Bronchoalveolar lavage fluid in Standardbred racehorses: Influence of unilateral/bilateral profiles and cut-off values on lower airway disease diagnosis

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Abstract

The aim of this study was to determine whether the lung side being sampled would significantly influence bronchoalveolar lavage (BAL) cytological profiles and subsequent diagnosis in Standardbred racehorses. One hundred and thirty-eight French Trotters in active training and racing were included in a prospective observational study. BAL was performed using videodendoscopy in both right and left lungs during summer meetings in 2011 (64 horses) and 2012 (74 horses). Cytological data performed 24 h later from right and left lungs were compared and specifically used to classify horses as affected with exercise-induced pulmonary haemorrhage (EIPH), inflammatory airway disease (IAD), or were ‘controls’. For IAD, cytological definition was based on two different cut off values.

Neutrophil percentages, haemosiderophage percentages and the haemosiderophage/macrophage (H/M) ratios were significantly higher in the right compared to the left lung. Measures of intra-class correlation coefficients revealed a fair agreement between left and right lungs for percentages of mast cells, eosinophils, and for the H/M ratio, and a moderate agreement for neutrophil percentages. Fair to moderate agreements were observed between left and right lungs for the diagnosis of IAD and/or EIPH based on kappa coefficients. When sampling one lung only, the risk of incorrectly classifying a horse as a ‘control’ increased with the use of the restraint cut-off values for IAD. As BAL from one lung is not representative of the other lung in the same horse, both lungs should be sampled for a better assessment of lung cellularity and for a precise diagnosis of lower airway diseases.

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Introduction

Lower respiratory tract diseases such as exercise-induced pulmonary haemorrhage (EIPH) and inflammatory airway disease (IAD) are common causes of poor performance in racehorses, reportedly due to impaired pulmonary gas exchange (McKane et al., 1995; Couetil and Denicola, 1999; Couroucé-Malblanc et al., 2002; Hinchcliff et al., 2005b; Sanchez et al., 2005).

IAD has been defined as a neutrophilic and/or mastocytic and/or eosinophilic mild lower airway inflammation affecting horses of any age, which might exhibit signs of intermittent cough and increased mucoid airway secretions, without evidence of severe respiratory signs at rest (Robinson, 2003; Couetil et al., 2007).

Although endoscopic and cytological evaluations of tracheal secretions have been commonly used in the diagnosis of IAD (Martin et al., 1999; Newton and Wood, 2002; Sanchez et al., 2005; Wood et al., 2005; Durando et al., 2006), a lack of agreement between tracheal and bronchoalveolar (BAL) cell populations (Derksen et al., 1989; Malikides et al., 2003) has led to BAL fluid (BALF) cytology or pulmonary function testing being recommended as the only accurate diagnostic methods in horses (Couetil et al., 2007).

EIPH is another common disease of racehorses resulting from stress failure of pulmonary capillaries occurring during maximal or sub-maximal exercise (Birks et al., 1997). Firstly described by the presence of post-exertional epistaxis in severe cases (Cook, 1974), different diagnostic methods for EIPH have been used. These include post-exercise tracheobronchoscopy evaluation of blood (Pascoe et al., 1981; MacNamara et al., 1990; Lapointe et al., 1994; Birks et al., 2002; Hinchcliff et al., 2005a; Costa et al., 2006).
Horses were not racing within 10 days following the sampling were sedated with IV romifidine, 0.04 mg/kg (Sedivet, Boehringer Ingelheim). For other horses racing within 10 days BAL was achieved with a nose twitch and without sedation because of drug testing considerations.

