INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 16, No. 1, p. 294-300, 2020

RESEARCH PAPER OPEN ACCESS

Multiple insecticide resistance in *Anopheles gambiae* (Diptera: Culicidae) from tori-bossito, republic of Benin

Yadouleton Anges[1,2-3*], Namountougou Moussa[4], Dramane Gado[1], Sanoussi Falilath[3], Badou Yvette[3], Hounkanrin Gildas[3], Agbanrin[3] Ramziyath[2], Baba-Moussa Lamine[5]

[1]*Ecole Normale Supérieure de Natitingou; Université Nationale des Sciences, Technologies, Ingénierie et Mathématiques (UNSTIM), Benin*

[2]*Centre de Recherche Entomologique de Cotonou, Benin*

[3]*Laboratoire des Fièvres Hémorragiques Virales et des Arbovirus du Bénin*

[4]*Université Nazi BONI/Institut de Recherche en Sciences de la Santé, Benin*

[5]*Laboratoire de Biologie ET de Typage Moléculaire en Microbiologie/Département de Biochimie ET de Biologie Cellulaire/Faculté des Sciences ET Techniques/Université d'AbomeyCalavi, Banin*

Key words: *Anopheles gambiae*, Insecticides, Resistance, Tori-Bossito, Benin

http://dx.doi.org/10.12692/ijb/16.1.294-300 Article published on January 30, 2020

Abstract

In order to detect the multiple insecticide resistance in *Anopheles gambiae* populations in the district of Tori-Bossito, southern Benin from June-September 2019, firstly adult females aged to 2-5 were subjected to susceptible test using impregnated papers (Permethrin 0.75%, delthamethrin 0.05%, DDT 4%, and bendiocarb 0.1%) following WHO testing protocol. Death and survival of *An. gambiae* populations from the test were screened for knock down resistance *(KDR)* and *acetylcholinesterase (Ace-1R)* mutations. Finally, biochemical analysis was done in order to detect Mixed Function Oxydase (MFO), non-specific esterase (NSE) and glutathione-S-transferases (GST) activity in individual 2–5 days old adult *An. gambiae* that had been reared from larvae and not previously exposed to insecticides. This research showed that *An. gambiae* populations from urban and rural areas were of resistance to DDT (2% as a means of mortality), permethrin (40%) and delthamethrin (72%) but fully susceptible bendiocarb. The kdr mutation due to the use of insecticides was the main resistance mechanism identified in these *An. gambiae* populations (0.72 as a means of frequency). The *Ace-1* mutation was found at a very low frequency (≤ 5%). Moreover, enzymatic activities (Esterase, Glutathione-s-transferase (GST) and P450 monooxygenase) in the wild population of *An. Gambiae* were significantly higher than the control strain ($P < 0,05$). This study provides clear evidence that there is a multiple insecticide resistance in *Anopheles gambiae* populations from Tori-Bossito. This will jeopardise the successful of fighting against malaria in this district.

* **Corresponding Author:** Yadouleton Anges ✉ anges33@yahoo.fr

Introduction

The burden of malaria remains the highest in sub-Saharan Africa, with 90% of cases and 92% of malaria deaths occurring in this region in 2017 (WHO, 2017). The use of Pyrethroid (PY) insecticides both in long-lasting insecticide nets (LLINs) and for indoor residual spraying (IRS) are the two methods against this disease. Indeed, malaria control mainly depends on pyrethroids, the only class of insecticide approved to be impregnated on mosquito nets, and it is also being widely used in IRS programs in Africa (WHO, 2014).

