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Bendiocarb resistance selection pressure: What happen after his massive use for Indoor residual spraying (IRS) in areas under universal coverage of long lasting insecticidal nets (LLINs) in southern Benin?

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Abstract
The present study was aimed to determine the relative impact of indoor residual spraying intervention on the development of insecticide resistance by looking for *Kdr* and *Ace*-1 mutations in *An. gambiae* s.l. as marker of insecticide pressure. *Anopheles* larvae were collected from Adjohoun, Dangbo, Misserete and Seme, four districts previously under IRS and in control area (Adjara) between June 2010 to November 2011. *An. gambiae* s.l. was tested to susceptibility to insecticide. The results revealed that *An. gambiae* s.l. showed a strong resistant to permethrin 0.75%, DDT 4% and deltamethrin 0.05% throughout the IRS period and after IRS cessation in all districts. But after 3 years of bendiocarb use in areas study, no resistance to this insecticide was recorded. Furthermore, the *Kdr* mutation frequency was very high, about 80% in 2010 and 2011. The *Ace*-1 alleles frequencies were homogeneous and worthless for two years in all four districts. This study demonstrates that *An. gambiae* s.l. in the Ouémé department have maintained and developed their resistance to pyrethroids, but are still susceptible to bendiocarb, mainly in districts under IRS.

Keywords: Bendiocarb resistance- *An. gambiae* s.l- IRS - Benin

1. Introduction

Mosquitoes transmit a wide range of human and animal pathogens, and insecticides are widely employed in their control [1]. The efficacy of these insecticides is influenced by the mosquitoes history past exposure. Long term exposure to a toxicant will eventually select for mutations conferring a level of resistance to that toxicant and indeed, insecticide resistant populations of mosquitoes (*An. gambiae* s.l and *An. funestus*) are now threatening the success of control programs [2].

In the lack of malaria vaccine and resistance to anti-malaria drug, one of the most effective controls of malaria is to limit malaria transmission by using Long Lasting Insecticide-treated Nets (LLINs) and Indoor Residual Spraying (IRS) [2, 3]. Indeed, twelve insecticides from four classes (organochlorines, organophosphates, carbamates and pyrethroids) have been recommended for IRS [2, 4], but pyrethroids are the only compounds licensed for the bed nets and other netting material for personal protection.

Unfortunately, resistance to pyrethroids has recently emerged both in West and East Africa and its rapid spread suggests that it will become a major hindrance to malaria control programs that target adult mosquitoes [5]. Therefore, to more reduce malaria transmission, our National Malaria Control Program (NMCP) has decided to adopt an integrated control and, in 2008 furthermore to LLINs, has implemented a vector control intervention based on indoor residual spraying (IRS) using bendiocarb. Four districts of high resistance of *Anopheles gambiae* to pyrethroids were sprayed with bendiocarb. More than 350,000 inhabitants have been protected [6].

Among target site resistance mechanisms, the most studied is knockdown resistance (*kdr*) associated with a single point mutation in the gene encoding the voltage-gated sodium channel is a common mechanism of resistance to both pyrethroids and DDT. This mutation results in a leucine to phenylalanine substitution found predominantly in West Africa, after termed (*kdr*-w) [7] or a leucine to serine substitution (*kdr*-e), which was originally identified in Kenya but is now found in several other African countries including Benin, Cameroon, Equatorial Guinea, Gabon, Angola and Uganda [8-10]. Both forms of *Kdr* presumably function through reducing the affinity of DDT and pyrethroids for their target site on the sodium channel [8].

One of other mechanism implicated in insecticide resistance is G119S mutation responsible for insensitive acetylcholinesterase resistance to insecticides and has recently been reported from natural populations of *Anopheles gambiae* in West Africa countries of the Ivory Coast, Benin and Burkina Faso [11-13]. This mutation confers resistance to organophosphate and carbamate insecticides by reducing the ability of these compounds to inhibit acetylcholinesterase (AChE) in nerve synapses [14, 15]. Its presence poses a potential problem for the control of the malaria vectors in the region. The G119S mutation is responsible for AChE insensitivity in several species of mosquito [15], and it has been extensively studied in natural populations of *Culex pipiens* in the south of France [16, 17]. Cross-resistance to carbamates and organophosphates can arise by insensitive AChE mechanism due to the glycine to serine substitution (G119S mutation) resulting from a single point mutation in the *ace-1* gene [14]. This mutation was found in both M and S molecular forms of *An. gambiae* [11, 15] but this mutation has a fitness cost.

