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Comparison of *Anopheles gambiae* and *Culex pipiens* acetylcholinesterase 1 biochemical properties

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ABSTRACT

Selection of insensitive acetylcholinesterase 1 (AChE1) has occurred in several mosquito species controlled with carbamate (CX) and organophosphate (OP) insecticides. In case of pyrethroid resistance, these insecticides represent an alternative for disease vector control program. Their heavy use in agriculture has selected resistant populations of *Anopheles gambiae* in West Africa. The evolution of resistance has to be studied to prevent, or at least slow down, the spread of resistant mosquito in wild populations. *An. gambiae* shares the same resistance mechanism to CX and OP insecticides as *Culex pipiens*, which was attributed to the G119S substitution in the AChE1 enzyme. By comparing resistant AChE1 from both species, we show here that similar resistance levels are obtained toward 10 insecticides of both classes. Moreover, similar AChE1 activity levels are recorded between either susceptible or resistant mosquitoes of both species. Enzymes belonging to both species seem thus to share identical properties. Consequently, we hypothesize that fitness cost associated with AChE1 insensitivity in *C. pipiens* mosquitoes should be similar in *An. gambiae* and thus be used in strategies to control resistant populations where malaria is prevalent.

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1. Introduction

Insecticide resistance is an impediment in the control of pests and vectors of human diseases and has emerged because of heavy insecticide treatments. Different resistance mechanisms (mostly target mutation or increased detoxication) have been selected in insects depending on the insecticide used.

Acetylcholinesterase (AChE, EC 3.1.1.7) is a synaptic enzyme that hydrolyzes the neurotransmitter acetylcholine to terminate nerve impulses. It is also involved in the development of the nervous system in vertebrates and invertebrates (Cousin et al., 2005; Grisaru et al., 1999). Organophosphorous and carbamate (OP and CX) insecticides are competitive inhibitors that irreversibly inhibit the AChE enzyme, blocking nervous transmission and leading to the death of the insect. Selection of a modified AChE less sensitive to these insecticides has been shown to be a common resistance mechanism and was observed in numerous arthropod pest species (Fournier and Mutéro, 1994).

Most insects possess two ace genes (*ace-1* paralogous to the *Drosophila melanogaster* gene and *ace-2* the orthologous one) except in true flies where *ace-2* is the unique acetylcholinesterase gene (Fournier

et al., 1989; Weill et al., 2002; Huchard et al., 2006). In true flies, AChE2 resistance is associated with combination of one to five potential mutations in the unique *ace-2* gene, all corresponding to residue substitutions around the active site (Mutéro et al., 1994; Menozzi et al., 2004). In mosquito species, *ace-1* codes the synaptic AChE responsible for the nervous system cholinesterase activity and thus for insecticide resistance while *ace-2* gene is not involved (Weill et al., 2002; Weill et al., 2003; Huchard et al., 2006). Resistance to OP and CX insecticides results mainly from a single mutation in the *ace-1* gene and, to date, only a few positions have been demonstrated to confer insensitivity, suggesting a high structural constraint of the enzyme (reviewed in Oakeshott et al., 2005).

The *Anopheles gambiae* complex consists of at least seven species among which is *An. gambiae* s.s.: the most efficient Afrotropical malaria vector. In West Africa, *An. gambiae* s.s. has been divided into five chromosomal forms designated with a non-Linear nomenclature: bamako, mopti, savanna, forest, and bissau (Coluzzi et al., 1985; Touré et al., 1994; Touré et al., 1998). Molecular studies revealed the existence of two genetic variants referred to as the molecular M and S forms (Favia et al., 1994; della Torre et al., 2001; Wondji et al., 2002). Both forms are anthropophilic and effective vectors of human malaria parasites.

