International Journal of Innovation and Applied Studies
ISSN 2028-9324 Vol. 17 No. 2 Jul. 2016, pp. 662-671

http://www.ijias.issr-journals.org/

Level of overexpression of metabolic enzymes (oxidase, esterases, and glutathion-S-transferase) involved in the resistance of vectors to insecticides in Benin

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ABSTRACT: Background: *Anopheles gambiae* resistance was accessed in different ecological areas in Benin. Insecticide resistance in *Anopheles gambiae* s.l is a major concern to malaria vector control programmes. In West Africa, resistance is mainly due to target–site insensitivity arising from a single point mutation. Metabolic-based resistance mechanisms have also been implicated and are currently being investigated in west Africa.

Methods: Anopheles mosquitoes were reared from larvae collected. Biochemical assays were also carried out to detect any increase in the activity of enzyme typically involved in insecticide metabolism (oxidase, esterase and glutathion-S-transferase).

Results: Biochemical assays suggest that resistance in this population is mediated bymetabolic resistance with elevated level of GST, MFO and NSE compared to a susceptible strain *An. gambiae Kisumu*.

Conclusions: Elevated levels of certains enzymes de detoxification with *An. gambiae* populations suggest *Anopheles gambiae* populations resistance includes target-site mutation and metabolic mechanism.

KEYWORDS: Resistance, mechanisms, Enzymes, *An. gambiae.*

1 INTRODUCTION

Insecticide resistance can involve avoidance behavior of the vector. In this so called behavioral resistance the vector does not come into contact with the insecticide. Insecticide resistance also involves a change in absorption or excretion of the insecticide, a target site mutation or overexpression of metabolic enzymes. In Benin, the last two mechanisms of resistance cited are broadly documented in malaria vectors. The most explored mechanism remains linked to mutations in the target sites. Many studies have shown that both main methods to control malaria vectors, IRS and LLINs, and agricultural practices involving the use of insecticide are responsible for the increase in the frequency of mutations *Kdr* and *Ace - 1R* [1-7]. . However, the causes of the decline in effectiveness in experimental huts of pyrethroid-treated nets observed in south of Benin are still not completely covered. It would seem that *kdr* is involved but direct evidence of an association of this mechanism with a metabolic resistance was not obvious [8]. Nevertheless we still lack data on the distribution of metabolic resistance in Benin and ecological and environmental factors favoring its emergence.

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Metabolic resistance consists of an overproduction of enzymes in the insect, allowing it to metabolize or break down insecticide molecules until they have no toxic effect on the target [9-10].These enzymes are esterases, monooxygenases in cytochromes P450 and glutathione-S-transferases [11]. Cytochromes P450 monooxygenases are the main family of enzyme responsible for the metabolism of pyrethroids in insects [12]. The overproduction of these enzymes was found in *An. gambiae* and *Culex quinquefasciatus* resistant to pyrethroids in association with *kdr* [13,8,14-15]. As for esterases, they are capable of catalyzing the hydrolysis of esters linkages like those contained in most organophosphates and carbamates.

Alpha and beta-esterases are responsible for resistance through the detoxification of organophosphates and carbamates in mosquitoes[16,9]. The esterase activity is also associated with resistance to pyrethroids [17]. The GST play an essential role in the detoxification of xenobiotics such as insecticides. The most important enzyme of this group is the DDTase which overproduction in some mosquitoes confers resistance to DDT [18-20]. In this study, we are interested in the distribution of the level of expression of metabolic enzymes involved in the insecticides resistance of vectors in different eco-geographical areas in the transect North-South in Benin. Data collected during this study will be used as a tool by the NMCP for the choice of vector control interventions and the implementation of management strategies of insecticide resistance of vectors.

2 MATERIALS AND METHODS

2.1 STUDY AREA

The study was carried out in 16 localities belonging to two of the 8 ecological zones throughout Benin: Pehunco and Tanguiéta located in indoor residual spraying of insecticide (IRS) and the cotton growing area in the Northwest of Benin; Adjohoun and Seme located in IRS and cereal growing zone in the South of Benin; Kandi and Parakou located in cotton zone in the Northeast of Benin; Houéyiho and Akron located in vegetable area in the South of Benin; Ifangni and Pobè located in cereal growing zone in the Southwest of Benin; Malanville (North Benin at the boarder of Niger Republic) and Bamè (Center Benin) located in rice-growing area; Vossa and Ladji located in flood zone in the South of Benin; Abomey-Calavi and Agblangandan located in urban area in the South of Benin.

