



Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm



Research paper

Selective cloning, characterization, and production of the *Culicoides nubeculosus* salivary gland allergen repertoire associated with equine insect bite hypersensitivity

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ARTICLE INFO

Article history:

Received 14 May 2010

Received in revised form 5 October 2010

Accepted 8 October 2010

Keywords:

Horse

IgE

Insect bite hypersensitivity

Culicoides nubeculosus

Phage surface display

Recombinant allergens

ABSTRACT

Salivary gland proteins of *Culicoides* spp. have been suggested to be among the main allergens inducing IgE-mediated insect bite hypersensitivity (IBH), an allergic dermatitis of the horse. The aim of our study was to identify, produce and characterize IgE-binding salivary gland proteins of *Culicoides nubeculosus* relevant for IBH by phage surface display technology. A cDNA library constructed with mRNA derived from *C. nubeculosus* salivary glands was displayed on the surface of filamentous phage M13 and enriched for clones binding serum IgE of IBH-affected horses. Ten cDNA inserts encoding putative salivary gland allergens were isolated and termed Cul n 2 to Cul n 11. However, nine cDNA sequences coded for truncated proteins as determined by database searches. The cDNA sequences were amplified by PCR, subcloned into high level expression vectors and expressed as hexahistidine-tagged fusion proteins in *Escherichia coli*. Preliminary ELISA results obtained with these fusions confirmed the specific binding to serum IgE of affected horses. Therefore, the putative complete open reading frames derived from BLAST analyses were isolated by RACE-PCR and subcloned into expression vectors. The full length proteins expressed in *Escherichia coli* showed molecular masses in the range of 15.5–68.7 kDa in SDS-PAGE in good agreement with the masses calculated from the predicted protein sequences. Western blot analyses of all recombinant allergens with a serum pool of IBH-affected horses showed their ability to specifically bind serum IgE of sensitized horses, and ELISA determinations yielded individual horse recognition patterns with a frequency of sensitization ranging from 13 to 57%, depending on the allergen tested. The *in vivo* relevance of eight of the recombinant allergens was demonstrated in intradermal skin testing. For the two characterized allergens Cul n 6 and Cul n 11, sensitized horses were not available for intradermal tests. Control horses without clinical signs of IBH did not develop any relevant immediate hypersensitivity reactions to the recombinant allergens. The major contribution of this study was to provide a repertoire of recombinant salivary gland allergens repertoire from *C. nubeculosus* potentially involved in the pathogenesis of

Abbreviations: aa, amino acids; cfu, colony forming units; *C. nubeculosus*, *Culicoides nubeculosus*; *C. sonorensis*, *Culicoides sonorensis*; *E. coli*, *Escherichia coli*; GSC, gene specific primer; IBH, insect bite hypersensitivity; mAb, monoclonal antibody; IDT, intradermal skin test; LPS, lipopolysaccharide; ORF, open reading frame; pfu, plaque forming units; SGE, salivary gland extract; *S. vittatum*, *Simulium vittatum*.

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IBH as a starting basis for the development of a component-resolved serologic diagnosis of IBH and, perhaps, for the development of single horse tailored specific immunotherapy depending on their component-resolved sensitization patterns.

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1. Introduction

Insect bite hypersensitivity (IBH), a chronic recurrent seasonal dermatitis of the horse, is strongly associated with hypersensitivity reactions against salivary gland allergens of biting insects (Hellberg et al., 2006, 2009; Langner et al., 2008). Although the disease, also known as summer eczema, is likely to result from a combination of inappropriate immune responses with contribution of both, genetic and environmental factors (Schurink et al., 2009; van Grevenhof et al., 2007), there is strong evidence for the involvement of IgE-mediated reactions in the pathogenesis of IBH (Hellberg et al., 2006; Langner et al., 2008). Mainly bites of insects of the genus *Culicoides* spp. (midge) and occasionally *Simulium* spp. (black fly) are considered as the offending agents (Marti et al., 2003; Schaffartzik et al., 2009), because avoidance of allergen exposure is an effective treatment strategy for IBH (Cunningham and Dunkel, 2008).

The worldwide prevalence of IBH in horses is estimated to be in the range of 5–10% (Brostrom et al., 1987; Halldorsdottir and Larsen, 1991; Bjornsdottir et al., 2006). However, in certain breeds and especially in horses of the Icelandic breed imported from Iceland to Europe the prevalence is significantly increased. Up to 50% of the Icelandic horses exported to the European continent develop IBH within two years as a consequence of exposure to *Culicoides* spp. which are not present in Iceland (Marti et al., 2003). In contrast only 5–10% of the horses born in Europe and breed from imported Icelandic horses suffer from IBH (Halldorsdottir et al., 1989; Halldorsdottir and Larsen, 1991) indicating an influence of genetic background, environmental exposure, and adaptive immune responses.

The clinical signs of IBH are severe itching affecting the base of the mane and tail and the withers (the feeding sites of *Culicoides* spp.), papules, broken hair, lichenification of the skin, and crust formation following intense scratching. This clinical picture is assumed to result from hypersensitivity reactions to allergens largely found in the saliva of biting midges (Hellberg et al., 2006; Wilson et al., 2001). The pivotal role of allergen-specific IgE in the pathogenesis of IBH has been demonstrated by a modified Prausnitz–Küstner experiment showing that the allergic reaction can be transferred to healthy animals using IgE purified from serum of IBH-affected horses (Wagner et al., 2006). As in other mammals, also in sensitized horses allergen re-exposure leads to cross-linking of high-affinity FcεRI bound IgE on effector cells and initiates a cascade of signalling events ending in effector cell degranulation and immediate release of potent mediators such as histamine and leukotrienes responsible for Type I hypersensitivity responses (Turner and Kinet, 1999).

