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Mosquitoes fauna diversity, *Plasmodium falciparum* infection and insecticide resistance status in malaria vectors in a lagoon area in Southern Benin, West Africa

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Abstract

Lagoon areas maintain ideal water conditions for mosquito breeding habitats and are thus environments with high risk of malaria transmission. In Benin, several administrative units, among which the Sô-Ava District, are located in lagoon areas. We conducted entomological surveys in this lagoon district from July 2014 to June 2015, in order to update existing information on biodiversity of mosquitoes, Plasmodium falciparum infection, and insecticide resistance status in malaria vectors. Our survey found that Culex quinquefasciatus and Mansonia africana were the most abundant species, and that Anopheles coluzzii represented the main malaria vector in this area, followed by Anopheles melas. Only Anopheles coluzzii was positive to Plasmudium falcimarum circum sporozoitic protein (4.2 %). An. gambiae s.l. were susceptible to chlorpyrifos-methyl and bendiocarb but resistant to all pyrethroids tested and to pyrimiphos-methyl. The average of kdr allelic frequency from July 2014 to June 2015 was 77.4% and that of ace[1] gene was less than 1%. We conclude that Anopheles coluzzii is the main malaria vector in the lagoon area we studied, somewhat contrary to our expectations. However, this malaria vector was resistant to insecticides used for bed net impregnation, even if the resistance level was lower than observed in other parts of Benin.

***Keys Words**: Malaria, vectors, Plasmodium, infection, resistance, lagoon, Benin*

1. Introduction

The past decade has seen a significant expansion of financial support and concomittant scale-up of malaria control tools (WHO, 2015). Despite the large reduction in malaria burden induced by such interventions, malaria is still a major public health concern, with an estimated 214 million cases (range: 149–303 million) and 438 000 deaths (range: 236 000–635 000) occurring in 2015 (WHO, 2015). In Benin, 1 044 235 malaria cases and 1869 deaths were reported in 2013 (WHO, 2015). In many malaria endemic areas such as Benin, Long Lasting Insecticidal treated Nets (LLIN) and Indoor Residual Spraying (IRS) represents the main interventions for malaria vector control (WHO, 2015). These insecticide-based methods depend on the susceptibility of vectors to insecticides and vectors behavior.

Widely documented insecticide resistances are a potential threat for the success of malaria vector control measures (N'Guessan *et al*., 2007; Ranson *et al*., 2009). The insecticide resistance mechanisms described so far rely on metabolic pathways and target site mutations that confer resistance to the four insecticide families used for malaria vector control (carbamates, organophosphates, organochlorines and pyrethroids) (Hemingway *et al*., 2004; Rivero *et al*., 2010). Moreover, non-compliance in the use of bed nets by inhabitants, changes in the composition of mosquito species, as well behavioral changes of mosquito have recently been shown to play a potential role in the low success of vectors control tools (Moiroux *et al*., 2012; Durnez *et al*., 2013; Wamae *et al*., 2015). Hence, it is necessary to assess the biodiversity and behavior of malaria vectors in endemic areas (such as Benin) and to identify how they have changed over time, in order to understand the entomological determinants of lower success rate of vector control measures.

Lagoon areas represent high risk environments for malaria transmission as their water bodies provide ideal breeding conditions for mosquito. In Benin, several administrative units, among which the villages of the Sô-Ava District, are located in lagoon areas, yet no recent entomological data for these villages are available. The most recent information on mosquito (Culicidae) fauna of

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this lagoon area date back to the 1990s. In this area, two malaria vectors species (*Anopheles melas* and *Anopheles gambiae*) were found sympatric with a sporozoitic index rate significantly lower, in cases where *An. melas* is the more abundant species (Akogbeto and Romano, 1999). Given the large scale implementation of insecticide based vector control (*e.i.* LLIN) and ongoing urbanization, an update of these data is highly desirable. Therefore, we conducted a one-year long entomological survey in order to assess the diversity and abundance of malaria vectors, their infection to circum sporozoitic protein (CSP), their insecticide resistance status and the prevalence known genetic variants conferring insecticide resistance.