However, the use of Pyrethroid insecticides both in long-lasting insecticide nets (LLINs) and for indoor residual spraying (IRS) has subjected malaria vectors, particularly *An. gambiae* (*sensu lato*) which is the predominant *Anopheles* species in Africa to the selection pressures (Dykes *et al.*, 2015). Indeed, reports from many Sub Saharan Africa countries such as Benin (Yadouleton *et al.*, 2018); Togo (Amoudji *et al.*, 2018); Mauritania (Mint *et al.*, 2018) showed that the main malaria vector *An. gambiae sensu lato* has developed strong resistance to many insecticides particularly the PYs. In fact, the resistance of *An. gambiae* S.l to PYs is streinghten by of the East and West know down mutation. Therefore, there is an urgent need to investigate alternatives to the current reliance on pyrethroids for malaria vector control. In the short term, other insecticide classes with different modes of action such as organophosphates (OPs) and carbamates (CMs) could be used either alone or in combination with pyrethroids for IRS or for impregnating bednets (Akogbeto *et al.*, 2010). However, resistance to both classes of insecticides has already been documented in natural *An. gambiae*s. L. populations (Aikpon *et al.*, 2013). This resistance to OPs and CMs in *An. gambiae*s.l. is due to a single point mutation in the acethlycholinesterase (Ace-1) gene encoding acetylcholinesterase, the target binding site of OPs and CMs, resulting in the substitution of a Glycine (GGC) into a Serine (AGC) at position 119 of the encoded protein (i.e. G119S mutation) (Weill *et al.*, 2003)**.** Moreover, many reports on the pyrethroid resistance in *An. gambiae*s.lin East and West Africa appears to be linked to the enzymatic mechanisms particularly to the increased monooxygenase activities, (Riveron *et al.*, 2013). Yadouleton *et al.* (2018) reported the presence of multiple mutations (kdr L1014F and Ace-1 G119S) which confere resistance to *An. gambiae* from vegetable farming and will jeopardize the fight against malaria in many Sub Saharan Africa countries. Additionally to these mutations, the role of metabolic-based detoxifying mechanisms in contributing to insecticide resistance in *An. gambiae* populations needs to be investigated in Benin.

The goal of this study was to assess the status of *An. gambiae S.l.* resistance to the four classes of insecticides, the presence of kdr L1014F, Ace-1 G119S mutations and the enzymatic activities in these populations of mosquitoes in the district of Tori-Bossito, South-East of Benin

Materials & methods

Study areas

The study was carried out in the district of Tori-Bossito southern Benin characterized by a continual practice of urban and peri-urban agriculture, with two rainy seasons (March- July and October- November) and two dry seasons (December-March and August-September). The annual mean rainfall is 1,500mm in July, relative humidity (RH) of 70% ± 5 and a minimum/maximum temperature ranging from 23 to 32°C. To have an idea on the multiple insecticide resistance in *An. gambiae* populations from this district, two points of mosquitoe collections were chosen (Fig. 1). One located in rural area (Manguevié) where agriculture practices are very important and the second is located in the urban area (Tokoli). The choice of these environments is justified by their particular bioclimatic characteristics and the use of insecticides or fertilizers in public health and agriculture in this district.

Mosquito collections

Mosquitoes larvae were collected during the dry (from February to March) and the rainy seasons (April-July). Larvae and pupae were collected using the dipping on breeding sites and then kept in separated labelled bottles related to each locality. Larvae samples was reared up to adult emergence at

the CREC (Centre de Recherche Entomologique de Cotonou, Benin) insectary for further susceptibility tests. Moreover, emerging adult females mosquitoes (F0) aged 6-8 days were fed with guinea pig blood and reared for emergence (F1) where adults 2-5 days were kept at -80 degrees for biochemical analysis.

