



Research paper

Bronchoalveolar lavage fluid cytokine, cytology and IgE allergen in horses with equine asthma

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ABSTRACT

The pathophysiology of equine asthma (EA) is still not fully described, but the involvement of an allergic reaction is strongly suspected. This theory has led to the use of allergen-specific immunoglobulin (IgE) tests to support a diagnosis of asthma. The objective of this descriptive study was to evaluate the correlation between four subgroups of EA (mastocytic mild equine asthma [MEA], neutrophilic MEA, mixed MEA, and severe equine asthma [SEA]), allergen specific IgE (measured in both serum and BALF) and mRNA expression of selected genes in bronchoalveolar lavage fluid (BALF). Serum and BALF were collected from 64 horses with a history of lower airway problems with or without poor performance. Differential cell counts from BALF were used to assign horses to one of four groups (mastocytic MEA; neutrophilic MEA, mixed MEA, and SEA). The expression of messenger RNA (mRNA) coding for IL4, IL5, IL8, IL10, TGFB, TNFA, toll-like receptor (TLR)4, IL1RA, IL1B, matrix metalloproteinase 8 (MMP8), TLR9, chemokine ligand 5 (CCL5) and cluster of differentiation (CD)14 in BALF were measured using reverse transcriptase (RT) quantitative PCR (qPCR). Allergen-specific IgE was measured in serum and BALF using an allergen-specific IgE ELISA test with the screening panel: house mites, storage mites, mould and pollen. As expected, the BALF neutrophil differential count correlated with mRNA expression of MMP-8 ($r = 0.611$, $p < 0.001$), TLR-4 ($r = 0.540$, $p < 0.001$), IL-1RA ($r = 0.490$, $p < 0.001$), IL-1 β ($r = 0.463$, $p < 0.001$) and IL-8 ($r = 0.302$, $p = 0.015$). Cytokine expression of IL-1 β ($p = 0.014$), MMP8 ($p = 0.028$) and IL-1RA ($p = 0.037$) was significantly higher in the SEA group compared to the MEA subgroups. The BALF mast cell count was correlated with allergen-specific IgE for insects ($r = 0.370$, $p = 0.002$) and pollen ($r = 0.313$, $p = 0.011$). Eosinophils in BALF were correlated with BALF mRNA expression of IL-4 ($r = 0.340$, $p = 0.006$) together with a significant correlation between BALF eosinophils and allergen-specific IgE for mites ($r = 0.930$, $p < 0.001$) and pollen in BALF ($r = 0.837$, $p < 0.001$). No correlation was found between allergen-specific IgE in serum and BALF for any of the allergen in the screening panel. Based on these results from allergen-specific IgE in horses with EA is not found in systemic circulation, and only the mastocytic and mixed subgroups of horses with EA had allergen-specific IgE in BALF. Further studies are needed to clarify the relationships identified here.

1. Introduction

The diagnosis of equine asthma (EA) is often based on a combination of clinical signs such as coughing and poor performance, and partly on endoscopic examination including mucus score and bronchoalveolar lavage fluid (BALF) cytology (Couetil et al., 2016). Equine asthma is

categorized into mild-moderate EA (MEA) and severe EA (SEA) based on the severity of the clinical signs, endoscopic examination findings, including mucus score (Gerber et al., 2004), and BALF cytology results. Affected horses have excessive tracheal mucus noted endoscopically and an increased number of inflammatory cells in BALF cytology (Couetil et al., 2016). Equine asthma can be further divided into

Abbreviations: ACTB, actin beta; BALF, bronchoalveolar lavage fluid; B2M, β 2-microglobulin; CCL, chemokine ligand; CD, cluster of differentiation; EA, equine asthma; HERBU, Heska Epsilon Receptor Binding Units; MMP, matrix metalloproteinase; MEA, mild equine asthma; RPL, ribosomal protein; RT, reverse transcriptase; SEA, severe equine asthma; TATABP, TATA box binding protein; TLR, toll like receptor

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subgroups based on the predominant cell type exhibited on BALF cytological examination. Different cell profiles in BALF are associated with different clinical symptoms, for example, mastocytic and eosinophilic MEA have been associated with airway hyperresponsiveness (Hare and Viel, 1998; Richard et al., 2009) and neutrophilic MEA with cough (Bedenice et al., 2008). The different subtypes of asthma have, to the authors knowledge, not been associated with different cytokine pathways (Richard et al., 2014).

The aetiology and pathogenesis of EA has not been clearly defined and various theories about the underlying immunologic cause have surfaced over time. The most common theories on the immune pathogenesis in EA involve a Th2 cytokine response, also seen in human allergic reactions (Lavoie et al., 2001; Cordeau et al., 2004; Beekman et al., 2012), a nonspecific Th1 innate immune response (Padoan et al., 2013; Richard et al., 2014; Ainsworth et al., 2003), or a combination of both (Niedzwiedz et al., 2016; Horohov et al., 2005). It has furthermore been suggested that some of these immune pathogenesises are linked to the BALF cell profile exhibited in the asthmatic horse (Richard and Robinson, 2016).

Several studies have evaluated the possible involvement of IgE in the EA syndrome (Verdon et al., 2019; Niedzwiedz et al., 2015; Kunzle et al., 2007) with discrepant results. However, the possibility of EA originating from an allergic response, has led to the development of a commercial allergy IgE specific test, the Allercept® screening test. The Allercept® screening test was adapted from use in humans, where allergic asthma is most common, and where eosinophils and IgE are known to be responsible for the clinical presentation (Schatz and Rosenwasser, 2014). It is an ELISA-based test, which determines the concentration of IgE in a sample using a recombinant human FcεR1α specific IgE.

