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Resistance to carbosulfan in *Anopheles gambiae* from Ivory Coast, based on reduced sensitivity of acetylcholinesterase

R. N'GUESSAN, F. DARRIET*, P. GUILLET†, P. CARNEVALE*, M. TRAORE-LAMIZANA, V. CORBEL*, A. A. KOFFI and F. CHANDRE

Centre Pierre Richet, Institut National Santé Publique, Bouaké, Côte d'Ivoire, *Laboratoire de Lutte contre les Insectes Nuisibles (LIN), Institut de Recherche pour le Développement (IRD, former ORSTOM), Montpellier, France and †Parasite & Vector Control CDS/CPE, World Health Organization, Geneva, Switzerland

Abstract. Resistance to carbosulfan, a carbamate insecticide, was detected in field populations of the malaria vector mosquito *Anopheles gambiae* Giles (Diptera: Culicidae) from two ecologically contrasted localities near Bouaké, Ivory Coast: rural M'bé with predominantly M form of *An. gambiae* susceptible to pyrethroids; suburban Yaokoffikro with predominantly S form of *An. gambiae* highly resistant to pyrethroids (96% kdr). The discriminating concentration of 0.4% carbosulfan (i.e. double the LC_{100}) was determined from bioassays with the susceptible *An. gambiae* Kisumu strain. Following exposure to the diagnostic dosage (0.4% carbosulfan for 1 h), mortality rates of female *An. gambiae* adults (reared from larvae collected from ricefields) were 62% and 29% of those from M'bé and Yaokoffikro, respectively, 24 h post-exposure. Exposure for 3 min to netting impregnated with the operational dosage of carbosulfan 200 mg/m^2 gave mortality rates of 88% of those from M'bé and only 12.2% for Yaokoffikro. In each case the control untreated mortality rate was insignificant. Biochemical assays to detect possible resistance mechanism(s) revealed the presence of insensitive AChE in populations of *An. gambiae* at both localities, more prevalent in the S form at Yaokoffikro than in M form at M'bé, as expected from bioassays results. Our study demonstrates the need to monitor carbamate resistance among populations of the *An. gambiae* complex in Africa, to determine its spread and anticipate vector control failure if these insecticides are employed.

Key words. *Anopheles gambiae*, M & S forms, acetylcholinesterase, carbamate resistance, carbosulfan, discriminating concentration, insecticide treated nets (ITNs), pyrethroid resistance, Côte d'Ivoire, Ivory Coast, West Africa.

Introduction

Malaria vector control in Africa relies heavily on DDT and pyrethroid insecticides (Zaim, 2002). Pyrethroid resistance in the most important malaria vector *Anopheles gambiae* is already widespread in several West African countries (Elissa *et al.*, 1993; Darriet *et al.*, 1997; Chandre *et al.*, 1999a), although the predominant kdr mechanism does not prevent the efficacy of pyrethroid-treated bednets (Darriet *et al.*, 2000). In South Africa, pyrethroid resistance in *An. funestus* Giles caused the failure of indoor residual house-spraying (IRS) with the pyrethroid deltamethrin (Hargreaves *et al.*, 2000) and threatens the efficacy of insecticide-treated nets, ITNs (Curtis, 1996).

Organophosphate and carbamate insecticides are the main alternatives to pyrethroids for controlling malaria

Correspondence: Dr. Fabrice Chandre and Raphael N'Guessan, LIN/IRD, 911 avenue Agropolis, B.P. 5045, 34032 Montpellier cedex 1, France. E-mail: fabrice.chandre@mpl.ird.fr

