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PYRETHROID CROSS RESISTANCE SPECTRUM AMONG POPULATIONS OF *ANOPHELES GAMBIAE* S.S. FROM CÔTE D'IVOIRE

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ABSTRACT. Field samples of *Anopheles gambiae* s.s. from Côte d'Ivoire were tested with 5 pyrethroids (cyfluthrin, λ-cyhalothrin, α-cypermethrin, deltamethrin, permethrin), 1 pseudo-pyrethroid (etofenprox), and an organochlorine (DDT). With the use of World Health Organization diagnostic tests, 5 out of 6 samples were found cross-resistant to these insecticides. A strong decrease in knockdown effect and mortality was also observed when testing deltamethrin-impregnated nettings. With a polymerase chain reaction amplification of specific alleles diagnostic test, resistance was found associated with the presence of a *kdr* mutation. The strong correlation between *kdr* allelic frequency and resistance to DDT or etofenprox indicated that *kdr* was the main resistance factor for these 2 insecticides. On the contrary, a lower correlation was observed between *kdr* frequency and resistance to 4 of the 5 pyrethroids tested, suggesting that another mechanism was also involved, likely a metabolic detoxification. These results point out the necessity to monitor pyrethroid resistance and the presence of *kdr* before implementation of any impregnated bed-net programs for malaria control.

KEY WORDS *Anopheles gambiae,* pyrethroid, resistance, *kdr,* malaria control, Côte d'Ivoire, West Africa

INTRODUCTION

Vector control is an important component of the World Health Organization (WHO) global strategy for malaria prevention. The main objective is to break the transmission of the parasite with indoor residual spraying or pyrethroid impregnated materials (bed-nets or curtains). Pyrethroids are preferably used for impregnation because they are highly effective and fast acting insecticides with a strong excito-repellent effect on mosquitoes.

Anopheles gambiae s.l. Giles is the main malaria vector in Africa, where 90% of the world cases occur. Resistance of *An. gambiae* s.s. to pyrethroids was first observed in Côte d'Ivoire (West Africa) by Elissa et al. (1993), who reported a significantly decreased mortality for permethrin and a lower susceptibility to the knockdown effect of deltamethrin and λ-cyhalothrin. More recently, knockdown resistance (*kdr*) to pyrethroids and DDT was observed in several countries from West Africa (Chandre et al. 1999). Investigations on the target site for pyrethroids and DDT have shown that this resistance was associated with a single point mutation on the gene coding for the sodium channel, resulting in the change of one amino acid (Martinez-Torres et al. 1998). A polymerase chain reaction (PCR) amplification of specific alleles (PASA) diagnostic test has been developed to identify the mutation on a single mosquito and to score for the genotype.

The current pyrethroid resistance observed in West Africa has been suspected to result mainly from the intensive use of DDT and, later on, pyrethroids for crop protection, especially cotton. Côte d'Ivoire, where resistance was 1st pointed out, is a country with intensive agriculture where huge amounts of insecticides have been used for many years. Therefore, we decided to carry out a survey in several localities from this country to verify whether resistance was widespread. Because the *kdr* mutation is known to induce a broad spectrum of cross-resistance to DDT and pyrethroids (Farnham 1973, 1977), we also tested other available pyrethroids usually regarded as possible alternatives in case of permethrin resistance. In addition to bioassays made with field-collected mosquitoes, we also evaluated the frequency of the *kdr* allele and compared it with the level of resistance to the different insecticides.

MATERIALS AND METHODS

Samples and strains of mosquitoes

Larvae of *An. gambiae* s.s. were collected in natural breeding sites from 6 localities of Côte d'Ivoire (Fig. 1). The samples from Abidjan were collected within the urban area of the capital of Côte d'Ivoire. Samples from Korhogo and Yaokoffikro were collected in periurban areas of these cities. The 3 other localities were sampled in rural areas, including Kafine and M'be from the southern savanna area and Zaïpobly from the forested area (Fig. 1).

