

Endoscopic findings, microbiological and cytological evaluation of tracheal aspirates in a population of Standardbred horses with poor performances

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ABSTRACT: Sixty-two Standardbred horses housed at the same racetrack, with history of reduced exercise tolerance, cough lasting for at least two weeks and/or prolonged recovery time were clinically examined. An endoscopic examination of the nasopharynx, larynx and trachea to the level of the carina was performed, amount of mucus in trachea was registered and samples of tracheal wash for cytological and microbiological examinations was collected. A strong statistical association between amount of mucus in trachea and neutrophils percentage in tracheal wash was found. Bacteria isolated included *Streptococcus equi* subsp. *zooepidemicus* (14 horses), *Streptococcus pneumoniae* (four horses), *S. mutans* (four horses), *S. equinus* (four horses) and *Burkholderia cepacia* (10 horses). *S. zooepidemicus* and *S. pneumoniae* were associated with elevated amount of mucus and increased neutrophilic percentage. *B. cepacia* was associated with cytological evidence of haemosiderophages but its role in racehorses needs further investigations. *Mycoplasma* spp. and *Pasteurella* spp. have not been isolated, suggesting that, as for the other putative causes of inflammatory airway diseases, infection could have a regional distribution among horse populations. This study shows that various types of airway inflammations exist in the examined population and that *S. zooepidemicus* and *S. pneumoniae* could play an important role in etiopathogenesis of airway inflammation in some horses. Particulate matter, pro-inflammatory agents or noxious gases present in the stables or on the track matter, could be the cause of inflammation in non infected horses.

Keywords: horse; tracheal wash; mucus; cytology; bacteriology; Streptococci

Poor performances and chronic cough are among the most usual complains reported by owners, trainers or drivers. RAO (Recurrent Airway Obstruction) or “heaves” is a well know allergic condition of mature to old horses (Couetil and Ward, 2003) and clinical signs include episode of severe respiratory distress, coughing and increased abdominal effort (Robinson et al., 1996).

IAD (Inflammatory Airway disease) is a syndrome that can be differentiated from RAO on the basis of aetiology, epidemiology and symptoms.

IAD can affect horses of any age (Couetil et al., 2007) but is more common in young racehorse in training and incidence decreases with age (Wood et al., 2005b). Clinical signs are subtle and include decreased performance in racing and training, mild exercise intolerance and chronic cough with

nasal discharge in few cases (MacNamara et al., 1990; Burrell et al., 1996; Christley et al., 2001; Robinson, 2003). Classically, affected horses do not show respiratory effort at rest, are not febrile and do not have any haematological abnormalities (Couetil et al., 2007). Endoscopic evaluation reveals variable amount of mucus in trachea containing increased proportion of neutrophils (Burrell et al., 1996; Moore et al., 1997; Christley et al., 2001; Robinson, 2003).

Independently from definition, several studies have been performed to better understand the causes of respiratory inflammation in young racehorses and a multifactorial aetiology has been hypothesized (Burrell et al., 1996).

Viral, bacterial (Burrell et al., 1996; Christley et al., 2001) and environmental agents (Burrell et al.,

1996; Holcombe et al., 2001; Couetil et al., 2007) are supposed to be involved, even though the exact aetiology and pathogenesis are not completely understood.

A statistical association between IAD and tracheal infection with *Streptococcus pneumoniae* (capsule type 3), *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*), *Actinobacillus* spp. and *Mycoplasma equirhinis* in British Thoroughbred has been found (Wood et al., 2005b). Since the prevalence and incidence of IAD, of *S. zooepidemicus* and *S. pneumoniae* decreased with age, it was supposed that these bacteria could play an important role in the pathogenesis of IAD in young horses. Specific association between upper and lower airway disease and *S. zooepidemicus* was highlighted also by other Authors (Burrell et al., 1996; Laus et al., 2007; Newton et al., 2008). On the other hand, several horses with IAD may result negative for culture of respiratory secretion (Chapman et al., 2000) and potentially pathogenic bacteria can be isolated from healthy horses (Sweeney et al., 1985). If the presence of bacteria in the trachea of affected horses indicates a primary infection or a secondary decreased clearance is therefore still unclear.

IAD is probably a broad spectrum definition including a wide range of respiratory disorders with uncertain diagnosis but characterized by airways inflammation.

Such a mild forms of airway inflammation are very common among Standardbred horse in Italy and are often cause of withdrawal from training and racing for weeks or months. The aim of this study was to verify the association among mucus accumulation, tracheal neutrophilia and presence of bacteria in poor performing Standardbred horses in a racetrack with high prevalence (49%) of chronically coughing horses.

