

Identification and Geographic Distribution of the *ACE-1^R* Mutation in the Malaria Vector *Anopheles gambiae* in South-Western Burkina Faso, West Africa

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Abstract. Resistance of *Anopheles gambiae* to organophosphate and carbamate insecticides was first reported in Côte d'Ivoire, West Africa. Subsequent studies revealed that it resulted from a single point mutation in the oxyanion hole of the acetylcholinesterase enzyme (*ace-1^R* mutation). We investigated the distribution and prevalence of the *ace-1^R* mutation in *An. gambiae* s.l. populations from seven locations in south-western Burkina Faso. The *ace-1^R* mutation was found in both M and S molecular forms of *An. gambiae* s.s., but it was absent in *An. arabiensis*. Its frequency ranged from 0.25 to 0.5 in S form and 0.04 to 0.13 in M form, though they were sympatric. The lack of homozygous resistance indicated a strong genetic cost associated with the mutation. These data suggest that organophosphate and carbamate resistance conferred by target site insensitivity is spreading in populations of *An. gambiae* s.s. from West Africa.

INTRODUCTION

Anopheles gambiae Giles, the major malaria vector in West Africa, has been subjected to selection pressure by DDT and, more recently, pyrethroids through use of these insecticides for both agricultural and public health purposes.^{1,2} The intensive application of these chemical insecticides led to the development of resistance in many insects including *Anopheles* mosquitoes. The first case of resistance to pyrethroids in *An. gambiae* was reported in 1993.³ This resistance results from a single point mutation (leucine TTA to phenylalanine TTT) in the gene that encodes the sodium channel, and confers the characteristic “knockdown resistance” or *kdr* phenotype.⁴ This mutation is widespread in West Africa and may compromise the use of pyrethroids for public health.^{1,5–7} Some alternative solutions to pyrethroids are therefore necessary.^{8,9}

Preliminary experiments have already been done to test organophosphates (OP) or carbamates (CX) as alternatives to pyrethroids on insecticide-treated nets.¹⁰ However, insensitivity of wild *An. gambiae* to both insecticides, which target acetylcholinesterase (AChE), has been recently reported in West Africa.¹¹ Sequencing of this target site identified a single mutation, (glycine to serine at position 119) in the oxyanion hole of the acetylcholinesterase enzymes that was associated with insensitivity of *An. gambiae* to OP and CX.¹² This mutation, named *ace-1_G119S* or *ace-1^R* (using the *Torpedo* nomenclature), was the same described in *Anopheles albimanus* Weidemann and *Culex pipiens* Say.^{13,14} A Polymerase Chain Reaction (PCR)-based assay was devised to detect its presence in *An. gambiae*, *An. Albimanus*, and *Cx. pipiens*.¹⁴

This study is designed to evaluate the presence and extent of the distribution of the *ace-1^R* mutation within and among *An. gambiae* s.l. populations in south-western Burkina Faso where pyrethroid resistance was also reported in *An. gambiae*.^{2,15}

MATERIALS AND METHODS

Study area. The study was carried out in seven locations in south-western Burkina Faso, based on their different patterns of insecticide use: i) VK7, a village located on the outskirts of rice fields (11°24'N; 4°24'E); ii) two cotton-growing areas, Samandeni (11°27'N; 4°27'E) and Seguere (11°30'N; 4°24'E); iii) two sites in the urban area of Bobo-Dioulasso (11°10'N; 4°17'E) where more than 95% of the households use coils frequently to protect themselves against mosquito bites: Dioullassoba (Bobo 1), located in the center of the city and Kuinima (Bobo 2) on the outskirts; iv) Yegueresso (11°09'N; 4°10'E) and Darsalamy (11°02'N; 4°21'E), two villages not far from the urban area of Bobo-Dioulasso. Yegueresso and Darsalamy are sites with limited use of insecticides because agriculture is done at the subsistence level (e.g., beans and maize). All of these sites were located in the same climatic area (annual rainfall 1,000 mm) (Figure 1).

