MICROBIOLOGICAL FINDINGS OF TRACHEOBRONCHIAL WASHES OF HEALTHY HORSES AND THOSE WITH RESPIRATORY DISEASES

ACHADOS MICROBIOLÓGICOS DO LAVADO TRAQUEOBRÔNQUICO DE EQUINOS CLINICAMENTE SADIOS E DAQUELES PORTADORES DE AFECÇÕES DO SISTEMA RESPIRATÓRIO

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SUMMARY

This study evaluated the microbiological aspects of tracheobronchial washes obtained through tracheal puncture of 25 healthy horses and 65 horses with respiratory distress admitted to the Hospital Veterinario (HOVET, USP). In the control group, only 8 of the 25 animals did not present bacterial growth in the obtained samples. Furthermore, the group that included eight horses with bacterial bronchopneumonia (12.3% of the animals studied) presented bacterial growth in 100% of the samples, with special attention to *Streptococcus* alpha-hemolyticus. The technique proved to be efficient, but more research is needed to improve its use as a complementary exam on the diagnosis of equine respiratory disorders.

KEYWORDS: Equines. Tracheobronchial washes. Microbiology.

RESUMO

Foram avaliados os achados microbiológicos do lavado traqueobrônquico, obtido por coleta transtraqueal, de 65 equinos com sintomas de alteração do sistema respiratório e de 25 equinos assintomáticos (grupo controle) encaminhados ao HOVET - USP. No grupo controle somente oito dos 25 animais não apresentaram crescimento de patógenos nas amostras obtidas, enquanto que no grupo que incluiu oito equinos portadores de broncopneumonia bacteriana (12,3%dos animais estudados) houve crescimento em 100% das amostras, destacando-se *Streptococcus* alfa-hemolítico. A técnica mostrou-se eficiente, porém mais estudos precisam ser conduzidos tanto em equinos saudáveis como naqueles portadores de afecções do sistema respiratório com o propósito de aperfeiçoar o uso de tal exame complementar no diagnóstico definitivo de doenças respiratórias em equinos.

PALAVRAS-CHAVE: Equinos. Lavado traqueobrônquico. Microbiologia.

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INTRODUCTION

Studies with athlete horses showed evidence that the respiratory system is a limiting factor for maximum performance, since any mild or moderate pulmonary dysfunction can interfere significantly in the aerobic metabolism (SANTOS et al., 2007). The disorders of the respiratory system are responsible for 42% of decreasing performance of athlete horses, second to diseases of the musculoskeletal system (SANTOS et al., 2007).

The mucous membrane lining of the respiratory tract of mammals is the site of intense activity of the organic defense mechanisms of both immune and inflammatory response, which manifest themselves through the action of phagocytes, lymphocytes and immunoglobulins. Thus, analysis of representative samples of respiratory tract secretions can provide extensive information about the nature of the changes that affect this system, be it infectious, parasitic or allergic (MAIR et al., 1987; FREEMAN et al., 1993). The methods investigated for this purpose include the aspirate (obtained via endoscopy), tracheobronchial and bronchoalveolar washes as well.

Pecora (1959) introduced the tracheal wash technique in humans to collect uncontaminated samples from respiratory tract secretions for cytological and bacteriological examination. This technique was later modified for horses by Mansmann & Knight (1972), who used percutaneously collected samples from more than 100 horses that had abnormal respiratory system and found a useful method to identify the disease causative agents. Since the catheter does not pass trough the nasal cavity and other parts of the respiratory tract, the risk of sample contamination by nasopharynx microbiota is eliminated (HEWSON & VIEL, 2002). Thus, it is possible to use the appropriate therapy based on culture and antibiogram results. However, to use this method as complementary examination tool, correct interpretation of the results is essential. The presence of plant spores and fungi in the tracheal aspirate is common and this does not necessarily indicate fungal infection, but it simply reflects environmental contamination or decreased mucociliary function. The presence of bacteria and lack of cytological infection, does not necessarily suggest that this finding is the causative agent of respiratory disease (BEECH, 1991b).