The objective of this study was to evaluate the correlation between 4 subgroups of EA (mastocytic MEA, neutrophilic MEA, mixed MEA, and SEA), allergen specific IgE, measured in both serum and BALF, and mRNA expression of selected genes in BALF. We hypothesised that IgE concentrations would be positively correlated with the percentage of BALF mast cells and eosinophils but not neutrophils. Furthermore, we hypothesised that BALF mRNA expression of Th2 cytokines but not Th1 would be positively correlated with one or multiple allergen specific forms of IgE. Finally, we hypothesised that no correlation between allergen specific IgE in serum and BALF would be found in horses with EA.

2. Materials and methods

2.1. Study population

Horses were prospectively selected among patients referred to the Large Animal Teaching Hospital, Denmark, with a history of lower airway problems and poor performance. Sixty-four horses with a mean age of 10.7 ($\pm$ 4.6) years representing eight different breeds were included in the study. The gender distribution was 27 mares, 35 geldings and 2 stallions. Inclusion criteria were history of poor performance with or without symptoms of lower airway problems in addition to abnormal BALF cytology. Exclusion criteria were abnormalities detected on haematology, upper airway pathology and/or increased rectal temperature. Ethical approval for the procedures was obtained from the University of Copenhagen Large Animal Teaching Hospital Ethical Committee. All samples were from client owned horses and were collected as a part of the routine diagnostic evaluation and with the owner's permission. The full study was approved by the Danish Council for Animal Experiments.

2.2. Sample collection

Blood samples were collected via jugular venipuncture in plain tubes before sedation. Samples were spun down after 10 min and serum

was frozen at -20°C for a maximum of 2 month before analysis. For the endoscopic exam, horses were sedated with 0.01 mg/kg detomidine (1.0 mg/ml, Orion Pharma, Denmark). The endoscope (3000 mm, dia. 9.8 mm, E-vet, Denmark) was inserted through the left nostril, and advanced to the fourth to sixth generation of bronchi in the right caudo-dorsal lung field. Two-hundred and fifty ml of sterile sodium chloride (0.9 %, Braun, Denmark) was inserted as one bolus and immediately manually aspirated with 60 ml syringes. The amount aspirated (44.0 ± 10.6) was recorded and eight ml of the BALF sample was transferred to an EDTA tube for cytological evaluation and to a plain tube for antibody screening test. The remaining sample was filtered into 50 ml tubes through a 100 nm strainer to remove mucus and other debris. The sample was centrifuged at 400g for ten minutes, the supernatant was removed and the cell pellet resuspended in PBS and centrifuged again. The pellet was resuspended in 3 ml sterile PBS (pH 7.2) transferred to PaxGene Blood RNA tube containing a proprietary solution to prevent RNA degradation (Qiagen, Denmark), kept at room temperature for 24 h, and then frozen at -80°C for later RNA extraction. The paxGene Blood RNA tube contain a patented blend solution that prevents degradation of RNA. The BALF plain tube sample for allergen screening was stored at -20°C until further processing.

2.3. Cytology

Samples were processed within 30 min of collection, 200 μl of BALF EDTA sample was transferred to the funnel of a cytospin (StatSpin® cytofuge, USA) and centrifuged for seven minutes at 93g. Samples were afterwards fixed in methanol (ChemSolute®Denamrk) and manually stained with May-Grünwald-Giemsa (Th. Geyer, Denmark). Five hundred cells were classified and counted by an experienced observer (SH) through light microscopy under high magnification ($400\times$). A modified 5-field leukocyte counting technique was used, counting 100 cells per field (Fernandez et al., 2013). Cells were classified as alveolar macrophages, lymphocytes, neutrophils, mast cells or eosinophils and were expressed in percentages of the total count. Reference values used for this study were neutrophils $\leq 5\%$, eosinophils $\leq 1\%$, and mast cells $\leq 2\%$ as described in Couetil et al. (2016).

2.4. Reverse transcription (RT) quantitative real-time PCR (qPCR)

2.4.1. RNA extraction

RNA from BALF samples was extracted from PAXgene tubes® as previously described (Vinther et al., 2015) using PAXgene Blood RNA Kit (Qiagen, Denmark) following the manufacturers protocol with a few modifications. To homogenise insoluble pellets, 1 ml acetylcysteine 200 mg/ml (solvidine vet, Virbac, Denmark) was added between the two centrifuge steps, and the pellet vortexed until dissolved. Contrasting with the manufacturer's protocol, 80 μl of proteinase K (PK, Qiagen, Denmark) were added to the solution. Concentration and purity of the total extracted RNA was measured by spectrophotometrically (Thermo Scientific, USA) at an OD of 260 nm and 280 nm, respectively. Concentration varied from 7.4 ng/ μl to 418.6 ng/ μl and all samples achieved a purity of A260/A280 between 1.96 and 2.10.

2.4.2. cDNA synthesis

Synthesis of cDNA was performed using QuantiTect Reverse Transcription Kit (Qiagen, Denmark). cDNA was synthesized in duplicate for each RNA sample. Purified RNA samples were incubated in gDNA Wipeout Buffer (Qiagen, Denmark) for removal of contaminating genomic DNA. Reverse transcription of RNA to cDNA took place in three consecutive steps. The RNA sample was mixed with master mix, consisting of Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix. The mixture was placed in a Biometra thermalcycler (Fischer scientific, Denmark). Reverse transcription activation occurred at 42°C prior to inactivation at 95°C .

The samples were then diluted 1:10 in low EDTA buffer

(Bionordika, Denmark) and stored at -20°C until further processing. Three negative reverse transcript (-RT) samples were made during the procedure for later use as control samples.

