Evaluation of variations in bronchoalveolar lavage fluid in horses with recurrent airway obstruction

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Objective—To determine variations in cytologic counts of bronchoalveolar lavage (BAL) fluid attributable to month of collection, first and second aliquots, and left and right lung sites in horses with recurrent airway obstruction (RAO).

Animals—5 horses with RAO and 5 healthy horses without respiratory tract disease.

Procedures—Horses were housed in a stable for 5 months prior to and throughout the study. Bronchoalveolar lavage fluid was collected from the right and left lung of each horse 3 times at monthly intervals (February, March, and April). Each BAL fluid collection was performed by use of 2 incremental instillations of 250 mL of isotonic saline (0.9% NaCl) solution in the same bronchial site. Analysis of BAL fluid included volume of BAL fluid recovered, a CBC, and differential cytologic counts.

Results—Volume of BAL fluid recovered and cytologic counts did not differ in horses with RAO across time or between right and left lungs, except for the number of mast cells. Horses with RAO had significantly lower volumes of BAL fluid recovered, significantly lower percentages of macrophages and lymphocytes, and significantly higher percentages of neutrophils than did healthy horses. Despite individual variation, all horses with RAO had >25% neutrophils throughout the study period.

Conclusions and Clinical Relevance—Despite variation among horses, BAL fluid cytologic counts were repeatable over short and long periods and samples can be used for longitudinal studies as a diagnostic tool of pulmonary inflammation in horses with RAO. (Am J Vet Res 2011;72:838–842)

Cytologic evaluation of BAL fluid is commonly used in clinical and research settings for the diagnosis and monitoring of diffuse lung diseases such as RAO (ie, heaves). Despite the fact there are guidelines on BAL techniques in horses with RAO and inflammatory airway disease, there is no general consensus on BAL technique, with wide variations in lavage volume and methods used by various research groups. It is assumed that a single sample of BAL fluid collected from any site is representative of an entire lung.

However, 1 study in healthy ponies and ponies with RAO in remission revealed variations in results of cytologic counts performed on BAL fluid obtained from the same animals at various times during a 4-week period. We suspect that some variations may be observed in cell populations in consecutive BAL samples obtained over time.

Controversy persists on the necessity to analyze 1 or several BAL aliquots for the diagnosis of lung diseases. In humans with bronchitis, the first aliquot is most representative of bronchial material (ie, contains concentrated numbers of neutrophils), whereas subsequent aliquots contain more bronchoalveolar secretions. Whether the same is true in horses remains controversial. For instance, investigators in 1 study found no significant difference in results of cytologic counts of BAL fluid for 3 sequential 100-ml lavages, compared with those of a single 300-ml lavage. In contrast, investigators in another study report that sequential lavages increased the volume of BAL fluid recovered and the proportion of macrophages in healthy horses. In that study, BAL fluid from 3 sequential 100-ml lavages was compared with BAL fluid from a single 300-ml lavage. Investigators in a third study reported that although variations were observed, there was no significant difference in nucleated cell counts, differential cell counts, and results of CBCs among sequential and pooled BAL fluid aliquots in clinically normal horses.
and horses with RAO. In that study, 300 mL of isotonic saline (0.9% NaCl) solution was introduced into a bronchus, and 3 sequential BAL fluid aliquots of 20 mL were collected and compared with pooled BAL fluid. Therefore, it was concluded that all aliquots were representative of the cell population of the lavaged lung segment.

It has been assumed that cell populations are similar in BAL fluid recovered from sites throughout the lungs in clinically normal horses. Investigators in an aforementioned study reported that there was no significant difference in cellular composition of BAL fluid between the right and left lungs in healthy horses, except for the number of mast cells, which was significantly higher in the left lung. Because RAO is a diffuse pulmonary disease, we postulated that cell populations in BAL fluid should be uniform in both lungs.

To our knowledge, cytologic analysis of BAL fluid collected repeatedly over time and comparison of results between the right and left lungs of horses with clinical RAO stabled in conditions with constant mold exposure have not been reported. Thus, the objectives of the study reported here were to examine variations in cell populations in BAL fluid collected at various times (February, March, and April) in healthy horses and horses with RAO, effects of repeated sample collections (first and second aliquots) on results of BAL fluid analysis, and differences in the cellular composition of BAL fluid between the right and left lungs. Our hypotheses were that temporal variations in cell populations in BAL fluid would be detected in horses with RAO, the second aliquot would have a lower neutrophil concentration than would the first aliquot, and no significant differences would be detected in samples of BAL fluid obtained from the right and left lungs.