Chronic atopic eczema in humans, a disease resembling IBH, results from a complex interaction between environmental factors, pharmacological abnormalities, skin barrier

dysfunction, susceptibility genes, and immunologic phenomena (Leung and Bieber, 2003), including a switch from a Th1 to a Th2 cytokine profile in lesional skin (Breuer et al., 2006). Perhaps the most efficient triggers for atopic eczema are allergens produced by skin colonizing yeasts of the genus *Malassezia* (Lindborg et al., 1999; Schmid-Grendelmeier et al., 2006). Similarly, chronic skin lesions of IBH-affected horses are mainly characterized by hyperkeratosis, infiltration with eosinophils, increased numbers of IgE-bearing effector cells, and increased expression of Th2 cytokines (Marti et al., 2008), and the most common triggers for IBH are assumed to be IgE-binding proteins present in salivary glands of *Culicoides* species. Western blot analyses of *Culicoides nubeculosus* salivary gland extracts (SGE) revealed at least ten IgE-binding protein bands potentially associated with IBH (Hellberg et al., 2006). Depending on the serum used variable, horse specific IgE-binding patterns with molecular masses ranging from 12 to 75 kDa were detected. Only one of these IgE-binding bands coding for an antigen 5-like allergen named Clu n 1, has been cloned, sequenced and expressed as recombinant protein (Schaffartzik et al., 2010) together with a homologous antigen 5-like allergen from *Simulium vittatum* (Sim v 1) (Schaffartzik et al., 2009).

In addition maltase of *Culicoides sonorensis* has been reported as an important allergen associated with IBH (Langner et al., 2009). To date they represent the only three allergens associated with IBH characterized at molecular level. However, the salivary gland proteome of *C. nubeculosus* is much larger as demonstrated by a mass spectrometry analysis of abundant salivary gland proteins which revealed at least 54 novel protein sequences including an unknown number of allergens (Russell et al., 2009). One of the major aims of this study was to clone, express and characterize at molecular level the repertoire of salivary gland allergens in a fast and efficient way. Therefore we used phage surface display technology which allows selective enrichment of cDNA expression products of interest (Crameri et al., 1994; Rhyner et al., 2004) previously used to identify whole allergen repertoires from various sources including *Aspergillus fumigatus*, *Malassezia furfur*, and peanut (for a review see Crameri and Kodzius, 2002). The major advantage of phage display over classical cloning systems results from the linkage between the phenotype, displayed as fusion to a phage coat protein, and the genotype, integrated as recombinant cDNA into the phage genome. This allows the survey of large libraries for the presence of specific clones using the discriminative power of affinity selection. Affinity enrichment is based on binding of phage to immobilized target molecules, in our case horse IgE, during consecutive rounds of selection. Thus each selected phage displaying a protein able to interact with serum IgE also contains the genetic information inserted as cDNA into the phage genome. Sequencing of the cDNAs elucidates the amino acid (aa) sequence of the displayed gene

product. The fast procedure allows, in a few days, screening of as much as 10^{11} single clones on a single well of a microtiter plate coated with horse IgE. Here we show that phage surface technology is a suitable method for the fast characterization of salivary gland allergen repertoires.

2. Materials and methods

2.1. Horses

Forty-five Icelandic horses and one Swiss warmblood horse with clinical signs of IBH and 29 clinically healthy horses without signs of IBH or other sensitizations (25 Icelandic, 3 Swiss warmbloods and 1 Shetland pony) all living in Switzerland were included in the study. The affected horses were chosen based on clinical signs and history (recurrent seasonal pruritus and skin lesions along the mane, tail and withers, with remission during the winter season). The healthy control horses did not show any clinical signs of IBH and were living in the same environment as the IBH-affected horses. The study was approved by ethical committee of the canton of Berne for animal experiments. Sera taken from all horses were kept at -80°C until use.

2.2. Construction of a *C. nubeculosus* cDNA library displayed on phage surface

Using a commercial kit, total mRNA was isolated from 1400 dissected salivary glands from laboratory bred female *C. nubeculosus* and used to synthesise cDNAs according to the standard protocol for full-length enriched cDNA synthesis starting from purified poly(A)⁺ RNA (Stratagene, La Jolla, USA). The cDNAs were cloned into *EcoRI/XhoI* restriction sites of the λ -ZAP II vector (Stratagene) after ligation with adaptors and the ligation mixture packaged *in vitro* to generate a primary library resulting in a complexity of 3×10^6 plaque-forming units (pfu). This library was amplified and used to construct a pJufO phage surface displayed library on the M13 phage surface. cDNAs from 3×10^9 cfu of the salivary gland *Culicoides* λ -Zap II library were excised *in vivo* by superinfection with ExAssist helper phage (Stratagene) to generate pBluescript[®]SK(–) phagemids. pBluescript[®]SK(–) phagemids were digested by *EcoRI/XhoI*, restriction digests separated on 1.5% agarose gels, inserts with sizes ranging from 500 to 2000 base pairs were purified, ligated into *EcoRI/XhoI* restricted, dephosphorylated pJufO vector and electroporated into *E. coli* XL-1 Blue (Stratagene). VCSM13 helper phage was added to generate a phage surface displayed cDNA library as described (Crameri et al., 2002). The primary size of the phagemid library titrated as ampicillin resistant colonies was 6.6×10^8 cfu.

2.3. Enrichment for IgE-binding phagemids by biopanning

500 ng/well mouse anti-horse IgE 3H10 or 1C12 mAbs (Wilson et al., 2006) were applied in 100 μl PBS to single wells of an ELISA plate overnight at 4°C to generate a specific IgE-ligand surface. Free sites were blocked with 1% milk powder, 0.05% Tween in PBS for 1 h. IgE from a pool

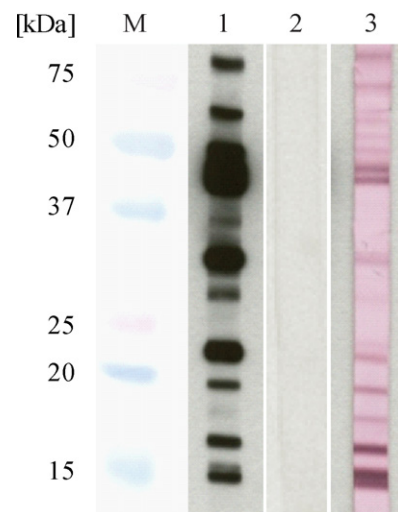


Fig. 1. Analysis of *C. nubeculosus* salivary gland extract (SGE) proteins after separation by SDS-PAGE, detection with mouse anti-horse IgE mAb 3H10, and visualization with HRP-labelled sheep anti-mouse-IgG. Lane 1: Separated SGE proteins developed with the serum IgE pool of IBH-affected horses used for screening of the phage surface displayed cDNA library. Lane 2: Separated SGE proteins developed for IgE with a serum pool of control horses not affected by IBH. Lane 3: Separated SGE proteins stained with colloidal gold. Molecular mass markers (M) are indicated on the left site.