2.4.3. Target genes

Relative gene expression in BALF cells was established by quantification of specific mRNA coding for the following genes: IL4, IL5, IL8, IL10, TGFB, TNFA, toll like receptor (TLR)4, IL1RA, IL1B, matrix metalloproteinase (MMP)8, TLR9, chemokine ligand 5 (CCL5) and cluster of differentiation (CD)14. Gene selection was based on relevance in relation to asthmatic and allergic reactions in horses and humans. Actin beta (ACTB), β 2-microglobulin (B2M), TATA box binding protein (TATAPB) and ribosomal protein (RPL) 32 were selected as reference genes after a NormFinder (Andersen et al., 2004) and GeNorm (Vandesompele et al., 2002) search. List of gene names and primer sequence are included in Table 2.

2.4.4. Amplification, exonuclease treatment and qPCR

Amplification, exonuclease treatment and qPCR were performed as previously described (Vinther et al., 2015). Briefly, Primer Mix TaqMan PreAmp Master Mix (Applied Biosystems, Denmark), low EDTA TE-buffer (VWR International, USA) were mixed with the cDNA and incubated in a thermocycler (Fischer Scientific™ Denmark) for 19 cycles (95°C for 10 min followed by 95°C for 15 s and 60°C for 4 min). Unincorporated primers were eliminated by exonuclease treatment using exonuclease I (New England Biolabs, USA). Previously amplified and exonuclease treated samples were diluted in low EDTA TE Buffer and stored at -20°C . A BioMark HD (Fluidigm Corporation, USA) was used to perform the qPCR, using the 192.24 dynamic array (Fluidigm Corporation, USA) combining 192 samples with 24 primer sets in 4608 separate qPCR reactions. Duplicated cDNA samples, -RT samples, mock samples and NTC samples all served as different quality control points for each process and were used for data processing and validation of the results.

2.5. Allergen screening

Frozen serum and BALF samples stored in plain tubes were shipped to the Heska Reference

Laboratory, Vet-allergy (Nibe, Denmark) for the equine Allercept® allergen screening panel. The screening panel consisted of a variety of moulds, mites, pollen, and insect extracts (Table 1). IgE concentration results were reported in Heska Epsilon Receptor Binding Units (HERBU), ranging from 0 to 2600 HERBU. The Heska Allercept® allergen test is designed to measure the concentration of allergen-specific IgE by a non-competitive, solid phase ELISA using a cloned human alpha receptor (FcεR1α), a molecule that binds specifically and exclusively to IgE (Stedman et al., 2001). It has previously shown reactivity with equine IgE (Frey et al., 2008; Niedzwiedz et al., 2015) and there is 64 % amino acid identity and a 76 % homology between the equine and human alpha chain has been described (Jensen-Jarolim et al., 2015).

Table 1

The Allergen screening panel used for the allergen specific IgE commercial ELISA test (Allercept®) using the human FcεR1α chain.

Allergens in screening panel:	
House mites:	<i>D. farina</i> , <i>D. pteronyssinus</i>
Storage mites:	<i>Tyrophagus</i> , <i>Acarus siro</i>
Mould:	<i>Alternaria</i> , <i>Penicillium</i> , <i>Rhizopus nigricans</i>
Pollen1:	<i>Fagus sylvatica</i> , <i>Salix caprea</i>
Pollen2:	<i>Rumex crispus</i> , <i>Urtica dioica</i> , <i>Dactylis glomerata</i>
Insects:	<i>Culicoides</i> , <i>Tabanus</i> , <i>Simulium</i> , <i>Culex</i>

2.6. Data processing and statistical analyses

Gene expression data were corrected for primer efficiencies using GenEx5 (MultiD, Sweden). Normalisation was performed using the reference genes *ACTB*, *B2M*, *RPL32* and *TATAPB*. A difference in quantification cycle (ΔCq) below ± 1.5 for the cDNA duplicates was accepted. For each gene, the expression level of the sample with the highest Cq value (lowest expression) was set to 1 and all other samples were scaled accordingly when transforming the data from Cq ($\log_2$) to relative expression.

Statistical analyses were performed using SAS 9.3 (SAS Institute, USA). Data was probed for associations between the quantitative measures of the genes encoding IL4, IL5, IL8, IL10, TGFB, TNFA, TLR4, IL1RA, IL1B, MMP8, TLR9, CCL5 and CD14 within BALF, the allergen-specific IgE concentrations in both serum and BALF, and BALF cytologic diagnosis. Log transformations of the quantitative gene outcome variables resulted in normal distribution and were used in the analyses. Correlations between continuous variables were assessed by PROC CORR (SAS Institute, USA) using Pearson's correlation coefficient (r). Associations between asthmatic groups were assessed by a mixed linear model using PROC MIXED (SAS Institute, USA) with a p-value < 0.05 .

3. Results

BALF cytology results was used as the gold standard for classification of horses as asthmatic (Couetil et al., 2016) and horses were assigned into one of four groups dependent on the cell types present in BALF (Table 3). Of the 64 horses examined, 25 horses (10.0 ± 3.9 years of age) were included in the mastocytic MEA group, nine horses (9.3 ± 4.1 years of age) were included in the neutrophilic MEA group, 21 horses (10.5 ± 3.9 years of age) were included in the mixed MEA group and nine horses (14.7 ± 6.2 years of age) were included in the SEA group. Horses diagnosed with SEA were significantly older ($p = 0.010$) than horses in the MEA groups.

