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The *kdr* pyrethroid resistance gene in *Anopheles gambiae*: tests of non-pyrethroid insecticides and a new detection method for the gene

C. Fanello[1], J.H. Kolaczinski[1, 2], D.J. Conway[1], P. Carnevale[2], C.F. Curtis[1]

[1] *London School of Hygiene and Tropical Medicine, London, UK;* [2] *Institut Pierre Richet, Bouaké, Côte d'Ivoire.*

Abstract. The organophosphate pirimiphos-methyl and the carbamate carbosulfan were evaluated in comparison to the pyrethroid alphacypermethrin and the 'near-pyrethroid' etofenprox against pyrethroid resistant *Anopheles gambiae* and *Culex* spp. in an experimental hut station located in central Côte d'Ivoire. Bednets were impregnated with the above mentioned compounds and randomly allocated to the huts. On 40 consecutive mornings, after sleepers had occupied the huts overnight, mosquitoes were collected from the huts, identified and scored as live or dead (including delayed mortality). *An. gambiae* s.l. that had been collected were tested for the presence of the *kdr* allele in heterozygous or homozygous form. Both non-pyrethroid treatments caused very high mortality, whereas mortality with alpha-cypermethrin and etofenprox generally did not differ from the levels observed with untreated control nets in this experiment. The nets had holes cut in them and there was considerable bloodfeeding on the sleepers, which was only significantly reduced for *An. gambiae* by carbosulfan and alpha-cypermethrin. PCR genotyping suggested that there was selection for the *kdr* resistance allele by the pyrethroid treated nets. Organophosphates and carbamates may therefore present an alternative to be used on bednets especially in areas of pyrethroid resistance, but the safety of these insecticides will have to be carefully considered.

Key words: *Anopheles gambiae*, pyrethroid resistance, *kdr*, insecticide-treated bednets, resistance management, organophosphates, carbamates.

Bednets treated with pyrethroid insecticides are currently being introduced in many areas where malaria vector control has not previously been attempted and in other areas as replacement for conventional house spraying.

Synthetic pyrethroid insecticides are favoured for impregnation, because of their low mammalian toxicity, bio-degradability, rapid killing and irritancy to mosquitoes, at low doses (Zerba, 1988). However, resistance to pyrethroid insecticides has been reported for populations of many agricultural and domestic insect pests and long been considered as a threat to the continued success of impregnated bednets (Curtis *et al.*, 1990; Curtis, 1996). Recently this type or resistance has also been documented in the malaria vector *Anopheles gambiae* s.l. from West Africa (Elissa *et al.*, 1993; Darriet *et al.*, 1997; Martinez-Torres *et al.*, 1998). The primary resistance mechanism in these populations is of the *kdr*-type, which gives broad spectrum resistance to all pyrethroids and DDT. However, the existing *kdr* resistance gene may not be sufficiently powerful to render treated nets ineffective (Guillet and Carnevale, in preparation).

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Correspondence: Dr Jan Kolaczinski, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK, Tel ++44 171 9272337, Fax ++44 171 6368739, e-mail: jan.kolaczinski@lshtm.ac.uk

In anticipation of the expected evolution of strong pyrethroid resistance in anopheline mosquitoes, a number of studies have been carried out to investigate possible resistance management alternatives (Curtis *et al.*, 1998). It was concluded that there is no obvious treatment for bednets to prevent or solve a potential pyrethroid resistance problem in *Anopheles* mosquitoes.

The main reasons for the lack of alternatives are: first the close contact of humans with the insecticide impregnated material and second the need for a fast acting, preferably excito-repellent, compound to achieve good personal protection which, in addition to reduction of the vector population in a community, underlies the effectiveness of treated nets. These demanding criteria for the selection of a suitable compound and the bad publicity that other classes of insecticides, such as the organophosphates, have received in recent years, have discouraged research into the use of non-pyrethroid insecticides on bednets, apart from the work of Brun and Sales (1976) with four organophosphates, and Miller *et al.* (1991) with pirimiphos methyl against susceptible *An. gambiae* populations.

