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Research paper

In vitro allergy tests compared to intradermal testing in horses with recurrent airway obstruction

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ABSTRACT

Recurrent airway obstruction (RAO) is a common condition in stabled horses characterised by small airway inflammation, airway neutrophilia and obstruction following exposure of susceptible horses to mouldy hay and straw and is thus regarded as a hypersensitivity reaction to mould spores. However, the role of IgE-mediated reactions in RAO remains unclear.

The aim of the study was to investigate with a serological IgE ELISA test (AllerceptTM), an *in vitro* sulfidoleukotriene (sLT) release assay (CAST[®]) and with intradermal testing (IDT) whether serum IgE and IgE-mediated reactions against various mould, mite and pollen extracts are associated with RAO. IDT reactions were evaluated at different times in order to detect IgE-mediated immediate type reactions (type I hypersensitivity reactions, 0.5–1 h), immune complex-mediated late type reactions (type III reactions, 4–10 h) and cell-mediated delayed type reactions (type IV hypersensitivity reactions 24–48 h).

In the serological test, overall the control horses displayed more positive reactions than the RAO-affected horses but the difference was not significant. Comparison of the measured IgE levels showed that the RAO-affected horses had slightly higher IgE levels against *Aspergillus fumigatus* than controls (35 and 16 AU, respectively, $p < 0.05$), but all values were below the cut off (150 AU) of the test. In the sLT release assay, seven positive reactions were observed in the RAO-affected horses and four in the controls but this difference was not significant.

A significantly higher proportion of late type IDT reactions was observed in RAO-affected horses compared to controls (25 of 238 possible reactions versus 12 of 238 possible reactions, respectively, $p < 0.05$). Interestingly, four RAO-affected but none of the control horses reacted with the recombinant mould allergen *A. fumigatus* 8 (rAsp f 8, $p < 0.05$), but only late phase and delayed type reactions were observed.

In all three tests the majority of the positive reactions was observed with the mite extracts (64%, 74% and 88% of all positive reactions, respectively) but none of the tests showed a significant difference between RAO-affected and control animals. Our findings do not support that IgE-mediated reactions are important in the pathogenesis of RAO. Further studies are needed to investigate whether sensitisation to mite allergens is of clinical relevance in the horse and to understand the role of immune reactions against rAsp f 8.

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1. Introduction

Recurrent airway obstruction (RAO) or heaves, is a common condition in mature stabled horses characterised by small airway inflammation, airway neutrophilia and obstruction following exposure of susceptible horses to mouldy hay and straw. The disease shares some similarities with human asthma. However, the role of IgE-mediated reactions and the specific allergens involved in RAO remains unclear. Earlier studies suggested that RAO is a hypersensitivity reaction to inhaled moulds present in hay and straw: skin tests, pulmonary challenge experiments and *in vitro* histamine release assays suggested that *Aspergillus fumigatus*, *Faenia rectivirgula* (McPherson et al., 1979; Derksen et al., 1988), *Alternaria* spp. (Eyre, 1972; Hare et al., 1999) and *Mucor* spp. (Dirschel et al., 1993) are implicated in the aetiology of RAO and that immediate, late phase and delayed hypersensitivity reaction types can occur (Halliwell et al., 1979; Evans et al., 1992; McGorum et al., 1993b).

The immunological basis of RAO still remains poorly understood. While a number of studies suggest that IgE-mediated immediate type allergic reactions (Halliwell et al., 1979; McPherson et al., 1979; Evans et al., 1992; McGorum et al., 1993c) and Th2-type cytokines are involved in RAO (Lavoie et al., 2001; Cordeau et al., 2004), others could not confirm these findings and suggest that other immunological mechanisms may be involved in the pathogenesis of RAO (Asmundsson et al., 1983; Giguere et al., 2002; Ainsworth et al., 2003). Results from intradermal testing (IDT) are also controversial: Schatzmann and Gerber (1972), Eyre (1972), Evans et al. (1992), McGorum et al. (1993a) as well as Lorch et al. (2001b) showed that RAO-affected horses as well as RAO-unaffected horses react to IDT with various allergens but no significant difference could be detected between both groups of horses. Conversely, in some other studies, RAO-affected horses reacted significantly more frequently to IDT than controls (Halliwell et al., 1979; McPherson et al., 1979; Jose-Cunilleras et al., 2001). These discrepancies can partly be explained by the fact that IDT and the allergens used are not standardised for the horse and findings from different investigations are thus difficult to compare. Conversely, horses with insect bite hypersensitivity (IBH), an allergic dermatosis of the horse, react more frequently in IDT after 1 h than controls with insect extracts, suggesting that immediate type reactions are often involved in IBH (Bravermann et al., 1983; Quinn et al., 1983; Fadok and Greiner, 1990). A recent study using the cellular antigen stimulation test (CAST[®]) further suggests that IgE-mediated reactions are often involved in IBH (Baselgia et al., 2006). This test was developed for the determination of sulfidoleukotrienes (sLT) released from peripheral blood leukocytes for the diagnosis of IgE-mediated allergic reactions (De Weck, 1993). A recent study has demonstrated that this test, with *Culicoides* extract as allergen, has a high specificity of 97% and a sensitivity of 78% (Baselgia et al., 2006) for *in vitro* diagnosis of IBH in the horse.

