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Impact of long lasting insecticidal nets and indoor residual sprayings on the knockdown resistance mutation in *Anopheles gambiae* s.s. in western Côte d'Ivoire

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ABSTRACT: Resistance occurred in *Anopheles* species to insecticides represents a threat for the success of malaria vector control. The impact of both long lasting insecticidal nets (LLINs) and indoor residual sprayings (IRS) with deltamethrin on the knockdown resistance (kdr) mutation conferring cross-resistance to pyrethroids and DDT was investigated in field *Anopheles gambiae* populations in Danané area in western Côte d'Ivoire.

The frequencies of the resistant allele at the kdr mutation L1014F locus were very low. Those frequencies increased on months 12th and 15th in mosquitoes from LLINs treated villages compared to those from untreated nets (UTNs) villages, whereas mosquitoes from IRS treated villages had showed an immediate impact with higher frequencies of the resistant allele and this was cancelled twelve months after.

The "M molecular form" of *An. gambiae s.s.* now formally named *An. coluzzii* Coetzee and Wilkerson sp.n. represented 74% versus 26% of the "S molecular form" assigned the nominotypical name *An. gambiae* Giles. No heterozygous individual MS was found. The kdr mutation L1014F was found only in the S form, although the S and M molecular forms were sympatric in Danané area.

Insecticidal pressure of LLINs on field *An. gambiae* in western Côte d'Ivoire was weak, but it lasted for a long time leading unavoidably to select resistant mosquitoes.

KEYWORDS: *Anopheles gambiae*, insecticide, resistance, allele, selection, Malaria, Côte d'Ivoire.

1 BACKGROUND

Malaria, transmitted by anopheline mosquitoes, is one of the world's most challenging public health problems. Malaria vector control aims to prevent the transmission of *Plasmodium* parasites by using essentially insecticide treated nets (ITNs) and/or indoor residual sprayings with insecticides (IRS). Many malaria vector control projects based upon sprayings were stopped because resistance to insecticides occurred in malaria mosquito populations [1] such as the A*nopheles gambiae* complex Giles 1902 [Diptera: Culicidae], a major malaria vector in Africa. Meanwhile, ITNs have proved their efficacy in reducing malaria morbidity and mortality [2], [3]. Then, long lasting insecticidal nets (LLINs) have been proposed and reported to protect against mosquitoes for several years [4].

To date, five (5) pyrethroids (α-cypermethrin, cyfluthrin, deltamethrin, λ-cyhalothrin, and permethrin) and one pseudopyrethroid (etofenprox) insecticides are recommended for net impregnation [5]. However, a lot of researches have reported pyrethroid resistance in several South Sahara African populations of *An. gambiae* sensu stricto [6], [7], [8], [9], [10].

This resistance induces a significant reduction in mosquito mortality after exposure to permethrin and lower knockdown effects of deltamethrin and λ-cyhalothrin [11], [12] representing a threat for the success of malaria vector control.

Investigations on the target site of action for pyrethroids show that the knockdown resistance (kdr) is mainly due to a single point mutation in the gene coding for the structure of the voltage-gated sodium channel. This mutation is the replacement of an adenine (TT***A***) to a thymine (TT***T***) [13], resulting in a change in amino acid from leucine to phenylalanine (L1014F) [14] or from leucine to serine (L1014S) [15], conferring cross-resistance to a wide range of pyrethroids and DDT [11]. It was initially detected in the S molecular form of *An. gambiae s.s.* from tropical Savanna areas [16], [12], [8]. In Benin and Ghana, this mutation has also been reported in the M molecular form [7], [17] and has spread in M form populations from dry Savanna areas [9].

The current survey was carried out in western Côte d'Ivoire to investigate the impact of both LLINs of Permanet® type and IRS with deltamethrin on pyrethroids and DDT cross-resistance in field *An. gambiae* populations.

2 MATERIAL AND METHODS

2.1 TREATMENTS IN STUDY SITES

The study involved 12 villages in the area of Danané city (7°15' N, 8°9' W, 365 m altitude) in western Côte d'Ivoire. Daily average temperature during the trial period was around 25°C with the minimum and maximum being 20°C and 34°C, respectively. Nets were rarely used in this area before the trial. Groups of three villages were constituted and a given treatment was applied to each group.

