Interaction of insecticide resistance genes in field populations of *Culex pipiens* (Diptera: Culicidae) from Italy in response to changing insecticide selection pressure

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Abstract

*Culex pipiens* Linnaeus larvae were collected from various locations in Italy and colonized as separate strains. These were analysed for elevated non-specific esterase activity and frequency of altered acetylcholinesterase (AChE) mechanisms of insecticide resistance, and bioassayed, to define the cross-resistance spectra conferred by these to organophosphorus and carbamate insecticides. These mechanisms were further characterized by polyacrylamide gel electrophoresis. Elevated esterase A1 (formerly known as Est 3A) which predominated in *C. pipiens* from Italy in 1985 had been replaced by two esterases, A2 and B2. Altered acetylcholinesterase was still present at high frequencies. Altered and normal acetylcholinesterase were distinguished by differential mobility on polyacrylamide gel electrophoresis. Levels of insecticide resistance were higher in the Lucca region of Italy than in other areas sampled, in response to intensive use of temephos and to a lesser extent chlorpyrifos, employed to reduce mosquito biting nuisance to tourists in this area.

Introduction

Control of the mosquito *Culex pipiens* Linnaeus in certain regions of Italy has been hindered by the development of insecticide resistance. Problems have arisen in localized tourist areas from mosquito biting nuisance (Anon., 1984, Breeden *et al.*, 1984). It has been shown that resistance in these mosquito populations is conferred by two mechanisms: elevation of non-specific esterase activity levels (Villani *et al.*, 1982) and altered acetylcholinesterase (AChE) (Villani & Hemingway, 1987), the prevalence of which was reported to be changing in response to continued insecticide selection pressure (Villani & Hemingway, 1987).

Alterations in insecticide sensitivity of the insect acetylcholinesterase is one of the major mechanisms causing broad spectrum organophosphate and carbamate resistance. Earlier work showed that the resistance levels conferred by this mechanism were related to the relative sensitivities of acetylcholinesterase to the different insecticides (Hemingway & Georgiou, 1983).

*C. pipiens* colonized from Lucca, Italy, along with two other species segregating for insensitive AChE, was used to develop a sensitive microtitre plate assay for detection of this resistance mechanism (ffrench-Constant & Bonning, 1989). The different genotypes for AChE in Italian *C. pipiens* could be differentiated on the basis of this assay (ffrench-Constant & Bonning, 1989). A microtitre plate assay has also been developed for the detec-
tion of elevated esterase activity and broad spectrum organophosphate resistance in *C. quinguefasciatus* Say from Sri Lanka (Peiris & Hemingway, 1990). The elevated esterases A2 and B2 segregating in this population are electrophoretically indistinguishable from esterase electromorphs seen in Italian *C. pipiens*, although other esterase electromorphs are also seen in *C. pipiens*.

The current resistance status of *C. pipiens* in Italy was assessed with respect to the altered AChE and elevated esterase resistance mechanisms by use of these rapid microtitre plate assays. The object of this study was to quantify the cross-resistance spectra and predict the likely outcome of the proposed spray strategies for future mosquito control in Italy.

**Methods**

*Culex pipiens* larvae were collected in Italy from different districts of Lucca, Tuscany (strains SA, CDM and PT); Fermoro, Marches (strains MP and PSG) and Orvieto, Umbria (strain CG). Larvae were collected by dipping (World Health Organization, 1975) and colonized in the laboratory for analysis of insecticide resistance mechanisms and for bioassay. The cross-resistance spectra of these strains were compared to ISS, an Italian laboratory susceptible strain. Electrophoretic classification of non-specific esterases was carried out with reference to laboratory standard strains of *C. pipiens* (Callaghan, 1989), and LUCCA, a strain colonized in 1985 from Lucca, Italy.

**Bioassays**

Bioassays were carried out by exposing 20 early fourth instar field-collected larvae to various concentrations of insecticide in 1 ml absolute alcohol, in 200 ml volumes of dechlorinated tap water (World Health Organization, 1981). Control larvae were exposed to 1 ml absolute alcohol alone in 200 ml water. Exposure was for a 24 hour period at 27°C and 39% relative humidity. At least two replicates were run for each insecticide concentration according to availability of larvae. Bioassays were also carried out on ISS, a laboratory susceptible strain of *C. pipiens*. LC50 and LC90 values were determined by log dosage-probit mortality regression analysis.

