



Evaluation of a diagnostic ELISA for insect bite hypersensitivity in horses using recombinant Obsoletus complex allergens



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ABSTRACT

Culicoides spp. of the Obsoletus complex belong to the most important species of midge, involved in causing insect bite hypersensitivity (IBH) in horses in The Netherlands. The aim of the current study was to evaluate seven different Obsoletus complex-derived recombinant allergens (Cul o 1–Cul o 7) and to compare these with Obsoletus complex whole body extract (WBE) in an IgE ELISA, using sera of 194 clinically-confirmed cases of IBH and 175 unaffected horses. The highest test accuracy was obtained with WBE, followed by Cul o 2, 3 and 5.

Two ELISAs with a combination of recombinant allergens, Combi-1 (Cul o 3, 5 and 7) and Combi-2 (Cul o 1, 2, 5 and 7) were additionally performed and both resulted in high test accuracies close to that obtained with WBE. Combi-1 resulted in the best sensitivity and specificity, both 89%. Both Combi-1 and Combi-2 performed less well with samples collected in winter, but over 70% of the IBH-affected horses could still be identified. In conclusion, a combination of three Obsoletus complex recombinant allergens (Cul o 3, 5 and 7) could potentially replace Obsoletus complex WBE in an IgE ELISA for diagnosis of IBH in horses.

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Introduction

Midges of the *Culicoides* spp. are the primary cause of a world-wide, seasonal recurrent skin allergy in horses, called insect bite hypersensitivity (IBH) (Marti et al., 2008). The welfare of affected horses is compromised as a result of severe itch, which leads to rubbing, scratching and localized hair loss, with excoriations and sometimes secondary infection (Riek, 1953; Schaffartzik et al., 2012). Affected horses generally develop IgE antibodies against allergens from *Culicoides* spp. (Anderson et al., 1993; Ferroglio et al., 2006; Hellberg et al., 2006).

Culicoides obsoletus is often reported as the most abundant midge species attracted to horses in different countries (Townley et al., 1984; Mullens et al., 2005; de Raat et al., 2008; van der Rijt et al., 2008). Whole body extracts (WBE) of this particular species have been shown to be more immunoreactive in different in vitro diagnostic tests for IBH, compared to WBE of other common non-native *Culicoides* spp. (van der Meide et al., 2012). Based on WBE

from this species, a diagnostic IgE ELISA with high sensitivity and specificity has been developed (van der Meide et al., 2012).

Culicoides obsoletus is one member of the Obsoletus complex, which, in central and northern Europe, also includes *C. scoticus* and *C. chiopterus* (Lehmann et al., 2012). *C. dewulfi* has previously been considered to belong to this group, due to its morphological similarity, but has been recently reassigned to a different taxonomic group (Schwenkenbecher et al., 2009). However, because female midges of all these species are difficult to distinguish, they are often referred to as the Obsoletus complex, when identification is based purely on morphology.

Insect WBE are complex mixtures, containing many proteins and other biological molecules. In common with other allergenic extract preparations, they can be contaminated with allergens from other sources and other unwanted components, including proteases, which can interfere with serological testing (Nelson et al., 1996; Chapman et al., 2000). Moreover, they are difficult to standardize and do not provide information about the precise allergen driving the pathological response. Additionally, Obsoletus complex insects still need to be collected from the wild, since they have not been successfully maintained in laboratory breeding colonies (Boorman, 1985). For these reasons, use of defined

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recombinant allergens is an attractive alternative to WBE. Furthermore, an individual's IgE reactivity profile can be determined more precisely with recombinant allergens, thus allowing component-resolved diagnostics (CRD). Subsequently, those allergens against which the animal has produced specific IgE antibodies can be selected for bespoke immunotherapy (Valenta et al., 1999).

We have previously identified, cloned and produced seven recombinant allergens from *Obsoletus* complex, based on similarities to previously described allergens for *C. nubeculosus* and *C. sonorensis* (Langner et al., 2009; Schaffartzik et al., 2010, 2011; van der Meide et al., 2013). We found that a maltase of *Obsoletus* complex (Cul o 1) resulted in more accurate diagnostic testing, compared to its homolog from *C. sonorensis*, a species that horses were not exposed to, thus showing the importance of using allergens from the most appropriate *Culicoides* species that feed on horses (van der Meide et al., 2013).

In the present study, we evaluated seven separate *Obsoletus* complex-derived recombinant allergens, alongside *Obsoletus* complex WBE, in an IgE ELISA, using sera from a large number of IBH-affected and unaffected horses. Two combinations that included several of these recombinant allergens were used to design an IgE ELISA with sensitivity and specificity comparable to an *Obsoletus* complex WBE IgE ELISA.