2. Material and methods

2.1. Study area

Sô-Ava district (6° 28' 00" N 2° 25' 00" E) is one of the eight Districts of the Atlantic Department in southern Benin with about 100 000 inhabitants and a surface area of 218 km². This district is located in Nokoue Lake, which is a permanent natural lake in the southeast of Benin that is influenced by the South Atlantic Ocean. The climate is essentially sub-equatorial, with two dry seasons (August-September and December-March), and two rainy seasons (April-July and October-November). The average annual rainfall is around 1200 mm. The average monthly temperatures vary between 22 and 35°C. The area is flooded from August to November.

2.2. Mosquitoes collection

Entomological surveys were performed between July 2014 and June 2015. Mosquitoes were collected during twelve surveys. At each survey, mosquitoes were collected during three consecutive days at eight different houses. After consent from the head of each household, mosquitoes were trapped from using light traps developed by the Center for Disease Control (CDC). Traps were placed inside each house, from 7:00 pm to 7:00 am, corresponding to the period from dusk to dawn. The traps were suspended from the ceiling, about 2 m above the ground. All mosquitoes were identified to species level under stereoscopic microscopes, according to morphological criteria in dichotomous keys (Edward, 1941; Gillies and Meillon, 1968; Gillies and Coetzee, 1987). Mosquitoes belonging to the *An. gambiae* complex were stored in individual tubes with silica gel and preserved at -20°C.

From January to March 2015, corresponding to the fall period, larvae of *An. gambiae* mosquito were collected in the study area. All larvae were brought back to laboratory of Centre de Recherche Entomologique de Cotonou (CREC) for rearing.

2.3. Mosquitoes treatments

Heads and thoraces of anopheline females were tested by enzyme-linked immunosorbent assays (ELISA) for detection of *P. falciparum* circumsporozoite protein (CSP), as previously described (Wirtz *et al*., 1987). Positive samples were identified by having an optical density 3 times as large as the average value for negative controls. Those having an optical density between 2 and 3 times the average were considered as unresolved and were tested a second time. The CSP index was calculated as the proportion of mosquitoes found to be positive for ELISA-CSP.

Genomic DNA was extracted from the abdomen of all mosquitoes using the extraction buffer Livak (Livak, 1984). The species was then identified by diagnostic PCR using the Scott protocol (Scott *et al*., 1993) and molecular characterization according to Santolamazza protocol (Favia *et al*., 2001; Santomalazza *et al*., 2008). The knock-down resistance mutation (*kdr*-west, L1014F) and acetylcholinesterase resistance mutation (*Ace1)* were detected by Taqman allelic discrimination assays as described by Bass (Bass *et al*., 2007).

2.4. Insecticide susceptibility test

Adult female mosquitoes (F0) reared from larvae brought back to the laboratory were used for insecticide susceptibility tests. WHO insecticide susceptibility test-kits and standard procedures (WHO, 2006) were used to monitor the susceptibility of wild *An. gambiae* populations to insecticides commonly used in public health and agriculture. Batches of 25 non-blood fed, 3-5 days old adult females were exposed to filter papers impregnated with 4% DDT (organochlorine), 0.1% bendiocarb (carbamate), 0.25% pyrimiphos-methyl (organochlorine), 0.4% chlorpyrifos-methyl (organochlorine), 0.75% permethrin, 0.05% alphacypermethrin (pyrethroids), 0.05% deltamethrin (pyrethroids) and to a non-treated paper as control. Insecticide papers were obtained from the WHO reference centre at the Vector Control Research Unit, University Sains Malaysia (WHO, 2001). For each test, 100 mosquitoes were exposed to treated and untreated (control) papers for 1 hour, with mortality recorded after 24 hours. WHO criteria were followed to classify populations as 'resistant' if less than 80% mortality was observed, as ''suspected resistant'' if mortality rates were between 80 and 97% and susceptible for mortality > 97% (WHO, 2001).

2.5. Data analysis

Malaria vectors abundance was compared between flood and fall periods using Kruskal-Wallis non-parametric test. Proportional data (CSP index, morality rate) were calculated with their confidence intervals and compared using chi² tests. Allelic frequencies of the kdr mutation were calculated per survey period and according to CSP status, and compared using Fischer exact tests implemented in Genepop software (Raymond and Rousset, 1995).

