

Results of cytological and microbiological examination using tracheal aspiration in race horses with lower respiratory tract disease

Sevim Kasap¹, Engin Kennerman¹, Huban Gocmen², Huseyin Cihan¹, Mihriban Ulgen³

¹Uludag University, Faculty of Veterinary Medicine, Department of Internal Medicine, Bursa, Turkey

²Near East University, Faculty of Veterinary Medicine, Department of Microbiology, Nicosia, Northern Cyprus, Turkey

³Uludag University, Faculty of Veterinary Medicine, Department of Microbiology, Bursa, Turkey

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Abstract

Respiratory tract disease is the second most common cause of poor performance in racehorses after musculoskeletal disease. Lower respiratory tract disorders (LRTD) are common in thoroughbred horses of all ages. The aim of this study was to investigate whether there was any association between the microbiological and cytological examinations. Fifty horses ranging in age from 2 to 6 years were examined. Horses with only upper respiratory tract abnormalities identified by endoscopy (at rest) were eliminated from the study and horses with LRTD were used in this study. Tracheal aspirate specimens were collected for cytological and microbiological examinations. Thirty six horses had positive and 14 horses had negative cultivation. The isolated bacteria included β -haemolytic *Streptococcus equi* subsp. *zooepidemicus* (38.8%), *Escherichia coli* (22.2%) and other bacteria that were isolated at rates ranging from 0.4 to 1.8%. Percentages of neutrophils, lymphocytes, eosinophils, macrophages and mast cells were evaluated in the cytological examination. The percentages of neutrophils were significantly higher in the samples with isolated bacteria ($35.75 \pm 2.60\%$) compared to the samples from which bacteria were not isolated ($16.79 \pm 2.36\%$) ($P < 0.001$). This study shows that *S. equi* subsp. *zooepidemicus* could play an important role in the etiopathogenesis of LRTD. It also demonstrates the importance of evaluating the microbiological findings of the tracheal aspirate specimens from horses suffering from respiratory infections, in addition to performing a detailed clinical examination and other complementary tests that focus on the respiratory system, such as endoscopy and cytology of the tracheal aspiration.

Equine, endoscopy, infection, Streptococcus, neutrophils

Respiratory tract disease is the second most common cause of poor performance in racehorses after musculoskeletal disease (Pascoe et al. 1981; Raphael et al. 1982). Lower respiratory tract disorders (LRTD) are common in thoroughbred horses of all ages. Although the presenting signs and symptoms vary with these disorders, they may include exercise intolerance, cough, nasal discharge, fever, respiratory distress, increased respiratory rate of effort, or generalized depression, inappetence, and weight loss. Amongst respiratory tract diseases, LRTD and the inflammatory airway disease have been discussed as common causes of poor performance, interruption of training and premature retirement in racehorses (Couetil et al. 2007). Careful clinical examination includes auscultation, and rebreathing often confirms the anatomic site of the problem.

Diagnostic testing, such as endoscopic examination, bronchoalveolar lavage, tracheal aspiration and wash with endoscopy, thoracic ultrasound, or thoracic radiography, are often indicated to confirm a suspected diagnosis (Davis et al. 2007).

Fibre optic endoscopy is an essential tool used to assess the equine respiratory tract. Endoscopic examination allows direct visualization of the upper respiratory tract, guttural pouches, trachea, and mainstem bronchi. Indications for endoscopic examination include

Address for correspondence:

Sevim Kasap
Department of Internal Medicine
Faculty of Veterinary Medicine
Uludag University, 16059, Bursa, Turkey

Phone: (+90 0224) 294 08 14
E-mail: etins@uludag.edu.tr
<http://actavet.vfu.cz/>

upper airway noise, inspiratory difficulty, poor exercise performance, and unilateral or bilateral nasal discharge (Hodgson and Hodgson 2003).

