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Full Length Research Paper

Entomological baseline data on malaria transmission and susceptibility of *Anopheles gambiae* to insecticides in preparation for Indoor Residual Spraying (IRS) in Atacora, (Benin)

Rock Aïkpon[1,2]*, Razaki Ossè[1,2], Renaud Govoetchan[1,2], Arthur Sovi[1,2], Frédéric Oké-Agbo[1] and Martin C. Akogbéto[1,2]

[1]Centre de Recherche Entomologique de Cotonou (CREC), Cotonou, Benin.
[2]Faculte des Sciences et Techniques de l'Université d'Abomey Calavi, Benin.

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To implement indoor residual spraying (IRS), the department of Atacora was selected in Benin. Entomological surveys were performed before IRS implementation. Mosquitoes were sampled by Pyrethrum spray catch and were identified morphologically and by molecular methods. The *Plasmodium falciparum* circumsporozoite indices were measured by enzyme linked immunosorbent assay (ELISA). Molecular detection of pyrethroid knock down resistance and that of insensitive acetylcholinesterase were performed. Susceptibility status of *Anopheles gambiae* was determined using World Health Organization (WHO) bioassay tests to various insecticides. *A. gambiae s.l.* was the main species harvested in houses (81.71%) and *A. gambiae s.s* is practically the only member that was found. Both M and S forms were in sympatry, but the molecular S form was predominant (94.42%). *A. gambiae s.l* were susceptible to bendiocarb but fully resistant to organochlorine (DDT), permethrin and deltamethrin. Entomological inoculation rate vectors (EIR) was 6 infectious bites per man per month on average during the study period. The average of *kdr* and *Ace-1* allelic frequency were 78 and 3%, respectively. *A. gambiae s.l* is characterized by a high endophilic behavior in Atacora, which is a good criterion for IRS implementation. The susceptibility to bendiocarb add to the low *Ace-1* mutation frequency found in *A. gambiae* populations could lead to the use of bendiocarb for IRS.

Key words: Entomological baseline data, IRS implementation, *Anopheles gambiae,* Atacora, Bénin.

INTRODUCTION

Malaria remains a major cause of morbidity and mortality in sub-Saharan Africa and represents one of the most critical public health challenges for Africa. More than two billion people around the world, particularly people living in South America, south-eastern Asia and sub-Saharan Africa, are at risk of contracting malaria. Besides, one million deaths are recorded yearly of which, 91% occur in sub-Saharan Africa (WHO, 2011). In Benin, in 2011, malaria was responsible for more than 1,753 deaths (MS, 2011). However, it incidence in Atacora in 2011 was

*Corresponding author. E-mail: rockypremier@yahoo.fr. Tel: (229) 97434183. Fax: (229) 21308860.

18.5%, which is higher than the national average (15.7%). The main malaria parasite was *Plasmodium falciparum* and was mainly transmitted by *Anopheles gambiae* s.*l*. This disease representing the primary cause of mortality and morbidity in health centres caused an enormous burden to health and economy in developing countries (WHO, 2011). Insecticide Treated Nets (ITNs) use represent the main approaches of malaria control (WHO, 2009). Household ITNs ownership reached more than 50% in several high burden African countries (WHO, 2009). Pyrethroids are the only insecticides used for net impregnation because of their strong efficacy, their fast acting effect at low doses and their low toxicity for mammals (Zaim et al., 2000). Unfortunately, resistance to pyrethroids in malaria vectors has spread across Africa and is now present in most of the countries where national malaria control programmes (NMCP) are implementing large scale distribution of long lasting insecticidal nets to populations at risk, that is children under five and pregnant women (Santolamazza et al., 2008).

There are numerous reports of resistance to pyrethroid throughout Africa (Elissa et al., 1993; Vulule et al., 1999; Koekemoer et al., 2002; Etang et al., 2006; Abdalla et al., 2008; Nwane et al., 2009; Bigoga et al., 2012). In Benin, the resistance of malaria vectors to pyrethroids observed first in Cotonou spread not only to central and southern regions of the country, but also in the northern localities (Akogbéto et al., 1999; Chandre et al., 1999; Corbel et al., 2007; Yadouleton et al., 2010).

