Original article

Antibody response against saliva antigens of *Anopheles gambiae* and *Aedes aegypti* in travellers in tropical Africa

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Abstract

Exposure to vectors of infectious diseases has been associated with antibody responses against salivary antigens of arthropods among people living in endemic areas. This immune response has been proposed as a surrogate marker of exposure to vectors appropriate for evaluating the protective efficacy of antivectorial devices. The existence and potential use of such antibody responses in travellers transiently exposed to *Plasmodium* or arbovirus vectors in tropical areas has never been investigated. The IgM and IgG antibody responses of 88 French soldiers against the saliva of *Anopheles gambiae* and *Aedes aegypti* were evaluated before and after a 5-month journey in tropical Africa. Antibody responses against *Anopheles* and *Aedes* saliva increased significantly in 41% and 15% of the individuals, respectively, and appeared to be specific to the mosquito genus. A proteomic and immunoproteomic analysis of anopheles and *Aedes* saliva allowed for the identification of some antigens that were recognized by most of the exposed individuals. These results suggest that antibody responses to the saliva of mosquitoes could be considered as specific surrogate markers of exposure of travellers to mosquito vectors that transmit arthropod borne infections.

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Keywords: malaria; arbovirus; vectors; saliva; antibody response; proteomic; travellers; *Anopheles gambiae; Aedes aegypti*

Abbreviations: SD, standard deviation; Ig, immunoglobulin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; OD and aOD, optical density and adjusted optical density; HRP, horse radish peroxidase; MS and MS/MS, mass spectrometry and tandem mass spectrometry; MALDI−TOF, matrix assisted laser desorption ionization−time-of-flight; PMF, peptide mass fingerprinting; 95% CI, 95% confidence interval; SAAnG, saliva antigens of *An. gambiae*; SAAea, saliva antigens of *Ae. aegypti.*

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1. Introduction

Malaria and arbovirosis, like dengue or yellow fever, are major threats in inter-tropical areas and responsible for 2—3 million deaths and 500 000 cases, respectively (WHO, http://www.who.int). Their agents are transmitted by mosquito vectors, like Anopheles gambiae for Plasmodii and Aedes aegypti for some arbovirus. Individual antivectorial devices like impregnated bed-nets, repellents and long-sleeved clothes are proposed to protect non-immune people travelling across endemic areas against mosquitoes vectors. However, the use of these antivectorial devices is often perceived as restrictive, and their efficacy in protecting travellers has not been thoroughly evaluated. Entomological methods are not designed for estimating individual exposure level to mosquito vector and their use is not practical for the evaluation of the efficacy of antivectorial devices among travellers.

In contrast, serological methods may provide an indirect means of estimating individual exposure to mosquito vectors. Several studies have shown that mosquito saliva is immunogenic. Early studies have demonstrated that allergic persons posed to anopheles bites, develop antibodies to anopheles salivary proteins [5,6]. An. gambiae anti-saliva IgG antibody responses have been shown to represent an immunological indicator of the level of P. falciparum transmission [6]. Association of the level of specific antibody responses to the saliva of arthropods with exposure to the corresponding vectors has also been demonstrated for Lyme disease [7], Chagas disease [8], leishmaniasis [9] and African trypanosomiasis [10]. These studies have focused on populations living in endemic areas, never on travellers transiently exposed to vectors. Therefore, it is unclear whether the saliva antigens of the main malaria and arbovirus vectors are immunogenic enough to trigger a detectable antibody response after only a few bites.

The objectives of this study were: (i) to analyse the development of antibody responses against saliva antigens of An. gambiae and Ae. aegypti in travellers who are transiently exposed to these vectors in endemic areas of tropical Africa; (ii) to evaluate the kinetics of these responses; and (iii) to characterize the antigenic saliva proteins from these two mosquito species. The antibody responses against the saliva antigens of mosquitoes could be analysed as a surrogate serological marker of individual exposure to vector bites in persons who are only transiently exposed and used for the evaluation of the efficacy of antivectorial devices among travellers.