In the past, several studies have investigated the role of IgE antibodies in the pathogenesis of equine allergic

diseases (Halliwell et al., 1993; Matthews et al., 1983; Schmallenbach et al., 1998). However, these investigations have been hindered by problems inherent to the use of polyclonal antibodies for detection of equine IgE. Recently, monoclonal antibodies (Wagner et al., 2003; Wilson et al., 2006) or other reagents such as the α chain of the IgE receptor (Lorch et al., 2001a) have allowed specific detection of IgE in horse serum but only few studies have been performed with these new reagents so far.

The purpose of the study reported here was to investigate with the CAST[®] and with one commercial assay for detection of antigen specific IgE levels, to what extent IgE-mediated reactions are involved in RAO and if these tests could be useful for *in vitro* diagnosis of RAO. Furthermore, the test results were compared to intradermal testing with the corresponding antigens. Intradermal testing was evaluated at different times to investigate whether, additionally to immediate type reactions, late type and delayed type reactions occurred with different frequencies in RAO and control horses.

2. Material and methods

2.1. Horses

Twenty-eight horses (14 RAO-affected and 14 controls) were included in this study, of which 17 horses (eight RAO-affected and nine controls) were from Switzerland and 11 horses (six RAO-affected and five controls) from Michigan State University, USA. The study was approved by the cantonal committee for animal experiments.

The Swiss horses either belonged to the Equine Clinic of the Faculty of Veterinary Medicine from the University of Berne or to the Swiss Equine Centre in Berne or were privately owned patients that had come to the Equine Clinic for diagnosis and treatment of RAO. A clinical examination including endoscopic examination and cytology of tracheobronchial secretions (TBS) was performed. The horses developed typical heaves symptoms such as cough, increased respiratory effort, tracheal mucus accumulation and increased neutrophil in TBS when housed in conventional stable environment and fed hay. None of the control horses had a history of respiratory disease for the last six months and neither the clinical examination nor the endoscopic examination or the TBS cytology revealed abnormal respiratory signs. The horses were either living in a conventional stable environment or had been withdrawn from hay and straw few days to maximum one month before testing and had not been subjected to any treatments such as glucocorticoids, antihistamines or bronchodilators.

All of the horses from Michigan State University had served previously as experimental animals in hay exposure protocols and either developed heaves exacerbations ($N=6$) according to the criteria defined by the International Workshop on Heaves (Robinson, 2001) or did not develop signs of RAO (control horses, $N=5$). The RAO horses from the USA were in remission when the tests were performed, but had been exposed to mouldy hay one to two months before blood sampling and IDT was performed.

The mean age of the 14 control horses (five mares and nine geldings) was 12 years (range 4–20 years) and of the 14 RAO horses (five mares and nine geldings) 16 years (range 7–24 years).

2.2. Blood samples

Prior to IDT, blood samples were collected by jugular venipuncture into sterile tubes containing a serum activating clot factor (Vacuette[®], Greiner, Huber u. Co., Reinach, Switzerland) and into sterile ACD-B vacuette tubes (Vacuette[®], Greiner) for the cellular antigen stimulation test. For preparation of serum the tubes were incubated 30 min at 37 °C and centrifuged 10 min. at 4 °C and 2500 rpm. The serum was placed in 1.5 ml polypropylene tubes (Sarstedt AG, Sevelen, Switzerland) and kept frozen at –80 °C until used.

2.3. Determination of allergen-specific serum IgE levels with a commercial test using the FcεRIα

The ELISA test was a commercial assay 'ALLERCEPT[™]' (CMG-Heska Allergy Products, 1700 Fribourg, Suisse) using the recombinant human FcεRIα chain to measure allergen-specific IgE. The test was performed as previously described (Lorch et al., 2001a) by CMG Heska laboratory in Fribourg, Switzerland. The laboratory was not aware of the IDT results or diagnosis. Twenty-four allergens included moulds extracts, mites extracts, tree extracts, weed extracts, grass extracts and one corn extract (Table 1). The allergen-specific IgE concentration was reported as a class score in allergy units (AU) ranging from 0 to 4000. A score of ≥150 AU units for an allergen was considered positive, as established by the manufacturers.

2.4. Cellular antigen stimulation test (CAST[®])

Eighteen allergens consisting of allergens and allergen-mixes and two recombinant *A. fumigatus* allergens, rAsp f 7 and rAsp f 8 (Cramer, 1998) were used in the CAST[®] (see Table 1). In addition, Concanavalin A (20 µg/ml; Sigma Products, Fluka Chemie GmbH, Buchs, Switzerland) was used as a positive control. All allergens, except rAsp f 7 and rAsp f 8, were purchased from a commercial company (CMG-Heska Allergy products, Fribourg, Switzerland). All the allergens were used at a final concentration of 50 µg/ml, except for rAsp f 7 and rAsp f 8, which were used at 20 µg/ml. The concentration of the allergen extracts to be used had been determined in pilot experiments that had shown that these were the concentrations that gave no background and induced sLT release from peripheral blood leukocytes in some horses. With lower antigen concentrations no sLT release was obtained.

