



IgE and IgG4 antibody responses to *Aedes* saliva in African children

Franck Remoue^{a,b,*}, Eric Alix^a, Sylvie Cornelie^a, Cheikh Sokhna^c,
Badara Cisse^{c,d}, Souleymane Doucoure^b, François Mouchet^a,
Denis Boulanger^a, François Simondon^a

^a Institut de Recherche pour le Développement (IRD) - UR024, "Epidémiologie et Prévention" Unit, Montpellier, France

^b Institut de Recherche pour le Développement (IRD) - UR024, "Epidémiologie et Prévention" Unit, Dakar, Senegal

^c Institut de Recherche pour le Développement (IRD) - UR77, "Paludologie Afro-tropicale" Unit, Dakar, Senegal

^d Département de Parasitologie, Faculté de Médecine, Université Cheikh Anta Diop (UCAD), Dakar, Senegal

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Abstract

Aedes mosquitoes are the major vectors of (re)-emerging infections including arboviruses (dengue, Chikungunya, yellow fever) in developing countries. Moreover, the emergence of *Aedes*-borne diseases in the developed world is currently a source of concern. Evaluation of human immune responses to *Aedes* bites could be a useful immuno-epidemiological tool for evaluating exposure to *Aedes*-borne diseases and thus predicting the risk of such emerging diseases. Specific IgE and IgG4 antibody (Ab) responses to *Aedes aegypti* saliva were evaluated in young Senegalese children living in an area of exposure to the *Aedes* vector. Specific IgE and IgG4 responses increased during rainy season of high exposure to *Aedes* bites. In addition, the evolution of anti-saliva isotype levels during the rainy season presented spatial heterogeneity between the studied villages. These preliminaries results support the potential approach of using anti-saliva Ab responses for evaluating exposure to *Aedes* vectors and risks of emerging arbovirus infections.

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1. Introduction

Aedes mosquitoes are the vectors of several diseases including arboviruses (dengue, Chikungunya, yellow fever) and parasites (filariasis). In developing countries,

these arthropod-borne diseases are considered to be public health problems while some are viewed as major (re)-emerging infections. Moreover, several such diseases threaten to re-emerge in the developed world where the current or potential presence of their vectors has been linked to the substantially increase human communications/transport to and from Africa, South America and Asia (Reiter et al., 2006). This has prompted development of surveillance systems in southern Europe, including networks set up to monitor *Aedes* populations in order to identify the risks of dengue and Chikungunya infections (Mackenzie et al., 2004; Ligon, 2006). Epi-

* Corresponding author at: Institut de Recherche pour le Développement (IRD) - UR024, "Epidémiologie et Prévention" Unit, Centre IRD de Hann - Route des Pères Maristes, B.P. 1386, CP 18524, Dakar, Senegal. Tel.: +221 849 35 55/35 51; fax: +221 832 43 07.

E-mail address: franck.remoue@ird.sn (F. Remoue).

URL: <http://www.mpl.ird.fr/epiprev> (F. Remoue).

demological tools for evaluating the risk of exposure to *Aedes* vector bites are thus urgently needed in both developing and developed countries.

The epidemiological evaluation of *Aedes*-borne diseases is currently based on pathogen detection in human populations and on entomological methods which indicate risks of transmission (Reiter et al., 2006; Mackenzie et al., 2004). The potential exposure of populations to *Aedes* bites is mainly evaluated by identification of breeding sites, capture of mosquitoes by traps, indoor spraying and human landing catches. Unfortunately, these methods have substantial limitations in terms of large-scale field application and may raise ethical concerns, especially for human landing catches. A simple, rapid method for evaluating of *Aedes* bite exposure is necessary to elucidate the risk of *Aedes*-transmitted diseases at population and individual levels.

