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ARTHROPODS AND MEDICAL ENTOMOLOGY - ORIGINAL PAPER

Characterization of IgE-binding proteins in the salivary glands of *Simulium nigrogilvum* (Diptera: Simuliidae)

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Abstract

Simulium dermatitis is an IgE-mediated skin reaction in animals and humans caused by the bites of black flies. Although *Simulium nigrogilvum* has been incriminated as the main human-biting black fly species in Thailand, information on its salivary allergens is lacking. Salivary gland extract of *S. nigrogilvum* females was subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis, and the separated components were applied onto nitrocellulose membranes for immunoblotting, which was performed by probing the protein blots with sera from 17 individuals who were allergic to the bites of *S. nigrogilvum*. IgE-reactive protein bands were characterized further by liquid chromatography-mass spectrometry (LC-MS/MS) analysis. Nine protein bands (79, 42, 32, 25, 24, 22, 15, 13, and 11 kDa) were recognized in the serum of the subjects. Four of the nine protein bands (32, 24, 15, and 11 kDa) showed IgE reactivity in all (100%) of the tested sera, and they were identified as salivary secreted antigen 5-related protein, salivary serine protease, erythema protein, and hypothetical secreted protein, respectively. Three other proteins, salivary serine protease (25 kDa), salivary D7 secreted protein (22 kDa), and hypothetical protein (13 kDa), reacted with > 50% of the sera. The relevance of the identified protein bands as allergens needs to be confirmed by using pure recombinant proteins, either in the in vivo skin prick test or in vitro detection of the specific IgE in the serum samples of allergic subjects. This will be useful for the rational design of component-resolved diagnosis and allergen immunotherapy for the allergy mediated by the bites of black flies.

Keywords *Simulium nigrogilvum* · *Simulium* dermatitis · IgE-binding proteins

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Introduction

Black flies (Diptera: Simuliidae) are small vicious blood-sucking insects that have a worldwide distribution (Adler and Crosskey 2018) and are able to vector several pathogens to humans and animals. In Africa and South America, black flies serve as vectors of the filarial nematode *Onchocerca volvulus*, the causative agent of human onchocerciasis or river blindness (Loum et al. 2017). Additionally, zoonotic onchocerciasis, which is caused by the *Onchocerca* species of animal origin, has been reported from many countries (Takaoka et al. 2012). The bites of black flies can cause localized dermatitis in both humans and animals due to an IgE-mediated reaction to salivary gland proteins (Hellberg et al. 2009; Schaffartzik et al. 2009, 2010, 2012; Chattopadhyay et al. 2014; Orange et al. 2004; Chiriac et al. 2016). This is not only a cause of annoyance to humans but also leads to economic loss through a reduced level of tourism and reduced efficiency of agricultural and industrial workers (Myburg and Nevil

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2003). Sarıözkan et al. (2014) calculated the economic impacts that occurred after the black fly outbreak of 2013 in the Cappadocia region of Turkey and revealed that approximately US$5.45 million was lost in tourist (hotels) and the livestock (dairy) industries.

At present, at least 110 black fly species classified in six subgenera of the genus *Simulium* are known to occur in Thailand and seven of the species, i.e., *S. asakoae*, *S. chamlongi*, *S. doipuiense*, *S. nigrogilvum*, *S. nodosum*, *S. rufibasis*, and *S. umphangense*, have been reported to bite humans (Choochote et al. 2005; Pramual et al. 2016; Takaoka et al. 2017). Human onchocerciasis or zoonotic onchocerciasis is non-existent in Thailand, but allergic reactions to black fly bites are of major concern. *Simulium nigrogilvum* is recognized as the main human-biting black fly species in Doi Inthanon and Doi Suthep-Pui National Parks in northern Thailand (Takaoka et al. 2003; Choochote et al. 2005; Ishii et al. 2008). Interestingly, the study of Fukuda et al. (2003) revealed that 0.72% of *S. nigrogilvum* collected in Doi Inthanon National Park were infected naturally with an unidentified species of filarial nematode, based on the recovery of third-stage filarial larvae from the thoraces of females.

