



Equine insect bite hypersensitivity: Immunoblot analysis of IgE and IgG subclass responses to *Culicoides nubeculosus* salivary gland extract

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Abstract

Insect bite hypersensitivity (IBH) is an allergic dermatitis of horses caused by IgE-mediated reactions to bites of *Culicoides* and sometimes *Simulium* spp. The allergens causing IBH are probably salivary gland proteins from these insects, but they have not yet been identified.

The aim of our study was to identify the number and molecular weight of salivary gland extract (SGE) proteins derived from *Culicoides nubeculosus* which are able to bind IgE antibodies (ab) from the sera of IBH-affected horses. Additionally, we sought to investigate the IgG subclass (IgGa, IgGb and IgGT) reactivity to these proteins.

Individual IgE and IgG subclass responses to proteins of *C. nubeculosus* SGE were evaluated by immunoblot in 42 IBH-affected and 26 healthy horses belonging to different groups (Icelandic horses born in Iceland, Icelandic horses and horses from different breeds born in mainland Europe). Additionally, the specific antibody response was studied before exposure to bites of *Culicoides* spp. and over a period of 3 years in a cohort of 10 Icelandic horses born in Iceland and imported to Switzerland.

Ten IgE-binding protein bands with approximate molecular weights of 75, 66, 52, 48, 47, 32, 22/21, 19, 15, 13/12 kDa were found in the SGE. Five of these bands bound IgE from 50% or more of the horse sera. Thirty-nine of the 42 IBH-affected horses but only 2 of the 26 healthy horses showed IgE-binding to the SGE ($p < 0.000001$). Similarly, more IBH-affected than healthy horses had IgGa ab binding to the *Culicoides* SGE (19/22 and 9/22, respectively, $p < 0.01$). Sera of IBH-affected horses

Abbreviations: ab, Antibody; *C. nubeculosus*, *Culicoides nubeculosus*; IBH, insect bite hypersensitivity; kDa, kilodalton; PVDF, polyvinylidene difluoride; SGE, salivary gland extract; SIT, specific immunotherapy

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contained IgE, IgGa and IgGT but not IgGb ab against significantly more protein bands than the sera of the healthy horses. The cohort of 10 Icelandic horses confirmed these results and showed that *Culicoides* SGE specific IgE correlates with onset of IBH. IBH-affected horses that were born in Iceland had IgGa and IgGT ab ($p \leq 0.01$) as well as IgE ab ($p = 0.06$) against a significantly higher number of SGE proteins than IBH-affected horses born in mainland Europe.

The present study shows that *Culicoides* SGE contains at least 10 potential allergens for IBH and that IBH-affected horses show a large variety of IgE-binding patterns in immunoblots. These findings are important for the future development of a specific immunotherapy with recombinant salivary gland allergens.

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1. Introduction

Bites of insects of the genus *Culicoides* can cause a seasonal recurrent dermatitis in predisposed horses, known as insect bite hypersensitivity (IBH) or summer eczema. IBH does not occur in Iceland supposedly due to the absence of *Culicoides* midges. However, up to 50% of Icelandic horses that were moved to the European continent and thereby exposed to bites of *Culicoides* develop IBH within 2 years of export (Marti et al., 2003). Interestingly, only 5–10% of the Icelandic horses born on the continent of Europe, yet produced from Icelandic horses that were imported from Iceland, develop IBH (Brostrom et al., 1987; Halldorsdottir and Larsen, 1991).

Due to the feeding sites of most female *Culicoides*, the mane, tail region and ventral midline can be affected. Affected horses suffer from severe pruritus and may present papules, broken hair, alopecia, crusts and lichenification of the skin. So far, treatment consists of attempted avoidance of exposure to the insects by stabling, the use of summer eczema blankets or repellents and, of symptomatic treatment with corticosteroids.

A number of studies indicate that IgE-antibodies are involved in this disease (Van der Haegen et al., 2001; Wilson et al., 2001). However, in the horse, it is not yet known, whether some IgG subclass ab are associated with allergic diseases and correlate with IgE levels as is the case in human allergic patients. For example, in human atopic patients, Pereira et al. (2005) showed recently that specific IgG4 levels to the house dust mite, *Blomia tropicalis*, were higher than in the control group and correlated significantly with specific IgE levels.

Although delayed type reactions have been described in intradermal skin testing (Halldorsdottir

et al., 1989) in most cases, immediate-type IgE-mediated reactions are involved in IBH (Halldorsdottir et al., 1989; Fadok and Foil, 1990; Fadok and Greiner, 1990). This suggests that specific immunotherapy (SIT) might be possible but the lack of purified allergens still hampers development of this treatment (Valenta et al., 1999). Hyposensitisation trials have been carried out with injections of *Culicoides* whole body extract by Anderson et al. (1996). These authors observed a decrease in clinical signs in treated horses. In contrast, a second study showed no difference between horses treated with a placebo and those treated with *Culicoides* whole body extract (Barbet et al., 1990).

The importance of the genus *Culicoides* as economically important vectors of arboviruses (blue-tongue) has already led to some investigations on their salivary transcriptomes (Campbell et al., 2005). However, the specific allergens that cause IBH, probably salivary gland antigens, have not been identified. Wilson et al. (2001) have shown that horses exposed to the bites of *Culicoides* have IgG binding to various antigens in immunoblots of a soluble extract of *Culicoides* thoraxes but there are no published data on immunoblots of *Culicoides* salivary gland extracts.

The main objective of this study was to identify the number and approximate molecular weight of IgE binding proteins in *Culicoides nubeculosus* salivary gland extract (SGE). In addition, we wanted to investigate which IgG subclasses are produced against *Culicoides* allergens. To determine whether specific IgE or IgG subclass responses are associated with sensitisation or tolerance to *Culicoides* bites, the specific humoral immune response was studied in a cohort of Icelandic horses before and after exposure to bites of *Culicoides* spp. Furthermore, we sought to investigate whether the quality or intensity of the

humoral immune response to *C. nubeculosus* SGE is different in horses born in Iceland from those born in Europe.

