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COMBINATION OF A NON-PYRETHROID INSECTICIDE AND A REPELLENT: A NEW APPROACH FOR CONTROLLING KNOCKDOWN-RESISTANT MOSQUITOES

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Abstract. Although pyrethroid-treated materials are a promising tool for the prevention and the control of dengue in the tropics, the development of pyrethroid resistance in the main mosquito vector (*Aedes aegypti*) may negate their use for personal and/or community protection. In that context, the efficacy of a mixture of a repellent (N,N-diethyl toluamide [DEET]) and a non-pyrethroid insecticide (propoxur) was investigated under laboratory conditions against both pyrethroid-susceptible and pyrethroid-resistant mosquitoes with the knockdown resistance (*kdr*) mutation. The results showed that a combination of propoxur and DEET induced a knockdown effect and mortality as high as deltamethrin (a standard pyrethroid) against the susceptible strain, and significantly higher efficacy against the pyrethroid-resistant strain. This could be explained mainly by the existence of a strong synergistic interaction between DEET and propoxur in mosquitoes. This study constitutes a first step towards an alternative strategy for improving mosquito control in areas with pyrethroid resistance.

INTRODUCTION

Pyrethroid insecticides represent important weapons against pests of both economic and medical importance. They share many properties with dichloro-diphenyl-trichloroethane (DDT), including a knock down and killing effect, resulting from action against the sodium channels of the peripheral and central nervous systems.[1] These products show remarkably high efficacy against insects but relatively low mammalian toxicity and low persistence in the environment.[2] Since the 1980s, pyrethroids have been widely used as residual sprays on house walls or on mosquito nets to control insects in the domestic environment.[3] Among these anti-vector measures, pyrethroid-treated nets have emerged in recent years as the most promising tool for reducing malaria mortality and morbidity, especially in children less than five years of age in disease-endemic areas in Africa.[4] Insecticide-treated materials (ITMs), which include plastic sheeting, curtains, hammocks, textiles, combat uniforms, or lids of water tanks, have increased importance in personal and community protection against pests and vectors that transmit malaria, typhus, or dengue.[5]

Unfortunately, the emergence of pyrethroid resistance in most mosquito species of public health importance represents a threat for sustainable vector control programs implemented in the tropics. The difficulties come from the fact that resistance to any pyrethroid generally confers cross-resistance to all others, thus limiting the number of effective alternatives suitable for vector control. The knockdown resistance (*kdr*) gene, which confers cross- resistance to DDT and pyrethroids, is now widely prevalent in mosquitoes of public health importance.[6–9] In *Anopheles gambiae* and *Culex quinquefasciatus,* the *kdr* mutation is conferred by a single amino acid change (one or both of the two known sites) in the axonal sodium channel insecticide-binding site, whereas knockdown resistance emerged from four amino acid substitutions in *Aedes aegypti*.[9,10]

The impact of the *kdr* mutation on the efficacy of ITMs has been the subject of numerous studies in Africa the past decade.[11–13] It has been generally observed that the *kdr* mutation was not sufficient to render pyrethroid-treated nets ineffective, which would result in a relatively high efficacy of impregnated bed nets in killing resistant mosquitoes, but not repelling them.[14,15] Such a finding was explained by the fact that resistant mosquitoes, which were less irritated by the insecticide, remained longer on the nets before finally receiving sufficient lethal doses by tarsal contact.[16] This low irritancy may represent a serious risk against personal protection. A recent experimental hut study carried out in Benin has shown that the proportion of blood-fed females exposed to permethrin-impregnated nets was significantly higher in resistant (R) mosquitoes (both in heterozygous [RS] and homozygous [RR] individuals) than in susceptible (S) ones.[13] Such findings, which need be confirmed with different insecticides and impregnated substrates, already strengthen the need for alternative chemicals and/or vector control strategies to maintain an effective barrier against pyrethroid-resistant mosquitoes. This is even more relevant for insecticide-treated fabrics or clothing that should maintain a fast-acting effect against resistant insects.

Since the number of new insecticides is drastically dwindling, an alternative strategy to maintain the global effectiveness of ITMs in areas of pyrethroid resistance may be the replacement of pyrethroids by other insecticides such as carbamates or organophosphates.[17] Although carbamate- or organophosphate-impregnated materials have shown efficacy against pyrethroid-resistant mosquitoes, their low excito-repellency allows mosquitoes to remain a sufficient time on the impregnated surface to take a blood meal.[11,18–20] Such findings may negate their use in textile or fabric impregnations.

