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Cloning of IgE-binding proteins from *Simulium vittatum* and their potential significance as allergens for equine insect bite hypersensitivity

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ABSTRACT

Insect bite hypersensitivity (IBH) is an allergic dermatitis of horses caused by bites of *Culicoides* and sometimes *Simulium* spp. The aim of this investigation was to identify *Simulium* allergens associated with IBH. A phage surface display cDNA library expressing recombinant *Simulium vittatum* salivary gland proteins was screened using sera of IBH-affected horses sensitized to *S. vittatum* salivary gland proteins as shown in immunoblot, resulting in the identification of seven cDNAs encoding IgE-binding proteins. The deduced amino acid sequences of these proteins showed sequence similarities to antigen 5 like protein (Sim v 1), to a serine protease inhibitor (Sim v 2), to two α -amylases (Sim v 3 and Sim v 4), and to three *S. vittatum* erythema proteins (SVEPs). The cDNA inserts were subcloned and expressed as [His]₆-tagged protein in *Escherichia coli* and purified using Ni²⁺-chelate affinity chromatography. Mice were immunised with the seven recombinant proteins and the antibodies tested against the recombinant proteins and salivary gland extract (SGE) of *S. vittatum* and *Culicoides nubeculosus* in immunoblot analyses.

r-Sim v 1 specific mouse Abs recognized a band of about 32 kDa in immunoblots of both *S. vittatum* and *C. nubeculosus* SGE, detectable also by serum IgE of IBH-affected horses. Preincubation of horse serum with r-Sim v 1 completely inhibited IgE binding to the 32 kDa band demonstrating the presence of cross-reactive antigen 5 like proteins in both SGE.

Determination of IgE levels against the r-Sim v proteins and crude *S. vittatum* extract by ELISA in sera from 25 IBH-affected and 20 control horses showed that IBH-affected horses had significantly higher IgE levels than controls against r-Sim v 1, 2, 3, 4 and *S. vittatum* extract, whereas the r-SVEP showed only marginal IgE binding. Further analyses showed that 60% of IBH-affected horses reacted to r-Sim v 1, suggesting that this could be a major allergen for IBH. Forty to twenty percent of the IBH-affected horses reacted with r-Sim v 2, 3 or 4. Combination of the results obtained with the 4 r-Sim v proteins showed that 92% of the IBH-affected but only 15% of the healthy horses had IgE levels against one or more of the 4 r-Sim v proteins. Seventy percent of the healthy horses had detectable IgE against *S. vittatum* extract, indicating a low specificity of the detection system used. Optimization of the ELISA system will be required to determine reliable cut-off values for the IBH-related allergens. Their *in vivo* relevance needs to be carefully assessed.

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Abbreviations: AP, alkaline phosphatase; *C. nubeculosus*, *Culicoides nubeculosus*; HRP, horse radish peroxidase; IBH, insect bite hypersensitivity; REU, relative ELISA units; SG, salivary glands; SGE, salivary gland extract; SVEP, *Simulium vittatum* erythema protein; *S. vittatum*, *Simulium vittatum*.

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1. Introduction

Insect bite hypersensitivity (IBH) is an allergic pruritic dermatosis frequently seen in horses. IBH symptoms can often be traced back to IgE-mediated, type I hypersensitivity reactions to bites of insects, mainly *Culicoides* (midges) and *Simulium* spp. (black flies). Most of the IBH-affected horses have IgE Abs against salivary gland proteins from *Culicoides* spp. (Wilson et al., 2001; Hellberg et al., 2006). They also show positive intradermal test (IDT) reactions with whole body extract and saliva of *Culicoides* species (Anderson et al., 1993; Langner et al., 2008) and/or *Simulium* whole body extracts (Fadok and Greiner, 1990). Moreover, peripheral blood leucocytes (PBL) of IBH-affected horses release sulfidoleukotrienes and histamine upon stimulation with crude *Culicoides nubeculosus* extract (Marti et al., 1999; Langner et al., 2008). However, a part of the IBH-affected horses showing a positive sulfidoleukotriene release after incubation of PBL with *Culicoides nubeculosus* whole body extract also reacted with *Simulium vittatum* extracts (Baselgia et al., 2006), suggesting the presence of cross-reactive structure shared between extracts of midges and black flies. In agreement with these observations, serum IgE of IBH-affected horses recognizes various protein bands, partly of comparable molecular mass, in *Culicoides* and *Simulium* salivary gland extract (SGE) (Hellberg et al., 2009). Presently, only whole body extract from these insects is available commercially. This has hampered research on the pathogenesis of IBH as well as the development of specific and sensitive diagnostic tests and efficient therapeutic approaches to cure the disease (Barbet et al., 1990; Morgan et al., 2007). One IBH-related allergen, a maltase from *C. sonorensis*, has recently been identified using a proteomics approach and expressed as recombinant protein (Langner et al., 2009). However, the studies by Hellberg et al. (2006) and Wilson et al. (2008) show that *Culicoides* SGE contains many IgE-binding proteins and allergens from *Simulium* spp. have not yet been identified.

In human allergy, phage surface display technology has proven to be a very efficient method for identifying whole allergen repertoires (Rhyner et al., 2004). The basic concept of phage-display links the phenotype, expressed as a fusion together with a phage surface coat protein, to its genetic information integrated into the phage genome (Crameri and Suter, 1993). This allows libraries to be screened using the power of affinity interaction between a solid phase-coated ligand and the phage library applied in fluid phase during multiple rounds of phage growth and selection. The advantage of phage-display libraries over conventional cDNA libraries is the 10^5 – 10^6 -fold selective enrichment of clones expressing proteins of interest (i.e. IgE binding). The small size of filamentous phage allows a very large number of independent phage (up to 10^{10} phage) to be screened in small volumes and in a short period of time using the discriminative power of affinity selection (Crameri et al., 1994; Kodzius et al., 2003). The purposes of this study were to test the performance of phage surface display for the identification of IgE-binding proteins from *S. vittatum* SG and to subclone, produce, and characterize potential allergens involved in the pathogenesis of IBH.