3.1. Equine asthma and cell types in BALF

A significant positive correlation between the ratio of BALF neutrophils and BALF mRNA expression of MMP8 ($r = 0.611$, $p < 0.001$), TLR4 ($r = 0.540$, $p < 0.001$), IL1RA ($r = 0.490$, $p < 0.001$), IL1B ($r = 0.463$, $p < 0.001$) and IL8 ($r = 0.302$, $p = 0.015$) respectively was found. A significant increase in BALF cytokine expression of IL1B ($p = 0.014$), MMP8 ($p = 0.028$) and IL1RA ($p = 0.037$) was found in the SEA group compared to the MEA groups. No correlation between BALF neutrophils and any of the screening panel allergen-specific IgE subtypes was found in either serum or BALF.

BALF eosinophil cell count and the BALF mRNA expression of IL4 ($r = 0.340$, $p = 0.006$) were significantly correlated. A positive correlation between BALF eosinophils and BALF allergen-specific IgE for mites ($r = 0.930$, $p < 0.001$) and pollen2 ($r = 0.837$, $p < 0.001$) was found.

A weak but significant correlation was found between the BALF mast cell ratio and allergen-specific IgE for insects ($r = 0.370$, $p = 0.002$) and pollen1 ($r = 0.313$, $p = 0.011$) in BALF.

3.2. Allergen panel

The Heska Allercept® screening test was performed on serum and BALF samples from each horse. Three horses (3/64) were negative for allergen-specific IgE in serum and 11 horses (11/64) had allergen-specific IgE in BALF. No correlations was identified in allergen panel testing results between serum and BALF. A significant correlation between mould-specific IgE in BALF, and IL5 ($r = -0.440$, $p < 0.001$) and CD14 ($r = 0.320$, $p = 0.010$) gene expression was found. Horses positive for pollen (both 1 and 2) -specific IgE in serum had significantly higher gene expression of CD14 ($p < 0.001$), MMP8 ($p = 0.012$) and

Table 2

List of genes and primer sequences used in this study.

	Gene of interest	Sequence (5' to 3')	Sequence (5' to 3')
ACTB	Actin, Beta	CAGTGGCATCCACGAACTA	AGCACTGTGTTGGCGTACAG
B2M	Beta-2-Microglobulin	TTACTCAGCTCACCAGCAG	ATTTCATCTCAGGCGGATG
CCL5	C-C Motif Chemokine Ligand 5	CCAGCAGTCGTCTTTGTAC	GGCCTCCAATCCTAGCTCAT
CD14	Cluster of differentiation 14	CTGCCCGTGGTGAGTAATCT	GGAGTTCTGGTCTTGCTGCT
IL1RA	Interleukin 1 Receptor Antagonist	ACAAATGTGGCTCCTCCAAG	TTCAGAGCGTCAGAAGTGC
IL1B	Interleukin 1 Beta	CAGCTCTCAGTGCTCAGTTTCTG	CATTGCCGCTGCAGTAAGT
IL10	Interleukin 10	GCCTTCAGTAAGCTCCAAGAG	CCCTAGGATGCTTCAGTTTTC
IL4	Interleukin 4	CTGCAAGGTGCTTCAACAG	TTGAGTTCTCTGCCAGTCC
IL5	Interleukin 5	TGTGGCCAACTATTCCAAA	TCTCCATCTTTCTCCTCCACA
IL5	Interleukin 5	TGTGGAGGAGAAAGATGGAGA	TCCGTGTCCACTCAGTGTT
IL8	Interleukin 8	CGTTTTTGAAGAGAGCTGAGG	GCTTGAAGTTTCATTGGCATC
MMP8	Matrix Metalloproteinase 8	CTGGAGATATGATAACCAAGACG	CTTGCTGGAAAATGCATCA
RPL32	Ribosomal protein 32	GTGCAACAAATCGTACTGTGC	GGGATTGGTGATTCTGATGG
TATABP	TATA box binding protein	CACCAGCAGTTTAGTAGTTATGAGC	AGGAGAACAAATCTGGGTTTGA
TLR4	Toll-like receptor 4	GCCAGGGAAGTCAACTCAA	TGGGAGACGATGCTCTTTTC
TLR9	Toll-like receptor 9	TGGTAATCTCTGAGCCCTGAC	GGGCCAGAAGAGGACACTC
TGFB	Transforming Growth Factor Beta	CTGTTTCAGCTCCACAGAGAAG	AGAAGTTGGCGTGGTAGCC
TNFA	Tumor Necrosis Factor Alpha	GTGTAGCAAAACCCCAAG	GGTTGTCTGTGAGCTTCACG

TLR4 ($p = 0.05$) in BALF than horses negative for pollen-specific IgE in serum.

4. Discussion

This is the first study where serological testing using an allergen-specific IgE test was performed on both serum and BALF in a group of asthmatic horses with different cytological profiles. We found that there was no relationship between allergen-specific IgE measured in serum and BALF, confirming one of our hypotheses. This finding suggests that local antibody production is not reflected systemically (Powe et al., 2010), and is consistent with previous work (Schmallenbach et al., 1998; Scharrenberg et al., 2010; Verdon et al., 2019), but contrasts with others that identified a correlation between mite-specific IgE and mould-specific IgE in serum of SEA-affected horses (Niedzwiedz et al., 2015; Kunzle et al., 2007; Tahon et al., 2009).

IL8 is a neutrophil-specific chemoattractant protein produced by pulmonary macrophages and endothelial cells (Franchini et al., 2000) and was positively correlated with increased BALF neutrophil percentage together with IL1B, IL10, TLR4, IL1RA and MMP8 in this study. Neutrophilic infiltration present in SEA has been established as an IL8 driven reaction (Nolen-Walston et al., 2013; Ainsworth et al., 2006, 2003). Furthermore, IL17 expressed by neutrophils initiates the inflammatory response by activation the expression of IL8, TNFA and IL1B among others (Ainsworth et al., 2006). In the present study, BALF MMP8 mRNA was significantly upregulated, and positively correlated with BALF neutrophils in the SEA group. In horses with SEA MMP8 was upregulated (Raulo et al., 2001) and studies have demonstrated a central role of MMPs in human asthma (Prikk et al., 2002; Gueders et al., 2005). Results from the present study showed that BALF mRNA expression of TLR4 was correlated with BALF neutrophil count, similar to a previous study where TLR4 was upregulated in horses with SEA

after a 2-week hay challenge (Ainsworth et al., 2006).