All tests were carried out either on nonbloodfed 2–5-day-old females emerged from field-collected larvae or on females emerged from the 1st progeny brood. Susceptibility of natural populations was compared with that of 2 laboratory strains of *An. gambiae* s.s. with the same lots of impregnated papers: Kisumu, a susceptible reference strain originated from Kenya and maintained for many years in the laboratory; and Kou, a selected resistant strain, originating from a natural pyrethroid- and

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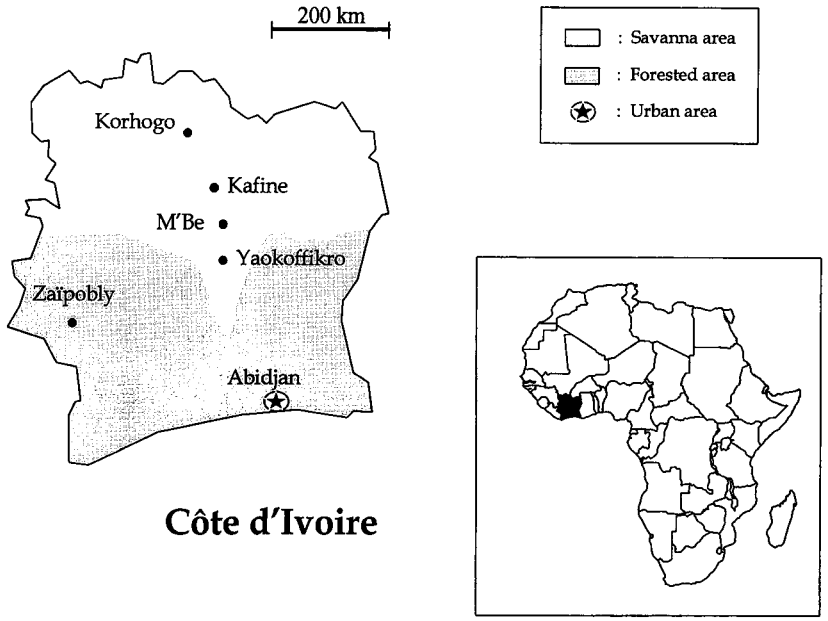

Fig. 1. Map of Côte d'Ivoire showing the localities of the 6 collecting sites of *Anopheles gambiae* s.s. situated either in the savanna area (white), forested area (shaded), or urban area (star).

DDT-resistant population collected in Burkina Faso in 1995. This strain was laboratory selected with permethrin and made homozygous for *kdr*. Colonies of both Kisumu and Kou strains were maintained at Bouake (Institut Pierre Richet, OCCGE) where all bioassays were made.

Resistance tests

Bioassays were carried out with WHO test kits for adult mosquitoes (World Health Organization 1970) with 7 insecticides of technical grade quality. Five were pyrethroids: cyfluthrin (92%) (Bayer, Leverkusen, Germany), λ-cyhalothrin (84.1%) (Zeneca, Bracknell, United Kingdom), α-cypermethrin (99.5%) (Phytagri, Geneva, Switzerland), deltamethrin (99.8%) (Agrevo, Berkhamsted, United Kingdom), and permethrin 25/75 (93.8%) (Agrevo). One insecticide was a nonester pyrethroid, etofenprox (99%) (Mitsui Toatsu, Tokyo, Japan), and 1 was an organochlorine, DDT (100%) (Sigma, St. Quentin Fallavier, France).

Impregnated papers were prepared in our laboratory with acetonic solutions and silicone oil (Dow Corning 556) as a carrier. Impregnations were made on the basis of 3.6 mg of oil per cm^2. Whatman filter paper sheets (12 × 15 cm) were impregnated with a mix of 0.7 ml of silicone oil and 1.3 ml of insecticide acetonic solution. Papers were stored at 4°C and were not used more than 3 times. The concentrations used were those recommended by WHO for deltamethrin (0.025%), λ-cyhalothrin (0.1%), permethrin (0.25%), and DDT (4%). Additional tests were made at higher concentrations for permethrin (1%). The 3 other insecticides, α-cypermethrin, cyfluthrin, and etofenprox, were used at 0.0025%, 0.05%, and 0.25% concentrations respectively, providing systematically 95–100% mortality for the susceptible reference strain. Then, for most insecticides, we considered the threshold for resistance below 95% mortality.