2.2 MOSQUITO COLLECTION AND REARING

An. gambiae larvae were collected in 2014 (rainy season) from ecological area. In each locality, larval collections (all instars) were carried out in larval development sites and specimens were pooled per locality. Larvae were brought back to the insectaries at Centre de Recherche Entomologique de Cotonou (CREC) where they were reared to adults under standard controlled conditions where they were maintained at 28 ± 2 °C and 72 ± 5% relative humidity. A laboratory susceptible strain of *An. gambiae* Kisumu was used as a reference strain to compare the susceptibility levels of the field populations. Upon emergence, mosquitoes were morphologically identified using identification keys and samples of 50 *An. gambiae* females of 2-5 days old were randomly picked from emerging mosquitoes from each locality and dry-frozen at −80°C for biochemical analysis.

2.3 BIOCHEMICAL ANALYSIS

Biochemical assays were performed to compare the levels of activity of mixed function oxidases (MFO), non-specific esterases (NSE) using a-naphtyl acetate as a substrate and glutathione S-transferases (GST) in the *An. gambiae* s.s. susceptible Kisumu and the 16 field population from 8 ecological areas. Mosquitoes used for the biochemical analysis have not been exposed to any insecticides prior to the assay. Detoxifying enzymes activities were measured on single mosquitoes (N = 50) from each test locality, which were stored at −80°C. All mosquitoes were tested according to the method described by Hemingway [21].

2.4 DATA ANALYSIS

Mean absorbance values of replicate wells for each tested mosquito were converted into enzyme activity and divided by the protein values. The median enzymatic activity was calculated for each test mosquito population and the distribution of enzyme activities was compared between the Kisumu reference strain and the field populations using non-parametric Mann-Whitney tests.

3 RESULTS

3.1 ACTIVITIES OF METABOLIC ENZYMES

Enzyme activities are represented by clouds of dots in the figures 1, 2 and 3. Each dot of the cloud represents the activity recorded in a mosquito. The average of the enzyme activities of each population was obtained by dividing the sum of the enzymatic activities of the entire population by the total number of mosquitoes tested.

3.2 OXIDASE

In cotton growing area of Parakou, the IRS and cotton growing area (Tanguiéta and Pehunco) as well as in vegetable areas (Akron and Houéyiho) or in the swampy area of Ladji, significantly high levels of oxidase activity were detected compared to the susceptible strain Kisumu ($p<0.05$). The average of the activity of cytochrome P450 was 16.04 10^{-2} nmol P450/min/mg protein at Akron, 17. 39 10^{-2} at Houéyiho, 17.37 10^{-2} Ladji, 15.36 10^{-2} at Parakou, 22.67 10^{-2} at Tanguiéta and 19.75 10^{-2} at Pehunco against 10.15 10^{-2} for the susceptible strain Kisumu. In the area of Ifangni, oxidase activity was high (13.62 10^{-2} nmol P450/min/mg protein) but not significantly different from that of Kisumu (figure 1).

