A significant increase in kdr in Anopheles gambiae is associated with an intensive vector control intervention in Burundi highlands

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Summary

OBJECTIVES AND METHODS In Burundi, the occurrence of the knock down resistance (kdr) mutation in Anopheles gambiae sensu lato (s.l.) was determined for six consecutive years within the framework of a vector control programme. Findings were also linked with the insecticide resistance status observed with bioassay in An. gambiae s.l. and An. funestus.

RESULTS The proportion of An. gambiae s.l. carrying the East Leu-Ser kdr mutation was 1% before the spraying intervention in 2002; by 2007 it was 86% in sprayed valleys and 67% in untreated valleys. Multivariate analysis showed that increased risk of carrying the kdr mutation is associated with spraying interventions, location and time. In bioassays conducted between 2005 and 2007 at five sites, An. funestus was susceptible to permethrin, deltamethrin and DDT. Anopheles gambiae s.l. remained susceptible or tolerant to deltamethrin and resistant to DDT and permethrin, but only when kdr allele carriers reached 90% of the population.

CONCLUSIONS The cross-resistance against DDT and permethrin in Karuzi suggests a possible kdr resistance mechanism. Nevertheless, the homozygous resistant genotype alone does not entirely explain the bioassay results, and other mechanisms conferring resistance cannot be ruled out. After exposure to all three insecticides, homozygote individuals for the kdr allele dominate among the surviving An. gambiae s.l. This confirms the potential selection pressure of pyrethroids on kdr mutation. However, the high occurrence of the kdr mutation, even at sites far from the sprayed areas, suggests a selection pressure other than that exerted by the vector control programme.

keywords Anopheles sp., knockdown resistance, insecticide resistance, indoor residual spraying, insecticide treated net, Burundi

Introduction

Vector control is an essential component of the WHO Global Strategy to roll back malaria. Many studies have shown the efficacy of indoor residual spraying (IRS) and insecticide treated net (ITN) in reducing malaria transmission and prevalence (Barutwanayo et al. 1991; Lengeler 2004; Protopopoff et al. 2007). However, these methods, especially ITNs, rely on the use of pyrethroid insecticides, and emergence of pyrethroid resistance in vector populations is a major concern for the sustainability of malaria prevention in Africa.

Resistance to pyrethroids in Anopheles gambiae sensu lato (s.l.) and to a lesser extent in An. funestus has become widespread in Africa (Vulule et al. 1994; Chandre et al. 1999; Hargreaves et al. 2000, 2003; Stump et al. 2004; Etang et al. 2006). Metabolic-based mechanisms and/or a mutation in the sodium channel insecticide target site are responsible for pyrethroid resistance in An. gambiae s.l. (Liu et al. 2006; Etang et al. 2007). Knockdown resistance (kdr) is caused by a single mutation in the sodium channel, resulting in a leucine to phenylalanine (West Africa mutation) or to serine (East Africa mutation) change. These two mutations have been held responsible for cross-resistance
against DDT and pyrethroid insecticides (Martinez-Torres et al. 1998; Ranson et al. 2000). However, the effect of knockdown resistance on the vector control efficacy remains uncertain. In some countries, ITNs can still provide individual protection against kdr resistant Anopheles populations (Darriet et al. 2000; Henry et al. 2003; Dabire et al. 2006) although more recently studies have shown reduced efficacy where the West African kdr mutation is high (Mahama et al. 2007; Sharp et al. 2007). The impact of the East African kdr mutation on intervention is unknown.

Resistance in the Anopheles species seems to be associated with the agricultural use of insecticides (Mouchet et al. 1998; Diabate et al. 2002). Nevertheless, evidence exists for the selection of kdr alleles associated with the massive use of ITNs or impregnated plastic sheeting (Stump et al. 2004; Diabate et al. 2006). For insecticide resistance management, it is essential to know where the selective pressure on Anopheles comes from.

A targeted vector control intervention combining IRS and ITN was carried out in the highland province of Karuzi (Burundi) between 2002 and 2005 (Protopopoff et al. 2007) with surveillance continuing for 2 additional years. The objective of this study was to determine the relative impact of these interventions on the development of insecticide resistance by monitoring the kdr mutation in An. gambiae s.l. as marker of insecticide pressure, and to link these findings with the insecticide resistance status observed in An. gambiae s.l. and An. funestus as defined by bioassays at the end of the intervention period. The occurrence of the kdr mutation in specimens (homozygote or heterozygote) was preferred to kdr allele frequency for statistical analysis purposes.