vectors (White, 1999; Najera & Zaim, 2002), having worked well for IRS in some situations (Najera *et al.*, 1967; Fontaine *et al.*, 1978) but not others (Kouznetsov, 1977; Molineaux & Gramiccia, 1980). In Burkina Faso, IRS with the carbamate carbosulfan resulted in more than 94% mortality of *An. gambiae and* greatly reduced its blood-feeding success in experimental huts (Klein & Darriet, 1989). Similar results were achieved in Ivory Coast when mosquito nets were treated with carbosulfan 200 mg/m^2 (Fanello *et al.*, 1999; Kolaczinski *et al.*, 2000). Trials of bednets treated with the organophosphate pirimiphos-methyl 1000 mg/m^2 achieved very high mortality rates (99–100%) of *An. gambiae* in The Gambia and Ivory Coast, but no reduction of blood-feeding in comparison to untreated nets (Miller *et al.*, 1991; Kolaczinski *et al.*, 2000). Combined 'two-in-one' treatment with pyrethroid plus carbosulfan on the same net was found to be very effective against pyrethroid resistant *An. gambiae* as well as the multiresistant pest mosquito *Culex quinquefasciatus* (Guillet *et al.*, 2001).

Acetylcholinesterase (AChE) is a common target for organophosphates and carbamates. These insecticides block transmission of nerve impulses by irreversible inhibition of AChE at cholinergic synapses, causing insect death. Cross-resistance to organophosphate and carbamate insecticides can arise from alteration of the AChE target site, making it less susceptible to these insecticides. This broad-spectrum resistance mechanism has occurred in several mosquito species, including the malaria vectors *Anopheles albimanus* Wiedemann (Ayad & Georghiou, 1975), *An. atroparvus* Van Theil (Hemingway, 1982) and *An. sacharovi* Favre (Hemingway *et al.*, 1985). Resistance to the carbamate propoxur was detected in *An. gambiae* populations from Bouaké area in Ivory Coast by Elissa *et al.* (1994).

Because organophosphates and carbamates are regarded as possible alternatives to pyrethroids for ITNs (Curtis *et al.*, 1998; Guillet *et al.*, 2001), it is necessary to assess the susceptibility status of potential target populations of Afrotropical mosquitoes. In the present study, the discriminating concentration of carbosulfan for testing susceptibility/resistance of adult *An. gambiae sensu stricto* was determined using a susceptible laboratory strain and field populations from near Bouaké in Ivory Coast. Efficacy of netting material treated with carbosulfan was assessed comparing field resistant strains and the susceptible laboratory strain. A significant level of carbamate resistance was detected in the field populations of *An. gambiae* s.s and biochemical assays have been carried out to identify the main resistance mechanism(s) involved.

Materials and methods

Mosquitoes

Adult females of the local *An. gambiae* s.s. populations were obtained from larvae collected in ricefields and reared to adulthood in the laboratory at CPR Bouaké under standard conditions (27 ± 2°C, 80% RH). Two localities were sampled:

- Yaokoffikro suburb of Bouaké. This population of *An. gambiae* s.s. is mostly of the S form (*sensu* della Torre *et al.*, 2001) and strongly resistant to pyrethroids with kdr mutation at allelic frequency of 0.96, determined by PCR (Martinez-Torres *et al.*, 1998).
- M'bé valley, 30 km north of Bouaké. This population of *An. gambiae* s.s. is mostly of the M form (*sensu* della Torre *et al.*, 2001) susceptible to pyrethroids with less than 0.04 kdr frequency.

For insecticide sensitivity tests, field samples were compared to a susceptible reference strain of *An. gambiae* s.s. originating from Kisumu, Kenya.

Determination of discriminating concentration for carbosulfan resistance

WHO (1981) test kits were used to assess the sensitivity of *An. gambiae* females to carbosulfan. Impregnated papers were prepared in our laboratory using technical grade carbosulfan (88.1% purity, provided by FMC, Philadelphia, PA, U.S.A.) dissolved via acetone in silicone oil 556 (Dow Corning, Midland, MI, U.S.A) as carrier. Treatment of the filter paper was made on the basis of 3.6 mg of oil per cm^2. Whatman filter paper sheets (12 × 15 cm) were impregnated with a mixture of 0.7 mL silicone oil + 1.3 mL carbosulfan acetonic solution. Papers were stored at 4°C and used no more than three times.

