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Evaluation of an IgE ELISA with *Culicoides* spp. extracts and recombinant salivary antigens for diagnosis of insect bite hypersensitivity in Warmblood horses

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ABSTRACT

Insect bite hypersensitivity (IBH) in horses represents an immunoglobulin E (IgE)-mediated hypersensitivity to salivary antigens from biting midges (*Culicoides* spp.). The aim of this study was to evaluate and compare the performances of IgE ELISAs using recombinant *Culicoides* spp. Obsoletus group salivary gland antigens or crude whole body extracts ('ObsWBE'), *C. nubeculosus* recombinant proteins (Culn1, 3, 4, 5, 7, 8 and 10) and Obsoletus group recombinant proteins (Culo1 and 2). IgE levels were measured in plasma of 343 Warmblood horses classified as IBH-affected ($n = 167$) and IBH-unaffected ($n = 176$) according to the owners' descriptions. IBH-affected horses were subdivided based on the severity of their clinical signs at sampling and whether or not their IBH history was considered to be classical. The accuracies of the tests increased when clinical signs at sampling were more pronounced or when the IBH history could be considered as classical. A combination of IgE levels against the three best performing *Culicoides* spp. recombinant proteins (Culn4, Culo1 and Culo2) and ObsWBE resulted in the best performing test. When IBH-affected horses showing a classical history of the disease and severe clinical signs were compared with IBH-unaffected horses, the Youden's index at the optimal cut-off for the three tests in combination was 0.67. This optimal cut-off had a sensitivity of 70%, a specificity of 97% and a total accuracy of 92%. The performance of the IgE ELISA was affected by the severity of IBH clinical signs at sampling and was improved when IgE levels against several recombinant proteins were combined.

Introduction

Insect bite hypersensitivity (IBH) in horses represents an IgE-mediated type I and/or type IV hypersensitivity to salivary antigens from biting midges (*Culicoides* spp.) and possibly other insects (Hellberg et al., 2006; Langner et al., 2008). Affected horses develop severe skin lesions, mainly along the mane and tail, due to self-mutilation in an attempt to alleviate pruritus (Scott and Miller, 2003).

Currently, the diagnosis of IBH in horses is based on a combination of clinical history, physical examination and exclusion of other conditions causing pruritus, but misdiagnosis occurs. The available diagnostic tests include: (1) intradermal testing (IDT) with *Culicoides* spp. preparations, which demonstrates mast cell activation (Ferroglio et al., 2006; Langner et al., 2008); (2) histamine release tests (HRT) or the sulphidoleucotriene release test (sLTRT), which utilise basophilic granulocytes from blood samples (Marti et al., 1999; Baselgia et al., 2006; Wagner et al., 2008; Peeters et al., 2009); and (3) serological tests that measure allergen-specific IgE levels (Frey et al., 2008; Langner et al., 2008; Peeters et al., 2012).

The use of an indirect ELISA to diagnose allergies is based on the hypothesis that allergic individuals display higher titres of allergen-specific IgE antibodies than non-allergic individuals (Mimura et al., 2004; Portengen et al., 2004). The most important advantage of serology-based tests compared to cell-based tests, such as the HRT, is that IgE levels can be measured in frozen serum or plasma samples. In addition, taking a blood sample is more owner-friendly than performing an intradermal skin test and skin tests in horses are more difficult to interpret than in dogs or human beings.

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Schaffartzik et al. (2011) cloned a *Culicoides nubeculosus* salivary gland allergen repertoire that was associated with IBH and evaluated an allergen-specific IgE ELISA in Icelandic horses with a history of severe IBH. Peeters et al. (2012) measured IgE levels against 10 *C. nubeculosus* r-allergens (Culn1–10) in the plasma of 115 Warmblood horses. However, the effect of the severity of the clinical signs at sampling on the diagnostic performances of these IgE ELISAs was not evaluated. In the present study, IgE levels were measured in the plasma of 343 Warmblood horses with no, mild or severe clinical signs of IBH at sampling. A panel of nine recombinant *C. nubeculosus* and Obsoletus group allergens, as well as *C. nubeculosus* and Obsoletus group extracts, were evaluated in an allergen-specific IgE ELISA and this information was combined in order to find the best combination of allergens for a serological in vitro diagnostic test.

Materials and methods

Horses and sampling

During the summers (June–September) of 2009, 2010 and 2011, blood samples were collected in Venosafe K2-EDTA tubes (Terumo) from 343 Warmblood horses in Belgium belonging to 109 owners. The study was approved retrospectively by the Ethical Committee for Animal Experiments of the Katholieke Universiteit Leuven (approval number P061-2012, date of approval 18 April 2012). Plasma was collected after centrifugation (10 min, 1500 *g*).