of sera from IBH-affected horses showing strong IgE binding to multiple bands in immunoblots of *C. nubeculosus* SGE (Fig. 1) was used for enrichment of phagemids displaying IgE-binding cDNA products. The sera were diluted 1:3 with blocking buffer, added to each well and incubated for 2 h at 37°C . After 3 washing steps with TBST (0.02 M Tris pH 7.5, 0.15 M NaCl, 0.01% Tween-20), 50 μl of the phagemid library diluted in 50 μl blocking buffer (6.6×10^{10} cfu) were applied to single wells and incubated for 2 h at 37°C . Each well was washed after the first panning round two times with TBST, one time with TBS and one time with ddH₂O. Binding phage were eluted with 100 μl 0.1 M glycine pH 2.2, removed and neutralized with 10 μl 1 M Tris pH 7.4. In the next step, the eluted phagemids were used to infect 5 ml exponentially growing *E. coli* XL1-Blue cells and superinfected with VCSM13 interference resistant helper phage (Stratagene) at a multiplicity of infection of 10 to generate phagemids for further rounds of panning. Starting from the second round of affinity selection the stringency of the washing steps was increased to five times with TBST, two times with TBS and one time with ddH₂O during the second round and ten times with TBST, three times with TBS and one time with ddH₂O for the third panning round.

2.4. DNA sequencing

Sequencing of cDNA inserts of the selected phagemids was carried out with the pJufO derived primer 5'-AACCGACCAGCTGGAAGACG, by an automated sequencing service provided by Microsynth GmbH (Balgach, Switzerland). Homology searches were performed with

Table 1

PCR primer used for cloning of the truncated recombinant *C. nubeculosus* allergens.

r-Protein	Primer
Cul n 2	5'-Primer 5'-atgggatcccatcaaggaaattatcacagca-3' 3'-Primer 3'-tcaaagctttatgacaaattggggttaagaattt-5'
Cul n 3	5'-Primer 5'-aggggatccctcatcatctaaaggttgac-3' 3'-Primer 3'-gcgctcgagctacgcatatgtgtcaagtagg-5'
Cul n 4	5'-Primer 5'-cttggatccaagcatttcagacatttaagcg-3' 3'-Primer 3'-taactcgagttataactttcatttaaacactcag-5'
Cul n 5	5'-Primer 5'-tgggatcccaaatggaatgaagataaaatt-3' 3'-Primer 3'-taaaagctttattttcttgacattttgtctc-5'
Cul n 6	5'-Primer 5'-aagggatcccttttacttcgctcaacattt-3' 3'-Primer 3'-taagaattcttaacggcaatgcaatgaatg-5'
Cul n 7	5'-Primer 5'-taaggatccttaccaggacacgtcacc-3' 3'-Primer 3'-tagctcgagctattttgcttgagctggaat-5'
Cul n 8	5'-Primer 5'-tgggatcccgacgaacaccaaagattt-3' 3'-Primer 3'-gaactcgagttattcatatagctcggatcac-5'
Cul n 9	5'-Primer 5'-ttggatccaaagatttacgtcagctgaaa-3' 3'-Primer 3'-taactcgagttattggcatcacgtttccat-5'
Cul n 10	5'-Primer 5'-aaaggatcccttagacaaagtatatgaaggat-3' 3'-Primer 3'-taaaagctttatttccctgtatatgttggg-5'
Cul n 11	5'-Primer 5'-tgggatcccgacttaccagtataaaattgtt-3' 3'-Primer 3'-taactcgagtttaattcaatctgtattgttc-5'

The recognition sites of *Bam*HI (ggatcc)/*Eco*RI (gaattc), *Hind*III (aagctt) and *Xho*I (ctcgag) restriction enzymes are underlined.

the sequence similarity search tool BLAST 2.18⁺ from the National Centre for Biotechnology Information, National Library of Medicine, National Institute of Health, Bethesda, MD, USA (Altschul et al., 1997). Putative signal peptide analyses were run by submitting the sequences to the SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>).

2.5. Cloning and production of recombinant proteins

The isolated cDNAs encoding (mostly truncated) putative salivary gland allergens were amplified by PCR using the primers listed in Table 1, subcloned as N-terminal fusion to a hexa-histidine tag into the high expression vector pET-17b (Novagen, San Diego, USA) restricted by *Bam*HI and *Eco*RI, *Xho*I or *Hind*III depending on the insert (Table 1) and transformed into *E. coli* BL21 DE 3 (Novagen, San Diego, USA). To produce the recombinant proteins, *E. coli* transformants carrying correct inserts were grown either at 32 °C or at 37 °C in 2xYT medium. When the cell concentration reached an OD₅₈₀ of 0.6, protein production was induced with 1 mM IPTG. After incubation for 4 or 6 h at 32 °C or 37 °C, 250 rpm, in 21 Erlenmeyer flasks, the *E. coli* cells were harvested by centrifugation at 10,000 × g for 30 min at 4 °C and stored at −20 °C. For protein purification cell pellets were resuspended in denaturing lysis buffer (6 M guanidine hydrochloride, 0.5 M NaCl, 20 mM Tris/HCl, 5 mM imidazol) and lysed under ultrasonic pulses. To remove insoluble material, lysates were centrifuged at 20,000 × g for 20 min at 4 °C and supernatants purified over Sephadex G-25 (GE Healthcare, Chalfont St Giles, UK)

to remove small molecular components interacting with further purification steps. Thereafter, the recombinant N-terminal (His)₆-fusions were purified under denaturing conditions by Ni-NTA (Ni²⁺-nitrilotriacetate)-affinity chromatography using 5 ml HisTrap FF columns (GE Healthcare) on an ÄktaPurifier (GE Healthcare). Proteins were eluted with 500 mM imidazol, dialysed against ultrapure water or PBS to obtain soluble protein preparations, and tested for their ability to bind serum IgE of IBH-affected horses (see below). Molecular size and purity were assessed in a SDS-PAGE on 12% gels (NuPage; 12% Bis-Tris; Invitrogen, Groningen, The Netherlands) and Coomassie blue staining. Salivary gland proteins separated by SDS-PAGE were stained with colloidal gold (Sasse and Gallagher, 2008) which is more sensitive than Coomassie blue staining.

