



# The use of tracheal wash and bronchoalveolar lavage in the clinical examination of coughing horses<sup>1</sup>

*O uso de lavado traqueal e broncoalveolar no exame clínico de cavalos com tosse*

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## Abstract

Our hypothesis is that coughing horses have airway inflammation. Fifty horses – 36 coughing (Co Group) and 14 control (C Group) – of different gender, age, and function were evaluated. Physical and endoscopic examinations, and cytological analyses of tracheal wash (TW) and bronchoalveolar lavage (BAL) fluids were undertaken. Higher grades of tracheal mucus were observed in coughing horses. TW fluid neutrophil count was higher ( $46.4\% \pm 30.8\%$  vs.  $19.5\% \pm 22.9\%$ ,  $p=0.003$ ) in the Co group than the C group. In BAL fluid, neutrophil count was higher in the Co group than in the C group ( $30.3\% \pm 27.3\%$  vs.  $5.0\% \pm 4.2\%$ ,  $p=0.001$ , respectively). BAL fluid cytological profile compatible with inflammatory airway disease (IAD), recurrent airway obstruction (RAO), and normal were evidenced in 14 (38.9%), 17 (47.2%), and 5 (13.9%) of the evaluated coughing horses. IAD was diagnosed in 6 horses of the C group. Cough is a clinical sign of airway inflammation in horses.

**Keywords:** Airway inflammation. Endoscopy. Equine. Macrophages. Neutrophils.

## Resumo

*A hipótese do presente estudo é que cavalos com tosse apresentam inflamação das vias aéreas. Foram avaliados 50 cavalos – 36 com tosse (Grupo T) e 14 controle (Grupo C) – de diferentes idades, sexo e função. Procedeu-se aos exames físico e endoscópico, bem como análise citológica dos fluidos dos lavados traqueal (LT)*

<sup>1</sup> Horses were evaluated in training facilities and at the Jockey Club of Paraná racetrack in the city of Curitiba in southern Brazil. The tracheal wash and bronchoalveolar lavage fluids were transported and analysed at the laboratory of the Equine Clinic of the Veterinary Hospital of the Pontifical Catholic University of Paraná (PUCPR). Preliminary results were presented at the meeting of the Brazilian Association of Equine Practitioners (2011, 2013).

e broncoalveolar (LBA). Observou-se escores de muco traqueal mais elevados nos cavalos com tosse. O número percentual de neutrófilos no LT foi maior no grupo T em relação ao C ( $46,4\% \pm 30,8\%$  vs.  $19,5\% \pm 22,9\%$ ,  $p=0,003$ ). No LBA, a contagem percentual de neutrófilos foi maior no grupo T em relação ao grupo C ( $30,3\% \pm 27,3\%$  vs.  $5,0\% \pm 4,2\%$ ,  $p=0,001$ , respectivamente). No fluido do LBA, perfil citológico compatível com doença inflamatória das vias aéreas (DIVA), obstrução recorrente das vias aéreas (ORVA), e normal foi evidenciado em 14 (38,9%), 17 (47,2%), e 5 (13,9%) dos cavalos do grupo T. Em seis cavalos do grupo C se evidenciou citologia de LBA compatível com DIVA. Conclui-se que tosse é um sinal clínico de inflamação das vias aéreas dos cavalos.

**Palavras-chave:** Endoscopia. Equino. Inflamação das vias aéreas. Macrófagos. Neutrófilos.

## Introduction

Cough is a physiologic non-immunologic mechanism for airway cleaning and protection. However, it is also responsible for considerable impairment of quality of life in humans (Yousaf et al., 2013).

In healthy horses, cough is rare or absent (Robinson, 2007). Therefore, the presence of cough has been associated with airway inflammation. Considering upper airway inflammation, cough was associated with greater scores of pharyngeal lymphoid hyperplasia in Thoroughbred (TB) racehorses (Christley et al., 2001), although this finding was not supported by another investigation (Koblinger et al., 2011). However, the association between cough and lower airway inflammation has been confirmed. Cough was associated with lower airway disease for more than one month (Burrell et al., 1996), with age of >7 years and >5% bronchoalveolar lavage (BAL) fluid neutrophil count (Bedenice et al., 2008), with high neutrophil percentage and increased prevalence of intracellular bacteria in tracheal mucus in coughing TB racehorses (Christley et al., 2001), and with oxidative stress and increased activity of platelet-activating factor in BAL fluid of young TB racehorses during race training (Michelotto et al., 2010).