Laboratory tests were made with lots of 25 adult females in WHO test tubes with at least 4 replicates per bioassay. Exposure time was 60 min with tubes maintained in the normal vertical position. After exposure, mosquitoes were kept under observation for 24 h, supplied with 10% honey solution, and maintained at a temperature around 26°C with about 80% RH. Mortality was read after this 24-h period.

Allelic frequency of *kdr* in field samples

PCR amplification for kdr *mutation:* Genotypes for *kdr* mutation were determined according to Martinez-Torres et al. (1998). For individual mosquitoes, DNA was extracted from 2–3 legs with 5% Chelex® in water solution. The PASA tests were made in buffer containing deoxynucleotides triphosphate, Taq DNA polymerase, the 2 primers for the control band, and the 2 specific primers for susceptible allele and *kdr* mutation. The lengths of amplified sequences were 293 bp for the control band, 195 bp for the *kdr* allele, and 137 bp for the susceptible allele, allowing an easy definition of genotypes.

PCR for identification of species: Although the *kdr* mutation has been found so far only in *An. gambiae* s.s., specimens from the field (except 1 locality, Korhogo) were identified with specific primers according to Scott et al. (1993).

Tests with deltamethrin-impregnated nettings

Assays were made with impregnated multifilament polyester nettings by the WHO standard protocol (World Health Organization 1996). Bed-nets were impregnated at the concentration of 25 mg/m^2 with deltamethrin 2.5% SC (K-Othrine) diluted in deionized water. Lots of 5 females were exposed for 3 min under WHO plastic cones. A total of 12–18 replicates were made for each test. The number of knockdown mosquitoes was evaluated 60 min after exposure.

Statistical analysis

In order to assess if *kdr* gene was the only factor responsible for resistance, the relationship between *kdr* and resistance was investigated. Coefficients of correlation were calculated between the *kdr* allelic frequency in different populations and the corresponding percentage of surviving mosquitoes for each insecticide. To determine if the correlation was significant, the null hypothesis ($r = 0$, i.e., variable uncorrelated) was tested by a t-test with $n - 2$ degrees of freedom ($t = \sqrt{[n - 2]/[1 - r^2]}$). The level of significance of each test was adjusted to take into account the other tests by the sequential procedure of Bonferroni (Rice 1989).

RESULTS

Resistance levels of field samples from Côte d'Ivoire

Mortality after 24 h for all insecticides ranged between 98% and 100% with the reference susceptible strain Kisumu (Fig. 2). On the contrary, much lower mortality rates were observed for the Kou resistant strain, with less than 15% mortality for all insecticides except λ-cyhalothrin.

Among the 6 field samples tested, only 1, Zaïpobly, displayed a normal susceptibility to deltamethrin and λ-cyhalothrin; however, it showed a lower mortality with permethrin 0.25%. This sample was not tested with other insecticides because the number of individuals found in the field was not enough. The M'be sample was resistant to several insecticides, but a high mortality was observed with λ-cyhalothrin and DDT. The 4 other samples were cross resistant to all tested pyrethroids, etofenprox, and DDT. When the samples were sorted according to their average mortality, the increasing order of resistance among samples was Abidjan, Kafine, Yaokoffikro, and Korhogo (Fig. 2). The latter even appeared nearly as resistant as the Kou selected strain.

Allelic frequency of *kdr* in natural populations

All specimens except those from Korhogo were identified by PCR and were found to be *An. gambiae* s.s. The *kdr* gene was detected in 5 out of 6 samples tested (Table 1) and was absent in Zaïpobly (26 individuals tested). In resistant populations, allelic frequencies of *kdr* ranged from 4% to 95%. The order of *kdr* allelic frequency was generally identical to the rank of resistance level obtained with bioassays.

To assess if *kdr* was the main factor conferring cross-resistance to all insecticides, attempts were made to correlate its allelic frequency with the mosquito survival rates for the different insecticides (Table 2). When each insecticide was considered separately, the correlation was significant at 95% with 6 out of the 7 tested (except α-cypermethrin). However, by the sequential Bonferroni procedure, which increases the statistical power of detecting false Ho (type I error) when simultaneous tests are made, a significant correlation at 95% was found for only 3 insecticides, λ-cyhalothrin, etofenprox, and DDT.