2.6. Production of antibodies against the recombinant proteins

Polyclonal antibodies specific for the *C. nubeculosus* recombinant proteins were produced at The Experimental Institute, University of Iceland, Keldur in mice according to the method of Overkamp et al. (1988). 20 µg of each purified recombinant protein dissolved in 250 µl complete Freund's adjuvant was injected intraperitoneally (i.p.). The injection was repeated on days 14 and 21 with incomplete Freund's adjuvant. On day 14 the mice were injected additionally with Pristane (Sigma, USA) i.p. The mice were kept under the protocol for maximally four weeks, thereafter euthanized, the ascetic fluid collected and tested for specificity with the corresponding recombinant protein and with *C. nubeculosus* SGE. Ascites fluids were produced at The Experimental Institute University of Iceland, Keldur and the experiments were approved by the national animal research committee of Iceland.

2.7. 5' rapid amplification of cDNAs (RACE)-PCR

Because most of the isolated cDNAs encoded truncated proteins, rapid amplification of cDNA ends was performed on total RNA from *C. nubeculosus*, using a 5'RACE-PCR system kit Version 2.0 (Invitrogen, Carlsbad, CA). All steps were conducted according to the manufacturer's protocol. In a first step, the first strand cDNAs were synthesized from total RNA by reverse transcription with gene-specific primers (GSP1) listed in Table 2, using SuperScript™ II reverse transcriptase (Invitrogen) as recommended. After removal of RNA by RNase (Invitrogen), cDNAs were purified from unincorporated dNTPs, gene-specific primers and proteins using S.N.A.P.™ columns (Invitrogen). A homopolymeric tail was then added to the 3'-end of the cDNAs to facilitate PCR amplification using *Taq* DNA polymerase, a nested, gene-specific primer (GSP2, Table 2) and a deoxynosine-containing anchor primer provided with the 5'RACE kit (Invitrogen). The PCR products were analyzed by gel electrophoresis, isolated from the gel and directly subcloned into the pET-17 b vector (Invitrogen) for sequence analysis and protein production.

BLAST analyses of the truncated sequences of Cul n 2, Cul n 8 and Cul n 10 revealed that the complete ORFs encoded

Table 2

Primers used for 5' RACE-PCR to obtain complete reading frames.

r-Protein	Primer ^c	
Cul n 2 ^a	5'-Primer	5'-catggatccatgtggtgaacgtgtaaatg-3'
Cul n 3	GSP1	5'-ctggacaatctgaac-3'
	GSP2	5'-cgtgtgaccacgactttatcttccctgtatc-3'
Cul n 4	GSP1	5'-atatcaaatgtaatttg-3'
	GSP2	5'-tcagggtacctgaacaggcgtaaaacacga-3'
Cul n 5	GSP1	5'-cttggacacctttgtc-3'
	GSP2	5'-tgggggtaccacactaaatattggtgtgtaatta-3'
Cul n 6 ^b	5'-primer	5'-catggatccatgaagctcttataatactttta-3'
Cul n 7	GSP1	5'-gggtcttaaggaaagt-3'
	GSP2	5'-accgggtaccggtgtgatcccgatgaggat-3'
Cul n 8 ^a	5'-primer	5'-catggatccatgataacatttaaaaaactaacac-3'
Cul n 9	GSP1	5'-acgttttccatcaag-3'
	GSP2	5'-ttcgtgtaccgaacttttgaccgtcagcattc-3'
Cul n 10 ^a	5'-primer	5'-catggatccatgcatcaaaaataaggactctg-3'
Cul n 11	GSP1	5'-caatttaaggtagct-3'
	GSP2	5'-ttgggtaccacacttttcaggattatattgtg-3'

^a The primers for the amplification of Cul n 2, Cul n 8 and Cul n 10 were designed according to the sequences with accession numbers ACM40915.1, ACM40914.1 and ACM40903.1, respectively, accessible from GenBank.

^b The primer for amplification of Cul n 6 was designed from the complete open reading frame obtained during this work (accession number HM145954).

^c The recognition sites of *KpnI* (ggatc)/*BamHI* (ggatcc) are underlined.

ing the proteins, although not yet described as allergens, were already deposited into GenBank under the accession numbers ACM40915.1, ACM40914.1 and ACM40903.1, respectively. According to these sequences specific primers (Table 2) were designed to isolate and subclone the complete ORFs of Cul n 2, Cul n 8 and Cul n 10.

2.8. Enzyme-linked immunosorbent assay (ELISA)

Recombinant proteins, 1 µg/ml diluted in 0.2 M Sodium carbonate-bicarbonate buffer pH 9.4 (Pierce) were coated for 2 h at 37 °C to polystyrene microtiter plates (MaxisorbTM; Nunc, Roskilde, Denmark). After washing with wash buffer PBS pH 7.4, 0.05% Tween 20, non-specific sites were blocked 1 h at 37 °C with 5% dry milk powder, 5% Tween 20 in PBS pH 7.4. Sera of IBH-affected horses and from control horses not affected by IBH were diluted 1:5 in blocking buffer, serially diluted 2-fold in duplicate through seven wells, and incubated overnight at 4 °C. After washing, secondary mouse anti-horse IgE 3H10 mAb (Wilson et al., 2006) at a concentration of 0.5 µg/ml was added to the wells followed by incubation for 2 h at 37 °C at 20 rpm on a shaker (model Polymax 1040, Heidolph GmbH & Co. KG, Schwabach, Germany). After extensive washing, alkaline-phosphatase-conjugated goat-anti mouse IgG (Jackson ImmunoResearch Laboratories Inc.; www.jacksonimmuno.com) diluted 1:1500 in blocking buffer was added for 1.5 h at 37 °C for detection of the secondary antibody. Finally ELISA plates were developed with 1.5 mg/ml disodium 4-nitrophenyl phosphate (Fluka, Buchs, Switzerland) in 1 M diethanolamine buffer at pH 9.8 for 1 h and absorbance measured at 405 nm and considered positive when the measured A_{405} value was more 3-fold the standard deviation + mean of the A_{405} value obtained for the healthy control horses.