**Methods**


In the central highland Karuzi province (2°55’–3°23’ S, 29°54’–30°21’ E), a 4 year vector control programme based on IRS and distribution of long lasting insecticidal nets (LNs; Permanet® I; Vestergaard Frandsen, Lausanne, Switzerland) was carried out between 2002 and 2005 (Protopopoff et al. 2007). Karuzi is a hilly area with a surface of 1457 km²; the valleys are at 1400–1680 m altitude. The temperature varies from 11 to 28 °C with an annual average of 19 °C. At this low temperature, the vectors are highly endophilic and clustered around the breeding sites in the valley bottom. Therefore, the intervention was targeted to valleys with the highest risk for malaria. IRS was carried out once a year in all human dwellings and cattle sheds of the targeted area (264 km², about 18 000 households) with the residual insecticides deltamethrin 5 Wettable Powder (WP) (from 2002 to 2004) and alpha cypermethrin 5WP (in 2005) at the dose of 25 mg a.i./m². Between 2002 and 2005, respectively, 754, 745, 1023 and 1080 kg of insecticide were used. Overall, 24 000 LNs were also distributed during the first year in household selected for spraying.

Between 2002 and 2007, two entomological surveys per year (in April-May and in November-December) using the pyrethrum spray catches took place to monitor adult Anopheles mosquitoes in treated and untreated areas (sampling was carried out at an altitude of 1396–1717 m) (Protopopoff et al. 2007). One baseline survey was conducted before the intervention (July 2002), eight surveys were done 3 and 9 months after the annual spray round and two surveys were carried out after the end of the intervention. For each survey, 25 clusters of four to eight houses were randomly chosen in the treated and untreated valleys. Specimens of An. gambiae s.l. were further analysed for the occurrence of the kdr mutation after molecular identification.

**WHO insecticide susceptibility bioassays**

Between 2005 and 2007, live indoor resting mosquitoes were collected by suction tubes in five sites to assess the resistance status of the vector species by WHO tube bioassay. Because only few mosquitoes could be collected in the treated province of Karuzi in 2005–2006, three sites were chosen in two communes of the neighbouring province of Gitega, just outside the treated area: commune Mutaho (site 1: 3°09’ S, 29°90’ E in 2003) and commune Gitega (site 2: 3°38’ S, 30°00’ E in 2005 and site 3: 3°42’ S, 30°02’ E in 2006). After the end of the spraying activities, a sufficient number of Anopheleses could be collected in two sites in Karuzi, one in a previously treated area (site 4: 3°01’ S, 30°16’ E) and one in untreated area (site 5: 3°00’ S, 30°19’ E) (Figure 1). Individual Anopheleses was identified using a simplified morphological key adapted from Gillies and Coetsee (1987). Morphologically identified An. gambiae s.l. and An. funestus were subjected to standard WHO (1998) bioassays with discriminative dosage of DDT (4%), permethrin (0.75%) and deltamethrin (0.05%). The bioassay kit, impregnated and control papers were supplied by Universiti Sains Malaysia, Penang, Malaysia. Anopheles mosquitoes were exposed to the insecticide for 1 h. Mortality was scored after a 24-h holding period during which the Anopheles had access to 10% sugar solution. Tests with control mortality above 10% were excluded. The bioassay results were divided into three mortality categories according to the WHO (1998) criteria: <80% 24-h post-exposure indicates resistance, 80–97% indicates potential resistance needing confirmation, ≥98% indicates a susceptible population.
Molecular identification and knockdown resistance detection

