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Evaluation of the Anti-Salivary IgG Response Specific to Anopheles Vectors of Malaria in Children under Five Seen in Health Care Settings in Areas of Low and High Insecticide Resistance in Southern Benin

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Abstract

The widespread use of long-lasting insecticidal nets (LLINs) is a major intervention method for malaria control. While coverage of LLINs has increased, there is a need for information on the operational effectiveness of nets deployed in the field in the context of malaria vector resistance to insecticides used for impregnation. The development of specific anti-malarial biomarkers to assess exposure to mosquito bites is an important development in evaluations. The aim of this study was to characterize the human IgG antibody response to Anopheles gSG6-P1 saliva, a salivary peptide antigen previously shown to be a relevant biomarker of human exposure to Anopheles bites, in order to assess the LLINs under field conditions in areas of low and high vector resistance to insecticides. We analyzed data from 240 randomly selected children (<5 years) of whom 70% were sleeping under LLINs in four (04) communes of Benin. No significant difference was observed in the expression of the anti-saliva IgG antibody in the different zones (high and low resistance zone) as well as in cases and controls ($p > 0.05$). Furthermore, the same find-

ing was observed for antibody expression in children whether or not they used LLINs on the day before the survey (p = 0.7724). Similarly, gender and especially age, major factors of variation in the adaptive immune response, did not have a significant effect on IgG expression.

Keywords

Case-Controls, LLINs, Malaria Morbidity, Pyrethroids Resistance, Antibodies, Bite Exposure, Biomarkers, Benin

1. Background

The protozoan parasite *Plasmodium*, which causes malaria and is transmitted by female Anopheles mosquitoes, continues to be a major problem in many countries. Although significant progress has been made toward its elimination in some previously endemic countries, malaria remains a serious public health problem [1]. The World Malaria Report 2020 estimated that in 2019, the global malaria burden included approximately 229 million reported cases and 409,000 deaths worldwide [2]. The progress made since the beginning of the millennium is truly astounding. However, as described in this report, this progress has stagnated for several years. Since 2017 [3], WHO had highlighted that global malaria control had reached a "crossroads" and that the key targets of the WHO Global Malaria Strategy would likely not be met. Three years later, progress is still stagnant. The WHO 2020 report estimates that the targets for reducing malaria incidence and mortality will be respectively missed by 37% and 22%. Several contexts explain this state of affairs, including vector resistance to insecticides and climate change [4].

Benin is one of the countries in the sub-Saharan region of Africa where the health indicators of the population are most alarming. The number of malaria cases recorded in health facilities is 2,599,896 in 2019 representing 46.8% of all consultations. Its incidence for both forms (simple and severe) is 212 per 1000 inhabitants (ASS/MS, 2019) [5].

For malaria, the EIR (Entomological Inoculation Rate) is the standard for measuring transmission intensity. EIRs are based on the number of mosquitoes caught and the proportion of mosquitoes infected with *Plasmodium* [6]. However, estimation of EIR is expensive and may be insufficient in some localities depending on the season or the level of transmission [7] [8]. Malaria is only contracted when *Plasmodium* spp sporozoites are injected into human skin by the bite of a female Anopheles mosquito with the mosquito's salivary proteins [8]. Previous studies have shown that a significant number of mosquito salivary proteins are immunogenic and capable of inducing antibody responses, primarily of the IgG isotype. These antibodies can reflect the intensity of human exposure to mosquito bites and are good indicators of the risk of infection with *Plasmodium* spp [9] [10] [11] [12]. The use of salivary glands and saliva antigens

has been previously validated as an indirect proxy for determining exposure to mosquito bites. The peptide, gSG6-P1, was designed from the original *An. gambiae* sequence. Specific IgG responses to this salivary peptide have been validated as a biomarker of human exposure [10] [12] [13] [14].