The high level of resistance to pyrethroid in *A. gambiae* in Africa, particularly in Benin (Corbel et al., 2007; Yadouleton et al., 2010) is one of the major challenges being faced by malaria vector control programmes. It is then urgent to find ways to manage this resistance. In this context, an alternative insecticide to pyrethroids in IRS strategy supported by the President Malaria Initiative launched since 2008 in 14 African countries including Benin could be on the way to resolve the problem of resistance to pyrethroids in *A. gambiae*. After experimental hut evaluation (phase II), the non-pyrethroid that is effective seems to be bendiocarb (carbamate) (Akogbéto et al., 2010).

A study conducted in Ouémé, in southern Benin showed that the first and second rounds of IRS using bendiocarb were successful with a drastic decrease in malaria transmission in areas under IRS (Akogbéto et al., 2011). In spite of this success, NMCP decided to move IRS from Ouémé to Atacora (northern Benin). As matter of fact, Ouémé is characterized by two periods of transmission which last all the year. Implementation of IRS in such area needs two rounds of intervention. Contrary to Ouémé is characterized by a single transmission period. This means that just one spray round per year would be sufficient to cover the period of transmission. However, no entomological data from Atacora was available. In order to collect baseline data relating to diversity and abundance of mosquitoes, malaria transmission and the prevalence of insecticide-resistance alleles in malaria vectors, entomological surveys were performed between September and October, 2010 in all the districts of Atacora before IRS implementation. The above mentioned baseline data are reported in this paper.

METHODOLOGY

Study area

The study was carried out in Atacora, a department located in north-west of Benin (Figure 1). This department covers an area of 31,665 km^2 and counts a total of 735,845 inhabitants including 146,309 children under 5 years old in 2011 (INSAE, 2009). It is located at 10° 18' 46'' N and 1° 23' 19'' E. There are fifty health facilities for nine districts. This department is characterized by a sub-equatorial climate, with only one dry season (December to May) and only one rainy season (July to November). The annual mean rainfall is 1,300 mm and the mean monthly temperature varies between 23 and 31°C. The department is irrigated by three major rivers: the Mekrou, the Pendjari and the Alibori. The major economic activity is agriculture and it is characterized by the production of cotton and millet, where various classes of pesticides are used for pest control.

Mosquito sampling

Larvae collection

Mosquito larvae were collected from September to October, 2010 at the end of the rainy season. All instars of larvae were collected using dipping method from a wide range of breeding sites (puddles, shallow wells, gutters and rice fields). Then all larvae were brought back to the laboratory of Centre de Recherche Entomologique de Cotonou (CREC) for rearing. After that, emerging adult female mosquitoes were used for insecticide susceptibility tests and finally a susceptible strain of *A. gambiae* (Kisumu) was used as reference strain for bioassays.

Pyrethrum spray catch (PSC)

Adult mosquito collections were carried out from September to November, 2010 in all the nine districts in Atacora. In addition to larvae collection, mosquitoes resting in the house were collected through morning spray catch (MSC) from 7 a.m. to 9 a.m. In all the nine districts, we collected mosquitoes resting indoors. In each district, two areas were selected: a central area more or less urbanized and rural areas. In each area**,** some ten homes were randomly selected for mosquito collection. Three sessions were performed per month. Morning pyrethrum spray catches were performed using pyrethrum spray Rambo® and white canvas spread on the floor to collect knocked down mosquitoes. Knocked down mosquitoes falling on white bed sheets were kept separately and later, kept in labeled tubes containing silica gel and frozen at -20°C for further laboratory analysis. This sampling method led to an accurate estimation of the total density of mosquito species in the houses and the proportion of female mosquitoes resting from the