The CAST[®]-2000 (Bühlmann Laboratories AG Schönenbuch, Switzerland) was performed as previously described (Baselgia et al., 2006) and following the manufacturer's instructions. Briefly: peripheral blood leukocytes were isolated from ACD-B anticoagulated blood, resuspended in stimulation buffer and incubated for 40 min at 37 °C with the various antigens (Table 1), the positive control or with stimulation buffer alone to determine spontaneous sLT

Table 1

Allergens used in the intradermal test (IDT), in the sulfidoleukotriene release assay (cellular antigen stimulation test, CAST[®]) and in the serological IgE ELISA (Allercept[™]). Allergens from the same source were used in CAST[®] and Allercept[™].

Allergens	IDT ^a	CAST ^{®b}	Allercept ^{™b}
Moulds			
<i>Aspergillus fumigatus</i>	×	×	×
Recombinant Asp f 7 ^c	×	×	
Recombinant Asp f 8 ^c	×	×	
<i>Penicillium notatum</i>	×	×	
<i>Penicillium-mix</i>			×
<i>Epicoccum</i> sp.			×
<i>Helminthosporium</i> sp.			×
<i>Fusarium</i> sp.			×
<i>Rhizopus</i> sp.			×
<i>Ustilago</i> sp.			×
<i>Mucor mucedo</i>	×		
<i>Cladosporium herbarum</i>	×	×	×
<i>Alternaria alternata</i>	×	×	×
Yeast			
<i>Candida albicans</i>	×	×	
Mites			
<i>Tyrophagus putrescentiae</i>	×	×	×
<i>Lepidoglyphus destructor</i>	×	×	
<i>Acarus siro</i>	×	×	×
<i>Dermatophagoides pteronyssinus</i>	×	×	1
<i>Dermatophagoides farinae</i>	×	×	1
<i>Glycophagus domesticus</i>		×	
Tree extracts			
Common Birch	2	×	×
Oak	2	×	
Willow			×
Hazel			×
Beech			×
Copperbeech	2		
Plane	2		
Weed extracts			
Ribwort	3	×	
Mugwort	3	×	×
Lamb's quarter			×
English plantain			×
Nettle	3		×
Yellow Dock			×
Dandelion	3		
Grass extracts			
Cocksfoot			×
Timothy grass	4		×
Eight-grass-mix		×	
Velvet grass	4		
Orchard grass	4		
Perennial grass	4		
Meadow grass	4		
Madow fescue	4		
Corn extracts			
Cultivated Rye	5		×
Mixed-grass extract	5		
Barley	5		
Oat	5		
Wheat	5		

(1) *D. farinae*–*D. pteronyssinus* mix; (2) Mixed-tree extract; (3) Mixed-weed extract (4) Mixed-grass extract; (5) Mixed-grass and corn extract.

^a Allergopharma AG, Therwil, Switzerland.

^b CMG-Heska Allergy products, Fribourg, Switzerland.

^c Prepared according to Cramer (1998).

release. SLT determination in the supernatant was carried out following the manufacturer's instructions using the CAST[®]-2000 ELISA. For all further evaluations the net values of stimulation were used, i.e. the spontaneous SLT release (= incubation with buffer only) was subtracted from the values obtained after stimulation with Concanavalin A or with the allergens extracts. Spontaneous SLT release was <185 pg/ml for all horses tested.

2.5. Intradermal testing

Seventeen allergens (Table 1) consisting of single allergens and allergen-mixes were used, including the two recombinant *A. fumigatus* allergens rAsp f 7 and rAsp f 8 (Crameri, 1998, 1999). In addition a positive control (histamine dihydrochloride 0.17 mg/ml, Allergopharma AG, Therwil, Switzerland) and a negative control (physiological saline solution) were used. All the allergens except rAsp f 7 and rAsp f 8, were purchased from Allergopharma AG. The allergen extracts from Allergopharma AG were selected because they have been developed for IDT. All allergens were tested at 2500 biological units (BU)/ml and 250 BU/ml except rAsp f 7, rAsp f 8 that were tested at 20 µg/ml.

Intradermal testing was performed on the flat surface of both shoulders. Hair was clipped and a permanent marker was used to delimit each injection area. Then, 0.1 ml of the test and control solutions was injected intradermally. Injection sites were evaluated 30 min, 1 h, 4 h, 8 h, 10 h, 24 h and 48 h after injection to investigate whether, additionally to immediate type reactions, late type and delayed type reactions occurred with different frequencies in RAO and control horses. Skin test reactions seen at 30 min to 1 h after injection represent type I hypersensitivity reactions, whereas those seen at 4–10 h are probably mediated by IgG antibodies and are type III immune complex-mediated hypersensitivity reactions or Arthus reactions (Roitt et al., 1986). In the horse, a limited number of biopsies of skin test reactions were examined. Histopathological findings were reported as being consistent with type I hypersensitivity at 30 min and type III hypersensitivity at 4–6 h (Halliwell et al., 1979). IgE-mediated reactions can also be followed by IgE-mediated late phase reactions, but usually, IgE-mediated late phase reactions to allergen skin testing develop in combination with immediate reactions (Roitt et al., 1986). Delayed type hypersensitivity reactions are cell-mediated and develop between 24 h and 72 h.

