Experimental and molecular genetic analysis of the impact of pyrethroid and non-pyrethroid insecticide impregnated bednets for mosquito control in an area of pyrethroid resistance

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Abstract

Experimental huts in Côte d’Ivoire were used to evaluate the pyrethroid alpha-cypermethrin, the non-ester pyrethroid etofenprox, the organophosphate pirimiphos-methyl and the carbamate carbosulfan on bednets against pyrethroid-resistant Anopheles gambiense Giles. To test for selection for the resistance gene by the treated nets, A. gambiae collected live or dead from the huts were kept and analysed for the presence of the kdr gene using a new polymerase chain reaction followed by sequence-specific oligonucleotide probing (PCR-SSOP) for kdr-genotyping. Deliberately holed bednets freshly treated with pirimiphos-methyl or carbosulfan caused over 90% kill of A. gambiae s.s. and Culex spp. However, the mortality with alpha-cypermethrin or etofenprox treated nets was similar to that with untreated nets. Bloodfeeding of A. gambiae s.s. on the sleepers under the nets was only significantly reduced by alpha-cypermethrin and carbosulfan. Tests of the residual activity of the bednets after seven months showed that pirimiphos-methyl had lost its efficacy while carbosulfan still performed well. Once again the pyrethroid treated nets gave similar results to the untreated nets. Selection for the kdr-allele by alpha-cypermethrin and etofenprox, but not by carbosulfan, was indicated by PCR-SSOP genotyping of mosquitoes. Thus carbamates such as carbosulfan, or organophosphates of longer persistence than pirimiphos-methyl and of low mammalian toxicity, would seem to be a promising alternative to be used on bednets, particularly in areas of pyrethroid resistance.

Introduction

Bednets impregnated with pyrethroid insecticide have been shown to reduce the risk of morbidity and mortality from malaria (Lengeler, 1998). The threat of pyrethroid resistance evolving in Anopheles mosquitoes (Diptera: Culicidae) and its impact on the large-scale use of impregnated bednets has long been recognized (Curtis et al., 1990, 1998a). Early reported pyrethroid resistance in anopheline mosquitoes does not seem to have had a pronounced effect on large-scale malaria control interventions, as it tended to be relatively localized and the resistance factor was not very high (Malcolm, 1988).

More recently Elissa et al. (1993) documented pyrethroid resistance in certain populations of Anopheles gambiae Giles (Culicidae) from Côte d’Ivoire, which was confirmed by Darriet et al. (1997), who also detected resistance in Burkina Faso. Chandre et al. (1999a) also report reduced susceptibility of A. gambiae from these countries as well as from Benin. Martinez-Torres et al. (1998) showed that this resistance is of the kdr type, involving a point mutation at codon 104 of the sodium channel gene, which gives broad-spectrum resistance to pyrethroids and cross-resistance to DDT. Its evolution in
Côte d'Ivoire is suggested by Chandre et al. (1999a) to be the result of selection by early use of DDT, as well as domestic and/or agricultural use of pyrethroids. Despite the fact that these existing pyrethroid resistance genes appear not to be sufficiently powerful to render nets treated with permethrin or deltamethrin ineffective (Guillet, 1998; Darriet et al., 1999), the evolution of a stronger resistance mechanism may be expected by addition of metabolic mechanisms or if an additional point mutation such as super-kdr arises, as reported in houseflies by Sawicki (1978).

To maintain the effectiveness of impregnated bednets it may become necessary to replace pyrethroids with compounds having a different mode of action. This study was designed to evaluate two non-pyrethroid insecticides on bednets, the organophosphate (OP) pirimiphos-methyl and the carbamate carbosulfan, against pyrethroid resistant A. gambiense in Côte d'Ivoire. These two compounds were chosen primarily because their mode of action is different from that of pyrethroids on a bednet and pirimiphos-methyl gave high mortality of susceptible A. gambiense in The Gambia (Miller et al., 1991). Good results were obtained for the carbamate-bendiocarb used on eave curtains in Tanzania but the manufacturers did not consider it safe enough for use on bednets (Curts et al., 1996). Thus in the present study, at the suggestion of Dr. F. Guillet, it was decided to test carbosulfan, with lower vertebrate toxicity, carbosulfan, which has given good results when sprayed on the walls of experimental huts (Darriet, 1998).

For comparison with the above non-pyrethroid treatments, we used two pyrethroid compounds, against which resistance due to the kdr-allele could be expected. These were alpha-cypermethrin, which has been reported as a cheap and effective insecticide treatment against pyrethroid susceptible A. gambiense (Luo et al., 1994; Jawara et al., 1998) and the non-ester pyrethroid etofenprox which was evaluated by Curtis et al. (1996) against susceptible A. gambiense in Tanzania and found to be somewhat less efficient compared to deltamethrin and lambda-cyhalothrin at a much lower dose.