One type of research hypothesizes that exposure to arthropod vector bites can be evaluated by analysis of the antibody (Ab) response to arthropod saliva in human populations potentially exposed to risk of infection (Remoue et al., 2005; Billingsley et al., 2006). Indeed, since the 1990s, it has been demonstrated that anti-salivary IgG responses can be used as markers of exposure to vectors such as *Ixodes dammini* ticks, vector of Lyme disease (Schwartz et al., 1990). In addition, specific IgG1 and IgG4 isotype responses to *Triatoma* saliva have been detected in Chagas disease (Nascimento et al., 2001). The Ab response to sandfly salivary antigens could represent an efficient indicator of exposure to *Leishmania* (Barral et al., 2000), and our team has recently used this approach for glossina species, vectors of human African trypanosomiasis (Poinsignon et al., 2007). The transient nature of mosquito feeding poses a problem in terms of the applicability of this approach to mosquito-transmitted diseases. However, similar relations have been observed for *Culex quinquefasciatus* and related *Wuchereria bancrofti* filariasis (Das et al., 1991) and for Ab responses to *Culex tarsalis* salivary gland antigens evaluated in chickens used as sentinel for arbovirus surveillance in California (Trevejo and Reeves, 2005). Recently, we showed that children from malaria-endemic area developed a specific IgG response to *Anopheles gambiae* saliva which was positively associated with the degree of exposure to the vector bites, evaluated by classical entomological studies (Remoue et al., 2006). In addition, high anti-saliva IgG levels were significantly associated with the outcome of malaria morbidity.

Major research on the immune response to *Aedes* saliva has been directed toward the study of allergy mechanisms induced in man by *Aedes* and *Culex* bites,

the latter being the most prevalent mosquitoes in developed countries (Finland, Canada). The main objectives of those studies were to develop new diagnostic tests for evaluating allergy risks induced by mosquito bites and for identifying salivary proteins for use in desensitization immunotherapy (Peng and Simons, 2004). In particular, studies showed that evaluation of the anti-saliva Ab response by quantitative and simple method (ELISA) could be a useful indicator of human exposure to *Aedes* bites in allergic patients (Brummer-Korvenkontio et al., 1997). In terms of isotype Ab responses, IgE and IgG4, isotypes frequently associated with allergy, were discriminative to evaluate the exposure to *Aedes* bites in adults (Brummer-Korvenkontio et al., 1994; Peng, 2002). An increase in human IgE and IgG4 anti-saliva Ab levels was reported during the season of *Aedes* exposure in Finland (Palosuo et al., 1997) and, especially, IgG4 Ab specific to *Aedes aegypti* saliva might be a adequate marker of intense exposure to *Aedes* bites (Reunala et al., 1994).

The objective of the present study was to evaluate using ELISA, specific IgE and IgG4 responses to whole saliva from *Ae. aegypti* in African young children living in a rural area of Senegal where this potential vector is present (Diallo et al., 1999; Thonnon et al., 1999). Immunological results were analysed according to (i) the rainy season (start and end) which corresponds to the highest season of bite exposure; (ii) the age of the children; (iii) the villages in this area which might present variations in the intensity of *Aedes* exposure.

2. Materials and methods

2.1. Studied population

The study was conducted in the Niakhar zone, a rural area of central Senegal, with frequent exposure to *Ae. aegypti* bites. This site is a dry savannah with rainy season from July to October; it is typical of the Sahelian and sub-Saharan regions of Africa.

The study was integrated into the framework of an anti-malaria treatment trial performed on 1200 children in 2003 (Cisse et al., 2006). Sera from a sub-sample of these children aged from 1- to 6-years-old ($n = 124$) were available at the start (July) and at the end (December) of the rainy season. Similar numbers of a sub-sample of these children ($n = 17$ – 19) from 7 villages (A–G in Fig. 3) in this area were included in the study. Selection of children's sera was randomized in order to select 6 children by year of birth from 1998 to 2002. The same children were followed in both July and December. The

indicated age of each child corresponded to the age at the start of the study in July.

Both the anti-malaria treatment trial and the present study followed ethical principles according to the Helsinki Declaration, and were approved by the ethical committees of the Ministry of Health of Senegal (August 2002 and May 2003, respectively) and the IRD (January 2004). Informed consent was obtained from the studied population.

Several epidemics of yellow fever have occurred in this area and infection by Chikungunya virus has been reported and both arboviruses are here transmitted by *Ae. aegypti* species (Thonnon et al., 1999). Indeed, epidemics of yellow fever, probably transmitted by this *Aedes* species (Mawlouth Diallo, personal communication) were recorded in 1995, 1996 and in September 2002 (Diallo et al., 2003). In this area, the rainy season corresponds to the season of highest exposure to *Ae. aegypti* bites (Thonnon et al., 1999).

