



Original Research

A Comparison of Intradermal Skin Testing and Serum Insect Allergen-specific IgE Determination in Horses With Insect Bite Hypersensitivity From 2008 to 2016

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ABSTRACT

New techniques in equine veterinary allergy testing include multiple allergen simultaneous tests (MASTs) that cover insect bite hypersensitivity (IBH), respiratory and atopic allergic diseases. The in vivo intradermal test (IDT) method is one of the most widely used techniques in veterinary practice, but recently, in vitro methods have been introduced as possible alternatives. This study was designed to assess the correlation between IDTs and MASTs based on IgE determination in serum. The study was performed on Malopolski breed horses with IBH ($n = 26$). IDTs were performed, and allergen-specific IgE serum concentrations were measured using a monoclonal anti-IgE antibody during the years 2008–2016. An allergen panel containing *Tabanus* spp., *Culicoides nubeculosus*, mosquito (*Culex* spp.), *Simulium equinum*, and *Stomoxys calcitrans* was used. This study shows good agreement and correlation (correlation coefficient ρ , 0.413–0.589) between the two tests for four (*Tabanus* spp., *Culicoides nubeculosus*, *Culex* spp., *Simulium equinum*) of the five tested allergens in horses with IBH. The overall sensitivity (Se) of the allergen-specific IgE insect horse panel was 72.6% (range, 30.8%–91.3%), the specificity (Sp) was 63.6% (range, 50%–70.1%), and the accuracy (Acc) was 73.3% (range, 48%–88.5%) compared with IDTs. This study found good agreement and correlation between IDTs and serological MASTs based on monoclonal antibodies for most of the determined allergens for horses with IBH.

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1. Introduction

Insect bite hypersensitivity (IBH) is the most common allergic skin disease of horses and manifests as chronic seasonal allergic dermatitis caused by bites from insects of the genera *Culicoides*, *Simulium*, *Stomoxys*, *Tabanus*, and *Culex* [1,2] and represents an IgE-mediated type I and/or type IV hypersensitivity to salivary antigens from biting midges (*Culicoides* spp.) and possibly other insects

[3–5]. Currently, the diagnosis of IBH in horses is based on a combination of clinical history, physical examination, and exclusion of other conditions causing pruritus [6,7]. The limited number of studies regarding IBH do not allow determination of an effective empirical therapy. Reliable and fast diagnostic methods are necessary to identify causative allergens associated with IBH in horses. The rapid development of diagnostic methods based on the determination of IgE antibody concentrations in equine veterinary practice has gradually replaced traditional in vivo methods as possible alternatives to intradermal tests (IDTs) for IBH diagnosis. A number of analytic measurements are used to promote more accurate diagnosis and better management of allergic subjects. Allergen skin testing is considered to be the most specific screening method for the diagnosis of allergic diseases in humans (prick test) and veterinary medicine (IDT). However, in vitro tests based on specific IgE determination in sera allow for allergen selection for allergen-specific immunotherapy (ASIT) as well. Allergen-specific

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IgE in sera can be used to confirm a clinical diagnosis but is not a predictor of IBH [8]. Various tests have been evaluated for the in vitro diagnosis of IBH to detect concurrent allergen-specific IgE [9–11]. Commonly, especially in European countries, immuno-enzymatic multiple allergen simultaneous tests (MASTs) have been used for the quantitative determination of IgE in humans and animals but are not based on FcεR1α-based IgE detection technology [12,13]. Owing to the low cost and simple technology for implementation in clinical allergology practice, in vitro tests have become useful tools for the identification of allergic factors.

The aim of this study was to evaluate the diagnostic value and clinical usefulness of IDTs and serological equine monoclonal antibody insect panels for horses with IBH by assessing the correlation between the test results.