The results against A. gambiense, when nets were freshly treated, were presented briefly by Fanello et al. (1999). This paper gives complete details, together with data on Culex quinquefasciatus Say (Culicidae) and also reports the results when all nets were re-tested after seven months to investigate the residual activity. Culex quinquefasciatus in this area has also been reported as pyrethroid resistant due to the presence of cytochrome P-450 enzymes and a kdr-type target site insensitivity (Chandre et al., 1998) as well as carbamate and organophosphate resistant, which is thought to be conferred by an insensitive AChE (Chandre et al., 1997).

To assess the degree to which the kdr resistance allele (codon 104F) protects individuals against being killed by the treated nets and therefore might promote the selection of the gene in the population, we compared the kdr allele frequency in mosquitoes recovered either live or dead from the huts.

**Materials and methods**

**Study area and experimental huts**

The insecticide treated bednets were evaluated in six experimental huts belonging to the Institut Pierre Richet at Yaokoffikro, in central Côte d'Ivoire. The experimental huts were located in a row about 5 m apart from each other beside rice fields. Each hut stands on a concrete base and is surrounded by a water-filled moat to attempt to keep scavengers of dead mosquitoes, such as ants or spiders, out of the huts. Access for the mosquitoes was only possible through the four 60 x 30 cm windows, that were designed to allow mosquito entry but inhibit exiting. The windows were constructed from pieces of plywood, fixed at an angle so as to create a funnel shape with an approximately 1 cm wide gap at the end of it. Mosquitoes that attempted to enter the hut had to fly upwards to pass through the gap, but downwards to exit. All huts were cleaned before the trial and bioassays with susceptible female A. gambiense (Kisumu strain) were carried out, to ensure that no insecticidal contamination had remained from previous trials.

**Bednets**

The mosquito nets used in the present study were green in colour, made of 100% polyester, 100 denier, 156 meshes per square inch and donated by the Siam Dutch Mosquito Netting Co., Bangkok, Thailand. They were 1.8 m long, 1.3 m wide and 1.5 m high, giving a total surface area of 11.6 m². To simulate a torn net and to allow for entry of mosquitoes into it, we adopted the procedure of F. Darriet (personal communication), as previously used for tests in these huts, of cutting two rows of 90 holes in the nets, about 2.5 x 2.5 cm in size and 4 cm apart.

**Insecticides**

The following four insecticidal treatments at the target dose indicated, as well as two untreated controls, were randomly allocated to the six experimental huts: (i) alpha-cypermethrin, 20 mg m⁻², Fastact 150 g kg⁻¹ WG, Cyanamid Agriculture Ltd; (ii) etofenprox, 200 mg m⁻², Vectron 100 g l⁻¹ EW, Mitsui Chemicals Inc.; (iii) carbosulfan, 200 mg m⁻², Marshal 200 g l⁻¹ CS, FMC Corporation; (iv) pirimiphos-methyl, 1000 mg m⁻², Actellic 500 g l⁻¹ EC, Zeneca. Impregnation of bednets with these compounds was carried out using the formula given by Pleass et al. (1993) to calculate the amount of insecticide needed for the dipping mixture. To confirm the amount of insecticide absorbed by each mosquito net, two additional 10 x 10 cm netting pieces were impregnated at the same time and sent for gas-chromatography by the WHO Pesticide Evaluation Scheme reference centre at Gembloux, Belgium, but were unfortunately lost in the post after leaving Geneva. We were therefore obliged to wait until the end of the trial before cutting further samples from the mosquito nets. These were used for bioassays with a susceptible strain of A. gambiense in London.

**Sleepers**

Six adult men were paid to sleep in the experimental huts every night of the trial, one per hut per night, and to collect mosquitoes every morning except on Sundays. They had been previously employed by the Institut Pierre Richet for this purpose and were familiar with the protocol for the collection of mosquitoes. They gave their informed consent to testing the treated nets and were provided with chloroquine anti-malaria prophylaxis, which is still the drug of choice in this area of Côte d'Ivoire.
Data collection