2. Materials and methods

2.1. Horses

In total, 68 horses living in Switzerland were investigated. The age of the horses ranged from 1 to 29 years with a mean age of 11.8 years for the 42 insect bite hypersensitivity (IBH)-affected horses, and a mean age of 11.5 years for the 26 healthy horses. Thirty-five of these 68 horses were Icelandic born, and exported to the European continent, of which 22 were affected with IBH and 13 were healthy. The mean time between import from Iceland and time when blood samples were taken was 7.7 years (1–15 years) for the healthy horses and 6.8 years (1–24 years) for the IBH-affected animals. Additionally, 20 horses of Icelandic breed, but born and raised outside Iceland (10 IBH-affected and 10 healthy) and 13 horses (10 IBH-affected and 3 healthy) belonging to various other breeds were included in the study.

Serum samples had all been taken during the IBH season, i.e. between April and November. The horses were from different stables in Switzerland and healthy control horses were selected randomly from the same stables where IBH-affected horses were living.

The diagnosis of IBH was based on clinical signs and history (recurrent seasonal pruritus and skin lesions along the typical anatomic sites, with remission in winter). Most of the affected horses were treated against IBH in various ways, such as covering with summer eczema blankets, stabling and or local application of lotions for insect and pruritus control.

The healthy (H) control horses had no clinical signs of pruritus or cutaneous lesions. These horses had no previous history of skin problems.

2.2. Study on the natural course of IBH in relation to the humoral antibody response

Additional to the above, in order to determine whether recognition of certain *Culicoides* SGE

antigens by IgE or IgG subclass ab is associated with subsequent sensitisation or tolerance to *Culicoides* bites, a cohort of 10 Icelandic horses was included in a separate study. Five of these horses had remained healthy and five had developed IBH. Sera had been taken from the animals in Iceland before their export to Switzerland, and then in June and October following importation into Switzerland, followed by samples taken in October of the second and third year after import into Switzerland.

2.3. Insects and salivary gland dissection

Adult *C. nubeculosus* were obtained from a laboratory bred colony (Institute for Animal Health, Pirbright, UK). Before dissection, the insects were cooled to about 4 °C to decrease their activity and induce anaesthesia. Females were selected and put into a single drop of 0.9% NaCl (Laboratorium Dr. G. Bichsel AG, 3800 Interlaken, Switzerland) on a glass slide. Dissection was carried out under a stereoscopic microscope at a 40× magnification by using extra-fine forceps and needles according to the methods of Watts (Watts, 1981). The salivary glands were transferred into a 1.5 ml polypropylene tube containing SDS-PAGE sample buffer (0.125 M Tris-Cl, 4% SDS, 20% (v/v) glycerol, 0.2 M DTT, 0.02% bromophenol blue pH 6.8) and kept on ice. The salivary gland extract was stored at –20 °C until used.

2.4. SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot

To disrupt the salivary glands and obtain a homogenous extract, samples were sonicated using a Fisherbrand sonifier homogenizer for 2 min and boiled at 100 °C for 3 min. Between these steps the tubes were kept on ice.

SDS-PAGE was performed using the discontinuous system according to Laemmli (1970) on preparative 10–20% Tris-HCl gels (BioRad, 4135 Reinach, Schweiz). Gels were run with Tris glycine buffer containing 0.25M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3 according to the manufacturers instructions in a Mini-Protein II apparatus (BioRad). Approximately, 20 salivary glands were loaded onto each gel. To estimate the molecular mass of the different protein bands, a pre-stained protein standard ("Precision

Plus”, BioRad) was used. Electrophoresis was performed at 80 V for 15 min and then at 140 V for additional 100 min.

After electrophoresis the proteins were electro-transferred to polyvinylidene difluoride (PVDF) membranes (BioRad) using a semi-dry electrophoretic transfer cell (Trans-Blot SD, BioRad) at 15 V for 38 min. Transfer buffer containing 39 mM glycine, 48 mM Tris, 0.00375% SDS, 20% methanol. The membranes were dried and cut into strips.

Horse serum was diluted 1:6 for detection of IgE ab or 1:50 for detection of IgG ab in washing buffer and added to the PVDF strips, which were first moistened in washing buffer. The strips were incubated at 4 °C overnight on a shaker. The following day the strips were washed in washing buffer five times for 5 min and incubated with a monoclonal mouse anti-horse-IgE ab (3H10, [Wilson et al., in press](#)) or monoclonal mouse anti-horse-IgG ab (anti-IgGa, anti-IgGb, anti-IgGT, Serotec GmbH, 40210 Düsseldorf, Germany) diluted 1:1000 and 1:2000 (anti-IgGT), respectively, and incubated for 3 h at room temperature. After an additional washing step, the strips were incubated for 2 h with a horse radish peroxidase (HRP) conjugated goat anti-mouse-IgG ab (Sigma–Aldrich, Buchs, Switzerland) diluted 1:10,000 in washing buffer. A final washing was carried out and the strips were developed with ECL Western blotting detection reagent (Amersham Biosciences) for 1 min.

Although genes coding for seven IgG isotypes in the horse have been found ([Wagner et al., 2004](#)), at present there are only ab available for recognition of four different equine IgG isotypes, one of which, the anti-IgGT ab, seems to be reacting with two different subclasses, IgG3 and IgG5 ([Wagner et al., 2003](#)).

According to the new nomenclature, anti-IgGa ab react with equine IgG1, anti-IgGb with IgG4 and anti-IgGc with equine IgG6. Preliminary experiments had shown that IgGc ab against SGE could not be detected with a commercially available ab (Serotec GmbH). We did thus detect IgG1, IgG4, IgG3 and 5, but not IgG6 ab in our study.

In the study of the natural course of IBH, all strips from one horse (different times and different isotypes) were always from the same gel, i.e. the amount of SGE loaded was the same on all strips tested with the serum from one horse. Additionally, to control technical differences, one sample of each of the 10 horses was tested simultaneously on the same blot.

