



A comparison of intradermal testing and detection of allergen-specific immunoglobulin E in serum by enzyme-linked immunosorbent assay in horses affected with skin hypersensitivity

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Abstract

Skin hypersensitivities (allergies) in horses are often diagnosed using clinical signs only. Intradermal testing or serological assays are diagnostic options to confirm the allergic nature of the disease and to identify the allergen(s). Our objective was to develop an allergen-specific enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody specific for horse IgE and to examine its potential for allergen detection in serum in comparison to intradermal testing. Intradermal testing with 61 allergen extracts was performed on 10 horses affected with skin hypersensitivity. Their sera were analyzed by ELISA for IgE antibodies to the same allergens. The kappa test of concordance was used for comparison of the results of both tests. Out of 61 allergen extracts, only two (Timothy and Quack) had kappa values greater than 0.60, suggesting a substantial agreement between skin testing and IgE ELISA. The statistical comparison of the remaining 59 allergens showed little or no concordance between the tests beyond chance. To identify parameters that may influence the sensitivity of the ELISA, the assay was modified to detect allergen-specific IgG and IgG(T) in serum, and the protein content in all allergen extracts was determined by SDS-PAGE. The commercial allergen extracts revealed a high variation in detectable protein. High concentrations of allergen-specific IgG in horse serum were found to compete with IgE for binding to the plates. In conclusion, an ELISA using whole serum and crude allergen preparations provides limited diagnostic information in horses. The reliable diagnosis of allergens in equine skin hypersensitivity is essential to improve allergen-specific treatments, such as hyposensitization, or the development of allergy vaccines.

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1. Introduction

Equine skin hypersensitivities, such as *Culicoides* hypersensitivity and atopic dermatitis (sweet itch, summer eczema), have important implications for the horse and its owner. The characteristic signs of these diseases include pruritus, alopecia and discomfort. The

clinical signs can become so severe that the animals injure their skin and become unable to be worked (Reik, 1953; Wilson et al., 2001; Van der Haegen et al., 2001). In horses, immediate skin reactions to allergens develop 15–30 min after allergen exposure (Wagner et al., 2006) and, due to similarities with human allergies which have been shown to be mediated by IgE (Ishizaka et al., 1966), these diseases in horses have long been thought to also have an allergic etiology (Matthews et al., 1983; Larsen et al., 1988; Anderson et al., 1993). Matthews et al. (1983) observed a “reagin-like antibody” upon injection of *Culicoides* extract similar to human IgE.

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Although polyclonal reagents to detect equine IgE have been developed previously (Suter and Fey, 1983; Halliwell et al., 1993; Marti et al., 1997), they were not widely available to investigate the role of IgE in equine allergic diseases for many years. Larsen et al. (1988) and Anderson et al. (1993) both conducted intradermal challenges of allergic and healthy horses by injecting suspected allergens and observed that allergic horses developed an immediate skin reaction typical of IgE mediated diseases. A study performed by Van der Haegen et al. (2001) found that horses affected by insect bite hypersensitivity had significantly more IgE-bearing cells in skin biopsies than healthy horses.

The recent development of highly specific monoclonal antibodies to detect equine IgE (Wagner et al., 2003) allowed us to show the causal role of IgE in skin hypersensitivity. Using a modified Prausnitz-Küstner experiment, we injected IgE and IgG from both allergic and healthy horses into the skin of clinically healthy horses. The allergic response was transferred to non-allergic horses using IgE from affected horses (Wagner et al., 2006).

The diagnosis of allergies is often based on clinical signs only. In addition, allergens responsible for disease can be determined by intradermal skin testing using extracts from environmental substances or by serological tests detecting allergen-specific antibodies in the serum of patients. Intradermal testing has been shown to be useful in identifying allergens in dogs and currently is often performed to diagnose skin hypersensitivity in horses. Several studies have shown that allergic horses react more frequently to intradermal injection of allergen extracts than healthy horses. This has been observed for several allergic diseases, including insect hypersensitivity (Larsen et al., 1988; Fadok and Greiner, 1990), atopic dermatitis and urticaria (Lorch et al., 2001a). Despite the results of these studies, the accuracy of intradermal testing has been under investigation for some time. Not only are the results of this test difficult to interpret (Lebis et al., 2002), but significant variation in skin reactivity to some antigens (Wong et al., 2005) and the “irritating nature” of certain extracts (Morris and Lindborg, 2003; Lebis et al., 2002) make intradermal testing an inconsistent diagnostic tool.

