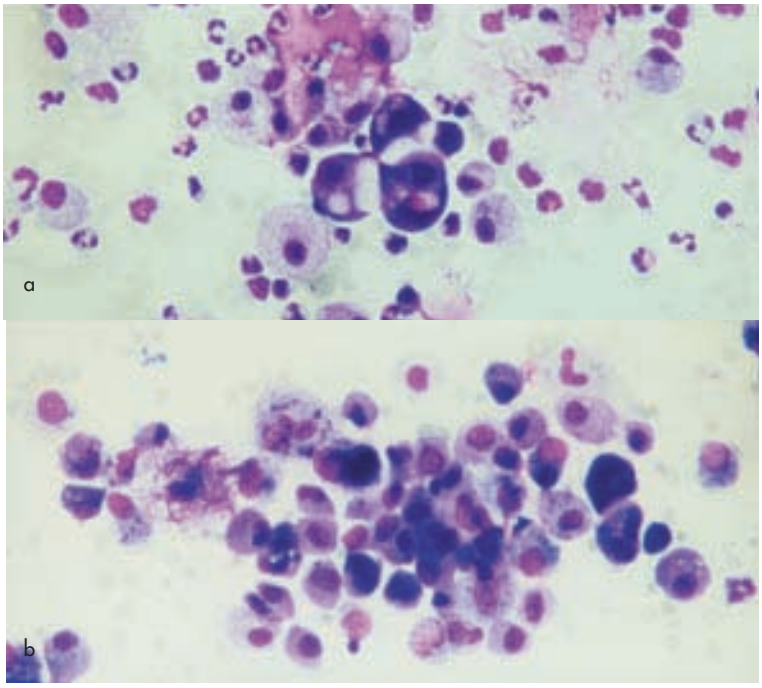

Figure 2. May–Grünwald Giemsa stain, screening of the bronchoalveolar fluid at low power magnification. a. Alveolar macrophages. The cytoplasm of alveolar macrophages may show a variable degree of vacuolization and may contain phagocytosed material such as carbon particles. A solitary, bacteria-loaded squamous epithelial cell is observed (left). b. Reactive type II pneumocytes arranged as a tissue fragment (centre). These cells are increased in diameter and display irregular edges, a high nuclear:cytoplasmatic ratio and gland-like inclusions.

cells can be found in textbooks of haematology and will not be discussed here.

MICROSCOPY

Cytocentrifuged preparations are first evaluated at low power magnification (objectives of x10) for the presence of foamy exudates that point to the diagnosis of *Pneumocystis carinii* pneumonia (*Figure 6*) (Stanley et al, 1988) and for detection of irregularly distributed SECs or BECs that may escape enumeration at high power magnification. Subsequently, the preparations are interpreted at high power magnification (objective x100, oil immersion). Slides are screened from the centre of the cytocentrifuge spot, and the fields are scanned following a circular pattern.

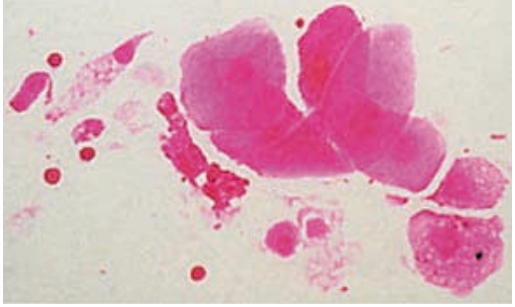

Figure 3. Gram stain, low power magnification. A sheet of squamous epithelial cells (upper right), a goblet cell and a ciliated cell (upper left corner), and two alveolar macrophages (lower right corner) are seen.

On MGG-stained preparations, we perform the differential cell counts on 500 consecutive nucleated cells, excluding epithelial cells. Neutrophil, eosinophil, basophil, lymphocyte and macrophage numbers are reported as a percentage of a 500-cell count. SECs and BECs are separately recorded and expressed as a percentage of the 500-cell count. The presence of damaged cells, red blood cells and intercellular debris is recorded semiquantitatively using a standardized score.

Cells containing organisms (infected cells) are enumerated on MGG-stained preparations. Both macrophages and neutrophils may contain phagocytosed microorganisms and the enumeration of both cell types must be considered. We express the quantification of infected cells as a percentage of the 500-cell count.