Given the importance of an accurate diagnosis to determine the most appropriate therapy for changes of the respiratory tract; it was deemed relevant to establish the main microbiological findings of tracheobronchial washes of healthy horses compared to those suffering from respiratory diseases, and associate the same with the results of the clinical, cytological and endoscopic examinations.

MATERIAL AND METHODS

Ninety horses, regardless of breed, gender and age, admitted to the Hospital Veterinário de Grandes Animais, of Faculdade de Medicina Veterinária e Zootecnia of Universidade de São Paulo (HOVET, USP) were evaluated. Of these, 25 were healthy and 65 were referred due to changes in the respiratory system. The horses underwent detailed clinical examination with special attention to the respiratory system and laryngoscopy as well. Depending on the diagnosis, horses were grouped into different categories. The control group consisted of healthy animals with no history or clinical signs of respiratory tract disease.

The percutaneous tracheobronchial wash was obtained according to the technique described by Mansmann & Knight (1972) and Fernandes et al. (2000). The resulting wash was bottled in two flasks, one sterile for microbiological test and the second non-sterile for cytological test performed at the Research Laboratory of the Departamento de Clínica Médica, FMVZ, USP, within four hours from collection, as recommended by McGorum & Dixon (1994).

The cytological evaluation of the tracheobronchial wash was performed using Rosenfeld staining as described by Fernandes et al. (2000)

For the microbiological test, the focus of this research, the material was plated on blood agar base DIFCO®, plus 10% defibrinated sheep blood on BHI DIFCO® in aerobic and microaerophilic atmosphere to isolate the bacteria. For the recovery of both fungi, mycelium and yeast, Sabouraud-dextrose DIFCO® was used at room temperature (26 ± 3°C) and 37°C, respectively. Samples were checked for microbial growth 24, 48, 72 and 96 hours after collection. After macroscopic examination, the colonies were microscopically examined by Gram staining for further biochemical identification. The bacteria were identified according to biochemical behavior by production of catalase, oxidase, coagulase, urease, DNAse, gas, indole and H_2S using glucose, lactose, tryptophan, lysine and Simmons citrate; oxidation-fermentation of sugars in Hugh-Leifson medium, Mac Conkey growth; motility in solid medium and hemolytic behavior on sheep blood agar according to Holt et al. (1986).

The flasks containing fungi isolates were checked daily during a 30-day post-culture period, and mycelium fungi were identified by macromorphology and further micromorphology using the microculture technique proposed by Barnett & Hunter (1972). Yeast fungi were identified by auxonogram according to criteria by Kreger-Van Rij (1984).

The experimental protocol was approved by the Bioethics Committee of the institution where the experiment was conducted.

RESULTS AND DISCUSSION

Although bronchoalveolar lavage is considered by some authors as the ideal method to study pulmonary cytology since it provides more accurate information (ITO et al., 2001), other authors (MORRIS, 1984; CHAPMAN et al., 2000; ODE et al., 2007) observed that transtracheal wash gives, in addition to cytology, important microbiological information. Ode et al. (2007) pointed out that transtracheal wash provides information on tracheal secretions and both lungs. It is

also a method of rapid diagnosis, precise and affordable. In this study, the applicability of transtracheal wash method to help the diagnosis of several changes of the respiratory tract is reassessed.

The microbiological results with respect to study groups are presented in Table 1.

The control group was formed by horses with no history of respiratory disease, representing 27.8% (25/90). On physical examination the only change observed was the presence of bilateral serous nasal discharge in 44% (11/25) of the horses, which was confirmed by endoscopy. Three horses presented lymphoid hyperplasia grade I. Wash cytology showed predominance of macrophages, which according to Beech (1991b), indicates a representative sample of posterior respiratory tract. Bacteriological results of the tracheobronchial wash from 8 horses had no growth. In the remaining 17 horses, the bacteria isolate had especially *Pseudomonas* sp. (52.9%) and *Staphylococcus* sp. coagulase negative (35.3%), as shown in Table 1. In the control group, where even healthy horses displayed some clinical, microbiological and endoscopic changes, the results corroborated the report by Santos et al. (2007), who observed that healthy young animals, in early training, had already shown some kind of respiratory disorder of infectious (*Streptococcus* sp.), allergic or neuromuscular nature (soft palate displacement, abnormal epiglottis, follicular hyperplasia). Thus, becoming evident the difficulty to establish a group of healthy horses.