2.9. Western blot analyses

2.9.1. Western blot analysis with recombinant proteins

0.5–1 µg of each recombinant protein were separated by SDS-PAGE on 12% Bis–Tris polyacrylamide gels (Invitrogen) under denaturing reducing conditions and transferred to Hybond-P polyvinylidene difluoride membranes (GE Healthcare). After blocking (5% skimmed milk in PBS/0.5% Tween 20) a serum pool of IBH-affected horses was diluted 1:4 in blocking buffer and incubated with the membrane overnight at 4 °C. The following day, the membrane was rinsed in washing buffer (20 mM Tris, 0.25 M NaCl, 0.125% Tween-20, pH7.5) 6 times for 5 min and incubated with mouse anti-horse IgE mAb 3H10 (0.5 µg/ml) for 3 h at room temperature. After an additional washing step, the membrane was incubated for 2 h with horseradish peroxidase (HRP), conjugated sheep anti-mouse-IgG antibody (GE Healthcare) diluted 1:10,000 in blocking buffer. Upon final washing, the membrane was developed with ECL Western blotting detection reagents (GE Healthcare) according to the manufacturer's protocol.

2.9.2. Western blot analysis with salivary gland extract

Salivary gland extract from laboratory bred *C. nubeculosus* midges, prepared as described (Hellberg et al., 2006), was separated by SDS-PAGE on 12% Bis–Tris polyacrylamide gels (Invitrogen), blotted onto Hybond-P polyvinylidene difluoride membranes as described above, and cut into strips. Protein bands of SGE were visualized with gold staining by immersion in the protogold stain kit (BB International, Cardiff, UK; www.bbighold.com). In a first experiment, two strips were incubated either with a serum pool of IBH-affected horses or with a serum pool of healthy control horses. After incubation overnight at 4 °C the strips were washed and incubated with mouse anti-horse IgE mAb 3H10 (0.5 mg/ml). In a second experiment, additional strips were incubated with mouse ascites fluid diluted 1:20 to 1:4000, depending on the ascites, to detect the corresponding protein. After a further washing step, alkaline phosphatase conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories Inc.; www.jacksonimmuno.com) diluted 1:1500 was added to the strips and subsequently developed with Nitro-Blue Tetrazolium Chloride/5-Bromo-4-Chloro-3'-Indolylphosphate p-Toluidine solution (NBT/BCIP, Roche Applied Science; <http://www.rocheapplied-science.com>) according to the manufacturer's instructions.

2.10. Intradermal skin test (IDT)

Seven control horses not affected by IBH and nine IBH-affected horses were chosen for intradermal skin tests performed during November 2009. Hair was clipped on the flat surface of the shoulder and the skin disinfected with 70% ethanol. Each injection area was labelled with a permanent marker. Two injections of 100 µl of every recombinant protein diluted to a concentration of 10 µg/ml and 1 µg/ml in ultrapure water, saline or PBS, depending on the solubility of the single allergens, was injected intradermally. Histamine (100 µl, 0.2 mg/ml) was used as positive, 100 µl sodium chloride or ultrapure water as neg-

ative control. Injection sites were evaluated by measuring the maximal longitudinal and transversal diameters of the wheal reaction with an electronic calliper 30 min and 4 h after injection and considered positive if the size of the wheal was at least half the size of the skin reaction induced by the histamine control (Wagner et al., 2006).

2.11. Statistical analyses

Statistical analyses were carried out with GraphPad Prism 5 (GraphPad Software; Inc.; La Jolla, CA). To compare horses' characteristics the unpaired *t* test was used. The level of significance was set at $p \leq 0.05$.

3. Results

3.1. Identification of salivary gland allergens of *C. nubeculosus*

Separation of SGE from *C. nubeculosus* midges on SDS-PAGE showed up to 12 protein bands after colloidal gold staining. Sera from IBH-affected horses react at least with ten different protein bands, whereas sera from control horses not affected by IBH do not show detectable IgE against the SGE proteins (Fig. 1).

Screening of a *C. nubeculosus* phage surface display library with serum IgE from IBH-affected horses yielded a large population of phage putatively expressing IgE-binding proteins. The phage population enriched by ligand/product interaction is likely to contain phage encoding different allergenic proteins. To test this assumption, DNA from randomly picked phage was extracted and the size of the inserts was determined by restriction enzyme analysis and agarose gel electrophoresis. Ten inserts of enriched phagemids carrying cDNAs of different lengths were sequenced to identify the encoded gene products. Except one clone containing a full length cDNA (Cul n 6), the sequences coded for truncated ORFs as shown by multiple BLAST analyses which identified the gene products as truncated forms of already known protein families sharing high sequence identity to *C. nubeculosus* and *C. sonorensis* salivary gland proteins, but not so far described as allergens (Table 3).

3.2. Expression and purification of the recombinant proteins

Cul n 6 without the signal peptide and the nine ORFs coding for truncated, IgE-selected *C. nubeculosus* salivary gland proteins were PCR amplified using gene-specific primers (Table 1) and subcloned into the high level expression vector pET-17b to generate N-terminally [His]₆-tagged fusions. The corresponding recombinant proteins were produced in *E. coli* BL-21 DE 3 cells and purified under denaturing conditions by gel-filtration chromatography and by Ni²⁺-chelate affinity chromatography. The size and the purity of the recombinant proteins were analyzed in SDS-PAGE. Because a *C. nubeculosus* named Cul n 1 has already been described (Schaffartzik et al., 2010), the putative allergens were formally termed Cul n 2 to Cul n 11 according to the recommendations of the international allergen nomenclature subcommittee for the classification of human allergens (www.allergen.org). To confirm the ability to bind specifically serum IgE, the recombinant proteins were tested with sera of IBH-affected and healthy control horses in ELISA experiments and all showed IgE-binding above background. Therefore, the missing N-terminal ends of the nine truncated ORFs were isolated by 5'RACE-PCR using gene-specific primers (Table 2) except for the truncated ORFs encoding Cul n 2, 8 and 10 which were completed by PCR using salivary gland mRNA and primers designed from the known sequence deposited into GenBank (Table 2). As reported in detail in Table 3, the proteins showed between 54 and 100% sequence identity to already described protein families. Because most of the full length proteins were expressed at low level in *E. coli* (data not shown), all cDNAs were PCR amplified using the gene specific primers reported in Table 2 which lack putative signal peptide sequences at the N-terminus of the proteins, subcloned in vector pET-17b, produced and purified as described above. The proteins were purified to apparent homogeneity as shown by SDS-PAGE and Coomassie blue staining (Fig. 2A). The estimated molecular masses (~15.5 kDa to ~68.7 kDa) for the recombinant fusion proteins were in good agreement with those calculated from the aa sequences except for Cul n 9 which showed a slightly higher apparent molecular weight in SDS-PAGE than those predicted from the aa sequence.