2. Materials and methods

2.1. Horses

Sera from six IBH-affected horses were used for screenings of an *S. vittatum* cDNA library displayed on phage surface. They were selected according to case history (seasonal recurrent dermatitis), characteristic clinical signs of IBH (see below) and positive sulfidoleukotriene release test with *S. vittatum* whole body extract (Baselgia et al., 2006). Additionally, these sera showed IgE binding to various protein bands in immunoblots of *S. vittatum* SGE (Hellberg et al., 2009).

To evaluate IgE binding to recombinant *S. vittatum* allergens, sera from 25 IBH-affected horses and from 20 healthy control horses were used in ELISA. The horses were from different stables in Switzerland and consisted of 37 horses of the Icelandic breed (21 IBH-affected and 16 healthy horses) and of eight Swiss Warmbloods and Franches-Montagnes horses (4 IBH-affected and 4 healthy horses). Healthy control horses were selected randomly from the same stables where IBH-affected horses were living.

The IBH-affected horses had pruritus, excoriations and thickening of the skin along the dorsal midline, localised mainly at the base of the mane and tail. Sometimes the ventral midline and/or the head were also affected. Most importantly, to be included in the study as an IBH-affected animal, the history of pruritus had to be recurrent and seasonal, i.e. occurring between March and October, followed by a period of remission in winter. All horses received treatments to alleviate IBH symptoms such as sweet itch blankets, and/or local application of various lotions. Horses receiving systemic corticosteroids were excluded from the study.

The healthy (H) control horses had no clinical signs of pruritus or cutaneous lesions, negative immunoblots to *S. vittatum* SGE and had no previous history of skin problems.

2.2. Construction and screening of a *S. vittatum* salivary gland cDNA library displayed on phage surface

A *S. vittatum* cDNA library (Cupp et al., 1998) prepared from SG mRNA was used to isolate *Simulium* SG cDNA inserts which were further subcloned into phagemid pJufO to generate a cDNA library displayed on the surface of filamentous phage M13 as described (Crameri et al., 1994, 2002). Serum IgE from IBH-affected horses was captured in microtiter plate wells coated with anti-equine IgE mAb (Wilson et al., 2006) and used as ligand for selective enrichment of allergen-displaying phage.

2.3. Identification of clones encoding *S. vittatum* SG proteins

Affinity enriched inserts carried by phages displaying potential IgE-binding proteins differing in length were sequenced by an automated sequencing service provided by Microsynth GmbH (Balgach, Switzerland) using vector derived primers. To establish possible relationships to previously published sequences, the sequence similarity search tool from the National Centre for Biotechnology

Information (NCBI) was used (BLAST 2.18+; Altschul et al., 1997). When necessary, sequence comparisons (alignments) were performed with CLUSTAL W (1.83; <http://www.ch.embnet.org/software/ClustalW.html>).

2.4. Expression and purification of recombinant *S. vittatum* proteins

Sequenced inserts were cloned into the high-level expression vector pQE30 (Qiagen; www.qiagen.com), expressed as His-tagged fusion proteins in *E. coli* M15/pREP4 (Qiagen) and purified under denaturing conditions by Ni²⁺-NTA chelate affinity chromatography according to the suppliers recommendations.

2.5. Production of antibodies against the recombinant proteins

Polyclonal antibodies specific for *S. vittatum* recombinant proteins were produced in mouse ascites fluid according to the method of Overkamp et al. (1988). Balb/c mice were injected intraperitoneally (i.p.) with 20 µg of purified protein dissolved in 250 µl Complete Freund's Adjuvant per mouse. The injection was repeated on days 14 and 21 with Incomplete Freund's Adjuvant. On day 14 the mice also received 500 µl Pristane (Sigma, USA) i.p. In the following weeks mice were sacrificed, the ascitic fluid collected and tested with the corresponding recombinant protein as well as with *S. vittatum* SGE in immunoblot.

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

Salivary glands (SG) were dissected from female *S. vittatum* and *C. nubeculosus* and used to prepare SG extracts (SGE; Hellberg et al., 2006). SDS-PAGE analyses were performed as described (Hellberg et al., 2006). Briefly, *C. nubeculosus* or *S. vittatum* SGE or the r-Sim v proteins were resolved by 12% SDS-PAGE using Mighty Small (Hoefer[®] Inc.; <http://www.hoeferinc.com/>) vertical electrophoresis unit, stained with Coomassie (for recombinant proteins) or transferred on a PVDF membrane (Bio-Rad Laboratories; www.bio-rad.com) using a semi-dry protein transfer cell (Bio-Rad Labs.) at 15 V for 30 min. Transfer buffer containing 39 mM glycine, 48 mM Tris, 0.00375% SDS, 20% methanol was used. The membranes were dried and cut into strips. Protein bands of SGE were visualized with a gold staining by immersion in the protogold stain kit (BB International, Cardiff, UK; www.bbigold.com) for approximately 20 min.

