

Catharina F. M. Linssen
Jan A. Jacobs
Jan S. A. G. Schouten
Walther N. K. A. van Mook
Graham Ramsay
Marjolein Drent

Influence of antibiotic therapy on the cytological diagnosis of ventilator-associated pneumonia

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C. F. M. Linssen (✉) · J. A. Jacobs
University Hospital Maastricht, Department
of Medical Microbiology,
P.O. Box 5800, 6202 AZ Maastricht,
The Netherlands
e-mail: klin@lmib.azm.nl

J. S. A. G. Schouten
University Hospital Maastricht, Department
of Epidemiology,
Maastricht, The Netherlands

J. S. A. G. Schouten
University Hospital Maastricht, Department
of Ophthalmology,
Maastricht, The Netherlands

W. N. K. A. van Mook · G. Ramsay
University Hospital Maastricht, Department
of Intensive Care Medicine,
Maastricht, The Netherlands

M. Drent
University Hospital Maastricht, Department
of Respiratory Medicine,
Maastricht, The Netherlands

Abstract Objective: To assess the influence of antibiotics on the value of various cytological parameters, and their combinations, in diagnosing ventilator-associated pneumonia (VAP). **Design:** Prospective study. **Setting:** The general intensive care unit (17 beds) of the University Hospital Maastricht. **Patients:** Three hundred and thirty-five episodes of clinically suspected VAP (defined by the clinical and radiological criteria previously described by Bonten et al.) in 282 patients were studied. **Interventions:** No additional interventions were conducted. **Measurements and results:** Bronchoalveolar lavage fluid cytology included a total cell count per millilitre, differential cell count and the percentage of infected cells (cells containing phagocytised organisms). Antibiotic therapy from 72 h prior to lavage was recorded. Areas under the curve (AUCs) of receiver operating characteristic curves were calculated for various cytological parameters and their combinations,

in patients with and without antibiotic therapy. In 126 episodes (37.6%) in 106 patients, VAP was confirmed. There was no difference in AUCs between patients with and without antibiotic therapy for any parameter studied. The most prominent AUCs were (for patient groups with and without antibiotics combined): total cell count, 0.65; percentage polymorphonuclear neutrophils, 0.71; and percentage infected cells, 0.90. The combination of percentage infected cells with any other cytological parameter did not increase the AUC. **Conclusion:** Antibiotic therapy did not influence the predictive value of the percentage infected cells in BALF in diagnosing VAP.

Keywords Pneumonia · Bronchoalveolar lavage · Cytology · Antibiotics

Introduction

Ventilator-associated pneumonia (VAP) is a common complication in intensive care (ICU) patients, represents up to 80% of the nosocomial infections in the ICU [1, 2] and is associated with high mortality rates [1–3].

Bronchoalveolar lavage (BAL) with quantitative culture is currently regarded as a reference method for the microbiologically confirmed diagnosis of VAP [4] since clinical presentation, radiology and laboratory parameters

are non-specific. However, results of BAL fluid (BALF) cultures can take up to 48 h. Because microscopic examination of BALF can be achieved within 2 h [5, 6], the diagnostic value of cytological parameters has been explored. Of the previously investigated parameters, such as the total cell count (TCC) [7–9], the percentage of polymorphonuclear neutrophils (PMNs) [8, 10] and the percentage of cells containing phagocytised organisms, also known as infected cells or cells with intracellular organisms (IC) [6, 11, 12], the last proved to be the most reliable.

However, few studies on the influence of antibiotic therapy on the percentage of IC have been published [13–15]. The previously performed studies used a relatively small study population [13], used different techniques for calculation of the percentage IC and had conflicting results [13–15]. Therefore, we set out to evaluate the influence of antibiotic therapy on the predictive value of combinations of cytological parameters of BALF in diagnosing VAP.