We further evaluated this organophosphate, as well as the carbamate carbosulfan, on bednets against *An. gambiae* in one of the areas of resistance in West Africa. For comparison we used the pyrethroid alphacypermethrin and the 'near-pyrethroid' etofenprox, against which resistance due to the *kdr*-allele could be expected.

In order to assess the degree to which the *kdr* re-

sistance allele protects individual insects against being killed by the treated nets, and therefore promotes the selection for the gene in the population, we compared the allele frequency in mosquitoes recovered live and dead from the huts.

Materials and methods

Experimental hut study

The study was carried out at the experimental hut station of the Institut Pierre Richet at Yaokoffikro, Bouaké region, central Côte d'Ivoire. The station consists of six experimental huts which are built in a row about 10 metres from the edge of rice fields. The huts were equiped with special windows, designed to allow mosquito entry but to prevent mosquitoes from exiting, and with a water-filled moat to exclude ants which might have consumed dead mosquitoes.

The following four insecticidal treatments as well as two untreated controls, were randomly allocated to the huts:

- alpha-cypermethrin, 20 mg/m², Fendona 15% WG (Cyanamid Agriculture Ltd)
- etofenprox, 200 mg/m², Vectron 10% EW (Mitsui Chemicals Inc)
- carbosulfan, 200 mg/m², Marshal 20% CS (FMC Corporation)
- pirimiphos-methyl, 1000 mg/m², Actellic 50% EC (Zeneca).

Treatments were not rotated between huts (unlike in the work of Curtis *et al.*, 1996, and Maxwell *et al.*, 1999) because we were advised that rotation involves a risk of cross-contamination between huts.

These dosages were applied to green mosquito nets (SiamDutch, Bangkok, Thailand) in each of which 180 holes (2.5×2.5 cm) were cut to simulate the poor condition in which nets are often found in villages. The pieces cut out of the nets have been sent for chemical analyses to check the actual doses on the nets, but these data are not yet available. Adult male sleepers occupied the nets each night and gave their informed consent to taking part in this trial. Each net remained in the same hut with the same sleeper throughout the trial.

PCR-SSOP method for *kdr* genotyping

A PCR method for detecting the *kdr* allele has been published by Martinez-Torres *et al.* (1998), based on the observation that this allele has a single base pair substitution causing a change from leucine to phenylalanine in domain II of the voltage-gated sodium channel protein. We developed a different method for detecting the *kdr* allele (Kolaczinski *et al.*, in preparation). From the gene sequence (EMBL Accession N. Y13592) we designed two primers to amplify the region with the point mutation. The amplified fragment was blotted onto nylon membranes and hybridised with two oligonucleotide probes, which are non-radioactively labelled (by 3'-incorporation of a digoxigenin-labeled nucleotide DIG), one complementary to the susceptibility allele and the other to the resistance allele. The DIG-labelled probes were detected after hybridisation by enzyme-linked immunoassay using antibody-conjugate, and detected by chemiluminescence. This method allowed large numbers of samples from the field to be efficiently and accurately genotyped as heterozygous or homozygous for the resistance or susceptibility allele.

Results and discussion

To evaluate the effectiveness of the different bednet treatments, the percentage blood-fed and dead were used as measures of personal protection and contribution to population reduction. The data for *An. gambiae* s.s. are shown in Table 1. The blood-feeding was only significantly reduced, compared to controls, by the alpha-cypermethrin and carbosulfan treatments, with the latter performing significantly better. The results for *Culex* spp. were similar (see Kolaczinski *et al.*, in preparation).

In terms of mortality caused by the treatments, both non-pyrethroids performed remarkably well: pirimiphos-methyl killed 100% of *An. gambiae* (Table 1) and it also killed almost 100% of *Culex* spp. entering the experimental huts. Carbosulfan performed almost as well (Table 1). The mortality with both alpha-cypermethrin and etofenprox was not significantly different from the mortality in the control huts. However, for unknown reasons this control mortality was much higher than normally

Table 1. Percentage of *Anopheles gambiae* s.s. blood-fed and dead (after 24 hours) collected from experimental huts over 40 nights. Note: Percentages in the same column sharing a superscript letter do not differ at the 5% level of significance.