Mouse ascites were diluted 1:500 and used for the detection of the corresponding SG proteins. For the detection of allergen-specific IgE, horse serum was diluted 1:6 in washing buffer (20 mM Tris, 0.25 M NaCl, 0.125% Tween-20, pH 7.5), added to the PVDF strips and incubated at 4 °C overnight on a shaker. The following day the strips were washed and incubated with a monoclonal mouse anti-horse-IgE mAb (0.5 µg/ml 3H10, Wilson et al., 2006), followed by incubation with a horse radish peroxidase (HRP) conjugated goat anti-mouse-IgG mAb (Sigma-Aldrich, <http://www.sigmaaldrich.com>) diluted 1:10,000

in washing buffer. After a final washing step the strips were developed with ECL Western blotting detection reagent (GE Healthcare; www.gehealthcare.com).

2.7. Inhibition immunoblot

Western blot strips of *C. nubeculosus* SGE (see above) were incubated with serum of an IBH-affected horse that recognized a distinct pattern of IgE-binding proteins differing in molecular weights. For inhibition, serum of the same horse was preincubated with 10 µg/ml r-Sim v 1 for 30 min at RT on a shaker, before addition to a Western blot strip of *C. nubeculosus* SGE. Blots were then developed as above with an anti-equine IgE mAb followed by an anti-mouse HRP-labelled antibody and bands visualized with ECL.

2.8. Allergen-specific IgE ELISA

Immunolon 2HB polystyrene microtitre plates (VWR International AG, <http://ch.vwr.com>) were coated by incubation at 37 °C for 2 h with 5 µg/ml (r-Sim v 4), 1 µg/ml (r-Sim v 1) or 0.5 µg/ml (r-Sim v 2 and 3) recombinant antigen or with 1 µg/ml *S. vittatum* whole body extracts in 0.2 M sodium carbonate–bicarbonate buffer pH 9.6 (Pierce Biotechnology, <http://www.pierce-net.com>). These concentrations had been chosen after pilot experiments where the antigen concentration gave the best signal to noise ratio and the best linearity of the curve of serially diluted ascites or serum samples. After coating, the plates were washed with PBS pH 7.4 (Calbiochem AG, <http://www.calbiochem.com>) containing 0.05% Tween-20 (Sigma-Aldrich, <http://www.sigmaaldrich.com>) and then blocked by incubation with PBS pH 7.4 containing 5% Tween-20 and 5% nonfat dry milk powder for 1 h at 37 °C. After washing, horse sera diluted 1:4 in blocking buffer were added. Mouse ascites specific for each r-Sim v proteins were used at dilutions between 1:500 and 1:8000 depending on the r-Sim v protein and the ascites used. Duplicate samples of the sera and ascites were then further serially diluted twofold in blocking buffer throughout the plate and incubated overnight at 4 °C. Following washing in PBS Tween, anti-equine IgE mAb 3H10 (0.5 µg/ml; Wilson et al., 2006) was added and incubated for 2 h at RT on a shaker. Following a further wash, a 1:1500 dilution of alkaline phosphatase (AP)-labeled goat anti-mouse Ab (Jackson ImmunoResearch Laboratories Inc.; www.jackson-immuno.com) was added for 90 min at RT on a shaker. After a final wash the ELISA was developed with 1 mg/ml p-nitrophenyl phosphate (Sigma-Aldrich) in 10% diethanolamine buffer at pH 9.8 (Sigma-Aldrich). Absorbance readings were measured at 405 nm with a Molecular Devices Reader (Sunnyvale, CA, USA). Serial dilutions of a reference horse serum positive for the tested allergen with an assigned value of 100 REU were used to generate a standard curve. The OD of the test samples were interpolated from the standard curve to give their IgE concentration in relative ELISA units (REU) and multiplied by the dilution factor. The concentration of the original serum samples was calculated as the average of the values from those dilutions where the concentration fell within the standard curve.

2.9. Inhibition ELISA

Immunolon 2HB polystyrene microtitre plates (VWR) were coated and blocked as described above. In a separate untreated 96-well plate (Sarstedt AG, 9475 Sevelen, Switzerland), serially diluted r-Sim v 1, 2, 4, r-SVEP or *E. coli* extract as a negative control, were incubated for 30 min at RT on a shaker with serum from individual horses sensitized to the respective r-Sim v proteins. The preincubated sera were transferred to the coated, blocked and washed ELISA plate and incubated at 4 °C overnight. After washing and development (see above), percentage of inhibition was calculated by setting the absorbance obtained for the serum preincubated without the r-Sim v proteins in the fluid phase as 0% inhibition. For graphics display, the calculated percentages were plotted against the antigen concentration in the fluid phase.

2.10. Statistical analyses

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

A preliminary analysis indicated that the allergen-specific IgE levels were not distributed normally, and medians and ranges were used subsequently for descriptive purposes.

For the graphs where a log scale was used, sera with IgE levels below the detection limit of the assay were given a value of 0.5 REU.

The nonparametric Mann–Whitney *U*-Test was used to compare continuous variables such as IgE levels against the various r-Sim v proteins or against *S. vittatum* whole body extract in IBH-affected and healthy horses.

To compare binary variables such as number of IBH-affected and healthy horses with IgE levels below or above an arbitrary cut-off, the 2-tailed Fisher's exact test was used.

The level of significance was set at $p \leq 0.05$.