Reactions were scored according to the longitudinal (D1) and the transversal (D2) wheel diameter as well as subjectively on a thickness scale of 1 to 6, where 1 was equal to the saline-induced reaction. The surface (S) of the wheal was calculated according to Nikolaizik et al. (2002) with the following formula: S: squared value of [(D1 + D2)/2]. Each surface was then multiplied with the factor corresponding to the thickness of its reaction. Results were considered as positive if the score of the wheal was at least half of the score of the histamine reaction read at 1 h. Only the first occurrence of a positive reaction is reported, i.e. for example, a reaction that starts at 30 min and remains positive for 8 h will only be reported once, as immediate

type reaction. This was done in order to distinguish IgE-mediated late phase reactions, which are usually preceded by an immediate type reaction, from immune complex late type reactions (type III) which are not preceded by an immediate type reaction. Intradermal tests were performed by the same person in Switzerland and in the USA and she was not aware of the clinical diagnosis of the horse.

2.6. Statistical analyses

Statistical analyses were carried out with the statistical software package NCSS 2001 (NCSS, Kaysville, Utah 84037, USA). A preliminary analysis indicated that allergen-specific IgE levels and released SLTs were not distributed normally, and medians and ranges were used subsequently for descriptive purposes.

The nonparametric Mann–Whitney *U*-test was used to compare continuous variables such as IgE levels and SLT release levels with the different allergens in RAO-affected and healthy horses. The analysis was performed both with all horses together as well as within groups from each country (Switzerland and USA).

To compare the proportion of RAO-affected and control horses with a positive or negative test result for IgE serology, SLT release and IDT or the proportion of positive IDT reactions at different times, the 2-tailed Fisher's exact test (2-sided hypothesis) was used. Again, the analysis was performed both with all horses together as well as within groups from each country (Switzerland and USA).

Overall *p*-values ≤0.05 were considered as statistically significant.

As there were no significant differences for any of the tested parameters between the horses from the USA and those from Switzerland, except that RAO-affected horses from the USA had significantly more positive IDT reactions in total than RAO-affected horses from Switzerland (26% of positive reactions at any time versus 15%, *p* < 0.05), the results from the horses from both countries are presented together.

3. Results

3.1. Allergen-specific IgE level determined with the FcεRIα-based assay (AllerceptTM)

Eight out of the 13 RAO horses (62%) had a positive IgE ELISA test for at least one allergen and five horses (38%) were negative to all allergens assayed. Among the 14 control horses 10 (71%) were IgE positive to at least 1 allergen (Table 2). Overall, the control horses also displayed more ELISA positive reactions (25 reactions in 14 horses, median = 2 positive reaction per horse) than the RAO horses (17 reactions in 13 horses, median = 1 positive reaction per horse) but the difference was not significant (Table 3). All horses had serum IgE levels against the mould extracts below the cut off of 150 AU (Table 3). Nevertheless, comparison of the measured IgE levels showed that the RAO-affected horses had slightly higher IgE levels against *A. fumigatus* than controls (35 and 16 AU, respectively, *p* < 0.05). Horses often had serum IgE levels against the mite

Table 2

Number of horses with recurrent airway obstruction (RAO) or controls, with a negative or positive test result to one or more allergens in IgE serology (Allercept™), cellular antigen stimulation test (CAST®) and intradermal testing (IDT). Skin reactions were read at different times: early reaction: start of reaction at 0.5–1 h; late reaction: start of reaction at 4–10 h; delayed reaction: start of reaction at 24–48 h.

Test used	Test result	Clinical diagnosis		
		RAO (N = 13 or 14)*	Controls (N = 14)	Fisher's exact test P
Allercept®	Negative	5 (38%)	4 (29%)	NS
	Positive (≥1 reactions)	8 (62%)	10 (71%)	
CAST®	Negative	9 (69%)	11 (79%)	NS
	Positive (≥1 reactions)	4 (31%)	3 (21%)	
IDT early	Negative	12 (86%)	9 (64%)	NS
	Positive (≥1 reactions)	2 (14%)	5 (36%)	
IDT late	Negative	4 (29%)	6 (43%)	NS
	Positive (≥1 reactions)	10 (71%)	8 (57%)	
IDT delayed	Negative	6 (43%)	8 (57%)	NS
	Positive (≥1 reactions)	8 (57%)	6 (43%)	

* 14 RAO-affected horses were tested in IDT but only 13 were tested in Allercept® and CAST®.

extracts that were above the cut off (Table 3): Over 50% of the horses had positive IgE levels to *Tyrophagus putrescentiae*, 15% had positive IgE levels to *Acarus siro* and 23% (RAO horses) or 43% (control horses) had positive IgE levels against *Dermatophagoides farinae*–*Dermatophagoides pteronyssinus* (Table 3). However, there were no significant differences between RAO-affected and control horses. Additionally, three RAO-affected and seven control horses had IgE against pollen extracts that were above the cut off of 150 AU (Table 3).