Materials and methods

BALF inclusion and laboratory processing

This prospective study was conducted at the University Hospital Maastricht during a 61-month period (from January 1999 to February 2004). The hospital has a general ICU of 17 beds. In this period all consecutive BALF samples of ICU patients with clinical suspicion of (a new episode of) VAP were included. Clinical suspicion of VAP was defined by the clinical and radiological criteria previously described by Bonten et al., i.e. rectal temperature $> 38^{\circ}\text{C}$ or $< 35.5^{\circ}\text{C}$, blood leukocytosis ($> 10 \times 10^3/\text{mm}^3$) and/or left shift or blood leukopenia ($< 3 \times 10^3/\text{mm}^3$), more than ten leukocytes per high-power field in Gram stain of tracheal aspirate, positive culture of tracheal aspirate and a new, persistent, or progressive infiltrate on chest radiography [16].

Sampling technique

The affected lung was determined by the radiological presence of infiltrate; if no apparent infiltrate was present, the lingula was lavaged [17]. A fiberoptic bronchoscope (Pentax FB-15H/FB-15X, Pentax Medicals, Tokyo, Japan) was introduced and “wedged” into the affected segmental or subsegmental bronchus. Sterile saline (0.9% NaCl, room temperature) was instilled in four aliquots of 50 ml, immediately aspirated and recovered. BALF samples were transported to the laboratory within 15 min after collection and processed immediately upon arrival at the laboratory.

Laboratory processing

The first fraction of BALF (bronchial fraction) was processed separately for mycobacterial analysis and the three remaining fractions were pooled for further analysis.

BALF samples were quantitatively cultured for bacteria and yeast using 2- μl and 10- μl pipettes [18]. After a total of 48 h, bacterial colonies were counted and identified according to standard methods and minimal inhibitory concentration (MIC) values for most commonly used antimicrobial agents were determined. Cyto-centrifuged preparations were made using Cytospin 3 apparatus

(Thermo Electron’s Anatomical Pathology Group, Astmoor, England) using a standardised protocol: speed 650 rpm, time 10 min, acceleration rate low. A differential cell count was done on May–Grünwald–Giemsa (MGG) stained preparations by counting 500 nucleated cells and identifying; alveolar macrophages (AMs), lymphocytes (Lym), polymorphonuclear neutrophils (PMNs), eosinophils (Eos) and mast cells (MC). IC were expressed as a percentage of the 500 nucleated cells counted [19]. The percentages of squamous epithelial cells and ciliated epithelial cells were counted on 500 nucleated cells and separately noted.

Rejection criteria

BALF samples were rejected if they fitted one of the following criteria: (a) recovered volume less than 20 ml; (b) TCC less than 60,000 cells/ml [9]; (c) presence of excessive amounts of intercellular debris or damaged nucleated cells [5]; (d) presence of more than 1% squamous epithelial cells [20, 21].

Definitions

BALF samples were defined as microbiologically confirmed VAP (VAP group) if the culture yielded organisms in quantities $\geq 10^4$ colony forming units (cfu)/ml. The remaining samples were categorised as non-VAP group. An episode was defined as the 14-day period starting at the time of BAL. For the percentage IC a cut-off value of 2% was used [11].

Antibiotic use

Antibiotic use was recorded for each episode, starting 72 h before BAL and including the day of BAL. We divided patients into two groups: (1) patients with antibiotic therapy and (2) patients without antibiotic therapy. The first group included patients on antibiotic therapy for more than 24 h prior to bronchoscopy. The second group included patients with no antibiotic therapy in the 72 h prior to bronchoscopy, those who were on antibiotic therapy for less than 24 h and those who received antibiotics that were not active against the isolated pathogen.

Statistical analysis

To compare the different cytological parameters for the VAP group versus the non-VAP group, *p* values were calculated using a Student *t*-test. To ascertain the value of different cells in diagnosing VAP, in both groups, receiver

operating characteristic (ROC) curves were plotted for prediction of VAP using the different cell types and their combinations [22].

