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Evaluation of the antibody response to *Anopheles* salivary antigens as a potential marker of risk of malaria

Franck Remoue[a,*], Badara Cisse[b,c], Fatou Ba[b], Cheikh Sokhna[b], Jean-Pierre Herve[d], Denis Boulanger[a], François Simondon[a]

[a] *Institut de Recherche pour le Développement (IRD), UR24 ''Epidémiologie et Prévention'' Unit, Centre IRD de Hann, BP 1386, Dakar, Sénégal*
[b] *Institut de Recherche pour le Développement (IRD), UR77 ''Paludologie Afro-tropicale'', Centre IRD de Hann, BP 1386, Dakar, Sénégal*
[c] *London School of Hygiene and Tropical Medicine, London WC1B 3DP, UK*
[d] *Institut de Recherche pour le Développement (IRD), UR16 ''Caractérisation et contrôle des populations de vecteurs'' Unit, Centre IRD, BP 64501, Montpellier 34394 Cedex 5, France*

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Summary The evaluation of human immune responses to arthropod bites may be a useful marker of exposure to vector-borne diseases, with applications to malaria, the most serious parasitic infection in humans. The specific antibody (Ab) IgG response to saliva obtained from *Anopheles gambiae* mosquitoes was evaluated in young children from an area of seasonal malaria transmission in Senegal. Specific IgG was higher in children who developed clinical *Plasmodium falciparum* malaria within the 3 months that followed than in those who did not ($P < 0.05$), and it increased significantly ($P < 0.0001$) with the level of *Anopheles* exposure, as evaluated by conventional entomological methods. These results suggest that evaluation of antisalivary Ab responses could be a useful approach for identifying a marker for the risk of malaria transmission.

1. Introduction

Malaria is one of the most important public health problems in the developing world. Each year, one million children under 5 years of age die of malaria, and over 75% of them live in sub-Saharan Africa (Breman et al., 2004).

* Corresponding author. Present address: Institut de Recherche pour le Développement (IRD), UR24 ''Epidémiologie et Prévention'' Unit, Centre IRD de Hann, Route des Pères Maristes, BP 1386, CP 18524, Dakar, Sénégal. Tel.: +221 849 35 55/35 51; fax: +221 832 43 07.
E-mail address: franck.remoue@ird.sn (F. Remoue).

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The evaluation of malaria transmission in areas at risk is based at the population level on entomological methods, and at the individual level on the detection of infection/morbidity in humans. The risk of malaria transmission can be assessed entomologically by evaluating the density of the *Anopheles* vector infected by *Plasmodium* (Rodhain and Perez, 1985). These data are then linked to the prevalence of infection/morbidity to evaluate transmission in a given population. Malaria transmission can also be assessed at the individual level based on rates of *Plasmodium* infection. In the 1990s, it was suggested that antibody (Ab) responses to *P. falciparum* proteins (e.g. merozoite and sporozoite surface antigens) could be used as serological markers of transmission (Ramasamy et al., 1994; Webster et al., 1992). These methods enabled the seasonal dynamics of transmission to be distinguished as well as differentiating between areas of high and low exposure to malaria. Major limitations to the use of Ab responses to blood-stage antigens when measuring exposure in children lie in their generally low titres and antigenic polymorphism (Ramasamy et al., 1994).

No methods for evaluating the risks of malaria exposure at the individual level are available as yet. Mortality and morbidity from malaria are closely linked to exposure of the human host to the *Anopheles* mosquito infected by *Plasmodium*. In Africa, *An. gambiae* is the major vector of *P. falciparum* infection, which induces the most serious clinical forms of malaria in young children (Breman et al., 2004). In terms of the three protagonists (pathogen—vector—human), few studies have dealt with the human—vector relationship, although the importance of this research topic was emphasised in results found in early research (Gordon and Crewe, 1948; Sykes, 1904). Older methods for identification of a marker for risk of malaria transmission can now benefit from recent developments in immunology and molecular biology. Since the middle of the 1990s, several studies have investigated the immune response of mammalian hosts to arthropod bites (Schoeler and Wikel, 2001). These were concerned primarily with veterinary vaccines, local immunomodulation by bites (*Phlebotomus* and ticks) and the development of allergies in humans (Palosuo et al., 1997; Ribeiro, 1995; Sandeman, 1996). Major advances in the study of human—vector immune relationships lie in the identification of immunogenic proteins secreted in the saliva of mosquitoes (principally *Aedes* and *Culex*) during the bite, and involvement of these proteins in allergic reactions (Brummer-Korvenkontio et al., 1997).

