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Characterization of *Aedes communis*, *Aedes aegypti* and *Anopheles stephensi* Mosquito Saliva Antigens by Immunoblotting

Abstract

Background: Mosquito bites cause immediate wheals and delayed bite papules in sensitized subjects having saliva-specific IgE and IgG4 antibodies. At present, mosquito saliva antigens are not well characterized. **Methods:** To identify immunogenic proteins in mosquito saliva and study their cross-reactivity we immunized mice with *Aedes communis*, *Aedes aegypti* and *Anopheles stephensi* bites. Immune sera were used in immunoblotting and immunoblot inhibition experiments. **Results:** The main *A. communis* saliva antigens were 22-, 30-, and 36-kD, *A. aegypti* saliva antigens 31-, 36-, 46- and 64- to 66-kD, and *A. stephensi* saliva antigen 46-kD proteins. Most of the saliva antigens appeared to be species-specific and only weak cross-reactivity was observed with heterologous immune sera. Distinct cross-reactivity was observed only between saliva proteins of *A. communis* and *Aedes punctator*, two taxonomically closely related species. Human IgE and IgG4 antibodies from mosquito-sensitive children bound to the same saliva proteins as antibodies from the immunized animals. **Conclusions:** This study disclosed several immunogenic proteins in *Aedes* and *Anopheles* mosquito saliva and suggests that these proteins can also be allergenic in man.

Key Words

Mosquitoes
Saliva proteins
Allergens
Immunoblotting
Cross-reactivity

Introduction

Mosquitoes are important vectors of many diseases. Besides transmitting pathogens mosquito saliva is antigenic and regularly elicits an immune response in the exposed host [1]. Saliva-specific IgE and IgG antibodies are formed, and immediate and delayed cutaneous bite reactions are common in sensitized people [2]. In contrast to bee and wasp venom allergy anaphylactic reactions to mosquito bites are rare [2]. We have previously shown by immunoblotting that 36-kD proteins from *Aedes communis* and *Aedes aegypti* saliva bind human IgE and IgG4 antibodies and could be the main saliva allergens in these

species [3, 4]. In addition, several previous mosquito bite challenge studies suggest that both species-specific and shared saliva antigens exist between different mosquito species [5–12].

In the present study we characterized mosquito saliva antigens in more detail by immunizing mice with the bites of *A. communis*, *A. aegypti* and *Anopheles stephensi* mosquitoes. In immunoblotting, homologous immune sera detected several saliva antigens. Immunoblotting with heterologous immune sera and immunoblot inhibition were used to study the cross-reactivity of saliva antigens. In addition, we examined whether the antigenic mosquito saliva proteins bind human IgE and IgG4 antibodies.

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Material and Methods

Mosquitoes

A. communis mosquitoes were collected as larvae in Southern Finland and reared to adults as previously described [13]. Adult *A. aegypti* and *A. stephensi* mosquitoes were laboratory strains from the Prince Leopold Institute of Tropical Medicine in Antwerpen, Belgium. Female mosquitoes were used for animal immunization. Saliva was collected from females and males of these species as earlier described [3]. In addition, saliva samples were obtained from *Aedes punctor*, *Culex pipiens*, *Culex molestus* and *Culex quinquefasciatus* mosquitoes. Adult female *A. punctor* mosquitoes were collected in a field in Southern Finland and identified under the stereomicroscope by postcoxal scales [14]. *Culex* species were laboratory strains from the London School of Hygiene and Tropical Medicine.

Animal Immunization

Immunizations were carried out by allowing the mosquitoes to feed through a small net cage as described earlier [13]. Two mice (NIH) were used for *A. communis*, 4 mice for *A. aegypti* and 3 mice for *A. stephensi* immunizations. The mean number of bites in one exposure was 7 (range 1–26). Serum samples were collected before and 75–240 days after the onset of immunization. A serum from a rabbit (New Zealand white) immunized with *A. communis* mosquito bites [13] was used to study the cross-reactivity between *A. communis*, *A. aegypti* and *A. punctor* saliva antigens. The animal experiments were performed according to the rules of the Institutional Animal Care and Use Committee. All sera were stored at –20°C before used.