Resistance associated with insensitive AChE1 in malaria vectors was first reported in *An. albimanus* in South and Central America (Ayad and Georghiou, 1975). In West Africa, propoxur resistance was first suspected in a population of *An. gambiae* from Ivory Coast (Elissa et al., 1994) and insensitive AChE1 was next confirmed as the resistance

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mechanism (N'Guessan et al., 2003). In these *Anopheles* species, AChE1 insensitivity is due to the same Gly-to-Ser substitution at position 119 (according to the *Torpedo californica* nomenclature, (Mas-soulié et al., 1992) as in *Culex pipiens* (Weill et al., 2003; Weill et al., 2004). To develop strategies of treatment that could delay or at least limit the spread of resistance to insecticides, it is important to understand how resistant allele affects life history traits in *An. gambiae* mosquitoes. According to the high homology in the amino acids sequence, we studied biochemical characteristics of the AChE1 from susceptible and resistant mosquitoes, comparing *An. gambiae* and *C. pipiens*.

2. Materials and methods

2.1. Mosquito samples

Two *An. gambiae* reference strains were used in comparison with two *C. pipiens* reference strains. The *An. gambiae* susceptible reference strain, Kisumu, was collected in Kenya in 1953 and has been maintained for many years under laboratory conditions. The resistant homozygous Acerkis strain was obtained by introgression of the resistant *ace-1* G119S allele into the Kisumu's genome through successive backcrosses. *ace-1* G119S allele was obtained from a sample of resistant *An. gambiae* population collected in Bobo-Dioulasso (Burkina Faso) in 2002 (Djogbénuou et al., 2007). Both strains share the same Kisumu genetic background and belong to the molecular S form. The *C. pipiens* reference strains are Slab, the susceptible one (Georghiou et al., 1966) and SR, the resistant homozygous G119S one (Berticat et al., 2002). Both strains share the same Slab genetic background.

2.2. Measure of individual AChE1 activity from mosquito heads

Each adult head was homogenised in 400 µL phosphate buffer (0.25 M, pH7) containing 1% Triton X-100. Homogenates were centrifuged (9000 g for 3 min) and 100 µL of the supernatant were used with 10 µL of ethanol (95%) for AChE1 activity measure. We then added 100 µL of 1.6 mM substrate, acetylthiocholine (Sigma, France), and AChE1 activity was estimated by measuring changes in optical density as described by Ellman et al. (1961). Colour development was measured at 412 nm for 15 min with a microplate reader ELx 800 and the analysis software KCjunior v1.41.4 (Bio-Tek Instruments, Inc.).

For each mosquito, the left wing was cut and measured from the notch to the wing tip as described by Van Handel and Day (1989), using a measuroscope (Measuroscope 10 Nikon, digital counter CM 6 S Nikon). Wing length was measured twice independently and correlation between both indicates good agreements ($R^2=0.97$). Thus, the mean of the two measures was used to correct activity by the individual body size.

2.3. AChE1 inhibition characteristics

Inhibition curves were performed by incubating 100 µL of mosquitoes extracts (see above) for 15 min. with 10 µL of insecticide solutions at various concentrations. All insecticides were purchased from CIL Luzeau (France) except eserine which was a gift from Dr. Leonetti J-P. (CBPS, CNRS UMR 5236, France). One hundred µL of substrate (1.6 mM acetylthiocholine) was then added and rate of hydrolysis was measured at 412 nm during 15 min. We analysed three to five replicates for each assay. The irreversible inhibition reaction is a pseudo-first order and the remaining activity follows the equation $\frac{[E]}{[E_0]} = e^{-k_i t [I_0]}$, when inhibitors are in excess compared to enzyme. k_i is the bimolecular rate constant, t represents time of incubation, and $[I_0]$ is the initial inhibitor concentration. Resistance ratios were calculated by dividing the k_i of the sensitive AChE1 by the k_i of the G119S AChE1.

