

# Identification of abundant proteins and potential allergens in *Culicoides nubeculosus* salivary glands

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## Abstract

IgE-mediated type 1 hypersensitivity reactions to the bites of insects are a common cause of skin disease in horses. Insect bite hypersensitivity (IBH) is most frequently associated with bites of *Culicoides* spp. and occurs in all parts of the world where horses and *Culicoides* coexist. The main allergens that cause IBH are probably some of the abundant proteins in the saliva of *Culicoides* associated with blood feeding. Western blots of *Culicoides* proteins separated by 1D gel-electrophoresis detected strong IgE responses in all horses with IBH to antigens in protein extracts from wild caught *Culicoides*, but only weak responses to salivary antigens from captive bred *C. nubeculosus* which may reflect important differences among allergens from different species of *Culicoides* or differences between thorax and salivary gland antigens. 2D electrophoresis and mass spectrometry were used to identify several of the abundant proteins in the saliva of *C. nubeculosus*. These included maltase, members of the D7 family, and several small, basic proteins associated with blood feeding. The most frequently detected IgE-binding proteins were in a group of proteins with  $pI > 8.5$  and mass 40–50 kDa. Mass spectrometry identified two of these allergenic proteins as similar to hyaluronidase and a heavily glycosylated protein of unknown function that have previously been identified in salivary glands of *C. sonorensis*.

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## 1. Introduction

Allergic reactions in horses to the bites of *Culicoides* spp. are common throughout the world except for a few locations such as Iceland where *Culicoides* are not found (Halldorsdottir and Larsen, 1991). Several reports have shown that horses with insect bite hypersensitivity (IBH) have positive reactions when challenged by intra-dermal injection with crude, whole body protein extracts of *Culicoides* (Halldorsdottir

et al., 1989; Kolm-Stark and Wagner, 2002; Quinn et al., 1983) and peripheral blood leukocytes of 87% of IBH but only 3% control horses released sulphidoleukotrienes after incubation with *C. nubeculosus* extract (Baselgia et al., 2006). The exact allergens present in *Culicoides* have not so far been identified, but abundant proteins in the saliva are likely candidates. Analysis of 708 ESTs from a cDNA library of *C. sonorensis* salivary gland mRNA detected a limited number of highly abundant sequences many of which had secretory signal sequences (Campbell et al., 2005). These sequences encode proteins some of which have anticoagulant or digestive functions related to blood feeding, but several of the most abundant transcripts currently have no

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identified function. Nevertheless, it would seem probable that these abundant transcripts encode proteins that will be present in concentrations sufficient to induce an immune response and so act as allergens. In a prior study we showed by immuno-histology that all horses exposed to *Culicoides* develop antibodies that react with *Culicoides* salivary glands (Wilson et al., 2001). Some horses also showed clear antibody staining of other organs including structures associated with the insect's eye, central nervous system and a population of large polygonal cells in the abdomen. Subsequent immuno-blotting of *C. nubeculosus* salivary gland extracts with serum from allergic and control horses confirmed that all exposed horses can have IgG antibodies to *Culicoides* antigens but only allergic horses have IgE antibodies (Hellberg et al., 2006). Overall the evidence supports the salivary gland as a source of the allergens that cause IBH, but is not clear to what extent nonsalivary gland-derived antigens may also be involved, possibly as insects become crushed into the inflamed skin when allergic horses rub in response bites.

In several studies of IBH, the test antigens have been derived from *C. nubeculosus*, a species that although widely distributed is not the most common in many locations. Whether or not the allergens of all *Culicoides* species are sufficiently conserved for every allergic horse to exhibit an equally severe reaction to their bites is not known. One study demonstrated that horses in Canada with IBH had positive reactions to both North American and to European varieties of *Culicoides*, supporting a conclusion that allergens are conserved between species (Anderson et al., 1993). In contrast, other studies have found that not all horses with IBH reacted to challenge with whole body extract from individual species of *Culicoides* (Halldorsdottir et al., 1989; Kolm-Stark and Wagner, 2002). This may indicate that individual horses can become sensitised to the *Culicoides* that are locally common without reacting to all species.

The central question remains: what are the identities of the exact antigens that horses with IBH are reacting to? In this paper we begin to address this question by identifying some of the most abundant proteins in salivary gland extracts and determining which commonly react with IgE in serum from allergic horses.

## 2. Materials and methods

### 2.1. Serum samples

Ten millilitres blood samples were collected by vacutainer from the jugular vein of 18 horses affected

with IBH; subjects included several different horse breeds including some Icelandic horses. Control samples were taken from three companions of the allergic horses that had been exposed to *Culicoides* as well as from three native Icelandic horses in Iceland that had never been exposed to *Culicoides*. The blood was allowed to clot, centrifuged and the serum removed then stored at  $-20^{\circ}\text{C}$ . IgE was affinity purified from horse serum using an anti-equine IgE monoclonal antibody 1C12 (Wilson et al., 2006) coupled to protein G sepharose using DMP cross-linker ([www.piercenet.com](http://www.piercenet.com)). Bound IgE was eluted in 0.2 M glycine pH 3, 1 M Tris was immediately added to the eluate to raise the pH to neutral. The concentration of IgE in the eluted samples was measured by ELISA as previously described (Wilson et al., 2006).

