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Original Research Article

The *L1014F kdr* Mutation in *Anopheles gambiae s.l.* Lambdacyhalothrin Resistant Populations from Kandi District in Northern Benin, West Africa

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Abstract

Anopheles gambiae, which is the main malaria vector in Benin has developed high level of resistance to pyrethroids (permethrin, deltamethrin and so on). This raises serious concerns to the future use of long-lasting insecticidal nets (LLINs). It is therefore important to assess the susceptibility status, resistance level in *Anopheles gambiae s.l.* to lambdacyhalothrin and to evaluate the presence of the *kdr* mutation within and among the *Anopheles gambiae s.l.* populations in this main malaria vector in Benin. Larvae and pupae of *Anopheles gambiae s.l.* mosquitoes were collected from the breeding sites in Kandi district. WHO susceptibility tests were conducted on unfed female mosquitoes aged 2–5 days old. WHO susceptibility tests were performed with impregnated papers with lambdacyhalothrin 0.05%. PCR techniques were used to detect species and *kdr* mutations. *Anopheles gambiae s.l.* populations from Kandi were resistant to lambdacyhalothrin 0.05% with mortality rate of 23.71%. PCR assay revealed that 100% of mosquitoes tested were *Anopheles gambiae s.s.* The frequency of *kdr* mutation in *Anopheles gambiae* Kandi was 91%. The phenotypic resistance status observed to lambdacyhalothrin may be due to *L1014F kdr* mutation. However, metabolic resistance mechanisms may also be involved in this resistance.

Keywords

Anopheles gambiae
Benin
L1014F kdr mutation
Lambdacyhalothrin
Malaria
Resistance

Introduction

In 2012, WHO issued the Global plan for insecticide resistance management in malaria vectors (GPIRM) (WHO, 2012), urging endemic countries to ensure timely entomological and resistance monitoring, and to develop and implement comprehensive insecticide resistance management (IRM) strategies (WHO, 2013a). To meet the target of universal access, WHO recommends that one LLIN be distributed for every two people at risk of malaria. Since many households have an odd number of members, the calculation needs to be adjusted when quantifying at the population level. Insecticide-

treated mosquito nets (ITNs) which include both LLINs and conventional nets treated with an insecticide work both on the individual level (by protecting the person sleeping under the net) and the community level (by extending the effect to an entire area) (WHO, 2013a).

During the past 10 years, the main factor driving the emergence and spread of insecticide resistance has been the heavy reliance on a single class of insecticide: the pyrethroids. The pyrethroids are both highly effective and the least expensive of the four classes of insecticides available for public health vector control. Preserving the efficacy of pyrethroids is an urgent global priority, because pyrethroids are the only class of insecticide available for use on LLINs, and most new products and compounds are still years away from entering the market.

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 *Anopheles 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.

The current study propose was to assess the susceptibility status, resistance level in *Anopheles gambiae s.l.* to lambdacyhalothrin and to evaluate the presence of the *kdr* mutation within and among the *Anopheles gambiae s.l.* populations from Kandi district in Benin, where pyrethroid resistance was also recently reported in *Anopheles gambiae* (Djègbé et al., 2011; Aïzoun et al., 2013a; Aïzoun et al., 2013b).

Materials and methods

Study area

The study area is located in the Republic of Benin (West Africa) and includes the department of Alibori. The Alibori department is located in the far north of Benin and the study was carried out more precisely in Kandi district, a cotton growing area. The choice of the study site took into account the economic activities of populations, their usual protection practices against mosquito bites, and peasant practices to control farming pests. The northern zone (Kandi) 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 of 1,300 mm.