2. Materials and methods

2.1. Population studied

The anti-saliva antibody response has been studied in 88 French soldiers (male, age mean ± SD: 24.3 ± 4.1 years, Caucasians) who travelled during a 5-month period in tropical Africa (Gabon and Côte d’Ivoire) where An. gambiae and Ae. aegypti are endemic [11,12]. Among them, 48 had previously travelled in countries where at least one of these mosquitoes is endemic. During their mission, they lived 64 and 36 days in urban and rural areas of Gabon, respectively, and 15 and 53 days in urban and rural areas of Côte d’Ivoire, respectively. The rural conditions in Côte d’Ivoire were mostly rough and they often slept in damaged houses or bivouacs. Blood samples were collected before their departure from France (T1), after 2 months in tropical Africa (T2), at the end of their mission (T3) and 3 months after returning to France (T4). Blood samples were obtained by venous puncture, centrifuged and sera were stored at −20 °C. In total, 88 and 87 blood samples were available for serological analysis at T1 and T3, respectively. Due to difficulties in obtaining saliva antigens, ELISA tests were done in a restricted number of randomly chosen samples: 35 and 33 samples for IgG response against saliva antigens of An. gambiae (SAAng) at T2 and T4, respectively, 41 pairs of sera for anti-SAAng IgM at T1 and T3, and 48 pairs of sera for IgG response against saliva antigens of Ae. aegypti (SAAea) at T1 and T3.

The volunteers used anti-malaria chemoprophylaxis (100 mg doxycycline per day during the mission and 4 weeks after its end) and individual antivectorial devices like impregnated bed-nets, repellents and long-sleeved battle dresses provided by the Army.

Blood samples were obtained twice at a 3—5-month intervals from 22 individuals living permanently in Marseille (France), i.e. an area free of An. gambiae or Ae. aegypti, and who had never travelled in countries that are endemic for these mosquitoes.

All participants gave their informed consent to take part in the study and the protocol was approved by Marseille-2 Ethical Committee.

2.2. Method of mosquito salivation

The An. gambiae and Ae. aegypti used in this study were the YAOUNDE and BORA-BORA reference strain, respectively, maintained under standard conditions. The mosquito salivation technique was performed as described by Remoue et al. [6]. Total protein concentrations was estimated from the absorbance values at 280 and 260 nm, using the formula (corresponding to 20 mosquitoes), lyophilized and stored at −20 °C without any anti-protease cocktail.

2.3. Enzyme-linked immunosorbent assay

The sera were tested by ELISA for the presence of IgG and IgM antibodies that bind to saliva. Mosquito saliva (1 μg/ml in phosphate-buffered saline (PBS)) was coated on flat-bottom microtitre plates maxisorb (Nunc, Denmark) overnight at 4 °C. Thereafter, the plates were blocked for 2 h at 37 °C with 200 μl of blocking buffer consisting of PBS solution plus 0.1% Tween-20 (Sigma Chemical Co., USA) and 3%
skimmed milk (Beckton, Dickinson Bioscience, USA), and washed three times with PBS plus 0.1% Tween-20 (PBS—
Tween). Sera, diluted 1/20 in blocking buffer, were incubated (100 μl/well) for 2 h at 37 °C, plates were then washed and
thereafter incubated at 37 °C for 1 h with 100 μl peroxidase-
conjugated goat anti-human IgG or IgM (Jackson Immunore-
search, USA) diluted 1/10000 in blocking buffer. Plates were
washed three times with PBS—Tween before incubating with
80 μl tetramethylbenzidine substrate (KPL, USA,) for
20 min at room temperature. The enzymatic reaction was
stopped by the addition of 50 μl 1 M H2SO4. Plates were
read at 450 nm with an ELISA reader (Versa Max® Turnable
Multiplate reader, Molecular Devices, UK).

