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TOPICAL APPLICATIONS OF PYRETHROID AND ORGANOPHOSPHATE MIXTURES REVEALED POSITIVE INTERACTIONS AGAINST PYRETHROID-RESISTANT *ANOPHELES GAMBIAE*

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ABSTRACT. The efficacy of a binary mixture of bifenthrin (pyrethroid) and chlorpyrifos-methyl (organophosphate) has been tested against susceptible and pyrethroid-resistant strains of *Anopheles gambiae* Giles, the major malaria vector in sub-Saharan Africa. Dose–mortality regression lines were determined for each insecticide alone and in mixtures by topical applications on adult female mosquitoes. A combination index (CI) was used to quantify the interactions occurring between the pyrethroid and organophosphate insecticides. The results revealed synergism at high doses against both susceptible ($0.7 > CI > 0.3$) and pyrethroid-resistant ($0.9 > CI > 0.7$) *An. gambiae.* These results suggest that insecticide mixtures may be an alternative strategy for vector control, especially in areas where mosquitoes are resistant to pyrethroids.

KEY WORDS Bifenthrin, chlorpyrifos-methyl, synergism, topical applications, *Anopheles gambiae*

INTRODUCTION

Pyrethroid insecticides are currently recommended for mosquito net impregnation in malaria control because of their low toxicity for humans, rapidity of action (knock-down effect), and repellent and irritating properties against adult mosquitoes (Zaim et al. 2000). However, the appearance of pyrethroid resistance in some *Anopheles* species constitutes a threat for the efficacy of treatments, especially in the case of *Anopheles gambiae* Giles, the major vector in sub-Saharan Africa (Chandre et al. 1999, Ranson et al. 2000). To limit the spread of resistance and to preserve the efficacy of impregnated mosquito nets, pyrethroids could be mixed with different chemicals having distinct modes of action, such as carbamates or organophosphates (OPs). Besides the fact that "multiple attacks" can be an efficient tool for the management of insecticide resistance in insects (Denholm and Rowland 1992), insecticide combination may also improve mosquito control when synergism occurs between the insecticides in a mixture. In such a case, the efficacy of a mixture is significantly superior to the combined effects of both insecticides, allowing a reduction of the total insecticide amount. Synergism has already been detected in several insects of agricultural or veterinarian importance (Hughes and Trevethan 1979, Koziol and Witkowski 1982, Ozaki et al. 1984, Asher et al. 1986, Campanhola and Plapp 1989, Gunning et al. 1999). For example, in *Helicoverpa armigera,* a main pest of cotton, mixtures of pyrethroids and OPs have shown important synergistic effects and prevented the spread of insecticide resistance in West Africa for more than 20 years (Martin et al. 2000).

Bifenthrin is a pyrethroid insecticide commonly used in public health for mosquito net impregnation according to the good results observed in the laboratory and in the field (Hougard et al. 2002). Synergism has been detected between bifenthrin and carbosulfan, a carbamate insecticide (Corbel et al. 2002), but, because of its transformation (loss of a group dibutylamino-thio) into a more toxic compound (WHO 1986), the use of carbofuran tends to be abandoned. Recently, chlorpyrifos-methyl, an OP insecticide much less toxic for humans and mammals, has been evaluated in the perspective of an association with bifenthrin. Some studies carried out in experimental huts in the Ivory Coast showed that chlorpyrifos-methyl was very effective against *An. gambiae* strongly resistant to pyrethroids on impregnated nets (N'Guessan et al. 2003). In the laboratory, a striking synergism also was observed between bifenthrin and chlorpyrifos-methyl in susceptible *An. gambiae* by the cone test method (Darriet et al. 2003), whereas no synergistic effect was detected in pyrethroid-resistant mosquitoes. Nevertheless, the cone test method is not appropriate to estimate the intrinsic toxicity of a mixture. Conversely, topical applications insure a permanent contact between insecticides and biological material, and allow a good appreciation of intrinsic toxicity. The purpose of this study was to determine by topical applications the efficacy of bifenthrin and chlorpyrifos-methyl mixtures against susceptible and pyrethroid-resistant *An. gambiae.*

MATERIALS AND METHODS

Mosquitoes: Two strains of *An. gambiae* were used during the study. The susceptible strain Kisumu, originating from Kenya, is free of any detectable insecticide resistance mechanism. The pyrethroid-resistant strain VKPR originated from the

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Kou Valley in Burkina Faso. This strain, which was already resistant to pyrethroids when it was collected in the field, was exposed to constant permethrin selections in the laboratory until becoming homozygous for the *Kdr* mutation (Darriet et al. 1997).