To overcome such limitations for personal protection, we propose an alternative concept to maintain the effectiveness of impregnated materials. This consists of associating a synthetic repellent with a non-pyrethroid insecticide to mimic similar or higher features of pyrethroids, especially irritancy, against pyrethroid-resistant mosquitoes. In this study, DEET, which is a classic synthetic repellent used since World War II for personal protection, was combined with propoxur, a carbamate insecticide, which has high insecticidal activity but low irritant properties against insects.[21,22] The objectives of this study were to compare the intrinsic efficacy of this non-pyrethroid DEET-propoxur mixture with a reference pyrethroid insecticide (deltamethrin), and to search for synergistic

interactions between these two compounds. Susceptible and *kdr*-resistant strains of *Ae. aegypti*, an important vector for arboviruses, were used for this study.

MATERIALS AND METHODS

Mosquitoes. Two laboratory strains of *Ae. aegypti* were used in this study. The susceptible Bora strain originated in French Polynesia and has no detectable insecticide resistance mechanism. The pyrethroid-resistant strain LHP originated in Vietnam and was already strongly resistant to permethrin when it was collected in the field. This strain has been maintained under constant permethrin selection at each generation and is now homozygous for the *kdr* gene (mutation L75W).[9] The resistant and susceptible strains were evaluated every three months for resistance status and the R genotype.

Insecticides and repellent. Bioassays were made with technical grade propoxur, DEET, and deltamethrin, the latter of which served as a reference for pyrethroids. The active ingredient of deltamethrin ((S)-alpha-cyano-3-phenoxybenzyl(1R,3R)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate) had a purity of 91.5% and contained at least 98% of the cis isomer. Propoxur (2-isopropoxyphenyl methylcarbamate) had a purity of 98.4%. DEET had a purity of 97% and contained a minimum of 95% of the meta isomer, the most effective molecule of DEET.

Substrates and treatment. Tarsal contact tests were conducted using filter paper treated with the technical grade of each insecticide and repellent. Filter papers were treated following a World Health Organization (WHO) protocol using acetone solutions of insecticide and silicone oil as the carrier.[23] The impregnation was done by dripping evenly onto the paper 2 mL of technical grade substance dissolved in acetone and silicone oil. The paper was dried for 12 hours before the test.

Tarsal contact with treated filter paper. The knockdown effect and mortality resulting from tarsal contact with treated filter paper were measured using WHO test kits against adult mosquitoes.[23] Concentrations were expressed in weight per weight percentage of active ingredient in silicone oil. Batches of 25 non–blood-fed female mosquitoes (2–5 days old) were introduced into holding tubes and maintained for 60 minutes at 27 ± 2°C at a relative humidity of 80 ± 10%. They were then transferred into exposure tubes and placed vertically for 60 minutes under subdued light. Since pyrethroids are fast-acting insecticides, the number of knocked-down mosquitoes at the bottom of the tubes was recorded every 10 minutes. Mortality was recorded 24 hours after exposure and corrected by the formula of Abbott[24] if necessary. Data were analyzed by the log-probit method of Finney[25] using Probit software.[26] Times after which 50% and 95% of mosquitoes were knocked down (KDT_{50} and KDT_{95}, respectively) and their 95% confidence intervals were estimated with Probit software. Each solution was tested four times and each test was repeated three times with different insect batches to take into account inter-test variability.

Irritability tests. Non–blood-fed female mosquitoes (2–5 days old) were individually introduced into plastic cones fitted with treated filter paper. After exposure 60 seconds, the time elapsed between the first landing and the next take off of the mosquito was recorded as the time for first take off.[27] Mosquitoes that did not take off at least once during a period of 256 seconds were discarded. For each test, 50 mosquitoes were tested individually. A simple program using the internal clock of a laptop computer has been developed in our laboratory in France to conduct this test and analyze the data. Mosquitoes were grouped by classes of first take off time and cumulative frequencies were used to calculate the time for which 50% and 95% of the mosquitoes take off (FT_{50} and FT_{95}, respectively) using Probit software. Fairly constant subdued lighting and air temperature (28 ± 2°C) were maintained during the test according to Hodjati and Curtis.[28]

Experimental design. Lethal concentrations of propoxur that induced 30% mortality (LC_{30}) were first determined for each strain of *Ae. aegypti* to allow better detection of synergistic interactions with DEET. The maximum irritant concentration of DEET was then determined on the susceptible strain. Therefore, the efficacy of DEET-propoxur mixtures, in terms of mortality, knockdown effect and irritancy, was compared with deltamethrin at the LC_{100} (chosen as a pyrethroid reference concentration). To detect any synergism between DEET and propoxur, we compared the results observed with the DEET-propoxur mixture with those theoretically expected in the absence of any interaction (uncorrelated joint action) between the two compounds.[29] The expected mortality was calculated by multiplying the survival rates of each compound tested separately and subtracting the result from 100%.