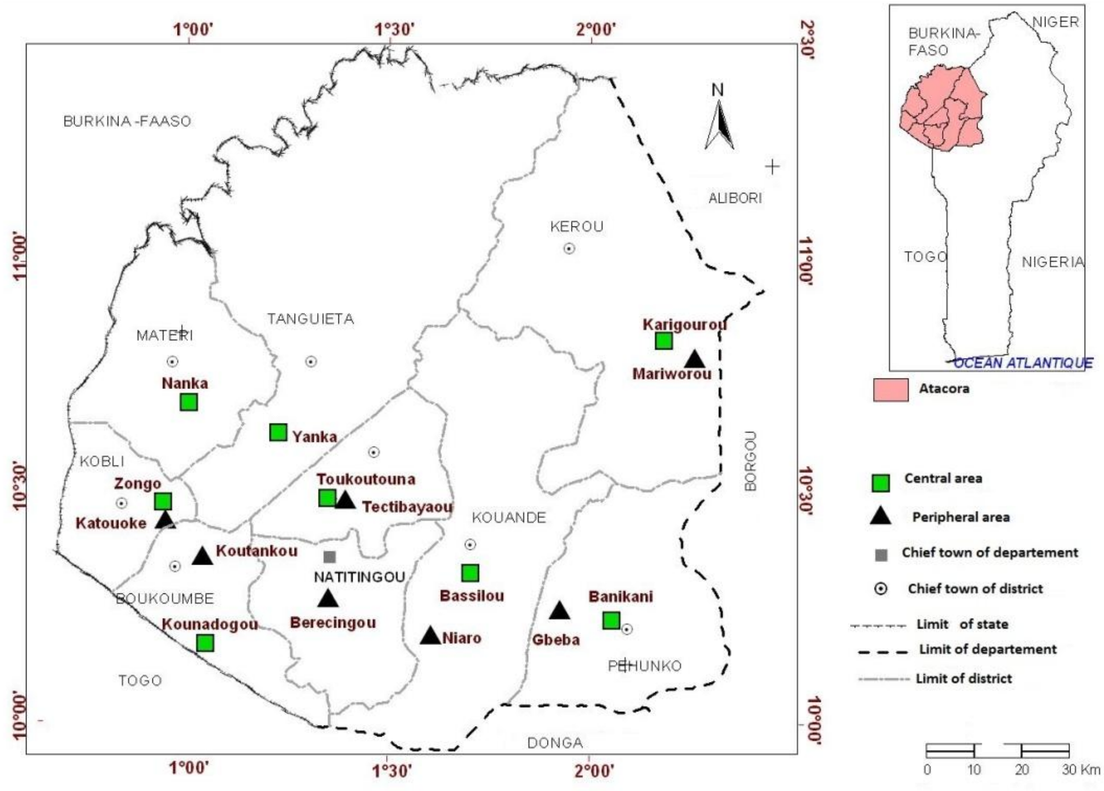

Figure 1. Map of study area.

houses. The recorded data were used to assess the human biting index (ma) and the entomological inoculation rate vectors (EIR).

Insecticide susceptibility tests

WHO insecticide susceptibility test-kits and standard procedures (WHO, 1998) were used to monitor the susceptibility of wild *A. gambiae* populations to the chemical groups of insecticides commonly used in public health and agriculture. Batches of 25 non-blood fed, 3 to 5 days old adult females were exposed to filter papers impregnated with 4% organochlorine (DDT), 0.1% bendiocarb (carbamate), 0.75% permethrin and 0.05% deltamethrin (pyrethroids). The number of mosquitoes knocked down was recorded every 5 min during exposure. After exposure, mosquitoes were supplied with glucose solution as food, and mortality was recorded 24 h post-exposure. Tests with untreated papers were systematically run as controls. WHO criteria were respected to classify populations as 'resistant' if less than 80% mortality was observed, as ''suspected resistant'' if mortality rates were between 80 and 97% and as 'susceptible' for mortality > 97% (WHO, 2001).

Laboratory processing

After each indoor pyrethrum spray, mosquitoes were sorted by genus as anopheline and culicine and counted. Anophelines were morphologically identified to species using taxonomic keys of Gillies and De Meillon (1968) and Gillies and Coetzee (1987). Ovaries from randomly selected female *A. gambiae s.l.* specimens captured by PSC were dissected to determine parity rate, by observing the coiling degree of ovarian tracheoles (Detinova and Gillies, 1964). Then, mosquito infectivity rates were determined from head and thorax of all female anopheline specimens by enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies against *Plasmodium falciparum* circumsporozoite protein (CSP) as described by Wirtz et al. (1987). The carcasses of these females (abdomens, wing and legs) were stored in individual tubes with silicagel and preserved at −20°C in the laboratory for identification of species and characterization of molecular forms within the *A. gambiae* complex as previously described (Scott et al., 1993; Favia et al., 2001).

Data analysis

The human biting index (ma) or *Anopheles* density (number of *Anopheles* per person and per night) and the circum sporozoite protein positive rate were calculated. The human biting rate was determined by dividing the number of mosquitoes fed and half gravid collected within a room by the number of sleepers in this room the night before morning spray catching. The circum sporozoite protein positive rate (% CS+) was calculated as the proportion of mosquitoes found to be positive for CSP. The entomological inoculation rate (EIR) was defined as *Anopheles* density by the CSP and estimated as the number of infectious bites per human per month. A Chi-square test with the MINITAB statistical software (Version 12.2) was used to compare the mortality rates among the

Table 1. Mosquito species caught by PSC from September to October, 2010 in the study area.