Treatment	Target dose (mg/m²)	% Blood-fed	% Dead	Total number
Control I	Untreated	41.3 ^{A}	27.0 ^{A}	126
Pirimiphos-methyl	1000	43.1 ^{A}	100 ^{B}	269
Etofenprox	200	52.9 ^{A}	20.0 ^{A,C}	140
Alpha-cypermethrin	20	28.0 ^{B}	18.5 ^{C}	378
Carbosulfan	200	15.2 ^{C}	92.4 ^{D}	158
Control II	Untreated	43.0 ^{A}	32.6 ^{A}	172

Table 2. Frequency of the *kdr* allele in *Anopheles gambiae* s.s. alive and dead collected from experimental huts.

Treatment	*kdr* frequency among		Number analysed		p (χ^2 or Fisher's)
	Live	Dead	Live	Dead	
Etofenprox	0.90	0.76	109	27	<0.01
Alpha-cypermethrin	0.92	0.81	283	67	<0.001
Carbosulfan	0.86	0.84	7	25	>0.5

found with untreated nets in these and other experimental huts (see Carnevale *et al.*, in preparation, and Curtis *et al.*, 1996).

The high mortality due to pirimiphos-methyl on nets was similar to that reported for pyrethroid-susceptible *An. gambiae* and *Mansonia* in The Gambia by Miller *et al.* (1991). However, they reported the pirimiphos-methyl impregnated nets to have a pungent odour, to feel sticky and to become dirty. A strong smell of the pirimiphos-methyl impregnated net was also observed in the present study, but the effect disappeared over a few weeks. Stickiness of the net was not observed and, by using green bednets, the accumulation of dirt was not apparent. The sleepers in the huts were enthusiastically in favour of the pirimiphos-methyl treatment and requested the chemical for treatment of their nets at home.

Evidence for selection for the *kdr* gene in *An. gambiae* s.s.

The PCR-SSOP described above, showed that the frequency of the *kdr* allele in *An. gambiae* s.s. was nearly 0.9. The mosquitoes caught were not in Hardy-Weinberg equilibrium at this locus, showing a deficit of heterozygotes. The distribution of the chromosomal forms of *An. gambiae* s.s. and causes of this disequilibrium are currently being investigated. In the huts where the etofenprox and alpha-cypermethrin nets were in use the frequency of the *kdr* allele was significantly higher ($p<0.01$) amongst surviving *An. gambiae* s.s. females than in those which died (Table 2), i.e. there was evidence for selection by the pyrethroid treated nets for the *kdr* gene. So far no significant evidence for selection for or against the *kdr* gene by the carbosulfan treated net has been found. Work is in progress to determine whether there was natural selection against the *kdr* gene in the huts with control nets where, as already mentioned, mortality was surprisingly high. The results will be reported by Kolacinzski *et al.*, in preparation.

The present study has shown that organophosphate and carbamate treatments of bednets can kill mosquitoes at least as well against pyrethroid-resistant mosquitoes as pyrethroids do against mosquitoes which are susceptible (Curtis *et al.*, 1996). Organophosphates and carbamates may present alternatives to be used on bednets especially in areas of pyrethroid resistance, but, because they would depend on mass mosquito killing and not prevention of blood feeding, they would only be likely to be effective in projects which achieved a high percentage coverage of the population with treated nets (e.g. Maxwell *et al.*, 1999).

There has been much concern about the toxicity of organophosphates over recent years, but it is important to emphasise that organophosphates cover a wide range of acetylcholinesterase inhibitors, ranging form nerve gases to chemicals used as pharmaceuticals or for treatment of human dwellings, grain stores and drinking water (Gallo and Lawryk, 1991; Karczmar, 1998). In our opinion they could be considered as possible alternatives for pyrethroids in view of the fact that, for every 180 insecticide treated nets used, one death of African children can be prevented per year (Lengeler, 1998) and this major life saver may be lost if resistance of a type which could prevent the effective functioning of pyrethorids were to become widespread.

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