Materials and methods

Preparation of *Obsoletus* complex WBE and recombinant allergens

Culicoides insects from the *Obsoletus* complex were collected from the wild and WBE prepared as previously described (van der Meide et al., 2012). Complimentary DNA (cDNA) encoding seven *Obsoletus* complex allergens (Cul o 1–Cul o 7, Genbank accession numbers KC339671–KC339677 respectively) were cloned and expressed in *E. coli* and recombinant proteins purified as previously described (van der Meide et al., 2013).

Horses and blood sampling

A total of 194 IBH-affected (105 Shetland ponies and 89 Icelandic horses) and 175 healthy controls (97 Shetland ponies and 78 Icelandic horses) were included in the study. Horses were selected according to a strict protocol, based on a case-control design previously described (Schurink et al., 2012, 2013). One hundred and three of the clinically confirmed IBH-affected horses and 100 healthy control horses had previously been used to evaluate a diagnostic ELISA (van der Meide et al., 2012). Fourteen clinically confirmed IBH-affected Shetland ponies with mild IBH clinical signs and 11 healthy control ponies were used to compare IgE ELISA results from serum collected during the IBH season (August 2011) versus serum collected off-season (March 2011). The remaining 180 IBH-affected horses that were used to evaluate the IgE ELISA showed clinical signs (mild to severe), while the 164 healthy controls did not show any signs of skin disease despite similar exposure to *Culicoides* spp.

IBH-affected horses and healthy controls were matched for various factors as described by Schurink et al. (2013). Most of the Icelandic horses were not imported, but born in The Netherlands (>85%). Blood samples from these horses were collected during Autumn of 2009 and 2010. All serum samples were frozen in aliquots not later than 24 h after blood sampling and stored at -20°C until use. Blood sampling was approved by the Board of Animal Ethics and Experiments of Wageningen University (experiment 2009055, approval date of 20 July 2009 and experiment 2010109, approval date of 30 August 2010).

ELISA

Specific serum IgE reactivity to WBE or recombinant Cul o 1–Cul o 7 was determined in an indirect ELISA as previously described (van der Meide et al., 2013). Briefly, Costar 384-well plates were coated with 20 μL /well of 10 $\mu\text{g}/\text{mL}$ *Obsoletus* complex WBE or recombinant allergen, except for Cul o 5, which was coated at 5 $\mu\text{g}/\text{mL}$. Two ELISAs with a combination of allergens were also performed; Combi-1 containing allergens Cul o 3, 5 and 7, coated as a mix at concentrations of 2.5 $\mu\text{g}/\text{mL}$, 1.25 $\mu\text{g}/\text{mL}$ and 2.5 $\mu\text{g}/\text{mL}$, respectively and Combi-2, containing allergens Cul o 1, -2, -5 and -7, coated as a mix at concentrations of 2.5 $\mu\text{g}/\text{mL}$, 2.5 $\mu\text{g}/\text{mL}$, 1.25 $\mu\text{g}/\text{mL}$ and 2.5 $\mu\text{g}/\text{mL}$, respectively.

After coating, wells were incubated with horse serum samples diluted 1:5 in a 1.5% casein buffer (Stereospecific Detection Technology). IgE binding was detected using a monoclonal murine anti-equine IgE (Clone 176; Wagner et al., 2003), followed by a polyclonal caprine anti-mouse IgG peroxidase conjugate (multispecies adsorbed; AbD Serotec). High sensitivity tetramethylbenzidine (Stereospecific Detection

Technology) was used as the substrate. Reactions were stopped with 1% hydrochloric acid. Absorbance was measured with a multi-mode microplate reader (SpectraMax M5, Molecular Devices) at a wave length of 450 nm corrected for 650 nm (optical density; OD₄₅₀). For each ELISA, sera of both IBH-affected and healthy horses were tested on the same plate, to avoid bias resulting from inter-assay variability. Optimal coating concentrations were determined prior to the experiment by titration.

SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were performed as previously described (van der Meide et al., 2012). For WBE, 20 μg protein/lane was loaded and recombinant allergens were pooled and 100 ng of individual allergen/lane was loaded on 15% SDS-PAGE gels. Separated proteins were transferred to nitrocellulose membranes (Protrans, Schleicher & Schuell Bioscience) by means of electrophoresis. IgE binding was detected with a monoclonal antibody against horse IgE (clone 176; Wagner et al., 2003), followed by caprine anti-mouse IgG horseradish peroxidase (Dako) 1:1000 in 5% non-fat cow's milk in Tris buffered saline (TBS)–Tween (10 mM Tris, 150 mM NaCl, pH 7.5, 0.05% (v/v) Tween 20).