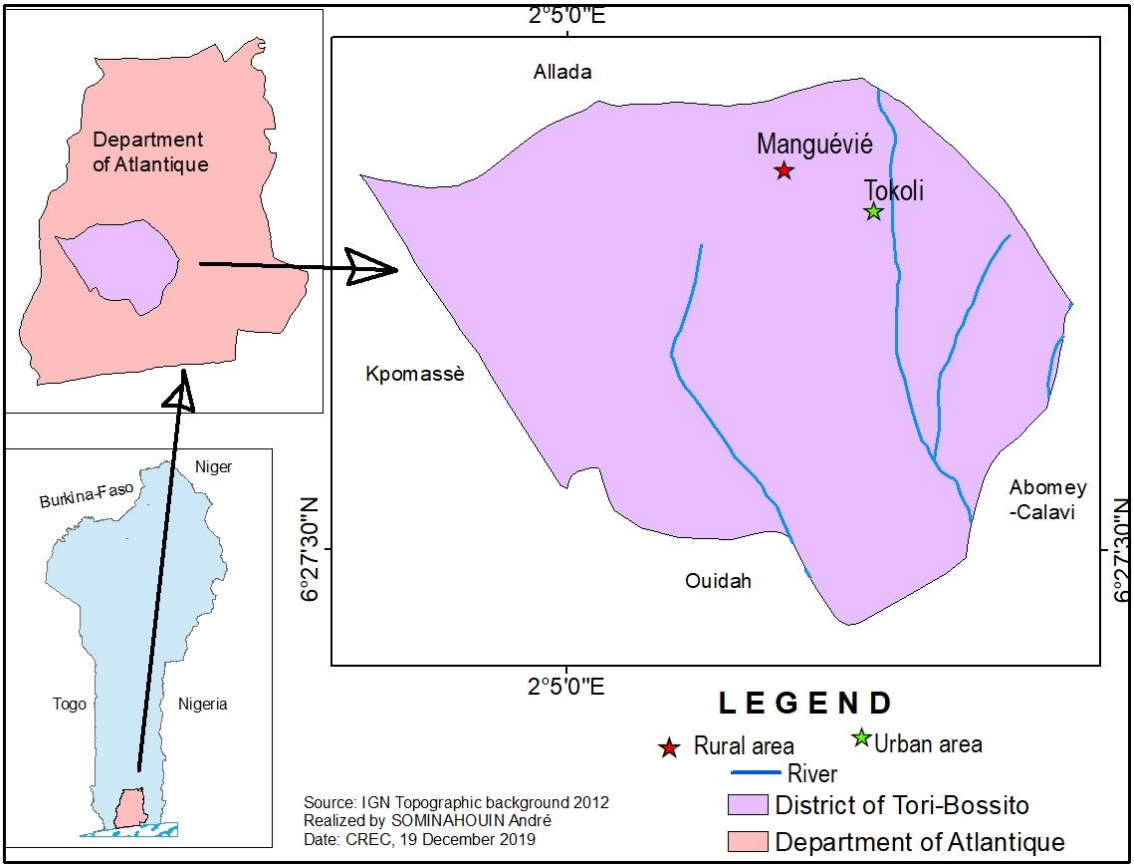

Fig. 1. Map of Benin showing the study area.

Insecticide susceptibility test

Females mosquitoes aged 2-5 days old were exposed to diagnostic doses of various insecticides for susceptibility tests using insecticide-impregnated papers, as described by the standard WHO testing protocol (WHO, 2016). The following insecticides were tested: deltamethrin (0.05%), permethrin (0.75%), DDT (4%) and bendiocarb (0.1%). For each treatment, five test tubes were used: one untreated paper as a control and four treated papers to expose mosquitoes. Control tubes contained filter papers impregnated with silicon oil (insecticide carrier) only, whereas treated papers were impregnated with diagnostic doses of insecticide plus carrier.

An average of twenty-five mosquitoes was introduced into each tube. Females of *An. gambiae* used in this study were exposed for one hour to insecticide-treated papers and monitored at different time intervals (10, 15, 20, 30, 45, 60 minutes) to record the "knock-down" times. After one-hour exposure, mosquitoes were transferred into holding tubes and provided with cotton wool wetted with a 10% honey solution. Mortalities were recorded after 24 hours and the susceptibility status of the population was graded according to the WHO recommended protocol (WHO, 2016). Dead and survived mosquitoes from this bioassay were separately kept in Carnoy solution at -20°C for further molecular characterization

Molecular characterization of Anopheles gambiae populations using PCR analysis

25-35 females of *An. gambiae* samples from the WHO bioassays were analysed at the molecular level. Polymerase chain reaction (PCR) was used to detect the presence of G119S mutation (Ace.1 gene) as described by Weill *et al.* (2003) and the knock down resistance (kdr) mutation as described by Syafruddin *et al.* (2010).

