Associations between clinical signs, endoscopic and cytological findings in equine bronchoalveolar lavage samples

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Abstract

Background: Endoscopy, including tracheal mucus score (TMS) and bronchoalveolar lavage (BAL) fluid cytology, are commonly used to diagnose equine asthma (EA). Large-scale studies comparing clinical signs, TMS and BAL cytology are warranted. Objectives: Investigate associations between clinical signs of EA, tracheal mucus score and BAL cytology. Study design: cross-sectional study Methods: Data from 604 horses, each horse having one BAL cytology sample evaluated at the laboratory service at the Large Animal Teaching Hospital in Denmark, with further information provided by an owner questionnaire and veterinarian performing the endoscopy and BAL. Results: Tracheal mucus score increased significantly with EA severity, BAL neutrophilia and owner reported coughing, but not with BAL mastocytosis. Owner complaint of coughing was associated with an increasing amount of BAL neutrophils, but not with an increase in BAL mast cells. Poor performance was not correlated to BAL neutrophils, mast cells or TMS. Percentage of BAL aspirated was found lower in the SEA group compared to the non-asthmatic and MEA groups. Main limitations: Several different veterinarians performed the BAL sampling, BAL samples were shipped and a 24 hour delay in processing of the sample. Not able to adjust for season as these data was not recorded. Conclusion: this large-scale study showed a correlation between TMS and increasing severity of lower airway inflammation, confirming TMS as a reliable diagnostic factor for EA. Owner complaint of coughing, was found to be associated with TMS and BAL neutrophilia, and poor performance without coughing is not a reliable parameter for EA.

Original research paper

Title: Associations between clinical signs, endoscopic and cytological findings in equine bronchoalveolar lavage samples

Running title: Endoscopic findings with equine asthma

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Summary

Background: Endoscopy, including tracheal mucus score (TMS) and bronchoalveolar lavage (BAL) fluid cytology, are commonly used to diagnose equine asthma (EA). Large-scale studies comparing clinical signs, TMS and BAL cytology are warranted.

Objectives: Investigate associations between clinical signs of EA, tracheal mucus score and BAL cytology.

Study design: cross-sectional study

Methods: Data from 604 horses, each horse having one BAL cytology sample evaluated at the laboratory service at the Large Animal Teaching Hospital in Denmark, with further information provided by an owner questionnaire and veterinarian performing the endoscopy and BAL.

Results: Tracheal mucus score increased significantly with EA severity, BAL neutrophilia and owner reported coughing, but not with BAL mastocytosis. Owner complaint of coughing was associated with an increasing amount of BAL neutrophils, but not with an increase in BAL mast cells. Poor performance was not correlated to BAL neutrophils, mast cells or TMS. Percentage of BAL aspirated was found lower in the SEA group compared to the non-asthmatic and MEA groups.

Main limitations: Several different veterinarians performed the BAL sampling, BAL samples were shipped and a 24 hour delay in processing of the sample. Not able to adjust for season as these data was not recorded.

Conclusion: this large-scale study showed a correlation between TMS and increasing severity of lower airway inflammation, confirming TMS as a reliable diagnostic factor for EA. Owner complaint of coughing, was found to be associated with TMS and BAL neutrophilia, and poor performance without coughing is not a reliable parameter for EA.

Key words: horse, severe equine asthma, mild-moderate equine asthma, tracheal mucus score, poor performance, cough

Introduction

Equine Asthma (EA) is a highly prevalent disease worldwide (Allen et al., 2006; Courouce-Malblanc et al., 2010; Robinson et al., 2006), and can be further divided into two phenotypes; severe equine asthma (SEA), affecting primarily older horses, and mild-moderate equine asthma (MEA) affecting horses of all ages (Couetil et al., 2016). Horses diagnosed with EA express different severities of coughing, poor performance and increased respiratory effort, either at exercise and/or at rest (Couetil et al., 2020; Koblinger et al., 2011; Robinson et al., 2003). A further subdivision of the MEA phenotype has been proposed to include mild EA for high performance horses with clinically unrecognized airway disease, as well as moderate EA of horses with clinical signs such as coughing and poor performance (Couetil et al., 2020).

