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Susceptibility to DDT and pyrethroids, and detection of knockdown resistance mutation in *Anopheles gambiae sensu lato* in Southern Togo

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ABSTRACT

Insecticides play an important role in malaria control programmes especially in Sub-Saharan Africa. However, the development of resistance by vectors to most commonly used public health insecticides has become a very crucial problem. DDT (Dichlorodiphenyltrichloroethane) was used worldwide to control malaria vectors and pyrethroids have been used in many vector control projects due to their known efficiency and lower toxicity to humans and non-target organisms. In West Africa, resistance to pyrethroid in the major malaria vector, *Anopheles gambiae*, had been reported in several countries. The last publication on this phenomenon in Togo was done in Togo in 2005. An update on the susceptibility of the malaria vector and characterization of its resistance mechanism becomes therefore crucial. Newly emerged female mosquitoes of two to five days old were selected and exposed to DDT 4%, Permethrin 1% and Deltamethrin 0.05%, the WHO diagnostic doses. These adult mosquitoes were from larvae of *Anopheles gambiae sensu lato* collected from rural and urban settings and reared in an insectary. A susceptible laboratory strain of *An. gambiae sensu stricto* was used as reference. Species identification was made using the morphological characteristics, PCR (polymerase chain reaction), and *Hha*I (*Haemophilus haemoliticus*) restriction digest. Knockdown resistance mutation screening was conducted on both living and dead mosquitoes. *An. gambiae sensu stricto* was the only sibling species of the complex present in Kovié and Lomé. The molecular M form was predominant in the two localities (almost 100%). High resistance level was observed in Lomé 1.19%, 56.45% and 41.17%; and in Kovié 0.91%, 55.55% and 67% mortality respectively to DDT 4%, Deltamethrin 0.05% and Permethrin 1% with a very high knockdown time (TKD_{50}). Up to 70% of resistant individuals have been obtained in the two localities. It is important to inspect these localities through routine tests in order to decide on possible alternative strategies to be used. This study showed that *An. gambiae* is highly resistant to both DDT and pyrethroids in Lomé and Kovié, with the presence of *kdr* allele.

Keyword: *Anopheles gambiae* s.s, DDT, pyrethroids, *kdr*, Southern Togo.

INTRODUCTION

During the World Health Organization's Global Malaria Eradication Program between 1955 and 1969, insecticide spraying led to significant, and in some instances, sustained reductions in the disease burden in many endemic regions, including countries on the Indian sub-continent and parts

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of South America (Trigg and Kondrachine, 1998; Cueto 2007). But very soon, resistance to insecticides has appeared in the major arthropod vectors from almost every genus and in 2001, it was reported that this concerned 540 species of arthropods, of which 198 were of medical and veterinary importance (Bills, 2001). The first case of DDT resistance was reported in *Aedes* mosquitoes in 1947 (Brown, 1986).

In many African countries, *Anopheles gambiae* s.l. is developing resistance to all classes of insecticides used for mosquito control (Etang et al., 2006; Djogbénou et al., 2007; Adasi and Hemingway, 2008; Ndjemaï et al., 2008; Kerah-Hinzoumbé et al., 2008; Djègbè et al., 2011; Dabire et al., 2012). Among these, pyrethroids are the only option for nets treatment due to their relative safety for humans at low dosage, excito-repellent properties, rapid rate of knock-down and killing effects (Zaim et al., 2000). The use of both DDT and more recently pyrethroids in the control of rice and cotton pests is likely to have contributed significantly to the development of resistance in *An. gambiae* from West Africa (Martinez-Torres et al., 1998; Chandre et al., 1999; Diabate et al., 2002; Yadouleton et al., 2011).

In order to control malaria, the National Malaria Control Programme (NMCP) of Togo has been promoting artemisinin-based combination therapy (ACT) since 2006 and a large scale use of Insecticide treated Nets (ITNs) as the main vector control tool but little is known on the vectors responsible for malaria transmission in the country. *An. gambiae* s.s, *An. arabiensis* and *An. melas* are known to be the main vector in malaria parasite transmission in Togo (Akogbéto and Di Deco, 1995; Ketoh et al., 2005; Ahadji-Dabla, 2006) and the first report on resistance was only well known in 2003 (Ketoh et al., 2005). Considering the burden of malaria in the country and the number of cases each year, a comprehensive knowledge of the factors underlying the resistance is needed for the implementation of efficient vector control programmes including resistance management strategies. There is therefore a need for countrywide and regular surveys to monitor the insecticide susceptibility status of major vectors, detect resistance genes and assess their implications on vector control activities (Kelly-Hope et al., 2008).