**Materials and Methods**

**Animals**—Ten adult (14- to 20-year-old) mixed-breed mares weighing 400 to 550 kg were used for the study. Five horses with RAO (RAO group; mean ± SD age, 16.4 ± 0.7 years; mean ± SD body weight, 484.0 ± 93.7 kg) and 5 horses without respiratory tract disease (control group; mean ± SD age, 17.8 ± 1.0 years; mean ± SD body weight, 460 ± 21.9 kg) from a university research herd were included in the study. Horses with RAO had a history of chronic respiratory tract disease and maximal change in P<sub>L</sub> > 15 cm H<sub>2</sub>O. Control horses were considered to be free of respiratory tract disease on the basis of history, physical examination findings, and results of pulmonary function measurements. For each horse, results of a CBC were within respective reference intervals, and endoscopy of the larynx and pharynx did not reveal remarkable abnormalities. The protocol for the study was approved by the Animal Care Committee of the Faculty of Veterinary Medicine at the University of Montreal.

Prior to the study, horses were conditioned to stand in stocks while wearing a face mask. Horses were housed in the same barn for 3 months before and throughout the study. Horses were fed dry timothy hay and a grain mix with added molasses (ie, sweet feed) twice daily. Straw was used for bedding. Management remained the same throughout the study. No medication other than anthelmintics was administered to the horses in the 5 months before or during the study.

**Pulmonary function measurements**—A tight-fitting face mask was placed over the nose of each horse. The mask was sealed with a rubber shoulder designed to avoid obstructing the nostrils. Flow rates were measured by use of a heated pneumotachograph and an associated differential pressure transducer attached to the mask. Electronic integration of the flow signal was used to measure tidal volume. Before and after each experiment, the system was calibrated by forcing air at known flow rates and volumes through the pneumotachograph by use of a blower-rotameter and 6-L calibrated syringe. Esophageal pressure was measured by use of a balloon distended with 5 mL of air to seal the end of a polyethylene catheter (internal diameter, 4.8 mm; outer diameter, 7.9 mm) placed in the distal third of the esophagus. The distance between the nares and distal third of the esophagus was visually approximated and was marked on the esophageal catheter. Pressure tracings were monitored to determine the precise position of the catheter as it was inserted. The catheter was placed so that there was maximal variation in P<sub>L</sub> and minimal cardiac artifacts. The length of the catheter that was inserted was recorded for each horse, and the same length was used thereafter. The catheter was connected to a differential pressure transducer that was calibrated by use of a water manometer before and immediately after each experiment. Transpulmonary pressure was defined as the difference between atmospheric and esophageal pressures. Signals from the transducers were amplified and passed through a digital-analog converter to a computer equipped with data acquisition and analysis software. The program provided measures of tidal volume, minute expiratory volume, respiratory rate, expiratory and inspiratory times, and the change in P<sub>L</sub> for each breath. Values of R<sub>L</sub> and E<sub>L</sub> were calculated by applying the data to the multiple regression equation for the single-compartment model of the lung as follows:

\[ P_L = (E_L \times \text{volume}) + (R_L \times \dot{V}) + K \]

where \( \dot{V} \) represents rate of airflow and K is the transpulmonary end-expiratory pressure. The coefficients of determination for the fit of the equation to the data were calculated for each breath. The mean data for each variable during 10 to 15 consecutive breaths were calculated. Results of the lung function of these horses have been reported elsewhere. All horses with RAO, but none of the control horses, had airway obstruction at all 3 sampling times. The horses with RAO had significantly higher minute expiratory volume, expiratory and inspiratory times, and change in P<sub>L</sub> for each breath. There was significant (P = 0.004) variation in E<sub>L</sub> in horses with RAO throughout the 3-month period. Individual changes in P<sub>L</sub> in horses with RAO remained > 15 cm H<sub>2</sub>O, whereas all control horses had changes in P<sub>L</sub> < 15 cm H<sub>2</sub>O during the study.

**BAL procedures**—Bronchoalveolar lavage fluid was collected in February, March, and April from the
left and right lungs of all horses. Horses were sedated with xylazine hydrochloride\(^1\) (0.6 to 1.0 mg/kg, IV) and butorphanol tartrate\(^2\) (20 µg/kg, IV). A fiber-optic flexible endoscope (length, 180 cm; end diameter, 14 mm) was passed through a nasal passage into the trachea and wedged in the distal aspect of the right lung. During passage of the endoscope through the airway, 50 to 100 mL of a solution of 0.5% lidocaine hydrochloride\(^6\) was instilled in small boluses to anesthetize the airway mucosa. A 250-mL bolus of warm (37°C) sterile isotonic saline solution was rapidly instilled in the bronchus and aspirated via the endoscope biopsy channel with a suction pump (vacuum pressure, 50 to 100 mm Hg). This was followed immediately by instillation of a second 250-mL bolus of warm sterile isotonic saline solution followed by aspiration. The fluid recovered after each aspiration was stored separately in siliconized glass vessels and kept on ice until analysis. The procedure then was repeated for the left lung. Analyses of BAL fluid (the first and second aliquots from both lungs) were performed within 2 hours after collection. All BAL procedures were performed between 1 PM and 4 PM.