### 2.2. Preparation of *Culicoides* protein extracts

Wild type *Culicoides* were captured with a pooter as they alighted to feed on horses. It is not possible to accurately identify *Culicoides* spp. solely on morphology of the females, however based on size and wing spot pattern the majority belonged to the *Obsoletus* group with lesser number of insects from the *Pulicaris* group ([www.iah.bbsrc.ac.uk/bluetongue/culicoides/index.html](http://www.iah.bbsrc.ac.uk/bluetongue/culicoides/index.html)). The insects were freeze dried, the thorax of dried insects was separated by dissection under a binocular microscope, placed in a small volume of PBS containing a cocktail of protease inhibitors ([www.sigma-aldrich.com](http://www.sigma-aldrich.com)). The whole thorax extract was prepared by freeze thawing the tissue three times then removing the insoluble material by spinning through a  $0.22\text{ }\mu\text{m}$  micro-centrifuge filter (Nanosep, [www.pall.com](http://www.pall.com)).

*C. nubeculosus* came from a laboratory bred colony maintained at Institute for Animal Health, Pirbright, Surrey, UK. Females were selected and put into Tris buffered saline (TBS) containing 0.05% Tween (Sigma) and dissected under a binocular microscope using extra-fine forceps and 30G needles. Salivary glands were transferred into 1.5 ml polypropylene tubes containing 10 mM Tris pH 7 and protease inhibitor cocktail (Sigma) in batches of 200 glands. The samples were frozen and thawed twice to ensure complete lysis then spun through a  $0.2\text{ }\mu\text{m}$  micro-centrifuge filter (Nanosep, Pall). The filtrate was then concentrated to  $20\text{ }\mu\text{l}$  in a 3 kDa cut-off micro-centrifuge filter, diluted in 1 ml 5 mM Tris buffer pH 7 and concentrated to  $20\text{ }\mu\text{l}$  three further times to ensure minimal residual salt concentration. The protein concentration and size distribution of the proteins was

measured by Experion micro-electrophoresis (Bio-Rad). The remainder of the samples were stored at  $-70^{\circ}\text{C}$  until use.

### 2.3. 1D SDS-PAGE

Proteins samples equivalent to five pairs of *C. nubeculosus* salivary glands or five wild midge thoraxes were diluted to  $3.75\ \mu\text{l}$  with water,  $2.5\ \mu\text{l}$   $4\times$  LDS Nu-page loading buffer per sample was added ([www.invitrogen.com](http://www.invitrogen.com)). The samples were loaded onto a 10-well 4–12% Nu-page bis-tris gel then run in MES buffer pH 7.3 at 200 V for 45 min according to the manufacturers protocol (Nu-page Invitrogen). At the end of the run the proteins were transferred to PVDF membranes in a Nu-page wet blot module (Invitrogen) using bis-tris buffer pH 7.2, 20% methanol, the transfer was run for 10 h at a constant 10 V. The blotted membranes were stained with ponceau-S to reveal the protein tracks and cut into strips.

### 2.4. 2D electrophoresis

Twenty microlitres of concentrated salivary gland extracts equivalent to approximately 200 glands were made up to  $200\ \mu\text{l}$  with 7 M urea, 2 M thiourea, 4% CHAPS, 0.002% Bromophenol blue, 0.5% (v/v) IPG buffer pH 3–11NL (GE Healthcare, [www.gelifesciences.com](http://www.gelifesciences.com)) and 1.2% (v/v) Destreak reagent (GE Healthcare). Samples were left at room temperature for 1 h prior to loading onto 11 cm immobiline DryStrip first dimension IPG strips pH3–11 nonlinear (GE Healthcare) by passive re-hydration.

Following overnight re-hydration, the strips were focussed using the Ettan IPGphor (GE Healthcare) according to the manufacturers instructions (in brief, 500 V for 1 h, 1000 V for 1 h and 8000 V for 2 h). The focussed IPG strips were then incubated for 15 min in 10 ml SDS equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% SDS, 0.002% Bromophenol blue) containing 100 mg DTT and for a further 15 min in 10 ml equilibration buffer containing 250 mg iodoacetamide.

The equilibrated strips were applied to the surface of vertical 12.5% SDS-polyacrylamide gels and proteins separated in the second dimension using the Ettan DALT II separation unit (GE Healthcare) at 0.2 W/gel for 1 h, 0.4 W/gel for 1 h and then 20 W/gel until completion.

