Insect Molecular Biology (2004) **13**(1), 1–7

The unique mutation in *ace-1* giving high insecticide resistance is easily detectable in mosquito vectors

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Abstract

High insecticide resistance resulting from insensitive acetylcholinesterase (AChE) has emerged in mosquitoes. A single mutation (G119S of the *ace-1* gene) explains this high resistance in *Culex pipiens* and in *Anopheles gambiae*. In order to provide better documentation of the *ace-1* gene and the effect of the G119S mutation, we present a three-dimension structure model of AChE, showing that this unique substitution is localized in the oxyanion hole, explaining the insecticide insensitivity and its interference with the enzyme catalytic functions. As the G119S creates a restriction site, a simple PCR test was devised to detect its presence in both *A. gambiae* and *C. pipiens*, two mosquito species belonging to different subfamilies (Culicinae and Anophelinae). It is possibile that this mutation also explains the high resistance found in other mosquitoes, and the present results indicate that the PCR test detects the G119S mutation in the malaria vector *A. albimanus*. The G119S has thus occurred independently at least four times in mosquitoes and this PCR test is probably of broad applicability within the Culicidae family.

Keywords: *Anopheles gambiae*, *Anopheles albimanus*, *Culex pipiens*, insecticide resistance, acetylcholinesterase mutation.

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Introduction

In insects, insensitive acetylcholinesterase (AChE) is a common resistance mechanism to organophosphate (OP) and carbamate insecticides (reviewed in Fournier & Mutéro, 1994). Insensitivity displayed by various resistant species is very variable, because different mutations may provide resistance and because distinct genes are coding the AChE target. The situation is simple in *Drosophila*, where only one *ace* gene (now called *ace-2*) exists in the genome. In other insects (*Aphis gossypii*, *Nephotettix cincticeps*, *Helicoverpa armigera* and probably others), including some Diptera species (*Culex tritaeniorynchus*, *Culex pipiens*), the gene homologous to the *Drosophila ace-2* is not involved in resistance (Malcolm *et al*., 1998; Menozzi, 2000; Tomita *et al*., 2000; Mori *et al*., 2001; Ren *et al*., 2002). Another *ace* gene (*ace-1*) was shown to be responsible for AChE insensitivity in resistant strains of two mosquito species, *Anopheles gambiae* and *Culex pipiens* (Weill *et al*., 2002, 2003). These two *ace* genes are the result of an ancient duplication probably older than the emergence of arthropods. The function of *ace-2*, in species where there is an additional *ace* gene (such as the Culicidae family), is unknown.

Mutations responsible for AChE insensitivity have been well described in species where *ace-2* codes for the insecticide target (AChE2). So far, this concerns only three Diptera species, *Drosophila melanogaster*, the housefly *Musca domestica* and the olive fruit fly *Bactrocera oleae* (Mutéro *et al*., 1994; Kozaki *et al*., 2001; Walsh *et al*., 2001; Vontas *et al*., 2002). For example, five distinct codons have been found mutated in *D. melanogaster* resistant strains, each mutation providing a low resistance ratio (Mutéro *et al*., 1994). This situation hampered the design of a molecular test. The recently discovered *ace-1* coding the insecticide target (AChE1) in mosquitoes seems to behave differently. In mosquitoes, a high level of AChE1 insensitivity has been described in, for example, *Anopheles albimanus* (Ayad & Georghiou, 1975), *A. gambiae* (N'Guessan *et al*., 2003; Weill *et al*., 2003) and *C. pipiens* (Bourguet *et al*., 1996a,b). It has been recently shown that the high insensitivity (hence resistance) displayed by *C. pipiens* and *A. gambiae* is due to the same glycine to serine substitution (or G119S, using the *Torpedo* nomenclature), resulting