3. Results

3.1. Screening of the *S. vittatum* phage surface display library

Biopanning of the *S. vittatum* phage surface display library resulted in selective enrichment of phage containing at least seven different sequences. Deduced amino acid sequences showed high identities to just

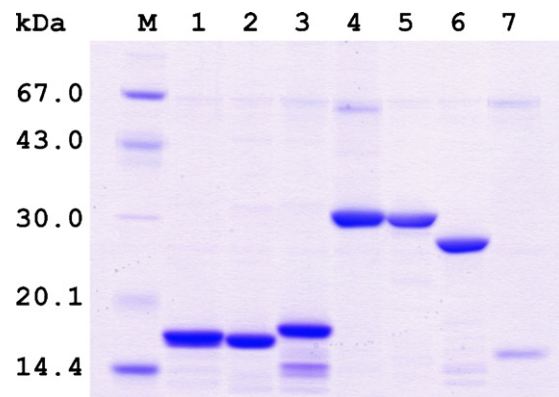


Fig. 1. SDS-PAGE and Coomassie stain of the *E. coli* expressed and Ni^{2+} purified recombinant *S. vittatum* proteins. Lane M: molecular weight marker. Lane 1: r-SVEP; lane 2: r-SVEP-9; lane 3: r-SVEP-10; lane 4: r-Sim v 1; lane 5: r-Sim v 3; lane 6: r-Sim v 4; lane 7: r-Sim v 2.

recently available sequences of the sialome of *S. vittatum* (Table 1). Sim v 1 showed 100% identity with the deduced amino acid sequence to an antigen 5 like protein, Sim v 2 100% identity to a Kunitz protease inhibitor, Sim v 3 has 98% identity to an α -amylase, and Sim v 4 98% identity to an additional α -amylase sequence from *S. vittatum* (Table 1). Three sequences with homology to erythema proteins were also identified. One of these erythema proteins had 100% identity at amino acid level to the SVEP (Cupp et al., 1998). Another protein shared 64% identity with SVEP at the amino acid level, while the third identified SVEP sequence had 64% identity to SVEP-5 (Table 1).

3.2. Expression and purification of r-*S. vittatum* proteins

The inserts encoding the seven different proteins were subcloned into the high-level expression vector pQE30, transformed into *E. coli* and sequences confirmed by nucleotide sequencing. Correct clones were used to produce the seven $[\text{His}]_6$ -tagged fusion proteins by IPTG induction. The recombinant proteins were purified by Ni^{2+} -chelate chromatography and refolded by dialysis. Except for the Kunitz protease inhibitor (Sim v 2) which could only be expressed at low levels, the r-*S. vittatum* proteins were found to be >95% pure as shown by denaturing SDS-PAGE and Coomassie blue staining (Fig. 1). The estimated molecular masses of the purified recombinant proteins were in good agreement with the sizes of the

Table 1

Characteristics of the recombinant *S. vittatum* proteins.

r-Protein	Accession no.	AA ^a	Complete/partial	MW ^a	Homology to <i>S. vittatum</i> sequences:	% AA identity with (protein-id no.)
Sim v 1	EU930215	257	Partial (21 AA missing at 5')	29.8 kDa	Antigen 5 like Protein	100% (ACH56843.1)
Sim v 2	EU930300	83	Partial (11 AA missing at 5')	9.6 kDa	Kunitz protease inhibitor	100% (ACH56928.1)
Sim v 3	EU930236	246	Partial	28 kDa	α -Amylase	98% (ACH56864.1)
Sim v 4	EU930294	241	Partial	26 kDa	α -Amylase	98% (ACH56922.1)
SVEP	U94515	133	Partial (19 AA missing at 5')	15.3 kDa	SVEP	100% (AAC26163)
SVEP-9	FJ655999	132	Partial	15 kDa	SVEP	64% (AAC26163)
SVEP-10	FJ655998	132	Partial	15.9 kDa	SVEP-5	64% (ACH56882.1)

^a Without cloning tag, calculated from the deduced amino acid sequence.

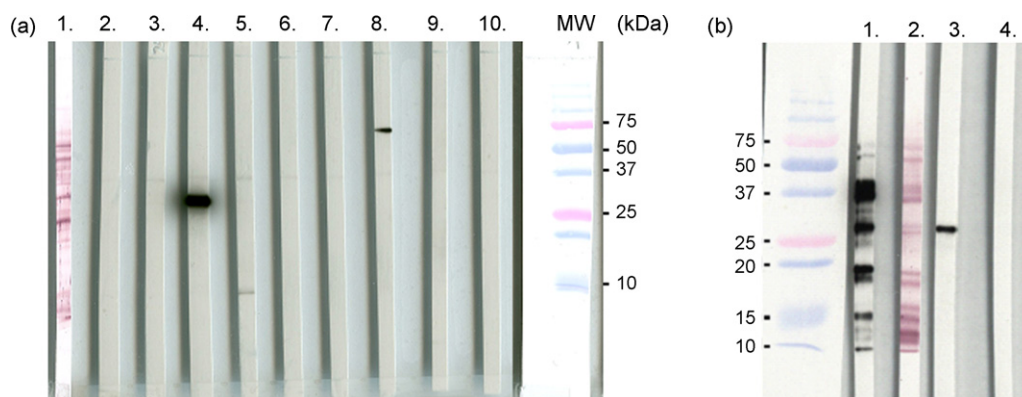


Fig. 2. (A) SDS-PAGE and Western blot of *Simulium vittatum* SGE. Lane 1: total protein stain with colloidal gold. Lanes 2–10: immunoblot stained with mouse antibodies specific for: lane 2: r-Sim v 2; lane 3: R-Sim v 4; lane 4: r-Sim v 1; lane 5: r-SVEP-10; lane 6: r-SVEP-9; lane 7: r-SVEP; lane 8: r-Sim v 3; lane 9: non-related antibody (negative control); lane 10: conjugate control. Molecular weights in kDa indicated on the right. (B) SDS-PAGE and Western blot of *Culicoides nubeculosus* SGE. Lane 1: immunoblot incubated with serum of an IBH-affected horse; IgE Abs detected with an anti-equine IgE mAb and HRP goat anti-mouse IgG; Lane 2: total protein stain with colloidal gold. Lane 3: immunoblot stained with mouse antibodies specific for r-Sim v 1 (same Ab as in lane 4 in (A)); lane 4: non-related antibody (negative control). Molecular weights in kDa indicated on the left.

fusion proteins predicted from the nucleotide sequence (Table 1).