On 40 consecutive mornings, after overnight use of the nets, all mosquitoes were collected from each hut by the man who had slept in that hut. The mosquitoes were identified, according to hut number and location in the hut or veranda, and scored as bloodfed/unfed and dead/alive. Semi-gravid and gravid mosquitoes were considered as unfed and male mosquitoes were not recorded. Live mosquitoes were put into plastic cups, provided with honey-soaked cotton wool and kept for a further 24 h. The majority of the mosquitoes caught in the experimental huts were Mansonia spp., Culic spp. or members of the A. gambiae complex. Occasionally we found a few Aedes spp., Anopheles funebris Giles, Anopheles pharoensis Theobald and Anopheles coustani Laveran (Culicidae), but they were too few to be included in the analysis. Due to unexplained high mortality of 62 and 72% in Mansonia spp. controls, this species was also not included in the present evaluation. All the A. gambiae s.s. that were collected during the baseline data and the trial weeks were kept for further processing in the laboratory in London, to determine species and kdr-resistance genotype and to estimate any selection pressure on the resistance gene exerted by the different treatments. After the trial had been completed, all bednets were left in the huts and a further 16 days of test were carried out seven months after the treatment of the nets.

kdr genotyping in natural populations of A. gambiae s.s.

The kdr (knock down resistance) allele is characterized by a single base pair substitution causing a change from leucine to phenylanine in codon 104 of the voltage-sensitive sodium channel protein sequence (EMBL accession number Y13592).

The primers of Martinez-Torres et al. (1998) were not used, but instead two primers were designed to amplify a 216 bp fragment from codon 76 to 128:

76-Forward Primer, 5'-TGGATTGAAATCAATGTGGGATTG-3',
128-Reverse Primer, 5'-TGGCCTTGTGTCAGACAAAGG-3'.

Oligonucleotide probes were designed, one complementary for the standard wildtype (kds) allele (104L) and one for the kdr allele (104F):

104L Probe, 5'-GGAAATTTAGTCGTAAGT-3',
104F Probe, 5'-GGAAATTCTGTAAGT-3'.

DNA from single specimens was extracted following the method of Collins et al. (1987), employing a single purification in phenol-chloroform 1:1 before precipitation in ethanol, with the DNA finally dissolved in 100 µl of H2O.

Polymerase chain reaction (PCR) amplification was performed in a total volume of 15 µl in each well of 96-well plates, with the following components: deoxynucleotides 0.1 mM each, forward primer and reverse primer 0.1 µM each, Taq polymerase (Biotaq Bioline) 0.75 u, 1 X Bioline amplification KCl reaction buffer (including MgCl2 1.5 mM), 2 µl genomic DNA. The amplification reaction consisted of one step at 94°C for 4 min; 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; with a final extension at 72°C for 4 min. Amplified DNA products were denatured for 2 min at 94°C, cooled to 4°C, and 1.5 µl dot-blotted onto replicate nylon membranes (MagnaGraph™) in 96-dot arrays. Membranes were first incubated in blocking solution (4 X SSPE, 0.1% Lauroylsarcosine, 1.0% milk powder) at 37°C for 30 min, and then probed with allele specific oligonucleotides at 53°C for 90 min. Probes were digoxigenin(DIG)-labelled (Boehringer Mannheim 3'-end labelling kit) and used at a final concentration of 2 nM in 5 ml of TAC hybridization solution (5 M Tetramethylammonium chloride, 50 mM tris-HCl pH 8, 0.1% SDS, 2 mM EDTA pH 8). Membranes were then washed for 2 X 10 min in 2 X SSPE 0.1% SDS at room temperature (low-stringency washes) and 2 X 10 min (high-stringency washes) in TAC solution at 56.5°C. Probes were detected using anti-DIG-AP Fab fragment conjugated with alkaline phosphatase and CSPD reagent as a substrate for alkaline phosphatase (Boehringer Mannheim) following manufacturer’s guidelines. Membranes were exposed to Hyperfilm-ECL for 30 min and films were developed and scored visually by two investigators examining films (fig. 1) independently and confirming results together. There were very few samples for which the scoring disagreed, and these were always repeated on a subsequent assay. These procedures allowed batches of several hundred mosquitoes to be confidently genotyped in an assay.

Statistical design and analysis

In the present study, the different nets were not rotated between huts to correct for possible variation in the attractiveness of individual huts or the sleepers in them. Instead, treatments were randomly allocated to huts and neither sleepers nor treatments were moved between huts throughout the trial period, to avoid the risk of...
contaminating huts with different insecticides (R. Darriet, personal communication). The excito-repellency measured by the number of mosquitoes collected outside the nets, the number blood fed and the mortality (tables 1 and 2) were expressed as proportions of the total catch on each night in the hut concerned. Analysis was based on a generalized linear model using GLIM. Proportions were analysed specifying a binomial distribution and log-link function, with significance tested against the z-distribution (Crawley, 1993). Comparisons between treatments were made by successively eliminating treatments from the overall comparison, which allowed all treatments to be compared with each other. The P-values were not adjusted where numbers caught inside the hut and the veranda under the personal communication). The excito-repellency measured specifying a binomial distribution and log-link function, with significance tested against the z-distribution (Crawley, 1993). Comparisons between treatments were made by multiple comparisons were made, but this was taken into consideration when interpreting results.