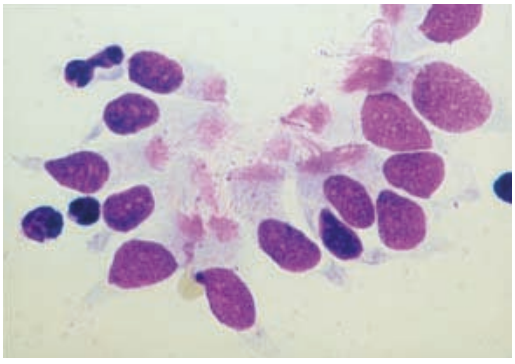

Figure 4. May–Grünwald Giemsa stain, high power magnification. Ciliated cells are columnar cells with a basally located nucleus. Cilia are lost when degenerative changes occur, but damaged cells may be recognized by their apical end plate.

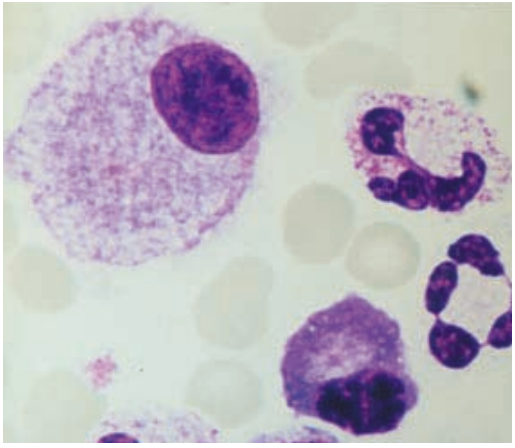

Figure 5. May–Grünwald Giemsa stain, high magnification. An alveolar macrophage (upper left corner), two neutrophils (right side) and a plasma cell (lower right corner) are seen. Plasma cells are exceptionally seen and may point to a non-infectious lung condition.

DIAGNOSTIC VALUE OF BAL FLUID CYTOLOGY IN ASSESSMENT OF VAP

In patients suspected of VAP, BAL fluid cytology provides criteria for specimen rejection and offers valuable evidence for the diagnosis of infectious pneumonia as well as for an alternative diagnosis.

Assessment of the BAL fluid quality

The presence of SECs is indicative of contamination by oropharyngeal flora, and most authors consider BAL fluid samples with ≥1% SECs as invalid for quantitative culture (Kahn and Jones, 1987; Gerbaux et al, 1998). We never reject such specimens, but consistently report the possibility of oropharyngeal contamination. Occasionally, we observed SECs in cases of aspiration pneumonia and in one patient, the presence of SECs pointed to a leaking endotracheal tube as a factor contributing to the development of VAP.

BECs represent bronchial tract contamination, and rejection criteria of ≥5% and ≥1% BECs have been proposed (Aubas et al, 1994; Gerbaux et al, 1998). Many ventilated patients have tracheobronchitis and BECs are easily overlooked when they are damaged or unevenly distributed over the cytocentrifuge spot. Therefore, we prefer the more stringent criterion of ≥1% BECs as an indication of bronchial contamination.

We do not reject these BAL fluid samples but consider them as endotracheal samples, for which conventional culture offers a good diagnostic sensitivity (but a low specificity) and a fair chance to identify the putative pathogen. In our experience, even BAL fluid samples that apparently contain only material from the bronchial level are still worth to be processed because they may reveal strictly pathogenic organisms such as *Mycobacterium tuberculosis* and *P. carinii*.

BAL FLUID CYTOLOGY IN THE PREDICTION OF VAP

BAL fluid cytological parameters that have been studied as predictive of VAP include the total cell count, the differential cell count and the number of infected cells.

The total cell count has been demonstrated to be significantly higher in BAL fluid samples from patients with VAP as compared to non-infected patients (Pugin et al, 1991; Allaouchiche et al, 1996). However, there remains a considerable overlap between the pneumonia and the non-pneumonia group, precluding accurate distinction. The total cell count of the BAL fluid also depends upon technical variables during bronchoscopy, so for this reason we and others (Timsit et al, 1995) use the total cell count as an additional quality control, i.e. low total cell counts (<60 000 cells/ml) are considered as indicative of technically invalid BAL fluid samples.

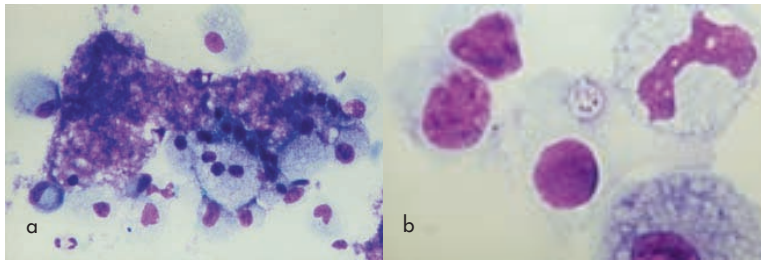

Figure 6. May–Grünwald Giemsa stain. Pneumocystis carinii *pneumonia. a. Low power magnification. Alveolar macrophages surrounding a prominent mass of exudate in the form of a foamy alveolar cast. b. High power magnification.* P. carinii *cyst with intracystic bodies (sporozoites). The cyst wall remains unstained and appears as a halo.*

