Parassitologia 41: 319-322, 1999

Current distribution of a pyrethroid resistance gene (*kdr*) in *Anopheles gambiae* complex from West Africa and further evidence for reproductive isolation of the Mopti form

F. Chandre[1], S. Manguin[1], C. Brengues[1], J. Dossou Yovo[2], F. Darriet[2], A. Diabate[3], P. Carnevale[2], P. Guillet[4]

[1] *LIN/IRD, Montpellier, France;* [2] *IPR/OCCGE, Bouaké, Côte d'Ivoire;* [3] *Centre Muraz, OCCGE, Bobo Dioulasso, Burkina Faso;* [4] *World Health Organization, Geneva, Switzerland.*

Abstract. In the field, the *kdr* mutation, involved in pyrethroid resistance, has been found widely distributed in the Savanna form of *Anopheles gambiae* s.s., but never in wild populations of the Mopti form or *An. arabiensis*, even in areas where both occur in sympatry with resistant Savanna populations. Under laboratory conditions, Mopti and Savanna forms were fully able to interbreed and the *kdr* mutation was transmissible from one form to the other. Both forms appeared to be exposed to pyrethroid selection pressure in the field. The absence of the *kdr* mutation in the Mopti form and the total lack of Mopti-Savanna heterozygotes in field populations provides further evidence of a pre-copulatory barrier to gene flow between these two forms. Molecular markers, including *kdr*, are powerful tools for studying population genetics and circulation of resistance genes, and should be used through an integrated approach for a better understanding of the speciation process.

Key words: *Anopheles gambiae* complex, Mopti form, resistance, pyrethroid, *kdr* mutation, West Africa.

Pyrethroid resistance due to the *kdr* mutation has been found widespread in several West African countries (Martinez-Torres *et al.*, 1998; Chandre *et al.*, 1999a). Its geographical distribution has been thoroughly investigated in Côte d'Ivoire where it occurs in most wild populations, occasionally at very high frequency (>90%) (Chandre *et al.*, 1999b). However, some populations showed deviation from Hardy Weinberg equilibrium (i.e. a deficit in heterozygotes). For a better understanding of the situation, allelic frequencies of *kdr* mutation were determined within the recognized forms of *Anopheles gambiae* s.s. including Savanna/Forest and Mopti. Since both Mopti and Savanna forms interbreed under laboratory conditions, back-cross experiments were done to evaluate whether the *kdr* mutation could be transmitted from one form to the other.

Materials and methods

Mosquitoes were collected in several localities from Côte d'Ivoire and Burkina Faso. In the former country, sampling was made related to rice cultivation practices: no rice (R0), 1 annual rice harvest (R1), and 2 irrigated rice harvests (R2) and in different environments from deep forest to savanna areas. Human landing females were collected in Côte d'Ivoire, whereas females in Burkina Faso were obtained from larval collections. All individuals were first identified morphologically and then by PCR (Scott *et al.*, 1993). Mopti and Savanna/Forest forms were identified by PCR-RFLP (Favia *et al.*, 1997). The *kdr* mutation was detected by PCR (Martinez-Torres *et al.*, 1998) and population genetic analysis was made using Genepop software (Raymond and Rousset, 1995). The level of significance of each test was corrected according to the sequential Bonferroni procedure (Rice, 1989).

In order to verify whether *kdr* gene was transmissible from one form to the other, a susceptible Savanna strain (*kdr* free, originated from Kenya) and resistant Mopti (homozygous for the *kdr* gene) were reciprocally crossed (SS males × RR females and SS females × RR males). This resistant Mopti strain was selected in the laboratory from a field sample that initially contained both susceptible Mopti and resistant Savanna forms.

The 8 possible back-crosses were tested (100 to 200 individuals per cage). From each progeny, 30 females and 10 males were identified by PCR-RFLP and genotyped for *kdr*.

The fitness cost associated with the *kdr* mutation was investigated in the field by comparing the *kdr* frequency between *P. falciparum* infected females of *An. gambiae* s.s. (salivary gland dissections confirmed by ELISA) and uninfected. This comparison was made in two areas of Côte d'Ivoire, including one in a savanna environment with a high pyrethroid resistance (*kdr* frequency >80%) and one in a forest environment with low resistance (<15%).

Invited contribution to the Malariology Centenary Conference "The malaria challenge after one hundred years of malariology" held in Rome at the Accademia Nazionale dei Lincei, 16-19 November 1998.

