



## Original Research Article

### Status of deltamethrin resistance in three *Anopheles gambiae sensu lato* populations from main ecological settings in Benin, West Africa

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In order to guide future malaria vector control interventions in Benin, it was useful to report the susceptibility status, pyrethroid resistance levels in *Anopheles gambiae s.l.*, the frequency and distribution of *kdr* “Leu-phe” mutation in malaria vectors. Larvae and pupae of *Anopheles gambiae s.l.* mosquitoes were collected from the breeding sites in Atlantic, collines and Borgou departments. WHO susceptibility tests were conducted on unfed female mosquitoes aged 2–5 days old. WHO bioassays were performed with impregnated papers of deltamethrin (0.05%). *An. gambiae* mosquitoes were identified to species using PCR techniques. Molecular assays were also carried out to identify *kdr* mutations in individual mosquitoes. *An. gambiae* Dassa-Zoume and Allada populations were resistant to deltamethrin whereas *An. gambiae* Parakou population resistance status required further investigation. PCR revealed 100% of mosquitoes tested were *Anopheles gambiae s.s.* The *L1014F kdr* mutation was found in *An. gambiae s.s.* Allada, Dassa-Zoume and Parakou at various allelic frequencies. The geographic distribution of vector susceptibility to pyrethroids is critically needed as it will provide baseline information for vector control. The presence, though at high frequency, of the West African *kdr* mutation in *Anopheles gambiae* Dassa-Zoume and Allada needs to be carefully monitored.

## Introduction

Malaria is a major health problem in Benin where it is the main cause of morbidity and mortality particularly among children under five and pregnant women. It is transmitted by *Anopheles* mosquitoes, and because there is currently no vaccine available, vector control is one of the most important means of malaria prevention. This vector control is generally done with insecticides.

In this country as across Africa, malaria control relies heavily on vector control through the use of insecticide-treated nets (ITN) and indoor residual spraying (IRS).

In West Africa, the main mechanism involved in pyrethroid-resistance in *An. gambiae* is caused by target site insensitivity through a knockdown resistance (*kdr*)-like

mutation caused by a single point mutation (Leu-Phe) in the para-sodium channel gene (Chandre *et al.*, 1999). Malaria vector resistance to insecticides in Benin is conferred by two main mechanisms: (1) alterations at site of action in the sodium channel, viz the *kdr* mutations and (2) an increase of detoxification and/or metabolism through high levels of multi-function oxidases (MFOs), non-specific esterases (NSEs) (Corbel *et al.*, 2007; Djogbénou *et al.*, 2009; Djègbé *et al.*, 2011; Aïzoun *et al.*, 2013a; Aïzoun *et al.*, 2013b).

Beninese National Malaria Control Programme has implemented large-scale and free distribution of LLIN since July 2011 through the entire country to increase coverage of LLINs. It is crucial that information on current status of *An. gambiae s.l.* resistance to pyrethroid being investigated. This will properly inform control programs of the most suitable insecticides to use and facilitate the design of appropriate resistance management strategies.

In this study, we report the assessment of the susceptibility status, insecticide resistance levels in *Anopheles gambiae s.l.* to deltamethrin and to evaluate the presence and extent of the distribution of the *kdr* mutation within and among these *An. gambiae s.l.* populations in the south-north transect Benin, where pyrethroid resistance was also recently reported in *An. gambiae* (Djègbé *et al.*, 2011; Aïzoun *et al.*, 2013a; Aïzoun *et al.*, 2013b).

## Materials and Methods

### Study area

The study was carried out in some localities; following a south-north transect Benin. Three contrasting localities of Benin were selected for mosquito collection on the basis

of variation in agricultural production, use of insecticides and/or ecological settings. The localities were: Dassa-Zoume a rice growing area located in the middle part of the country. Allada is a cereal growing area (maize, ground-nut and so on) located in the south part of the country. Parakou, an urban vegetable growing area located in the north of Benin. The choice of the study sites took into account the economic activities of populations, their usual protection practices against mosquito bites, and peasant practices to control farming pests. These factors have a direct impact on the development of insecticide resistance in the local mosquito vectors.

The southern region is characterized by a tropical Guinean climate with two rainy seasons (April–July and September–November) with a mean annual rainfall over 1,500 mm. The middle part of the country is characterized by a Sudano Guinean climate with two rainy seasons (March–July and August) with an average rainfall of 1,000 mm per year. The northern zone is characterized by a Sudanian climate with only one rainy season per year (May to October) and one dry season (November–April). The temperature ranged from 22 to 33°C with the annual mean rainfall which is 1,300 mm.

### Mosquito collection

*An. gambiae s.l.* mosquitoes were collected from April–July and September–November 2012 during the rainy season in Allada locality selected in south Benin. Larvae and pupae were collected in Allada district within both padding and village using the dipping method on several breeding sites (brick pits, pools, marshes, streams, ditches, pits dug for plastering traditional huts, puddles of water, water pockets caused by the gutters).