Three villages (Finneu, Bouenneu and Danta) were provided with LLINs, with each sleeping unit receiving a bed-net. Thus, one thousand eight hundred and five (1,805) LLINs were distributed in May 2001. These nets represented a total amount of 1,290.575 g of deltamethrin (55 mg/m² x 13 m² x 1,805) used to cover the three villages. One thousand six hundred and ninety five (1,695) nets of the same material without insecticide were also given to sleeping units in three other villages (Seileu, Pepleu and Zoleu), which served as controls to the villages provided with LLINs.

Indoor residual sprayings (IRS) with deltamethrin WG 20 mg/m² on walls of every house were done in three other villages (Yotta, Gbontégleu and Vétouo). The spraying operation was repeated six months later in November 2001 with the same insecticide formulation at the same concentration, in these same villages. The amounts of deltamethrin used for IRS in May and in November 2001 were 755 and 737 g of deltamethrin, respectively. The first IRS covered 37,510 m² while the second one covered 32,010 m². Three other villages (Bepleu, Biétouo and Méantouo) where no intervention was carried out served as controls.

2.2 NETS AND INSECTICIDES PROVISION

Permanet® is a long lasting insecticidal net (LLIN) made of a polyester net and treated at the factory level with deltamethrin at a concentration of 55 mg/m². The dosage is twice higher than that of hand-treated nets (25 mg/m²). Permanet® is reported to be active for up to 20 standard washes and its biological activity lasts as long as the net itself, 3 to 5 years. These nets were made of white multifilament polyester fabric (75 deniers, 156 meshes, 12 x 13 holes/in^2, Vestergaard Frandsen A/S, Copenhagen, Denmark).

Indoor residual sprayings were done using deltamethrin (K-Othrine®) WG 250 (25% ww, Sanofi-Aventis® formerly Aventis®, Paris, France).

2.3 *AN. GAMBIAE* COLLECTIONS AND STORAGE

Field adult mosquitoes were separately collected inside and outside doors of selected households just before nets distribution and the first IRS, from May 2001 to July 2002 at three months intervals in all the 12 villages. They were identified morphologically and only females of *An. gambiae sensu lato* were selected. Those mosquitoes were then dissected to examine the ovaries to distinguish parous and nulliparous [18], [19]. The mosquitos were dried over silica gel and were then stored at -20°C for later analysis.

Two reference laboratory strains of *An. gambiae s.s.* were used for PCR analysis. The "Kisumu" susceptible reference strain, originated from Kenya and the "Vk per" resistant strain, originated from Valley of Kou in Burkina Faso. The later strain is resistant to pyrethroids and DDT, with more than 70% kdr allelic frequency (unpublished data).

The Kisumu strain is S molecular form of *An. gambiae s.s.*, while the Vk per is M form. Both Kisumu and Vk per were reared in laboratory conditions (25±2°C temperature, 70-80% R.H.), in separated room in insectary.

2.4 GENOTYPIC IDENTIFICATION

Total DNA of Individual mosquito was extracted [20] and used to perform three Polymerase Chain Reaction (PCR). DNA from each specimen was first analyzed using the *An. gambiae* species specific PCR [21]. Then, the resistant or susceptible allele at the kdr mutation L1014F locus was test-detected [14]. The DNA of all mosquitoes identified as *An. gambiae s.s.* was subjected to PCR-PASA assay to distinguish the M and S molecular forms of *An. gambiae s.s.* [22].

2.5 DATA ANALYSIS

Populations of mosquitoes from villages were pooled together according to the origin of mosquitoes i.e. LLINs, IRS, UTNs and Controls. The resistant allele frequencies at the kdr mutation L1014F locus were calculated using GENEPOP software version 3.2a [23]. The impact of LLINs and IRS during this survey was performed by comparing the genotypic frequencies. Genotypic differentiation was then tested between the populations of mosquitoes collected from LLINs and IRS treated villages and those from UTNs and Controls villages, respectively, basing on the G-test of Goudet [24]. Conformity to Hardy-Weinberg equilibrium was tested [25] and Fis index showing deviation from random mating was estimated basing on Weir and Cockerham model [26].