Each serum was tested in duplicate and in control wells
without saliva. When the optical densities (OD) in the control
well were higher than 0.25, or when one duplicate had a signal
33% higher or more than the other, serum was reanalysed.

Inter-assay variations in OD values of the negative and pos-
itive controls ranged within 25% of their mean values.

2.4. Statistical analysis

The levels of IgG and IgM antibodies were expressed as
adjusted OD (aOD) calculated for each serum as the mean
OD value with saliva minus the OD value of the control wells,
i.e. without saliva.

Individual variations in IgG and IgM antibody responses
were assessed by OD difference between pairs of sera col-
clected at 3—5-month intervals, i.e. OD at the end of the period
minus OD at the beginning of the period. The lower limit of
a significant increase in IgG or IgM responses between the
beginning and the end of the mission was defined as the mean 
+ 3.09 × SD of the difference in OD estimated for 22
pairs of sera from the unexposed individuals. Assuming a
Gaussian distribution, values higher than these cut-offs
were supposed to be observed in less than 0.1% of pairs of
sera.

The Mann—Whitney test, Spearman’s rank correlation co-
efficient and Wilcoxon matched-pairs signed-ranks test were
computed when appropriate with STATA version 9.0 (Stata-
corp, USA). Incidence rates of significant increase in IgG or
IgM responses were estimated with their exact confidence
intervals for binomial variables.

2.5. SDS-PAGE and Western blot

Three samples corresponding to lyophilized saliva extracts
from 60 mosquitoes (120 μg and 60 μg of saliva proteins of
An. gambiae and Ae. aegypti, respectively) were reconstituted
in 30 μl of distilled water. Mosquito saliva samples were sus-
cessed in Tris buffer containing 5% (w/v) SDS (Sigma),
reduced with 1% (w/v) dithiothreitol (Sigma) and boiled for
5 min before protein being separated by 12% SDS-PAGE in
a Mini PROTEAN II (BioRad, Hercules, USA). Gels were
either stained with Coomassie brilliant blue (CBB) G-250
(Sigma) or silver nitrate (Sigma) as previously described
[13,14], and were either used for band removal and trypsin
digestion for protein sequencing, or for transfer to a nitrocellu-
lose membrane (0.45 μm, Amersham Pharmacia, France) by
semydri blotting (0.8 mA/cm²) [15]. Blots were saturated with
5% w/v skimmed milk, and each membrane was cut into
15—25 strips, 3—4 mm wide. Western blot was carried
out with human sera diluted 1/100 in PBS containing 0.2%
v/v Tween-20 with 5% w/v skimmed milk. After an overnight
incubation at +4 °C, blots were incubated with anti-human
IgG horse radish peroxidase (HRP) conjugated antibody
1/5000 (Sigma), and revealed on X-ray films (Amersham
Pharmacia) using an enhanced chemiluminescence kit (Roche,
Switzerland). Immune profiles were analysed when two inde-
pendent assays produced identical patterns. Stained gels and
autoradiographs were digitalized using a high-resolution scan-
er (Molecular Dynamics Personal Densitometer, Amersham
Pharmacia Biotech) and analysed using LumiAnalyst 3.1
software (Roche).

2.6. In-gel digestion and mass spectrometry (MS) analysis

Excised plugs from silver- or CBB-stained gels, were pre-
pared as described previously by Gharahdaghi et al. [14] and
Shevchenko et al. [16]. Proteins were digested overnight at
37 °C by sequencing-grade trypsin (12.5 μg/ml; Promega,
Madison, USA) in 50 mM NH4HCO3. The resulting peptides
were extracted and lyophilized.