Resistance in the Anopheles species seems to be associated with the agricultural use of insecticides [18, 19]. Nevertheless, evidence exists for the selection of *Kdr* alleles associated with the massive use of ITNs or impregnated plastic sheeting [20, 21]. For insecticide resistance management, it is essential to know where the selective pressure on Anopheles comes from. Indeed, several authors reported that the use of insecticides in households and pesticides in agricultural settings has greatly increased selection pressure leading to the emergence of insecticide resistance in malaria vectors [22-24].

From 2008 to 2010, four rounds of IRS were implemented in southern Benin using bendiocarb. After the last round implementation in August 2010, the program was moved and continuing in Atacora district in northern Benin. Several recent studies reported that *An. gambiae* is highly resistant to pyrethroids and DDT, but not to bendiocarb in Benin [25-27]. Knowing that, evolution of insecticide resistance is a phenomenon of time, it is not excluded the increased of resistance event after cessation of IRS. It is therefore important to investigate the status of insecticide resistance in *An. gambiae* in southern Benin after IRS cessation. In addition to the *Kdr* mutation, which is the mechanism more investigated and linked to pyrethroids resistance, it's important to know if after a massive use of bendiocarb, the *Ace*.1 allele has or not been selected. The objective of this study was to determine the relative impact of these interventions on the development of insecticide resistance by looking for *Kdr* and *Ace*-1 mutations in *An. gambiae* s.l. as marker of insecticide pressure, and to link these findings with the insecticide resistance status observed in *An. gambiae* s.l. by bioassays in areas previously under IRS intervention.

2. Material and Methods

2.1 Study area

The present study was carried out from June 2010 to November 2011 in four districts of Ouémé department (Adjohoun, Dangbo, Missérété and Sèmè) in southern Benin, where a large scale programme of IRS has been implemented (Fig 1). Malaria transmission is stable in the Ouémé region, which is irrigated by the river 'Ouémé', Lake 'Nokoué' and the lagoon of 'Porto-Novo'. This district is essentially characterized by sub-equatorial climate, with two dry seasons (August-September and December-March) and two rainy seasons (April-July and October-November). The annual mean rainfall is 1,500 mm in July, relative humidity (RH) of 70% ± 5 and the average monthly temperature vary between 23 and 32°C. The data from these four districts previously under IRS intervention were compared with those of a control area (district of Adjara) with the same characteristics but where no house was sprayed.

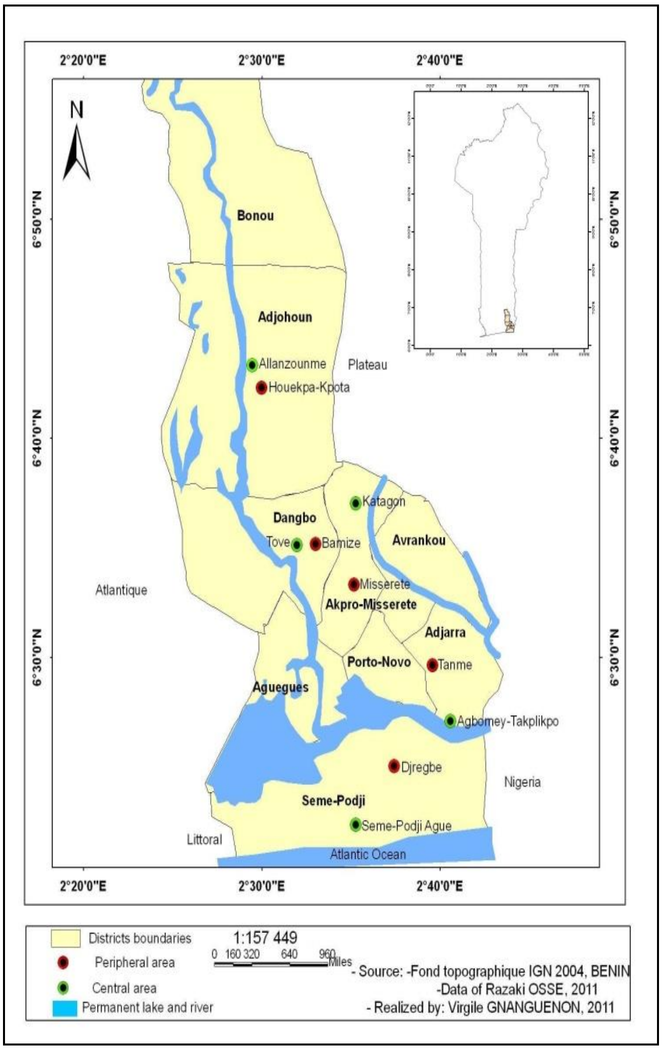

Fig 1: Map of the study area

2.2 Insecticide and household indoor walls spraying

Bendiocarb 80%WP (Wettable powder) was selected and was used to spray the walls of households situated in IRS area. The application dose was 0.4 g/m2 of bendiocarb on walls of houses. From 2008 to 2010, four rounds of IRS were made. After the last round in August 2010, IRS was stopped in Ouémé department and transferred in northern Benin. The four applications were implemented by volunteers selected from the local community and trained by the Research Triangle Institute (RTI) team, the implementing partner of the U.S. Agency for International Development. According to RTI, the coverage rate of IRS was more than 90% for each of the first three rounds.

