Characterization of Insensitive Acetylcholinesterase (*ace-1^R*) in *Anopheles gambiae* (Diptera: Culicidae): Resistance Levels and Dominance

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ABSTRACT Characterization of insecticide resistance provides data on the evolutionary processes involved in the adaptation of insects to environmental changes. Studying the dominance status and resistance level represents a great interest, in terms of understanding resistance evolution in the field to eventually adapt vector control. Resistance and dominance levels conferred by the G119S mutation of acetylcholinesterase (*ace-1^R*) of the mosquito *Anopheles gambiae* s.s. (Diptera: Culicidae) were studied for various insecticides belonging to different classes, using strains sharing the same genetic background. Our survey shows that the homozygote resistant strain AcerKis displayed a very high resistance level to various carbamates (range 3,000- to 5,000-fold) compared with that of various organophosphates (range 12- to 30-fold). Furthermore, the dominance status varied between semi-recessivity with fenitrothion and chlorpyrifos methyl insecticides to semidominance with temephos, carbosulfan, and propoxur. These results indicate that this resistance mechanism could spread rapidly in the field and then compromise the use of organophosphate and carbamate compounds in public health. This study underlines the necessity to monitor the *ace-1^R* mutation in natural populations before planning and implementing malaria control programs based on the use of these insecticides.

KEY WORDS *Anopheles gambiae*, insecticides, resistance, dominance, acetylcholinesterase

In 2001, resistance to insecticides concerned 540 species of arthropods, of which 198 were of medical and veterinary importance (Bills 2001). This report was all the more worrying because insecticides play a major role in the control of insect pests and vectors of human diseases. In Africa, pyrethroid-impregnated nets are now widely used to reduce malaria morbidity and mortality in tropical countries. Unfortunately, in West Africa, resistance to pyrethroids is widespread in *Anopheles gambiae* s.s. (Chandre et al. 1999) and *Anopheles arabiensis* Patton (Diabate et al. 2004), two major malaria vectors in sub-Saharan Africa. An alternative strategy to maintain the global effectiveness of insecticide-treated nets should be the use of other insecticides such as carbamates (CX) and organophosphates (OP), alone or in combination with pyrethroids. These insecticides inactivate acetylcholinesterase (AChE; EC 3.1.1.7), an enzyme responsible for neurotransmitter degradation at the cholinergic nerve synapse. However, resistance to OP and CX based on reduced sensitivity of AChE1 has recently been detected among *An. gambiae* from Côte d'Ivoire (N'Guessan et al. 2003, Weill et al. 2003). It was also shown that the AChE1 insensitivity displayed by *An. gambiae*, *Anopheles albimanus* Wiedemann, and *Culex pipiens* L. was due to the same glycine-serine substitution at position 119 (mutation G119S using torpedo nomenclature) resulting from a single point mutation GGC to AGC in the *ace-1* gene (Weill et al. 2003, 2004).

A prerequisite for establishing resistance management strategies is to understand the factors influencing the evolution of resistance. Among these factors, resistance level to various insecticides and dominance/recessiveness status are important. For a given resistance mechanism, the resistance level may largely vary depending on insecticides, even within the same inhibitors family. The dominance level describes the relationships between the phenotypes of the three genotypes: heterozygotes and susceptible and resistant homozygotes (Sved and Mayo 1970). Studying dominance is of great interest not only in terms of vector control, because a distinct amount of insecticide would be necessary to kill heterozygotes depending on dominance status, but also in terms of resistance evolution in the field. The diffusion of resistance will be faster if the resistant allele is dominant instead of recessive. The dominance level of insecticide resis-

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tance provided by insensitive AChE1 has been intensively studied in many mosquitoes such as *Cx. pipiens* (Bourguet et al. 1996a), *Culex tritaeniorhynchus* (Takahashi and Yasutomi 1987), and *An. albimanus* (Ayad and Georghiou 1975). Resistance to CX and OP is usually codominant to dominant. A similar feature is expected for *An. gambiae*, although no data are presently available on this species.

Our major goal was to characterize insecticide resistance resulting from insensitive AChE1 in *An. gambiae.* We created a reference strain of *An. gambiae*, sharing the same genetic background as the Kisumu susceptible reference strain but homozygote resistant for the G119S mutation at the locus *ace-1.* This strain allowed us to evaluate precisely the phenotypic effects of the *ace-1* mutation without any interactions of other genes that could have been selected in natural populations and that could have modified the expression of resistant allele.