Mosquito collections. Larvae of *An. gambiae* s.l. were collected in October 2005 (end of the rainy season) and March 2006 (dry season) and were transferred to the IRSS (Institut Régional des Sciences de Santé) laboratory for adult emergence. *An. gambiae* adults were visually sorted according to morphologic identification keys and were kept at –20°C for molecular analysis.

DNA extraction and PCR identification of the *An. gambiae* M and S. Genomic DNA was extracted from individual mosquitoes using the protocol slightly modified from Collins and others.¹⁶ Species of the *An. gambiae* complex and molecular forms of *An. gambiae* s.s. were identified using the methods of Scott and others and Favia and others, respectively.^{17,18}

PCR identification of *ace-1^R* mutations of acetylcholinesterase gene of *An. gambiae*. The *ace-1* gene was amplified by PCR with Ex3AGdir 5'GATCGTGGACACCGTGTTCG3' and Ex3AGrev 5'AGGATGGCCCGCTGGAACAG3' oligonucleotide primers.¹⁴ PCR was performed in 25 µL volumes containing 2.5 µL of 10X *Taq* DNA polymerase buffer, 200 µM deoxynucleoside triphosphate (dNTP), 0.1 U of *Taq* DNA polymerase (Qiagen, France), 10 pmol of each primer, and 1 to 10 ng of extracted DNA. PCR conditions included an initial denaturation step at 94°C for 5 min followed by thirty

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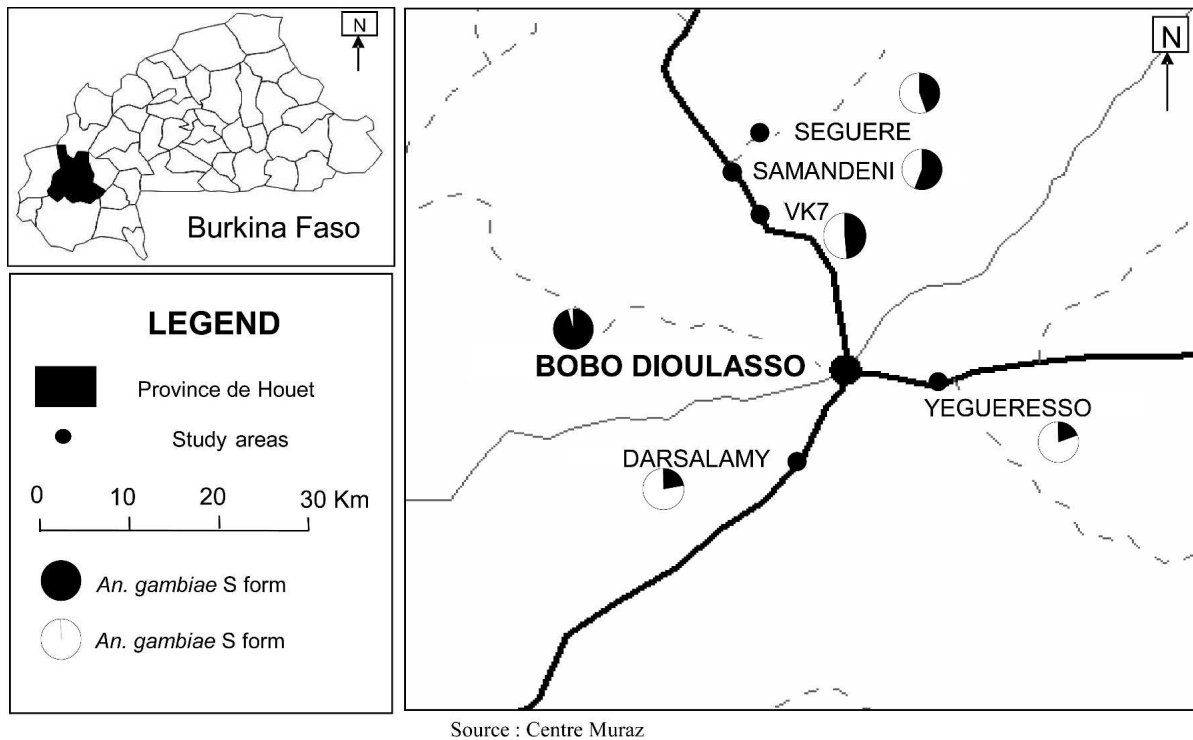


