

BRIEF COMMUNICATION

Serum concentrations of allergen-specific IgE in horses with equine recurrent airway obstruction and healthy controls assessed by ELISA

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Background: Equine recurrent airway obstruction (RAO), also known as heaves, is one of the most common respiratory problems in older horses. When RAO-affected horses stay pastured or in a dust-free environment for a prolonged time, clinical signs as well as airway inflammation wane. A number of environmental, immunologic, infectious, and genetic factors play an important role in the pathogenesis of RAO, and the immunologic basis of this disease is still poorly understood.

Objectives: The aim of this study was to investigate the concentrations of allergen-specific IgE in the serum of horses suffering from RAO and healthy controls.

Material and methods: The study included a group of 14 adult Polish Konik horses, kept in a standardized environment, and divided into 2 groups: 7 horses which did not have any respiratory problems comprised the control group and 7 horses with a history of RAO constituted the study group. A clinical and laboratory evaluation, endoscopic examination, and bronchoalveolar lavage (BAL) were performed in all horses. Sera of all horses were tested against allergens from 9 molds and 3 mites using the Heska Allercept assay.

Results: In the serologic tests, a statistically significant difference between both groups was found for specific IgE against mites, wherein *Tyrophagus putrescentia* correlated most clearly with RAO. There was no difference between groups for IgE specific against molds.

Conclusion: On the basis of our observations and results, we conclude that RAO is associated with increased serum concentrations of specific serum IgE against mites, in particular *T. putrescentia*.

Equine recurrent airway obstruction (RAO), also known as heaves, is one of the most common respiratory problems in older horses. It is characterized by airway obstruction, excessive mucus accumulation, and influx of neutrophils into the bronchial tree. Clinical signs including cough, nasal discharge, exercise intolerance, and increased respiratory effort develop as a result of exposure to and inhalation of aeroallergens, such as hay mold and barn dust. When RAO-affected horses stay pastured or in a dust-free environment for a prolonged time, clinical signs as well as airway inflammation wane. Once susceptible animals return to the stable and a dusty environment, respiratory problems reoccur.¹

A number of environmental, immunologic, infectious, and genetic factors play an important role in the pathogenesis of RAO.¹ The immunologic basis of this disease is still poorly understood and requires further studies. While some studies suggest that IgE-mediated immediate allergic reactions are involved in RAO, other studies do not confirm these findings and suggest that there may be other immunologic mechanisms involved in its pathogenesis.¹ The development of RAO has been shown to be associated with an inflammatory response dependent on T-helper lymphocytes type 2 and IgE antibody mediated by inflammatory cytokines such as IL 4, IL 5, and IL 13.²

Many studies have been carried out in horses with regard to specific IgE in the serum and fluid obtained during bronchoalveolar lavage (BALF). However, the results were varied. Few studies showed an increased concentration of IgE in RAO-affected horses against the most common allergens originating from molds such as *Aspergillus fumigatus* and *Faenia rectivigula* in BALF, compared with the control group.^{3–5} Other studies showed no difference in the concentrations of specific IgE in equine serum between the control group and horses with RAO.⁶ Similar studies on human asthma patients have shown a strong correlation between the disease and high concentrations of specific IgE against common mold aeroallergens.⁷ Moreover, previous studies have documented *Aspergillus fumigatus*, *Faenia rectivigula*, *Alternaria* spp, and *Mucor* spp as possible etiologic agents of RAO.⁸ Although the molds listed are the most common pathogens involved in the development of RAO, the role of mite sensitization in this disease still remains unclear.⁶

Therefore, the aim of our study was to investigate and compare the concentrations of common allergen-specific IgE using an ELISA method in the serum of Polish Konik horses with equine RAO and healthy controls. This study was approved by the 2nd Local Ethics Committee on Animal Experimentation in Wrocław (resolution No 1/2012).

Fourteen adult Polish Konik horses housed in the same environment were used in this study. Thirteen horses belonged to the Polish Academy of Sciences Research Station for Ecological Agriculture and Preserve Animal Breeding in Popielno, and one to a private breeder. Seven horses were used as the control group and were between 5 and 13 years old with no evidence of respiratory tract disease, of which 5 were castrated males (geldings) and 2 were mares. The study group consisted of 4 geldings and 3 mares, 7–14 years old with clinical signs of RAO. RAO was diagnosed based on the history, specific clinical signs like chronic cough, nasal discharge, labored breathing, and the results of a careful physical examination, arterial blood gas analysis, and cytologic examination of BALF obtained during endoscopy.¹ No horses from either group had evidence of respiratory infection. This was based on CBC results, plasma fibrinogen and haptoglobin concentrations, as well as a cytologic examination of specimens obtained from small airways.

