Insect bite hypersensitivity in the horse: Comparison of IgE-binding proteins in salivary gland extracts from *Simulium vittatum* and *Culicoides nubeculosus*

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1. Introduction

Insect bite hypersensitivity (IBH) is an IgE-mediated allergic dermatitis of horses caused by bites of insects such as *Culicoides* or *Simulium* spp. The aim of the present study was to compare the IgE-binding pattern of sera of IBH-affected horses to *Culicoides nubeculosus* and *Simulium vittatum* salivary gland extracts (SGE). Individual IgE responses to proteins of *S. vittatum* and *C. nubeculosus* SGEs were evaluated in 15 IBH-affected and three healthy horses on immunoblots. Fourteen out of the 15 IBH-affected but none of the healthy horses showed individual IgE binding patterns to seven and six main protein bands in *C. nubeculosus* and *S. vittatum* SGE, respectively. These 14 sera showed IgE-binding to proteins from SGE of both *C. nubeculosus* and *S. vittatum*, but they reacted with fewer protein bands derived from *S. vittatum* than from *C. nubeculosus* SGE. Sera showing IgE-binding to a 32 kDa band from *C. nubeculosus* always bound to a 32 kDa band from *S. vittatum*. Similarly, all sera binding to a 70 kDa band from *C. nubeculosus* reacted with a corresponding band in *S. vittatum* SGE. The 70 kDa bands from *S. vittatum* and *C. nubeculosus* were identified by mass spectrometry as heat shock protein-70-cognate-3.
intradermal tests with *Simulium* extracts (Fadok and Greiner, 1990). These data suggest that at least some IBH allergens are also present in black flies.

Consequently, the aim of the present study was to investigate by immunoblots, whether salivary gland extracts of *S. vittatum* contain IgE-binding proteins and to compare the number and approximate molecular weights of IgE-binding bands in salivary gland extracts of *S. vittatum* and *C. nubeculosus*.

### 2. Materials and methods

#### 2.1. Horses

Blood samples were taken from 15 IBH-affected and three healthy horses living in the same environment as the IBH-affected animals. 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 and followed by a period of remission in winter. All horses were treated for IBH, receiving a variety of treatments such as sweet itch blankets, and/or local application of various lotions. The horses selected had not received systemic corticosteroids. The healthy (H) control horses had no clinical signs of pruritus or cutaneous lesions. These horses had no previous history of skin problems. All IBH-affected horses but none of the controls had a positive *in vitro* sulfidoleukotriene release assay with extracts from *C. nubeculosus* and *S. vittatum* (Baselgia et al., 2006).

#### 2.2. Salivary glands

Salivary glands (SG) from *S. vittatum* and *C. nubeculosus* were obtained by dissection following the method of Watts (1981), as described in Hellberg et al. (2006). Only the females were taken, because the males do not feed on blood. The glands were stored in sample buffer (0.125 M Tris–HCl, 4% SDS; 20%, v/v, glycerol, 0.2 M DTT, 0.02% bromophenyl blue, pH 6.8) at −20 °C until used.

#### 2.3. Electrophoresis

SDS-PAGE electrophoresis was carried out according to Laemmli (1970) with 10–20% gradient Tris–HCl gels (Bio-Rad Laboratories; www.bio-rad.com), run under denaturing–reducing conditions. Before loading, the salivary gland extracts were sonicated and boiled for 3 min. The SGE from *C. nubeculosus* (equivalent of 10 SGs per lane) was loaded

![Image](https://example.com/image.png)
alternately with the SGE from *S. vittatum* (equivalent of 3 SGs per lane). A preliminary analysis with Coomassie blue staining of the gel had shown that with these SG numbers a staining of similar intensity would be obtained with both insect species (black flies have larger SGs than midges). Pre-stained protein standard (Bio-Rad Laboratories) was used to estimate the molecular mass of the different proteins.

For better separation of the 70 kDa bands excised from the SDS-PAGE gels for mass spectrometry, SGEs were also run on 7.5% SDS-PAGE and stained with Coomassie blue. The 70 kDa bands were then cut out of the gels with sterile scalpel blades and the N-terminal amino acid sequences were determined by mass spectrometry after “in gel” digestion, offered as a service by the Analytical Unit.

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Department of Biochemistry, University of Zürich (Prof. P. Hunziker). First, peptide mass fingerprint measurement by MALDI-TOF was carried out. However, no significant protein matches were found using the data obtained with MALDI. Next, liquid chromatography and tandem mass spectrometry (LC/MS/MS) measurements were carried out and peptides resulting from it were searched against the database using protein BLAST (NCBI; Altschul et al., 1997).

2.4. Western blot

The gel separated proteins were electro-transferred to polyvinylidene difluoride (PVDF) membranes using a semi-dry electrophoretic transfer cell (Bio-Rad Laboratories). After blotting, the membranes were cut into strips containing SGEs from both *C. nubeculosus* and *S. vittatum*.