A relationship between lower airway inflammation, poor performance, Tracheal mucus score (TMS) and coughing has been noted in both small and large-scale studies (Bedenice et al., 2008; Christley et al., 2001; Fogarty and Buckley, 1991; Holcombe et al., 2006; Lavoie et al., 2011; Widmer et al., 2009). Other, large-scale studies are warranted to investigate associations between clinical signs and diagnostics.

Horse owner questionnaires have shown to be reliable for identifying horses with SEA, whereby the horse owner assessed respiratory signs index (HOARSI) has been validated (Hotchkiss et al., 2006) and used in EA populations (Laumen et al., 2010). Besides owner reported clinical signs, endoscopic examination including scoring of tracheal mucus (Gerber et al., 2004b) and bronchoalveolar lavage (BAL) is considered the gold standard in EA diagnostics (Bond et al., 2018; Couetil et al., 2016). For example, tracheal mucus score is reported to increase with both MEA and SEA cases, poor performance and coughing, and is thus a reliable, important parameter of the endoscopic examination (Almeida et al., 2015; Cardwell et al., 2011; Gerber et al., 2004a; Holcombe et al., 2006; Koblinger et al., 2011; Pirie et al., 2016; Widmer et al., 2009). Tracheal

mucus score between MEA subtypes has been evaluated sparsely, and an increased amount of mucus has been found associated only with the mixed MEA subtype (Nolen-Walston et al., 2013).

The amount of BAL fluid obtained after instilling a volume of 250-500 ml, and manually aspiration, is expected to be between 50-70 percent of the instilled fluid volume (Couetil et al., 2016). The volume aspirated has been found to be lower in horses diagnosed with SEA compared to healthy horses (Couëtil et al., 2005; Jean et al., 2011), but no difference in volume aspirated has been found between MEA subtypes (Richard et al., 2014). This present study investigated the associations between stabling factors, clinical signs of EA, TMS and BAL cytology, including a large group of horses. It was hypothesized that 1) Clinical signs of coughing and poor performance would be associated with BAL neutrophilia, BAL mastocytosis and an increased TMS. 2) The TMS score will increase with an increase in BAL neutrophils and severity of EA diagnosis, but not with an increase in BAL mast cells.3) Volume of BAL aspirated in percentage of BAL instilled would decrease with EA disease severity and lastly, 4) Stabling environment and roughage fed would be associated with the EA diagnosis.

Materials and methods

Data

A cross-sectional study was performed including 604 BAL samples, from clinical cases, submitted by referring veterinarians all over Denmark during the period between 2016-2019, to the diagnostic service at the University of Copenhagen. A questionnaire and further information were provided from both the horse owner and referring veterinarian about the horse, clinical symptoms and BAL procedure. Duplicate samples were excluded from the analysis. Furthermore, data were excluded if another BAL volume than 200-250 ml was used, if shipping had been delayed or if the sample was of a non-diagnostic quality.

Horse and housing variables

Based on questionnaire data obtained from the horse owners, the following variables were collected including age and breed of the horse, along with the clinical history. For the analyses, horses were categorized into three age-groups 0-6 years, 7-14 years and >14 years.

The owners were also requested for information about the stabling facilities/housing types (box, loose housing), bedding material (straw, shavings) and roughage fed (dry hay, soaked hay, silage).

Clinical parameters

Since the two main complaints reported by the owners were poor performance and coughing, then the dataset further sub-divided horses into four groups based on clinical symptoms:

Group 1: no clinical symptoms,

Group 2: poor performance,

Group 3: coughing,

Group 4: both poor performance and coughing (Table 1).

Referring veterinarians were requested for the clinical examination (temperature, pulse, respiration and lung auscultation) and details of the endoscopic examination, including: TMS, BAL volume as well as BAL volume aspirated.

Referring veterinarians were given a protocol on how to perform the endoscopic examination, including how to score mucus and the BAL volume to use (Appendix 1).