The resistance also termed *kdr* (knock down resistance) which resulted from a single point mutation of a leucine amino acid to phenylalanine (Leu/Phe) was identified in West Africa (Martinez-Torres et al., 1998). In East Africa, a second *kdr* mutation in the same amino acid resulted in leucine-serine (Leu/Ser) substitution (Ranson et al., 2000) was also identified. In both cases, this resistance occurs with DDT and pyrethroids and the sodium channel gene is the target site. Both insecticides are commonly used in Togo for crop protection (DESA, 1990) and pyrethroids are incorporated in the new generation nets known as Long Lasting Insecticide Treated Nets (LLINs) used in malaria vector control through large-scale distribution programmes. With regard to the role of DDT and pyrethroids, it was important to carry out this study to determine DDT and pyrethroids susceptibility status and resistance mechanism in malaria vectors in southern Togo for a sustainable vector control programme implementation.

MATERIALS AND METHODS

Study sites

The study was carried out in November and December 2009 in Lomé and Kovié (Figure 1). Both localities belong to the Southern coastal region (Maritime region) with an annual rainfall of about 1000 mm; the mean temperature is 28 ºC. Kovié is a rural setting located in an area where irrigated rice-growing is the main agricultural activity. Mosquitoes were sampled near the Zio River in a rice field (N 06°20’87’’; 01°7’24’’ E). Lomé, the capital city of Togo, is the urban setting located on the Gulf of Guinea (06° 07’30’’N; 01° 13’34’’E) and covers an area of 333km^2. The poor

urbanization management of Lomé over the past years has created favourable conditions for the development of mosquito species. The climatic conditions in Lomé are similar to those in Kovié. Insecticides are essentially used for market gardening in the outskirts of Lomé (DESA, 1990; Mondédji, 2010).

Mosquito sampling

Wild larvae of *Anopheles gambiae* s.l. mosquitoes were collected from anophelines breeding sites in each locality. All the larvae from each location were then locally reared to adults stage. Emerging adults were provided with a 10% sugar solution. Adult mosquitoes were sexed and morphologically identified (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987) and only females *An. gambiae* s.l. were used for insecticide susceptibility tests. A laboratory susceptible reference strain of *An. gambiae* (Kisumu) was used to compare the susceptibility levels of wild populations.

Susceptibility tests

Bioassays were carried out using WHO test kits for adult mosquitoes (WHO, 1998). Impregnated papers were provided by "Laboratoire de lutte contre les Insectes Nuisibles" of IRD (Montpellier, France), a WHO Collaborating Centre. Two to five day-old female mosquitoes were selected and exposed to diagnostic doses of DDT (4%), permethrin (1%) and deltamethrin (0.05%). Each test on a batch of 20-25 mosquitoes was replicated four times with different mosquitoes to account for inter-batch variability. The number of knock down mosquitoes was recorded every 10 min during exposure. Controls made up of Kisumu susceptible and wild mosquitoes were exposed to papers treated with solvent only. The mosquitoes were transferred into WHO observation insecticide free tubes and maintained on sucrose solution after 1 h exposure. Mortality was recorded 24 h post exposure. Dead and survivor mosquitoes were stored separately at −20 °C on silica gel for PCR analysis. The knockdown times for 50% (KDT_{50}) and 95% (KDT_{95}) of tested mosquitoes were calculated.

Molecular identification and detection of *kdr* mutations

For each locality a representative sample drawn from the unexposed mosquitoes, bioassays controls (Lomé: N= 125; Kovié: N= 70), being tested were identified according to species and molecular forms using the PCR-RFLP (Polymerase Chain Reaction – Restriction Fragment Length Polymorphism) technique (Fanello et al., 2002) after DNA extraction according to Collins et al. (1987). Polymerase chain reaction diagnostic tests for detection of *kdr* "Leu-Phe" mutation were done on all the surviving and eventually on the dead individuals (Martinez-Torres et al., 1998).