2.4. Statistical analysis

The effect of the size and the species of each genotype on the total AChE1 activity (OD) was analysed using a GLM model with Gaussian error. Three independent variables were considered: *species*, a qualitative variable with two modalities (*C. pipiens* and *An. gambiae*); *genotype*, a qualitative variable with two modalities (*susceptible* and *resistant*) and *size*, a quantitative variable. The initial linear model assumes OD to depend on: *species+genotype+size+species.genotype+genotype.size+size.species+species.genotype.size* (with "+" denoting additive effects and dots denoting the interactions between variables). This model was subsequently simplified following a step-by-step AIC-based procedure. The effects of the variables retained in the minimal model were then tested using F-tests and normality of residuals was checked. All these analyses were performed using R software (v2.0.1, www.r-project.org).

2.5. Three-dimensional modelling

Three-dimensional structures of AChE1 were created by automated homology modelling as previously described (Weill et al., 2004). The structural templates used were AChE from *Torpedo californica* [pdb: 1EA5; (Sussman et al., 1991)] and from *Drosophila melanogaster* [pdb: 1DX4; (Harel et al., 2000)]. The alpha-carbon skeleton of the modelled 3D structure of AChE1 was superimposed on that of the AChE of *T. californica*. RMS deviation is 1.1 Å on 528 carbon atoms.

3. Results

3.1. *Culex pipiens* and *Anopheles gambiae* AChE1 homology

We compared the primary sequence of AChE1 from susceptible *C. pipiens* and *An. gambiae*, since previous studies showed that the G119S is the only one substitution responsible for AChE1 insensitivity in resistant mosquitoes of the two species (Weill et al., 2003; 2004). We found 93.3% homology identifying 36 different amino acids in the total 536 amino acids of the mature protein (Fig. 1A). Fig. 1B represents the *C. pipiens* AChE1 structural model based on that of *Torpedo californica* structure (PDB: 1EA5) showing dissimilarities with *An. gambiae* AChE1. The model indicates that all these differences are located at the periphery of the enzyme, far from the active site and its entrance. They do not belong to important cholinesterase sites, such as the catalytic triad (S200, E327 and H440), the peripheral anionic site (D72, Y121, W279 and Y334), the choline binding site (W84, Y130, Y330 and F331), the acyl binding pocket (F288 and F290) and the oxyanion hole (G118, G119, A201) (Gibney et al., 1990; Sussman et al., 1991; Harel et al., 1992; Ordentlich et al., 1993; Vellom et al., 1993). Thus, these 36 amino acids are not likely to have any function in the catalytic or in the binding process, and AChE1 from both species should display similar kinetic properties.

3.2. Inhibition of AChE1 activity by various insecticides

We analysed inhibition characteristics of AChE1 from the susceptible (Kisumu) and resistant G119S (Acerkis) *An. gambiae* strains in comparison with the susceptible (Slab) and G119S resistant (SR) *C. pipiens* strains (Fig. 2). The measure of residual AChE1 activity in presence of insecticides showed that inhibition patterns from both species were identical for all insecticides tested (aldicarb, eserine, pirimicarb, propoxur, dichlorvos, chlorpyrifos-oxon, fenitroxon, malaoxon, paraoxon-ethyl and paraoxon-methyl). We performed non-linear regression to determine the bimolecular velocity rate constant (k_i). Variations in k_i values within susceptible or resistant strains were recorded and resistance ratios for both species were very