Following the same protocol, horses with abnormal breathing, representing 72.2% (65/90) were grouped based on clinical and laboratory findings.

Group 1 consisted of 10 horses suffering from morphological and functional changes of the anterior respiratory tract (15.4%), that covered right (2) and left (6) laryngeal hemiplegia; dorsal displacement of the soft palate (1) and hypoplasia epiglottis (1). Physical examination detected noisy breathing during physical exercise and decreased athletic performance. Endoscopy confirmed paralysis of the left arytenoid cartilage in six cases, the presence of serous/mucous discharge (4/10) and displacement of palate (2/10). The wash cytology showed no significant difference in the cell population detected in the control group. Bacteriological test of the tracheobronchial wash showed predominant growth of *Pseudomonas* sp. and *Staphylococcus* sp. coagulase positive (Table 1). No growth was observed in samples from two horses.

Group 2 consisted of 21 horses suffering from bacterial infections of the anterior respiratory tract (32.3%), including cleft palate (1), fibrous osteodystrophy (1), myiasis of the nasal cavity (1), submandibular phlegmon (1) and bacterial infections of the anterior respiratory tract (17). Clinical examination detected no seromucous or purulent nasal discharge in six horses, while all the others had. Bronchoscopy results displayed mucopurulent secretion (14/17) and hyperemia of the mucous membranes (14/17). Wash cytology results showed decreased macrophages and increased neutrophils in response, probably, to a bacterial infection. Bacteriological results of tracheobronchial wash showed predominant growth of *Staphylococcus* sp. coagulase negative, *Bacillus* sp. and *Enterobacter* sp. (Table 1). There was no growth in samples obtained from 10 horses.

Group 3 consisted of 12 horses suffering from viral infections of the anterior respiratory tract (18.6%). Clinical examination detected bilateral nasal serous discharge, confirmed by bronchoscopy. Patient history highlighted no complaints by the owners. Wash cytology showed lower concentration of macrophages compared to control group, and the presence of ciliated cylindrical epithelial cells, possibly showing aggression of the epithelium by the viral agent (VIEL & HEWSON, 2003). Micro-serum-neutralization tests were performed to check for equine viral arteritis and hemagglutination inhibition test for equine influenza and herpes virus type A1, A2 Kentucky and A2 Miami. Eight horses were positive for HVE and four positive for influenza A1. None was positive for viral arteritis. Bacteriological tests of tracheobronchial wash showed predominant growth of *Bacillus* sp. and *Staphylococcus* sp. coagulase positive (Table 1). It is believed that the presence of bacteria in this group was by secondary contamination. There was no growth in samples from 5 horses.

Group 4 consisted of 10 horses suffering from recurrent airway obstruction, RAO (15.4%). Physical examination detected coughing, mucous nasal discharge and auscultatory changes. Bronchoscopy showed mucous discharge in the anterior respiratory tract in 80% of the horses. Wash cytology results showed predominance of neutrophils, similar to that already described for horses of group 2. The bacteriological tests of tracheobronchial wash showed predominance of *Bacillus* sp. (Table 1). There was no growth in samples from 5 horses.

Group 5 consisted of 8 horses suffering from bacterial pneumonia (12.3%). During physical examination, purulent nasal discharge, increased body temperature and auscultatory changes were observed. Bronchoscopy showed the presence of purulent nasal discharge in the anterior tract, hyperemia of mucous membranes and lymphoid hyperplasia. Just as in groups 2 and 4, wash cytological evaluation showed predominance of neutrophils. According to Hodgson & Hodgson (2003) it is not possible to associate increased number of neutrophils present in the tracheobronchial wash alone to a specific respiratory disease, it is necessary to to link this information to other clinical and laboratory tests. Bacteriological examination of the tracheobronchial wash showed predominance of *Streptococcus* alpha-hemolytic and *Staphylococcus* sp. coagulase negative (Table 1), with 100% of the samples showing bacterial growth.