In the field of vector-borne diseases, antisalivary IgG responses can be used as a marker for exposure to ticks, the vectors of Lyme disease (Schwartz et al., 1990). Specific IgG1 and IgG4 isotypes of antibodies to *Triatoma* salivary antigens have also been detected in individuals exposed to Chagas disease (Nascimento et al., 2001), and the Ab response to sandfly salivary gland antigens may be a useful marker of exposure to leishmaniasis (Barral et al., 2000). In those studies, the value of antisalivary Ab responses as a marker of exposure to disease transmission was evaluated in comparison with detection of the pathogen by serodiagnosis after infection of study subjects. To our knowledge, no study has yet compared the antisalivary Ab response with entomological data.

Several earlier studies evaluated the Ab response to *Anopheles* salivary proteins. Specific IgE and IgG4 responses to saliva of *An. stephensi* were identified by an immunoblotting method in exposed individuals with allergies (Brummer-Korvenkontio et al., 1997). Another study showed that an inflammatory protein from *An. stephensi* saliva is recognised by specific IgG Ab from individuals exposed to malaria (Owhashi et al., 2001). Some of those studies suggested that Ab responses to mosquito salivary proteins are species specific (*Anopheles*, *Culex* or *Aedes*), indicating little cross-reactivity between salivary proteins from different arthropods (Matsuoka et al., 1997).

The objective of the present study was to measure specific IgG responses to whole salivary extract from *An. gambiae* s.l. in young children living in a malaria-endemic area in rural Senegal. The immunological results obtained were compared with: (i) the outcome of malaria attacks within the 3 months that followed; and (ii) conventional entomological measurements of the intensity of exposure to *An. gambiae* s.l. bites. The evolution of Ab responses according to age and during the course of the transmission season was followed.

2. Materials and methods

2.1. Study population

The study was carried out in Niakhar, a rural area of central Senegal situated 115 km east of Dakar. The population is almost exclusively from the Serer ethnic group. The site is a dry savannah with approximately 400 mm of rain recorded from June to September. This study area is typical of the Sahel and sub-Sahel regions of Africa, as malaria is unstable, with a season of transmission

mainly from September to November. There are clear-cut seasonal variations in prevalence and parasite rates. Among children aged 0—4 years, the *P. falciparum* rate is 22% in February (mid-dry season), 14% in June (start of wet season) and 65% in November (end of wet season) (Robert et al., 1998).

2.2. Malaria surveillance and study sample

The study was integrated into the framework of a trial of antimalaria treatment performed in 1200 children aged 6 weeks to 5 years. As a component of this study, children were under active surveillance for malaria morbidity. Clinical malaria (malaria attacks) was defined as an axillary temperature $\geq 38\,^{\circ}C$ and the presence of *P. falciparum* infection detected by thick smear examination (density >3000 parasites/μl of blood). Sera were available from a subsample of children from the antimalaria treatment trial at the start (September 2002; $n = 448$) and at the end of malaria transmission (December 2002; $n = 272$). Analysis of the immune response according to malaria morbidity was performed on sera from children belonging to the control group ($n = 222$) who did not receive preventive antimalaria treatment.

Both the trial of antimalaria treatment 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). The antimalaria trial was approved by the ethical committee from London School of Hygiene and Tropical Medicine in June 2002. Informed consent was obtained from the study population.