Mosquito sampling

Anopheles gambiae s.l. mosquitoes were collected from May to October 2012 during the rainy season in Kandi district selected in northern Benin. Larvae and pupae were collected in this district within both padding and town 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). Once, larvae and pupae collected, they were then kept in labeled bottles related to the Kandi district surveyed. Otherwise, larvae collected from multiple breeding sites were pooled together 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. *Anopheles gambiae* Kisumu, a reference susceptible strain was used as a control for the bioassay tests. We used Kisumu more precisely to confirm the quality of treated or impregnated papers and then to calculate the resistance ratio. 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

Female *Anopheles gambiae s.l.* aged 2 to 5 days old were exposed to WHO diagnostic dosage of lambdacyhalothrin 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 min. and mortalities at 24 hrs post treatment 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 lambdacyhalothrin, an insecticide of same class as permethrin to check if there was already resistance to this product in the district surveyed.

PCR detection of species and the *kdr* mutation

At the end of WHO bioassays, polymerase chain reaction tests for species identification (Scott et al., 1993) was performed to identify the members of *Anopheles gambiae* complex collected in Kandi district. PCR for the detection of the *kdr* "Leu-phe" mutation was carried out on dead and alive *Anopheles 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, 2013b) 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 rate in control tube was less than 5% (Abbott WS, 1987). Molecular results (*kdr* frequencies) were correlated with the results of insecticide susceptibility tests performed with WHO method from the district surveyed.

ANOVA test was performed with mortality rate as the dependent variable and the locality as a covariate. ANOVA test was also performed with *kdr* frequency as the dependent variable and the locality as a covariate. The knocked-down times for 50% and 95% of tested mosquitoes (Kdt_{50} and Kdt_{95}) were estimated using SPSS version 16.0 (SPSS Inc., Chicago, IL). The resistance ratio (RR_{50}) was determined relative to the Kisumu susceptible strain. This was obtained by dividing the Kdt_{50} of wild strain to the Kdt_{50} of the susceptible strain. The software R-2.15.2. (R Development Core Team, 2011) was used for the statistical analysis.

Results

Susceptibility of *Anopheles gambiae s.l.* populations to lambdacyhalothrin

Fig. 1 shows that Kisumu strain (control) confirmed its susceptibility status as a reference strain. We used Kisumu to confirm the quality of treated or impregnated papers and then to calculate the resistance ratio. The 24 h mortality recording shows that female mosquitoes of *Anopheles gambiae* Kisumu which were exposed to WHO papers impregnated with lambdacyhalothrin 0.05% were susceptible to this product with the mortality rate of 100%.

Regarding *Anopheles gambiae s.l.* populations from Kandi, they were resistant to lambdacyhalothrin with the mortality rate of 23.71% (Fig. 1). Univariate logistic regression, performed with mortality rate as the dependent variable and locality as a covariate with ANOVA test showed that the phenotypic resistance to lambdacyhalothrin was associated with the locality ($p < 0.05$). Univariate logistic regression, performed with *kdr* frequency as the dependent variable and locality as a covariate with ANOVA test, also showed that high *kdr* frequency was associated with the locality ($p < 0.05$).

Determination of resistance ratio (RR)

The resistance ratio (RR_{50}) of the wild populations of *Anopheles gambiae s.l.* from Kandi with regard to lambdacyhalothrin was higher than 1 (Table 1).
The knocked-down time (Kdt_{50}) of *Anopheles gambiae s.l.* from Kandi with regard to lambdacyhalothrin was 54.704 min. versus 13.602 min. for *Anopheles gambiae s.l.* Kisumu susceptible reference strain. The resistance ratio (RR_{50}) was 4.021.

The same remark was made with Kdt_{95} value obtained with these same wild populations of *Anopheles gambiae s.l.* using the same insecticide. The resistance ratio (RR_{95}) obtained with *Anopheles*

gambiae s.l. from Kandi with regard to lambdacyhalothrin was 3.874.

Species *Anopheles gambiae*

Mosquitoes from WHO bioassay were analysed by PCR for identification of sibling species among *Anopheles 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 *Anopheles gambiae s.s.* Kandi at allelic frequency of 91% (Table 2).