Group 6 consisted of only one horse carrying a parasitic bronchopneumonia (1.5%). The horse had a history of cough, and clinical examination showed presence of crackles during auscultation of the right lung. Endoscopy showed the presence of purulent secretions in the anterior respiratory tract. Cytology of the tracheobronchial wash was rich in eosinophils, associated to lack of growth in the wash. The parasite found in the wash was identified as *Dictiocaulus*

arnfield, as described by George et al. (1981) and Britt & Preston (1985).

Group 7 consisted of only one horse carrying allergy caused by inhaled particles (1.5%). The horse had dry cough and presence of bilateral nasal secretion. Endoscopy showed purulent discharge in the anterior respiratory tract. Eosinophils were the predominant cells in the tracheobronchial wash of this horse, similar to described by Michelotto Junior (2008). The bacteriological examination showed predominance of *Micrococcus luteus, Nocardia* sp. and *Pseudomonas aeruginosa* (Table 1).

Finally, group 8 consisted of 2 horses suffering from exercise induced pulmonary hemorrhage, EIPH (3.0%). Physical examination showed drop in performance, cough and auscultatory changes. Endoscopy showed mucous secretion in the anterior respiratory tract. It was also noted the presence of hemosiderophages, similar to the findings reported by Hegedus et al. (2007). No bacterial growth was observed in this wash.

Table 1 - Main microbiological results of the tracheobronchial washes of horses admitted to HOVET, USP, distributed according to clinical diagnosis established by clinical and complementary examinations.

Group	Clinical Diagnosis	Microbiological findings
Control	Asymptomatic (healthy horses)	*Pseudomonas* sp. (9/17)* *Staphylococcus* coag.neg. (6/17) *Streptococcus sp* alpha hemolytic (5/17) *Bacillus* sp. (4/17) *Nocardia* sp. (2/17) *Acinetobacter* sp., *Enterobacter* sp., *Enterococcus* sp., *Escherichia coli*, *Micrococcus luteus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Rhodococcus equi*, *Staphylococcus sp* coagulase positive, *Streptococcus equi* (1/17)
1	Morphofunctional changes of anterior respiratory tract	*Pseudomonas* sp., *Staphylococcus sp* coagulase positive (3/8) *Bacillus* sp., *Bacillus subtilis*, *Nocardia* sp, *Streptococcus sp* alpha hemolytic (2/8) *Corynebacterium* sp., *Enterococcus faecium*, *Enterobacter* sp., *Micrococcus* sp., *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Streptococcus sp* beta hemolytic (1/8)
2	Bacterial infections of anterior respiratory tract	*Staphylococcus sp* coagulase negative (4/11) *Bacillus* sp., *Enterobacter* sp. (3/11) *Acinetobacter* sp., *Micrococcus luteus*, *Nocardia* sp., *Pseudomonas aeruginosa*, *Pseudomonas* sp., *Streptococcus sp alpha* hemolytic (2/11) *Acinetobacter calcoaceticus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Micrococcus* sp., *Rhodococcus equi* (1/11)
3	Viral infections of anterior respiratory tract	*Bacillus* sp., *Staphylococcus sp* coagulase positive (3/7) *Klebsiella pneumoniae*, *Proteus* sp., *Pseudomonas* sp. (2/7) *Escherichia coli*, *Micrococcus luteus, Staphylococcus sp* coagulase negative (1/7)
4	ORVA	*Bacillus* sp., *Enterobacter* sp., *Micrococcus* sp., *Nocardia* sp., *Proteus* sp., *Pseudomonas aeruginosa, Pseudomonas* sp., *Staphylococcus sp* coagulase negative, *Serratia marcecens* (1/5)
5	Bacterial bronchopneumonia	*Streptococcus sp* alpha hemolytic (3/8) *Staphylococcus sp* coagulase negative, *Streptococcus zooepidemicus* (2/8) *Bacillus* sp., *Klebsiella pneumoniae*, *Proteus* sp., *Pseudomonas aeruginosa*, *Pseudomonas* sp., *Rodococcus* sp., *Staphylococcus* sp., *Streptococcus equi*, *Streptococcus sp* beta hemolytic (1/8)
6	Parasitic bronchopneumonia	----
7	Allergy by inhaled particles	*Micrococcus luteus*, *Nocardia*, *Pseudomonas aeruginosa* (1/1)
8	HPIE	----

* In the column "Microbiological findings", the number between parenthesis is the number of isolated pathogen with respect to total number of horses that presented bacterial growth in the washes. In some cultures more than one bacteria was isolated.