Tests were performed with batches of 25 unfed females of *An. gambiae*, 3–5 days old, four replicates per concentration. Mosquitoes were exposed to the insecticide treated papers for 60 min at 27 ± 1°C and 80% RH. After scoring knockdown, all the exposed mosquitoes were transferred to the observation tube of the test kit, supplied with honey solution and held for 24 h before scoring mortality. Batches exposed to untreated papers were used as control.

As the first step, a range of carbosulfan concentrations was tested to determine the minimum dosage to consistently kill 100% of the susceptible strain. Using various lots of impregnated papers and rearing batches of the Kisumu strain, three replicate sets of serial concentrations were tested using 1 h exposure, totalling ~300 females per concentration. Mortality was calculated as the mean ± standard deviation of the three consecutive tests. In keeping with WHO (1998) practice, the discriminating concentration was set as twice the minimum concentration that systematically kills 100% of susceptible mosquitoes. Once determined, this diagnostic concentration was tested on *An. gambiae* females of the two field populations (M'bé and Yaokoffikro) in comparison with the Kisumu strain used as control.

Efficacy of carbosulfan on netting material

Polyester multifilament 100 denier netting (SiamDutch Mosquito Netting Co., Bangkok, Thailand) was treated with carbosulfan 25% microencapsulated formulation, Marshal® CS (FMC, Philadelphia, PA, U.S.A). Each piece of netting 1 m^2 in area was dipped in 35 mL of water with insecticide formulation at appropriate concentration to give the predetermined treatment rate of 200 mg/m^2. Control netting was dipped in water alone. Test groups of five female mosquitoes, 3–5 days old and non-bloodfed, were exposed to the netting for 3 min under plastic bioassay cones (WHO, 1975). Tests were replicated 10 times, totalling ~50 females tested from each strain of *An. gambiae*. After 3 min exposure, mosquitoes were kept at 28°C and 80% RH in plastic cups supplied with 10% honey food on cotton wool. Mortality was recorded 24 h post-exposure.

Acetylcholinesterase assay procedure

Samples of female mosquitoes (not previously exposed to carbosulfan or any other insecticide) from the same batches used for bioassays (see above) were frozen at −80°C for biochemical analysis. Microplate AChE assays followed the protocol of Hemingway (1998) adapted from Ellman *et al.* (1961). Mosquitoes were individually ground in 200 μL of distilled water. For each mosquito, 25 μL of homogenate was placed in two wells of a microplate. 145 μL of phosphate buffer (0.1 M, pH 7.8) containing 1% Triton and 10 μL DTNB (0.01 M) was added to each well. For each mosquito, 25 μL of substrate acetylthiocholine iodide (0.014 M) was added to the first well (uninhibited activity), and 25 μL of substrate acetylthiocholine iodide (0.014 M) plus propoxur (inhibited activity) was added to the second well. The percentage of AChE inhibition by propoxur was calculated for each mosquito as 1 − (activity rate in propoxur inhibited well/activity rate in uninhibited well).

Preliminary tests were made to determine the propoxur concentration providing 80% to 100% inhibition of AChE activity in females of the susceptible Kisumu strain; thus the concentration of 3.10^{-5} M propoxur was adopted. Levels of AChE inhibition by propoxur were then compared for samples of 90–100 females from each of the three *An. gambiae* populations (Kisumu, M'bé, Yaokoffikro).

Results

Determination of discriminating concentration for resistance to carbosulfan

Exposure for 60 min to concentrations of 0.2% or 0.4% carbosulfan consistently caused 100% mortality of *An. gambiae* Kisumu females, whereas some mosquitoes survived for 24 h following exposure to 0.1% or lower concentrations of carbosulfan in silicone oil on filter paper (Table 1). The concentration of 0.4% was therefore adopted (i.e. double the minimum concentration causing 100% mortality) as the discriminating concentration for this study.

