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 gambiae* 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

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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. gambiae* 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. gambiae* 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 (Curtis *et al*., 1996). Thus in the present study, at the suggestion of Dr P. Guillet, it was decided to test a carbamate 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 bednet treatment against pyrethroid susceptible *A. gambiae* (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. gambiae* in Tanzania and found to be somewhat less efficient compared to deltamethrin and lambda-cyhalothrin at a much lower dose.

The results against *A. gambiae*, 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 × 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. gambiae* (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 m^2. 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 × 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}, Fastac 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 × 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 we used for bioassays with a susceptible strain of *A. gambiae* 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., *Culex* spp. or members of the *A. gambiae* complex. Occasionally we found a few *Aedes* spp., *Anopheles funestus* 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 phenylananine 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′ -TGGATTGAATCAATGTGGGATTG-3′,
128-Reverse Primer, 5′ -TGCCGTTGGTGCAGACAAGG- 3′.

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

104L Probe, 5′ -GGAAATTT**A**GTCGTAAGT- 3′,
104F Probe, 5′ -GGAAATTT**T**GTCGTAAGT 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 H_2O.

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 × Bioline amplification KCl reaction buffer (including $MgCl_2$ 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 × 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 TMAC hybridization solution (3 M Tetramethylammonium chloride, 50 mM tris/HCl pH 8, 0.1% SDS, 2 mM EDTA pH 8). Membranes were then washed for 2 × 10 min in 2 × SSPE/0.1% SDS at room temperature (low-stringency washes) and 2 × 10 min (high-stringency washes) in TMAC 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

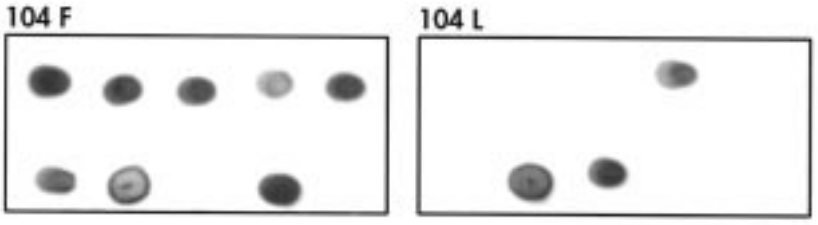

Fig. 1. A portion of developed duplicate membranes showing examples of mosquito genotypes for the pyrethroid resistance *kdr* allele (sodium channel codon 104 *F*) and the susceptibility (*kds*) allele (104 *L*) in *Anopheles gambiae*. The possible diploid genotypes are *FF*, *FL* or *LL*. Results shown are, top row (left to right): *FF*, *FF*, *FF*, *FL*, *FF*; bottom row (left to right) *FF*, *FL*, *LL*, *FF*, -ve control.

contaminating huts with different insecticides (F. Darriet, personal communication). The excito-repellency measured by the number of mosquitoes collected outside 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 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 bloodfed 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 bloodfed *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.

Species	Treatment	Dose (mg m^{-2})	Total number	% outside net*	95% confidence interval	% bloodfed	95% confidence interval	% dead after 24 h	95% confidence interval
A. gambiae	Control I	untreated	126	45.2^{A}	(35.8–55.1)	Controls pooled**		Controls pooled**	
	Control II	untreated	172	70.2^{B}	(62.2–77.1)	42.4 ^{A}	(33.0–52.4)	30.3 ^{A}	(24.5–36.8)
	Pirimiphos-methyl	1000	269	73.2^{B,C}	(67.7–78.1)	43.1 ^{A}	(32.9–54.0)	100 ^{B}	–
	Etofenprox	200	140	95.0^{D}	(90.0–97.6)	52.9 ^{A}	(38.6–65.0)	20.0 ^{A,C}	(13.1–30.1)
	Alpha-cypermethrin	20	378	78.8^{C,E}	(74.1–83.0)	28.0 ^{B}	(21.5–35.7)	18.5 ^{C}	(14.2–23.8)
	Carbosulfan	200	158	86.7^{E}	(79.8–91.5)	15.2 ^{C}	(7.9–27.3)	92.4 ^{D}	(86.7–95.8)
Culex spp.	Control I	untreated	117	53.0^{A}	(42.4–63.3)	Controls pooled**		Controls pooled**	
	Control II	untreated	122	73.9^{B}	(63.7–82.1)	38.1 ^{A}	(29.4–47.6)	28.5 ^{A}	(22.3–35.6)
	Pirimiphos-methyl	1000	236	85.2^{C}	(79.0–89.8)	44.5 ^{A}	(35.4–53.9)	99.6 ^{B}	(96.6–99.9)
	Etofenprox	200	169	96.4^{D}	(91.4–98.6)	50.9 ^{A}	(40.8–60.9)	19.5 ^{A,E}	(13.2–27.8)
	Alpha-cypermethrin	20	211	70.1^{B}	(62.1–77.1)	39.8 ^{A}	(32.7–52.6)	10.9 ^{C,E}	(7.0–16.6)
	Carbosulfan	200	161	79.5^{B,C}	(71.2–85.9)	19.3 ^{B}	(11.7–30.0)	95.7 ^{D}	(90.4–98.1)