Human Sera

Three sera from Finnish, Kenyan and Mexican mosquito-sensitive children with saliva-specific IgE and IgG4 antibodies to *A. communis* or *A. aegypti* [4] were used in comparison with animal-immune sera. In addition, sera from 3 children were studied for occurrence of saliva-specific IgE and IgG4 antibodies to *A. stephensi*. All sera were stored at –20°C before use.

Electrophoresis

Saliva samples were electrophoresed in Laemmli's [15] 12% SDS-PAGE. Water saliva solution was diluted 1:2 to electrophoresis sample buffer containing β -mercaptoethanol and 4.6 μ l were loaded per mm² of 12% page gel (corresponding 1.7 μ l water-saliva solution/mm strip studied). Otherwise electrophoresis was carried out as described earlier [3].

Immunoblotting

Saliva samples from different mosquito species were analyzed against the sera of species-specific mosquito bite-immunized animals, except *A. punctor* saliva which was analyzed only with sera of *A. communis*-immunized rabbit. Immunoblotting to polyvinylidene difluoride membrane (Millipore) was carried out for 2 h at 100 V in melting ice. To study the total saliva proteins the blotted membrane was stained with colloidal gold kit (Bio-Rad). For immunoblot studies the membrane was cut into 3-mm-wide strips, washed 3 times for 5 min in Tris-buffered saline (TBS)–0.1% Tween, blocked for 1 h with 5% milk in TBS and incubated overnight with serum dilutions 1:500 for rabbit and mouse IgG, 1:4 for human IgE and for 1:10 for human IgG4. After washing twice for 1 min and 3 times for 5 min in TBS-Tween, alkaline phosphate (AP) conjugated polyclonal goat anti rabbit IgG (1:500) and goat anti mouse IgG (1:500) antibodies (Zymed) or biotinylated polyclonal goat anti-human IgE (1:250; Kirkegaard & Perry) and mouse

Table 1. Molecular weights of the proteins found in the saliva of different mosquito species after SDS-PAGE and colloidal gold staining

Mosquito species	Molecular weights, kD					
<i>A. communis</i>	16	22	30	36	48	55 64–70
<i>A. punctor</i>	16	22	30	38		55 64–70
<i>A. aegypti</i>	16	19	31	36, 38	46	55 64–70
<i>A. stephensi</i>	15	24	27	33	40, 44	55 64–70
<i>C. pipiens</i>	17	23		33	37	64–66
<i>C. molestus</i>	17			33	37	64–66
<i>C. quinquefasciatus</i>	17			33	37	64–66

monoclonal antihuman IgG4 (1:250; Zymed) antibodies were added in 5% milk in TBS and incubated for 30 min. The strips were washed as before and the biotinylated antibodies were further incubated for 30 min with AP-conjugated streptavidin (1:1,000) in TBS. After final washings in TBS-Tween, the substrate for AP was added (Bio-Rad). The reaction was stopped by rinsing the strips in water.

Immunoblot Inhibition

Immunoblot inhibition experiments were performed with the sera of *A. communis*- and *A. aegypti*-immunized mice. The immune sera were titrated against homologous saliva antigen. The highest reactive IgG antibody dilution (1:1,000 for *A. communis* sera, 1:5,000 for *A. aegypti* sera) was selected for inhibition studies. These were performed by preincubating the diluted mice immune sera with 1 ml of *A. communis* and *A. aegypti* saliva samples (400 μ g/ml, measured by the bichinchonic acid method, Pierce, III., USA) in 5% milk-TBS for 2 h. Preincubated sera were then immunoblotted by using *A. communis* and *A. aegypti* saliva antigen strips.

Results

Electrophoretic Characterization of Saliva Proteins

Molecular weights of the main proteins found in the saliva of female *Aedes*, *Anopheles* and *Culex* mosquito species are given in table 1. Colloidal gold staining disclosed several protein bands ranging from 14 to 68 kD. Saliva from *A. communis*, *A. aegypti* and *A. stephensi* showed both low (15–31 kD) and high (64–70 kD) molecular weight proteins (fig. 1). A few bands were broad and seemed to be clusters of several proteins. Saliva from the *Culex* species showed 17-, 33-, 37- and 64- to 66-kD bands.