3.3. Reactivity of the mouse antibodies raised against the recombinant *S. vittatum* proteins

All mouse ascites reacted with the recombinant proteins against which they had been raised as expected. Additionally, the Abs produced against the r-antigen 5 protein (r-Sim v 1) and the α -amylase (r-Sim v 3) bound to native *S. vittatum* SG proteins (Fig. 2A). The r-Sim v 1 specific Abs bound to a band of about 32 kDa in the *S. vittatum* SGE. Notably, the antibodies produced against r-Sim v 1 also bound to a protein band from *C. nubeculosus*

SGE of a similar molecular weight (Fig. 2B) which was also able to bind serum IgE from some IBH-affected horses (Fig. 2B). The Abs specific for r-Sim v 3 recognized a band of about 65 kDa in the immunoblot of *S. vittatum* SGE but, unlike the r-Sim v 1 specific mouse Abs, did not bind to any protein band in immunoblots of *C. nubeculosus* SGE.

3.4. Inhibition immunoblot

The inhibition experiment showed that preincubation of a horse serum with r-Sim v 1 completely abrogated binding to the 32 kDa protein band in immunoblots of *C. nubeculosus* SGE (Fig. 3), suggesting that the antigen 5 like proteins from *S. vittatum* and *C. nubeculosus* are cross-reactive.

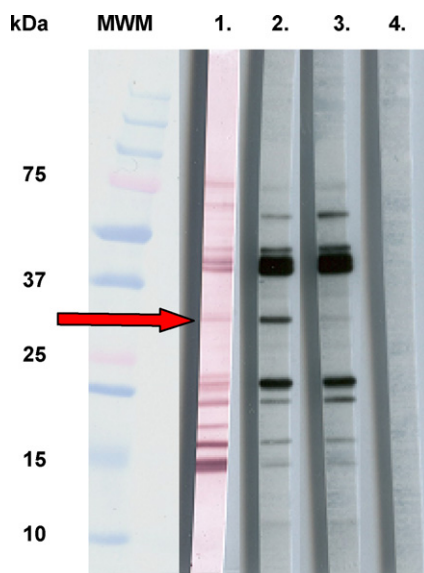


Fig. 3. Inhibition immunoblot: SDS-PAGE and Western blot of *Culicoides nubeculosus* SGE. Lane 1: total protein stain with colloidal gold; lane 2: immunoblot incubated with serum of an IBH-affected horse; IgE Abs detected with an anti-equine IgE mAb and goat anti-mouse IgG; lane 3: immunoblot with serum of an IBH-affected horses, after preincubation with r-Sim v 1 (10 μ g/ml); Lane 4: conjugate control. Molecular weights in kDa indicated on the left. The arrow points to the band to which IgE binding is inhibited after preincubation of the horse serum with r-Sim v 1.

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CS-Ag5B      MIRNLSVLFLFTICISFALSTDFCNRLCKRQTGPQTFTYMKHIGCRHNGRNANTCPRDAK
Sim v 1      -----DVTQDYCSEEWQCPEK-----RPHIACRNNNAFVSSCPDRDQ
              :: *:*.:: *: :.          **:*.*. .:****:

CS-Ag5B      ILPMSTKRKNLILKVHNRRLRNKVALGLLPGFPAARMPILRWDDLAYLAELNVKQCQMK
Sim v 1      LVPMTSTKRKNLLMLHNRMRNKVALGKLEGYQQAQKMPILQWDDLAYLAELNVKTCKFA
              ::*****:* :*:***** * *: :* *****:***** *::

CS-Ag5B      HDECRTDKFKYAGQNSAYTMGTQKN--SVRIMKLIRAWFSEHENATASFMDKYRDHPQ
Sim v 1      HDSCRNTKKYRTAGQNLAYGARS DKYKPLAQALKDMMRAFFNEYKDANMTYIKAYQNHDK
              **,***.::: **** * : : : : :.:**.*.:::*. :.:. *:.*:

CS-Ag5B      GRVIGHLLL-----
Sim v 1      GKQIGHFTQLVSDRTRNVGCAISRYTEWSPQYQMDMKTLLACDYSFTNIWKEPVYETGP
              *: ***:

CS-Ag5B      -----
Sim v 1      TAGSGCKAGRHNVYKGLCKASEDVPYQNFYTWLWPQNRG

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Fig. 4. Alignment of the deduced amino acid sequences of the antigen 5 like proteins of *C. sonorensis* (CS-Ag5B; accession no. AAU06471), and *S. vittatum* (Sim v 1). Identities are marked by stars (*) and conservative exchanges by columns (:). Gaps due to alignment are indicated by dotted lines (---).

Comparison of the deduced amino acid sequences of antigen 5 like protein from *S. vittatum* and *C. sonorensis* shows that they share only an overall sequence identity of 48% including different conserved stretches of 7–14 identical amino acids (Fig. 4).

3.5. IgE binding of horse sera to the recombinant *S. vittatum* proteins

Each of the five horse sera independently used for screenings of the *S. vittatum* phage library had detectable r-antigen-specific serum IgE for the proteins enriched using the respective horse serum.

Although the two horse sera that allowed enrichment of three SVEP r-proteins showed relatively high IgE binding to the r-proteins, determination of IgE levels in the sera of 24 other IBH-affected and 20 healthy control horses showed only marginal IgE binding without significant differences in median IgE levels of IBH-affected horses compared to the healthy control horse group. Therefore, these three r-SVEP proteins were not further investigated.