Horses were subdivided according to the criteria shown in Table 1. The first level of classification was based on owner information ('status'). IBH-unaffected horses (status = 0) had no history of skin problems (class 1, $n = 176$). Alternatively, if a horse ever had clinical signs, it was classified as IBH-affected (status = 1, $n = 167$). All IBH-unaffected horses were at least 3 years old and stabled with IBH-affected horses.

IBH-affected horses were subdivided on the basis of the severity of IBH clinical signs at sampling ('class'). Horses were examined for the presence or absence of the following clinical signs: (1) thickening of the skin at the base of the mane; (2) IBH lesions along the mane; and (3) IBH lesions along the tail (numerous papules, tufted hair, hyperaesthesia). IBH-affected horses without clinical signs at sampling were classified into class 2 ($n = 66$), horses with mild clinical signs at sampling were classified into class 3 (one or two clinical signs, $n = 50$) and horses with severe clinical signs were classified into class 4 (all three signs, $n = 51$). A last classification was based on whether or not the IBH history could be considered as 'classical' or 'non-classical' ('class_AB'). All classically IBH-affected horses (classes 2A, 3A and 4A) had clinical signs typical of IBH that were recurrent for at least 2 years, even if these clinical signs could be suppressed by preventive measures (e.g. blankets, insect repellents and/or itch-reducing products). An IBH-affected horse was classified as 'non-classical' (classes 2B, 3B and 4B) if it was the first season in which IBH clinical signs were observed, if the clinical signs were not recurrent, if the age at which the first clinical signs were observed was high (>7 years; Broström et al., 1987) or if the only IBH signs ever observed were lesions along the tail (Table 1).

Production of recombinant Culicoides spp. proteins and preparation of extracts

Culicoides spp. recombinant proteins (r-allergens) were produced as described by Schaffartzik et al. (2011). A pilot study including 115/343 Warmblood horses used in this study had shown that few serum samples contained IgE against Culn2, 6 or 9 above the detection limit of the ELISA (Peeters et al., 2012); thus, these r-allergens were not evaluated further. Seven *C. nubeculosus* (Culn1, 3, 4, 5, 7, 8 and 10; Schaffartzik et al., 2010, 2011; Peeters et al., 2012) and two Obsoletus group salivary gland r-allergens (Culo1 and 2) were used. The sequences of Culo1 and Culo2 (GenBank JX512273 and JX512274) were derived from a cDNA library constructed using mRNA from salivary glands of Obsoletus group midges and were identified as homologues of IgE-reactive *C. nubeculosus* salivary proteins (Russel et al., 2009). Culo1 has ~43% identity to Culn9, whereas Culo2 has no direct homologue in the Culn allergens.

Culicoides spp. were caught on a horse farm in Zürich, Switzerland, using Onderstepoort ultraviolet-light suction traps (Venter and Meiswinkel, 1994), with the exception that the insects were caught alive and then killed by freezing. The biting midges were classified at group level using a stereomicroscope based on wing pattern and morphological features (Goffredo and Meiswinkel, 2004).

Members of the Obsoletus group (i.e. *C. obsoletus*, *C. scoticus*, *C. chiopterus* and *C. dewulfi*) were pooled together and extracts were prepared as described in Marti et al. (1999). A pilot study comparing *C. nubeculosus* and Obsoletus Group whole body and thorax extracts had markedly higher IgE binding by *C. nubeculosus* thorax extract than *C. nubeculosus* whole body extracts. This difference was not found when comparing Obsoletus Group thorax and whole body extracts (data not shown). Because of limited availability of the Obsoletus Group midges, whole body extracts was used from this Group. Female biting midges (whole body) from the Obsoletus group or female *C. nubeculosus* (thorax and head, abdomen removed) were added to 1.5 mL Eppendorf tubes with 0.9% sodium chloride (~300 midges in 0.3 mL sodium chloride) and beads of a TeSeE Purification Kit tube (BioRad). The extracts were stored at −80° C. Whole body extracts from biting midges of the Obsoletus group are herein abbreviated as 'ObsWBE' and the thorax extracts of *C. nubeculosus* are abbreviated as 'NubTH' (head and thorax, body removed).