First, each sample was analysed at least two times by matrix
assisted laser desorption ionization—time-of-flight (MALDI—
TOF) with a MALDI—TOF Etan-Pro (Amersham Bionsciences,
Sweden) mass spectrometer. When necessary, the samples were
further analysed using tandem mass spectrometry (MS/MS) on
a LCQTM-Deca-XP-Plus ion trap mass spectrometer (Thermo-
Finnigan, USA) equipped with a LCQTM nanospray ionization
source.

For MALDI—TOF analysis, peptides were resuspended in
10 μl 0.1% TFA, desalted on ZipTip C18-microcolumns
(Millipore, USA) and eluted with z-cyano-4-hydroxycinnamic
acid (3 mg/ml) in 50% (v/v) acetonitrile directly onto the
MALDI target. Spectra were calibrated using monoisotopic
MH+ ion from two peptide standards (trypsin autodigestion
products: 842.510 and 2211.1046 Da). Peptide mass finger-
printing (PMF) database searching was carried out using Pro-
found (ProteoMetrics, USA) and Mascot (Matrix Science Ltd,
UK) software that query comprehensive sequence databases
(VectorBase, http://vectorbase.org/).

For MS/MS analysis, nano-liquid separation of peptides was
carried out using an Etan—MDLC chromatographic system in
high throughput configuration and piloted by Unicorn 5.01
software (Amersham Bioncsiences). Ten microlitres of digest
was first trapped on a Zorbax 300SB-C18 5 μm × 0.3 mm and
eluted at a flow rate of approximately 200 nI/min, 3.5 μm,
150 × 0.075 mm by a linear gradient of eluant B (0.1% formic
acid, 84% acetonitrile) in eluant A (1% formic acid). A spray
voltage of 1.8 kV was applied to the liquid junction via an in-
union high voltage contact coupled to a silicaTip emitter (New
Objective). Operation of the mass spectrometer was fully
automated using the Excalibur-1.3 data system (ThermoFinnigan). Continuous cycles of one full scan (m/z 500—1700) followed by three data-dependent MS/MS measurements at 35% normalized collision energy were done. MS/MS measurements were allowed for the three most intense precursor ions with a maximum rejection time limit of 1 min. All MS/MS spectra were sequence database searched using Bioworks 3.1 (Thermo-Finnigan) or Mascott software. The MS/MS spectra were searched against the non-redundant \textit{Ae. aegypti} and \textit{An. gambiae} database (Vectorbase) using the following parameters: precursor-ion mass tolerance of 1.5 Da, fragment ion tolerance of 1.0 Da with methionine oxidation and cysteine carboxamidomethylation specified as differential modifications and a maximum of one missed cleavage site allowed.

3. Results

The development and the kinetics of antibody responses against saliva antigens of \textit{An. gambiae} and \textit{Ae. aegypti} have been analysed in travellers transiently exposed to these vectors in endemic areas of tropical Africa.

3.1. The serologic IgG signature of transient exposure to \textit{An. gambiae} is rapid and transitory, the one to \textit{Ae. aegypti} is more sustained

The IgG response against saliva antigens of \textit{An. gambiae} (SAAng) increased significantly from T1 to T3 (mean change in aOD $+0.11$, 95% confidence interval, 95% CI T1 T3 T4 $0.06$ to $0.17$ $P<0.0001$ $P=0.034$ $P<0.0005$ $P=0.84$).

![Fig. 1. Box plot graphs of IgG antibody response of French soldiers against the saliva antigens of Anopheles gambiae ($n=88$) and Aedes aegypti ($n=49$) before (T1), at the end (T3) and 3 months after (T4) a 5-month travel period in tropical Africa (Gabon, Côte d’Ivoire). aOD, individual mean OD value of wells with antigen minus OD value of wells without antigen. Lines of the boxes represent 75th percentile, median and 25th percentile of the individual average aOD values; whiskers represent the lower and upper adjacent values, and dots represent outside values. Differences between T1 and T3 and between T3 and T4 have been tested using Mann–Whitney test. $p$-values are indicated.]