Tracheal washing is a diagnostic procedure used in equine practice for respiratory disease because tracheal aspirates (TA) can provide clinicians with important cytological and microbiological information (Chapman et al. 2000; Takizawa et al. 2005). Bronchoalveolar lavage (BAL) is another diagnostic procedure for respiratory disease in horses, and cytological, biochemical and microbiological examinations of BAL fluid (BALF) provide even more accurate information to assist with the diagnosis of pneumonia (Hobo et al. 1997; Ito et al. 2001). Takizawa et al. (2005) suggested that the diagnostic value of TA obtained using endoscopes from young horses immediately after transportation is high for cytological examinations but extremely low with respect to bacteriological examination. According to these previous studies, BALF may provide more precise information than TA when diagnosing pneumonia in horses (Ode et al. 2007). However, some authors favoured TA because it is easier and cheaper to obtain, they believe that the sample contains secretions and cells originating from all areas of the lung (McGorum et al. 1994) and they can collect it free of outside contamination for bacteriological examination (Christley et al. 1999; Darien et al. 1990).

Normally, the trachea and bronchi (airways) contain small numbers of bacteria that are considered to be transient contaminants in the process of being removed by clearance mechanisms. When the pulmonary defence mechanism is overwhelmed, aspirated bacteria from the oropharynx may proliferate and cause pneumonia. The most commonly involved gram-positive bacteria are *Streptococcus equi* subsp. *zooepidemicus* (β -haemolytic) (Chapman et al. 2000; Christley et al 2001; Newton et al. 2003), *Staphylococcus aureus* and *Streptococcus pneumoniae* (α -haemolytic) (Chapman et al. 2000; Christley et al 2001). The most frequent gram-negative isolates are *Pasteurella*, *Actinobacillus* spp. (Chapman et al. 2000; Christley et al 2001; Newton et al. 2003), *Escherichia coli* (Debroy et al. 2008; Racklyeft and Love 2000), *Klebsiella pneumoniae*, *Bordetella bronchiseptica* (Davis et al 2007), and *Enterobacter* spp. (Sweeney et al. 1991).

In this study, we performed cytological and microbiological examinations using tracheal aspiration in 50 racehorses with LRTD. We also investigated whether there was any association between the microbiological and cytological examinations.

Materials and Methods

Animals

A total of 19 Arabic and 31 thoroughbred horses (total 50) ranging from 2 to 6 years of age were examined. Horses with only upper respiratory tract abnormalities that were identified by endoscopy were eliminated from the study and only horses with upper and LRTD were included. All horses were presented with a cough, nasal discharge and poor performance, and they were either in race training or racing. The physical examination, which included cardiac and thoracic auscultation, was normal in all cases, as was the lameness examination. All applications were performed under the control and approval of the University of Uludag Ethics Committee, in accordance with the Animal Welfare Guidelines (2009-10/06).

Clinical examination

Fifty racehorses demonstrated strong lower respiratory tract abnormalities. A weighted clinical score was provided for each subject, based on information from the history, physical examination, and endoscopic findings in the upper and lower airways at rest (Doucet and Viel 2002). The minimum clinical score was 5 (Table 1).

Endoscopic examination and tracheal aspirate cytology

Tracheal aspirates were obtained from 50 horses at the early stage of LRTD. The horses were restrained in the stocks, and a twitch was applied to the upper lip. A 3 m flexible endoscope (VetVu® VFS300, The Veterinarian's Endoscope, USA) was passed through one nostril to the pharynx and through the rima glottidis into the trachea. The endoscope was advanced through the straight mid-cervical trachea until the curvature of the distal trachea was observed. A single lumen polypropylene catheter tip was positioned just proximal to the tracheal bifurcation, and 50 ml of sterile 0.9% saline was injected into the tracheal lumen; as much fluid as possible was aspirated under endoscopic visualisation. Samples were submitted to the laboratory and processed within 30 min of collection.

Table 1. Case history, physical examination, and endoscopic evaluation used to calculate the clinical score of the lower respiratory tract.