Insecticides: Bioassays were carried out by using a technical grade of bifenthrin (93.5%) provided by FMC Corp. (Princeton, NJ). Bifenthrin ((2-methyl[1,1′-biphenyl]-3-yl) methyl 3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethyl-cyclopropanecarboxylate) is a non-α-cyano pyrethroid that is particularly effective against mosquitoes but not toxic for mammals at the recommended dosage (toxicity for the rat: median lethal dose [LD_{50}] for ingestion = 54.5 mg/kg and LD_{50} for contact > 2,000 mg/kg; Tomlin 2000). Bifenthrin acts by contact or ingestion and is not persistent in the environment. As is true for all pyrethroids, bifenthrin is a neurotoxic compound that acts on the neuronal cell membrane. Its targeting on the voltage-gated sodium channels induces a strong modification of ionic currents circulating through the membrane and a disruption of the propagation of nerve impulses (Salgado et al. 1983).

The technical grade of chlorpyrifos-methyl (97.5%) used in our study was provided by Dow AgroScience (Norfolk, United Kingdom). Chlorpyrifos-methyl (0,0-dimethyl 0-3,5,6-trichloro-2-pyridyl phosphorothioate) is an OP compound that presents, comparatively with other anti-cholinesterasic insecticides, no toxicity for mammals at the recommended dosage (toxicity for the rat: LD_{50} for ingestion > 3,000 mg/kg and LD_{50} for contact > 2,000 mg/kg; Tomlin 2000). Chlorpyrifos-methyl acts by contact, ingestion, or inhalation. After its transformation into the oxon-form by mixed function oxidase in vivo (Champ 1985), chlorpyrifos-methyl inhibits acetylcholinesterase activity, leading to a blockage of synaptic transmission (Eldefrawi 1976).

Topical applications: Topical applications allow a good estimation of the intrinsic toxicity of an insecticide by excluding all other effects linked to the mosquito behavior, especially when exposed to irritating and repellent insecticides. Bifenthrin and chlorpyrifos-methyl were 1st tested separately against both susceptible and pyrethroid-resistant strains of *An. gambiae,* then in a binary mixture at a constant ratio determined by the ratio of the LD_{50}s. Five to 8 doses were used for each test to provide a range of mortality from 0 to 100%. Each test was conducted in triplicate to test different generations of mosquitoes. Two- to 5-day-old nonbloodfed females were 1st anesthetized by extended contact with carbon dioxide, then deposited on a refrigerating plate at 4°C by samples of 25 individuals. The refrigerating plate allows maintaining anesthesia during manipulation. A serial of 2 samples of 25 females (n = 50) was used for each dose of insecticide. A volume of 0.1 μl of insecticide solution at the required concentration, prepared previously by dilution in acetone, was next deposited on the upper part of the pronotum of females by using a microcapillary tube. Two samples of 25 females on which a volume of 0.1 μl of pure acetone was deposited served as control. After each test, females were transferred into plastic cups provided with honey-soaked cotton (honey diluted at 10% in osmotic water). Cups containing females were maintained at standard temperature (27°C) and humidity (80%) conditions. Mortality rates were recorded 24 h after the tests. Data were analyzed according to the method of Finney (1971) by using Probit® software (Raymond et al. 1997), which provides an estimation of the LD_{50} and LD_{95} with their 95% confidence intervals. Data (LD_{50} and LD_{95}) initially expressed in nanograms of insecticide per female were next converted into nanograms of insecticide per milligrams of female to compare different mosquito generations and strains. Results were corrected by the formula of Abbott (1925) in the case of a control mortality superior to 5%.

Analysis of insecticide interactions: The existence of synergism between bifenthrin and chlorpyrifos-methyl was determined by the calculation of a combination index (CI) according to the method of Chou and Talalay (1984) by using CalcuSyn® software (Chou and Hayball 1996). This isobologram-based method is particularly well adapted to analysis of multiple drug effects (Tallarida 2002) and is more powerful than the cotoxicity-coefficient method (Sun and Johnson 1960) used in previous work for detecting synergism in pests of agricultural importance. The CI gives a quantitative measure of the interactions (synergism, antagonism, and summation) occurring between insecticides on a given strain. For 2 insecticides with independent modes of action (which is the case for bifenthrin and chlorpyrifos-methyl), the CI is calculated for a mortality x by the following formula:

$$CI_x = \frac{LD_x^{bi(m)}}{LD_x^{bi}} + \frac{LD_x^{cm(m)}}{LD_x^{cm}} + \frac{LD_x^{bi(m)} \times LD_x^{cm(m)}}{LD_x^{bi} \times LD_x^{cm}}$$

where $LD_x^{bi(m)}$ and $LD_x^{cm(m)}$ designate the doses of bifenthrin and chlorpyrifos-methyl, respectively, inducing a mortality x in mixture and LD_x^{bi} and LD_x^{cm} designate the doses of bifenthrin and chlorpyrifos-methyl inducing the same mortality x when used alone. A CI = 1, < 1, and > 1 indicates an additive effect, a synergistic effect, and an antagonistic effect, respectively.

RESULTS

Susceptible strain Kisumu

For each test, the mortality rate was less than 15% in the control batches. The (log) dose–(probit) mortality regression lines of bifenthrin and chlorpyrifos, alone and in mixture, are shown in Fig. 1. The LD_{50} and LD_{95}, respectively, were 0.092

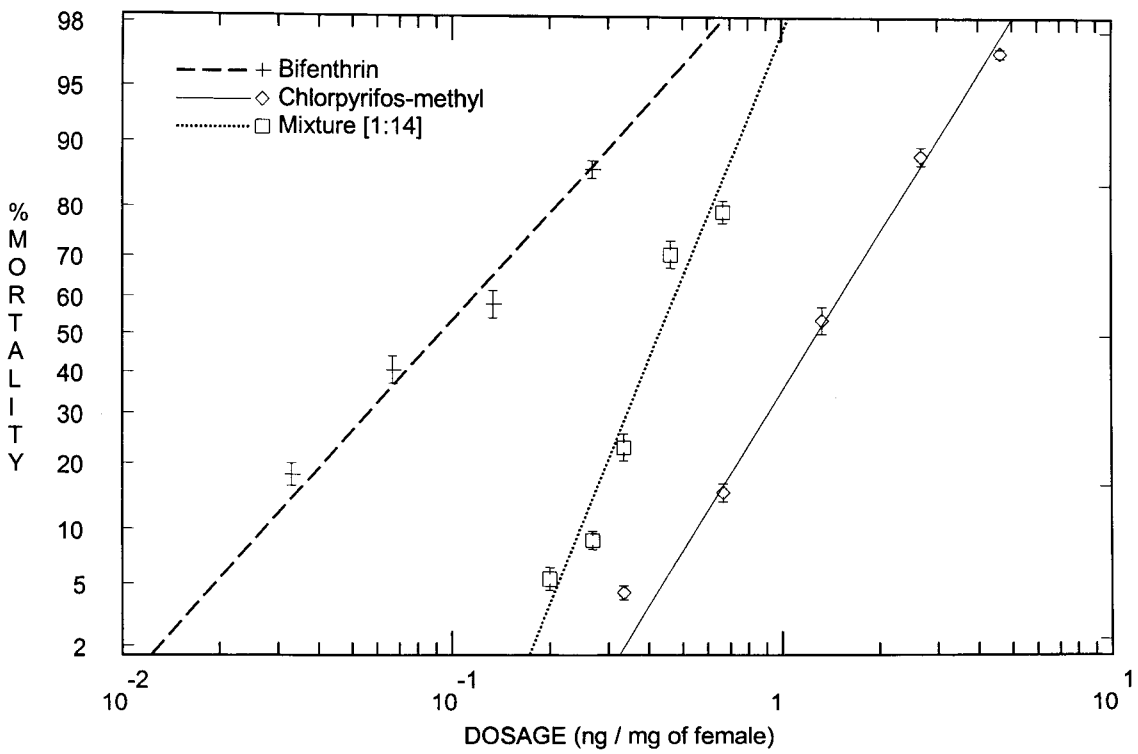

Fig. 1. Dose–mortality regression lines of bifenthrin and chlopyrifos-methyl, alone and in combination, on the susceptible strain of *Anopheles gambiae* (Kisumu).