MATERIAL AND METHODS

Population

The total population at the racetrack included 350 horses and about 200 of them were less than four years old. All owners and/or drivers and the resident veterinaries were interviewed for poor performances in association with respiratory symptoms in young horses. History, clinical examination and application of re-breathing bag (to elicit cough reflex) of these horses resulted in a prevalence of

poor performing and chronically coughing young horses of 49% (97 horses). A pretty large numbers of histories correlated the beginning of coughing with the introduction of the horse in the racetrack.

Animals

Sixty-two Standardbred horses in training with history of reduced exercise tolerance and/or prolonged recovery time and cough lasting from at least two weeks were selected. All horses examined were housed at the same racetrack, permanently indoor and in conventional stable environment (bedded on straw and fed hay). All animals were regularly vaccinated, treated for gastrointestinal parasite and trained on the same track surfaces, made of a plastic polymer. The horses' age ranged from 2–4 years. Animals presenting systemic signs of diseases like fever or depression or showing increased respiratory effort at rest, were excluded from the study. Both clinical and endoscopical evaluations were carried out in an examination room belonging to the racetrack complex.

Clinical and endoscopical evaluation of horses

Horses were clinically examined for upper and lower respiratory tract diseases.

An endoscopical examination of the nasopharynx, larynx and trachea to the level of the carina was performed (Mercury Endoscopia Italiana, L = 320 cm, ϕ = 0.98 cm, Italy) and animals with evidence of upper airway dysfunction were excluded from the study. Horses were restrained with the only use of a twitchnose. Mucus accumulation in the trachea was classified using the following scoring system suggested by Gerber et al. (2004):

Score 0: no mucus

Score 1: small, singular drops of mucus

Score 2: larger, confluent drops of mucus

Score 3: streams of mucus

Score 4: large streams or pools of mucus covering more than 1/3 of tracheal diameter (Figure 1).

To perform tracheal wash (TW), the endoscope was retracted to the mid-tracheal level and 40 ml of sterile and prewarmed saline solution infused via a sterile catheter passed in the accessory channel of the endoscope. Advancing caudally in the trachea the pool of saline solution was then individuated,

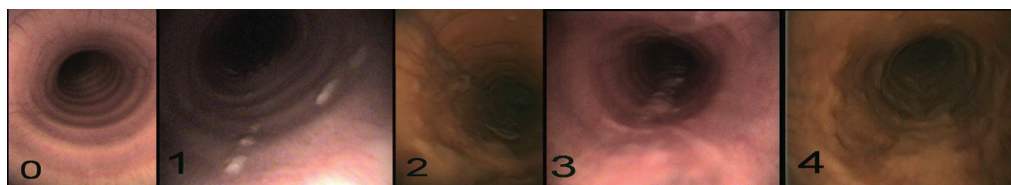


Figure 1. Different amount of mucus in trachea and relative scores

collected using a 50 ml sterile syringe and transferred in sterile tubes stored on ice. All samples were brought to the laboratories within two hours from collection.

The endoscope was cleaned with an iodopovidone solution, rinsed with sterile saline solution and, lastly, with sterile distilled water before the following horse examination.

Bacteriological examination

An aliquot (100 µl) of TW fluid was cultured on Columbia blood Agar with and without Streptococcus Selective Supplement, MacConkey Agar, Baird-Parker Agar and Plate Count Agar (Oxoid, Milan, Italy). Plates were incubated in aerobic and anaerobic conditions at 37°C for 48 h. The colonies were subcultured and identified by using biochemical gallery (RapID™ System, Oxoid, Milan, Italy), serological (Streptex®, Oxoid, Milan, Italy) or PCR techniques (Preziuso et al., 2007).

An equal amount, 100 µl, of TW fluid was used for *Mycoplasma* isolation using Mycoplasma Broth with Mycoplasma Selective Supplement – G (Oxoid, Milan, Italy) incubated at 4°C for 24h. After centrifugation at $2\,000 \times g$ for 20 min, the supernatant was added to Mycoplasma broth with 20% of equine sterile serum and incubated at 37°C for 72 h. Finally, 100 µl of supernatant were inoculated into Mycoplasma Agar (Oxoid, Milan, Italy) with 20% of equine serum and plates were incubated for 10 days at 37°C in humid chamber with 10% CO₂.