Allergen-specific IgE ELISA

The allergen-specific IgE ELISA was performed as described by Schaffartzik et al. (2011) and Peeters et al. (2012), with the following modifications. Every sample was tested in duplicate. A negative control, a blank and at least five positive controls were tested in duplicate on every plate. The optical density (OD_{405}) values of each plate were multiplied after blank correction with a correction factor specific for that plate. The correction factor was calculated as the sum of the OD_{405} values of all positive controls of that plate divided by the sum of the OD_{405} values of all positive controls of one of the plates used as a reference plate. The corrected OD_{405} values were used as values for the IgE level. The same batch of r-allergens or extracts was used to test all horses. The cut-off for a detectable IgE level against an r-allergen or extract was arbitrarily defined as a corrected OD_{405} value equal to 0.2. The term 'CulSumOf3_Obs' was used when the IgE levels against Culn4, Culo1 and Culo2 were added to the IgE levels against ObsWBE.

Statistical analysis

Assumptions of normality were checked using an SAS9.2 macro (MULTNORM, SAS). Non-parametric tests for location differences between levels of a factor were conducted using a Kruskal–Wallis test calculated with PROC NPAR1WAY, based on Wilcoxon scores and post hoc corrected for multiple comparisons (Siegal and Castellan, 1988). To evaluate the performance of the diagnostic tests, non-parametric receiver operating curve (ROC) analysis was performed and calculations of the area under the empirical curve (AUC) were performed using PROC LOGISTIC. The ROC-CONTRAST statement compared ROC models based on a non-parametric approach (De Long et al., 1988). The function 'ordROC' of the package 'NonbinROC' in R was used to evaluate the performance of a diagnostic test when non-binary classifiers were used (Obuchowski, 2005; Nguyen, 2007). The STEPDISC procedure in SAS 9.2 was used to perform a stepwise discriminant analysis. To select an optimal

Table 1
Overview of horses in the study.

First classification		Second classification		Third classification			
Status	Status	Status + clinical signs at sampling	Clinical signs at sampling[a]	Status + clinical signs at sampling + 'classical' IBH	Classical IBH[b]	Age (years)	Minimum age (years)
IBH-unaffected ($n = 176$)	0	Class 1 ($n = 176$)	No	Class 1 ($n = 176$)	–	10.4 ± 5.7	3
IBH-affected ($n = 167$)	1	Class 2 ($n = 66$)	No	Class 2A ($n = 34$)	Yes	8.7 ± 5.0	3
				Class 2B ($n = 32$)	No	8.1 ± 4.9	2
		Class 3 ($n = 51$)	Mild	Class 3A ($n = 36$)	Yes	8.1 ± 4.2	3
				Class 3B ($n = 15$)	No	5.5 ± 4.3	2
		Class 4 ($n = 50$)	Severe	Class 4A ($n = 39$)	Yes	6.2 ± 4.1	2
				Class 4B ($n = 11$)	No	4.8 ± 4.9	1

[a] No, no clinical signs at sampling; Mild, thickening of the skin at the base of the mane and/or lesions along the mane and/or lesions along the tail; Severe, thickening of the skin at the base of the mane and lesions on the mane and on the tail.

[B] Yes, 'Classical' insect bite hypersensitivity (IBH)-affected horses had IBH clinical signs that were recurrent for at least 2 years, even if these clinical signs could be suppressed by preventive measures; No, 'Non-classical' means it was the first season IBH clinical signs were observed or the clinical signs were not recurrent or the age at which the first clinical signs were observed was high (>7 years) or the IBH clinical signs were dubious.

cut-off, a macro in SAS was used (SNSP_TRADEOFF; Lambert and Lipkovich, 2008). Cut-offs that maximised the Youden's index were selected as optimal cut-offs.

Results

IgE level in insect bite hypersensitivity-affected vs. unaffected horses

The percentages of horses with IgE levels above the ELISA detection limit against all specific r-allergens or extracts was significantly higher for IBH-affected horses (4–45%) than IBH-unaffected horses (1–9%; Fig. 1A, all P values <0.05). Seventy-two per cent of IBH-unaffected horses did not have detectable IgE levels against any r-allergen, compared to only 30% of IBH-affected horses (Fig. 2, $P < 0.001$). Ninety-one per cent of IBH-unaffected horses did not have detectable IgE levels against any of the *Culicoides* extracts, compared to 61% of IBH-affected horses ($P < 0.001$).

There were significant differences between IBH-affected and IBH-unaffected horses in the percentages of horses with detectable IgE levels against two or more r-allergens (55% vs. 15%, $P < 0.0001$), against Obsoletus group whole body extracts (34% vs. 7%, $P < 0.0001$) and against *C. nubeculosus* thorax extracts (26% vs. 3%, $P < 0.0001$).