BALF neutrophil cell count correlated with mRNA expression of the IL1RA gene, an endogenous antagonist of the pro-inflammatory cytokine IL1B. Furthermore, IL1RA was found to be upregulated in the SEA group compared to the MEA groups. Similar to this study, an upregulation of IL1RA and IL1B have previously been found in EA horses (Giguere et al., 2002; Padoan et al., 2013; Beekman et al., 2012; Hughes et al., 2011).

We found a positive correlation between the BALF mRNA expression of IL4, BALF eosinophils and allergen-specific IgE against mites and pollen2 measured in BALF. It may be that an allergic reaction to mites or pollen2, in the presence of IL10 and IL4, leads to stimulation of BALF neutrophils in asthmatic horses, increasing the expression of IL1RA. Supporting this hypothesis, a human study revealed that IL4 stimulated neutrophils in the presence of IL10 lead to an increased release of IL1RA (Crepaldi et al., 2002). IL10 is involved in the differentiation of CD4⁺ cells to Th2 cells and subsequently the production of the Th2 type cytokine IL4. Th2 cells regulate IgE production through release of IL4 and IL13, function through the production of IL5 (Matucci et al., 2018), and are involved in hypersensitivity reactions (Robinson et al., 1992).

Another interesting role for the BALF neutrophil has been suggested in the allergic asthma syndrome of humans. In vivo release of eosinophil cationic protein from neutrophils has been verified (Monteseirin et al., 2007), leading to speculations that the neutrophil could act as a regulator of the eosinophil and IgE in hypersensitivity.

The presence of eosinophils in BALF is characterized as one of three subtypes of MEA (Couetil et al., 2016; Hare and Viel, 1998; Moore et al., 1995). Previous studies found an increase in BALF eosinophils in relation to respirable dust exposure (Ivester et al., 2014) and during summer months (Riihimäki et al., 2008). In the present study, only one horse, a three-year-old Friesian had an increased BALF eosinophil count (17.4 %); this horse had normal eosinophils on haematology and a low

Table 3

Bronchoalveolar lavage fluid (BALF) differential cell count distribution. Horses were divided into subgroups based on the BALF cytology. Cell counts are represented as interquartile range.

	Mastocytic MEA (n = 25)	Neutrophilic MEA (n = 9)	Mixed MEA (n = 21)	SEA (n = 9)	Total (n = 64)
Alveolar macrophages	60.5 (53.3–67.5)	54.5 (51.5–62.5)	54.5 (51.5–62.5)	37.5 (27.0–47.8)	56.0 (49.6–63.8)
Lymphocytes	29.0 (24.5–36.0)	32.0 (26.8–34.3)	25.0 (20.5–34.8)	19.0 (15.8–32.5)	28.8 (21.8–35)
Neutrophils	2.0 (1.5–3.5)	12.5 (9.3–15.0)	9.5 (6.3–12.3)	31.5 (27.0–42.3)	7.0 (2.5–12.9)
Mast cells	4.5(3.5–6.0)	0.5(0.0–1.5)	4.5(3.0–6.0)	2.0(0.5–4.0)	4.0(2.0–5.5)
Eosinophils	0.0(0.0–0.0)	0.0(0.0–0.5)	0.0(0.0–1.5)	0.0(0.0–0.8)	0.0(0.0–0.5)

MEA: mild to moderate equine asthma, SEA: Severe equine asthma.
500 cells were counted in each sample.

faecal egg count of 100 egg/gram faeces (reference interval 0–500 egg/gram faeces, Nielsen et al., 2010). Further studies are needed on mastocytic and eosinophilic subtypes of MEA to further evaluate an association with allergen-specific IgE in BALF, but these preliminary results confirm our hypothesis.

In this study, the BALF mRNA expression of CD14 showed a positive correlation with BALF mould-specific IgE. CD14 is the primary receptor for LPS, and was shown to be upregulated in SEA (Wagner et al., 2013). In a human study, CD14 protein expression correlated with airway eosinophils and the Th2 type cytokine IL13 in asthmatics (Alexis et al., 2001; Virchow et al., 1998). A study in mice showed that exposure to LPS suppressed the FcεR1 IgE surface receptor on mast cells causing a transient loss of sensitivity to IgE activation (Wang et al., 2017). In this study, a correlation between allergen-specific IgE for mould in BALF and the mRNA expression of the CD14 receptor gene was found, which agrees with previous studies suggesting a central role for this receptor in local allergic reactions (Eguiluz-Gracia et al., 2016).

The test used in this study is an ELISA that uses the human FcεR1α chain to bind IgE specifically. IgE is responsible for the immediate hypersensitivity reaction, but other immunoglobulins such as subclasses of IgG may also be involved in the immunopathogenesis of EA. Other studies have shown an upregulation of both IgE and IgG in asthmatic horses, (Schmallenbach et al., 1998; Eder et al., 2000). A limitation of the present study was that only IgE was measured.