In the same way, the expected KDT and FT for the mixture was calculated by multiplying the percentage of mosquitoes that were not knocked down (air-landed mosquitoes) at each time and subtracting the results from 100%. Synergism occurred when the observed results were significantly higher than the expected one. Conversely, when the observed results were significantly lower than the expected one, there was antagonism.

Statistical analysis. Mortality rates for DEET and propoxur alone and combined were compared with Yates' corrected chi-square test at 0.05% level of significance. The differences between two KDT_{50-95} and two FT_{50-95} values were considered significant if their 95% confidence intervals did not overlap.

RESULTS

Preliminary data. The maximum irritant concentration of 1% DEET induced no mortality and no knockdown effect (Tables 1 and 2). The LC_{30}s of propoxur were 0.02% and 0.01% for the Bora and LHP strains, respectively. At this concentration, propoxur induced no knockdown effect and only low irritancy against both mosquito strains (Tables 2 and 3). The LC_{100} of deltamethrin was estimated to be 0.014% for the Bora strain. The DEET-propoxur mixtures tested on mosquitoes were 0.02% propoxur plus 1% DEET for the Bora strain and 0.01% propoxur plus 1% DEET for the LHP strain. All results (mortality, knockdown effect, and irritancy) obtained with each insecticide and repellent, alone or in mixture, are summarized in Tables 1, 2 and 3.

Comparative efficacy between deltamethrin and the DEET-propoxur mixture. The mortality rate of the Bora strain (100%) when tested with deltamethrin did not differ significantly from that with DEET-propoxur mixture (96%) ($\chi^2 = 2.30$, degrees of freedom [df] = 1, $P = 0.13$) (Table 1), but the knockdown effect was significantly higher than with

TABLE 1

Mortality at 24 hours of susceptible (Bora) and kdr-resistant (LHP) *Aedes aegypti* mosquitoes exposed for one hour to papers impregnated with deltamethrin, propoxur, and DEET, separate or combined*

		Insecticidal effect			
		Dose, %	No.	% mortality	χ^2 (*P*)
Ae. aegypti (Bora)	Control	–	300	No effect	–
	Propoxur LC_{30}	0.02	300	34.0	–
	DEET	1	300	No effect	–
	Mixture (observed)	0.02 + 1	300	96.0	81.78† (< 0.0001)
	Mixture (expected)	–	–	34.0	–
	Deltaméthrine LC_{100}	0.014	300	100.0	2.30‡ (0.1297)
Ae. aegypti (LHP)	Control	–	300	No effect	–
	Propoxur LC_{30}	0.01	275	34.5	–
	DEET	1	300	No effect	–
	Mixture (observed)	0.01 + 1	300	94.6	76.51† (< 0.0001)
	Mixture (expected)	–	–	34.5	–
	Deltamethrin LC^{100}	0.014	300	9.3	144.73‡ (< 0.00001)

* Kdr = knock down resistance; DEET = N, N-diethyl-toluamide; LC = lethal concentration.
† Chi square and its propability between expected and observed mortalities.
‡ Chi square and its propability between deltamethrin and mixture-induced mortalities.

the DEET-propoxur mixture (both at the KDT_{50} and KDT_{95} levels) (Table 2). Irritancy of deltamethrin was greater than that of the DEET-propoxur mixture at the FT_{50} level, but not significantly different at the FT_{95} level (Table 3).

The mortality rate of the LHP strain when tested with deltamethrin was fairly low (9%), but remained high (94%) with the DEET-propoxur mixture (χ^2 = 144.73, df = 1, P < 0.0001) (Table 1). In addition, the knockdown effect of deltamethrin was not observed in the LHP strain, but was high in the Bora strain (Table 2). The irritant properties of deltamethrin and the DEET-propoxur mixture did not differ significantly from each other at both the FT_{50} and FT_{95} levels (confidence intervals overlapped) (Table 3).

