Gram stain of bronchoalveolar lavage fluid in the early diagnosis of ventilator-associated pneumonia

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To assess the usefulness of the Gram stain in the early diagnosis of ventilator-associated pneumonia (VAP), we performed 146 protected specimen brushings (PSB) and bronchoalveolar lavages (BAL) in 118 patients suspected of having nosocomial pneumonia. Gram stain and counts of infected cells were performed in all samples from BAL fluid. A final diagnosis of pneumonia was established in 51 patients and there was no infection in 95 cases. A threshold of 2% of infected cells was used to distinguish between VAP and the group without VAP (sensitivity 86.3%, specificity 78.9%, positive predictive value 68.7% and negative predictive value 91.4%); there was good agreement with the final diagnosis (kappa statistic 0.616; concordance 81.5%). Regarding detection of bacteria using the Gram stain, we found a sensitivity of 90.2%, specificity 73.7%, positive predictive value 64.8% and negative predictive value 93.3%; there was moderate agreement with the final diagnosis (kappa statistic 0.586; concordance 79.4%). In the VAP group, we analysed the degree of qualitative agreement between Gram stain and PSB quantitative cultures: the correlation was complete in 51% (26 of 51 VAP), partial in 39.2% (20 of 51 VAP) and there was no correlation in 9.8% (five of 51 VAP). We conclude that the Gram stain is useful for rapid diagnosis of VAP but unreliable for early adaptation of empiric therapy.

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Ventilator-associated pneumonia (VAP) is a frequent and severe complication in patients undergoing mechanical ventilation in the intensive care unit. The exact incidence of VAP has yet to be established, although reports have indicated a range of 9–60%. Unfortunately, the optimal technique for diagnosis of VAP has not been determined. Protected specimen brushings (PSB) is a method with a sensitivity of 90% and a specificity of 75% using 10³ colony-forming units (cfu)/ml as a threshold. Quantitative cultures of bacteria in bronchoalveolar lavage (BAL) fluid may also be a reliable method.

Large numbers of patients who do not have bacterial pneumonia are exposed to expensive and ineffective antibiotics that increase the risk of colonization, with the potential emergence of multi-resistant micro-organisms. In fact, prior antimicrobial chemotherapy increases the rate of pneumonia caused by multi-resistant organisms such as Pseudomonas aeruginosa or methicillin-resistant staphylococci. Because appropriate antibiotic therapy may significantly increase survival of patients with pneumonia undergoing ventilation and decrease toxicity and cost of treatment, rapid identification of ICU patients requiring antimicrobial therapy and accurate selection of such antibiotics represent important clinical goals.

The aim of our study was to analyse the reliability of Gram stain of BAL fluid for rapid diagnosis and treatment of VAP.

Patients and methods

We studied 118 patients undergoing mechanical ventilation over a 2-yr period at the Intensive Care Unit (ICU), Edouard Herriot Hospital, Lyon (France). All patients were suspected of having nosocomial pneumonia: all had a fever (≥38.5°C), purulent tracheal aspirates, leucocytosis (≥12 000 cells mm⁻³) and new or persistent radiographic lung infiltrates unrelated to cardiogenic causes.

Eighty-five procedures were performed in patients who had not received antimicrobial therapy during the previous 3 days; 61 procedures were performed in patients who had received antibiotics (for more than 72 h). No patient received topical prophylactic antibiotics.

Sampling technique

We performed 146 BAL and PSB by fibreoptic bronchoscopy (Olympus BF P 20D, Olympus Optical Corp. of
America, New Hyde Park, NY, USA). During the procedures, 100% oxygen was administered and patients were sedated with midazolam; pancuronium was used to achieve neuromuscular block. Topical anaesthesia was not used. Heart rate, arterial pressure and arterial oxygen saturation were monitored during the investigation.