Biochemical analysis

60 adult females of the wild populations of *An. gambiae s.s.* from the study site (Fig. 1) were kept at -80 degrees and were subjected to biochemical based on the methods decribed by Penilla *al.* (1998) to compare the levels of activity of mixed function oxidases (MFO), non-specific esterases (NSE) using α-naphtyl acetate as a substrate and glutathione S-transferases (GST) to the laboratory Kisumu susceptible reference strain.

Individual mosquitoes were homogenized in 200 µl ml distilled water. Each of 10 ml of the homogenate was used for monooxygenase, glutathion S-transferase and protein assay. The other twenty µl ml of homogenate was used for esterases.

Glutathione -S-transferase (GST) assay

10µl of each homogenate was transferred to a microplate well followed by 200µl of the GSH/CDNB working solution which was prepared by adding 0.060g of glutathione solution(GSH) in 20 ml of Phospahte sodium buffer 0.1M and 0.013gr (in 1ml of methanol) 1-chloro-2,4-dinitrobenzene (CDNB). The plates were read after 5 mins with the ELISA plate reader at a wave length of 340nM. GST activity was expressed as:mmoles/ min/ mg protein

Monooxygenase (Cytochrome p450) assay

10µl of homogenate were placed in separate of microtitre plate followed by addition of 80µl 0.625M potassium phosphate buffer (pH 7.2). Ten mg of 3,3,5'5', Tetramethyl Benzidine(TMBZ) in 5 ml methanol were prepared and a 15ml of 0.25 M sodium acetate buffer (pH 5.0) was prepared. Two hundred µl of the above TMBZ solution was added in to each well followed by 25µl of 3% hydrogen peroxide. The plate was read after 2 hours at 630nm.

Esterase assay

20µl of homogenated were placed in separate wells of microtitre plate. 200µl of 0.3mm Alpha/Beta napthyl acetate were added to each well. Leave the plate at room temperature for 1 min and then added 50µl of fast garnet. After 30 minutes, enzyme activity was determined as an OD value by microplate reader at450nm.

Protein assay

The total protein content of individual mosquitoes was determined using the Bio –Rad Protein Assay Kit (Bio –Rad Laboratories) in order to detect the differences in size among individuals that might require correction factors for the enzyme assays

Data interpretation

According to WHO criteria, a mosquito population was considered resistant whether the 24-hour mortality was <90%. Resistance was suspected when mortality was between 90% and 98% and the population was susceptible when the mortality was >98.

Biochemical assay data (enzymatic activity per mg protein, levels of MFO, NSE and GST between Kisumu and field populations *An. gambiae s.s.*) were compared using Mann-Whitney non-parametric *U*-test (Statistica software).

Results

Resistance status

A total of 600 adult females of *An. gambiae* collected from the two points and exposed to impregnated papers with discriminating doses of permethrin (0.75%), deltamethrin (0.05%), DDT (4%) and bendiocarb (0.1%) showed that all populations of *An. gambiae* mosquitoes were fully resistant to DDT (2% as average of mortality), permethrin (40%) and deltamethrin (72%). However, the same populations of *An. gambiae* from the two points of the study site were fully susceptible to bendiocarb.

Resistance mutations

The knock down mutation (kdr) was present in all *An. gambiae* populations collected from the two points of the study site. The average of kdr frequency is 0.75 in rural and 0.7 in urban areas (Table 1). The Ace-1 mutation was found in *An. gambiae* populations collected from the different points but at very low frequency (from 3% to5%) (Table 1).

