Veterinary Immunology and Immunopathology 153 (2013) 227–239

Contents lists available at SciVerse ScienceDirect

Veterinary Immunology and Immunopathology

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

Research paper

Cloning and expression of candidate allergens from *Culicoides obsoletus* for diagnosis of insect bite hypersensitivity in horses

Nathalie M.A. van der Meide[a], Nathalie Roders[a], Marianne M. Sloet van Oldruitenborgh-Oosterbaan[b], Peter J. Schaap[c], Monique M. van Oers[d], Wolfgang Leibold[e], Huub F.J. Savelkoul[a], Edwin Tijhaar[a,*]

[a] *Cell Biology and Immunology Group, Wageningen University, Wageningen, The Netherlands*
[b] *Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands*
[c] *Laboratory of Systems and Synthetic Biology, Wageningen University, Wageningen, The Netherlands*
[d] *Laboratory of Virology, Wageningen University, Wageningen, The Netherlands*
[e] *Immunology Unit, University of Veterinary Medicine Hannover, Hannover, Germany*

ARTICLE INFO

Article history:
Received 18 December 2012
Received in revised form 27 February 2013
Accepted 7 March 2013

Keywords:
Horse (Equine) IgE
Insect bite hypersensitivity
ELISA
Culicoides obsoletus
Recombinant allergens

ABSTRACT

Insect bite hypersensitivity (IBH) is an IgE-mediated (Type I) hypersensitivity reaction induced by allergens from biting midges of the *Culicoides* spp. The aim of the present study was to identify, clone and express recombinant allergens from *C. obsoletus*, the main species found feeding on horses in the Netherlands, by sequence homology searches on the *C. obsoletus* specific RNA database, with previously described allergens from *C. nubeculosus* and *C. sonorensis.* BLAST searches with these described allergens resulted in similarity hits with 7 genes coding for *C. obsoletus* allergens. These allergens were expressed as hexahistidine tagged recombinant proteins in *E. coli*. Allergens were termed Cul o 1–Cul o 7. A maltase (Cul o 1) plus Cul s 1 (maltase of *C. sonorensis*) were additionally expressed in insect cells using the baculovirus expression system to compare homologous allergens from different species produced with different expression systems in diagnostic *in vitro* and *in vivo* tests.

We demonstrate that IBH affected horses in the Netherlands show higher IgE levels to Cul o 1 than to Cul s 1, as determined by an IgE ELISA. Furthermore, we show that Cul o 1 produced in *E. coli* is at least as suitable for *in vitro* diagnosis of IBH affected horses as Cul o 1 produced in the baculovirus/insect cell expression system.

The resulting proteins were evaluated for their ability to discriminate IBH affected and healthy horses by ELISA and intradermal testing. The frequency of positive test results by ELISA within IBH affected horses ranged from 38% to 67% for the different allergens. When results of IgE-binding to Cul o 1–Cul o 7 were combined the test had a sensitivity of 92% and specificity of 85%. The capability of the allergens to induce Type I hypersensitivity reaction in IBH affected horses was demonstrated by an intradermal test.

The results show that *E. coli* expressed recombinant allergens from *C. obsoletus* are valuable tools to determine the allergen specific sensitisation profile (component resolved diagnosis) in horses with IBH in countries were *C. obsoletus* is the most abundant species and may facilitate in the development of future immunotherapy.

© 2013 Elsevier B.V. All rights reserved.

Abbreviations: aa, amino acids; *C. nubeculosus*, *Culicoides nubeculosus*; *C. obsoletus*, *Culicoides obsoletus*; *C. sonorensis*, *Culicoides sonorensis*; *C. variipenis*, *Culicoides variipenis*; *E. coli*, *Escherichia coli*; IBH, insect bite hypersensitivity; MOI, multiplicity of infection; $TCID_{50}$, 50% tissue culture infective dose; WBE, whole body extract.

* Corresponding author at: Cell Biology and Immunology Group, P.O. Box 338, 6700 AH Wageningen, The Netherlands. Tel.: +31 317 483967; fax: +31 317 483962.
E-mail address: Edwin.tijhaar@wur.nl (E. Tijhaar).

0165-2427/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetimm.2013.03.005

1. Introduction

Insect bite hypersensitivity (IBH) is an IgE-mediated (Type I) hypersensitivity reaction found worldwide and is believed to be mainly induced by allergens from biting midges of the *Culicoides* spp., although other insect species, such as *Simulium* and *Stomoxys* spp. were also described to be of importance (Baselgia et al., 2006; Fadok and Greiner, 1990; Quinn et al., 1983; Wagner et al., 2006). Although IBH is not life threatening, it causes a lot of discomfort to affected horses, due to the self-mutilation to alleviate the symptoms of pruritus.

Current methods to prevent IBH include insect avoidance by stabling the horses during dusk and dawn when midges are most active, use of blankets to cover the horse, and insect repellents. Skin-related symptoms are sometimes suppressed by the use of topically applied corticosteroids. Some treatment options, like anti-histamines, dietary supplements and ointments are available on the market, but studies on horses using these treatments have shown inconsistent results (Olsén et al., 2010; van den Boom et al., 2010, 2011).

Immunotherapy could alleviate the symptoms for horses suffering from IBH and some trials have already been carried out with *Culicoides* whole body extracts, but with varying results (Anderson et al., 1996; Barbet et al., 1990). Anderson et al. (1996), however did see a clear reduction of clinical signs in most and even a complete disappearance in some horses after immunotherapy with *C. variipenis* extract. The quantity of insects required to obtain these results though was very high, being more than 10,000 insects per horse. The use of purified allergens from *Culicoides* insects could simplify and improve future immunotherapeutic approaches.

Several studies have shown that many proteins from *Culicoides* can bind IgE in IBH-affected horses and are therefore potential allergens (Hellberg et al., 2006; Wilson et al., 2008).

Several allergens have now been identified and produced as recombinant proteins. These allergens were derived from the laboratory bred *Culicoides* species, *C. sonorensis* and *C. nubeculosus* (Langner et al., 2009; Schaffartzik et al., 2010, 2011).

The first allergen associated with IBH was identified to be a 66 kDa maltase from *C. sonorensis* and was expressed as recombinant protein (Cul s 1) *via* the baculovirus expression system (Langner et al., 2009). This recombinant protein was found to be highly allergenic in both *in vivo* and *in vitro* tests.

Later, a gene encoding an antigen 5-like allergen was cloned, sequenced and expressed as recombinant protein. This was the first described allergen from *C. nubeculosus* and was therefore designated Cul n 1 (Schaffartzik et al., 2010). In addition, using phage display technology, a set of 10 recombinant salivary gland allergens from *C. nubeculosus* was produced and named Cul n 2–Cul n 11. These allergens were recognized by IgE of 13% to 56.5% of IBH-affected horses, depending on the allergen (Schaffartzik et al., 2011).

Although many *Culicoides* species have been described to be associated with IBH, apparently *C. obsoletus* is the most important species in the Netherlands in relation to IBH, as this is by far the most abundant *Culicoides* species found feeding on horses in this country (van der Rijt et al., 2008). Furthermore, a comparison for *in vitro* diagnosis of IBH using whole body extracts of *C. sonorensis*, *C. nubeculosus* and *C. obsoletus*, showed the importance of using *C. obsoletus* for sensitive and specific diagnostics for horses in the Netherlands. An ELISA with *C. obsoletus* WBE was developed with high sensitivity and specificity that can be used both with sera collected in the *Culicoides* active season as well as with sera collected in the off season (Meulenbroeks et al., 2013; van der Meide et al., 2012).

C. obsoletus insects still need to be collected from the wild, because they are not available from laboratory bred colonies and are difficult to rear (Boorman, 1985). Therefore recombinant allergens would be an attractive alternative for whole body extracts for diagnostic assays.

The aim of this study was to develop recombinant allergens from *C. obsoletus* that can be used to improve diagnosis and may be applied in immunotherapy for horses that are exposed to *C. obsoletus.* Furthermore, we compared the reactivity of recombinant maltase from *C. obsoletus* (Cul o 1) with that of *C. sonorensis* (Cul s 1) (Langner et al., 2009). These allergens were expressed both in an *E. coli* as well as a baculovirus expression system, to evaluate whether these allergens are more allergenic when expressed in an eukaryotic system, due to possible post translational modifications. Additional *C. obsoletus* genes homologous to previous described allergens from *C. nubeculosus* were cloned and expressed as recombinant proteins in *E.coli*. These proteins were evaluated for their ability to discriminate IBH and healthy horses *in vitro* by ELISA and their ability to induce immediate and delayed type skin reactions *in vivo*.

2. Materials and methods

2.1. Insects

Wild *C. obsoletus* insects were collected in the Netherlands and whole body extracts (WBE) were prepared as previously described (van der Meide et al., 2012). A small fraction (±5%) of the insects was checked under a stereo microscope and indeed only contained *C. obsoletus*. Identification of *C. obsoletus* was based on size and wing patterns (Campbell and Pelham-Clinton, 1960)

2.2. Horses

A total of 217 Shetland ponies and Icelandic horses located in different regions of The Netherlands were included in this study. Pairs of clinically confirmed IBH affected and healthy control horses kept at the same location were formed (Schurink et al., 2009). Seven IBH affected Icelandic horses that showed recurrent symptoms for at least several years and 7 healthy controls from the same pasture were selected for the intradermal skin test. The remaining 203 horses (76 Icelandic horses and 127 Shetland ponies) were used to evaluate *E. coli* expressed Cul o 1–Cul o 7 and Cul s 1 in a diagnostic ELISA.

Blood samples were taken from all horses and serum was frozen in aliquots not later than 24 h after blood sampling and stored at −20 °C until use. The experimental procedures were approved by the Board on Animal Ethics and Experiments from Wageningen University.

2.3. cDNA synthesis

RNA was isolated from ±200 wild-caught *C. obsoletus* insects by homogenization in TRIzol® (Invitrogen, Paisley, UK) according to the instructions of the manufacturer. The concentration was measured on a spectrophotometer (Nanodrop Technologies) at OD_{260nm}. The integrity of RNA was analysed by electrophoresis on a 1% agarose gel. Total RNA was stored at −80 °C until use.

Prior to cDNA synthesis, a DNase treatment was performed by mixing 2 μg RNA with 1 μl 10× DNAse I Reaction buffer, DNAse I (1 U/μl) and DEPC-treated water up to a final volume of 10 μl. The mixture was incubated for 15 min at room temperature (RT), followed by the inactivation of DNAse I by adding 25 mM EDTA (Merck, Darmstadt, Germany) and heating for 10 min at 65 °C. Synthesis of cDNA was performed using the Superscript III First Strand Synthesis system (Invitrogen, Paisley, UK), according to manufacturer instructions. Briefly, 300 ng random primers (Invitrogen, Cat. no. 48190-011, 300 ng/μl) and 10 mM dNTPs were added to the DNase I-treated RNA sample. The mixture was heated at 65 °C for 5 min and held on ice for a minimum of one minute. 5× first strand buffer (Invitrogen), 0.1 M DTT (Invitrogen), RNase OUT Inhibitor (Invitrogen, Cat. no. 10777-019, 40 U/μl), and SuperScript III (Invitrogen, 200 U/μl) was added to the mixture and incubated for 10 min at RT, followed by incubation for 50–60 min at 50 °C. The reaction was terminated by heating at 70 °C for 15 min.