The median IgE levels against r-Sim v 1, 2, 3, and 4 in sera from 25 IBH-affected and 20 control horses are

shown in Table 2 and the distribution of the IgE levels against these r-Sim v proteins in Fig. 5. IBH-affected horses had significantly higher IgE levels than healthy control horses against r-Sim v 1 ($p < 0.001$), r-Sim v 2 ($p < 0.001$), r-Sim v 3 ($p < 0.01$) and r-Sim v 4 ($p = 0.05$). The healthy horses that had detectable IgE against r-Sim v 1, 3, or 4 only had low IgE levels (< 5 REU) against these r-Sim v proteins, while IgE levels against r-Sim v 2 were somewhat higher in the healthy horses (< 21 REU). Comparison of the test results as binary variables (positive/negative result) using an arbitrary cut-off, set at the mean value of the healthy horses + 3 standard deviations showed that the highest number of IBH-affected horses with a positive result was found for r-Sim v 1 (15 out of 25 IBH horses versus 1 out of 20 healthy horses, $p \leq 0.001$), followed by r-Sim v 3 (11/25 IBH and 1/20 healthy horses, $p \leq 0.01$), r-Sim v 2 (8/25 IBH and 0/20 healthy horses, $p \leq 0.01$) and r-Sim v 4 (5/25 IBH and 0/20 healthy horses, $p \leq 0.05$). Combination of the results obtained with the 4 r-Sim v proteins showed that 92% of the IBH-affected but only 15% of the healthy horses had IgE above the cut-off against one or more of the 4 r-Sim v proteins ($p \leq 0.000001$) (Table 2).

Table 2

IgE binding of sera of IBH-affected and healthy horses to recombinant *Simulium vittatum* (r-Sim v) proteins and crude *Simulium vittatum* extract as determined by ELISA. For the r-Sim v proteins, the mean value of the healthy control horses + 3 standard deviation was used as arbitrary cut-off to discriminate between a positive or negative test result.

Name:	r-Sim v protein with homology to	Median IgE levels (REU) (range)			No. of positive test results (N/total)		
		Healthy (N = 20)	IBH (N = 25)	P (U-Test)	Healthy	IBH	P (Fisher's)
r-Sim v 1	Antigen 5 like Protein	0 (0–1.7)	4 (0–227)	≤ 0.001	1/20	15/25	≤ 0.001
r-Sim v 2	Kunitz protease inhibitor	0 (0–18)	13 (0–75)	≤ 0.001	0/20	8/25	≤ 0.01
r-Sim v 3	α -Amylase	0 (0–3.3)	0 (0–172)	≤ 0.01	1/20	11/25	≤ 0.01
r-Sim v 4	α -Amylase	0 (0–4.2)	0 (0–75)	≤ 0.05	0/20	5/25	≤ 0.05
No of horses with detectable specific IgE							
<i>S. vittatum</i> whole body extract		3.4 (0–28)	36 (0–293)	≤ 0.0001	14/20	25/25	≤ 0.01
No of horses with a positive test result with one or more of the 4 r-Sim v proteins							
r-Sim v 1–4					3/20	23/25	≤ 0.000001