The trachea was aspirated before introducing the bronchoscope. The PSB was inserted in the bronchoscope channel. The bronchoscope was advanced through an adaptor (Bodai Suction Safe Y, Sontek Medical, Lexington, MA, USA) into the desired bronchial segment, as suspected from the chest radiograph. The PSB catheter was then advanced out of the bronchoscope, and the polyethylene glycol plug on the tip was ejected by protruding the brush (Ventimed, Marsannay-la-Côte, France) which was manipulated and rotated to be placed in contact with the secretions. The brush was retracted and the catheter was removed. The extremity of the brush was placed into a sterile tube which contained saline 1 ml. The sample was transported to the laboratory within 20 min of collection. BAL was performed by injecting 2 or 3 aliquots of sterile saline 50 ml through the bronchoscope. The fluid was then withdrawn by hand suction into syringes for infusion. Approximately 30% of instilled fluid was retrieved. BAL fluid was filtered and pooled. Gram and Giemsa stains, counts of polymorphonuclear neutrophils (PMN) and infected cells were performed using standard methods.

Bacteriological analysis

The tube which contained the brush was vortexed and the fluid was diluted to obtain concentrations of $10^{-1}$, $10^{-3}$ and $10^{-5}$. The sample was inoculated on Columbia agar, chocolate agar, trypticase soy, McConkey and Sabouraud agar. Bacterial colonies were counted and identified using conventional techniques.

Cytological analysis

The cytology of BAL was performed on a homogeneous sample. This sample was centrifuged for 10 min at 8000 g and BAL fluid was stained using Gram and Wright stains. For BAL specimens, a total cell count was performed on aliquots of resuspended BAL using a haemocytometer counting chamber. Slides were stained with Wright and Gram stains. A differential cell count (squamous epithelial cells, alveolar macrophages and neutrophils) was obtained by counting 100 cells twice on the Wright stain slide at low magnification ($\times 25$). Screening for micro-organisms was performed at high magnification (1000-fold) on the Wright stain slide. If this was positive, Gram stain was then examined at high magnification ($\times 100$) to determine the morphology and percentage of infected cells (intra-cyttoplasmic micro-organisms within macrophages and neutrophils) by counting 100 cells. In order to minimize the variability dependent on the operator’s skill, all of the cytological procedures were performed by the same microbiologist.

The percentage of ciliated cells was noted in BAL samples. Samples containing more than 1% ciliated cells were considered as contaminated and excluded.

Diagnostic categories

A diagnosis of pneumonia was based on positive results of PSB culture (cut-off $\geq 10^3$ cfu ml$^{-1}$) and clinical outcome consistent with bacterial pneumonia while receiving appropriate antibiotic therapy for organisms cultured at a significant growth in PSB. Pneumonia was excluded if the following criteria were fulfilled: negative or non-significant growth in culture of PSB and full recovery without antibiotic therapy or without changes in antimicrobial therapy initiated at least 72 h before the appearance of radiological infiltrates or diagnosis of another disease of the chest accounting for the chest radiograph abnormality.

The therapeutic strategy based on the results of the determination of infected cells in BAL fluid was to give immediate empirical antimicrobial therapy if the percentage of infected cells was $\geq 2\%^{10}$ (based on previous studies). Patients were also treated if PSB $\geq 10^3$ cfu ml$^{-1}$. When the percentage of infected cells was $< 2\%$, no immediate empirical antimicrobial therapy was instituted unless there was evidence of septic shock or severe hypoxaemia. In most cases, patients were treated initially with cefazidime and vancomycin according to the bacterial ecology of our ICU and the results of the Gram stain. Antimicrobial chemotherapy was secondarily adapted to the results of PSB cultures.

Statistical analysis

Results are expressed as mean (SD) or median (range). Sensitivity, specificity, and positive and negative predictive values were estimated using standard formulae. Comparison between groups was made using the Mann–Whitney $U$ test. $P<0.05$ was considered significant.

The degree of concordance between cytological examination of the BAL sample and final diagnosis was established using the Cohen–Kappa coefficient. Kappa values greater than 0.81 were considered to indicate very good agreement, values of 0.61–0.80 good agreement, values of 0.41–0.60 moderate agreement, values of 0.21–0.40 fair agreement and values less than 0.20 poor agreement.

Results

We studied 118 consecutive patients (mean age 52 (range 18–82) yr, mean simplified acute physiologic score (SAPS)14 (4), 78 men and 40 women). A total of 146 BAL and PSB were performed; all patients who underwent several bronchoscopic procedures had negative or no significant growth on the first quantitative cultures of PSB.