*An. gambiae s.l.* mosquitoes were also collected from July to November 2012 in Dassa-Zoume district selected in the centre part of the country. *Anopheles* pre-imaginal stages (L1 to L4 instars) were collected via ladles within rice farms, Lema, from Dassa-Zoumè. Due to that the farms are irrigated, breeding sites are present throughout the year and we therefore assumed that the larvae collected in the study period were representative of the population that could be found during other periods of the year.

*An. gambiae s.l.* mosquitoes were also collected from May to October 2012 during the rainy season in Parakou district selected in north Benin. *Anopheles* pre-imaginal stages (L1 to L4 instars) were collected via ladles within vegetable farms from Parakou. In these three districts, larvae and pupae were collected using the dipping on breeding sites and then kept in separated labeled bottles related to each locality. Otherwise, larvae collected from multiple breeding sites were pooled together related to each locality then re-distributed evenly in development trays containing tap water.

Larvae were provided access to powdered TetraFin® fish food, and were reared to adults under insectary conditions of 25±2°C and 70 to 80% relative humidity at Centre de Recherche Entomologique de Cotonou (CREC) located in Akpakpa, in Cotonou district. The samples were reared up to adult emergence at the CREC insectary. *An. gambiae* Kisumu, a reference susceptible strain was used as a control for the bioassay tests.

Susceptibility tests were done following WHO protocol on unfed females mosquitoes aged 2–5 days old reared from larval and pupal collections. All susceptibility tests were conducted in the CREC laboratory at 25±2°C and 70 to 80% relative humidity.

### **Testing insecticide susceptibility**

Females *An. gambiae* aged 2 to 5 days old were exposed to WHO diagnostic dosage of deltamethrin 0.05% according to the WHO protocol (WHO, 1998). Thus, an aspirator was used to introduce 20 to 25 unfed female mosquitoes into five WHO holding tubes (four tests and one control) that contained untreated papers. They were then gently blown into the exposure tubes containing the insecticide impregnated papers. After one-hour exposure, mosquitoes were transferred back into holding tubes and provided with cotton wool moistened with a 10% honey solution. The number of mosquitoes “knocked down” at 60 minutes and mortalities at 24 hours were recorded following the WHO protocol (WHO, 1998). Dead and surviving mosquitoes were separately stored in individual tubes with silica gel and preserved at -20°C in the laboratory, for further molecular characterization. We used deltamethrin, an insecticide of same class as permethrin to check if there was resistance to this product in districts surveyed.

### **PCR detection of species and the *kdr* mutation**

At the end of WHO bioassays, a polymerase chain reaction test for species identification (Scott et al., 1993) was performed to identify the members of *An. gambiae* complex collected from each site. PCR for the detection of the *kdr* “Leu-phe” mutation was carried out on dead and alive *An. gambiae* mosquitoes as described by Martinez-Torres *et al.* (1998).

### **Statistical analysis**

The resistance status of mosquito samples was determined according to the latest WHO criteria (WHO, 2013) as follows:

- Mortality rates between 98%-100% indicate full susceptibility
- Mortality rates between 90%-97% require further investigation
- Mortality rates < 90%, the population is considered resistant to the tested insecticides.

Abbott's formula was not used in this study for the correction of mortality rates in test-tubes because the mortality rates in control tube were less than 5% (Abbott, 1987).

Molecular results (*kdr* frequencies) were correlated with the results of insecticide susceptibility tests performed with WHO method from each of the districts surveyed. ANOVA test was performed with mortality rate as the dependent variable and the localities as a covariate. ANOVA test was also performed with *kdr* frequency as the dependent variable and the localities as a covariate.

## Results and Discussion

### Resistance status

Table 1 shows that Kisumu strain (control) confirmed its susceptibility status as a reference strain. The 24 hours mortality recording shows that female mosquitoes of *Anopheles gambiae* Kisumu which were exposed to WHO papers impregnated with deltamethrin 0.05% were susceptible to this product with the mortality rate of 100%. Regarding *An. gambiae* Dassa-Zoume and Allada populations, they were resistance to deltamethrin with the mortality rates of 82% and 55% respectively. The mortality rate obtained with *An. gambiae* Parakou populations was 97.5% and requires further investigation. The resistance level in *An. gambiae* Allada populations was higher than the one observed with *An. gambiae* Dassa-Zoume and Parakou populations (Table 1).

Univariate logistic regression, performed with mortality rate as the dependent variable and localities as a covariate with ANOVA test showed that the phenotypic resistance to deltamethrin was associated with the localities ( $p < 0.05$ ) except Parakou locality ( $p > 0.05$ ) on the one hand. Univariate logistic regression, performed with *kdr* frequency as the dependent variable and localities as a covariate with ANOVA test, showed that high *kdr* frequency was associated with the localities ( $p < 0.05$ ) on the other hand.

### Species *Anopheles gambiae*

Mosquitoes from WHO bioassay were analysed by PCR for identification of sibling species among *An. gambiae s.l.* complex. PCR revealed 100% of mosquitoes tested were *Anopheles gambiae s.s.* (Table 2).