3 RESULTS

3.1 EVOLUTION OF THE RESISTANT ALLELE AT THE KDR MUTATION L1014F LOCUS IN MOSQUITOES FROM TREATED VERSUS CONTROL VILLAGES

One thousand seven hundred sixty five (1765) individuals of *An. gambiae s.s.* were testdetected for the kdr mutation. The frequencies of the resistant allele at the kdr mutation L1014F locus were very low (0-0.16), though they significantly increased into mosquitoes' populations from LLINs and IRS treated villages compared to those from the villages with untreated nets (UTNs) and controls, respectively for the whole survey (P = 0.009 and P = 0.017, respectively) (Table).

There was no genotypic differentiation between mosquitoes' populations from LLINs and UTNs villages up to month 9^{th} (P (dG) > 0.05), whereas mosquitoes from IRS treated villages were significantly different from the controls (P (dG) < 0.05). However, on months 12^{th} and 15^{th} mosquitoes' populations from LLINs treated villages showed significant genotypic differentiation with higher frequencies of the kdr mutation compared to those from UTNs villages (P (dG) < 0.05), while no significant genotypic differentiation was observed between mosquitoes' populations from IRS treated villages compared to those from controls (P (dG) > 0.05).

Table 1. Knockdown resistance (kdr) mutation in An.gambiae s.s. in western Côte d'Ivoire

Months	Origin	N	F (kdr)	P (dG)	P (HW)	Fis (W & C)
0 (May 2001)	UTNs	62	0,03	0,11	1,00	-0,03
	LLINs	110	0,08		0,02	0,28
	Controls	65	0	$<10^{-2}$	1,00	0
	IRS	84	0,08		$<10^{-2}$	0,54
3 (August 2001)	UTNs	112	0,03	0,57	$<10^{-2}$	0,66
	LLINs	170	0,04		$<10^{-2}$	0,41
	Controls	127	0,01	0,02	$<10^{-2}$	1,00
	IRS	159	0,05		$<10^{-2}$	0,37
6 (November 2001)	UTNs	89	0,02	1,00	0,03	0,49
	LLINs	35	0,03		1,00	-0,02
	Controls	68	0	0,01	1,00	0
	IRS	28	0,13		$<10^{-2}$	0,84
9 (January 2002)	UTNs	27	0	0,15	1,00	0
	LLINs	18	0,06		1,00	-0,03
	Controls	54	0	-	1,00	0
	IRS	-	-		-	-
12 (April 2002)	UTNs	83	0,02	$<10^{-2}$	1,00	-0,01
	LLINs	35	0,14		0,01	0,54
	Controls	58	0,10	0,49	0,01	0,45
	IRS	22	0,16		0,43	0,17
15 (July 2002)	UTNs	91	0,04	$<10^{-2}$	0,15	0,22
	LLINs	78	0,16		0,02	0,29
	Controls	83	0,08	0,64	0,01	0,26
	IRS	107	0,10		0,01	0,32

Months (date): month 0 – months 15th; Origin: origin of mosquitoes i.e. from villages treated with LLINs, IRS, UTNs and Controls, respectively; N: number of mosquitoes test-detected; F (kdr): frequency of the kdr allele; P (dG): exact probability of genotypic differentiation between populations; P (HW): exact probability of Hardy-Weinberg equilibrium; Fis (W & C): index of Wright, difference to panmixis according to Weir and Cockerham; 95% confidence intervals for all exact probability.

3.2 HARDY-WEINBERG EQUILIBRIUM TESTED AT THE KDR MUTATION L1014F LOCUS

Thirteen (13) field *An. gambiae* mosquitoes' populations out of the twenty three (23) analyzed were not in Hardy-Weinberg equilibrium (P (HW) < 0.05) for the whole trial, with Fis (W & C) > 0 in most of cases, indicating systematic lack of heterozygous individuals at the kdr mutation locus (Table).

Only the S molecular form of *An. gambiae s.s.* carried the kdr mutation during the trial, i.e. one hundred nine (109) heterozygous and thirty nine (39) homozygous resistant individuals for the kdr mutation. One population of the twenty three (23) field *An. gambiae s.s.* mosquitoes' populations formerly analyzed did not contain any S molecular form. The study of Hardy-Weinberg equilibrium into solely these S molecular form of *An. gambiae s.s.* showed that only two (2) populations (i.e. mosquitoes from UTNs and LLINs treated villages on months 3rd) over twenty two (22) analyzed were not in Hardy-Weinberg equilibrium. The differences to panmixis for the two populations were low (P (HW) = 0.03 and P (HW) = 0.04, respectively) and they still indicated lack of heterozygous individuals (Fis (W & C) = 0.62 and Fis (W & C) = 0.34, respectively).