Immunoblot Characterization of Saliva Antigens

The antigenic proteins found in the saliva of different mosquito species with homologous mice immune sera are listed in table 2. In the saliva of female *A. communis* the strongest antibody binding was seen against 22-, 30- and 36-kD, in *A. aegypti* 31-, 36-, 46-, and 64- to 66-kD and in *A.*

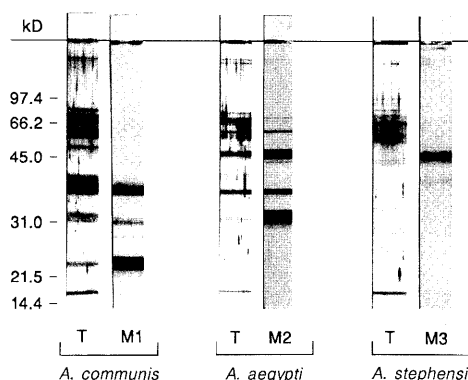


Fig. 1. Total colloidal gold staining (T) and immunoblotting (M1–M3) of saliva antigens from *A. communis*, *A. aegypti* and *A. stephensi* mosquitoes after SDS-PAGE. Several antigens were detected in the saliva of the three mosquito species with immune sera (M1–M3) of homologous mice.

stephensi 46-kD antigens (fig. 1). No antigens were detected in the saliva of male *A. communis* and *A. aegypti* mosquitoes.

Human IgE and IgG4 antibodies bound to the 22-, 30- and 36-kD antigens in the saliva of *A. communis* and to the 31-, 36-, 46- and 64- to 66-kD antigens in the saliva of *A. aegypti* (fig. 2). The *A. stephensi* 46-kD saliva protein bound IgE antibodies from two human sera and 36- and 68-kD proteins from one human serum (fig. 2).

Cross-Reactivity of Saliva Antigens

Immunoblotting with heterologous mice immune sera was used to study the presence of putative cross-reacting antigens in the saliva of *A. communis*, *A. aegypti* and *A. stephensi* mosquitoes. The results are shown in table 2. Immunoblot reactivity to the main saliva antigens was always weaker with heterologous than with homologous immune sera. Overall, only few and usually faint bands were seen with heterologous immune sera. *A. communis* immune sera did not detect any antigens in *A. aegypti* or *A. stephensi* saliva. In contrast, *A. aegypti* immune sera showed a weak binding to *A. communis* 30-kD (one serum), 36-kD (two sera) and 54-kD (one serum) saliva proteins, and also to *A. stephensi* 54-kD (one serum) protein. *A. stephensi* immune sera reacted weakly to *A. communis* 64- to 66-kD (one serum) and 54-kD (two sera) saliva proteins, and to *A. aegypti* 36-kD (two sera) and 54-kD (one serum) proteins.

Table 2. Molecular weights and cross-reactivity of the main saliva antigens of *A. communis*, *A. aegypti* and *A. stephensi* mosquitoes studied with mice immune sera in immunoblotting

Immune sera	<i>A. communis</i> saliva	<i>A. aegypti</i> saliva	<i>A. stephensi</i> saliva
<i>A. communis</i>	22, 30, 36	NR	NR
<i>A. aegypti</i>	30, 36, 54	31, 36, 46, 54 54, 64, 66	54
<i>A. stephensi</i>	54, 64–66	36, 54	15, 40, 46, 54 64–68, > 200

Bold figures represent a strong reaction. Shaded areas represent reactions with homologous immune sera. NR = No reaction.

Immunoblot inhibition experiments, too, did not disclose cross-reactivity between the saliva antigens of *A. communis* and *A. aegypti*. Saliva of *A. communis* did not inhibit binding of *A. aegypti* immune sera to homologous saliva antigens, and the same was true for *A. aegypti* saliva in *A. communis* antibody inhibition test (fig. 3).

Additional immunoblot experiments with *A. communis*, *A. aegypti* and *A. stephensi* mice immune sera did not show any reactivity against the saliva antigens of *Culex* species [data not shown]. When *A. communis* rabbit immune serum was tested, it showed weak binding to *A. aegypti* 31-, 36- and 66-kD saliva antigens and strong binding to several *A. punctator* saliva antigens (fig. 4).