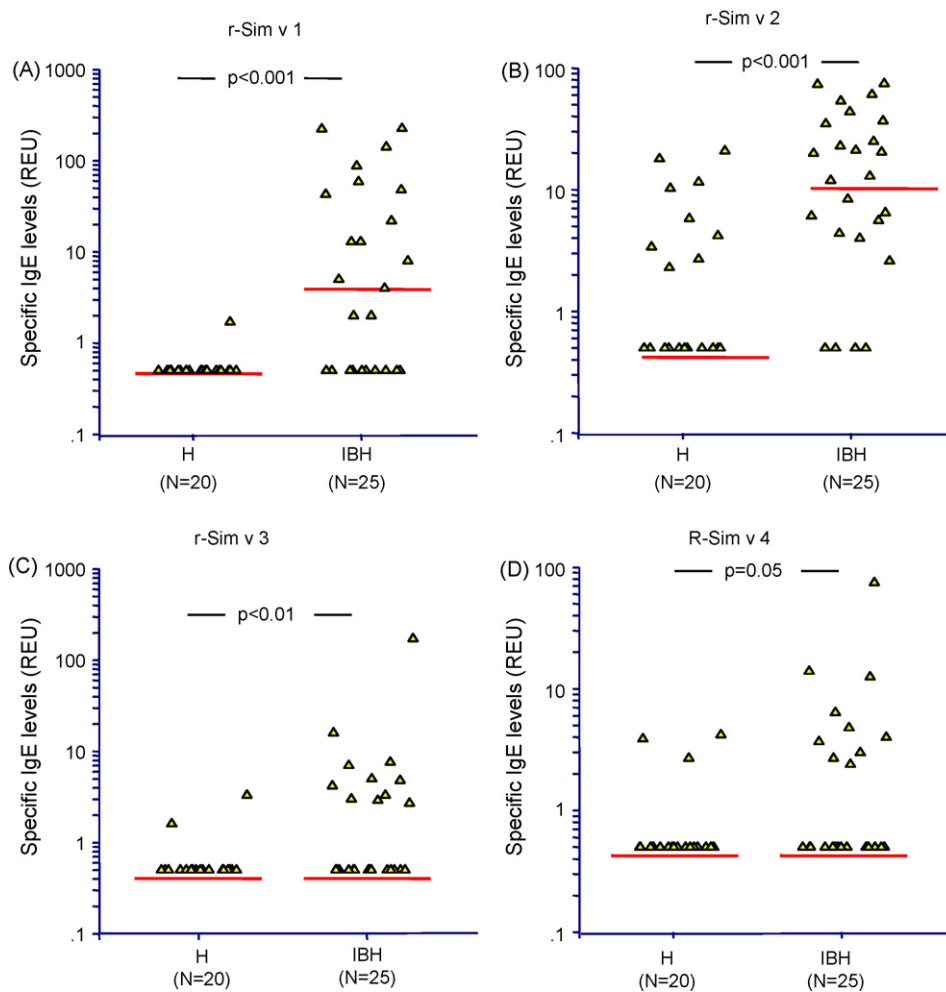


Fig. 5. (A–D) Comparison of IgE levels against recombinant *S. vittatum* proteins expressed in relative ELISA Units (REU) and shown on a log scale, in sera of IBH-affected and healthy control horses. Red lines indicate the medians. Statistical significance (Mann–Whitney *U*-Test) of the differences between both groups of horses is shown. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

IBH-affected horses also had significantly higher IgE levels against the crude *S. vittatum* extract than the controls (Table 2 and Fig. 6). However, the differences were less clear than with the r-Sim v proteins, as 100% of the IBH-affected but also 70% of the 20 healthy horses had detectable IgE against the extract (Table 2). These results indicate the presence of unspecific IgE-binding substances in the whole body extracts because the sera of healthy horses do not recognize IgE-binding proteins in Western blot analyses of SGE separated by SDS-PAGE (Hellberg et al., 2009). Therefore, IgE determinations based on whole body extracts might lead to false positive diagnoses, or by increasing the cut-off, to a low sensitivity of the ELISA assay.

3.6. Inhibition ELISA

Preincubation of horse sera showing IgE binding to a recombinant allergen with the corresponding r-protein resulted in a concentration dependent reduction of the IgE binding, while preincubation with an irrelevant r-protein

did not or only marginally ($\leq 10\%$) inhibited IgE binding of these sera to the coated r-protein, as exemplified for r-Sim v 3 in Fig. 7. Notably, binding of serum with IgE Abs to r-Sim v 4 was inhibited to 60% by preincubation with 10 $\mu\text{g}/\text{ml}$ r-Sim v 4 in the fluid phase but not by preincubation with r-Sim v 3, the other r- α -amylase protein used at the same concentration indicating that the α -amylases are not cross-reactive (data not shown).

4. Discussion

The aim of our study was to identify potential allergens from *S. vittatum* associated with IBH by phage surface display technology. The method is well-established for the isolation of allergens involved in human diseases (Rhyner et al., 2002, 2004), however some prerequisites for a successful screening such as high affinity mAbs specific for equine IgE were not available until recently (Wilson et al., 2006). Attempts with phage surface display technology using a chicken Ab raised against a recombinant fragment of the equine IgE CH3–4 specific for native equine IgE

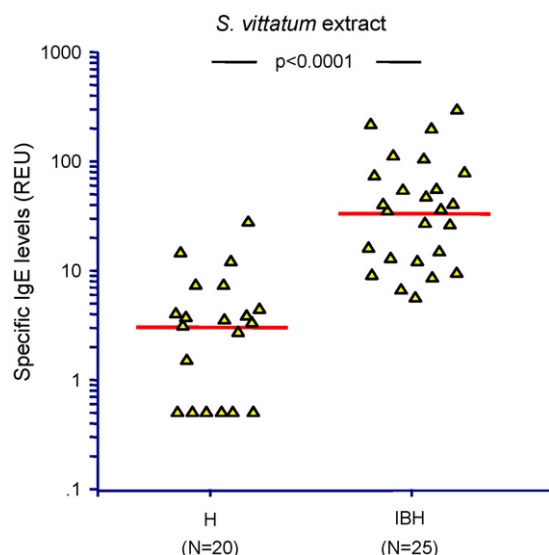


Fig. 6. Comparison of IgE levels against *S. vittatum* whole body extract, expressed in relative ELISA Units (REU) and shown on a log scale, in sera of IBH-affected and healthy control horses. Red lines indicate the medians. Statistical significance (Mann–Whitney *U*-Test) of the differences between groups of horses is shown. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

(Marti et al., 1997), but probably with a rather low affinity, failed. Previous studies (Althaus et al., 2004; Hellberg et al., 2006; Langner et al., 2008) have also demonstrated the need to use salivary gland libraries instead of libraries made from whole bodies. As serum IgE of IBH-affected horses recognize several protein bands in immunoblots with *S. vittatum* SGE (Hellberg et al., 2009) and a cDNA library from *S. vittatum* SG was already available, we decided to test the performance of phage surface display technology for the isolation of *S. vittatum* SG allergens.

Screening of this phage surface display library has resulted in the identification of one r-protein which could be a major allergen (r-Sim v 1) for IBH as 60% of the IBH-

affected animals showed IgE binding to this r-protein. Only one out of the 20 healthy horses showed low IgE binding to this r-Sim v 1 protein. Interestingly, r-Sim v 1 is homologous to antigen 5 like proteins from *Aedes*, *Culex* and *Phlebotomus* spp. and shares 48% identity to an antigen 5 like protein from *C. sonorensis*. Antigen 5 protein is one of the major venom allergens of vespids and fire ants (King and Spangfort, 2000; Suck et al., 2000) and the antigen 5 like protein from *C. sonorensis* was therefore considered also as a potential allergen by Campbell et al. (2005), who first identified EST sequences coding for antigen 5 like proteins from SGs of these insects.