Correspondence: Dr Pierre Guillet, World Health Organization, Division of Communicable Diseases Prevention and Control, 1211 Geneva 27, Switzerland, Tel ++41 22 7913860, Fax ++41 22 7914777, e-mail: guilletp@who.ch

Results and discussion

More than 1,000 mosquitoes were both identified taxonomically by PCR and genotyped for *kdr* (Table 1). Among them, 478 were identified as belonging to the Mopti or Savanna/Forest forms. In 5 out of 6 areas where resistant *An. gambiae* s.s. and *An. arabiensis* were in sympatry, the *kdr* mutation was not found in *An. arabiensis*. Pyrethroid susceptibility of the latter species was also confirmed by bioassays. Among the 19 populations of *An gambiae* s.s. tested, 4 deviated significantly from Hardy-Weinberg equilibrium. However, within the Savanna/Forest forms, all populations were at equilibrium. Of the 149 Mopti individuals screened from the field, none was found with the *kdr* mutation, although Mopti populations were commonly found in sympatry with the resistant Savanna form (8 mixed populations, with Savanna *kdr* frequency from 17 to 100%).

Isozyme analysis comparing three populations, one of Mopti (Mbé) and two resistant Savanna (Kafiné and Yaokoffikro) on the basis of 14 enzymatic systems controlled by 25 loci, showed little differentiation between the populations with a value of the F-statistic (Fst) of 0.042. In addition, unbiased genetic distances of Nei (1978) were low, with values ranging from 0.003 to 0.008. As found by Cianchi *et al.* (1983) on different natural populations, the genetic distance between Mopti and Savanna forms was very low and no discriminant alleles by multilocus analysis were found. As stated by Cianchi *et al.* (1985), sibling species identification by multilocus electrophoresis becomes less efficient when their origin is quite recent, and their genetic divergence at the structural gene level is still minimal.

Backcrossed hybrids from homozygous resistant (RR) Mopti and homozygous susceptible (SS) Savanna, yielded allelic frequencies for taxonomic status and *kdr*. The *kdr* mutation was not sex-linked since frequencies did not differ significantly from Mendelian autosomal expectation. It is freely transmitted from one form to the other under laboratory conditions. In addition, this experiment confirmed that both forms, Savanna and Mopti, were able to freely interbreed in the laboratory.

If *kdr* mutation reduces fitness, one possible effect at the adult stage would be the reduction of life expectancy. Since vectorial capacity is closely related to life expectancy, it would be expected that the frequency of the *kdr* mutation in infected females would be significantly lower than in uninfected ones.

Results in Table 2 showed that difference in genotype frequencies of infected and uninfected individuals was not significant ($P>0.05$, Goudet exact test; Goudet *et al.*, 1996). *Kdr* mutation was apparently not found associated with a fitness cost. Thus, *kdr* individuals appear to live as long as susceptible ones. Under laboratory conditions, the *kdr* gene was

Table 1. Frequencies of *kdr* mutation among species of *An. gambiae* s.l. and forms of *An. gambiae* s.s. within field samples of mosquitoes from Côte d'Ivoire and Burkina Faso. G. savanna, Guinean savanna; S. savanna, Sahelian savanna; Rice cult., presence or absence of ricefields; % and (N), frequency of each species/form and number of mosquitoes tested; F(*kdr*), allelic frequency of *kdr* mutation; HW, P value from exact test for conformity to Hardy-Weinberg ratio: values still significant ($P<0.05$), when taking into account multiple tests, are indicated in bold characters; ND, not determined.