Before the study, all horses were kept on pasture or in a stable with wood shavings as bedding. An acute crisis of RAO was induced by placing the horses into a poorly ventilated stable, bedding them on straw and feeding them hay with a visible mold growth for 48 hours prior to the examination. This environment

has previously been shown to induce airway inflammation and RAO exacerbation in affected horses. The response to the environmental challenge was confirmed based on a clinical RAO score and additional testing, including an endoscopic examination, BALF cytology, arterial blood gas analysis, and venous blood sampling.

Blood samples were obtained by venipuncture of the jugular vein using a 20 mL syringe and 20 Ga needle (Kruuse, Langeskov, Denmark). Blood samples were centrifuged for 10 minutes at 5000g within one hour after collection; serum was separated from packed cells and then stored at –80°C for approximately 5 months before analysis, without any freeze–thaw cycles in between.

At initiation of the study, the horses underwent a full clinical examination. Clinical RAO scores were based on guidelines previously described.⁹ Endoscopy of the airways and bronchoalveolar lavage were performed. Each horse was sedated with 0.01 mg/kg of detomidine (Domosedan, Orion Corporation, Espoo, Finland) and 0.01 mg/kg of butorphanol (Butomidor; Richter Pharma AG, Wels, Austria). In some cases, a twitch was also used to subdue the animals. A 1.8 m long, 10 mm diameter endoscope (Karl Storz GmbH, Tuttlingen, Germany) was passed through the nasal passage into trachea. Using visual guidance, the endoscope was passed until it wedged in the peripheral, right dorsal bronchial tree. Bronchoalveolar lavage was performed by instilling 250 mL of sterile saline (0.9% NaCl) at body temperature through the endoscope working channel into a bronchus using a 60 mL syringe, and reaspirating BALF using gentle suction through a syringe. The amount of recovered fluid was recorded, and BALF for each individual horse was pooled in a sterile plastic specimen cup, placed on ice and processed within 2 hours after collection.

For cytologic examination of BALF, a 10 mL aliquot was centrifuged at 300g for 10 minutes using a centrifuge (Beckman Coulter Allegra x-22; Beckman Coulter Inc., CA, Brea, USA) and the smear of the sediment was stained with Wright's stain. A 400-cell leukocyte differential count was performed; epithelial cells were not included in the differential count.

Frozen serum samples were sent to Heska AG (Fribourg, Switzerland) to determine allergen-specific IgE. In vitro ELISA technology using a human α chain of the high-affinity mast cell receptor for IgE (Fc ϵ R1 α) was used to test reactivity against 12 different regional allergens, typical for north-eastern Europe. Details of this method have been described elsewhere.¹⁰ Briefly, the test is based on the use of cloned human α chains, reactive with equine IgE and quantified by a color

reaction. After sample incubation and a washing procedure, a streptavidin-alkaline phosphatase-conjugated FcεR1α was added. The intensity of the blue color was measured photometrically. This semi-quantitative assay was considered positive when the optical density (OD) measured at 405 nm was > 150 ELISA absorption units (EAU), a cutoff determined by Heska Corp. The observed optical density values were compared with the standard optical density curve established from average results of repeated experiments with a pooled positive reference serum standard. Samples were tested against 12 whole-year allergens, comprising 9 molds and 3 mites.

Analysis using the Anderson–Darling test indicated that IgE concentrations were not distributed normally. Data are presented as median, 25th and 75th percentiles. The nonparametric *U* Mann–Whitney test was used to analyze the data. All analyses were performed using v. 10.0 of STATISTICA software (Stat Soft, Tulsa, OK, USA). A *P*-value of < .05 was considered statistically significant.

Detailed data describing the results of the clinical scores and the endoscopic examination are presented in Table 1. The results of the hematology and screening biochemistry did not indicate any systemic illnesses (data not shown). All horses from the control group had cough, nostril flare, and abdominal lift scores < 1. Sporadic coughing and slightly flared nostrils were the only abnormalities observed in the control horses. There was a significant difference (*P* < .05) in all clinical variables between the control and the study groups. Horses included in the study group had a median clinical score around 2. The components that had the most significant impact on the final results were cough, nostril flare, and abdominal lift.