It is particularly true that pyrethroids, the class of insecticide used on ITNs show an increasing inability of insecticides to kill malaria vectors and it is worrisome because insecticide-based interventions are vital to prevent malaria deaths and illnesses in African children. It is estimated that insecticide-treated nets (ITNs) used more than 50% in 2016 were responsible for 78% of the 663 million clinical malaria cases averted in sub-Saharan Africa since 2001 [15] [16]. However, it is unclear how increasing insecticide resistance will affect the malaria burden in Africa. Mathematical models predict an increase in malaria incidence, but actual evidence of this increase is lacking [17]. The limited data available at malaria control programs in South Africa, Equatorial Guinea, and Sudan suggest an impact of resistance on malaria burden, but these examples involve indoor residual spraying of insecticides rather than LLINs, and in none of the LLIN cases is the evidence conclusive [18]-[22]. There have been no convincing examples of failure to control malaria with LLINs due to pyrethroid resistance.

Two recent trials showed that in areas of resistance, nets that incorporated either the synergist piperonyl butoxide (PBO) or another active ingredient in addition to a pyrethroid were more effective than other conventional nets [23] [24]. Although the trials did not attempt to assess whether standard ITNs provide protection in a pyrethroid-resistant zone, they showed that they were more effective than conventional ITNs. It will take time to deploy a new class of nets in all areas with a similar resistance profile. In the meantime, the question of whether conventional ITNs continue to provide sufficient protection against malaria is urgent.

The current study is part of a program to investigate the impact of insecticide resistance [25] [26]. Results published to date show that children who sleep under ITNs have a lower risk of malaria infection as measured by cross-sectional surveys, and that children who sleep under ITNs experience a lower rate of clinical malaria episodes as measured by active follow-ups [22] [27] [28]. The present study evaluated in a case-control approach the specific anti-salivary IgG response of malaria vector *Anopheles* in children under five who were users or non-users of LLINs seen in health care settings in areas of low and high insecticide resistance. There is a need to use validated biomarkers that better express bite exposures to assess the impact of vector insecticide resistance. In case children as well as in control children who are mosquito net users or non-users, from low or high resistance areas, variations in these biomarkers will better inform the differences. In addition, it was assessed whether the gSG6-P1 peptide assay for anti-salivary antibodies might be a better marker useful for detecting the implications of vector resistance in different settings in Benin.

2. Materials and Methods

2.1. Study Area, Sample Selection

The samples used in this study were collected as part of a case-control study whose objective was to evaluate the effect of LLIN use on malaria morbidity in clusters of low and high vector resistance to pyrethroids [29]. Dried blood drops (DBD) on Whatman® 903 paper were collected for passive case detection, conducted between December 2011 and July 2012 in four hospitals in four communes (Pobè, Kétou, Sakété, and Ifangni) of the Plateau department [29].

2.2. Type of Study, Sampling and Study Population

This is a cross-sectional case-control study with one control for one case that started in November 2011 and ended in July 2012 in the above-mentioned communes. The epidemiological results were published in 2022 by Tokponnon *et al.* [29].

In these communes, clusters with low and high insecticide resistance of malaria vectors where the coverage of LLINs distributed to the population is greater than or equal to 80% were selected. According to the level of resistance, we compared the data obtained in the two groups of children (cases and controls) and also evaluated the influence of resistance in the two R+ and R+++ areas (Sovi *et al.*, 2014). This prior study provided information on the resistance status of malaria vectors in the villages of origin of the children to be included in the study. A total of 32 clusters, 16 of which were high and 16 low resistance, were selected for recruitment of children.

The overall sample size was estimated at 240 children under 5 years of age, with 60 children per health center.

The sample consisted of all children under five years of age residing in the study localities for at least six months, who were seen in medical consultations or in vaccination services and whose parents had given their informed consent. We recruited the first 120 children under 5 years of age with fever and the first 120 children under 5 years of age without fever who were seen in medical consultation. Four reference health facilities were identified in the four communes to house our work. These were the Health Centers of Kétou and Ifangni, and the Hospitals of Sakété and Pobè. Recruitment in each health facility involved the first 30 children under 5 years of age with fever and the first 30 children under 5 years of age without fever.

A questionnaire is asked to the children's companions on the use or not of LLINs and a thick drop sample is taken to assess malaria infestation.

2.3. Preparation of Confetti

A filter paper was made per patient for the determination of anti-saliva antibodies.