Cytology

After cytocentrifugation, the TW samples were smeared, air-dried, stained with May-Grundwald-Giemsa and observed under a microscope using a 100× magnification. The numbers of neutrophils were estimated by counting 300 cells and the percentages higher than 30% were considered sign of inflammation.

Statistical analysis

Results were elaborated by χ^2 test with Yates correction factor. The level of significance was established at $P < 0.05$ for all the tests.

RESULTS

There were no significant differences among symptoms observed apart from little variability concerning the rate of cough.

Mucus scores detected at endoscopic examination and their correlations with neutrophilic inflammation and total bacterial count are reported in Table 1.

The previous table shows as 20 out of 24 horses (83.3%) with mucous score > 1 had an high neutrophils percentage in TW, revealing a strong statistical association between amount of mucous in trachea and neutrophils percentage in TW ($P < 0.05$). Furthermore, the increasing of mucous score

Table 1. Mucus score correlated with neutrophilic inflammation and total bacterial load

Mucus score	Number of horses	Horses with neutrophilic inflammation	Total bacterial load mean (CFU/ml)
0	18	0	149×10^2
1	20	4	394×10^2
2	18	14	$1\,194 \times 10^2$
3	2	2	$4\,940 \times 10^2$
4	4	4	$3\,417 \times 10^2$
Total	62	24	

Table 2. Association between neutrophils percentage and total bacterial load

Number of horses	Neutrophilic inflammation	Total bacterial load mean (CFU/ml)
37	no	309×10^2
25	yes	$1\,666 \times 10^2$

is pretty consistent with the increasing of total bacterial count ($P < 0.05$).

The association between neutrophilic inflammation and total bacterial load is reported in Table 2.

Among β -haemolytic Streptococci, *S. zooepidemicus* has been isolated from TW fluid of 14 horses. The correlation between these results and other parameters of the same horses are summarized in Table 3.

There was not a statistical correlation for *S. zooepidemicus* neither with mucus score nor with neutrophils percentage.

Four horses were positives for *Streptococcus pneumoniae* and the association with mucus amount and neutrophil percentage is showed in Table 4.

S. zooepidemicus and *S. pneumoniae* were never isolated together in the same horse.

Other α -haemolytic streptococci isolated were *S. mutans* (from four horses) and *S. equinus* (from four horses).

From ten horses *Burkholderia cepacea*, without association with neutrophil percentage or mucus score, has been isolated. Two samples resulted sterile.

Table 3. Neutrophils percentage and mucus score in horses positive for *S. zooepidemicus*

Horse ID	<i>S. zooepidemicus</i> (CFU/ml)	Total bacterial count (CFU/ml)	Neutrophils percentage	Mucus score
48	1×10^2	16×10^2	46	1
19	1×10^2	14×10^2	57	1
51	8×10^2	52×10^2	8	0
30	7×10^2	49×10^2	6	0
33	68×10^2	710×10^2	25	0
11	70×10^2	670×10^2	21	0
44	138×10^2	138×10^2	70	2
3	144×10^2	144×10^2	77	2
52	$3\,580 \times 10^2$	$4\,642 \times 10^2$	50	3
14	$3\,700 \times 10^2$	$5\,237 \times 10^2$	53	3
60	$4\,280 \times 10^2$	$6\,244 \times 10^2$	72	4
15	$4\,350 \times 10^2$	$7\,401 \times 10^2$	70	4
61	$6\,980 \times 10^2$	$9\,420 \times 10^2$	79	2
16	$7\,860 \times 10^2$	$10\,120 \times 10^2$	81	2

Table 4. Neutrophils percentage and mucus score in horses positive for *S. pneumoniae*

Horse ID	<i>S. pneumoniae</i> (CFU/ml)	Total bacterial count (CFU/ml)	Neutrophils percentage	Mucus score
32	890×10^2	$1\,012 \times 10^2$	21	2
59	560×10^2	815×10^2	29	0
39	1×10^2	3×10^2	56	4
24	1×10^2	18×10^2	58	4

From none of the horses other potentially pathogenic bacteria, included *Mycoplasma* spp., have been isolated, and no haematological abnormalities were found. Results about isolation of normal flora has not been reported.

DISCUSSION

The 62 examined horses represent the 64% of the total young coughing horses (97) and can be considered representative of the coughing population. Therefore, it is possible to carried out both general and specific considerations.