3. Results

3.1. Mosquitoesdiversity

From July 2014 to June 2015, 5609 mosquitoes were collected belonging to nine species (*Aedes aegypti, Anopheles gambiae s.l., Anopheles pharoensis, Anopheles ziemanni, Culex decens, Culex fatigans, Culex nebulosus, Culex quinquefasciatus and Mansonia africana*) (table I). *Culex quinquefasciatus* and *Mansonia africana* were the most abundant species. Overall, 859 malaria vectors were caught from which, 773 were successfully tested for species diagnostic PCR. Malaria vectors were more abundant during the fall period, but not significantly different from during flood periods (Kruskal-Wallis khi² = 0.33, df = 1, p-value = 0.56). *Anopheles coluzzii* was the main malaria vector in the study area (763 individuals), followed by *Anopheles melas* (6 individuals) and *Anopheles gambiae s.s.* (4 individuals).

Table I: Mosquitoes diversity

	Flood		Fall		Total
	Total	N per month	Total	N per month	
Aedesaegypti	2	1	4	0	**6**
Anophelesgambiae	163	54	696	77	**859**
Anophelespharoensis	23	8	46	5	**69**
Anophelesziemanni	225	75	94	10	**319**
Culex decens	76	25	569	63	**645**
Culex fatigans	0	0	5	1	**5**
Culex nebulosus	12	4	108	12	**120**
Culex quinquefasciatus	944	315	943	105	**1887**
Mansoniaafricana	1390	463	309	34	**1699**
Total	**2835**	945	**2774**	308	**5609**

3.2. CSP index in malaria vectors

ELISA for detection of *P. falciparum* CSP was carried out on 859 malaria vectors of which 36 were positive, corresponding to 4.2 ± 1,3 % (table II). Only *An. coluzzii* was positive to CSP. The CSP index for the fall period was higher than that for the flood period (1.8% *vs* 4.7%), but this difference was not statistically significant (chi² = 2.59, df = 1, p-value = 0.11).

Table II : CSP infection rate in malaria vectors

	Flood	Fall	**Total**
N tested	163	696	**859**
N CSP+	3	33	**36**
CSP infection rate	**1.8**[a]	**4.7**[a]	**4.2**
CI95%	2.1	1.6	1.3

Numbers sharing the same letter are not significantly different

3.3. Resistance status in An. gambiae s.l.

An. gambiae s.l. tested were susceptible to chlorpyrifos-methyl (mortality rate = 100%) and to bendiocarb (mortality rate = 99%). In contrast, vectors were resistant to DDT (mortality rate = 1%), to permethrin (mortality rate = 20%), to deltamethrin (mortality rate = 45%), to alphacypermethrin (mortality rate = 20%) and to pyrimiphos-methyl (mortality rate = 40%) (figures 1 and 2).

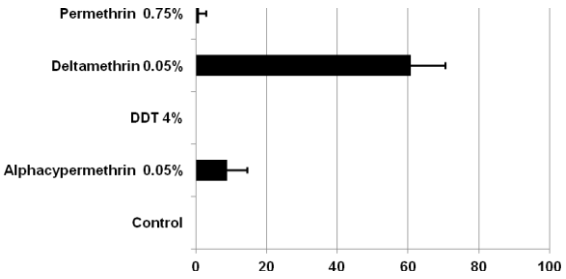

Figure 1: KD60 rate (%) of *Anopheles gambiae s. l.* after exposure to insecticides

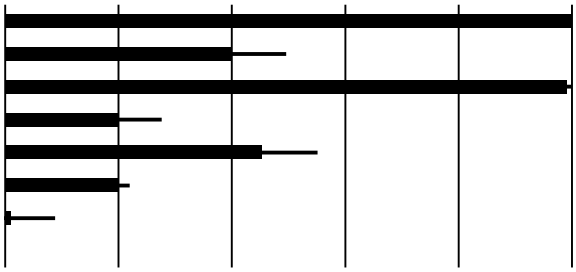

Figure 2 : Mortality rate (%) of *Anopheles gambiae* 24 hours after exposure to insecticides

3.4. Kdr and ace 1 resistance gene status in An. gambiae s.l.

The average allelic frequency of *kdr* from July 2014 to June 2015 was 77.4%, with no significant variation throughout the year (chi² = 0.50, df = 1, p-value = 0.47) (table III). The *kdr* allelic frequency for CSP positive and CSP negative were 71.21% and 70.35%, respectively (table IV). No significant difference was observed between these frequencies (chi² = 0.004, df = 1, p-value = 0.95). Concerning *ace*[1] gene, only one *Anopheles coluzzii* of the 770 *Anopheles gambiae s.l.* tested successfully was heterozygote for the mutation.