2.3. Entomological analysis

Entomological data were collected each month between September and December 2002 in 11 villages in the Niakhar area. The mosquito population was evaluated using capture by light traps (CDC miniature light trap) placed from 19:00 hours to 07:00 hours for two consecutive nights in each village (four houses/village/night) for every month during the study. Traps were arranged in the bedroom near two 'human bait' sleeping under a bed net. The collection by CDC light traps has been demonstrated to be an efficient method for evaluating malaria transmission by *An. gambiae* and for use as a substitute for the human-landing method (Mathenge et al., 2004; Sadanandane et al., 2004). During the transmission season, 2383 female Culicidae mosquitoes were collected, 924 of which were *Anopheles* species, with 93.3% belonging to the *An. gambiae* complex. Evaluation of the number of infected *An. gambiae* (ELISA of anti-CSP Ab) indicated that 3.3% of mosquitoes were positive during the entire transmission season. The bites/human/night were calculated from the count of *An. gambiae* s.l. by village and by month. According to the data obtained in September, the 11 study villages were classified into three groups with different intensities of exposure to *An. gambiae* bites: low (L; 7 villages), medium (M; 1 village) and high (H; 3 villages).

2.4. Method of *Anopheles* salivation

The mosquito salivation technique was perfected in our laboratory in order to collect whole saliva extract (WSE) from uninfected female *An. gambiae* s.l. This technique is based on a capillary method developed previously by H. Brummer-Korvenkontio (Brummer-Korvenkontio et al., 1997). Briefly, 10-day-old uninfected *An. gambiae* s.s. females (bred in an insectary) were sedated with CO_2 and their legs and wings were removed. The mosquitoes had been selected 2 days after the last of their two blood feedings on rabbits. The proboscis of the mosquito was placed in a conventional plastic pipette tip containing 10 μl of H_2O that had been previously fixed on a glass slide by adhesive cellotape, as shown in Figure 1.

Salivation was increased by topical application of 0.7 μl of 0.25% malathion in acetone to the thorax region, as previously described (Brummer-Korvenkontio et al., 1997). After 1 h of salivation at room temperature, the liquid in the tip (saliva + H_2O) was collected and pooled with other

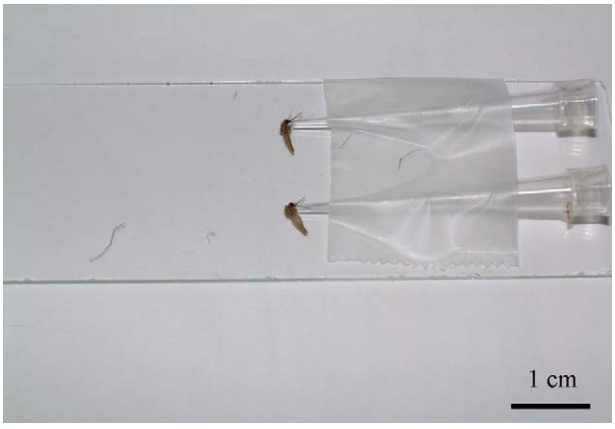

Figure 1 Collection of saliva from *Anopheles gambiae*. The method is described in Section 2.4.

mosquito saliva and the quantity of proteins was evaluated by the bicinchoninic acid method (Pierce, Rockford, IL, USA). Despite variability, this technique of salivation yielded 100—400 μg/ml of salivary proteins corresponding to 20—75 ng/mosquito. This WSE was then used for immunological evaluation.