For mini-gel samples of *Culicoides* proteins equivalent to 10 salivary glands each were focused using the ZOOM IEF system Invitrogen with 4–10 pH linear gradient strips. The voltage gradient was ramped to 175 V over 15 min then ramped to 2000 V over 45 min

and thereafter run at constant 2000 V for 90 min at a maximum current of 5 mA/strip. On completion of the run the strips were equilibrated into LDS loading buffer containing Nu-page reducing agent for 15 min then in LDS buffer containing 125 mM iodoacetimide for a further 15 min. The equilibrated strips were loaded on a 4–12% bis-tris gradient gel, then run at 200 V for 45 min in MES buffer (Invitrogen).

After protein separation, gels were either fixed and stained for total protein using SYPRO ruby protein gel stain (Invitrogen), according to the manufacturer's instructions, or transferred to PVDF membrane using Nu-page transfer module as described above.

### 2.5. Amino terminal sequencing and mass spectroscopy

Protein spots of interest were excised from the gel using the Investigator ProPic automated spot picker and digested with trypsin using the ProGest automated digestion unit (Perkin-Elmer Life Sciences, [www.perkinelmer.com](http://www.perkinelmer.com)). The resulting peptides were analysed using a 4700 MALDI-Tof/Tof mass spectrometer ([www.appliedbiosystems.com](http://www.appliedbiosystems.com)) to give a peptide mass fingerprint and peptide sequence information, which was searched against the MSDB database using the Mascot search engine from Matrix Science to identify the protein.

For N-terminal sequencing, proteins were transferred from the 2D gel onto PVDF membrane using a semi-dry transfer blotter. The membranes were stained with Coomassie brilliant blue, selected spots were excised from the membrane and sequenced on an Applied Biosystems Procise 492 cLC using standard cycles.

### 2.6. Western blotting

Blots of *Culicoides* proteins were blocked with 5% non-fat cow's milk in 10 mM Tris 150 mM NaCl (TBS) pH 7.5. The membranes were incubated overnight with affinity purified IgE from allergic and control horses diluted to a standard concentration of 100 ng/ml in TBS 0.05% Tween, 5% non-fat milk. Following washing in several changes of TBS 0.05% Tween buffer the membranes were incubated for 2 h with purified monoclonal anti-equine IgE 3H10 (Wilson et al., 2006) then donkey anti-mouse IgG horseradish peroxidase absorbed with equine serum proteins ([www.jacksonimmuno.com](http://www.jacksonimmuno.com)). The membranes were washed in TBS Tween and developed with ECL chemiluminescent substrate using Amersham Hyperfilm ECL (GE Healthcare).

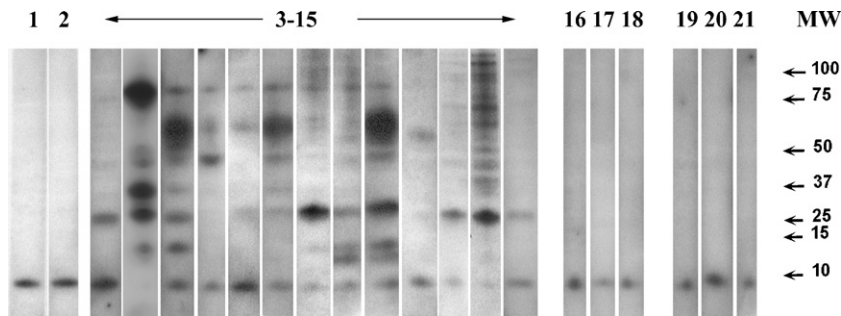


Fig. 1. Western blot of whole thorax extract from wild type *Culicoides* probed with 100 ng/ml equine IgE. Lane 1 anti-mouse peroxidase conjugate. Lane 2 monoclonal anti-equine IgE and anti-mouse peroxidase conjugate. Lanes 3–15 probed with samples of 100 ng/ml affinity purified IgE from horses with *Culicoides* hypersensitivity. Lanes 16–18 probed with IgE from healthy horses exposed to *Culicoides*. Lanes 19–21 probed with IgE from Icelandic horses never exposed to *Culicoides*.

### 3. Results

#### 3.1. 1D blotting

The results from blots of wild type midge whole thorax probed with affinity purified equine IgE antibodies are shown in Fig. 1. Conjugate control lanes 1 and 2 show that the donkey anti-mouse antibody cross-reacted with a small peptide in all the blots which acts as an alignment marker for the other strips. Neither the healthy horses exposed to *Culicoides* bites nor any of the Icelandic horses that had never been bitten had IgE antibodies to *Culicoides* proteins. In contrast all of the allergic horses had IgE antibodies that recognised proteins on the blots. Films for these blot strips were exposed for times varying between 2 min and 10 min during development. Individual sera show considerable variation in intensity of their reaction, therefore, the data in Fig. 1 shows some of the highly reactive sera that have been exposed for 2 min compared to the weakest samples which have been exposed for 10 min. As well as varying in intensity the individual allergic horses reacted to different number of proteins in midge thorax with sizes varying from 15 kDa to 100 kDa.