Most studies reported the percentage of BAL fluid neutrophils as being significantly higher in patients with VAP compared to those without VAP (Solé-Violán et al, 1994; Allaouchiche et al, 1996). An attempt to convert this information into a discriminatory test was not successful, as no useful threshold could be defined (Aubas et al, 1994). Further, the number of neutrophils also increases in pulmonary processes that are inflammatory but non-infectious, such as in the early phase of the adult respiratory distress syndrome (ARDS) (Nakos et al, 1998). Therefore we believe that the BAL fluid neutrophil percentage is more useful in its negative prediction. In our experience, a low neutrophil number (or conversely a high number of macrophages) is a valuable argument for exclusion of VAP. This has been clearly shown by Kirtland et al (1997) who found that a level of <50% neutrophils had a 100% negative predictive value for VAP as defined by histological criteria.

Infected cells are also more frequently found in BAL fluid samples of patients with VAP than in patients without VAP. The cut-off values for infected cells cited in the literature range from 2% (Allaouchiche et al, 1996) to 25% (Chastre et al, 1988). These differences may depend upon the patient population studied, the stain used and the cell type enumerated, but few data are available on the technical aspects of this test. We compared the test characteristics of the MGG, Gram and Acridine Orange stains for enumeration of infected cells and found that the MGG-stained preparations offered the best interobserver agreement and reproducibility (*Figures 7* and *8*) (De Brauwer et al, 1999).

The reported cut-off values display a high specificity combined with a lower sensitivity. Most of the studies used quantitative BAL fluid cultures as the validating standard for VAP. The

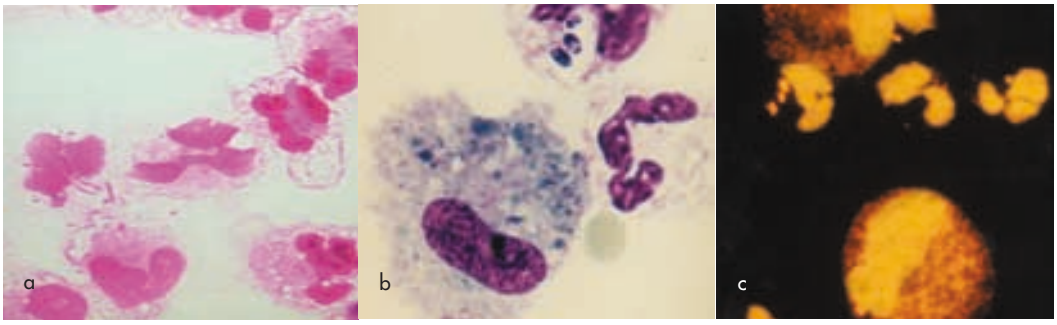

*Figure 7. Enumeration of infected cells, high power field. a. Gram stain shows Gram-negative rods (*Pseudomonas aeruginosa *and* Haemophilus influenzae*). b. May–Grünwald Giemsa stain reveals yeast forms (*Candida albicans*). c. Acridine Orange stain shows both alveolar macrophages and neutrophils containing cocci (*Staphylococcus aureus*).*

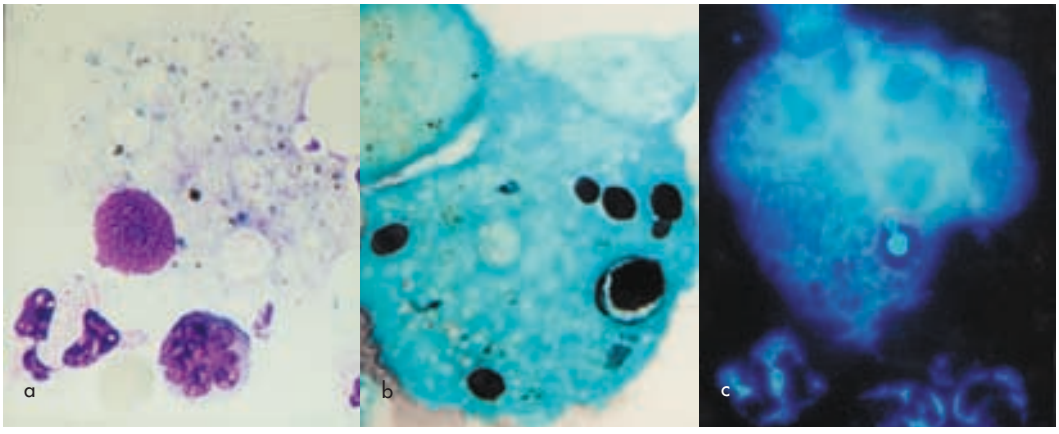

Figure 8. Cryptococcus neoformans *yeasts intracellularly located in a macrophage (high power magnification). Encapsulated yeasts are barely discernable on (a) the May–Grünwald Giemsa stain, and are detected by their negatively staining capsule. b. The methenamine silver nitrate stain and (c) the fluorescent brightener Leukophor clearly show encapsulated, budding yeasts.*