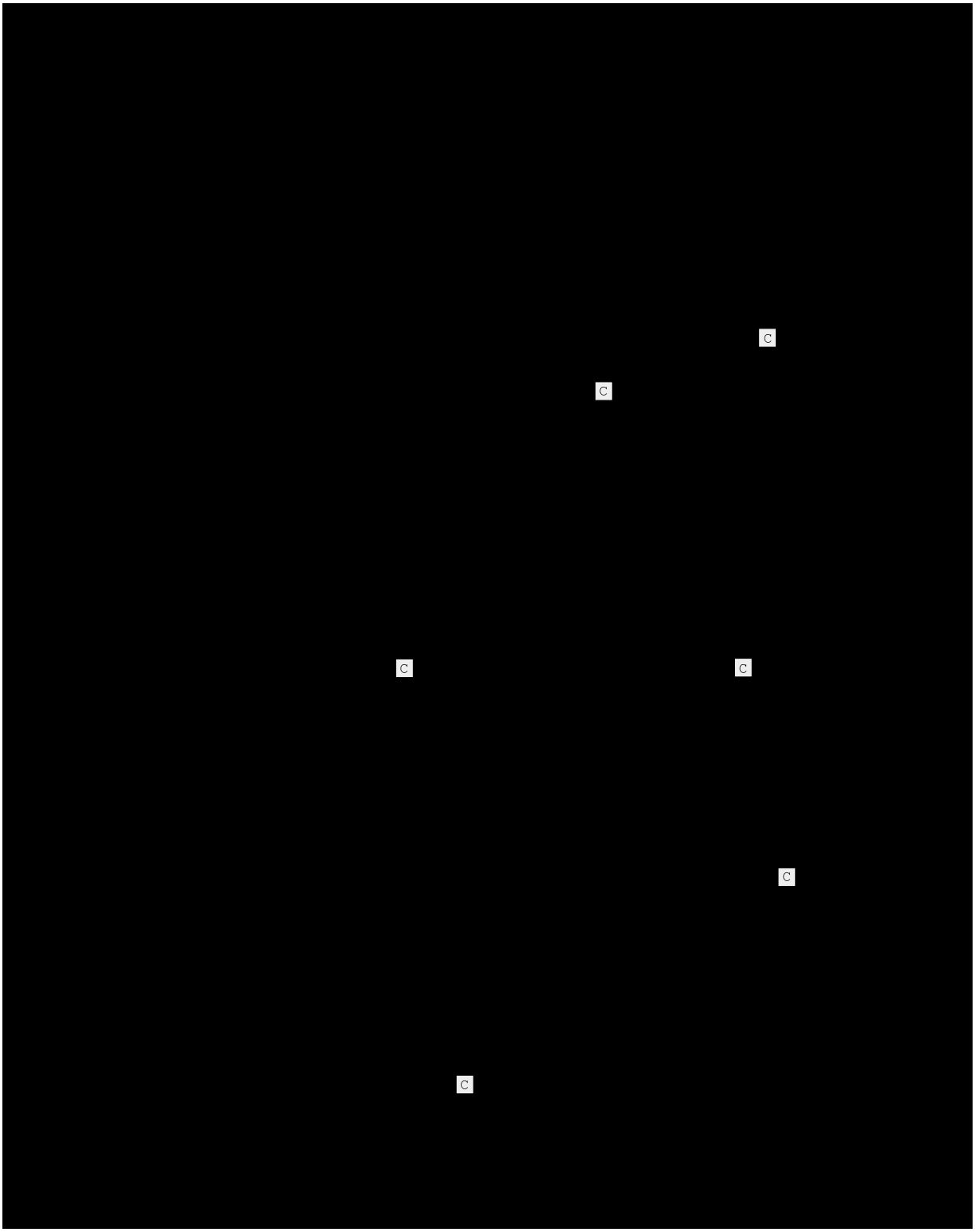

Figure 1. Alignment of *ace-1* coding cDNA and AChE1 proteins of *A. gambiae* susceptible (KISUMU) and resistant (YAO) strains. The position of each intron is indicated by a triangle. The three residues composing the catalytic triad S200, E327 and H440 are indicated by a boxed letter (the amino acid numbering is that of *Torpedo*). W84 from the choline binding site is underlined. Each intrachain disulphide bridge is indicated by dotted lines between two cysteins (shaded boxes). The 119 codon is in bold type. The arrows indicate the position of the oligonucleotides used for the diagnostic test. The *Alu*I restriction site used in the diagnostic test is indicated by an ellipse.

from a single point mutation GGC to AGC in the gene *ace-1* (Weill *et al*., 2003). Moreover, within the *C. pipiens* species, this mutation arose at least twice independently, once in the temperate (*C. p. pipiens*) and once in the tropical form (*C. p. quinquefasciatus*).