Analysis of proteins in *C. nubeculosus* salivary glands by micro-electrophoresis, detected a peak corresponding to the most abundant proteins with a mass 15–17 kDa. Several smaller peaks corresponding to less abundant proteins were detected at 20–25 kDa, 45–50 kDa and 67 kDa (Fig. 2, lane 2). The total protein concentration of the salivary gland extract was 1238 ng/μl. Four microlitres was loaded onto each lane and separated by SDS-PAGE equivalent to between 20 ng and 200 ng for each individual protein peak. Fig. 2 (lanes 3–7) shows five of the samples of IgE from allergic horses that gave the most intense staining on whole thorax extracts of wild type midges tested by

Western blot on *C. nubeculosus* salivary gland extracts. The strongest reactions occurred to the 45–50 kDa band but close examination of the blots revealed several other fainter bands. Overall the reactions with *C. nubeculosus* salivary proteins were weak compared to the whole wild type thorax proteins, suggesting that the IgE antibody responses of horses to proteins from different species of midge may vary. Both the number and molecular weight range of the saliva proteins reacting with IgE were less than that seen using whole thorax, which supports a role for nonsalivary proteins as allergens.

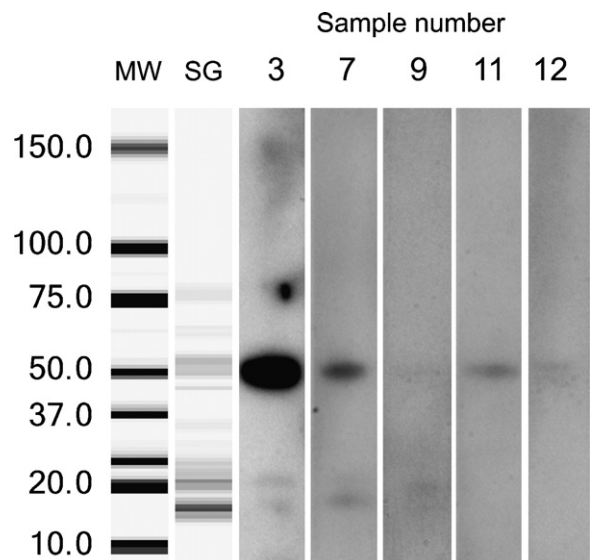


Fig. 2. Lane MW position of molecular weight markers in Experion electrophoresis 'virtual gel' output. Lane SG, 'virtual gel' output of *C. nubeculosus* salivary gland extract separated by Experion electrophoresis. The remaining lanes are Western blots of *C. nubeculosus* salivary gland proteins probed with affinity purified 100 ng/ml IgE from horses with *Culicoides* hypersensitivity, each is labelled with the number of the corresponding lane in Fig. 1.

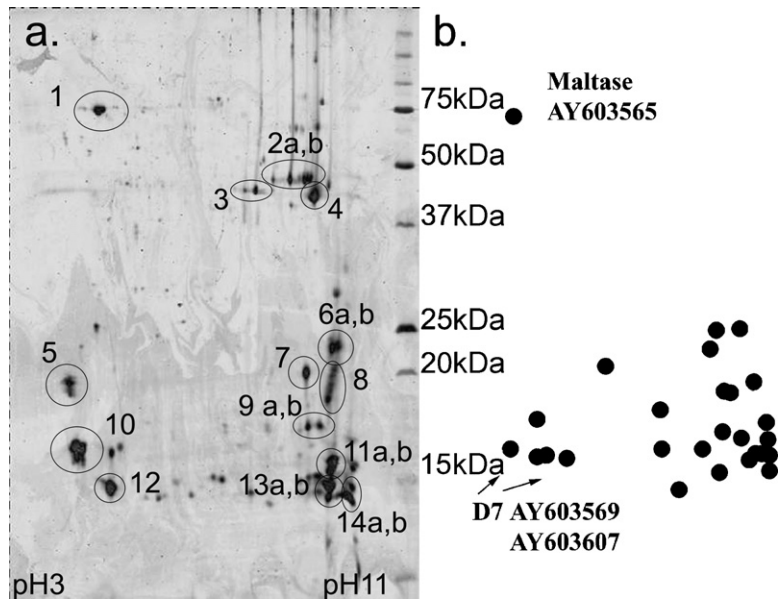


Fig. 3. (a) A Sypro-Ruby stained gel of protein extracted from 100 *C. nubeculosus* salivary glands by 2D electrophoresis. The range of pI from 3 to 11 is indicated across the bottom and the molecular weight markers appear on the right-hand side with the corresponding mass written to the right of the main bands. Spots selected for mass spectroscopy are numbered. (b) A scale drawing showing the predicted pI and mass of proteins encoded by the 27 most abundant ESTs from *C. sonorensis* salivary glands (Campbell et al., 2005). Only a few of the predicted proteins from *C. sonorensis* can be matched with confidence to actual protein spots in *C. nubeculosus* saliva. Also note the absence of any predicted proteins from *C. sonorensis* between 37 kDa and 50 kDa with a pI of 8.5–10.

