Resistance to Organophosphorous Insecticides in *Culex pipiens quinquefasciatus* (Diptera: Culicidae) from Martinique

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J. Med. Entomol. 32(2): 77–82 (1995)

ABSTRACT Before beginning a widespread control program against *Culex pipiens quinquefasciatus* in Martinique, resistance to temephos, chlorpyrifos, and two organophosphorous insecticides, was investigated at seven breeding sites. At LC_{95}, populations exhibited resistance ratios between 6.9 and 11.6 for temephos and between 6.4 and 51.4 for chlorpyrifos. Overproduced esterases A2-B2 and B1, known to be involved in organophosphorous-resistance, were present at all breeding sites; esterases A2-B2 frequency was >50% at all sites but one; and esterase B1 frequency was <7%. Experimental treatment of three breeding sites with temephos induced no significant increase in resistance, but our esterase studies indicated a significant increase in the frequencies of esterase B1 and of a new highly active esterase C2. These results indicate that a large-scale *C. p. quinquefasciatus* control program with organophosphorous insecticides will induce a rapid increase of these resistance genes throughout Martinique. However, this may not necessarily result in high levels of resistance, because, at present, the level of gene amplification of esterase B still appears to be low.

KEY WORDS resistance, esterases, organophosphates

SIMILAR TO MANY Caribbean islands, Martinique has been subjected to intensive mosquito control aimed at malaria vectors, *Anopheles aquasalis* Curry and *Anopheles albimanus* Wiedemann, and, more recently, the dengue vector *Aedes aegypti* (L.). Malaria vector control was started around 1947 using 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) and 1,2,3,4,5,6-hexachlorocyclohexane (HCH), and the disease has been absent from the island since 1961–1963. An *A. aegypti* eradication program began in 1968 is still underway. It is based on intensive chemical control of the larval and adult stages using organophosphorous insecticides complemented by physical control. Despite significant financial investments, the possibility of dengue epidemics has remained high, and progress depends on the permanent cooperation of the population in restricting potential breeding sites. Information campaigns are undertaken regularly, but their effect is always short-lived, mainly because destroying *A. aegypti* breeding sites does not significantly reduce the mosquito nuisance caused mostly by *Culex pipiens quinquefasciatus* Say. Indeed, in some areas of Fort-de-France and Sainte-Luce, up to 250 *C. p. quinquefasciatus* bites per man were registered between 1800 and 2400 hours (A.Y., unpublished data).

To involve the Martinique population in dengue control and to decrease mosquito nuisance level, it was decided to undertake *C. p. quinquefasciatus* control with organophosphorous insecticides. Such control also may prevent the reintroduction of Bancroftian filariasis, a disease agent transmitted by *C. p. quinquefasciatus* in the Caribbean region, which has not been reported in Martinique for the last 30 yr. The current study was conducted in May 1990, a few months before starting the control program in January 1991. It had three purposes: (1) to determine the status of resistance of larval populations to temephos and chlorpyrifos, (2) to identify organophosphorous insecticide-resistance genes, if present, and (3) to forecast how organophosphorous insecticide-resistance levels and gene frequencies will evolve by conducting a control experiment on a few breeding sites.

Materials and Methods

Mosquitoes were collected as larvae or pupae at seven breeding sites (six localities) on the island of Martinique in May 1990 (Fig. 1). Larvae were reared to adults under standard laboratory conditions; 2–3-d-old adults were stored in liquid nitrogen until further processed.

Bioassays were conducted on fourth instars according to the procedure of Raymond et al. (1986), using temephos (technical grade, 96.4% purity, American Cyanamid, Princeton, NJ) and chlorpyrifos (technical grade, 98% purity, Dow Chemical, Midland, MI). The S-LAB strain, isolated by Georghiou et al. (1966) and maintained in Montpellier since 1988, was tested simultaneously for reference purposes. Mortality data were analyzed using the log-probit computer program of Raymond et al. (1993), based on Finney (1971).