2.4. Cloning of C. obsoletus cDNA's encoding potential allergens

cDNA of *C. obsoletus* was synthesized using random primers, modified and enriched for attachment to the Illumina flowcell (HiSeq2000, Illumina) at the Genome Analysis Facility of the Universitair Medisch Centrum Groningen (UMCG), The Netherlands. One 100-cycle paired-end lane was sequenced. *De novo* sequence assembly was done with Velvet (version 1.1.04) (Zerbino and Birney, 2008) using a coverage cut-off of 5 and a minimum contig length of 200 yielding 26936 cDNA contigs (manuscript in preparation). The cDNA contigs were used as an input to create a *C. obsoletus* specific RNA blast database. A protein BLAST with the sequence coding for Cul s 1 was performed on this RNA database to find the homologous gene *for C. obsoletus* and specific primers were designed.

Meanwhile, new allergens from *C. nubeculosus* were identified (Schaffartzik et al., 2010, 2011) and similarity searches with the sequences coding for these genes were performed as well using BLAST on the *C. obsoletus* RNA database. Six complete sequences coding for genes homologous to, respectively, Cul n 1 (antigen 5 like protein), Cul n 2 (hyaluronidase), Cul n 4 (secreted salivary protein), Cul n 7 (unknown salivary protein), Cul n 9 (D7-related salivary protein) and Cul n 11 (trypsin) were found and specific primers for these allergens from *C. obsoletus* were designed. The primers are listed in Table 1, set 1.

The 7 allergens were termed according to the recommendation of the allergen nomenclature sub-committee (www.allergen.org) and according to their MW; Cul o 1 (homologous to Cul s 1), Cul o 2 (homologous to Cul n 2), Cul o 3 (homologous to Cul n 1), Cul o 4 (homologous to Cul n 11), Cul o 5 (homologous to Cul n 7), Cul o 6 (homologous to Cul n 9) and Cul o 7 (homologous to Cul n 4). The recombinant proteins with their characteristics are listed in Table 2.

Signal peptides for each allergen were predicted by submitting the sequences to SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP). To clone the *C. obsoletus* allergens in expression vectors, the sequence corresponding to the mature part of the allergens was amplified from *C. obsoletus* cDNA by nested PCR (for primers see Table 1) using KOD hot start DNA polymerase according to manufacturer's instructions (Novagen, Nottingham, UK). The inner primer pairs contained additional restriction sites (Table 1, set 2) for cloning of the PCR products into the corresponding sites of the used expression vectors. For expression by *E. coli* the obtained PCR products were ligated into a pET15b (Novagen) derived vector, pET15bNew, restricted by *BamHI* and *XhoI* or *HindIII*, downstream of the sequence coding for a hexahistidine tag. The mature part of Cul s 1 was ordered as a synthetic gene (Mr. Gene, Regensburg, Germany) codon optimized for *E.coli* expression and directly cloned between the *BamHI*- and *HindIII*-sites of pET15bNew.

Searches for homology within the database were performed using the BLAST method http://blast.ncbi.nlm.nih.gov/Blast. Nucleotide sequences were translated using the BCM search launcher translation tool (http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html) and aligned with Clustal W (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The cloned cDNAs encoding for Cul s 1 or Cul o 1 including the N-terminal hexahistidine tag, were sub-cloned into pFB-HBM, a derivative of the pFastBac1vector (Invitrogen) downstream of a honey bee mellitin (HBM) signal sequence (Kaba et al., 2004), kindly provided by Dr. P. Sondermeyer, MSD Animal Health, Boxmeer, NL, for subsequent expression in the baculovirus/insect cell expression system.

2.5. E. coli expression and purification

The isolated pET15bNew plasmids encoding the different *Culicoides* antigens were used to transform *E. coli* BL21_CodonPLus (DE3) (Agilent Technologies, Amsterdam, The Netherlands). Proteins were expressed similarly as described by (van der Aa et al., 2010).

2.6. Baculovirus expression and purification of Cul o 1 and Cul s 1

Recombinant *Autographa californica* nucelopolyedrovirus (AcMNPV) encoding Cul o 1 and Cul s 1 with N-terminal hexahistidine tag were generated using the Bac-to-Bac system (Invitrogen). Briefly, the pFB-HBM

Table 1
Primers used to amplify cDNA's coding for allergens Cul o 1–Cul o 7. Set 2 includes primers to amplify the mature part of the protein including restriction sites (underlined).

Cul o 1	set 1	Fw	5′-GAAGAATGATAATTTTCAAAACATT-3′
		Rv	5′-TTATTGTTCATATTGTCCGGGA-3′
	set 2	Fw	5′-CGCGGATCCCCTCAGGGAGCTCGTGAA-3′
		Rv	5′-GCGCTCGAGTTATTGTTCATATTGTCCGGGATC-3′
Cul o 2	set 1	Fw	5′-TACACTTGTTGTGTGTTTCTG-3′
		Rv	5′-CTCTACATCATAACATAGATAATAATG-3′
	set 2	Fw	5′-CGCGGATCCCAACATCTGATTCAGGCAGA-3′
		Rv	5′-GCGCTCGAGTACATCATAACATAGATAATAATG-3′
Cul o 3	set 1	Fw	5′-GAAAATGTTTCGTATTTGTTTATTTAC-3′
		Rv	5′-TATTTGATGGCAAAACTCTGTT-3′
	set 2	Fw	5′-CGCGGATCCACAGATTTTTGTGACCGAAAGC-3′
		Rv	5′-GCGAAGCTTCCAATTACGGTAAAAATGGCA-3′
Cul o 4	set 1	Fw	5′-GCAATAATATCGGCTGTTTATGC-3′
		Rv	5′-TTAGTTCATGTTTTGCGTTATCC-3′
	set 2	Fw	5′-CGCGGATCCGCTGATCATCAATCGC-3′
		Rv	5′-GCGAAGCTTTTAGTTCATGTTTTGCG-3′
Cul o 5	set 1	Fw	5′-GCGTAATCTTAGGAGGAAT-3′
		Rv	5′-TGAATAGATCATCATTTCT-3′
	set 2	Fw	5′-CGCGGATCCTTTGATTTAAGTGATG-3′
		Rv	5′-GCGCTCGAGTCATCATTTCTTTATTT-3′
Cul o 6	set 1	Fw	5′-CCGTTACTTGCCGTTATTGG-3′
		Rv	5′-TCATTATAGACCTTCGTCAAC-3′
	set 2	Fw	5′-CGCGGATCCTCATTCGTTCCAAAGGAGAG-3′
		Rv	5′-GCGAAGCTTTTATATTGTATTCGGTTTTCCATC-3′
Cul o 7	set 1	Fw	5′-GTTCTCCGTGTCTAAGCTATC-3′
		Rv	5′-TTAATGAAGAGCTGCGAGTG-3′
	set 2	Fw	5′-CGCGGATCCTGCTCCTCAATGGCAAATAAG-3′
		Rv	5′-GCGAAGCTTTTAATGAAGAGCTGCG-3′

recombinant plasmids containing either Cul o 1 or Cul s 1 cDNA were used to transform DH10ß competent cells containing an AcMNPV bacmid with a chitinase and cathepsin deletion (Kaba et al., 2004) followed by isolation of the recombinant bacmid DNA. Screening of transposition-positive constructs was done by PCR with bacmid and gene specific primers.

Preliminary experiments revealed that an optimal production was obtained with a multiplicity of infection (MOI) of 10 $TCID_{50}$ units/cell 72 h after infection.

The recombinant bacmid DNAs were used to transfect Sf9 cells with CELL FECTIN reagent (Invitrogen) using standard protocols and the virus was harvested 72 h later, followed by amplification of the viral stock on Sf9 cells in suspension cultures with a cell density of 2×10^6 viable cells/ml in serum free medium (Sf-900 II SFM, Invitrogen) to produce working viral stocks without serum. The titer of the viral stocks was determined by an End-Point Dilution Assay using the Sf-9 Easy Titre cell line, encoding EGFP under control of the polyhedrin promoter (Hopkins and Esposito, 2009). Immediately after titer determination, Sf9 cells in suspension cultures (5× 60 ml) were infected at a density of 2×10^6 cells/ml with a multiplicity of infection (MOI) of 10 50% Tissue Culture Infective Dose ($TCID_{50}$) units/cell. At 72 h post infection, the cell suspensions were centrifuged at 1000 g for 10 min at RT and supernatant and cells were collected. The cell-free supernatants were concentrated using Centricon Plus-70 centrifugal Filter Devices (Merck Millipore, Billerica, Massachusetts, US) and subsequently Cul o 1 and Cul s 1 were purified on Ni^{2+}charged chelating sepharose (Fast Flow, GE Healthcare, Uppsala, Sweden) columns. Proteins were eluted with 500 mM imidazol and afterwards dialysed against PBS.

2.7. SDS-Page and Western blot analysis of recombinant proteins

To confirm localization and to analyze the purification of the baculovirus expressed recombinant proteins, samples were analysed by SDS-PAGE (12.5% gel) and immunoblotting. To analyze the purity of the recombinant proteins, dialyzed eluted fractions were loaded on a 12.5% SDS-PAGE and the gel was stained with Pierce Silver Stain Kit (Pierce, Rockford, USA) for the baculovirus recombinant proteins or stained with Gelcode Blue Stain Reagent (Pierce) for the *E. coli* recombinant proteins.

Table 2
Characteristics of the *C. obsoletus* allergens.

Allergen	Genbank accesion nr	MW	Homolog	% aa identity
Cul o 1	KC339671	66.8	Maltase (Cul s 1) AY 603565	78%
Cul o 2	KC339672	42.3	Hyaluronidase (Cul n 2) HM 145950	75%
Cul o 3	KC339673	27.9	Antigen 5 like protein (Cul n 1) EU 978899	70%
Cul o 4	KC339674	27.1	Trypsin (Cul n 11) HM 145959	51%
Cul o 5	KC339675	17.9	Unknown salivary protein (Cul n 7) HM 145955	41%
Cul o 6	KC339676	15.2	D7-related salivary protein (Cul n 9) HM 145957	40%
Cul o 7	KC339677	15.0	Secreted salivary protein (Cul n 4) HM 145952	33%

For immunoblotting, nitrocellulose membranes (Protran, Schleicher & Schuell Bioscience GmbH, Dassel, Germany) were blocked in 5% non-fat cow's milk in Tris buffered saline (TBS)-Tween (10 mM Tris/HCL, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20) for 1 h at RT and then incubated for 1.5 hour with an anti-His6 tag specific monoclonal antibody that was earlier developed in house. Membranes were then incubated for 1 h with goat anti-mouse IgG horseradish peroxidase (Dako, 1:1000 in milk powder/TBS-Tween). Between each incubation step, membranes were washed three times with TBS-Tween. Signal was detected by development with an enhanced chemiluminescence (ECL) western blotting detection reagent (GE Healthcare) according to the manufacturer's protocol and visualized by the use of Lumni-fil chemiluminescent Detection Film (Roche, Woerden, The Netherlands).