### 3.2. Identification of abundant salivary gland proteins in *C. nubeculosus*

Fig. 3a shows a negative image of a Sypro-Ruby stained maxi gel on which proteins from 100 salivary glands have been separated by 2D electrophoresis, further resolving the bands seen in 1D gels. Fig. 3b which shows the predicted locations of secretory proteins encoded by the abundant salivary gland mRNA transcripts identified in an EST library from *C. sonorensis* (Campbell et al., 2005). A few of the predicted mass/pI locations in Fig. 3b can be matched to actual spots on the *C. nubeculosus* 2D gel, most clearly spot 1 matches the predicted position of maltase (AY603565) and spots 10 and 12 match the predicted location of the abundant D7 proteins (AY603569 and AY603607). There are a large number of small proteins with a high pI but they are too close together to be matched with any confidence. Notably there are no abundant transcripts from *C. sonorensis* that yield predicted protein with a pI > 8.5 and a mass between 37 kDa and 50 kDa that could correspond to the actual proteins spots 2–4 in *C. nubeculosus* saliva. This information along with a combination of data from amino terminal sequencing and mass spectroscopy were used to identify several homologous proteins in *C.*

*nubeculosus* saliva (Table 1). For mass spectroscopy protein scores of over 40 were considered to be significant, a more precise identification was available in some cases where ion scores confirmed the identity of the peptide amino acid sequence.

In addition amino terminal sequence data were obtained from two spots, in both cases the leader peptide had been cleaved from the protein product in the saliva. The remainder of the spots gave no N-terminal sequence data probably due to modified terminal amino acids blocking the sequencing reaction. Although many of the other spots had mass spectroscopy hits on *Culicoides* salivary gland proteins, the protein scores were too low to assign them identities. Spots 2b and 3 are included despite their low protein score, as a separate study has confirmed the presence of proteins homologous to AY603639, and AY603640 with a mass of 40 kDa secreted in *C. nubeculosus* saliva (Langner et al., 2007). Spot 4 is also included as there is only partial hyaluronidase sequence data to match and the low protein score would therefore be expected.

### 3.3. Western blotting

The results of Western blots of 2D maxi gels with serum samples from four horses that had reacted strongly

Table 1  
Identity of abundant protein in salivary glands of *C. nubeculosus*

| Spot number <sup>a</sup> | N-terminal  | MS proteins score | MS/MS sequence (ion score C.I.%)                                                  | Accession number <sup>b</sup> |
|--------------------------|-------------|-------------------|-----------------------------------------------------------------------------------|-------------------------------|
| 1                        | NA          | 223               | SSAWEWNEER (50, 100)<br>NLPPTNWVSAFR (79, 100)<br>DDPGEGYRNDYYIWHPGKPNP (54, 100) | Maltase AY603565              |
| 2b                       | Blocked     | 24                | NA                                                                                | AY603640                      |
| 3                        | Blocked     | 36                | NA                                                                                | AY603639                      |
| 4                        | Blocked     | 19                | NA                                                                                | AY603562                      |
| 10                       | DEAPSGDQYDT | NA                | NA                                                                                | D7 AY603569                   |
| 11a                      | NA          | 99                | GGLQEAAANR (19, 99.9)<br>YYVLTPDDKLGSYLK (48, 100)                                | AY603631/AY603632             |
| 11b                      | NA          | 41                | NA                                                                                | AY603632/AY603631             |
| 12                       | AKVLPKGTIEE | 15                | NA                                                                                | D7 AY603607/AY603608          |
| 13b                      | NA          | 45                | NA                                                                                | AY603579                      |
| 14b                      | NA          | 44                | NKCFVEAGR (14, 99.9)                                                              | AY603637/AY603638             |

<sup>a</sup> Spot number refers to those shown in Fig. 3a.

<sup>b</sup> Accession number refers to homologous EST from *C. sonorensis* (Campbell et al., 2005). MS protein scores of greater than 40 are considered significant. Spots 10 and 12 are included on the basis of N-terminal data. Spots 2a and 3 are included as there is published data corroborating their identity (Langner et al., 2007). Spot 4 is included as the available sequence data is partial therefore a low score would be expected.

in 1D gels of *C. nubeculosus* salivary proteins are shown in Fig. 4. None of these horses recognised the abundant acidic proteins corresponding to maltase or either of the small acidic D7 proteins. Three of the horses (a–c) all reacted with members of the group of basic antigens with a mass of around 40–50 kDa that are shown in Fig. 3 as spots 2a, 2b, 3 and 4. Horse (a) also recognised a cluster of medium-sized basic proteins designated as spot 8.