The results of this study confirm a strong statistical association between neutrophilic inflammation and accumulation of mucus in trachea. Increased number of neutrophils could be the cause of mucus production but infectious agents and some environmental contaminants can directly trigger it (Robinson, 2003; Gerber et al., 2004). Has been demonstrated that mucus in trachea is associated with decreased performances (Holcombe et al., 2006) but it is a matter of concern if streptococci or other bacteria could be a causative agent of the increasing in tracheal secretions in airways inflammation affected horses. The result of this study shows a fairly agreement between the mucus score and quantitative analysis for *S. zooepidemicus* and these results could indicate that mucus in trachea is elicited by a specific bacteria. On the other hand, a similar correlation between mucus score and total bacterial load was found. Therefore, it could be possible that the decreased clearance in lower and upper airway consequent to inflammation, led to a bacterial accumulation in the airways. In this case, mucus accumulation would be the cause and not the effect of bacteria colonisation of the airways. Nevertheless, some observations should be done: although a large number of horses having high mucus score and/or neutrophil percentage were negative for *S. zooepidemicus* in trachea, among the 14 positive horses, the higher *S. zooepidemicus* load belong to the horses with mucus score > 2. Furthermore, 10 out of 14 positive horses had cytological evidences of inflammation with the higher neutrophil percentage (81%) associated at the highest bacterial load ($7\,860 \times 10^2$) in horse number 16. Similar findings in thoroughbred were also reported (Chapman et al., 2000). In horse number 3, with evident endoscopical and cytological signs of inflammation, *S. zooepidemicus* was the

only bacteria isolated. Based on this findings, even though a statistical association were not found, we can not exclude a role of *S. zooepidemicus* in the pathogenesis of IAD, as a causative or, more probably, as a perpetuating agent of inflammation in Standardbred horses.

Tracheal infection with *S. pneumoniae* has been statistically associated with IAD in British Racehorse (Wood et al., 2005a). In horses of the present work, *S. pneumoniae* was associated with increased mucus amount (Table 4) but, unexpectedly, bacterial count showed almost a reversed correlation with neutrophilic inflammation and mucus. *Mycoplasma felis* and *Mycoplasma equirhinis* are frequently isolated in British Thoroughbred with clinical respiratory disease (Newton et al., 2003; Wood et al., 2005a), but has never been isolated in Standardbred horses of this study. The different etiological forms of IAD, depending on feeding, housing, preventive medicine practice and, most important, different distribution of infectious agents (Couetil et al., 2007) could be an explanation for variability of these results.

Burkholderia cepacia is the major pathogens that colonize the airway surface and cause progressive respiratory failure and high mortality in human being, especially in cystic fibrosis patients (Treerat et al., 2008) and usually is not isolated from healthy people (Hyde and Humphreys, 1997). Differently from *Burkholderia mallei*, its role is unknown in horses. *B. cepacia* was isolated from 10 horses, without association with neutrophil percentage or mucus score. Recent study showed that *Burkholderia cenocepacia*, a member of the *B. cepacia* complex, require a source of iron to colonize the lung and to satisfy its nutritional requirements and that is the only one able to extract iron from ferritin (Whitby et al., 2006). None of the five horses had history or clinical signs of exercise induced pulmonary haemorrhage (EPEI) but some haemosiderophages (macrophages containing a ferritin polymer called hemosiderin) in their tracheal secretions were found at cytological examination. This is a normal finding also in healthy racehorses but could be interesting to establish the epidemiology of *B. cepacia* among horses and to further investigate the relationship between *B. cepacia* and respiratory horse diseases.

A load of pathogenic bacteria greater than 10^2 CFU/ml, in the presence of neutrophilic inflammation, is supposed to be a cut-off value to warrant administration of antibiotic therapy (Christley and

Rush, 2007). All horses positive for *S. zooepidemicus* or *S. pneumoniae* with cytological signs of inflammation had a bacterial load $\geq 10^2$ CFU/ml and can be considered affected by so called *bacterial IAD*. Nevertheless, from this point of view, this study failed in found a common or, at least, a more diffuse causes for so high prevalence of coughing horses. Eighteen horses did not show any mucus in trachea in spite of the presence of cough and poor performances. These horses could be affected by a deeper problem in respiratory tract, like bronchioles inflammation and bronchospasmus, without involvement of trachea. This cases and other horses with various grades of mucus and/or signs of cytological inflammation without signs of infection, could be affected by the other subcategory, called *non-bacterial IAD*. Inhalation of particulate matter, pro-inflammatory agents or noxious gases are reasonable causes for non infectious coughing horse. To test airborne endotoxin concentration in the stables where horses are housed and in the racetrack matter where horses are trained, will be the second step of the present study.

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