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Original Research Article

Cyfluthrin resistance in *Anopheles gambiae s.l.* populations from the cotton growing area of Hlassoé in Glazoué district in the central part of Benin, West Africa

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A B S T R A C T

In order to guide future malaria vector control interventions in Benin, it was useful to report the susceptibility status, pyrethroid resistance level in *Anopheles gambiae s.l.*, the frequency of *kdr* "Leu-phe" mutation in malaria vectors. Larvae and pupae of *Anopheles gambiae s.l.* mosquitoes were collected from the breeding sites in collines department. WHO susceptibility tests were conducted on unfed female mosquitoes aged 2–5 days old. WHO bioassays were performed with impregnated papers with Cyfluthrin (0.15%). *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 s.l.* populations from Hlassoé were resistant to Cyfluthrin. PCR revealed 100% of mosquitoes tested were *Anopheles gambiae s.s.* The *L1014F kdr* mutation was found in *An. gambiae s.s.* Hlassoé at allelic frequency of 1. The carrying of *Kdr* gene by a mosquito is not systematically synonymous of insecticide phenotype resistance. However, the presence, though at high frequency, of the West African *kdr* mutation in *Anopheles gambiae* populations from Hlassoé needs to be carefully monitored.

Introduction

World Health Organization (WHO) recommends a multi-pronged strategy to control and eliminate malaria, which includes vector control interventions, preventive therapies, diagnostic testing, treatment with quality-assured artemisinin-based combination therapies (ACTs), and strong malaria surveillance. Effective malaria control and elimination requires strong and well-funded National Malaria Control Programmes (NMCPs), tailored national and regional strategies, extensive applied and operational research, and a close collaboration among partners in the global malaria and development community. Achieving effective scale-up of malaria interventions also requires significant human resources at national, district and community levels, and the regular training of malaria programme staff (WHO, 2013a).

Even in areas where resistance has been identified, LLINs continue to provide some level of protection by acting as a physical barrier against disease vectors. Countries should therefore continue to promote the goal of universal LLIN coverage. In areas with high levels of LLIN coverage in which pyrethroid resistance is identified, WHO recommends the deployment of focal IRS with a non-pyrethroid insecticide. The presence of a non-pyrethroid on wall surfaces reduces the probability that pyrethroid resistance will spread (WHO, 2013a).

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.

The current study propose was to assess the susceptibility status, resistance level in *Anopheles gambiae s.l.* to cyfluthrin and to evaluate the presence of the *kdr* mutation within and among the *An. gambiae s.l.* populations from Glazoué district in Benin, country where pyrethroid resistance was also recently reported in *An. gambiae s.l.* (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 Collines. The Collines department is located in the central part of Benin and the study was carried out more precisely in Glazoué district in the cotton growing area of Hlassoé. 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 central 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.

Mosquito collection

An. gambiae s.l. mosquitoes were collected from March to July 2012 during the first rainy season in Glazoué district selected in the central part of the country. *Anopheles* pre-imaginal stages (L1 to L4 instars) were collected via ladles within cotton farms from Hlassoé using the dipping method on several breeding sites. Once, larvae and pupae collected, they were then kept in labeled bottles related to the Glazoué 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. *An. gambiae s.l.* 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 s.l.* aged 2 to 5 days old were exposed to WHO diagnostic dosage of cyfluthrin 0.15% 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 post treatment were recorded following the WHO protocol (WHO, 1998). Dead and surviving mosquitoes were separately stored in individual tubes with silicagel and preserved at -20°C in the laboratory, for further molecular characterization. We used cyfluthrin, 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 *An. gambiae* complex collected in Glazoué district. 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, 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 rates in control tube was less than 5% (Abbott WS, 1987).

Molecular results (*kdr* frequency) 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 localitiy as a covariate. A logistic regression was performed with survivors' rate as interest variable and the carrying or not of *Kdr* gene variable as variation source. The software R-2.15.2. (R Development Core Team, 2011) was used for the statistical analysis.

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 s.l.* Kisumu which were exposed to WHO papers impregnated with cyfluthrin 0.15% were susceptible to this product with the mortality rate of 100%. Regarding *An. gambiae s.l.* populations from Hlassoé, they were resistance to cyfluthrin with the mortality rate of 82% (Table 1). Univariate logistic regression, performed with mortality rate as the dependent variable and locality as a covariate with ANOVA test showed that the cyfluthrin phenotypic resistance 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). A logistic regression was also performed with survivors' rate as interest variable and the carrying or not of *Kdr* gene variable as variation source and showed that the carrying of *Kdr* gene by a mosquito is not systematically synonymous of cyfluthrin phenotype resistance (OR=1.3 [0.7-2.41], p= 0.411).

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.* Hlassoé at allelic frequency of 100% (Table 2).

Anopheles gambiae s.l. populations from Hlassoé were resistant to cyfluthrin and the resistance level recorded may be explained by increased use of various insecticidal products (including pyrethroids) for crop protection, mainly for cotton protection. In fact, Akogbéto *et al.* (2006) showed that after pesticide treatments in agricultural settings, residues of insecticides get into mosquito breeding sites. These residues have lethal effects on larvae of some populations of mosquito whereas they exert a selective pressure on other populations, leading to a gradual tolerance of insecticide concentrations and to the emergence of resistant populations.

The alterations at site of action in the sodium channel, viz the *kdr* mutations in *Anopheles gambiae s.l.* mosquitoes from Hlassoé was not likely the only resistance mechanism involved in these mosquitoes populations. In fact, very high *kdr* allelic frequency was observed in *An. gambiae s.s.* Hlassoé populations and might likely be explained by absence of metabolic-based resistance.

The *kdr* frequency recorded in *Anopheles gambiae* populations from Glazoué in 2008 were 0.10 [Djogbenou *et al.*, unpublished data]. In the current study, the *kdr* frequency recorded in these same *Anopheles gambiae* populations were 1. 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 *An. gambiae* following a nationwide distribution of long-lasting insecticide-treated nets in Niger (Czeher *et al.*, 2008) and in Benin (Aïzoun *et al.*, 2014a). The carrying of *Kdr* gene by a mosquito is not systematically synonymous of insecticide phenotype resistance.

In the current study, all *Anopheles gambiae* Hlassoé specimens tested were *Anopheles gambiae s.s.* No *An. arabiensis* mosquitoes were found. Similar remark was already made with *An. gambiae* populations from Kandi resistant to lambdacyhalothrin in northern Benin (Aïzoun and Azondekon, 2014b). This result showed that *An. arabiensis* populations from Glazoué district tend to decline after four years. In fact, Djogbénou *et al.* [unpublished data] have found 91.9% of *An. arabiensis* among *Anopheles gambiae* complex in this district in 2008.

The current study clearly shows that the carrying of *Kdr* gene by a mosquito is not systematically synonymous of insecticide phenotype resistance. However, the presence, though at high frequency, of the West African *kdr* mutation in *Anopheles gambiae* populations from Hlassoé needs to be carefully monitored.

Table.1 Percentage of dead *Anopheles gambiae s.l.* observed after 1 hour exposure to WHO papers impregnated with cyfluthrin 0.15% in Hlassoé locality.

Populations	Insecticides	Number tested	% Mortality	Resistance status
Kisumu (Control)	Cyfluthrin	92	100	S
Hlassoé	Cyfluthrin	100	82	R

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*)
Hlassoé	35	35	35	0	0	1

Ag: *An. gambiae s.s.*

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