![Fig. 2. IgG antibody response against the saliva antigens of Anopheles gambiae and Aedes aegypti of French soldiers before (A) any exposure to these mosquitoes ($n=15$) and after (B) a 5-month travel period in tropical Africa (Gabon, Côte d’Ivoire) where the two mosquitoes are endemic ($n=48$). aOD, individual mean OD value of wells with antigen minus OD value of wells without antigen. Regression lines for pairs of aOD values corresponding to the IgG antibody responses against the saliva antigens of \textit{Anopheles gambiae} and \textit{Aedes aegypti} are drawn. The Spearman’s rank correlation coefficient were rho $=0.58$, $p$-value $=0.023$ before (A) any exposure and rho $=0.19$, $p$-value $=0.21$ after (B) exposure to the two vectors.]

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+0.16; n = 87; signed-ranks test, p < 0.0001; Fig. 1) and decreased significantly from T3 to T4 (−0.03, 95% CI −0.06 to −0.003; n = 32; p = 0.034). It increased significantly from T1 to T2, i.e. after 2 months in Tropical Africa (+0.05, 95% CI +0.0 to +0.11; n = 35; p = 0.002) and from T2 to T3 (+0.05, 95% CI +0.02 to +0.09; n = 34; p = 0.002).

There was a significant increase in the IgG response against saliva antigens of Ae. aegypti (SAAea) between T1 and T3 (+0.09, 95% CI +0.04 to +0.14; n = 48; p < 0.0005; Fig. 1) and then after a non-significant decrease between T3 and T4 (−0.01, 95% CI −0.05 to +0.03; n = 32).

The anti-SAAng IgM response did not significantly change from T1 to T3 (−0.001, 95% CI −0.02 to +0.02; n = 41). Due to difficulties in obtaining saliva antigens of Aedes aegypti, the anti-SAAea IgM response was not analysed.

There was no significant difference in aOD for anti-SAAng and anti-SAAea IgG at T1 (n = 88) and T3 (n = 87) between individuals who had or had not a previous experience of travelling abroad in countries endemic for An. gambiae or Ae. aegypti before the mission.

3.2. IgG responses to saliva antigens of An. gambiae and Ae. aegypti: significant post-exposure increases and little cross-reactivity

Among soldiers who had no previous exposure to An. gambiae or Ae. aegypti, the aOD values for anti-SAAng and anti-SAAea IgG were significantly correlated at T1, i.e. before any exposure (Fig. 2A; n = 15, Spearman’s rho = 0.58; p = 0.023). That correlation was not significant among soldiers who had been exposed to An. gambiae and Ae. aegypti, i.e. at T3 (Fig. 2B; n = 48; rho = 0.19; p = 0.21).

The analysis of the individual variations in anti-SAAng and anti-SAAea antibody responses between T1 and T3 was aimed to estimate the incidence rate of significant individual increases in aOD during the travel. The incidence rate of significant antibody responses between T1 and T3 were 41% (36/87; 95CI: 31–52%), 17% (7/41; 95CI: 7–32%) and 15% (7/48; 95CI: 6–28%) for anti-SAAng IgG, anti-SAAng IgM and anti-SAAea IgG, respectively. Among the 48 individuals whose serum could be tested at T1 and T3 for anti-SAAng and anti-SAAea IgG, 15, one and six had a significant immune response against SAAng only, against SAAea only and against both saliva antigens, respectively. Among these individuals, there was no significant difference between those who had or had not previous exposure to An. gambiae or Ae. aegypti.

3.3. Fifteen or less proteins have been identified in An. gambiae and Ae. aegypti saliva

Saliva proteins separated by SDS-PAGE and silver stained revealed 15 and 10 protein bands from An. gambiae and Ae. aegypti, respectively, with a molecular weight ranging from 13 to >300 kDa (Fig. 3). Mass spectrometry identified 14 and 10 distinct proteins of An. gambiae and Ae. aegypti saliva, respectively (Table 1). Most of them belong to known salivary gland protein families, e.g. apyrase and nucleotidase families that are involved in anti-clotting reactions.