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0022-2585/95/0077–0082$02.00/0

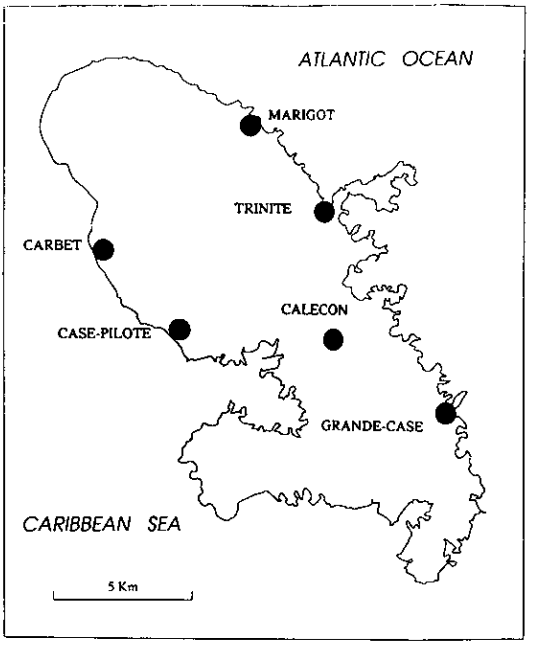

Fig. 1. *C. p. quinquefasciatus* populations sampled on Martinique, May 1990.

Resistance genes were analyzed for adults emerging from field-collected larvae and in a few instances from larvae that had survived in bioassays. Acetylcholinesterase (AChE) genotypes were identified with the microplate test of Raymond & Marquine (1994). Each microplate contained three reference mosquitoes: one Ace^{SS} from the S-LAB strain, one Ace^{RR} from the MSE strain (Raymond et al. 1986), and one Ace^{SR}, which was the offspring of a MSE × S-LAB cross. Over-produced esterases were characterized by starch gel electrophoresis (Pasteur et al. 1988), on the basis of their staining intensity in the presence of α- and β-naphthyl acetates and their electrophoretic mobilities. Reference strains included BARRIOL with esterase A1 (N.P. & M.R., unpublished data), TEM-R with esterase B1 (Georghiou et al. 1980), SELAX with esterases A2 and B2 (Wirth et al. 1990), and VIM with esterases A4 and B4 (Poirié et al. 1992).

Modifications of organophosphorous-resistance levels and organophosphorous-resistance gene frequencies after temephos treatments were investigated in breeding sites at Carbet, Marigot, and Trinité. Each site was visited regularly during 3 wk. At each visit, preimaginal abundance was estimated, larvae were collected for bioassays and resistance gene analyses, and the breeding sites were treated with 1% temephos sand granules at the recommended World Health Organization dose (1 mg/liter). Abundance of each preimaginal stage (egg rafts, first, second, third and fourth instars, and pupae) was rated from the number of each stage that could be collected by two persons during a half-hour search (0, no collection of a particular stage; 1, 2, and 3, collection of a few tens, a few hundred, and many thousands, respectively).

Results

Resistance of *C. p. quinquefasciatus* larvae from Martinique. Bioassays were conducted on six samples (Table 1). Log-probit mortality curves were linear ($P > 0.05$) in all samples, except the Case-Pilote-Haut sample tested for temephos and the Marigot sample tested for chlorpyrifos. The

Table 1. Characteristics of resistance to temephos and chlorpyrifos in larval populations from Martinique and in susceptible S-LAB strain