Mortality rates of field-collected An. gambiae *exposed to carbosulfan diagnostic dosage*

Using 1 h exposure to carbosulfan 0.4% concentration as the diagnostic dosage for susceptibility/resistance tests, applied to *An. gambiae* females reared from field-collected larvae, in March and April 2000 we observed mortality rates of 62% for those from M'bé and only 29% for those sampled from Yaokoffikro, compared with 100% mortality of susceptible Kisumu females and negligible control mortalities (Table 2).

Efficacy of carbosulfan impregnated netting

Following 3 min exposure of *An. gambiae* females to netting impregnated with carbosulfan 200 mg/m^2, the mortality rates within 24 h were 100% for the Kisumu susceptible reference strain, compared with 88% for those from M'bé and only 12% for those from Yaokoffikro (Table 2).

Biochemical assay

Using 3.10^{-5} M propoxur, the inhibited fraction of AChE was over 80% for all individuals of the susceptible Kisumu strain (Fig. 1) and 75% of individuals showed 90–100% inhibition. Significantly lower mean levels of

Table 1. Mortality rates (%) of the susceptible strain of *An. gambiae* Kisumu, within 24 h following 1 h exposure to the specified concentration of carbosulfan on impregnated papers. n = number of females tested.

	Untreated papers		Carbosulfan concentration (%)									
			0.025		0.05		0.1		0.2		0.4	
Replicate	*n*	Mortality	*n*	Mortality	*n*	Mortality	*n*	Mortality	*n*	Mortality	*n*	Mortality
R1	94	2.1	101	17.8	98	42.8	94	92.5	102	100	99	100
R2	96	3.1	100	25.0	101	55.4	99	99.0	103	100	88	100
R3	100	7.0	101	20.8	97	53.4	97	100	104	100	82	100
Total	290	4.1	302	21.2	296	50.7	290	97.2	309	100	269	100

Table 2. Mortality rates (%) of susceptible strain Kisumu and wild samples of *An. gambiae* from Yaokoffikro and M'bé after 1 h exposure to papers impregnated with diagnostic concentration of carbosulfan (0.4%) compared with 3 min exposure to netting impregnated with carbosulfan (200 mg/m^2). n = number of females tested.

		Kisumu		M'bé		Yaokoffikro	
		n	Mortality	*n*	Mortality	*n*	Mortality
Impregnated paper	Control	103	8.7	78	2.6	97	0
	Carbosulfan 0.4%	99	100	95	62.1	99	29.3
Impregnated netting	Control	57	3.5	51	2.0	51	2.0
	Carbosulfan (200 mg/m^2)	59	100	50	88.0	49	12.2

AChE inhibition were recorded for the samples from M'bé and Yaokoffikro (Mann–Whitney *U*-test, $P < 0.0001$), associated with their partial survival of the diagnostic dosage of carbosulfan. Whereas 51% of the M'bé population had more than 90% inhibition (indicating susceptibility), 19% showed less than 80% reduction of AChE activity (indicating resistance). For the Yaokoffikro sample, only 10% of individuals had >90% inhibition of AChE (susceptibles), whereas 40% had less than 80% inhibition (resistance), with one mosquito showing only 29% inhibition. This reduced inhibition suggests the presence of an altered AChE responsible for carbamate resistance in those proportions of the field populations.