The challenges and limitations of intradermal testing make serological tests, such as an ELISA, appear to be a promising alternative for diagnosing equine allergic diseases. In dogs, several studies have shown that allergen-specific IgE concentration in serum can be used to identify allergies (Mueller et al., 1999; Foster et al., 2003). DeBoer and Hillier (2001), however, noted that the accuracy of these tests has not been shown, and, thus,

these tests are primarily used by clinicians as diagnostic aids and not definitive allergy tests. IgE concentrations have also been used to diagnose allergies in humans (Bernard et al., 2003). However, Purohit et al. (2005) found that, on a molecular level, allergen-specific serum IgE levels in humans are not necessarily related to the biological sensitivity as determined by cellular and *in vivo* tests. Little has been done to examine the usefulness of ELISA testing for equine allergies. Kalina et al. (2003) conducted a study investigating the effectiveness of an ELISA using polyclonal anti-IgE obtained from a peptide-based IgE. Halliwell et al. (1993) developed an ELISA to detect antibodies specific to *Micropolyspora faeni* and *Aspergillus fumigatus* in bronchoalveolar lavage fluid samples and found that allergen-specific IgE and IgA levels were higher in horses affected with recurrent airway obstruction. Eder et al. (2000) also developed an allergen-specific IgE ELISA using a chicken antibody to equine IgE. They tested sera from horses suffering from chronic bronchitis using both crude extracts and recombinant allergens. They concluded that ELISA using “complex protein extracts” is not a useful method for detecting IgE in horse serum.

To the best of our knowledge, only a single study by Lorch et al. (2001b) exists comparing skin testing results to serological allergy testing using three different allergen-specific assays (a commercial assay for testing dog and cat serum using a recombinant human FcεRIα chain, an ELISA using polyclonal rabbit anti-horse IgE, and a radioallergosorbent test). They found that none of the serum assays produced results similar to those obtained by intradermal testing. Despite this, several companies currently do offer serological testing for allergic diseases. In general, no scientific literature is available for these assays. Our objective was to re-examine the usefulness of allergen-specific IgE detection in serum by ELISA in comparison to the established method of intradermal testing in horses with skin hypersensitivity. For the ELISA, we used commercial allergen extracts to coat the plates and, in contrast to the study by Lorch et al. (2001b), a monoclonal antibody to equine IgE for detection of serum IgE. This antibody was previously described to specifically detect equine IgE and exhibited no cross-reactivity with IgG in horse serum (Wagner et al., 2003, 2006).

2. Materials and methods

2.1. Horses

Ten adult horses with recurrent signs of skin hypersensitivity were used in this study. One allergic

horse was from the experimental herd of the Baker Institute for Animal Health at Cornell University. The remaining horses were patients at the Cornell University Hospital for Animals. Five of these horses were mares and five were geldings, all between 4 and 23 years of age. All horses had a history of pruritus, urticaria, or both. Eight horses had non-seasonal disease with significant worsening of their clinical signs during the spring and summer. Two horses had seasonal disease during spring, summer, and fall. Their clinical signs lessened noticeably with the disappearance of insects at frost but persisted for another 4–6 weeks. All horses had generalized involvement of their skin. All experimental procedures were approved by the Animal Care Committee of Cornell University and were in accordance with the guidelines established by the NIH.

2.2. SDS-PAGE of allergen extracts

To determine the protein concentration in the allergen extracts, the Bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) was performed following the manufacturer's instructions. In addition, the allergen extracts were separated SDS-PAGE. A total of 15 μ l (containing 3–11 mg/ml protein) of each allergen extract was separated under non-reducing conditions using 8–16% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA). After separation, the gels were stained using Coomassie Brilliant Blue.

2.3. Intradermal skin testing

Intradermal skin testing was performed by one investigator (Dr. Miller). Histamine (27.5 μ g/ml) and saline (both Greer Laboratories, Lenoir, NC) were used as positive and negative controls, respectively. These controls and 61 allergens (Greer Laboratories, Lenoir, NC) (Table 1) were injected into the skin of 10 allergic horses. Horses were clipped at the lateral neck and 100 μ l of each allergen extract were injected i.d. at 1000 PNU/ml. After 15–20 min, the reaction of the skin was examined. Based on the size of the wheal by visual examination and palpation, a score (0–4) was applied to each injection site. A score of 0 was given for wheals equivalent to those seen for the negative control (saline) and a score of 4 was given to wheals matching those seen for the positive control (histamine). A score of 2 was given to wheals that were intermediate between the two controls. Wheals with a score of 2 or greater were considered positive reactions.