Results

Total number caught and excito-repellency with freshly treated nets

There were major differences between the total number of A. gambiae and Culex spp. caught in the six huts (table 1), which we attribute mainly to differences in the attractiveness of the sleepers in the huts. We cannot separate such effects from possible deterrency due to the net treatments nor to differences in location of huts, because the nets were not rotated between the huts. Due to the relatively small size of the experimental huts, leaving only about 50 cm between net and the outer walls, it was decided to pool the numbers caught inside the hut and the veranda under the category ‘outside net’, for statistical analysis. Differences between huts were therefore analysed by comparing total (live + dead) numbers caught outside and inside the net on 40 consecutive mornings as a proportion of the total number of mosquitoes caught in all three locations. The proportion of A. gambiae and Culex outside the net (table 1) is shown as an indication of excito-repellency due to the net treatments in preventing entry through the holes in the net or inducing exit. However, the results could be affected by a treatment that causes rapid mortality before mosquitoes had a chance to respond to excito-repellent stimuli.

The proportions of A. gambiae s.s. and Culex spp. collected outside the etofenprox treated net, was significantly greater than for any of the other treatments or the two controls (P < 0.001). Carbosulfan and alpha-cypermethrin treated nets also had significant excito-repellent effects but pirimiphos-methyl had no such effect.

Bloodfeeding and mortality with freshly treated nets

To compare the efficacy of the four treatments we used percentage blooded and dead (after 24 h) as the measures for personal protection and contribution to population reduction in the community, respectively. We found that alpha-cypermethrin and carbosulfan both significantly reduced the proportion of blooded A. gambiae compared to controls (respective P values: 0.026, 0.001), with the latter treatment performing significantly better than the former (table 1). Mortality of A. gambiae with the control nets was unexpectedly high, possibly because of rough handling by the sleepers/collectors in these huts. The mortality caused by the four treatments differed significantly between the pyrethroid and the non-pyrethroid treatments. Both etofenprox and alpha-cypermethrin appeared to be associated with less mortality than the controls, though only for the latter was this significant (P = 0.003). The OP pirimiphos-methyl was, however, found to perform exceptionally well in killing A. gambiae, causing 100% kill over 40 consecutive days. The carbamate carbosulfan was also extremely effective at killing, causing an overall mortality of 92%, which was significantly less than for pirimiphos-methyl (P = 0.003) but much higher than for the other treatments and controls.

Results obtained for Culex spp. (table 1) were similar to those for A. gambiae, with bloodfeeding compared to controls only being significantly reduced by carbosulfan (P = 0.008). Once again, control mortality was high and the two non-pyrethroids caused similar mortality of Culex spp. as for A. gambiae. Pirimiphos-methyl caused almost 100% mortality

Table 1. Numbers of Anopheles gambiae s.s. and Culex spp. collected from experimental huts over 40 nights soon after the nets were treated.