Impact of resistance on mortality with deltamethrin-impregnated nettings

Tests were made with the 2 most resistant samples, Korhogo and Yaokoffikro (Table 3). Sixty minutes after the 3-min insecticide exposure, knockdown mosquitoes ranged from only 4% for those from Korhogo to 21% from Yaokoffikro in comparison with 100% for the susceptible strain (Kisumu). After 24 h, no mortality was observed for the Korhogo specimens and only 10% for the Yaokoffikro ones versus 100% for the susceptible strain.

DISCUSSION

Anopheles gambiae s.s. from only 1 (Zaïpobly) of the 6 study sites sampled were fully susceptible to pyrethroids. However, in this locality, 13% survived at permethrin 0.25%, which is comparable with susceptible populations. In fact, the diagnostic concentration of 0.25% proposed by WHO for permethrin is too low when using the recommended isomeric ratio of 25% cis/75% trans. In the Gambia, 9% survival with permethrin 0.25% (isomeric ratio not known) was observed in a population considered as susceptible (Hemingway et al. 1995). A mortality rate of 80%–90% was also observed in wild susceptible populations from various countries in Africa, which corresponded to the mortality range of a susceptible laboratory strain (Chandre et al. 1999). In this study, the highest mortality rates were systematically observed with λ-cyhalothrin 0.1%, most likely because this diagnostic concentration currently proposed by WHO (1995) was too high. When the M'be sam-