Populations	LD_{50}, mg/liter[a]	Slope[b]	χ^2 (df)	RR[c] at LD_{50}	RR[c] at LD_{95}
		Temephos			
S-LAB	0.0018 (0.0017 –0.0019)	5.56 (0.39)	6.63 (4)	—	—
Case-Pilote-H	0.010[d] —	—	6.55 (1)	5.6[d]	8.3[d]
Case-Pilote-B	0.0056 (0.0046 –0.0069)	2.42 (0.25)	3.10 (2)	3.1*	7.6*
Carbet	0.0065 (0.0049 –0.0082)	2.06 (0.25)	3.38 (2)	3.6*	11.6*
Marigot	0.0052 (0.0042 –0.0065)	2.47 (0.28)	0.24 (2)	2.9*	6.9*
Trinité	0.0081 (0.0067 –0.0097)	3.13 (0.38)	3.08 (1)	4.5*	7.7*
Caleçon	0.0057 (0.0045 –0.0071)	2.38 (0.32)	2.71 (1)	3.2*	8.0*
		Chlorpyrifos			
S-LAB	0.0009 (0.00085–0.00109)	4.58 (0.76)	0.15 (1)	—	—
Case-Pilote-H	0.0066 (0.0053 –0.0081)	2.80 (0.35)	0.003 (1)	6.4*	6.4*
Case-Pilote-B	0.0065 (0.0053 –0.0079)	2.48 (0.25)	1.42 (2)	6.9*	13.9*
Carbet	0.0066 (0.0054 –0.0081)	2.47 (0.25)	2.03 (1)	7.0*	14.2*
Marigot	0.017[d] —	—	12.83 (1)	19[d]	25[d]
Trinité	0.0096 (0.0067 –0.022)	2.01 (0.61)	3.2 (1)	10.3*	29.4*
Caleçon	0.0076 (0.0052 –0.016)	1.42 (0.45)	5.2 (1)	8.1*	51.4*

[a] 95% Confidence limits in parentheses.
[b] Standard deviation of slope.
[c] $LD_{50} = LD_{95}$ when parallelism of mortality line with S-LAB is not rejected at the 5% level. *, RR different from 1 at $P < 0.05$.
[d] Estimated visually.

Table 2. Frequency of mosquitoes with different over-produced esterases in *C. p. quinquefasciatus* adults emerging from field-collected larvae

Populations	*Ace*		*Est-1*		*Est-2*					*Est-1–Est-2*[c]
	RS[a]	(n)[b]	C2[a]	(n)[b]	B2[a]	B1[a]	B2/B1[a]	None[a]	(n)[b]	
Case-Pilote-H	0.03	(37)	0.74	(69)	0.54	0.04	0	0.42	(69)	0.300
Case-Pilote-B	—		0.83	(46)	0.67	0.07	0	0.26	(46)	0.343
Carbet	0	(29)	0.75	(65)	0.52	0.05	0	0.43	(65)	0.175
Marigot	0	(29)	0.52	(61)	0.70	0	0	0.30	(61)	0.512
Trinité	—		0.55	(64)	0.89	0	0	0.11	(64)	0.087
Grande Case	—		0.62	(68)	0.16	0	0	0.84	(68)	0.0001
Caleçon	—		0.65	(60)	0.77	0	0	0.23	(60)	0.346

[a] Frequency.
[b] Sample size.
[c] Probability of nonrandom association of esterase C2 with an esterase B (B1 or B2 or both).

chlorpyrifos mortality line of the Case-Pilote-Haut sample was parallel to that of the S-LAB reference strain ($P > 0.05$), whereas other samples displayed a higher resistance ratio at LC_{95} than at LC_{50}. For temephos, resistance ratios varied between 2.9 and 5.6 at LC_{50}, and between 6.9 and 11.6 at LC_{95}; for chlorpyrifos between 6.4 and 19 at LC_{50}, and between 6.4 and 51.4 at LC_{95}.

Identification of Resistance Genes in Adults Emerging from Collected Larvae. An AChE with reduced insensitivity in the presence of propoxur was observed in one mosquito (*Ace*^{SR}) among the 37 tested from Case-Pilote-Haut, but in none of the 58 tested from the Marigot and Carbet sites. To detect the presence of the *Ace*^{R} allele in the Case-Pilote-Haut sample, adults emerging from larvae that survived 24 h exposures to 0.01 mg/liter temephos in bioassays were tested; none carried *Ace*^{R}.