Materials and Methods

Mosquito Strains. Kisumu, a susceptible reference strain of *An. gambiae* s.s. originated from Kenya and maintained for many years under laboratory conditions, was used to compare bioassays results.

The strain AcerKis was obtained by introgression of the resistant *ace-1*^{R} locus into the Kisumu's genome through successive backcrosses. *ace-1*^{R} was obtained from a sample of resistant *An. gambiae* population collected in Bobo-Dioulasso (Burkina Faso) in 2002. The Bobo-Dioulasso sample was mass selected by exposing late third and early fourth instars to propoxur (CX). A discriminating dose of propoxur (10 mg/liter) was applied to kill all susceptible homozygous and to select the resistant homozygous and heterozygous. The first offspring (G1) was obtained by crossing selected males with females of Kisumu strain. The G1 offspring was thus composed of only susceptible homozygous and heterozygous individuals. G1 larvae were mass selected as described previously and resistant adult males were backcrossed with Kisumu females. The same protocol, i.e., larval selection + backcross with Kisumu females was repeated for 19 generations. After the 19th generation, the strain was inbred and selected with propoxur for four additional generations to increase the *ace-1*^{R} frequency. Finally the purification of *ace-1*^{RR} homozygous was performed. Pupae were individualized, and emerged adults (one male per three females) were distributed in plastic cups and allowed to mate for 7 d. After mating, the males of each cup were removed and their genotypes for at the locus *ace-1* determined according to the polymerase chain reaction (PCR) diagnosis test of Weill et al. (2004). Only the females that were mated with homozygous *ace-1*^{RR} males were kept and allowed to blood-feed. After 5 d, females were individually placed in plastic cups to lay their eggs. Females were then killed and their genotypes for *ace-1* determined. The AcerKis strain was established by keeping only the progeny of homozygous *ace-1*^{RR} females.

Larval Bioassays. Resistance characteristics of the two strains (Kisumu and AcerKis) and their F1 progeny (AcerKis males × Kisumu females) were analyzed with bioassays done in plastics cups on late third and early fourth instars. Seven insecticides of technical grade quality were used: two carbamates: propoxur (98.8% of purity) and carbosulfan (90.8% of purity); three organophosphates: chlorpyrifos methyl (97.5% of purity), fenitrothion (94.3% of purity), and temephos (96% of purity); one pyrethroid: deltamethrin (100% of purity); and one organochlorine: DDT (73.1% of purity). Insecticide solutions were made in 90% ethanol and stored at 4°C for no >2 mo.

A set of 20 larvae was incubated in 99 ml of distilled water, to which 1 ml of insecticide solution at the required concentration was added. Five replicates were done for each concentration. Six to eight insecticide concentrations providing a range of mortality between 0 and 100% were done for each insecticide tested. Mortality was recorded after a 24-h exposure. Controls were made with 1 ml of ethanol, and mortality never exceeded 5%. Temperature was maintained at 27 ± 2°C during bioassays.

Data Analysis. Mortality data were analyzed using the log-probit software Win DL (CIRAD-CA 1999) based on Finney (1971). This software calculates the lethal concentration for 50 or 95% of individuals (LC_{50}, LC_{95}), the probability that the relation between log (concentration) and probit (mortality) is fitted by a straight line, and the slope of the line. In addition to probit regression analysis, this program allows the comparison of probit lines by testing parallelism of slopes. Two regression lines are considered identical when the hypothesis of parallelism is not rejected at the 5% threshold (by using a chi-square test) and the confidence intervals of their LC overlapped. Resistance level at a given mortality was defined by the ratios LC_R / LC_S where LC_S and LC_R are the insecticide concentrations needed to obtain the same mortality for susceptible and resistant strains.

Dominance of Insecticide Resistance. In our study, the phenotypic character studied was "insecticide resistance". The dominance was estimated by comparing the LC_{50} of heterozygotes to the LC_{50} values of parental strains. Dominance was calculated using the Bourguet et al. (1996b) formula to obtain 0–1 range:

$$D = [LC_{50}(RS) - LC_{50}(SS)] / [LC_{50}(RR) - LC_{50}(SS)].$$

When RS and RR genotypes have the same LC_{50}, then D = 1 and R is dominant over S. When RS and SS have the same LC_{50}, then D = 0 and R is recessive over S. Finally, when RS has an intermediate LC_{50}, D = 0.5 and alleles R and S are co-dominant. Five dominance categories were defined according to Georghiou (1969): 1) recessive when $D \approx 0$, 2) semirecessive when $0 < D < 0.5$, 3) codominant when $D \approx 0.5$, 4) semidominant when $0.5 < D < 1$, and 5) dominant when $D \approx 1$.