There was no significant difference between levels of IgE against Culn8 in IBH-affected and IBH-unaffected horses ($P = 0.09$), whereas IgE levels against all extracts and all other r-allergens were significantly higher in plasma of IBH-affected horses (status = 1) than IBH-unaffected horses (status = 0) ($P < 0.05$; Table 2). To verify that IgE levels against certain *Culicoides* spp. r-allergens could differentiate IBH-affected and IBH-unaffected horses, ROC analysis were performed with 'status' as classifier; AUCs ranged from 0.55 (Culn8) to 0.76 (Culn4). The r-allergens that differentiated best between IBH-affected and unaffected horses were Culn4, Culo1 and Culo2 (AUC = 0.76, 0.75 and 0.72, respectively); these AUCs were significantly higher than the AUCs of the other antigens ($P < 0.005$). AUCs for IgE levels against NubTH and against ObsWBE were 0.72 for both extracts.

A stepwise discriminant analysis indicated that combining the three best performing antigens (Culn4, Culo1 and Culo2), together with the IgE level against ObsWBE, would result in the best diagnostic test (CulSumOf3_Obs). The AUC of CulSumOf3_Obs (AUC = 0.8) was significantly higher than the AUCs of the IgE levels against all r-antigens, including Culn4, Culo1 and Culo2, and was significantly higher than the AUCs of both *Culicoides* spp. extracts ($P < 0.05$).

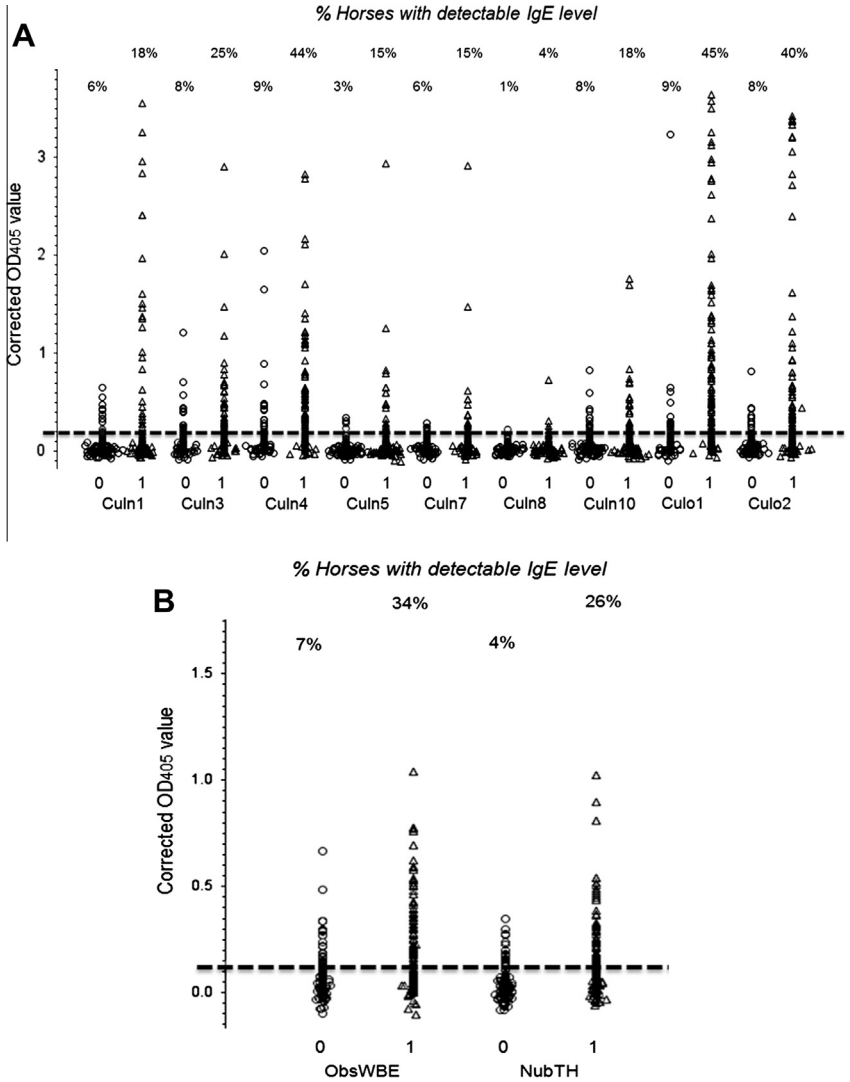

Fig. 1. IgE levels, shown as corrected optical density (OD) values. (A) IgE levels against seven *Culicoides nubeculosus* proteins (Culn1, 3, 4, 5, 7, 8 and 10) and two *Culicoides obsoletus* proteins (Culo1 and 2). (B) IgE levels against *C. obsoletus* whole body extracts ('ObsWBE') and *C. nubeculosus* thorax extracts ('NubTH'). Insect bite hypersensitivity (IBH)-affected horses (status 1) are represented with a triangle; a circle is used for IBH-unaffected horses (status 0). A horizontal line represents the cut-off defined for detectable IgE level. The percentages of IBH-affected and IBH-unaffected horses that had detectable IgE level against a specific r-allergen are shown at the top of the figure.