2.2. Method of *Aedes* salivation

The mosquito salivation technique was improved in order to collect whole saliva from uninfected female *Ae. aegypti*, bred in an insectarium (IRD, Montpellier, France), as previously described for *Anopheles* mosquitoes (Remoue et al., 2006). Briefly, mosquitoes were sedated with CO₂ and their legs and wings were removed. Their probosces were placed in a conventional plastic tip fixed on a glass slide and containing 10 µl of Hepes–NaCl buffer. The salivation process was increased by topical application of 0.25% malathion in acetone to the thorax region. After 1h of salivation at room temperature, the liquid in the tip (saliva + buffer) was collected and pooled with other mosquito saliva and the quantity of proteins was evaluated by the bicinchoninic acid method (BCA, Pierce, Rockford, IL, USA). This technique yielded a similar concentration of salivary proteins in each of the various *Ae. aegypti* salivation sessions corresponding to 10–90 µg/ml and a mean of 50 µg/ml. This saliva solution was then stored frozen at –20 °C before use for immunological evaluation.

2.3. Evaluation of human IgE and IgG4 Ab levels

An enzyme-linked immunosorbent assay (ELISA) was carried out using saliva from uninfected *Ae. aegypti* and sera were tested for IgE and IgG4 antibodies. Saliva (1 µg/ml in carbonate/bicarbonate buffer) was coated on 96-well plates (Nunc, Roskilde, Denmark) for 150 min at 37 °C. Plates were blocked using PBS buffer containing 0.5% gelatin (Merck, Darmstadt, Ger-

many). Individual sera were incubated in duplicate at 4 °C overnight at a 1/10 dilution (in PBS-Tween 0.1%) for IgE and at 1/20 for IgG4 analysis. All ELISA conditions were determined as the optimum after several preliminary experiments. Mouse biotinylated mAbs to human IgG4 and to human IgE (BD Pharmingen, San Diego, CA) were incubated at a 1/500 and 1/250 dilution (90 min at 37 °C), respectively. Peroxidase-conjugated streptavidin (Amersham, Les Ulis, France) for IgG4 and peroxidase-conjugated extravidin (Sigma, St. Louis, MO) for IgE were then added (1/1000; 30 min at 37 °C). Colorimetric development was carried out using ABTS (2,2'-azino-bis(3-ethylbenzthiazoline 6-sulfonic acid) diammonium; Sigma, St. Louis, MO, USA) in 50 mM citrate buffer (pH 4) containing 0.003% H₂O₂, and absorbance (OD) was measured at 405 nm. Identical ELISAs were performed in parallel on a negative pool of sera from Europeans (*n* = 30) with no known exposure to *Ae. aegypti* mosquito bites and positive control sera were used to validate each ELISA plate. In addition, the absence of significant Ab detection in wells without antigens was verified for sera presenting positive OD values. Individual results were expressed as ΔOD value calculated according to the formula: ΔOD = OD_x – OD_n, where OD_x represented the individual OD value of exposed individuals and OD_n was the arithmetic mean of the individual OD value for negative control individuals (OD_n values for IgG4 = 0.089 and for IgE = 0.181). The reproducibility of OD-positive values from responders in the children studied was verified later in 2 assays. A subject was considered as an “immune responder” if his/her OD_x was higher than the OD_n + (3S.D.) value.

2.4. Statistical analysis

All data were analyzed with the Graph Pad Prism® (Graph Pad Software, San Diego, USA). After verifying that values in each group did not assume a Gaussian distribution, the difference in the Ab level between more than 2 groups was analyzed by the Kruskal–Wallis test. The non-parametric Wilcoxon matched pair test was used to compare paired sera between July and December. All differences were considered significant at *P* < 0.05.

3. Results

3.1. Ab responses and rainy season

The IgE and IgG4 Ab responses specific to *Ae. aegypti* whole saliva were evaluated at the start (July) and at the end (December) of the rainy season (Fig. 1). A similar