Table 1. Log-concentration and probit-mortality data for different insecticides of *An. gambiae* Kisumu and AcerKis

Insecticide	Strain	LC_{50}[a]	95% CI	LC_{95}[a]	95% CI	χ^2	P[b]	RR50[c]
Propoxur	Kisumu	0.027	0.0176–0.0274	0.0621	0.0527–0.0756	2.7	0.44	
	AcerKis	144.3	120.8–163.5	411	346.0–539.2	4	0.26	5340
Carbosulfan	Kisumu	0.000398	0.000339–0.000449	0.00115	0.00101–0.00141	9	0.12	
	AcerKis	1.11	1.00–1.57	11.6	10.2–17.0	5.6	0.34	2780
Temephos	Kisumu	0.000417	0.000320–0.000530	0.00185	0.00118–0.00483	4.7	0.10	
	AcerKis	0.0128	0.0105–0.0151	0.0609	0.0469–0.0896	5.7	0.21	30.6
Fenitrothion	Kisumu	0.0015	0.00107–0.00192	0.012	0.00893–0.0186	3.3	0.51	
	AcerKis	0.0431	0.0295–0.0552	0.264	0.205–0.388	9.4	0.09	28.7
Chlorpyrifos methyl	Kisumu	0.000902	0.000766–0.00105	0.00372	0.00480–0.00306	7.5	0.11	
	AcerKis	0.0107	0.00909–0.0143	0.0579	0.0321–0.232	2	0.73	12.0

[a] LC_{50} and LC_{95} are lethal concentrations in milligrams per liter and their associated 95% confidence intervals.
[b] Probability associated with linear adjustment (linearity of dose–mortality response is rejected when $P < 0.05$).
[c] RR50 is resistance ratio at LC_{50} = [LC_{50} (Acerkis)/LC_{50} (Kisumu)].

Results

Resistance Level of AcerKis to Carbamates and Organophosphates. For all bioassays with carbamates and organophosphates (Table 1), the relationships between log-concentration and probit-mortality were well fitted by straight lines for both strains ($P > 0.05$). Furthermore, the parallelism of Kisumu and AcerKis mortality lines was not rejected ($\chi^2 = 0.09$, df = 1, $P = 0.76$) for propoxur, ($\chi^2 = 0.12$, df = 1, $P = 0.73$), for carbosulfan, ($\chi^2 = 0.11$, df = 1, $P = 0.74$) for temephos, ($\chi^2 = 1.48$, df = 1, $P = 0.22$) for fenitrothion and ($\chi^2 = 1.00$, df = 1, $P = 0.32$) for chlorpyrifos methyl.

The LC_{50} values of AcerKis with propoxur and carbosulfan were 144 and 1.11 mg/liter, respectively. These values corresponded to very high resistance level, ≈5,000-fold for propoxur and ≈3,000-fold for carbosulfan. Conversely, the AcerKis strain displayed a lower resistance to organophosphates. The LC_{50} values of AcerKis were 12.8 μg/liter for temephos, 43.1 μg/liter for fenitrothion, and 10.7 μg/liter for chlorpyrifos methyl. The resistance level of AcerKis ranged from 12- to 40-fold for the tested OP.

Susceptibility of AcerKis to Deltamethrin and DDT. For DDT and deltamethrin (Table 2), the relationships between log-concentration and probit-mortality were well fitted by straight lines for both strains ($P > 0.05$). As for CX and OP, the parallelism of Kisumu and AcerKis mortality lines was not rejected for DDT ($\chi^2 = 1.25$, df = 1, $P = 0.26$) and deltamethrin ($\chi^2 = 0.01$, df = 1, $P > 0.2$).

The LC_{50} of AcerKis was 16 μg/liter for DDT and 0.34 μg /liter for deltamethrin. Even if the LC_{50} of AcerKis to DDT was slightly higher than the LC_{50} of Kisumu, this was not the case for LC_{95}s. The susceptibility of both strains to deltamethrin was identical.

Level of Dominance of *ace-1^R*. As for parental strain, the relationships between log-concentrations and probit-mortality for the F1 progeny (AcerKis males × Kisumu females) were statistically well fitted by straight lines ($P > 0.05$). The results in Table 3 show that D was higher than 0.5 for propoxur, carbosulfan and temephos (semidominance) and lower than 0.5 for fenitrothion and chlorpyrifos methyl (semirecessive).