2.4. ELISA to detect allergen-specific IgE in horse serum

An ELISA was developed to detect allergen-specific IgE in the serum of allergic horses. Identical allergen preparations were used for both ELISA and skin testing. ELISA plates (Maxisorb, Nunc International, Rochester, NY) were coated overnight at 4 °C with each of the allergen extracts in a dilution of 1:100 (corresponding to 30–110 μ g/ml protein as determined by the BCA assay) in sodium carbonate buffer containing 15 mmol Na_2CO_3 and 35 mmol NaHCO_3 at pH 9.6. The following day, the plates were washed three times with wash buffer (2.5 mmol NaH_2PO_4 , 7.5 mmol Na_2HPO_4 , 145 mmol NaCl and 0.05% (v/v) Tween 20 at pH 7.2). Serial dilutions of serum (ranging from 1:2 to 1:32) were added and incubated for 90 min at room temperature. For a negative control (background), wash buffer was added for each allergen at this step. After another wash step, the plates were incubated for 1 h with the monoclonal anti-IgE134 antibody (Wagner et al., 2003) using cell culture supernatant diluted 1:10 in wash buffer (approximately 1 μ g/ml anti-IgE134). The plates were again washed and then incubated with a polyclonal peroxidase-conjugated goat-anti-mouse IgG(H + L) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 45 min. The antibody was diluted 1:10,000 in wash buffer. After a final wash step, the substrate solution (0.01% (v/v) H_2O_2 and 130 μ g/ml 3,3',5,5'-tetramethylbenzidine in citrate buffer consisting of 33.3 mmol citric acid and 66.7 mmol NaH_2PO_4 at pH 5.0) was added. The plates were incubated for 15 min in the dark. Then, 0.5 M sulfuric acid was added to stop the reaction. The plates were read in an automated microplate reader (BioTek Instruments, Inc., Winooski, VT) at a wavelength of 450 nm and reference wavelength of 650 nm. The computer program KC junior was used for evaluation of samples. ELISA reactions were considered positive if a sample had an optical density greater than 0.1 and double the background value.

2.5. ELISA to detect allergen-specific IgG in horse serum

To detect allergen-specific IgG in horse serum, ELISAs were conducted as described above using serial serum dilutions ranging from 1:50 to 1:800 and the monoclonal antibodies, CVS 39 and CVS 40 (both 1:10), to detect IgGb and IgG(T), respectively. Both antibodies were kindly provided by Dr. Paul Lunn. All other steps were performed as described above.

Table 1
Results of skin testing and allergen-specific IgE ELISA for 10 allergic horses