BAL was performed using a flexible 3.2 m long, 12.8 mm tip diameter videolaryngoscope (Optomed). The endoscope was first introduced without sedation in the ventral meatus of the left nostril to assess laryngeal function according to the Havermeyer grading system (Dixon et al., 2003). Pharyngitis was also scored using a grade 1–4 scale (Raker and Boles, 1978). If sedation was necessary and feasible based on above considerations, the endoscope was then removed and romifidine was injected IV. Following sedation, the videolaryngoscope was reintroduced through the left nostril, via the pharynx into the trachea, and randomly directed into the left or right main stem bronchus until wedged in a distal bronchus. When sedation was not used, this procedure was conducted immediately following laryngeal assessment. Tracheal mucous accumulation (grade 1–5 scale) and tracheal septum thickness (grade 1–2 scale) were also recorded (Gerber et al., 2004; Koch et al., 2007). A total of 250 mL of sterile isotonic saline solution was instilled into the bronchus via the endoscope biopsy channel, which was previously pre-filled with 20 mL of saline. A first 125 mL bolus was instilled, using two 60 mL pre-filled syringes. Immediately after instillation of the second syringe, aspiration was manually performed with the same syringe used for injection. The first 20 mL of aspirated liquid, corresponding to the volume of the endoscope biopsy channel that did not reach the lung, was discarded. The residual liquid was then collected in one or two syringes, depending on the volume harvested. The second 125 mL bolus of isotonic saline was similarly injected and collected. At the end of lavage in the first lung side, the endoscope instrument channel was cleaned with a 30 mL bolus of isotonic saline. Then, the endoscope was moved back to the carina and introduced in the contralateral lung and lavage procedure was repeated. The volume of liquid collected and macroscopic assessment (colour, turbidity, and presence of foam) were recorded, and syringes were pooled in a metallic bowl for each lung side. A sample was taken from both pools and kept into EDTA tubes.

BAL fluid analysis

BAL samples were preserved in EDTA and kept at room temperature. The volume recovered was sufficient for analysis of all 276 samples (138 horses > 2), which were then investigated. At reception in the laboratory, within 24 h of collection, 200 µL of fluid were immediately cytocentrifuged (80 g, 10 min) (Shandon CytoSpin, Thermo Scientific) and stained with May–Gruenwald–Giemsa (MGG). Left and right samples were then processed simultaneously for each horse. Differential cell count was performed on 300 cells, and the number of each cell type was expressed as a percentage of total nucleated cells. Epithelial cells were not included in the differential count.

Case definition

Horses were considered as ‘controls’ according to the formal definition of inflammatory airway disease (IAD) when BAL cytological profiles were ≤5% neutrophils, ≤2% mast cells and ≤1% eosinophils (Robinson, 2003; Couetil et al., 2007). Horses with values above the cut-off for any of these three cell types in at least one lung were considered to have evidence of IAD. An alternative definition of ‘control’ horses, based on higher cut-off values of neutrophils (>10%), mast cells (>5%) and eosinophils (>5%) was also used in this study (Hare and Viel, 1998; Hughes et al., 2003; Richard et al., 2010a; Beekman et al., 2011; Kohlinger et al., 2011; Wasko et al., 2011).

The ratio of BAL haemosiderophagelboutique (H/M) ≥ 20% was considered as evidence of previous episode of EIPH (Richard et al., 2010a,b).

Statistical analysis

Normality of continuous data distribution was evaluated using the Shapiro–Wilk W test. The different variables were not normally distributed with data presented as median, 1st–3rd quartile, unless stated otherwise. Because of the large number of horses, data were log 10 transformed to normalise distribution. Multiple comparisons were performed using one-way ANOVA with Tukey post hoc test, and independent Student’s t test. A paired t test was used when comparing left to right lungs. Associations between cell percentages from both lungs were evaluated using Pearson’s correlation coefficient and linear regression analysis (equation: \( y = a \times x + b \), respectively. For this, 95% confidence interval (CI) of the slope (a) and intercept (b) of the regression line should include the ideal regression (\( y = 1 \times x + 0 \)), if the investigated parameters are equivalent. Measures of agreement for numerical and categorical (`control’, ‘IAD’, ‘EIPH’) variables were evaluated using the intra-class correlation coefficient (ICC) and the Cohen’s kappa coefficient (\( \kappa \)), respectively. Both

Materials and methods

Horses

One hundred and thirty-eight French Trotter horses (76 geldings, 58 females and 4 males), aged 3–9 years (mean 4.7 ± 1.6 years old), were included in the study. Horses came from 11 different training stables, and were sampled during summer race meetings in 2011 (64 horses) and 2012 (74 horses). They were all involved in active training and had raced within 1 month of sampling.