Enzymatic resistance in Anopheles gambiae populations

Biochemical assay showed a significantly higher level of GST activity from the wild populations of an

gambiae from the study site compared to susceptible Kisumu strain (Mann Whitney test, P>0.05) (Fig. 2). The same trend was observed with Monooxygenase (Cytochrome p450). (Fig. 3). For esterase activity (α and β esterase), the means of optical density values in the populations of mosquitoes from Tori-Bossito (Fig. 4a and 4b) were not significantly higher compared to the reference susceptible Kisumu strain (P>0.05).

Table 1. The frequency of Kdr and Ace-1R mutations in *Anopheles gambiae s.s.* from the district of Tori-Bossito.

		Kdr Mutation				Ace-1 Mutation			
Study site	Localities	SS	RS	RR	F(R)	SS	RS	RR	F(R)
Rural area	Manguevié	4	41	48	0.75	75	05	0	0.03
Urban area	Tokoli	3	38	32	0.71	86	04	0	0.02

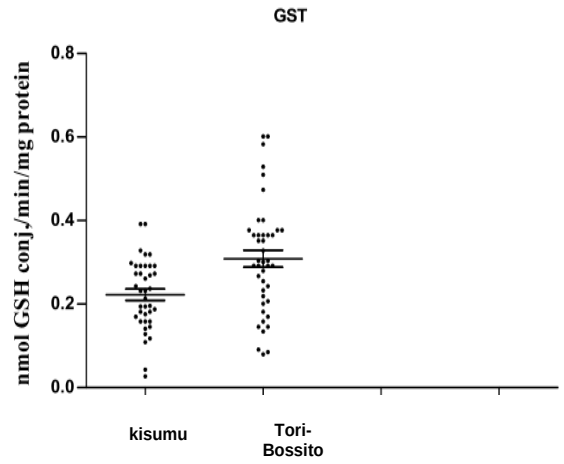

Fig. 2. Gluthation activity of *Anopheles gambiae* populations from Tori-Bossito.

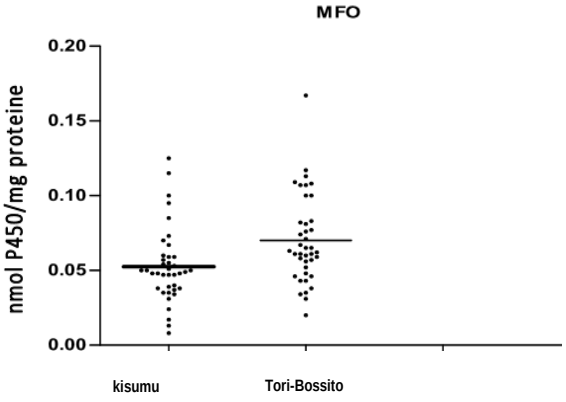

Fig. 3. Mixed Function Oxidases activity of *Anopheles gambiae* populations from Tori-Bossito.

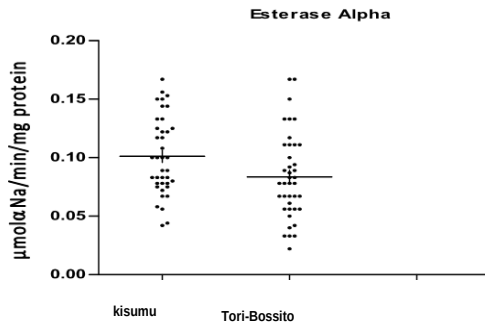

Fig. 4a. Alpha-esterase activity of *Anopheles gambiae* populations from Tori-Bossito.