Species	Nb	%
Aedes aegypti	11	1.04
Aedes gr. Palpalis	5	0.47
Aedes longipalpis	2	0.19
Aedes vittatus	5	0.47
Anopheles broheri	6	0.57
Anopheles funestus	13	1.23
Anopheles gambiae	862	81.71
Anopheles pharoensis	7	0.66
Culex annulioris	3	0.28
Culex fatigans	45	4.27
Culex gr decens	9	0.85
Culex nebulosus	3	0.28
Culex quinquefasciatus	72	6,82
Mansonia africana	12	1,14
Total	1055	

localities. The genotypic differentiation of *kdr* and *Ace1* loci was tested using the Fischer exact test implemented in GenePop software (Raymond and Rousset, 1995), and the Fisher test was used to compare these frequencies. An analysis of variance (ANOVA) was performed to compare the entomological estimates (ma, EIR, CSP) among the sites.

Ethical consideration

Permission was sought from inhabitants to perform collections in their rooms. In addition, community consent had been obtained beforehand in all the villages. This study was approved by the Ethical Committee of the Ministry of Health in Benin.

RESULTS

Culicidae diversity and endophily

A total of 1,055 mosquitoes belonging to 14 different species were caught. They included 84.71% (888) *A. gambiae s.l.*, 2.18% (23) *Aedes* spp., 12.51% (132) *Culex* spp., and 1.14% (12) *Mansonia africana* (Table 1). During the period of study, anopheline species were the most important species collected in all the districts except Tanguiéta where *Culex* spp. was rather very important. The ovary physiological state of *A. gambiae s.l* collected from PSC were mostly fed, half-gravid and gravid, reflecting a strong endophily of this species (Table 2). The percentage of female fed, half-gravid and gravid, was higher than 80% in most of the districts ($p > 0.05$). Vector density by human habitation was on average 10 females in the nine districts.

Vectors infection to CSP and malaria transmission risk

Table 3 shows the circumsporozoite protein positive rate (%CS+) and the EIR at the study sites. Results from this section showed that the risk of malaria transmission was very high in the nine districts. In fact, the average of the sporozoite rate in the nine districts is 6.63% (53 positive thoraces on 800). Among the nine districts, the highest rate was obtained in Natitingou (55.6%) ($p < 0.05$). The average of EIR was 6 infectious bites per person per month (6 bi/p/m). The highest EIR was found in the districts of Cobly and Matéri, with 10 bi/p/m, which means that in these localities every inhabitant receives on infected bite every three days during the study period.

Resistance status

Figure 2 shows the resistance status of *A. gambiae s.l* populations collected in the various districts at Atacora. From this study, it appears that results are similar in the nine districts. *A. gambiae s.l* populations were susceptible to bendiocarb, with mortality rates ranging between 95 and 100%. For this carbamate, no significant difference was noticed from a district to the other ($p > 0.05$). On the other hand, *A. gambiae* was resistant to deltamethrin with mortality rate ranging between 27 and 54%. Moreover, *A. gambiae* was fully resistant to DDT and permethrin with a mortality rate which did not exceed 18%.

Species and molecular forms of *Anopheles gambiae*

Mosquitoes from PSC were analysed by polymerase chain reaction (PCR) for identification of sibling species among *A. gambiae* complex and molecular M and S forms of *A. gambiae s.s*. In all districts, *A. gambiae s.s* was predominant (99.49%). Only one specimen of *A. arabiensis* was found in Kérou (Table 4). Both M and S forms were found in sympatry, but the molecular S form was predominant (94.42%).

Kdr resistance gene status in *A. gambiae*

The average of *kdr* allelic frequency from September to October, 2010 was 78%. *kdr* allelic frequency was very high (98%) in Cobly and Kouandé.

Insensitive acetylcholinesterase gene status in *An. gambiae s.l*

In all the nine districts, the allelic frequency of this gene

Table 2. Vectors distribution and the physiological state of their abdomen.