2.3 Mosquito sampling

Wild *An. gambiae s.l.* mosquitoes were collected as larvae from range of breeding sites representative of the diversity of productive anophelines development sites in each locality. Larvae and pupae collections were conducted in each of the four districts previously under IRS intervention and in control area (district of Adjara) during the rainy season between June to July 2010, June to July 2011 and October to November 2011. All larvae and pupae from each location were kept in separated labelled bottles related to each locality and then pooled together and reared locally until emergence. Emerging adult were provided with a 10% honey solution. Adult mosquitoes were sexed and identified morphologically [28] and only females *An. gambiae s.l.* were used for insecticide susceptibility tests.

2.4 Susceptibility bioassays

Insecticide susceptibility tests were performed using the WHO standard procedures and test kits for adult mosquitoes [29]. Two to five days old, non-blood fed adult females morphologically identified as *An.gambiae* s.l. were exposed to discriminating dosages of 4% DDT, 0.05% deltamethrin, 0.75% permethrin and bendiocarb (0.1%) using WHO papers (obtained from Vector Control Research Unit, School of biological Sciences, University Sains Malaysia) and bioassay tubes for one hour. We tested deltamethrin because of an overlap country wide distribution of PermaNets by the NMCP. DDT was tested because of its intensive use in the past as well as to assess cross-resistance with pyrethroids in *Anopheles* populations. Bendiocarb was the insecticide used in the IRS area. Impregnated papers were stored at +4°C and were not used more than 4 times.

Briefly, for each tested insecticide, four replicates of 20-25 unfed females were exposed to diagnostic doses of various insecticides for susceptibility tests. Number of mosquitoes knocked down during exposure was recorded at different time intervals (10, 15, 20, 30, 45, 60 minutes). After 60 min exposure, mosquitoes were transferred into holding tubes with untreated papers and were placed under observation (at 25°C and 80% of humidity) and provided with cotton wools wet with a 10% honey solution. The proportions of survivors and dead mosquitoes were recorded 24 hours post exposure. The same number of mosquitoes was exposed to insecticide free papers as controls. Dead and survived mosquitoes from this bioassay were separately kept in eppendorf tube containing silicagel and stored at -20ºC for further molecular characterization.

2.5 Molecular characterization of Anopheles populations using PCR analysis

A polymerase chain reaction (PCR) assay was carried out on alives females from bioassays and mosquitoes were confirmed to belong to *An. gambiae* s.l. prior determined by morphological characters [28]. In each locality, 20-28 females of *An. gambiae* samples from the WHO bioassays were analysed per year at the molecular level. DNA was extracted from individual adults [30]. PCR analysis for species identification [31] was performed to identify various members of *An. gambiae* complex collected in each site. The next set of PCR focused on molecular forms using PCR-RFLP [32], which involved only *An. gambiae* s.s. The last series of PCRs determined the presence of *Kdr* mutations in *An. gambiae* ss. Populations, as described by Martinez-Torres *et al.* [33]. The PCR-RFLP diagnostic test was used to detect the presence of G119S mutation (*Ace*.1 gene) as described by Weill *et al.* [15].

2.6 Statistical analysis

Data were analysed using Excel and the R statistical package, version 2.11.1. Results from the insecticide susceptibility bioassays were evaluated according to the recommendations of the WHO [29]. Mortality rates of *An. gambiae* mosquitoes exposed to different insecticide impregnated papers according to the different periods were compared with chi-square test. To assess changes in the frequency of *Kdr* gene in *An. gambiae* from 2010 to 2011, the univariate logistic regression with the software R was used. This regression takes *Kdr* frequencies of the different localities as the dependent variable and year with the terms 2010 and 2011 as covariate.