2.5. Immunoblot

The strips were incubated overnight at 4 °C with horse sera diluted 1:6 in buffer containing 20 mM Tris, 0.25 M NaCl, 0.125% Tween-20. The bound IgE antibodies were detected with a monoclonal mouse anti-horse IgE (Wilson et al., 2006), followed by an alkaline phosphatase conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories Inc.; www.jacksonimmuno.com). Development of the strips was performed with NBT/BCIP solution (Roche Applied Science; https://www.roche-applied-science.com) according to the manufacturers’ instructions.

3. Results and discussion

All but one of the 15 IBH-affected horses showed IgE-binding to distinct protein bands of the *C. nubeculosus* and *S. vittatum* SGEs. No IgE-binding to the SGEs was observed with the sera of the three healthy horses. The sera bound to seven main protein bands in the *C. nubeculosus* SGE. These had MW between 13 and 70 kDa (Table 1), in agreement with a previous study (Hellberg et al., 2006). Between 20 and 80% of the sera of IBH-affected horses showed IgE-binding to the respective bands (Table 1). Six main protein
bands showing IgE-binding were found in the *S. vittatum* SGE. They had molecular weights ranging from 12 to 70 kDa. The horse sera showed IgE-binding to both SGs, but the IgE-binding pattern of the individual horses to the different bands usually differed between the *C. nubeculosus* and *S. vittatum* SGE (Table 1). Overall, horse sera reacted less frequently with protein bands derived from SGs of *S. vittatum* than *C. nubeculosus* (Table 1).

Interestingly, the eight sera reacting with the sharp, intensely stained 70 kDa band from *C. nubeculosus* also showed IgE-binding to the 70 kDa band from *S. vittatum* (Table 1 and illustrated in Fig. 1). Similarly, the six horse sera binding to the 32 kDa band from *C. nubeculosus* also showed IgE-binding to a band of similar molecular weight in *S. vittatum* SGE (Table 1 and Fig. 1), suggesting that there are probably some conserved or cross-reacting antigens in midges and black flies, as postulated previously by Baselgia et al. (2006). The presence of a 32 kDa cross-reactive antigen in *S. vittatum* and *C. nubeculosus* SGE is further supported by Schaffartzik et al. in this issue. The 70 kDa protein band from each insect species but not the 32 kDa or the other protein bands could be separated sufficiently well in 1D SDS-PAGE to be cut out of the gel with a high likelihood of isolating a single protein band. Mass spectrometry allowed the identification of these 70 kDa proteins from both *C. nubeculosus* and *S. vittatum* as heat shock 70 cognate proteins. With the 70 kDa band from *S. vittatum*, three motifs of 9, 13 and 22 amino acids, were identical to the heat shock protein 70 cognate (Hsc70) gene from the insects *Anopheles gambiae* and *Drosophila melanogaster* and nearly identical to the same gene from *Bombbyx mori* and *Spodoptera frugiperda* (Fig. 2). With the 70 kDa band from *C. nubeculosus*, three motifs of 9, 11 and 13 amino acids were also identical to the Hsc70 gene from the insect species mentioned above. Furthermore, one of these peptides was identical to two EST clones from *Culicoides sonorensis* (Fig. 2). These results confirm that there are some shared IgE-binding proteins in both *S. vittatum* and *C. nubeculosus*. This supports our assumption that reactivity to *Simulium* may be secondary to sensitisation to *Culicoides* SG antigens. Proteins with sequence similarities to heat shock protein (HSP) 70 from pollen, moulds and *Malassezia* have been reported as IgE-binding antigens in humans (Zhang et al., 1996; Shen et al., 1997; Gruehn et al., 2003; Andersson et al., 2004), suggesting that the identified Hsc70 may indeed be a candidate allergen for IBH. However, as these proteins show high degrees of similarity to HSP proteins from mammals, it is not known yet if they represent cross-reactive structures. The Hsc70 from the insects described above has 82% identity at the protein level to HSP 70 from the horse and other mammals. Expression of HSPs are upregulated in inflamed or damaged skin (Ghoreishi, 2000) and might thus result in IgE auto-antibodies against HSP, cross-reacting with HSP 70 from other sources. Further studies are necessary to investigate whether these antibodies could contribute to exacerbation of the skin inflammation in IBH-affected horses.

In conclusion, this study has shown that most IBH-affected but not healthy control horses show IgE-binding to salivary gland proteins from both *C. nubeculosus* and *S. vittatum*. Some but not all of the IgE-binding proteins from both insect SGs seem to share common epitopes as shown by comparison of the IgE-binding pattern of horse sera to the SGE of both insect extracts. This is confirmed for one protein, which was identified as a heat shock protein 70. Cloning and expression of this Hsc70 protein will allow testing to determine whether this protein is an important allergen for IBH. Further studies using 2D electrophoresis or molecular techniques are needed to identify the other allergens for IBH in saliva of midges and black flies.

Conflict of interest

None.

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