Horse (d) recognised several proteins from the cluster of medium-sized basic proteins including a spot 8 and possibly also spots 7 and 9 although it is difficult to assign the exact spots with confidence.

To expand the number of horses tested we went on to repeat this procedure with “-D” mini-blot loaded with 10 salivary glands. In total we probed a further 14 mini-blot with IgE from allergic horses but overall the results were disappointing with only three horses giving readable reactions, in all cases this was against the same group of basic proteins with a mass of around 40–50 kDa as those detected in large format blots.

To show the limited extent to which the abundant D7 proteins are identical an alignment of the sequence AY603607 of a D7 from *C. sonorensis* with SG55 a homologous D7 from *C. nubeculosus* (C. Russell, unpublished) is shown in Fig. 5. Although 60% of the amino acids in this sequence are identical between the two proteins, there are few stretches of more than six identical amino acids in a row.

#### 4. Discussion

Hypersensitivity to insect bites is probably the commonest skin disease in horses, with an incidence

varying from 3% to 60% depending on the population studied (Braverman et al., 1983; Brostrom et al., 1987; Halldorsdottir and Larsen, 1991; Littlewood, 1998; McCaig, 1973; Riek, 1953; van Grevenhof et al., 2007). Because accurate diagnosis and possible immunotherapy will depend on having the correct antigens it is important to identify the exact proteins to which horses react. This task is complicated by prior evidence that not all allergens are exclusively derived from the insect's saliva (Wilson et al., 2001) and that not all horses react to every species of *Culicoides* (Halldorsdottir et al., 1989; Wilson et al., 2001). Either of these considerations could provide an explanation for the results reported here, in which all IDH horses had IgE antibodies to proteins in thorax preparations from native *Culicoides* (captured as they alighted on the horses to feed), but only a weak IgE response to salivary gland proteins extracted from *C. nubeculosus* (a species widely distributed in Europe though not common in our captured population).

Lack of binding to salivary gland proteins could be due to inadequate sensitivity of our blotting protocol but this does not seem likely. Preliminary studies showed our anti-IgE reagents are able to detect less than 3 ng of equine IgE in dot-blot. Analysis of the total protein concentration and the amount of individual salivary gland proteins in spots on 2D gels showed them to be in the range that is easily detected by chemiluminescent substrate. Previous studies have detected IgG antibodies in allergic horse serum which could block IgE binding (Hellberg et al., 2006; Wilson et al., 2001), we therefore used affinity purified IgE to probe our Western blots and avoid potential problems with IgG blocking antibodies.