3. Results

3.1 Mosquito species identification

247 *An. gambiae* s.l. samples were assayed by PCR. The results of molecular tests performed with *An. gambiae* s.l. survivors from the insecticide susceptibility bioassays in 2010 and 2011 revealed the presence of two species: *An. gambiae* and *An. coluzzii*. Almost all mosquitoes analyzed in 2010 are *An. coluzzii* (100%). On the other hand, except the Sèmè district (100% of *An. coluzzii*), populations of *An. gambiae* s.l analyzed in 2011 are composed of *An. coluzzii* and *An. gambiae* with a predominance of the *An. coluzzii* (average 90%) regardless the district (Table 1).

Table 1: Species identification within *Anopheles gambiae* complex and the frequency of *Kdr* and *Ace*-1R mutations in *Anopheles gambiae* s.l in areas previously under IRS in Benin

Locality	Years	Tested Number	Species			*Kdr* Mutation				*Ace-1* Mutation			
			Am	Ac	Ag	RR	RS	SS	F(R)	RR	RS	SS	F(R)
Adjohoun	2010	24	0	24	0	16	8	0	0,83*	0	0	24	0
	2011	23	0	21	2	12	8	0	0,80*	0	0	23	0
Dangbo	2010	25	0	25	0	19	6	0	0,88*	0	0	25	0
	2011	20	0	18	2	10	5	0	0,83*	0	0	20	0
Missérété	2010	22	0	22	0	18	4	0	0,91*	0	0	22	0
	2011	26	0	21	2	15	6	0	0,86*	0	0	26	0
Sèmè	2010	25	0	25	0	20	5	0	0,9*	0	0	25	0
	2011	28	0	28	0	18	7	0	0,86*	0	0	28	0
Adjara (Ctrl)	2010	27	0	27	0	21	6	0	0,89*	0	0	27	0
	2011	27	0	26	1	25	2	0	0,96*	0	1	26	0,02

Am = *An. melas*; Ac = *An. coluzzii;* Ag = *An. gambiae*; Ctrl= control; (*) Frequencies with the same symbol by district superscript are not significantly different (p>0.05).

3.2 Bioassays results

According to WHO recommendations, 98–100% mosquito mortality indicates susceptibility, 90–98% suggests potential resistance that needs to be confirmed, and < 90% mortality suggests resistance [29]. Based on these criteria, *An. gambiae* s.l. mosquitoes from Adjohoun, Dangbo, Missérété and Sèmè showed a phenotypic resistant to permethrin 0.75% in 2010 (during IRS) and June-July 2011 (after stopping the IRS) (Fig 2, 3, 4 and 5). In November 2011, this resistance was also observed, but the mortality rate increased showing then, the decrease of phenotypic resistance status in Adjohoun (74.60% against 11% in 2010), Dangbo (65% against 16.92% in 2010) and Missérété (65.59% against 16.12% in 2010). This drop in level of resistance to permethrin was also manifested in Sèmè and Adjara where *An. gambiae* s.l. showed resistance towards permethrin in November 2011 (Fig 5 and 6). The susceptibility level recorded during this period is significantly different from that obtained in 2010 and in June-July 2011 in all districts including the control area ($p<0.05$).

With DDT, strong resistance was recorded throughout the IRS period and after IRS cessation in all districts and in the control area (Fig 2, 3, 4, 5 and 6). But in the districts of Missérété and Sèmè, we observed a significant difference between the mortalities rate recorded in June and November 2011 ($p<0.05$) (Fig 4 and 5).

Before 2010, *An. gambiae* s.l. was fully susceptible to deltamethrin in all districts including the control. But from 2010-2011, the susceptibility tests show a decreased level of susceptibility or complete resistance of *An. gambiae* s.l. towards this insecticide. Furthermore, the mosquitoes susceptibility collected in 2011 is significantly different from that recorded in 2010 ($p<0.05$) (Fig 2, 4, 5 and 6). On the other hand, in the Dangbo district, no significant difference was observed between the mortality rates of two years ($p>0.05$) (Fig 3). The emergence of *An. gambiae* s.l. phenotypic resistance to deltamethrin in 2011 was observed not only in areas previously under IRS but also in the control area.

After 3 years of bendiocarb use in Ouémé areas, no resistance to this insecticide was recorded in the intervention districts except in Adjohoun where a suspicion of resistance was noted in November 2011 (90% of mortality rate) (Fig 2, 3, 4 and 5). However, this mortality rate obtained in November 2011 in Adjohoun is significantly different from those recorded in July 2010 and June-July 2011 (100%) (Fig 2).