Statistical analysis

The Mann–Whitney *U* test was used to compare OD_{450nm} values or log₂ dilutions of the standard from IBH-affected horses with those from healthy horses against WBE or allergens using IBM SPSS statistics, version 19. Results were considered significant at $P < 0.05$.

The overall diagnostic performance of each ELISA (allergens and WBE) was determined from receiver-operating characteristic (ROC) curves (Sackett et al., 1991), computed in IBM SPSS statistics, version 19. The cut-off value of each ELISA was set as the value that corresponded to a balanced optimal sensitivity and specificity.

The predicted probability of IBH status (IBH or healthy) for a certain OD_{450nm} value expressed as log₂ IgE titres was determined by logistic regression. The analysis was performed with PROC LOGISTIC function of SAS (SAS Inc, version 9.2).

Results

IgE ELISAs with *Obsoletus* complex allergens

ROC analysis was performed on the IgE ELISA data of all individual recombinant allergens and WBE of *Obsoletus* complex tested on 194 IBH-affected and 175 healthy control Shetland ponies and Icelandic horses. The test accuracies (area under the curve [AUC], sensitivity, specificity) were obtained from the analysis and the cut-off values determined by these ROC curves (Table 1, Fig. 1). The highest test accuracy based on the AUC was obtained for WBE, followed by Cul o 2 and Cul o 3.

For all allergens and WBE, IBH-affected horses had significantly higher OD_{450nm} values than healthy horses (Fig. 2; $P < 0.001$). The greatest proportion of IBH-affected horses testing positive, based on the cut-off determined by the ROC curve, was obtained for WBE (86%). With the individual allergens, Cul o 3 resulted in the greatest proportion of IBH-affected horses (85%) testing positive and with Cul o 1 the lowest percentage of IBH-affected horses testing positive (76%). The proportion of false-positive tested horses within the healthy control group ranged from 15% to 24% among the recombinant allergens.

Nearly half (46%) of the IBH-affected horses had positive test results with all seven allergens, followed by 21% of these horses reacting to six allergens and 16% to five allergens (Fig. 3). Only 1% of IBH-affected horses failed to show any reactivity to the allergens tested. In contrast, most healthy horses (61%) demonstrated negative test result against each of the allergens, followed by 21% of control horses reacting to one allergen and 11% to two allergens. Only 1% of the healthy horses had positive test results with all seven recombinant allergens.

Western blot analysis

Four IBH-affected horses that were tested in the ELISAs showed high OD_{450nm} values with WBE, but did not have IgE reactivity with any of the seven allergens or a relatively low OD_{450nm} value for just one allergen, suggesting that other allergens present in the WBE

Table 1

Test accuracy parameters for indirect IgE ELISAs on sera of IBH-affected and healthy control horses using Obsoletus complex whole body extract (WBE) or recombinant allergens (Cul o 1–Cul o 7).

	WBE	Cul o 1	Cul o 2	Cul o 3	Cul o 4	Cul o 5	Cul o 6	Cul o 7	Combi-1	Combi-2
AUC	0.95	0.85	0.90	0.90	0.86	0.88	0.83	0.86	0.94	0.92
Threshold ^a	0.32	0.15	0.41	0.37	0.15	0.40	0.17	0.12	0.57	0.54
Cut-off std c ^b	–	–	–	–	–	–	–	–	2.5	2.7
Sensitivity (%)	86	76	83	85	78	82	77	80	89	86
Specificity (%)	86	76	84	85	79	81	77	80	89	86

^a Threshold value for OD_{450nm} above which samples are considered positive, corresponding to balanced optimal sensitivity and specificity.

^b Cut-off std c: The cut-off value that corresponds to a balanced optimal sensitivity and specificity, expressed as log₂ IgE titre of the standard that can be used as a reference in future ELISAs when using the serum pool of 17 IBH-affected horses to prepare a standard curve of 2 × dilution steps.