Discussion

To better understand the impact of *ace-1^R* allele on the phenotypic traits of *An. gambiae*, we evaluated the resistance level it conferred and its dominance status by using two strains sharing the same genetic background. For a given allele of an introgressed strain, we can define the probability P that, at the end of i backcrosses, this allele is still associated with the selected resistance allele, i.e., no recombination event has occurred between the two genes. If r is the recombination rate between both genes, then $P = (1 - r)^i$. This allows the computation of the genetic distance around the selected gene, which has not been replaced by the Kisumu's genome, e.g., around $1 - (e^{(\ln(\alpha)/i)})$, with α being the risk level. This leads to a distance of 15 cM for 19 backcrossing generations at the 0.05 risk level (Berticat et al. 2002).

Although the mutation *ace-1* G119S provided cross-resistance to organophosphates and carbamates, the resistance level greatly varied between both insecti-

Table 2. Log-concentration and probit-mortality data for DDT and deltamethrin of *An. gambiae* Kisumu and AcerKis

Insecticide	Strain	LC_{50}[a]	95% CI	LC_{95}[a]	95% CI	χ^2	P[b]	RR50[c]
DDT	Kisumu	0.0116	0.0104–0.0129	0.0465	0.0378–0.0609	3.7	0.60	
	AcerKis	0.0157	0.0141–0.0175	0.0544	0.0446–0.0709	2.1	0.72	1.4
Deltamethrin	Kisumu	0.000375	0.000313–0.000430	0.0013	0.00106–0.00177	3.2	0.53	
	AcerKis	0.000337	0.000271–0.000394	0.0012	0.000989–0.00162	6.7	0.15	0.9

[a] LC_{50} and LC_{95} are lethal concentrations in milligrams per liter and their associated 95% confidence intervals.
[b] Probability associated with linear adjustment (linearity of dose–mortality response is rejected when $P < 0.05$).
[c] RR50 is resistance ratio at LC_{50} = [LC_{50} (Acerkis)/LC_{50} (Kisumu)].

Table 3. Log-concentration and probit-mortality data for different insecticides of F1 individuals (male AcerKis × female Kisumu) and dominance levels of resistance conferred by different insecticides

Insecticide	LC_{50}[a]	95% CI	χ^2	P[b]	RR50[c]	D	Dominance level
Propoxur	93.0	77.9–107.4	8	0.24	3439	0.64	Semidominant
Carbosulfan	0.599	0.411–0.779	6.8	0.24	1503	0.54	Semidominant
Temephos	0.00791	0.00652–0.00929	6.2	0.19	19	0.61	Semidominant
Fenitrothion	0.0122	0.00795–0.0166	3.6	0.30	8.12	0.26	Semirecessive
Chlorpyrifos methyl	0.00336	0.00262–0.00396	5.3	0.14	3.72	0.25	Semirecessive

[a] LC_{50} and LC_{95} are lethal concentrations in milligrams per liter and their associated 95% confidence intervals.
[b] Probability associated with linear adjustment (linearity of dose–mortality response is rejected when $P < 0.05$).
[c] RR50 is resistance ratio at LC_{50} = [LC_{50} (F_1)/LC_{50} (Kisumu)].

cide families. The homozygote resistant strain of *An. gambiae* AcerKis displayed a very high resistance level to carbamates, ranging from 3,000- to 5,000-fold, compared with that of organophosphates, ranging from 10- to 40-fold. A similar observation was previously made with a resistant strain of *Cx. quinquefasciatus* from Côte d'Ivoire (Super-Car) from which the same G119S mutation produced 50- to 100-fold less resistance to organophosphates than carbamates (Chandre et al. 1997). The measure of AChE1 activities from several strains of *Cx. pipiens* having the G119S mutation showed that the ratios of inhibition constants (k_i) between AChE1 purified from susceptible strain and that from resistant strains were 300,000-fold for propoxur (carbamate) but only 10- to 400-fold for organophosphates (Bourguet et al. 1996a).