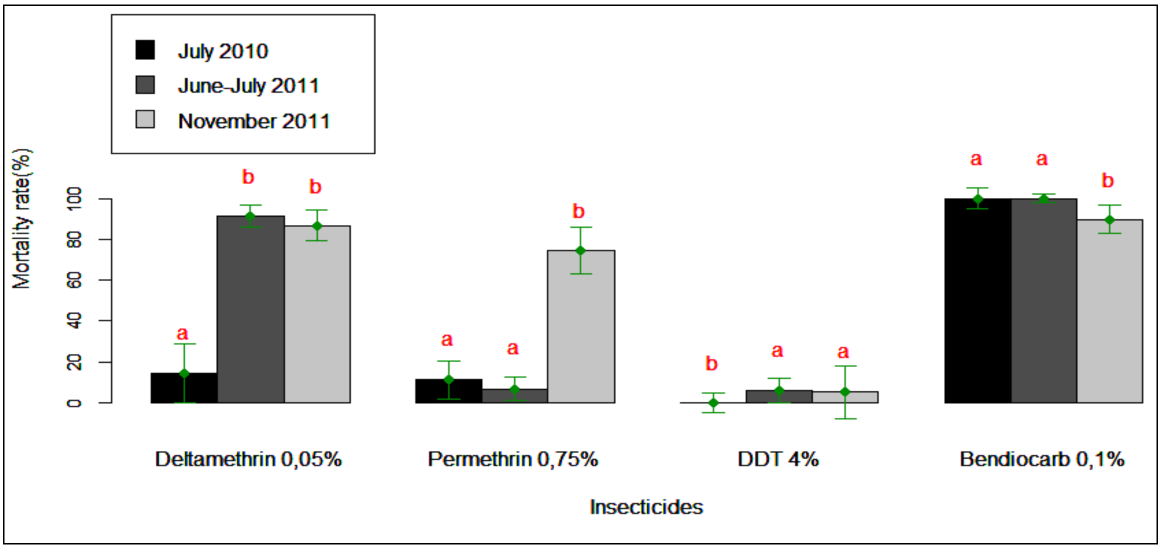

Fig 2: *An. gambiae* mortality rate to different papers impregnated observed during the IRS campaign and after stopping IRS in Adjohoun district.

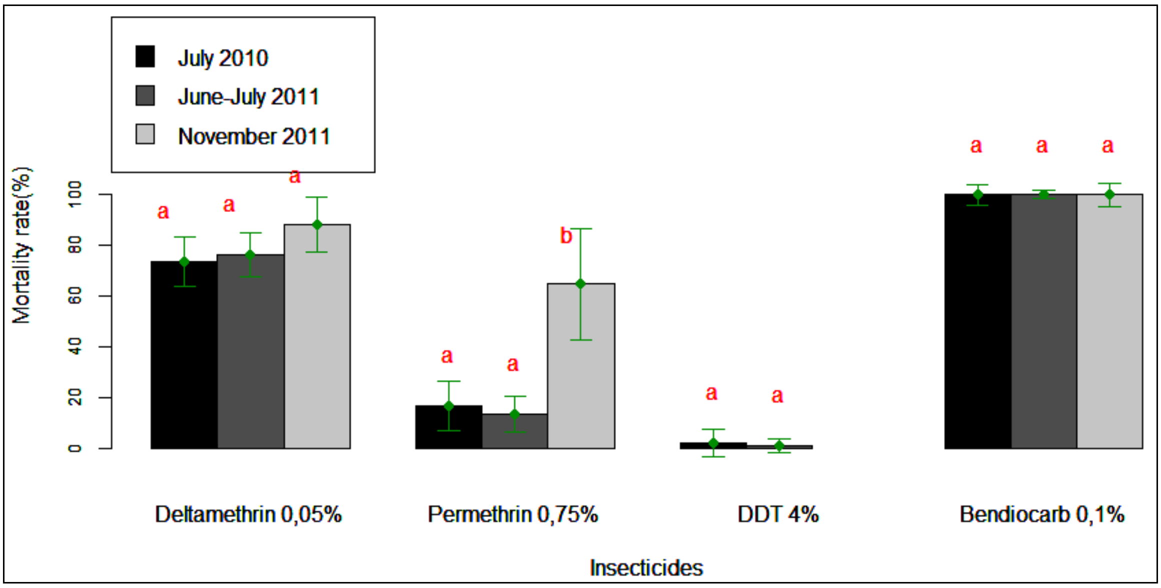

Fig 3: *An. gambiae* mortality rate to different papers impregnated observed during the IRS campaign and after stopping IRS Dangbo district.