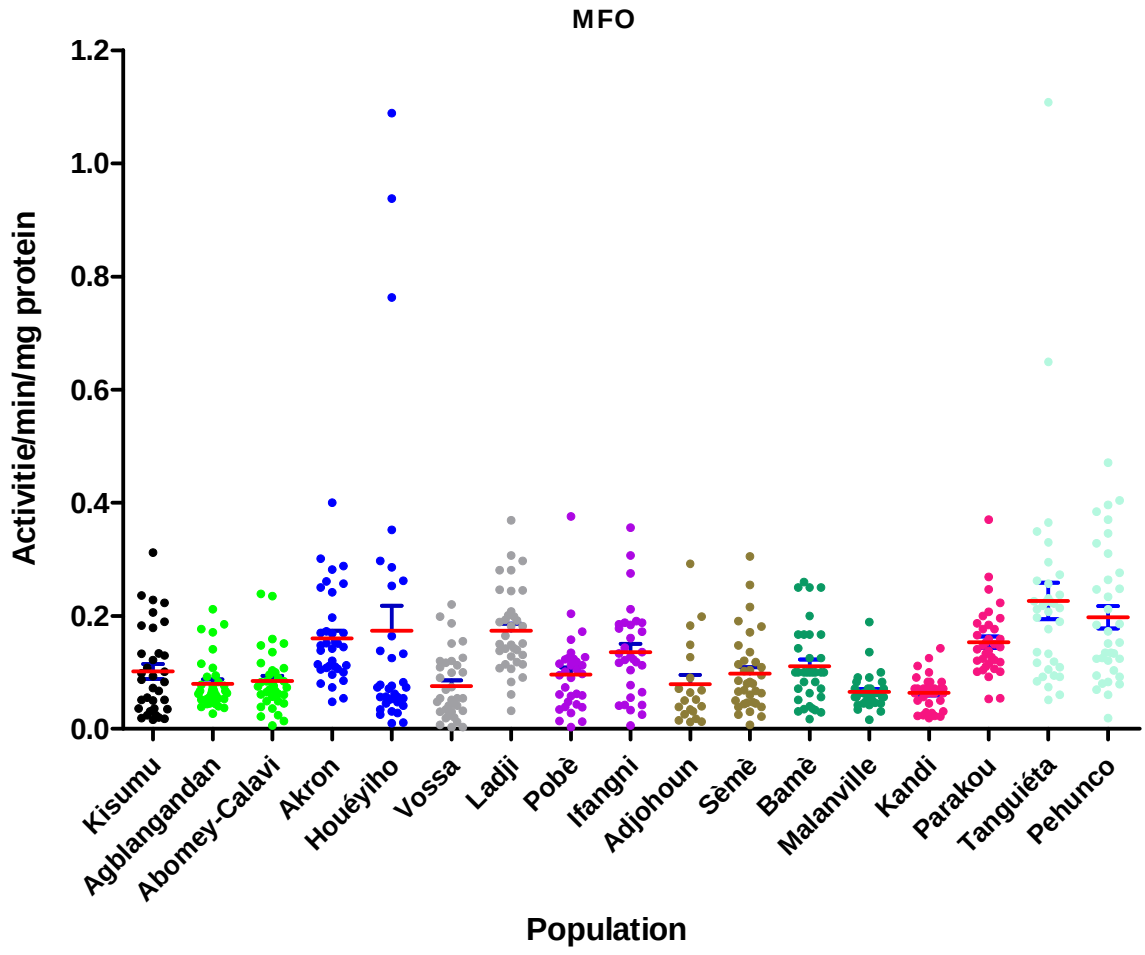

Figure 1: Oxidase activity of Anopheles gambiae populations from different ecological zones along the Northern - South transect in Benin

3.3 ESTERASE

Biochemical tests on mosquito specimen revealed significantly high levels of esterase (α and β esterase) activity at Pobe (14.6 10^{-2} nmol/min/mg protein), Houéyiho (8. 77 10^{-2}), Kandi (12.53 10^{-2}) and Tanguiéta (15.85 10^{-2}) compared to Kisumu

(7.65 10^{-2} nmol/min/mg protein) ($p<0.05$). The mosquitoes populations from the localities of Parakou (8.12 10^{-2} nmol/min/mg protein) and Akron (8.58 10^{-2} nmol/min/mg protein) exhibit high level of esterase activities although it was not significantly different from that of Kisumu (figure 2a and 2b).