3.2. Cellular antigen stimulation test (CAST®)

Overall four RAO-affected animals and three controls had sLT release above 200 pg/ml with at least one of the various allergens tested (Table 2). While all horses, except one, had a positive sLT release (> 250 pg/ml) with Concanavalin A, used as positive control, only few positive CAST® results were obtained with the allergen extracts (Table 4). In total, seven positive reaction were observed in the RAO horses and four in the controls. In this test too, the

Table 3

Allergen-specific IgE levels (expressed in allergy units) against various allergens in sera from 13 horses with recurrent airway obstruction (RAO) and from 14 controls, measured with the Allercept™ assay. A cut off of 150 AU was used to classify the test result as positive or negative. N (%) = number and percentage of horses with a positive test result.

Allergen extract	RAO (N = 13)			Controls (N = 14)			Mann–Whitney U-test
	Median	Range	N(%) +	Median	Range	N(%) +	RAO versus controls
Moulds							
<i>A. fumigatus</i>	35	7–113	0	16	2–85	0	0.049
<i>Penicillium mix</i>	26	3–51	0	20	0–48	0	ns
<i>Epicoccum sp.</i>	0	0–25	0	0	0–19	0	ns
<i>Helminthosporium sp.</i>	0	0	0	0	0	0	ns
<i>Fusarium sp.</i>	23	9–105	0	16	0–110	0	ns
<i>Rhizopus sp.</i>	0	0	0	0	0	0	ns
<i>Ustilago sp.</i>	13	3–61	0	9	0–40	0	ns
<i>C. herbarum</i>	22	1–98	0	9	0–61	0	ns
<i>A. alternata</i>	40	0–92	0	12	1–64	0	ns
Mites							
<i>T. putrescentiae</i>	163	93–993	8 (62%)	219	54–512	10 (71%)	ns
<i>A. siro</i>	69	16–367	2 (15%)	97	25–379	2 (14%)	ns
<i>D. farinae</i> – <i>D. pteronyssinus</i>	105	29–1393	3 (23%)	119	39–707	6 (43%)	ns
Pollens							
Common birch	0	0	0	0	0	0	ns
Willow	0	0	0	0	0	0	ns
Hazel	0	0	0	0	0	0	ns
Beech	0	0	0	0	0	0	ns
Mugwort	0	0–38	0	0	0–30	0	ns
Lamb's quarter	0	0–42	0	0	0–38	0	ns
English Plantain	0	0	0	0	0	0	ns
Nettle	0	0	0	0	0	0	ns
Yellow Dock	45	0–234	3 (23%)	25	0–358	3 (21%)	ns
Cocksfoot	0	0–177	1 (8%)	17	0–785	2 (14%)	ns
Timothy grass	2	0–104	0	11	0–583	1 (7%)	ns
Cultivated Rye	0	0–82	0	0	0–671	1 (7%)	ns
Median no (range) of positive reactions per horse			2 (0–4)			1(0–4)	

Table 4

Results from the cellular antigen stimulation test (CAST[®]). Sulfidoleukotriene (sLT) generation (pg/ml) by peripheral blood leukocytes from horses with recurrent airway obstruction (RAO) and controls after incubation with various antigen extracts. A cut off of 200 pg/ml released sLT was used to classify the test result as positive or negative. N (%) + = number and percentage of horses with a positive test result.

Stimulans or allergen extract	RAO (N = 13)			Controls (N = 14)			Mann–Whitney U-test RAO versus controls
	Median	Range	N(%) +	Median	Range	N(%) +	
Concanavalin A:	1286	256–3422	13 (100%)	1153	0–2989	13 (93%)	ns
Moulds							
<i>A. fumigatus</i>	5	0–163	0	0	0–43	0	ns
rAsp f 7	0	0–36	0	0	0–115	0	ns
rAsp f 8	0	0	0	0	0–116	0	0.049
<i>A. alternata</i>	0	0–45	0	0	0–16	0	ns
<i>P. notatum</i>	0	0–113	0	0	0–36	0	ns
<i>C. herbarum</i>	0	0–201	1 (8%)	0	0–36	0	ns
Yeast							
<i>C. albicans</i>	0	0–35	0	0	0	0	ns
Mites							
<i>D. farinae</i>	0	0–141	0	12	0–1240	1 (7%)	ns
<i>T. putrescentiae</i>	31	0–131	0	21	0–85	0	ns
<i>L. destructor</i>	39	0–210	1 (8%)	39	0–129	0	ns
<i>G. domesticus</i>	58	0–184	0	80	0–132	0	ns
<i>A. siro</i>	27	0–187	0	17	0–184	0	ns
<i>D. pteronyssinus</i>	16	0–527	4 (31%)	33	0–1431	3 (21%)	ns
Pollens							
Oak	0	0–58	0	0	0–25	0	ns
Ribwort	0	0–115	0	0	0–133	0	ns
Mugwort	0	0–487	1 (8%)	3	0–168	0	ns
Common Birch	0	0–56	0	0	0–154	0	ns
Eight-grass-mix	0	0	0	0	0–180	0	ns
Total			7			4	

horses reacted mainly with allergens of the mite family (Table 4). Additionally, one RAO-affected horse reacted with the mold *Cladosporium herbarum* and one with mugwort.