Technique for preparation of confetti

The filter paper with 5 circles of 12 mm diameter was impregnated with 5

drops of blood taken from each spot. The confetti was air-dried for 24 hours, stored in individual plastic bags and then in airtight straps (containing a desiccant, silica gel) and a humidity control at room temperature until the genotypic and ELISA tests were performed and transported to the CREC/IRD laboratories for storage and handling.

It should be noted that each DBS is labeled with the date of preparation, the initials of the name and surname of the individual and his identification number, according to the following **Figure 1**.

2.4. Conservation of Filter Papers

Blood samples on filter paper (FP) are dried completely to prevent fungal growth. The technician must ensure that each filter paper has the same information as the thick drip slide. The moisture indicator should be checked periodically (once a week) in case it changes color and is replaced routinely.

2.5. Assay of Human IgG Ab Levels to gSG6-P1 Peptide by ELISA

This was done according to the technique of Fontenille described by Beier *et al.* (1998) and SOP N˚13 [30]. We prepare the "Blood Meal" Buffer (to be kept at +4˚C, 1 to 2 weeks) for 1/2 liter, take 2.5 g of casein in 50 ml of 0.1 N NaOH.

For each selected child (N = 240, including 120 cases with temperature above 37.5˚C and 120 controls without fever), a standardized DBS (1 cm in diameter) was eluted by incubation in 350 µL of phosphate-buffered saline (PBS-Tween 0.1%) at +4˚C for 48 hours. Enzyme-linked immunosorbent assay (ELISA) was performed on the eluates to assess the level of IgG responses to the gSG6-P1 peptide antigen, as described previously [13]. Briefly, the wells, of Maxisorp plate (Nunc, Roskilde, Denmark), were coated with gSG6-P1 (20 µg/mL) in PBS. After washing (distilled water + 0.1% Tween), the DBS eluate from each child was incubated in an incubator at 37˚C. This sensitized plate was then incubated (in duplicate) at +4˚C overnight at a 1/40 dilution (PBS-Tween 1%). This optimal dilution was determined by preliminary experiments. A biotinylated mouse antibody to human IgG (BD Pharmingen, San Diego, CA) was incubated at a 1/1000 dilution in PBS with 1% Tween (1.5 hours at 37˚C) and streptavidin (1/1,000; 1 hour at 37˚C) conjugated to peroxidase (Amersham, Les Ulis, France). Colorimetric development was performed using ABTS (2,2'-azino-bis (3-ethylbenzothiazoline6-sulfonic acid) diammonium; Sigma, St Louis, MO) in 50 mM citrate buffer (pH = 4)

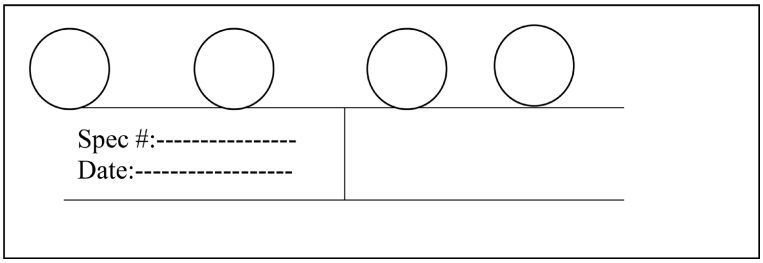

Figure 1. Illustration of a confetti paper.

containing 0.003% H_2O_2. Optical density (OD) was measured at 405 nm after 2 hours at room temperature in the dark. In parallel, each tested sample was evaluated in a blank well containing no gSG6-P1 antigen (ODn) to measure non-specific reactions. Known positive controls were included on each ELISA plate to control for plate variation and assay reproducibility. Specific anti-gSG6-P1 antibodies were also assessed in individuals not exposed to Anopheles (N = 12; negative control from France) to quantify the non-specific background of the ELISA. Individual results were expressed as the ΔOD value: ΔOD =ODx−ODn, where ODx represents the average of the individual ODs in the two antigen wells OD the two antigen wells [13] [31] [32].

2.6. Ethical Considerations

The study was submitted to the National Ethics Committee for Health Research of Benin, which gave its approval (Agreement N˚ 007 of May 25, 2010). Informed consent was obtained in writing from the parents of the children included in the study.