2.5. Assay of human Ab levels

An ELISA was carried out using WSE from uninfected mosquitoes, and sera were tested for IgG antibodies. WSE (0.1 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, Germany). Individual sera were incubated in duplicate at 4 °C overnight at a 1/80 dilution (in PBS—Tween-0.1%). This dilution was determined as the optimum after several preliminary experiments. Mouse biotinylated mAb to human IgG (BD Pharmingen, San Diego, CA, USA) was incubated at a 1/1000 dilution (90 min at 37 °C). Peroxidase-conjugated streptavidin (1/1000; 30 min at 37 °C) was then added (Amersham, Les Ulis, France). Colorimetric development was carried out using ABTS (2,2′-azinobis (3-ethylbenzthiazoline 6-sulfonic acid) diammonium; Sigma, St Louis, MO, USA) in 50 mM citrate buffer (pH 4) containing 0.003% H_2O_2, and absorbance (OD) was measured at 405 nm. Identical ELISA was performed in parallel on sera of 30 Europeans not exposed to malaria transmission. Individual results were expressed as △OD value calculated according to the formula: $\triangle OD = OD_x - OD_n$, where OD_x represented the individual OD value of exposed individuals and OD_n was the arithmetic mean of the individual OD value for the 30 control individuals (OD_n value for IgG = 0.233). The reproducibility of OD-positive values from IgG responders in the study children was verified in three later assays. A subject was considered an 'immune responder' if his OD_x was higher than the $OD_n + (3 \times SD)$ value.

2.6. Statistical analysis

After verifying that values in each group did not assume a Gaussian distribution, the difference in the Ab level between more than two groups was analysed by the Kruskal—Wallis test. The non-parametric Mann—Whitney *U*-test was used for comparison between the two independent groups, whilst the Wilcoxon matched pair test was used to compare paired sera between September and December. All differences were considered significant at $P < 0.05$.

3. Results

3.1. Ab levels and age

Specific IgG responses to *An. gambiae* s.l. WSE were evaluated in September in children aged 6 weeks to 5 years and analysed by age group (Figure 2). In the study area, 70.2% of children were immune responders. The median value of antisalivary IgG Ab levels differed significantly according to age ($P = 0.0001$); they were highest in young children (1 and 2 years old) and declined progressively up to 5 years of age. In contrast, very low specific IgG responses were detected in children <1 year, particularly among those in the 2—6 months age group, in which only one child was an IgG responder.

3.2. Ab levels and malaria morbidity

Anti-WSE IgG levels in September were related to the occurrence of clinical malaria in study children during the 3 months that followed (September to December). During that period of time, 90 malaria attacks were observed among the 222 children followed for malaria morbidity.

Since IgG Ab levels were very low in children under 1 year of age, the analysis was stratified according to children who were over and under 1

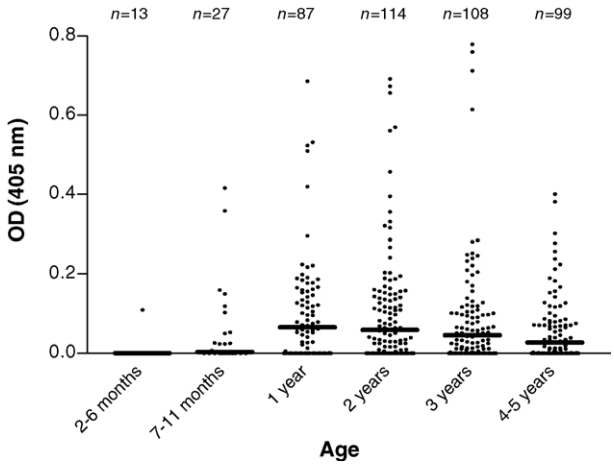

Figure 2 Evaluation of IgG antibodies to *Anopheles gambiae* saliva according to age in children from an endemic area. Individual absorbance (OD) results obtained in September are shown for children aged 6 weeks to 5 years ($n = 448$). Bars indicate the median value for each age group. Children over 5 years ($n = 6$) were regrouped for analysis with children aged 4 years.