Location	Ecological zone	Rice cult.	*An. gambiae* s.s.			Savanna/Forest forms			Mopti form		*An. arabiensis*	
			% and (No.)	F(*kdr*)	HW	% and (No.)	F(*kdr*)	HW	% and (No.)	F(*kdr*)	% and (No.)	F(*kdr*)
Côte d'Ivoire												
Mbe	G. savanna	Yes	100 (67)	0.04	0.0740	6.4 (4)	0.63	1	93.6 (63)	0	0	–
Kafine	G. savanna	Yes	100 (77)	0.94	**0.0009**	84.2 (16)	0.90	1	15.8 (3)	0	0	–
Yaokoffikro	Peri urban	Yes	100 (56)	0.96	0.0880	100 (56)	0.96	1	0	–	0	–
Korhogo	G. savanna	Yes	100 (91)	0.84	0.0600	100 (65)	0.82	0.0200	0	–	0	–
Kabolo	G. savanna	No (R0)	100 (29)	0.31	**<0.0001**	44.8 (13)	0.69	0.0330	55.2 (16)	0	0	–
Tioroniaradougou	G. savanna	Yes (R1)	100 (29)	0.83	0.0170	100 (29)	0.83	0.0170	0	–	0	–
Nombolo	G. savanna	Yes (R2)	100 (29)	0.81	0.0360	89.7 (26)	0.90	1	10.3 (3)	0	0	–
Nambekaha	G. savanna	Yes (R2)	100 (30)	0.88	1	100 (30)	0.88	1	0	–	0	–
Fapaha	G. savanna	Yes (R1)	100 (28)	0.73	0.3500	100 (28)	0.73	1	0	–	0	–
Danane	Forest	Yes	100 (24)	0.21	0.5400	83.3 (20)	0.25	0.2800	16.7 (4)	0	0	–
Guiglo	Forest	Yes	100 (85)	0.10	0.5900	59.8 (49)	0.17	1	40.2 (33)	0	0	–
Abidjan	Urban	No	100 (27)	0.39	1	ND	–	–	ND	–	0	–
Burkina Faso												
Sabou	S. savanna	No	92.1 (70)	0.29	1	ND	–	–	ND	–	7.9 (6)	0
Boromo	Peri urban	No	64.1 (25)	0.08	1	ND	–	–	ND	–	35.9 (14)	0
Kuiti	S. savanna	No	82.1 (23)	0.02	1	ND	–	–	ND	–	17.9 (5)	0
Hounde	Peri urban	Yes	94.7 (161)	0.04	**0.0013**	72.7 (8)	0.50	0.2200	27.3 (3)	0	5.3 (9)	0
Kou Valley	S. savanna	Yes	98.0 (100)	0.02	**<0.0001**	7.7 (2)	1	1	92.3 (24)	0	2.0 (2)	0
Bobo	Urban	No	100 (11)	0.95	1	100 (11)	0.95	1	0	–	0	–
Ouagadougou	Urban	No	60.6 (43)	0	1	ND	–	–	ND	–	39.4 (28)	0

Table 2. Comparison of genotypes for *kdr* between infected and uninfected females from savanna and forest areas of Côte d'Ivoire. F(*kdr*), allelic frequency of *kdr* mutation; P value for significance test between *kdr* genotypes of infected and uninfected; NS, non-significant.

Samples	RR	RS	SS	F(*kdr*)	*P*
High resistance area (savanna)					
Infected	34	6	5	0.822	
Uninfected	31	12	1	0.841	0.869 (NS)
Low resistance area (forest)					
Infected	1	3	26	0.083	
Uninfected	0	8	23	0.129	0.573 (NS)

maintained at a low frequency over numerous generations in the absence of any insecticide pressure. Consequently, it is reasonable to assume that the *kdr* mutation would be maintained in the Mopti form under field conditions if introduced by natural interbreeding from the resistant Savanna form.

Why did the Savanna and not the Mopti form develop resistance, although they live in the same environment? Since the *kdr* mutation is fully transmissible from one form to the other under laboratory conditions, why has it so far never been found in Mopti field populations?

Both Mopti and Savanna populations are presumably exposed to pyrethroid selection pressure in the study area. This is obvious for the Savanna form considering the extremely high *kdr* frequencies commonly observed in its natural populations. In the field, Mopti females are also likely to be exposed to selection pressure. Indeed, contrary to expectations, in northern Côte d'Ivoire during July-August, this form accounted for 55% of females collected in R0 area, zero % in R1, and only 5.2% in R2, although the Mopti form is more commonly associated with rice fields. This association was confirmed in the same area, in an experimental rice field station not surrounded by villages (Mbe, see Table 1), where the frequency of the Mopti form was over 80%.

Sociological KAP surveys (Audibert, unpublished data) have shown that rural populations in R1 areas were using large amount of household pyrethroids for personal protection (coils and aerosols), 3 times more than in R0 and twice more than in R2. Cotton is a more profitable crop than rice and since it is extensively grown in R1, unlike R0 (no cultivation) and R2 (essentially rice), people in R1 make more money and consequently can afford personal protection. Therefore, absence or low prevalence of the Mopti form in some rice field areas can be explained by selection pressure exerted on susceptible adults by household pyrethroids and probably also on larvae by agricultural insecticides.