BAL fluid analysis is the preferred cytological evaluation for the diagnosis of inflammatory airway disease (IAD) and recurrent airway obstruction (RAO) (Robinson 2001; Couëtil et al., 2007). Although tracheal wash (TW) is controversial for the investigation of IAD and RAO because of its poor correlation with BAL, it is widely used for the diagnosis of airway inflammation because it is easily performed during endoscopic examination and gives information from different segments of the lower airways. Consequently, for investigation of complex

cases such as coughing horses, TW and BAL should be used in combination with assessment of airway health (Hodgson and Hodgson, 2003).

However, investigation of different airway levels of coughing horses using TW and BAL, as well as the correlation among the findings of physical examination, endoscopy, and TW and BAL fluid cytology, has not been demonstrated. Therefore, the hypothesis of the present study was that coughing horses have an inflammatory process in the lower airways, and that physical examination, endoscopy, and cytological analyses both of TW and BAL fluids are effective in definitive diagnosis.

## Materials and methods

### Subjects

The study population comprised 50 horses, aged  $10.7 \pm 3.9$  years (2–16 years) and weighing 410–500 kg, and included Brazilian jumping horses, Lusitanians, Crioulos, Quarter horses, Thoroughbreds, and mixed breeds of both genders. Horses with a history of recurrent or persistent cough without fever were included in the coughing group (Co group;  $n = 36$ ), and 14 horses without history of cough or other sign of airway disease and with normal activity levels were equally evaluated for comparison and included in the control group (C group;  $n = 14$ ). All the evaluated horses were regularly dewormed and were vaccinated for EHV-1, EHV-4, and equine influenza.

This study was approved by the Committee on Animal Experimentation of the Pontifícia Universidade Católica do Paraná (PUCPR), Curitiba, Brazil (registered as number 542), and was in

accordance with the Guiding Principles in the Care and Use of Animals (APS, 2015).

### Physical examination

Physical examination included evaluation of body temperature, nasal discharge, lymph nodes, and respiration, together with cardiac, tracheal, and pulmonary auscultation. The upper limit for respiratory rate was considered 20 breaths/min (Bedenice et al., 2008).

### Bronchoscopic examination and tracheal wash collection and processing

Horses were sedated with acepromazine 0.03 mg/kg IM (Acepran 1%; Univet Laboratory, São Paulo, Brazil) followed by xylazine 0.3 mg/kg IV (Sedazine; Fort Dodge Laboratory, São Paulo, Brazil) 30 min later. Bronchoscopic examination was performed using a flexible fiberscope (Olympus CF-140-L; Olympus, Lake Success, NY, USA) introduced through one nare and advanced to the tracheal bifurcation. For the purpose of the present study, pharyngeal lymphoid hyperplasia (PLH) was graded from I to IV (Raker and Bowles, 1978), and tracheal mucus was graded from 0 to 5 (Gerber et al., 2004). For TW, the fiberscope was positioned proximal to the carina, a polyethylene tube was passed through the working channel of the equipment, and 30 mL sterile 0.9% sodium chloride (saline) solution was instilled and immediately aspirated (Mansmann and Knight, 1972). The recovered TW fluid was centrifuged (Centrifuge 5810 R; Eppendorf, Bloomington, IN, USA) at 340 g for 6 min at room temperature. Ten microliters of the cell pellet were used for the preparation of cytological smears. Slides were stained with Romanowski stain (Panótico, Laborclin, Pinhais, Brazil), and 300 cells were counted at 1000× magnification.

Normal was considered <10% lymphocytes, <1% eosinophils, and <20% neutrophils (Malikides et al., 2003).