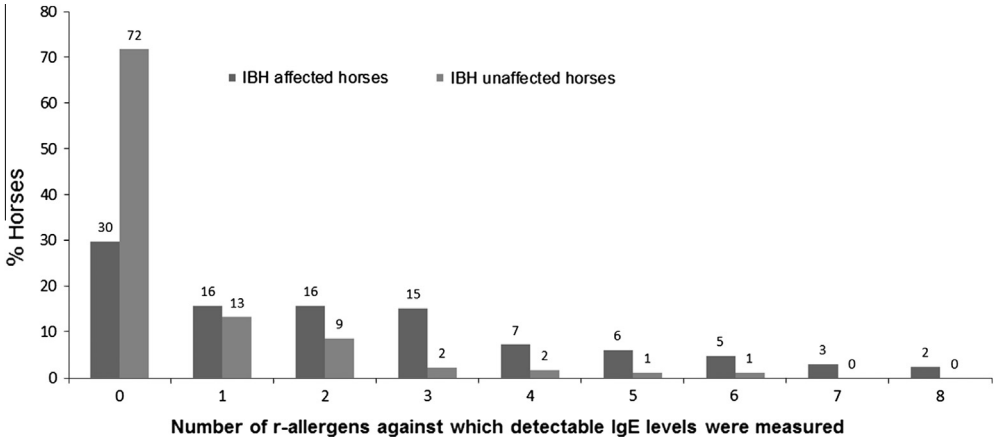

Fig. 2. Percentages of insect bite hypersensitivity (IBH)-affected and IBH-unaffected horses that had detectable IgE levels against a certain number of r-allergens (range 0–8). The *X*-axis shows the number of r-allergens against which a detectable IgE level was measured (range 0–8). The *Y*-axis shows the percentages of horses (of all IBH-affected and IBH-unaffected horses) that had a detectable IgE level against a certain number of r-allergens.

Table 2
Area under the curve (AUC) and pairwise accuracies for different allergens and for the combination CulSumOf3_Obs.

	Class 1 vs. 2 + 3 + 4	No clinical signs 1 vs. 2	Mild clinical signs 1 vs. 3	Severe clinical signs 1 vs. 4	Non-classical 1 vs. 2B + 3B + 4B	Classical 1 vs. 2A + 3A + 4A	Classical and severe clinical signs 1 vs. 4A
Culn1	**0.62***	0.57	**0.65***	**0.64***	0.59	**0.62***	**0.66***
Culn3	**0.62***	**0.62***	0.61	**0.65***	**0.64***	**0.60***	0.60
Culn4	**0.76***	**0.71***	**0.76***	**0.76***	**0.68***	**0.77***	**0.78***
Culn5	**0.59***	0.59	0.60	0.59	**0.62***	0.57	0.59
Culn7	**0.60***	0.54	**0.68***	**0.63***	**0.61***	**0.60***	0.59
Culn8	0.55	0.51	**0.63***	0.53	0.57	0.54	0.52
Culn10	**0.60***	0.54	**0.64***	0.59	**0.60***	0.58	0.57
Culo1	**0.75***	**0.64***	**0.75***	**0.86***	**0.68***	**0.77***	**0.88***
Culo2	**0.72***	**0.67***	**0.74***	**0.73***	**0.68***	**0.71***	**0.74***
ObsWBE	**0.72***	**0.66***	**0.70***	**0.80***	**0.66***	**0.74***	**0.84***
NubTH	**0.72***	**0.65***	**0.71***	**0.79***	**0.67***	**0.74***	**0.86***
CulSumOf3_Obs	**0.80***	**0.73***	**0.80***	**0.86***	**0.72***	**0.83***	**0.88***

'ObsWBE' and 'NubTH' represent IgE levels against Obsoletus group whole body extracts and *C. nubeculosus* thorax extract, respectively. 'CulSumOf3_Obs' combines the IgE level against Culn4, Culo1 and Culo2 with ObsWBE. When a significant difference between categories was found (*P* value of Kruskal–Wallis after post hoc correction for multiple comparisons <0.05), an asterisk was used.