Table 3

Characteristics of the cloned *C. nubeculosus* allergens.

r-Protein	GenBank accession no. ^a	AA	MW (kDa)	Homology to (NCBI accession number)	% AA identity	% positive sera
Cul n 2	HM145950	403	46.7	Hyaluronidase (ACM40915.1) ^b	98%	56.5%
Cul n 3	HM145951	391	44.6	Putative cysteine endopeptidase (ACM40897.1) ^b	82%	47.8%
Cul n 4	HM145952	153	17.5	Secreted salivary protein (ACM40874.1) ^b	99%	45.7%
Cul n 5	HM145953	406	45.7	Secreted salivary protein (ACM40903.1) ^b	54%	43.5%
Cul n 6	HM145954	147	16.9	Secreted salivary protein (ACM40905.1) ^b	99%	34.8%
Cul n 7	HM145955	186	20.9	Unknown salivary protein (AUU06522.1) ^c	77%	30.4%
Cul n 8	HM145956	603	68.7	Maltase (ACM40914.1) ^b	97%	21.7%
Cul n 9	HM145957	142	15.5	D7-related salivary protein (ACM40878.1) ^b	100%	26.1%
Cul n 10	HM145958	420	47.8	Secreted salivary protein (ACM40903.1) ^b	95%	15.2%
Cul n 11	HM145959	275	30.1	Trypsin (ACM40875.1) ^b	68%	13.0%

^a Generated during this work.

^b Homology to reported proteins sequences from *Culicoides nubeculosus*.

^c Homology to a *Culicoides sonorensis* salivary gland protein without known function.

Please cite this article in press as: Schaffartzik, A., et al., Selective cloning, characterization, and production of the *Culicoides nubeculosus* salivary gland allergen repertoire associated with equine insect bite hypersensitivity. Vet. Immunol. Immunopathol. (2010), doi:10.1016/j.vetimm.2010.10.015

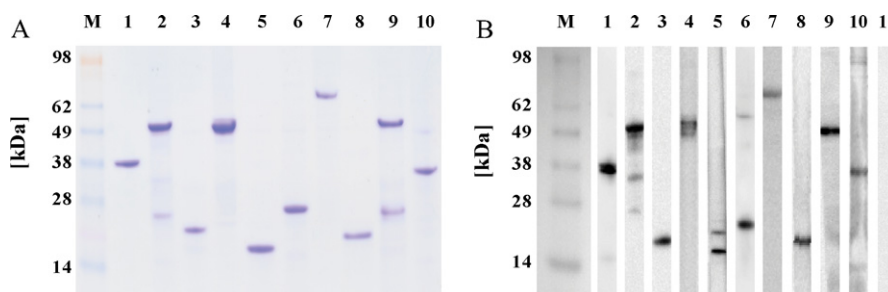


Fig. 2. (A) SDS-PAGE and Coomassie blue stain of *E. coli*-expressed Cul n 2 to Cul n 11 (lanes 1–10). (B) Western blot analysis of Cul n 2 to Cul n 11 (lanes 2–10) with a serum pool of IBH-affected horses. Lane 11: *C. nubeculosus* SGE developed with a serum pool of control horses without signs of IBH was used as a negative control. Molecular mass markers (M) are indicated on the left site.

3.3. Demonstration of specific IgE-binding and analysis of the prevalence of sensitization against the recombinant allergens

Specific IgE-binding to the recombinant Cul n-allergens was demonstrated by Western blot analyses using a pool of five sera from IBH-affected horses. All recombinant proteins were able to bind serum IgE from affected horses but were not recognized by serum of healthy control horses (Fig. 2B).

The IgE-binding frequency to the recombinant allergens was determined by ELISA using sera of 46 IBH-affected horses and 29 control horses (Fig. 3). The results were expressed as absorbance at OD_{405nm} (A_{405}) and were considered positive when the measured A_{405} value was more 3-fold the standard deviation + mean of the A_{405} value obtained for the healthy control horses. The frequency of IgE-sensitization against the single salivary gland allergens varies between 13 and 57% among the horses suffering from IBH as summarized in Table 3. To confirm that we have identified most of the important salivary gland proteins associated with IBH, we calculated the number of horses which react with at least one of the eleven allergens available, including the Cul n 1 allergen described in an earlier report (Schaffartzik et al., 2010). The recombinant salivary gland allergens detected 45 of the 46 IBH-affected horses resulting in a diagnostic sensitivity of 98%.

3.4. Binding of the mouse antibodies raised against the recombinant *C. nubeculosus* proteins

All mouse ascites raised against the recombinant allergens recognized protein bands of the expected size in Western blot analyses with *C. nubeculosus* SGE, demonstrating that the cloned proteins are indeed salivary gland allergens of the midge protein bands corresponding to the expected size (Fig. 4).

3.5. Intradermal skin test

The ability of the recombinant antigens to induce type I hypersensitivity reactions was investigated *in vivo* by IDT performed on nine IBH-affected horses and seven healthy control horses. For each recombinant protein, except Cul n 6 and Cul n 11 where we were not able to obtain permission from the owners to test the horse sensitized to these

allergens, one to six IBH-affected horses showing positive ELISA results to the corresponding Cul n allergens were challenged. All eight recombinant proteins tested were able to elicit type I skin reactions in IBH-affected horses 30 min post-injection which were significantly greater than in control horses (mean diameter IBH 11.2 mm, mean diameter healthy horses 4.0 mm, $p < 0.0001$, Table 4). However, as described in the literature (Wagner et al., 2009), delayed