Mean duration of mechanical ventilation was 15 (4) days. The primary indications for ventilatory support were: multisystem trauma patients ($n=32$), postoperative respiratory failure ($n=29$), exacerbation of chronic obstructive
pulmonary disease (n = 16), severe sepsis (n = 12), multiple organ failure (n = 9), neurological emergencies (n = 12), other pulmonary diseases (n = 5) and acute pancreatitis (n = 3).

The diagnosis of bacterial pneumonia was established in 51 cases; polymicrobial growth was seen in 47% of patients (24 of 51). There was no bacterial pneumonia in 95 patients.

All bronchoscopies were accomplished without complications (no hypoxaemia, no pneumothorax and no haemorrhage). We did not observe any major haemodynamic changes during or after the procedure in any patient.

Cytology
None of the samples had more than 1% squamous epithelial cells. Total cell count was significantly higher in infected patients compared with patients not infected (median 815×10^3 ml^-1 (range 16–200,000×10^3 ml^-1) vs 3275×10^3 ml^-1 (18–19,600×10^3 ml^-1) (P<0.001). The proportion of PMN in BAL fluid was significantly higher in the group with VAP (90 (35 to 98)% compared with those not infected (80 (0–98)% (P<0.01). The infected cell count was made on 100 cells. The percentage of infected cells was significantly higher in VAP (13.39 (12.73)% (95% confidence interval (CI) 9.81–16.97) vs 1.25 (3.59)% (95% CI 0.52–1.98)) (P<0.0001). For absolute number of infected cells, results were 10430 (9967) (95% CI 6639–14221) for VAP vs 3275×10^3 for no VAP, respectively (P<0.0001).

Using a cut-off of 2% infected cells on the Wright stain to predict VAP, gave a sensitivity of 86.3%, specificity 78.9%, positive predictive value 68.7% and negative predictive value 91.4%. There was good agreement between the final diagnosis and the infected cell count (kappa statistic 0.616; concordance 81.5%).

Bacteriology
A Gram stain was performed on all BAL samples. First, we compared the final diagnosis with the presence or absence of bacteria on Gram stain (in BAL fluid): sensitivity and specificity were 90.2% and 73.7%, respectively; positive and negative predictive values were 64.8% and 93.3%, respectively. There was moderate agreement between the final diagnosis and the Gram stain (kappa statistic 0.586; concordance 79.4%).

Afterwards, we established the degree of qualitative correlation between the Gram stain and PSB quantitative cultures. We defined three degrees of correlation: complete correlation, partial correlation and absence of correlation. For example, there was complete correlation when we found gram-negative bacilli with the Gram stain and Pseudomonas aeruginosa in PSB quantitative cultures; a partial correlation when we found gram-negative bacilli and gram-positive cocci with the Gram stain and Staphylococcus aureus in PSB quantitative cultures and no correlation when we found gram-negative bacilli with the Gram stain and Staphylococcus aureus in PSB quantitative cultures.

<table>
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<tr>
<th>Bacteria identified on Gram stain</th>
<th>Yes</th>
<th>No</th>
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<tbody>
<tr>
<td>VAP group</td>
<td>46 (86.9% with IC ≥2%)</td>
<td>5 (80% with IC &lt;2%)</td>
</tr>
<tr>
<td>Control group</td>
<td>25 (41.6% with IC ≥2%)</td>
<td>70 (85.9% with IC &lt;2%)</td>
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In the VAP group, the correlation was complete in 51% (26 of 51 VAP), partial in 39.2% (20 of 51 VAP) and there was no correlation in 9.8% (five of 51 VAP). In the group without VAP, there was total agreement in 74.7% (71 of 95 samples) and no agreement in 25.3% (24 of 95 samples).

We then searched for a quantitative relationship between the Gram stain and PSB quantitative cultures, that is between the number of bacilli and/or cocci in the Gram stain and the number of bacteria yielded in quantitative cultures: the relationship was poor.

Finally, we analysed the concordance between the Gram stain and infected cell count (Table 1). We found that most of the true positives and true negatives were consistent (86.9% and 85.9%, respectively), but a lot of false negatives and nearly half of the false positives were consistently wrong (80% and 41.6%, respectively): this is a problem because some VAP are not recognized early when the Gram stain is negative and the infected cell count is <2%.