FIGURE 1. The study areas map showing the relative frequency of M and S molecular forms of *An. gambiae* s.s.

five cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. Fifteen microliters of this product were digested with 5 U of *AluI* restriction enzyme (Promega, France) in a final volume of 25 μ L. The PCR amplification products were analyzed by electrophoresis onto a 2% agarose gel stained with ethidium bromide and visualized under UV light.

Statistical analysis. Conformity of *ace-1^R* frequency with Hardy Weinberg expectations was tested for each population using the exact probability test.¹⁹

RESULTS

The numbers and species of mosquitoes collected are shown in Table 1. A total of 492 mosquitoes were identified to species level and to molecular form (Table 1). Two species of the *An. gambiae* complex were recorded and found in sympatry in six locations out of seven. Overall *An. gambiae* s.s. was predominant with 61%, but *An. arabiensis* (Patton) occurred at a significantly higher frequency in Darsalamy, Yegueresso, Bobo Dioulasso, and Seguer (> 58% in these breeding sites) as opposed to Samandeni (4%) and VK7 (0%). The two molecular forms of *An. gambiae* s.s. were sympatric in all locations, except in Bobo 2 where only S form was identified (Table 1). The S form predominated in urban areas at frequencies of 95–100%. The M form was predominant in Darsalamy and Yegueresso.

The *ace-1^R* mutation was detected in *An. gambiae* s.s. only and was present in all sites, regardless of the different patterns of insecticide use. Its frequency was higher in the urban, cotton, and rice-growing areas, compared with Yegueresso and Darsalamy where insecticide use is limited (Table 2). The *ace-1^R* allele is present in both M and S molecular forms of

An. gambiae but is more frequent in the S form (Table 2). In addition, few specimens analyzed were homozygous for the *ace-1^R* mutation (only 3 of 131 resistant mosquitoes recorded from all sites). A significant departure from Hardy Weinberg equilibrium was observed in some samples because of a strong deficit of homozygous-resistant individuals (Table 2).

DISCUSSION

Pyrethroid-treated bednets remain one of the major tools for malaria vector control in tropical areas. However, the spectrum of resistance to pyrethroids calls for alternative and complementary solutions. Even if they are not recommended for bednets impregnation, OP and CX should be considered as some alternatives for indoor residual spraying. Resistance of *An. gambiae* s.s. to OP and CX has been reported by N'Guessan and others¹¹ using bioassay techniques. This study demonstrated the need to monitor OP and CX resistance

TABLE 1

Frequencies in percentage of *Anopheles gambiae* s.s., S and M molecular forms, and *Anopheles arabiensis* at seven sites in southwestern Burkina Faso.

Area	Site	N	<i>Anopheles gambiae</i> s.s.			<i>Anopheles arabiensis</i> total (%)
			S-form (%)	M-form (%)	Total (%)	
Urban	Bobo 1	60	95.8	4.2	40.0	60.0
	Bobo 2	87	100	0.0	31.0	69.0
Cotton	Samandeni	47	55.6	44.4	95.7	4.3
	Seguer	43	44.4	55.6	41.9	58.1
Ricefield	VK7	166	48.2	51.8	100	0.0
Other sites	Darsalamy	45	22.2	77.8	20.0	80.0
	Yegueresso	44	20.0	80.0	22.7	77.3

* N: number of mosquito tested by PCR analysis.

TABLE 2
Frequencies of the *ace-1^R* allele and genotypes in the *Anopheles gambiae* s.s. and S and M molecular forms.