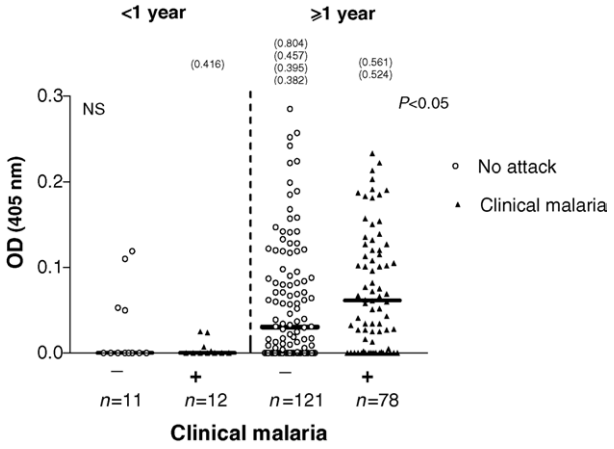

Figure 3 Antisalivary IgG according to malaria morbidity. The results of individual absorbance (OD) values in September are shown according to subsequent detection of clinical malaria for the age groups <1 year and ≥1 year. Bars indicate the median value for each group. Numbers in parentheses on the figure above the dot plots indicate values above OD = 0.3. Statistical significances between groups in relation to subsequent occurrence of clinical malaria are indicated for each age group (non-parametric Mann—Whitney *U*-test).

year of age (Figure 3). The median IgG Ab level was significantly higher in children aged 1—5 years who developed clinical malaria within the next 3 months than in those who did not ($P = 0.027$). Within that age range, the percent of clinical malaria did not significantly differ between age groups (data not shown). In particular, children aged 1—2 years presented higher antisaliva IgG responses but did not develop more clinical attacks compared with other age groups.

3.3. Ab levels and exposure to *An. gambiae* bites

The study population was divided into three groups of villages according to the intensity of their exposure to *An. gambiae* s.l. in September (Table 1). In the months that followed, from October to December, the intensity of exposure to *An. gambiae* mosquitoes progressively decreased in each of the three groups: October (L = 1.39 ± 1.02 [mean ± SD bites/human/night]; M = 4; H = 14.92 ± 8.72); November (L = 0.96 ± 1.37; M = 3.25 ± 1.5; H = 6.58 ± 1.22); December (L = 0.21 ± 0.36; M = 0.25 ± 0.35; H = 0.67 ± 0.94).

In September, the median of anti-WSE IgG Ab levels differed significantly ($P < 0.0001$) between the three groups of exposure (Figure 4A). The median Ab response increased gradually with the intensity of exposure to *An. gambiae* s.l. bites. Subanalysis taking into account age showed that antisalivary IgG Ab levels differed significantly between the three groups, with different levels of exposure for the 1 year and 2 year age groups ($P = 0.0001$ and $P = 0.0032$, respectively); differences between groups were non-significant in the 3 year and 4—5 year age groups. These results suggest that specific IgG responses are closely associated with exposure to *An. gambiae* bites, and that differences can be detected most readily in children aged 1—2 years.

Anti-WSE IgG responses in September (start of the transmission season) and in December (end of the transmission season) were compared in the same children ($n = 272$; Figure 4B). Specific IgG responses were significantly lower in December compared with September in the groups with low

Table 1 Characteristics of the study population according to entomological data

	Intensity of exposure to *An. gambiae* s.l.[a]		
	Low	Medium	High
Entomological data[b]			
No. of collected *Anopheles gambiae*[c]	5.14	67	124.33
Bites/human/night (mean ± SD)	1.29 ± 1.11	16.75 ± 3.18	31.08 ± 3.47
Characteristics of the population			
September			
No.	281	50	117
Age, mean ± SD (years)	2.42 ± 1.3	2.34 ± 1.21	2.09 ± 1.25
December			
No.	174	35	63
Age, mean ± SD (years)	2.82 ± 1.07	2.51 ± 1.01	2.67 ± 0.97

[a] The 11 study villages were classified into three groups according to the intensity of exposure to *An. gambiae* s.l.: low, 7 villages; medium, 1 village; high, 3 villages.
[b] Data are only for September.
[c] *An. gambiae* s.l. were collected by light traps as described in Section 2.3. The indicated number represents the mean/village group. The number of bites/human/night was calculated as described in Section 2.3.