Limitations of this study includes the lack of a control group, as all horses were given a diagnosis of either MEA or SEA. Sampling took place over a one-year period, and at different time point in each horse's disease progression. While time of sampling in relative to disease progression (Halliwell et al., 1993), housing (Hansen et al., 2019) and season (Hansen et al., 2018; Wilkolek et al., 2014) is important, these factors could not be assessed in this study. Additionally, intradermal skin testing, used intensively for diagnosing insect bite hypersensitivity, was not used in this study, and its utility in diagnosing MEA has not been evaluated (Wilkolek et al., 2019).

In conclusion, BALF mRNA expression of several proinflammatory factors including IL1B, IL8, IL1RA, MMP8 and TLR4 was found to correlate with the expression of neutrophils in asthmatic horses with neutrophilic cell profiles. BALF mRNA expression of IL4 was significantly and positively correlated to BALF eosinophils and BALF allergen-specific IgE against mites and pollen2. Further studies including more horses diagnosed with mastocytic and eosinophilic subtypes of MEA are needed to confirm this finding. Similarly, further testing is needed to validate the Equine Allercapt® test as a diagnostic tool for EA.

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References

- Ainsworth, D.M., Grunig, G., Matychak, M.B., Young, J., Wagner, B., Erb, H.N., Antczak, D.F., 2003. Recurrent airway obstruction (RAO) in horses is characterized by IFN-γ and IL-8 production in bronchoalveolar lavage cells. *Vet. Immunol. Immunopathol.* 96, 83–91.
- Ainsworth, Dorothy M., Wagner, Bettina, Franchini, Marco, Grünig, Gabriele, Erb, Hollis N., Jean-Yin, Tan., 2006. Time-dependent alterations in gene expression of interleukin-8 in the bronchial epithelium of horses with recurrent airway obstruction. *AJVR* 67, 669–677.
- Alexis, N., Eldridge, M., Reed, W., Bromberg, P., Peden, D.B., 2001. CD14-dependent airway neutrophil response to inhaled LPS: role of atopy. *J. Allergy Clin. Immunol.* 107, 31–35.
- Andersen, C.L., Jensen, J.L., Orntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* 64, 5245–5250.
- Bedenice, D., Mazan, M.R., Hoffman, A.M., 2008. Association between cough and cytology of bronchoalveolar lavage fluid and pulmonary function in horses diagnosed with inflammatory airway disease. *J. Vet. Intern. Med.* 22, 1022–1028.
- Beekman, L., Tohver, T., Leguillette, R., 2012. Comparison of cytokine mRNA expression in the bronchoalveolar lavage fluid of horses with inflammatory airway disease and bronchoalveolar lavage mastocytosis or neutrophilia using REST software analysis. *J. Vet. Intern. Med.* 26, 153–161.
- Cordeau, M.E., Joubert, P., Dewachi, O., Hamid, Q., Lavoie, J.P., 2004. IL-4, IL-5 and IFN-γ mRNA expression in pulmonary lymphocytes in equine heaves. *Vet. Immunol. Immunopathol.* 97, 87–96.
- Couetil, L.L., Cardwell, J.M., Gerber, V., Lavoie, J.P., Leguillette, R., Richard, E.A., 2016. Inflammatory airway disease of horses-revised consensus statement. *J. Vet. Intern. Med.* 30, 503–515.
- Crepaldi, L., Silveri, L., Calzetti, F., Pinardi, C., Cassatella, M.A., 2002. Molecular basis of the synergistic production of IL-1 receptor antagonist by human neutrophils stimulated with IL-4 and IL-10. *Int. Immunol.* 14, 1145–1153.
- Eder, C., Cramer, R., Mayer, C., Eicher, R., Straub, R., Gerber, H., Lazary, S., Marti, E., 2000. Allergen-specific IgE levels against crude mould and storage mite extracts and recombinant mould allergens in sera from horses affected with chronic bronchitis. *Vet. Immunol. Immunopathol.* 73, 241–253.
- Eguiluz-Gracia, I., Bosco, A., Dollner, R., Melum, G.R., Lexberg, M.H., Jones, A.C., Dheyaaldeen, S.A., Holt, P.G., Baekkevold, E.S., Jahnsen, F.L., 2016. Rapid recruitment of CD14(+) monocytes in experimentally induced allergic rhinitis in human subjects. *J. Allergy Clin. Immunol.* 137, 1872–1881 e12.
- Fernandez, N.J., Hecker, K.G., Gilroy, C.V., Warren, A.L., Leguillette, R., 2013. Reliability of 400-cell and 5-field leukocyte differential counts for equine bronchoalveolar lavage fluid. *Vet. Clin. Pathol.* 42, 92–98.
- Franchini, M., Gill, U., von Fellenberg, R., Bracher, V.D., 2000. Interleukin-8 concentration and neutrophil chemotactic activity in bronchoalveolar lavage fluid of horses with chronic obstructive pulmonary disease following exposure to hay. *Am. J. Vet. Res.* 61, 1369–1374.