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>Dose (mg m⁻²)</th>
<th>Total number</th>
<th>% outside net</th>
<th>95% confidence interval</th>
<th>% blooded</th>
<th>95% confidence interval</th>
<th>% dead after 24 h</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control I</td>
<td>untreated</td>
<td>126</td>
<td>45.2</td>
<td>(35.8-55.1)</td>
<td>53.2</td>
<td>(37.5-62.6)</td>
<td>4.3</td>
<td>(2.5-6.0)</td>
</tr>
<tr>
<td>A. gambiae</td>
<td>Pirimiphos-methyl</td>
<td>1000</td>
<td>269</td>
<td>73.0</td>
<td>(67.7-78.1)</td>
<td>43.1</td>
<td>(33.5-52.8)</td>
<td>15.2</td>
<td>(11.1-19.4)</td>
</tr>
<tr>
<td>Culex spp.</td>
<td>Etofenprox</td>
<td>200</td>
<td>140</td>
<td>95.0</td>
<td>(90.0-97.6)</td>
<td>52.9</td>
<td>(43.8-61.9)</td>
<td>13.1</td>
<td>(11.3-15.0)</td>
</tr>
<tr>
<td></td>
<td>Carbosulfan</td>
<td>20</td>
<td>378</td>
<td>78.6</td>
<td>(74.1-83.0)</td>
<td>28.0</td>
<td>(21.5-35.7)</td>
<td>18.5</td>
<td>(14.2-23.8)</td>
</tr>
<tr>
<td></td>
<td>Control II</td>
<td>untreated</td>
<td>172</td>
<td>70.2</td>
<td>(62.2-77.1)</td>
<td>42.4</td>
<td>(33.0-52.4)</td>
<td>50.9</td>
<td>(40.8-60.9)</td>
</tr>
<tr>
<td></td>
<td>Pirimiphos-methyl</td>
<td>1000</td>
<td>258</td>
<td>72.3</td>
<td>(68.3-76.4)</td>
<td>45.1</td>
<td>(36.8-53.4)</td>
<td>19.5</td>
<td>(14.8-24.2)</td>
</tr>
<tr>
<td></td>
<td>Etofenprox</td>
<td>200</td>
<td>140</td>
<td>95.0</td>
<td>(90.0-97.6)</td>
<td>52.9</td>
<td>(43.8-61.9)</td>
<td>13.1</td>
<td>(11.3-15.0)</td>
</tr>
<tr>
<td></td>
<td>Carbosulfan</td>
<td>20</td>
<td>378</td>
<td>78.6</td>
<td>(74.1-83.0)</td>
<td>28.0</td>
<td>(21.5-35.7)</td>
<td>18.5</td>
<td>(14.2-23.8)</td>
</tr>
<tr>
<td></td>
<td>Control III</td>
<td>untreated</td>
<td>117</td>
<td>53.0</td>
<td>(42.4-63.3)</td>
<td>38.1</td>
<td>(29.4-46.7)</td>
<td>28.5</td>
<td>(22.3-35.6)</td>
</tr>
<tr>
<td>Culex spp.</td>
<td>Pirimiphos-methyl</td>
<td>1000</td>
<td>236</td>
<td>82.2</td>
<td>(79.0-89.8)</td>
<td>44.5</td>
<td>(35.3-53.9)</td>
<td>9.6</td>
<td>(6.6-12.6)</td>
</tr>
<tr>
<td></td>
<td>Etofenprox</td>
<td>200</td>
<td>169</td>
<td>96.4</td>
<td>(91.4-98.6)</td>
<td>50.9</td>
<td>(40.8-60.9)</td>
<td>19.5</td>
<td>(13.2-27.5)</td>
</tr>
<tr>
<td></td>
<td>Alpha-cypermethrin</td>
<td>20</td>
<td>211</td>
<td>70.1</td>
<td>(62.1-77.1)</td>
<td>39.8</td>
<td>(32.7-46.0)</td>
<td>10.4</td>
<td>(7.0-16.6)</td>
</tr>
<tr>
<td></td>
<td>Carbosulfan</td>
<td>20</td>
<td>161</td>
<td>79.9</td>
<td>(71.2-85.9)</td>
<td>19.3</td>
<td>(11.7-30.0)</td>
<td>92.3</td>
<td>(86.7-97.5)</td>
</tr>
</tbody>
</table>

Percentages in the same column sharing a superscript letter do not differ at the 5% level of significance.

*Percentage outside net refers to total percentage, i.e. live and dead.
**Controls that did not differ significantly from each other at the 5% level of significance were pooled for analysis; the mean value for the pooled control is shown.
Insecticide-treated bednets against pyrethroid resistant mosquitoes

Table 2. Numbers of Anopheles gambiae s.s. and Culex spp. collected from experimental huts over 16 nights, seven months after net treatment.

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>Dose (mg m⁻²)</th>
<th>Total number</th>
<th>% outside net*</th>
<th>95% confidence interval</th>
<th>% blooded</th>
<th>95% confidence interval</th>
<th>% dead after 24 h</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. gambiae</em></td>
<td>Control I</td>
<td>untreated</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control II</td>
<td>untreated</td>
<td>95</td>
<td>48.8B</td>
<td>(38.4-59.2)</td>
<td>44.6A</td>
<td>(33.6-56.2)</td>
<td>2.5B</td>
<td>(0.7-8.2)</td>
</tr>
<tr>
<td></td>
<td>Pirimiphos-methyl</td>
<td>1000</td>
<td>100</td>
<td>31.0B</td>
<td>(22.0-41.7)</td>
<td>75.0B</td>
<td>(62.9-84.2)</td>
<td>5.0B</td>
<td>(1.7-13.5)</td>
</tr>
<tr>
<td></td>
<td>Etofenprox</td>
<td>200</td>
<td>77</td>
<td>75.3C</td>
<td>(62.6-84.8)</td>
<td>64.4C</td>
<td>(54.1-77.1)</td>
<td>2.6A</td>
<td>(0.5-12.7)</td>
</tr>
<tr>
<td></td>
<td>Alpha-cypermethrin</td>
<td>20</td>
<td>101</td>
<td>69.6C</td>
<td>(58.0-79.1)</td>
<td>52.9AC</td>
<td>(41.1-64.5)</td>
<td>2.0A</td>
<td>(0.3-11.0)</td>
</tr>
<tr>
<td></td>
<td>Carbosulfan</td>
<td>200</td>
<td>56</td>
<td>84.2C</td>
<td>(69.7-92.6)</td>
<td>10.5D</td>
<td>(4.0-25.0)</td>
<td>66.7B</td>
<td>(49.4-80.4)</td>
</tr>
</tbody>
</table>