Discussion

The discriminating concentration of 0.4% carbosulfan to detect resistance in *An. gambiae* was determined as double the LC_{100} of 0.2% for the susceptible reference strain (Kisumu) with the standard WHO (1975, 1981) test procedure for measuring dose–response of adult mosquitoes. According to WHO (1998) criteria, mortality rates of 98–100% recorded 24 h post-exposure for 1 h to the discriminating concentration indicate susceptibility, whereas <80% mortality indicates resistance. Thus, using the diagnostic dosage of 1 h exposure to 0.4% carbosulfan, populations of *An. gambiae* from M'bé and Yaokoffikro were found to be partially resistant to carbosulfan with 38% and 71%, respectively, surviving the resistance test. These frequencies of resistance were corroborated by incomplete mortality rates (M'bé 88%, Yaokoffikro 12%, compared with 100% for susceptible Kisumu strain) following 3 min exposure to netting impregnated with carbosulfan 200 mg/m^2. Evidently the resistance frequency is much higher at suburban Yaokoffikro than at rural M'bé and is apparently associated with an altered AChE mechanism.

Our results confirmed carbamate resistance first detected by Elissa *et al.* (1994) in *An. gambiae* populations from Bouaké and surrounding areas. They suggested that it might be a side-effect of crop-spraying or household use of carbamates. Because *An. gambiae* breeds in ricefields

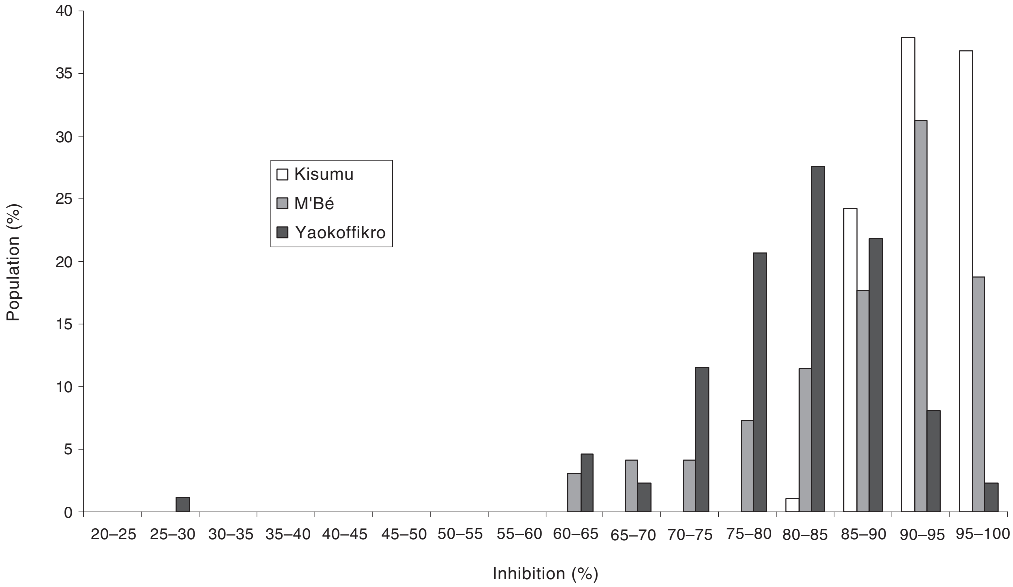

Fig. 1. Frequency distributions of acetylcholinesterase inhibition rates for samples of *An. gambiae* s.s. from Yaokoffikro and M'bé (adult females reared from field-collected larvae) and the susceptible Kisumu reference strain, using 3.10^{-5} M final propoxur concentration (n = 90–98 females tested per sample).

(at early stage of the cultivation) and habitually rests in houses of Bouaké urban and peri-urban areas, it may be exposed to considerable selection pressure by agricultural insecticides and/or domestic aerosols and mosquito coils. Reviewing the impact of agriculture on vector resistance, Mouchet (1988) noted at least 15 malaria vector species for which resistance was directly linked to agricultural treatments. For example, multiple resistance in *An. sacharovi* from southern Turkey has been attributed to heavy usage of organophosphates and carbamates in agriculture (Davidson, 1982), because these insecticides were not used for mosquito control in that area. Similar agrochemical selection scenarios have also been described for *An. nigerrimus* in Sri Lanka (Hemingway *et al*., 1986) and *An. albimanus* in Central America (Georghiou, 1990) and Mexico (Penilla *et al*., 1998). Moreover, pyrethroid resistance in West African *An. gambiae* is thought to have been selected by agricultural treatments, especially those applied to cotton (Chandre *et al*., 2001). In the Bouaké area, large amounts of organophosphate and carbamate insecticides are applied annually to crops for control of pests which are not affected by pyrethroids, in the framework of pest resistance management. This could at least partly explain the development of carbosulfan resistance among *An. gambiae* populations exposed to such agrochemical practices in this part of Ivory Coast. It should be noted that neither carbamates nor organophosphates have so far been used for malaria vector control in this part of West Africa.