Results

Numbers of samples, demographic data and microbiologic findings

During the study period, 394 consecutive BALF samples were obtained from 394 episodes of suspected VAP in 316 patients. Fifty-nine (14.9%) samples were rejected, leaving 335 samples obtained from 282 patients for inclusion. Figure 1 shows an algorithm for the patients included in

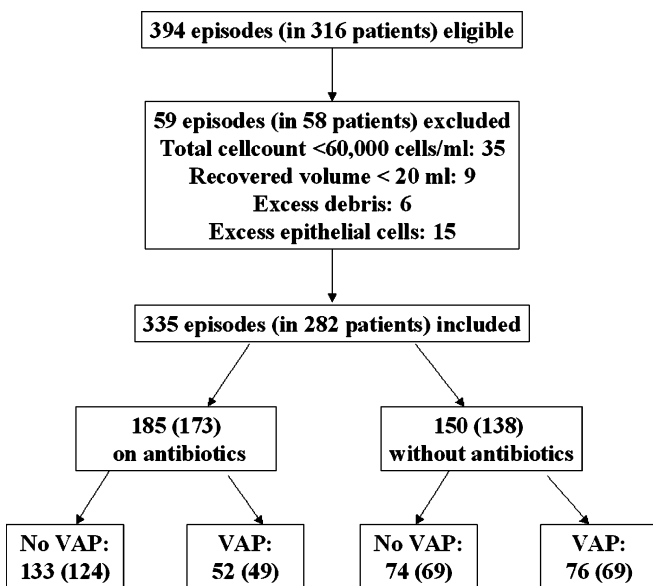


Fig. 1 An algorithm for the samples included in our study. Numbers of patients are shown in brackets

the study. Table 1 shows the baseline characteristics of the study patients. The median age of the patients was 60 years (range 15–87 years), and the male-to-female ratio was 2.2:1. A total of 185 (55.2%) episodes were included from 166 patients on antibiotics at the time of bronchoscopy; the remaining 150 (44.8%) episodes occurred in 138 patients who were not on antibiotics at the time of bronchoscopy. In 23 patients with multiple episodes of suspected VAP at least one episode of suspected VAP was whilst the patient was receiving antibiotic therapy and at least one without. VAP was microbiologically confirmed using culture as a reference test in 126 (37.6%) episodes in 106 patients. In 40.5% (51/126) of these episodes, the patient was already receiving antibiotics at the time of the BAL. Table 2 gives an overview of the antibiotics used and whether mono-therapy or combination therapy was given up to 72 h before BAL.

Cytological data

The TCC, the percentages of PMNs, IC in PMNs, IC in AMs and IC (total) were significantly higher in the VAP group, whilst the percentages of AMs, Lym and Eos were significantly higher in the non-VAP group. In 186 samples, IC were observed: in 151 samples (81.2%) only PMNs with phagocytised micro-organisms were found, in 2 samples (1.0%) only AMs with micro-organisms, and in 33 (17.8%) the IC comprised both PMNs and AMs.

Predictive value of cytological parameters

Table 3 shows the predictive value of the various cytological parameters for the diagnosis of VAP, expressed as the AUC of their ROC curves. The TCC, the percentage PMNs and the percentage IC reached the highest AUC values. Combinations of these parameters, however, did not re-

Table 1 Baseline characteristics of the study patients

| | VAP (n = 106) | Non-VAP (n = 176) | p value |
|------------------------------------|------------------|----------------------|----------|
| Characteristics | | | |
| Age (\pm SD) | 61 (\pm 18) | 60 (\pm 16) | p = 0.7 |
| Gender (%) | | | p = 0.06 |
| Male | 79 (74.5) | 112 (63.6) | |
| Female | 27 (25.5) | 64 (36.4) | |
| Diagnosis on admittance to ICU (%) | | | p = 0.03 |
| Cardiac disease | 8 (7.5) | 19 (10.8) | |
| Pulmonary disease | 19 (18.0) | 43 (24.4) | |
| Underlying malignancy | 5 (4.7) | 18 (10.2) | |
| Trauma | 18 (17.0) | 14 (8.0) | |
| Surgery | 14 (13.2) | 25 (14.2) | |
| Vascular surgery | 18 (17.0) | 21 (12.0) | |
| Neurological | 16 (15.1) | 13 (7.4) | |
| Other | 8 (7.5) | 23 (13.0) | |