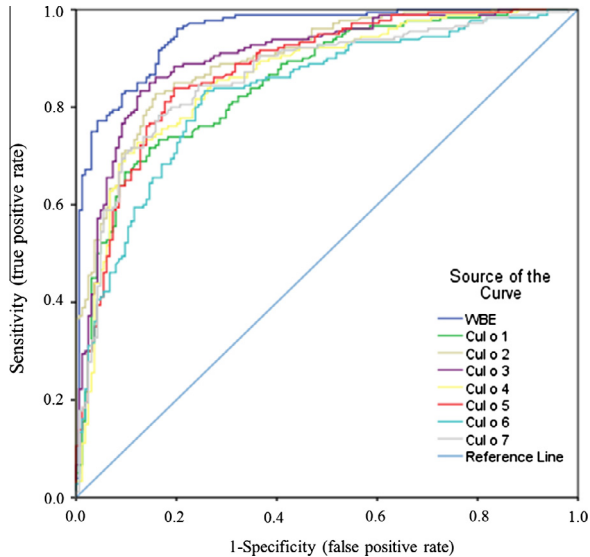


Fig. 1. ROC curves of IgE ELISA against Obsoletus complex WBE and recombinant allergens (Cul o 1–Cul o 7) on serum samples from 180 IBH-affected and 164 healthy horses.

were responsible for the high signal in this ELISA. Western blot analysis, using Obsoletus complex WBE and a pool of all seven

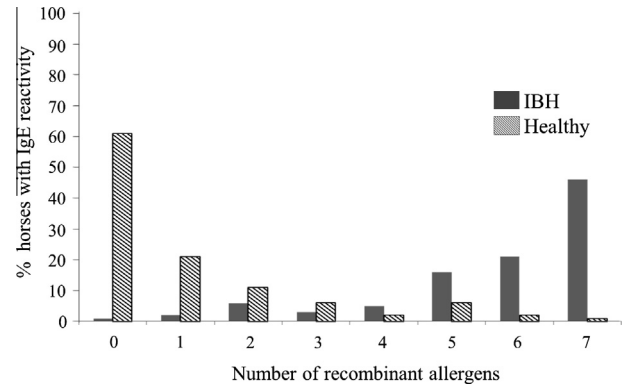


Fig. 3. Percentages (y-axis) of IBH-affected and healthy horses against the number of different recombinant allergens to which they react (x-axis). IgE reactivity is defined as an OD_{450nm} value in the IgE ELISA above the positive threshold, determined by the ROC curves.

recombinant allergens, was performed on these horses to evaluate the presence of other allergens in the extract. Sera from all four horses showed IgE-reactivity with a protein of around 15 kDa in the WBE and three of the horses also reacted to a band of around 20 kDa (Fig. 4). Two horses also showed IgE reactivity with some higher molecular weight proteins present in the WBE and with Cul o 4 in the pool of recombinant allergens, based on its size.

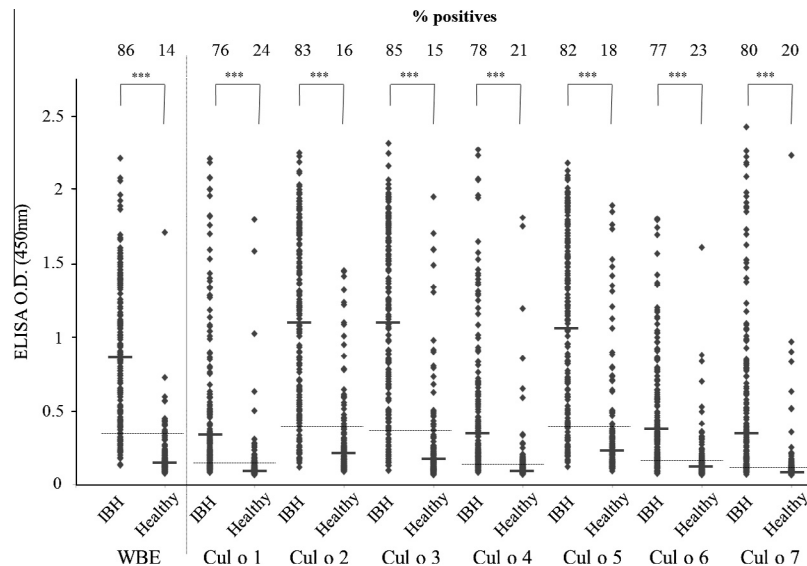


Fig. 2. IgE reactivity presented as OD_{450nm} values against Obsoletus complex WBE and seven recombinant allergens (Cul o 1–Cul o 7) from sera of 180 IBH-affected and 164 healthy horses. Individual values are shown, with the bold horizontal line crossing each group representing the median. The fine horizontal lines crossing the two groups (IBH and healthy) within each ELISA represent the positive threshold, determined by the ROC curves. The percentage of positive horses for each ELISA is indicated on top of the figure. ***Significant difference ($P < 0.0001$).