Fig. 1: Percentage of dead *Anopheles gambiae* observed after 1 h exposure to WHO papers impregnated with lambdacyhalothrin 0.05% in Kandi district in comparison with control (Kisumu).

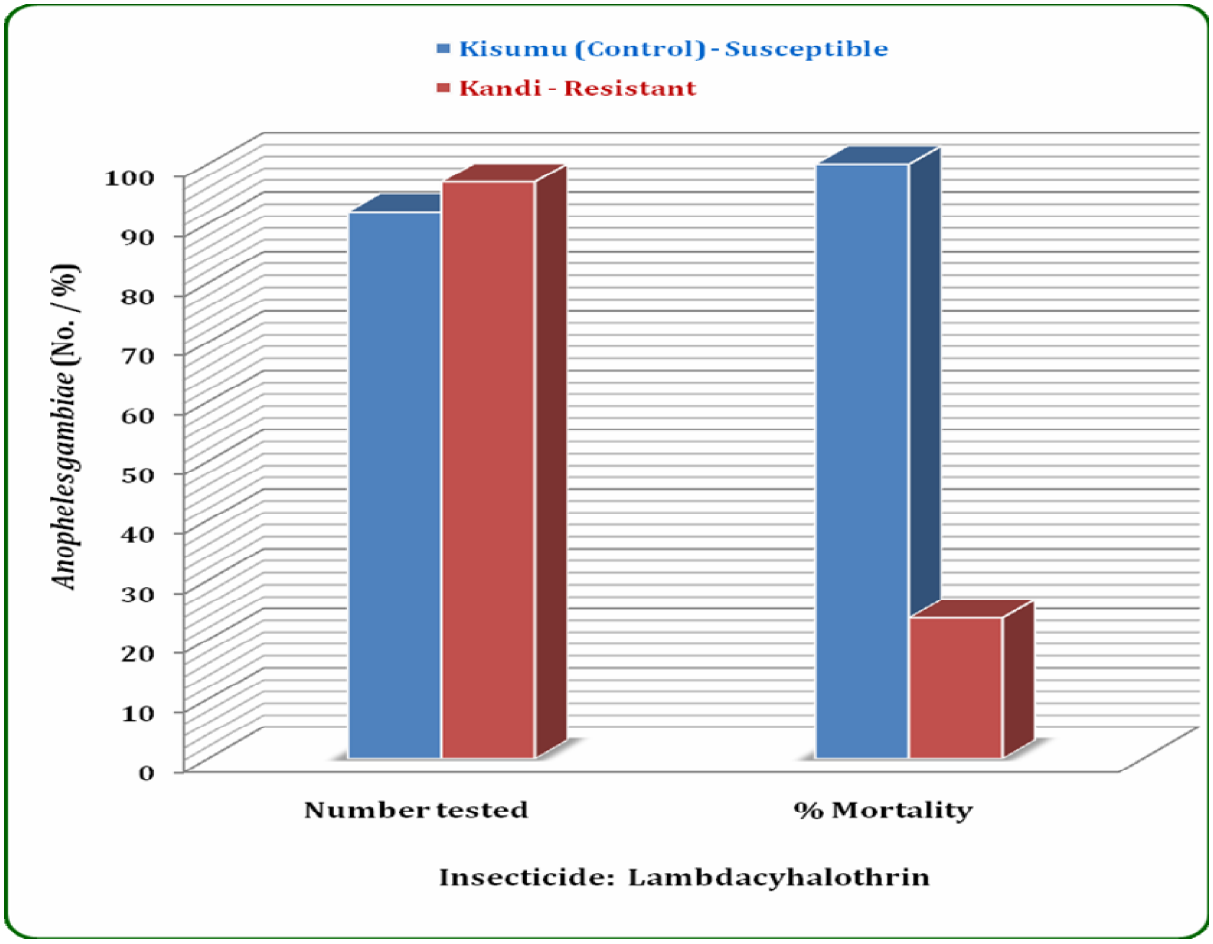

Table 1. Resistance ratio RR_{50} and RR_{95} with regard to *Anopheles gambiae* Kandi and Kisumu populations' susceptibility to lambdacyhalothrin.