VAP, ventilator-associated pneumonia; SD, standard deviation; ICU, intensive care unit

Table 2 Antibiomechanical treatment within 3 days prior to bronchoscopy with BAL, for 335 episodes of suspected VAP

| | VAP | Non-VAP | Total | <i>p</i> value |
|--|-----|---------|-------|-----------------|
| No antibiotics | 75 | 75 | 150 | <i>p</i> = 0.1 |
| Single antibiotics (<i>n</i> = 134) | | | | <i>p</i> = 0.07 |
| β-Lactam antibiotics | 33 | 77 | 110 | |
| Carbapenem | 1 | 1 | 2 | |
| Fluoroquinolones | 1 | 4 | 5 | |
| Glycopeptides | 0 | 4 | 4 | |
| Macrolides | 2 | 3 | 5 | |
| Trimethoprim-sulfa | 3 | 5 | 8 | |
| Combination antibiotics (<i>n</i> = 51) | | | | <i>p</i> = 0.7 |
| β-Lactam antibiotic + aminoglycoside | 4 | 12 | 16 | |
| β-Lactam antibiotics + macrolide | 4 | 1 | 5 | |
| β-Lactam antibiotics + fluoroquinolones | 1 | 7 | 8 | |
| Other ^a | 3 | 19 | 22 | |

^a Combinations of two or more of the following antibiotics: trimethoprim-sulfa, clindamycin, metronidazole, rifampicin, glycopeptides, fluoroquinolones and macrolides
BAL, bronchoalveolar lavage; VAP, ventilator-associated pneumonia

Table 3 Predictive value of cytologic parameters for the prediction of VAP, expressed as area under the curve (AUC) of their ROC curve

| Cytologic parameter | Total (<i>n</i> = 335) | With antibiotics (<i>n</i> = 185) | Without antibiotics (<i>n</i> = 150) |
|---------------------------------------|-------------------------|------------------------------------|---------------------------------------|
| Total cell count | 0.647 | 0.655 | 0.611 |
| Differential cell count (%) | | | |
| Alveolar macrophages | 0.313 | 0.349 | 0.257 |
| Lymphocytes | 0.381 | 0.380 | 0.353 |
| Polymorphonuclear neutrophils | 0.705 | 0.675 | 0.756 |
| Eosinophils | 0.589 | 0.439 | 0.372 |
| Mast cells | 0.557 | 0.444 | 0.444 |
| Infected cells ^a (%) | 0.904 | 0.905 | 0.900 |
| Combination of cytologic parameters | | | |
| Infected cells (%) + PMNs (%) | 0.892 | 0.901 | 0.893 |
| Infected cells (%) + Total cell count | 0.890 | 0.900 | 0.890 |

^a Polymorphonuclear neutrophils and alveolar macrophages combined
PMNs, polymorphonuclear neutrophils; VAP, ventilator-associated pneumonia; ROC, receiver operating characteristic

sult in an improvement of AUC, nor did any combination of other cytological parameters (Table 3). Antibiotic therapy in 72 h prior to performance of BAL did not influence the predictive value of the IC for the diagnosis of VAP (Table 3). The highest AUC was reached by the percentage IC (PMNs and AMs combined) as a single parameter. Antibiotic therapy in 72 h prior to performance of BAL did not influence the predictive value of the IC for the diagnosis VAP.