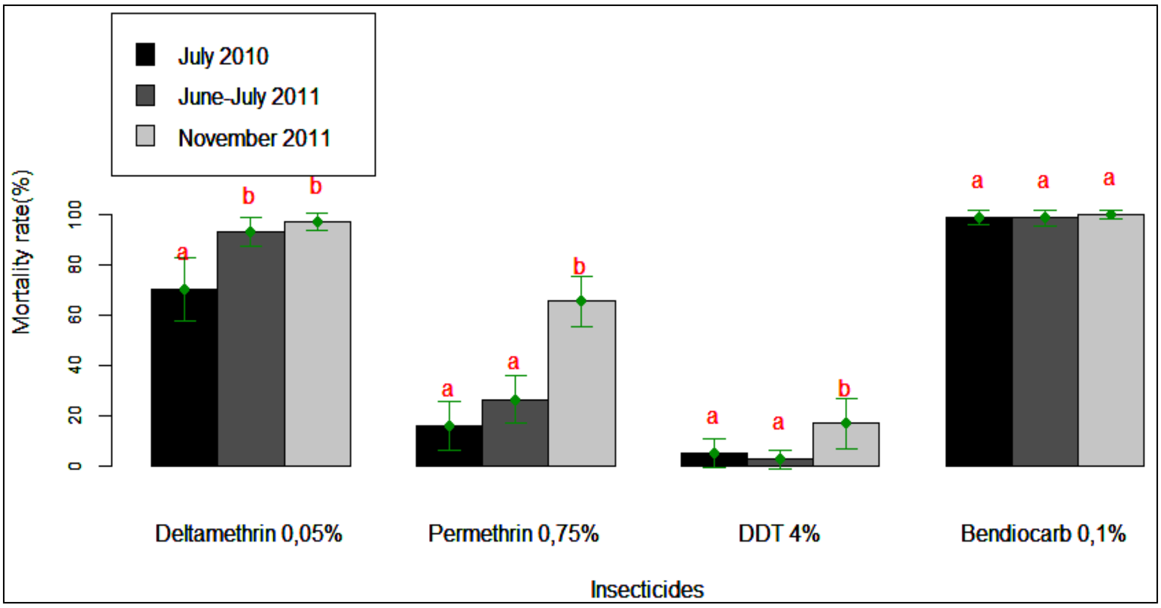

Fig 4: *An. gambiae* mortality rate to different papers impregnated observed during the IRS campaign and after stopping IRS in Missérété district.

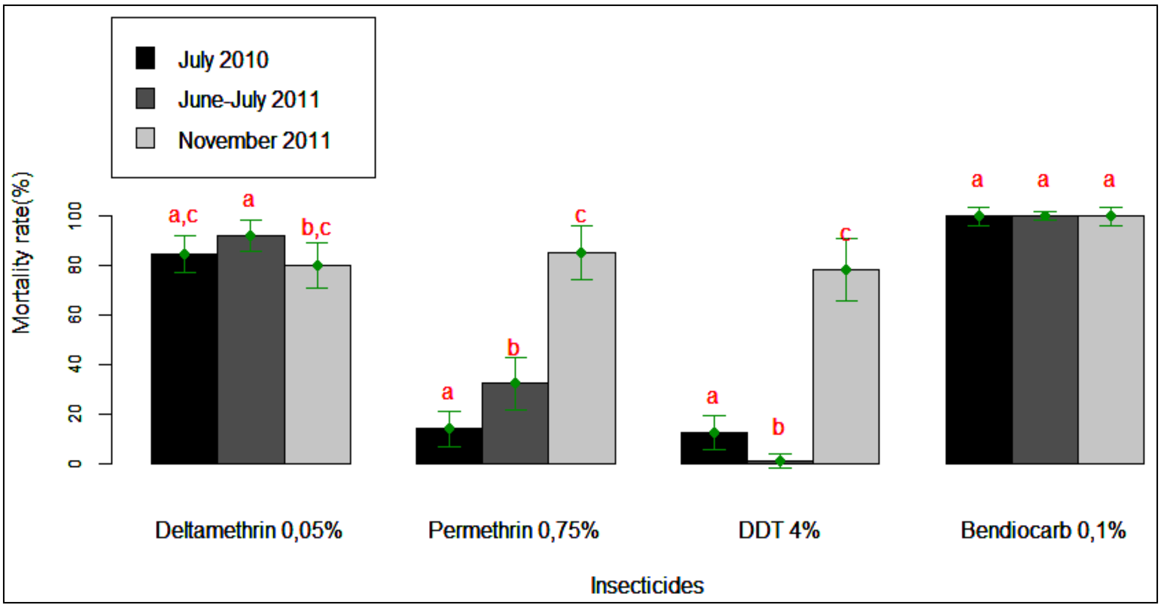

Fig 5: *An. gambiae* mortality rate to different papers impregnated observed during the IRS campaign and after stopping IRS in Sèmè district.

