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Determination of *Ace.1* Genotypes in Single Mosquitoes: Toward an Ecumenical Biochemical Test

DENIS BOURGUET, NICOLE PASTEUR, JUAN BISSET,* AND MICHEL RAYMOND

*Institut des Sciences de l'Evolution (UMR 5554), Lab. Génétique et Environnement, Université Montpellier II (C.C. 065), F-34095, Montpellier Cedex 05, France; and *Instituto de Medicina Tropical "Pedro Kouri," Apartado 601, Mariano 13, La Habana, Cuba*

The occurrence of two acetylcholinesterases, AChE1 and AChE2, in the mosquito *Culex pipiens* has been recently documented. Resistance to organophosphates and carbamates due to target insensitivity is the result of a qualitative change of only AChE1, encoded by the *Ace.1* gene. Because AChE1 and AChE2 differ in their sensitivity to inhibitors, *Ace.1* genotypes can be misclassified by previous tests. We describe a new rapid microplate test that allows unambiguous identification of *Ace.1* genotypes. This test involves comparing AChE activities in the absence of insecticide and in the presence of two propoxur concentrations: a low concentration that inhibits only the sensitive AChE1 and a higher concentration that inhibits also AChE2 but not the insensitive AChE1 responsible of insecticide resistance. This comparison allows the identification of the three *Ace.1* genotypes: resistant ($Ace.1^{RR}$), susceptible ($Ace.1^{SS}$) homozygotes, and heterozygotes ($Ace.1^{RS}$). The similarity of propoxur sensitivity of modified AChE1s found in various resistant strains from the United States, Europe, and Africa indicates that this test is probably suitable for all the *Ace.1* alleles described so far in *C. pipiens*.

INTRODUCTION

In insects, acetylcholinesterase (AChE) is a key enzyme in cholinergic systems where it hydrolyzes the neurotransmitter responsible for nerve transmission (1). This enzyme is the main target of two major insecticide classes: carbamates and organophosphates (2). Intensive pest controls have selected less sensitive AChEs in more than 25 arthropod species including several mosquitoes (for review see Fournier and Mutéro (3)). In the *Culex pipiens* complex, this resistance mechanism has been detected in numerous places (Table 1).

Several single-mosquito tests to determine *Ace* genotypes have been developed using either microplate assays (4–9) or dot-blot tests (10). All of these techniques rely on the comparison of the AChE activity in the absence and in the presence of an insecticide at concentrations that inhibit sensitive AChEs but not insensitive ones. The comparison of inhibited and uninhibited AChE activities using a chromogenic substrate allows a distinction to be made between the three genotypes: resis-

tant Ace^{RR}, susceptible Ace^{SS} homozygotes, and heterozygotes Ace^{RS}.

All of these tests assume that a single AChE is present in mosquitoes. This is the case for *Drosophila*, and studies conducted on several species of Diptera, Lepidoptera, Orthoptera, and Coleoptera, also gave no evidence that there was more than one AChE in other insects. Thus, the occurrence of a single AChE was thought to be general in all insects (reviewed by Toutant (1)). However, two recent reports indicate a more complex situation in *C. pipiens*. First, two AChE enzymes (AChE1 and AChE2) differing in substrate specificity and in sensitivity to inhibitors have been recently characterized (11), and only AChE1 has been shown to be involved in insecticide resistance. Based on the available literature on AChE and on some recent results obtained at the molecular level, Bourguet *et al.* (11) suggested that AChE1 and AChE2 are the products of two distinct genes, namely *Ace.1* and *Ace.2*. Second, the activity pattern of AChE in mosquitoes from the Caribbean is best explained by a duplication of *Ace.1* (12).

TABLE 1
Reports of Insensitive AChE in Mosquitoes
of the Culex Pipiens Complex

Localization	References
Europe	
France	4, 23–24
Portugal	15
Italy	25, 26
Greece	Bourguet, unpublished
Cyprus	17
Spain	20
Africa	
Tanzania	27
Tunisia	16
Ivory Coast	Chandre, unpublished
Asia	
Burma	22
Caribbean	
Cuba	18
Martinique	28

The occurrence of two AChEs in *C. pipiens* mosquitoes has important consequences. Most of the insensitive AChEs are highly resistant to propoxur, and consequently this carbamate has been used for genotype determinations (4–10). This technique may lead to potential misclassification of mosquito *Ace.1* genotypes, because the *C. pipiens* AChE2 has also a low propoxur sensitivity (11). In the present paper (i) we described a new method allowing unambiguous discrimination between *Ace.1* genotypes, and (ii) we discuss the generalization of this new test by studying seven strains from various geographical origins possessing an insensitive AChE1.