Data confidentiality and anonymity were respected. Health check-ups and treatment of uncomplicated malaria in the targeted children were free of charge. An information note presenting the objectives, benefits, risks, and inconveniences of the study was read to the study participants.

2.7. Data Management and Analysis

Data were entered independently in duplicate into a database using Epi data software. Data analyses were done with SPSS 16.0 software on the one hand and GraphPad Prism5 statistical software® (GraphPad, San Diego, CA, USA) for the evaluation of the median of anti-saliva antibody levels. Differences in proportions were analyzed by the test of equality of two proportions. The distribution of ADOs is not normally (Gaussian) distributed. Thus, nonparametric tests were used to compare antibody levels. The Mann-Whitney Test was used for comparison of antibody levels between two independent groups, and the nonparametric Kruskal-Wallis test was used to compare ADOs between more than two groups. All differences were considered significant at $p < 0.05$.

3. Results

3.1. Socio-Demographic Characteristics of the Children and Net Used

The socio-demographic characteristics of the children included in this study are similar to those described in previously published epidemiological studies [29]. Thus, 240 children were recruited from the 4 health facilities participating in the study. A total of 185 children tested negative among the children under five years of age included in the study. The 120 cases and 120 controls were well identified. The median age of the children was 21 months (minimum 1 and maximum 59 months). The sex ratio (M/F) was 1.01. The 120 children referred

to as cases (i.e., with an axillary temperature greater than 37˚5) suffering from malaria and the 120 others referred to as controls were selected at random according to their origin and progressively according to their order of arrival at the health center on the list of cases and controls. Their mothers or guardians were asked to fill out a questionnaire. Of the parents or caregivers of children at the health center, 169 (70.4%) reported that their children had slept under LLINs the day before they arrived at the health center. This rate represents 70.8% (85 children) among cases and 70.0% (84 children) among controls with no significant difference ($p = 0.8$). When considering the status of the villages, the rate of use is 68.0% in the high resistance zone and 74.2% (69 children) in the low resistance zone with no significant difference ($p = 0.3$). The use of impregnated mosquito nets the day before the survey, the frequency of use of impregnated mosquito nets during the two weeks preceding the day of the survey, the availability of impregnated mosquito nets at the household level, and the use of other means of protection against mosquito bites were the same in both groups of children (cases and controls) ($p > 0.05$) in both low and high vector resistance localities [29].

3.2. Specific Anti-Salivary IgG Response

We assessed levels of anti-gSG6-P1 antibodies to malaria in children to determine their level of exposure to mosquito bites. We analyzed and presented the results of anti-gSG6-P1 IgG assays, by ELISA, of 240 individuals (120 cases and 120 controls constituting the population of this study). This biomarker is thought to better explain variations in exposure or human-vector contact. These analyses were done taking into account the use of LLINs, the clinical status of the children, the presence or absence of the parasite, the status of the clusters found, and the communes in which the health centers used for data collection were located.

3.3. Anti-Saliva IgG Response by Low and High Vector Resistance Status and LLIN Use by All Children

For the "resistance" effect, we compared the profile of individual IgG responses of subjects in low-resistance clusters ($n = 93$; 38.75%) with those in high-resistance clusters ($n = 130$; 61.25%), across all ages of children. The evolution of median-specific IgG levels between children was similar (**Figure 2(a)**). This suggested that vector resistance at the cluster level does not affect the anti-saliva IgG response and that specific IgG levels were similar in both groups ($p = 0.4146$). **Figure 2(b)** shows that the median antibody level was similar in children whether or not they used LLINs the day before the survey ($p = 0.7724$).

In this study, gender and especially age, major factors of variation in the adaptive immune response, do not have a significant effect on the development of the Ac (IgG) response.

3.4. Anti-Saliva IgG Response by Infection Status of Individuals and Study Sites

We tested whether individuals infected with *P. falciparum* (the only plasmodial

species diagnosed in this study) had different profiles or levels of specific IgG response to *An. gambiae* total saliva. For this purpose, we took all the samples and compared the specific IgG levels between uninfected individuals (n= 185) and *P. falciparum* infected individuals (n= 55). The results of this comparison are shown in Figure 3(b).