2.8. ELISA

Specific IgE serum levels against the recombinant *C. obsoletus* allergens (Cul o 1–Cul o 7) and Cul s 1 were determined in an indirect ELISA as previously described (van der Meide et al., 2012). Briefly, wells (384 wells plates) were coated with 20 μl/well of 10 μg/ml of recombinant protein overnight at 4 °C, followed by incubation with horse serum samples diluted 1:5 in a 1.5% casein buffer (SDT GmbH, Baesweiler, Germany). IgE binding was detected by mouse monoclonal anti-equine IgE-176 (Wagner et al., 2003), followed by goat anti-mouse peroxidase conjugate (multispecies adsorbed, AbD Serotec, Oxford, UK). High sensitivity tetramethylbenzidine (SDT GmbH) was used as a substrate. The reaction was stopped with 1% HCL. Absorbance was measured with a multi-mode microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, US) at a wave length of 450 nm corrected for 650 nm. For each allergen, sera of all the IBH affected and healthy horses were tested on the same 384-wells plate to avoid "between plate" OD value differences. Wells without horse serum, but all the other components showed background OD450 nm values < 0.07.

The cut-off for positive IgE-binding was set at the mean +3 times the standard deviation of the OD_{450nm} reading of the healthy horses. The OD_{450nm} values of the healthy horses that were evidently positive (>1.0, arbitrarily decided) were left out to determine this cut-off, but were taken into consideration to determine false positives.

2.9. Intradermal test

An intradermal test was performed on seven clinically confirmed IBH affected and seven healthy control Icelandic horses to test the capability of the *Culicoides* recombinant allergens to induce Type I (IgE mediated) hypersensitivity reactions. All horses were living under the same circumstances (bedding, feeding, pasture) for at least one year The middle region of the neck of the horses was clipped. The injection sites were indicated with a permanent marker and were placed about 5 cm apart. One concentration (10 μg/ml) per recombinant allergen or *C. obsoletus* WBE were injected intradermal in a volume of 0.1 ml (Sloet van Oldruitenborgh-Oosterbaan et al., 2009). Histamine phosphate was used as a positive control (10 mg/ml) for a positive wheal reaction and a phosphate buffer as a negative control (Artuvetrin, Artu Biologicals, Almere, The Netherlands). The wheal diameter was measured in millimeters at 30 min and 1 h (immediate phase reaction) and 4 h and 24 h (late phase cell-mediated reaction).

Skin reactions were considered positive when the absolute wheal diameter was larger than the highest wheal diameter provoked by the negative control at the same time point.

2.10. Statistical analysis

IBM SPSS statistics, version 19, software program was used for statistical analysis. The non-parametric Mann–Whitney *U* test was used to compare OD_{450nm} values from IBH-affected horses with those from healthy controls against Cul o 1 and Cul s 1 and to compare wheal diameters from IBH affected horses and healthy horses after the intradermal test. Results were considered significant at $p \leq 0.05$.

3. Results

3.1. Cloning of Cul o 1 cDNA

A *C. obsoletus* specific RNA blast database was used to identify the gene encoding for maltase with the sequence encoding for the maltase of *C. sonorensis* (Cul s 1). Specific primers were designed and used to amplify the gene encoding for the maltase (Cul o 1).

To detect a putative polymorphism in the gene encoding for Cul o 1 in a *C. obsoletus* population, ten independent PCRs were performed on the cDNA from a pool of *Culicoides obsoletus* insects. Three isoforms were found with only minor differences on the amino acid level. Alignment of the most detected Cul o 1 isoform with the published Cul s 1 maltase revealed an identity of 78% on amino acid level (Supplementary Fig. 1).

3.2. E. coli expression and purification of Cul o 1 and Cul s 1

Cul o 1 and Cul s 1, expressed in *E. coli*, were found to be in the insoluble fractions (inclusion bodies) and their molecular masses were in agreement with the expected size (69 kDa). After purification and refolding, a yield of 10.6 mg/L bacterial culture was obtained for Cul o 1 and 11.2 mg/L bacterial culture for Cul s 1. The proteins were found to be >95% (Fig. 1).

3.3. Baculovirus expression and purification of Cul o 1 and Cul s 1

The Bac-to-Bac system was used for the production of recombinant Cul o 1 and Cul s 1 in a baculovirus expression system. Immunoblot analysis with an anti-6× His tag specific antibody showed that the recombinant Cul o 1 and Cul s 1 were mainly found in the cell-free supernatant (Fig. 2A). After purification, a substantial reduction of recombinant

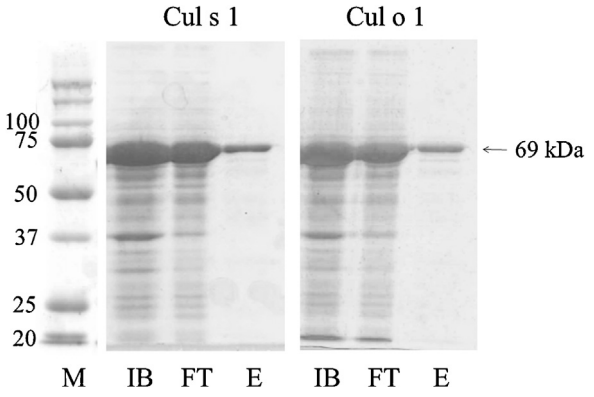


Fig. 1. Purification of *E. coli* expressed Cul s 1 and Cul o 1. M = marker, IB = solved inclusion bodies, FT = flow through (unbound proteins), E = refolded and dialysed eluted fraction. Proteins were separated by SDS-PAGE (12.5% gel) and stained with Gelcode coomassie blue-stain.

protein was observed in the flow through and bound proteins were recovered by elution with 500 mM imidazole (Fig. 2B). Eluted Cul o 1 and Cul s 1 were found to be >95% pure (Fig. 2C). A yield of approximately 100 μg/10^9 cells was obtained for both Cul s 1 and Cul o 1.

3.4. Determination of IgE antibodies against E. coli expressed Cul o 1 and Cul s 1

An initial IgE and IgG ELISA with 10 IBH affected and 10 healthy control horses with all three Cul o 1 isoforms did not show any clear differences in OD_{450nm} values (data not shown) and therefore the isoform that was most abundant (isoform 3) was chosen as the antigen in further experiments.

Sera of 103 IBH affected and 100 healthy horses were evaluated for IgE levels (OD_{450nm} values) against Cul o 1 and Cul s 1 in an indirect ELISA. Test results were considered positive when above the cut-off level (mean + 3 times the standard deviation of IgE levels of healthy horses).

Almost double the percentage of positive tested IBH affected horses was obtained with Cul o 1 (43%) compared to Cul s 1 (22%). A significant difference between the OD_{450nm} values of IBH affected and healthy horses was observed for both Cul o 1 and Cul s 1 ($p < 0.001$), however OD_{450nm} values obtained with Cul o 1 for IBH affected horses were significantly higher compared to OD_{450nm} values obtained with Cul s 1 ($p < 0.001$, Fig. 3).

3.5. Determination of IgE antibodies against baculovirus expressed Cul o 1 and Cul s 1 versus E. coli expressed Cul o 1 and Cul s 1

To compare the IgE levels (OD_{450nm} values) against baculovirus expressed Cul o 1 and Cul s 1 with IgE levels against *E. coli* expressed Cul o 1 and Cul s 1, an additional IgE ELISA was performed.

Because of the low yield of baculovirus expressed Cul o 1 and Cul s 1, only a small number of horses could be tested by ELISA. These horses were selected to belong to one of the following groups: Group A) Seven horses that were clinically IBH affected and previously tested positive in ELISA with WBE as well as with *E. coli* Cul o 1; Group B) Seven horses that were clinically IBH affected and previously tested positive in ELISA with WBE, but negative with *E. coli* Cul o 1; Group C) Ten horses that were clinically healthy and previously tested negative ELISA with WBE as well as with *E. coli* Cul o 1 (Fig. 4).

Horses from group A on average showed similar IgE levels (OD_{450nm} values) against *E. coli* expressed Cul o 1 as against baculovirus expressed Cul o 1. However, IgE levels against baculovirus expressed Cul s 1 were higher compared to *E. coli* expressed Cul s 1. All horses in group A, except for 1 horse with Cul s 1, had a positive test result with both recombinant proteins regardless of the used expression system.

Horses from group B, that were previously tested positive on WBE but negative on *E. coli* expressed Cul o 1, on average had higher IgE levels against the baculovirus expressed Cul o 1 and Cul s 1 and the OD values of all these horses were above the cut-off level. However, clinically healthy horses (group C) showed similar IgE levels against these baculovirus expressed proteins. In contrast, IgE levels against the *E.coli* expressed proteins were not above the cut-off level, suggesting non-specfic reaction with baculovirus expressed recombinant Cul o 1 and Cul s 1 (Fig. 4).

3.6. Amplification of Cul o 2–Cul o 7 cDNA

Six complete genes coding for *C. obsoletus* allergens homologous to Cul n 1, Cul n 2, Cul n 4, Cul n 7, Cul n 9 and Cul n 11 (Schaffartzik et al., 2010, 2011) were found in a *C. obsoletus* RNA database. Specific primers were designed and used to amplify these genes from *C. obsoletus* cDNA.

Alignments of the allergens Cul o 2–Cul o 7 with their similar *C. nubeculosus* homologous allergens showed global similarity with amino acid identities ranging from 33 to 75%, depending on the alignment (Supplementary Figs. 2–7).

3.7. E. coli expression and purification of Cul o 2–Cul o 7

Cul o 2–Cul o 7 were successfully expressed in *E. coli*. The recombinant proteins were found to be in the insoluble fractions and were dissolved, purified, refolded and dialysed (Fig. 5A). All *E. coli* expressed proteins were found to be >95% pure (Fig. 5B). The sizes of the proteins that were purified were in good agreement with the expected sizes based on the calculation from the amino acid sequences including the hexahistidine tag.

3.8. Determination of IgE antibodies against E. coli expressed Cul o 2–Cul o 7

Sera of 103 IBH affected and 100 healthy horses were evaluated for IgE levels (OD_{450nm} values) against *E. coli* expressed Cul o 2- Cul o 7. Test results were considered positive when above the cut-off level (*i.e.* mean + 3 times the standard deviation of IgE levels of healthy horses).