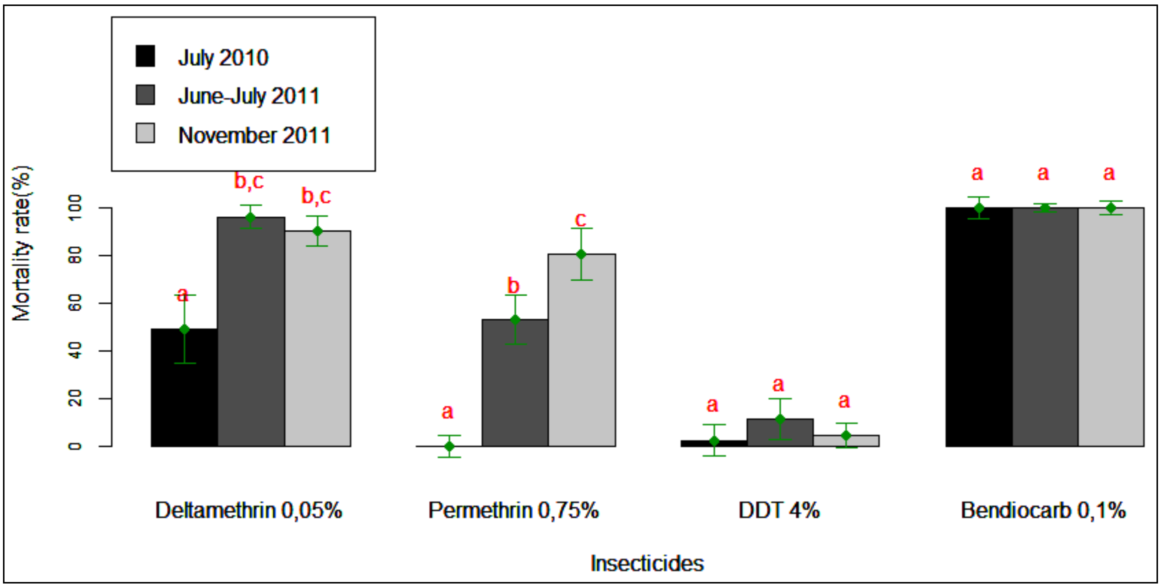

Fig 6: *An. gambiae* to different papers impregnated observed during the IRS campaign and after stopping IRS in Control district (The rates of the same superscript letter per district are not significantly different $p>0.05$).

3.3 Detection of target site resistance genes

Allele and genotype frequencies at the *Kdr* and *Ace*.1 loci are shown in Table 1. From the resistance mechanisms investigated, the *Kdr* mutation seems to play important role in this resistance observed in all districts included the control district with a very high frequency, about 80% in 2010 and 2011 (Table 1). But no significant difference was noted between the *Kdr* frequencies of two years in all districts (p>0.05).

The *Ace*-1 alleles frequencies are homogeneous and worthless for two years in all four districts previously under IRS except in the control area where a low frequency was observed (0.02) (Table 1).

4. Discussion

This study demonstrates temporal variation in susceptibility of *An. gambiae* s.l. populations to different classes of insecticides commonly used in public health and agriculture, in an area previously under IRS in Southern Benin. We underline that precaution was took when doing bioassays. Time variation in insecticide susceptibility is important to acknowledge because it can seriously bias the outcome of transversal susceptibility assays that are to be conducted prior to the implementation of any large scale malaria vector control initiative [34], and could thus lead to erroneous choices of insecticides.

The species composition of *Anopheles gambiae* complex found during this study did not differ from previous records in Benin [26, 35]. It was shown that, in addition to the *Anopheles coluzzii*, which is the only specie found in 2010 (during IRS), in 2011 the *An. gambiae* (3.7% to 10%) was also found but in very low percentage in the districts after IRS cessation and in the control area. This is the first time that this species was met at a relatively "high" frequency. Until 2010, *An. gambiae* had never been found in the department of Ouémé. This could be due to the effects of climate change. Indeed, increasingly, the world is faced with the phenomenon of global warming and decreasing rainfall, which could have corollary the reduction in permanent and semi-permanent breeding sites favorable of *An. coluzzii* to profit of temporary breeding sites favorable to development of the *An. gambiae*. The same trend was found in some localities of Benin, Mali, Nigeria [36-38].