**Biochemical assays**

Individual adult mosquitoes were homogenized in 200 µl 20 mM phosphate buffer, pH 7.2. 30 µl of this was placed in duplicate in microtitre plates and assayed for the AChE resistance genotype by the method of French-Constant & Bonning (1989). For each mosquito homogenate, rates of reaction in the presence and absence of insecticide were compared in order to differentiate resistance genotypes: 25 µl of substrate, acetylthiocholine iodide (ASCII), or substrate and inhibitor (100 mM propoxur) was added to 30 µl homogenate and 0.5 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in PB/Triton (0.1 M phosphate buffer pH 7.8, containing 1% Triton X-100) in a final volume of 200 µl. Reaction rates were measured on a Vmax kinetic microplate reader at 405 nm over a period of 5 minutes. Graphs of optical density over time, and reaction rates are produced for each well of the microtitre plate. Inhibited AChE activity plotted against uninhibited activity shows a negative correlation between activity and insensitivity of AChE. Genotypes of individual insects were deduced from these data (French-Constant & Bonning, 1989).

A further 10 µl of mosquito homogenate was incubated in 200 µl of 0.3 mM β-naphtyl acetate in 20 mM phosphate buffer, pH 7.2 for 10 minutes at room temperature before 40 µl of dye solution (0.3% Fast Blue B salt in 3.5% sodium dodecyl sulphate) was added to stop the reaction. Absorbance was read at 490 nm and the amount of product formed calculated from a β-naphtol standard curve.

**Polyacrylamide gel electrophoresis**

Non-specific esterases present in field populations were classified by non-denaturing polyacrylamide gel electrophoresis (PAGE). 7.5% acrylamide gels with a 4% stacking layer, were run in Tris-borate EDTA electrode buffer (0.1M Tris/0.07M boric acid/2.5mM EDTA), pH 8 for 4 hours at 20 V. Individual adult mosquitoes homogenized in 30 µl 20 mM phosphate buffer pH 7, were mixed with 20 µl glycerol and xylene cyanol, and loaded on to the gels. Gels, stained in 1 mM α-naphtyl acetate and 1 mM β-naphtyl acetate in 100 ml 0.1 M phosphate buffer pH 6, with 0.025% Fast Blue B salt, were fixed and preserved in 7% acetic acid. Non-specific esterase bands were identified by reference to laboratory standard strains of *Culex pipiens* run at the same time (Callaghan, 1989).

For analysis of AChE by PAGE, up to six adult mosquitoes of the same AChE genotype, as determined by microtitre plate assay, were homogenized in 30 µl 20 mM phosphate buffer pH 7 containing 1% Triton X-100. 20 µl glycerol was added to samples, which were loaded on to the gels. Gels, stained in 1 mM α-naphtyl acetate and 1 mM β-naphtyl acetate in 100 ml 0.1 M phosphate buffer pH 6, were run for 15 hours at 200 V in Tris-borate EDTA buffer containing 0.1% (w/v) Triton X-100, and stained for AChE using acetyltiocholine iodide as substrate (Karnovsky & Roots, 1964).

**Results**

**Bioassay**

The highest resistance ratio of all compounds tested in all strains was to temephos (table 1). The highest level of temephos resistance, 385-fold at the LC50 level, was found in the SA strain. All three collections from the Lucca region had temephos resistance which was 1-2 orders of magnitude higher than that in the other regions. Resistance was also detected to chlorpyrifos and malathion in the strains from the Lucca area, but not at significant levels at the LC50 in the strains from other regions. The levels of malathion and chlorpyrifos resistance were higher in the SA strain, than the CDM and PT strains from Lucca, which reflects the higher gene frequency of AChE and non-specific esterase activity levels in this strain compared to the other two strains (table 2). Only the SA strain showed a low level of propoxur resistance at the LC50 level, while the MP strain was 14-fold less tolerant to propoxur than the reference strain.