Data analysis

The resistance/susceptibility status of the tested populations was determined for each insecticide according to WHO criteria (WHO, 1998). According to these criteria, a resistant population is defined by mortality rates less than 80% after a 24 h observation period while mortality rates greater than 98% are indicative of susceptible populations. Mortality rates between 80-98% suggest a suspected resistance that requires confirmation. The various times for 50% and 95% knockdown (KDT_{50} and KDT_{95}) of tested mosquitoes were estimated using Win DL (Giner et al., 1999), a log-time probit software based on Finney (1971). When the control mortality was between 5 and 20%, the mortality rate in tested samples was corrected using Abbott formula (Abbott, 1925):

$$\text{Susceptibility} = \frac{100 - \%\text{Test Mortality}}{100 - \%\text{ Control Mortality}} \text{X } 100$$

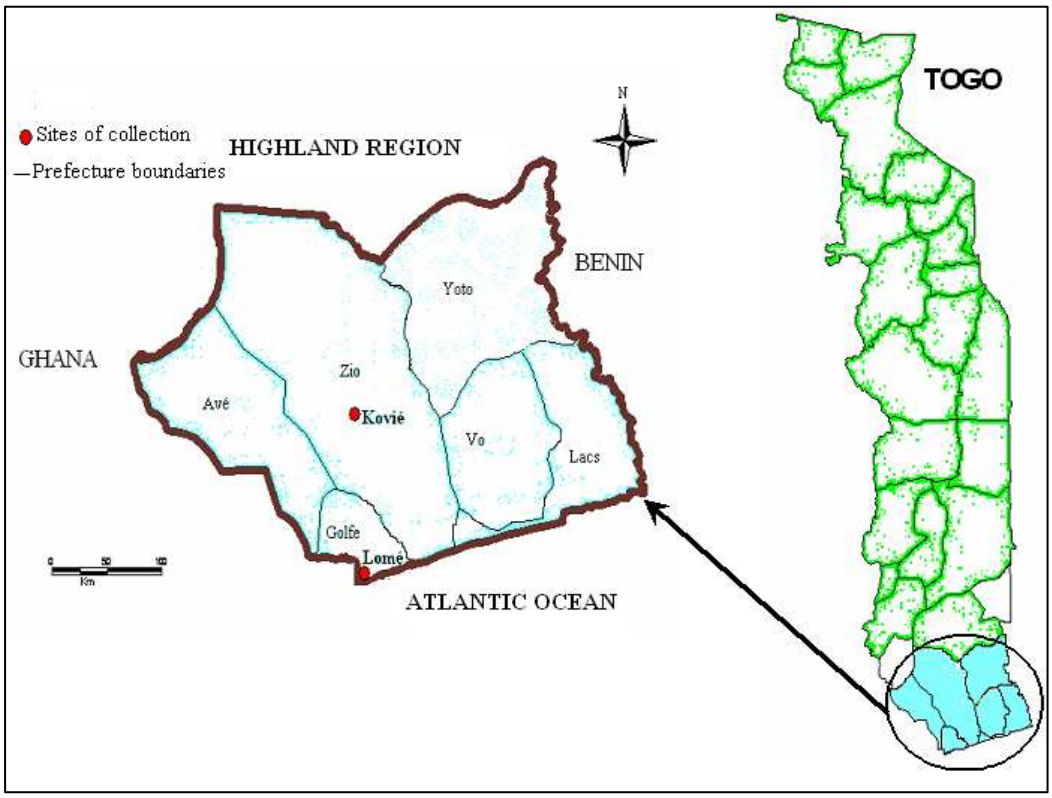

Figure 1: Map of southern Togo showing the study sites (Kovié and Lomé).

RESULTS

Susceptibility Tests

A total of 607 wild population of *An. gambiae* complex were tested including that of DDT4%. Because of the very few mortality observed with DDT4% (1.19% in Lomé and 0.91% in Kovié), its knockdown times are not taken into consideration here. Only the mortality rates are presented in Figure 2. The reference strain showed 100% mortality at diagnostic concentrations of DDT, deltaméthrin and permethrin. No complete susceptibility to pyrethroids was recorded in both localities. All populations showed resistance to both pyrethroids tested with mortality of 56.45% in Lomé and 55.55% in Kovié for deltamethrin (Table 1) and 41.17% in Lomé and 66.99% in Kovié for permethrin (Table 2).

Resistance to deltamethrin and permethrin in Lomé were respectively 2.75 fold ($KDT_{50}R$) and 11.89 as against 3.34 and 4.15 in Kovié. Pyrethroid resistance seems to be associated to knockdown times; less than 85% of the mosquitoes were knocked down at the end of the exposure period for both insecticides. This showed increased resistance among the populations (Tables 1 and 2). The mortality is however less than 70% with each insecticide in the two localities.

Mosquito species, molecular forms and *kdr* mutation

About 195 individuals of *An. gambiae* s.l samples examined by PCR were successfully identified to species and molecular forms. Only *An. gambiae* s.s was obtained; No other species of the complex was identified in the sampling sites. The characterization by *Hha*I restriction digest to distinguish between the molecular M and S forms revealed that the M form was

predominant in Lomé (>99%), only one hybrid specimen M-S (0.8%) was identified while in Kovié, 100% M form were identified (Table 3).