Total nucleated cells were counted in a BAL fluid sample (dilution, 1:50) by use of a hemacytometer. Films of the BAL fluid were prepared by means of centrifugation\(^7\) (90 \(\times\) g for 10 minutes) and stained with a modified Wright stain.\(^7\) A differential cytologic count was made on samples of at least 400 cells; epithelial cells were not included in the differential cytologic count.

**Data analysis**—Cytologic counts of BAL fluid were analyzed with repeated-measures linear models with lung (left vs right), aliquot (first vs second), and time (February, March, or April) as within-subject factors and group (control vs RAO) as a between-subject factor. Models all included interaction terms between group and the other factors. Tukey post hoc tests were used to compare pairs of means. Cytologic counts of mast cells, eosinophils, epithelial cells, and RBCs were skewed heavily to the right. Therefore, these variables were dichotomized (present vs absent) and a repeated-measures logistic regression with the aforementioned factors was used to analyze the results. Intraclass correlation reliability coefficients were calculated for each BAL measurement by use of restricted maximum likelihood estimation. The coefficient represented the proportion of total variance accounted for by variation between lungs, over time, and between groups in hierarchic models of the data. A value close to 1 indicated high repeatability of results between aliquots. Values of \(P < 0.05\) were considered significant for all analyses.

**Results**

The intraclass correlation reliability coefficient was evaluated for all BAL measurements, and the coefficient indicated good repeatability of data for the volume of BAL fluid recovered and for percentages of neutrophils, macrophages, and lymphocytes (Table 1). In horses with RAO, mast cells were present and there was significantly lower volume of BAL fluid recovered, lower percentages of macrophages and lymphocytes, and higher percentage of neutrophils throughout the study period, compared with results for control horses (Table 2).

Significantly higher percentages of neutrophils and lower percentages of macrophages were detected in the first aliquot, compared with results for the second aliquot, for horses in the control and RAO groups. There were significant differences in the presence of mast cells in both lungs for control horses and horses with RAO. In all control horses, except for 1, the first aliquot in each monthly evaluation contained < 25% neutrophils. However, the second aliquot of all control horses contained < 25% neutrophils at all monthly evaluations.

**Discussion**

Cytologic evaluation of BAL fluid is commonly used for the diagnosis of diseases in the bronchoalveolar space of horses in clinical and research settings. It is assumed that a single sample of BAL fluid is representative of an entire lung. However, although there is good agreement between sequential BAL fluid samples obtained from clinically normal humans,\(^{10}\) variations can be found in repeated samples obtained from humans with diseased lungs.\(^\)\(^3\) In clinically normal horses, consistency of cytologic counts in BAL fluid has varied among studies, with some investigators finding no variation,\(^{2,11,12}\) whereas others finding some degree of variation.\(^{1,5,6,8,9}\)

The present study was conducted to determine the possible contribution of month of sample collection to variations in differential cytologic counts in horses housed in a stable. In addition, we evaluated variations attributable to lavage volume and lung sites in horses with RAO and horses without respiratory tract disease. As expected, horses with RAO had significantly lower volumes of BAL fluid recovered, lower percentages of macrophages and lymphocytes, and significantly higher percentages of neutrophils than did control horses.

There were significantly higher percentages of neutrophils and lower percentages of macrophages in the