The fact that the same G119S mutation arose at least three times independently, twice in *C. pipiens* and once in *A. gambiae*, has two implications. First, a molecular test for detecting this mutation will be an invaluable tool for population surveys and monitoring resistance in the field, particularly for *A. gambiae*, which is the main malaria vector throughout Africa. Second, there is the possibility that the same mutation explains the high resistance found in other mosquito species.

The aim of this work is three-fold. First, to provide better documentation of the *ace-1* gene and the effect of the G119S mutation; second, to present a simple molecular test to detect this mutation in single individuals; and third, to evaluate whether the G119S mutation explains the high resistance found in some other mosquito species.

Results

ace-1 *cDNA sequence of* A. gambiae

Genomic sequences of the *ace-1* gene of *A. gambiae* susceptible (KISUMU) and resistant (YAO) strains were acquired by direct sequencing of PCR products. PCR oligonucleotides were designed on the *A. gambiae* sequence as found in the *in silico* genome (Weill *et al*., 2002). Coding cDNA sequences were then deduced and aligned (Fig. 1). The G119S mutation responsible for the high carbamate AChE1 insensitivity (Weill *et al*., 2003) is located in the third coding exon (which corresponds to the fifth exon, as there are two non-coding exons in the 5′ region).

How might the G119S mutation interfere with the insecticide binding?

A three-dimensional (3D) model was applied to *A. gambiae* sequence. Indeed, the sequence similarities between the AChE of *Torpedo californicus* and the AChE1 of *A. gambiae* are close enough for a 3D model to produce a superimposition of the active sites, in which it can be seen that the mutated residue G119S is located within the active gorge of the enzyme (Fig. 2). Moreover, S119 is close enough to the catalytic residues to alter sterically the presentation of substrates and site inhibitors, as the serine abuts the oxyanion hole (Silman *et al*., 1999). The steric effect is further suspected as substrates and inhibitors are overlying in the 3D model.

Single mosquito test to detect the G119S mutation

The presence of the G119S creates a *Alu*I restriction site in the *ace-1* gene of resistant individuals (Fig. 1). This was used to design a PCR/RFLP test that detects the presence of the G119S mutation in single mosquitoes. Two primers located in the third coding exon, on each side of the position 119 (Moustdir1 and Moustrev1) generated a 194 bp fragment by PCR on genomic DNA, which is cut by the *Alu*I restriction enzyme only in resistant mosquitoes (Fig. 1). We designed degenerate oligonucleotides that could amplify specifically *ace-1* in either *A. gambiae* or *C. pipiens* genomic DNA. Individual mosquitoes were first sorted into three sets, according to the sensitivity of their AChE: set 1 with only sensitive AChE (homozygous susceptible), set 2 with only insensitive AChE (homozygous resistant) and set 3 with both types of AChE (heterozygous) (Fig. 3A). The presence of the serine conferring an *Alu*I site was observed in all *A. gambiae* YAO ($N = 20$) and the M'bé ($N = 20$) population and also in *C. pipiens* ($N = 40$) resistant mosquitoes (homozygous or heterozygous) but in none of the susceptible mosquitoes tested ($N = 60$). In the M'bé population, the two molecular forms of *A. gambiae* s.s. (S and M, Favia *et al*., 1997) coexist, and we found the serine mutation in both.