Molecular assays detected homozygote L1014F *kdr* mutation in 130 mosquitoes that is 99 (79.2%) from Lomé and 31 (73.8%) from Kovié. Twenty five (25) and eight (8) individuals, *kdr/kds* were obtained respectively in Lomé and Kovié. Only 7.14% and 0.8% of these specimens carried the susceptible allele respectively in Kovié and Lomé (Table 4).

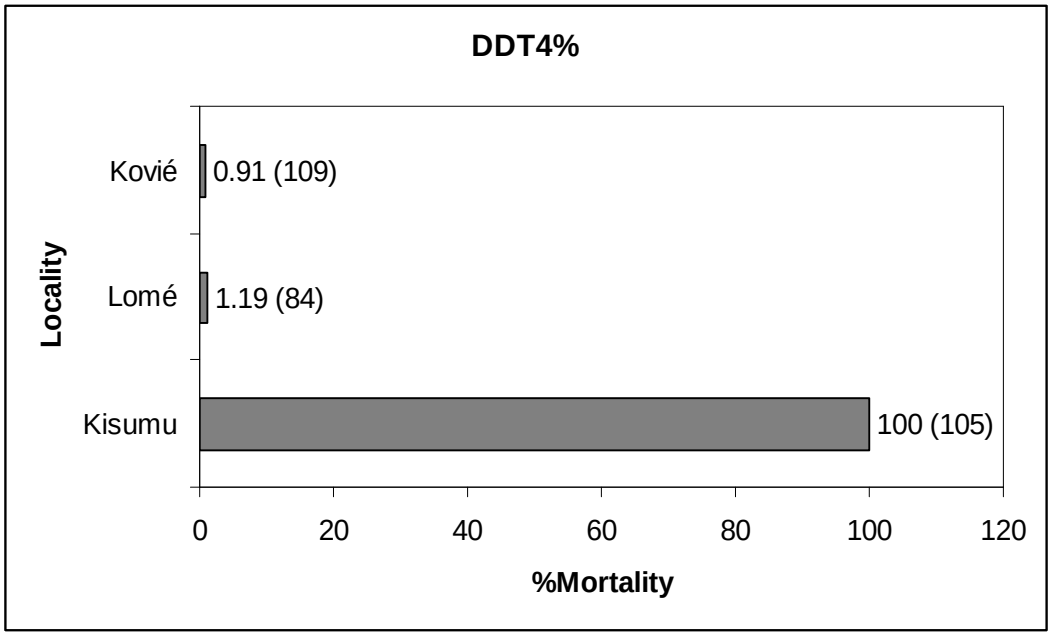

Figure 2: Mortality recorded with DDT 4% in Lomé and in Kovié. Number of individuals in parenthesis.

Table 1: Knockdown times (KDT) and mortality rates of *An. gambiae* s.l. populations exposed to deltamethrin 0.05%.

Site	N	Mortality (%)	%KD after 1h exposure	KDT_{50} in min (95% CI)	KDT_{95} in min (95% CI)	$KDT_{50}R$*
Lomé	101	56.45**	81.18	44.38 (39.94-49.41)	79.32 (66.17-115.26)	2.75
Kovié	108	55.55**	50	53.78 (50.65-57.95)	109.1 (93.45-136.88)	3.34
Kisumu susceptible strain	103	100	100	16.09 (10.72-20.18)	35.59 (28.25-54.66)	

* $KDT_{50}R$: KDT_{50} of the tested population divided by KDT_{50} of the Kisumu susceptible strain
**: Resistant (WHO, 1998)

Table 2: Knockdown times (KDT) and mortality rates of *An. gambiae* s.l. populations exposed to permethrin1%.

Site	N	Mortality (%)	%KD after 1h exposure	KDT_{50} in min (95% CI)	KDT_{95} in min (95% CI)	$KDT_{50}R$*
Lomé	102	41.17**	6.86	194.32 (107.59-473.09)	699.5 (231.1-3057)	11.89
Kovié	103	66.99**	32.03	67.96 (63.18-79.62)	100.32 (83.88-153.64)	4.15
Kisumu susceptible strain	100	100	100	16.34 (11.15-20.3)	34.46 (27.7-52.57)	

* $KDT_{50}R$: KDT_{50} of the tested population divided by KDT_{50} of the Kisumu susceptible strain
** Resistant (WHO, 1998)

Table 3: Distribution of the molecular forms of *An. gambiae* s.s. in Lomé and in Kovié.