2. Materials and Methods

2.1. Horses

This study was performed on 26 IBH-affected Malopolski horses (14 females, 12 males). Some animals were privately owned, and some were patients of the Sub-Department of Clinical Diagnostics and Veterinary Dermatology at the University of Life Sciences in Lublin (referral clinic), Poland. All horses lived in the same rural area and were kept loose in group housing stables during the winter and kept in pastures and stables during the summer. The age range of the animals was 2–18 years (median age, 7.8 years). All horses had typical clinical signs of IBH and a clinical history of recurrent seasonal (summer) pruritus and dermatitis affecting the mane and tail area, and the dorsal and sometimes the ventral midline. Other causes of pruritic skin diseases, such as parasitic, fungal, and bacterial infections and adverse food reactions, were excluded using appropriate diagnostic tests and therapy (trichography, multiple skin scraping samples from various locations, cytological examinations, fungal and bacterial cultures, elimination diets, and deworming).

2.2. Intradermal Tests

Intradermal tests were performed between September and December during the years 2008–2016 in unsedated horses in the middle region of the neck. The hair was clipped, the skin was cleaned with 70% alcohol, and a permanent marker was used to indicate the injection sites, which were placed approximately 5 cm apart. Seven injections of 0.1 mL using insect whole-body extract were placed intradermally using a 25 G needle: histamine was used as a positive control at a concentration of 1:1000 w/v, a phosphate buffer was used as a negative control, and *Tabanus* spp., *Culicoides nubeculosus*, *Culex* spp., *Simulium equinum*, and *Stomoxys calcitrans* extracts were injected (Agroskin RTU 20; Agrolabo Horse Panel, Scarmagio, Italy). The wheals were evaluated at 0.5 hours, 4 hours, and 24 hours (Langner 2008). The wheal diameter was assessed semiquantitatively. The firmness of the wheal was evaluated using a scale from 0 to +++ as proposed by Lebis [14]: 0 = no palpable reaction, +/- = very flat reaction with a poorly defined outline, + = reaction with barely palpable thickness, ++ = reaction with obvious thickness, and +++ = reaction with same thickness as the histamine reference.

2.3. Serological Tests

Sample collection and IgE measurements were performed during the summer season (between June and September). A volume of 5 mL of peripheral blood was collected by jugular venipuncture from each horse and centrifuged for 10 minutes at 4500 g. Serum

samples were kept cool at 4°C without any freeze-thaw cycles until an assay was performed (5–12 hours after blood collection). The serum allergen-specific IgE concentrations were determined using insect whole-body extract and monoclonal anti-IgE antibody (Polycheck Allergie NF Horse Panel, BioCheck GmbH, Münster, Germany) according to the manufacturer's instructions. The Northern and Central European allergen panel was composed of 15 (including *Tabanus* spp., *Culicoides nubeculosus*, mosquito *Culex* spp., *Simulium equinum*, and *Stomoxys calcitrans*) individual allergens and five allergen mixtures. In accordance with the guidelines provided by the manufacturer of the test, the reactions (allergy classes) were interpreted as negative (<1 kU/l; NR, class 0), mild positive (1.0–2.0 kU/l; MPR, class 1), moderate positive (2.0–20 kU/l; MR, class 2), and strongly positive (>20 kU/l; SR, class 3). No corticosteroids or antihistamines were administered for at least 6 weeks before each scheduled test.

2.4. Statistical Analysis

The correlations between IDT results (wheal diameter) and allergen-specific IgE serum concentrations (kU/L) were analyzed using the Spearman-rank correlation test. All analyses were carried out using computer software (Statistica, version 10.0; Statsoft, Tulsa, Oklahoma, USA). A *P* value < .05 was considered statistically significant. The sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), and accuracy (Acc) were determined for each allergen individually and were calculated using the following formulae: $Se = a/a + c \times 100\%$, $Sp = d/b + d \times 100\%$, $PPV = a/a + b \times 100\%$, $NPV = d/c + d \times 100\%$, and $Acc = a + d / a + b + c + d \times 100\%$, where a = positive IgE and positive IDT, b = positive IgE and negative IDT, c = negative IgE and positive IDT, and d = negative IgE and negative IDT. All investigations involving the use of animals were approved by the local ethics committee.