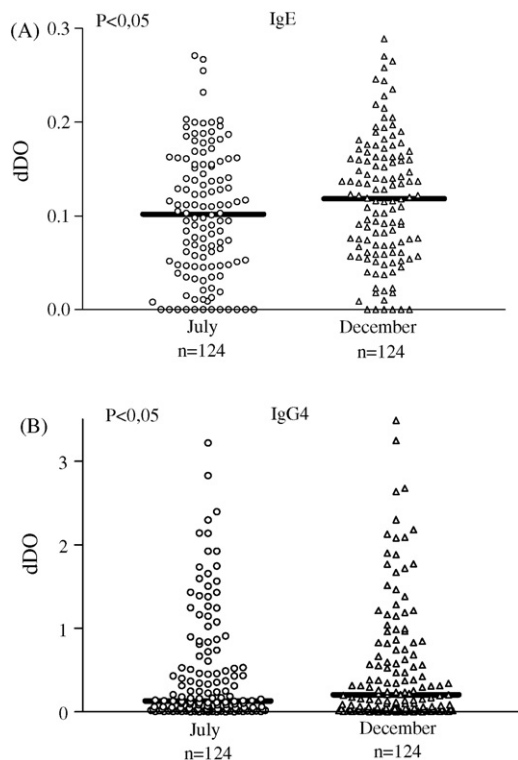


Fig. 1. Evolution of specific IgE and IgG4 antibodies to *Aedes* saliva between the start (July) and the end (December) of the rainy season. Individual OD results (Δ OD as described in Section 2) are presented for the same children ($n = 124$) in July ($\circ$) and December (Δ). Bars indicate the median value for each group. Statistical significances between July and December are indicated for each isotype (non-parametric Wilcoxon matched pair test).

proportion of responders were observed for specific IgE in July (50.81%) and in December (57.26%). For the IgG4 isotype, the percentage of responders appeared to increase during the rainy season from 47.2% in July to 56.25% in December but this difference was not significant. In term of intensity of specific Ab response (OD value), a similar evolution in anti-saliva IgE and IgG4 responses was observed, since the median value of Ab levels was significantly higher in December than in July ($P = 0.0015$ and $P = 0.0068$, respectively), despite substantial dispersion of OD values. These results suggest that the intensity of responses of both isotypes increased during the rainy season whereas the rates of Ab responders were similar.

3.2. Ab responses and age

Anti-saliva IgE and IgG4 responses were evaluated in children 1- to-6-years-old and then analyzed by age group. Results are presented for December, at the end

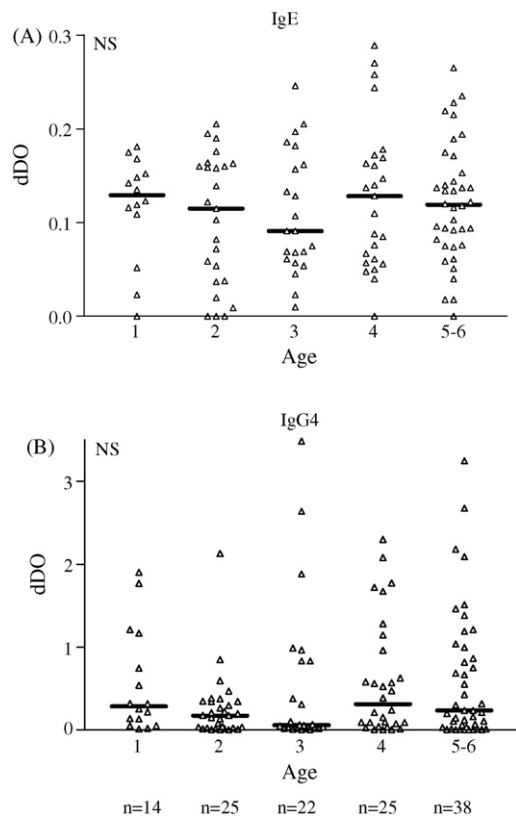


Fig. 2. Anti-saliva IgE and IgG4 antibodies according to age of children. Individual OD results (Δ OD as described in Section 2) obtained in December are shown for children aged 1-to-5-years-old. Bars indicate the median value for each age group. Children over 6 were regrouped for analysis with 5-years-old. Statistical significances between all age groups are indicated (non-parametric Kruskal–Wallis test).

of the season of highest exposure to *Ae. aegypti* bites (Fig. 2). Similar results according to age groups were observed in July (data not shown). No clearly significant effect of age on Ab levels was observed. However, a trend indicated that the median value of anti-saliva IgE and IgG4 was lower, although not significantly, in the 3-year-old age group compared to other age groups.

The evolution of specific isotype Ab levels during the rainy season was analyzed in each age group (data not shown). The anti-saliva IgE response was significantly higher in December compared to July only for the 3-year-old ($P = 0.0421$) and 4-year-old ($P = 0.0284$) groups whereas the specific IgG4 response was higher in December for the 3 youngest groups ($P = 0.0079$ for 1-year-olds; $P = 0.0246$ 2-year-olds; $P = 0.0303$ for 3-year-olds). These results suggest that the impact of the rainy season on anti-saliva Ab responses appeared to be age- and probably isotype-dependent.