IgE ELISAs with two different allergen combinations

The greatest number of IBH-affected horses could be discriminated from healthy horses with ELISAs using allergens Cul o 2, Cul o 3 or Cul o 5. However, Cul o 2 and Cul o 3 gave similar results in terms of the horses that tested positive. Therefore two additional ELISAs were performed with a combination of either Cul o 2 with Cul o 5 and Cul o 7 (Combi-1) or Cul o 3 with Cul o 5, Cul o 7 and additionally Cul o 1 (Combi-2). Cul o 1 was included, because addition of this allergen was found to increase the number of IBH-affected horses testing positive.

A pool of equal volumes of sera from 17 IBH-affected horses with previously determined high *Culicoides* allergen-specific IgE reactivity was used as a reference serum to generate a standard curve with doubling dilutions, starting from 1:2.5 (serum:sample buffer). For the multivalent ELISAs, the OD_{450nm} values of individual animal serum samples were converted to the titre of the corresponding dilution of the reference serum, run on the same ELISA plate that resulted in the same OD_{450nm} value. The greatest reference serum dilution that still gave an OD_{450nm} above the cut-off value (OD_{450nm} = 0.1, average of wells without serum incubation + 3 SD) was assigned a titre of 1. This was obtained at a 1280× dilution of the reference serum, which sets the titre of the undiluted reference serum at 1280. This reference serum can be used in future ELISAs to convert OD_{450nm} values to IgE titres of the standard dilutions to avoid test result differences caused by environmental or other factors influencing the ELISA results.

A high test accuracy from the ROC curves was observed for both Combi-1 (Cul o 3, 5 and 7) and Combi-2 (Cul o 1, 2, 5 and 7), which came close to the assay using WBE, with slightly better results for Combi-1 (Fig 5, Table 1). IgE values for IBH-affected horses were significantly higher than those of healthy horses (Fig. 6). The greatest sensitivity and specificity (89% for each) was obtained with Combi-1.

Seasonality of IgE reactivity

For 14 IBH-affected horses and 11 healthy horses, serum samples taken during the IBH-season (August) and off-season (March) were available. To test the applicability of the multivalent ELISAs to discriminate between cases and controls, when samples were obtained in the off-season, additional serological testing was

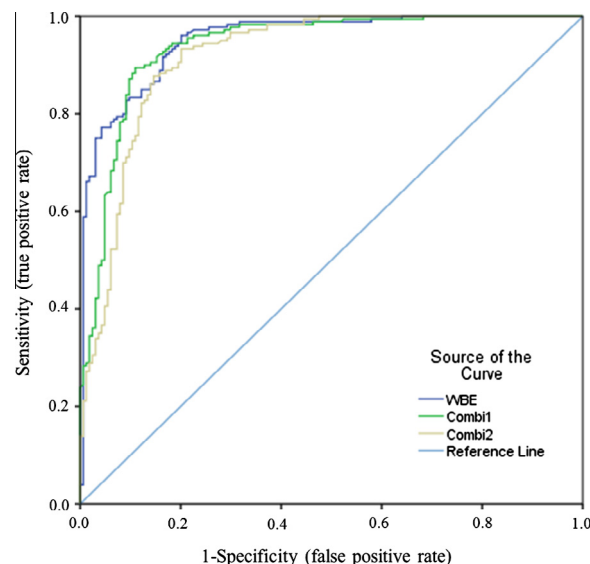


Fig. 5. ROC curves of IgE ELISA against *Obsoletus* complex WBE and a combination of recombinant allergens: Combi-1 (Cul o 3, Cul o 5 and Cul o 7) and Combi-2 (Cul o 1, Cul o 2, Cul o 5 and Cul o 7) on serum samples of 180 IBH-affected and 164 healthy horses.