2.5. Statistical analyses

Statistical analyses were carried out with the statistical software package NCSS 2001 (NCSS, Kaysville, UT 84037, USA).

The two-tailed Fisher’s exact test was used to compare the proportion of IBH-affected and control horses showing either IgE, IgGa, IgGb or IgGT ab binding to any ([Table 1](#)) or a defined single protein band ([Figs. 2 and 4](#)) of the *C. nubeculosus* SGE. In a second analysis, the number of protein bands identified with the different sera was assessed and compared between IBH and control horses. Due to the non-normality of the data, median/ranges and the Mann–Whitney *U*-test were used ([Table 2](#)). The same test was used to compare immuno-reactivity between horses of different origin (Icelandic breed born in Iceland, Icelandic breed born in Europe and other breeds; [Table 2](#)).

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

Table 1

Number of insect bite hypersensitivity (IBH)-affected and healthy (H) horses with serum antibodies binding to at least one protein band in immunoblots of *C. nubeculosus* salivary gland extract

Antibody isotype	Number of horses with ab binding to at least one protein band		Significance Fisher’s exact test (<i>p</i>)
	IBH pos/total	H pos/total	
IgE	39/42	2/24	<0.000001
IgGa	19/22	9/22	<0.01
IgGb	18/22	17/22	Ns
IgGT	22/22	18/22	Ns

Table 2

Median number of bands on western blots from a *C. nubeculosus* salivary gland extract binding antibodies from serum of insect bite hypersensitivity (IBH)-affected and healthy horses belonging to various groups

Groups	IgE		IgGa		IgGb		IgGT	
	IBH	H	IBH	H	IBH	H	IBH	H
1. Icelandic breed born in Iceland and imported to Switzerland								
<i>N</i>	22	13	12	12	12	12	12	12
Median	4.5	0	5.5	0.5	2	1	4	2
Range	1–8	0–1	2–8	0–5	0–4	0–5	3–8	0–3
<i>p</i> (H vs. IBH)	<0.00001		<0.001		ns		<0.0001	
2. Icelandic breed born in Europe								
<i>N</i>	10	10	10	10	10	10	10	10
Median	3	0	2	0	2.5	1	2.5	1
Range	1–5	0–1	0–5	0–1	1–4	0–3	1–7	0–2
<i>p</i> (H vs. IBH)	<0.0001		≤0.01		0.06		<0.01	
3. Other breeds born in Europe								
<i>N</i>	10	3						
Median	3	0	nt	nt	nt	nt	nt	nt
Range	0–6							
<i>p</i> (H vs. IBH)	0.06							
All together								
<i>N</i>	42	26	22	22	22	22	22	22
Median	3.5	0	4	0	2	1	4	1
Range	0–8	0–1	0–8	0–5	1–4	0–5	1–8	0–3
<i>p</i> (H vs. IBH)	<0.00001		<0.0001		ns		<0.0001	
Comparison between groups								
<i>p</i> (1 vs. 2)	0.06	ns	<0.01	ns	ns	ns	≤0.01	<0.05
<i>p</i> (1 vs. 3)	0.06	ns						
<i>p</i> (2 vs. 3)	ns	ns						

ns, not significant; nt, not tested.

3. Results

3.1. Comparison of the immuno-reactivity of IBH-affected and healthy control horses

3.1.1. IgE antibodies

Immunoblots with sera of IBH-affected horses resulted in individual patterns of IgE ab-binding: some sera reacted with up to eight salivary gland (SG) proteins whereas others reacted only with one band (Fig. 1A). Overall, 39 of the 42 IBH-affected horses showed IgE-binding to SGE antigens. Only 2 of the 26 healthy horses showed marginal IgE antibody reactions against the SGE (Table 1).

On average, IBH-affected horses had IgE ab against a median number of 3.5 different protein bands (Table 2). The 48, 47, 22, 15 and 13 kDa protein bands from *C. nubeculosus* showed IgE-binding in

50% or more of the IBH-affected horses (Fig. 2), suggesting that these proteins constitute the major allergens from *C. nubeculosus*. The 32 and 19 kDa bands also seem to be important allergens for IBH as they each bound IgE ab from nearly one third of the IBH-affected horses (12 out of 42, Fig. 2). Seven out of the 42 IBH-affected horses and 1 of the healthy horses reacted with the 75 kDa band. Interestingly, the healthy horse showing IgE-binding to the 75 kDa band (Fig. 1, strip 21) developed IBH few months after blood sampling.

3.1.2. IgG subclass antibodies

Sera from both IBH-affected and healthy horses contained IgGa, IgGb and IgGT ab binding to the *Culicoides* salivary gland extract. IgG ab bound to protein bands of similar molecular weight as IgE ab (Fig. 1), but the binding pattern of every horse was

