LONGITUDINAL SURVEY OF KNOCKDOWN RESISTANCE TO PYRETHROID (KDR) IN MALI, WEST AFRICA, AND EVIDENCE OF ITS EMERGENCE IN THE BAMAKO FORM OF ANOPHELES GAMBIAE S.S.

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Abstract. Studies aimed at monitoring the spread of knockdown resistance to pyrethroids (kdr) in time and space are particularly useful for detecting barriers to gene flow among the chromosomal and molecular forms of Anopheles gambiae. We used a recently developed polymerase chain reaction assay to estimate changes in kdr frequency that occurred in several mixed-form populations from Mali, West Africa, in the past decade. We found that the kdr allele significantly increased in frequency in most populations but was still absent from the M molecular form. Importantly, within the S molecular form, kdr was detected for the first time in the Bamako chromosomal form. These results provide important insights on the patterns of spread and emergence of pyrethroid knockdown resistance in West Africa.

INTRODUCTION

Malaria still accounts for 300–500 million clinical cases and 1.5–3 million deaths per year in Africa.1,2 The major vectors of malaria in this region are the members of the Anopheles gambiae complex that comprises at least seven sibling species, among which An. arabiensis and An. gambiae sensu stricto have the broadest spatial distribution and are the most abundant.3,4 An. gambiae s.s. has been further subdivided into five chromosomal forms based on extensive polytene chromosome studies.5–7 These forms have been designated with the non-Linnean nomenclature: Savanna, Bamako, Mopti, Forest, and Bissau and occupy specific ecological zones.5,8 More recently, molecular studies of the ribosomal DNA region on the X chromosome revealed a fixed difference between the Mopti form and Savanna and Bamako form in Mali.9 In this region, the so-called M IGS type was found in near perfect linkage disequilibrium with the inversion karyotypes characteristic of the Mopti form, whereas the S-type is common to the Savanna and Bamako forms. This difference suggests that the Mopti form may be completely reproductively isolated from the other two forms.9,10 In other parts of West Africa, the distribution of M and S molecular types are not linked to chromosomal forms but are still thought to characterize partial or completely isolated populations.11–14 Understanding patterns of gene flow within and between complex populations of An. gambiae is important for evaluating the feasibility of genetic vector control projects15 and can help predict the spread of resistance to pesticides.

Current vector control projects in Africa rely heavily on the use of pesticides for insecticide-treated nets (ITNs) and for indoor residual spraying (IRS).16,17 Several studies have shown the efficacy of ITN programs for curbing malaria incidence.18–20 Pyrethroid compounds are the only insecticides approved for such use because of their lower toxicity to mammals and comparatively better excito-repellent and speed of killing effects than organochlorines (DDT), organophosphates (e.g., malathion, fenitrothion), and carbamates (e.g., propoxur, sevin).21,22 Unfortunately, resistance to pyrethroids has recently emerged both in West and East Africa and its rapid spread suggests that it will become a major hindrance to malaria control programs that target adult mosquitoes.22 A common form of resistance to pyrethroids is the knockdown resistance (kdr), which is linked to a single nucleotide substitution in the voltage gate of the sodium channel gene that results in target site resistance to pyrethroids and cross-resistance to DDT.23 In West Africa, the substitution results in a change from leucine to phenylalanine (TTA to TTT)24 and was first detected in populations of the Savanna chromosomal form and S molecular form in coastal Ivory Coast.25 Since then, the kdr allele has also been detected in Savanna populations from Benin,26 Burkina Faso,27 Ghana,29 Nigeria,30 and Mali.31 Agricultural spraying of cotton and rice fields is thought to have driven the emergence of kdr in some populations, where frequencies of resistance higher than 90% are common.31,28 In East Africa, kdr resistance is caused by a substitution from leucine to serine (TTA to TCA) affecting the same codon as the substitution observed in West Africa.23 Thus far, it has only been detected in Kenya where it is thought to have emerged as a result of DDT use to control agricultural pests as well as mosquitoes and Glossina flies (tsetse flies).32 An increase in the level of resistance has recently been observed in some areas of Kenya as a result of ITN programs.33 Because of the rapid spread of kdr resistance in West Africa and its emergence or re-emergence in East Africa, large-scale surveys of the current distribution of resistance throughout the geographical range of the major vectors are critical to successful ITN and IRS projects. Comparisons between the distribution of the kdr allele and pyrethroid resistance as detected by in vivo assays are also important for uncovering additional mechanisms of resistance.34