Tracheal mucus score (TMS) for each horse was based on a qualitative ordinal scoring system ranging 0-5 (Gerber et al., 2004b) (0 = no mucus, 1 = small blobs, 2 = multiple blobs, 3 = stream-forming, 4 = pool-forming and 5 = profuse amount). A TMS [?]2 was considered acceptable in healthy sports horses (Couetil et al., 2016). Initial BAL volume used was standardized to 200-250 ml, and the amount aspirated as a

percentage of the volume instilled was calculated. Based on the results, horses were sub-divided normal BAL cytology or diagnosed with either MEA or SEA based on alternative BAL cytology reference values (Couetil et al., 2016) and differences in the aspirated volume between EA diagnoses were explored.

Laboratory procedure

The laboratory analyses of the BAL samples shipped by veterinarians from all over Denmark, including cytospin preparations and differential cell counts, were performed by the diagnostic laboratory services at the Large Animal Teaching Hospital in Denmark. The samples were shipped on ice, stored in EDTA tubes, and received by the laboratory within 24 hours. A two-hundred µl aliquot of the BAL EDTA sample was instilled into the funnel of a cytospin (StatSpin® Cytofuge, USA) and centrifuged at 93 g for seven minutes. The cytospin samples were fixed in methanol (ChemSolute®, Th. Geyer, Denmark) and stained with May-Grünwald-Giemsa (Merck, Germany). Slides were examined under light microscopy at 400 x magnification using the five-field counting technique (Fernandez et al., 2013) by experienced observers.

Four-hundred cells were counted and classified as percentages of alveolar macrophages, lymphocytes, neutrophils, mast cells or eosinophils.

Asthma types

Based on the BAL differential cell counts, horses were diagnosed as either with normal BAL cytology, MEA or SEA (Couetil et al., 2016). Horses with normal BAL cytology were defined by a BAL neutrophil count below 10%, a mast cell or eosinophil cell count below 5%; MEA horses by a neutrophil cell count between 10-25%, and/or a mast cell and/or eosinophil cell count above 5% and SEA horses by a neutrophil cell count above 25%. Mastocytic MEA was defined as horses with BAL mast cells count above 5% mast cells, and with BAL neutrophils below 10% and eosinophils below 5%; neutrophilic MEA referred to horses with a BAL neutrophil count between 10-25 % with both BAL mast cells and eosinophils being below 5%.

Statistical analysis

All data analyses were done using the program R version 4.0.2. All data were examined and found to be non-parametric by Shapiro-Wilk's test, visual evaluation of QQ plots and density plots. Descriptive analysis, along with quantile sets, were calculated and box plots were created to describe and visualize the data. The non-parametric Kruskal-Wallis test and Games-Howell post hoc test were used to examine statistically significant differences between the ordinal test groups for continuous outcome variables (BAL cytology and BAL volume instilled and aspirated). Associations between ordinal variables TMS, clinical signs, asthma types, housing factors and age groups were assessed by Chi-square tests of independence. All p values were assessed at a significance level of 5%.

Results

Descriptive analyses

During the data collection period, a total of 737 samples were received at the diagnostic laboratory. Data control was performed and duplicates were removed, as only the first sample from a horse was included. Finally, six hundred and four BAL samples were included, originating from 70 different veterinary practices around Denmark. Forty-seven different horse breeds were represented, with warmblood (n=108) and Icelandic horse (n=89) being the two most common breeds. The mean age of the total population was 10.5 (±4.5) years (Table 1). Bronchoalveolar lavage differential cell counts were done for all horse samples included (Table 2).

Clinical signs

An anamnesis of poor performance without concomitant coughing was not associated with TMS (p=0.060). Horses without an anamnesis of coughing and poor performance, and horses with only poor performance had significantly lower levels of BAL neutrophils compared to the group of horses with coughing (p=0.009 and p=0.001, respectively) and the group of horses with both poor performance and coughing (p=0.005

and p<0.001, respectively). No differences were found between horses with clinical signs coughing or poor performance and the percentage of BAL mast cells (p=0.129).