### Detection of resistance genes

The *L1014F kdr* mutation was found in *An. gambiae s.s.* Parakou, Dassa-Zoume and Allada at various allelic frequencies. These frequencies were 0.74%, 1% and 0.73% respectively (Table 2). The management of insecticide resistance is a major issue, which must interest the different National Malaria Control Programmes. This management requires two kinds of information: sound knowledge of the mechanisms of resistance and a thorough resistance monitoring programme (Aizoun *et al.*, 2013a).

*Anopheles gambiae* natural populations have developed resistance to deltamethrin in the different bio-climatic areas surveyed in the south-north transect Benin except *Anopheles gambiae* Parakou for which further investigations are required before its resistance status clarification. The resistance levels to deltamethrin observed in *Anopheles gambiae* Allada and Dassa-Zoume populations were higher than the one

observed with *Anopheles gambiae* Parakou. Similar results were also already observed by (Akogbeto *et al.*, unpublished data) in the framework of « *Multilateral initiative of malaria* » (MIM) network in West Africa. In fact, Allada, a cereal growing area and Dassa-Zoume, a rice growing area are two localities where no insecticidal products are generally used to control agricultural pests comparatively to the vegetable growing area of Parakou where various insecticidal products are used for this purpose.

Very high *kdr* allelic frequency was observed in *An. gambiae s.s.* Dassa-Zoume populations and might likely be explained by absence of metabolic-based resistance. According to Corbel *et al.*, (2007), in the vegetable growing area of Parakou, the low level of pyrethroid-resistance observed in *An. gambiae s.l.* was explained by the presence of (i) a relatively high proportion of susceptible *An. arabiensis* mosquitoes, (ii) a rather low *kdr* allelic frequency in *An. gambiae s.s.* and (iii) the absence of metabolic-based resistance. But, in the current study, all *Anopheles gambiae* Parakou specimens tested were *Anopheles gambiae s.s.* No *An. arabiensis* mosquitoes were found. This result showed that *An. arabiensis* populations from vegetable growing area of Parakou tend to decline after five years. The main reason of this decrease in *An. arabiensis* Parakou populations was that the proportion of *An. arabiensis* mosquitoes from Parakou found by Corbel *et al.*, (2007) was almost susceptible. The *kdr* frequency recorded in *Anopheles gambiae* Parakou populations was 0.74 in the current study. The *kdr* frequency recorded in the same *Anopheles gambiae* populations in 2007 by Corbel *et al.*, (2007) was 0.20. In the same way, the *kdr* frequencies in *Anopheles gambiae* Dassa-Zoume and Allada populations in 2008 were 0.46 and 0.33 respectively

(Djogbenou *et al.*, unpublished data). In the current study, the *kdr* frequencies recorded in these same *Anopheles gambiae* populations were 1 and 0.73 respectively. These results showed that *kdr* frequency in these *Anopheles gambiae* populations has significantly increased after five years. This is consistent with previous observations reporting an increase of the *kdr L1014F* frequency in *An. gambiae* following a nationwide distribution of long-lasting insecticide-treated nets in Niger (Czeher *et al.*, 2008).

The *kdr* frequency in *Anopheles gambiae* Dassa-Zoume populations recorded in this study was higher than the one observed in *Anopheles gambiae* Parakou. Similar results were also already observed by (Akogbeto *et al.*, unpublished data) in the framework of « *Multilateral initiative of malaria* » (MIM) network in West Africa. By contrast to the finding of Diabaté *et al.*, (2002) in Burkina Faso, the agricultural use of insecticide was not always a source of selection pressure for resistance in *An. gambiae s.l.* as *kdr* frequency in *Anopheles gambiae* Dassa-Zoume was higher than the one observed in *Anopheles gambiae* Parakou. In fact, Dassa-Zoume is a rice growing area where no insecticidal products are generally used to control agricultural pests comparatively to the vegetable growing area of Parakou where various insecticidal products are used for this purpose.

The current study clearly shows that the geographic distribution of vector susceptibility to pyrethroids is critically needed as it will provide baseline information for vector control. The presence, though at high frequency, of the West African *kdr* mutation in *Anopheles gambiae* Dassa-Zoume and Allada needs to be carefully monitored.

**Table.1** Percentage of dead *Anopheles gambiae* observed after 1 hour exposure to WHO papers impregnated with deltamethrin 0.05% in Parakou, Dassa-Zoume and Allada localities

Bio-climatic areas	Localities	Insecticides	Number tested	% Mortality	Resistance Status
	Kisumu	Deltamethrin	92	100	S
Sudanian	Parakou	Deltamethrin	80	97.5	r
Sudano-guinean	Dassa-Zoume	Deltamethrin	100	82	R
Guinean	Allada	Deltamethrin	100	55	R
R: Resistant	S: Susceptible		r: require further investigation		

**Table.2** Species identification and *kdr* frequency in *Anopheles gambiae s.l.* from WHO bioassays

Locality	Number tested	Species Ag	<i>Kdr</i> mutation			
			RR	RS	SS	F( <i>Kdr</i> )
Parakou	40	40	21	17	2	0.74
Dassa-Zoume	35	35	35	0	0	1
Allada	47	47	26	17	4	0.73

Ag: *An. gambiae s.s.*

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