Studies of the distribution and spread of the kdr allele are also useful for assessing the presence or absence of barriers to gene flow among populations and are thus particularly suited for studying reproductive isolation between the chromosomal and molecular forms of An. gambiae s.s.35,36 The fact that early studies found high kdr frequencies in several S-form Savanna populations but not in sympatric M forms confirmed the existence of strong reproductive barriers between forms in West Africa.36,37 However, the kdr mutation was later de-
ected in M-form populations from Benin,26 Burkina Faso,27,28 and Ghana.29 Weill and others26 sequenced part of the sodium-channel gene of M and S individuals from Ivory Coast and Benin and identified two substitutions characteristic of all resistant M- and S-form individuals. This pattern suggests that the kdr allele might occur in the M form through recent introgression from resistant S-form populations.30 Paradoxically, on the Island of Bioko in Equatorial Guinea, the kdr allele was recently detected in the M form but not in the sympatric S form.31 In this case, sequencing of the sodium channel did not suggest introgression between forms but, rather, independent emergence of kdr in the M form and strong reproductive barriers between the M and S forms.32 In Mali, West Africa, Fanello and others33 used the distribution of the kdr allele to infer the level of reproductive isolation between sympatric S-form populations of the Bamako and Savanna chromosomal forms. In their study, the kdr allele was only found in the S-form Savanna chromosomal form, suggesting that it is strongly isolated from sympatric S-form Bamako populations in addition to being isolated from S-form Mopti populations.34 However, one limitation of that study was the relatively small number of Bamako individuals assayed for kdr.35

To assess whether the kdr allele is still increasing in frequency in Mali and whether it is still limited to the Savanna chromosomal form of An. gambiae s.s., we conducted a comparatively larger longitudinal survey of the distribution of the kdr allele in Savanna, Bamako, and Mopti form populations from locations where two or more forms co-occurred. We assayed ~1,000 DNA samples from field collections made between 1993 and 2004 for the presence of the kdr allele using the fluorescent polymerase chain reaction (PCR) developed by Tripet and others.36 This method facilitates the detection of kdr in old samples with poor DNA quality by using fluorescent primers with comparatively much higher amplification efficiency than previous PCR-based methods and by visualizing the amplified alleles on an automated sequencer.37 The results underscore the usefulness of the kdr allele as a tool for evaluating reproductive isolation between the chromosomal forms of An. gambiae and provide new insights on the patterns of spread and emergence of pyrethroid knockdown resistance in West Africa.

MATERIALS AND METHODS

Study sites. Adult females were collected by aspiration from huts during the rainy season in July 1993 and September 2004 in Banambani; August 1993, August 2002, and September 2004 in Selenkenyi; July 1996, August 2002, and September 2003 in Soulouba; and July 1996 and October 2002 in Pimperena (Figure 1). In addition, in 2002, a large number of third- and fourth-instar larvae were collected from puddles, potholes and swamps in the village of Banambani. A randomly picked subsample of these larvae was used in this study.

The villages of Banambani, Selenkenyi, and Soulouba are located in western Mali in the discontinuous habitat characteristic of the upper tributaries of the Niger River. This area features rocky outcrops and valleys and enjoys comparatively higher rainfalls (North Soudan Savanna ecological zone) than the northwestern part of the country (Sahelian and Saharian zones). The villages of Banambani and Soulouba are charac-

FIGURE 1. Geographical location of the villages of Banambani, Pimperena, Selenkenyi, and Soulouba where An. gambiae s.s. populations were sampled at various time-points between 1993 and 2004. The dashed lines roughly delimit four zones characterized by the presence of different chromosomal forms of An. gambiae. In zone S and M, the Savanna and Mopti forms are, each time, the only forms to be found. In contrast, in the M and S, and the M, S, and B zones, the Mopti and Savanna or Mopti, Savanna, and Bamako forms co-occur at various densities; hence, these zones could potentially act as corridors for the passage of pyrethroid resistance between forms.

terized by small-scale vegetable and cereal cultivation. Pimperena is located in the southern region, the rainiest area of Mali (South Soudan zone), and a region of intensive and extensive agriculture. The village of Selenkenyi is intermediate with regard to the extent of cultivations in the area. In the rainy season, An. gambiae populations from Selenkenyi are characterized by high proportions of Bamako and Mopti individuals, whereas the Savanna form occurs at low frequency (0–10%). The Savanna form is common in Banambani (10–40%) and in Soulouba (60–80%) where it co-occurs with the Mopti (5–25%) and Bamako forms (10–40%).8 In Pimperena, the Savanna form dominates with the Mopti form, occurring only at very low frequencies (0–5%).8