Doi Pha Hom Pok National Park is the northernmost national park in Thailand. It has the second highest mountain in the country, at approximately 2285 m above sea level. The park covers more than 500 km^2 of mountainous terrain in the Daen Lao Range on the Thai-Myanmar border (Srisuka et al. 2015). Nature trekking and camping are popular activities of tourists. Therefore, many tourists and park inhabitants are affected by the bites of *S. nigrogilvum*. However, knowledge of allergens in the salivary glands of black flies in Thailand is lacking. Therefore, this study aimed to identify IgE-binding proteins (allergens) in extracts from the salivary glands of *S. nigrogilvum* by using IgE immunoblotting and LC-MS/MS analysis.

Materials and methods

Field collection of *S. nigrogilvum* and preparation salivary gland extracts

Individuals of *S. nigrogilvum* were captured in Ban Lek, Fang District, Chiang Mai Province, northern Thailand. Collections were performed using insect nets to capture both landing and flying insects. Captured black flies were kept in net-covered paper cups with a pad of cotton wool soaked with 5% sucrose solution placed on top and stored in a humid container. The specimens were transported to the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, for species identification, which was performed using published keys (Edwards 1934; Takaoka 1979; Takaoka and Suzuki 1984; Takaoka and Davies 1995; Takaoka and Choochote 2004; Takaoka et al. 2017).

Black flies were anesthetized on ice and placed in phosphate-buffered saline (PBS) at pH 7.2; their salivary glands were dissected out with needles under a stereomicroscope (× 4) and transferred to microcentrifuge tubes with a small volume of PBS containing a cocktail of protease inhibitors (Roche Diagnostics, Risch-Rotkreuz, Switzerland). The salivary glands were homogenized using sonicator. Homogenates were centrifuged at 12,000*g* for 5 min. Supernatants were kept in small aliquots at − 80 °C in single-use vials.

Serum samples

Blood serum was collected from 17 people (10 villagers living in the collection areas and seven tourists visiting the same locations) with a history of bites from *S. nigrogilvum* and current skin lesions, wheal lesions with a hemorrhagic center (Fig. 1a) rapidly enlarging to > 10 mm with a sore, painful, and persistent itching and erythematous and mildly edematous papules observed within 24 h after being bitten (Fig. 1b). The serum samples of five healthy subjects who had not been bitten by insects and had no history of allergic reactions were used as negative controls. The serum samples collected from clotted blood taken from all subjects were stored at − 20 °C until use.

IgE ELISA

Wells of microtiter plates were coated with 100 μl of salivary gland extract (10 μg/ml in 50-mM carbonate-bicarbonate buffer at pH 9.6) and incubated at 37 °C for at least 16 h. After coating, the plate was washed with phosphate buffer solution (PBS) containing 0.05% Tween 20 (PBST), blocked with 1% bovine serum albumin (BSA) in PBST, and washed

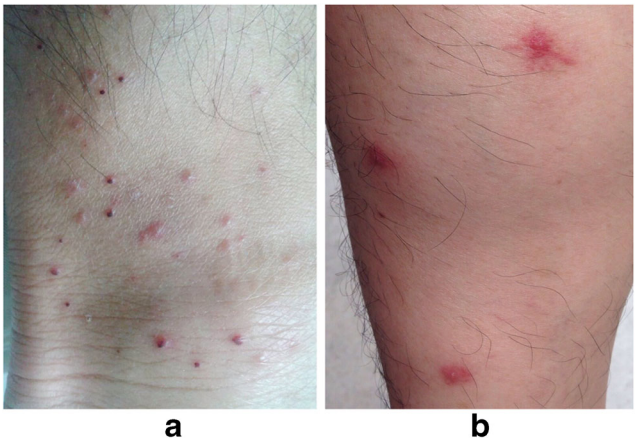

Fig. 1 Representation of the *S. nigrogilvum* biting site. **a** Small wheals with hemorrhagic lesion at the center. **b** Rapidly enlarged wheals and erythematous and mildly edematous papules