Tracheal mucus score

A TMS was available for 448 horses, and a significant association between TMS and asthma types was found (p<0.001). The largest contributions to the differences between the observed and expected values in the Chi-square test were due to horses with normal BAL cytology having a TMS of 1 (14.8%), while horses with SEA had a higher frequency of TMS score 4 (15.7%) and score 5 (14%). The TMS plotted against the different BAL cytology diagnosis in shown in figure 1.

The further specification of horses diagnosed with MEA and differentiated into MEA neutrophilic, MEA mastocytic, or MEA mixed diagnoses revealed significant differences in TMS (p<0.001) (Table 2). Horses with normal BAL cytology had a higher frequency of lower TMS scores compared to horse with SEA, with a contribution of horses with normal BAL cytology /TMS scores 0-1 of 13.8% vs. SEA/TMS score 4-5 of 26.7%. Amongst the differentiated asthma types, the MEA mastocytic accounted for the highest proportion of differences in TMS scores with a total contribution of 9%, followed by MEA neutrophilic 6%, MEA eosinophilic 3% and MEA mixed 1.6%. No significant association between TMS and BAL mast cell count was found.

BAL volume aspirated

The BAL volume aspirated was available for 540 horses. No significant difference was found for the group of horses with normal BAL cytology values compared with the group of horses with MEA (p=0.076), but significantly higher compared to the group of horses with SEA (50.0% vs. 40.0%, p=0.035) (table 2).

No significant difference between the BAL volume aspirated and the age of the horse was found, neither when horses were sub-divided into age groups (p=0.139).

Housing

Information on housing (single box or loose-housed in groups) was collected for 523 horses. The Chi- square test revealed dependency between housing and EA groups (p=0.011). Of the horses in loose-housed groups 28% had normal BAL cytology, 34% had MEA and 38% had SEA. Of the horses housed in single boxes, 19% had normal BAL cytology, 49% had MEA and 32% had SEA diagnosis (Figure 2). Information on roughage fed (divided into dry hay n=186, soaked hay n =56 or haylage n=254) was available for 496 horses, the Chi-square test revealed dependency between roughage fed and EA groups (p<0.001). Of the horses with the SEA diagnosis, 56% were fed hay, 14% were fed soaked hay and 30% were fed haylage at the point of examination. For the horses with the MEA diagnosis, 27% were fed hay, 10% were fed soaked hay and 63% were fed haylage.

Discussion

The results from this large-scale study including BAL samples from 604 horses adds to existing knowledge that coughing is the most important owner reported clinical sign with regard to EA, as coughing was found to be associated both with an increase in both TMS and BAL neutrophils. Furthermore, TMS showed, alongside with coughing, significant associations with both EA diagnosis, and BAL neutrophils.

Horses with an anamnesis of coughing, or a combination of coughing and poor performance, had a significantly higher TMS and a higher percentage of BAL neutrophils than horses reported solely with poor performance. Several studies have found associations between coughing, lower airway neutrophilia and TMS in both sport horses and racehorses (Almeida et al., 2015; Bedenice et al., 2008; Bosshard and Gerber, 2014; Leguillette et al., 2016; Wasko et al., 2011). In the present study, poor performance was found to have significantly lower TMS than the groups with complaints of coughing; this can be explained by the multifactorial nature of poor performance (Ellis et al., 2022), as well as uncertainties regarding both the poor performance definition and owner reliability (Richard et al., 2010; Salz et al., 2016). BAL mast cells were not associated with neither coughing nor poor performance in this study. Mastocytic MEA has been described as an allergic asthma

type, with primarily a T_2 helper cell response (Beekman et al., 2012; Hansen et al., 2020), and with clinical signs primarily associated with airway hyper-responsiveness (Secombe et al., 2019). Mastocytic MEA was previously found not to be associated with coughing (Bedenice et al., 2008), in line with the results from this study/our present findings.