Identification of karyotypes and molecular forms. Polytenic chromosome preparations were made from the ovaries of half-gravid females, and DNA was extracted from the female head and thorax using established protocols.40–42 The chromosomal forms of An. gambiae and An. arabiensis were distinguished by scoring chromosomes by light microscopy. Individuals were assigned to the Bamako, Savanna, and Mopti forms of An. gambiae s.s. on the basis of their karyotypic arrangement using the classification presented by Touré and others8 with one modification. Chromosomal arrangements referred to as “hybrid” karyotypes in their publication,8 are referred to here, as “atypical arrangements” because it is now known that such arrangements do not characterize true hybrids between forms but rather rare arrangements within the established chromosomal forms.10 Individuals presented here as Bamako individuals were all homozygous for inversions j, c, and u, and polymorphic for b as described elsewhere.8 The IGS r-DNA type of all samples was determined using established PCR diagnostics.40,43

Kdr detection by PCR elongation assay. PCR elongation assays were conducted as described previously.36 The optimized PCR reaction mix for detecting the Leu-Phe substitution included 0.25 μL of the AGSWA primer (5’-GGCCACTGTAGTGATAGGAAATTTA-3’) labeled with
green fluorescence (5′-Hex modification), 0.12 μL of the AGRWA primer (5′-GGCCACTGTA GTGATAGGAAATT TT-3′) labeled with blue fluorescence (5′-Fam), 0.25 μL of the AGREV primer (5′-GCAAGGTAA GAAAAAGTTAGCA-3′), 0.45 μL of dNTPs (10 mmol/L), and 0.125 μL of Taq (Ependorf) and 1 μL of DNA template in a 25-μL reaction with 10× buffer, 5× PCR enhancer (Ependorf), and 2.5 μL magnesium chloride (25 mmol/L). PCR amplifications were done on MJ Research PTC-200 thermal cycler (MJ Research, Watertown, MA) and included an initial 2 minutes at 95°C, followed by 25 cycles of 1 minute at 95°C, 30 seconds at 63°C, 30 seconds at 72°C, and a final extension step at 72°C for 5 minutes. PCR products were diluted in H2O 10–40 times before they were mixed with Genescan 400HD size standard (Applied Biosystems, Foster City, CA) and run on an ABI 3100 capillary sequencer (Applied Biosystems). The gels were analyzed using the ABI PRISM Genescan Analysis Software (Applied Biosystems). Individuals were genotyped for kdr based on the presence and intensity of the allele specific green and blue peaks at size 110 bp as described elsewhere.39

**Statistical analyses of changes in kdr frequency.** Temporal changes in the frequency of susceptible ss, resistant rr, and heterozygote rs individuals were analyzed using logistic regression analyses. The variable collection date was considered an ordinal variable. All statistical analyses were performed using the JMP statistical software.44

**RESULTS**

A total of 944 of 970 specimens (97.3%) were successfully scored for the presence of the kdr allele using the PCR elongation assay with fluorescence.30 Five hundred thirty-eight individuals were of the S molecular form and 406 of the M molecular form. Among S form individuals, 133 were further characterized cytogenetically as belonging to the Bamako chromosomal form, 194 were of the Savanna form, and 7 exhibited atypical chromosomal arrangements that could not be assigned to either of the chromosomal forms (Table 1). The remaining 204 individuals were only characterized molecularly. Among M-form individuals, 247 were of the Mopti chromosomal form, 7 were chromosomal arrangements atypical of the Mopti form, and the remaining 152 individuals were only characterized molecularly (Table 1).