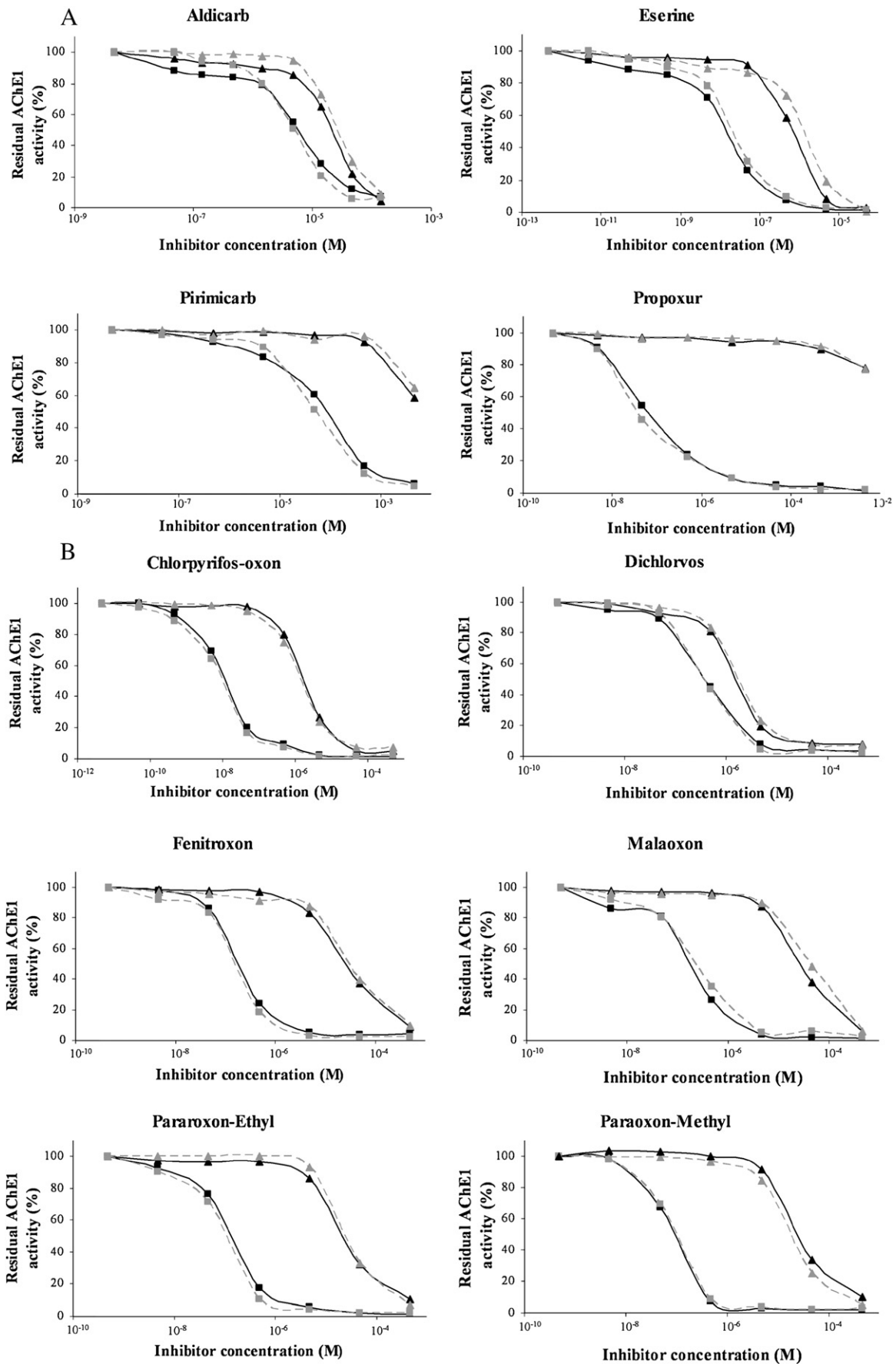


Fig. 2. Residual AChE1 activities of susceptible (squares) and resistant (triangles) mosquito head extract measured in presence of increasing dose of insecticides. Comparison between *An. gambiae* (black symbols) and *C. pipiens* (grey symbols) AChE1. (A) Carbamate insecticides; (B) Organophosphate insecticides.