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.

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

Species	Treatment	Dose (mg m^{-2})	Total number	% outside net*	95% confidence interval	% bloodfed	95% confidence interval	% dead after 24 h	95% confidence interval
	Control I	untreated	26	Controls pooled**		Controls pooled**		Controls pooled**	
	Control II	untreated	95	48.8^{A}	(38.4–59.2)	44.6 ^{A}	(33.6 – 56.2)	2.5 ^{A}	(0.7–8.2)
A. gambiae	Pirimiphos-methyl	1000	100	31.0^{B}	(22.0–41.7)	75.0 ^{B}	(62.9–84.2)	5.0 ^{A}	(1.7–13.5)
	Etofenprox	200	77	75.3^{C}	(62.6–84.8)	64.9 ^{B,C}	(54.1–77.1)	2.6 ^{A}	(0.5–12.7)
	Alpha-cypermethrin	20	101	69.6^{C}	(58.0–79.1)	52.9 ^{A,C}	(41.1–64.5)	2.0 ^{A}	(0.3–11.0)
	Carbosulfan	200	56	84.2^{C}	(69.7–92.6)	10.5 ^{D}	(4.0–25.0)	66.7 ^{B}	(49.4–80.4)
	Control I	untreated	98	Controls pooled**		Controls pooled**		Controls pooled**	
	Control II	untreated	97	56.4^{A}	(48.1–63.9)	51.0 ^{A}	(38.3–63.6)	3.6 ^{A}	(1.5–8.1)
Culex spp.	Pirimiphos-methyl	1000	155	58.4^{A,B}	(49.3–67.0)	76.6 ^{B}	(61.6–87.0)	4.5 ^{A}	(1.8–11.2)
	Etofenprox	200	138	60.4^{A,B}	(51.4–68.8)	71.2 ^{B}	(54.1–83.8)	2.9 ^{A}	(0.8–9.4)
	Alpha-cypermethrin	20	139	69.1^{B}	(59.6–77.1)	63.3 ^{A,B}	(48.1–76.2)	3.6 ^{A}	(1.0–12.3)
	Carbosulfan	200	81	85. 2^{C}	(73.9–92.1)	19.8 ^{C}	(8.4–39.7)	44.4 ^{B}	(29.5–60.5)

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.

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* in netting covered 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 (KT_{50}) 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 KT_{50} 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.

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.

Treatment	Dose (mg m^{-2})	Number live			Number dead		
		kdr/kdr	*kdr/kds*	*kds/kds*	*kdr/kdr*	*kdr/kds*	*kds/kds*
Etofenprox	200	92	13	4	17	7	3
Alpha-cypermethrin	20	245	28	10	49	11	7
Carbosulfan	200	6	–	1	19	4	2
Control I and II	untreated	105	17	10	52	17	2

Discussion

In experimental hut studies, the percent protection of sleepers under a net may be measured by the number of bloodfed 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 Weerasooriya *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 (Magesa *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 Guillet (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 4. *kdr* allele frequencies calculated from the data in table 3.

Treatment	Dose (mg m^{-2})	*kdr* frequency among		P from χ^2 Yates or Fisher's exact test	Number analysed	
		Live	Dead		Live	Dead
Etofenprox	200	0.90	0.76	P=0.008	109	27
Alpha-cypermethrin	20	0.92	0.81	P=0.0009	283	67
Carbosulfan	200	0.86	0.84	Fisher P=0.5526	7	25
Control I and II	untreated	0.86	0.85	P=0.949	132	71

Numbers of the *kds/kds* homozygotes were too small to allow χ^2 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

Chandre, F., Darriet, F., Doannio, J.M.C., Rivière, F., Pasteur, N. & Guillet, P. (1997) Distribution of organophosphate and carbamate resistance in *Culex pipiens quinquefasciatus* (Diptera: Culicidae) in West Africa. *Journal of Medical Entomology* **34**, 664–671.