Electrophoretic studies of single adult homogenates disclosed four esterases of high activity: esterases A2, B1, and B2, which are overproduced (Mouchès et al. 1987), and a new esterase, designated here as esterase C2. Esterase C2 hydrolyzes preferentially α-naphthyl acetate and migrates slightly faster than the highly active esterase C1 recently observed in mainland France (Rivet et al. 1993) and in Corsica (Raymond & Marquine 1994). Esterase C2 presumably is encoded by the *Est-1* locus described by de Stordeur (1976), whereas esterases B1 and B2 are encoded by the *Est-2* locus, and esterase A2 by the *Est-3* locus (Pasteur et al. 1981, Wirth et al. 1990). Among the 433 mosquitoes tested, esterases A2 and B2 were found together in 257 mosquitoes, esterase B2 was alone in 2 mosquitoes, and esterase A2 alone in 1. The esterase B2 identification recently has been confirmed (M.R., unpublished data) by constructing the restriction map of the amplified DNA region encompassing the structural gene in mosquitoes derived from the Trinité collection (TRIN strain homozygous for the presence of esterase B2). This map was strictly identical to that of mosquitoes from the SELAX reference strain (Raymond et al. 1991).

Esterase B2 (and the associated esterase A2) and esterase C2 were observed at all breeding sites (frequency range, 0.16–0.89 for B2 and 0.52–0.83 for C2). In contrast, esterase B1 was detected at a low frequency (0.04–0.07) only at sites from the west coast (Case-Pilote-H, Case-Pilote-B, and Carbet). Esterase C2 and esterases B (B1 or B2, or both) were associated randomly at all sites ($P > 0.05$) except at Grande-Case where mosquitoes with an esterase C2 significantly ($P < 0.001$) lacked an esterase B (Table 2).

Evolution of Populations Following Temephos Treatments. The population abundance at three breeding sites (Carbet, Marigot, and Trinité) was estimated at 2-d intervals (day 1 and day 3) before the first temephos treatment (Table 3). The absence of variation indicated that the population age structure was stationary; i.e., new egg rafts were oviposited regularly.

Temephos was applied first on day 3. Three days later (day 6), the abundance in egg rafts, L1, and L2 was not different from days 1 and 3, but there were very few L3 and no L4 at all sites (Table 3). A few pupae were observed at Carbet. The large decrease in L3, L4, and pupae abundance indicated that all or most of the larvae that should have reached these stages on day 6 had been killed by the treatment on day 3. Egg rafts, L1, L2, and possibly L3 came from eggs deposited after the day 3 treatment. In contrast, Carbet pupae probably were derived from larvae that had survived the day 3-treatment. Adults emerging from pupae collected at Carbet displayed a significant ($P < 0.01$) increase in the frequency of esterases A2-B2 and B1 (both known to be involved in temephos resistance) as compared with adults derived from larvae collected on day 1; they also showed a significant ($P < 0.02$) association of esterase C2 and esterases B (B1 or B2, or both) which was absent on day 1. Carbet L1 and L2 collected on day 6 were also reared in the laboratory for bioassays on L4 and esterase analyses on adults. As compared with day 1, there was no significant difference ($P > 0.05$) in the mortalities registered with temephos and chlorpyrifos. Although all highly active ester-

Table 3. Larval abundance, resistance levels, and resistance gene frequencies in *C. p. quinquefasciatus* at breeding sites treated with 1 mg/liter temephos sand granules