Fungal examination of the tracheobronchial wash from all groups, of both healthy horses and the ones suffering from diseases of the respiratory system, showed that *Aspergillus* sp. was predominant.

According to Takizawa et al. (2005), diagnosis of the bacterial respiratory diseases in horses usually includes auscultation of the airways, measurement of body temperature and hemogram (CBC) as well. These same authors point out that little is known about the diagnostic value of cytological and especially microbiological data of bronchoalveolar and tracheal washes for several diseases , among which fever resulting from transportation.

In 1985, Sweeney et al. evaluated tracheobronchial aspirate of 53 healthy Thoroughbred horses in activity and 36 pasture raised healthy PSI horses. There was no bacterial growth in 74% of the horses of the first group, while 24% showed growth of bacterial isolates and only 8% showed aerobic bacterial growth with recognized pathogenicity. In the second group, 28% did not show bacterial growth, while 64% showed growth of bacterial isolates and 8% showed aerobic bacterial growth with recognized pathogenicity. In the present study, there was no bacterial growth in 32% of the samples from the healthy horse's group, similar to the values reported by Sweeney et al. (1985) for pasture raised horses, probably because most of the horses in this study were raised in semi-intensive or extensive systems.

In 1991, Sweeney et al. evaluated tracheal aspirates of 327 horses with pneumonia or pleuropneumonia. There was aerobic bacterial growth in 221 cases (67.6%), particularly *Streptococcus* sp. beta-hemolytic, *Pasteurella* sp., *Escherichia coli* and *Enterobacter* sp., while in 6 cases it was observed anaerobic bacterial growth, *Bacteroides* sp. and *Clostridium* sp., in addition to mixed infections in six horses and no growth in the remaining horses. In group 5 of the present study, with horses suffering from bacterial bronchopneumonia, bacterial growth was observed in 100% of the samples, but differently from the previous one, the main isolate was *Streptococcus* sp. alpha-hemolytic. In 2007, Ode et al. assessed the rate of isolation in bronchoalveolar wash of 33 horses from 43 horses with pneumonia, *Streptococcus zooepidemicus* was isolated in 39.5% of the samples, *E. coli* in 11.3% and Gram negative anaerobic bacteria in 7.0%.

Streptococcus zooepidemicus and *S. pneumoniae* were the main bacteria isolated from the tracheobronchial washes of horses that carried inflammatory diseases of the lower airways (WOOD et al., 2005). Takizawa et al. (2005) also reported *Streptococcus zooepidemicus* as the main single agent (83.3%) isolated from tracheobronchial washes of 29 racing horses after long distance transportation; however, the bacterial growth was not correlated with the cytological findings indicative of pulmonary inflammation, such as the presence of neutrophils. Similarly, Morris (1984) and Chapman et al. (2000) suggested a relationship between the inflammation of the respiratory tract and the isolated bacteria. However, Sweeney et al. (1985) and Darien et al. (1990) reported that bacteria can be isolated from the tracheobronchial washes of healthy horses, a fact that was also confirmed in the present study where bacteria such as *Pseudomonas* sp. and *Staphylococcus* sp coagulase positive were isolated from the tracheobronchial washes of the control group, formed by healthy horses.

CONCLUSIONS

The results of the present study suggest the importance of the evaluation of microbiological findings of the tracheobronchial washes of horses suffering from respiratory infections together with detailed clinical examination and other complementary tests focused on the respiratory system, such as endoscopy and cytology of the tracheal wash. Thus, establishing a definitive diagnosis and the most appropriate therapy becomes feasible. Further studies are needed in both healthy horses and others affected by disorders of the respiratory system with the purpose of improving microbiological evaluation of the tracheobronchial wash and make it routine when establishing clinical diagnosis of horses carrying respiratory disorders.

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