Chandre, F., Darriet, F., Darder, M., Cuany, A., Doannio, J.M.C., Pasteur, N. & Guillet, P. (1998) Pyrethroid resistance in *Culex quinquefasciatus* from West Africa. *Medical and Veterinary Entomology* **12**, 359–366.

Chandre, F., Darriet, F., Manga, L., Akogbeto, M., Faye, O., Mouchet, J. & Guillet, P. (1999a) Status of pyrethroid resistance in *Anopheles gambiae* sensu lato. *Bulletin of the World Health Organization* **77**, 230–234.

Chandre, F., Darriet, F., Manguin, S., Brengues, C., Carnevale, P. & Guillet, P. (1999b) Pyrethroid cross resistance spectrum among populations of *Anopheles gambiae* s.s. from Côte d'Ivoire. *Journal of the American Mosquito Control Association* **15**, 53–59.

Collins, F.H., Mendez, M.A., Rasmussen, M.O., Mehaffey, P.C., Besansky, N.J. & Finnerty, V. (1987) A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. *American Journal of Tropical Medicine and Hygiene* **37**, 37–41.

Crawley, M.J. (1993) *GLIM for ecologists*. 379 pp. Oxford, Blackwell Scientific Publications.

Curtis, C.F. (1993) Workshop on bednets at the International Congress of Tropical Medicine. *Japanese Journal of Sanitary Zoology* **44**, 65–68.

Curtis, C.F., Hill, N., Ulloa, M. & Magesa, S. (1990) The possible impact of resistance on the effectiveness of pyrethroid-impregnated bednets. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **84**, 455.

Curtis, C.F., Myamba, J. & Wilkes, T.J. (1996) Comparison of different insecticides and fabrics for anti-mosquito bednets and curtains. *Medical and Veterinary Entomology* **10**, 1–11.

Curtis, C.F., Miller, J.E., Hodjati, M.H., Kolaczinski, J.H. & Kasumba, I. (1998a) Can anything be done to maintain the effectiveness of pyrethroid-impregnated bednets against malaria vectors? *Philosophical Transactions of the Royal Society of London B*, **353**, 1769–1775.

Curtis, C.F., Maxwell, C.A., Finch, R.J. & Njunwa, K.J. (1998b) A comparison of use of a pyrethroid either for house spraying or for bednet treatment against malaria vectors. *Tropical Medicine and International Health* **3**, 619–631.

Darriet, F. (1998) *La lutte contre les moustiques nuisants et vecteurs de maladies*. 114 pp. Karthala-ORSTOM Editions, ISBN: 2-86537-864-0.

Darriet, F., Guillet, P., Chandre, F., Guessan, R.N., Doannio, J.M.C., Rivière, F. & Carnevale, P. (1997) Présence et évolution de la résistance aux pyrethrinoides et au DDT chez deux populations d'*Anopheles gambiae* s.s. d'Afrique de l'Ouest. WHO/CTD/VBC/97.1001.

Darriet, F., Guillet, P., N'Guessan, R.N., Doannio, J.M.C., Koffi, A.A., Konan, L.Y. & Carnevale, P. (1999) The impact of permethrin and deltamethrin resistance in *Anopheles gambiae* s.s. on the efficacy of insecticide-treated mosquito nets. WHO/VBC 99.1002.

Elissa, N., Mouchet, J., Rivière, F., Meunier, J.-Y. & Yao, K. (1993) Resistance of *Anopheles gambiae* s.s. to pyrethroids in Côte d'Ivoire. *Annales de la Société Belge de Médécine Tropicale* **73**, 291–294.

Fanello, C., Kolaczinski, J., Conway, D., Carnevale, P. & Curtis, C.F. (1999) The *kdr* pyrethroid resistance gene in *Anopheles gambiae*: test of non-pyrethroid insecticides and improvement of the detection method for the gene. *Parassitologia* **41**, 323–326.

Guillet, P. (1998) Implications of knock-down resistance (kdr) development on the use of impregnated bednets. pp. 45–48 in *Report of 1st Meeting of Global Collaboration for Development of Pesticides for Public Health, 14th–15th of October 1998*. CTD/WHOPES/GCDPP/98.1. World Health Organization, Geneva.

Hemingway, J. (1995) Efficacy of etofenprox against insecticide susceptible and resistant mosquito strains containing characterised resistance mechanisms. *Medical and Veterinary Entomology* **9**, 423–426.