Additional data on false-negative and false-positive results

Table 4 shows the quantitative culture results versus the infected cell count. Based on an IC count below the 2% cut-off value, 26 BALF samples of the VAP group (20.6%) were incorrectly assigned to the non-VAP group. In 50% of these patients (13/26), BAL was performed whilst the patient was already receiving antibiotic treatment. Most (17/26, 65.4%) of these false-negative samples

Table 4 Quantitative culture results versus the infected cell count in patients with and without previous antibiotic therapy

| Quantitative culture | Previous antibiotic therapy (<i>n</i> = 185) | | No previous antibiotic therapy (<i>n</i> = 150) | |
|--------------------------|---|---------|--|---------|
| | IC ≥ 2% | IC < 2% | IC ≥ 2% | IC < 2% |
| < 10 ³ cfu/ml | 2 | 83 | 1 | 40 |
| 10 ³ cfu/ml | 6 | 43 | 7 | 27 |
| 10 ⁴ cfu/ml | 16 | 9 | 23 | 8 |
| ≥ 10 ³ cfu/ml | 22 | 4 | 39 | 5 |

IC, infected cell count; cfu/ml, colony-forming units per millilitre

Table 5 Micro-organisms recovered in episodes of VAP (quantitative cultures $\geq 10^4$ cfu/ml) matched by the numbers of infected cells (< 2% or $\geq 2\%$)

| Quantitative culture $\geq 10^4$ cfu/ml Micro-organism | IC < 2% | IC $\geq 2\%$ | Total ^a |
|---|---------|---------------|--------------------|
| <i>Pseudomonas aeruginosa</i> | 7 | 30 | 37 |
| <i>Staphylococcus aureus</i> | 2 | 27 | 29 |
| <i>Enterobacteriaceae</i> ^b | 4 | 22 | 26 |
| Oral flora | 4 | 17 | 21 |
| <i>Haemophilus influenzae</i> | 4 | 12 | 16 |
| <i>Streptococcus pneumoniae</i> | 0 | 6 | 6 |
| Other streptococci | 2 | 6 | 8 |
| Other ^c | 6 | 21 | 27 |

^a Numbers exceed total of BALF samples because in 31/126 samples, multiple organisms were recovered in quantities $\geq 10^4$ cfu/ml

^b Including *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae*

^c Including *Acinetobacter baumannii*, *Stenotrophomonas maltophilia* and *Moraxella catharrhalis*

showed culture results around the 10^4 cfu/ml threshold. The micro-organisms involved did not differ from those recovered in the true-positive group (Table 5), and 6 out of 7 micro-organisms recovered were susceptible to the antibiotic the patients was receiving at the time of the BAL. In 17 (65.4%) of these false-negative samples, the MGG stained preparations revealed mucus plugs compiled of PMNs and micro-organisms. The mucus plugs were irregularly distributed over the cytopsin spot, and in seven samples infected cells were observed within these plugs. Bacteria cultured most often from samples containing these plugs were *Pseudomonas aeruginosa* (8/17, 47.1%) and *Staphylococcus aureus* (2/17, 11.8%). There was no significant difference in the percentages of squamous epithelial cells and bronchial epithelial cells between the false-negative samples and the true-negative samples.

A total of 209 (62.4%) BALF samples had a quantitative culture below 10^4 cfu/ml (Table 4). Of these samples 16 (7.7%) showed $\geq 2\%$ infected cells and were therefore considered as false-positive results. Their median percentage IC was 3.4% (range 2.0–35.0%) and in six samples, the percentage IC exceeded 5% (range 6.4–35.0%). In half of the patients (8/16) with false-positive results, antibiotic therapy was started before BAL was performed. In four out of eight samples from patients already on antibiotic therapy, BAL yielded a micro-organism susceptible to the given antibiotic therapy. One sample yielded no growth and the remaining three samples yielded bacteria resistant to the antibiotic therapy. Thirteen (13/16, 81.3%), of the false-positive samples reached a quantitative culture result just below the cut-off value of 10^4 cfu/ml.