MATERIALS AND METHODS

Insects

Eight mosquito strains were used: S-LAB, an insecticide susceptible reference strain from California (13) and seven resistant strains homozygous for an insensitive AChE1: (i) MSE, collected from southern France in 1979 (14, 15); (ii) PRAIAS, collected from Portugal in 1993 (15); (iii) ESPRO, collected in Tunisia in 1993 (16); (iv) SUPERCAR col-

lected from Ivory Coast in 1994 (Chandre, unpublished results); (v) ACE-R collected in Cyprus in 1993 (17); (vi) MRES, collected in Cuba in 1987 (12, 18); and (vii) MARTINIQUE, collected in Martinique in 1993 (12).

To obtain heterozygous individuals, resistant males of each strain were mass-crossed with S-LAB females. Offspring were designated as MSE-F1, PRAIAS-F1, ESPRO-F1, SUPERCAR-F1, ACE-R-F1, MRES-F1, and MARTINIQUE-F1 depending on the resistant strain used as male parent.

AChE Assays

Enzyme preparation. For each strain, around 1000 mosquitoes were mass-homogenized in 20 ml extraction buffer (20 mM Tris, pH 7.0, containing 0.1% Triton X-100). Homogenates were centrifuged at 10,000g for 5 min. Supernatants were dialyzed against extraction buffer and used for the measurement of AChE activity. Activities were evaluated by monitoring the hydrolysis of acetylthiocholine iodide (AcSCh) in the presence of 5,5′-dithio*bis*-2-nitrobenzoic (DTNB) (19).

Evaluation of catalytic parameters. For MSE and S-LAB, the bimolecular rate constant (ki) for three carbamates (eserine, aldicarb, and propoxur) and two oxon forms of OPs (malaoxon and paraoxon) was estimated for AChE1 and AChE2 following the dilution method of Aldridge (20). Briefly, supernatants were incubated with the inhibitor for various times before adding 100 μl of these inhibition mixtures into 100 μl of a substrate–reagent solution (final concentration: 1.7 mM DTNB; 2.5 mM AcSCh). The plot of the ln of residual activity (Vi/Vo) against time, for a given concentration, is linear when AChE1 and AChE2 have the same ki. When their kis are different, the plot is biphasic. The slope of each line divided by the inhibitor concentration gives the respective ki of each AChE. Using the same procedure, ki of insensitive AChE1 was estimated for propoxur in all other resistant strains.

Residual AChE activities. They were estimated in presence of increasing concentra-

tions of propoxur. (larval homogenates were used because of higher quantity of AChE2, unpublished data). Around 50 larvae were mass-homogenized in 5 ml of extraction buffer (see above). Homogenates were centrifuged at 10,000g for 5 min and supernatants were used to measure AChE activities. Different concentrations of propoxur were added to 100 μl of supernatant. One hundred microliters of the substrate reagent (see above) was added after 15 min of incubation. Residual AChE activities were measured at different concentrations of propoxur and compared to AChE activity in the absence of insecticide.

Description of the Test to Identify Ace.1 Genotypes

Principle. The test compares AChE activities from three identical equal aliquots taken from single mosquito homogenates. The first aliquot measures the AChE activity in the absence of insecticide (A1), which corresponds to AChE1 (sensitive and/or insensitive) plus AChE2 activities. The second aliquot establishes the AChE activity (A2) at a concentration of insecticide (C2) that inhibits only the sensitive AChE1. Finally, aliquot 3 measures the AChE activity (A3) at a concentration of insecticide (C3) that inhibits AChE2 and sensitive AChE1, but not the insensitive AChE1.

Stock solutions. Solution A can be either 20 mM Tris–HCl or 100 mM sodium phosphate, pH 7.0, both containing 1% Triton X-100. Solution B is 25 mM sodium phosphate, pH 7.0, containing 0.2 mM DTNB and 0.35 mM sodium bicarbonate.