| *Culex* spp. | Control I | untreated | 98         |               |                         |           |                         |                  |                         |
|              | Control II | untreated | 97         | 56.4A         | (48.1-63.9)            | 51.0A     | (38.3-63.6)             | 3.6A             | (1.5-8.1)               |
|              | Pirimiphos-methyl | 1000 | 153         | 58.4A         | (49.5-67.0)            | 76.6B     | (61.6-87.0)             | 2.6A             | (1.8-11.2)              |
|              | Etofenprox   | 200        | 138         | 60.4A-B       | (51.4-69.6)            | 71.2B     | (64.1-83.8)             | 2.9A             | (0.8-9.4)               |
|              | Alpha-cypermethrin | 20   | 139         | 69.1B         | (59.6-77.1)            | 63.3AB    | (48.1-76.2)             | 3.6A             | (1.0-12.3)              |
|              | Carbosulfan  | 200        | 81          | 85.2C         | (73.9-92.1)            | 19.8C     | (8.4-39.7)              | 44.4B            | (29.5-60.5)             |

Percentages in the same column sharing a superscript letter do not differ at the 5% level of significance.
+ Controls outside net refers to total percentage, i.e. live and dead.
** Controls that did not differ significantly from each other at the 5% level of significance were pooled for analysis; the mean value for the pooled control is shown.

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Data over the 40 days and was significantly different from all other treatments and controls (*P* < 0.001). The overall mortality of *Culex* spp. caused by carbosulfan was found to be just significantly less than for pirimiphos-methyl (*P* = 0.043). Mortality caused by each of the pyrethroid insecticides was again found to be less than for controls, with alphacypermethrin causing least kill, being significantly lower than control mortality (*P* < 0.001).

Residual efficacy of insecticidal treatments seven months after initial impregnation

Our initial results showing the poor performance of the alpha-cypermethrin and etofenprox nets against this population of pyrethroid resistant *A. gambiae* and *Culex* spp. were confirmed during re-testing of the treated nets. For both *A. gambiae* and *Culex* spp., percentage mortality did not differ significantly from controls and percentage bloodfeeding was similar to or higher than controls (table 2). However, in this test the control mortality was satisfactorily low.

As confirmed by bioassays with samples cut from both the etofenprox and alpha-cypermethrin treated nets nine months after impregnation, the poor performance of these two pyrethroid insecticides in the field was not due to the absence of insecticidal activity. Exposure of susceptible *A. gambiae* insecticide-treated wire-spheres and observation of the times for knockdown of samples of 11 newly emerged female mosquitoes showed that the mean of the median knockdown time (KTₙₐₙ) for the nine-month-old, 20 mg alpha-cypermethrin m⁻² net was 21.9 min, which is somewhat longer than the time of 14.1 min, obtained for a net newly impregnated with the same formulation and dose (O. Akinpelu, unpublished). The KTₙₐₙ value for etofenprox was 20.6 min, indicating that both pyrethroid treatments were of similar knockdown efficacy. Mortality of the knocked down mosquitoes after 24 h was, however, significantly different for the two treatments (*P* < 0.001), etofenprox causing 98% kill whereas alpha-cypermethrin only caused 36%. Controls exposed to untreated netting for 50 min showed no knockdown or mortality. Three minute exposure to both the nine-month-old etofenprox and alpha-cypermethrin treated netting pieces, as well as to a piece from the carbosulfan net, all failed to cause more than 5% mortality.

Presumably because of its volatility, pirimiphos-methyl had lost its activity over the seven months and performed similarly in the hut tests to controls and the two pyrethroid treatments. Only carbosulfan was found to have maintained its activity, still performing significantly better in killing and preventing bloodfeeding when compared to controls or any of the treatments. Compared to the efficacy when freshly applied (table 1), carbosulfan was found to cause less mortality, the difference being just significant for *A. gambiae* (*P* = 0.049) but highly significant for *Culex* spp. (*P* < 0.001). There was, however, no significant reduction in its effect in preventing bloodfeeding (*P* = 0.68 and 0.54, respectively).