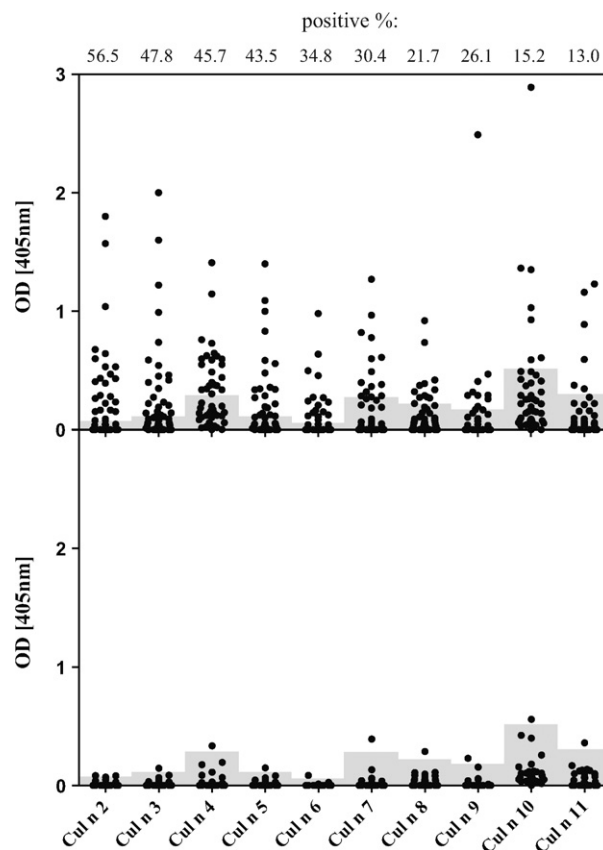


Fig. 3. Specific serum IgE against recombinant Cul n 2 to Cul n 11 determined by solid phase ELISA. Sera from 46 IBH-affected horses (upper panel) and 29 healthy control horses (lower panel) were analyzed. The cut-off values for positive results are highlighted in gray and correspond to 3-fold the standard deviation + mean of the healthy control horses. The frequency of positive horses for each single allergen (%) is indicated on the top.

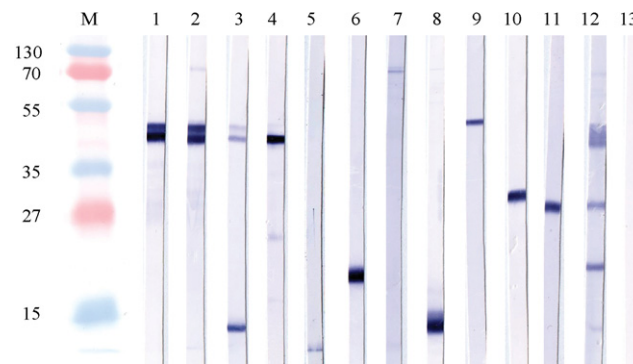


Fig. 4. SDS-PAGE and Western blot of *C. nubeculosus* SGE. Immunoblot stained with mouse ascites fluid specific for: Lane 1: r-Cul n 2; lane 2: r-Cul n 3; lane 3: r-Cul n 4; lane 4: r-Cul n 5; lane 5: r-Cul n 6; lane 6: r-Cul n 7; lane 7: r-Cul n 8; lane 8: r-Cul n 9; lane 9: r-Cul n 10; lane 10: r-Cul n 11; lane 11: r-Cul n 1; lane 12: immunoblot incubated with serum of an IBH-affected horse; lane 13: SGE stained with a non-related antibody (negative control). All strips were incubated with alkaline phosphates conjugated goat anti-mouse IgG antibody for visualization of the detected protein bands.

Table 4

Immediate (30 min) and delayed (4 h) hyperreactivity reactions of IBH-affected horses and healthy control horses to eight recombinant proteins.

Preparation ^a	IBH-affected horses				Control horses			
30 min	No. of horses ^b	Pos	Neg	Average diameter (mm) ^c	No. of horses	Pos	Neg	Average diameter (mm)
Saline	9			0.8 ± 0.8	6			6.7 ± 2.2
Histamine	9	9		18.8 ± 1.0	6	6		20.0 ± 1.6
Cul n 2	3	3	0	14.0 ± 1.0	4	0	4	5.0 ± 2.9
Cul n 5	3	2	1	12.0 ± 2.4	4	0	4	2.8 ± 2.8
Cul n 7	4	4	0	10.6 ± 0.4	5	0	5	3.8 ± 2.3
Cul n 8	3	2	1	10.5 ± 0.5	5	0	5	4.4 ± 2.7
Cul n 9	1	1	0	11 ± 0.0	3	0	3	3.0 ± 3.0
Cul n 10	6	4	2	9.7 ± 0.4	6	0	6	1.3 ± 1.3
ddH ₂ O	5			2.4 ± 1.5	5			3.8 ± 2.4
Histamine	5	5		19.2 ± 1.6	5	5		21.4 ± 1.2
Cul n 3	4	4	0	12.0 ± 1.2	5	0	5	5.8 ± 2.4
Cul n 4	4	2	2	9.5 ± 0.6	5	0	5	5.6 ± 2.3
Preparation	IBH-affected horses				Control horses			
4 h	No. of horses ^b	Pos	Neg	Average diameter (mm) ^c	No. of horses	Pos	Neg	Average diameter (mm)
Saline	9			0.8 ± 0.8	6			1.5 ± 1.5
Histamine	9	9		16.9 ± 0.8	6	6		19.2 ± 1.8
Cul n 2	3	2	1	13.0 ± 3.5	4	0	4	2.8 ± 2.8
Cul n 5	3	3	0	16.0 ± 2.0	4	3	1	12.5 ± 1.0
Cul n 7	4	3	1	9.8 ± 3.6	5	2	3	8.2 ± 2.2
Cul n 8	2	2	0	11.5 ± 0.5	5	3	2	9.6 ± 2.5
Cul n 9	1	1	0	10.0 ± 0.0	3	1	2	11.0 ± 0.6
Cul n 10	6	4	2	10.0 ± 2.7	6	0	6	3.8 ± 2.4
ddH ₂ O	5			6.4 ± 1.6	5			4.2 ± 2.6
Histamine	5	5		18.4 ± 1.0	5	5		20.2 ± 1.6
Cul n 3	4	2	2	12.0 ± 0.9	5	2	3	10.6 ± 0.7
Cul n 4	4	2	2	15.3 ± 2.0	5	3	2	13.0 ± 0.7

^a Cul n 2, 5, 7, 8, 9 and 10 were dissolved in saline; Cul n 3 and 4 in ddH₂O.

^b Number of horses tested.

^c Diameter of skin reactions for each group of horses in mm (mean ± standard error).

type reactions 4 h post-injection were observed also in some healthy control horses (Table 4).