3.4. A few saliva antigens of An. gambiae and Ae. aegypti were recognized by IgG of travellers

Antigenicity of saliva proteins was evaluated by immunoblots using sera from non-exposed (n = 5) and exposed individuals having high anti-SAAng (n = 11) or/and anti-SAAea (n = 6) IgG response at T3. Sera from all exposed subjects recognized a similar small group of An. gambiae saliva proteins whose molecular weight was in the range 12–18 kDa, 33–40 kDa or 64–77 kDa (Fig. 4). Most sera from exposed subjects recognized an Ae. aegypti saliva immunogenic band of 45 kDa. The profiles of these recognized Ae. aegypti and An. gambiae proteins were completely different. Sera from unexposed individuals recognized some Ae. aegypti or An. gambiae proteins that differed from those recognized by sera of exposed individuals.

4. Discussion

Our data show that travellers transiently exposed to An. gambiae and Ae. aegypti bites developed IgG antibody responses...
Table 1
Protein identification of *Anopheles gambiae* and *Aedes aegypti* saliva by mass spectrometry (MALDI–TOF and MS/MS) from the Mascott database

<table>
<thead>
<tr>
<th>Band</th>
<th>Protein name</th>
<th>Family</th>
<th>Ensembl and/or uniprotKB/TrEMBL</th>
<th>Accession number</th>
<th><em>M</em>&lt;sub&gt;r&lt;/sub&gt; (Da)</th>
<th>MALDI-TOF analysis</th>
<th>MS/MS analysis</th>
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<td>Number of sequences</td>
</tr>
</tbody>
</table>

**Aedes aegypti**

1. Hypothetical protein 5’Nucleotidase family
   - Accession: AAEEL009968-PA gi
   - *M*<sub>r</sub>: 25240
   - MALDI-TOF: 2, 0.01, 12.3

2. 30 kDa salivary gland allergen Aed a 3
   - Accession: AAEEL010235-PA and ALL3_AEDE
   - *M*<sub>r</sub>: 30000
   - MALDI-TOF: 3, 2.2, 12.3

3. 30 kDa salivary gland allergen Aed a 3
   - Accession: AAEEL010235-PA and ALL3_AEDE
   - *M*<sub>r</sub>: 30000
   - MALDI-TOF: 4, 1.2, 12.3

   - Actin
     - Accession: AAEEL011197-PA and Q4PKE5
     - *M*<sub>r</sub>: 41822
     - MALDI-TOF: 4, 0.417, 15.9

4. Inosine–uridine preferring nucleoside hydrolase
   - Accession: AAEEL006485-PA and Q8T9V9
   - *M*<sub>r</sub>: 37906
   - MALDI-TOF: 3, 4, 15.9

5. n.i.
6. Salivary serpin antiFXA
   - Accession: AAEEL002704-PC and Q1HOGR_AEDE
   - *M*<sub>r</sub>: 47040
   - MALDI-TOF: 2, 7.7

7. Beclin
   - Accession: AAEEL010427-PA and Q16T27
   - *M*<sub>r</sub>: 49600
   - MALDI-TOF: 3, 0.00, 7.7

8. D7 family
   - Accession: AAEEL006424-PA and D7_AEDE
   - *M*<sub>r</sub>: 37028
   - MALDI-TOF: 2, 15.9

9. Long form D7Bch1 salivary protein or allergen Aed a 2
   - Accession: AAEEL011197-PA and Q4PKE5
   - *M*<sub>r</sub>: 41822
   - MALDI-TOF: 4, 0.417, 15.9

10. n.d.
11. Salivary apyrase or allergen Aed a 1
   - Accession: AAEEL006347-PA and APY_AEDE
   - *M*<sub>r</sub>: 36000
   - MALDI-TOF: 3, 0.908, 5