<table>
<thead>
<tr>
<th>Strain</th>
<th>CDM</th>
<th>SA</th>
<th>PT</th>
<th>Fermoro</th>
<th>PSG</th>
<th>MP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Table 1: of field reference</td>
<td>Populati</td>
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</table>

**Altered AChE**

Altered AChE strains from Lucca quency of 0.52 in specific esterase a The resistance gen esterase activity n CDM and PT su significantly differ This is reflected if seen in these strains, as the mechanism AChE was absent Fermoro and Orvieto armoury of insect specific esterase at
Table 1. Larval LC50 values with 95% confidence limits, LC90 values and resistance ratios from bioassays of field populations of Culex pipiens from Italy. Resistance ratios were calculated at the LC50 level with reference to the laboratory strain ISS.

<table>
<thead>
<tr>
<th>Population</th>
<th>Propoxur</th>
<th>Malathion</th>
<th>Temephos</th>
<th>Chlorpyrifos</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDM</td>
<td>34</td>
<td>(28-40)</td>
<td>190</td>
<td>(150-230)</td>
</tr>
<tr>
<td></td>
<td>138</td>
<td>0.89</td>
<td>322</td>
<td>6.3</td>
</tr>
<tr>
<td>SA</td>
<td>54</td>
<td>(44-46)</td>
<td>460</td>
<td>(380-560)</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>1.4</td>
<td>574</td>
<td>15</td>
</tr>
<tr>
<td>PT</td>
<td>27</td>
<td>(22-33)</td>
<td>160</td>
<td>(130-200)</td>
</tr>
<tr>
<td></td>
<td>132</td>
<td>0.71</td>
<td>277</td>
<td>5.3</td>
</tr>
<tr>
<td>PSG</td>
<td>28</td>
<td>(23-33)</td>
<td>17</td>
<td>(8-37)</td>
</tr>
<tr>
<td></td>
<td>114</td>
<td>0.74</td>
<td>386</td>
<td>0.37</td>
</tr>
<tr>
<td>MP</td>
<td>3</td>
<td>(2.3-3.4)</td>
<td>38</td>
<td>(31-46)</td>
</tr>
<tr>
<td></td>
<td>167</td>
<td>0.074</td>
<td>152</td>
<td>1.3</td>
</tr>
<tr>
<td>ISS</td>
<td>38</td>
<td>(32-45)</td>
<td>30</td>
<td>(25-36)</td>
</tr>
<tr>
<td></td>
<td>193</td>
<td>—</td>
<td>130</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 2. The relative frequency of AChE alleles and mean levels of non-specific esterase activity in field populations of Culex pipiens from different regions of Italy.

<table>
<thead>
<tr>
<th>Strain</th>
<th>AChE Genotype</th>
<th>Number tested</th>
<th>R allele frequency</th>
<th>Mean β-naphthol production/min/mosquito (±s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS RS RR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lucca</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDM</td>
<td>0.66 0.30 0.04</td>
<td>334</td>
<td>0.1886</td>
<td>0.003 ± 0.003</td>
</tr>
<tr>
<td>SA</td>
<td>0.16 0.64 0.19</td>
<td>313</td>
<td>0.5176</td>
<td>0.008 ± 0.005</td>
</tr>
<tr>
<td>PT</td>
<td>0.77 0.21 0.015</td>
<td>334</td>
<td>0.1198</td>
<td>0.005 ± 0.004</td>
</tr>
<tr>
<td>Ferro</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSG</td>
<td>1.00 0.00 0.00</td>
<td>302</td>
<td>0.0000</td>
<td>0.003 ± 0.002</td>
</tr>
<tr>
<td>MP</td>
<td>1.00 0.00 0.00</td>
<td>114</td>
<td>0.0000</td>
<td>0.004 ± 0.003</td>
</tr>
</tbody>
</table>