4. Discussion

IBH is known since a long time as a seasonal skin disease of the horse and earlier studies have shown that IBH is associated with type I hypersensitivity reactions to *Culicoides* spp. and sometimes also other insects (Fadok and Greiner, 1990). First experiments based on Western blot analyses showed that some salivary gland proteins of the insects

are indeed IgE-binding proteins (Hellberg et al., 2006), but the identity of the allergens associated with IBH remained unknown until the group of Langner reported cloning, production and characterization of a 66 kDa protein of *C. sonorensis* identified as maltase (Langner et al., 2009), an enzyme known to elicit allergic reactions also in humans (Horner et al., 2008). In parallel we used phage surface display technology to identify the allergen repertoire of salivary glands from black fly *S. vittatum* (Schaffartzik et al., 2009). Among these we isolated an antigen 5 like protein (Sim v 1) which belong to the ag-5 allergen family involved

in human insect sting allergy (King and Spangfort, 2000) also present in *C. nubeculosus* SGE (Cul n 1; Schaffartzik et al., 2010). However, SGE of *C. nubeculosus* contains at last ten biochemically uncharacterized IgE-binding protein bands as shown by Western blot analyses (Hellberg et al., 2006). The aim of our study was to clone the SGE allergen repertoire of *C. nubeculosus* by phage surface display technology, produce the corresponding proteins, and verify their allergenicity *in vitro* and *in vivo*. Analysis of SGE with a complex serum pool from IBH-affected horses revealed ten clearly resolved IgE-binding protein bands with molecular masses ranging from ca. 12 to 75 kDa (Fig. 1). Screening of a salivary gland cDNA library of *C. nubeculosus* displayed on the phage surface with serum IgE of IBH-affected horses yielded ten clones carrying different cDNA potentially coding for allergens. BLAST analyses showed that nine of the ten sequences encoded truncated salivary gland proteins not yet described as allergens (Table 1), whereas one clone contained the complete ORF coding for a secreted *C. nubeculosus* salivary gland protein of unknown function (Accession No. ACM40905.1; Russell et al., 2009). In a first step all ten cDNAs were subcloned into a high level protein expression vector, produced, purified and tested for IgE-binding capacity in ELISA. Because all truncated proteins were able to bind IgE from IBH-affected horses, we completed all ORFs by RACE-PCR, subcloned the complete reading frames without leader sequences into high level expression vectors, produced and purified all proteins (Fig. 2A). In a second step, the IgE-binding capacity of the proteins was confirmed by Western blot analysis (Fig. 2B). The prevalence of IgE sensitization of IBH-affected horses to the full length recombinant proteins determined by ELISA ranged from 13.0 to 56.5%, depending on the allergen tested (Fig. 3), whereas healthy horses did not show any relevant IgE-binding to the recombinant allergens. Except one, all IBH-affected horses reacted to at least one allergen demonstrating that the cloned *C. nubeculosus* SG allergen repertoire is suitable for a highly specific and sensitive component resolved diagnosis of IBH. To be able to identify which SGE protein bands correspond to the cloned allergens, mice were immunized with the recombinant allergens and ascites fluids produced. The ascites antibodies reacted with protein bands in Western blot analyses of *C. nubeculosus* SGE (Fig. 4) with a similar molecular weight of those recognized by serum IgE of IBH-affected horses (Fig. 1), demonstrating the presence of the allergens in the glands of the insect.

These findings clearly demonstrate that serum IgE of IBH-affected horses recognize recombinant salivary proteins *in vitro*. However, the results do not provide any information about the *in vivo* allergenic potential of the cloned proteins. Therefore, IBH-affected horses and healthy control horses were challenged intradermally with the recombinant allergens. Cul n 6 and Cul n 11 could not be investigated *in vivo*, because the owners of horses sensitized to these allergens refused the permission to perform the skin tests. However, the remaining eight proteins elicited immediate wheal reactions after 30 min in IBH-affected horses, but not in healthy controls (Table 4). After 4 h, the reaction wheals persisted and in several cases they even increased. In addition at this time point also some

healthy controls showed positive reactions to the recombinant proteins (Table 4). These results are not unexpected because positive, non-IgE mediated reactions to allergen challenges in healthy horses which can be due to many factors are reported in the literature (Wagner et al., 2009). In our case some of the allergens were poorly soluble in physiological solutions like PBS or NaCl and were therefore diluted in ultrapure H₂O for skin testing. Ultrapure H₂O does not reflect the physiological conditions in the skin and might lead to cell lysis and delayed inflammatory responses due to its hypotonic character. However, also 4 h post challenge IBH-affected horses still showed a more pronounced wheal reaction to the allergens than non-affected control horses (Table 4).

Sequence analyses revealed a strong homology of the cloned allergens with the sequences of already known SG proteins of *C. nubeculosus* and *C. sonorensis* not yet described to be allergenic (Table 3). Interestingly, some *C. nubeculosus* allergens show extended sequences homologies to protein families known to induce allergy in humans. This is particularly true for Cul n 2 which code for a hyaluronidase known to play a pivotal role in bee venom allergy (Soldatova et al., 1998), for Cul n 3 exhibiting sequence homology to cysteine proteases which represent major allergens of house dust mites (Shakib et al., 2008; Thomas and Hales, 2007), and for the Cul n 8-encoded maltase, known to elicit allergic reactions in humans (Horner et al., 2008).

The salivary gland protein termed Cul n 9 shares sequence homology to a member of D7-related proteins of unknown function present in the saliva or salivary glands of numerous female blood-sucking insects. D7 protein of *Aedes aegypti* is involved in mosquito allergies in humans (Malafronte Rdos et al., 2003). However, the role and function of all identified salivary gland proteins has to be investigated more deeply, to improve the understanding of their involvement in the pathogenesis of allergic disease.

In conclusion, the identification of ten recombinant proteins which bind serum IgE and elicit positive skin tests in IBH-affected horses, together with the earlier described ag5-like Cul n 1 allergen (Schaffartzik et al., 2010), provide a more or less complete *C. nubeculosus* SG allergen repertoire with a great potential to improve the diagnostic sensitivity and specificity for IBH. Highly pure recombinant allergens will form a solid basis for the study of allergic reactions involved in IBH, for the development of highly sensitive diagnostic kits, and for the development of tailored multi-component vaccines for a component resolved immunotherapy of IBH.

Conflicts of interest

None.

Acknowledgments

Work at SIAF was supported by the Swiss National Science Foundation grant no. 320030.127618/1. Work at the Vetsuisse Faculty of Berne was supported by a grant of the Department of Clinical Veterinary Medicine of the Vetsuisse Faculty of Berne, by the Swiss National Science

Foundation grant no. 31-116803/1, by the Agricultural Productivity Fund of Iceland, and by the Icelandic Research Fund. The cooperation of the horse owners is gratefully appreciated. We thank Dr. W. Hellberg for helping dissecting *Culicoides* salivary glands.

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