Differential cell counts in canine cytocentrifuged bronchoalveolar lavage fluid: a study on reliable enumeration of each cell type

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Key Words
Bronchoalveolar lavage, cell count, cytology, pulmonary disease

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Background: Bronchoalveolar lavage (BAL) allows cell recovery from the lower respiratory tract; differential cell counts of BAL fluid gives important information in the assessment of various bronchial and pulmonary diseases. To the best of our knowledge no study has investigated the relation between the number of cells counted and the reproducibility of BAL fluid differential cell counts.

Objective: The purpose of this study was to investigate using statistical methods how many cells should be counted in cytocentrifuged BAL fluid preparations in order to obtain a reliable enumeration of each cell type.

Methods: BAL fluid samples from dogs with suspected bronchopulmonary disease were obtained during fiberoptic bronchoscopy with a standardized protocol. Differential cell counts were performed on May–Grünwald–Giemsa-stained cytocentrifuged preparations by 2 independent observers. Reproducibility for the enumeration of each cell type was expressed as the intraclass correlation coefficient. We considered a threshold level of $Z_{0.90}$ to be high and a threshold level of $Z_{0.85}$ to be adequate.

Results: Forty BAL fluid samples were included in the study. For neutrophils, alveolar macrophages, and eosinophils high reproducibility was reached by counting 200 cells; adequate reproducibility was reached for lymphocytes and bronchial epithelial cells by counting 500 cells.

Conclusions: A 500-cell differential count is required for all types of cells to be quantified with adequate reproducibility in canine cytocentrifuged BAL fluid samples.

Introduction

Bronchoalveolar lavage (BAL) is a procedure carried out to recover cells, fluids, and etiologic agents from the lower respiratory tract and alveolar spaces. Both in human and veterinary medicine, changes in the BAL fluid profile may reflect pathologic processes within lower airways and lung parenchyma. Identification and quantification of the different cell types have been demonstrated to be useful and often fundamental in the diagnostic workup of pulmonary diseases, particularly in alveolar and interstitial lung diseases and suspected infectious diseases.

Perhaps more than with any other type of cytologic specimen, the choice of processing technique for lavage fluid may affect both the quantity and the type of cells available for microscopic study. For this reason caution should be exercised when comparing differential cell counts obtained from different laboratories, because processing methods may differ considerably. It is important to reproduce another laboratory’s method exactly when intending to compare data and it is also important to practice consistent and standardized collection and processing techniques within one’s own institution when developing normative data.

Several technical factors can potentially affect total and differential cell counts in BAL fluid: the quantity of instilled fluid, number of instilled aliquots, retrieval method, concentration technique, and number of cells counted. While the Task Group on BAL of the European Society of Pneumology, in accordance with the guidelines of American Society of Pneumology,
Each cell type.

standard of reproducibility for the relative number of
fuged canine BAL fluid preparations to obtain a high
number of cells that must be counted in cytocentri-
fore, the aim of the present study was to determine the
differential cell counts in BAL fluid from dogs. There-
number of cells counted and the reproducibility of
 systematically investigated the relation between the
tempered cells in human BAL fluid, no study has sys-
ics. Cell counts were determined on unprocessed fluid
2
Vet Clin Pathol
using an improved Neubauer hemocytometer (Bodan-
Cell counts were determined on unprocessed fluid
2
Vet Clin Pathol
was withdrawn by a sterile suction device (ASP-A00-
by syringe. A few seconds after each injection the fluid
of equal volume (1 mL/kg body weight) were instilled,
chloride solution was instilled through the working
nonbacteriostatic, prewarmed (37°C), 0.9% sodium
epithelial cells.
utrophils, eosinophils, lymphocytes, and bronchial
expression as the mean number per 100 cells counted
counted. The differential cell count was recorded after
Only well-preserved and fully evaluable cells were