### Bronchoalveolar lavage fluid collection and processing

BAL fluid collection was performed after bronchoscopy and TW. An equine BAL catheter

(V-PBAL-300; Cook Vet Products, Hamburg, Germany) was blindly introduced through a nare with concomitant instillation of a solution containing lidocaine hydrochloride 0.5% (Xylestesin; Cristália Laboratory, Itapira, SP, Brazil) (without epinephrine) for patient comfort. When the catheter had lodged in a bronchus, the cuff was inflated with 10 mL air, and 300 mL prewarmed (37.0 °C) sterile saline solution was instilled and immediately aspirated. BAL fluid was transferred to plastic bottles, maintained on ice until arrival at the laboratory, and processed in less than 1 h. The recovered BAL fluid was centrifuged at 340 g for 6 min at room temperature. The cell pellet was resuspended in 1 mL saline for the performance of total cell count in a Neubauer chamber. Ten microliters of the cell pellet were used for the preparation of cytological smears. Slides were stained with Romanowski stain, and 300 cells were counted at 1000× magnification.

Normal was considered  $\pm 60\%$  macrophages,  $\pm 35\%$  lymphocytes, <5% neutrophils, <1% eosinophils, and <2% mast cells (Michelotto et al., 2013). Considering the diagnosis based on the BAL fluid differential cytological examination, the 50 evaluated horses were categorised as normal ( $\leq 5\%$  neutrophils), IAD ( $\leq 20\%$  neutrophils), and RAO ( $> 20\%$  neutrophils) (Couëtil et al., 2007).

### Statistical analysis

Normality of data distribution was evaluated by the D'Agostino and Pearson test. Comparisons between groups were performed using the Mann-Whitney test and Spearman test was used for correlations, using the software GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) and presented as mean  $\pm$  SD.  $p < 0.05$  was considered significant.

### Results

Mean age of the horses was  $10.9 \pm 3.9$  and  $9.9 \pm 3.9$  years for the Co and C groups, respectively. Most of the horses were housed in conventional stables with wood shavings as bedding, although five horses in the Co group were maintained under field conditions.

Fourteen (38.9%) horses in the Co group displayed a respiratory rate of >20 breaths/min, and mean respiratory rate was higher in the Co than C group ( $21.1 \pm 6.3$  vs.  $15.6 \pm 3.4$  breaths/min,  $p=0.030$ ). Moreover, crackles or wheezes were auscultated in 12 (33.3%) horses in the Co and one (7.1%) horse in the C group.

Upper airway bronchoscopic evaluation was considered normal in both groups, as mean grades of PLH were 1.4 and 1.5 for C and Co groups. Grade 3 PLH was observed in four of the evaluated horses of the Co and one of the C groups, all of them were 2-year-old TB. Additionally, tracheal mucus score did not differ between the Co ( $2.8 \pm 1.3$ ) and C ( $2.0 \pm 1.0$ ) groups ( $p=0.057$ ), although higher mucus scores of 4 and 5 were seen only in horses of the Co group.

In the TW analysis, mean neutrophil percentage was significantly higher in the Co than C group

( $46.4\% \pm 30.8\%$  vs.  $19.5\% \pm 22.9\%$ ,  $p=0.003$ ) (Table 1). Considering the adopted upper limits for neutrophil and eosinophil counts in equine TW fluid, 61.1% (22/36) horses in the Co group had TW results compatible with airway inflammation; 81.8% (18/22) of these displayed neutrophilia, 9.1% (2/22) eosinophilia, 9.1% (2/22) both neutrophilia and eosinophilia. Neutrophilia was observed in 28.6% (4/14) horses in the C group. There was a positive correlation between a neutrophil count of >20% in TW fluid and a tracheal mucus score of  $\geq 2$  ( $p=0.030$ ,  $r = 0.3419$ ) or  $\geq 3$  ( $p=0.013$ ,  $r = 0.3850$ ).