Prior to any procedure, each horse was submitted to a thorough clinical examination in order to ensure that no obvious clinical abnormality was present. Venous blood samples were collected in the morning between 0600 and 0730 h and before feeding, for a complete haematological and biochemical assessment to rule out any systemic disease.

The study was approved by the regional Animal Ethic Committee (CEEA,2012.179) and all owners signed a consent form.
coefficients indicated high repeatability of results between lungs for cell populations if the value was close to 1. Values of $P < 0.05$ were considered significant for all analyses.

Results

All horses were considered as healthy based on clinical examination. Twenty horses presented a pharyngeal grade $\geq 3/4$. Ten horses presented a grade of laryngeal hemiplegia of $3/4$ at rest, while none exhibited a grade 4/4. Six horses presented a tracheal mucus score $\geq 3/5$. The BAL endpoints (turbidity and colour) and recovery percentage of BALF depending on cytological profiles are represented in Supplementary Table 1. The volume of fluid recovered was not significantly different between right (median, 110.5; 1st quartile, 100.0 – 3rd quartile, 124.8 mL) and left lungs (112.0, 100.0–128.0 mL) lung, while no significant correlation was found between lungs ($R = 0.06$; CI $-0.11$ to 0.22). No disease-, age- or gender-related differences were observed for BAL recovery volume (Supplementary Table 2).

Total cell counts (220, 150–350 and 200, 140–300 cells/mm$^3$, respectively) were not significantly different amongst lungs. Associations between total cell counts and cytological profiles are represented in Supplementary Table 1. No significant correlation was found between BALF recovery volume and cellularity in right and left lungs ($R = 0.24$; CI 0.06–0.39, and $R = 0.23$; CI 0.06–0.39, respectively). Total cell count was also not influenced by either age or gender (Supplementary Table 2).

The mean neutrophil percentage was significantly higher in BALF of the right lung, compared to the left lung (Fig. 1a; Table 1). Neutrophil proportions were higher in the right compared to the left lung in 81 horses (58.7%), and higher in the left compared to the right lung in 50 horses (36.2%). Mean haemosiderophage percentages were significantly higher in BALF of the right lung, compared to the left lung (Fig. 1a; Table 1), as was the H/M ratio (7.0, 0.0–24.25% in the right lung, and 4.0, 0.0–17.0% in the left lung). Haemosiderophage proportions were higher in the right compared to the left lung in 66 horses (47.8%), and higher in the left compared to the right lung in 36 horses (26.1%).

Differential cell counts for the other cell types were not significantly different amongst lungs (Fig. 1b; Table 1). Contralateral differences for neutrophil percentages, haemosiderophage percentages, and H/M ratio were not significantly influenced by contralateral differences in the volume being retrieved (Supplementary Table 3). Tracheal mucus score was not significantly different among groups (IAD, EIPH and controls; Supplementary Table 4). Tracheal mucus was not significantly correlated with BALF neutrophil percentage in either right ($R = 0.15$; CI $-0.03$–0.31) or left lung ($R = 0.14$; CI $-0.03$–0.31). Cytological profiles (IAD, EIPH and Controls) were also not significantly influenced by age (Supplementary Table 5).

Significant correlations ($P < 0.001$) between lungs were found for percentages of neutrophils ($R = 0.84$; CI 0.78–0.88), mast cells ($R = 0.60$; CI 0.48–0.70) and eosinophils ($R = 0.40$; CI 0.25–0.54), as well as for H/M ratio ($R = 0.57$; CI 0.44–0.67). When considering linear regression between cell percentages from left and right lung BALF, 95% CI of the slope and the intercept did not respectively include the value 1 and 0, for any cell type (Table 2). Based on the measures of intra-class correlation coefficient (ICC) (Table 3, Supplementary Fig. 1), moderate agreement was found for neutrophil percentages in BALF harvested from left and right lungs, while a fair agreement was found for percentages of mast cells, eosinophils, and H/M ratio.