Area	Site	<i>Anopheles gambiae</i> s.s.		S-form					M-form				
		F(<i>ace-1^R</i>) & (N)	HW	RR	RS	SS	F(<i>ace-1^R</i>)	HW	RR	RS	SS	F(<i>ace-1^R</i>)	HW
Urban	Bobo 1	0.35 (24)	0.175	1	15	7	0.37	0.093	0	0	1	0	—
	Bobo 2	0.33 (27)	0.024	0	18	9	0.33	0.024	0	0	0	0	—
Cotton	Samandeni	0.19 (45)	0.320	0	17	8	0.34	0.021	0	0	20	0	—
	Seguere	0.17 (18)	1	0	6	2	0.38	0.441	0	0	10	0	—
Rice field	VK7	0.24 (166)	0.011	2	69	9	0.46	0.000	0	6	80	0.04	1
Other sites	Darsalamy	0.11 (9)	1	0	2	0	0.50	1	0	0	7	0	—
	Yegueresso	0.15 (10)	1	0	1	1	0.25	—	0	2	6	0.13	1

F (*ace-1^R*) values = frequencies of the *ace-1^R* mutation.

HW = the Exact Probability for rejecting Hardy-Weinberg equilibrium.

(N) = is the number of *An. gambiae* s.s.

among populations of the *An. gambiae* complex in Africa, to determine its spread and anticipate vector control failure where these insecticides are used. Since the development of a molecular test to detect the *ace-1^R* mutation in *An. gambiae*,¹⁴ this study is the first that reports the distribution of the *ace-1^R* mutation in populations of *An. gambiae* s.l. across areas with different patterns of insecticide use in Burkina Faso.

The use of insecticides for crop protection may explain the level of OP and CX resistance observed in some of our rural areas. Diabate and others² showed that agricultural use of insecticides in Burkina Faso was involved in the development of pyrethroid resistance in *An. gambiae* s.l.. In the cotton-growing areas, farmers use huge amounts of insecticides to avoid substantial yield reduction of their crops. Even if the selection pressure is much lower in rice field areas, a high frequency of resistant individuals was observed in VK7. VK7 is located at the periphery of rice fields and the resistant mosquitoes of the S form found in VK7 might have migrated from surrounding cotton areas as was the case for the *kdr* mutation in the same village.² In the urban area of Bobo-Dioulasso the presence of *ace-1^R* should be partly explained by the use of household insecticides, which sometimes contain carbamate. In 1993 in Bouake, Côte d'Ivoire, Elissa and others³ attributed the presence of permethrin resistance to the widespread use of pyrethroids in households. However, during our larval collections in Bobo-Dioulasso, we also noticed important vegetable growing areas within town where farmers apply large amounts of insecticides for crop protection (personal observations). Otherwise the results from the survey conducted in 2002 by Diabate and others² showed high levels of pyrethroid resistance (*kdr* mutation) up to 95.6% in the same area of Bobo 1. In Yegueresso and Darsalamy where limited amounts of insecticide is used to protect crops for subsistence, the level of resistance observed (0.11 for Darsalamy and 0.15 for Yegueresso) in mosquitoes may have been due to a migration from the urban areas of Bobo Dioulasso into these villages.

The molecular M and S forms of *An. gambiae* are incipient species.²⁰ They display a pattern of habitat segregation and a strong assortative mating in nature that results in a strong barrier to gene flow.^{21–23} The *ace-1^R* mutation was detected in both M and S molecular forms of *An. gambiae* s.s. but at different frequencies. It was significantly higher in the S form, probably reflecting the differences in the insecticide selection pressures the two forms experience in their natural habitats. It is not clear from our data whether the OP and Cx resistance gene was selected in one form and arose later on in the second one through genetic introgression as it has been shown with

the *kdr* mutation. Further studies to clarify this point are needed and eventually that may help to better understand the gene flow pattern between the two forms in natural populations. Although *An. arabiensis* was found in sympatry with the two molecular forms of *An. gambiae*, the *ace-1^R* mutation was never found in this mosquito species suggesting that gene flow between *An. arabiensis* and *An. gambiae* is strongly reduced.