Figure 3(a) shows that the median level of *An. gambiae* total saliva specific IgG was not significantly lower in uninfected individuals compared to those infected with *P. falciparum* (p = 0.1152; non-parametric Mann-Whitney test).

Large variabilities were observed at study site (Figure 2(a)). The medians of the graph on the left clearly indicate that the exposure levels are different and position Pobè as the commune with the highest level according to the Kruskal Wallis test (p = 0.03).

In addition, the individual specific IgG levels of the infected individuals were

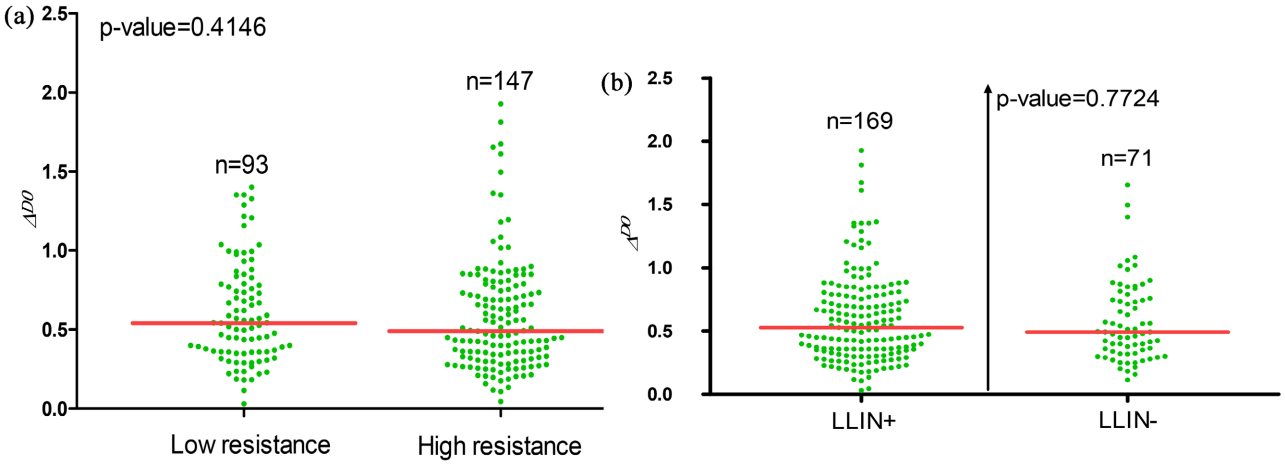

Figure 2. Total anti-saliva IgG response in 240 individuals according to high and low resistance zone and LLIN use. (a) IgG response according to area; (b) IgG response according to net usage.

