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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.

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 develop immunoglobulin G4 (IgG4) and IgE to mosquito saliva antigens [1–4]. Recently, studies have demonstrated that people living in malaria endemic areas, who are constantly exposed 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 $1.55 \times A_{280} - 0.76 \times A_{260}$, and the concentration adjusted at 400 µg/ml for *Anopheles* and 200 µg/ml for *Aedes.* Mosquito saliva samples were pooled, aliquoted in 100 µl (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%

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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 Immunoresearch, 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 H_2SO_4. 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 positive 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 collected 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 coefficient and Wilcoxon matched-pairs signed-ranks test were computed when appropriate with STATA version 9.0 (StataCorp, 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 suspended 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 brillant 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 nitrocellulose membrane (0.45 µm, Amersham Pharmacia, France) by semidry blotting (0.8 mA/cm^2) [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 independent assays produced identical patterns. Stained gels and autoradiographs were digitalized using a high-resolution scanner (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 prepared 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 NH_4HCO_3. 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 Ettan-Pro (Amersham Biosciences, 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 α-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 fingerprinting (PMF) database searching was carried out using Profound (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 Ettan–MDLC chromatographic system in high throughput configuration and piloted by Unicorn 5.01 software (Amersham Biosciences). Ten microlitres of digest was first trapped on a Zorbax 300SB-C18 5 × 0.3 mm and eluted at a flow rate of approximately 200 nl/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

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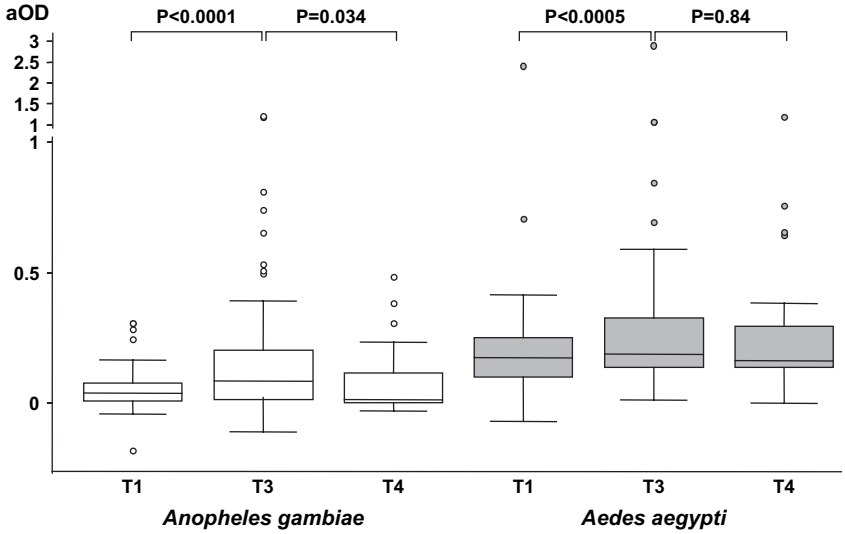

Fig. 1. Box plot graphs of IgG antibody response of French soldiers against the saliva antigens of *Anopheles gambiae* ($n = 88$) and *Aedes aegypti* ($n = 49$) before (T1), at the end (T3) and 3 months after (T4) a 5-month travel period in tropical Africa (Gabon, Côte d'Ivoire). aOD, individual mean 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.

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 (ThermoFinnigan) or Mascott software. The MS/MS spectra were searched against the non-redundant *Ae. aegypti* and *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 carboxyamidomethylation 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 *An. gambiae* and *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 An. gambiae *is rapid and transitory, the one to* Ae. aegypti *is more sustained*

The IgG response against saliva antigens of *An. gambiae* (SAAng) increased significantly from T1 to T3 (mean change in aOD +0.11, 95% confidence interval, 95% CI +0.06 to

