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SHORT REPORT: FIRST REPORT OF KNOCKDOWN MUTATIONS IN THE MALARIA VECTOR *ANOPHELES GAMBIAE* FROM CAMEROON

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Abstract. We report the first finding of the knockdown Leu-Phe and Leu-Ser mutations associated with resistance to pyrethroids and DDT insecticides in the malaria mosquito *Anopheles gambiae* from Cameroon. The Leu-Phe mutation was found in both the M and S molecular forms of *An. gambiae*. Importantly, two specimens of the S molecular form were found to carry both mutations in a heterozygous state.

The spread of pyrethroid resistance in *Anopheles gambiae* is a major concern for malaria prevention in Africa, which essentially relies on the use of insecticide treated materials or indoor residual spraying.[1] Resistance mechanisms include metabolic detoxification through increased enzyme activities and target site insensitivity due to mutations at the DNA level.[2] One of the most well-characterized pyrethroid resistance mechanisms in *An. gambiae* is due to a single nucleotide polymorphism in the gene encoding subunit 2 of the sodium channel. This mutation, known as the knockdown (*kdr*) mutation, leads to the substitution of a leucine (TTA) for phenylalanine (TTT) and is widespread in west Africa, where it is strongly associated with the S molecular form of *An. gambiae*.[3,4]

More recently, a second *kdr* mutation in the same amino acid, changing the leucine (TTA) to a serine (TCA) was described from east African populations.[5] Although data on malaria vector susceptibility to insecticides are scarce for countries in central Africa, recent surveys conducted in Cameroon demonstrated various levels of resistance in *An. gambiae* populations from Cameroon, but *kdr* mutations were never detected.[6–8] Here, we report heterogeneous patterns of resistance to DDT and pyrethroids in three areas in Cameroon. We also provide the first evidence for *kdr* mutations in both the M and S forms of *An. gambiae* s.s.

The survey was undertaken between May 2003 and September 2004. Mosquito larvae were collected in three areas: 1) Foumbot (5°31′N, 10°37′E), an area of intensive gardening in the mountain grasslands of the western region (altitude = 1,100 meters, mean annual rainfall = 1,721 mm), 2) Bonassama district (3°51′N, 10°08′E) in Douala, a city in the northern coastal region (altitude = 18 meters, mean annual rainfall = 4,110 mm), and 3) Campo (2°22′N, 9°50′E) in the southern coastal region (altitude = 25 meters, mean annual rainfall = 2,804 mm). Field-collected larvae were reared to adults, and emerging specimens were identified morphologically.[9] Susceptibility of field *An. gambiae* populations to 4% DDT, 1% permethrin, 0.05% deltamethrin, or 0.05% lambdacyhalothrin was assessed using World Health Organization (Geneva, Switzerland) test kits according to standardized procedures.[10]

Times for 50% knockdown (KdT_{50}) and mortality rates 24 hours post-exposure are shown in Table 1. Resistance to DDT was observed in Foumbot and Douala with less than 35% mortality rates and increased KdT_{50} up to the loss of the knockdown effect in Foumbot. This population was also resistant to permethrin and deltamethrin (50–90% mortality) while in Douala, mosquitoes were resistant to lambdacyhalothrin (90% mortality) but susceptible to deltamethrin. In Campo, a slight decrease in susceptibility to DDT and permethrin (> 95% mortality) was observed.

DNA was extracted from survivors to the bioassays and from at least 30 (unexposed) control specimens per site. Mosquito species and molecular forms were simultaneously determined using a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay.[11] All mosquitoes tested were *An. gambiae* s.s., in agreement with the known geographic distribution of species within the *An. gambiae* complex in Africa.[12] Only the S molecular form was found in the Foumbot sample (n = 60), while all specimens from Douala were of the M form (n = 80). In Campo, both molecular forms were found (n = 60) and no hybrid was observed, as previously reported in Cameroon.[13] The S form was predominant (46 of 60 = 77%) over the M form (14 of 60 = 23%).