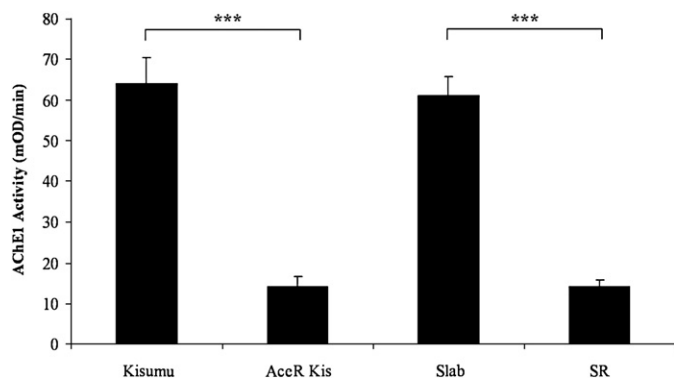


Fig. 3. Average AChE1 activity in mosquito head from *An. gambiae* susceptible and resistant strains (Kisumu (N=30) and Acerkis (N=24), respectively) and *C. pipiens* susceptible and resistant strains (Slab (N=23) and SR (N=28), respectively). Difference between susceptible *An. gambiae* and *C. pipiens* mosquitoes ($P=0.26$) and between resistant *An. gambiae* and *C. pipiens* ($P=0.79$) were not significant but residual AChE1 activity between susceptible and resistant mosquitoes were highly different ($P<0.001$).

3.3. Total AChE1 activity in mosquito head

Mosquito heads were individually grounded and total AChE1 activity was measured for *An. gambiae* strains: Kisumu and Acerkis (N=30 and N=24, respectively); and *C. pipiens* strains: Slab and SR (N=23 and N=28, respectively). Wing lengths were measured to control for the body size. Only an effect of genotype variable on the total AChE1 activity was significantly detected through the model tested (*species.genotype.size*, $F_{1,97}=1.71$, $P=0.19$; *species.genotype*, $F_{1,97}=0.41$, $P=0.52$; *genotype.size*, $F_{1,97}=0.62$, $P=0.43$; *species.size*, $F_{1,97}=0.96$, $P=0.33$; *genotype*, $F_{1,97}=1672.3$, $P<0.001$; *size*, $F_{1,97}=0.20$, $P=0.65$; *species*, $F_{1,97}=0.067$, $P=0.79$) (Fig. 3). Average AChE1 activity was similar for susceptible (61 ± 1.1 OD/min) or resistant (14 ± 0.3 OD/min) mosquito of the two species. Thus, the G119S substitution is responsible for the same decrease in AChE1 activity (about 77%), whatever the species considered here. This reinforces the assumption that none of the 36 amino acid substitutions between both species affect important structural features.

4. Discussion

We report here a comparison of the AChE1 biochemical properties from two distinct mosquito species: *An. gambiae* and *C. pipiens*. To this aim, we studied inhibition characteristics of AChE1 to various insecticides and total activity in several individuals.

Inhibition rates between wild type and resistant AChE1 were found similar in both species. Inhibition constants (k_i) determined for 10 insecticides (CX and OP) were also found similar in the two species. Furthermore, Van Handel and Day (1989) have demonstrated the correlation between total protein amount in an individual mosquito and its wing length. Wing length was thus measured to correct the total AChE1 activity by the body size. No significant difference was found when comparing AChE1 total activity in either susceptible or in resistant individuals from both species. Similar AChE1 activity in individual mosquitoes together with the same resistance ratios are good arguments to assert that enzymes from both species are very closely related and share kinetic properties.

Many studies have pointed out that most of the resistance genes are associated with deleterious effects (reviewed in Roush and McKenzie, 1987). In *C. pipiens* resistant mosquitoes, the G119S substitution in the AChE1 active site drastically reduces catalytic efficiency for its natural substrate, as shown by the decrease in AChE1 activity in individual mosquito (Bourguet et al., 1996). The reduced total AChE1 activity in resistant individual may be responsible for the observed fitness cost

since decrease in AChE activity is known to alter development in insects (Hoffmann et al., 1992). Some affected life history traits such as increased development time, higher susceptibility to predation and infection, or decreased male reproductive success have been observed in *C. pipiens* resistant mosquitoes (Raymond et al., 2001; Berticat et al., 2002; Berticat et al., 2004; Duron et al., 2006). Our data disclosed a reduction of AChE1 activity in resistant individuals similar in both species (Fig. 3). Thus, insecticide resistance is expected to be associated with a fitness cost in *An. gambiae* as well, decreasing resistant allele frequency in non-treated areas, due to competition with susceptible mosquitoes. However, the fitness cost associated with the G119S AChE1 genotype in *An. gambiae* remains to be characterized. Already, preliminary field studies have indicated that the frequency of resistant homozygous individuals for the G119S mutation was extremely low within populations of *An. gambiae* from Burkina Faso, even in samples displaying a frequency of heterozygotes higher than 50% (Djogbénou et al., 2008).