Effect of 'severity of clinical signs' and 'insect bite hypersensitivity history'

The performance of CulSumOf3_Obs improved when the clinical signs at sampling were more pronounced (Table 2). The pairwise accuracy of CulSumOf3_Obs (0.86 ± 0.04) was higher between classes 1 and 4 (IBH-unaffected vs. IBH-affected with severe clinical signs) compared to the pairwise accuracy (0.80 ± 0.02) between classes 1 and 3 (IBH-affected with mild clinical signs) and between classes 1 and 2 (IBH-affected without clinical signs at sampling; pairwise accuracy = 0.73 ± 0.02).

In addition, the performance of CulSumOf3_Obs significantly improved when the IBH history could be considered as classical (accuracy = 0.83 ± 0.04) compared to when the IgE level of IBH-unaffected horses was compared to non-classical IBH-affected horses (accuracy = 0.72 ± 0.02). The accuracy of CulSumOf3_Obs increased to 0.88 (±0.04) when the IgE level of IBH-unaffected (class 1) horses was compared to IBH-affected horses with severe clinical signs and a classical IBH history (class 4A).

Performance parameters

All performance parameters of CulSumOf3_Obs increased when the test was evaluated on the ability to differentiate between class 1 and class 4A compared to when the test was evaluated on the ability to differentiate between IBH-unaffected (status 0, class 1) and IBH-affected horses (status 1, class 2 + 3 + 4) (Table 3). Total accuracy and Youden's indexes increased from 74% to 92% and from 47% to 67% respectively (Table 3). The Youden's index at the optimal cut-off was 0.67 when comparing class 1 with class 4A. This optimal cut-off corresponded with a sensitivity of 70%, a specificity of 97%, a total accuracy of 92%, a negative predictive value (NPV) of 94% and a positive predictive value (PPV) of 84%. The distribution of CulSumOf3_Obs is represented in Fig. 3.

Discussion

To date, no reliable serodiagnostic tests for equine IBH are available commercially. In this study, we evaluated the performance of an allergen-specific IgE ELISA using different r-allergens as well as extracts to confirm the diagnosis of IBH in Warmblood horses. The clinical diagnosis in combination with IBH history was used as the gold standard because the presence or absence of a well-documented history and clinical examination is more reliable for diagnosis of IBH than IDT (Morris and Lindborg, 2003). Although professional inspectors may be more objective compared to horse owners, the owner has experience of the horse's status over a longer period of time and may be able to provide complete information (Eriksson et al.,

Table 3
Overview of performance parameters at optimal cut-offs for CulSumOf3_Obs.

	IBH-unaffected vs. IBH-affected Class 1 vs. classes 2 + 3 + 4	IBH-unaffected vs. classical IBH-affected Class 1 vs. classes 2A + 3A + 4A	IBH-unaffected vs. classical IBH-affected with severe clinical signs Class 1 vs. class 4A
Pairwise accuracy (AUC)	0.80 ± 0.02	0.83 ± 0.03	0.88 ± 0.04
Optimal cut-off	0.30	0.44	1.12
Youden's index	0.47	0.5	0.67
Sensitivity (%)	77	72	70
Specificity (%)	70	78	97
Total accuracy (%)	74	76	92
Negative predictive value (%)	77	82	94
Positive predictive value (%)	80	66	84

Optimal cut-off selection is based on maximising the Youden's index. Different classifiers were used: (1) the first column represents the comparisons of horses with status 0 (class 1) against horses with status 1 (class 2 + 3 + 4); (2) the second column summarises the results when horses of class 1 were compared with all 'classical' IBH-affected horses (class 2A + 3A + 4A); and (3) the third column gives the parameters when horses of class 1 were compared with horses of class 4A.
IBH, insect bite hypersensitivity; AUC, area under the curve.