In 146 procedures, we noted 95 patients without pneumonia and 51 cases with pneumonia: 16 patients had gram-negative bacilli, 12 had gram-positive cocci (principally Staphylococcus aureus) and 23 were polymicrobial infections (gram-negative bacilli and gram-positive cocci).

Discussion
Ventilator-associated pneumonia (VAP) presents a diagnostic challenge in the care of ICU patients as it appears that better treatment of this infection may have a major impact on associated morbidity and mortality.

Luna and colleagues studied the selection of antibiotics and outcome of patients with VAP. When adequate antibiotic therapy was initiated early, the mortality rate was reduced if this empiric therapy was appropriate compared with when this therapy was inadequate or no therapy was given. Alvarez-Lerma showed that in a high percentage of patients with VAP, their initial empirical antibiotic treatment had to be modified because of inadequate antibiotic coverage of microorganisms.

To solve the problem of delay, we studied methods that give early results in an attempt to determine their usefulness. These methods were Gram stain and the infected cell count of BAL fluid. For bacteria, the Gram stain is clearly the most frequently used procedure and provides morphological information that can be used in the empirical selection of antibiotics for therapy. Several articles have studied Gram stain of BAL fluid. Solé-Violán and colleagues found a
correlation between Gram stain, intracellular microorganisms and both PSB and BAL culture results. Meduri and colleagues\textsuperscript{10} studied protected BAL (PBAL) and PSB. In the control group, Gram stains of the PBAL effluents were negative for all but one patient who had an endobronchial lesion and significant bacterial growth. Gram stain of the PSB specimens was negative in all patients. In the group with pneumonia, Gram stains of the PBAL cytospin specimens were positive in all but one patient and the stains correctly identified the organisms that grew at significant concentrations. Recently, Prekates and colleagues,\textsuperscript{18} in a group of 75 patients, found that the Gram stain of BAL fluid for VAP diagnosis had a sensitivity of 77\%, specificity of 87\%, positive predictive value of 71\% and negative predictive value of 90\%. For Aubas and colleagues,\textsuperscript{19} the presence of bacteria on Gram stain was significantly more frequent in the pneumonia group (28 patients). For a diagnosis of pneumonia, the presence of bacteria on BAL smears showed a sensitivity of 67.8\% and a specificity of 82.7\%. Meduri and colleagues\textsuperscript{20} studied 94 ARDS (adult respiratory distress syndrome) patients with suspected VAP who underwent 172 bronchoscopies (344 BAL). Gram stain had a sensitivity of 54\% and a specificity of 87\% and Giemsa stain (>2\% infected cells) had a sensitivity of 46\% and specificity of 93\%. Croce and colleagues\textsuperscript{21} performed 443 bronchoscopies with BAL in 232 patients. Gram stain identified gram-positive organisms in 80\% of patients with gram-positive VAP and 40\% of patients with gram-negative VAP. Gram stain identified gram-negative organisms in 52\% of patients with gram-positive VAP and 77\% with gram-negative VAP. In this study, Gram stain of BAL effluent correlated poorly with quantitative cultures and was not reliable for dictating empiric therapy.

Our study showed that the Gram stain is useful for identifying patients with VAP with good reliability (for the presence or absence of bacteria on Gram stain (in BAL fluid), sensitivity and specificity were 90.2\% and 73.7\%, respectively; positive and negative predictive values were 64.8\% and 93.3\%, respectively). There was moderate agreement between the final diagnosis and Gram stain (kappa statistic 0.586; concordance 79.4\%). However, these results had limited accuracy when used to select appropriate antibiotic therapy (in the VAP group the correlation between Gram stain and PSB quantitative cultures was complete in 51\% (26 of 51 VAP), partial in 39.2\% (20 of 51 VAP) and there was no correlation in 9.8\% (five of 51 VAP)).