The average recovery of the bronchoalveolar lavage fluid (BALF) was 56.6% in the control horses

and 43.6% in the study group (*P* < .001). The median percentage of nondegenerate neutrophils in BALF was significantly higher in RAO-affected horses than in the control group (*P* < .001; Table 1). The BALF median percentage of macrophages was significantly lower in RAO-affected horses than in the control group (*P* < .001; Table 1).

All control horses tested negative for all IgE-tested allergens, except one horse that was positive for *Alternaria alata* with specific IgE serum concentration > 150 EAU. Likewise, the horses from the study group had IgE concentrations against mold below the cutoff point and no statistical difference between the groups was found. However, all study horses had IgE antibody titers considered positive for different mites. We found specific IgE > 150 EAU directed against *Tyrophagus putrescentia* in 5 horses (Figure 1), *Dermatophagoides farinae/pteronyssinus* in 3 horses (Figure 2), and *Acarus siro* in 2 horses (Figure 3). A significant difference between the concentrations of IgE against mite extracts in RAO-affected horses (*P* < .05 in all cases) was found when compared to the control group. Detailed results are provided in Table 2.

The aim of our study was to investigate serum concentrations of allergen-specific IgE against the most common molds and mites using commercially available tests both in RAO-affected horses and healthy controls living in the same standardized environment. While the allergic background of equine RAO is undisputed, the IgE-mediated mechanism in the

Table 1. Results of bronchoalveolar lavage fluid (BALF) cytology, clinical assessment, and blood gas analysis in healthy horses and recurrent airway obstruction (RAO)-affected horses.

	Healthy (n = 7)	RAO-affected (n=7)
Clinical score*	2.0 (2 and 2)	6 (5 and 6)
PaO ₂ (mmHg)*	91 (85 and 107)	96 (91 and 108)
PaCO ₂ (mmHg)†	45 (43 and 46)	45.5 (44 and 50)
BALF Neutrophils (%)*	5.1 (4.1 and 5.3)	59.8 (51.3 and 64.8)
BALF Lymphocytes (%)*	41 (38.5 and 45.9)	38.1 (34.8 and 41.1)
BALF Macrophages (%)*	55.8 (49.8 and 59.1)	32.8 (25.9 and 35.7)
BALF Eosinophils (%)*	0.4 (0.2 and 0.5)	0 (0 and 0)
BALF Mast cells (%)†	0.1 (0 and 0.3)	0 (0 and 0)

Data are expressed as median and 25th and 75th percentiles.

*Statistically significant differences (*P* < .05)

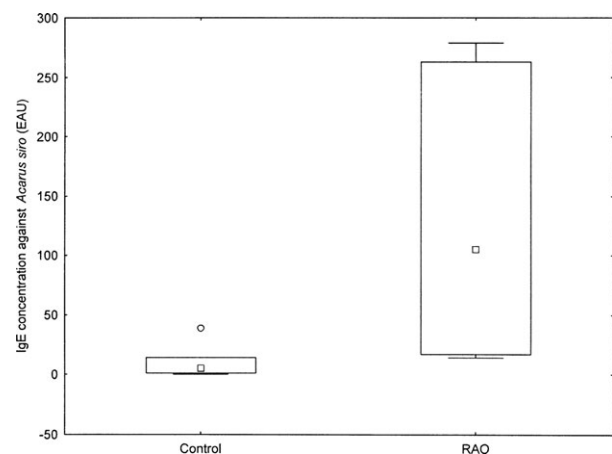


Figure 1. Concentration of IgE antibody expressed in ELISA absorption units (EAU) against *Acarus siro* in sera from 7 horses with recurrent airway obstruction (RAO) and from 7 controls, measured with the Heska Allercept assay (*P* < .05). The center square of the box plots marks the median of the sample. The edges of the box mark the 1st and 3rd quartiles. The horizontal line extends from the minimum to the maximum value. The circle indicates an outlier.