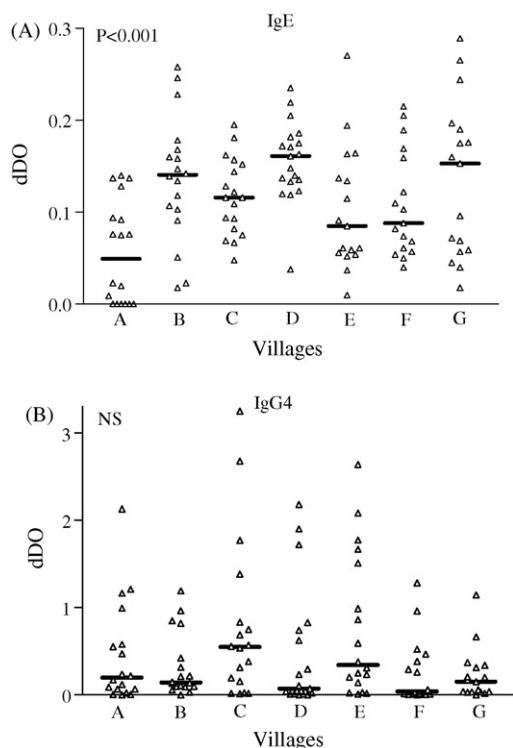


Fig. 3. Anti-saliva IgE and IgG4 antibodies according to village. Individual OD results (ΔOD as described in Section 2) obtained in December ($n = 17$ – 19 by villages) are shown according to 7 villages (A–G) in the Niakhar area. Bars indicate the median value. Statistical significances between all villages are indicated (non-parametric Kruskal–Wallis test).

3.3. Ab responses and villages

The differences in IgE and IgG4 responses to *Ae. aegypti* saliva in the 7 villages of the studied area were analyzed for the month of December (Fig. 3). Considerable variations for both isotypes were observed according to village. Specific IgE responses differed significantly according to village ($P = 0.0003$), whereas no statistical difference was observed for the IgG4 isotype, even though median Ab level values were higher in villages C and E compared to others. In addition, no close association between isotype responses was observed in any village. Indeed, although both isotype responses were similarly high in village C, anti-saliva IgE was higher in villages B and D, whereas the IgG4 Ab level was low.

Interestingly, the specific IgE response was significantly higher in December than in July, only in villages A ($P = 0.0107$), D ($P = 0.0017$) and F ($P = 0.0317$), whereas the increase in the IgG4 response in December was observed only in village D ($P = 0.0053$). Taken together, these results suggest that the level of anti-saliva IgE and

IgG4 differed according to village and that the increasing evolution of specific Ab responses during the rainy season was dependent both on the isotype and on the village.

These results in 7 villages are presented in a map of the Niakhar study area showing the median value of specific anti-IgE (green) and IgG4 (blue) responses (Fig. 4). This “immunological” map indicates the potential use of the anti-saliva Ab tool under epidemiological conditions.

4. Discussion

In the present study, we detected IgE and IgG4 responses to *Aedes* whole saliva in young African children living in an area exposed to *Ae. aegypti* bites. To our knowledge, this is the first immuno-epidemiological evaluation, on a large scale, of anti-saliva Ab responses in African individuals exposed to *Aedes* vector. A similar percentage of responders was observed in both July and December months. In contrast, despite the dispersion of OD values, similar evolution in the intensity of the two specific isotype responses was observed during the rainy season since IgE and IgG4 Abs levels were higher at the end (December) than at the start (July) of the season. These results suggest that the intensity, but not the outcome (% of responders) of anti-saliva IgE and IgG4 responses increased during the season of high exposure to *Aedes* bites. This strengthens the concept that anti-saliva Ab responses could be a marker of exposure to *Aedes* bites in African children. This hypothesis was previously supported by studies on European adults in Finland, where the anti-saliva IgE and IgG4 levels increased with seasonal exposure to mosquito bites (Palosuo et al., 1997). In addition, IgE and IgG4 responses to 3 major salivary proteins of *Ae. aegypti* were detected by immuno-blot in Kenyan children (Reunala et al., 1994). The latter study also indicated that the occurrence of anti-saliva IgG4 could be an indicator of intense mosquito bite exposure.