Discussion

In the present study, total protein staining detected many proteins in the saliva of *Aedes*, *Anopheles* and *Culex* mosquitoes. These findings are in agreement with previous studies characterizing *A. communis*, *A. aegypti* and *C. molestus* saliva proteins [3, 16, 17]. To study antigenic properties of saliva proteins mice were immunized with the bites of *A. communis*, *A. aegypti* and *A. stephensi*. Immunoblotting showed that the main *A. communis* saliva antigens were 22-, 30- and 36-kD proteins. The same saliva proteins bound also human IgE and IgG4 antibodies, a finding which is consistent with our previous studies in mosquito-bite-sensitive children and adults [3, 4]. The main *A. aegypti* saliva antigens detected by mice immune sera were 31-, 36-, 46- and 64- to 66-kD proteins, which also bound human IgE and IgG4 antibodies. These results are in agreement with our previous immunoblot studies with human IgE antibodies from mosquito-bite-sensitive children [4], and also with

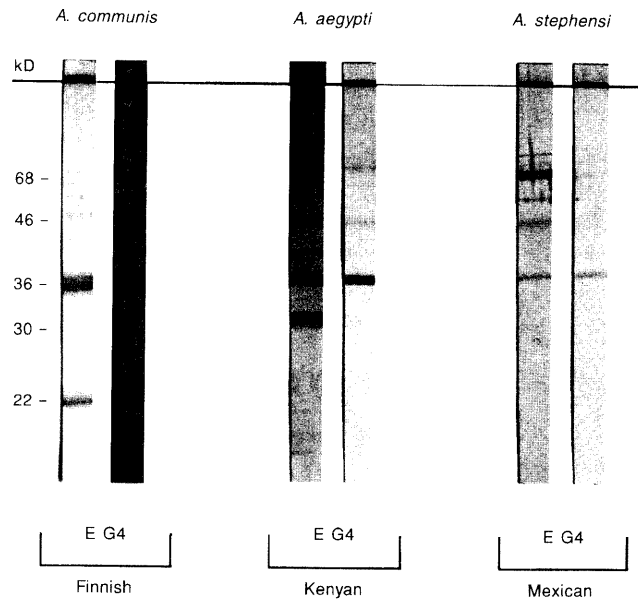


Fig. 2. Immunoblotting showing human IgE (E) and IgG4 (G4) antibody binding to *A. communis*, *A. aegypti* and *A. stephensi* saliva antigens. Sera were obtained from Finnish, Kenyan and Mexican mosquito-bite-sensitive children.

gel electrophoresis studies reported by Racioppi and Spielman [16]. Recently, James et al. [18] isolated a gene expressing a 37-kD protein from *A. aegypti* salivary glands. The 37-kD protein could well be same as the 36-kD antigen found in the present study in the saliva of *A. aegypti*. In the saliva of *A. stephensi* we found several immunogenic proteins of which the main antigen seemed to be a 46-kD protein. Human IgE antibodies bound also to this protein suggesting that the 46-kD *A. stephensi* saliva protein is also allergenic in man.

At present, the function of the mosquito saliva antigens is unknown. Female mosquitoes need blood to complete the development of their ovaries, whereas males do not take blood meals. We could find saliva antigens only in the female but not in the male *A. communis* which suggests that these proteins are involved in blood feeding. A salivary gland-specific protein, 63-kD apyrase-inhibiting platelet aggregation, has recently been sequenced from female *A. aegypti* salivary glands [19]. This protein could be one of the 64- to 66-kD *A. aegypti* saliva antigens found in the present study.

Theoretically cutaneous reactions to mosquito bites may be either species-specific or due to cross-reactivity between

saliva antigens of various species. All previous data considering the cross-reactivity of saliva antigens is based merely on the observation of skin reactivity during mosquito bite exposures. Mellanby [5] and other investigators [6, 7] documented in adult subjects that the first exposure to *A. aegypti* could cause bite reactions which were possibly due to previous sensitization from other mosquito species. Bite challenge studies performed by Killby and Silverman [11] presented some evidence that cutaneous reactivity to the bites could also be specific to the mosquito genera. In accordance with this view, we have shown with passive transfer experiments that IgE-mediated cross-reactivity can occur between the bites of *A. aegypti* and other *Aedes* species [20]. In the present immunoblotting and immunoblot inhibition experiments with mice immune sera, the cross-reactivity between the saliva antigens of *A. communis* and *A. aegypti* however, seemed to be weak. Some *A. aegypti* immune sera reacted weakly to *A. communis* saliva 30- and 36-kD antigens but not vice versa. However, *A. communis* rabbit immune sera detected a faint binding to *A. aegypti* 31-, 36- and 66-kD saliva antigens. Overall, these findings suggest that there is a weak in vitro cross-reactivity between *A. communis* 30- to 36- and 64- to 66-kD and *A. aegypti* 31-,