Regarding classification of different cytological profiles as being control or IAD (Table 4), fair to moderate agreements were observed according to $\kappa$ coefficients. Based on the formal definition, 129/138 (93.5%) of the horses met the cytological inclusion criterion for IAD: 103 (74.7%) in both lungs, and 26 (18.8%) within one lung only. Nine horses (6.5%) were definitively classified as non-IAD horses ($\kappa = 0.30$; CI 0.09–0.50). Of the 35 horses with non-IAD cytological profiles for at least one of the BAL fluids, 26 were classified as IAD from the other lung (13 at left, 13 at right), and nine horses were ‘real’ controls. Thus, 13/35 (37.1%) IAD affected horses would have incorrectly been classified as ‘controls’ when considering either right or left lung only.

Based on the alternative definition, 78 horses (56.5%) met the cytological inclusion criteria for IAD: 52 horses (37.7%) in both lungs, and 26 (18.8%) within one lung only. Sixty horses (43.5%) were classified as controls ($\kappa = 0.62$; CI 0.49–0.75). From the 86 horses with non-IAD cytological profiles for at least one of the BAL fluids, 26 were classified as IAD from the other lung, whereas 60 horses were definitively ‘controls’. Thus, respectively, 15/86 (17.4%) and 11/86 (12.8%) horses would have incorrectly been classified as controls when considering either right or left lung only.

For EIPH, moderate agreement between left and right lungs was also found ($\kappa = 0.49$; CI 0.32–0.66). Forty-four horses (31.9%) met the cytological inclusion criteria for EIPH diagnosis: 19 horses (13.8%) in both lungs, 25 (18.1%) within one lung only. Ninety-four horses (68.1%) were classified as non-EIPH. Among the 119 horses with non-EIPH cytological profiles for at least one of the BAL fluids, respectively 8 (6.7%) and 17 (14.3%) EIPH affected horses would have incorrectly been classified as controls when considering either right or left lung only.

Discussion

To our knowledge, this is the first study in which BAL has been performed in both lungs on a large population of Standardbred racehorses. Comparison between left and right lungs revealed significant differences for neutrophil percentages, haemosiderophage percentages and H/M ratios, as these cells were more prevalent in the right compared to the left lung. Such lung differences have not been previously described, which may probably be explained by the large population of horses included in the present study. On the other hand, the mast cell population was not significantly different between right and left lungs, unlike reports from previous studies (Sweeney et al., 1992; Jean et al., 2011). However, enumeration of mast cells has recently been demonstrated to be unreliable when the standard 400-cell differential counting method was used (Fernandez et al., 2013), because of the small proportion of these cells in BALF. Reliability of mast cells and eosinophils may thus be improved in further studies by using techniques permitting the evaluation of larger numbers of cells (Fernandez et al., 2013).

It has been reported that a BAL performed blindly with a cuffed tube mostly (even if not systematically) results in the right lung being sampled (Rush and Mair, 2004; Deniau et al., 2010). Similarly, aspiration bronchopneumonia mainly affects the right lung in horses because of the straighter disposition of the right main stem bronchus (Ainsworth and Hacker, 2004). This anatomical feature might also be responsible for a greater exposition of the right lung to allergens and organisms, and could partially explain the higher proportion of BALF neutrophil percentages found in the right lung. There is, to our knowledge, no physiological evidence that pulmonary haemorrhage occurs any differently within the left or right lung.

BAL is accepted as gold standard diagnostic method to detect IAD in horses, since tracheal examination (Couetil et al., 2007) or evaluation of clinical signs (Wascko et al., 2011) are not sensitive enough. A reliable definition of normal BAL cytology is thus crucial in order to accurately classify horses as ‘controls’ or ‘IAD’ in an observational study. When the formal definition of IAD was applied, only 9/138 (6.5%) of the horses in our study were definitively...
classified as controls based on BALF cytological profiles. Furthermore, even if significant correlations were found between the right and left lung, the agreement was only fair to moderate for all cell types based on logistic linear regression and ICC coefficient.