3.3 GENETIC COST OF THE KDR MUTATION AND IMPACT ON MOSQUITOES BLOOD FEEDING BEHAVIOUR

There was no significant genotypic differentiation between field parous and nulliparous individuals of *An. gambiae s.s.* during the whole trial (P (dG) > 0.05).

There was also no significant genotypic differentiation between field mosquitoes collected neither outside nor inside of houses (P (dG) > 0.05).

3.4 VARIATION OF M AND S MOLECULAR FORMS OF *AN. GAMBIAE S.S.*

The mosquitoes test-detected for the kdr mutation, were analyzed for the M and S molecular forms of *An. gambiae s.s.* Thus, on the one thousand six hundred forty height (1648) individuals genotyped, four hundred thirty two (432) individuals were S molecular form of *An. gambiae s.s.* representing 26% versus 74% of M form and no heterozygous individual MS was found. The M and S forms were sympatric (Figure). The frequencies of S form individuals decreased between May and November 2001, corresponding to the dry season. Then, they increased until July 2002, corresponding to the rainy season in western Côte d'Ivoire.

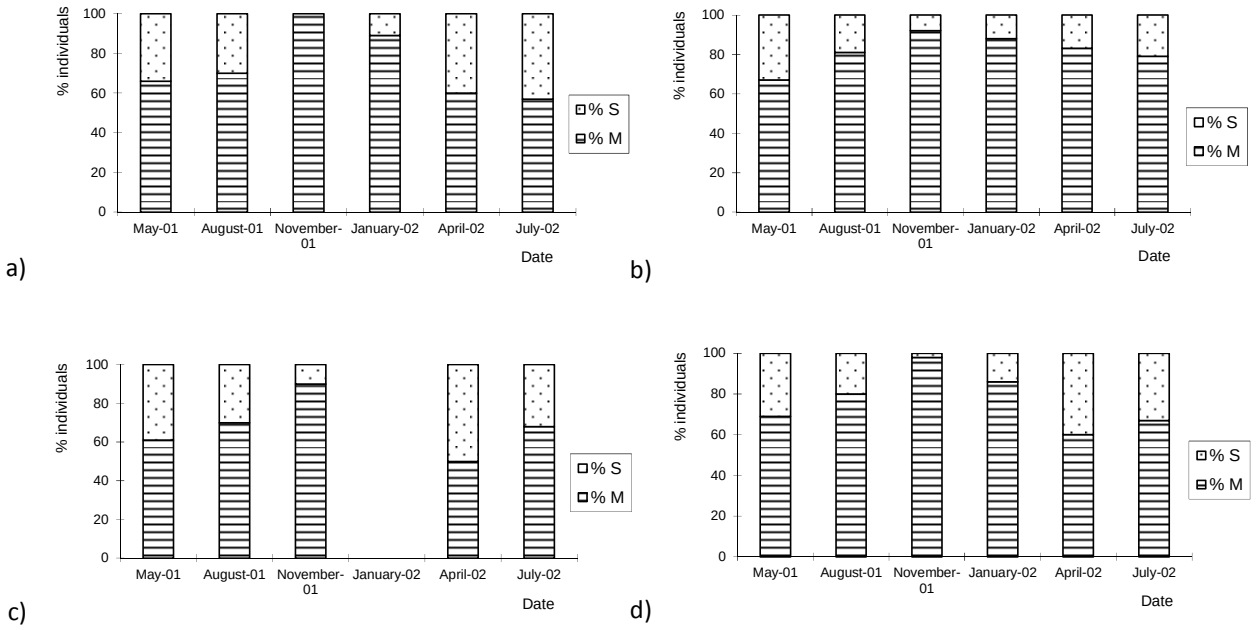

Fig. 1. Variation of M and S molecular forms of An. gambiae s.s. from villages treated with (a) LLINs; (b) UTNs; (c) IRS and (d) controls in Western Côte d'Ivoire

4 DISCUSSION

The frequencies of the resistant allele at the kdr mutation L1014F locus in Danané area in western Côte d'Ivoire were low (< 0.20). The current results also revealed that LLINs induced an increase of the kdr allele frequencies on months 12^{th}, whereas mosquitoes from IRS treated villages had showed an immediate impact with higher frequencies of the resistant allele and this was cancelled twelve months after.