In the present study, no direct estimate of exposure to *Aedes* bites was available, and only indirect ecological arguments are presented. This may partly explain the distribution of OD values and the high level of responders. It is likely that most children are exposed to *Aedes* bites throughout the year, but that the density of the *Aedes* population increases during the rainy season. Indeed, it was shown in Senegal that the rainy season began with heavy rains which flooded the *Aedes* breeding grounds (Mondet et al., 2005). Following this, only the adult *Aedes* population increased during the rainy season.

Our results showed no significant differences between age groups in the two isotype specific responses, whether

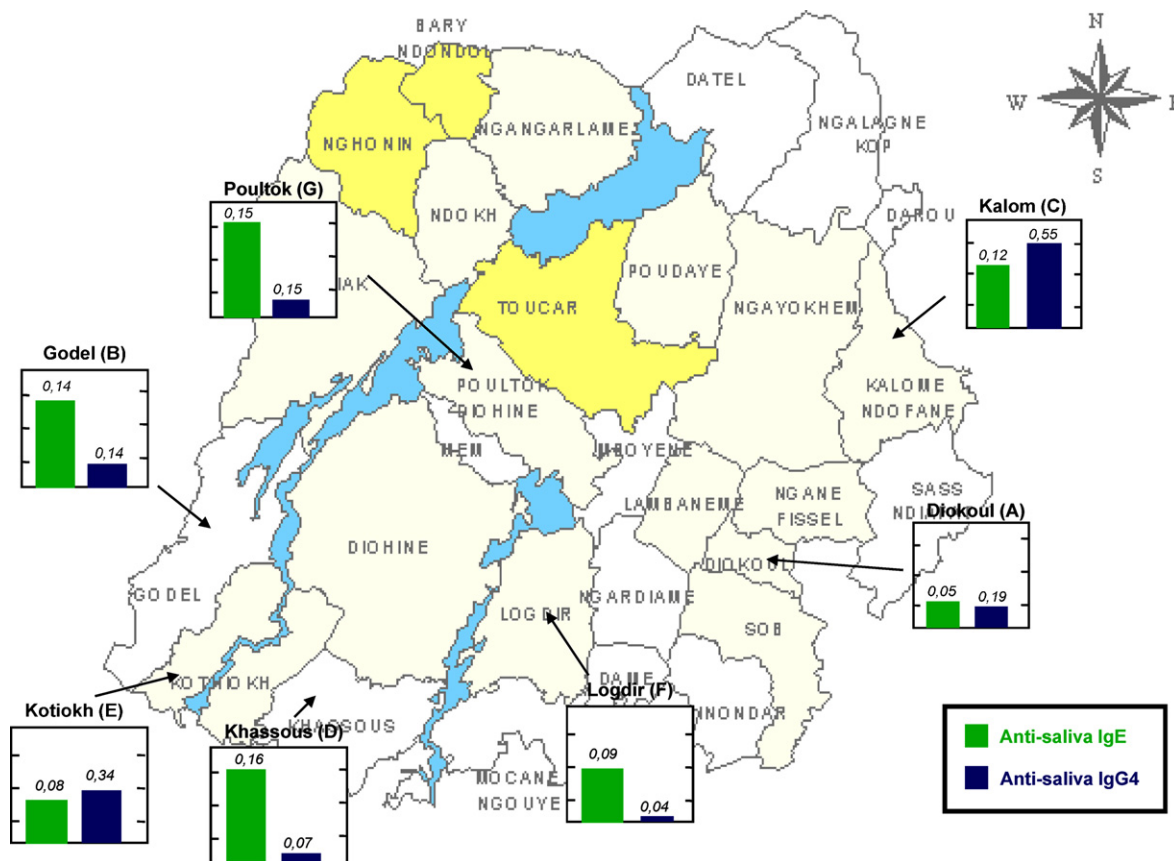


Fig. 4. Epidemiological map of IgE and IgG4 antibodies to *Aedes* saliva in the Niakhar area. The median of specific IgE (green) and IgG4 (blue) responses by village is presented on the map of the Niakhar area. Numbers above each histogram indicate the median value. Village name and corresponding letter (A–G, Fig. 3) are noticed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