Districts	Villages	Nb of houses sprayed	*A. gambiae*						*A. funestus*				
			Unfed	Fed	Half Gr	Gr	Total	Density/House	Unfed	Fed	Half Gr	Gr	Total
Pehunco	Gbéba	7	8	64	3	7	82	11.71	-	1	-	-	1
	Banikani	6	4	11	3	5	23	3.83	-	-	-	-	-
Kouandé	Niaro	5		70		1	71	14.20	-	3	-	-	3
	Bassilou	5		8	1		9	1.80	-	-	-	-	-
Cobly	Zongo	7	10	122	30	19	181	25.86	-	2	-	-	2
	katouokè	6	20	120	28	5	173	28.83	-	-	-	-	-
Boukoumbé	Coutankou	5	5	111	30	29	175	35	-	1	1	-	2
	Kounadogou	2	1	4	2	5	12	6	-	1	-	2	3
Tanguiéta	Yanka	7	3	21	1		25	3.57	-	-	-	-	-
	Thanwassaka	5	2	17		1	20	4	-	1	-	-	1
Toukountouna	Sanga	6	8	22		3	33	5.50	-	-	-	-	-
	Tectibayaou	4	13	15			28	7	-	1	-	-	1
Natitingou	Bérécingou	5	1	8		2	11	2.20	-	-	-	-	-
Matéri	Nanka	10		17		2	19	1.90	-	-	-	-	-
Kérou	Karigourou	4		1			1	0.25	-	-	-	-	-
	Maréworou	5		16			16	3.20	-	-	-	-	-
Total		89	75	610	98	79	862	9.69	0	10	1	2	13

was low. The average of *Ace-1* allelic frequency from September to October, 2010 was 3% and no homozygous individuals RR were found (Table 4 and Figure 3).

DISCUSSION

The present study provides entomological base-line data in Atacora for the first time. 14 different species of mosquito were collected during our investigation. A similar result was obtained by Huttel (1950) in southern Benin. As a matter of fact, he got the same species by using the same

Table 3. *A. gambiae* infectivity to circumsporozoïtic antigen (CS) and Entomological Inoculation Rate (EIR) in Atacora.

Locality	N tested	CS+	% CS+	Nb of Sleepers	ma	EIR (bi /p/m)
Pehunco	93	2	2.15[a] [00.26-07.55]	43	2.16[b] [01.75-02.65]	1.4[a] [01.06-01.80]
Kouandé	79	9	11.39[b] [05.34-20.53]	45	1.76[b] [01.49-02.19]	6[c] [05.30-06.76]
Cobly	330	15	4.55[a] [02.57-07.39]	44	7.5[c] [06.71-08.36]	10.23[d] [09.30-11.22]
Boukoumbé	153	3	1.96[a] [00.41-05.62]	22	6.95[c] [05.89-08.15]	4.09[b] [03.29-05.03]
Tanguiéta	44	4	9.09[b] [02.53-21.67]	20	2.2[b] [01.60-02.95]	6[c] [05.35-07.18]
Toukountouna	58	5	8.62[b] [02.86-18.98]	24	2.42[b] [01.83-03.12]	6.25[c] [05.29-07.34]
Natitingou	9	5	55.56[d] [21.20-86.30]	18	0.5[a] [00.23-00.95]	8.33[d] [07.46-09.78]
Matéri	17	5	29.41[c] [10.31-55.96]	15	1.13[a] [00.66-01.32]	10[d] [08.46-11.74]
Kérou	17	5	29.41[c] [10.31-55.96]	34	0.5[a] [00.29-00.80]	4.41[b] [03.73-05.18]
Total	800	53	6.63	265	3.02	6

%CS+: the circum sporozoite protein positive rate; bi/p/m: infective bites per person per month; ma: Number of bites per person per night.

method. However, more species were supposed to be collected if the PSC method was associated with other catch methods such as Human Landing Catch (HLC), Center for Disease Control (CDC) and BG sentinel traps. In fact, a study conducted in southern Benin the same year by Lingenfelser et al. (2010), using HLC with conventional aspirators, showed the presence of 24 species belonging to 6 genera of mosquito. Therefore, the choice of sampling method influenced the entomological data recorded. A report conducted in Senegal by Ndiath et al. (2011) on the sampling method concluded that the sampling technique has to be chosen according to the vector studied and the aim of the study.

A. gambiae, the main malaria vector in sub-Saharan Africa, is the main species harvested in houses (81.71%). This predominance of *A. gambiae* is probably due to the low urbanization level of the districts. Indeed, unlike urban areas, in rural areas, larval habitats are less polluted and therefore, favorable for the production of anophelines; hence the higher production of anopheline fauna in rural areas. *A. funestus* was also present in the department but in small proportion (1.23%), probably due to the presence of dams which surface was covered by vegetation suitable to *A. funestus* development.