12. Ecotropic viral integration site
    - Accession: AAEEL009993-PA and Q4VQ31_AEDE
    - *M*<sub>r</sub>: 345879
    - MALDI-TOF: 5, 2.9

**Anopheles gambiae**

13. gSG6 protein
    - Accession: Q9BIH5
    - *M*<sub>r</sub>: 13095
    - MALDI-TOF: 9, 0.046, 59

14. D7-related 2 protein
    - Accession: Q9UB31
    - *M*<sub>r</sub>: 18469
    - MALDI-TOF: 6, 0.023, 37

15. D7-related 3 protein
    - Accession: Q9UB32
    - *M*<sub>r</sub>: 18692
    - MALDI-TOF: 3, 0.043, 26

16. D7-related 4 protein
    - Accession: Q9BIH3
    - *M*<sub>r</sub>: 19332
    - MALDI-TOF: 6, 0.018, 33

17. Long form D7 salivary protein
    - Accession: Q8WR35
    - *M*<sub>r</sub>: 35767
    - MALDI-TOF: 10, 0.00084, 34
against saliva antigens from these two unrelated mosquitoes in about 41% and 15% of the individuals, respectively. The IgG antibody response to SAAng appeared to be rapid and short-lived. The increases and decreases in these antibody responses were similar to those observed in subjects living in southern Finland before and after the mosquito season [17] and among Senegalese children [6]. The averaged anti-SAAng IgM antibody aODs did not differ significantly between T1 and T3, possibly because IgM response is short-lived. The anti-SAAea IgG increased similarly to the anti-SAAng IgG but did not decrease as rapidly after the end of exposure. Furthermore, only 15% of the exposed individuals developed a significant increase in anti-SAAea IgG. The difference between the incidences in anti-SAAng and anti-SAAea antibody responses could be explained by a difference in immunogenicity of saliva antigens, in the immunoglobulin isotype of antibodies (i.e. anti-SAAea IgE and anti-SAAng IgG) or in exposure. Indeed, soldiers spent more time in rural areas where *An. gambiae* is usually more prevalent than in urban areas where *Ae. aegypti* is usually more common. Differences in exposure, attractiveness for mosquitoes or use of antivectorial devices may also explain differences in antibody responses between individuals. However, no entomological data was collected in the present study to support these hypotheses of differences in exposure.

Doxycycline is thought to transiently reduce antibody responses [18] and may have depressed the observed antibody responses to saliva antigens.

*An. gambiae* and *Ae. aegypti* saliva were used as antigens instead of salivary gland extracts [19] because we were interested in the antibody response to secreted proteins possibly subjected to post-transductional modifications, including enzymatic activation, and not to structural proteins of salivary glands or unactivated proenzymes. Difficulties associated with collecting large quantities of saliva and preserving it from enzyme degradation have limited the extent of our serological analysis.

Most of the *An. gambiae* saliva proteins identified in our study have been previously identified by salivary gland transcripts analysis [20,21] or proteomic methods [19]. We identified SG4 and SG5 [22] proteins in saliva, which have not been previously identified in salivary gland extract [19]. Other proteins like D7-related-1 (gi:31222471), D7-related-5 (gi:18378603), trio (gi:18389917) and SG1 (gi:4210615) have been identified in salivary gland extract but not in the saliva of our study. These differences could be attributed to the low quantity of proteins collected in saliva or to differences in the relative quantity of proteins present in one band. It is also possible that these proteins are not secreted or are degraded before or shortly after salivation. For example, SG1 protein has been proposed to be a sporozoite receptor and localized to the basal lamina of salivary glands [22]. Therefore, it could not be secreted in the saliva despite being identified in salivary gland extracts.