Possible cross-resistance between carbosulfan and pyrethroids was recently suggested for *An. stephensi* and for an *An. gambiae* strain originating from Burkina Faso and further selected for permethrin resistance (Asidi & Curtis, 2001). Such cross-resistance was detected in *An. funestus* from South Africa (Brooke *et al*., 2001). Cross-resistance between organophosphates and pyrethroids in mosquito species such as *An. albimanus* (Brogdon & Barber, 1987) and *Culex quinquefasciatus* (Bisset *et al*., 1997, 1998) has been attributed to increased detoxification mechanisms. Overproduced esterases in the aphid *Myzus persicae* confer a broad cross-resistance spectrum including organophosphates, carbamates and pyrethroids (Devonshire & Moores, 1982). Because carbamates and pyrethroids have different modes of action, the limited cross-resistance of the Burkina strain of *An. gambiae* could be caused by metabolic detoxification enhanced by permethrin selection. Although a detoxification mechanism (e.g. esterase, glutathione S-transferase or oxygenase, none of which has been investigated in our samples) may also be involved in the carbosulfan resistance described in this paper, insensitive AChE is expected to be the main mechanism in our field populations of *An. gambiae*, because the M'bé population (M form) is susceptible to most pyrethroids (Chandre *et al*., 1999b; Koffi *et al*., 1999), whereas the kdr mechanism of altered nerve target site has been implicated for pyrethroid-resistance in the Yaokoffikro population (Martinez-Torres *et al*., 1998).

Because of the pyrethroid resistance arising among major malaria vector populations in several parts of Africa (Chandre *et al*., 1999a; Hargreaves *et al*., 2000; Ranson *et al*., 2000; Brooke *et al*., 2001), increasing consideration is being given to the use of alternative insecticides such as carbamates or organophosphates for the treatment of mosquito nets. In view of the strong resistance level usually conferred by AChE insensitivity, the prevalence of the type of resistance that we have detected in *An. gambiae* might be a serious operational obstacle to use of these insecticide classes. Fortunately, however, experimental hut studies at the same localities (M'bé and Yaokoffikro) have shown that despite this AChE-based resistance, bednets treated with carbosulfan were very effective in killing mosquitoes and reducing their blood-feeding (Fanello *et al*., 1999; Kolaczinski *et al*., 2000; Guillet *et al*., 2001). A similarly favourable phenomenon has been observed with pyrethroid resistance induced by the kdr mutation in *An. gambiae* from the same zone. Although kdr induces changes in the behaviour of mosquitoes exposed to pyrethroid-treated substrates (Chandre *et al*., 2000), nets treated with various pyrethroids (bifenthrin, deltamethrin, lambdacyhalothrin, permethrin) were as effective in preventing blood-feeding and reducing malaria morbidity as in areas where mosquitoes were susceptible (Fanello *et al*., 1999; Henry *et al*., 1999; Darriet *et al*., 2000; Dossou-Yovo *et al*., 2000; Kolaczinski *et al*., 2000; Guillet *et al*., 2001). Despite these encouraging preliminary results, the situation regarding carbamate and organophosphate resistance in Afrotropical malaria vectors requires monitoring, especially if such insecticides are to be used for IRS or ITN programmes where cross-resistance issues will be a great challenge to operational resistance management strategies.

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