Anopheles arabiensis was also very poorly represented in our samples; one specimen was collected in Kérou whereas fifteen years before Akogbeto and Di Deco (1995) reported its presence in sympatry with *A. gambiae* s.s in northern Benin. The absence of *A. arabiensis* during the study period may appear strange. Indeed, the long drought that characterizes Atacora (Sudanian climate), and the presence of a large dry savannah area, satisfy the conditions for the development of *A. arabiensis*. In an article, Djogbénou et al. (2010) reported *A. arabiensis* disappearance in Atacora, especially in Tanguiéta and Natitingou between 2006 and 2007, without explaining the causes of this disappearance. In reality, it was not a problem of disappearance, but a problem of sampling and duration of the study instead.

Regarding our study, the catches were performed inside human dwellings while *A. arabiensis* endophilic trends is low. However, it is possible that the action of man on the environment be unfavorable to the development of *A. arabiensis* in this area. A study on the dynamics of *A. arabiensis* and its distribution showed that within 25 years, *A. arabiensis* moved from latitude 10° 10' N (northern Benin) to latitude 6° 40' N (southern Benin). This change of environment is made possible through deforestation, thus creating favorable conditions for the development of *A. arabiensis* in the central and southern Benin. As a matter of fact, shelters for its development are threatened in the north by a severe drought. During this period, the absence of pasturage in the north led to the movement of this species to the center and the south of Benin through transhumance. Climate change, therefore, favored the movement of *A. arabiensis* to the south. This new distribution of *A. arabiensis* should help the NMCP to adapt to its vector control programme. Molecular analyses performed on the same samples showed that the S form is more represented. This confirms that the S form of *A. gambiae* depends on dry savannah areas as pointed out by studies carried out in West Africa and which reported similar results in similar bioecological zones in Nigeria, Cameroon and Burkina Faso (Wondji et al., 2002; Onyabe et al., 2003; Dabiré et al., 2009). IRS implementation as vector control strategy is based on endophilic behavior of vector populations and their preference to rest inside houses. This is why the high vectors population collected indoors is an asset for the IRS implementation in Atacora. Indeed, the endophilic behavior of vectors facilitates their contact with the insecticide. The second argument for the need to strengthen