Mosquito preparation. Each mosquito (with or without its abdomen) is homogenized in an Eppendorf tube with a glass pestle in 400 μl of solution A. Tubes are centrifuged at 10,000g for 2 min. The supernatants are used for AChE activity measures.

Procedure. To determine *Ace.1* genotypes, three wells (H1, H2, and H3) of a microtitration plate are needed to determine A1, A2, and A3, respectively. One hundred microliters of mosquito extract are added to each well. Then, 10 μl of alcohol, 10 μl of ninefold C2,

and 10 μl of ninefold C3 are added to H1, H2, and H3, respectively. C2 and C3 are determined as described under Results and Discussion. The microtitre plate is left 15 min at room temperature before the addition of 100 μl of solution B (plus 2.5 mM acetylthiocholine) to each well. Reading can be done kinetically but may also be done visually depending on the available equipment:

(i) Kinetically: Rates of reaction is measured after 1 min (this ensures linearity during the recording period) on a Spectramax 250 (Molecular Devices) at 412 nm over a period of 10 min. Graphs of optical density over time for all the wells can be recorded.

(ii) Visually: Readings can be done several times between 15 min and 1 hr.

RESULTS AND DISCUSSION

In larval and adult mosquitoes, AChE2 and AChE1 enzymes coexist in the thorax as well as in the head (11), so that it is impossible to separate them. As a consequence, *Ace.1* genotype determination requires the use of discriminative concentrations (i.e., concentrations that inhibit differentially AChE2 and sensitive and insensitive AChE1).

Determination of the Discriminative Concentrations

The inhibition constants of AChE1 and AChE2 for different inhibitors are given in Table 2 for MSE and S-LAB strains. As previously found, AChE2 has similar inhibitor sensitivities in both strains (11). Conversely, AChE1 sensitivity was lower in MSE than in S-LAB, which is congruent with insecticide resistance levels displayed by MSE (14, 15). The ki ratio between sensitive AChE1 of S-LAB and insensitive AChE1 of MSE depends on the compound considered and varied from 6.7 for malaoxon to 290,000 for propoxur. Propoxur is the only insecticide that allowed a clear discrimination of three AChEs (AChE2, sensitive and insensitive AChE1) as each one displayed a different sensitivity to this compound (Table 2). This property enabled two

TABLE 2

Bimolecular Rate Constants (ki) for AChE1 and AChE2 of MSE and S-LAB
for Different Organophosphates and Carbamates

Inhibitors		ki (mM^{-1} min^{-1})		
		S-LAB (susceptible)	MSE (resistant)	Ratio MSE/ S-LAB
Propoxur	AChE1	145	0.0005	290,000
	AChE2	0.008	0.009	0.93
Aldicarb	AChE1	6.04	0.58	10
	AChE2	6.04	6.2	0.97
Eserine	AChE1	15,000	61	246
	AChE2	98	110	0.89
Malaoxon	AChE1	1.2	0.18	6.7
	AChE2	0.117	0.105	1.11
Paraoxon	AChE1	159	0.37	430
	AChE2	159	162	0.98

discriminating propoxur concentrations to be defined: C2, inhibiting only the sensitive AChE1, and C3, inhibiting both the sensitive AChE1 and AChE2 but not the insensitive AChE1. These concentrations were determined by recording the residual AChE activities of MSE, S-LAB, and MSE-F1 larvae in the presence of increasing concentrations of propoxur (Fig. 1). The biphasic curves observed in the two homozygous strains correspond to the differential inhibition of AChE2 and sensitive (for S-LAB) or insensitive (for MSE) AChE1s. For heterozygotes (MSE-F1), which carry a mixture of the three AChE enzyme, the curve is triphasic. These curves
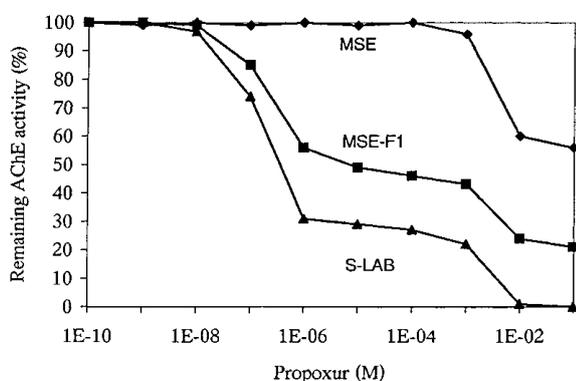