Hossain, M.I., Curtis, C.F. & Heekin, J.P. (1989) Assays of permethrin-impregnated fabrics and bioassays with mosquitoes (Diptera: Culicidae). *Bulletin of Entomological Research* **79**, 299–308.

Jana-Kara, B.R., Jihullah, W.A., Shahi, B., Dev, V., Curtis, C.F. & Sharma, V.P. (1995) Deltamethrin impregnated bednets against *Anopheles minimus* transmitted malaria in Assam, India. *Journal of Tropical Medicine and Hygiene* **98**, 73–83.

Jawara, M., McBeath, J., Lines, J.D., Pinder, M., Sanyang, F. & Greenwood, B.M. (1998) Comparison of bednets treated with alphacypermethrin, permethrin or lambdacyhalothrin against *Anopheles gambiae* in The Gambia. *Medical and Veterinary Entomology* **12**, 60–66.

Lengeler, C. (1998) Insecticide treated bednets and curtains for malaria control (Cochrane Review). in *The Cochrane Library*, Issue 3. Oxford, Update Software.

Luo, D., Lu, D., Yao, R., Li, P., Huo, X., Li, A., Wen, L., Ge, C., Zhang, S. & Huo, H. (1994) Alphamethrin-impregnated bed nets for malaria and mosquito control in China. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **88**, 625–628.

Magesa, S.M., Wilkes, T.J., Mnzava, A.E.P., Njunwa, K.J., Myamba, J., Kivuyo, M.D.P., Hill, N., Lines, J.D. & Curtis, C.F. (1991) Trial of pyrethroid impregnated bednets in an area of Tanzania holoendemic for malaria: Part 2. Effects on the malaria vector population. *Acta Tropica* **49**, 97–108.

Magnin, M., Marboutin, E. & Pasteur, N. (1988) Insecticide resistance in *Culex quinquefasciatus* (Diptera: Culicidae) in West Africa. *Journal of Medical Entomology* **25**, 99–104.

Malcolm, C.A. (1988) Current status of pyrethroid resistance in anophelines. *Parasitology Today* **4**, S13–S15.

Martinez-Torres, D., Chandre, F., Williamson, M.S., Darriet, F., Bergé, J.B., Devonshire, A.L., Guillet, P., Pasteur, N. & Pauron, D. (1998) Molecular characterisation of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae* s.s. *Insect Molecular Biology* **7**, 179–184.

Miller, J.E. & Gibson, G. (1994) Behavioural response of host-seeking mosquitoes (Diptera: Culicidae) to insecticide-impregnated bed netting: a new approach to insecticide bioassays. *Journal of Medical Entomology* **31**, 114–126.

Miller, J.E., Lindsay, S.W. & Armstrong, J.R.M. (1991) Experimental hut trials of bednets impregnated with synthetic pyrethroid or organophosphate insecticides for mosquito control in The Gambia. *Medical and Veterinary Entomology* **5**, 465–476.

Pates, H.V. & Curtis, C.F. (in press) Mosquito behaviour in relation to control. *Ch. 12 in* Costantini, C. (*Ed.*) *Mosquito Behaviour.*

Pleass, R.J., Armstrong, J.R.M., Curtis, C.F., Jawara, M. & Lindsay, S.W. (1993) Comparison of permethrin treatments for bednets in The Gambia. *Bulletin of Entomological Research* **83**, 133–140.

Sawicki, R.M. (1978) Unusual response of DDT-resistant houseflies to carbinol analogues of DDT. *Nature* **275**, 443–444.

Tippe, A. (1993) Sind Pyrethroide unbedenklich? Zur Bewertung experimenteller Befunde. *Zentralblatt für Hygiene und Umweltmedizin* **194**, 342–359.

Tomlin, C. (1994) *The pesticide manual.* 1341 pp. British Crop Protection Council, Farnham, UK and The Royal Society of Chemistry, Cambridge, UK.

Weerasooriya, M.V., Munasinghe, C.S., Mudalige, M.P.S., Curtis, C.F. & Samarawickrema, W.A. (1996) Comparative efficacy of house curtains impregnated with permethrin, lambdacyhalothrin or bendiocarb against the vector of bancroftian filariasis, *Culex quinquefasciatus*, in Matara, Sri Lanka. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **90**, 103–104.

Zimicki, S. (1996) Promotion in sub-Saharan Africa. pp. 129–174 *in* Lengeler, C., Cattani, J. & de Savigny, D. (*Eds*) *Net gain.* Ottawa, Canada, International Development Research Centre, Geneva, Switzerland, and World Health Organization.

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