The only reasonable explanation for the complete absence of the *kdr* mutation in the Mopti populations, although sympatric with highly resistant Savanna populations and both forms are exposed to pyrethroid selection pressure, is that the Mopti form is genetically isolated from the Savanna form by a strong barrier to gene flow. Since both forms interbreed under laboratory conditions and the *kdr* gene is freely transmitted from one form to the other, isolation must be at the pre-copulatory stage. In addition, this isolation was also confirmed during our study, as also reported by Favia *et al.* (1997), by the complete absence of heterozygotes by the RFLP type diagnostic of the forms in field populations (although heterozygotes were systematically and undoubtedly identified in crossing experiments).

Detection of gene flow based on the presence of backcross progeny in field populations, through chromosome examination, usually requires a very large number of samples, especially when these flow rates are very low. Our results showed that the *kdr* mutation is a sensitive marker with which to detect possible gene flow when the mutation is highly selected as in our study area. The presence of a strong barrier to gene flow is confirmed through the pyrethroid resistance pattern, which further reinforced Coluzzi's hypothesis that the Mopti form should be regarded as a distinct species separated from the Savanna-Forest form (Coluzzi *et al.*, 1985; Touré *et al.*, 1994).

Since the Mopti form is widely distributed in forested areas of south-eastern Côte d'Ivoire, more individuals from this area will be identified and tested for the *kdr* mutation in order to further evaluate the isolation between the Mopti and Savanna-Forest forms. Since *kdr* is also widespread at a high frequency in southern Benin (around Cotonou), it would be interesting to use it as a marker to investigate the degree of genetical isolation between *An. melas* and the Forest form of *An. gambiae* s.s., which are sympatric in this area.

The use of new molecular markers allows a better understanding not only of population genetics, but also of resistance gene flow, as well as the speciation process. Since species ecology plays an important role in the dynamics of resistance, further studies on the occurrence of resistance genes and their possible evolution should be done through an integrated approach combining population dynamics and its regulatory factors in natural environments.

Acknowledgements

This study was funded by WARDA (West African Rice Development Association) and WHO/TDR (Task Force on impregnated materials, Project No. 970523).

References

Chandre F, Darriet F, Manga L, Akogbeto M, Faye O, Mouchet J, Guillet P (1999a). Status of pyrethroid resistance in *Anopheles gambiae* s.l. Bull Wld Hlth Org 77: 230-234.

Chandre F, Darriet F, Manguin S, Brengues C, Carnevale P, Guillet P (1999b). Pyrethroid cross resistance spectrum among populations of *Anopheles gambiae* s.s. from Côte d'Ivoire. J Am Mosq Control Ass 15: 53-59.

Cianchi R, Villani F, Touré YT, Petrarca V, Bullini L (1983). Electrophoresis study of different chromosomal forms within *Anopheles gambiae* s.s. Parassitologia 25: 239-241.

Cianchi R, Urbanelli S, Villani F, Sabatini A, Bullini L (1985). Electrophoresis study in mosquitoes: recent advances. Parassitologia 27: 157-167.

Coluzzi M, Petrarca V, Di Deco MA (1985). Chromosomal inversion intergradation and incipient speciation in *Anopheles gambiae*. Boll Zool 52: 45-63.

Favia G, della Torre A, Bagayoko M, Lanfrancotti A, Sagnon N'F, Touré YT, Coluzzi M (1997). Molecular identification of sympatric chromosomal forms of *Anopheles gambiae* and further evidence of their reproductive isolation. Insect Mol Biol 6: 377-383.

Goudet J, Raymond M, De Meeüs T, Rousset F (1996). Testing differentiation in diploid populations. Genetics 144: 1933-1940.

Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Berge JB, Devonshire AL, Guillet P, Pasteur N, Pauron D (1998). Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae* s.s. Insect Mol Biol 7: 179-184.

Nei M (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583-590.

Raymond M, Rousset F (1995). GENEPOP (version 1.2); population genetics software for exact tests and eucumenicism. J Heredity 86: 248-249.

Rice WR (1989). Analyzing tables of statistical tests. Evolution 43: 223-225.

Scott JA, Brogdon WG, Collins FH (1993). Identification of single specimens of *Anopheles gambiae* complex by the polymerase chain reaction. Am J Trop Med Hyg 49: 520-529.

Touré YT, Petrarca V, Traoré SF, Coulibaly A, Maïga HM, Sankaré O, Sow M, Di Deco MA, Coluzzi M (1994). Ecological genetic studies in the chromosomal form Mopti of *Anopheles gambiae* s.s. in Mali, West Africa. Genetica 94: 213-223.