3.3. Intradermal skin testing

Except for the recombinant allergens, used at a concentration of 20 µg/ml, IDT was performed at concentrations of 250 and 2500 U/ml with the allergen extracts. As *Candida albicans* often induced very strong reactions (diameter of the reaction up to 8 cm) at a concentration of 2500 U/ml, this concentration was only used in 12 controls and eight RAO horses. Positive reactions were still often observed with the lower concentration of 250 U/ml. With the other allergen extracts, no positive IDT reactions were observed at a concentration of 250 U/ml except for positive IDT reactions to *D. pteronyssinus* and *D. farinae* in one RAO horse and to *T. putrescentiae* in one RAO and one control horse. Except for *C. albicans*, only the data of the IDT with 2500 U/ml are thus presented for the allergen extracts.

The score of the wheals after intradermal injection of histamine was not significantly different between RAO-affected and control horses (at 1 h, medians values of 18.75 and 20.8, respectively, $p > 0.1$), although individual variation was observed (wheal score ranging from 11 to 38).

Overall, 13 of the 14 RAO horses and 11 of the 14 controls reacted with at least one allergen in IDT. The total number of reactions at any time (early [0.5–1 h], late

[4–10 h] and delayed [24–48 h] type reactions) was higher in RAO horses than in controls, as the 14 RAO horses showed in total 42 positive IDT reactions of 238 possible reactions while only 28 positive reactions of 238 were observed in the 14 control horses. This difference, however, was not significant. Interestingly, this difference was mainly due to late phase reactions that occurred twice as frequently in RAO horses than in controls (25 versus 12 reactions, $p < 0.05$, Table 5a). However, there was no significant difference between both groups of horses when the proportion of horses with one or more late phase reaction was compared (Table 2). Delayed type reactions were also observed more frequently in RAO horses than in controls (22 versus 14 reactions, $p > 0.1$, Table 5a), but the difference was not significant. Surprisingly, immediate type reactions were rarely observed (Tables 2 and 5a): they were only observed in two RAO horses (two reactions) and in five controls (six reactions). This difference was not significant. Except for one allergen (*Penicillium notatum*), immediate type reactions were only observed with the mite extracts, *T. putrescentiae*, *A. siro* and *D. farinae*. Most of the late phase reactions were also due to the mite extracts, except for two reactions caused by the recombinant mould allergens rAsp f 7 and rAsp f 8 in one RAO horse (Table 5a). Usually the recombinant *A. fumigatus* allergens induced delayed type reactions (24–48 h) and delayed type reactions were also induced by the yeast *C. albicans*. RAO-affected horses reacted significantly more frequently than controls with the higher concentration of *C. albicans* (2500 U/ml), as seven of eight tested RAO-affected horses

Table 5a

Number of positive intradermal reactions to 17 allergens at different times in horses with recurrent airway obstruction (RAO) and controls. In each group there were 238 possible positive reactions (17 allergens $\times$ 14 horses).

Allergens	RAO (N = 14)				Controls (N = 14)			
	Early 0.5–1 h	Late 4–10 h	Delayed 24–48 h	Total	Early 0.5–1 h	Late 4–10 h	Delayed 24–48 h	Total
Moulds								
<i>A. fumigatus</i>	–	–	–	–	–	–	–	–
<i>P. notatum</i>	–	–	–	–	1	–	–	1
<i>M. mucedo</i>	–	–	–	–	–	–	–	–
<i>C. herbarum</i>	–	–	–	–	–	–	–	–
<i>A. alternata</i>	–	–	–	–	–	–	–	–
Recombinant Asp f								
rAsp f 7	–	1	5	6	–	–	3	3
rAsp f 8	–	1	3	4 [*]	–	–	–	0 [*]
Yeast								
<i>C. albicans</i> 250 U/ml	–	–	7	7	–	–	4	4
<i>C. albicans</i> 2500 U/ml ^a	–	–	7	7 [*]	–	–	4	4 [*]
Mites								
<i>T. putrescenciae</i>	2	6	–	8	1	1	2	4
<i>L. destructor</i>	–	2	–	2	–	1	1	2
<i>A. siro</i>	–	5	–	5	1	2	–	3
<i>D. pteronyssinus</i>	–	3	–	3	2	1	–	3
<i>D. farinae</i>	–	7	–	7	1	7	–	8
Mixed-tree extract	–	–	–	–	–	–	–	–
Mixed-weed extract	–	–	–	–	–	–	–	–
Mixed-grass extract	–	–	–	–	–	–	–	–
Mixed-grass and corn extract	–	–	–	–	–	–	–	–
Total	2	25 [*]	22	42	6	12 [*]	14	28