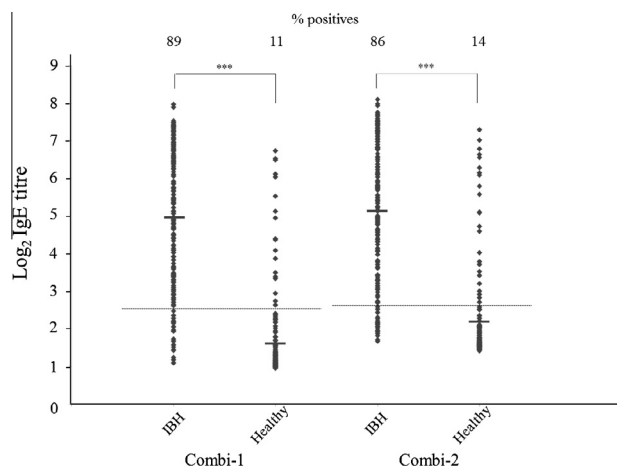


Fig. 6. IgE levels presented as log₂ IgE titres of the standard (a pool of 17 IBH-affected horses), against a combination of recombinant allergens: Combi-1 (Cul o 3, Cul o 5 and Cul o 7) and Combi-2 (Cul o 1, Cul o 2, Cul o 5 and Cul o 7) from sera of 180-IBH affected and 164 healthy horses. Individual values are shown, with the bold horizontal lines crossing each group representing the median. The fine horizontal lines crossing the two groups (IBH and healthy) within each ELISA represent the positive threshold, determined by the ROC curves. The percentage of positive tested horses for each ELISA is indicated on top of the figure. ***Significant difference ($P < 0.0001$).

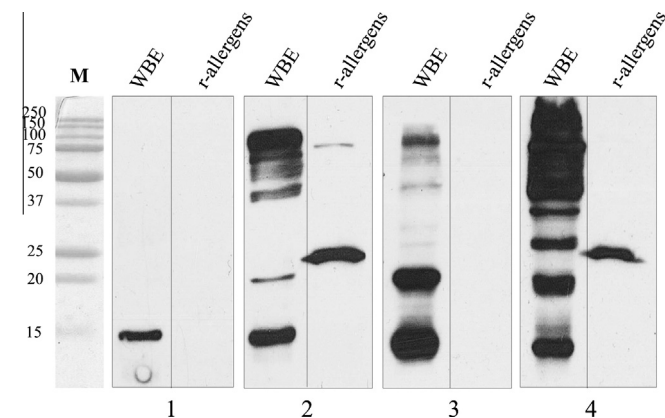


Fig. 4. Western blot analysis of four serum samples (1–4) from IBH-affected horses that tested positive in the WBE IgE ELISA, but negative against the recombinant allergens. The sera were used for western blot analysis using *C. obsoletus* WBE (20 µg) or a pool of all recombinant allergens (r-allergens = Cul o 1–Cul o 7, 100 ng each). Proteins were separated on 15% SDS-PAGE gels and transferred to nitrocellulose membranes. Binding of IgE from horse sera was detected with an anti-equine IgE mouse mAb and HRP goat anti-mouse IgG. The molecular weight marker (M) is indicated on the left in kDa.

performed. *Obsoletus* complex WBE was included as a positive control, because we have previously reported no significant difference between OD_{450nm} values in serum obtained in the off-season compared to the IBH season (Meulenbroeks et al., 2013). A significant decrease in the IgE titre was observed with off-season sera from IBH-affected horses for both Combi-1 and Combi-2 ELISA, but not for WBE (Fig. 7). Although a significant decrease was observed for the entire IBH-affected group, for some horses there was only a small decrease in the IgE titre observed during the off-season. Horses showing a clear decrease in IgE titre for Combi-1, also showed the same for Combi-2. For WBE, some horses showed an increase in the IgE titre during the off-season.

Predicted probability of IBH-status related to IgE titres of Combi-1 ELISA

Since the best results were obtained when using the Combi-1 ELISA, logistic regression was performed to predict the probability of the IBH-status (i.e. IBH-affected or healthy) in relation to IgE titres from the Combi-1 ELISA. The predicted probabilities of both IBH outcomes related to $\log_2$ IgE titres are shown in Fig. 8. With values corresponding to a $\log_2$ IgE titre of approximately 5.0 or higher the probability of being IBH affected is 90–100%, whereas values corresponding to a $\log_2$ IgE titre of approximately 1.8 or lower the probability of being healthy is 80–90%. At the inflection point at a $\log_2$ IgE titre of approximately 3.0 the probability of being IBH affected or healthy is equal.

Discussion

Insect bite hypersensitivity is an IgE-mediated allergic skin disease in horses caused by the bites of *Culicoides* spp. (Wagner et al., 2006). We previously reported a *Culicoides* spp. from the *Obsoletus* complex to be particularly important, to be used for reliable diagnosis of IBH in countries where this is the main midge species found feeding on horses (van der Meide et al., 2012).

Although we have previously described an IgE ELISA with high sensitivity and specificity using *Obsoletus* complex WBE, to date there is no such test available using recombinant allergens, which would provide an attractive alternative for WBE, eliminating the need for collection of insects from the wild. The use of recombinant proteins is easier to standardize than crude extracts to avoid batch to batch variation. Furthermore, testing of individual recombinant allergens would enable component-resolved diagnosis of IBH in horses and tailor-made immunotherapy. In the present study, we evaluated the IgE reactivity to WBE, seven individual *Obsoletus* complex recombinant allergens and a combination of selected allergens, in an IgE ELISA for the diagnosis of IBH in horses.