A similar study performed in Korhogo area in northern Côte d'Ivoire showed that the frequencies of the resistant allele at the kdr mutation L1014F locus were very high (>0.80) [10]. Those results revealed that λ-cyhalothrin-treated nets significantly increased the kdr allele frequencies in mosquitoes from treated villages after 14 months of treatment. In western Kenya, the frequencies of the resistant allele at the kdr mutation L1014S locus increased twice (from 4% to 8%) between 1987 and 2001 in *An. gambiae* populations with permethrin-treated nets [27]. However, impregnated nets pressure on those mosquitoes was weak, but it lasted for a long time leading unavoidably to select resistant mosquitoes.

The kdr mutation L1014F was found only in the S molecular form of *An. gambiae* s.s. in Danané area. Conversely, in southern forest area in Benin, the M molecular form of *An. gambiae* s.s. was reported to carry the kdr mutation L1014F by introgression from the S form

[7]. A recent study in Port-Bouët in southern Côte d'Ivoire revealed the kdr mutation L1014F with high frequencies (0.70) in the M molecular form [28].

The frequencies of S form individuals decreased in the dry season but, increased in the rainy season during the survey. This is well supported by the ecology of each of the M and S molecular forms. The M larval habitats tend to be rice fields with high predator density, whereas the S form habitats are associated with seasonal rainfall that are largely unpolluted and predator free in west Africa [29]. The high frequencies of the S form carrying the resistant allele at the kdr mutation L1014F locus induced intrinsic variation with relatively high frequency of this allele during the rainy season without any impact on the survey, since all villages were in the same climatic area (7°15' N, 8°9' W, 365 m altitude).

Thirteen (13) field *An. gambiae* mosquitoes' populations out of the twenty three (23) analyzed were not in Hardy-Weinberg equilibrium, whereas almost all populations of the S molecular form analyzed solely were in Hardy-Weinberg equilibrium. The high frequencies of the S form carrying the resistant allele during the rainy season led to more field populations in Hardy-Weinberg equilibrium at the kdr mutation L1014F locus.

No heterozygous MS was observed, although the S and M molecular forms were sympatric in Danané area, suggesting that the specimens M and S belong to two different sub-species of *An. gambiae* s.s. A former study in Cameroon, using microsatellites also revealed the absence of hybrid MS, and outlined that the genetic distances between the M and S molecular forms from a same village were higher than those between the villages [30]. Indeed, a strong differentiation based on single-nucleotide polymorphism (SNP) analysis was reported between the M and S forms only in the pericentromeric region of the X chromosome that contains the molecular form specific marker locus, with only a few other loci showing minor differences [31].

The level of hybridization between M and S forms is variable in West Africa. While, a very low frequency of hybrids (less than 2%) was observed in Burkina Faso, Côte d'Ivoire, Nigeria and Benin [29], [32], a relatively high frequency of hybrids (up to 24%) was recorded in Gambia and Guinea Bissau [33], [34]. However, the M and S molecular forms interbreed in laboratory conditions conforming that the phenomenon of speciation involved is ongoing and did not induce yet a genetic incompatibility [35]. The "M molecular form" of *An. gambiae s.s.* is now formally named *An. coluzzii* Coetzee and Wilkerson sp.n. and the "S form" is assigned the nominotypical name *An. gambiae* Giles [36].

5 CONCLUSIONS

Insecticidal pressure of LLINs on field *An. gambiae* in western Côte d'Ivoire was weak. During the survey, the frequencies of the resistant allele at the kdr mutation L1014F locus were very low, though they significantly increased into mosquitoes' populations from LLINs and IRS treated villages compared to those from the villages with untreated nets (UTNs) and controls, confirming that LLINs select resistant mosquitoes.

However, there was no significant genotypic differentiation between field parous and nulliparous individuals of *An. gambiae* during the whole trial, highlighting the absence of genetic cost of the kdr mutation in terms of lifetime duration of mosquitoes under the hypothesis that parous mosquitoes are older than nulliparous ones.

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