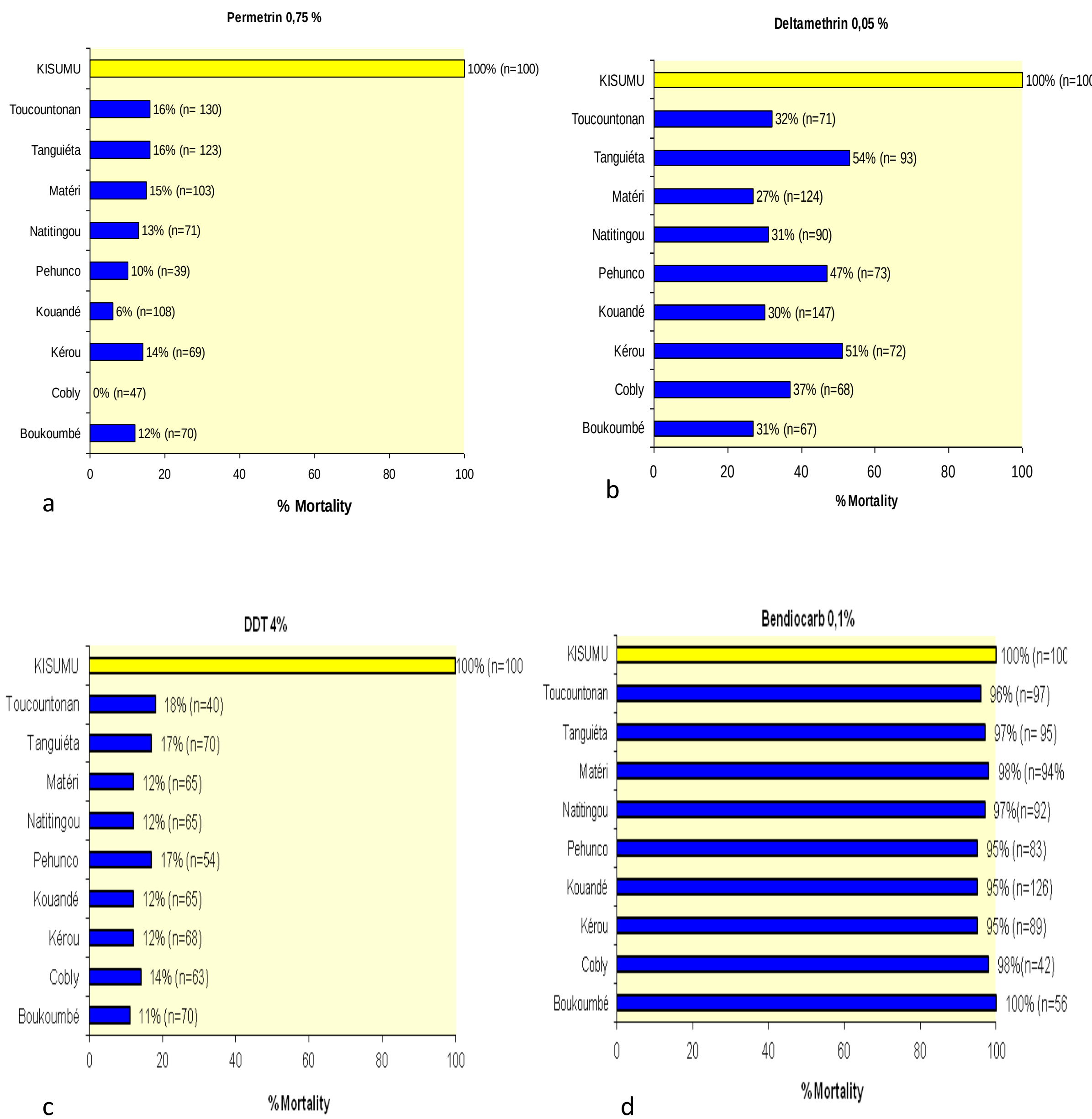

Figure 2. Insecticide susceptibility status of *An. gambiae s.l.* in the nine districts in Atacora.

the fight against malaria in Atacora by a rapid intervention effect on the vectorial capacity can be justified by the very high circumsporozoitic index in Atacora. The entomological inoculation rate recorded during the study (6 infected bites per man per month) was higher than that obtained in southern Benin (Djènontin et al., 2010) and in north-east of Benin (Yadouleton et al., 2010). The high level of this EIR could be explained by the low coverage of LLINs. In fact, a survey conducted in Atacora in 2010 on the use of bednets by the populations showed that very few people had a bednet (1 net for 5 people) and those who had it, would not use it frequently because of the strong heat (Aïkpon, personal communication).

Data recorded from the susceptibility test performed on *A. gambiae* showed a high level of resistance to permethrin, deltamethrin and DDT but susceptibility to bendiocarb. The emergence of resistance to pyrethroid in *A. gambiae* has become a serious concern for the success of malaria control in the last decades. In fact, in Benin, pyrethroids have been extensively introduced in

Table 4. Species and molecular forms of *An. gambiae* and *kdr* and *Ace.1* allelic frequency.

Locality	Species		Molecular forms		*kdr* mutation				*Ace-1* mutation			
	Aa	Ag	M	S	RR	RS	SS	F(*kdr*)	RR	RS	SS	F(*Ace-1*)
Pehunco	0	28	1	27	10	18	0	0.68	0	2	26	0.04
Kouandé	0	27	0	27	26	1	0	0.98	0	1	26	0.02
Cobly	0	21	2	19	20	1	0	0.98	0	1	20	0.02
Boukoumbé	0	27	0	27	10	17	0	0.69	0	0	27	0.00
Tanguiéta	0	26	6	20	20	4	2	0.85	0	0	26	0.00
Toukountounan	0	26	0	26	13	11	2	0.71	0	1	25	0.02
Natitingou	0	9	0	9	3	3	3	0.50	0	1	8	0.06
Matéri	0	17	0	17	10	5	2	0.74	0	1	16	0.03
Kérou	1	16	2	14	11	2	3	0.75	0	3	13	0.09