Insecticide resistance in Culex pipiens

Altered AChE and non-specific esterase activity

Altered AChE was present in the CDM, SA and PT strains from Lucca, with the highest resistance allele frequency of 0.52 in the SA strain (table 2). Elevated non-specific esterase activity was most pronounced in SA. The resistance gene frequencies for AChE, and elevated esterase activity means were not different between the CDM and PT strains, but the AChE frequency was significantly different from that of the SA strain (table 2). This is reflected in the low level of propoxur resistance seen in these strains at the LC90, compared to the SA strain, as the altered AChE but not the elevated esterase mechanism will confer propoxur resistance. Altered AChE was absent in strains MP, PSG and CG from the Fermo and Orvieto areas, despite intensive use of an armory of insecticides in the former region. Non-specific esterase amplification was not apparent in either strain. AChE activity was incubated in acrylamide gels with a 4% Triton X-100, 20 μl homogenates were loaded on to a polyacrylamide gel. The altered AChE stained less intensely than the normal band. Altered AChE was present in the Fermo and Orvieto samples but were not elevated (fig. 1).

Polyacrylamide gel electrophoresis

The non-specific esterases present in the Lucca populations were A1, A2, B1, B2 and B4. Esterases A2 and B2 were particularly elevated in SA as seen by PAGE. Esterases B2, B3 and B4 were present in the Fermo and Orvieto samples but were not elevated (fig. 1).

On PAGE of AChE a single band was seen in both resistant and susceptible adult homogenates. Normal and altered AChE could be distinguished by their differential mobility (fig. 2). The relative mobility of altered AChE with reference to the running edge of the gel was 0.26, as opposed to 0.27 for normal AChE run on the same gel. The altered AChE stained less intensely than the normal AChE when the same numbers of mosquitoes were loaded on to the gel.
In Lucca, mos- 

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(Abate) was applied 
season of 1987. In 

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the three organo- 
and bendiocar- 
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similar, but an a- 
tance mechanism 
in 1984 which wa- 
ificantly whil- 
(Villani & Heming- 
the 1988 spra) 
treatment, there w- 
resistance in C. pi- 
the earlier collect- 
chlorpyrifos and 
1985 collection. It 
appears to have 
erved in 1985. H 
pared to strain M 
1985) which sug- 

Fig. 1. Non-specific esterases in individual mosquitoes from different regions of Italy. 7.5% polyacrylamide gel electrophoresis of C. pipiens from Italy stained for non-specific esterases. A: 3 replicates of (1) LUCCA; 2 replicates of (2) MP (3) PSG (4) reference strain with esterases A2 and B2. B: 3 replicates of (1) SA; 2 replicates of (2) PT (3) CDM (4) reference strain with esterases A2, B2 and B3. C: Diagrammatic representation of non-specific esterases in field populations of C. pipiens from Italy.
Insecticide resistance in Culex pipiens

Fig. 2. Differential migration on polyacrylamide gel electrophoresis, of altered and normal AChE from Culex pipiens from Italy. 7.5% polyacrylamide gel containing 1% Triton X-100. Two replicates of (1) and (3) altered AChE; (2) and (4) normal AChE, showing reduced migration of altered AChE (R/R mosquitoes) compared with normal AChE (S/S mosquitoes).

Discussion

Insecticide usage

In Lucca, mosquito control problems arose as insecticide resistance developed in C. pipiens. The larvicide chlorpyrifos had been used since 1970, the overall concentrations and quantity being increased dramatically in response to reduced efficacy. In 1986 and 1987, temephos (Abate) was applied, at 80 mg/l weekly during the spray season of 1987. In 1988 temephos was again employed.

Bioassay data

Resistance levels at the LC50 for malathion, chlorpyrifos and temephos were calculated in collections from the field populations around Lucca in 1982, 1984 and 1985 (Villani et al., 1986; Villani & Hemingway, 1987). In the 1982 material there was resistance to chlorpyrifos, malathion and temephos, but not to the carbamates, propoxur and bendiocarb. In 1984, the resistance levels to the three organophosphorous insecticides had increased, and there was also evidence of resistance to both propoxur and bendiocarb. The frequency of elevated esterase A1 (Est 3A) in the 1982 and 1984 populations was similar, but an altered acetylcholinesterase-based resistance mechanism was also segregating in the population in 1984 which was not detected in 1982. In the 1985 field collection the frequency of esterase A1 had dropped significantly while that of the altered AChE had risen (Villani & Hemingway, 1987). In comparison, at the start of the 1988 spray season, after two years of temephos treatment, there was a 20 to 45-fold increase in temephos resistance in C. pipiens from the Lucca area compared to the earlier collections, and a 0.7- and 0.35-fold decline in chlorpyrifos and malathion resistance compared to the 1985 collection. The level of propoxur resistance also appears to have declined significantly from that observed in 1985. However, the Italian reference strain ISS has higher LC50 and LC90 values for propoxur compared to strain MP and other Culex spp. (Amin & White, 1985) which suggests this strain may not be propoxur susceptible, and will lead to an underestimate of the level of propoxur resistance in the other Italian field strains. An additional resistance mechanism, such as mixed function oxidases, may be present in the ISS strain.