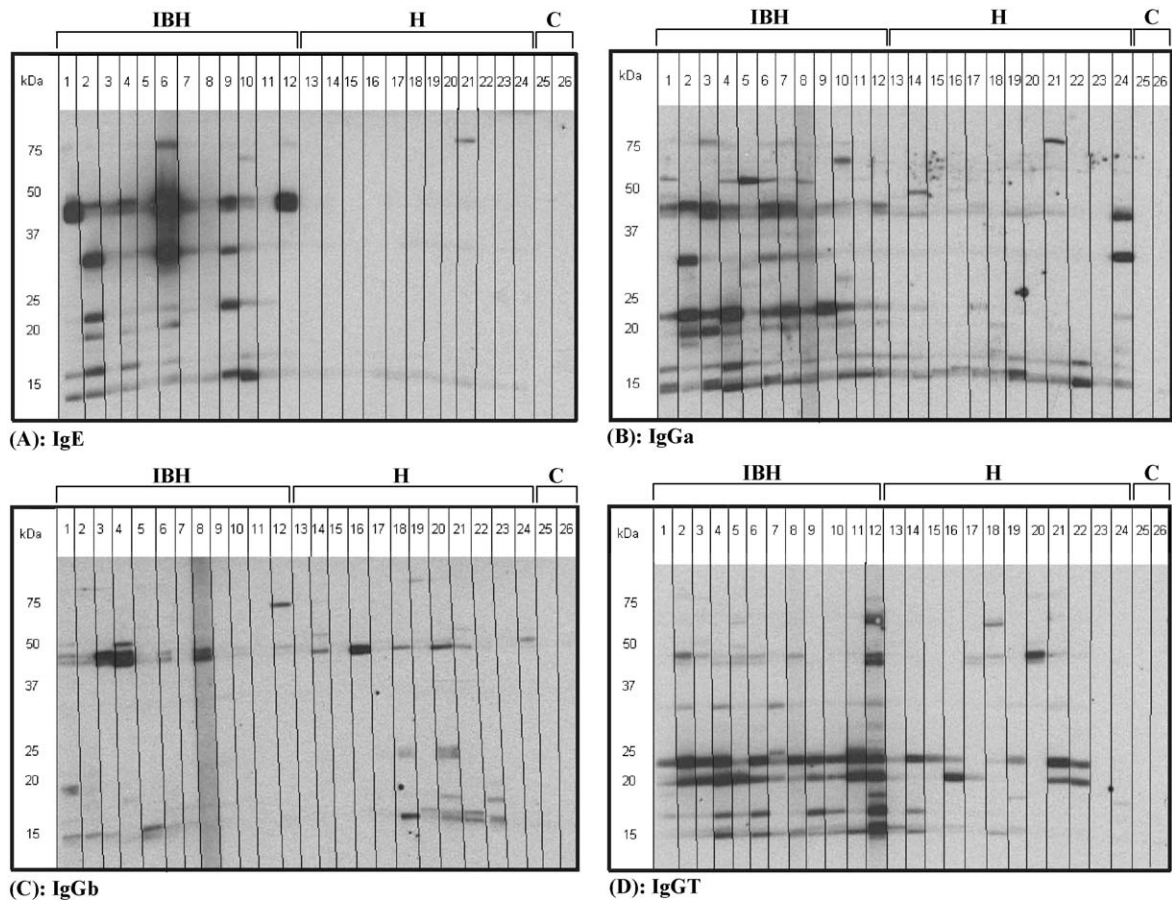


Fig. 1. Example of immunoblot analysis of (A) IgE, (B) IgGa, (C) IgGb and (D) IgGT antibodies in sera of Icelandic horses born in Iceland and imported to Switzerland, binding to salivary gland proteins derived from *Culicoides nubeculosus*. Protein standard in kiloDalton on the left side. Lanes 1–12: Horses with insect bite hypersensitivity (IBH). Lanes 13–24: Healthy horses (H). Lane 21: Horse that was healthy at the time of blood sampling but that developed IBH few months later. Lanes 25 and 26: Negative controls (C). Lane 25: Respective anti-IgE or IgG subclass monoclonal ab and HRP-conjugate anti-mouse ab. Lane 26: Negative control: HRP-conjugate anti-mouse ab only.

individual and differences were seen depending on the antibody subclass. Due to the limited availability of salivary gland extract, only 22 IBH-affected and 22 healthy horses were tested for three IgG subclasses.

Nineteen (86%) of the 22 IBH-affected horses, but only 9 (41%) of the 22 control horses had IgGa ab against at least one *C. nubeculosus* SGE antigen ($p < 0.01$, Table 1). As shown in Table 2, horses with IBH also had IgGa ab against a significantly higher number of protein bands (median 4, range 0–8) than control horses (median 0, range 0–5). Significant differences between IBH-affected and control horses

were found with the 52, 48, 32, 22, 19 and 13 kDa bands (Fig. 2).

The binding of IgGb ab to salivary gland proteins seemed less intense and less frequent than binding of the other IgG subclasses (Fig. 1). Seventeen of the 22 IBH-affected horses and 18 of the 22 healthy animals had IgGb ab against at least one SG protein band (Table 1). On average, healthy horses had IgGb ab against one (range 0–5) and IBH-affected horses against two (range 0–4) protein bands (Table 2, ns). A significant difference between IBH-affected and healthy horses was only observed for the 48 kDa band, as 9 of the 22 affected but only two of the 22

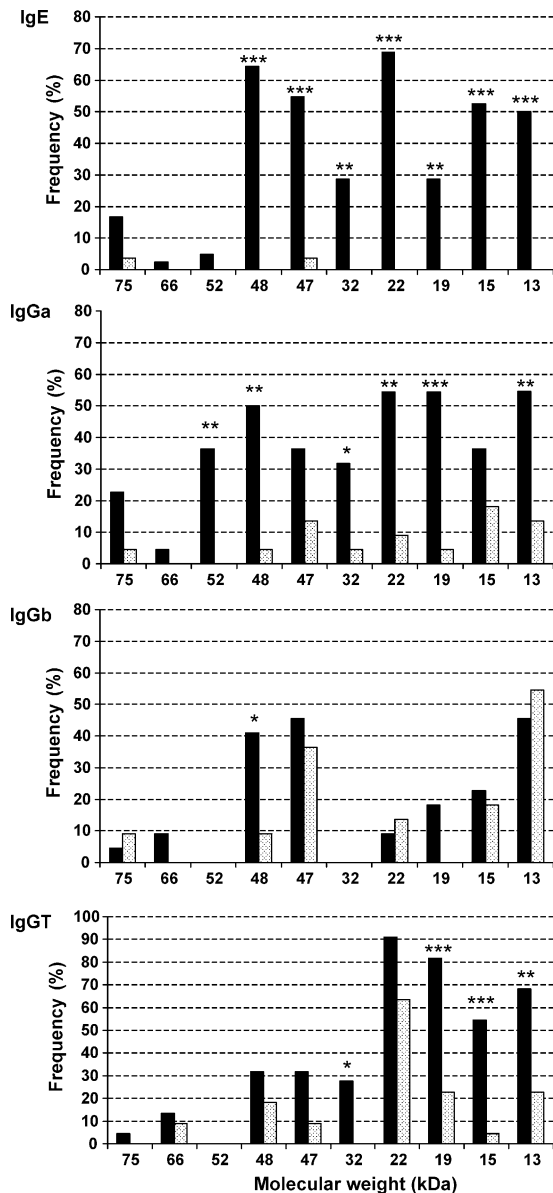


Fig. 2. Frequency (%) of antigenic components derived from *C. nubeculosus* salivary gland extract recognized by IgE, IgGa, IgGb and IgGT antibodies in immunoblotting in sera of IBH-affected (■) and healthy control horses (□). Statistical significance of the differences between both groups of horses is shown by: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

healthy horses showed IgGb-binding to this protein band (Fig. 2; $p < 0.05$). Furthermore, IgGb ab also bound to a smaller number of bands than IgE or IgGa ab (Fig. 2).