(0.081–0.103) and 0.44 (0.36–0.56) ng/mg for bifenthrin and 1.27 (1.14–1.41) and 3.74 (3.23–4.55) ng/mg for chlorpyrifos-methyl. A binary mixture of bifenthrin and chlorpyrifos-methyl was realized at the ratio 1:14 by considering the values of the LD_{50} obtained with this strain. The LD_{50} and LD_{95}, respectively, of the mixture were 0.43 (0.37–0.48) and 0.87 (0.65–1.17) ng/mg. Table 1 gives an estimation of the CI of the mixture for increasing doses and mortality levels. The CI is close to 1 with low mortality, then decreases to 0.4 at the LD_{95}. According to Chou and Hayball (1996), such results indicate a striking synergism between bifenthrin and chlorpyrifos-methyl at high doses ($0.7 > CI > 0.3$).

Pyrethroid-resistant strain VKPR

For each test, the mortality rate was less than 6% in the control batches. The (log) dose–(probit) mor-

Table 1. Combination index (CI) of the bifenthrin and chlorpyrifos-methyl mixture on the susceptible strain (Kisumu) and the pyrethroid-resistant strain (VKPR) of *Anopheles gambiae* (range shows 95% confidence limits).

	Kisumu strain		VKPR strain	
% mortality (lethal dose level)	Dose of 1:14 insecticide mixture (ng/mg of female)	CI	Dose of 1:2 insecticide mixture (ng/mg of female)	CI
10	0.25	1.03 (0.85–1.22)	0.79	1.01 (0.91–1.11)
20	0.31	0.89 (0.76–1.02)	0.96	0.94 (0.86–1.01)
30	0.35	0.81 (0.71–0.91)	1.10	0.89 (0.83–0.96)
40	0.40	0.74 (0.66–0.83)	1.23	0.86 (0.80–0.92)
50	0.44	0.69 (0.61–0.77)	1.36	0.83 (0.78–0.88)
60	0.49	0.64 (0.57–0.71)	1.50	0.81 (0.76–0.86)
70	0.55	0.59 (0.52–0.66)	1.67	0.78 (0.73–0.83)
80	0.63	0.54 (0.47–0.61)	1.91	0.76 (0.71–0.81)
90	0.78	0.47 (0.39–0.54)	2.33	0.72 (0.66–0.77)
95	0.96	0.41 (0.33–0.48)	2.80	0.69 (0.63–0.75)
99	1.48	0.31 (0.23–0.38)	4.20	0.64 (0.56–0.72)

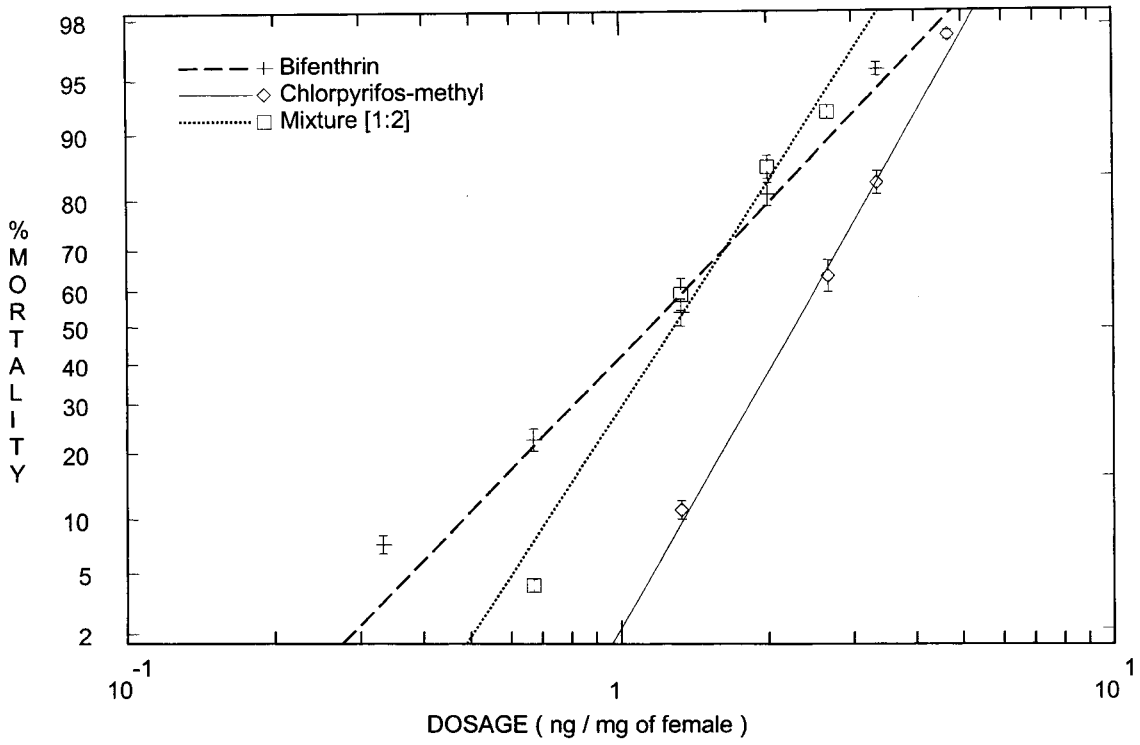

Fig. 2. Dose–mortality regression lines of bifenthrin and chlorpyrifos-methyl, alone and in combination, on the pyrethroid-resistant strain of *Anopheles gambiae* (VKPR).