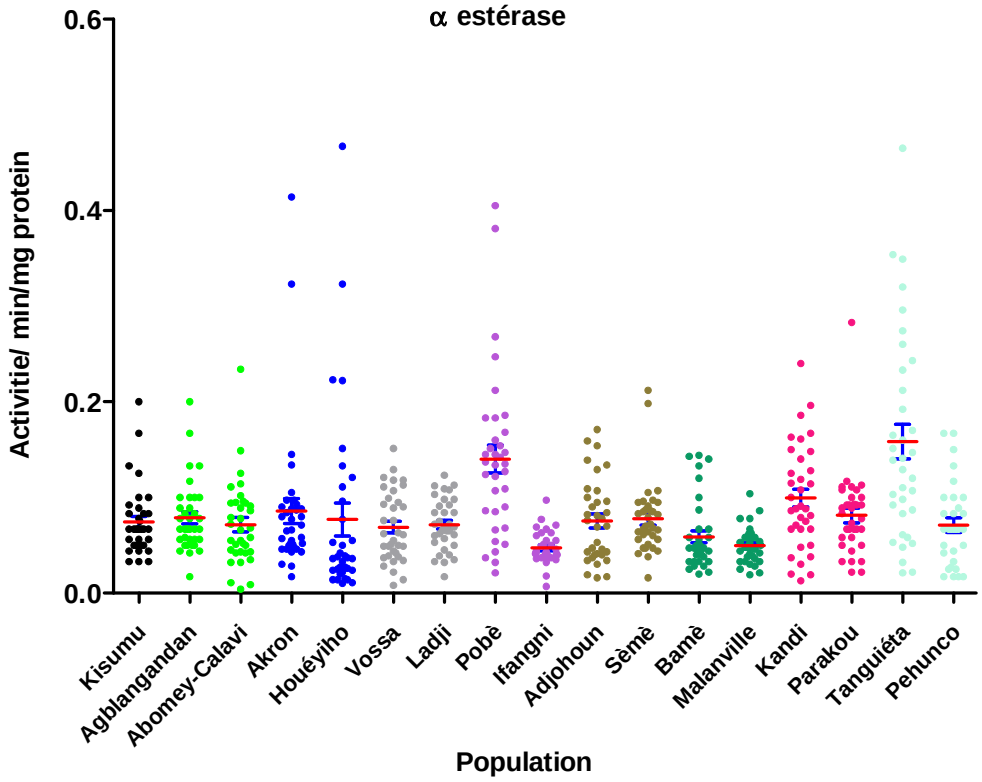

Figure 2a: Alpha-esterase activity of Anopheles gambiae populations from different ecological zones along the Northern-South transect in Benin

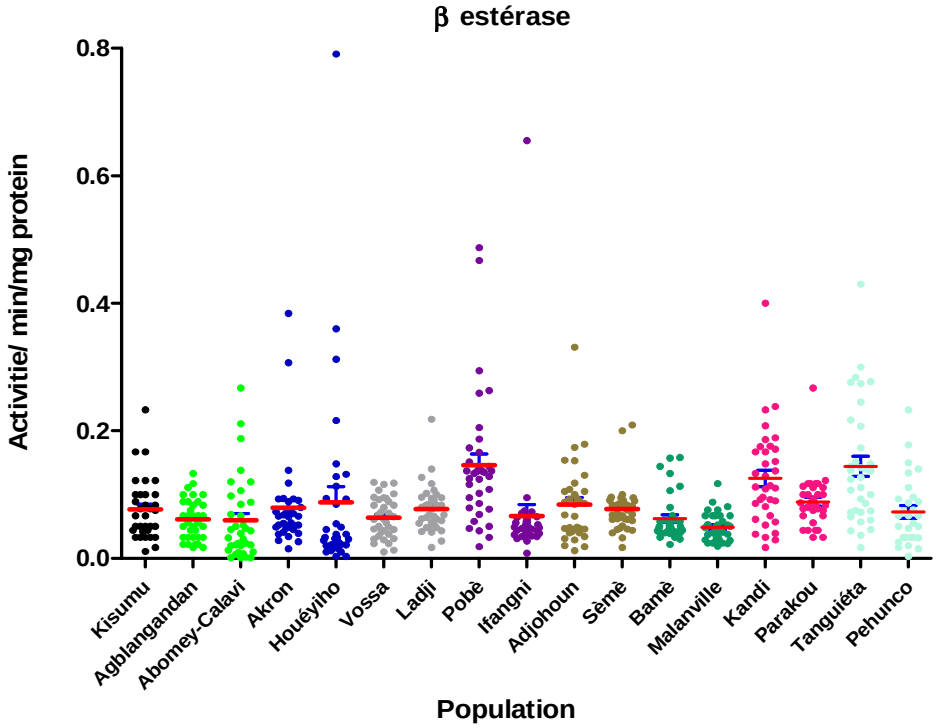

Figure 2b: Beta-esterase activity of Anopheles gambiae populations from different ecological zones along the Northern- South transect in Benin

