Chapter 4

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

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Submitted

Abstract

Objective
To assess the influence of antibiotics on the value of various cytological parameters, and their combinations in diagnosing ventilator-associated pneumonia.

Study design
This prospective study was conducted at the general intensive care unit (17 beds) of the University Hospital Maastricht. A total of 335 episodes of clinically suspected ventilator-associated pneumonia (VAP) (defined by the clinical and radiological criteria previously described by Bonten *et al.*) in 282 patients were studied.

Results
Bronchoalveolar lavage fluid (BALF) cytology included a total cell count/ml, differential cell count and the percentage of infected cells (IC, cells containing phagocytised organisms). Antibiotic therapy from 72 hours prior to lavage was recorded. Areas under the Curve (AUC) of Receiver Operating Characteristic curves were calculated for various cytological parameters and their combinations, in patients with and without antibiotic therapy. In 126 (37.6%) episodes in 106 patients VAP was confirmed. There was no difference in AUCs in patients with or without antibiotic therapy for any parameter studied. Most prominent AUCs were (for patient groups with and without antibiotics combined): total cell count: 0.65, percentage polymorphonuclear neutrophils: 0.71 and percentage IC: 0.90. The combination of percentage IC with any other cytological parameter did not increase the AUC. At a threshold of 2%, the percentage infected cells had an 86.2% positive and an 88.1% negative predictive value for VAP.

Conclusion
Antibiotic therapy did not influence the predictive value of the percentage infected cells in BALF in diagnosing VAP.

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[3,4].
Bronchoalveolar lavage (BAL) with quantitative culture is currently regarded as the reference method for the diagnosis VAP[5] since clinical presentation, radiology and laboratory parameters are non-specific. However, results of BAL fluid (BALF) cultures can take up to 48 hours. Because microscopic examination of BALF can be achieved within two hours[6,7], the diagnostic value of cytological parameters has been explored. These parameters include the total cell count (TCC)[8-10], the percentage of polymorphonuclear neutrophils (PMNs)[9,11] and the percentage of cells containing phagocytised organisms, also known as infected cells or cells with intracellular organisms (IC)[7,9,12,13]. However, the influence of antibiotic therapy on the percentage of IC has not been studied thoroughly. The previously performed studies used a relatively small study population[14], used different techniques for calculation of the percentage IC and resulted in conflicting results[14-16]. Therefore, we evaluated the influence of antibiotic therapy on the predictive value of combinations of cytological parameters of BALF in diagnosing VAP. In addition, we established the most accurate cut-off values of these parameters for the diagnosis of VAP.

Materials and methods

BALF inclusion and laboratory processing

This prospective study was conducted at the University Hospital Maastricht during a 61-month period (January 1999 until 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 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°C or <35.5°C, blood leukocytosis (>10×10^3/mm^3) and/or left shift or blood leukopenia (<3×10^3/mm^3), more than ten leukocytes in Gram-stain of tracheal aspirate (in high-power field), positive culture of tracheal aspirate and a new, persistent, or progressive infiltrate on chest radiograph[17].

Sampling technique

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 minutes 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 work-up was conducted as previously described[6] and included a total cell count, differential cell count and quantitative culture. BALF samples were quantitatively cultured for bacteria and yeast using 2 and 10 μl pipettes[18]. After 48 hours, bacterial colonies were counted and identified according to standard methods and minimal inhibitory concentration (MIC) values for most commonly used antimicrobial agents were determined. Cytocentrifuged preparations were made using Cytospin 3 apparatus (Thermo Electron's Anatomical Pathology Group, Astmoor, England) using a standardised protocol[6]. A differential cell count was done on May-Grünwald Giemsa (MGG) stained preparations by counting 500 nucleated cells and identifying; alveolar macrophages (AMs), lymphocytes (Lyms), poly-morphonuclear neutrophils (PMNs), eosinophils (Eos) and mast cells (MCs). IC were expressed as a percentage of 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: i) recovered volume less than 20 ml, ii) total cell count less than 60.000 cells/ml[10], ii) presence of excessive amounts of intercellular debris or damaged nucleated cells[6] iv) presence of more than 1% squamous epithelial cells[20,21].