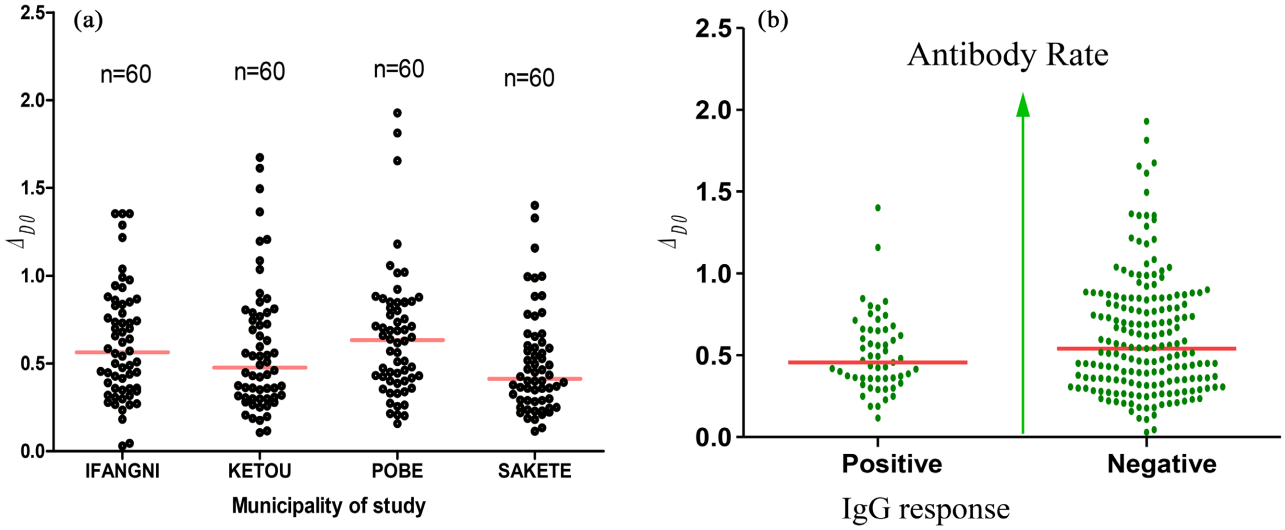

Figure 3. Total anti-salivary IgG response in 240 individuals by site and by infected and uninfected. (a) IgG response according to district; (b) IgG response according to tick drop.

mostly above 0.250 (Reaction Threshold); this was not the case for the non-infected individuals in whom several OD (individual) < 0.250 were observed.

3.5. Anti-Saliva IgG Response by Individual Infection Status and Study Sites

The results of individual gSG6-P1-specific IgG responses in cases and controls are shown in **Figure 4**. No differences in exposure level were observed in cases and controls according to the level of insecticide resistance of the vectors. The Kruskal Wallis test (p = 0.7724) noted no difference in exposure levels even with LLIN use.

4. Discussion

IgG anti-salivary antibodies assessed at the health center level in children under five years of age did not demonstrate the impact of vector resistance on LLIN efficacy. Information collected at the four health facilities is only a reflection of the situation that would normally be seen in the population under real conditions of ITN use in contexts of high or low vector resistance.

The study data showed a high heterogeneity in the specific IgG response to this salivary peptide between individuals, 1) within cases and controls; 2) between the 4 study sites; 3) between the different localities of origin of the children, among those who use or do not use LLINs or even between individuals with or without a positive thick drop. These observations suggest that in these locations, important differences in the level of human-vector contact observed could be influenced by several factors and individual [33] and/or household behaviors. For example, the use of LLINs [34] and the movement of populations from one locality to another, where higher exposure to Anopheles [33], may significantly increase/decrease the likelihood of contact between human hosts and malaria vectors and explain some of the observed immunological findings. But some epidemiological factors such as genetic co-infection, nutritional parameters, could

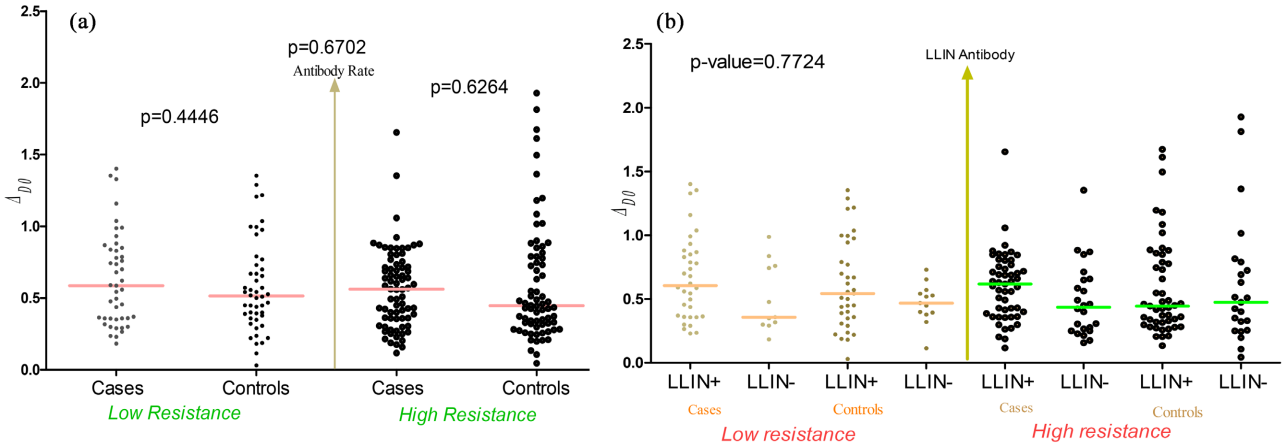

Figure 4. Total anti-salivary IgG response in 240 individuals following cases and controls according to whether or not they used LLINs in the two types of localities. (a) IgG response according to case or control and zone; (b) IgG response according to user and zone.

not be excluded.