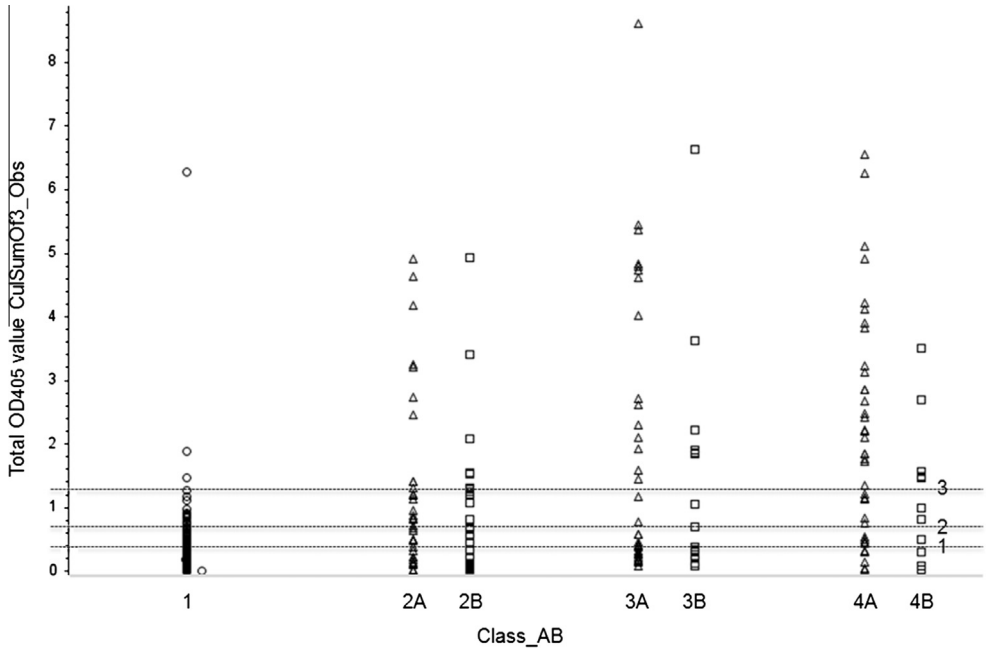

Fig. 3. Distribution of 'CulSumOf3_Obs': insect bite hypersensitivity (IBH)-unaffected horses (class 1) are shown with circles, IBH-affected horses with classical IBH history (class 2A, 3A and 4A) with triangles and IBH-affected horses with non-classical IBH history (class 2B, 3B and 4B) with squares. Three optimal cut-offs are visualised with horizontal lines: (1) optimal cut-off if class 1 is compared to 2 + 3 + 4; (2) optimal cut-off when class 1 is compared to 2A + 3A + 4A; and (3) optimal cut-off when class 1 is compared to 4A.

2008). Therefore, classification of IBH-affected and unaffected horses was based on owner descriptions. IBH-unaffected horses were stabled in the same environment as IBH-affected horses, so the overall exposure to *Culicoides* spp. was similar for both groups.

The best performing in vitro diagnostic tests are HRT and sLTRT (Baselgia et al., 2006; Langner et al., 2008; Wagner et al., 2008). The HRT reported by Langner et al. (2008) had a sensitivity and specificity of 100% when *Culicoides* spp. saliva extracts were used, but their study included only 16 horses. Baselgia et al. (2006) evaluated a sLTRT (cellular antigen stimulation test, CAST) based on test results from 314 horses; the highest diagnostic sensitivity and specificity levels were attained when using *C. nubeculosus* whole body extract (78% and 97%, respectively). The HRT evaluated by Wagner et al. (2008) had a sensitivity and specificity at the optimal cut-off of 76% and 55%, respectively, with an AUC of 0.68.

A sensitivity of 78% and specificity of 100% using *Culicoides* spp. whole body extract or saliva has been described for an IDT in horses (Langner et al., 2008). However, IDT has limitations for use as a routine clinical test, including the need to immobilise animals for intradermal injections (Pastorello, 1993; Codner and Tinker, 1995; Hensel et al., 2004).

Until recently, because of poor performances, serological tests were not suitable for establishing a diagnosis of IBH (Frey et al., 2008; Langner et al., 2008). Langner et al. (2008) reported sensitivities of 44% and 22%, corresponding to a specificity of 100%, for an IgE-specific ELISA with *C. nubeculosus* whole body extracts or saliva, respectively. In our study, a sensitivity of 70%, corresponding to a specificity of 97%, was found when comparing IBH-unaffected horses with IBH-affected horses with clear clinical signs and a classical IBH history. The low sensitivity of serological tests is due to low IgE serum levels, since most IgE is bound to the surface of mast cells and basophils. The sensitivity of CulSumOf3_Obs can be increased by lowering the cut-off, resulting in a lower specificity. For example, when lowering the cut-off, a sensitivity of 90%, corresponding to a specificity of 70%, was obtained.