Total nucleated cell count of the BAL fluid was similar in both groups (Table 1). Differential cytologic evaluation of the BAL fluid showed lower mean lymphocyte percentage and higher neutrophil percentage in the Co than C group ( $25.8\% \pm 17.3\%$

**Table 1** - Tracheal wash (TW) and bronchoalveolar lavage (BAL) fluid cytology of coughing (Co, n = 36) and control (C, n = 14) horses

	TNCC (cells/ $\mu$ L of BALF)	Macroph %	Lympho %	Neutro %	Eosino %	Mast Cells %	Epithelial Cells %
C group	NA	34.5	13.5	19.5	0.5	0.01	31.8
(TW)		$\pm 14.5$	$\pm 4.8$	$\pm 23.0$	$\pm 0.9$	$\pm 0.1$	$\pm 25.0$
Co group	NA	26.2	10.9	46.4	4.2	0.2	11.8
(TW)		$\pm 16.8$	$\pm 5.6$	$\pm 30.8^x$	$\pm 13.2$	$\pm 0.4$	$\pm 16.1^y$
C group	82.7	44.9	48.8	5.0	0.3	0.1	NT
(BAL)	$\pm 78.1$	$\pm 14.4$	$\pm 14.7$	$\pm 4.2$	$\pm 0.5$	$\pm 0.3$	
Co group	93.0	40.2	25.8	30.3	2.9	0.1	NT
(BAL)	$\pm 107.4$	$\pm 17.8$	$\pm 17.3^z$	$\pm 27.3^w$	$\pm 7.4$	$\pm 0.3$	

Legend: TNCC, total nucleated cell count; Macroph, macrophages; Lympho, lymphocytes; Neutro, neutrophils; Eosino, eosinophils; NT, not tested.

Note: Results are expressed as mean  $\pm$  SD;  $^x$ ,  $^y$ ,  $p=0.003$  vs. C group TW;  $^z$ ,  $^w$ ,  $p=0.004$  and  $^w$ ,  $^p=0.001$  vs. C group BAL.

vs.  $48.8\% \pm 14.7\%$ ,  $p=0.004$ , and  $30.3\% \pm 27.3\%$  vs.  $5.0\% \pm 4.2\%$ ,  $p=0.001$ , respectively) (Table 1).

Considering normal values of equine BAL fluid as  $<1\%$  eosinophils and  $<5\%$  neutrophils, there were  $86.1\%$  (31/36) horses in the Co group with pulmonary inflammation; from these, neutrophilia was observed in  $64.5\%$  (20/31), eosinophilia in  $9.7\%$  (3/31), and both in  $25.8\%$  (8/31). The remaining five horses in the Co group had BAL fluid cell counts within normal limits. In the C group, an increase in mean neutrophil or eosinophil percentile count was found in  $42.9\%$  (6/14) horses. Additionally, three horses (two in the Co group and one in the C group) showed TW fluid neutrophil counts of  $21.0\%$ ,  $22.3\%$ , and  $23.7\%$  with BAL fluid neutrophil counts of  $<5\%$ .

In the Co group, diagnoses compatible with IAD, RAO, and normal were ascertained in  $38.9\%$  (14/36),  $47.2\%$  (17/36), and  $13.9\%$  (5/36) horses, respectively. Furthermore, the six horses in the C group that showed abnormal BAL fluid analyses were diagnosed with IAD.

Considering the diagnoses in the 50 evaluated horses, concurrence between the TW and BAL diagnoses was observed in  $66.0\%$  (33/50) horses. Of the 26 horses (22 in the Co group and four in the C group) that showed an inflammatory profile on TW fluid analysis,  $88.5\%$  (23/26) displayed IAD or RAO on the BAL fluid evaluation. Moreover, of 15 horses with normal TW fluid differential cytology,  $60.0\%$  (9/15),  $26.7\%$  (4/15), and  $13.3\%$  (2/15) were diagnosed as normal, IAD, and RAO, respectively, on BAL fluid evaluation. Furthermore, TW fluid neutrophilia ( $>20\%$ ) was not correlated with BAL fluid neutrophilia of  $>5\%$  ( $p=0.06$ ).

Considering the nine horses (eight in the Co group and one in the C group) that showed an eosinophil count in the BAL fluid of  $>1\%$  ( $1.3\%$ – $30.6\%$ ), three showed eosinophilia in both BAL ( $22.0\%$ ,  $26.0\%$ , and  $30.6\%$ ) and TW ( $24.0\%$ ,  $59.0\%$ , and  $39.0\%$ , respectively) fluids, while the remaining six horses (with a BAL fluid eosinophil count of  $1.3\%$ – $5.7\%$ ) had normal TW fluid cytologic results.