*An. gambiae* s.l. and *An. funestus* mosquitoes were morphologically identified. Samples of *An. gambiae* complex collected during the surveys and for the bioassays were tested using a Polymerase Chain Reaction (PCR) adapted from Scott *et al.* (1993) to distinguish the different member species. M and S molecular forms (Favia *et al.* 1997) were identified on 119 *An. gambiae* sensu stricto (s.s.) with different *kdr* genotypes collected during the surveys. On the whole, 222 *An. funestus* from the bioassays were identified following the protocol of Garros *et al.* (2004) to distinguish the different *kdr* genotypes and described in Verhaeghen *et al.* (2006). A Fluorescence Resonance Energy Transfer/Melt Curve Analysis assay (FRET/MCA) (Verhaeghen *et al.* 2006) was used to detect the East and West African *kdr* mutation in all the *An. gambiae* s.l. that survived the bioassay tests and in a fraction (1/3) of the dead mosquitoes. The FRET/MCA technique was also used for quality control of the allele-specific PCR on a sample of the survey specimens (*n* = 264) and to check for the possible occurrence of the West African *kdr* mutation (*n* = 1082, combination of surveys and bioassays). Homozygote and heterozygote *An. gambiae* s.l. for the *kdr* mutation are presented as RR and RS and absence of *kdr* mutation by SS.

Statistical analysis

The proportion of *An. gambiae* s.l. collected in the spray-catch surveys that had either the homozygous resistant (RR) or heterozygous (RS) *kdr* genotype was analysed in a robust multivariate logistic regression in Stata 9 (Stata-Corporation, Lakeway, Texas, USA, version 9.2). Communes, year of collection (two surveys a year) and vector control activities (intervention vs. control valleys) were used as discrete explanatory variables. Clusters were defined as primary sampling units and sampling weights were used to correct for the proportion of the mosquitoes tested from each house. Genotype frequencies between dead and alive *An. gambiae* s.l. in bioassays were compared using the software GENEPOP (version 3.4; Laboratoire de Génétique et environment, Montpellier, France). The global estimation of the *kdr* occurrence in the *An. gambiae* s.l. population was obtained from a weighted average of the proportions of dead and alive *An. gambiae* s.l. carrying the *kdr* allele.

Results

*kdr* mutation during the intervention and post-intervention periods

We caught 9473 *An. gambiae* s.l. females during the eleven surveys. On specimen identified by PCR (*n* = 4225) only 74 (1.8%) were *An. arabiensis*. Only the molecular S form of *An. gambiae* s.s. was found. None of the *An. arabiensis* tested (*n* = 36) carried the East or West African *kdr* mutation. Using the FRET/MCA, the West African *kdr* mutation was not identified in the screened *An. gambiae* s.s. The quality control, done with the FRET/MCA, showed only one discrepancy (*n* = 264) for the East African *kdr* mutation with the result of allele-specific PCR. Before the start of the intervention, the East African *kdr* allele was detected in 1% (4/404) of the *An. gambiae* s.l. and only in heterozygous genotypes. Between 2002 and 2004 and in the intervention valleys, the *kdr* mutation...
increased 3 months after the spray round and decreased 6 months later (Figure 1). However, from 2005 onwards, the number of kdr carriers steadily raised in both treated and control valleys.

As shown in Figure 2 (first 3 blocks), the SS genotype in An. gambiae s.l. was predominant in the entire province from 2002 to 2004. Homozygote for the kdr mutation (RR) appeared by the end of 2004. Between 2006 and 2007, RR and RS genotypes became predominant in most districts. Location (communes), time (year) and spraying were positively associated by multivariate analysis with the proportion of the An. gambiae s.l. carrying the kdr mutation (Table 1). Treated valleys were at greater risk to have An. gambiae s.s. carrying the kdr mutation (OR: 2.7, 95% CI: 1.4–5.2). When compared with the year 2002, this risk increased significantly after 2004, and peaked in 2007 (OR: 168.6, 95% CI: 70.2–405.1).

Resistance status of Anopheles gambiae sensu lato and Anopheles funestus as defined by bioassays

Morphological identification was good, as only 2 specimens of 711 molecularly tested An. gambiae s.l and 4 of 222 An. funestus (1.8%) were misclassified. Anopheles arabiensis comprised only 1% (7/709) of the An. gambiae complex. Anopheles funestus was almost susceptible (Table 2) to 4% DDT, 0.75% permethrin and 0.05% deltamethrin (mortality >95%); An. gambiae s.l. was susceptible to deltamethrin at all sites, except at site 4 (Karuzi), where possible resistance can occur. Outside the province, only suspected permethrin resistance (mortality >80%) was observed for An. gambiae s.l. except for site 4 in Karuzi, where high permethrin resistance was detected (mortality of 57%). DDT resistance was similarly high in Karuzi (site 4 and site 5), and possible resistance is observed in site 3 (Table 2).