Recently, van der Meide et al. (2012) described an allergen-specific IgE ELISA using *C. obsoletus* extract including 103 IBH-affected and 100 IBH-unaffected Shetland and Icelandic horses with an area under the curve of 0.97, corresponding to a sensitivity and specificity of 93.2% and 90.0%, respectively. Although *C. obsoletus* is the most important *Culicoides* spp. attracted to horses in Belgium (Losson et al., 2007), horses included in our study seem to react less to *C. obsoletus* extract. Since extracts were prepared differently, it is difficult to compare performances between the present study and that of van der Meide et al. (2012). In addition, the higher sensitivity of the ELISA described in van der Meide et al. (2012) may be due to the different study populations. It has been shown that IBH-affected horses imported from Iceland display stronger T helper 2 (Th2) and IgE responses against *Culicoides* spp. allergens than other horses (Hellberg et al., 2006; Hamza et al., 2007). Furthermore, no information on how horses were kept was provided by van der Meide et al. (2012). Since ponies and Icelandic horses are often kept more in the field than Warmblood horses, there may have been more contact with *Culicoides* spp., resulting in a more stimulated allergen-specific IgE response.

Until now, the effect of the severity of IBH clinical signs at sampling on the performance of serological tests has not been examined (Hellberg et al., 2006; Frey et al., 2008; Langner et al., 2008; van der Meide et al., 2012). Warmblood horses are often kept inside or are treated with preventive measures when showing clinical signs. Clinical signs of IBH are thus often mild, even during summer. Since there is no clear distinction between exposed and unexposed horses, the effect of preventive measures could not be investigated in the present study, but an effect of the severity of clinical signs was clearly shown. The performances of the diagnostic tests were evaluated in horses with severe, mild and no clinical signs at sampling. The accuracies of the IgE tests increased when clinical signs at sampling were more pronounced. It seems likely that a higher concentration of free *Culicoides* spp.-specific IgE in plasma correlates with a higher amount of *Culicoides* spp.-specific IgE bound to mast cells and basophils (Wagner et al., 2006). Baselgia et al. (2006) also described a difference between the percentages of horses with a positive CAST result when blood was sampled during the IBH season (97%) compared to sampling at the end of winter, i.e. ~5 months after the last allergen exposure (74%). Furthermore, the risk of misdiagnosis is higher in the group without clinical signs, since diagnosis is only based on IBH history and owner statement. This might also explain the lower accuracies in this group of horses.

For diagnostic purposes, testing IgE levels against Culn4, Culo1, Culo2 and ObsWBE extracts is sufficient. However, the sensitisation patterns are different between horses, since detectable IgE levels against the tested r-allergens were measured in different combinations. Detectable IgE levels against the tested r-allergens were measured in many different combinations in this study. In addition, the sensitisation pattern may differ between breeds of horses. For example, Culn2 was excluded from this study based on a pilot study including 115 Warmblood horses, since few serum samples contained IgE levels above the detection limit (Peeters et al., 2012). However, 48% of Icelandic horses had detectable IgE levels against this r-allergen (Schaffartzik et al., 2011). These differences in sensitisation pattern may be explained by genetic differences and/or differences in *Culicoides* contact. In terms of immunotherapy, it is important to know the specific antigens against which a horse is sensitised. Tools such as the allergen-specific IgE ELISA could be useful to identify the specific causative allergens.

The additional use of r-allergens improved the performance of the allergen-specific IgE ELISA as compared to using only natural extracts. CulSumOf3_Obs had a significantly higher accuracy (0.80) than ObsWBE (0.72) and NubTH (0.72). Several studies have reported similar findings (Virtanen et al., 1996; Bohle and Vieths, 2004; Asero et al., 2007). When using extracts, the specific allergens might be missing, their concentration might be too low or non-allergenic proteins or carbohydrates could bind IgE non-specifically (Schmid-Grendelmeier and Crameri, 2001). Moreover allergen extracts are difficult to standardise (Schaffartzik et al., 2012). When combining IgE levels against Culn4, Culo1 and Culo3 (CulSumOf3), a specificity of 97% and a sensitivity of 57% were obtained when comparing unaffected horses with IBH-affected horses with a classical IBH history and clear clinical signs. In this study, 30% of IBH-affected horses did not have detectable IgE levels against any r-allergens.

Conclusions

CulSumOf3_Obs combines allergen-specific IgE levels against *C. nubeculosus* r-allergen Culn4 and Obsoletus group r-allergens Culo1 and Culo2 with IgE levels against Obsoletus group whole body extracts. When clinical signs of IBH at sampling were mild or absent, the performance of the test decreased. Additional research is required to optimise diagnostic tests to support the diagnosis of IBH susceptibility when clinical signs are less clear at sampling and to compare performances of diagnostic tests in different breeds. The identification of other major IBH allergens might result in an increase in the performance of the allergen-specific IgE ELISA as a diagnostic test for IBH.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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