Definitions

BALF samples were defined as VAP if the culture yielded organisms in quantities $\geq 10^4$ colony forming units (cfu)/ml. The remaining samples were categorised as non-VAP. An episode was defined as the period starting at the time of BAL until 14 days thereafter.

Antibiotic use

Antibiotic use was recorded for each episode, starting 72 hours 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 hours prior to bronchoscopy. The second group included patients with no antibiotic therapy in the 72 hours prior to bronchoscopy, those who were on antibiotic therapy for less than 24 hours 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 Operation Characteristic (ROC) curves were plotted for prediction VAP using the different cell types and their combinations[22]. In addition, the most reliable cut-off value for the diagnosis of VAP of these parameters was determined.

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. Table 4.1 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 using antibiotics at the time of bronchoscopy, the remaining 150 (44.8%) episodes were from 138 patients who were not using 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 4.2 gives an overview of the antibiotics used and whether mono-therapy or combination therapy was given up to 72 hours before BAL.

Table 4.1 Baseline characteristics of study patients.

	VAP (n=106)	Non-VAP (n=176)
Characteristics		
Age (± SD)	61 (± 18)	60 (± 16)
Gender (%)		
Male	79 (74.5)	112 (63.6)
Female	27 (25.5)	64 (36.4))
Diagnosis on admittance ICU (%)		
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)

Table 4.2 Antimicrobial treatment within 3 days prior to bronchoscopy with BAL, for 335 episodes of suspected VAP.

	VAP	Non-VAP	Total
No antibiotics	75	75	150
Single antibiotic (n = 134)			
β-lactam antibiotics	33	77	110
Carbapenem	1	1	2
Fluoquinolones	1	4	5
Glycopeptides	0	4	4
Macrolides	2	3	5
Trimethroprim-Sulfa	3	5	8
Combination antibiotics (n = 51)			
β-lactam antibiotic + aminoglycoside	4	12	16
β-lactam antibiotic + macrolide	4	1	5
β-lactam antibiotic + fluochinolon	1	7	8
Other[a]	3	19	22

[a] Combinations of two or more of the following antibiotics: trimethroprim-sulfa, clindamycin, metronidazol, rifampicin, glycopeptides, fluochinolones and macrolides. BAL=bronchoalveolar lavage, VAP=ventilator-associated pneumonia.

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

Predictive value of cytological parameters

Table 4.3 shows the predictive value of the various cytological parameters for the diagnosis of VAP, expressed as the AUC of their ROC curves. The total cell count, the percentage PMNs and the percentage IC reached the highest AUC-values. Combinations of these parameters however did not result in an improvement of AUC, nor did any combination of other cytological parameters (Table 4.3). Antibiotic therapy in 72 hours prior to performance of BAL did not influence the predictive value of the IC for the diagnosis VAP (Table 4.3). The highest AUC was reached by the percentage IC (PMNs and AMs combined) as a single parameter. Antibiotic therapy in 72 hours prior to performance of BAL did not influence the predictive value of the IC for the diagnosis VAP. The best cut-off value for the diagnosis of VAP was obtained at 2%, resulting in a sensitivity of 79.4% (95% confidence interval (C.I): 75.4-83.4%) and a specificity of 92.3% (95% C.I.: 88.0-96.6%). With a VAP prevalence of 37.6% this amounted to a positive predictive value of 86.2% and a negative predictive value of 88.1%.

Table 4.3 Predictive value of cytologic parameters for the prediction of VAP, expressed as area under the curve (AUC) of their ROC curve.

Cytologic parameter	Total (n=335)	With antibiotics (n=185)	Without antibiotics (n=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 Operation Characteristic.

Additional data on false-negative and false-positive results

Based on an IC count below the 2% cut-off value, 26 (20.6%) BALF samples of the VAP group were incorrectly assigned to the non-VAP group. In fifty percent 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 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 4.4), 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 cytospin 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.

Table 4.4 Micro-organisms recovered in episodes of VAP (quantitative cultures $\geq 10^4$ cfu/ml) matched by the numbers of infected cells (<2% or ≥2%).

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

[a] Numbers outreach 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*, *Klebsiella pneumoniae*; [c] Including *Acinetobacter baumanii*, *Stenotrophomonas maltophilia* and *Moraxella catharrhalis*.