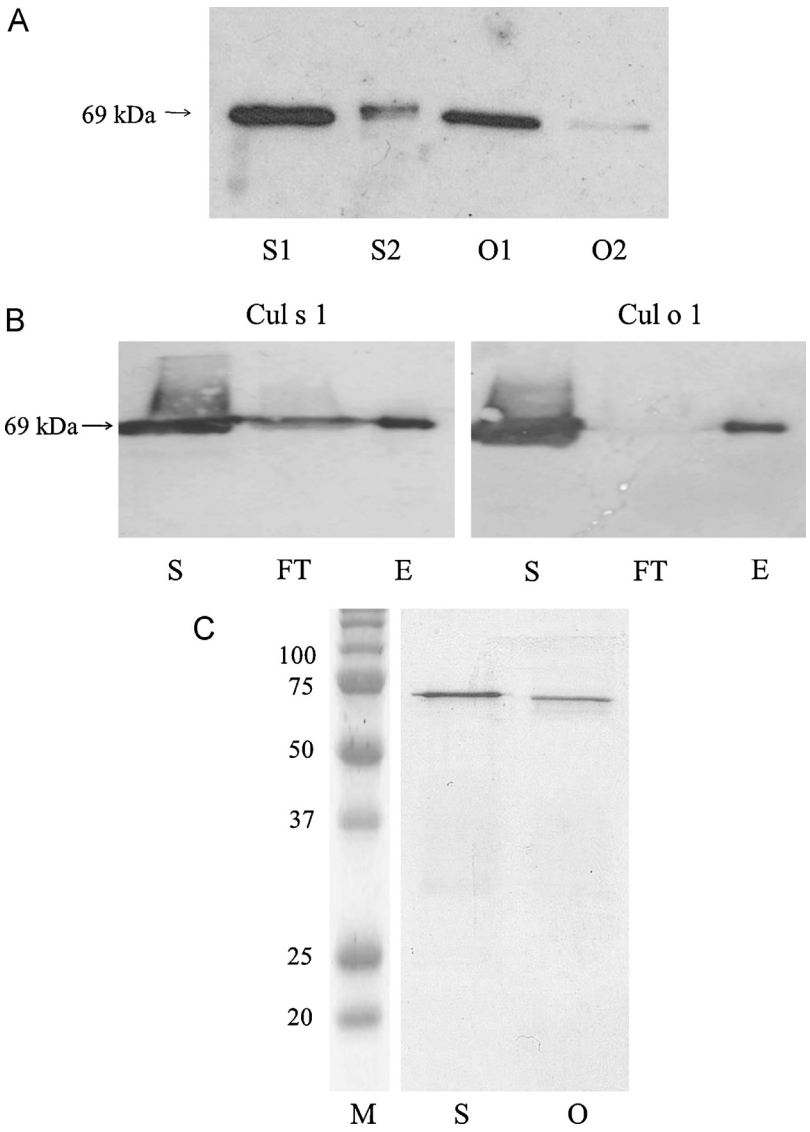


Fig. 2. (A) Western blot analysis of cell-free supernantants (1) and cell lysates (2) of 72 h post infection with recombinant baculovirus expressing Cul o1 or Cul s1. The presence of Cul o 1 (O) and Cul s 1 (S) was detected by an anti-[His]6 antibody, followed by horseradish peroxidase-labelled detection antibody. (B) Western blot analysis of purified baculovirus expressed Cul o 1 and Cul s 1 from the cell-free supernatants. S: concentrated supernatant prior to purification; FT: flow-through (unbound proteins); E: eluted fraction after dialysis. (C) Silverstaining of purified baculovirus expressed Cul s 1 (S) and Cul o 1 (O).

The frequency of positive test results varied between the allergens, with Cul o 6 resulting in the lowest percentage of positive tested IBH affected horses (38%) and Cul o 5 the highest percentage of positive tested IBH affected horses (67%). Percentages of positive tested clinically healthy horses were between 2 and 6%, depending on the allergen (Fig. 6).

From the 103 IBH affected horses, 95 reacted with at least one of the *E. coli* recombinant allergens (Cul o 1–Cul o 7) while 15 out of 100 healthy horses showed IgE reactivity, resulting in a test sensitivity of 92% and specificity of 85%. The eight horses that were tested negative with the allergens were also tested negative using WBE, except for one horse (results not shown) (van der Meide et al., 2012)).

3.9. Intradermal test

An intradermal test was performed on 7 IBH affected horses and 7 healthy horses to test whether recombinant allergens and *C. obsoletus* WBE were able to induce type I hypersensitivity (IgE mediated) immediate (0.5 h and 1 h) and late phase (4 h and 24 h) skin reactions *in vivo*.

At 30 min post injection all horses showed a clearly visible wheal at the histamine injection site, which remained visible until 4 h after injection (Table 3). After 24 h these wheals had disappeared. The wheal diameters induced by the histamine injection and the negative control (saline) did not differ significantly between IBH affected and healthy horses for any of the time points.

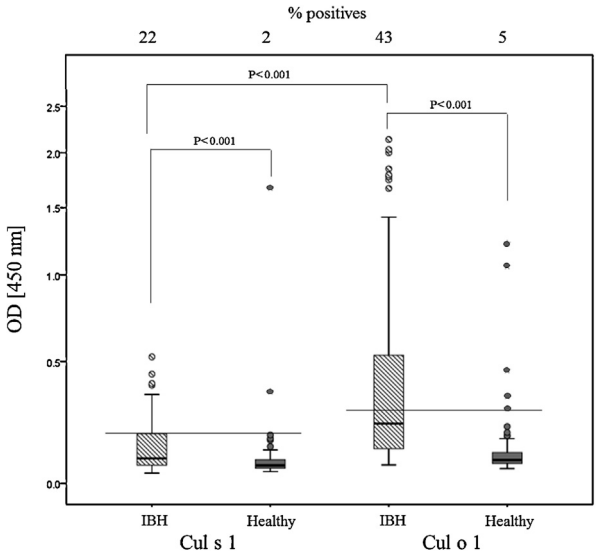


Fig. 3. IgE levels presented as OD_{450} values against *E. coli* expressed Cul s 1 and Cul o 1 in sera diluted 1:5 of 103 IBH affected horses and 100 healthy control horses. Results are presented in box plots. The horizontal lines inside of the box is the median of the measurements. The horizontal lines crossing the box plots are the cut-off values determined by the mean OD_{450} values of the healthy horses + 3 × standard deviation. The bottom and top of the box are the 25th and 75th percentile, respectively. The end of the whiskers represent the minimum and maximum value. The dots represent the outliers. Lines connecting two box plots denote a statistically significant difference with the *p* value on top. The percentage of positive tested horses for each allergen is indicated on the top.

The most noticeable skin reactions for WBE and allergens were observed after 1 h and 4 h (Table 3, Fig. 7). All IBH affected horses showed positive skin reactions for WBE from time point 1 h onwards, while only 1 healthy horse reacted positive at 0.5 and 24 h and 2 healthy horses at 1 h and 4 h. There was a significant difference between IBH affected and healthy horses for WBE at each time point ($p < 0.01$).

For all allergens at least one IBH affected horse showed a positive type I hypersensitivity skin reaction, but for Cul o 2 this was only the case in the late phase (at 24 h) of the reaction (Table 3). The highest number of IBH affected horses with positive skin reactions was observed for the baculovirus expressed allergens and Cul o 4 and Cul o 5. For these allergens, positive skin reactions were also observed for 1 or 2 healthy horses at different time points.

A significant difference between IBH affected and healthy horses was only observed with the wheal diameters provoked by Cul o 4 after 1 h. However, the means of the wheal diameters of the IBH affected horses were always larger than the ones of the healthy horses and for each allergen and WBE.

There was no significant difference between the wheal diameters provoked by Cul o 1 *versus* Cul s 1 for either the baculovirus or *E. coli* expressed variants. Although more horses were tested positive with the baculovirus expressed Cul o 1 or Cul s1 compared to *E. coli* expressed Cul o 1 or Cul s 1, there was no significant difference between the wheal diameters provoked by baculovirus *versus E. coli* produced proteins.

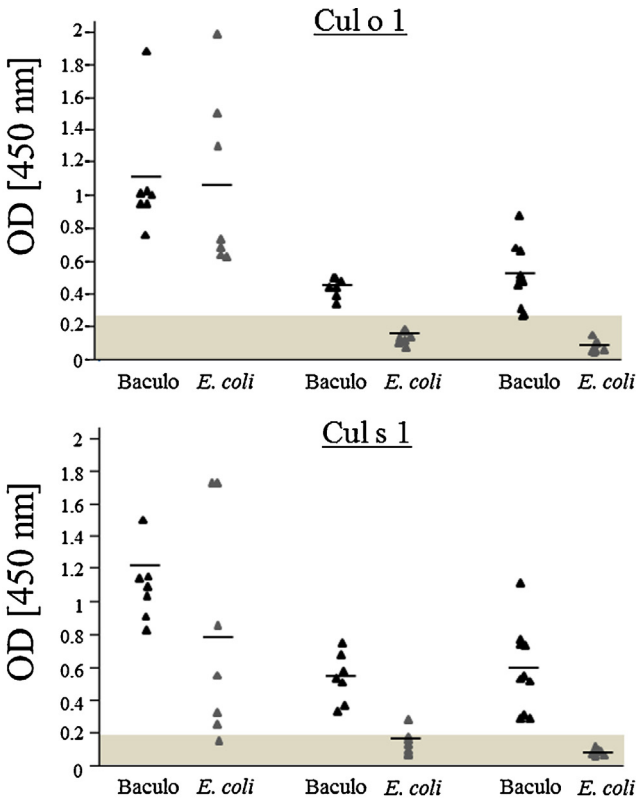
4. Discussion

In this study we report the identification and production of 7 *C. obsoletus* recombinant allergens that can be used to diagnose IBH with a sensitivity of 92% and specificity of 85% when combining the results of each single allergen IgE ELISA. A maltase from *C. sonorensis* (Cul s 1) was the first allergen described to be associated with IBH (Langner et al., 2009), but *C. sonorensis* is not native in the Netherlands. To evaluate if the maltase from *C. obsoletus* (Cul o 1) is better for IBH diagnosis in the Netherlands than Cul s 1, both were expressed in *E.coli* as well as in a baculovirus/insect cell expression system. Horses with IBH showed a higher average IgE-binding to *E.coli* produced Cul o 1 than to Cul s 1 and more IBH affected horses scored positive with Cul o 1 (43%) than with Cul s 1 (22%). These findings are in agreement with previously reported data, where ELISA with WBE of *C. obsoletus* resulted in better diagnosis than such an ELISA with WBE of *C. sonorensis* (van der Meide et al., 2012). Some horses did show IgE-binding with Cul s 1, which is most likely due to cross-reactivity between shared epitopes between Cul o 1 and Cul s 1, since the horses that were used in this study are unlikely to be exposed to the North-American *C. sonorensis*. Reactivity to Cul s 1 was also observed previously in a histamine release test and intradermal skin test using horses from Germany that most likely have not been exposed to *C. sonorensis* (Langner et al., 2009). The maltase from *C. nubeculosus* (Cul n 8) was used in a similar ELISA as described here by (Schaffartzik et al., 2011) and resulted in a positive test result for 21.7% of the IBH-affected horses, comparable to the reactivity that we found for Cul s 1, whereas we found over 40% of our horses to be tested positive with Cul o 1. Although we tested different horses, it is likely that for these horses in Switzerland better results would be obtained with the maltase from *C. obsoletus* as it is also one of the most abundant species found in this country (Casati et al., 2009).

To compare potential differences between the immunoreactivity between the maltase expressed in a prokaryotic system and the same maltase expressed in an eukaryotic system, we produced this allergen in both *E. coli* as well as a baculovirus expression system. The

E. coli system has the advantage that it is easy to handle and often results in high expression levels of the recombinant protein. However proteins expressed in *E. coli* often accumulate in insoluble inclusion bodies, and therefore require chemical refolding procedures to obtain the protein in a native, biological active form (Dudler et al., 1992; Schmidt and Hoffman, 2002). The baculovirus expression system is characterized by post-translational processing and therefore often results in the production of (glycosylated) biologically active soluble proteins (Schmidt and Hoffman, 2002).