again. The serum was diluted to 1:2 in 1% BSA in PBST, added to the wells, and incubated overnight at 4 °C. After washing, peroxidase-conjugated mouse anti-human IgE (SouthernBiotech, Birmingham, AL, USA) was added to each well and incubated at 37 °C for 1 h before washing again. SureBlue Reverse™ Tetramethylbenzidine (TMB) Microwell Peroxidase Substrate (Kirkegaard & Perry Lab Inc., Gaithersburg, MD, USA) was used as a substrate after the last washing. The reactions were terminated using H_2SO_4, and absorbance was measured within 1 h at 450 nm. Every sample was tested in triplicate. The samples were considered positive when the OD_{450nm} value measured more than the mean of OD_{450nm} + 3-fold the standard deviations (SD) obtained from sera of the healthy controls.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 15% polyacrylamide separating gel and 4% stacking gel cast in a mini-PROTEAN Tetra Cell with PowerPac HC Power Supply, 200/240 V (Bio-Rad Laboratories, Hercules, CA, USA) at 100 V for 1.30 h. The salivary gland extract was mixed with SDS loading buffer (Novagen®) 1:4 (*v*/*v*), boiled for 5 min, and loaded on the stacking gel. The molecular weight marker (PageRuler™ Prestained Protein Ladder, Thermo Scientific, Rockford, IL, USA) was applied in one gel slot as reference. The electrophoresed gels were stained with Coomassie Brilliant Blue G-250 dye (CBB). The stained gels were photographed with Image Scanner III (GE Healthcare, UK).

Immunoblotting

After SDS-PAGE, the separated proteins were electrotransferred to the nitrocellulose membrane, using the Mini Transblot System (Bio-Rad Laboratories) at 100 V for 60 min. The membranes were washed with PBST, blocked with blocking buffer (Pierce™ Clear Milk Blocking Buffer, Thermo Scientific) at 25 °C for 1 h, and cut into strips. After three washes with PBST, the strips were reacted with individual serum (1:10) at 4 °C overnight and incubated with 1:2000 mouse anti-human IgE (SouthernBiotech) at 25 °C for 3 h. Finally, TMB Insoluble (Merck, Kenilworth, NJ, USA) was used as a detection reagent.

Identification of IgE-binding proteins by LC-MS/MS analysis and database search

The CBB-stained protein bands corresponding to those recognized by IgE in the IgE immunoblotting were excised from the gel using a sterile surgical blade with aseptic technique. The gel plugs were digested with trypsin and subjected to an Ultimate 3000 LC system (Dionex, Surrey, UK) coupled to MicroToF Q II mass spectrometer (Bruker Optik GmbH, Bremen, Germany). Peptide spectra were analyzed and searched for orthologous proteins against the non-redundant NCBI database using MASCOT 2.2 (Matrix Science, Boston, MA, USA).

Ethical consideration

The study protocol of this work was approved by the Research Ethics Committee (PAR-2558-03360, No. 521/2015), Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

Results

IgE ELISA

Specific serum IgE against *S. nigrogilvum* salivary gland extract was detected in all of the 17 subjects with a history of black fly bites. The serum collected from the seven tourists visiting the *S. nigrogilvum* collection areas, and 10 samples from the villagers living there, had significantly higher OD_{450nm} values than those of the healthy controls ($p < 0.05$) (Fig. 2). Furthermore, the OD_{450nm} of serum samples from the seven tourists was significantly lower than the OD_{450nm} of 10 samples from the inhabitants ($p < 0.05$) (Fig. 2). The serum samples from the 17 subjects who had a history of *S. nigrogilvum* bites were ELISA positive, i.e., they showed OD_{450nm} higher than the mean + 3SD (0.0732) of the healthy subjects.