The increase in resistance for temephos, but not the other organophosphates and carbamates in the 1988 collection suggests that a further mechanism, other than the esterase A1 and altered AChE, has been selected as a result of chlorpyrifos replacement with temephos for mosquito control in the Lucca region.

Frequency of the altered AChE allele

In 1985 a high frequency of the altered AChE allele was recorded in the Lucca area, although it was not present in collection sites 106 km from Lucca (Villani & Hemingway, 1987). A much lower frequency of the altered AChE mechanism was found in the 1988 collected insects from Lucca. The lower frequency of the AChE mechanism was probably a consequence of the delay in the onset of insecticide treatment in 1988. In the period between the end of spraying in 1987 and the start of the 1988 spray season, the frequency of the resistant AChE allele should have dropped due both to a reduction in fitness compared to the susceptible allele (Bonning & Hemmingway, in press), and to migration of C. pipiens from untreated areas outside Lucca, which are still homozygous for the susceptible AChE allele. The frequency of the insecticide insensitive AChE allele rose to 1.0 again in the Lucca area by the end of the 1988 spray season.

Status of non-specific esterase activity

In Lucca, esterase A1 was reported to be elevated in 1985, but present at a lower frequency in 1986. In 1988, esterase A1 was still present in the Lucca population, but not in an elevated state. In strain SA (Saint Alessio—a suburb of Lucca), A2 and B2 showed elevated activity, but the increase in activity was lower than that for A1 in C. pipiens in 1985. Resistance mechanisms alter in response to changes in both quantity and type of insecticide applied. The exact responses of different non-specific esterases to selection pressure from different insecticides are poorly understood. In Italian C. pipiens chlorpyrifos appears to have selected elevation of esterase A1 activity in conjunction with altered AChE, and the replacement of chlorpyrifos with temephos has then selected for elevation of esterases A2 and B2 as well as altered AChE, while elevated A1 has been lost from the field population. Elevated non-specific esterase activity may confer greater organophosphate resistance than altered AChE in the heterozygous state (Villani & Hemingway, 1987), or may sequester or hydrolyse sufficient inhibitor to prevent inhibition of AChE in mosquitoes heterozygous for the AChE mechanism.

Microtitre plate tests for detection of insecticide resistance

Microtitre plate tests for elevated non-specific esterase activity and altered AChE resistance mechanisms can be used to detect and monitor insecticide resistance in field populations of mosquitoes. The levels of organophosphate resistance in C. pipiens in Italy defined by conventional World Health Organization bioassay
techniques correlated with altered AChE gene frequency and non-specific esterase levels obtained by use of the microtitre plate assays. The sensitivity of the microtitre plate tests is also of considerable value in improving detection of resistance development in its early stages. This is particularly true of the altered AChE assay where genotypes can be differentiated. If conventional bioassays are used to detect resistance in the field using log-dosage probit mortality regression, a low resistance gene frequency has only a minimal effect on the LC50 values and resistance can be missed. This is apparent with the propoxur resistance in this strain indicated the potential for resistance in its early stages. This is particularly true of the altered AChE assay where genotypes can be differentiated.

Detection of low frequencies of insensitive AChE genotypes in Italian C. pipiens gives a similar warning of the probability of developing high levels of carbamate and organophosphate resistance. In addition, these biochemical assays are applicable to any stage in the life cycle of the insect and complement standard bioassay techniques by providing information about the expected cross-resistance patterns. For the elevated non-specific esterase activity and altered AChE resistance mechanisms, the resistance is independent of the adult insect age.

Acknowledgements

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References


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