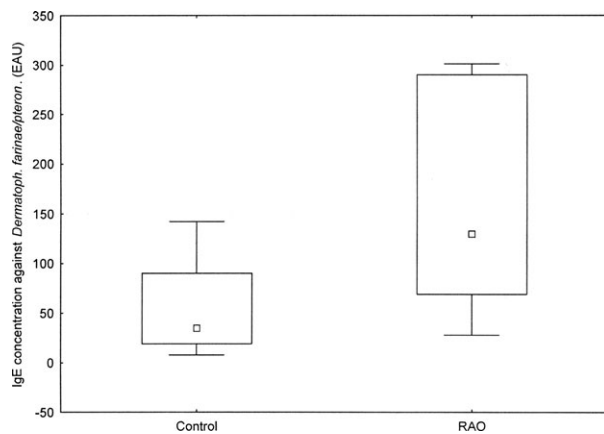


Figure 2. Concentration of IgE antibody expressed in ELISA absorption units (EAU) against *Dermatophagoides farinae/pteronyssinus* in sera from 7 horses with recurrent airway obstruction (RAO) and from 7 controls, measured with the Hesca Allercept assay ($P < .05$). The center square of the box plots marks the median of the sample. The edges of the box mark the 1st and 3rd quartiles. The horizontal line extends from the minimum to the maximum value.

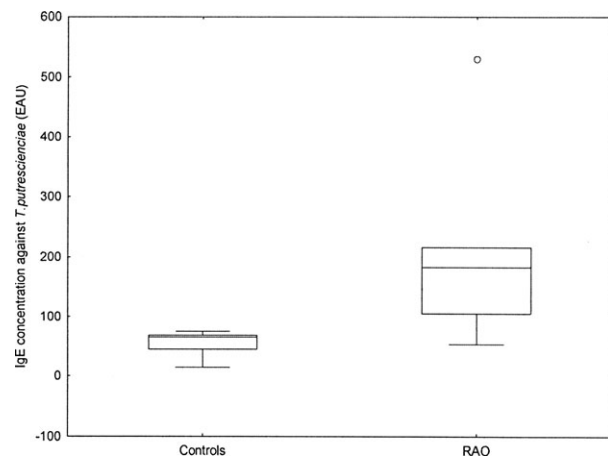


Figure 3. Concentration of IgE antibody expressed in ELISA absorption units (EAU) against *Tyrophagus putrescentiae* in sera from 7 horses with recurrent airway obstruction (RAO) and from 7 controls, measured with the Hesca Allercept assay ($P < .05$). The center horizontal line of the box plots marks the median of the sample. The edges of the box mark the 1st and 3rd quartiles. The horizontal line extends from the minimum to the maximum value. The circle indicates an outlier.

pathogenesis of RAO still remains unclear. A few studies conducted to date which evaluated serum-specific IgE titers in horses with RAO have produced inconsistent results.^{3–6} Moreover, the comparison of these studies is difficult due to the different testing methods used.

Determining the level of serum-specific antibodies is widely used in the diagnosis of allergic diseases in

people and other living organisms, including dogs and horses.^{11,12} Most tests are carried out using extracts containing the most commonly occurring allergens, such as molds, insects, mites, pollens, and yeasts. Considering that an inhalation of aeroallergens from bedding or hay is likely to be involved in the etiology of RAO in horses, it seems reasonable to investigate the

Table 2. Allergen-specific IgE concentrations expressed in ELISA absorption units (EAU) against various allergens in sera from 7 horses with recurrent airway obstruction (RAO) and from 7 controls, measured with the Heska Allercept assay.

Allergen extract	Control				RAO-affected				Mann–Whitney <i>U</i> test
	Median	25 th and 75 th percentiles	Range	n ⁺	Median	25 th and 75 th percentiles	n ⁺	Range	<i>P</i>
Mites									
<i>Acarus siro</i>	5	1–14	0–39	0	105	17–263	2	14–279	.006
<i>Dermatophadoides ferinae/pteronysinus</i>	35	19–90	8–142	0	130	69–290	3	28–301	.04
<i>Tyrophagus putrescentia</i>	65	45–68	14–75	0	183	105–216	5	53–529	.01
Molds									
<i>Alternaria alata</i>	0	0–12	0–225	1	14	0–17	0	0–26	NS
<i>Aspergillus fumigatus</i>	1	0–27	0–76	0	22	3–29	0	0–42	NS
<i>Cladosporium herbarum</i>	0	0–87	0–95	0	0	0–14	0	0–19	NS
<i>Epicoccum nigrum purpureum</i>	6	2–210	0–47	0	28	0–34	0	0–36	NS
<i>Helminthosporum halodes</i>	0	0–8	0–94	0	0	0–12	0	0–22	NS
<i>Penicillium notatum</i>	5	1–32	0–88	0	8	0–28	0	0–29	NS
<i>Fusarium culmorum</i>	4	0–27	0–42	0	0	0–18	0	0–32	NS
<i>Ustilago tritici</i>	0	0–24	0–56	0	29	0–38	0	0–45	NS
<i>Rhizopus nigricans</i>	0	0–19	0–23	0	0	0–13	0	0–37	NS