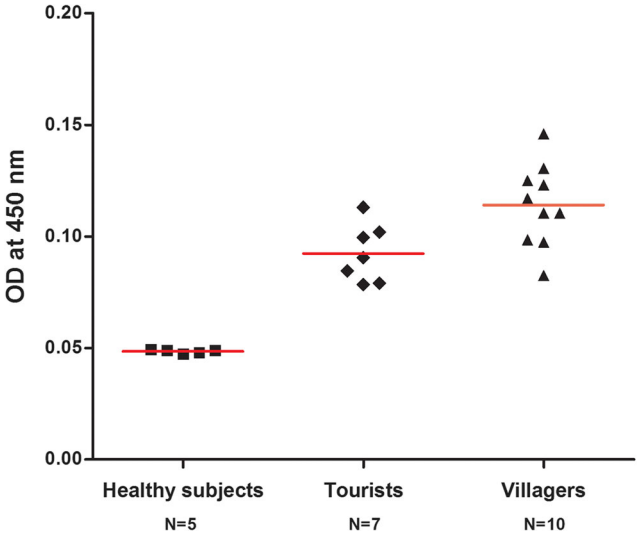

Fig. 2 Specific IgE serum level against salivary gland extracts from *S. nigrogilvum* determined by ELISA. Analysis of sera from healthy subjects (■) and subjects with a history of *S. nigrogilvum* bites and skin lesions (tourists (♦) and villagers (▲)). OD_{450nm} values greater than the mean + 3SD of OD_{450nm} of healthy subjects were considered positive

SDS-PAGE of *S. nigrogilvum* salivary glands and IgE-immunoblotting

The SDS-PAGE-separated *S. nigrogilvum* salivary gland extract revealed at least 13 major protein bands and several minor bands with molecular weights between 10 and 180 kDa (lane A, Fig. 3). To identify IgE-binding proteins, immunoblotting was performed using positive and negative serum samples from IgE ELISA to probe the protein blots with the SDS-PAGE-separated salivary gland extract. The results of the immunoblotting are shown in Fig. 3: nine protein bands with molecular weights of 79, 42, 32, 25, 24, 22, 15, 13, and 11 kD were recognized in the ELISA-positive samples. The proteins with molecular weights of 32, 24, 15, and 11 kDa were detected in all of the 17 ELISA-positive subjects. The proteins with molecular weights of 25, 22, and 13 kDa were detected in more than 50% of the ELISA-positive subjects, whereas the proteins with molecular weights of 72 and 42 kDa were detected in only one subject, number 17. The ELISA-negative serum samples did not show IgE reactivity.

LC-MS/MS analysis for the IgE-binding protein

The results of LC-MS/MS analysis are shown in Table 1. The major allergens with molecular weights of 32, 24, 15, and 11 kDa showed that 100% of the IgE binding matched the salivary secreted antigen-related protein, salivary serine protease, erythema protein, and hypothetical secreted protein in the database corresponded with 100, 75, 100, and 83% identity to *S. vittatum* salivary gland proteins, respectively. The protein bands with molecular weights of 25, 22, and 13 kDa matched the salivary serine protease, salivary D7 secreted protein, and the hypothetical protein of *S. vittatum*, respectively. The minor allergens with molecular weights of 79 and 42 kDa matched the hypothetical protein and salivary serine protease, respectively.

Discussion

The bites of black flies that cause *Simulium* dermatitis are a worldwide problem for animals and humans (Chiriac et al. 2016; Youssefi et al. 2008; Borah et al. 2012). Equine insect bite hypersensitivity is associated with an IgE-mediated reaction against black fly salivary gland proteins (Schaffartzik et al. 2009, 2010). During blood feeding of black flies, a variety of proteins and active molecules contained in the saliva are pumped into the wound, i.e., a small cavity cut into the skin of the host (Sutcliffe and McIver 1984). The site of the bite quickly appears as a small ecchymosis with a blood crust, followed in a few hours by painful, persistent itching that lasts for several days or weeks, and can result in secondary infections (Gudgel and Grauer 1954). A skin biopsy at the site of the black fly bite displays orthokeratinization of the epidermis, granular degeneration and focal fragmentation of the collagen fibers, interstitial edema with small vesicles, and cellular infiltration with lymphocytes and eosinophils at the dermis (Gudgel and Grauer 1954; Youssefi et al. 2008; Schaffartzik et al. 2009, 2010; Kim and Lockey 2010; Borah et al. 2012; Oliveira-Filho et al. 2012; Chiriac et al. 2016). Occasionally, a bite can result in black fly fever, with symptoms of headache, fever, nausea, vomiting, malaise, and lymphadenopathy