Borderline culture results

Of the 335 BALF samples obtained, 225 (67%) yielded one or more micro-organisms (Table 4). A large proportion (139/225, 61.7%) showed quantitative culture results approaching the cut-off value: quantitative culture results of 10^3 cfu/ml and 10^4 cfu/ml were found in respectively

83 and 55 samples. There was an equal distribution over episodes with and without antibiotic therapy (74/185, 40.0% vs. 65/150, 43.4%). Lowering the cut-off value for the quantitative culture from 10^4 cfu/ml to 10^3 cfu/ml for episodes with antibiotic therapy resulted in a large increase in false-negative infective cell counts. The sensitivity decreased from 79.4% (95% CI 75.4–83.4%) to 60.6% (95% CI 57.1–64.1%) with a slight increase in specificity from 92.3% (95% CI 88.0–96.6%) to 93.8% (95% CI 89.4–98.1%).

Discussion

The main, and clinically relevant, finding of the present study is that antibiotic therapy in the period of 72 h prior to performance of BAL under suspicion of VAP did not influence the reliability of the infected cell count in BALF in the prediction of microbiologically confirmed VAP. Among the cytological parameters of BALF cytocentrifuged preparations, the percentage IC most accurately predicted the presence of VAP. Moreover, combining percentage IC with any other cytological parameters did not increase the predictive value for VAP.

The microscopic evaluation of the percentage of IC in BALF has proven to be a reliable tool in the diagnosis of VAP [6, 8, 9, 13, 15, 23, 24]. However, there are large differences in the methodology and in the cut-off values used in these different studies, making comparison of the study results difficult. Furthermore, conflicting results have been published concerning antibiotic influence on this parameter. The method used in this study was highly standardised [19]. Three parameters (TCC, percentage PMNs and percentage IC) were found to have significantly higher values in patients with VAP than in the non-VAP group. Of these, the AUC for percentage IC appeared to display the greatest difference. Combining the different cytological parameters, however, did not improve the predictive value in diagnosing VAP. This can be explained by the high AUC of the percentage IC on its own, as well as by the

number of other clinical conditions that cause elevations of the percentage PMNs in ventilated patients [25]. No difference was found in the predictive value of cytological parameters between patients with and without antibiotic therapy at the time of the BAL (started up to 72 h before). This is in contrast with previous studies [13, 15]. Dotson et al. found that both current antibiotic use and a recent history of antibiotic use negatively influenced the recovery of IC. In their group of seven patients with VAP already on antibiotic therapy before bronchoscopy, they found only two patients (28%) with IC in BALF. Furthermore, the micro-organisms recovered from these two patients were resistant to the antibiotic regime administered. In our study, 74.5% (38/51) of episodes of VAP were correctly predicted by IC in the group receiving antibiotics. This significant difference could in part be explained by the size of the research group. Dotson et al. investigated 49 episodes (31 without antibiotic therapy, 18 with recent antibiotic therapy), whereas our study included 335 episodes of VAP. A second possible explanation is the cut-off value they used for IC, namely 7%. In the present study 2% IC yielded the best predictive value; with higher cut-off values the sensitivity declined and specificity increased. Sirvent et al. [15] evaluated protected bronchoalveolar mini-lavage fluid and found a decline in sensitivity and specificity of IC for the prediction of VAP in patients receiving antibiotic therapy up to 72 h before BAL. Moreover, Sirvent et al. [15] found a high percentage (16/65, 22.2%) of *Haemophilus influenzae* as causative organisms in their patients with VAP, compared to 12.6% (16/126) in the present study. Since *H. influenzae*, due to its small size and fragile appearance, can easily be missed in both MGG- and Gram-stained preparations, we hypothesised that this could attribute to the difference in sensitivity between the two studies. However, the authors did not mention the organisms involved in the false-negative results [15]. The findings of the present study are in line with those of Timsit et al. [14], who found no difference in sensitivity and specificity of the IC count between patients with and without antibiotic therapy in the 48 h before BAL.