At sites 1 to 5, the kdr mutation was present in 64.9%, 22.6%, 25.2%, 97.6% and 89.6% of the An. gambiae s.l. specimens. The RR genotype was largely predominant in site 4 and 5 (Figure 2, block 4). No kdr mutations were observed in An. arabiensis (n = 6). The frequency of kdr genotype in dead and alive mosquitoes 24 h post-exposure and by insecticide is presented in Figure 3. The proportion of kdr genotypes were significantly different between survivors and non-survivors and this for all insecticides tested. In mosquitoes that survived the frequency of RR genotype was 75%, 93% and 100% after exposure to permethrin, DDT and deltamethrin, respectively. Furthermore, SS genotype was mostly found in dead Anopheles, although RR genotype occurred also in dead An. gambiae s.l.

Discussion

Selection of the knockdown resistance mutation in West Africa has been mainly attributed to the intensive use of DDT and pyrethroids in agriculture and to DDT-based vector control campaigns of the 1950s (Akogbeto et al. 2005; Tia et al. 2006).

Before vector control, the East African kdr mutation occurred in 1% of An. gambiae s.l. in Karuzi. Between 2002 and 2004, its frequency increased temporarily 3 months after each spray round and fell to baseline values 9 months later. This phenomenon lasted only for 2 years. Indeed, from the second half of 2004 onwards, a steady increase of the kdr mutation carriers was observed, in both treated and untreated valleys. The increase was higher in treated valleys, reaching 60% in less than 3 years. It has been argued that IRS exerts a much stronger selective pressure than ITNs for insecticide resistance because resistant fed females would fly away from treated surfaces of sprayed houses while unfed females searching for a blood meal would have repeated and longer contacts on ITNs and would be killed as readily as susceptible ones (Chandre et al. 2000; Diabate et al. 2006). Indeed, in our study the occurrence of the kdr mutation was not significantly different in houses having at least one ITN than in those with no ITN. The high percentage of resistant homozygous An. gambiae s.l. alive after exposure to deltamethrin in the bioassays could indicate the strong selective pressure exerted by the IRS, although this conclusion is based only on seven survivors An. gambiae s.l.

The spread of resistance genes in a treated region will depend on the initial kdr frequency, the degree of dominance of kdr allele and the importance of migration relative to the selection pressure (May & Dobson 1986). The steady increase of the prevalence of the kdr mutation observed in both treated and untreated valleys may be explained by several factors. The kdr mutation may have migrated from treated to untreated valleys, explaining the parallel increase in these areas, although this occurred only after the third IRS round. Conversely, the greater frequency of kdr in An. gambiae specimens in treated valleys, despite the fact that they are interspersed with untreated valleys, suggests restricted migration of An. gambiae s.l. preventing a massive influx of susceptible individuals from the untreated areas. Once the kdr allele frequency reaches a certain threshold, and this combined with a drastic decrease of vector densities by IRS, an exponential increase of the resistant forms can be observed in a short period of time. May & Dobson (1986) stated that when the dominance of the resistant allele is low (<0.5), which is the cases for the kdr allele (0.41 reported in Culex pipiens and
Figure 2 Occurrence of the East African Leu-Ser kdr mutation in *Anopheles gambiae* s.l. in intervention (I) and control (C) valleys between 2002 and 2007. Arrows represent the spraying times. The global estimation of the *kdr* occurrence in the *An. gambiae* s.l. population was obtained from a weighted average of the proportion of mosquitoes tested from each house.
Aedes aegypti exposed to permethrin (Bourguet & Raymond 1998), the system settles to a state of high kdr frequency if migration is small and selection overcomes gene flow. If migration is restricted, the selection pressure in the untreated valleys may be caused by selection pressure other than the one induced by IRS. Indeed, the high occurrence of kdr mutation observed in the neighbouring province (up to 69% in Mutaho site 1 in 2005), far from the treated valleys, suggests that selection of the resistant form has been caused by pyrethroids used for other purposes than the IRS, although it is difficult to identify the specific activity with the present study. The only record of massive insecticide use in this area was in 1956 when all the houses of Burundi up to an altitude of 2000 m were treated with DDT (Coosemans 1985). Since then, no specific vector control has taken place in the highlands. Domestic use of insecticide (mosquito coils, aerosols) was rare or non-existent during the study period. In this region, the only official record of insecticide use was treating coffee crops for export with lambda-cyhalothrin.