Interaction between DEET and propoxur. The DEET-propoxur mixture showed a significantly higher mortality rate (96%) in the Bora strain than expected (34%) when testing the hypothesis of an uncorrelated joint action of the two compounds (χ^2 = 81.78; df = 1, P < 0.0001) (Table 1). Such results indicate a strong synergism between propoxur and DEET. Moreover, the existence of a knockdown effect with the DEET-propoxur mixture also provided evidence for a striking synergism between these two compounds since DEET and propoxur tested separately did not induce any knockdown effect (Table 2). However, irritancy of the DEET-propoxur mixture was not significantly different from that theoretically expected, indicating an additive effect for this parameter (Table 3).

A synergistic interaction was also observed between DEET and propoxur in the LHP strain for both the mortality rate (94.5% versus 34.5%; χ^2 = 76.51, df = 1, P < 0.0001) (Table 1) and the knockdown effect (KDT_{50} = 41.5 minutes) (Table 2). Conversely, irritancy of the DEET-propoxur mixture was significantly lower than that theoretically expected, indicating an antagonistic interaction between these two compounds (confidence intervals did not overlap) (Table 3).

DISCUSSION

The combination of propoxur (at the LC_{30}) and DEET (at a sub-lethal dose) showed irritant properties as high as del-

TABLE 2

Knock-down times (KDt_{50} and KDt_{95}) of susceptible (Bora) and kdr-resistant (LHP) *Aedes aegypti* mosquitoes exposed for one hour to papers impregnated with deltamethrin, propoxur, and DEET, separate or combined*

		Knock-down effect					
		Dose, %	No.	Kdt_{50} (min)	95% CI	Kdt_{95} (min)	95% CI
Ae. aegypti (Bora)	Control	–	300	No effect	–	No effect	–
	Propoxur LC_{30}	0.02	300	No effect	–	No effect	–
	DEET	1	300	No effect	–	No effect	–
	Mixture (observed)	0.02 + 1	300	36.6	35.7–37.4	54.8	52.9–57.0
	Mixture (expected)	–	–	–	–	–	–
	Deltamethrin LC_{100}	0.014	300	23.8	21.6–26.2	38.3	32.5–45.1
Ae. aegypti (LHP)	Control	–	300	No effect	–	No effect	–
	Propoxur LC_{30}	0.01	300	No effect	–	No effect	–
	DEET	1	300	No effect	–	No effect	–
	Mixture (observed)	0.01 + 1	300	41.5	40.6–42.4	60.1	58.1–62.5
	Mixture (expected)	–	–	–	–	–	–
	Deltamethrin LC_{100}	0.014	300	No effect	–	No effect	–

* KDt_{50} = 50% knock-down time; KDt_{90} = 90% knock-down time; DEET = N, N-diethyl-toluamide; CI = confidence interval; LC = lethal concentration.

TABLE 3

Time of first take-off (FT_{50} and FT_{95}) of susceptible (Bora) and kdr-resistant (LHP) *Aedes aegypti* mosquitoes exposed to papers impregnated with deltamethrin, propoxur, and DEET, separate or combined.*

		Irritant effect					
		Dose, %	No.	FT_{50} (sec)	95% CI	FT_{95} (sec)	95% CI
Ae. aegypti (Bora)	Control	–	163	251	188.8–363.9	6,256.1	3,122.2–16,269.7
	Propoxur LC_{30}	0.02	150	133.9	108.8–172.3	2,508.1	1,486.7–4,993.3
	DEET	1	150	24.2	21.9–26.8	128.2	107.6–157.4
	Mixture (observed)	0.02 + 1	150	21.5	18.7–24.6	88.9	69.7–114.7
	Mixture (expected)	–	–	19.2	17.0–21.7	96.4	78.7–123.3
	Deltamethrin LC_{100}	0.014	150	15.4	13.9–17.1	86.9	72.9–106.8
A. aegypti (LHP)	Control	–	150	81.2	63.8–104.0	739.3	379.0–1,501.8
	Propoxur LC_{30}	0.01	153	62.5	54.0–73.6	856.9	607.3–1,308.0
	DEET	1	150	23.6	19.3–28.9	172.1	116.1–257.8
	Mixture (observed)	0.01 + 1	152	23.6	21.2–26.2	159.3	133.2–195.8
	Mixture (expected)	–	–	15.8	14.0–17.9	101.6	81.1–133.2
	Deltamethrin LC_{100}	0.014	152	24.1	21.5–27.0	183.2	148.0–236.1

* FT_{50} = 50% take-off time; FT_{95} = 95% take-off time; DEET = N, N-diethyl-toluamide; CI = confidence interval; LC = lethal concentration.

tamethrin (at the LC_{100}) against both Bora and LHP mosquito strains, although irritancy was slightly lower against LHP mosquitoes. In the LHP strain, the decrease in irritancy with deltamethrin might be due to the presence of the *kdr* mutation, although it probably results from a more complex phenomenon for the DEET-propoxur combination. It is likely that the massive knockdown effect observed with the DEET-propoxur mixture disrupted the time for first take off of mosquitoes, since knocked-down mosquitoes were unable to fly. This phenomenon probably explained the antagonistic interaction in irritancy observed between propoxur and DEET in the LHP strain.