Classification of horses as ‘IAD’ or ‘control’ led to a fair agreement between both lungs, meaning that a high percentage of horses would have been falsely classified as controls when considering one lung only. Interestingly, when the alternative definition was used, the classification disclosed 60 (43.5%) control horses and a slightly better agreement between lungs, compared to those classified by the formal definition. This finding is in accordance with other reports that considered the threshold for normal neutrophil percentage in BAL cytology as being too restrictive (Richard et al., 2010a,b; Koblinger et al., 2011; Wasko et al., 2011).

We also used higher cut-off values for normal mast cell and eosinophil percentages, which appeared to be more relevant for

**Table 1**

Descriptive statistics of the cytological data from both lungs of 138 horses.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Lung side</th>
<th>Median (%)</th>
<th>1st Quartile (%)</th>
<th>3rd Quartile (%)</th>
<th>Minimum (%)</th>
<th>Maximum (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>Right</td>
<td>10</td>
<td>5.0</td>
<td>20.0</td>
<td>1.4</td>
<td>76.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>9.0</td>
<td>5.0</td>
<td>15.0</td>
<td>2.0</td>
<td>69.0</td>
<td></td>
</tr>
<tr>
<td>Mast cells</td>
<td>Right</td>
<td>1.0</td>
<td>0</td>
<td>2.0</td>
<td>0</td>
<td>20.6</td>
<td>0.066</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>1.7</td>
<td>0</td>
<td>2.0</td>
<td>0</td>
<td>42.0</td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td>Right</td>
<td>0</td>
<td>0</td>
<td>2.0</td>
<td>0</td>
<td>35.7</td>
<td>0.162</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>0</td>
<td>0</td>
<td>2.0</td>
<td>0</td>
<td>26.0</td>
<td></td>
</tr>
<tr>
<td>Haemosiderophages</td>
<td>Right</td>
<td>3.0</td>
<td>0</td>
<td>9.0</td>
<td>0</td>
<td>60.0</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>2.0</td>
<td>0</td>
<td>6.0</td>
<td>0</td>
<td>50.0</td>
<td></td>
</tr>
</tbody>
</table>

![Fig. 1](a and b) Differential BAL cell count (median; 1st–3rd quartile) from both lungs expressed as percentages of total nucleated cells (n = 138). R, right; L, left; Neutro, neutrophils; Macro, macrophages; Lymph, lymphocytes; Mast, mast cells; Eos, eosinophils; Haem, haemosiderophages; Giant, giant cells (macrophage lineage). *Significant difference at P < 0.001.
this population of racehorses in training and racing (Hare and Viel, 1998; Beekman et al., 2011; Hughes et al., 2011). This alternative definition allowed us to increase the agreement between right and left lungs, and thus to limit the probability of incorrectly considering an IAD-affected horse as being a control, when based on the cytology from one lung only.

Our results further highlight the importance of an accurate understanding of the statistical analyses, which should always be interpreted with caution in terms of clinical significance. For instance, it might be expected that the presence of 'outliers' with very high neutrophil counts would modify the statistical differences between left and right lungs. However, these extreme values did not significantly influence the overall outcome of the analysis.

Furthermore, correlations of cytological profiles between left and right lungs appear acceptable, but linear regression confirms that both lungs may not be considered as equivalent. Some limitations of the statistical analyses may also be related to the multiple possible definitions of a syndrome mainly diagnosed based on three different cell types. Indeed, the $\kappa$ coefficient evaluates the reliability between left and right lungs only for the final diagnosis of IAD. This coefficient might however overestimate the agreement between lungs, since it does not take into account the different sub-types of IAD. Horses with IAD may then exhibit increased proportions of one cell type (i.e. neutrophils) in the left lung, and increased proportions of another cell type (i.e. mast cells) in the right lung.