Site	Day[a]	Temephos[b] treatment	Abundance of[c]						% mortalities[d]						Mosquitoes with					
									Temephos			Chlorpyrifos			*Est-1*	*Est-2*[e]				
			E	L1	L2	L3	L4	P	0.003	0.010	0.030	0.003	0.010	0.030	C2	B2	B1	B1B2	None	*n*
Carbet	D1	No	1	3	3	3	3	3	20	73	90	12	65	98	0.75	0.52	0.05	0	0.43	65
	D3	Yes	1	3	3	3	3	3	—	—	—	—	—	—	—	—	—	—	—	—
	D6	No	1	3	3	0	0	1							0.90[f]	0.70[f]	0.10[f]	0.05[f]	0.15[f]	20[f]
									30	78	100	54	93	100	0.85	0.68	0.14	0	0.18	73
	D9	Yes	1	3	2	3	3	1	6	54	97	—	—	—	0.98	0.67	0	0	0.33	43
															0.95[g]	0.74[g]	0.12[g]	0.05[g]	0.09[g]	43[g]
	D13	No	1	2	2	1	1	0	—	—	—	—	—	—	—	—	—	—	—	—
	D20	No	1	2	1	1	1	2	—	—	—	—	—	—	—	—	—	—	—	—
Marigot	D1	No	2	3	3	3	3	3	28	78	97	18	87	100	0.52	0.70	0	0	0.30	61
	D3	Yes	2	3	3	3	3	3	—	—	—	—	—	—	—	—	—	—	—	—
	D6	No	2	3	3	1	0	0	—	—	—	—	—	—	—	—	—	—	—	—
	D9	Yes	2	3	3	3	3	1	37	83	100	30	90	100	0.62	0.41	0.07	0.03	0.49	61
	D13	Yes	1	1	1	3	3	0	24	77	100	31	78	100	0.90	0.50	0.26	0.05	0.19	58
	D16	No	0	0	0	0	0	0	—	—	—	—	—	—	—	—	—	—	—	—
	D20	No	1	2	2	2	1	0	—	—	—	—	—	—	—	—	—	—	—	—
Trinité	D1	No	1	3	3	3	3	3	9	62	96	15	54	100	0.55	0.89	0	0	0.11	64
	D3	Yes	1	3	3	3	3	3	—	—	—	—	—	—	—	—	—	—	—	—
	D6	No	2	3	3	1	0	0	—	—	—	—	—	—	—	—	—	—	—	—
	D9	Yes	2	3	3	3	3	1	15	58	90	10	50	92	0.52	0.78	0.08	0.14	0	59
	D13	No	1	1	0	1	1	1	—	—	—	—	—	—	0.31	0.93	0.06	0	0	16
	D16	Yes	1	2	0	2	3	2	—	—	—	—	—	—	—	—	—	—	—	—
	D20	No	1	1	1	1	1	1	—	—	—	—	—	—	—	—	—	—	—	—

[a] Days of observation, e.g., D1, day 1; D3, day 3.
[b] All treatments were done after collecting preimaginal stages.
[c] E, egg rafts; L1, L2, L3, and L4, first, second, third, and fourth instars, respectively; P, pupae; abundances are rated from 0 to 3, with 0, none; 1, a few tens; 2, a few hundreds; and 3, extremely numerous. Numbers underlined indicate the stages from which L4 was derived for bioassays and adults for resistance gene identification unless otherwise indicated.
[d] Mean mortalities observed on 2–3 replicates of 20 larvae each; doses are expressed in mg/liter.
[e] All mosquitoes with esterase B2 also possessed esterase A2.
[f] Data from adults emerged from collected pupae.
[g] Data from adults emerged from larvae exposed to 0.01 mg/liter temephos in bioassay.

ases increased in frequency, only the increase in esterase C2 was significant ($P < 0.01$). Because no treatment was done on day 6, larvae present at each site continued to develop until day 9. On day 9, abundance of egg rafts and larvae was similar to that of day 1, but pupae were still in low number. L3 and L4 larvae were collected at the three breeding sites and reared in laboratory conditions for bioassays and esterase analyses. At the three sites, temephos and chlorpyrifos mortalities showed no significant ($P > 0.05$) change as compared with day 1. Increases in highly active esterases were significant for adults from Carbet for esterase C2 ($P < 0.001$) and from Marigot and Trinité for esterase B1 ($P < 0.01$ and $P < 0.001$, respectively).

The second temephos treatment was applied on day 9 (after collecting larvae). Three days later (day 13), the abundance of eggs, L1, L2, and pupae was reduced greatly at the three sites as compared with day 1 or 6. The number of L3 and L4 also was low at Carbet and Trinité but not at Marigot. As previously, L3 and L4 were collected and reared in the laboratory for bioassays and electrophoresis analyses. Bioassays conducted on Marigot again showed no significant ($P > 0.05$) decrease in temephos or chlorpyrifos mortalities as compared with day 1. Analyses of highly active esterases showed a significant increase at Marigot for esterase C2 ($P < 0.001$) and esterase B1 ($P < 0.001$) as compared with day 1, and an absence of mosquitoes lacking a highly active esterase.