<table>
<thead>
<tr>
<th>Variable</th>
<th>Intraclass correlation reliability coefficient</th>
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</thead>
<tbody>
<tr>
<td>Volume of BAL fluid recovered</td>
<td>0.76</td>
</tr>
<tr>
<td>Total cell count</td>
<td>0.49</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.95</td>
</tr>
<tr>
<td>Macrophages</td>
<td>0.85</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.90</td>
</tr>
<tr>
<td>Macrophage-to-lymphocyte ratio</td>
<td>0.48</td>
</tr>
<tr>
<td>Mast cells</td>
<td>0.34</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.51</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>0.34</td>
</tr>
<tr>
<td>RBCs</td>
<td>0.32</td>
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*Bronchoalveolar lavage fluid was collected in February, March, and April from the left and right lungs of each horse. For each BAL fluid collection, fluid for analysis was obtained by use of 2 instillations of 250 mL of isotonic saline (0.9% NaCl) solution (aliquots 1 and 2) at the same bronchial site for a lung. The coefficient represents the proportion of the total variance accounted for by variation between lungs, among months, and between groups in hierarchic models of the data. A value close to 1 indicates high repeatability of the results between aliquots.
In control horses (36.7%), compared with that recovered in horses with RAO, the amount of BAL fluid recovered was significantly greater (Table 2). The volume of BAL fluid recovered ranged from 50% to 60% as previously reported.11 In addition, the volume infused collapses small airways during expiration (possibly exacerbated by the aspiration of BAL fluid), and the net flow of fluid from the pulmonary circulation may contribute to variations in the volume of BAL fluid recovered because fluid movement in the respiratory tract is complex13 and has not been completely elucidated. The variable and lower amount of BAL fluid recovered in horses with RAO suggests that differential cytologic counts rather than absolute cell counts should be used for cytologic analysis of BAL fluid in horses.

Relative neutrophil counts in BAL fluid did not differ significantly throughout the 3-month study period or between lungs in horses in the RAO and control groups. Analysis of the data indicated that despite some individual variations, results of BAL fluid analyses in horses with clinical RAO could be diagnostic of pulmonary neutrophilia during 3 consecutive monthly evaluations. We used the threshold of > 25% neutrophils in the BAL to determine a status of pulmonary neutrophilia, as has been reported in the literature.3 This threshold is able to differentiate horses with RAO from healthy horses, as determined by lung function.14 However, the observed temporal individual variations should be taken into consideration when performing quantitative studies (evaluation of a treatment over time) involving cytologic analysis of BAL fluid in horses with RAO.

We also observed pulmonary neutrophilia, which was not associated with signs of respiratory distress, throughout the 3 consecutive monthly evaluations in 1 control horse. However, the relative neutrophil counts in the BAL fluid of this horse were < 25% in the second aliquot for the 3 consecutive monthly evaluations. Similar results have also been reported11,15,16 by other authors who also observed pulmonary neutrophilia in healthy horses housed in stables. Considering that we detected pulmonary neutrophilia in a control horse for the first aliquot but not for the second aliquot, analysis of the second BAL fluid sample may be more appropriate for use in differentiating between healthy horses and horses with RAO.

We detected significantly more neutrophils and fewer macrophages in the first aliquot than in the second aliquot for horses in the control and RAO groups. There were significant differences between the left and right lungs for the presence of mast cells in horses in the control and RAO groups. Also, mast cells were present significantly more often in lungs of control horses than in lungs of horses with RAO. All horses with RAO, except for 1, had > 25% neutrophils in the BAL fluid. This finding. A decrease in neutrophils in the second aliquot has been reported8,9 in humans with asthma. It has been suggested that the first aliquot does not reach the caudal limit of the small airways and bronchoalveolar area and thus samples represent primarily the bronchial airways. The second aliquot purportedly diffuses farther into the airways to reach the alveolar spaces. We postulate that the second aliquot would be a more representative means of cytologic evaluation of the bronchoalveolar area in horses.

In the study reported here, there was no significant difference between the cell populations in BAL fluid obtained from the right and left lungs, except for the presence of mast cells, which was significantly different between lungs for horses in the control and RAO groups. Despite individual variation of cell populations
in horses in the control and RAO groups, the results are in agreement with those reported for healthy horses\(^1,2\) and horses with RAO.\(^3\) We have no explanation for the mast cell findings, but the difference between lungs does not change the ability to differentiate between horses in the control and RAO groups. We assumed that the cell population of healthy horses and horses with RAO is essentially uniform between both lungs. Analysis of these results suggests that results obtained are unlikely to be influenced by the site of collection, such as when BAL is performed by use of a tube and the exact sample location is not known. We propose that it is appropriate to collect BAL fluid from various sites in the lungs during future studies involving collection of BAL fluid samples in horses.

We concluded that for horses housed in a stable, there was not a significant difference in the percentages for cell populations in BAL fluid between horses without respiratory tract disease and horses with RAO among months nor between the left and right lungs. Despite individual variation, a threshold of > 25% neutrophils in the second aliquot of BAL fluid could be used to successfully differentiate horses with RAO from control horses. However, individual variation should be taken into consideration when performing quantitative studies that involve cytologic analysis of BAL fluid obtained from horses with clinical RAO.

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