## Discussion

The present study aimed to clarify clinical and clinicopathologic findings in the coughing horse,

and the importance of clinical evaluation using both cytological techniques of TW and BAL.

Although cough is a frequently observed clinical sign, it might not be perceived as an early indication of airway allergy in horses of all ages. By comparing two groups of horses, with and without cough, with similar mean ages, we demonstrated that the presence of cough was associated with an inflammatory process in the airways, with detection in both TW and BAL in most of the cases. Nonseptic airway inflammation is common in athletic horses; therefore, investigation requires a complete and detailed protocol as well as consideration of other causes of cough, such as cardiac conditions.

Most of the evaluated horses in the present study were maintained under conventional stable management with exposure to environmental allergens, as previously demonstrated in horses in similar conditions (Woods et al., 1993; Clements and Pirie, 2007; Berndt et al., 2010). The association between cough, airway inflammation, and stable management practices favouring inhalation of allergens has been described previously (Burrell et al., 1996; Berndt et al., 2010). Jackson et al. (2000) reported that clinical condition and pulmonary function of horses with RAO improved significantly after 30 days of being turned out to pasture, and Clements and Pirie (2007) showed that respirable dust concentration was reduced by soaking hay. On the other hand, horses living in pasture conditions can also display airway inflammation (Robinson et al., 2006). In fact, five of the horses in our study maintained on pastures displayed cough together with airway inflammation, which could have resulted from climatic conditions (Robinson et al., 2006) or inhalation of allergens present in the pasture. These five horses could have been withdrawn from the study because they resided in a different environment from the other horses, but our focus was on cough, and it is important to alert clinicians that it may be a sign of airway inflammation even if the horse is maintained in good environmental conditions.

The involvement of microorganisms in the aetiology of cough was not investigated in the present study, even though cough together with increased prevalence of neutrophilia and intracellular bacteria in TW fluid has been described in TB racehorses (Christley et al., 2001). Nevertheless, none of the evaluated horses displayed clinical signs of airway



infection observed as enlarged lymph nodes, coloured nasal discharge, or fever.

Methodically performed physical examination yields normal or inconclusive results in most cases, as demonstrated for IAD horses (Lessa et al., 2011). In the present study, 38.9% of the evaluated horses had increased respiratory rate considering >20 breaths/min, and 33.3% abnormal lung sounds. Obvious clinical signs at rest might be expected in RAO horses, but thoracic auscultation might not reveal abnormalities in cases of IAD (Couëtil et al., 2007; Bedenice et al., 2008). Therefore, ancillary diagnostic techniques are important in a thorough clinical examination of horses with cough.

Airway endoscopy for evaluation of upper and lower airways is common practice in equine examination and the first step to investigate a horse suspected of airway inflammation after the clinical examination. Concerning the upper airway, PLH seemed to not influence cough in the evaluated horses because the mean age of the horses of both groups was approximately 10 years. PLH declines to zero in horses of 6 years and older (Hobo et al., 1995), corroborating with the observation of five grade 3 horses in the present study, three of which were 2-year-old TB.

However, endoscopic observation of increased quantity of tracheal mucus is considered a hallmark sign of airway inflammation (Sweeney et al., 1992). Increased grades of tracheal mucus were associated with impaired performance in racehorses (Holcombe et al., 2006), as well as reduced exercise capacity in performance horses (Widmer et al., 2009). Interestingly, in the present study coughing and non-coughing horses displayed increased quantities of mucus in the trachea, and the mean grade of mucus was similar in both groups. Probably, mean age, stable environment, and dietary hay influenced this observation in the study horses as they represent risk factors for increased tracheal mucus (Couëtil et al., 2007; Widmer et al., 2009).