The sensitivity and specificity of the WBE IgE ELISA was found to be somewhat lower than that previously reported (van der Meide et al., 2012). The same horses were included in the present study, but additional cases and controls, selected using the same criteria, were included. More healthy horses were found to

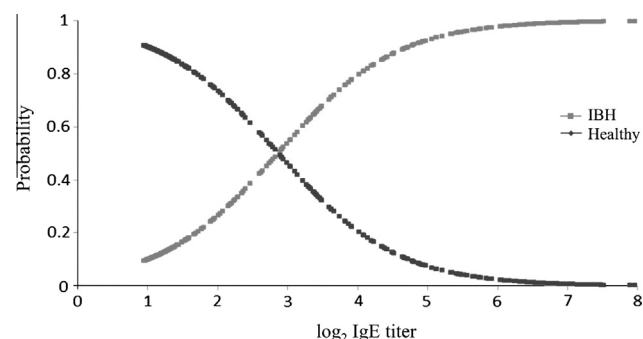


Fig. 8. Estimated probability to be IBH-affected or healthy in relation to the $\log_2$ IgE titre against Combi-1 (Cul o 3, Cul o 5 and Cul o 7). Determined by logistic regression analysis of the obtained ELISA data.

demonstrate evidence of allergen sensitization. Furthermore, clinically-confirmed IBH-affected horses within the group, supplementing the original case cohort, showed lower IgE levels, below the cut-off value against WBE. However, most of these IBH-affected horses did show IgE reactivity with one or more of the single allergens, suggesting that the clinical signs present in these horses is caused by IBH and not by some other skin condition.

The healthy horses used in this study that tested positive for allergen-specific IgE, did not show clinical signs of allergic skin disease, but some of these horses demonstrated clear IgE reactivity against several recombinant allergens as well as against WBE. The ELISA described here only detects the presence of circulating IgE and does not determine whether the allergens detected can cause cross-linkage of FcεR1-bound IgE antibodies on basophils and mast cells, which is necessary for clinical signs to occur. Horses have been reported that show evidence of sensitization to *Culicoides* spp. allergens, but without showing clinical signs (Wagner et al., 2009). Similarly, high levels of allergen-specific serum IgE has been reported in clinically-healthy dogs (Sævik et al., 2003). Why these animals do not progress from the sensitization phase to overt allergic disease is still unknown, but regulatory immune mechanisms might play a role. Regulatory T cells have been shown to suppress mast cell activation in mice (Gri et al., 2008) and there

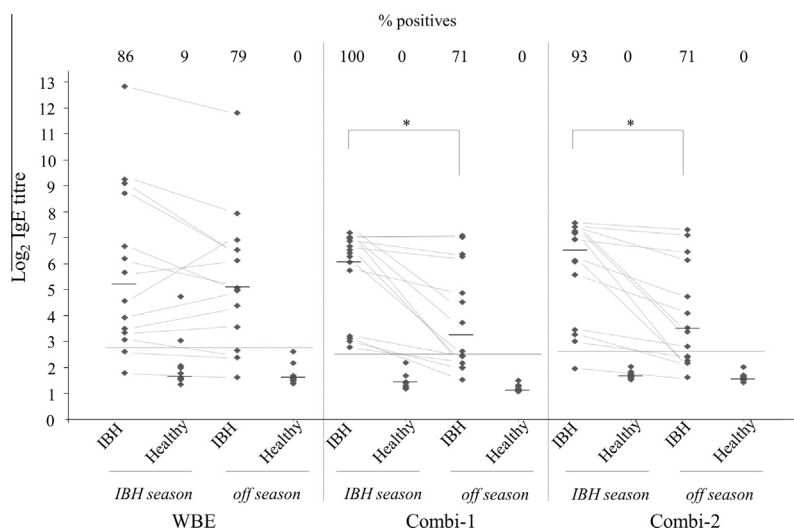


Fig. 7. IgE values presented as $\log_2$ IgE titres of the standard (a pool of 17 IBH-affected horses) against *Obsoletus* complex WBE and combinations of recombinant allergens: Combi-1 (Cul o 3, Cul o 5 and Cul o 7); Combi-2 (Cul o 1, Cul o 2, Cul o 5 and Cul o 7) from sera collected during IBH season and off-season of 14 IBH-affected and 11 healthy horses. The bold horizontal line crossing each group represents the median. The fine horizontal lines crossing the four groups (IBH and healthy for both IBH season and off-season) within each ELISA represent the positive threshold, determined by ROC curves. Lines connecting two data-points denote measurements for the same horse. The percentage of positive tested horses for each ELISA is indicated on top of the figure. *Significant difference ($P < 0.05$).

is evidence suggesting that healthy horses have a higher number of regulatory T cells compared to IBH-affected horses (Heimann et al., 2011; Hamza et al., 2012).