Owner questionnaires have been widely included in studies of lower airway inflammation, and the owner reliability to detect SEA was found to be excellent, whereas owners' ability to detect MEA was limited to a degree that they could not differentiate between MEA and healthy horses (Laumen et al., 2010; Wasko et al., 2011). In the present study, 24 horses diagnosed with SEA were not reported to cough; this can reflect a bias, as some of the horses were only used for pasture pets with limited owner recognition of coughing.

The TMS used in this study is based on a published study (Gerber et al., 2004b) and validated (Leguillette et al., 2016), as well as widely used in the EA literature (Holcombe et al., 2006; Robinson et al., 2006). Furthermore, the latest consensus statement on MEA includes a TMS [?] 2/5 for sports horses and [?]1/5 for racehorses as one of the diagnostic criteria for MEA (Couetil et al., 2016).

In this study, TMS increased from the group of horses with normal BAL cytology, to horses with the MEA and SEA diagnoses. Furthermore, TMS increased with elevated BAL neutrophil cell percentages, in agreement with previous studies (Koblinger et al., 2011; Wysocka and Klucinski, 2014, 2015). A recent study reported poor sensitivity of the TMS as diagnostic criteria for MEA (Dauvillier et al., 2019).This in line with our findings, as no correlation was identified between TMS and the amount of BAL mast cells. In this study, differences were found between the MEA subtypes, in line with a study by Nolen-Walston et al. (2013) whom found that horses with the mixed MEA diagnosis were more likely to have an increased TMS than control horses; likewise, a study by Wysocka and Klucinski (2015) reported a negative correlation between TMS and*eosinophilic MEA* .

This study found a significantly lower volume aspirated in SEA horses than horses with normal BAL cytology and the MEA diagnosis, this is in line with a study by Couetil et al. (2005) that found significant less volume aspirated before treatment than after treatment for a group of EA horses. In the present study, no age-related effect was found with the BAL volume aspirated. Using a larger instilled volume with subsequent manual aspiration of BAL fluid compared to mechanical suction would both significantly increase aspirated BAL volume (Orard et al., 2016), whereas the use of N-butylscopolammonium bromide did not have any effect on BAL volume aspirated (Bowser et al., 2018).

In this study, a positive correlation was found between higher BAL neutrophil cell percentages and increasing age. This highly likely reflect that SEA is predominantly a disease of older horses as this is in agreement with the literature reporting SEA predominantly as a disease of older horses (Couetil et al., 2016; Jocelyn et al., 2018; Robinson, 2001). The population of horses with BAL cytology within normal limits, no age-related changes in BAL differential cell count has been identified (Christmann et al., 2009; Hansen et al., 2013), except one single study showing a decrease in BAL lymphocyte count with increasing age (Gerber et al., 2003). Mild-moderate equine asthma is a more prevalent disease in high performance horses and pleasure horses of all ages (Couetil et al., 2016); in line with this, the results from our study found no age-related changes with regard to BAL mast cells.

Results from this study demonstrated that housing type and roughage fed were dependent on the stabling environment. Of hoses with the SEA diagnosis, 32% were stabled in a single box and 56% were fed hay. Feeding hay has been associated with airway inflammation (Olave et al., 2021), whereby soaking or steaming of the hay, or changing the roughage to haylage has been recommended (Clements and Pirie, 2007a, b; Dauvillier et al., 2019). Loose-housing is often recommended for horses with airway inflammation, but the specific design of the actual loose housing system needs to be considered in order to determine if such conditions are beneficial (Hansen et al., 2019).

Limitations of the study included the heterogeneity of the sampling, several different veterinarians included, shipping of the samples as well as the inability to adjust for year and month of sampling (data not available) add to limitations. With regard to year and month of sampling, a previous study found no effect of season

on TMS (Nolen-Walston et al., 2013) and with equally high MEA diagnosis year round (Davis and Sheats, 2019).

In conclusion, this large-scale study found that owner reported coughing was significantly associated with TMS and BAL neutrophilia. Furthermore, TMS increased with severity of BAL neutrophils and EA diagnosis. BAL mast cells and mastocytic EA was not associated with either owner complaints, TMS, or ageing. Although, several of the study results have been reported previously, none of these studies included a sample size and diversity of horse population matching the present study.