in July or in December. One trend indicated that anti-saliva IgE and IgG4 responses were lower in the 3-year-old age group. Despite the strong individual differences observed in each age group, the evolution of the median of anti-saliva isotype Ab levels during the rainy season seemed to be age-dependent. Indeed, both IgE and IgG4 responses increased significantly in the 3-year-old group, whereas only IgE Ab levels increased in the 4-year-old group and IgG4 in the 1- and 2-year-old groups. These results suggest that the influence of age on the occurrence of the anti-saliva Ab response during the seasonal exposure to *Aedes* bites could be isotype-dependent. Age-dependent Ab responses to mosquito saliva have been previously observed by our team during exposure to *Anopheles* in malaria (Remoue et al., 2006). Although the two isotype could be associated as a marker of *Aedes* exposure, we hypothesize that the anti-saliva IgG4 response is better adapted to very young children (1–3 years-old) whereas the IgE isotype could be an indicator in older children (3–4 years-old). This

hypothesis remains to be confirmed by studies in various areas of *Aedes* exposure and within a larger population which would include exposed adolescent and adult populations. Indeed, our study may have lacked statistical significance because of the small sample size by age group.

The present study indicated considerable variations in anti-saliva IgE and IgG4 Abs levels according to the village. Strong individual differences in Ab responses were observed in each village but higher median Ab levels were detected in villages B, C, D and G compared to others areas. Our results according to the rainy season and to the literature appeared to indicate that anti-saliva IgE and IgG4 responses could be associated to the exposure of *Aedes* bites. For these reasons, we can hypothesize that these villages (B, C, D and G) were likely to have been more strongly exposed to *Aedes* vector than others studied in this area. However, some differences related to IgE and IgG4 isotypes were observed in the present study. Indeed, high levels of both isotypes were detected

in village C whereas a high IgE response was observed in villages B, D and G. In this preliminary study, it was difficult to distinguish a particular isotype of interest as a marker of exposure to *Aedes* bite. A comparison between anti-saliva Ab responses and entomological data, along with the identification of breeding sites (evaluating the level of exposure to *Ae. aegypti*) is thus of particular relevance. Nevertheless, our results indicate variations in anti-saliva IgE and IgG4 responses according to the season of exposure and possibly according to the village, thus confirming the need to evaluate anti-saliva Ab responses as markers of exposure to *Ae. aegypti* bites and the ensuing risk of *Aedes*-borne diseases in human populations.

Here we have evaluated IgE and IgG4 responses to whole saliva of *Ae. aegypti*. The assessment of other isotypes and of Ab against specific salivary proteins of *Aedes* will be necessary to define an efficient marker of exposure. In particular, the evaluation of Ab responses to *Ae. aegypti* salivary proteins, identified as diagnostic tests for evaluating risks of mosquito allergies (Simons and Peng, 2001; Peng et al., 1998) is under investigation. It would thus be of interest to evaluate the IgE and IgG4 responses to these “allergy” proteins in African children exposed to *Aedes* vector. Another research project could involve identifying immunogenic proteins by immuno-proteomic analysis combining mass spectrometry with immuno-blot of sera of exposed African children. This technology is particularly relevant for identifying immunogenic salivary proteins specific to the *Aedes* genus (uncommon with other mosquitoes) and specific to *Ae. aegypti* species compared to other major *Aedes* (*Ae. albopictus*, *Ae. vexans*, etc.). Indeed, it has been previously observed that *Aedes* immunogenic proteins may be species-shared whereas others proteins are species-specific (Peng et al., 1998). This type of research opens up new pathways for identification of markers of exposure to common *Aedes* genera versus a specific indicator of *Aedes* species bites, such as *Ae. aegypti* or *Ae. albopictus*, the major vector of arboviruses (dengue, yellow fever, Chikungunya). A research orientation might then examine the potential association between the anti-saliva Ab response and the outcome of infection/morbidity linked to arboviruses or parasite transmitted by *Aedes* species. Indeed, preliminary results on *Anopheles* exposure in children suggested that the Ab response to *A. gambiae* saliva could be a predictive marker of malaria morbidity (Remoue et al., 2006).

The present study lends support to the use of the Ab response to mosquito salivary proteins as a potential indicator of exposure to vector bite. In particular, it represents a step to demonstrate that the evalua-

tion of Ab levels to *Aedes* saliva could be a valuable immuno-epidemiological tool for monitoring an exposed population and for evaluating the risk of diseases transmitted by the *Aedes* mosquito. In this way, it could be used to elaborate an epidemiological map of risks of vector-borne disease and to be integrated to several anti-vector strategies for determining the efficacy of intervention strategies such as use of impregnated bed-net and insecticides.

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