Excito-repellency of the insecticides seven months after impregnation was observed to be highest for carbosulfan, though the difference was not significant compared to results obtained with etofenprox or alpha-cypermethrin against *A. gambiae*. The proportion of mosquitoes collected outside the etofenprox and alpha-cypermethrin nets was less than during the initial test but higher than for controls, though this difference was not significant for *Culex* spp. In response to etofenprox, Pirimiphos-methyl was again found to have no excito-repellent effect, with the feeding rate of *A. gambiae* being significantly greater than with controls.

Selection for the kdr resistance allele

The results of kdr genotyping of 721 *A. gambiae* from the experimental huts are shown in tables 3 and 4. There was evidence for a significantly higher kdr frequency in those that survived the pyrethroids than in those that died on them. There was no evidence for selection for or against kdr in the control huts, where we suspect that the surprisingly high mortality may have been due to rough handling. There was also no evidence for selection by carbosulfan, though the numbers available for testing were small.
Discussion

In experimental hut studies, the percent protection of sleepers under a net may be measured by the number of blooded mosquitoes collected inside the hut, net, exit trap and veranda as a proportion of the overall number that had visited the hut in the course of the night. The present study showed that damaged bednets with a large number of holes and impregnated with the pyrethroid etofenprox or the OP pirimiphos-methyl did not provide protection against being bitten by pyrethroid resistant *A. gambiae* and *Culex* spp.

Because etofenprox is structurally different from pyrethroids, it had earlier been hoped that it would maintain its activity against pyrethroid resistant mosquitoes. However it was later found that in the laboratory pyrethroid resistant *Anopheles stephensi* Liston and *C. quinquefasciatus* showed cross-resistance to etofenprox (Curtis, 1993; Hemingway, 1995). Although etofenprox failed in the hut trials to prevent bloodfeeding, it was the most effective of the treatments in driving mosquitoes out of the net in the trial soon after impregnation (Table 1).

The observed failure of the OP pirimiphos-methyl to have any impact on bloodfeeding by either *A. gambiae* or *Culex* spp. is consistent with results obtained by Miller et al. (1991) who tested this compound against susceptible *A. gambiae* in The Gambia and with later observations of little or no excito-repellency in wind tunnel experiments (Miller & Gibson, 1994).

In contrast to etofenprox and pirimiphos-methyl, a significant reduction (by 50-65%) in bloodfeeding of *A. gambiae* s.s. and *Culex* was observed with the carbosulfan treated net compared to controls. The pyrethroid alpha-cypermethrin performed less well, significantly reducing bloodfeeding of *A. gambiae*, but not that of *Culex* spp.

Carbosulfan has not previously been evaluated as a bednet treatment, but results obtained in the present study may be compared with those of Weerasekoriya et al. (1996) against *C. quinquefasciatus* in Sri Lanka, who found that curtains treated with the carbamate bendiocarb were inferior to pyrethroid treated curtains in keeping mosquitoes out of houses. In our study, carbosulfan still provided good protection against being bitten by *A. gambiae* and *Culex* spp. seven months after net treatment and its overall performance on physically damaged nets was comparable to some of the most efficient pyrethroid treatments against pyrethroid susceptible mosquitoes (Curtis et al., 1996).

Besides the personal protection effect exerted by impregnated bednets, most, but not all, studies show a marked reduction in the infective population when insecticidal bednets are used extensively in a community (see review by Pates & Curtis, in press). In the present study, insecticidal activity of the bednets against *A. gambiae* was, however, found only with the two non-pyrethroids when freshly applied and solely with carbosulfan after seven months.

High insecticidal activity with pirimiphos-methyl and carbosulfan treated nets was observed not only against *A. gambiae* but also against *Culex* spp. in the present study. Previously, *Culex* mosquitoes have been reported as having a high tolerance to tarsal contact with pyrethroid treated netting (Hossain et al., 1989). This is consistent with an overall lack of population reduction of *Culex* spp. in response to widespread use of pyrethroid impregnated bednets (Magee et al., 1991; Jana-Kara et al., 1995; Curtis et al., 1998b). By contrast, the present study shows that high mortality of *Culex* can be achieved, using fresh deposits of pirimiphos-methyl or carbosulfan deposits up to seven months old on nets, despite reported resistance of *C. quinquefasciatus* to carbamates and organophosphates (as well as pyrethroids) in the area (Magnin et al., 1988; Chandre et al., 1997, 1998). High efficacy against *Culex* spp. may result in an increased acceptance of treated nets, as many people are primarily interested in protection from nuisance biting by *Culex* (see Zimicki, 1996).