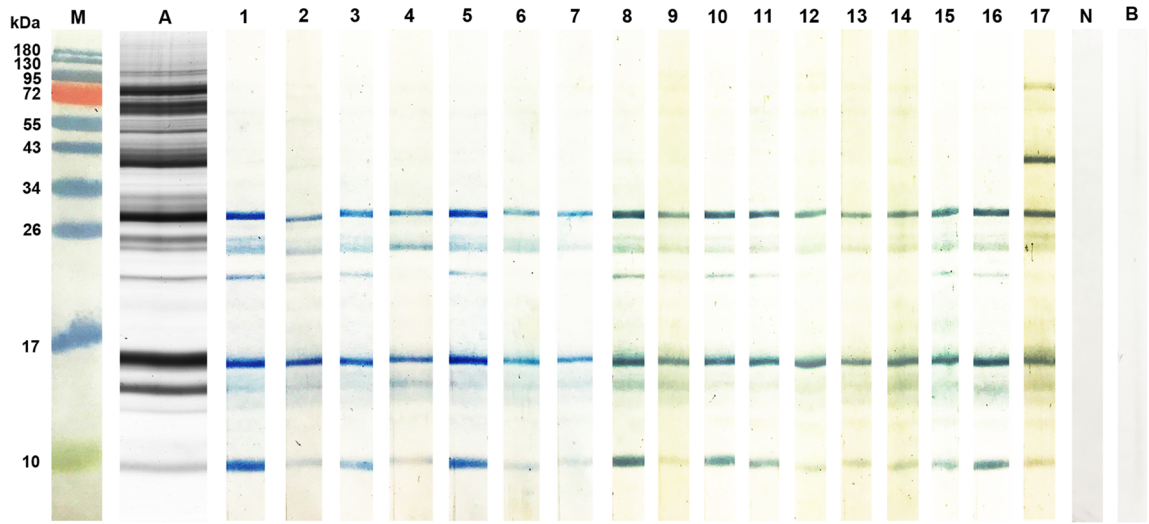

Fig. 3 SDS-PAGE and immunoblotting of salivary gland extracts of *S. nigrogilvum*, using sera from 17 subjects with a history of *S. nigrogilvum* bites, skin lesions, and positivity by IgE ELISA. Lane M, molecular weight marker; lane A, SDS-PAGE-separated salivary gland extract stained with CBB; lanes 1–7, immunoblots of serum samples from seven tourists who visited the *S. nigrogilvum* collection areas and were bitten by black flies; lanes 8–17, representative immunoblots of serum samples from 10 villagers living in the *S. nigrogilvum* collection areas; lane N, immunoblot of a serum sample from a healthy subject (as shown by ELISA-negative serum);lane B, blank (the NC blot was probed with PB

Table 1 List of IgE-binding proteins detected in the salivary gland extracts of *S. nigrogilvum*, identified by LC-MS/MS

No.	Molecular weight (kDa)	Accession number	Protein	Source	Identity (%)	Frequency (%)
1	79	ACH56860.1	Hypothetical protein, partial	*S. vittatum*	100	1/17 (5.88)
2	42	ACH56854.1	Salivary serine protease	*S. vittatum*	75	1/17 (5.88)
3	32	ACH56843.1	Salivary secreted antigen 5-related protein	*S. vittatum*	100	17/17 (100)
4	25	ACH56854.1	Salivary serine protease	*S. vittatum*	75	9/17 (52.94)
5	24	ACH56854.1	Salivary serine protease	*S. vittatum*	75	17/17 (100)
6	22	ACH56919.1	Salivary D7 secreted protein	*S. vittatum*	100	9/17 (52.94)
7	15	AAC26163.1	Erythema protein	*S. vittatum*	100	17/17 (100)
8	13	ACH56838.1	Hypothetical protein, partial	*S. vittatum*	100	10/17 (58.82)
9	11	ACH56829.1	Hypothetical secreted protein	*S. vittatum*	83	17/17 (100)

(Myburg and Nevil 2003). Recently, Schnellbacher et al. (2012) showed that several bird species experience acute lethargy, generalized subcutaneous petechiae, vasculitis, and death caused by the bites of *S. meridionale*. Black fly bites are associated with IgE-mediated hypersensitivity in animals (Hellberg et al. 2009; Schaffartzik et al. 2009, 2010, 2012), but acute, severe hypersensitive reactions have not been reported in humans.