Except for four healthy horses, IgGT ab against *C. nubeculosus* SG proteins were found in the sera of all horses (Table 1, healthy versus IBH ns). However, IBH-affected horses had IgGT ab against a significantly higher number of protein bands than healthy horses (medians = 4 and 1, respectively, $p < 0.0001$; Table 2). Significant differences between IBH-affected and control horses were mainly found with the lower molecular weight proteins, i.e. the 32, 19, 15 and 13 kDa bands (Figs. 1D and 2).

3.2. Comparison of the immuno-reactivity of horses from different origins

Sera of IBH-affected horses born in Iceland and exported to Switzerland showed IgE-binding to a higher number of protein bands than sera from horses born in Europe belonging to the Icelandic or to other breeds (median number of 4.5 versus 3 IgE-binding bands, respectively, $p = 0.06$; Table 2). In the group of Icelandic horses born in Europe, the 48 and 47 kDa bands were not as immuno-reactive as in the group of horses born in Iceland as only 40% versus 82% and 10% versus 77% of the sera contained IgE ab binding to these bands, respectively ($p < 0.05$ and $p < 0.001$, respectively). Furthermore, all 32 IBH-affected horses belonging to the Icelandic breed had detectable IgE ab against at least one protein band, while three of the 10 IBH-affected horses belonging to other breeds, although having a clinical history of IBH, did not show detectable IgE binding to any SG protein (Table 2). We also made the subjective observation that the signal obtained with sera from horses of Icelandic origin was stronger than in the other horses.

Interestingly, IBH-affected horses born in Iceland also had IgGa ab binding to significantly more protein bands than Icelandic horses born in Europe (5.5 versus 2, $p < 0.01$; Table 2). In particular, there was significantly more frequent IgGa ab binding to the 75 kDa (42% versus 0%, $p < 0.05$) and to the 48 kDa (75% versus 20%, $p < 0.05$) bands (data not shown). The same was seen with the IgGT ab subclass: IBH-affected horses with Icelandic origin had IgGT ab binding to a higher number of protein bands than IBH-affected Icelandic horses born in Europe (4 and 2.5, respectively, $p < 0.05$, Table 2). This was not the case for IgGb ab (2 versus 2.5, ns Table 2).