Baculovirus expression can display better IgE-binding to certain allergens compared to *E. coli* expressed allergens (McDermott et al., 2000; Soldatova et al., 1998). However, some studies report no difference between *E. coli* and baculovirus expressed allergens in relation to their IgE-binding properties *in vitro* or skin prick reactivity *in vivo* in allergic individuals (Kronqvist et al., 2000; Olsson et al., 1998) and an *E. coli* expressed allergen has shown to have similar



Group	A	B	C
Clinical signs	+	+	-
ELISA WBE *C. obsoletus*	+	+	-
ELISA *E. Coli* Cul o 1	+	-	-

Fig. 4. IgE levels presented as OD_{450} values against Cul o 1 (upper graph) and Cul s 1 (lower graph) expressed in baculovirus or *E. coli* of (A) seven clinically IBH affected horses, tested positive with *C. obsoletus* WBE as well as for *E. coli* Cul o 1; (B) seven clinically IBH affected horses, tested positive with *C. obsoletus* WBE, but negative for *E. coli* Cul o 1. (C) Ten clinically healthy horses, tested negative with *C. obsoletus* WBE as well as for *E. coli* Cul o 1. The cut-off level is highlighted in light gray and determined by the mean OD_{450} values of the healthy horses + 3 × standard deviation. The triangles represent the individual values. The horizontal lines are the means of the IgE levels.

Table 3
Results intradermal skin test of seven IBH affected horses and seven healthy control horses to *C. obsoletus* WBE and recombinant allergens.

Injection	IBH affected horses				Healthy horses			
	Number of positives[a]				Number of positives[a]			
	0.5 h	1 h	4 h	24 h	0.5 h	1 h	4 h	24 h
Saline	0	0	0	0	0	0	0	0
Histamine	7	7	7	0	7	7	7	0
WBE	5	7	7	7	1	2	2	1
Cul s 1 *E. coli*	1	3	2	1	0	0	0	0
Cul o 1 *E. coli*	0	5	1	0	0	0	0	0
Cul s 1 *Baculo*	2	4	4	1	0	0	2	1
Cul o 1 *Baculo*	2	3	4	0	0	0	1	0
Cul o 2	0	0	0	1	0	0	0	0
Cul o 3	0	2	2	0	0	0	0	0
Cul o 4	2	5	4	4	1	0	1	1
Cul o 5	2	3	6	5	0	1	2	1
Cul o 6	1	1	1	1	0	0	0	0
Cul o 7	2	3	3	3	0	0	0	0

[a] Cut-off = highest wheal diameter provoked by saline.

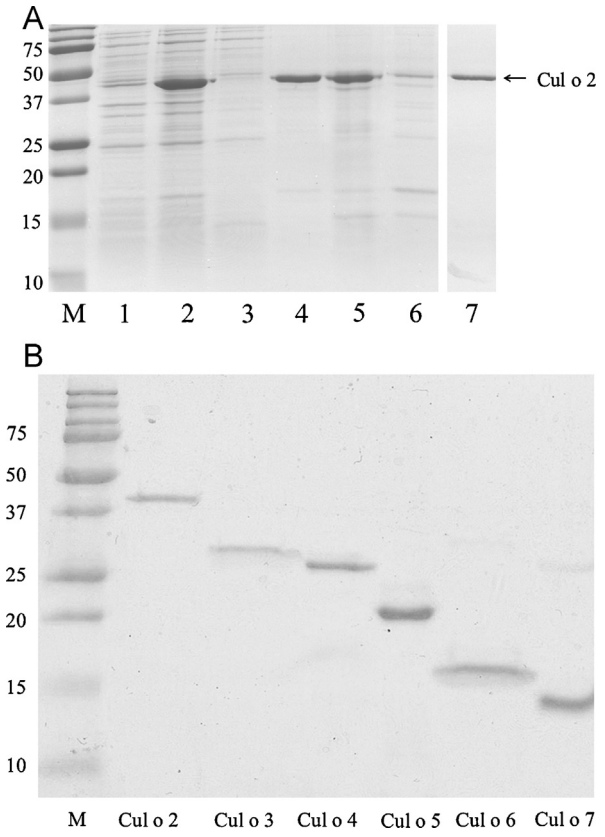


Fig. 5. (A) Typical example of induction and purification of *E. coli* expressed protein (Cul o 2). (1) Uninduced protein; (2) induced with IPTG; (3) soluble fraction; (4) insoluble fraction (inclusion bodies); (5) solved inclusion bodies; (6) flow through (unbound proteins); (7) refolded and dialysed eluted fraction. Proteins were separated by SDS-PAGE (12.5% gel) and stained with Gelcode coomassie blue-stain. B. SDS-PAGE (12.5% gel) and Gelcode coomassie blue-stain of the *E. coli* expressed proteins Cul o 2 t/m Cul o 7.

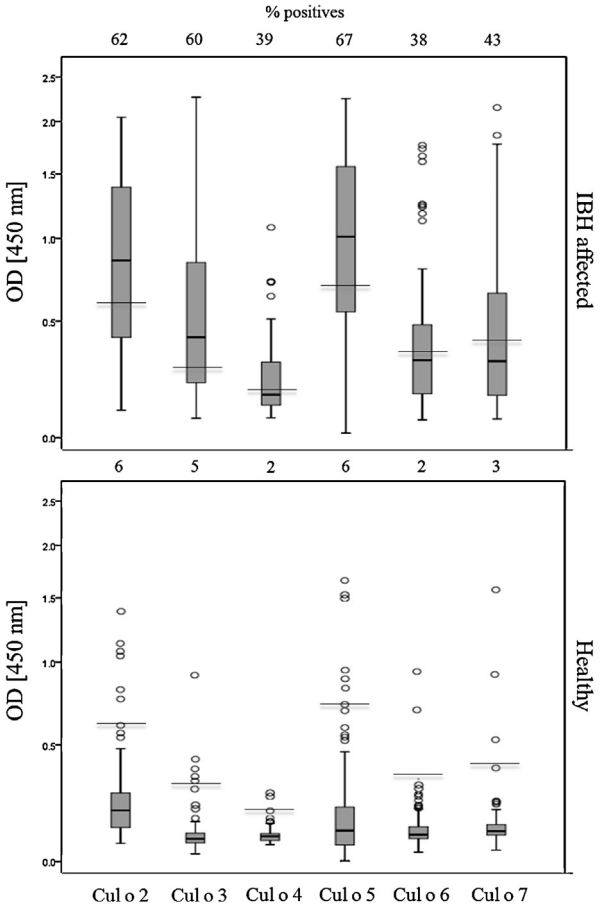


Fig. 6. IgE levels presented as OD_{450} values against Cul o 2–Cul o 7 in sera diluted 1:5 of 103 IBH affected horses and 100 healthy control horses. Results are presented in box plots. The horizontal lines inside of the box is the median of the measurements. The horizontal lines crossing the box plots are the cut-off values determined by the mean OD_{450} values of the healthy horses + three times the standard deviation (excluding OD values above 1.0). The bottom and top of the box are the 25th and 75th percentile, respectively. The end of the whiskers represent the minimum and maximum value. The dots represent the outliers. The percentage of positive tested horses for each allergen is indicated on the top.

immunological properties as its natural form (Dudler et al., 1992). This suggests that for some allergens glycosylation and correct folding obtained by an eukaryotic system might be important for biological activity, but for other allergens a prokaryotic system might be sufficient. Here, we report Cul o 1 and Cul s 1 expressed in *E. coli* to be at least as suitable in IgE-binding compared to the same allergens expressed in the baculovirus expression system in horses with IBH.

The high background values within the healthy horses tested in ELISA with baculovirus expressed Cul o 1 and Cul s 1, compared to E. coli expressed proteins, might be due to antibodies to N-glycans present on the baculovirus expressed allergens. Insect cells produce structural different N-glycans than mammalian cells (Harrison and Jarvis, 2006). Horses are constantly exposed to different types of insects and therefore the presence of pre-existing antibodies against insect produced N-glycans is not unlikely. This kind of antibody will not bind to the unglycosilated *E. coli* expressed allergens. Also non-specific binding to minor contaminations in the purified protein fraction can not be excluded, but the allergens seemed pure as shown by SDS-PAGE and silverstaining

Because of the easy-handling of *E. coli* cells and the high expression of Cul o 1 and Cul s 1 compared to the baculovirus expression system, 6 additional allergens from *C. obsoletus* were expressed solely in *E. coli*. These allergens were found based on similarity searches within the *C. obsoletus* RNA database using previously described allergens from *C. nubeculosus* (Schaffartzik et al., 2010, 2011). Homologous genes of four described allergens from *C. nubeculosus* were not found in the RNA database. Either homologous genes for these proteins are not present in the *C. obsoletus* specific RNA database or the database is not yet complete.

Sequence analysis revealed different percentages of amino acid similarities between the individual cloned allergens and the homologous allergens from *C. nubeculosus* or *C. sonorensis* (Langner et al., 2009; Schaffartzik et al., 2010, 2011). The highest similarity was found for maltase and lowest similarity with a secreted salivary protein (Cul n 4, 33%). Even though Cul o 1 and Cul s 1 have a high amino

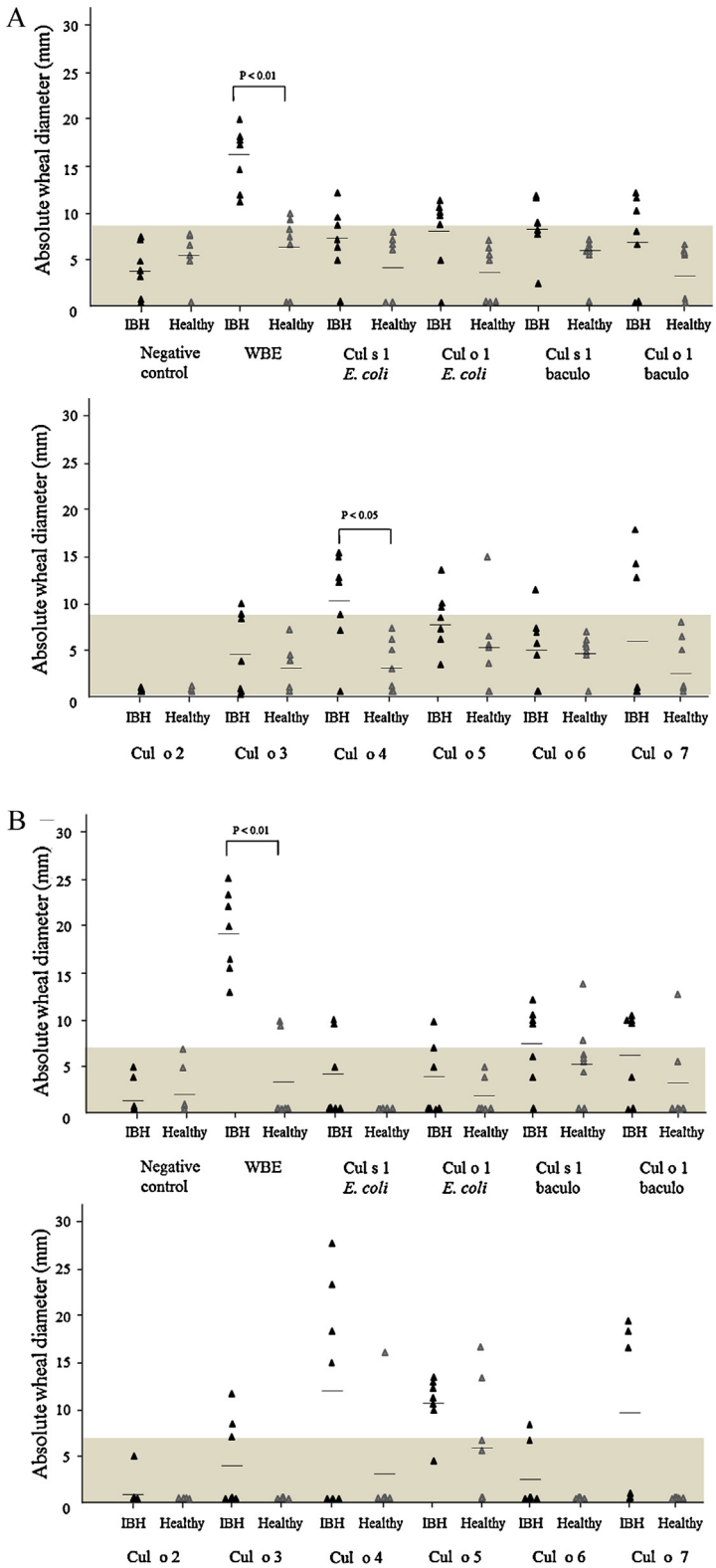


Fig. 7. Absolute wheal diameters in 7 IBH affected and 7 healthy horses following injection with *Culicoides* WBE or allergen (10 μg/ml) after 1 h (A) or 4 h (B). The cut-off level is highlighted in light gray and arbitrarily determined by the negative control wheal diameters. The triangles represent the individual values. The horizontal lines are the means of the wheal diameters.