FIG. 1. *Residual AChE activity of larval mosquito homogenates of S-LAB, MSE, and MSE-F1, in the presence of increasing propoxur concentrations.*

show that the sensitive AChE1 from S-LAB and MSE-F1 is completely inhibited by 10^{-4} M propoxur, whereas AChE2 and insensitive AChE1 are unaffected. Consequently, this concentration (10^{-4} M) was chosen as C2. In S-LAB, no residual AChE activity was recorded at 10^{-2} M propoxur indicating that AChE2 is completely inhibited. The remaining AChE activity in MSE and MSE-F1 in the presence of 10^{-2} M propoxur is therefore due to the insensitive AChE1. This second concentration was chosen as C3.

Microtiter Plate Test on Single Mosquitoes

AChE activities of S-LAB, MSE-F1, and MSE single adult mosquitoes were analyzed in the absence of propoxur (A1) and in the presence of propoxur at concentration C2 (A2) and C3 (A3) (Table 3). As expected, in susceptible S-LAB individuals $Ace.1^{SS}$, A3 activity is equal or almost equal to zero due to the complete inhibition of both AChE2 and sensitive AChE1, and A2 (due to the AChE2 enzyme) represents less than 20% of the total activity (A1). In resistant MSE individuals $Ace.1^{RR}$, A1, and A2 activities are identical, which is congruent with the absence of sensitive AChE1. The small reduction of A3 activity as compared to A1 and A2 activities is

	AChE activity in mD0 min^{-1} (±SE)		
	A1	A2	A3
S-LAB	97 ± 24	8 ± 1	0
MSE-F1	65 ± 15	23 ± 4	18 ± 3
MSE	32 ± 5.0	32 ± 5	28 ± 3

TABLE 4
Bimolecular Rate Constants (ki) of AChE1 for Propoxur for the Reference Susceptible Strain (S-LAB) and for Several Resistant Strains from Various Geographical Origins

Resistant strain	Origin	ki (M^{-1} min^{-1})
S-LAB	California	145,000
MSE	Southern France	0.50
ESPRO	Tunisia	0.63
PRAIAS	Portugal	0.58
MRES	Cuba	0.72
MARTINQUE	Martinique	0.65
SUPERCAR	Ivory Coast	0.46
ACE-R	Cyprus	670

probably due to inhibition of the AChE2 enzyme. Finally, for *Ace.1^{RS}* mosquitoes (MSE-F1), the presence of an A3 activity is due to the insensitive AChE1 fraction and the lower A2 compared to A1 is due to the sensitive AChE1 fraction. An example of a typical output from the kinetic plate reader is given in Fig. 2.

Toward an Ecumenical Test?

The test described above allows a perfect discrimination of *Ace.1* genotypes for the strains studied. In order to evaluate if this test could be applied to other situations, six resistant
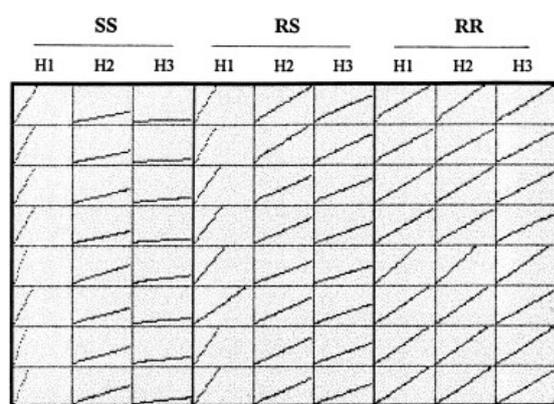