accepted culture cut-off point of 10^4 colony forming units/ml has originally been proposed in a concern to avoid underdiagnosis of VAP (Meduri and Chastre, 1992), and consequently implicates a number of false-positive pneumonias (Meduri et al, 1998). Moreover, several authors (Aubas et al, 1994; Timsit et al, 1995) used an exclusion criterion of ≥5% ciliated cells and this may be too low to rule out bronchial contamination. Consequently, the current 'gold' culture standard for VAP is probably too sensitive, explaining at least in part the lower sensitivity of the infected cell counts. Using the quantitative bacteriological burden of the BAL fluid (and not just the culture threshold) as an endpoint for comparison, Meduri et al (1998) found that the percentages of neutrophils and infected cells were related to the severity of pneumonia and accurately expressed the degree of local inflammation.

Conflicting results have been reported with regard to the influence of previous and current antibiotics on the recovery rate of infected cells. Dotson and Pingleton (1993) reported a decrease in the recovery rate, but this was not confirmed by Souweine et al (1998). As up to one third of patients suspected of VAP are receiving antibiotics before bronchoscopy (Allaouchiche et al, 1996), this issue should be further studied, with special attention to the influence of antibiotics that are not or only partially effective against the putative pathogen.

In view of all these factors, cut-off points for infected cells need to be established in the local clinical setting. A cut-off value that provides better sensitivity with 100% specificity would be preferred, as this figure contributes most to the positive predictive value, i.e. the confirmation of VAP. For the infected cell count, we presently apply a threshold value of ≥2%.

Detection of non-infectious lung conditions

Non-infectious conditions such as alveolar haemorrhage, drug-induced pulmonary toxicity, ARDS, or aspiration of gastric contents may have a clinical presentation similar to that of VAP (Timsit et al, 1996). Cytological findings such as the presence of activated lymphocytes, plasma cells, >5% eosinophils, a preponderance of foamy macrophages and reactive type II pneumocytes may point the clinician towards the diagnosis of such a condition (*Figures 2* and *5*) (Jacobs et al, 1999).

Although these findings are sensitive for non-infectious lung conditions, they are not pathognomonic. Notable infectious conditions in which BAL fluid cytology may display one of these findings are infections caused by *P. carinii* and *M. tuberculosis* (Baughman et al, 1991; Smith et al, 1988).

Prediction of the combined cytological data

It is clear that BAL fluid cytology offers valuable arguments for predicting pneumonia and may also point to a non-infectious lung condition. However, none of the parameters in itself constitutes a perfect discriminatory test. We suggest that a combination of the probability rates for multiple cytological parameters may provide additional information above the cut-off point of a single parameter, but this possibility has not yet been evaluated.

CONCLUSION

In BAL fluid cytology, the percentage of neutrophils and the infected cell count are diagnostic clues in the assessment of patients suspected of VAP. The influence of prior antimicrobial therapy on the infected cell count needs to be studied, and the evaluation of a predictive model encompassing multiple cytological parameters needs to be considered.

The above described protocol is readily applicable in the traditional microbiological setting and provides results within a 2-hour turnover time. In our experience, BAL fluid microscopy has favoured the cooperation with the intensive care physicians and pulmonologists, it has expanded our insights in the microbiology of VAP, and, most importantly, it has contributed significantly to daily patient care. HM

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KEY POINTS

- Bronchoalveolar lavage (BAL) fluid is unsuitable for reliable quantitative culture if >1% squamous epithelial cells or >1% bronchial epithelial cells are present on cytological examination.
- A low total cell count (<60 000/ml) is indicative of a technically invalid BAL fluid.
- A neutrophil count <50% rules out ventilator-associated pneumonia (VAP).
- Elevated infected cell count (≥2%) argues for VAP.
- Neutrophil and infected cell counts reflect degree of local infection
- Presence of activated lymphocytes, plasma cells, >5% eosinophils, reactive type II pneumocytes, and a preponderance of foamy macrophages point to the diagnosis of a non-infectious lung condition (e.g. drug toxicity, adult respiratory distress syndrome, aspiration of gastric contents).