	Case history	Absent	Present
Poor performance (reported by trainer)		0	1
Physical examination			
Nasal discharge/coughing or other related respiratory signs		0	3
Epistaxis		0	1
Inappetence, weakness		0	1
Physical examination			
Temperature >38 °C		0	1
Respiratory rate >16/dk		0	1
Nasal discharge		0	3
Abdominal effort at rest		0	1
Abnormal lung sounds on auscultation		0	2
Other problems on physical examination		0	1
Endoscopic evaluation			
Haemorrhage		0	1
Other upper airway disorders		0	2
Hyperaemia of the airways		0	3
Mucoid discharge		0	1
Purulent discharge		0	3
Oedema		0	1

of biochemical tests) and API systems (API rapid ID 32 Strep, API Staph, API 20E, BioMerieux Industry, Italy).

Statistical analysis

The comparison of cytological examination between the samples with positive bacteriology and the samples without isolated bacteria was analysed via independent sample *t*-test. All the statistical analyses were performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Results were expressed as mean \pm standard error and *P* value < 0.05 was considered as significant.

Results

Clinical findings

The respiratory rate was over 10–16 breaths per min, and the body temperature was over 37.6–38.0 °C. Cough and nasal discharge were present in most cases. Nine horses had no cough, and hardened vesicular sound was present in 7 horses.

Cytological findings

The percentage of macrophages and neutrophils was 51.46% and 30.40%, respectively, in the cytological results of TA (Table 3). The percentage of neutrophils and macrophages was 35.75% and 47.06%, respectively, in samples with positive bacterial culture whereas the percentage of neutrophils and macrophages was 16.79% and 60.79%, respectively, in TAs with negative bacterial culture (Table 3). The percentages of neutrophils were significantly higher in the samples with isolated bacteria compared with samples without positive cultivation ($P < 0.001$).

Bacteriological findings

A positive bacterial culture of TA was found in 36 of 50 horses. Fourteen bacterial species, including facultative aerobic organisms, gram-positive bacteria and six additional

Smears made from cytocentrifuged aliquots from samples were stained with Diff-Quik® (Baxter, Illinois) and of 100 inflammatory cells were counted. Cells in the specimens were classified into neutrophils, macrophages, lymphocytes, eosinophils and mast cells.

Bacteriologic examination

Tracheal aspirate sample from each horse was examined bacteriologically. The samples were preserved without disruption of the cooling chain and transmitted to the Microbiology Laboratory at the Faculty of Veterinary Medicine, Uludag University. For bacterial isolation, a 10 ml aliquot of TA was centrifuged for 10 min at 2000 \times g. They were inoculated to general, selective and differential media for bacteriological isolation. Columbia agar with 5% sheep blood, tryptic soy agar as general media, MacConkey agar, eosin methylene blue agar as differential agars and ceftazidime-novobiocin Mueller-Hinton agar, chocolate agar, and Edward's agar as selective media were used. All media were incubated both aerobically and anaerobically at 37 °C for 24–48 h. The selective media were incubated for up to 5 days and controlled daily.

Bacterial identification of pure cultures was performed with routine methods (colony morphology, haemolysis characteristics, microscopy morphology, Gram staining and catalase, coagulase, and oxidase tests as some

Table 3. Cytological findings in the tracheal aspirates obtained from racehorses with lower respiratory tract disease.