FIG. 2. *Graphical output from kinetic plate reader for S-LAB (SS, Ace.1^{SS}), MSE-F1 (RS, Ace.1^{RS}) and MSE (RR: Ace.1^{RR}) Culex pipiens. Seventy-two graphs of optical density (maximum limit = 0.1) against time (5 min overall) of a microtiter plate. Horizontal wells H1 (with no propoxur), H2 (with 10^{-4} M propoxur) and H3 (with 10^{-2} M propoxur) represent aliquots from the same mosquito.*

strains from various geographic origins, and all homozygous for an insensitive AChE, were analyzed (Table 4). All of these strains except ACE-R displayed an insensitive AChE1 with a similar sensitivity to propoxur. These strains were crossed with S-LAB in order to obtain *Ace.1^{RS}* genotypes, and microtiter plate tests were performed on single adults as described above. For European (except ACE-R) and African strains, the test gave a perfect discrimination between the three *Ace.1* genotypes (details not shown), indicating that the present test could be used in these areas.

In the two strains from Caribbean islands (Martinique and Cuba), the distinction between *Ace.1^{RR}* and *Ace.1^{RS}* was not possible because MARTINIQUE and MRES insects carry a mixture of sensitive and insensitive AChE1s probably due to the duplication of *Ace.1* (12). Thus, when C2 is applied, AChE activity of resistant homozygotes and F1s heterozygotes are partially inhibited, preventing the discrimination of the two genotypes. Consequently, it seems difficult to devise a reliable biochemical test to establish the *Ace.1* genotypes of Caribbean mosquitoes. However, the proportions of *Ace.1^{SS}* and (*Ace.1^{RS}* + *Ace.1^{RR}*) genotypes can be correctly estimated with the present test, and *Ace.1^{R}* and *Ace.1^{S}* frequencies could be computed assuming Hardy–Weinberg equilibrium although, with the various selection pressures acting at this locus (21), a cautious use of the test is advised.
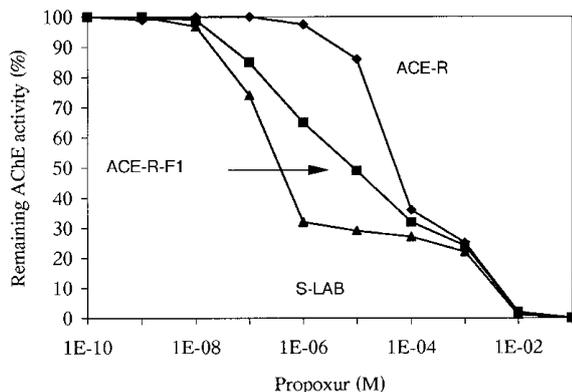
FIG. 3. *Residual AChE activity of mosquito larval homogenates of S-LAB, ACE-R, and ACE-R-F1 in the presence of increasing concentrations of propoxur.*

Our test failed to characterize *Ace.1* genotypes from the ACE-R strain derived from a natural population collected in Cyprus (17). The insensitive *Ace.1* allele in this strain is much more sensitive to propoxur than the other ones (Table 4) and is consequently fully inhibited by C3 propoxur. Comparisons of residual AChE activities for S-LAB, ACE-R, and ACE-R-F1 as a function of propoxur concentrations (Fig. 3) show that $10^{-6} M$ propoxur could be used to discriminate the three genotypes. However, at this concentration, AChE2 will not be inhibited, leading to a potential misdiscrimination between *Ace.1^{SS}* and *Ace.1^{RS}*. As a consequence it may be safer to determine the frequency of *Ace.1^{RR}* and (*Ace.1^{RS}* + *Ace.1^{RR}*) genotypes; like for Caribbean populations, allelic frequencies of *Ace.1^{S}* and *Ace.1^{R}* estimated by assuming Hardy–Weinberg equilibrium should be considered with caution.

In conclusion our test seems to be a useful tool for determining *Ace.1* genotypes in numerous populations from different parts of the world. It has been recently used in the Ivory Coast on *Culex p. quinquefasciatus* mosquitoes collected in the field and the results are consistent with the level of propoxur resistance determined by insecticide bioassays (Chandre, personal communication). Finally, we cannot ignore the fact that some insensitive AChE1 alleles could not be detected by the present test, in particular if they display a low insensitivity to propoxur (see, e.g., (22)). In these cases, it will be necessary to find another insecticide for which the three AChEs have different sensitivities. This makes the determination of catalytic properties of insensitive AChE1 a necessary step before extensive field investigations.

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