Prevalence of EIPH mostly depends on the method used for diagnosis. Criteria for classification of EIPH based on haemosiderophage detection differs among other studies: several authors considered the sole presence of haemosiderophages as being evidence of prior haemorrhage (Fogarty and Buckley, 1991; McKane et al., 1993; Ferrucci et al., 2009; Michelotto et al., 2011). In the present study, the cut off value of 20% was used for the H/M ratio, as previously suggested (Richard et al., 2010a,b). Haemosiderophage percentages and the H/M ratio were significantly higher in the right compared to the left lung, and moderate agreement only was found between right and left lungs when diagnosing EIPH. As for IAD, a non-negligible proportion of horses would have been incorrectly classified as control with the evaluation of one lung only, especially when the left lung was sampled.

BAL is often considered to be an invasive method requiring sedation. According to some authors, this can hinder its routine clinical use (Cardwell et al., 2011; Derksen et al., 2011) or limits its use to a small number of horses (Wasko et al., 2011). In the present study, considerations relating to drug testing and proximity to racing prevented administration of sedation in many horses. Also, for doping considerations, local anaesthesia was not used to desensitise the lower airways when passing the endoscope. A previous report concluded that administration of lidocaine reduced the severity of coughing but did not modify either volume or composition of BALF when compared to those animals in which lidocaine was not used (Westermann et al., 2005).

To our knowledge, there is no study comparing cytological characteristics when BAL has been performed with or without the use of alpha2 agonists. In the present study, several (but not all) horses did cough moderately when the endoscope was passed either in the trachea or the bronchi. No cough was further noticed during fluid instillation and aspiration.

It has also been reported that the time leading to a significant reduction in total nucleated cell count and cell viability of unfixed BALF samples decreased as the storage temperature increased (Pickles et al., 2002b). Samples were kept in EDTA-tubes in the present study, then despatched at ambient temperature to the laboratory, with left and right samples of each horse being simultaneously stained within 24 h. One of the limitations might then be the timing between sampling and processing. However, a recent study demonstrated that there was no significant difference in total and differential cell counts when cytological evaluation was performed either immediately or after a 24 h period, whatever the conservation temperature (refrigerated or ambient temperature) (Deniau et al., 2010).

Interpretation of BALF cytological results may vary depending on the volume of liquid infused and conservation conditions of the samples (Sweeney et al., 1992; McGorum et al., 1993). In the present study, a total volume of 250 mL of isotonic saline was used. This volume represents the minimum recommended by the International Workshop on Equine Chronic Airway Disease (Robinson, 2001) within the lungs.
The volume of fluid retrieved was not significantly different between lungs and did not influence BAL cellularity. Also, the volume recovered was not related to the cytological profile of disease (EIPH, IAD). Bronchoconstriction associated with RAO is thought to be responsible for a reduced BAL recovery volume (Sweeney et al., 1992; Jean et al., 2011). Even if some degree of bronchoconstriction has previously been demonstrated in IAD affected horses (Richard et al., 2009) this seems not to have been sufficiently marked to be associated with reduced BAL recovery volume in our study. Several horses exhibited very high values of neutrophil percentages in BALF. These horses were unlikely to be affected with RAO since none of them presented increased respiratory effort at rest. However, neither a respiratory function test nor BAL cytology from the firstly sampled lung reveals a normal profile. Furthermore, the risk of incorrectly considering a horse as a ‘control’ when cytology from the firstly sampled lung reveals a normal profile. Therefore, the presence of tracheal mucus and BALF cytology (Richard et al., 2010b). The lack of influence of age on BAL cytology has also been shown in previous studies (Gerber et al., 2003; Mallikides et al., 2003; Koblinger et al., 2011; Sad et al., 2013).

Conclusions

Our results demonstrate that BAL performed on one lung may not be representative of the other lung in the same horse. Sampling one lung only is sufficient for diagnosing IAD and/or EIPH when BAL cytological analysis reveals increased cell populations consistent with the disease. Sampling the opposite lung is however necessary to definitively classify the horse as being a ‘control’ when cytology from the firstly sampled lung reveals a normal profile. Furthermore, the risk of incorrectly considering a horse as a ‘control’ is increased when using restrictive cut-off values for cell populations. Both lung sides should therefore be sampled for (1) a better assessment of lung cellularity and (2) a more precise diagnosis of lower airway disease.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tvjl.2013.10.013.

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