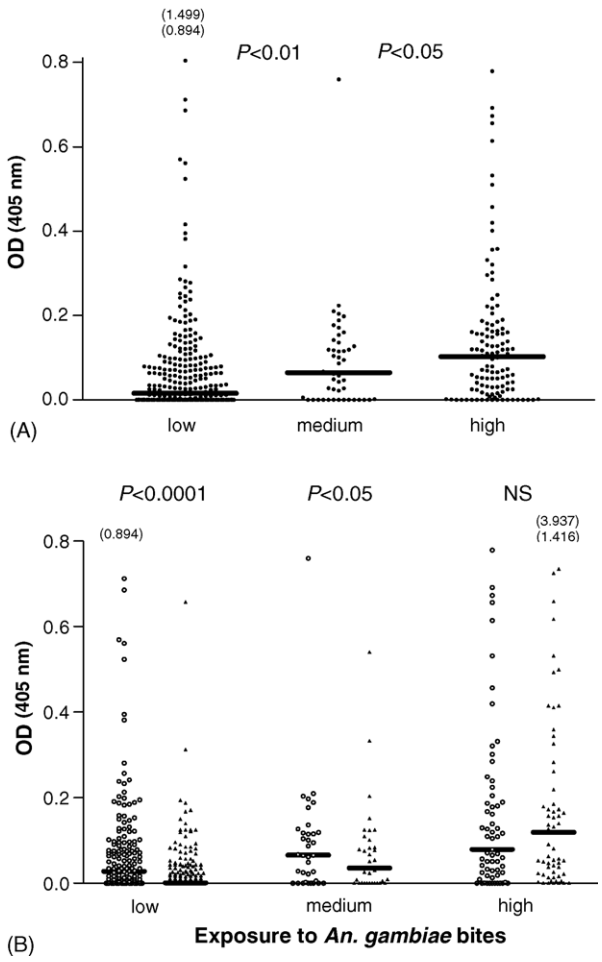

Figure 4 Antisalivary IgG according to intensity of exposure to *Anopheles gambiae* bites. (A) Individual absorbance (OD) values in September are shown for the three groups with different levels of exposure (as defined in Table 1). (B) Comparison of antibody levels between September (◯) and December (▲) for the same children ($n = 272$). Bars indicate the median value for each group. Numbers in parentheses on the figure above the dot plots indicate values above OD = 0.8. Statistical significances between each group by non-parametric Mann—Whitney *U*-test (A) and between September and December values by Wilcoxon matched pair test (B) are indicated.

or medium levels of exposure to *An. gambiae* bites. In contrast, IgG responses were not significantly higher in December than in September in the high exposure group.

4. Discussion

In the present study, we observed that the IgG Ab response to *An. gambiae* WSE in September was higher in children who developed clinical malaria within the next 3 months than in those who did not. In addition, we showed that the anti-WSE IgG level was positively associated with the intensity of exposure to mosquito bites, as evaluated by classical entomological data.

Possible limitations of our results might hypothetically lie in the nature of the *Anopheles* species saliva. In our study, the IgG response was evaluated with respect to WSE obtained from uninfected *An. gambiae* s.s., whereas *An. arabiensis* may be the major vector in this setting, as indicated by previous work in the study area (Robert et al., 1998). However, although different species, there is strong homogeneity within the *An. gambiae* s.l. complex (Holt et al., 2002). Thus, marked differences in the immunogenic profile were not anticipated. Furthermore, the only species of the *An. gambiae* complex that is currently bred in insectaries is *An. gambiae* s.s., thereby only WSE from this species is available. One possible limitation might lie in a lack of power in detecting an association with morbidity, but this was not the case in our study. Further work should take this aspect into account. In addition, the Ab response to salivary antigens from *An. gambiae* might show immune cross-reactivity with salivary proteins from other major mosquitoes such as *Aedes aegypti* and *Culex quinquefasciatus*. We felt that the ELISA technique could not be used in our study to respond to this hypothesis, even in the light of previous studies in animal models showing very low immune cross-reactivity in ELISA with WSE from these major mosquito species (Matsuoka et al., 1997).