The high relative frequencies of *An. arabiensis* in our mosquito samples across the study area were surprising. Previous studies in the same area have recorded a maximum of 8% of *An. arabiensis* in contrast to the present study where *An. arabiensis* represented 60% of the total collection.^{2,21} Coluzzi and others²⁴ first reported the penetration of this mosquito species into urban settings in southern Nigeria. Many other studies confirmed these observations later in different places drawing attention to the rapid extension of *An. arabiensis* into urban areas.^{25,26} Further studies are needed to understand the current distribution of *An. arabiensis* in West African cities.

The insensitivity of acetylcholinesterase to OP and CX leads to a reduction of the normal enzyme function. Several studies have suggested that the biochemical change introduced by G119S results in a fitness reduction on life-history traits associated with the mutation. This phenomenon was observed with *Cx. pipiens*. Numerous life-history traits are modified in insecticide-resistant individuals as compared with susceptible ones (e.g., an increased larval development time and mortality, a lower male reproductive success, and a lower female survival during overwintering).^{27,28} The data obtained from the field samples in the present study indicate that the frequency of resistant homozygous individuals (*ace-1^{RR}*) is extremely low in adult females (1% homozygous resistant versus 45% heterozygous). The Hardy-Weinberg test showed an excess of heterozygotes. This drastic lack of resistant homozygous *An. gambiae* in the field is consistent with the hypothesis of a high fitness cost associated with the G119S mutation in this species. Additional studies are required to explore this hypothesis through laboratory experiments comparing different life-history traits of the resistant allele, to understand the low frequency of resistant homozygous specimens recorded in the field populations.

In conclusion, we report here that this mutation occurs in many specimens of *An. gambiae* s.s. found in South-Western Burkina Faso in addition to the case in Bouake in Côte d'Ivoire. All these findings suggest that OP and CX resistance may be widespread throughout West Africa. This wide distribution of *ace-1^R* mutation in West Africa theoretically could result either from the spread of the single mutation or from the independent occurrence of the same or different muta-

tions in many countries. Although the rate of mutations generating resistance genes among mosquitoes is unknown, we can consider that the migration including passive transportation of mosquitoes and gene flow play major roles in the dispersion of resistance genes between distant populations.²⁹ Further genetic studies will be performed on mosquito samples to investigate gene flow between Bouake in Côte d'Ivoire and other towns in Burkina Faso. However mosquitoes with resistance for both *kdr* and *ace-1^R* mutations were found in the field. The presence of both resistance mechanisms (*kdr* and *ace-1^R*) in mosquitoes may allow them to be simultaneously resistant to pyrethroids, carbamates, and organophosphates. Although there is no miraculous short-term solution to this resistance problem, it is important for program managers to better understand resistance issues and to promote good practices in chemical-based vector control. Indeed it is essential to use public health insecticides in such a way that they are safe, effective, and affordable, while taking into account resistance problems. This work like others underlines the relationship between vector resistance and the use of agricultural insecticides. It is clear that closer collaboration between resistance experts in agriculture and public health is needed. Public health agencies can definitely benefit from the extensive experience gained by the agricultural sector in promoting integrated pest-management principles as well as disseminating simple and pragmatic guidelines for insecticide resistance management.

Received May 26, 2007. Accepted for publication October 31, 2007.

Acknowledgments: We thank J. B. Ouedraogo, the head of IRSS, for his support during the study. Our thanks also go to A. Ouattara for his participation in mosquito sample collection. Finally, we thank Sébastien Koudénoukpo of the Centre de Recherche Entomologique de Cotonou for his help with the genotyping.

Financial support: This study and Luc Djogbénu were supported by a grant from Institut de Recherche pour le Développement (IRD).

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