A total of 209 (62.4%) BALF samples had a quantitative culture below 10^4 cfu/ml. Of these samples 16 (7.7%) showed ≥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, it 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, BALF 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

Out of the 335 BALF samples obtained, 225 (67%) yielded one or more micro-organisms. A large percentage (138/225, 61.3%) 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 (83/185, 39.5% versus 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 of false negative infective cell counts. The sensitivity decreased from 79.4% (95% C.I: 75.4-83.4%) to 60.6% (95% C.I: 57.1–64.1%) with a slight increase in specificity from 92.3% (95% C.I.: 88.0-96.6%) to 93.8 (95% C.I: 89.4-98.1%).

Discussion

The main, and clinically relevant finding of the present study is that, antibiotic therapy in 72 hours 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 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.

Several cytological parameters have been investigated in order to achieve a fast and reliable tool in the diagnosis of VAP. Both the TCC and the percentage of PMNs have demonstrated to be significantly higher in BALF samples from patients with VAP as compared to non-VAP[8,9,23,24]. Due to the overlap between the two groups however a clear distinction on the basis of both TCC and percentage PMNs was not possible, as confirmed in the present study.

The microscopic evaluation of the percentage of IC in BALF has proven to be a reliable diagnostic tool in the diagnosing VAP[7,9,10,12-15]. 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 be significantly higher in patients with VAP, compared to non-VAP. Out of these, the AUC for percentage IC appeared to be the highest. 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]. The best combination of sensitivity and specificity was reached at a cut-off value of 2% IC, which is in agreement with previous studies[9,10].

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 hours before). This is in contrast with previous studies[14,15]. Dotson *et al.*[14] found both current antibiotic use as well as a recent history of antibiotic use to negatively influence the recovery of IC. In their group of seven patients with VAP already on antibiotic therapy before bronchoscopy, they found only two (28%) patients with IC in BALF. Furthermore, the micro-organisms recovered from these two patients were resistant to the antibiotic regime administered to these patients. In our study, 74.5% (38/51) of VAPs 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 seven percent. In the present study two percent IC yielded the best predictive value, and when using higher cut-off values the sensitivity declines while increasing specificity. 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 hours before BAL. However, they also found a percentage of 2% IC or more to be the best cut-off value for both patients with and without antibiotic therapy at time of 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 hypothesized that this could attribute to the difference in sensitivity between both 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 Timsit *et al.*[16] who found no difference in sensitivity and specificity of the IC count in patients with and without antibiotic therapy in the 48 hours before BAL.

The presence of plugs containing numerous bacteria may explain in part for 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 the observed plugs to be initial foci of pneumonia and they 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 has been chosen in order to prevent undiagnosed VAPs and consequently may overestimate the number of VAPs[27]. In this way, 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 that 2% IC actually are to be considered as non-VAPs. Some authors suggested to decrease the cut-off value to 10^3 in case of antibiotic therapy in the preceding 72-24 hours of lavage[28,29]. The present study does not subscribe 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 without antibiotic therapy. Furthermore, the sensitivity for IC in predicting VAP decreased from almost 80% to 60%, with only a slight increase in specificity.

No apparent clarification was present to explain for the 7.7% false positive results. Irrespective of the use of antibiotics, the high number of percentage IC in many the false-positive samples however gives concern with regard to the exclusion of the diagnosis 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 independently of the culture threshold as a criterion. This approach has also been implemented in daily practice in our intensive care[13,30-34]. 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 (thus 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%).

In the present study neither the occurrence of other conditions associated with PMNs influx in the ICU 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[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 constructed for use in the assessment of interstitial lung diseases[23].

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 hospitals. The presently used stains and techniques are within reach of all microbiology laboratory. If the MGG stain is not available, laboratories may also rely upon the Gram-stain as an alternative, as this latter stain 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 and this

precludes the need for more specialised cytological expertise of the laboratory staff.

In conclusion, antibiotic therapy in the 72 hours preceding the BAL did not influence the predictive value of cytological BALF parameters in the microscopic diagnosis VAP. The percentage of IC appears to be the most important parameter for distinguishing VAP from a non-VAP condition. A cut-off value of 2% could be ascertained using a standardised method. Combining the percentage IC with any other cytological parameter did not revealed better predictive values.

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