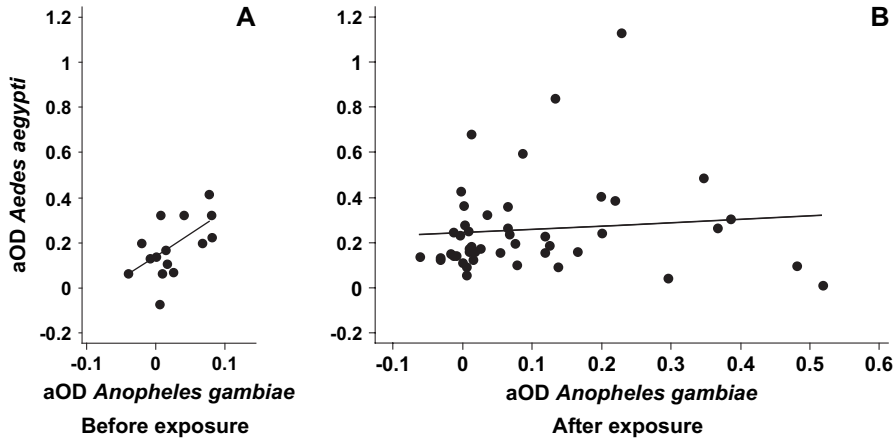

Fig. 2. IgG antibody response against the saliva antigens of *Anopheles gambiae* and *Aedes aegypti* of French soldiers before (A) any exposure to these mosquitoes ($n = 15$) and after (B) a 5-month travel period in tropical Africa (Gabon, Côte d'Ivoire) where the two mosquitoes are endemic ($n = 48$). 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 *Anopheles gambiae* and *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.

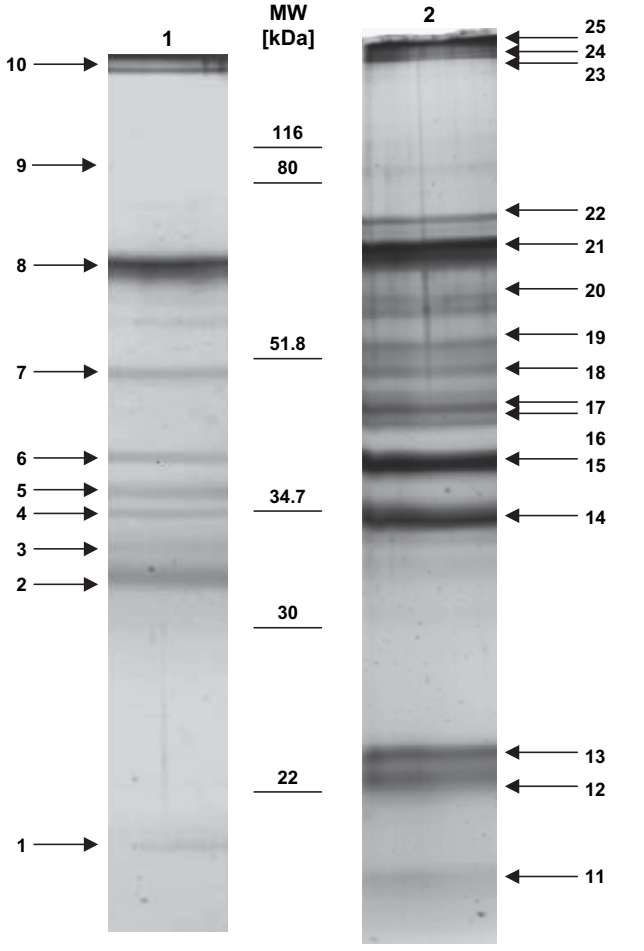

Fig. 3. *Aedes aegypti* (1) and *Anopheles gambiae* (2) saliva SDS-PAGE analysis (12%). Numbers refer to Table 1 of protein identification. Standard protein markers (BioRad, Hercules, CA, USA) range from 22 to 116 kDa.