Leukocyte cells	Mean value in bacterial isolated cases (n = 36) ± SE*	Mean value in non-bacterial isolated cases (n = 14) ± SE*	General (n = 50) ± SE*
Neutrophil (%)	35.75 ± 2.60***	16.79 ± 2.36	30.40 ± 2.31
Lymphocyte (%)	15.08 ± 1.17**	21.50 ± 2.14	16.84 ± 1.10
Eosinophil (%)	1.94 ± 0.45	1.57 ± 0.30	0.80 ± 0.20
Macrophage (%)	47.06 ± 2.22***	60.79 ± 2.82	51.46 ± 1.95
Mast (%)	4.33 ± 2.23	1.00 ± 0.00	0.26 ± 0.16

*SE: standard error

Difference of leukocyte cells percentage between bacterial isolated cases and non-bacterial isolated cases

(** $P < 0.05$ and *** $P < 0.001$).

species of gram-negative bacteria, including facultative anaerobic and aerobic bacteria, were isolated (Table 2). β -haemolytic *S. equi* subsp. *zooeidemicus* predominated (38.8%), followed by *E. coli* (22.2%). Other bacteria were isolated at rates ranging from 0.4 to 1.8%.

Table 2. Bacterial isolation from the tracheal aspiration fluid obtained from racehorses with lower respiratory diseases.

Aerobic/FA* bacteria	Number of bacteria (n)
Aerobic/FA Gram+ cocci	
<i>Streptococcus equi</i> subsp. <i>zooeidemicus</i>	14
α -haemolytic <i>Streptococcus</i> spp. α -haemolytic	5
<i>Streptococcus dysgalactiae</i> ssp. <i>equisimilis</i>	1
α -haemolytic <i>Streptococcus uberis</i>	1
<i>Staphylococcus</i> sp.	1
<i>Staphylococcus lentus</i>	4
<i>Staphylococcus gallinarum</i>	1
<i>Staphylococcus equorum</i>	1
<i>Staphylococcus sciuri</i>	1
<i>Staphylococcus vitulus</i>	1
<i>Staphylococcus haemolyticus</i>	2
<i>Staphylococcus xylosus</i>	1
<i>Staphylococcus saprophyticus</i>	1
β -haemolytic <i>Streptococcus pyogenes</i>	1
Aerobic/FA Gram- bacilli	
<i>E. coli</i>	8
<i>Enterobacter aerogenes</i>	2
<i>Enterobacter cloacae</i>	3
<i>Klebsiella pneumoniae</i> ssp. <i>pneumoniae</i>	1
<i>Stenotrophomonas maltophilia</i> (<i>Pseudomonas maltophilia</i>)	1
<i>Shewanella putrefaciens</i>	1
Total number of bacteria	51

*FA: facultative anaerobic

Discussion

Poor performances and cough are among the most frequently symptoms reported (by owners, trainers or drivers) in association with LRTD (Laus et al. 2009). Laus et al. (2009) found that 64% of examined horses suffered from coughing, and reported coughing as a major clinical finding of LRTD. In this study, we found an even higher percentage of horses suffering from cough, namely 82% (41 out of 50). Coughing is known to be a relatively specific indicator of LRTD and a potential symptom of pneumonia, pleuritis, viral and bacterial respiratory infections, exercise-induced pulmonary haemorrhage and chronic obstructive pulmonary disease (Bailey et al. 1999).

In equine practice, tracheal aspiration is a diagnostic procedure for LRTD because TA can provide clinicians with important cytological and microbiological information (Chapman et al. 2000). Tracheal aspiration can be easily performed through fibre optic endoscopy (Hodgson and Hodgson 2003; Santos et al. 2007). We performed

the tracheal aspiration technique using fibre optic endoscopy in horses at the stable, without local anaesthesia.