The results of this study revealed nine protein bands with molecular weights of 79, 42, 32, 25, 24, 22, 15, 13, and 11 kDa are present in the salivary gland extracts of *S. nigrogilvum*, and they were recognized by IgE in sera of subject allergic to black fly bites. No IgE-binding proteins were observed in the sera of healthy subjects. The protein bands with molecular weights of 32, 24, 15, and 11 kDa were detected by IgE in all serum samples of allergic subjects, identified by LC-MS/MS as salivary secreted antigen 5-related protein, salivary serine protease, erythema protein, and hypothetical secreted protein, respectively, indicating that these salivary proteins are major allergens contained in the saliva of *Simulium* species. The proteins with molecular weights of 25, 22, and 13 kDa reacted to the IgE in more than 50% of the serum samples obtained from allergic subjects; those proteins were identified as a salivary serine protease, salivary D7 secreted protein, and hypothetical protein that is present in the saliva of *S. vittatum*, respectively. Therefore, these seven proteins appear to be the major allergens contained in the saliva of *S. nigrogilvum*.

Among these seven allergenic proteins, only the salivary secreted antigen 5-related protein was reported as the major allergen of *S. vittatum*, namely "Sim v 1" in horses and it shared a high degree of sequence identity and homology with antigen 5 protein of other insects, including *Culicoides sonorensis* (Diptera: Ceratopogonidae), *Aedes* and *Culex* species (Diptera: Culicidae) and *Phlebotomus* species (Diptera: Psychodidae) (Schaffartzik et al. 2009, 2010). The antigen 5 protein belongs to the ag-5 protein family and is reported to be the major allergen of stinging insects (Sookrung et al. 2014; Potiwat and Sitcharungsi 2015; Srisong et al. 2016; Tomsitz and Brockow 2017). The function of this protein is still unknown, but its potential as a major allergen for horses has been demonstrated (Schaffartzik et al. 2009). This study found salivary serine protease enzymes in the bands with molecular weights of 42, 25, and 24 kDa. A previous study of sialotranscriptomes in *S. vittatum* reported that a variety of transcript encodings of these enzymes have been discovered, e.g., elastase, collagenase, and hyaluronidase. These enzymes help to create a larger blood pool and inhibit coagulation for successful blood feeding (Andersen et al. 2009). A vasodilation protein, erythema, has been found in many black fly species, including *S. nigrogilvum*, with 100% identity and molecular weight of 15 kDa, which is concordant with previous studies of other black flies (Cupp et al. 1994; Andersen et al. 2009; Ribeiro et al. 2010; Chagas et al. 2011). The protein band with a molecular weight of 22 kDa was identified as a salivary D7 secreted protein and showed greater 50% reactivity in allergic sera, which is in agreement with findings of Peng et al. (1998), who demonstrated that D7 proteins of mosquitoes can cause allergic reactions in humans. Of interest, only one of the 17 black fly allergic sera was bound to two additional protein bands, the hypothetical protein (72 kDa) and salivary serine protease (42 kDa). This may be due to variations of individual immune responses to allergens. The hypothetical proteins, with molecular weights of 79, 13, and 11 kDa, are shown to be allergens in this study. It would be worthwhile to further study and identify their molecular structures and functions. Previous studies by Schaffartzik et al. (2009) revealed cross-reacting allergens of *S. vittatum* and *Culicoides nubeculosus*. Therefore, the possibility of cross-reaction of IgE-binding proteins of other human-biting black flies, and salivary gland extracts of other insects against that of *S. nigrogilvum*, should be investigated.

In summary, nine proteins in the salivary gland extract of *S. nigrogilvum* were detected by IgE in the sera of the people who exhibited allergic reactions to the bites of black flies. Seven of the proteins bound with IgE of sera from > 50–

100% of allergic subjects, indicating that they are major allergens. However, the separated protein bands obtained from SDS-PAGE may be a mixture of proteins that have the same molecular weight, and in consequence, 2-DE immunoblotting (Gonzalez-Buitrago et al. 2007) or use of recombinant proteins in skin prick tests and/or detection of specific IgE to the proteins in serum samples should be performed (Cupp et al. 1998; Schaffartzik et al. 2009, 2010). The information gained from this study should be useful in the design of both component-resolved diagnostics and therapeutics of allergies to black fly bites.

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