A strong synergism was reported between an insecticide and a repellent. The level of synergy detected with this nonpyrethroid DEET-propoxur mixture was higher than those previously observed between pyrethroids and carbamates (e.g., propoxur) or organophosphates against *Anopheles* mosquitoes.[30,31] The most intriguing result was the manifestation of a knockdown effect induced by compounds other than pyrethroids, especially against LHP mosquitoes. Such findings are of practical importance since the knockdown effect is an essential characteristic in personal protection against mosquito bites.

The physiologic mechanisms responsible for synergistic interactions between DEET and propoxur remain unclear. The mode of action of carbamates is well known (inhibition of acetylcholinesterase), but that of DEET has not been elucidated.[32] Davis proposed several assumptions to explain the effect of DEET in insects[33]: 1) inhibition of an attraction signal; 2) inversion of attraction signal perception for an irritant message; 3) activation of a receptor system that could mediate a competing or inappropriate behavior pattern; 4) activation of a noxious odor receptor; and 5) activation of different receptors that could mediate various behavior patterns, indicating that repellents are interfering with the sensory information system.

In this study, we used filter papers without any vertebrate host. DEET acts not only by inducing a disruption of an attraction signal, but also by generating a physiologic perturbation in mosquitoes. Indeed, preliminary bioassays have shown that a DEET concentration range of 5–10% induced mortality and a knockdown effect as great as pyrethroids (Pennetier C, unpublished data). In addition, electrophysiologic tests with dorsal unpaired median neurons of American cockroach (*Periplaneta americana*) showed that DEET induced a strong neurotoxic effect (Lapied B, unpublished data). As previously observed by Corbel and others[34] with insecticide combinations, synergism between DEET and propoxur may be the result of a general physiologic disruption involving different target sites in the central nervous system. Further investigations are now in progress to determine precisely the mode of action of DEET alone and in combination with other compounds on insect physiology.

Another possible explanation for the observed synergism is detoxification by enzymes in insects. One component of the DEET-propoxur mixture may interfere with the detoxification of the other, thereby increasing the toxicity of the two compounds.[35,36] For example, synergism between organophosphates and pyrethroids was caused by sequestration of organophosphates by esterases, which prevented the degradation of pyrethroids.[37] Although DEET does not share ester bonds, other enzymes such as oxidases have been shown to be involved in its detoxification and could therefore play a role in synergism.[38–40]

In conclusion, a DEET-propoxur mixture may be a new promising tool for vector control because pyrethroid resistance is now widely prevalent in mosquitoes worldwide, especially in Africa. The control of *Ae. aegypti*, which is based mainly on pyrethroid sprays or impregnated materials, is being threatened by pyrethroid resistance. A combination of DEET and propoxur (or others carbamates) may improve personal protection against *kdr*-resistant mosquitoes and contribute to a better management of pyrethroid resistance. Another use for such a mixture stems from the fact that the $Ace.1^R$ mutation (G119S), which confers cross-resistance to organophosphates and carbamates, has never been observed in *Ae. aegypti*, and is unlikely to appear since it requires a double mutation.[41,42] In contrast to *Ae. aegypti*, the $Ace.1^R$ mutation has been found in *An. gambiae,* the main malaria vector in Africa and in *Cx. quinquefasciatus*, the main urban mosquito in tropical areas.[43] It will be interesting to investigate the impact of the $Ace.1^R$ gene on the efficacy of this DEET-propoxur mixture. Since DEET is a volatile compound, there is an urgent need to search for an adequate formulation that could extend the residual effect of this combination in impregnated materials. Recent investigations in

the laboratory have shown that efficacy of a DEET-based formulation (Insect Ecran®; Osler, Paris, France) persisted for at least 45 days on netting against pyrethroid-resistant *Cx. quinquefasciatus* (N'Guessan R, unpublished data). These findings constitute a first step towards an alternative strategy of combating vectors of human diseases.

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