Further investigation is needed of the poor performance which we observed against *A. gambiae* in response to both alpha-cypermethrin and etofenprox. These data contrast with those of Guillot (1998) and Darriet et al. (1999) who reported from Benin and Côte d'Ivoire that whether *kdr* frequencies are high or low, has little or no influence on the

Table 3. Numbers of the *kdr/kdr, kdr/kds* and *kds/kds* genotypes in *Anopheles gambiae* s.s. alive and dead collected from experimental huts during the test soon after the nets were treated.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg m⁻²)</th>
<th>Number live</th>
<th>Number dead</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kdr/kdr</td>
<td>kdr/kds</td>
<td>kds/kds</td>
</tr>
<tr>
<td>Etofenprox</td>
<td>200</td>
<td>92</td>
<td>13</td>
</tr>
<tr>
<td>Alpha-cypermethrin</td>
<td>20</td>
<td>245</td>
<td>26</td>
</tr>
<tr>
<td>Carbosulfan</td>
<td>200</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>Control I and</td>
<td>untreated</td>
<td>105</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 4. *kdr* allele frequencies calculated from the data in table 3.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg m⁻²)</th>
<th><em>kdr</em> frequency among</th>
<th>P from χ² Yates or Fisher's exact test</th>
<th>Number analysed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Live</td>
<td>Dead</td>
<td></td>
</tr>
<tr>
<td>Etofenprox</td>
<td>200</td>
<td>0.90</td>
<td>0.76</td>
<td><em>P</em>=0.008</td>
</tr>
<tr>
<td>Alpha-cypermethrin</td>
<td>20</td>
<td>0.92</td>
<td>0.81</td>
<td><em>P</em>=0.0009</td>
</tr>
<tr>
<td>Carbosulfan</td>
<td>200</td>
<td>0.86</td>
<td>0.84</td>
<td>Fisher <em>P</em>=0.5526</td>
</tr>
<tr>
<td>Control I and</td>
<td>untreated</td>
<td>0.86</td>
<td>0.85</td>
<td><em>P</em>=0.949</td>
</tr>
</tbody>
</table>

Numbers of the *kds/kds* homozygotes were too small to allow χ² tests on the numbers of each genotype; therefore significance tests were carried out on numbers of *kdr* and *kds* genes among the live and dead mosquitoes.
efficacy of bednets treated with permethrin or deltamethrin. The data of Chandre et al. (1999b) suggests that the kdr gene is particularly effective in protecting the insect against being killed by alpha-cypermethrin. However, these tests were done with papers impregnated with 0.0025% alpha-cypermethrin, i.e. much less than the WHO recommended discriminating dose for deltamethrin, but the doses used for net treatment with these compounds are the same.

We hope to clarify the situation by a side-by-side test in the same set of huts of nets treated with deltamethrin, alpha-cypermethrin and carbosulfan. Our results suggest that carbosulfan should be considered as a substitute for pyrethroids to delay, or respond to, emergence of a pyrethroid resistance problem. There may be reluctance to use anything other than pyrethroids for net treatment because of perceived risks of human toxicity. However, it is worth pointing out that the oral LD50 values for deltamethrin and carbosulfan are 135 mg kg⁻¹ and 250 mg kg⁻¹ respectively and that both compounds have the same allowable daily intake of 0.01 mg kg⁻¹ (Tomlin, 1994). Also, some consider pyrethroids seriously hazardous (Tippe, 1993). It is important that an objective risk-benefit analysis is carried out on all effective net treatments, keeping in view the reductions in all-cause child mortality which they can achieve (Lengeler, 1998).

The polymerase chain reaction followed by sequence-specific oligonucleotide probing (PCR-SSOP) method of detection of the kdr gene was found very convenient for processing large numbers of mosquitoes, especially because the method does not require electrophoresis. For examination of whether resistance exists in a population at low frequency, it has the advantage over bioassays that the latter may not detect heterozygotes because of recessiveness of the gene. In the present project it allowed examination of the resistance frequency in mosquitoes, which were already dead when collected. This provided evidence for selection for kdr by contact with the pyrethroid treated nets. Thus, although it is considered that treated nets were not responsible for the emergence of kdr in this population (Elissa et al., 1993; Chandre et al., 1999a), it appears that continued use of pyrethroid treated nets could enhance its frequency.

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References


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