Category ^a	Antigen	Number of ST ^b positive and		Number of ST ^b negative and		Kappa value
		ELISA positive	ELISA negative	ELISA positive	ELISA negative	
I	Cat Dander	1	1	3	5	0.18
	Red Cedar	0	2	1	7	-0.15
	Birch	0	3	1	6	-0.25
II	Timothy	1	1	0	8	0.62
	Orchard	0	2	2	6	-0.25
	Rye	0	1	0	9	0.00
	Fescue	0	2	1	7	-0.15
	Beech	0	2	0	8	0.00
	Mixed Feather	0	4	1	5	-0.19
III	Quack	1	1	0	8	0.62
	Sweet Vernal	0	2	1	7	-0.15
	Brome	0	1	0	9	0.00
	Elm	0	0	0	10	0.00
	Bayberry	0	1	1	8	-0.11
	Lamb's Quarter	0	7	0	3	0.00
	Kentucky Blue	0	3	1	6	-0.18
	Red Top	1	2	1	6	0.21
	Maple/Elder	0	3	0	7	0.00
	Eastern Oak	0	0	0	10	0.00
	Dust Mite Mix	0	6	0	4	0.00
	Kochia	0	6	0	4	0.00
	Marsh Elder	1	4	0	5	0.20
	Pigweed	0	2	0	8	0.00
	Ragweed	0	3	0	7	0.00
	Mugwort	0	3	1	6	-0.18
	Botrytis	0	1	0	9	0.00
	Helminthosporium	0	1	1	8	-0.11
	D. ptermyssinus	0	2	0	8	0.00
	D. farinae	0	2	0	8	0.00
IV	Aspergillus	0	0	1	9	0.00
	White Alder	0	0	0	10	0.00
	Dandelion	0	3	0	7	0.00
	Sorrel/Dock	0	3	0	7	0.00
	Wormwood	0	4	0	6	0.00
	Alfalfa	0	5	1	4	-0.20
	Plantain	0	3	0	7	0.00
	House Dust	0	8	0	2	0.00
	Goldenrod	1	4	0	5	0.20
	Alternaria	0	0	3	7	0.00
	Walnut	0	1	0	9	0.00
	Rhizopus	2	4	0	4	0.29
	Ash	0	1	0	9	0.00
V	Poplar	0	1	1	8	-0.11
	Stemphylium	0	2	1	7	-0.15
	Sycamore	0	1	1	8	-0.11
	Phoma	0	0	0	10	0.00
	Cocklebur	0	2	0	8	0.00
	Cephalosporium	0	0	0	10	0.00
	Epicoccum	0	0	3	7	-0.15
	Fusarium	0	2	1	7	-0.18
	Mucor	1	1	1	7	0.27
	Cladosporium	1	1	2	6	0.27
	Penicillium	0	0	1	9	0.00
	Pullularia	0	0	1	9	0.00
	Human Dander	2	2	0	6	0.55
	White Pine	0	1	3	6	-0.18
	Curvularia	0	0	3	7	0.00
	Tyrophagus	1	7	0	2	-0.05
	Acaris siro	1	6	0	3	0.09
	Malessezia	0	1	2	7	-0.15
	Culicoides	1	7	1	1	-0.18

Shading indicates concordant results. Allergens with kappa values indicating substantial agreement are boxed.

^aCategories based on separation of the extracts using SDS-PAGE. I = Single Strong Band, II = 2–3 Strong Bands, III = 1 or more Weak Bands, IV = Smear, V = No Protein Visible.

^bST = intradermal skin testing.

2.6. Statistical analysis

Positive skin testing results from 61 allergens were compared with the ELISA results using the kappa test of concordance (Dohoo et al., 2003) to determine the concordance between the tests. For each allergen, the number of horses for which the ELISA and skin tests produced the same results were analyzed with the expected frequency of these agreements occurring by chance. Kappa values were calculated using the formula:

$$K = \frac{\sum a - \sum ef}{N - \sum ef}$$

where $\sum a$ is the sum of the agreements (both positive and negative) between the two tests, $\sum ef$ the expected frequency of these agreements occurring by chance, and N is the number of horses being compared. Kappa values greater than 0.60 were considered important.

3. Results

3.1. Comparison of results obtained by intradermal skin testing and by detection of allergen-specific IgE in serum by ELISA

An allergen-specific IgE ELISA was conducted on sera from 10 horses with clinical signs of skin hypersensitivities using 61 allergens. In addition, intradermal testing with the same allergens was performed on these horses after serum samples were obtained. Allergens identified by both ELISA and intradermal testing (concordant positive results) are shaded in Table 1. Only 6 out of 10 horses had concordant positive results for a few allergens. For statistical analysis, the kappa test of concordance was used to determine the concordance of the tests for each of the allergens. Only two (timothy and quack) of the 61 allergens had kappa values greater than 0.60, suggesting a substantial agreement for those allergens. For the remaining 59 allergens, the ELISA and skin testing did not provide comparable results. The following experiments were performed to define parameters that might influence the ELISA and provide explanations for the discrepancy between the results.