3.4 GST

Figure 3 shows high level of activity of GST in mosquito populations from the urban area (Agblangandan), vegetable growing area (Akron), swampy area (Ladji), cereal growing area (Pobè), IRS area (Seme) and IRS-cotton area (Tanguiéta and Pehunco) compared to Kisumu ($p < 0.05$). The GST activity averaged was 38.46 10^{-2} nmol GSH/min/mg protein in Kisumu strain against 73.19 10^{-2} at Agblangandan, 222.1 10^{-2} at Akron, 119. 4 10^{-2} at Ladji, 50. 73 10^{-2} at Pobè, 62. 32 10^{-2} at Seme, 118. 2 10^{-2} at Tanguiéta and 93.53 10^{-2} at Pehunco. In addition, at Parakou (cotton growing area), strong GST activity (46.98 10^{-2} nmol GSH/min/mg protein) was recorded although not significant compared to Kisumu ($p>0.05$).

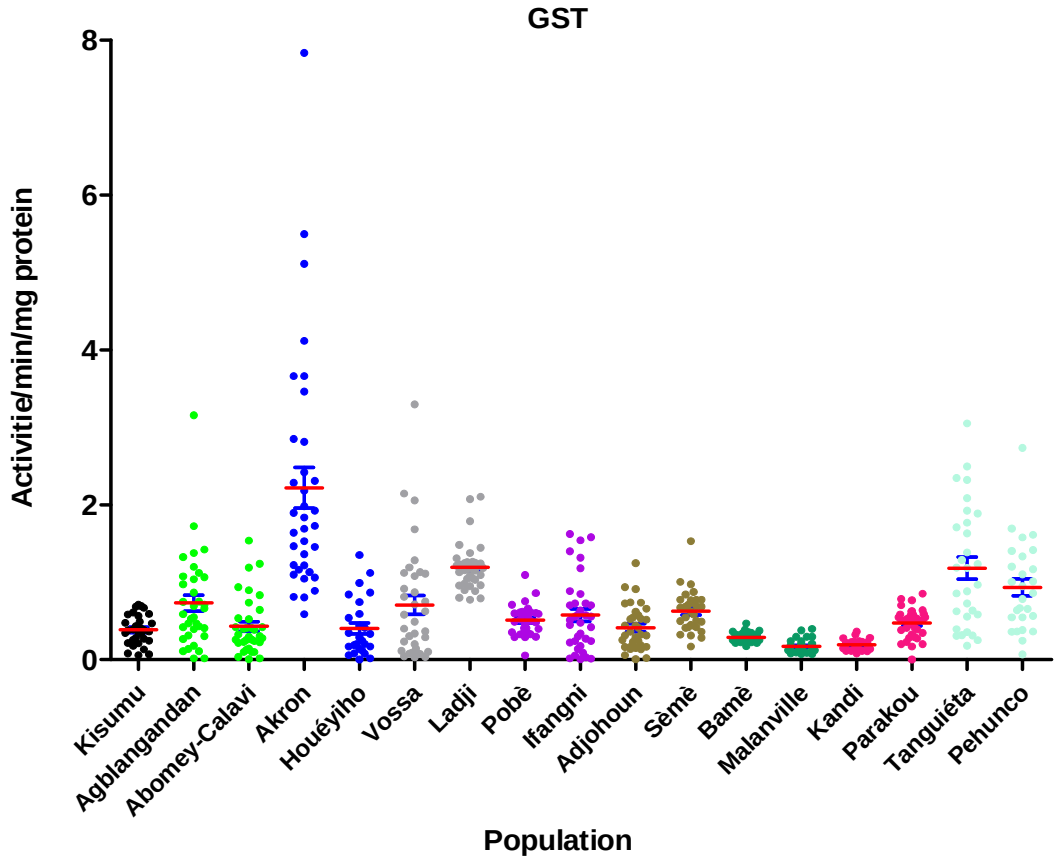

Figure 3: GST activity of the populations of Anopheles gambiae from different ecological zones along the Northern-South transect in Benin