3. Results

The obtained positive results for IDTs and the mean values and ranges of allergen-specific IgE serum concentrations in IBH horses for each insect allergen are shown in Table 1. A positive correlation was found between IDT results and serological test results for 4 of 5 insect allergens. The highest test correlation was reported for *Tabanus* spp. and *Culicoides nubeculosus*. There was no correlation between the tests for *Stomoxys calcitrans*. The Se, Sp, PPV, NPV, and Acc were varied and depended on the type of allergen (Table 2). Using a mean IDT cutoff diameter of at least + and an IgE cutoff value ≥ 0.9 kU/L, the overall Se was 72.6% (range, 30.8%–91.3%), and the Sp was 63.6% (range, 50%–70.1%). The overall Acc of the Polycheck Horse Insect Panel Test and IDTs was 73.3% (range, 48%–88.5%).

The most prevalent positive reaction (PR) on the serological test and the highest insect-specific IgE concentrations were observed for allergens to *Tabanus* spp. (85%) and *Culicoides nubeculosus* (81%), with a high percentage of moderate reactions. The concordance and discordance between the two tests varied depending on the type of allergen. Twenty-one PRs on the serological test were found among 23 horses with positive early IDT reactions for *Tabanus* spp. (91%), 20 of 24 horses for *Culicoides* spp. (83%), 14 of 17 horses for *Simulium* spp. (82%), 6 of 9 horses for *Culex* spp. (67%), and 4 of 10 horses for *Stomoxys* spp. (40%).

4. Discussion

Currently, unlike in humans, dogs, and cats, in studies concerning allergic horses, there is little data regarding the reliability of serological tests based on long-term clinical evaluation. Recent

Table 1

Correlation analysis between IgE concentrations (kU/L) and the results of IDTs (+, ++, +++) showing the number and percentage of horses with IBH with PRs on the IDT and PRs, mean values (MV), and ranges (R) on the serological MAST.

Allergen	IDT (n = 26)			MAST IgE	P-Value	Spearman's Rho
	N (%) PR			N (%) PR		
	Early (0.5 hr	MV (R) kU/L	Delayed (24 hr)	MV (R) kU/L		
<i>Tabanus</i> spp.	23 (88)	20 (70)	7 (27)	22 (85) 4.98 (0.7–16)	$P = .0015^*$	0.589069
<i>Culicoides nubeculosus</i>	24 (92)	22 (85)	13 (50)	21 (81) 2.15 (0.3–9.2)	$P = .002^*$	0.567976
Mosquito (<i>Culex</i> spp.)	9 (35)	4 (15)	3 (12)	11 (42) 1.6 (0.2–6.5)	$P = .02^*$	0.451754
<i>Simulium equinum</i>	17 (65)	9 (35)	2 (8)	17 (65) 1.88 (0.3–6.1)	$P = .036^*$	0.413111
<i>Stomoxys calcitrans</i>	10 (38)	7 (27)	3 (12)	9 (35) 1.12 (0–6.4)	$P = .89$	−0.027959

Abbreviations: IDT, intradermal test; IBH, insect bite hypersensitivity; MAST, multiple allergen simultaneous test; PR, positive reaction.

Significant differences were indicated by * ($P < .05$).

years have brought new perspectives on diagnosing allergic diseases. Intradermal tests and histamine release tests are the most sensitive, specific, and confirmatory biological assays that resemble the natural hypersensitivity reaction [5,15]. However, the in vitro measurement of allergen-specific IgE is the laboratory equivalent of clinical skin testing and is gradually replacing IDT techniques. Serological assays using allergen-specific IgE detection could be an alternative to IDTs in horses, but the Acc, Se, and Sp of these testing methods may vary in terms of the type of causative allergen. Most of these data are obtained from serological tests using an FcεR1α-based ELISA. Multiple allergen simultaneous tests are increasingly used in daily allergological practice, and their diagnostic value in medicine can be assessed by comparing these tests with other sensitive methods, such as in vivo prick tests or in vitro ImmunoCAP systems. The present study assessed the diagnostic value of MASTs compared with IDTs in the diagnosis of IBH in horses.