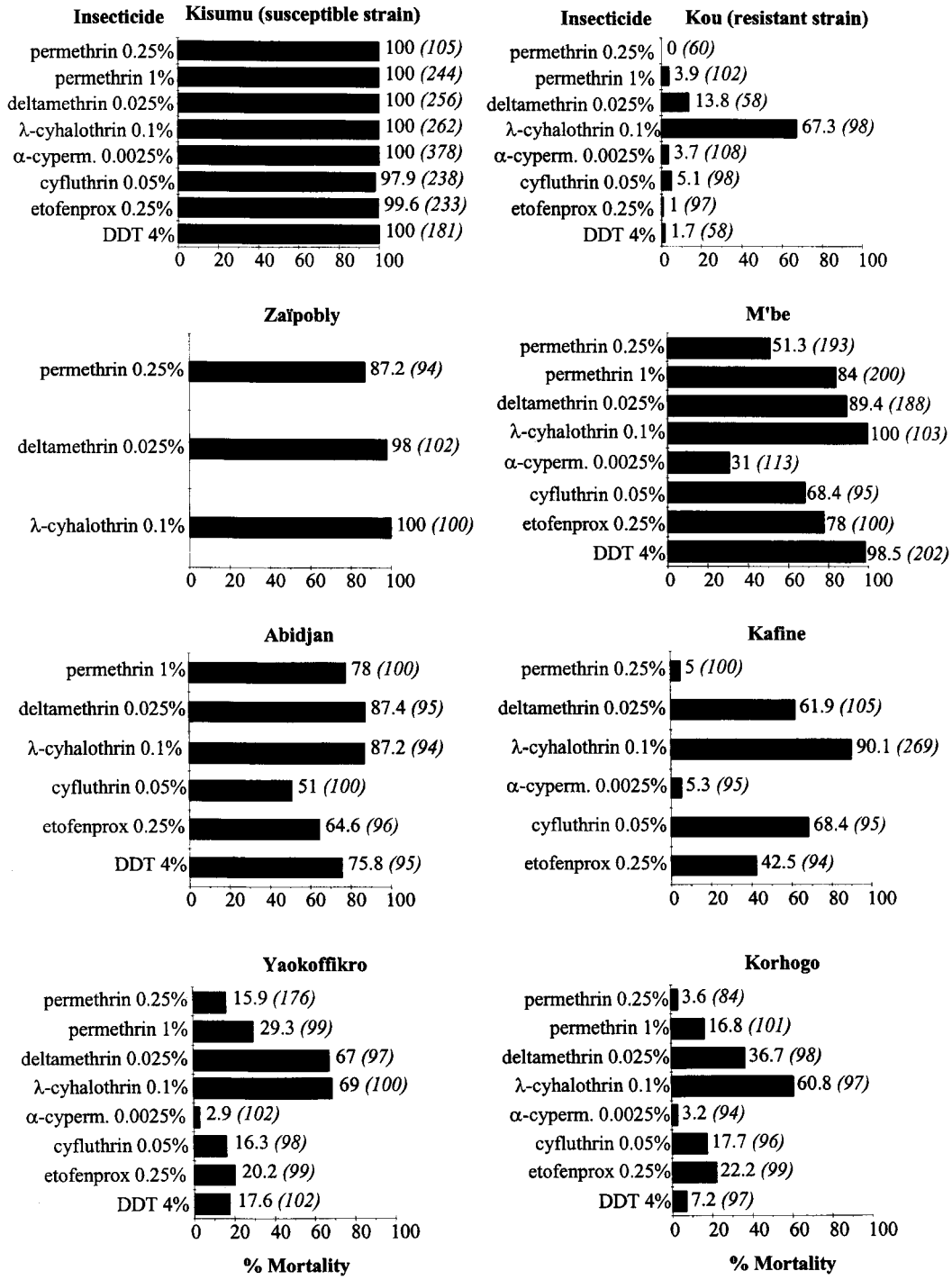

Fig. 2. Percentages of mortality to WHO tests with different insecticides for 6 field samples of *Anopheles gambiae* s.s., a susceptible reference strain (Kisumu), and a resistant one (Kou). Number of mosquitoes assayed in brackets.

Table 1. Genotypes of *Anopheles gambiae* and allelic frequency of *kdr* in natural populations of Côte d'Ivoire.

Sample	Genotype SS	RS	RR	F(R)[1]	n[2]
Zaïpobly	26	0	0	0%	26
M'be	63	3	1	3.7%	67
Abidjan	10	13	4	38.9%	27
Kafine	12	6	10	46.4%	28
Yaokoffikro	5	3	22	78.3%	30
Korhogo	1	1	28	95.0%	30

[1] Allelic frequency of *kdr* in %.
[2] Number of mosquitoes assayed.

ple was tested, a low but detectable resistance level to most of the pyrethroid insecticides was found. However, this sample would have been regarded as fully susceptible if tested only with λ-cyhalothrin.

Furthermore, a collaborative study was initiated by WHO after this study to determine the proper diagnostic concentrations of pyrethroids for the most important mosquito vector species. In the framework of this study, our laboratory (WHO Collaborating Centre for Vector Control) has found that the mean mortality rate with λ-cyhalothrin 0.01% was 98.7% with susceptible *An. gambiae* s.s. (4 replicates, 400 individuals tested, impregnated papers provided by WHO). The concentrations providing 100% mortality ranged from 0.01% to 0.025%. Therefore, the concentration of 0.1% mentioned above is obviously too high, and the correct diagnostic concentration for λ-cyhalothrin should be 0.05% (twice our highest concentration value). Similarly, the correct diagnostic concentration for permethrin 25/75 would be 1% instead of the 0.25% proposed by WHO using permethrin with a different isomeric ratio. In accordance with both Hemingway (1995) and our present study, the concentration used for etofenprox (0.25%) was shown to be discriminant for anophelines. These results underline the necessity to standardize the case of diagnostic concentrations for monitoring mosquito resistance in the field.

Table 3. Knockdown (Kd) and mortality of *Anopheles gambiae* after 3-min exposure to deltamethrin nettings (25 mg/m²) for 2 field-resistant samples (Korhogo, Yaokoffikro) and susceptible reference strain (Kisumu).

Sample	Kd at 60 min	Mortality	n
Kisumu	100%	100%	60
Korhogo	3.9%	1.3%	77
Yaokoffikro	21.3%	10.1%	89

A broad spectrum of resistance to various pyrethroids and DDT was conferred by the *kdr* mutation, as shown from the results obtained with the Kou strain, homozygous for *kdr*. The cross-resistance pattern observed in 5 populations from Côte d'Ivoire was also clearly associated with the presence of the *kdr* mutation. The strong correlation observed between *kdr* allelic frequency and survival of mosquitoes with DDT, λ-cyhalothrin, and etofenprox indicated that *kdr* was the main resistance factor for these 3 insecticides.