3.2. SDS-PAGE analysis of allergen extracts

The BCA assay was used to determine the protein concentration of the extracts, containing 3–11 mg/ml of protein according to this assay. In addition, SDS-PAGE

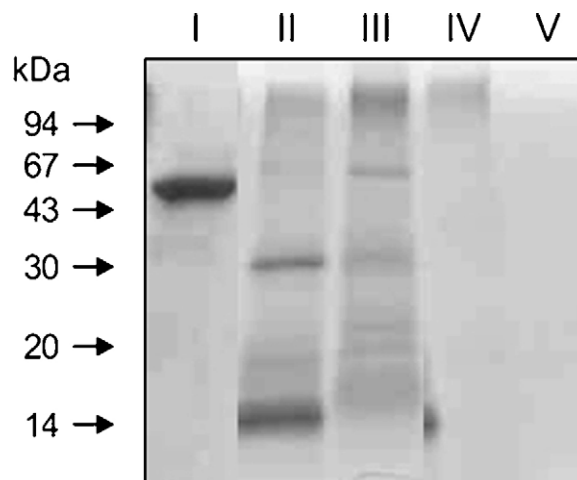


Fig. 1. Allergen extracts were separated in 8–16% gradient gels by SDS-PAGE under non-reducing conditions. Selected allergen preparations are shown. I, Cat dander; II, Timothy; III, Brome; IV, Alfalfa; V, Culicoides.

was performed under non-reducing conditions to visualize the proteins in the allergen extracts. The allergens listed in Table 1 have been categorized based on the protein content in the extracts. Fig. 1 contains an example of an SDS-gel and each allergen category. Three of the 61 extracts examined showed a single strong band after SDS-PAGE (category I). Six extracts contained 2–3 strong bands (category II) and 20 extracts contained several weak bands (category III). For the remaining 32 allergens, a weak smear (category IV) was detected or no visible bands (category V) were observed. Thus, around 50% of the allergens contained almost no detectable proteins in the separation range (10–300 kDa) of the gels. These extracts may have contained small proteins that were lost during the gel run and contributed to the protein concentration of the extracts determined in the BCA assay.

3.3. Detection of allergen-specific IgG in horse serum

IgG antibodies in serum were expected to detect allergens coated to the ELISA plate and this could reduce the binding of IgE antibodies by competition for allergen binding sites on the plate. IgGb and IgG(T) antibodies represent the major IgG subclasses in equine serum. Thus, they were assumed to be the major source of potential allergen-specific IgG antibodies. Allergen-specific IgGb and IgG(T) were measured for six of the serum samples by ELISA. With the exception of Horse B's negative results for IgG(T), IgG antibodies were detected between 2 and 60 times more often than IgE

Table 2

Comparison of allergen-specific IgE, IgGb, and IgG(T) antibodies detected in horse serum by ELISA

Horse	IgE		IgGb		IgG(T)	
	Positive results ^a	Range of titers ^b	Positive results ^a	Range of titers ^b	Positive results ^a	Range of titers ^b
A	4	1:2–1:8	49	1:50–1:800	41	1:50–1:800
B	1	1:2	61	1:50–1:800	0	n/a
C	16	1:2–1:32	42	1:50–1: >800	33	1:50–1:800
D	10	1:2–1:32	61	1:100–1:800	61	1:200–1:800
E	12	1:2–1:16	59	1:50–1:800	60	1:50–1:200
F	2	1:32	59	1:400–1: >800	61	1:800–1: >800
Median (min/max)	7 (1/16)		59 (42/61)		51 (0/61)	

^a The number of positive results is shown from a total of 61 allergens used in the assay.^b Range of end-point titers for all positive allergens for each horse.

antibodies (Table 2). In addition, IgG antibodies were found at much higher dilutions of serum than those of IgE. This suggests that IgG antibodies to various allergen extracts exist in horse serum in much higher concentrations than IgE. When serum samples are used, the allergen-specific IgG antibodies are likely to compete with the less concentrated IgE in the ELISA.

4. Discussion

Horses are exposed to a wide variety of potential allergens in their environments. Thus, it can often be challenging to determine which allergen is responsible when a horse develops skin hypersensitivity. Intradermal skin testing and serological allergen assays are diagnostic methods available to aid the veterinarian in identifying substances responsible for allergic disease. However, only one study exists that compares these methods for equine allergy diagnostics (Lorch et al., 2001b).