Other authors have reported discrepancies between the Gram stain and quantitative cultures of pulmonary samples. Papazian and colleagues studied the Gram stain in BAL,\textsuperscript{6} mini-BAL and bronchial blind sampling (BBS). The results of each technique were compared with histology and culture of lung tissue specimens obtained by surgical pneumonectomies in 28 patients who died after at least 72 h of mechanical ventilation. Pathological specimens were positive in 13 patients and negative in 15. When only VAP with positive lung culture was considered (histological signs of bronchopneumonia with positive lung tissue culture), the sensitivity of Gram staining on BAL, mini-BAL and BBS was 56\%, 44\% and 56\%, respectively. When all samples were considered, the sensitivity and specificity of the determination of the percentage of infected cells were low (less than 70\%), whatever the sampling technique. Kollef and colleagues\textsuperscript{22} reported poor diagnostic agreement between BAL fluid Gram stain results and microbiologically confirmed gram-negative pneumonia (kappa statistic 0.35; concordance 71.8\%).

Determination of the percentage of infected cells from BAL is a reliable method for rapid diagnosis of VAP. Chastre and colleagues\textsuperscript{23} related the usefulness of BAL cytology with the search for intracellular micro-organisms to assert the early diagnosis of nosocomial pneumonia. With a threshold of 7\% of infected cells in a study of 61 patients (14 had pneumonia) there was good prediction of infection (sensitivity 86\%, specificity 96\%). The cut-off values differed between investigators. Pugin and colleagues\textsuperscript{24} detected polymorphonuclear neutrophils with infected cells in 11 of 15 patients with infection (73\%) and no infected cells in 25 patients without bacterial pneumonia. Meduri and colleagues, using a protected transbronchoscopic balloon-tipped catheter,\textsuperscript{10} found infected cells in more than 2\% of alveolar cells in 11 of 13 patients with bacterial pneumonia and in none of 33 patients without bacterial pneumonia. Solé-Violán and colleagues\textsuperscript{13} used a threshold of 4\%, giving a sensitivity of 62.5\% and a specificity of 100\%, in a study of 33 BAL. In a study of 80 BAL (28 pneumonias), Aubas and colleagues,\textsuperscript{19} using a receiver operator characteristic (ROC) curve, attempted to define a threshold for infected cells in pneumonia, but the area under the curve was 0.718 and no cut-off value could be defined. Using a cut-off value of 5\%, sensitivity was 39.3\% and specificity was 98\%. In our study, the presence of infected cells in 2\% on the Giemsa stain corresponded to a sensitivity of 86.3\%, specificity of 78.9\%, positive predictive value of 68.7\% and negative predictive value of 91.4\%. There was good agreement between the final diagnosis and infected cell count (kappa statistic 0.616; concordance 81.5\%).

In our study, PSB was considered the reference method. However, the question of a gold standard for evaluating the diagnosis of VAP remains problematic. Recent studies report discrepancies between histology and bacteriological cultures. Corley and colleagues\textsuperscript{25} studied 39 patients who died after a mean time of 14 days of mechanical ventilation. A postmortem open lung biopsy was performed in all patients. The tissue was reviewed independently by four pathologists who categorized the slides from each patient as showing or not showing pneumonia. However, the prevalence of histological pneumonia, as determined by each of the four pathologists, varied from 18 to 38\%. Kirtland and colleagues\textsuperscript{26} found that neither the bacterial density from the four airway quantitative cultures nor the bacterial density from quantitative culture of lung
Gram stain of BAL fluid

parenchyma accurately separated the histological pneumonia and non-pneumonia groups. Solé-Violán and colleagues compared bronchoscopic diagnostic techniques with histological findings in nine organ donors without suspected pneumonia immediately after death. Seven of the nine organ donors without clinical evidence of pulmonary infection and not receiving antibiotic therapy showed histological features of bronchopneumonia.

Some authors have studied other techniques to improve the accuracy of direct examination of BAL fluid. We have reported the reliability of a DNA probe to rapidly identify patients with Staphylococcus aureus VAP. We found agreement between quantitative cultures and probes in 96.3% of cases. Kollef and colleagues studied the accuracy of increased concentrations of endotoxin in BAL fluid for a diagnosis of gram-negative pneumonia. They suggested that a concentration of endotoxin in BAL fluid >5 EU ml\(^{-1}\) is superior to the Gram stain examination for rapid identification of patients with gram-negative pneumonia.

In summary, the Gram stain was useful for rapid diagnosis of VAP but not reliable for early adaptation of empiric therapy. In case of VAP, other techniques are warranted to improve early antibiotic treatment.

References