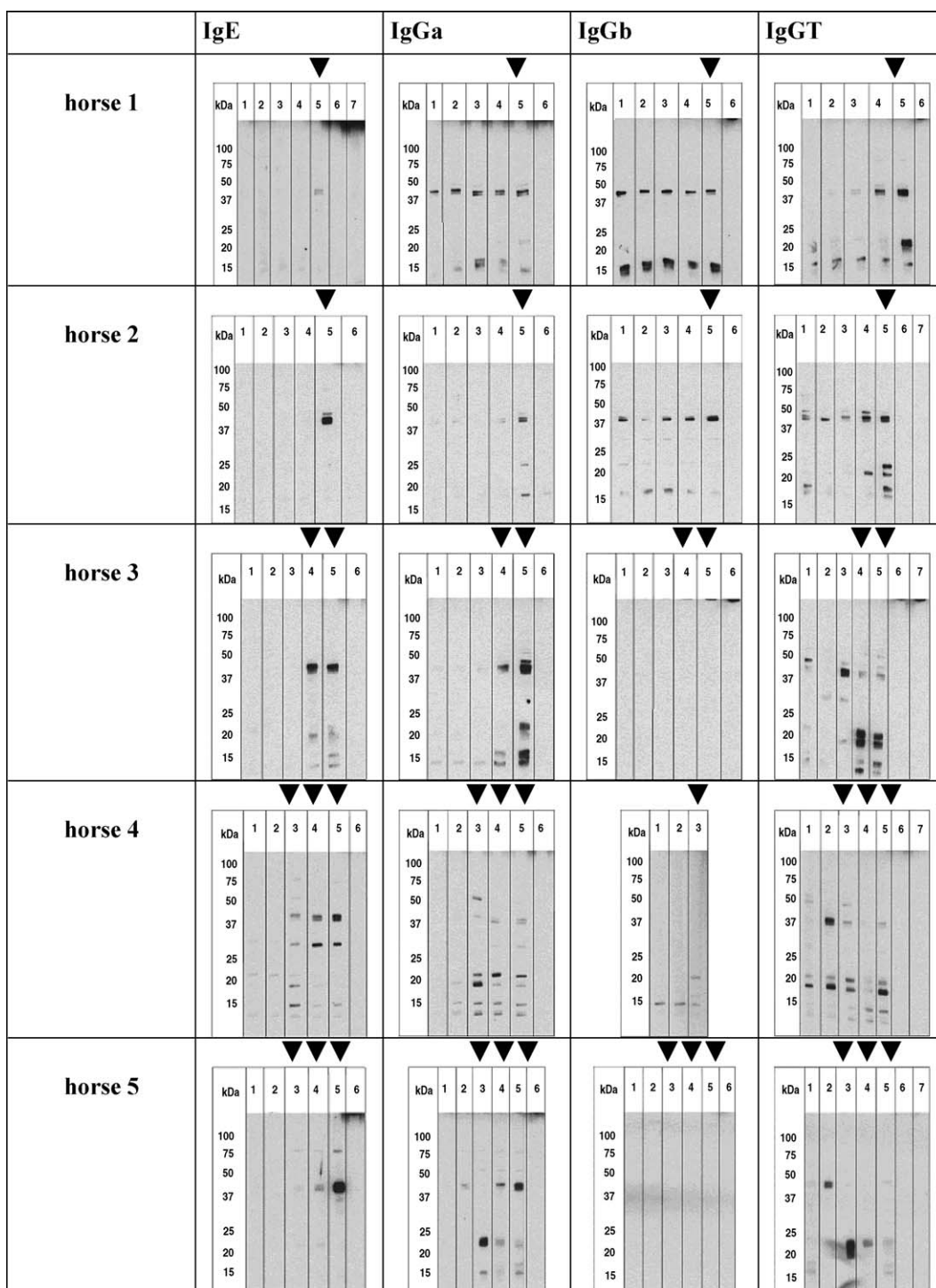


Fig. 3. Study on the natural course of IBH: immunoblot analysis of IgE, IgGa, IgGb and IgGT antibodies binding to salivary gland proteins derived from *Culicoides nubeculosus*. Immunoblots from five horses that developed insect bite hypersensitivity. Arrows indicate clinical signs of

3.3. Study on the natural course of the allergy

The five IBH-affected horses investigated in this additional study had developed IBH between 1 and 2 years after importation from Iceland into Switzerland. The five horses that had remained healthy over the sampling period had been imported into Switzerland 3, 2.5 and 2 years before the last blood sample was taken without developing clinical signs of IBH.

3.3.1. IgE antibodies

In all IBH-affected horses, detection of IgE ab correlated with onset of clinical signs (Fig. 3, clinical signs of IBH marked with arrows), except for one horse serum that showed a weak reaction against a 22 kDa protein before onset of the disease and already with the serum taken in Iceland (Fig. 3, horse 4). The bands with molecular weights of 47 and 48 kDa usually gave the strongest signal, but other bands could also be seen (Fig. 3). Every horse had its own characteristic IgE-binding pattern for every year of blood sampling. In October 03, 60% and in October 04 all horses that developed IBH showed IgE binding to the 48 and 47 kDa bands (Figs. 3 and 4A), while none of the horses that remained healthy showed IgE binding to the *C. nubeculosus* SGE (Fig. 4A).

3.3.2. IgG subclass antibodies

Differences in the binding pattern of IgG ab to SGE of *Culicoides* were observed depending on the antibody subclass.

Marginal IgGa binding to some protein bands was already observed in sera collected in Iceland, both in horses that developed IBH and in those that remained healthy (Figs. 3 and 4B). However, while IgGa reactivity remained more or less unchanged over time in the group of healthy horses (mainly with the 47 kDa band), the proportion of horses showing IgGa binding to various protein bands increased over time in the group of horses that developed IBH (Fig. 4B). In October 04, 100% of the horses that developed IBH reacted with the 48, 47, 22 and 12 kDa bands and 60%

of these horses also reacted with the 19 and 15 kDa bands. The proportion of horses whose serum reacted with these protein bands was significantly higher in IBH-affected than in healthy horses in October 04, and for some of these protein bands the difference was already significant for earlier time points, starting in October 02. The binding pattern was unique for every horse, and the intensity of the signals and the pattern also varied but to a lesser extent between years (Fig. 3).

Two healthy and one IBH-affected horse (Fig. 3, horse 1 as an example of an IBH-affected animal) had strong IgGb binding to some bands at every time of blood sampling. The sera of all the other horses showed only low level IgGb binding to the SGE and no increase of reactivity could be observed over time. There were no significant differences between the horses that developed IBH and those that remained healthy (Fig. 4C).

A number of the healthy horses, as well as of the IBH-affected ones, had IgGT ab to the *C. nubeculosus* SGE already in Iceland and, also at the other sampling periods (Figs. 3 and 4D). However, over time, more horses of the IBH group showed IgGT binding to various protein bands than in the healthy group, but the difference between both groups was only significant in October 04 for 15 and 12 kDa bands (Fig. 4D).

To check for technical differences due to immunoblotting, the final serum samples from each of the 10 horses were tested on strips of the same western blot and the differences in IgE-binding patterns observed and approximate differences in the signal intensity between horses confirmed (data not shown).

4. Discussion

We found 10 protein bands with MWs ranging from 12 to 75 kDa in the SGE of *C. nubeculosus*, which bound IgE from sera of IBH-affected horses. The 48, 47, 22, 15 and 13 kDa protein bands seem to be major allergens for IBH, as they bound IgE ab from $\geq 50\%$ of the sera from IBH-affected horses. Reactivity to the 32

IBH. Protein standard in kiloDalton on the left. Lane 1: Sera taken in Iceland before export. Lane 2: Sera taken in June following importation into Switzerland. Lane 3: Sera taken in October following importation into Switzerland. Lanes 4 and 5: Sera taken in October of the second and third year following importation into Switzerland. Lanes 6: Negative controls (respective anti-IgE or IgG subclass monoclonal ab and HRP-conjugate anti-mouse ab). Lane 7: Negative control (HRP-conjugate anti-mouse ab only).