The genome and a catalogue of the salivary gland transcripts of *Ae. aegypti* are now available [23]. Our mass spectrometry analysis allowed the identification of 11 saliva

<table>
<thead>
<tr>
<th>Band name</th>
<th>Accession number</th>
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<th>Number of matching peptides</th>
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The band name corresponds to the name indicated on the gel in Fig. 3. The identities of the bands, their accession number, their family and theoretical Mr value, as well as the number of matching peptides and the corresponding percentage of sequence coverage are indicated. n.i., no identification.
proteins from *Ae. aegypti* including three proteins considered to be allergen factors (Aed-a-1, -2 and -3) [24–26]. Two of them (Aed-a-1 and -2) belong to the 5′ nucleotidase and D7 family that are also present in *An. gambiae* saliva.

The immune response to mosquito saliva is complex, as indicated by Western blot data. Some antigens may elicit a response pattern that differs from one individual to another while other antigens are recognized by the sera of most of the exposed individuals.

Sera from exposed subjects recognized *An. gambiae* saliva proteins of 12–18 kDa, 33–40 kDa and 64 kDa, which might, according to their molecular weight, correspond to gSG6 (band #11), SG1b (band #18), SG1-like 3 (band #16 and 17) and 5′-nucleotidase (band #21), respectively. Short forms of the D7 salivary protein of *An. gambiae* (∼17 kDa) have been recognized as antigenic in other studies [1,5]. Sera from exposed subjects recognized an *Ae. aegypti* saliva protein of 45 kDa that is similar in size to the protein recognized by allergic persons in *Ae. aegypti* mosquito extract [4] and to Aed-a-X1 [27].

Previous studies have suggested that antibody responses to mosquito salivary proteins are genus-specific, indicating little cross-reactivity between salivary proteins from different arthropods (i.e. *Anopheles, Culex* or *Aedes*) [28] or that both species-shared and species-specific allergens exist [1,29]. The significant correlation between low anti-SAAng and anti-SAAea IgG responses we observed before any exposure to these two unrelated mosquitoes suggest that: (i) cross-reactions may exist to saliva antigens from other mosquitoes, e.g. mosquitoes that are endemic in France; and (ii) antibody responses to saliva antigens could be non-specific to mosquito genus. However, the following results suggest that there is at least some mosquito genus specificity in IgG antibody responses: (i) the absence of a significant correlation between IgG antibody responses after exposure to *An. gambiae* and/or *Ae. aegypti* bites; (ii) the different patterns of significant increases in IgG antibody to *An. gambiae* or *Ae. aegypti* saliva during the mission; and (iii) the differences in antigenicity of saliva proteins revealed by immunoblots. Some of the saliva proteins of *An. gambiae* recognized by the sera of exposed individuals (SG1 family proteins and gSG6 protein) have orthologues in other anopheline mosquitoes and may be specific immunological markers of exposure to this mosquito genus [30]. Other recognized proteins have orthologues in the salivary glands of many other mosquitoes, including *Aedes* (e.g. 5′-nucleotidase and D7 salivary protein family).

In conclusion, the saliva of *An. gambiae* and *Ae. aegypti* are immunogenic enough for individuals transiently exposed to the bites of these two unrelated vectors to develop IgG and IgM antibody responses that are specific to the mosquito genus. However, additional investigations are necessary to identify saliva antigens that could be used for the serological evaluation of exposure to mosquito bites and efficacy of antivectorial measures.

Fig. 4. Antibody responses of subjects transiently exposed or unexposed to *Aedes aegypti* and *Anopheles gambiae* saliva. IgG immune profiles obtained with sera collected from eleven exposed subjects (lines identified by letters from a to l) and five unexposed subjects (lines identified by numbers from 1 to 5) were tested against *Ae. aegypti* and *An. gambiae* salivary proteins separated by 12% SDS-PAGE and blotted onto a nitrocellulose membrane. Prestained SDS-PAGE standards low-range (BioRad, Hercules, CA, USA) protein marker ranges from 22 to 114 kDa.

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References