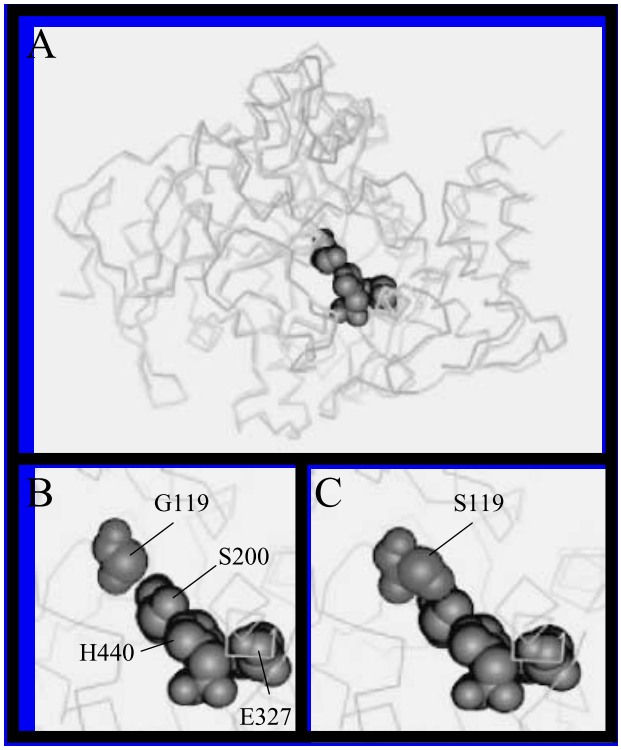

Figure 2. Position of the G119S mutation in *ace-1* of *A. gambiae*. (A) The alpha-carbon skeleton of the modelled 3D structure of *ace-1* overlain on that of the AChE of *T. californicus* (pdb 1EA5); RMS deviation of 1.1 Å on 528 carbon atoms. The catalytic triad (S200, H440, E327) is shown as van der Waals spheres. The view is down the catalytic gorge. (B) A close-up of the catalytic triad of *ace-1* of the susceptible strain of *A. gambiae* showing G119 at the edge of the oxyanion hole. (C) A close-up of the catalytic triad of *ace-1* of the resistant strain of *A. gambiae* showing S119. The alpha-carbons of G118 and S119 are displaced 0.5 Å from those shown in B; all other differences in the active site are negligible. The catalytic S200 hydroxyl is within hydrogen-bonding distance of either H440 or S119.