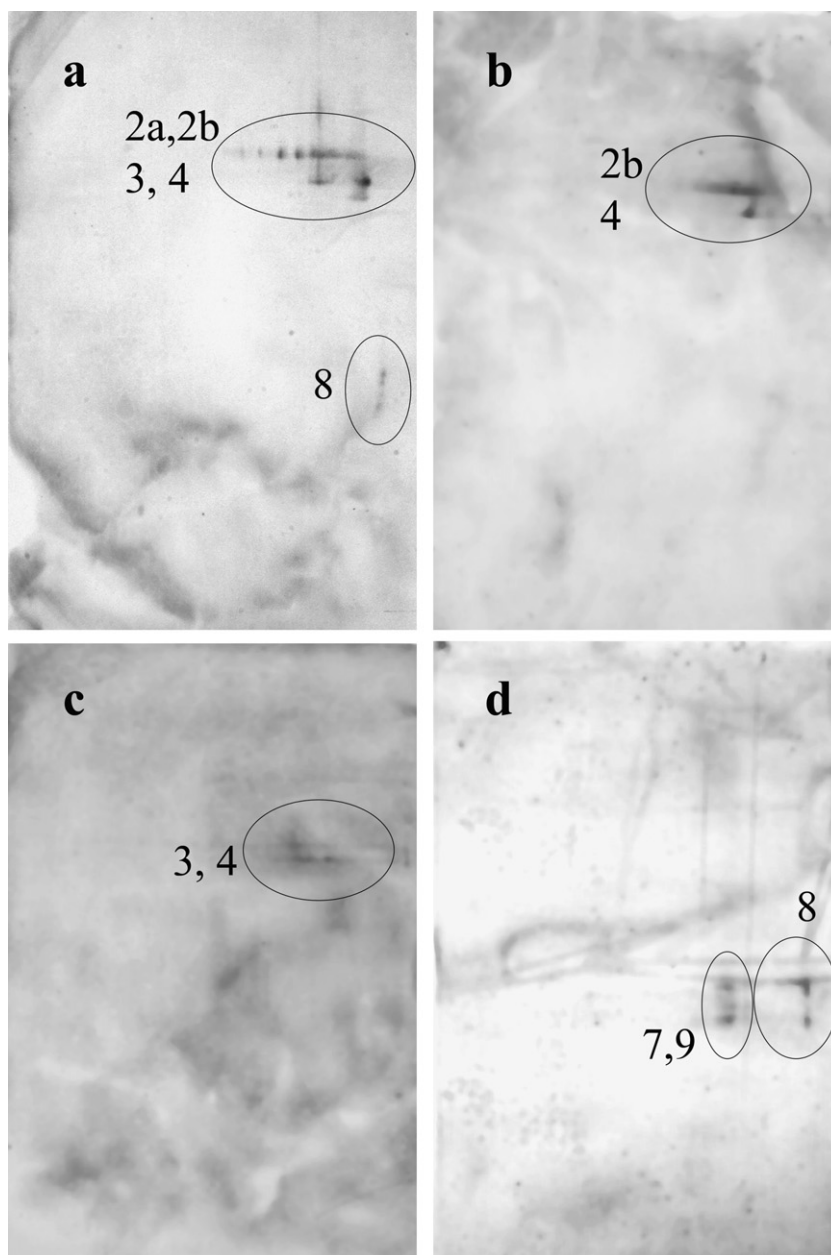


Fig. 4. Western blots of proteins extracted from 25 *C. nubeculosus* salivary glands separated by 2D electrophoresis. Blots were probed with 100 ng/ml affinity purified IgE from four horses with *Culicoides* hypersensitivity. The positive proteins are ringed and numbered according to the spots in Fig. 3a. Panel a is probed with IgE corresponding to lane 3 in Fig. 1. Panels b, c and d correspond to IgE from lanes 7, 9 and 11, respectively.

The concentration of 100 ng/ml IgE was sufficient to give a strong signal in blots of thorax proteins but the signal in blots of purified *C. nubeculosus* remained weak and increasing the concentration of IgE led to problems with high background binding.

Several horses had strong reactions to proteins in thorax preps with a molecular weight in excess of the abundant salivary gland proteins, supporting a role for

extra-salivary antigens as important allergens; alternatively these thorax bands may all be due to cross-reacting IgE antibodies. Cross-reactions in Western blots commonly generate false positive results, for example the cross-reactive band of affinity purified anti-mouse conjugate with a 10 kDa peptide band in *Culicoides* thorax preps. As only the allergic horses had IgE antibodies to *Culicoides* extract, we cannot

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AY603607  MALKTIKVLVLLTVAIAYIPNHVECSNMRTVEVHHQCLAKVLPGKTIEEASWDQVKEAID 60
SG55      MTSGNICKILVLTVAITIIIPNFVECAET-SVLRKQCLAKVLPGKTIEDVEWNNVKKEAIE 59
          *: . * *:*****: **,**: : : :*:***** * :. *:*****:

AY603607  NGNRDYQCFILCELTNLNMLKSGVQVQTDSEPLHPALGAKLTECANMKVDADSKNAKDS 120
SG55      NDNREFQCFILCELTNLNMLKSDGAVQTNGNPLEPALGDKISECSNLELYQDNCKAAKNA 119
          *.:*:*****:***:*.***: .**.*** * :*:*: : : *.* ***:

AY603607  AQCIINVTAELGKYYEVEGIFQKEWKNFDESGKQIVWNN 159
SG55      ASCLIEATHKLEKYYEVENIFQAEWKKFENSGKLEWNN 158
          *:*.*: . * :*****,* ***:*:*: * **

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Fig. 5. Clustal alignment of AY603607, a D7 protein sequence from *C. sonorensis* (Campbell et al., 2005) with SG55 an equivalent D7 sequence from *C. nubeculosus*.

conclude that all horse IgE is cross-reactive with *Culicoides* antigens, but it remains possible that once a horse is sensitised to *Culicoides*, its saliva protein specific IgE antibodies cross-react with other proteins in the thorax. Some proteins in *Culicoides* saliva may be expressed at sites outside the salivary gland, for example trypsin-like serine proteases are found in both saliva and mid-gut (Campbell et al., 2005), but as many of the most abundant proteins in saliva have specialised functions associated with blood feeding it is not clear what role they could have in other insect tissues. Alternatively, as all of the allergens detected in 2D blots had a high *pI*, the IgE antibodies may cross-react with basic epitopes such as those found in some abundant ribosome associated proteins. Carbohydrates also act as cross-reactive epitopes, which may account for a proportion of the bands seen in blots of thorax, particularly as insect proteins are glycosylated differently from those of mammals (Altmann, 2007). Cross-reactions between carbohydrate epitopes have been reported to cause allergy to multiple insect species in humans (Hemmer et al., 2001), similarly allergic horses often react to insects in different genera (Fadok and Greiner, 1990). In one study carried out in Switzerland 75% of horses sensitised to *Culicoides* also showed reactivity with an Icelandic species of *Simulium* (Baselgia et al., 2006). There are no reports of horses developing a primary allergy to *Simulium* in Iceland and so the observed reactions may represent a cross-reaction following sensitisation to *Culicoides*.