Moreover, airway inflammation in coughing horses was confirmed through TW fluid cytology, a first step in cytological diagnosis easily performed during endoscopic evaluation, which gives information from different regions of distal airways (Malikides et al., 2003). The Co group showed remarkable neutrophilia on TW fluid analysis, while the C group showed a neutrophil count of <20%, considered the upper limit of normal. Although cough was considered

a nonsensitive measure of lower airway disease (Burrell et al., 1996), in the present study, grades 4 and 5 tracheal mucus were observed only in the Co group, and increased amount of tracheal mucus was positively associated with increased neutrophil percentage in the TW fluid in this group, concurring with the study by Christley et al. (2001) that evaluated coughing TB racehorses. However, TW fluid analysis is not usually considered in the diagnosis of IAD and RAO, because an absolute correlation has not been observed between BAL and TW fluid cytology results and so far no conclusive data have been found relating TW fluid analysis with performance in horses (Robinson, 2001; Couëtil et al., 2007). A correlation between increased percentage of neutrophils in TW fluid and decreased spirometric indices of pulmonary function has been reported in horses with poor racing performance (Evans et al., 2011), demonstrating that TW fluid neutrophilia might be initial evidence of pulmonary inflammation in coughing horses. Here, most of the horses with an inflammatory TW fluid profile were confirmed as IAD or RAO on BAL fluid evaluation, evidencing a role for TW cytology in airway investigation of horses. However, four horses in the C group also showed TW fluid neutrophilia. Previously, in the evaluation of 66 TB racehorses, 27% showed more than 20% neutrophils on tracheobronchial cytology; in addition, >20% neutrophils were found in the TW fluid of more than 70% of evaluated pleasure horses in Michigan (Robinson et al., 2006). Indeed, one previous study by our group evaluating 27 Quarter horses after a three-barrel racing competition revealed a mean relative neutrophil count of 23.1% on TW fluid analysis (Michelotto et al., 2007). Considering these findings, as well as the fact that three horses in the present study showed 21.0%, 22.3%, and 23.7% neutrophils in TW fluid together with <5% neutrophils in BAL fluid, we suggest that the upper limit for relative neutrophil count in the TW fluid of normal horses be considered at least 24%, although its validity must be investigated in further studies.

BAL fluid differential cytology confirmed the presence of pulmonary inflammation in the coughing horses; the Co group displayed a higher percentage of neutrophils than the C group, which remained below 5%, considered the upper limit in normal horses (Lessa et al., 2011; Michelotto et al., 2013). Similarly, cough was previously correlated with >5% BAL fluid neutrophils in IAD horses (Bedenice et al.,

2008), and with increased BAL fluid neutrophil count in RAO horses (Tilley et al., 2012). In the C group, the six horses that showed BAL fluid modification were diagnosed with IAD, which shows the nonspecific and mild clinical presentation of this disease and emphasises the importance of BAL in definitive diagnosis as previously demonstrated for police horses (Lessa et al., 2011).

Cell populations have been shown to differ between TW and BAL fluids, so both techniques were combined in this study, as previously suggested for airway cytological examination (Hodgson and Hodgson, 2003). Previous studies investigating cough applied the following techniques: endoscopy and TW (Burrell et al., 1996); history, endoscopy, and TW (Christley et al., 2001); pulmonary mechanical function, bronchoprovocation, and BAL (Bedenice et al., 2008); and clinical examination, endoscopy, thoracic radiography, and BAL (Tilley et al., 2012). Studies that used both TW and BAL cytological analyses for investigation of horses showing poor performance (Malikides et al., 2003) or chronic lung disease (Derksen et al., 1989) resulted in at most 63.0% correlation between TW and BAL fluids in differential cytology, similar to the present study. Our results show that both techniques must be used together for a more specific diagnosis, as previously mentioned (Malikides et al., 2003).

Hypothesizing 24% as the upper limit for TW fluid neutrophil count in normal horses, a positive correlation was observed between >24% TW fluid neutrophils and >5% BAL fluid neutrophils ( $p=0.003$ ,  $r = 0.4412$ ). This correlation was not found with a cut-off value of 20% for TW fluid neutrophils.

Considering the five coughing horses maintained on pasture, IAD was diagnosed in two and RAO in three. In these horses, clinical exacerbation occurred during summer and autumn. Summer-pasture airway obstruction is more prevalent during hot months, which are more favourable for fungal development as well as grass pollination. Although this was not investigated for the present cases, cough seems to be an important clinical manifestation.

## Conclusion

Cough is confirmed as an essential clinical sign associated with airway inflammation. A methodical

clinical evaluation including physical examination, airway endoscopy, and cytology evaluation of TW and BAL fluid samples should be conducted for accurate diagnosis.

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