Authors of a study of British racehorses indicated that *S. zooepidemicus* and *S. pneumoniae* play important aetiological roles in the pathogenesis of inflammatory airway disease in young horses and isolated aerobic bacteria such as *E. coli*, *Enterobacter* spp. and anaerobic bacteria such as *Bacteriodes* spp., and *Clostridium* spp. (Sweeney et al. 1991). However, *S. pneumoniae* was not isolated from TA in Japan (Newton and Wood 1999), which indicates that the presence of *S. pneumoniae* can vary geographically. In this study, all TA samples were examined with bacteriological methods and aerobic and facultative anaerobic bacteria as pure and mixed culture were isolated. *Streptococcus zooepidemicus* (27.4%) and *E. coli* (15.6%) were isolated primarily from racehorses with LRTD. On the other hand, α - and β -haemolytic *Streptococcus* spp., coagulase negative *Staphylococcus* spp., *K. pneumoniae*, and *Enterobacter* spp. as aerobic/facultative anaerobic gram-negative bacteria were isolated from tracheal samples. These bacteria play mainly the role as opportunistic pathogens in LRTD. Bond et al. (2017) showed differentiation of lower respiratory tract microbiota between healthy and asthma horses. The genera *Pseudomonas* (14.57%), *Streptococcus* (1.79%) and *Staphylococcus* (3.6 %) belong to the Firmicutes phylum were observed in healthy horses. However, they reported that the abundance of *Streptococcus* spp. was increased in horses with mild asthma. In this study, *Streptococcus* species were the dominant bacteria that may be pathogenic or non-pathogenic in the lower respiratory tract and may cause the main disease or opportunistic infection in horses.

Several reports on LRTD suggest a relationship between respiratory organ inflammation and the bacteria isolated from TA specimens or between neutrophil and bacterial counts in TA specimens (Medica et al. 2010). According to Hodgson and Hodgson (2003), it is not possible to associate an increased number of neutrophils present in the tracheobronchial wash alone to a specific respiratory disease; it is necessary to link this information to other clinical and laboratory tests. According to Takizawa et al. (2005), cytological diagnosis of the tracheal aspirations revealed that observed cells were predominantly represented by neutrophils in 6 of 29 samples (samples with positive bacterial culture in the cytological examination). On the contrary, in the remaining 23 samples, most of the observed cells were pulmonary alveolar macrophages, epithelial cells, or lymphocytes, and neutrophils were rarely or not at all observed (samples with negative bacterial culture in the cytological examination). In this study, a number of different species of bacteria were isolated from 36 tracheal fluid samples. In a comparison between samples with and without bacteria, it appeared that there were differences in the numbers of inflammatory cells. The percentages of neutrophils were significantly higher in samples with positive bacterial culture ($P < 0.001$).

Several reports have suggested a relationship between cytological and clinical examinations (Beech 1975; Fernandes et al. 2011). Fernandes et al. (2011) have reported that TA cytology results showed decreased macrophages and increased neutrophils in response to a bacterial infection. In the same study, the cytological results showed a predominance of macrophages in the control group, with no clinical signs. Beech (1975) has reported that mononuclear cells were increased, according to a cytological examination of healthy horses. There was no control group in the present study, but the percentage of macrophages was higher in the horses with low clinical scores and no bacterial infections compared with the horses with bacterial infections. These results corresponded to the other studies. In this study, bacterial isolation was performed for 50 horses with clinical signs of LRTD; these horses also had higher clinical score compared to the others and 36 of them had positive cultivation. The percentages of neutrophils were significantly higher in the samples with isolated bacteria compared with samples without positive cultivation ($P < 0.001$). While an association between bacterial infection and lower airway inflammation

has been described (Burrell et al. 1996), environmental exposure may also play a major role in this syndrome. Stabled horses can be exposed to very high levels of organic dust that contains a variety of mould and other components capable of inducing airway inflammation (Woods et al 1993; McGorum et al 1998). Authors of this study think that inadequate ventilation and shelter condition can lead to LRTD in horses without positive bacteriology. More detailed studies on this subject are planned.

In conclusion, many studies have emphasized the importance of cytological examination in racehorses in order to determine the cause of cough and decrease in sportive performance. Using the TA technique, airway samples can be obtained without difficulty, resulting in complete understanding of the airway status in horses. In addition, the results of the present study suggest the importance of evaluating the microbiological findings of TA samples in horses suffering from respiratory infections, along with performing a detailed clinical examination and other complementary tests that focus on the respiratory system, such as endoscopy and cytology of the TA. Thus, establishing a definitive diagnosis and the most appropriate therapy becomes feasible for LRTD.

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