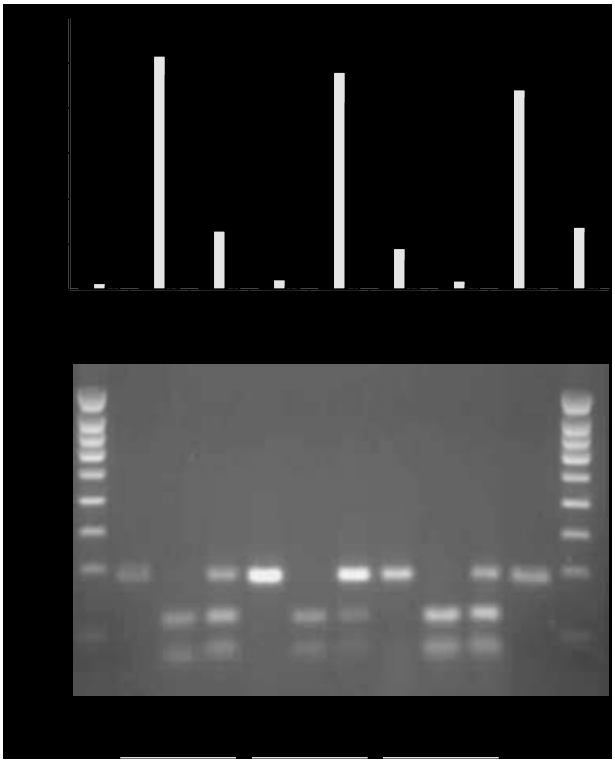

Figure 3. Detecting G119S in single individuals. (A) Relative AChE residual activity measured in the absence of insecticide (black), or in the presence of 10^{-4} M (grey) or 10^{-2} M (dots) of propoxur (a carbamate insecticide), for the three possible genotypes (SS, RR or RS) in each species. (B) Diagnostic PCR-RFLP test to detect the G119S mutation in *A. gambiae*, *C. pipiens* and *A. albimanus* individual mosquitoes. Genomic DNA amplification with the degenerated primers Moustdir1 and Moustrev1 produced a 194 bp fragment (lane C), which is undigested by *Alu*I for susceptible homozygous mosquitoes (SS), and cut into two fragments (74 bp and 120 bp) for homozygous resistant (RR). Heterozygous individuals (RS) display a combined pattern.

Application to Anopheles albimanus

The strain FEST, which is polymorphic for resistance owing to high AChE insensitivity, was used. Individual mosquitoes were sorted into three sets, as previously done for *C. pipiens* and *A. gambiae* (Fig. 3A). Genomic DNA was extracted individually, and the above PCR diagnostic test was performed on each mosquito (Fig. 3B). The concordance between insensitive AChE and the presence of the G119S mutation, as detected by the PCR test, was 100% ($N = 10$). This was confirmed by sequencing the PCR product (Fig. 4). The AChE activity measured biochemically in adults is therefore due to AChE1 coded by *ace-1*.

Discussion

High AChE insensitivity in *A. albimanus*, a malaria vector in Central America, is the result of the same mutation G119S found in two other mosquito species, *Culex pipiens* and *A. gambiae*. This mutation has thus occurred at least four times independently, as two distinct occurrences have been documented in *C. pipiens*. This mutation was also detected in M and S molecular forms of *A. gambiae* s.s. in a sample from the Ivory Coast. M and S are considered to represent two incipient species within *A. gambiae* s.s., as gene flow between them is absent or very rare when they coexist in sympatry (Chandre *et al*., 1999; Weill *et al*., 2000; della Torre *et al*., 2002). It is, however, not known if the co-occurrence of this mutation in M and S is due to two independent mutational events or to an introgression between one molecular form and the other. This situation suggests that there are few possible locations, perhaps only one, at which to enhance dramatically AChE1 insensitivity towards OP or carbamate insecticides. This mutation, located in the