There is conflicting data available in the literature with respect to the importance of using local midge species when testing horses for allergy. In several studies, hypersensitivity to *Culicoides* has been confirmed by intra-dermal challenge with crude extracts of insect proteins. The reported incidence of positive reactions ranged from 80% to 100% where extracts of *Culicoides* spp. native to the region in which the test horses originated were used (Fadok and Greiner, 1990; Ferroglio et al., 2006). Also allergic horses in Canada showed positive reactions to intra-dermal challenge with *Culicoides* extracts from both native and exotic

European species (Anderson et al., 1993). In contrast the proportion of Norwegian horses reacting to each of the extracts from four different native midge species varied from 5/14 to 12/12 (Halldorsdottir et al., 1989) and a study carried out in Austria using extracts of captive bred *C. variipennis* (native to North America) found that most of the allergic horses did not react to allergen at a dilution below the threshold at which healthy control horse also reacted (Kolm-Stark and Wagner, 2002). Currently it is hard to reconcile all of the conflicting evidence that many IBH horses react to a wide range of antigens from *Culicoides* and other insects while others can be refractory to all but a single species of midge also many studies found a few horses with symptoms of IBH that did not react to any antigen tested. These difficulties must in part reflect the use of different reagents and methods to diagnose IBH, which in turn highlights a need to develop standardised reagents.

One approach to this is the identification of the major allergens that can then be generated in vitro as recombinant proteins, to this end we have attempted to identify the proteins in saliva of *C. nubeculosus* that most commonly react with IgE from horses with IBH. Apart from one horse which reacted to intermediate size basic proteins, all of the horses that had positive reactions with antigens in 2D blots bound antigens identified as spots 2–4 on maxi gels or their equivalent in mini gels. Unfortunately all of these spots were N-terminal blocked and none matched to any of the predicted mass/*pI* data of abundant transcripts in the *C. sonorensis* cDNA library. Data from the mass spectroscopy did suggest spots 2a and 3 were homologous to AY603639 and AY603640, nearly identical versions of a predicted novel protein of unknown function with a predicted mass of only 29 kDa and a *pI* of 8.7. However, as the sequences contain over 30 serine and threonine residues that are potentially glycosylated additional sugars may account for the extra mass seen in the 2D gels. These same proteins have been identified in 1D gels of *C. nubeculosus* saliva with a mass of 44 kDa providing corroboration for their identity (Langner et al., 2007).

Spot 4 gave a weak match with hyaluronidase, which is a known allergen in wasp and bee venom (Hemmer et al., 2001). There is only a partial sequence of *C. sonorensis* hyaluronidase in the database so a low protein score would be expected, but the predicted weight of this intact enzyme from bee venom is 44 kDa which matches well with the observed position of spot 4. In addition hyaluronidase activity at an apparent molecular weight of about 40 kDa is detectable in *C. nubeculosus* salivary gland extract by substrate gel zymography (Wilson, unpublished observation).

The failure to detect any IgE antibodies that bound proteins smaller than 20 kDa such as the D7 proteins was surprising as they are very abundant and were visible as clear focused spots in both large and small format gels. D7 proteins from mosquito saliva have been shown to be human allergens (Peng et al., 2006) and spots corresponding to the *Culicoides* D7 proteins were clearly identifiable on all gels. However, the harsh and irreversible denaturing of proteins in 2D gels will disrupt their native conformation and leave only linear epitopes available for antibody binding. Comparison of D7 sequences from *C. sonorensis* and *C. nubeculosus* showed that although they are 60% identical in amino acid sequence there are few identical stretches of sufficient length to form linear antibody binding epitopes which are conserved between *Culicoides* spp. If this level of identity reflected in other proteins it would also account for the limited success we had in matching *C. nubeculosus* proteins to their homologous counterparts in *C. sonorensis* by mass spectroscopy. We are currently deriving more sequences from a *C. nubeculosus* cDNA library which will enable more accurate identification of the proteins spots from the mass spectroscopy data. Ultimately the expression of these recombinant proteins in *Baculovirus* may enable the production of several abundant *Culicoides* salivary proteins including D7 in their native folding which will be used to reassess the salivary gland proteins as allergens in future studies.

In conclusion our results confirm that horses with IBH all have IgE antibodies to insect proteins while healthy horses do not. However whether horses react equally to salivary gland proteins from all species of *Culicoides* and the role of nonsalivary proteins as allergens remains unresolved. Nevertheless we have been able to identify several abundant proteins in *C. nubeculosus* saliva that are allergens in some horses and we believe that a screening a library of recombinant proteins from several species of midge will be the best approach to resolving these issues and to address the further question of T-cell immunity in IBH.

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