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

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Table 1
Protein identification of *Anopheles gambiae* and *Aedes aegypti* saliva by mass spectrometry (MALDI−TOF and MS/MS) from the Mascott database

Band number	Protein name	Family	Ensembl and/or uniprotKB/TrEMBL	Accession number	M_r (Da)	MALDI-TOF analysis			MS/MS analysis	
						Number of peptides	Score (profound)	Sequence coverage %	Number of peptides	Sequences
Aedes aegypti										
1	Hypothetical protein	5′Nucleotidase family	AAEL009968-PA	gi| 108873892	25240	2	0.01	12.3		
2	30 kDa salivary gland allergen Aed a 3		AAEL010235-PA and ALL3_AEDAE	gi| 14423642	30000				3	R.VPVVEAIGR.I; R. SALNNDLQSEVR.V; K.VDHIQSEYLR.S
3	30 kDa salivary gland allergen Aed a 3		AAEL010235-PA and ALL3_AEDAE	gi| 14423643	30000				4	R.VPVVEAIGR.I; R.QVVALLDKDTK.V; K.VDHIQSEYLR.S R.SALNNDLQSEVR.V
	Actin		AAEL011197-PA and Q4PKE5	gi| 108872510	41822				4	R.AVFPSIVGRPR.H; K.AGFAGDDAPR.A; R.VAPEEHPVLLTEAPLNPK.A; K.SYELPDGQVITIGNER.F
4	Inosine−uridine preferring nucleoside hydrolase	Nucleotidase	AAEL006485-PB and Q8T9V9	gi| 108877688	37906				3	R.NSVFKEPK.N; R.YKADVELAGK.V; K.SEIAGIYILGGNR.N
	Long form D7Bclu1 salivary protein or allergen Aed a 2	D7 family	AAEL006424-PA and D7_AEDAE	gi| 118216	37028				2	R.TGLYDPVAQK.F; K.FDASVIQEQFK.A
5	n.i.									
6	Salivary serpin antiFXA	Serpins	AAEL002704-PC and Q1HQG8_AEDAE	gi| 121959062	47040				2	R.IPQFGLQTTVPGR.Q; K.VFEQGQDVALGEIVQK.M
7	Beclin		AAEL010427-PA and Q16T27	gi| 108873379	49600	3	0.00	7.7		
	Actin		AAEL011197-PA and Q4PKE5	gi| 108872510	41822	4	0.417	15.9		
8	Salivary apyrase or allergen Aed a 1	5′Nucleotidase family	AAEL006347-PA and APY_AEDAE	gi| 1703351	63240	3	0.918	5	2	K.EAEYYIVVPSYLADGK.D; K.VTLSNAVEAVR.R
9	Ecotropic viral integration site		AAEL013460-PA	gi| 108870049	95190	7	0.253	10.9		
10	SG1	SG1 family	AAEL009993-PA and Q4VQB1_AEDAE	gi| 66828491	345879	5	0.397	2.9	2	R.YTGQEFDEETGLYNYHAR.L; R.FSFPLVDLQGR.M
Anopheles gambiae										
11	gSG6 protein	Salivary gland	Q9BIH5	gi|13537666	13095	9	0.046	59		
12	D7-related 2 protein	D7 family	Q9UB31	gi| 4538889	18469	6	0.023	37	4	K.MQTSDPFDMNR.V; K.NAVDYNELLK.A; K.ANTFYTCFLGTSSLAGFK.N; K.ESVLLELLQR.V
13	D7-related 3 protein	D7 family	Q9UB32	gi| 17016226	18692	3	0.043	26	2	K.YAVDYVELLR.A; K.LDMGTTFNAGQVSALMK.Q
	D7-related 4 protein	D7 family	Q9BIH3	gi| 17016228	19332	6	0.018	33	3	K.CIGECVQVPTSER.A; R.ALDFVYEDGRGDYHK.L; R.YTAEFVQIMK.D
14	Long form D7salivary protein	D7 family	Q8WR35	Q8WR35	35767	10	0.00084	34		