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Conflict of interest: no conflict of interest to declare.

Ethical approval: Research ethics committee approval was granted from the University of Copenhagen

Informed consent: Explicit owner informed consent for inclusion of samples from animals in this study was not sought but owners were aware that excess material from clinical samples would be retained for research; in general, all owners have the option to opt out of research.

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Figure legends

Figure 1: Distribution of tracheal mucus scores among horses with normal bronchoalveolar lavage (BAL) cytology, with mild-moderate equine asthma (MEA) diagnosis, based on reference values >10% neutrophils, >5% mast cells or eosinophils, and severe equine asthma (SEA) with reference values >25% neutrophils. Significant differences were found between all groups.

Figure 2: The distribution of horses with normal BAL cytology, mild-moderate equine asthma (MEA) and severe equine asthma (SEA) sub-divided into horses stabled in individual boxes and horses in loose-housing systems.

Table 1: Horses included in the study, sub-divided into groups based on owner complaints (coughing, poor performance). For each group, age and bronchoalveolar lavage (BAL) cytology results are shown, cytological diagnoses are based on reference values for mild-moderate equine asthma >10% neutrophils, >5% mast cells or eosinophils, and SEA >25% neutrophils.

	No coughing or poor performance	Poor performance	cough	Poor performance and cough
Number of horses	63	297	435	225

	No coughing or poor performance	Poor performance	cough	Poor performance and cough
Age	9.4 (±4.2)	10.7 (±4.4)	10.8 (±4.7)	11.0 (±4.5)
Normal BAL cytology	24	60	83	43
MEA	46	141	186	96
SEA	24	93	165	83

BAL = bronchoalveolar lavage; MEA = mild-moderate equine asthma; SEA = severe equine asthma. Age showed as mean and standard deviation.

Table 2: Bronchoalveolar lavage (BAL) cytology results for the included horses, and divided into groups based on cytological diagnosis, based on reference values for mild-moderate equine asthma >10% neutrophils, >5% mast cells or eosinophils, and SEA >25% neutrophils.

	Normal BAL cytology	MEA	Mastocytic MEA	Neutrophilic MEA	Mixed MEA
Number of horses	122	283	79	136	64
Age (mean std)	10.1 (±4.5)	10.2 (±4.8)	9.4 (±4.7)	10.8 (±5.0)	9.9 (±4.5)
% BAL aspirated	50.0 (32.7-60.0)	44.0 (28.9-54.0)	50.0 (30.0-55.6)	41.7 (28.9-50.0)	40.0-(25.0-50.0)
Mucus score (0-5)	1 (1-2)	2 (1-3)	2 (1-3)	2 (1-3)	2 (1-3)
% BAL macrophages	68.8 (48.9-76.5)	58.0 (52.0-65.0)	66.0 (59.5-72.5)	57.0 (52.5-62.0)	52.5 (46.6-55.8)
% BAL lymphocytes	24.5 (16.9-32.5)	22.5 (16.5-27.5)	21.5 (15.0-27.5)	23.0 (16.6-27.5)	23.0 (19.0-29.4)
% BAL neutrophils	5.0 (2.0-7.0)	14.0 (7.5-19.0)	4.0 (2.0-7.0)	17.0 (13.5-21.0)	16.0 (13.0-19.0)
% BAL mast cells	2.3 (1.0-3.5)	5.0 (2.0-7.0)	7.0 (6.0-9.0)	2.0 (1.0-3.0)	7.0 (5.5-8.8)
% BAL eosinophils	0.0 (0.0-0.5)	0.5 (0.0-1.0)	0.0 (0.0-0.5)	0.5 (0.0-0.5)	0.8 (0.0-

BAL = bronchoalveolar lavage; MEA = mild-moderate equine asthma; SEA = severe equine asthma. Age reported as mean and standard deviation, all other parameters are reported by median and 25-75 interquartile range due to data not being normally distributed.