Insecticide	Kdt_{50} Kandi	Kdt_{50} Kisumu	RR_{50}	Kdt_{95} Kandi	Kdt_{95}Kisumu	RR_{95}
Lambdacyhalothrin	54.704	13.602	4.021	94.639	24.425	3.874

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

			kdr mutation			
Locality	Number tested	Species Ag	RR	RS	SS	F(*kdr*)
Kandi	48	48	39	9	0	0.91
Ag: *Anopheles gambiae s.s.*						

Discussion

In the current study, the Kdt_{50} and Kdt_{95} values obtained with *Anopheles gambiae s.l.* Kisumu susceptible reference strain were lower than those recorded with the wild populations of *Anopheles gambiae s.l.* from Kandi. Therefore, *Anopheles gambiae s.l.* Kandi populations took more time to die when they were exposed to lambdacyhalothrin comparatively to Kisumu susceptible strain.

Lambdacyhalothrin resistance in *Anopheles gambiae s.l.* populations from Kandi may be explained by increased use of various insecticidal products (organophosphates, pyrethroids, and so on) for crop protection, mainly for cotton protection, but the amount applied is far higher than that consumed in public health against malaria vectors (Chandre et al., 1999). Lambdacyhalothrin resistance in *Anopheles gambiae s.l.* populations from Kandi may also be explained by increased use of household insecticide and availability of xenobiotics for larval breeding sites in the urban area. They were one of the possible factors selecting for pyrethroid resistance in *Anopheles gambiae s.l.* in urban areas (Aïzoun et al., 2014a) even if Kandi district is less developed comparatively to the districts from southern Benin such as Cotonou and Porto-Novo districts. In fact, the same remark was already made with *Anopheles gambiae s.l.* populations from Akron and Suru-léré resistant to lambdacyhalothrin (Aïzoun et al., 2014b).

The alterations at site of action in the sodium channel, *viz.*, the *kdr* mutations in *Anopheles gambiae s.l.* mosquitoes from Kandi was not the only resistance mechanism involved in these mosquitoes populations. A recent study carried out by Aïzoun et al. (2013b) on *Anopheles gambiae s.l.* populations from Kandi in Alibori department showed that the synergist assay with PBO, an inhibitor of Cytochrome P450 monooxygenases, indicated that this enzyme family plays a role in the deltamethrin resistance observed in Kandi. So, there were multiple mechanisms in pyrethroid resistance in *Anopheles gambiae s.l.* populations from Kandi. The *kdr* frequency recorded in *Anopheles gambiae* populations from Kandi was 0.91 in the current study. The *kdr* frequency recorded in the same *Anopheles gambiae* populations in 2008 by Djogbénou et al. [unpublished data] was 0.63. These results showed that *kdr* frequency in these *Anopheles gambiae* populations has significantly increased after four years. This is consistent with previous observations reporting an increase of the *kdr L1014F* frequency in *Anopheles gambiae* following a nationwide distribution of long-lasting insecticide-treated nets in Niger (Czeher et al., 2008) and in Benin (Aïzoun et al., 2014a).

In the current study, all *Anopheles gambiae* Kandi specimens tested were *Anopheles gambiae s.s.* No *Anopheles arabiensis* mosquitoes were found. Similar remark was already made with *Anopheles gambiae* populations from Kandi susceptible to both to carbamates and organophosphates (Aïzoun et al., 2013c). This result showed that *Anopheles arabiensis* populations from Kandi district tend to decline after four years. In fact, Djogbénou et al. [unpublished data] have found 47% of *Anopheles arabiensis* among *Anopheles gambiae* complex in this district in 2008.

The current study clearly shows that the phenotypic resistance status observed to lambdacyhalothrin may be due to *L1014F kdr* mutation. However, metabolic resistance mechanisms may also be involved in this resistance.

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