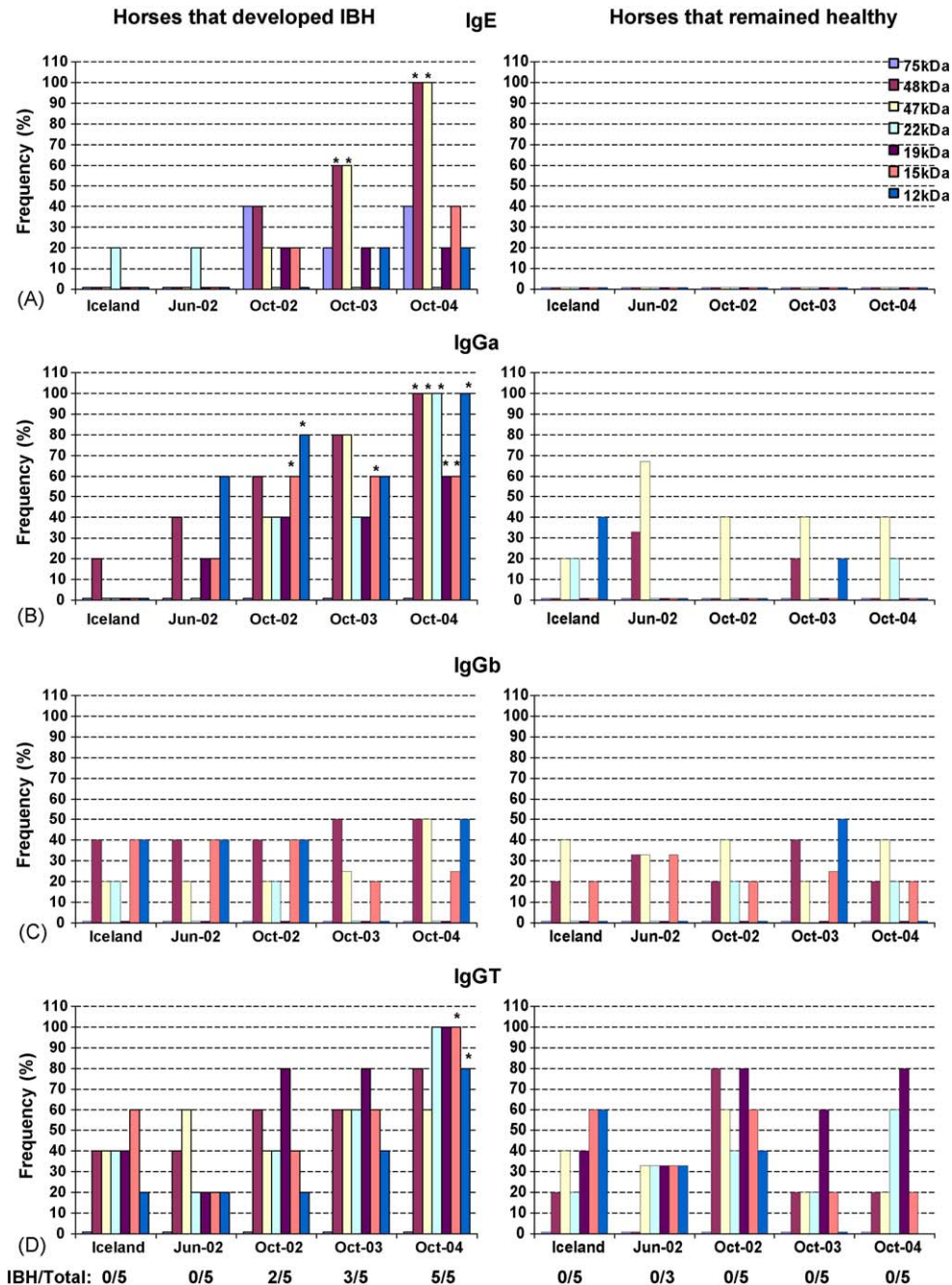


Fig. 4. Study on the natural course of insect bite hypersensitivity (IBH): frequency (%) of IBH-affected (left) and control horses (right) showing IgE (A), IgGa (B), IgGb (C) and IgGT (D) binding to the main antigenic components of salivary gland proteins derived from *Culicoides nubeculosus* in immunoblots. Horses (five that developed IBH and five that remained healthy) were followed over a period of 3 years. Additionally, sera were tested before export from Iceland to continental Europe. *Significant difference (Fisher's exact test; $p < 0.05$) for antibody-binding to corresponding protein band tested at the same given time point between IBH-affected and control horses.

and 19 kDa protein bands was seen less frequently (in 30% of the IBH-affected horses) but the difference between healthy and affected horses was significant in this case, too. With the sera selected for this study, therefore, determination of the presence of IgE ab against *C. nubeculosus* SGE seems to enable the reliable sero-diagnosis of IBH, as 93% of the IBH-affected horses showed IgE-binding to the *C. nubeculosus* SGE while the same proportion of healthy horses did not show detectable IgE binding to the SGE (Table 1). Only two apparently healthy horses had IgE ab binding to either a 75 or a 47 kDa protein, and one of these horses developed IBH a few months after blood sampling. Unfortunately, the lack of availability of *C. nubeculosus* SGE will not allow this method to become a widely available test for the in vitro diagnosis of IBH.

Only 3 out of the 42 tested IBH-affected horses, had no detectable IgE ab to any protein band of *C. nubeculosus* SGE. Interestingly, none of these three horses belonged to the Icelandic breed. It is possible that these horses were sensitized to the bites of other insects, that the specific IgE ab levels were below the detection limit of the assay, or that IBH was not due to IgE-mediated reactions in these horses. Previous studies have indicated that type IV hypersensitivity reactions can occur alone or in combination with IgE-mediated type I hypersensitivity reactions in some cases of IBH (Halldorsdottir et al., 1989).

Significantly more proteins of the SGE were recognized by IgGa and IgGT ab but not by IgGb ab in the group of allergic horses than in the healthy control group (Table 2). These findings suggest that these two antibody subclasses and in particular IgGa ab, are associated with allergic sensitisation in the horse. In mouse models of allergic disorders or in human allergies it has been shown that some of the IgG subclass ab were increased in allergic patients (Brummer-Korvenkontio et al., 1994; Peng and Simons, 2004).

Interestingly, our investigation showed that only the smaller salivary gland proteins (≤ 32 kDa) were recognized more often by IgGT ab from IBH-affected horses as compared with controls, while significant differences between IBH-affected and control horses were found with antigens ranging between 52 and 13 kDa for IgGa ab, and between 48 and 13 kDa for IgE ab. In a mouse model Chen et al. (1998) showed

that in vivo allergenicity of the recombinant mosquito allergen rAed a 2 induced a significant increase in both IgE and IgG1 (Th2-driven ab) but not IgG2a (Th1-driven ab). Therefore, the structure of the allergen epitopes might be decisive for the subclass of IgG ab being produced.