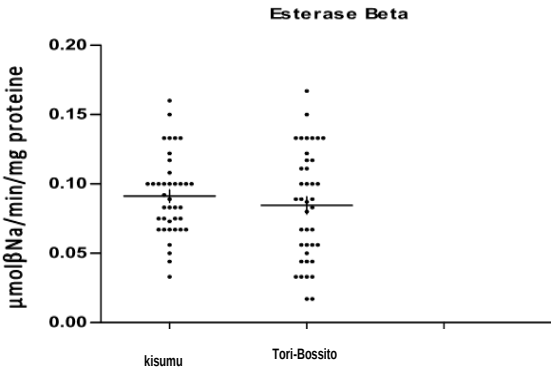

Fig. 4b. Beta-esterase activity of *Anopheles gambiae* populations from Tori-Bossito.

Discussion

The study showed a wide distribution of resistance in *An. gambiae s.l.* to permethrin, deltamethrin and DDT in the district of Tori-Bossito whereas all samples of *An. gambiae* tested were fully susceptible to bendiocarb. The widespread resistance to DDT, permethrin and deltamethrin in this district can be explained by a long-standing, massive use of DDT house-spraying in several districts of the country during the WHO malaria eradication programme in the 1950s (Joncour, 1959). In addition, agriculture practices which used enough quantity of insecticide is one of the major factors that contributes to a large distribution of pyrethroid resistance in *An. gambiae* s.l in this district. A recent report by Yadouleton *et al.* (2018) has confirmed that urban farming in Benin has enormously contributed to the emergence of resistance in *Anopheles* populations. As reported by Akogbeto *et al.* (2005) some populations of *An. gambiae* lay their eggs in breeding sites containing insecticide residues.

A study in vegetable farming has shown that such activities in urban areas directly led to an improper use of insecticides to control vegetable pests, thus exerting a huge selection pressure on mosquito larval population. Moreover, the liberalization of the pesticide sector and the increased cost of pesticides registration have incited most of the farmers to illegally procure insecticides and an uncontrolled use of these chemicals in Benin.

This factor has also contributed to the emergence of insecticide resistance in *An. gambiae* populations (Yadouleton *et al*., 2009). This explain the high frequency of kdr mutation in these *An. gambiae* populations from Tori-Bossito. The low frequency of Ace-1 mutation found in *An. gambiae* populations from this district showed that less quantity of carbamate and organophosphate are used or not in agriculture and in public health in this district.

While insecticide resistance associated with kdr is well studied at the physiological, behavioural and population level, much less is known about the enzymes associated with metabolic resistance. One route of metabolic resistance is through up-regulation of detoxification enzymes. Findings from the present study showed an increase level of GST and monooxygenase P450 activities in the wild populations of *An. gambiae* compared to the susceptible Kisumu strain. In fact, the high level of GST in the wild population of *An. gambiae* can be explained by the high resistance that *An. gambiae* populations from this district developped against DDT and PY.

In addition, the high monooxygenase P450 activity in the wild populations of *An. gambiae* is one of the consequence of the high frequency of the kdr gene mutation observed in *An. gambiae* population from this district. Since these two metabolic genes found at high level compared to the susceptible Kisumu strain, it would be important to quantify these genes in the future using the qPcr technique. Moreover, the overexpression of the two enzymes seemed to be clear that only kdr mutation can not explain the insecticide resistance found in *An. gambiae* populations in this district.

Conclusion

The emergence of PY resistance in *An. gambiae* has become a serious concern for the success of malaria control in the last decades. This study provides clear evidence that the use of insecticides by local farmers for crops protection is one factor that impacts negatively the immune system of mosquito which has led to the emergence of insecticide resistance in malaria vectors.

The current findings will help for decision-making in the National Malaria Control Programme especially in the choice of insecticide to be used during the campaigns of Indoor Residual Spraying (IRS) in this part of Benin.

Acknowledgement

This work is financially supported by SNIS. I am grateful to Houndeton Geraldo for technical assistance.

Competing interests

The authors declare that they have no competing interests.