To the best of our knowledge, this is the first study to determine the Acc of MAST for assessing the etiology of equine IBH. Previous studies have shown very significant differences between the concentrations of IgE in sera of the same individuals during in-season and out-of-season exposure to allergens. Determining IgE in the off-season or during the asymptomatic period can result in false negative reactions [16]. A decrease in serum IgE concentrations during the asymptomatic period has been shown in the case of recombinant allergens [17]; however, with whole-body extracts, such differences have not been shown [18]. In our study, allergen-specific IgE concentrations were evaluated the period immediately after exposure to insect allergens in horses with a seasonal form of IBH in environments with ubiquitous insects. Thus, serological testing was performed during the symptomatic period before the treatment was undertaken, whereas IDTs were performed after suitable drug withdrawal during remission in the fall and winter. Our study showed a correlation between in vivo and in vitro tests for most insect allergens with high Se for *Tabanus*

(91.3%), *Culicoides* (83.3%), and *Simulium* (82.4%), and moderately Sp serological tests for *Culex* (70.1%), *Tabanus* and *Simulium* (66.7%), and *Culicoides* (50%). In the assessed tests, the probability of misdiagnosing an allergic horse as healthy (false negative) was low, while a false positive result in IBH-free ones was more likely. These results are consistent with the research of many authors, which has described that healthy horses had positive results on serological tests [17,19,20]; however, allergic horses react more severely to insect allergens than normal ones [20,21]. In addition, IgG antibodies in MAST can induce false positive results if cross-reactive detection reagents are used in the assay and create competition for IgE binding to the allergens [22]. IgG can also activate skin mast cells and might have a potential role in allergic conditions in horses [3]. Clinically healthy horses also often temporarily develop allergen-specific IgE antibodies [7].

Currently, the Acc of serological tests is estimated using recombinant allergens and whole-body insect extracts. The Se and Sp of ELISA based on recombinant allergens *Culicoides* were estimated to be near 90% [17]. A high Sp of such tests is obtained at the expense of lower Se, which reduces the risk of “hyperreactivity” of these tests. A Se of 44% and 22%, for an IgE-specific ELISA with *C. nubeculosus* whole-body extracts or saliva, respectively, corresponding to a Sp of 100%, has been described by Langner et al. Peeters et al. reported moderate Se (70%) and high Sp (97%) of tests for *Culicoides* spp. allergens detected by ELISA. However, lowering the cutoff point induced an increase in Se and a decrease in Sp of the test. Thus, one objective in deciding on a cutoff point is to achieve a proper balance between false positives and false negatives. In our study, the proportion of Se and Sp was overstated for the benefit of Se. Our research shows that the cutoff point should be increased to increase the Sp of the test. However, given that serological tests are performed in allergic horses to use specific immunotherapy, false negative reactions may result in a failure to choose certain allergens, which can determine the effectiveness of

Table 2

Se, Sp, PPV, NPV and Acc of allergen-specific IgE MASTs compared with those of IDTs.

	<i>Tabanus</i> spp.	<i>Culicoides nubeculosus</i>	Mosquito (<i>Culex</i> spp.)	<i>Simulium equinum</i>	<i>Stomoxys calcitrans</i>	Mean Value
Se (%)	91.3	83.3	75	82.4	30.8	72.6
Sp (%)	66.7	50	70.1	66.7	64.3	63.6
PPV (%)	95.5	95.2	54.5	82.4	44.4	74.4
NPV (%)	50	20	85.7	66.7	50	54.5
Acc (%)	88.5	80.8	72	76.9	48	73.3

Abbreviations: IDT, intradermal test; MAST, multiple allergen simultaneous test; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; Acc, accuracy.

ASIT in horses. The appropriate cutoff point and use of relevant recombinant allergens significantly affect the reliability and Acc of serological tests.

In conclusion, immunoenzymatic MASTs show good correlation with IDTs and moderate Acc, high Se, and moderate Sp for etiologic diagnosis in horses with insect sensitization. Multiple allergen simultaneous tests cannot be used to distinguish IBH-affected from non-IBH horses because of possibility of the numerous false PRs and a moderate risk of false negative reactions. Despite some discrepancies between the in vivo and in vitro procedures, the IgE system has been expected to demonstrate acceptable concordance with IDTs and high enough sensitivity in horses and shows high value for selecting allergens for specific immunotherapy. Multiple allergen simultaneous tests should be interpreted in accordance with clinical signs and history. Multiple allergen simultaneous tests should be performed in horses with a history and clinical signs highly suggestive of insect allergy, but because of their lower specificity, it should not be used as a screening test in horses with pruritic skin diseases. Future studies should focus on determining clear cutoff values for IgE to insect allergens.

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