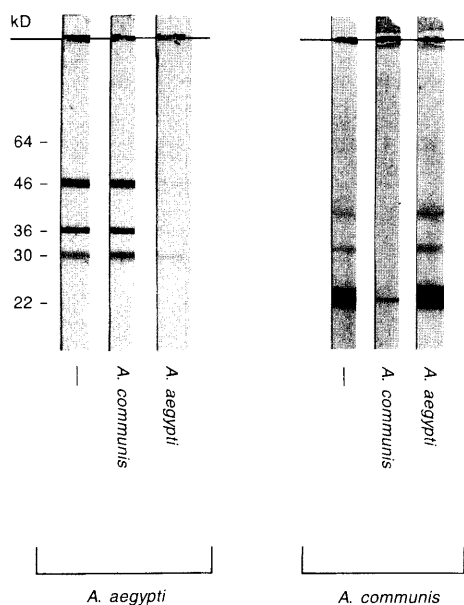


Fig. 3. Immunoblot inhibition experiments showing marked inhibition with homologous but not with heterologous mosquito saliva. *A. aegypti* (a) and *A. communis* (b) mouse immune sera were preincubated for 2 h with saliva antigens before immunoblotting. First columns show controls preincubated without saliva antigens.

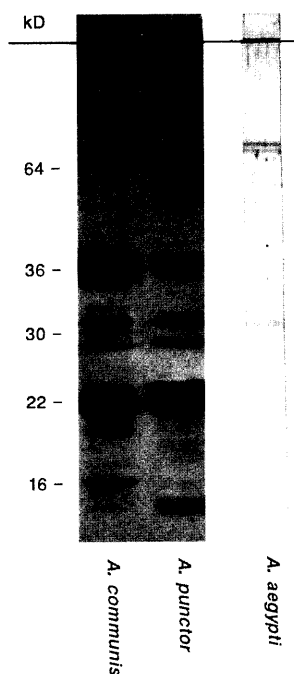


Fig. 4. Immunoblotting with *A. communis* rabbit immune serum shows cross-reactivity between *A. communis* and *A. punctor* saliva antigens, whereas only a weak reactivity was seen against *A. aegypti* saliva.

36- and 66-kD saliva antigens. It is of interest that no cross-reactivity was seen with *A. communis* 22-kD saliva protein which is one of the main saliva allergens of this species [3, 4, 20].

In contrast to weak cross-reactivity observed between the saliva antigens of *A. communis* and *A. aegypti*, the saliva of the third *Aedes* species, *A. punctor*, strongly bound *A. communis* antibodies from rabbit immune sera. *A. communis* and *A. punctor* are taxonomically and geographically closely related; they belong to the *Ochlerotatus* subgenus of *Aedes* mosquitoes and are prevalent in northern Europe while *A. aegypti* is a subtropical species [21]. It seems evident, therefore, that even within one mosquito genus, *Aedes*, the saliva antigens may show considerable variation. On the other hand, some degree of cross-reactivity may occur between the saliva antigens of even unrelated mosquito species. Supporting this, the present study showed that

A. stephensi immune sera reacted weakly with the *A. aegypti* 36-kD saliva antigen. However, *A. aegypti* immune sera did not recognize the main *A. stephensi* 46-kD saliva antigen. In agreement with these seemingly contradictory immunoblot results, French and West [12] showed that *A. stephensi*-sensitized animals reacted to *A. aegypti* bites but not vice versa. These findings suggest that depending on the immunogenic properties of the saliva antigens involved in primary sensitization the cross-reactivity may expand even to unrelated mosquito species.

In the present study we could clearly show that human IgE and IgG4 antibodies bound to the main immunogenic saliva antigens of *A. communis*, *A. aegypti* and *A. stephensi*. These findings confirm our previous immunoblot results with *Aedes* mosquito saliva in mosquito-sensitive subjects [3, 4] and extend the data to the immunogenic and allergenic properties of the saliva of *Anopheles* mosquitoes. Addi-

tional studies measuring cutaneous reactivity to mosquito bites in man with simultaneous saliva-specific antibody measurements [22, 23] are needed to further characterize the degree of cross-reactivity between medically important mosquito species.

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