^a – only 12 controls and 8 RAO horses tested with 2500 U/ml; these results are not included in Total as results of 250 U/ml are already included for *C. albicans*

^{*} RAO versus controls: $p < 0.05$

and four of the controls showed a positive delayed type reaction. Interestingly, only RAO-affected horses reacted with rAsp f 8: four out of the fourteen RAO-affected and none of the control horses reacted with this recombinant allergen ($p < 0.05$) (Table 5a)

3.4. Discussion

The aim of our study was to investigate with commercially available tests, whether IgE-mediated reactions are involved in RAO and whether these tests could be useful in the diagnosis of RAO and the identification of the relevant allergens for a subsequent specific immunotherapy. Results from these two tests were compared both to the clinical diagnosis (RAO or healthy) and to IDT results. The tests that were used detect IgE-mediated reactions at different levels: while the Allercept™ assays detect free allergen-specific IgE antibodies in serum, the cellular antigen stimulation test detects allergic sensitisation further down the allergic cascade; degranulation of sensitised basophilic granulocytes is induced *in vitro* through crosslinking of cell surface bound IgE with the allergen. The Allercept™ is now widely used for allergy diagnosis in the dog (Foster et al., 2003) and is also used in the horse (Lorch et al., 2001a). A preliminary investigation in our laboratory had also shown that this reagent recognises native equine serum IgE in immunoblot (data not shown) specifically. The CAST® is a human allergy test which has been adapted for use in the horse. It seems to be a useful test for detection of IgE-mediated allergic reactions

in the horse, as it has a good sensitivity (78%) and very high specificity (97%) in the diagnosis of insect bite hypersensitivity (Baselgia et al., 2006). However, the present study suggests that the CAST® is not a useful test for diagnosis of RAO as only few horses (4 of 13 RAO-affected and 3 of 14 controls) showed a positive result. This was not due to a too low proportion of basophils in the periphery, as PBLs from all but one horse showed sLT release above the cut off after stimulation with Concanavalin A used as positive control (Table 4). Interestingly, the number of horses with a positive IDT at 30 min to 1 h was also low, as only 2 of the 14 horses with RAO and 5 of the 14 controls showed an immediate type skin reaction. The results from these two tests thus suggest that IgE-mediated reactions against the allergens used in the CAST and IDT were rare in the horses included in our study.

More horses showed a positive result in the serological test: 62% of the RAO-affected horses and 71% of the controls had IgE levels above the cut off of 150 AU. However, to our knowledge, the cut off of the Allercept™ has not been determined for the horse. Larger numbers of horses would need to be tested to determine the best cut off for the horse. However, evaluation of the continuous data (Table 3) suggests that increasing the cut off would not improve the accuracy of this test for diagnosis of RAO, as control horses also had high IgE levels against the same allergens as the RAO-affected horses. Thus, in the present study, none of these two *in vitro* tests seems useful in the diagnosis of RAO, as there were no significant differences between the groups of RAO-affected and control horses.

These findings are confirmed by the results of the IDT, which revealed few immediate type reactions. Most of the positive IDT reactions appeared after 4 h and sometimes 24 h and were thus late and delayed type reactions (Table 5a). Because determination of the heights of the IDT reaction can be regarded as a subjective criterion, evaluation of the IDT was also performed using only the surface of the wheal (Supplementary Table 5b). This resulted in an increase of the number of positive reactions in both RAO-affected (eight additional positive IDT reactions) and healthy horses (18 additional positive IDT reactions) but there were no significant differences between RAO-affected and healthy horses for the proportion of positive IDT reactions at any time. Surprisingly and in contrast to earlier studies (Halliwell et al., 1979; McPherson et al., 1979), only very few positive reactions were observed with the mould antigens in all three tests. The only significant difference that we found was that RAO horses had significantly higher IgE levels against *A. fumigatus* than controls. However, this result has to be interpreted carefully, as all values were low: all horses had IgE levels below the cut off of 150 AU. These findings confirm those from Lorch et al. (2001a) who also showed a low sensitivity of the FcεRIα-based ELISA for mould allergens. We cannot conclude from our study, whether the *A. fumigatus* extract did not contain allergens relevant to the horse in sufficient quantity or whether hay fed to horses presently is of better quality (i.e. less mouldy) than in earlier days and that sensitisation to moulds is thus much less frequent. However, the horses from Michigan State University had been exposed to mouldy hay one to two months before blood sampling and IDT and none of them was positive with mould extracts either. Mould extracts are complex mixtures of a large number of proteins and glycoproteins. The allergenic proteins represent only a small percentage of the total protein content and the composition of the extracts varies between preparations. Therefore, it may also be that the mould extracts used in this study did not contain allergens relevant to the horse in sufficient concentration. This would then be true for the mould extracts from the different companies that were used in IDT, CAST[®] and serology. The recombinant *A. fumigatus* allergens rAsp f 7 and rAsp f 8 were included in IDT and CAST[®] because an earlier investigation had shown that horses with RAO have significantly more often IgE and IgG antibodies against these two recombinant mould allergens than controls (Eder et al., 2000). More RAO-affected horses than controls reacted with rAsp f 7 in IDT (6 of the 14 RAO-affected horses versus 3 of the 14 controls) but the difference was not significant. Interestingly, 4 out of the 14 RAO-affected animals but none of the controls showed a positive IDT with rAsp f 8 ($p < 0.05$). However, no immediate type but only late phase or delayed reactions were observed after IDT with the recombinant allergens and no positive sLT release was induced. These results are in accordance with a study by Wong et al. (2005), who only found significant differences between RAO-affected and control horses 24 h after intradermal injection of *A. fumigatus* extract but not at 1 h or 4 h after intradermal injection of this allergen extract. These findings suggest that IgE levels against these