Mice immunised with r-Sim v 1 produced Abs which reacted not only with the immunogen but also with a SGE protein from *S. vittatum*, recognized also by IgE from sera of IBH-affected horses, providing the first evidence of a possible involvement of r-Sim v 1 in IBH (Fig. 2). Immunoblots with r-Sim v 1 specific mouse Abs on both *S. vittatum* and *C. nubeculosus* SGEs showed the presence of a cross-reactive protein band of about 32 kDa in SGE extracts from both insect species (Fig. 2). This was confirmed by inhibition experiments: addition of r-Sim v 1 to serum of a horse showing IgE binding to the 32 kDa protein band present in *C. nubeculosus* SGE selectively abrogated IgE binding of the horse serum to this particular band (Fig. 3). This finding thus supports our previous hypothesis that *S. vittatum* and *C. nubeculosus* produce cross-reactive allergens. Cloning and expression of the antigen 5 like protein from *C. nubeculosus* is in progress and will, together with the available r-Sim v 1 protein, allow an experimental verification this hypothesis.

We also found significant differences between IBH-affected and healthy horses for three additional r-Sim v proteins. However, the proportion of IBH-affected horses showing strong IgE-responses to r-Sim v 3 and 4 was lower compared to r-Sim v 1. A possible explanation for the low IgE binding capacity of r-Sim v 3 and 4 could be related to an incomplete folding of the truncated sequences used for the production of the recombinant proteins, although minor allergens are often present in complex extracts. Immunoblot of the r-Sim v 3 specific mouse Abs on SGE indicate a molecular mass of about 65 kDa for the full length protein, while the molecular mass of r-Sim v 3 was only 28 kDa. However, the complete sequences of α -amylases from *S. vittatum* are still not completely elucidated (Table 1). A high percentage of the sera from IBH horses showed IgE binding to the truncated r-Sim v 2 (11 amino acids missing at the 5' end). Although the difference between IgE binding detected in IBH-affected horses and healthy controls was statistically significant, also about half of the healthy control horses tested showed detectable serum IgE levels against r-Sim v 2. At present it is not clear why the background of the IgE determinations against r-Sim v 2 in sera of healthy horses is much higher than those obtained using r-Sim v 1. One possible explanation could be that r-Sim v 2 is expressed at very low levels in *E. coli*. The low expression yield hampers the purification of the recombinant protein by affinity chromatography resulting in preparations containing relevant amounts of *E. coli* proteins (Fig. 1, lane 7). This could explain the rather high proportion of healthy horses

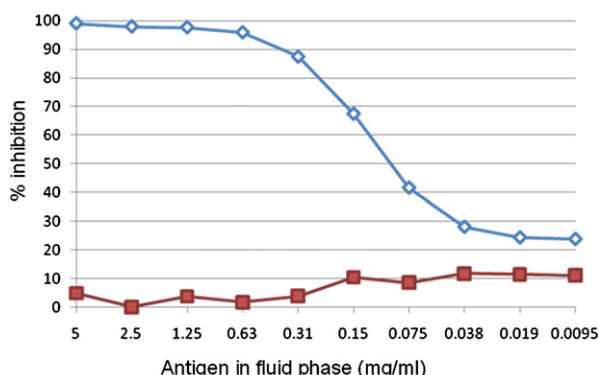


Fig. 7. Inhibition of IgE binding to solid phase-coated r-Sim v 3. Serum from a horse with IgE Abs against r-Sim v 3 was preincubated with decreasing amounts of r-Sim v 3 (◇) or r-SVEP (□) as negative control. Preincubated serum samples were transferred to wells coated with r-Sim v 3 and residual IgE binding measured by antigen-specific ELISA.

showing IgE binding to r-Sim v 2, which could be due to IgE raised against contaminant *E. coli* proteins. However, binding of serum IgE of an IBH-affected horse with high levels of r-Sim v 2-specific IgE could be inhibited to 80% by preincubation of the serum with r-Sim v 2 but not with an irrelevant r-protein, demonstrating the specificity of the reaction (data not shown).

Interestingly, the r-Sim v proteins for which we found significant differences in IgE binding between IBH-affected and healthy horses all belong to protein families known to induce allergy in humans. This is the case for the antigen 5 like protein (r-Sim v 1), for serine protease inhibitors (r-Sim v 2) and for α -amylases (r-Sim v 3 and 4). Interestingly α -amylase and serine protease inhibitors are known allergens in food-related asthma and have been recently isolated from wheat by phage surface display technology (Weichel et al., 2006).

Although the ELISA needs to be optimized and cut-off levels defined using a larger set of clinically well-defined IBH-affected and healthy horses, these results already show that the use of pure r-allergens instead of crude *S. vittatum* can improve the performance of a serological test for IBH. Our results obtained with the crude *S. vittatum* extract confirm the lack of specificity and sensitivity of IgE determination against crude allergen extract (Frey et al., 2008; Langner et al., 2008; Morgan et al., 2007). We expect that the use of r-*Culicoides* allergens will lead to a better serological test for IBH, as various studies suggest that the most important allergens for IBH are probably originated from *Culicoides* spp. (Baselgia et al., 2006; Marti et al., 2008).

In conclusion, this study has shown that phage surface display technology using SG libraries and high affinity mAbs for equine IgE is an efficient method to isolate allergens associated with IBH. One potential major *Simulium* and *Culicoides* allergen and three potential minor *Simulium* allergens have been identified in a short time demonstrating the power of phage surface display technology for the isolation of relevant pathogenesis-related allergens and will now be used to identify and produce *Culicoides* allergens. Further work will also be required to complete the truncated cDNA sequences obtained, to produce the full length allergens and to confirm their biological relevance in intradermal tests, *in vitro* histamine/sulfidoleukotriene release and lymphocyte proliferation assays.

Conflict of interest

None.

Acknowledgments

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