tality regression lines of bifenthrin and chlorpyrifos-methyl, alone and in mixture, are shown in Fig. 2. The LD_{50} and LD_{95}, respectively, were 1.15 (1.05–1.25) and 3.52 (3.05–4.24) ng/mg for bifenthrin and 2.25 (2.11–2.40) and 4.41 (4.06–4.89) ng/mg for chlorpyrifos-methyl. A high level of bifenthrin resistance was noted with the VKPR strain in comparison with the Kisumu strain (about 12 times at the LD_{50}). A binary mixture of bifenthrin and chlorpyrifos-methyl was realized at the ratio 1:2 by considering the values of the LD_{50} obtained with this strain. The LD_{50} and LD_{95}, respectively, of the mixture were 1.29 (1.18–1.38) and 2.74 (2.47–3.13) ng/mg. The CI values were close to 1 with low mortality, then decreased to 0.7 at the LD_{95} (Table 1). According to Chou and Hayball (1996), a moderate synergism was noted between bifenthrin and chlorpyrifos-methyl at high doses ($0.9 > CI > 0.7$).

DISCUSSION

The decrease of bifenthrin efficacy on the pyrethroid-resistant strain VKPR, compared to the susceptible strain Kisumu, indicates the presence of a specific resistance mechanism to pyrethroids. The synergistic effect detected on the susceptible strain between bifenthrin and chlorpyrifos-methyl at high doses confirms the results of Darriet et al. (2003) with the cone test method. Conversely, the moderate synergism observed with the resistant strain was not previously observed by Darriet et al. (2003), with only a simple additive effect being detected. These differences could be related to the cone test method used by these authors, which did not allow permanent contact between the mosquito and the insecticides. In this case, the excito-repellent effect of combined bifenthrin and chlorpyrifos-methyl–impregnated nets may limit the time of contact of the mosquito with the treated surface, hiding the synergistic effect observed by topical applications. Therefore, mosquito behavior seems to be an important factor able to modify the nature of interactions occurring between insecticides.

Higher levels of synergism between pyrethroids and OPs were previously detected in a pyrethroid-resistant strain of *H. armigera* from West Africa (Martin et al. 2003). One explanation for the higher toxicity of these insecticide mixtures in *H. armigera* than in *An. gambiae* is that mono-oxygenases, overproduced in the resistant strain of *H. armigera,* may enhance the activation of thiophosphates to the oxon-form (Champ 1985). Consequently, binding of OPs to mono-oxygenases 1st activates the molecule and 2nd may prevent binding and degradation of pyrethroids by enzyme–substrate competition (Martin et al. 2003). Biochemical assays carried out in our laboratory did not reveal significant differences in oxidase or esterase activity between the

susceptible and the pyrethroid-resistant strains (unpublished data), and it is likely that such results explain the lower toxicity of pyrethroid–OP mixtures in these mosquitoes.

Nevertheless, the existence of synergism between OPs and pyrethroids in pyrethroid-resistant *Anopheles* suggests that use of insecticide mixtures may be a promising strategy for the management of insecticide resistance in malaria vectors. In West Africa, the association of carbamate or OP insecticides with pyrethroids (in mosaics or in mixtures) allowed certain limitations encountered during treatments to be overcome (Guillet et al. 2001, Hougard et al. 2003). Moreover, a recent experimental hut study carried out in the Ivory Coast showed that pyrethroid and carbamate "two-in-one"–treated nets did not select for an insensitive acetylcholinesterase, unlike mosquito nets treated with carbamate alone (Corbel et al. 2003). Field studies are now being undertaken in Benin and Cameroon to assess the impact of different insecticide combinations in terms of personal protection and management of insecticide resistance in mosquitoes. Such findings may or may not allow consideration of an operational application of insecticide mixtures within the framework of malaria control programs in Africa.

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