4 MAPPING OF METABOLIC RESISTANCE IN BENIN

Table I, show the enzyme activities and the distribution of oxidases and the GST, alpha and beta esterases in different localities of the study area. The results obtained show a significant activity of oxidase more in the North than in the South of Benin (table I), The highest GST activity was recorded in the vegetable area (Akron) (table I). Overall, we have noticed non significant esterase activity in all localities with the exception of Houeyiho, Pobe, Kandi and Tanguieta (table I),

Tableau I : Activités oxydases, estérases et GST des populations de Anopheles gambiae dans les zones d'étude

Populations	Oxydase		Estérase			GST	
	Mean Act (nmol P450/min/mg protein)	Statut	Mean Act α (µmol-a naph/ min/mg protein)	Mean Act β (µmol-a naph/ min/mg protein)	Statut	Mean Act (nmol GSH conj/min/mg protein)	Status
Kisumu	0,1015		0,07409	0,07655		0,3846	
Agblangandan	0,07966	-	0,07883	0,06117	-	0,7319	**+**
Abomey-Calavi	0,08454	-	0,07149	0,05929	-	0,4295	-
Akron	0,1604	**+**	0,08589	0,07897	-	2,221	**+**
Houéyiho	0,1739	+	0,07694	0,08774	**+**	0,4042	-
Vossa	0,07566	-	0,06897	0,06389	-	0,7078	-
Ladji	0,1737	**+**	0,07146	0,0774	-	1,194	**+**
Pobè	0,09572	-	0,1401	0,146	**+**	0,5073	**+**
Ifangni	0,1362	-	0,0473	0,06635	-	0,575	-
Adjohoun	0,07865	-	0,07538	0,08459	-	0,4137	-
Sèmè	0,09749	-	0,07766	0,07694	-	0,6232	**+**
Bamè	0,1106	-	0,0588	0,06223	-	0,2901	-
Malanville	0,06549	-	0,04949	0,04871	-	0,1723	-
Kandi	0,06389	-	0,09929	0,1253	**+**	0,1902	-
Parakou	0,1536	**+**	0,08124	0,08871	-	0,4698	-
Tanguiéta	0,2267	**+**	0,1585	0,1442	**+**	1,182	**+**
Pehunco	0,1975	**+**	0,07113	0,07269	-	0,9353	**+**

Mean Act = Mean activity ; + = significantly high compared to Kisumu (p<0,05)

5 DISCUSSION

The resistance to insecticides is a growing concern for the African countries especially malaria endemic countries. The findings of this study have shown the presence of monooxygenases in cytochrome P450, esterase and the GST in the populations of *An. gambiae* from different localities surveyed. Over expression of such metabolic enzymes was also observed in some locations.

In general, low esterase activity was recorded in the whole of the prospected localities except in Pobe, Houeyiho, Kandi and Tanguieta where high level was recorded. This strong esterase activity could be due to the selection pressure on mosquito larvae by important use of insecticides by farmers to control pests of cotton and vegetable crops[22-23]. and also maybe by other xenobiotics. Esterases are enzymes that hydrolyze the ester, including organophosphates and carbamates linkages.(24) Through this action, the insecticides become ineffective as prevented from entering the body of the insect and reaching its target. Thus, the high production of this enzyme because of the esterase gene duplication is a mechanism offering resistance to organophosphates and carbamates in an important number of arthropod species including mosquitoes, ticks, aphids and cockroaches [25]. In such case, the overexpression of this enzyme in populations of *An. gambiae* of Tanguiéta could be a threat to the effectiveness of the pirimiphos methy based IRS campaign (Actellic EC, an organophosphorus) implementation in that area. Furthermore, this overproduction of esterases in Tanguieta confirms the work of Aizoun *et al*. (2013b) [26]. who had already suspected the involvement of esterases resistance in *An. gambiae* (Tanguiéta population) to organophosphates following CDC tests with the DEF Synergist. The resistance to carbamate is therefore sufficiently pronounced in Atacora department since the work of Aïkpon *et al*. (2014) [27]. had previously shown an increase in the frequency of the *Ace - 1R* mutation. In addition, recent studies using microarrays have shown that esterases play no role or very little in resistance to pyrethroids and DDT in populations of *An. gambiae* [14,28-29].