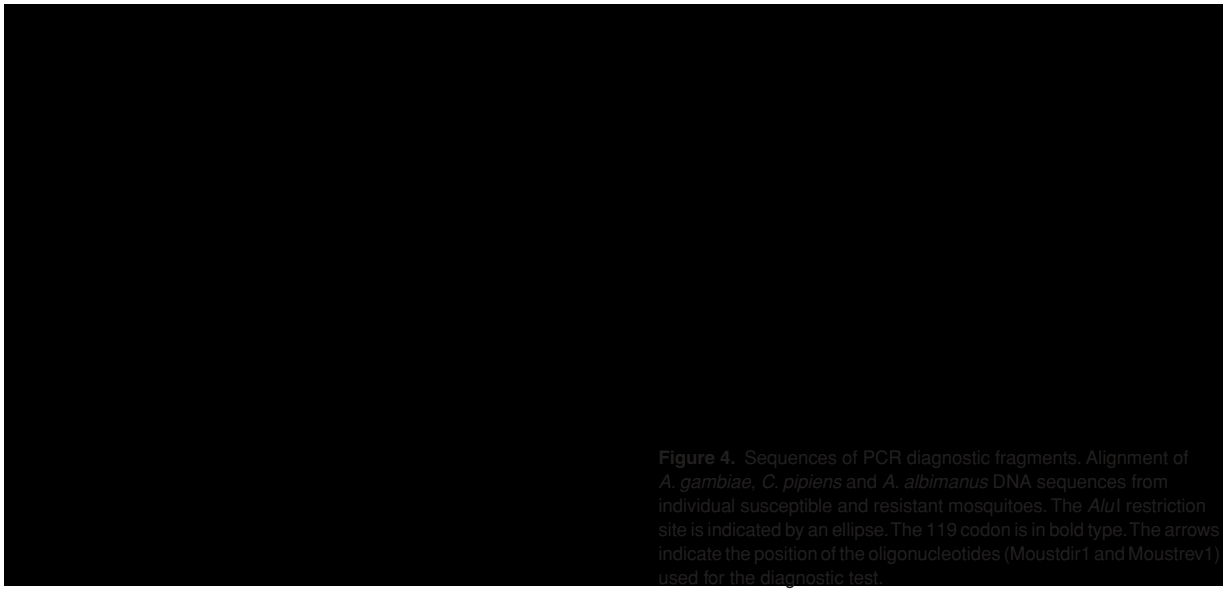

Figure 4. Sequences of PCR diagnostic fragments. Alignment of *A. gambiae*, *C. pipiens* and *A. albimanus* DNA sequences from individual susceptible and resistant mosquitoes. The *Alu*I restriction site is indicated by an ellipse. The 119 codon is in bold type. The arrows indicate the position of the oligonucleotides (Moustdir1 and Moustrev1) used for the diagnostic test.

oxyanion hole, alters the active site, and the serine at position 119 reduces the access to the catalytic triads. Interestingly, mutations at the same residue are known to alter substrate specificity in vertebrate butyrylcholinesterase (Lockridge *et al*., 1997) and insect carboxylesterase E3 (Newcomb *et al*., 1997), and in both cases OP hydrolysis is enhanced. The reduction is substantial for the insecticide (thus the insensitivity), but it affects also the binding of acetylcholine, its natural substrate. Thus the insensitivity towards the insecticide leads to a reduction of the normal enzyme function. In *C. pipiens*, this reduction is *c*. 60% (Bourguet *et al*., 1997) and is also substantial, although not quantified, in *A. albimanus* (Hemingway *et al*., 1986; ffrench-Constant & Bonning, 1989; Dary *et al*., 1991). The biochemical change introduced by G119S is translated into a fitness reduction (fitness cost) associated with the mutation, which has been estimated in *C. pipiens* to be *c*. 11% during the breeding season, and 50–60% during overwintering (review in Raymond *et al*., 2001). A similar feature is expected for *A. gambiae* and *A. albimanus*, although no data are presently available in these species. Preliminary results obtained with field samples from the Ivory Coast indicate that the frequency of resistant homozygous individuals is extremely low in adult females even for samples where the frequency of heterozygosity is around 75%. Incidentally, a drastic decrease of *A. albimanus* with an insensitive AChE1 in the field, after the reduction in agriculture insecticide use in Central America (Hemingway *et al*., 1997), is consistent with the hypothesis of a high fitness cost of the G119S mutation in this species.

The PCR test was devised to detect this particular mutation in both *A. gambiae* and *C. pipiens*, two mosquitoes belonging to different subfamilies (Culicinae and Anophelinae) and the present results show that it works on a third species, *A. albimanus*. This indicates that it has probably a broad applicability within the Culicidae family. For the three species presently considered, resistant individuals display a similarly high insensitivity towards carbamate insecticide. For example, the ki ratio, or (ki susceptible strain)/(ki resistant strain), using the insecticide propoxur, is far higher than 1000 for *C. pipiens*, *A. gambiae* and *A. albimanus* (Ayad & Georghiou, 1975; Bourguet *et al*., 1997; Weill *et al*., 2003). Thus it is suspected that any other mosquito species displaying such high AChE insensitivity will also possess the same G119S mutation, which could eventually be detected by the present PCR test. By contrast, resistant individuals displaying a lower AChE insensitivity may carry an alternative mutation, which remains to be identified. This is probably the case for resistant *C. pipiens* from Cyprus (propoxur ki ratio = 223), and perhaps for a malaria vector in the Middle East, *A. sacharovi* (propoxur ki ratio probably << 1000) (Hemingway *et al*., 1985; Bourguet *et al*., 1997).