References

Aikpon R, Osse R, Govoetchan R, Sovi A, Oke-Agbo F, Akogbeto MC. 2013. Entomological baseline data on malaria transmission and susceptibility of *Anopheles gambiae* to insecticides in preparation for indoor residual spraying (IRS) in Atacora, (Benin). Journal of Parasitology and Vector Biology **5,** 102-111.

Akogbéto M, Djouaka R, Noukpo H. 2005. Use of agricultural insecticides in Benin Bulletin de la Société de Pathologie Exotique **98,** 400-405.

Akogbéto M, Padonou G, Bankolé H, Kindé Gazard D, Gbédjissi G. 2011. Dramatic decline of malaria transmission after implementation of large-scale Indoor Residual Spraying using bendiocarb in Bénin, West Africa, an area of high *Anopheles gambiae* resistance to pyrethroids. American Journal of Tropical Medicine and Hygiene **85,** 586-593.

Amoudji AD, Ahadji-Dabla KM, Hien AS, Apétogbo YG, Yaméogo B, Soma DD, Bamogo R, Atcha- Oubou RT, Dabiré RK, Ketoh GK. 2019. Insecticide resistance profiles of *Anopheles gambiae* s.l. in Togo and genetic mechanisms involved, during 3-year survey: is there any need for resistance management. Malaria Journal **18,** 177-190.

Dykes C, Kushwah R, Das M, Sharma S, Bhatt R, Veer V. 2015. Knockdown resistance (kdr) mutations in Indian Anopheles culicifacies populations. Parasitology Vectors **8,** 333-340.

Joncour G. 1959. Lutte anti-palustre au Dahomey. Rapport nº 13: Ministère de la Santé Publique **11,** 72-105.

Mint Lekweiry K, Ould Ahmedou Salem MS, Basco LK, Briolant S, Hafid JE, Ould Mohamed Salem Boukhary A. 2015. Malaria in Mauritania: retrospective and prospective overview. Malaria Journal **14,** 100-109.

Penilla RP, Rodriguez AD, Hemingway J, Torres JL, Arrendo-Jimenez JI, Rodriguez MH. 1998. Resistance management strategies in malaria vector mosquito control. Baseline data for a large scale field trial against *Anopheles albimanus* in Mexico. Medical and Veterinary Entomology **12,** 217-233

Riveron JM, Yunta C, Ibrahim SS, Djouaka R, Irving H. 2014. A single mutation in the GSTe2 gene allows tracking of metabolically-based insecticide resistance in a major malaria vector. Genome Biology **15,** 27-36.

Syafruddin D, Hidayati AP, Asih PB, Hawley WA, Sukowati S, Lobo NF. 2010. Detection of 1014F kdr mutation in four major Anopheline malaria vectors in Indonesia. Malaria Journal **9,** 315-322.

Weill M, Lutfalla G, Mogensen K. 2003. Insecticide resistance in mosquito vectors. Nature **423,** 136-137.

WHO. 2014. WHO Guidance for Countries on Combining Indoor Residual Spraying and Long-lasting Insecticidal Nets. https://www.who.int /malaria/publications/atoz/who-guidance-combining –irs -llins/en/

WHO. 2016. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. http:// www.who.int

WHO. 2017. Malaria fact sheet. Geneva. https://www.who.int/en/news-room/fact-sheets

Yadouleton Anges, Lamine Baba-Moussa, Jean-Robert Klotoe, Tenin Coulibaly, Tchibozo Carine, Roland Tossou, Fabrice Ahissou and Martin Akogbeto. 2018. Detection of multiple insecticide resistance mechanisms in *Anopheles gambiae* s.l. populations from the vegetable farming area of Houeyiho, Southern Benin, West Africa. International Journal of Mosquito Research **5,** 21-23.

Yadouleton AW, Asidi A, Djouaka RF, Braïma J, Agossou CD, Akogbeto MC. 2009. Development of vegetable farming: a cause of the emergence of insecticide resistance in populations of *Anopheles gambiae* in urban areas of Benin. Malaria Journal **8,** 103-10.