NS indicates not significant.

Data are expressed as median, percentiles, range and number of positive horses (n⁺) with cutoff > 150 EAU.

most commonly inhaled pollutants, which include molds and mites.¹ In our study, we screened equine serum for 12 allergen-specific IgE, including 9 molds and 3 mites.

The only individual with a positive result above the cutoff point against *Aspergillus fumigatus* was a horse from the control group. Our study differs from previous studies, which showed a statistically significant difference in the concentrations of *Aspergillus*-specific IgE antibodies between control and RAO horses. *A. fumigatus* has been shown to be an important etiologic factor in the development of equine RAO.¹² This fungus produces small conidia and spores that can remain suspended in the environment for long periods of time. Consequently, these factors greatly increase the probability of the mold being inhaled by stabled horses. Other studies using intradermal skin testing with molds and fungal allergen extracts conducted in RAO and control horses showed positive reactions in the RAO-affected horses to *A. fumigatus* antigens at 4 and 24 hours after the injection.¹³

Even though many of the above-mentioned studies show participation of these molds in the pathophysiology of RAO, the results of our study strongly suggest that *A. fumigatus* is not involved in the etiology of this disease. Considering that all horses were housed under identical conditions and were fed the same, poor quality moldy hay, we can conclude that this result, although contrary to previously reported research, is likely reliable. Namely, in the present study, we found no significant difference between the concentration of mold-specific IgE between RAO-affected and control horses. The only positive result above the cutoff point originated from a control horse.

Interestingly, our study showed a statistically significant difference between RAO and control horses with regard to serum concentrations of allergen-specific IgE against mites. Only RAO-affected horses had specific IgE concentrations > 150 EAU, the cutoff point. Five horses tested positive for *T. putrescentiae*. These results were characterized by the highest median score of all tested mite allergens. It is widely recognized that the prevalence and severity of allergic respiratory diseases such as asthma or rhinitis in people and companion animals, like dogs and cats, are increasing in the developed world. This necessitates a greater focus on their causative agents.¹⁴ It is thought that these diseases involve sensitization to allergens from mites or other arthropods. A wide range of arthropods can produce allergic reactions, whose characteristics depend on the route of exposure.

Our study clearly differed from previous studies, which showed sensitization to mites in both groups of

horses.⁶ In that study, over 50% of horses from both groups had positive reactions to *T. putrescentiae*. Moreover, almost 23% of horses from the RAO group and 43% of control horses were positive for *D. farinae*, whereas 15% of horses from both groups were positive for *Acarus siro*. The results of another study also showed elevated serum-specific IgE against house dust mites in healthy horses. Positive results were most commonly obtained for IgE against *D. farinae/pteronyssinus*, whereas all horses were negative for *T. putrescentiae*.¹⁰ However, the usefulness of this study in the evaluation of our results is limited, as no horses with a respiratory problem were included in this research. In only one equine study was there an association between respiratory disease and mites; however, this study had no control group.¹⁵ Our study suggests that respiratory problems in the RAO-affected horses may be due to mite sensitization as the increase in concentration of mite-specific IgE in the RAO-affected horses is statistically significant compared with the healthy controls.

In conclusion, a serologic investigation of Polish Konik horses living in the same environment shows that RAO may be associated with high concentrations of specific serum IgE against mites, particularly *T. putrescentiae*. To the authors' knowledge, this is the first study to show an association between RAO in horses and a hypersensitivity to mites. However, due to the relatively small population of horses used in this study, further research is required to confirm an association between RAO and forage mites. One should take into consideration the fact that the results obtained during serologic testing are of limited value in the diagnosis of RAO and can be used only to identify a cause of sensitization in susceptible horses.

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