acid similarity (78%), Cul o 1 clearly has a higher IgE reactivity with IBH affected horses compared to Cul s 1. We found lower sequence similarities for the allergens Cul o 2–Cul o 7 with their homologous *C. nubeculosus* allergen (33–75%) and therefore the difference between reactivity with IgE for these allergens might even be higher when compared with their homologous allergen derived from *C. nubeculosus*. We already previously reported *C. obsoletus* to have more IgE-binding proteins compared to *C. nubeculosus* and *C. sonorensis* as detected by western blot analysis in all tested IBH affected horses (van der Meide et al., 2012).

The IgE-binding capacity of each allergen was determined by ELISA for both IBH affected as wells as healthy horses. IBH-affected horses that were tested positive ranged from 38 to 67%, depending on the allergen. Although we tested different horses, we report higher percentages of positive tested horses with our *C. obsoletus* allergens, than was reported for the corresponding allergen from *C. nubeculosus* by Schaffartzik et al. (2010, 2011). However, to be more conclusive about the cross-reactivity between these allergens, they should be tested together in the same ELISA for the same horses.

When IgE-binding ELISA results to Cul o 1 to Cul o 7 are combined, a sensitivity of 92% and a specificity of 85% is obtained, which is close to the sensitivity and specificity obtained with the same ELISA using *C. obsoletus* WBE (van der Meide et al., 2012). Horses that did not show positive test results with any of the seven recombinant allergens, also did not show positive test results with WBE (data not shown). A possible explanation for the lack of reactivity, might be that these horses are sensitised to other specific allergens *e.g.* from other insects that were able to cause IBH but were not included in this study. Schaffartzik et al. (2011) reported 11 allergens from *C. nubeculosus*, while we were able to express 7 allergens based on similarity searches with these allergens. Cul n 3 and Cul n 5 were both reported to show IgE reactivity with a large percentage of horses (Schaffartzik et al., 2011) and therefore similar *C. obsoletus* allergens might exist that are crucial for diagnosis of some horses by ELISA. However we were not yet able to find complete genes coding for the proteins similar to Cul n 3 and Cul n 5 in the *C. obsoletus* RNA database and were therefore not able to clone and express these allergens.

Thus, our findings show that serum IgE of IBH affected horses recognize the *E. coli* produced *C. obsoletus* allergens and can therefore be used as a diagnostic tool for IBH in horses where *C. obsoletus* is the most abundant species. However, to provide information about the capability of the recombinant allergens to induce a Type I hypersensitivity reaction (immediate and late phase) *in vivo*, an intradermal test was performed. Type I skin reactions were observed for all allergens within IBH affected horses.

Some healthy horse showed type I skin reactions with WBE, Cul o 4, Cul o 5 and the baculovirus expressed allergens. These horses might be asymptomatic cases of IBH. Indeed, type I hypersensitivity reactions after intradermal injection with *Culicoides* allergens are more often reported in healthy horses (Schaffartzik et al., 2010, 2011; Sloet van Oldruitenborgh-Oosterbaan et al., 2009; Wagner et al., 2009). Thus, horses might be sensitized to *Culicoides* allergens without showing clinical signs of IBH. On the whole, greater skin reaction in response to the recombinant allergens were observed for IBH affected horses compared to healthy horses. Interestingly, Type I skin reactions in IBH affected horses, provoked by WBE or individual allergens were stronger after 1 and 4 h than after 30 min. Other studies also reported greater wheals after 1 and 4 h compared to 30 min after intradermal injections with *Culicoides* WBE (Halldórdsóttir et al., 1989; Sloet van Oldruitenborgh-Oosterbaan et al., 2009). This suggests that the immediate reaction induced by degranulation of mast cells in horses provoked by allergens of *Culicoides* spp probably peaks after 30 min. Wheals measured at 4 h are more likely to be caused by the influx of other inflammatory cells, which contribute to the late phase of the response.

In conclusion, the 7 recombinant allergens Cul o 1–Cul o 7, expressed in *E. coli*, provide essential tools to determine IgE-binding of horses to individual components of *C. obsoletus* and are capable to induce immediate and delayed skin reactions in horses *in vivo*. These allergens can be useful to determine the sensitisation profile (component resolved diagnosis) of horses and may contribute to the development of allergen-specific immunotherapy for individual horses suffering from IBH.

Acknowledgements

The authors want to thank all horse owners for their cooperation. We would like to thank the owners of Stal Stónas, Hannah van Litsenburg en Bart Savenije, for their cooperation with the intradermal test. We thank dr. Bettina Wagner for supplying monoclonal anti-horse IgE 176, Marleen Scheer for participating in the collection of blood samples, Anouk Schurink for helping collecting *Culicoides obsoletus*, and Els Roode, Christine van Altena, Johanna Roth and Vera Korneeva for technical support.

This work is financially supported by the Dutch Technology Foundation STW (STW-NWO), the Dutch Federation of horse breeding ('s-Hertogenbosch, The Netherlands) and ALK-Abelló/Artu Biologicals (Almere, The Netherlands).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2013.03.005.

References

Anderson, G.S., Belton, P., Jahren, E., Lange, H., Kleider, N., 1996. Immunotherapy trial for horses in British Columbia with *Culicoides* (Diptera: *Ceratopogonidae*) hypersensitivity. J. Med. Entomol. 33, 458–466.

Barbet, J.O.Y.L., Bevier, D., Greiner, E., 1990. Specific immunotherapy in the treatment of *Culicoides* hypersensitive horses: a double blind study. Equine Vet. J. 22, 232–235.

Baselgia, S., Doherr, M.G., Mellor, R., Torsteinsdottir, S., Jermann, T., Zurbriggen, A., Jungi, T., Marti, E., 2006. Evaluation of an *in vitro* sulphidoleukotriene release test for diagnosis of insect bite hypersensitivity in horses. Equine Vet. J. 38, 40–46.

Boorman, J., 1985. Rearing *Culicoides obsoletus* (Diptera, Ceratopogonidae) on agar cultures of nematodes. Prog. Clin. Biol. Res. 178, 229–231.

Campbell, J.A., Pelham-Clinton, E.C., 1960. A taxonomic review of the British species of "Culicoides" Latreille (Diptera, Ceratopogonidae). Royal Society of Edinburgh, Edinburgh.

Casati, S., Racloz, V., Delecolle, J.C., Kuhn, M., Mathis, A., Griot, C., Staerk, K.D.C., Vanzetti, T., 2009. An investigation on the *Culicoides* species composition at seven sites in southern Switzerland. Med. Vet. Entomol. 23, 93–98.

Dudler, T., Chen, W.Q., Wang, S.S., Schneider, T., Annand, R.R., Dempcy, R.O., Crameri, R., Gmachl, M., Suter, M., Gelb, M.H., 1992. High-level expression in *Escherichia coli* and rapid purification of enzymatically active honey bee venom phospholipase A2. Biochim. Biophys. Acta 1165, 201–210.

Fadok, V.A., Greiner, E.C., 1990. Equine insect hypersensitivity: skin test and biopsy results correlated with clinical data. Equine Vet. J. 22, 236–240.

Halldórdsóttir, S., Larsen, H.J., Mehl, R., 1989. Intradermal challenge of Icelandic horses with extracts of four species of the genus *Culicoides*. Res. Vet. Sci. 47, 283–287.

Harrison, R.L., Jarvis, D.L., 2006. Protein N-glycosylation in the baculovirus-insect cell expression system and engineering of insect cells to produce "mammalianized" recombinant glycoproteins. In: Bonning, B.C. (Ed.), Insect Viruses: Biotechnological Applications, vol. 68. , pp. 159–191.

Hellberg, W., Wilson, A.D., Mellor, P., Doherr, M.G., Torsteinsdottir, S., Zurbriggen, A., Jungi, T., Marti, E., 2006. Equine insect bite hypersensitivity: immunoblot analysis of IgE and IgG subclass responses to *Culicoides nubeculosus* salivary gland extract. Vet. Immunol. Immunopathol. 113, 99–112.

Hopkins, R.F., Esposito, D., 2009. A rapid method for titrating baculovirus stocks using the Sf-9 easy titer cell line. Biotechniques 47, 785–787.

Kaba, S.A., Salcedo, A.M., Wafula, P.O., Vlak, J.M., van Oers, M.M., 2004. Development of a chitinase and v-cathepsin negative bacmid for improved integrity of secreted recombinant proteins. J. Virol. Methods 122, 113–118.

Kronqvist, M., Johansson, E., Magnusson, C.G.M., Olsson, S., Eriksson, T.L.J., Gafvelin, G., Van Hage-Hamsten, M., 2000. Skin prick test and serological analysis with recombinant group 2 allergens of the dust mites L-destructor and T-putrescentiae. Clin. Exp. Allergy 30, 670–676.

Langner, K.F.A., Jarvis, D.L., Nimtz, M., Heselhaus, J.E., McHolland, L.E., Leibold, W., Drolet, B.S., 2009. Identification, expression and characterisation of a major salivary allergen (Cul s 1) of the biting midge *Culicoides sonorensis* relevant for summer eczema in horses. Int. J. Parasitol. 39, 243–250.

McDermott, M.J., Weber, E., Hunter, S., Stedman, K.E., Best, E., Frank, C.R., Wang, R., Escudero, J., Kuner, J., McCall, C., 2000. Identification, cloning, and characterization of a major cat flea salivary allergen (Cte f 1). Mol. Immunol. 37, 361–375.

Meulenbroeks, C., van der Meide, N.M.A., Zaiss, D.M.W., Sloet van Oldruitenborgh-Oosterbaan, M.M., van der Lugt, J.J., Smak, J., Rutten, V.P.M.G., Willemse, T., 2013. Seasonal differences in cytokine expression in the skin of Shetland ponies suffering from insect bite hypersensitivity. Vet. Immunol. Immunopathol. 151, 147–156.