The presence of plugs containing numerous bacteria may explain in part the false-negative samples. As these samples did not contain high numbers of squamous and/or bronchial epithelial cells, these plugs did not represent bronchial or oropharyngeal contamination. In histological studies, it has been shown that early stages of pneumonia are associated with focal accumulation of PMNs in the smaller bronchi, with spreading of PMNs throughout the lung in advanced pneumonia [26]. Hence, we hypothesised that the observed plugs were initial foci of pneumonia and should be actively looked for in the low-power field setting (magnification 100×). An alternative explanation may be the quantitative culture threshold used for defining VAP: the cut-off value of 10^4 cfu/ml was chosen in order to prevent failure to diagnose VAP and consequently may have

overestimated the frequency of VAP [27]. In this regard, it is interesting that nearly two thirds of false-negative samples had quantitative cultures close to the 10^4 cfu/ml threshold. It is possible that some of the culture-positive samples with less than 2% IC should actually be considered as non-VAP. Some authors have suggested decreasing the cut-off value to 10^3 in the case of antibiotic therapy in the preceding 72–24 h of lavage [28, 29]. The present study does not support this view since no significant difference was found in the distribution of quantitative culture results (excluding the episodes which did not yield any bacteria in BALF) between patients with and those without antibiotic therapy. Furthermore, the sensitivity for IC in predicting VAP decreased from almost 80% to 60%, with only a slight increase in specificity.

There was no obvious explanation for the 7.7% false-positive results. Irrespective of the use of antibiotics, the high percentage of IC in many of the false-positive samples, however, gives rise to concern with regard to the exclusion of VAP when the culture threshold is not reached. In this respect, it is interesting that recent studies define VAP not only by using the 10^4 cfu/ml culture threshold but also by incorporating the 2% cut-off value of IC as a criterion independently of the culture threshold. This approach has also been implemented in daily practice in our ICU [24, 30–34]. Supportive of this approach is the fact that more than 80% of the false positives reached a quantitative culture result just below the cut-off value. Using the above-cited combination of criteria to define VAP (i.e. combining the 10^4 cfu/ml culture threshold and the 2% IC value), the number of VAP episodes in the present study would have increased from 126/335 (37.6%) to 137/335 (41.0%).

A limitation of the presented study is the lack of a gold standard for the diagnosis VAP. Instead, a “semi-gold” standard, namely the quantitative culture of BALF, was used. Unfortunately, this “semi-gold standard” itself is influenced by antibiotic therapy. Therefore, it was only possible to study the influence of antibiotic therapy on the infected cell count in microbiologically confirmed VAP (with quantitative cultures exceeding the cut-off value of 10^4 cfu/ml) (since autopsy as a gold standard is not practically useful).

In the present study neither the occurrence of other conditions associated with influx of PMNs in the IC population nor the episodes with borderline quantitative culture results were studied. Therefore, an additional study is required to address the presence of qualitative cytological parameters suggesting non-infectious conditions, such as eosinophils, foamy alveolar macrophages, activated lymphocytes, plasma cells and reactive type II pneumocytes (RPII cells) [35]. The presence of RPII cells has been associated with severe alveolar damage. A prospective evaluation of cytological parameters in different non-infectious conditions should be established to enable the construction of a predictive computer model in line with that con-

structured for use in the assessment of interstitial lung diseases [36].

The present findings are relevant for ICUs in other hospitals, since the study population represents a heterogeneous group of intensive care patients comparable to that seen in general ICUs in many other institutions. The presently used stains and techniques are within the scope of any microbiology laboratory. If the MGG stain is not available, laboratories may fall back on the Gram stain, as the latter also enables reliable detection of the percentage IC in BALF samples [19]. Since the percentage

IC encompasses both PMNs and AMs, no distinction between these cell types is required, precluding the need for more specialised cytological expertise on the part of the laboratory staff.

In conclusion, antibiotic therapy in the 72 h preceding the BAL did not influence the predictive value of cytological BALF parameters in the microscopic diagnosis of VAP. The percentage of IC appears to be the most important parameter for distinguishing VAP from non-VAP conditions. Combining the percentage IC with any other cytological parameter did not result in better predictive values.

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