Materials and Methods

Animals and lavage procedure

For this prospective study, BAL fluid samples from dogs
presented to the “San Marco” Private Veterinary Clinic
between January 2007 and June 2008 with clinical
and imaging results suspicious for bronchial and
bronchopulmonary disease were obtained using a
standardized protocol. BAL was performed during
fiberoptic bronchoscopy under general anesthesia us-
ing a 2.7 mm outer diameter bronchoscope (Fibro-
scope 60003VB, Karl Storz, Tuttlingen, Germany) in
small dogs (≤ 10 kg) and a 5.5 mm outer diameter fib-
roscope (Fibroscope 60003VB) in large dogs (> 10 kg).
The instrument was inserted through a T-adapter be-
tween the tracheal tube and the tubing of the anesthe-
sia machine allowing patient oxygenation during
endoscopic examination and BAL fluid recovery. The
bronchial location of the BAL was at the discre-
tion of the bronchoscopist, but was generally the
lung segment most affected by disease based on imag-
ing changes or visual appearance at bronchoscopy.
The tip of the endoscope was passed into a distal
bronchus until a snug fit was achieved. Sterile,
nonbacteriostatic, prewarmed (37°C), 0.9% sodium
carbonate solution was instilled through the working
channel of the bronchoscope. In total, 2 aliquots of
equal volume (1 mL/kg body weight) were instilled,
by syringe. A few seconds after each injection the fluid
was withdrawn by a sterile suction device (ASP-A00-
01, Meditalia, Palermo, Italy) set at low aspiration
pressure (~ 60 mmHg). Fluid retrieved from both
instilled aliquots was combined into a single sample
for analysis.

Differential cell counts

Total nucleated cell counts (TNCC) and slide prepara-
tion were performed within 1 hour of fluid collection.
Cell counts were determined on unprocessed fluid
using an improved Neubauer hemocytometer (Bodan-
chimica, Cagliari, Italy). For cytologic evaluation, cyto-
centrifuged slides were prepared (Aerospray Slide
Stainer/Cytocentrifuge 7150, Wescor, Logan, UT, USA).
A 150 μL aliquot of well-suspended fluid was placed in
the centrifugation chamber and centrifuged at 850 rpm
(311 g) for 8 minutes using a fast absorption filter
(Cytopad Absorption Pads Cat. No. SS-111, Wescor).
The samples were air dried and stained with
May–Gr¨unwald–Giemsa in an automatic slide stainer
(Aerospray Slide Stainer/Cytocentrifuge 7150, We-
scor). Because a low TNCC may indicate that the fluid
collected is more likely bronchial than bronchoalveo-
lar, samples with a TNCC ≤ 250/μL were excluded
from analysis; the TNCC was not otherwise used in the
analysis. In addition, samples with an excessive num-
ber of RBCs (based on microscopic examination) or
mucus that interfered with nucleated cell identifica-
tion were also excluded.

The cytocentrifuged preparations of BAL fluid
were examined in a blinded fashion by 2 European
College of Veterinary Clinical Pathology board-
certified pathologists (D.D.L. and C.M.) using a × 100
oil immersion objective with a grid reticle that divided
the observation field into 4 identical sections. A differ-
ential cell count was performed on 500 nucleated cells
in a circular pattern around the center of the cell area.
Only well-preserved and fully evaluable cells were
counted. The differential cell count was recorded after
100, 200, 300, 400, and 500 cells were counted using
dedicated software (BAL2000 Freeware Version 3.0,
available at http://www.nickels.fi/). Cell counts were
expressed as the mean number per 100 cells counted
(equivalent to the percentage). Cell types included in
the differential count were alveolar macrophages, ne-
utrophils, eosinophils, lymphocytes, and bronchial
epithelial cells.

Statistical analysis

Variance analysis and intraclass correlation coefficients
(ICC) were calculated for each combination of cell type
(6 cell types considered) at each interval of 100 cells
counted (5 intervals). The ICC was used to indicate the
degree of absolute agreement among measurements
on selected samples and to define how many cells
should be counted, in this case by the 2 observers, to
obtain a reliable enumeration of each cell type. An ICC
may be between 0.0 and 1.0; a high ICC indicates little
difference between the scores assigned by the 2 ob-
servers. We arbitrarily considered a threshold of ≥ 0.90
to be high reproducibility and a threshold of ≥ 0.85 to
be adequate reproducibility.
Variance components to be used in calculating the ICC were estimated by a 1-way random effects model according to McGraw and Wong:

\[ x_{ij} = \mu + a_i + b_i + (ab)_{ij} + e_{ij} \]

where \( x_{ij} \) is the \( i \)th observation made by the \( j \)th rater; \( \mu \) is the overall population mean of the count; \( a_i \) is the observer effect (judge); \( b_i \) is the difference among counts on same sample (target); \( (ab)_{ij} \) is the interaction effect; and \( e_{ij} \) is the residual error. In this study, \( (ab)_{ij} \) and \( e_{ij} \) were not separately estimable because there was only 1 observation per cell. From this model we get a typical ANOVA table (Table 1) from which it is possible to obtain the variance components for calculating the ICC according to this formula:

\[ ICC = \frac{BMS - EMS}{BMS + (JMS - EMS)/n} \]

where BMS indicates between target mean square, EMS indicates error mean square, JMS indicates mean square for judges (observers), and \( n \) indicates the number of observed samples.