A combination of only three allergens in the Combi-1 ELISA (Cul o 3, 5 and 7) resulted in a sensitivity and specificity (both 89%) slightly higher than that obtained with WBE. The corresponding probability curve can be used for individual-tested horses to determine, for a certain titre, the probability that the horse is affected with IBH.

Interestingly, nearly 90% of IBH-affected horses showed a positive test result with four or more recombinant allergens, whereas only 11% of healthy horses did, suggesting that IgE reactivity to a larger number of allergens implies a greater chance of truly having IBH. Those IBH-affected horses that did not demonstrate IgE reactivity with any of the recombinant allergens, but did have IgE reactivity to WBE, specifically reacted with proteins of 15 and 20 kDa. A protein of 20 kDa has already been implicated as an important allergen in IBH-affected horses (van der Meide et al., 2012) and the identity of these additional putative allergens is currently under investigation.

We compared IgE values of sera obtained in the IBH season with those of sera taken from the same horses during the off-season. As was previously observed with the WBE IgE ELISA (Meulenbroeks et al., 2013), there was no significant difference between in-season and off-season sera. Human beings suffering from hay fever have also been reported to have high titres of pollen-specific IgE even in the winter months (Smurthwaite et al., 2001). However, when we performed the IgE ELISA with a combination of three or four allergens, we found a significant decrease in IgE reactivity during the off-season, although this decrease was not consistent for all horses. A decrease in the test sensitivity during off-season was also observed in a sulfidoleukotriene release assay performed by Basaglia et al. (2006).

The discrepancy between the WBE and the recombinant allergen IgE ELISAs might be due to different amounts of coated antigens. The total protein concentrations used for the WBE and Combi-1 ELISAs were 10 µg/mL and 6.25 µg/mL, respectively. However, in the case of the Combi-1 ELISA, this consisted of a mixture of specific recombinant allergens. In contrast, only a small fraction of all the proteins in the WBE will actually consist of allergens, which will have to compete with all the other proteins in the WBE for binding to the surface of the well. Therefore, in comparison to the recombinant allergen ELISAs, only a small amount of each individual allergen will become coated in the WBE ELISA. A reduction in IgE titre in the off-season might, therefore, not be directly reflected in a reduction in absorbance value in the WBE ELISA, because the specific IgE might still be in excess of the amount of coated allergen.

The fact that horses have relatively high IgE concentrations in their serum, compared to other species, including humans (Wilson et al., 2006), will make this phenomenon more likely. The signal obtained in the WBE IgE ELISA is still high, in spite of the low amounts of the individual allergens, because the WBE contains many different allergens that all contribute to the total IgE binding. Coating with purified recombinant allergens can result in reduced ELISA absorbance values, because allergen-specific IgE in serum is much less likely to be present in excess of much higher amounts of coated allergens. Nevertheless, we were still able to detect high IgE reactivity off-season in most IBH-affected horses, even with recombinant allergens; therefore it seems that the production of IgE is maintained, even in the absence of exposure to *Culicoides* insects.

There is evidence that at least some IgE-secreting plasma cells in allergic individuals are long-lived and that these cells can survive in the bone marrow, lymph nodes or spleen for many years (Holt et al., 1984; Manz et al., 1997; Slifka et al., 1998; Luger

et al., 2009). Therefore, the maintenance of production of IgE might be explained by such long-lived plasma cells, specific for *Culicoides* spp. allergens. Since serum IgE levels in healthy horses also seemed to decrease during the off-season, a lower positive threshold might be more appropriate in the IgE ELISA, when testing samples collected in the winter season.

Conclusions

A combination of three recombinant *Obsoletus* complex allergens (Cul o 3, 5 and 7) in a multivalent IgE ELISA can be used for diagnostic testing of horses for IBH with a high sensitivity and specificity. The individual allergens reported in the present study could also be useful for developing bespoke immunotherapy, since most of the IBH-affected horses show IgE reactivity with at least one of these allergens.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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