The molecular mechanisms of allergy (Ishizaka et al., 1966; Kinet, 1999) and the recent proof of the integral role of IgE in mediating equine skin hypersensitivities (Wagner et al., 2006) suggests that serological assays using detection of allergen-specific IgE could be an alternative to intradermal testing in horses. Blood sampling is a simpler procedure than intradermal skin testing and provides a convenient diagnostic tool for identifying causal allergens in skin hypersensitivities. Kalina et al. (2003) used anti-serum to detect allergen-specific antibodies by ELISA and found a correlation between positive ELISA results and clinical allergy. In our experiments, many of the commercial allergen preparations which are made for intradermal testing were found to be crude extracts. The concentration of specific allergens in the preparations is unknown and, since many were shown to contain

several different proteins, other components in the extracts can interfere with allergen coating in the ELISA, consequently decreasing the amount of allergen available in the test. Thus, most of these allergen extracts cannot be recommended for coating of plates or other matrices for allergen-specific IgE detection in serum. If pure, recombinant allergens were available for serological assays, their sensitivity would likely be improved. Eder et al. (2000) compared crude extracts and recombinant mould allergens to detect mould-specific IgE antibodies in sera from horses with recurrent airway obstruction. No differences were observed between affected horses and healthy horses using crude extracts. In contrast, significantly more allergen-specific IgE was detectable in allergic horses than healthy horses when using recombinant allergens.

Another aspect that influences the sensitivity of serological assays is the high concentration of IgG in serum. IgG antibodies can induce false positive results if cross-reactive detection reagents are used in the assay and also create competition for IgE binding to the allergens. Although the monoclonal anti-equine IgE used for detection in the ELISA described here has been previously shown to have no cross-reactivity to IgG (Wagner et al., 2003), IgE is a minor isotype in serum and the higher concentrations of allergen-specific IgGb and IgG(T) in serum compete with IgE for binding to allergens. It is not yet known whether these allergen-specific IgG antibodies contribute to allergic diseases in the horse. Our recent data showed that most IgG isotypes do not mediate skin reactions, however anti-IgG(T) did induce immediate intradermal reactions, indicating that it may play a role in the allergic response (Wagner et al., 2006).

Some companies do offer serological assays for allergen-specific IgE detection after IgG depletion from horse serum to improve the performance of the test and

to reduce the competitive effects of IgG antibodies described above. The IgG depletion is often performed by using Protein A or G columns. For horse serum, both affinity columns are not capable to deplete all IgGs. Protein G binds to most IgG isotypes in horse serum (Sheoran and Holmes, 1996). However, Protein G does not bind a major fraction of the equine IgG(T) (Wagner et al., 2006). IgG(T) is more than 1000-fold more concentrated in horse serum than IgE (Wagner, 2006). In this study, we found that IgG(T) binds to multiple allergens in the ELISA. We depleted IgG from horse serum by Protein G to improve the ELISA. The 'IgG-depleted' serum was then used in the allergen ELISA. The results obtained by ELISA for the sera before and after IgG depletion did not differ (data not shown). This suggested that IgG depletion by Protein G is not an effective method to improve allergen-specific IgE detection in horse serum.

Intradermal testing provides clinicians with an *in vivo* test to identify the allergen(s) responsible for disease. The reaction tests the ability of intradermal mast cells to release inflammatory mediators in the presence of allergens, and thus resembles the pathological mechanism in skin hypersensitivity. The reaction is mediated by allergen-specific IgE molecules bound to high-affinity IgE receptors on the surface of mast cells (Kinet, 1999). Since intradermal testing involves injecting the allergens directly into the skin, the mast cells are able to identify specific allergens even in crude extracts and respond. Intradermal testing is certainly not a perfect diagnostic test, since, as discussed previously, it often produces variable results and false positives. However, when crude extracts are used, intradermal testing does have an advantage over serological assays due to the mast cells' ability to select the specific allergen via cell surface bound IgE antibodies. Another aspect that could influence the discrepancies observed for skin testing and ELISA are the different half-lives of IgE in serum and IgE bound to mast cell surfaces. IgE in serum has a quick turnover rate of around 3 days, while the half-life of IgE bound to IgE receptors on mast cells is approximately 2 weeks. In the absence of a seasonal allergen in the environment, the allergen-specific IgE concentration might decrease below the detection limit of the ELISA, while the same allergen still results in a positive skin test reaction.

In conclusion, our data suggests that an ELISA using whole serum and crude allergen extracts provides us with limited diagnostic information and should not be exclusively relied upon to identify allergens in skin hypersensitivity. The current serological methods to detect allergens in allergic diseases of the horse require

better validation, improved allergen preparations, and reagents with confirmed specificity to equine IgE. While the accuracy of serological tests cannot be definitively determined since intradermal testing is not infallible (DeBoer and Hillier, 2001), our results indicate that caution should be taken in trying to substitute either of these tests for the other.

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