The Marigot breeding site was treated again on day 13 after larvae had been collected. Preimaginal stages were not detected 3 d later (day 16) and only a few were collected 6 d later (day 20). The Trinité site was not treated on day 13, and preimaginal stages were a little more abundant on day 16 than on day 13; the temephos treatment done on day 16 again induced the decrease of preimaginal stages 3 d later (day 20).

Discussion

Culex pipiens quinquefasciatus populations from Martinique displayed a low, but consistent, resistance to temephos and chlorpyrifos organophosphorous insecticides which was caused, in part, by the presence of esterases A2-B2 previously found in many countries in North America, Africa, Asia, and Europe (Raymond et al. 1991), and esterase B1 known in North America and Cuba (Pasteur &

Georghiou 1989; Bisset et al. 1990, 1991). In addition, esterase C2, a new esterase with high activity toward α-naphthyl acetate, was found at high frequency. The significant increase in frequency of esterase C2 at breeding sites controlled with temephos strongly indicates that it also may confer resistance to this insecticide. Possibly an AChE of reduced sensitivity is present on Martinique, as indicated by our identification of one *Ace*^{SR} genotype. However, the biochemical test used was adjusted for detecting AChE variants from France and may need modifications when used in other countries. Recent work on AChE variants in *Drosophila melanogaster* (Meigen) has revealed a large spectrum of variability in sensitivity to organophosphorous insecticides (Fournier et al. 1993), which points out that, depending on the variants present in a population, both the nature and the dose of insecticide used in biochemical assays must be carefully established.

Trial control of breeding sites with temephos was very effective, because it strongly decreased larval abundance in the three sites. However, it did not prevent rapid recolonization by oviposition. In fact, recolonization decreased only after the second treatment, indicating that at least 6 d may be required for registering a decrease in egg laying by females. The recolonization of breeding sites and the presence of L2 3 d after treatment, further indicated that within 24 h temephos rapidly decreased to a concentration that allowed the survival of a large number of larvae.

Although larvae collected after temephos treatment displayed no significant increase in resistance, the increase in frequency of highly active esterases indicated that they were undergoing to temephos selection. These data indicate that a large-scale *C. p. quinquefasciatus* control program with organophosphorous insecticides will induce a rapid increase in resistance gene frequencies throughout Martinique. Esterases B1 and esterases A2-B2 are known to confer a 1,000- and a 100-fold resistance to temephos, respectively. However, these levels of resistance were reached only in laboratory strains undergoing intense selection every generation (for ≈20 yr for esterase B1 and ≈10 yr for esterases A2-B2). This resistance is mainly caused by sequestration of the insecticide (Cuany et al. 1993) and is, therefore, a function of esterase overproduction. Esterase B overproduction is caused by amplification of the structural gene (Mouchès et al. 1986, Raymond et al. 1989) and depends on the number of gene copies in each amplification system. The resistance levels observed in Martinique are much lower than those observed in laboratory strains, indicating that gene amplification levels also are much lower. Therefore, before reaching the amplification levels of laboratory strains, mosquitoes may be controlled by organophosphorous insecticides, possibly for several years. To better understand this evolution in the field, and to optimize insecticide uses, a constant monitoring of resistance genes is now under way, using both biochemical assays and bioassays. In addition, investigations have been undertaken to identify insecticides to which the resistant genotypes present on Martinique are susceptible.

In conclusion, although *C. p. quinquefasciatus* populations have never been controlled in Martinique, they nevertheless contain resistance genes, several of which previously were described in other countries (Raymond et al. 1991). The presence of these genes at high frequencies (often >50%) indicates that this mosquito has been submitted to organophosphorous insecticides selection. This selection may be the result of insecticide use in agriculture or for domestic purposes (aerosol insecticides are widely used by the population for protection against mosquito biting). It also is very likely that *A. aegypti* control may have had an important effect, because the two species share ≈30% of the same breeding sites.

Acknowledgments

This work was supported in part by grant no. 89-412 from the Commission de Coordination de la Recherche dans les Départements d'Outre-Mer. This is Institut des Sciences de l'Evolution publication no. 94-078.

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Received for publication 30 November 1993; accepted 15 July 1994.