**Distribution of kdr allele among forms.** The kdr allele was found in all four localities and all years in individuals of the S molecular form except for the Selenkenyi samples collected in 1993 (Table 1; Figure 2). With all years and locations combined, an average of 26.1% of the S-form individuals

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**Table 1**

Number of individuals of S-form Bamako (Bam) and Savanna (Sav) chromosomal form individuals as well as M-form Mopti (Mop) chromosomal form individuals genotyped for kdr in the villages of Banambani, Pimperena, Selenkenyi, and Soulouba from 1993 to 2004

<table>
<thead>
<tr>
<th>Locality</th>
<th>Year</th>
<th>kdr</th>
<th>Bam</th>
<th>Sav</th>
<th>Atyp</th>
<th>Mol</th>
<th>Freq</th>
<th>Total</th>
<th>Mop</th>
<th>Atyp</th>
<th>Mol</th>
<th>Total</th>
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<tr>
<td></td>
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*Within S and M molecular forms, a few individuals exhibited arrangements that were not typical of any chromosomal forms (Atyp), and some individuals were only typed molecularly (Mol). The frequency of homozygote resistant r/r, susceptible s/s, and heterozygotes r/s is also indicated.*
carried one or two copies of the kdr allele (Table 1). Kdr was found at particularly high frequencies in the village of Pimperena, where homozygote-resistant individuals accounted for 80% in 1996 and 69% of individuals in 2002 (Table 1; Figure 2). In none of the years and none of the locations was kdr detected in sympatric populations of the M molecular form (Table 1; Figure 2).

Within the S molecular form, the majority of individuals bearing the kdr allele were of the Savanna chromosomal form (Table 1). However, kdr was also detected for the first time in the Bamako chromosomal form. Two individuals heterozygote for kdr were detected out of 31 samples collected in 2004 from Selenkenyi (6.5%). In the village of Soulouba, one heterozygous individual was found out of two individuals sampled in 1996 (50%). In the same location in 2003, two heterozygous individuals and one homozygous individual were found out of 16 Bamako individuals (18.8%). In 2004, no kdr carriers were found but sample size was again small (N = 4; Table 1; Figure 3).

**Temporal changes in kdr frequencies.** The frequency of homozygous-resistant rr and/or heterozygous rs individuals in the S molecular form increased significantly between 1993 and 2004 in the village of Banambani (logistic regression: $R^2 = 0.071$, df = 4, $\chi^2 = 13.15$, $P = 0.011$) and Selenkenyi (logistic regression: $R^2 = 0.165$, df = 4, $\chi^2 = 11.76$, $P = 0.019$; Table 1; Figure 2). A significant increase in the number of homozygous-resistant individuals was also observed in Soulouba between 1996 and 2003 (logistic regression: $R^2 = 0.044$, df = 4, $\chi^2 = 18.35$, $P = 0.001$). Note that the 1993 Banambani collection had a high proportion of Bamako chromosomal form, and this partly accounted for the extremely low kdr frequency (0.025%) observed that year compared with the 27.7% and 26% found in 2002 and 2004. When only Savanna individuals were considered (data not available for 2002), the kdr frequency increased significantly from 10% in 1993 to 54.5% in 2004 (logistic regression: $R^2 = 0.194$, df = 2, $\chi^2 = 6.49$, $P = 0.039$; Table 1; Figure 2). In Selenkenyi, no Savanna individuals were analyzed in 1993, but the kdr frequency in the Savanna form tended to increase from 15% in 2002 to 60% in 2004 (logistic regression: $R^2 = 0.208$, df = 2, $\chi^2 = 5.78$, $P = 0.056$). In this case, the small sample size was responsible for the lack of significance. In Soulouba, kdr frequency in the Savanna samples was 34.8% in 1996, 34.6% in 2002, and increased to 52.4% in 2003 (Logistic regression: $R^2 = 0.051$, df = 4, $\chi^2 = 12.71$, $P = 0.013$). In Pimperena, where the Savanna chromosomal form is the only S-form present, the frequencies of heterozygote and homozygote S-form individuals remained high and unchanged between 1996 (89.0%) and 2002 (83.0%; logistic regression: $R^2 = 0.016$, df = 2, $\chi^2 = 2.49$, $P = 0.287$; Table 1; Figure 2).
DISCUSSION