The dominance of a resistance gene is a fundamental character for the evolution of insecticide resistance in natural populations. The selection of resistance in the field for a dominant allele will be faster than for a recessive allele (Lenormand and Raymond 1998). The level of dominance in case of insecticide resistance measures the relative position of mortality lines of heterozygotes compared with both susceptible and resistant parental homozygotes (Bourguet and Raymond 1998). Generally, it is not possible to predict the level of dominance of a resistance gene, unless the precise physiological role of this gene and its mode of interaction with the insecticide are known. In enzymatic resistance, the metabolic theory proposed by Wright, which takes into account kinetics properties of metabolic systems, predicts resistance to be dominant or codominant (Kacser and Burns 1981, Keightley 1996). However, for target site insensitivity (e.g., receptors and ion channels), a relevant understanding has to be sought case by case (Bourguet and Raymond 1998).

Several studies have shown that AChE1 activity in wild arthropods is in large excess compared with the minimum AChE1 activity compatible with life under laboratory conditions. This minimum activity is usually <30% (e.g., 25% for *Drosophila;* Hoffmann et al. 1992) and 2.3% for *Tetranychus urticae* Koch (Smissaert et al. 1975). When an insecticide is applied at a concentration that kills susceptible homozygotes, 50% of the AChE1 of heterozygotes corresponding to the entire susceptible counterpart was inhibited. Their insensitive counterpart was more or less inhibited depending on the affinity between the insecticide and the modified target site. This explains why differences in AChEl activity produce variation in dominance level (Bourguet et al. 1997). Moreover, Bourguet et al. (1996b) demonstrated that dominance of *ace-1*^{R} was not a fixed parameter but can be influenced by environmental conditions such as water depth, shape of plastic cups used for bioassays. All our bioassays being done in the same conditions, it was likely that the differences observed in dominance level relied more on insecticide specificity rather than on environmental factors.

Resistance management strategies are mainly based on the rational use of the compounds already available, especially in public health because the number of insecticides is very limited. CX and OP are the main alternatives for indoor residual spraying or larval treatments against mosquitoes in case of pyrethroid resistance. Some experimental studies were done with bed-nets impregnated with CX or OP either alone or in association with pyrethroids (Fanello et al. 1999, Guillet et al. 2001). Although these kinds of bed-nets are still at an experimental stage, the presence of *ace-1*^{R} in *An. gambiae* should be taken into consideration for their further development. Similar to pyrethroids the *kdr* mutation, the presence of a*ce-1*^{R} did not necessary mean that bed-nets impregnated CX or OP will be ineffective, because several factors such as the mosquito behavior influence the efficacy of vector control methods (Chandre et al. 2000).

Preliminary field studies indicated that the frequency of resistant homozygous individuals for *ace-1*^{R} was extremely low within populations of *An. gambiae* from Côte d'Ivoire or Burkina Faso even for samples in which the frequency of heterozygotes was >50%. This suggested a high fitness cost of the mutation under natural conditions, compared with the resistant allele *ace-1*^{R} codes for an AChE1 whose catalytic properties are significantly reduced toward the natural substrate (Weill et al. 2004). These modifications disturb the normal functioning of the synapses and may have an impact on the metabolism or the behavior of mosquitoes. This explains why insensitive AChE1 is often associated with an important fitness cost (Raymond et al. 2001). Consequently, in area where the resistant allele a*ce-1*^{R} is present, resistant mosquitoes will be mainly at heterozygote state (*ace-1*^{RS}). The fact that this mutation is recessive with some OP is of primary importance in the framework of vector control operations, because according to the insecticide,

the phenotype of heterozygotes will be more or less close to the phenotype of susceptible individuals. This is particularly true for chlorpyrifos methyl for which it was shown that under field conditions in experimental huts the *ace-1*^{R} allele did not give effective resistance (Asidi et al. 2005) to bed-nets treated with this OP.

The combination of insecticides using rotations, mosaics or mixtures is a possible way to overcome insecticide resistance within malaria vectors not only for indoor residual sprayings (Hemingway et al. 1997, Penilla et al. 2006) but also for impregnated nets (Hougard et al. 2002). This is all the more interesting that in some case insecticides can have synergistic interactions particularly against resistant mosquitoes (Corbel et al. 2004). However, combinations can sometimes have antagonistic effect as it was demonstrated using carbamate and pyrethroid for *C. pipiens* with *ace-1*^{R} mutation (Corbel et al. 2004). With the rapid extension of pyrethroid resistance in the main malaria vectors from Africa and the various resistance mechanisms involved (metabolic resistance and kdr), it is important to envisage resistance management strategies now. Thus, the knowledge of the *ace-1*^{R} effects on phenotypes of *An. gambiae* will help us to strengthen the basic and operational researches on the development of strategies that will use organophosphates or carbamates as alternatives against pyrethroids-resistant malaria vectors in the field.

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