Experimental procedures

Mosquito populations

Kisumu is a susceptible reference strain of *A. gambiae* s.s. (100% S molecular form) originated from Kenya and maintained for many years under laboratory conditions.

Yao is a strain of *A. gambiae* s.s. (100% S molecular form) originated from a peri-urban area of Bouaké (Ivory Coast). This strain was initially strongly resistant to carbamates (N'Guessan *et al*., 2003) and further selected with carbamates for fifteen generations under laboratory conditions. M'bé is a field sample of *A. gambiae* s.s. (95% M and 5% S molecular forms) collected as adults in 1998 in rice fields, 35 km north of Bouaké. The FEST strain of *A. albimanus* was used. It was originally collected in 1974 from El Salvador, Central America (Hemingway *et al*., 1986). When assayed, this strain was polymorphic for AChE insensitivity. For *C. pipiens*, we used the susceptible reference strain S-LAB (Georghiou *et al*., 1966) and the homozygous resistant strain displaying an insensitive AChE (Berticat *et al*., 2002).

Three-dimensional modelling

Three-dimensional structures of *ace-1* were created by automated homology modelling using the program MODELLER (Sali & Blundell, 1993) in the Quanta98 package (Accelerys, San Diego CA, USA), with default settings for all calculations. The structural templates used were *ace-2* from *D. melanogaster* (pdb: 1DX4) and *ace* from *T. californicus* (pdb: 1EA5). With gaps and insertions both templates possess about 40–50% amino acid homology with each other and with *ace-1*. The initial models generated were subsequently refined by iterative modelling until the interactive energies (electrostatic and Van der Waals) of the residues in the subdomains surrounding the active site had been minimized to a level compatible with that found in the two template molecules; and the side chains of the amino acids adopted rotamer conformations compatible with their main chain angles. No local modifications were introduced at any point of the modelling of the wild-type *ace-1*. The refined model of *ace-1*, overlain on the templates according to matched secondary structures with the alignment that was used to generate the initial structure of *ace-1*, gave the following rms deviations: *ace-2 D. melanogaster*: *ace*, *T. californicus*: 1.6 Å (314 matched carbon alpha atoms); *ace-1*, *A. gambiae*: *ace*, *T. californicus*: 0.7 Å (316 matched carbon alpha atoms); *ace-1*, *A. gambiae*: *ace-2*, *D. melanogaster*: 1.3 Å (318 matched carbon alpha atoms). The G119S mutation was introduced into the sequence of wild-type AChE1 and subjected to a single cycle of modelling. The side-chain conformation of S119 varied among the models generated. The model obtained has adopted a conformation within hydrogen-bonding distance of the catalytic S200.

Accession numbers

AGA515150, AGA488492: *ace-1* Kisumu
AGA515149, AGA515148: *ace-1* Yao
AJ566402: *A. albimanus* susceptible
AJ566403: *A. albimanus* resistant

DNA diagnostic test for the G119S *mutation in single mosquitoes*

Mosquito genomic DNA was extracted from single mosquitoes according to Rogers & Bendich (1988). DNA was then PCR amplified

with the degenerated primers Moustdir1 5′CCGGGNGCSACYATGTGGAA3′ and Moustrev1 5′ACGATMACGTTCTCYTCCGA3′ for thirty cycles (94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min). The PCR fragments were digested with *Alu*I restriction enzyme according to the manufacturer's instructions and fractionated on a 2% agarose gel. AChE activity and propoxur sensitivity tests were performed according to Bourguet *et al.* (1996b). In order to check the identity of the amplified fragments, sequences were performed directly on PCR products using the Big Dye terminator kit (Applied Biosystem).

Acknowledgements

We are grateful to Nicole Pasteur for critical reading the manuscript, C. Bernard, C. Berticat and P. Fort for technical help, R. N'Guessan, A. Koffi and Y. Konan for help in mosquito collection, and V. Durand for help with the literature search. This investigation received financial support from Ministère de la Recherche (PAL+ no. 2002-45) and CNRS 'Post-séquençage anophèle'. This is publication ISEM 2003.XXX.

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