It is widely known that the below 6-year-old age group presents the highest morbidity/mortality from malaria (Breman et al., 2004). However, the relationship between the anti-WSE Ab response and morbidity does not seem to be explained by a possible age-dependent effect, as no significant variation in morbidity by age was found between 1 year and 5 years (data not shown).

It has been demonstrated in several studies in various endemic areas that collection of *Anopheles* by CDC light traps is an efficient method for measuring bite intensity, for evaluating malaria transmission by *An. gambiae* and also for use as a substitute for the human-landing method, although underestimation of CDC traps cannot be excluded when evaluating mosquito density (Mathenge et al., 2004; Sadanandane et al., 2004). Our results of entomological—immunological relationships may thus be underestimated; however, in each case they show the same proportionality between the three entomological groups.

We also showed that the specific IgG response was significantly lower in December than in September in the groups with low or medium levels of

exposure, whereas specific IgG levels were similar in the high exposure group. In the high exposure group, entomological data indicated that *An. gambiae* were collected between October and December, with a progressive decrease in the count with time. In that group, the high IgG responses to WSE observed in September and December are likely to result from a high level of exposure to *An. gambiae* bites throughout the transmission season. The marked decline seen in Ab concentrations over a period of 3 months in children with low or medium levels of exposure suggests that IgG induced by salivary antigens have only a short half-life.

To our knowledge, this study is the first to show that anti-*An. gambiae* salivary Ab responses may be related to exposure to malaria, and thus represent a possible means of elaborating an immunoepidemiological marker. Given the above mentioned limitations, further studies are needed to characterise immunogenic proteins specific to the *An. gambiae* species (versus other major mosquito species) and possibly to *P. falciparum*-infected mosquitoes. The use of immunoproteomic technology for identifying a saliva 'immunome' could help in achieving this objective as a follow-up to the recent sequencing of the whole *An. gambiae* genome (Holt et al., 2002). The identification of salivary proteins specific to infected *Anopheles* bites could be an efficient way of determining an immunological marker of the intensity of infected bites. Indeed, it has been suggested that infection of the mosquito by a pathogen could influence the expression and/or secretion of salivary proteins (Ribeiro, 1995). The time needed for probing, blood feeding and salivation are increased in infected *Anopheles* to allow efficient inhibition of the localised human reaction and therefore to favour pathogen transmission (James and Rossignol, 1991; Rossignol et al., 1984). For example, it has been reported that secretion of apyrase, an inhibitor of platelet aggregation, was reduced in infected *Anopheles* compared with uninfected mosquitoes (Rossignol et al., 1984). Taken together, these data suggest that the induced Ab responses to specific salivary proteins after exposure to infected *An. gambiae* might be different from responses to uninfected bites. This hypothesis is currently under investigation.

The present study indicates that analysis of the antisaliva Ab response may thus be considered a potential approach to identification of an immunological indicator of the level of malaria transmission. These initial results will require confirmation in future studies in several endemic areas in which malaria transmission differs in intensity (hypoendemic, mesoendemic and hyperendemic) and in terms of exposure to various *Anopheles* species.

This immunoepidemiological marker could thus be used to identify populations at high risk of exposure to malaria transmission, allowing targeting of adequate antimalaria interventions. It could also serve as a direct criterion of efficacy in evaluation of different vector control strategies in malaria, including treated bed nets, and might be useful for adjusting the results of other efficacy trials according to the individual intensity of exposure to *Anopheles* bites. A simple quantitative marker (ELISA) of individual exposure to the risk of malaria transmission and/or predictive of morbidity can be easily used under field conditions and for studying transmission in other vector-borne diseases.

Conflicts of interest statement
The authors have no conflicts of interest concerning the work reported in this paper.

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