- Frey, R., Bergvall, K., Egenvall, A., 2008. Allergen-specific IgE in Icelandic horses with insect bite hypersensitivity and healthy controls, assessed by Fcεp1α-based serology. *Vet. Immunol. Immunopathol.* 126, 102–109.
- Gerber, V., Straub, R., Marti, E., Hauptman, J., Herholz, C., King, M., Imhof, A., Tahon, L., Robinson, N.E., 2004. Endoscopic scoring of mucus quantity and quality: observer and horse variance and relationship to inflammation, mucus viscoelasticity and volume. *EVJ* 36, 576–582.
- Giguere, S., Viel, L., Lee, E., MacKay, R.J., Hernandez, J., Franchini, M., 2002. Cytokine induction in pulmonary airways of horses with heaves and effect of therapy with inhaled fluticasone propionate. *Vet. Immunol. Immunopathol.* 85, 147–158.
- Gueders, M.M., Balbin, M., Rocks, N., Foidart, J.M., Gosset, P., Louis, R., Shapiro, S., Lopez-Otin, C., Noel, A., Cataldo, D.D., 2005. Matrix metalloproteinase-8 deficiency promotes granulocytic allergen-induced airway inflammation. *J. Immunol.* 175, 2589–2597.
- Halliwell, R.E., McGorum, B.C., Irving, P., Dixon, P.M., 1993. Local and systemic antibody production in horses affected with chronic obstructive pulmonary disease. *Vet. Immunol. Immunopathol.* 38, 201–215.
- Hansen, S., Honoré, M.L., Riihimäki, M., Pringle, J., Ammentorp, A.H., Fjeldborg, J., 2018. Seasonal variation in tracheal mucous and bronchoalveolar lavage cytology for adult clinically healthy stabled horses. *J. Equine Vet. Sci.* 71, 1–5.
- Hansen, S., Klintoe, K., Austevoll, M., Baptiste, K.E., Fjeldborg, J., 2019. Equine airway inflammation in loose-housing management compared with pasture and conventional stabling. *Vet. Rec.* <https://doi.org/10.1136/vr.104580>.
- Hare, J.E., Viel, L., 1998. Pulmonary eosinophilia associated with increased airway responsiveness in young racing horses. *J. Vet. Intern. Med.* 12, 163–170.
- Horohov, D.W., Beadle, R.E., Mouch, S., Pourciau, S.S., 2005. Temporal regulation of cytokine mRNA expression in equine recurrent airway obstruction. *Vet. Immunol. Immunopathol.* 108, 237–245.
- Hughes, K.J., Nicolson, L., Da Costa, N., Franklin, S.H., Allen, K.J., Dunham, S.P., 2011. Evaluation of cytokine mRNA expression in bronchoalveolar lavage cells from horses with inflammatory airway disease. *Vet. Immunol. Immunopathol.* 140, 82–89.
- Ivester, K.M., Couetil, L.L., Moore, G.E., Zimmerman, N.J., Raskin, R.E., 2014. Environmental exposures and airway inflammation in young thoroughbred horses. *J. Vet. Intern. Med.* 28, 918–924.
- Jensen-Jarolim, E., Einhorn, L., Herrmann, I., Thalhammer, J.G., Panakova, L., 2015. Pollen allergies in humans and their dogs, cats and horses: differences and similarities. *Clin. Transl. Allergy* 5, 15.
- Kunze, F., Gerber, V., Van Der Haegen, A., Wampfler, B., Straub, R., Marti, E., 2007. IgE-bearing cells in bronchoalveolar lavage fluid and allergen-specific IgE levels in sera from RAO-affected horses. *J. Vet. Med. A Physiol. Pathol. Clin. Med.* 54, 40–47.
- Lavoie, J.P., Maghni, K., Desnoyers, M., Taha, R., Martin, J.G., Hamid, Q.A., 2001. Neutrophilic airway inflammation in horses with heaves is characterized by a Th2-type cytokine profile. *Am. J. Respir. Crit. Care Med.* 164, 1410–1413.
- Matucci, A., Valtaggio, A., Maggi, E., Kasujee, I., 2018. Is IgE or eosinophils the key player in allergic asthma pathogenesis? Are we asking the right question? *Respir. Res.* 19, 113.
- Monteseirin, J., Vega, A., Chacon, P., Camacho, M.J., El Bekay, R., Asturias, J.A., Martínez, A., Guardia, P., Perez-Cano, R., Conde, J., 2007. Neutrophils as a novel source of eosinophil cationic protein in IgE-mediated processes. *J. Immunol.* 179, 2634–2641.
- Moore, B.R., Krakowka, S., Robertson, J.T., Cummins, J.M., 1995. Cytologic evaluation of bronchoalveolar lavage fluid obtained from standardbred racehorses with inflammatory airway disease. *Am. J. Vet. Res.* 56, 562–567.
- Niedzwiedz, A., Borowicz, H., Kubiak, K., Nicpon, J., Skrzypczak, P., Jaworski, Z., Cegielski, M., Nicpon, J., 2016. Evaluation of serum cytokine levels in recurrent airway obstruction. *Pol. J. Vet. Sci.* 19, 785–791.
- Niedzwiedz, A., Jaworski, Z., Kubiak, K., 2015. Serum concentrations of allergen-specific IgE in horses with equine recurrent airway obstruction and healthy controls assessed by ELISA. *Vet. Clin. Pathol.* 44, 391–396.
- Nielsen, M.K., Baptiste, K.E., Tolliver, S.C., Collins, S.S., Lyons, E.T., 2010. Analysis of