Control of vector borne diseases use different methods depending on physiological, behavioural and ecological features of the vector. The use of larvicides is a method of choice in vector control but is usually not applicable to *An. gambiae* because of its small, widely dispersed and transient larval habitats. Instead, malaria control in Africa is mainly based on the use of indoor residual spraying (IRS) and insecticide treated nets (ITN) with pyrethroid insecticides essentially because of their knockdown effect, their excito-repellent properties and their low mammalian toxicity (Zaim et al., 2000). Recently, insecticide-treated plastic sheeting (ITPS) has been developed as an alternative to IRS to overcome the logistic, technical and operational constraints. ITPS is used as a wall lining in conventional habitations to reduce mosquito longevity. It has been shown to have apparent protective effect against susceptible phenotypes but little protection was observed against homozygotes for the knockdown resistance (*kdr*) (Diabaté et al., 2006). The emergence and spread of *kdr* resistance among *An. gambiae* should burden the large scale programmes of impregnated net distribution that are promoted all over African countries. Resistance developed by 15 malaria vector species was directly linked to insecticide treatments for crop protection (Mouchet, 1988). This increases the difficulty for implementation of resistance management strategies by public health operations. Therefore, to maintain ITN effectiveness, mixture using non-pyrethroid insecticides such as OP and CX should then represent a good alternative. Indeed, experimental hut studies using combination of an OP and a repellent impregnated nets are giving promising results for *An. gambiae* control in West Africa (Pennetier et al., 2007).

A resistance control strategy has been modelled which takes into account gene flow, size of treated area and a number of selection coefficient (dominance, fitness cost and insecticide selection pressure) (Lenormand and Raymond, 1998). This strategy consists in localizing insecticide treatments on restricted areas closed to non-treated areas (or areas treated with insecticides directed against another target), allowing competition between susceptible and resistant mosquitoes by migration. However, the efficiency of such a strategy relies on a high fitness cost associated with the resistant genotype. Different insecticides may be applied in rotation or in mosaic. An experimental hut trial has been conducted to test a combination of a pyrethroid and carbamate insecticides "two-in-one" treated nets in comparison with nets treated with one insecticide alone (Guillet et al., 2001). Corbel et al. (2003) have demonstrated that there is no selection of G119S resistant mosquitoes compared with nets treated with only carbamate insecticide. Moreover, similar results were obtained either with mosaic or mixture. The latter has the advantage to require lower concentration of both insecticides.

Here, we show that insensitivity to aldicarb and dichlorvos insecticides is weak. Thus, these insecticides could provide a better control of resistant populations associated with the G119S AChE1. However, dichlorvos may select other AChE1 mutations, such as the

F290V substitution found in *C. pipiens* from Cyprus Island or the F331W substitution found in *C. tritaeniorhynchus* from China where treatments relied mainly on these insecticides (Alout et al., 2007a,b). Laboratory investigations have to be performed to determine resistance level of *An. gambiae* mosquitoes to various insecticides available or which could be used in a next future by Public Health and to study the pleiotropic effects associated with resistance genes. This will help to develop strategies that will use wisely CX or OP as alternatives to control resistant populations of malaria vectors.

Given the importance of the vector control against malaria disease, there is an urgent need of field and laboratory surveys of insecticide resistance. Characterization of fine biochemical interactions between insecticides and resistant target sites will contribute to identify or to design new insecticides that should improve effectiveness of resistance management strategies against resistant *Anopheles* species in tropical region.

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