Olsén, L., Bondesson, U., Broström, H., Olsson, U., Mazogi, B., Sundqvist, M., Tjälve, H., Ingvast-Larsson, C., 2010. Pharmacokinetics and effects of cetirizine in horses with insect bite hypersensitivity. Vet. J. 187, 347–351.

Olsson, S., Van Hage-Hamsten, M., Whitley, P., Johansson, E., Hoffman, D.R., Gafvelin, G., Schmidt, M., 1998. Expression of two isoforms of Lep d 2, the major allergen of Lepidoglyphus destructor, in both prokaryotic and eukaryotic systems. Clin. Exp. Allergy 28, 984–991.

Quinn, P., Baker, K., Morrow, A., 1983. Sweet itch: responses of clinically normal and affected horses to intradermal challenge with extracts of biting insects. Equine Vet. J. 15, 266–272.

Schaffartzik, A., Marti, E., Crameri, R., Rhyner, C., 2010. Cloning, production and characterization of antigen 5 like proteins from *Simulium vittatum* and *Culicoides nubeculosus*, the first cross-reactive allergen associated with equine insect bite hypersensitivity. Vet. Immunol. Immunopathol. 137, 76–83.

Schaffartzik, A., Marti, E., Torsteinsdottir, S., Mellor, P.S., Crameri, R., Rhyner, C., 2011. Selective cloning; characterization; and production of the *Culicoides nubeculosus* salivary gland allergen repertoire associated with equine insect bite hypersensitivity. Vet. Immunol. Immunopathol. 139, 200–209.

Schmidt, M., Hoffman, D.R., 2002. Expression systems for production of recombinant allergens. Int. Arch. Allergy Immunol. 128, 264–270.

Schurink, A., van Grevenhof, E.M., Ducro, B.J., van Arendonk, J.A.M., 2009. Heritability and repeatability of insect bite hypersensitivity in Dutch Shetland breeding mares. J. Anim. Sci. 87, 484–490.

Sloet van Oldruitenborgh-Oosterbaan, M.M., Van Poppel, M., De Raat, I.J., Van Den Boom, R., Savelkoul, H.F.J., 2009. Intradermal testing of horses with and without insect bite hypersensitivity in the Netherlands using an extract of native *Culicoides* species. Vet. Dermatol. 20, 607–614.

Soldatova, L.N., Crameri, R., Gmachl, M., Kemeny, D.M., Schmidt, M., Weber, M., Mueller, U.R., 1998. Superior biologic activity of the recombinant bee venom allergen hyaluronidase expressed in baculovirus-infected insect cells as compared with *Escherichia coli*. J. Allergy Clin. Immunol. 101, 691–698.

van den Boom, R., Driessen, F., Streumer, S.J., Sloet van Oldruitenborgh-Oosterbaan, M.M., 2010. The effect of a supplement containing sunflower oil, vitamins, amino acids, and peptides on the severity of symptoms in horses suffering insect bite hypersensitivity. Tijdschr. Diergeneeskd. 135, 520–525.

van den Boom, R., Kempenaars, M., Sloet van Oldruitenborgh-Oosterbaan, M.M., 2011. The healing effects of a topical phytogenic ointment on insect bite hypersensitivity lesions in horses. Tijdschr. Diergeneeskd. 136, 20–26.

van der Aa, L.M., Chadzinska, M., Tijhaar, E., Boudinot, P., Verburg-van Kemenade, B.M.L., 2010. CXCL8 chemokines in teleost fish: two lineages with distinct expression profiles during early phases of inflammation. PLOS ONE 5, doi:10.1371/journal.pone.0012384.

van der Meide, N.M.A., Meulenbroeks, C., van Altena, C., Schurink, A., Ducro, B.J., Wagner, B., Leibold, W., Rohwer, J., Jacobs, F., Sloet van Oldruitenborgh-Oosterbaan, M.M., Savelkoul, H.F.J., Tijhaar, E., 2012. Culicoides obsoletus extract relevant for diagnostics of insect bite hypersensitivity in horses. Vet. Immunol. Immunopathol. 149, 245–254.

van der Rijt, R., van den Boom, R., Jongema, Y., Sloet Oldruitenborgh-Oosterbaan, M.M., 2008. Culicoides species attracted to horses with and without insect hypersensitivity. The Veterinary Journal 178, 91–97.

Wagner, B., Miller Jr., W.H., Erb, H.N., Lunn, D.P., Antczak, D.F., 2009. Sensitization of skin mast cells with IgE antibodies to *Culicoides* allergens occurs frequently in clinically healthy horses. Vet. Immunol. Immunopathol. 132, 53–61.

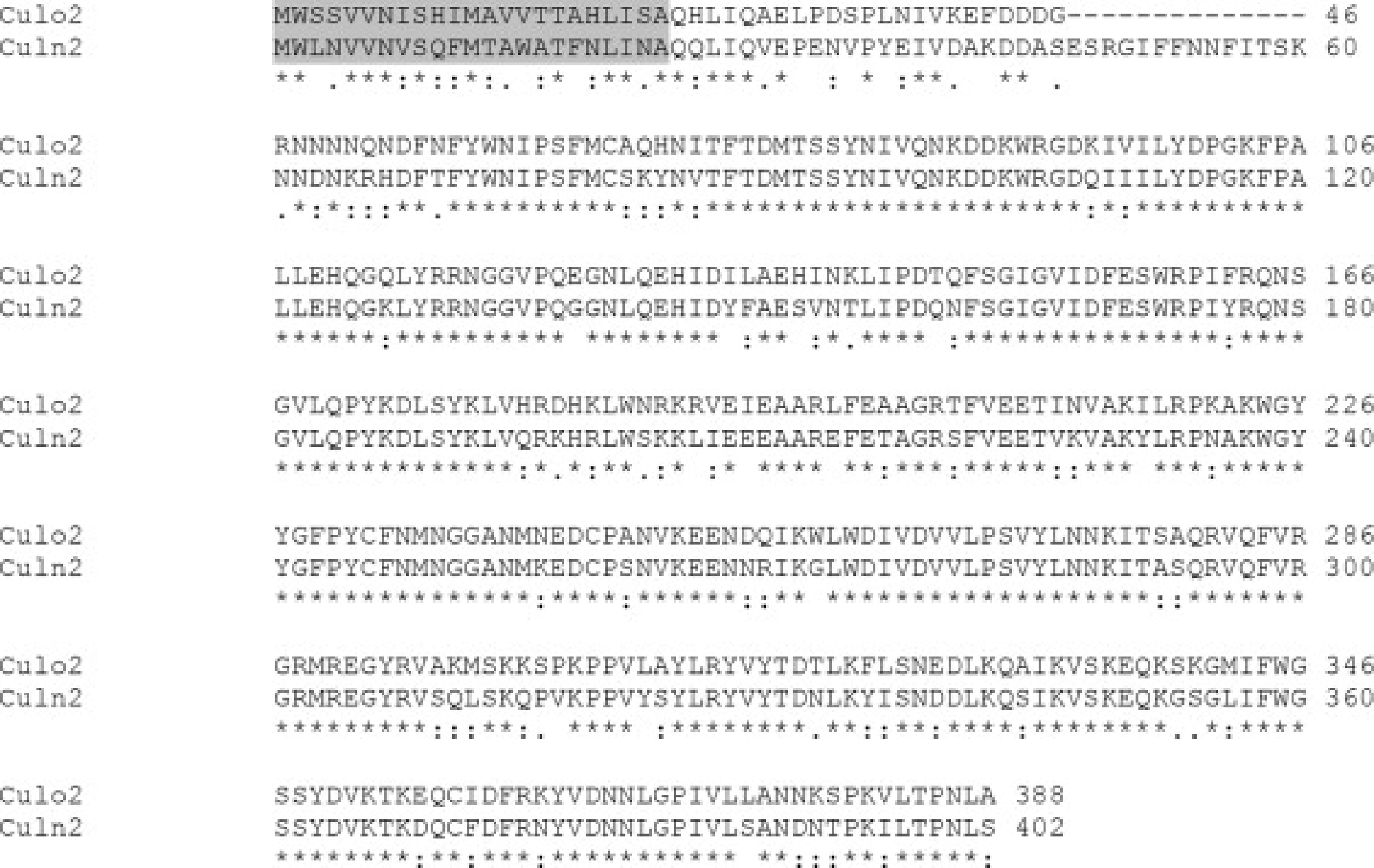
Wagner, B., Miller, W.H., Morgan, E.E., Hillegas, J.M., Erb, H.N., Leibold, W., Antczak, D.F., 2006. IgE and IgG antibodies in skin allergy of the horse. Vet. Res. 37, 813–825.

Wagner, B., Radbruch, A., Rohwer, J., Leibold, W., 2003. Monoclonal anti-equine IgE antibodies with specificity for different epitopes on the immunoglobulin heavy chain of native IgE. Vet. Immunol. Immunopathol. 92, 45–60.

Wilson, A.D., Heesom, K.J., Mawby, W.J., Mellor, P.S., Russell, C.L., 2008. Identification of abundant proteins and potential allergens in *Culicoides nubeculosus* salivary glands. Vet. Immunol. Immunopathol. 122, 94–103.