The *kdr* genotypes of survivors were determined using the recently described hot ligation assay (HOLA).[14] A high frequency of the *kdr* Leu-Phe mutation was observed in S form mosquitoes from Foumbot; 21 of 27 survivors showed the resistant allele in the homozygous state. Similar findings were

TABLE 1

Knockdown times and mortality rates in field populations of *Anopheles gambiae* s.s. in Cameroon to 4% DDT, 1% permethrin and 0.05% deltamethrin or 0.05% lambdacyhalothrin*

Sites	Insecticides	No.	KdT_{50} (95% CI)	Mortality (%)
Foumbot	4% DDT	162	No Kd	6.8
	1% permethrin	159	61.0 (55.3–75.2)	54.7
	0.05% deltamethrin	165	22.5 (21.7–23.3)	87.9
Douala	4% DDT	100	58.0 (53.3–64.7)	32.3
	0.05% lambdacyhalothrin	99	11.7 (11.0–12.3)	90.9
	0.05% deltamethrin	100	7.3 (6.7–7.8)	100
Campo	4% DDT	157	29.1 (21.8–33.6)	96.8
	1% permethrin	159	10.0 (8.4–11.5)	99.4
	0.05% deltamethrin	158	8.4 (5.8–10.7)	100

* KdT_{50} = knockdown time (minutes) for 50% of mosquitoes; CI = confidence interval; mortality = mortality rate 24 hours post-exposure.

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TABLE 2

Knockdown (*Kdr*) genotypes in M and S molecular forms of *Anopheles gambiae* s.s. surviving exposure to insecticides in Cameroon

Kdr genotype*	Foumbot (S form)	Douala (M form)	Campo (S form)
TTA/TTA (SS)	1	7	2
TTA/TTT (SRw)	1	3	4
TTA/TCA (SRe)	1	0	0
TTT/TTT (RwRw)	3	0	0
TTT/TCA (RwRe)	2	0	0
TCA/TCA (ReRe)	1	0	0
Total	9	10	6

* S = susceptible allele, Rw = kdr leucine-phenylalanine allele, Re = kdr leucine-serine allele.

reported in several west African countries.[4,7,15,16] Moreover, two specimens from this sample displayed both *kdr* Leu-Phe and Leu-Ser alleles in a heterozygous state. The *kdr* Leu-Phe allele was also observed in S form mosquitoes from Campo and in the M form from Douala, although at lower frequencies (6 of 21 and 3 of 41 specimens, respectively) and only in the heterozygous state. The PCR products of 25 specimens from the three areas were cloned using the Topo TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced using primer T7 to confirm results obtained with the HOLA assay on a representative sample. As expected, the resistant alleles were characterized by an A → T or T → C substitution (Table 2), leading to Leu-Phe and Leu-Ser substitutions, respectively, at the protein level. These results validated the HOLA protocol for use in *An. gambiae* field specimens from Cameroon. Investigations are underway to provide more precise assessment of the frequency and geographic distribution of both *kdr* mutations in wild *An. gambiae* populations from Cameroon.

Our data suggest that DDT and pyrethroid resistance in *An. gambiae* from Foumbot is mainly due to *kdr* mutations. However, lower allelic frequencies of *kdr* in coastal areas indicate that other resistance mechanisms are involved, such as metabolic detoxification, especially in Douala where high levels of glutathione S-transferase and esterase activities were previously reported (Etang J, unpublished data). Together with recent reports of the *kdr* Leu-Phe mutation in the M form of *An. gambiae* from neighboring Benin and the Central African Republic,[17] Nigeria,[18] and the island of Bioko,[8] the present report from Cameroon confirms the ongoing spread of *kdr*-based pyrethroid resistance in Africa. This emphasizes the need for close monitoring of resistance to elaborate appropriate strategies for malaria vector control.

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