The biomarker results reinforce previous results that validated this salivary peptide of *An. gambiae* as a biomarker to assess the level of human exposure to malaria vectors in different contexts of exposure and malaria transmission [35] [36] [37] [38]. Median levels that do not differ by vector resistance levels and by LLIN and non-LLIN users suggest that in localities, the level of LLIN availability is high and that populations that would not use LLINs have other means of protection such as aerosol cans, coils, and others. However, the results should be interpreted with great caution, given the large discrepancy between the number of infected and uninfected children and the small sample size of those infected (n = 55). This study then highlights the relevance of the use of this biological marker peptide for the evaluation, of the population exposure to bites and the effectiveness of vector control strategies as it has been demonstrated in other localities [36] [38]. It was observed that the average levels of exposure to vector bites were significantly different between communes and could be due to the fact that not all populations of a locality necessarily attend the reference health facility in the area. The immunological results obtained on the evaluation of the impact of vector resistance on LLIN efficacy confirm the epidemiological data that did not show any difference between the two categories of localities. The results of the transmission assessment work conducted in the same period in the study area in a short duration had not noted any difference between high and low resistance localities as well as other epidemiological data of the area [29] [39] [40].

Our method of assessing specific IgG antibodies did not establish a relationship between the median level of these antibodies, the use of LLINs and the level of insecticide resistance of the vectors. Furthermore, the resistance of *An. gambiae* s.l. to pyrethroid insecticides in the region should not be overlooked. Ideally, it would have been interesting to have a free resistant area versus a fully susceptible area to demonstrate the impact of the level of transmission expressed by biomarkers such as anti-malarial antibodies and the level of insecticide resistance in the areas in order to assess the operational effectiveness of vector control interventions that the LLINs. This is not the situation, and we used the median mortality rate to discriminate between the two types of zone, which is a limitation of this type of evaluation.

This study is a cross-sectional survey has limitations. First, there was no direct observation of the protection offered by LLINs in children and the information collected was declarative from parents and caregivers of children at the health center. Instead, it should be inferred at the ecological level from the fact that, although LLINs are the primary malaria vector control tool in the study area, malaria prevalence was not higher in villages where vector resistance was higher. Second, there are many interesting aspects to this study. Cases and controls were identified in the community, simultaneously from the same at-risk population. Data collected in health centers may be a source of information bias, particularly with respect to exposure variables. Because LLINs were distributed free of charge,

mothers may lie (prevarication bias) about their child's exposure, especially at the health center level when children were sick to receive care. Third, in addition to personal protection, LLINs can also provide protection through mass effect, killing mosquitoes, which reduces the longevity and density of local mosquitoes and provides protection to all community members, including those who do not use LLINs. The study was unable to differentiate between these two types of protection. Fourth, the measure of insecticide resistance was based on the WHO insecticide susceptibility test, simply measuring the frequency of resistant vectors.

Measures of resistance intensity based on a dose-response relationship that uses resistance intensity might be more informative, but were not practical at the scale of this study [29] [38].

5. Conclusion

The evaluation of the anti-salivary IgG response specific to Anopheles vectors of malaria in children under five seen in health care settings in areas of low and high insecticide resistance did not reveal the impact of resistance on the operational effectiveness of the use of long-lasting insecticide-treated nets. No significant difference was observed in the expression of the anti-saliva IgG antibody in the different zones (high and low resistance zone) as well as in cases and controls ($p > 0.05$). Furthermore, the same finding was observed for antibody expression in children whether or not they used LLINs on the day before the survey ($p = 0.7724$). Similarly, gender and especially age, major factors of variation in the adaptive immune response, did not have a significant effect on IgG expression.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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