Little is known about the function of IgG subclasses in horses so far. Studies by Nelson et al. (1998) indicate that after infection with influenza IgGa (IgG1) and IgGb (IgG4) sub-isotypes are produced while vaccination with an influenza vaccine only leads to an IgGT (IgG3 and 5) ab response. Our present investigation suggests that IgGb ab are only marginally produced after exposure to *Culicoides* bites, while IgGT ab seem to be induced in all horses after exposure to the bites of these insects, although to a lesser extent in horses without IBH. Interestingly, production of IgGa (IgG1) ab against salivary gland antigens seems to be associated to some extent with IBH and presence of antigen-specific IgE ab. This finding was confirmed in the study of the natural course of the disease. IgGc (IgG6) ab binding to the SGE could not be detected with a commercially available supernatant (Serotec GmbH) specific for equine IgGc. We do not know if this is due to the absence of IgGc ab against salivary gland antigens in the sera tested or to a technical problem, such as a low titre in the supernatant. Further studies will be needed to clarify which IgG subclasses are associated with allergic reactions in the horse.

The results obtained with the study on the natural course of the allergy showed again that every horse has its own antibody-binding pattern in the immunoblot, but even more interestingly, this pattern also varied depending on the time points of sampling. Importantly, regardless of the antigens detected in SGE, IgE ab appearance correlated with onset of severe clinical signs of IBH, confirming the previous results. However, IgG ab against some proteins of the SGE were already present in the sera taken in Iceland although *Culicoides* are not native there. This might be explained by the presence of species-shared proteins in the saliva of different insect groups, as, contrarily to *Culicoides* spp., *Simulium vittatum*, another species of biting insect, is found in Iceland (Johannsson, 1988). Both *Culicoides* (family Ceratopogonidae) and the *Simuliidae* are families of the Nematocera, a sub-order

of the Diptera (Brohmer, 1994). It is possible that their close phylogenetic relationship and similar mechanism of blood feeding has led to similar salivary ingredients. The presence of species-shared allergens in the saliva of different mosquito species has been demonstrated in mosquito allergy in humans (Peng and Simons, 1997; Peng et al., 1998). Furthermore, Baselgia et al. (2006) have demonstrated that a large proportion of IBH-affected horses are sensitised against both *C. nubeculosus* and *S. vittatum* whole body extract, suggesting that there may be some conserved or cross-reacting allergens in both insect species. Preliminary investigations by immunoblotting support this hypothesis: some horse sera contain IgE ab binding to protein bands of the same molecular weight in immunoblots of *C. nubeculosus* and *S. vittatum* SGE (data not shown). However, only further investigations using 2D electrophoresis and mass spectrometry will allow to tell whether these two insect species contain conserved antigens. The absence of IBH in Iceland, although *S. vittatum* are found there, could be explained by the fact that duration of insect exposure in Iceland is much shorter than in most of continental Europe. The level of biting in Iceland may therefore be insufficient for the development of IBH but could be enough to induce the production of IgG ab.

It is still unknown why the prevalence of IBH is significantly higher in Icelandic horses born in Iceland and exported to continental Europe than in Icelandic horses born in continental Europe. Although genetic factors may play a role, this significant difference cannot be explained by genetic factors only, as the Icelandic horses born in mainland Europe are offspring of the imported Icelandic sires and dams and a large proportion of these horses used for breeding are affected by IBH. Maternal effects may contribute to this difference in IBH-prevalence: Jenmalm and Bjorksten (1999) have investigated the development of IgG subclass ab to allergens in early childhood in humans and showed that maternally derived ab modulate immune responses. They postulated that the time, route and level of exposure to an allergen, as well as maternally transferred immunity might be decisive in determining whether sensitisation or tolerance will ensue. The absence of *Culicoides* species from Iceland hampers the possibility of developing any immune response modulation

in Icelandic horses born in that country although there might be some cross reactive allergens from black flies (*Simuliidae*), but the differences in the immune responses of horses of Icelandic origin to Icelandic horses born outside Iceland could reflect the different varieties of their allergen exposure. To find out whether there were differences in the antigen-specific humoral immune response between these two groups of horses we compared IgE and IgG subclass ab binding to *Culicoides* SGE, in horses born in Iceland and imported to Switzerland and in horses born in Europe belonging to the Icelandic or other breeds. While no differences were found between groups of healthy horses, horses with IBH that were born in Iceland had IgGa and IgGT ab binding to a significantly higher number of SGE antigens than horses born in Europe and this difference nearly reached the level of significance ($p = 0.06$) for SGE specific IgE ab. Furthermore, Icelandic horses born in Iceland showed IgE-binding to the 47 and 48 kDa bands more frequently than Icelandic horses born in continental Europe. Further studies are needed to understand the immunological mechanisms responsible for this increased specific immune response in Icelandic horses exposed to bites of *Culicoides* spp. for the first time as adults.

In conclusion, our results have shown that at least 10 different *C. nubeculosus* SGE proteins can bind IgE ab from sera of IBH-affected horses and are thus the potential allergens for IBH. Besides IgE ab, IgG ab of the IgGa and IgGT subclasses are produced against *Culicoides* salivary gland antigens and are associated to some extent with clinical signs of IBH. Significant differences in the humoral immune response against *C. nubeculosus* SGE were seen in a group of horses with a high prevalence of IBH, i.e. horses born in Iceland and exposed to bites of midges for the first time as adults, compared to horses with a low prevalence of IBH, i.e. horses born in Europe that have been exposed to bites of midges since the first year of life.

Every horse showed a unique pattern of antibody binding in the immunoblot. This finding is important for the future development of specific immunotherapy with recombinant salivary gland allergens, as it indicates that the recombinant allergens required will vary from horse to horse. This means that a patient tailored immunotherapy will have to be

designed, based on the results of a preceding allergy test.

This study is a first step towards elucidation of the different allergens causing IBH and contributes to a better knowledge of the pathogenesis of this disease. Further investigations are needed to characterize the allergens present in the saliva of *C. nubeculosus*.

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