Site	M	M-S	S	Total
Lomé	124 (99.2%)	1 (0.8%)	0	125
Kovié	70 (100%)	0	0	70

Table 4: Distribution of the *kdr* allele in Lomé and in Kovié.

Site	RR	RS	SS	Total
Lomé	99 (79.2%)	25 (20%)	1 (0.8%)	125
Kovié	31 (73.8%)	8 (19.04%)	3 (7.14%)	42

DISCUSSION

This study revealed the existence of DDT, permethrin and deltamethrin resistance in *An. gambiae* s.l from southern Togo. The very high resistance level of *An. gambiae* to DDT observed could be attributed to the extensive use of DDT in the past. In fact, some years after the discovering of this organochlorine insecticide, the colonial administration in Togo decided to use it for vectors control in Lomé the capital city. This led to the establishment in 1952 of the "Malaria Control Service" that later became the "Malaria National Service" and is now the National Malaria Control Programme (NMCP). In addition, the development of market gardening since 1986, year of rapid extension of Lomé, reinforced the use of organochlorines and pyrethroids for pests control the in sector. Indeed, coils and bomb sprays are also frequently used for personal protection against mosquito bites (Amoudji, 2011). Moreover, the implantation of illicit insecticides selling markets in the country especially around rice cultivation areas, contributed to the increase in the resistance. In many African countries, resistance to pyrethroids has been attributed to extensive use of these compounds in agriculture (Diabate et al., 2002; Yadouleton et al., 2011; Antonio-Nkondjio et al., 2011). The insecticide is drawn in the water that is the larvae breeding site. Further studies involving social scientists, chemical ecologists and environmental biologists would be needed to document the amount, frequency and diversity of insecticides used in these areas in order to further explore the putative selective pressures leading to the selection of insecticide resistance in malaria vectors. In this study,

pyrethroid resistance matches *kdr* mutation, this is proven by the high knockdown time ratios ($KDT_{50}R$). This situation was also reported in many West African countries where high *kdr* frequencies of >90% have been detected in *An. gambiae* in Côte d'Ivoire, Burkina Faso, Benin, Ghana and Nigeria (Yawson et al., 2004; Awolola et al., 2005; Corbel et al., 2007; Djogbénou et al., 2007; Dabire et al., 2012, Yadouleton et al., 2011; Oduola et al., 2010; Koffi et al., 2012). It has also been detected recently in Togo (Ketoh et al., 2009). The *kdr* mutation has also been reported in other sub-Saharan African countries such as Equatorial Guinea and Cameroon (Reimer et al., 2005, Etang et al., 2006, Nwane et al., 2009). Though the resistance is observed in this study, deltamethrin was the most effective and had the least resistance ratio at comparable toxicity.

An. gambiae exists as a species complex, with seven sibling species that are closely related and morphologically indistinguishable by routine taxonomic methods, and yet distinctly different with respect to ecological and behavioural characteristics and vectorial competence (White, 1974). Among them *An. gambiae* s.s. and *An. arabiensis* are the major vectors of malaria transmission in West Africa, with the former being more efficient due to its high degree of anthropophily (Pates et al., 2001; Besansky et al., 2004). Recently, Ketoh et al. (2009) reported that *An. gambiae* s.s was more abundant in Lomé, where its two molecular forms were found to occur in sympatry with *An. arabiensis*. In line with this study, *An. arabiensis* was not found basically because of the sites where the larvae were collected. The molecular M form of *An. gambiae s.s* was highly predominant and represented >99% of all samples analyzed. Previously, a study showed the S form is predominant in the highland region (Ketoh, personal communication.). The role of habitat selection by species preferentially choosing and occupying a non-random set of available habitats greatly influences species interactions and is of major importance for the interpretation of spatial and temporal distributions of populations and a variety of other ecological phenomena such as species divergence and diversification (Morris, 2003). This invariably influences the spatio-temporal distribution of malaria vectors in sub-Saharan Africa where different eco-geographical zones exist (Adasi and Hemingway, 2008). The M-S hybrid form observed in Lomé was about 0.8%. Hybrids are naturally rare, but heterogamous insemination of ~1% has been observed (Tripet et al., 2001), demonstrating the existence of incomplete premating barriers (Della Torre et al., 1997).

The level of pyrethroid resistance observed in *An. gambiae s.s.* populations in this study has implications for the ITN component of the nation's malaria control programme. This awareness could guide the choice of appropriate LLINs in large scale distribution and the doses of pyrethroids for bed net treatment. Furthermore, we would recommend that insecticide susceptibility studies should be conducted in other regions of the country.

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