Confidence of intervals were estimated by bootstrap method. A trellis graph was used to illustrate how the ICC changed with an increase in the number of counted cells. All data analysis was done using “R” software.

Results

BAL fluid samples were obtained from 56 dogs, of which 40 were included in the study. Five samples were excluded for excessive RBCs, 6 samples were excluded for excessive mucus, and 5 samples were excluded because of a TNCC < 250/μL.

Based on the ICC, a differential cell count using 200 cells resulted in high reproducibility for macrophages, neutrophils, and eosinophils; counting > 200 cells resulted in only a minor increase in reproducibility for these cell types (Figure 1). High reproducibility was not reached even by counting 500 cells for lymphocytes and epithelial cells. Adequate reproducibility was achieved for the enumeration of all cell types by counting 500 cells. The results of the 500-cell differential counts obtained by the 2 observers were tabulated (Table 2).

Discussion

A great deal of variability is present in BAL differential cell counts among healthy dogs and dogs with bronchopulmonary disease and increased variability is added by inconsistency in collection techniques and specimen processing. In this study we determined the number of cells required in a differential cell count to obtain reproducible differential cell counts in BAL fluid. Reproducibility was expressed as the ICC, which indicates the degree of absolute agreement among

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Observer 1</th>
<th>Observer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>207.06 ± 118.28</td>
<td>175.81 ± 114.92</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>203.81 ± 150.82</td>
<td>213.68 ± 158.65</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>49.77 ± 83.08</td>
<td>56.1 ± 93.99</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>29.48 ± 35.36</td>
<td>45.84 ± 41.12</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>9.74 ± 23.91</td>
<td>8.42 ± 22.27</td>
</tr>
</tbody>
</table>

Data are mean ± SD of the number of cells counted.
measurements made on randomly selected samples by 2 observers. To minimize variability, for example, in using BALs in research investigations where only small differences are tolerated between various measurements, a high standard (ICC ≥ 0.9) of reproducibility would be desirable, whereas an ICC threshold of ≥ 0.85 may be considered adequate for diagnostic purposes.

The reproducibility of neutrophil counts was the highest, with an adequate ICC threshold reached by counting 100 cells. This can be explained by the fact that these cells are easily identified and were present in the highest mean percentage in BAL fluid samples. Canine eosinophils also are easily recognizable and consequently adequate reproducibility was reached by counting 200 cells in spite of the relatively low mean number of eosinophils in our BAL samples. Compared with neutrophils and eosinophils, alveolar macrophages had a lower ICC threshold, probably because of the high mean relative percentage of alveolar macrophages in the BAL samples. Lymphocyte counts had the lowest ICC values and therefore the lowest reproducibility among inflammatory cells. A possible explanation can be found in the difficulty in distinguishing large activated lymphocytes from alveolar macrophages. We hypothesize that this, in combination with the greater fragility of lymphocytes and their overall low mean percentage might have accounted for the lower agreement between the 2 observers in the present study.

Bronchial epithelial cells should be found in extremely low numbers in canine BAL samples, especially if alveolar washing is performed atraumatically. A high number of bronchial cells can artifactually increase the TNCC and for this reason must be counted but recorded separately for diagnostic purposes. We included bronchial epithelial cells in the differential cell count only for statistical reasons, as in our laboratory these cells are recorded separately in daily practice according to official recommendations of Task Group on BAL of the European Society of Pneumology. Adequate but not high reproducibility of epithelial cell counts was reached by counting 500 cells. Although they are easy to recognize, bronchial cells are generally present in a very low numbers and therefore are often irregularly distributed in the cytocentrifuged specimen. In addition, they tend to occur not only as single cells but often in small clusters. For this reason, their number is strongly affected by the areas examined, which may explain the irregular ICC values obtained in this study.

In summary, the results of this study defined the number of total cells that must be counted in BAL fluid to obtain a reliable differential cell count. Even if adequate enumeration of alveolar macrophages, neutrophils, and eosinophils can be achieved by counting 200 cells, acceptable reproducibility can be achieved for all cell types only by counting 500 cells.

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References