	gVAG protein	Antigen 5 family	O97413	gi|34556108	28913	8	0.007	40	
15	ENSANGP00000017629		Q7QA52	gi|55240805	36227	5	0.083	17	
16	Salivary gland 1-like 3 protein	SG1 family	Q8WR33	gi|18389895	30971	10	7.70E-06	36	
17	Salivary gland 1-like 3 protein	SG1 family	Q8WR33	gi|18389895	30971	7	0.026	25	
18	gSG1b protein	SG1 family	Q9BIH6	gi|74848221	43625	10	0.0055	30	
19	n.i.								
20	n.i.								
21	5′-Nucleotidase	Enzyme	Q9UB34	gi|4582528	63459	13	8.80E-08	29	
22	ENSANGP00000017682 str pest	Amylase	Q7PYT9	gi|55233274	63491	10	0.026	18	
23	ENSANGP00000028598	Salivary gland	Q5TUS2	gi|55243829	136447	8	0.126	10	
24	SG4	Salivary gland	Q5XLG7	gi|54066728	389184	21	0.0024	8	
25	SG5	Salivary gland	Q5XLG6	gi|54066769	384673	15	0.0022	6	

The band name corresponds to the name indicated on the gels in Fig. 3. The identities of the bands, their accession number, their family and theoretical M_r value, as well as the number of matching peptides and corresponding percentage of sequence coverage are listed for MALDI–TOF analysis. For MS/MS analysis, the number of matching peptides and the corresponding sequences are indicated. n.i., no identification.

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

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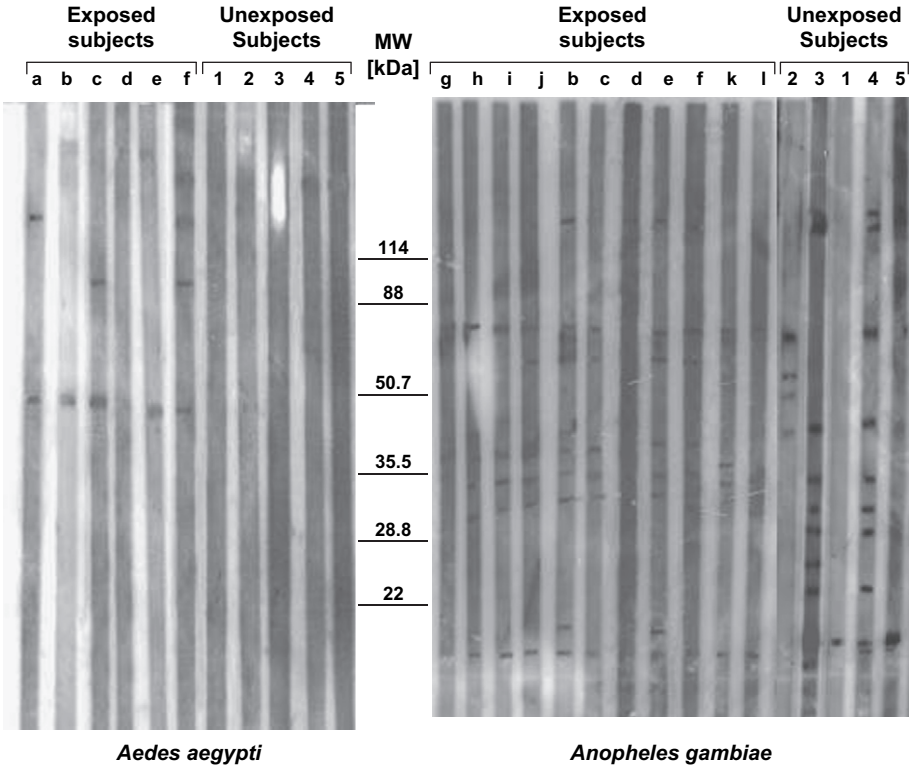

Fig. 4. Antibody responses of subjects transiently exposed or unexposed to *Aedes aegypti* and *Anopheles gambiae* saliva. IgG immune profiles obtained with sera collected from eleven exposed subjects (lines identified by letters from a to l) and five unexposed subjects (lines identified by numbers from 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.

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* ($\approx$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 anti-vectorial measures.

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