Our results also showed overexpression of monooxygenases in cytochrome P450 in populations of *An. gambiae* of Akron, Houéyiho, Ladji, Parakou, Tanguiéta, and Pehunco. Oxidase were often involved in the detoxification of pyrethroids in *Anopheles gambiae* [30-31]. (The monooxygenase catalyze the transfer of an oxygen atom to the substrate thus leading to the formation of a molecule of water. In public health, large-scale use of LLINs and the huge amounts of pyrethroids used in agriculture could be responsible for the overproduction of the recorded oxidases. The findings of this study also correlate with that of Djouaka *et al*. (2011) in natural populations of *An. funestus* at Pahou [32].. Furthermore, the high amounts of oxidase found in Akron correlate with the findings of 13Corbel *et al*. (2007) and 3Djegbe *et al*. (2011). Nevertheless, it is

worth noting that the biochemical tests only give an overall estimate of the enzymatic activity of cytochrome P450 and it is possible that different genes of this gene family are expressed differentially in mosquito populations tested as demonstrated by studies using the technique of microarray [14,27].

Previous studies [33,5], showed high frequencies of the kdr gene in the different localities of this study. A combination of this gene (kdr) and metabolic resistance mechanisms in mosquitoes in the areas of study would be a threat for successful malaria vector control strategies and therefore be catastrophic for our current NMCP as mosquitoes show resistance against to all insecticides used by this program. Thus, the simultaneous presence of the kdr gene and oxidase could confer greater resistance to mosquitoes. To that end, it should be possible, to preserve the effectiveness of the LLINs by combining synergists such as PBO (product intended to inhibit the action of the oxidases) alone or in mixture in the fibres of the nets. Furthermore, the glutathione-S-Transferase activity in anopheles mosquitoes in the study sites shows great variability. However, the strong GST activities within wild populations of An. gambiae from eight sites (Akron, Agblangandan, Ladji, Pobè, Seme, Tanguiéta and Pehunco) out of the 16 confirms the resistance observed by[13]. Corbel et al. (2007) in An. gambiae to DDT from North to South Benin. These results are consistent with those obtained by [32].Djouaka et al. (2011) in An. funestus. These authors have shown that the high rate of GST expression is due to overexpression of the gene GSTe2. It is therefore important to pursue the research so as to find out the involved genes in the surveyed areas. [25] have shown that the GST may play a minor role in the resistance to pyrethroids by detoxification of the products of pyrethroid-induced lipid peroxidation. Thus, the GST may therefore be complemental to the phenotypic effect of kdr mutations to increase the resistance to pyrethroids and DDT levels and broaden the spectrum of resistance to independent compounds [34]. (35 Vontas et al., 2001).

Finally, the lack of overexpression of metabolic enzymes in rice-growing areas could be explained by the absence of the use of insecticides and other factors that can contribute to the overproduction of metabolic enzymes in these populations of mosquitoes.

6 CONCLUSION

This study shows that metabolic resistance is involved in the insecticides resistance mechanism in malaria vector in Benin. The presence of multiple resistance mechanisms in *An. gambiae* in Benin may hinder the success of the malaria vector control strategies based on the LLINs and IRS although concrete proof on the operational impact of vector control tools still need to be documented in depth. The resistance of vectors to insecticides being a dynamic phenomenon, we recommend regular monitoring in order to update the map of the distribution of metabolic resistance in Benin.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

BK, VG, AR and MA designed the study. BK, RA, AS, UF, ZS and RAY carried out the field activities. BK and BA analyzed the data. BK drafted the manuscript. MA, SA, RO and AY critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

ACKNOWLEDGMENT

We are grateful to the President's Malaria Initiative (PMI) which supported financially this study through USAID. We also thank the Ministry of Higher Education and Scientific Research (MESRS) and the team of CREC for their technical assistance during field work and the laboratory.

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