The insecticide susceptibility data indicate high levels of resistance to DDT in *An. gambiae* s.l at all localities, although varying level of mortality were observed. In Benin Republic, Akogbéto and Yacoubou [25] suspected that DDT resistance, of *An. gambiae* s.l. was related to the massive use of DDT for house-spraying applications in several villages from 1953 to 1960 during WHO programmes of malaria eradication [23]. In agriculture, this insecticide was also intensively used in cotton growing areas. DDT is currently not used in Benin, but resistance is still largely present.

This study revealed the existence of permethrin and deltamethrin resistance in several *An. gambiae* s.l. populations from southern Benin. Indeed, before 2010, *An. gambiae* s.l. was fully susceptible to deltamethrin. But from 2010-2011, the susceptibility tests showed a high resistance of *An. gambiae* s.l. to deltamethrin. The emergence of *An. gambiae* s.l. resistance to deltamethrin in 2011 was observed not only in areas previously under IRS, but also in the control area. The development of resistance to deltamethrin which is perhaps not due to IRS is certainly due to the widespread use of LLINs in Benin in recent years and a higher utilization of agricultural inputs especially in fields cotton and in market gardens [24, 38].

These various levels of insecticide susceptibility may reflect differential insecticide selection pressure exerted on field mosquito populations. In many African countries, resistance to pyrethroids has been attributed to extensive use of these compounds in agriculture [18, 39], resistance levels being more important in cotton cultivation areas than in others agricultural schemes [19, 25, 40]. This is consistent with pyrethroid resistance being detected in these areas. Insecticide use for vector control interventions and/or personal protection against nuisances has been suggested as an additional putative source of selective pressure for pyrethroid resistance in malaria vectors, especially in urban cities and irrigated areas [19, 20]. Indeed, in these districts, in addition to the recently introduced ITNs, coils and bomb sprays are frequently used for personal protection [41]. Further studies involving social scientists, chemical ecologists and environmental biologists would be needed to document the amount, frequency and diversity of insecticides used in these areas in order to explore in greater details the putative selective pressures leading to the selection of insecticide resistance in malaria vectors.

Pyrethroid resistance observed in *An. gambiae* s.l. during the three years in areas previously under IRS is associated with the increased of kdr alleles. Indeed, the *Kdr* mutation, responsible for resistance to DDT and pyrethroids seem to be the main mechanism of resistance identified in populations of *An. gambiae* s.l. examined in this study. The allele frequency of this mutation is very high in all districts and in the control. But it’s obvious that, in front of this high phenotypic resistance, kdr only can’t be the resistance mechanism involved. The existence of other resistance like metabolic resistance (not investigated in this study) could have a significant impact on the effectiveness of LLIN [42].

Full susceptibility to bendiocarb was recorded in all samples tested, except in Adjohoun, where in November 2011, some level of tolerance to bendiocarb was observed. These results confirm those of previous study conducted in Benin [26, 43, 44] and in Bioko [45]. This susceptibility to bendiocarb was also confirmed by the absence of molecular resistance towards the carbamates (only 0.02 like frequency in control district). This mutation only observed in control area may be due to the fitness cost linked to *Ace*-1 mutation in presence of treatment [15]. Thus IRS implementation using bendiocarb (carbamate) showed a great benefice because of his effectiveness demonstrated by Akogbéto *et al.* [6] and this insecticide seem to not have impact on resistance. The low frequency observed in Adjara might be explained by the fact that in these areas, carbamate and organophosphate insecticides were not mostly used by farmers for crop protection. The results confirm also, those of Corbel *et al.* [38], Djogbenou *et al.* [11], Yadouleton *et al.* [35] and Padonou *et al.* [26] that previously showed a low frequency of the *Ace*-1 allele in malaria vectors populations Benin. This is particularly relevant to strengthen vector control campaigns using Indoor Residual Spraying based on carbamate and/or organophosphate as alternatives to pyrethroids, which are currently used by the NMCP in several areas of Benin. However, it is necessary to regularly monitor the evolution of resistance linked to this gene for any change. Indeed, the establishment of the phenomenon of resistance is a problem of time. Biological, genetic and environmental factors may intervene in modulating resistance to insecticides.

5. Conclusion

This study underlines the variability of phenotypic resistance level in *An. gambiae* s.l from southern Benin to insecticides after IRS cessation. The populations of *An. gambiae* s.l. in the Ouémé department have maintained and developed their resistance to pyrethroids, but are still susceptible to bendiocarb, mainly in districts under IRS. The IRS intervention by the massive use of bendiocarb has not exerted a selection pressure on malaria vectors. This may be due to the fitness cost linked to *Ace*-1 in presence of treatment.

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