On the contrary, the correlation for other tested insecticides was not as significant, although an increase of *kdr* frequency was clearly associated with a decreased mortality to pyrethroids. Although this poor correlation could be attributed to the small number of samples considered, it suggested, above all, that *kdr* was not the only resistance mechanism involved. More interestingly, although the *kdr* frequency in the M'be sample was low (4%) and mortality with DDT high as expected (98.5%), a high survival rate was observed with α-cypermethrin (69%). Considering that for several years DDT has not been used in Côte d'Ivoire, the high resistance observed indicates that the *An. gambiae* s.s. populations were submitted to a strong pyrethroid selection pressure. Possibly, this pressure selected more specific pyrethroid resistance mechanisms, such as metabolic detoxification. Oxidase-based metabolism through P450 cytochromes and, to a lesser extent, esterases

Table 2. Correlation coefficients between *kdr* allelic frequency (Table 1) and corresponding percentage of population survival rate (Fig. 2) for different insecticides.

Insecticide	Correlation coefficient	t-value	$P(t)$[1]	n[2]
Cyfluthrin 0.05%	0.883	3.257	0.0236	5
λ-Cyhalothrin 0.1%	0.977*	9.098	0.0004	6
α-Cypermethrin 0.0025%	0.898	2.880	0.0512	4
Deltamethrin 0.025%	0.853	3.263	0.0155	6
Permethrin 0.25%	0.899	3.554	0.0190	5
Permethrin 1%	0.959	4.780	0.0206	4
Etofenprox 0.25%	0.953*	5.445	0.0061	5
DDT 4%	0.986*	8.379	0.0070	4

[1] Probability of t-value with $n - 2$ df.
[2] Number of samples.
* Correlation coefficient significantly different from 0 at 95% level after sequential Bonferroni procedure.

is commonly involved in pyrethroid resistance. Pyrethroid detoxification mechanisms may not confer resistance to DDT and etofenprox (nonester pyrethroid), which have different chemical structures and metabolic pathways. This may explain why resistance to these 2 insecticides was strongly correlated to *kdr* frequencies and resistance to pyrethroids was not (except for λ-cyhalothrin because the concentration used was too high). Ongoing studies are aimed to identify the other resistance mechanism(s) involved.

As previously suggested, it is likely that the *kdr* mutation in West Africa was inherited from DDT resistance, itself induced by the agricultural use of organochlorine insecticides (Chandre et al. 1999). This resistance has probably been further enhanced by the intensive use of pyrethroids for crop protection and domestic hygiene. Although α-cypermethrin has never been used in Côte d'Ivoire for mosquito control, resistance was observed even in the M'be sample, where resistance to other pyrethroids was much lower. However, this is not surprising considering that cypermethrin accounts for 80–90% of the overall quantity of pyrethroids used for several years for cotton, with about 1 million liters applied annually in this country (Vassal, personal communication).

Pyrethroid resistance in Côte d'Ivoire should definitely be regarded as a threat for the implementation of malaria control programs with impregnated bed-nets. Field-collected mosquitoes from resistance areas were no longer killed when exposed for 3 min to nettings impregnated with deltamethrin. In addition, resistant individuals are much less susceptible to the knockdown and excito-repellent effects of pyrethroids (unpublished data). Several large-scale control programs with pyrethroid-impregnated bed-nets, such as in the Gambia (Hemingway et al. 1995) or in China (Cheng et al. 1995), did not result in the selection of resistance among anopheline populations in these areas. However, 1 case of permethrin resistance was observed in East Africa in a permethrin-impregnated bed-net area (Vulule et al. 1994). The problem may be quite different when resistance genes are already present in target populations, as found in Côte d'Ivoire. Interestingly, in 1 of the sampled localities (Kafine), the allelic frequency of *kdr* increased from 46.4% to 92.5% in only 1 year after distribution of permethrin-impregnated bed-nets (Doannio et al. 1997). However, in the same time, the malaria morbidity of children was reduced by more than 50%, which is in the order range of results obtained with impregnated bed-nets in other parts of Africa (Choi et al. 1995). These results underline the necessity to monitor pyrethroid resistance and the presence of *kdr* gene before planning and implementing malaria control programs based on the use of impregnated materials.

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