The results presented here provide the first evidence of the presence of knockdown resistance to pyrethroid insecticides (*kdr*) in the Bamako chromosomal form of *An. gambiae* s.s. In a previous study conducted in Mali, Fanello and others detected the *kdr* allele solely in the Savanna chromosomal form. They found that the *kdr* allele was already present in the village of Banambani in 1987 and that its frequency increased steadily from 2.8% in 1987 to 62.1% in 2000. In this study, we found similar evidence of the rapid spread of *kdr* in the Savanna form in Banambani between 1993 and 2004. The village features small-scale vegetable cultivation, and pyrethroid application is restricted to limited individual use. The increase observed in such setting is not unique as we report a sharp increase in resistance in Soulobua, another village characterized by low agricultural and domestic pyrethroid use. In these two locations, the selection pressure associated with limited applications was presumably strong enough to drive the *kdr* allele to frequencies higher than 50%. Typical use of pyrethroids in the two studied villages include punctual treatment of plots of vegetables or cereals against herbivorous insect pests, occasional indoor spraying aimed at reducing mosquito burden, indoor “spray catches” conducted for scientific research, as well as some use of pyrethroid-treated bednets. In contrast, the area of Pimperena is characterized by intensive and extensive cotton cultivation combined with widespread application of organophosphates and pyrethroids targeting herbivorous insects. As expected, the frequency of the *kdr* allele in that area was already high in 1996 and remained above 80%. Not surprisingly, in Selenkenyi, where the extent of cultivation and pyrethroid use is intermediate, the frequency of *kdr* within the Savanna form was also observed to be intermediate. Note that because *kdr* confers cross-resistance to DDT, it is not clear how much the past and possibly recent use of DDT in agriculture has affected the current distribution of *kdr* in Mali and possibly other West-African countries.

That the *kdr* allele was found in several Bamako populations from Mali suggests that the frequency of resistant individuals in mixed S-form Savanna and Bamako populations could reach new levels in the near future. Indeed, in the western part of the country where it is associated with riverine zones of the upper Niger River, the Bamako form makes up a large proportion of *An. gambiae* populations throughout the rainy season. The consequences of such an increase in *kdr* frequency for current and future vector control projects in the area remains to be evaluated. Our data from Selenkenyi suggest that the *kdr* allele emerged only recently in the Bamako form. However, because of small sample sizes we cannot be sure if that was also the case for the Bamako form in Soulouba nor that the *kdr* allele is currently absent in that form in Banambani.

The observation that *kdr* is now found in the Bamako form but is still absent from the Mopti form suggests different levels of reproductive isolation between the three chromosomal forms in Mali. Laboratory studies have shown that the *kdr* allele can readily pass from the Savanna to the Mopti form through experimental interbreeding. This is also true for the r-DNA locus on the basis of which the molecular forms have been described. Because some M/S hybrids have been found in nature, it is thought that reproductive isolation can only be maintained if hybrids exhibit reduced fitness. Thus, the fact that after more than a decade of existence in the Savanna form *kdr* is still not found in the Mopti form may reflect either strong selection against M/S hybrids at the r-DNA locus or strong selection against *r/s* hybrids at the *kdr* locus, or possibly both. The two loci are located near the centromeres of the second and X chromosomes, two regions of the genome that are differentiated between the M and S forms and may contain genes responsible for speciation and ecological adaptation. Their physical location may thus hinder introgression more difficult because of selection against recombinants. The *kdr* allele has nevertheless recently been found at low frequency in several M-form populations co-occurring with resistant S-form Savanna and Forest populations. Evidence from patterns of polymorphisms in the upstream sequence of the sodium channel gene strongly suggests that its emergence is caused by introgression between the two molecular forms. Furthermore, the geographical and chromosomal form distribution of M form populations that bear the *kdr* mutation suggests that introgression occurred several times rather than in a single introgression event followed by a genetic sweep. Importantly, the presence of alternative pyrethroid detoxification mechanisms detected in some M-form populations lacking *kdr* might reduce the fitness advantage conferred by the *kdr* allele in areas of heavy pyrethroid use and thus decrease the likelihood of *kdr* introgression from resistant S-form populations. Further studies should determine whether such a mechanism is responsible for preventing the introgression of *kdr* between the M and S forms in Mali.

Although we suggest that *kdr* occurred in the Bamako form through introgression from the Savanna form, we cannot strictly exclude the possibility that it occurred independently because susceptible Bamako populations and resistant Savanna chromosomal forms are identical with regard to sequence polymorphisms in the sodium channel gene. Consequently, sequencing in this case cannot be used to distinguish between these two alternate hypotheses. The introgression hypothesis, however, is the most plausible one and is compatible with the lack of differentiation observed between the Savanna and Bamako forms at neutral microsatellite loci (Tripet pers. obs.) and at the r-DNA locus. In the context of prospective transgenic vector control projects, current introgression would alleviate at least some of the hurdles associated with spreading beneficial genes through the complex *An. gambiae* populations of the upper Niger River area. More prosaically, classic vector control measures such as ITN and IRS could face a rapid decrease in efficacy in that area.

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