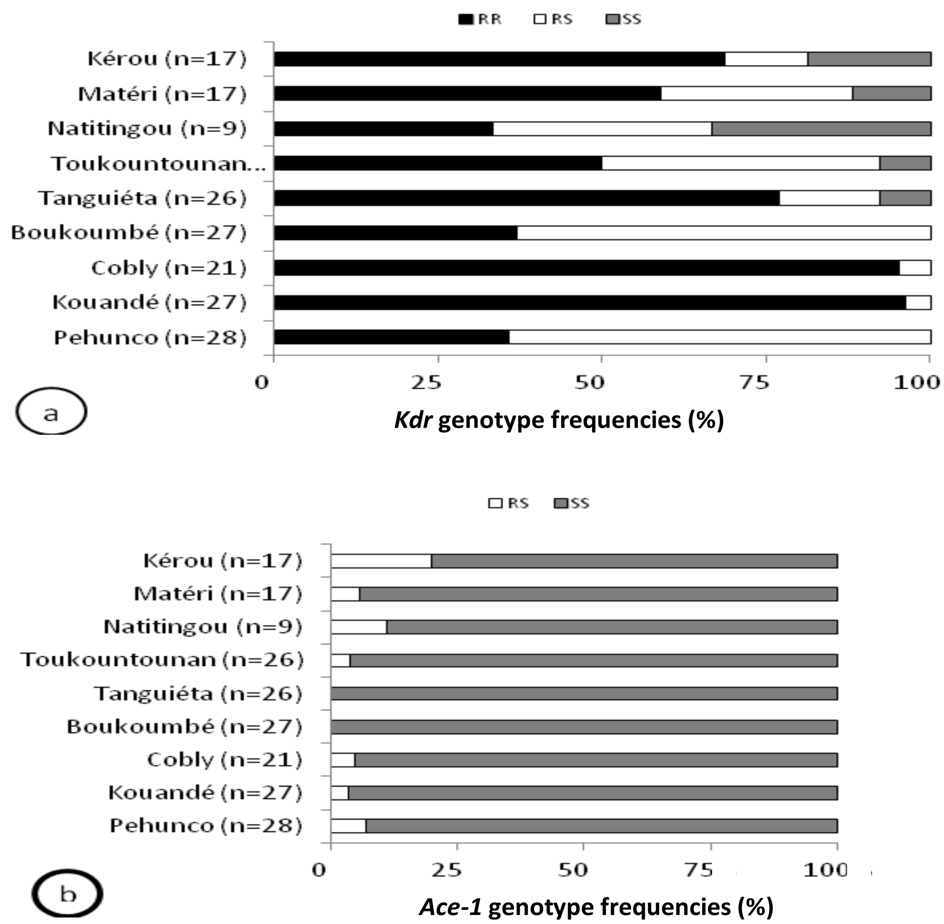

Figure 3. *kdr* (a) and *Ace-1* (b) allelic frequency.

agriculture since 1980s (Prudent et al., 2006). This factor is probably one of the causes of the selection of high resistance in *A. gambiae* to pyrethroids, with a high allelic frequency of the knock down resistance (*kdr*) mutation level. The *kdr* which seems to be the main mechanism conferring resistance in *A. gambiae* to pyrethroids (Leu-Phe *kdr* mutation) in West Africa was found in mosquito samples collected in different sites. The allelic frequency found in our study area was higher than what was reported in southern Benin by Djènontin et al. (2010), and similar to those reported in Parakou, a town in the north-east of Benin by Yadouleton et al. (2011). This would certainly explain the fact that most of the localities in northern Benin are cotton growing and use many pesticides. This massive use of insecticide selects the resistance of malaria vectors (Akogbéto et al., 2005; Dabiré et al., 2008; Yadouleton et al., 2009).

The *Ace-1* mutation was also found but at a very low frequency (< 0.1). Several reports in southern Benin (Yadouleton et al., 2009; Djènontin et al., 2010; Padonou et al., 2012) confirmed the low allelic frequency of this mutation. Basing on the information above related to the susceptibility tests, and for better management of resistance to insecticide in *A. gambiae* populations, the low frequency of *Ace-1* recorded in all populations encourages the use of bendiocarb as an alternative insecticide to pyrethroids for IRS in the study areas. A previous study conducted in southern Benin (Akogbéto et al., 2010) had shown bendiocarb as alternative to pyrethroids. The author showed that the use of this insecticide for IRS had drastically reduced malaria transmission.

Conclusion

This study shows that *A. gambiae s.l* was the most abundant species found in the nine districts. *A. gambiae s.s* was practically the only member of the complex found responsible for malaria transmission in Atacora. Besides, the high antigen CSP positivity rate observed proves the vulnerability of populations to malaria transmission and constitutes challenge not only for the reinforcement of existing control strategies (LLINs), but also resorting to other complementary strategies such as IRS. The susceptibility to bendiocarb added to the low frequency of *Ace-1* mutation found in *A. gambiae* populations could lead to the use of bendiocarb as a potential alternative against resistance of *A. gambiae* to pyrethroid in the nine districts for IRS.

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