Table III : *kdr* frequency in malaria vectors

	Flood	Fall	**Total**
RR	90	309	**399**
RS	38	248	**286**
SS	8	7	15
Freq	**80.15**[a]	**76.77**[a]	**77.43**

Numbers sharing the same letter are not significantly different

Table IV :*kdr* frequency according to the infection to CSP

	CSP+	CSP-
RR	17	382
RS	13	273
SS	3	82
Freq	**71.21**[a]	**70.35**[a]

Numbers sharing the same letter are not significantly different

4. Discussion

The present study found that *Anopheles coluzzii* is the main malaria vector in the Sô-Ava district. Moreover, our exposure tests showed that this vector is resistant to all pyrethroid insecticides, but susceptible to organophosphate and carbamates.

Nine different species were caught during the surveys. This number is lower than that observed in other settings in Benin Djènontin *et al*., 2010; Padonou *et al*, 2011). The lower number of mosquitoes species observed in the present study is probably due to the specific environmental conditions of the study area. The relative abundance of *Mansonia africana* and *Culex quinquefasciatus* observed was expected. Indeed, these mosquitoes are adapted to larval breeding sites with vegetation and polluted larval breeding sites respectively and such breeding sites are frequent in the study area. Given that the Sô-Ava district is located in a permanent natural lake influenced by the Atlantic Ocean, the proportion of *Anopheles melas* observed in the study is lower than was expected. Indeed, a previous study reported in an 80% proportion of *Anopheles melas* in neighbouring lagoon areas (Akogbeto and Romano, 1999; Akogbeto, 2000). The lower proportion of this secondary vector observed in the Sô-Ava villages could be due to the urbanized nature of this area (Akogbeto, 2000), which has probably led to the proliferation of breeding sites of *Anopheles coluzzii* to the detriment of those of *Anopheles melas*. *Anopheles melas* is known to have a sporozoitic index significantly lower than that of *Anopheles coluzzii* (Akogbeto and Romano, 1999; Akogbeto, 2000; Diop *et al*. 2002). Therefore, the relative proliferation of breeding sites of *Anopheles coluzzii* is a potential health concern, considering ongoing malaria transmission in this area. Any subsequent increase in malaria vectors in this area may have been masked by the large scale implementation of vectors control tools (*e.g.* Long Lasting Insecticidal Nets). Indeed, since the last decade, the National Malaria Control Program of Benin had implemented various LLIN mass distribution campaigns. A recent study carried out in this study area showed a general high possession of LLIN by the pregnant women and more than 8 out of 10 women correctly used it (Hounhonnou *et al*, in prep).

Malaria vectors were resistant to all pyrethroid insecticides tested, but the mortality rates observed were higher than that observed in southern Benin, indicating a lower pyrethroid resistance level. Moreover, the *kdr* allelic frequency was ~80% in the study area vs ~100% in Cotonou, a neighbouring District. This trend could be due to lower use of insecticides linked to agriculture, as fishing is the most important economic activity in the study area. The insecticide resistance selection in this area could be due to the domestic use of insecticide for vectors control. In neighbouring Districts, an increase activity of the vegetable farming has led to the use of insecticide in an improper manner to control vegetable pests and then a huge selection pressure on mosquito population. Resistant mosquitoes from such areas could come in the study area to increase insecticide resistance prevalence in malaria vectors (Yadouleton *et al*., 2009). Pesticide residues from these areas could also contribute directly to insecticide resistance selection in the study area. Further investigations are needed to clarify the origin of insecticide resistance in the study area.

We conclude that *Anopheles coluzzii* is the main malaria vector in the lagoon area we studied, somewhat contrary to our expectations. However, this malaria vector was resistant to insecticides used for bed net impregnation, even if the resistance level was lower than observed in other parts of Benin. Investigations on the origin of insecticide resistance selection in malaria vectors in the study area are needed.

5. Authors' contributions

AD, DC and CP conceived of the study. AD and BZ participated in data collection. JA and BZ carried out bioassays in laboratory. AD participated in the analysis and interpretation of data. The manuscript was drafted by AD. All authors read and approved the final manuscript.

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