- multiyear studies in horses in Kentucky to ascertain whether counts of eggs and larvae per gram of feces are reliable indicators of numbers of strongyles and ascarids present. *Vet. Parasitol.* 174, 77–84.
- Nolen-Walston, R.D., Harris, M., Agnew, M.E., Martin, B.B., Reef, V.B., Boston, R.C., Davidson, E.J., 2013. Clinical and diagnostic features of inflammatory airway disease subtypes in horses examined because of poor performance: 98 cases (2004–2010). *J. Am. Vet. Med. Assoc.* 242, 1138–1145.
- Padoan, E., Ferrareso, S., Pegolo, S., Castagnaro, M., Barnini, C., Bargelloni, L., 2013. Real time RT-PCR analysis of inflammatory mediator expression in recurrent airway obstruction-affected horses. *Vet. Immunol. Immunopathol.* 156, 190–199.
- Powe, D.G., Bonnin, A.J., Jones, N.S., 2010. 'Entopy': local allergy paradigm. *Clin. Exp. Allergy* 40, 987–997.
- Prikk, K., Maisi, P., Pirila, E., Reintam, M.A., Salo, T., Sorsa, T., Sepper, R., 2002. Airway obstruction correlates with collagenase-2 (MMP-8) expression and activation in bronchial asthma. *Lab. Invest.* 82, 1535–1545.
- Raulo, S.M., Sorsa, T.A., Kiili, M.T., Maisi, P.S., 2001. Evaluation of collagenase activity, matrix metalloproteinase-8, and matrix metalloproteinase-13 in horses with chronic obstructive pulmonary disease. *Am. J. Vet. Res.* 62, 1142–1148.
- Richard, E.A., Depecker, M., Defontis, M., Leleu, C., Fortier, G., Pitel, P.H., Courouche-Malblanc, A., 2014. Cytokine concentrations in Bronchoalveolar lavage fluid from horses with neutrophilic inflammatory airway disease. *J. Vet. Intern. Med.* 28 (6), 1838–1844. <https://doi.org/10.1111/jvim.12464>.
- Richard, E.A., Fortier, G.D., Denoix, J.M., Art, T., Lekeux, P.M., Van Erck, E., 2009. Influence of subclinical inflammatory airway disease on equine respiratory function evaluated by impulse oscillometry. *EVJ* 41, 384–389.
- Richard, E.A., Robinson, N.E., 2016. Inflammatory airway disease congress: one syndrome, multiple pathways: a Dorothy Russell havemeyer symposium. *EVE* 28, 9–12.
- Riihimäki, M., Raine, A., Elfman, L., Pringle, J., 2008. Markers of respiratory inflammation in horses in relation to seasonal changes in air quality in a conventional racing stable. *Can. J. Vet. Res.* 72, 432–439.
- Robinson, D.S., Hamid, Q., Ying, S., Tsicopoulos, A., Barkans, J., Bentley, A.M., Corrigan, C., Durham, S.R., Kay, A.B., 1992. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N. Engl. J. Med.* 326, 298–304.
- Scharrenberg, A., Gerber, V., Swinburne, J.E., Wilson, A.D., Klukowska-Rotzler, J., Laumen, E., Marti, E., 2010. IgE, IgG, IgG(T) serum antibody levels in offspring of two sires affected with equine recurrent airway obstruction. *Anim. Genet.* 41 (Suppl 2), 131–137.
- Schatz, M., Rosenwasser, L., 2014. The allergic asthma phenotype. *J. Allergy Clin. Immunol. Pract.* 2, 645–648 quiz 49.
- Schmallenbach, K.H., Rahman, I., Sasse, H.H., Dixon, P.M., Halliwell, R.E., McGorum, B.C., Crameri, R., Miller, H.R., 1998. Studies on pulmonary and systemic *Aspergillus fumigatus*-specific IgE and IgG antibodies in horses affected with chronic obstructive pulmonary disease (COPD). *Vet. Immunol. Immunopathol.* 66, 245–256.
- Stedman, K., Lee, K., Hunter, S., Rivoire, B., McCall, C., Wassom, D., 2001. Measurement of canine IgE using the alpha chain of the human high affinity IgE receptor. *Vet. Immunol. Immunopathol.* 78, 349–355.
- Tahon, L., Baselgia, S., Gerber, V., Doherr, M.G., Straub, R., Robinson, N.E., Marti, E., 2009. In vitro allergy tests compared to intradermal testing in horses with recurrent airway obstruction. *Vet. Immunol. Immunopathol.* 127, 85–93.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3 RESEARCH0034.
- Verdon, M., Lanz, S., Rhyner, C., Gerber, V., Marti, E., 2019. Allergen-specific immunoglobulin E in sera of horses affected with insect bite hypersensitivity, severe equine asthma or both conditions. *J. Vet. Intern. Med.* 33, 266–274.
- Vinther, A.M., Skovgaard, K., Heegaard, P.M., Andersen, P.H., 2015. Dynamic expression of leukocyte innate immune genes in whole blood from horses with lipopolysaccharide-induced acute systemic inflammation. *BMC Vet. Res.* 11, 134.
- Virchow Jr., J.C., Julius, P., Matthys, H., Kroegel, C., Luttmann, W., 1998. CD14 expression and soluble CD14 after segmental allergen provocation in atopic asthma. *Eur. Respir. J.* 11, 317–323.
- Wagner, B., Ainsworth, D.M., Freer, H., 2013. Analysis of soluble CD14 and its use as a biomarker in neonatal foals with septicemia and horses with recurrent airway obstruction. *Vet. Immunol. Immunopathol.* 155, 124–128.
- Wang, N., McKell, M., Dang, A., Yamani, A., Waggoner, L., Vanoni, S., Noah, T., Wu, D., Kordowski, A., Kohl, J., Hoebe, K., Divanovic, S., Hogan, S.P., 2017. Lipopolysaccharide suppresses IgE-mast cell-mediated reactions. *Clin. Exp. Allergy* 47, 1574–1585.
- Wilkolek, P.M., Pomorski, Z.J., Szczepanik, M.P., Adamek, L., Pluta, M., Taszkun, I., Golynski, M., Rozwod, A., Sitkowski, W., 2014. Assessment of serum levels of allergen-specific immunoglobulin E in different seasons and breeds in healthy horses. *Pol. J. Vet. Sci.* 17, 331–337.
- Wilkolek, P., Szczepanik, M., Sitkowski, W., Adamek, L., Pluta, M., Taszkun, I., Golynski, M., 2019. A comparison of intradermal skin testing and serum insect allergen-specific IgE determination in horses with insect bite hypersensitivity from 2008 to 2016. *J. Equine Vet. Sci.* 75, 65–68.