Zerbino, D.R., Birney, E., 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. 18, 821–829.

```
Culo1           MIIFKTLTILISSACLVLTVPQGAREKDWWETGNFYQVYPRSFMDSDGDGIGDLKGVQQK 60
Culs1           MIPFKKLTILLSIACSVLAAPEGAREKDWWEIGNFYQVYPRSFMDSDGDGVGDLKGISEK 60
                ** **.****:* ** **:.*:********* ********************:*****:.:*

Culo1           VAYLKEIGMDGVWLSPIFDSPMADFGYDISNFTKVYPKFGDMQSIQELIDECNKNDIKLI 120
Culs1           VGYLKEIGMDGVWLSPIFDSPMADFGYDISNFTKVFPQFGDLSSIDELVAEFNKKDMKLI 120
                *.*************************************:*:***:.**:**: * **:*:***

Culo1           LDFVPNHSSDECEWFKKSVKRDPYYSDFYIWHPGKPNPNGGRNLPPTNWVSAFRSSAWEW 180
Culs1           LDFVPNHTSDQCEWFKKSIQRDPEYNDYYIWHPGKPNPDGGRNLPPTNWVSAFRSSAWEW 180
                *******:**:********::*** *.*:**********:************************

Culo1           NDERQEYYLHQFLAAQPDLNYRNPEVVKTMKNVLKFWLNKGINGFRIDAVPYLFEVGPDA 240
Culs1           NEERGEYYLHQFLAQQPDLNYRNPKVVETMKNVLRFWLSKGINGFRIDAVPYLFEVGPDA 240
                *:** ********* *********:**:******:***.**********************

Culo1           SGNYPDEPLTYKCADPLSQCYYDHIYTQMRSETVNMVTQWRTVMDDWQKENGGPTRVLLV 300
Culs1           NGNYPDEIETHACSDPLSQCYLYHDYTQNRPETFEMVTEWRATLEEFKQKNGGPTRVLMV 300
                .******  *: *:*******  * *** *.**.:***:**:.:::::::********:*

Culo1           EAYAPLAKVVEIYGENGKLNGAEFPFNFEFLNYLGPKSDAADFKAIIDNYLATIPQGSTP 360
Culs1           EAYAPLTKVIQIYGQNGQLNGAQIPFNFEFLNNLGAVSNARDFKDVIDNYLSTIPEGATP 360
                ******:**::***:**:****::******** **. *:* *** :*****:***:*:**

Culo1           NWVQGNHDQHRSASRLGPQKADAVNMLLQVLPGAAITYYGEELAMEDVFIPWYRTVDPQA 420
Culs1           NWVQGNHDQHRSASRLGPQKADAVNMLLQVLPGAAVTYYGEELAMEDVFVPWSRTVDPQA 420
                ************************************:**************:** *******

Culo1           CTTDPVTFHKYSRDPARTPMIWDDQRNAGFSNANSTWLPVGPDFRSNNVELQRSERSSHL 480
Culs1           CTTDPNIFHAKSRDPARTPMIWTSQKNAGFSSSNYTWLPTGPDYRKNNVEVQRSQRGSHL 480
                *****  **  *********** .*:*****.:* ****.***:*.****:***:*.***

Culo1           NIFKKLMQLRKQDILMYGDYDSVLLNDDVLAVKRELSNNRTLIAVLNLGFTEQMVKLSTI 540
Culs1           NIFKKLTQLRKQDILMYGTYDSYLANDDVLVIKREIENNRTLIAVLNLGFTEQVVNLNLN 540
                ****** *********** *** * *****.:***:.*****************:*:*.

Culo1           S-QWQIPQVMEVYTASVGSGMFERQPVYTDEVYVAAGIGVVLDYQVGKRLPQPRGDDPGQ 599
Culs1           DRDWKVPERMEVATASVNAGMFERQPVVTSEVYVSAGVGVVLDYQVGRQIPEPRGDDPGL 600
                . :*::*: *** ****.:******** *.****:**:*********::*:*******

Culo1           YEQ 602
Culs1           YE- 602
                **
```


Culo2 MWSSVVNISHIMAVVTTAHLISAQHLIQAELPDSPLNIVKEFDDDG---------- 46
Culn2 MWLNVVNVSQFMTAWATFNLINAQQLIQVEPENVPYEIVDAKDDASESRGIFFNNFITSK 60
Culo2 RNNNNQNDFNFYWNIPSFMCAQHNITFTDMTSSYNIVQNKDDKWRGDKIVILYDPGKFPA 106
Culn2 NNDNKRHDFTFYWNIPSFMCSKYNVTFTDMTSSYNIVQNKDDKWRGDQIIILYDPGKFPA 120
Culo2 LLEHQGQLYRRNGGVPQEGNLQEHIDILAEHINKLIPDTQFSGIGVIDFESWRPIFRQNS 166
Culn2 LLEHQGKLYRRNGGVPQGGNLQEHIDYFAESVNTLIPDQNFSGIGVIDFESWRPIYRQNS 180
Culo2 GVLQPYKDLSYKLVHRDHKLWNRKRVEIEAARLFEAAGRTFVEETINVAKILRPKAKWGY 226
Culn2 GVLQPYKDLSYKLVQRKHRLWSKKLIEEEAAREFETAGRSFVEETVKVAKYLRPNAKWGY 240
Culo2 YGFPYCFNMNGGANMNEDCPANVKEENDQIKWLWDIVDVVLPSVYLNNKITSAQRVQFVR 286
Culn2 YGFPYCFNMNGGANMKEDCPSNVKEENNRIKGLWDIVDVVLPSVYLNNKITASQRVQFVR 300
Culo2 GRMREGYRVAKMSKKSPKPPVLAYLRYVYTDTLKFLSNEDLKQAIKVSKEQKSKGMIFWG 346
Culn2 GRMREGYRVSQLSKQPVKPPVYSYLRYVYTDNLKYISNDDLKQSIKVSKEQKGSGLIFWG 360
Culo2 SSYDVKTKEQCIDFRKYVDNNLGPIVLLANNKSPKVLTPNLA 388
Culn2 SSYDVKTKDQCFDFRNYVDNNLGPIVLSANDNTPKILTPNLS 402

```
Culo3       MFRICLFTVL--CVNFVVATDFCDRKLCRRQIEPNVYQNIPHIGCNHDGRNSPACPSDAK 58
Culn1       MIKKLSIVILFSCISFVLSTNFCNKDLCKRQNGPQSFTYLKHIGCRHTGKNANTCPRDAK 60
            *::   :.:*  *:.**::*:**::.**:**  *: :  : ****.* *:*: :** ***

Culo3       ILPMSTKRKNLILRVHNRLRNKVALGQLPGYPKAVRMPILRWDDELAYLAELNVKQCEMK 118
Culn1       ILPMSTKRKNLILKVHNRLRNKVALGKLPGFPKAARMPILRWDDELAYLAELNVKQCKME 120
            **************:************:***:***.**********************:*:

Culo3       HDQCRNTDKFRYAGQNLAYIGGGKEPNAVRIKTLVRAWFDEYKDANSSFIDKYRSHPNGK 178
Culn1       HDQCRNTDKFKYAGQNLAYTMGTPQKNAVRIKKLIRAWFKEHENATASFIGKYRDHPQGR 180
            **********:********  *  : ******.*:****.*:::*.:***.***.**:*:

Culo3       AIGHFTAMVQDRTDTVGCAILRHTKNTYFFLACNYSFTNMVKDKVYTRGAKSCSKCRTGC 238
Culn1       VIGHFTAMIQDRTDTVGCAILRHSGNKYFFLACNYGFTNMMNQKVYTKSNKSCSKCRTGC 240
            .*******:***************: *.********.****::****:. **********

Culo3       SPVYKGLCKPHEYVNPDPDEDLD 261
Culn1       SKVYKGLCAAHEYVNPDPYEDLD 263
            * ****** .******** ****
```

```
Culo4       MNIKSTIIVIAIISAVYAADHQSQKAVAVPSYTYRRP--STKIIGGAPAFSHQFPWQASI 58
Culn11      MKSLKFIFIFIAFSVLEVYCNEVQEVIAVPPRNYTKPRYSIKIVGGSPAREHQFPWQAS- 59
            *:  . *:::  :*.: .  :: *:.:***. .* :*  * **:**:** .******** 

Culo4       TVTACSGDWCSLCGGSLISRKHVLTAAHCTKGLSSFTIGLGSNTRNRPAVTVVAKSKTEH 118
Culn11      -VTSCSEKSCLICGGSLISRRYVLTAAHCTAGLSEFQIGVGSTDRNRPAFSVFSYGKIEH 118
             **:** . * :********::******** ***.* **:**. *****.:*.: .* ** 

Culo4       PKYNPESLANDVSIITLSLNVNLNNNIKVISLANSGIGTLVNRNAFVSGYGKTSSSSEGS 178
Culn11      PQYNPEKLFNDIAILKLPRPIRFIKGVETIKLARNNV-SYTNQNATLSGYGKTSSSGPVS 177
            *:****.* **::*:.*.  :.: :.::.*.**...: : .*:** :*********.  * 

Culo4       NTLNYLSMRLISNSDCYKVFGPQIYSTTLCAVARSSVHKNVCSGDSGGPLVIKRNGNYVQ 238
Culn11      DKLNFVDMRIISNQECANEYS-IVRNTTLCALGRNRSVENACQGDSGGPLVVKERSSFVQ 236
            :.**::.**:***.:* : :.  : .*****:.*.   :*.*.********:*....:** 

Culo4       VGIVSFVAKVGCDAGFPSGYARVSSFRNWITQNMN-- 273
Culn11      VGIVSFVAKVGCEYGYPSGYARVSSFYKWIANNTDLN 273
            ************: *:********** :**::* :
```

```
Culo5           MKRVILGGIILFLTSKFVLSFDLSDALPGHITDDMTTTEKPTNVAAATADFSDDDLLAVI 60
Culn7           -MQAITVAIILILSVVFVGCFDLRDALPGHVTDDQTTTVKPG-VVQRTADFSDDDLWAVL 58
                  :.*  .***:*:  ** .*** ******:*** *** **  *.  ********* **:

Culo5           NESKKKVKSS---DVKPTNKLLTNVKNKLESVDFKKIDQKVASLLIPIYKKASEAILNWI 117
Culn7           KEDNKKSKSSSGSQAKDKQTILKTMQAKLKSIKFDQVDTKLASRISSPYVQSANSVMQWI 118
                :*.:** ***   :.* .:.:*.::: **:*:.*.::* *:** : . * :::::::**

Culo5           SMTGAAIETSPCFEKIDRIFTKILQDTSKYFTPARAN--IAQKSYVEKLTKALSTIRQCI 175
Culn7           SSITNIKKVVNCFDNIEIYFQSIINEASNNYTKKKVQLQVVEKQFKEKLYATLSSTRQCL 178
                *      :.  **:*:  * .*::::*: :*  :.:  :.:*.: ***  :**: ***:

Culo5           ISKIKK- 181
Culn7           ISRLKAK 185

                **::*
```

```
Culo6           MKFLIPLLAVIGLISCS--FVPKESDYDTNNLKKVQKCELKHQVNDSEKAIWWAWKIPAK 58
Culn9           MKLIILIFTIIGLALCAPDEVPSGDQYDTDNLLIVRDCEEEKDLPAAEKAEWWDWKVPAN 60
                **::* ::::***  *:   **. .:***:**  *:.** ::::  :*** ** **:**:

Culo6           PARCYLACVMSAFKWIDKKGNPKLD-AIKKSYKAVGHEDSAPNQQFIKKNCGRKLPKNPC 117
Culn9           PTECYIDCIFQKYGWLSGEGGSIVNSAVEASYAAVGHSNPS------LASCN--PSKSGC 112
                *:.**: *::. : *:. :*.. :: *:: ** ****.:.:        .*.   .*. *

Culo6           DKAILLYACFLVRSENLQHFKDAFDGKPNTI 148
Culn9           SKADELYACLLN--ADGQKFKDAFDGKRDAK 141
                .**  ****:*    : *:******** ::
```

```
Culo7          MKFSVSKLSLLLCITILCICFATAAPQWQISELSEQSNVIKCSNENNFGIYKELCQFLKK 60
Culn4          MKFPT----FLILAFFLSLYISSTASRRKHFRHLKRIEAANDCPAKNSGTYQKVCKQLQK 56
               ***..    :*:   :*.: ::::*.: :  .  :: :. : .  :* * *:::*: *:*

Culo7          IYIKAPDEDLGSYLRGGLQSAANRLLDPTVTLPKNTLKNVEDCMKNFQAVINEYNVVALK 120
Culn4          YYVLTPDDKLGSYLKGGLQEAANRVLTPVSKSDKITFDIVQNCLKNFQVMVNKHNKEALR 116
                *: :**:.*****:****.****:* *. .  * *:. *::*:****.::*::*  **:

Culo7          KYQECDDQCAKQAGQLFENDVSKTAGRMGDCIVSLAALH 159
Culn4          KYRECKKECFTEVGKEFSSALDKTGVQIAECLNESL--- 152

               **:**.:* .:.*: *.. :.**. ::.:*: .
```