recombinant allergens described in previous studies (Eder et al., 2000) may have been a bystander reaction, i.e. that IgG antibodies against these allergens may be significant for the pathogenesis of RAO but that additionally, IgE antibodies are also produced. However, these IgE antibodies do not bind to mast cells and are thus not relevant for the pathogenesis of RAO. Further investigations are needed to understand why and what kind of immune reaction horses with RAO mount against rAsp f 8, which is a ribosomal P2 protein from *A. fumigatus* (Cramer, 1998). Results of IDT with the higher concentration of *C. albicans* also deserve further studies as more RAO-affected than controls horses showed a positive delayed type reaction. Similar findings have been reported by Jose-Cunilleras et al. (2001).

Our study suggests that horses, like other species such as the dog (Foster et al., 2003), are often sensitised to mite allergens, since in all three tests the majority of the positive reactions was observed with the mite extracts. We exclude that the positive reactions were non-specific reactions or that positive IDT was due to irritation, as there was a lot of variation in reactivity between horses. Reportedly, pelleted feeds and grains, in particular, can contain large amounts of forage mites and this could explain why horses are sensitised to various mite antigens. However, in the present study we found no significant difference with the mite extracts between RAO and control horses with any of the three tests used. Further studies are needed to investigate whether sensitisation to forage and dust mites is clinically relevant in other equine diseases, such as for instance, urticaria and heel scratch.

Overall, all three tests lead to the same conclusion although different allergen sources were used.

In conclusion, the present study suggests that IgE-mediated, immediate type reactions are not the major immunological mechanism leading to RAO. Results from IDT suggest that late phase and delayed reactions may play a more important role than immediate type reactions in the pathogenesis of RAO. These tests are thus of limited value for diagnosis of RAO, although they allow to identify sensitisation in the horse. All three tests show that RAO-affected as well as clinically healthy horses are sensitised frequently to various mite extracts. Further studies are needed to investigate the clinical significance of sensitisation to mites in the horse.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.vetimm.2008.09.021](https://doi.org/10.1016/j.vetimm.2008.09.021).

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Table 5b Number of positive intradermal reactions to 17 allergens at different times in horses with recurrent airway obstruction (RAO) and controls. In each group there were 238 possible positive reactions (17 allergens $\times$ 14 horses). Additional positives (+x) when only the surface of the wheal is considered (positive = at least half the size of the wheal of the histamine reaction) and not multiplied by the thickness of the skin reaction.

Allergens	[0,2-5]RAO (N = 14)				[0,6-9]
	Early 0.5– 1 h	Late 4– 10 h	Delayed 24– 48 h	Total	Early 0.5– 1 h
[0,1-9]Moulds					
<i>A. fumigatus</i>		–	–	–	–
<i>P. notatum</i>		+1		+1	1
<i>M.ucedo</i>					
<i>C. herbarum</i>					
<i>A. alternata</i>					
[0,1-9]					
[0,1-9]Recombinant Asp f					
rAsp f 7		1	5	6	

rAsp f 8 [0,1-9]		1	3	4	
[0,1-9]Yeast <i>C. albicans</i> 250 U/ml			7	7	
<i>C. albicans</i> 2500 U/ml^a [0,1-9]			7	7 [*]	
[0,1-9]Mites <i>T. putrescenciae</i>	2	6 + 2		8 + 2	1 + 1
<i>L. destuctor</i>		2 + 1		2 + 1	
<i>A. siro</i>		5 + 1		5 + 1	1
<i>D. pteronyssinus</i>		3		3	2 + 1
<i>D. farinae</i>		7 + 2		7 + 3	1 + 1
	+ 1				
Mixed-tree extract					
Mixed-weed extract					
Mixed-grass extract					

Mixed-grass and corn extract [0,1-9]					
Total	2 + 1	25 + 7	22	42 + 8	6 + 3

^a1 only 12 controls and 8 RAO horses tested with 2500 U/ml; these results are not included in Total as results of 250 U/ml are already included for *C. albicans*.

*RAO versus controls: $p < 0.05$.