### Table 1
Multivariate analysis showing the risk (OR) to have Anopheles gambiae sensu lato (s.l.) carrying the kdr allele (either in the heterozygous or homozygous form) in relation to vector control activities, location and time

<table>
<thead>
<tr>
<th>Occurrence of kdr mutation (%)</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td><strong>Valleys</strong></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>1233</td>
</tr>
<tr>
<td>Treated</td>
<td>566</td>
</tr>
<tr>
<td>Net used</td>
<td>0</td>
</tr>
<tr>
<td>≤1</td>
<td>135</td>
</tr>
<tr>
<td><strong>Communes</strong></td>
<td></td>
</tr>
<tr>
<td>Mutumba</td>
<td>554</td>
</tr>
<tr>
<td>Shombo</td>
<td>428</td>
</tr>
<tr>
<td>Buhiga</td>
<td>415</td>
</tr>
<tr>
<td>Nyabikere</td>
<td>169</td>
</tr>
<tr>
<td>Bugenyuzi</td>
<td>93</td>
</tr>
<tr>
<td>Gitaramuka</td>
<td>140</td>
</tr>
<tr>
<td><strong>Years</strong></td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>395</td>
</tr>
<tr>
<td>2003</td>
<td>220</td>
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<tr>
<td>2004</td>
<td>377</td>
</tr>
<tr>
<td>2005</td>
<td>411</td>
</tr>
<tr>
<td>2006</td>
<td>268</td>
</tr>
<tr>
<td>2007</td>
<td>128</td>
</tr>
</tbody>
</table>

### Table 2
WHO susceptibility test results on Anopheles funestus and Anopheles gambiae sensu lato (s.l.), reporting the percentage mortality 24 h post-exposure in different sites

<table>
<thead>
<tr>
<th>Species</th>
<th>Locations</th>
<th>Sites no.* (years)</th>
<th>n</th>
<th>% Mortality (no.)</th>
<th>n</th>
<th>% Mortality (no.)</th>
<th>n</th>
<th>% Mortality (no.)</th>
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<tbody>
<tr>
<td><strong>An. funestus</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutaho</td>
<td>1 (2005)</td>
<td>99</td>
<td>98 (97)</td>
<td>94</td>
<td>99 (93)</td>
<td>104</td>
<td>100 (104)</td>
<td></td>
</tr>
<tr>
<td>Gitega</td>
<td>2 (2005)</td>
<td>–</td>
<td>–</td>
<td>60</td>
<td>100 (60)</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 (2006)</td>
<td>92</td>
<td>98 (90)</td>
<td>86</td>
<td>97 (83)</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Karuzi</td>
<td>4 (2007)</td>
<td>96</td>
<td>97 (93)</td>
<td>94</td>
<td>99 (93)</td>
<td>86</td>
<td>100 (86)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 (2007)</td>
<td>81</td>
<td>95 (77)</td>
<td>83</td>
<td>100 (83)</td>
<td>101</td>
<td>100 (101)</td>
<td></td>
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<tr>
<td><strong>An. gambiae</strong></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Mutaho</td>
<td>1 (2005)</td>
<td>102</td>
<td>98 (100)</td>
<td>153</td>
<td>87 (133)</td>
<td>80</td>
<td>99 (79)</td>
<td></td>
</tr>
<tr>
<td>Gitega</td>
<td>2 (2005)</td>
<td>31</td>
<td>100 (31)</td>
<td>83</td>
<td>93 (77)</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>3 (2006)</td>
<td>101</td>
<td>96 (99)</td>
<td>107</td>
<td>84 (90)</td>
<td>101</td>
<td>100 (101)</td>
<td></td>
</tr>
<tr>
<td>Karuzi</td>
<td>4 (2007)</td>
<td>98</td>
<td>58 (57)</td>
<td>189</td>
<td>57 (108)</td>
<td>177</td>
<td>94 (167)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 (2007)</td>
<td>19</td>
<td>79 (15)</td>
<td>–</td>
<td>–</td>
<td>20</td>
<td>100 (20)</td>
<td></td>
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</tbody>
</table>

*Site location can be found on Figure 2 part bioassays, n = sample size.
The West African kdr mutation has been held responsible for the decrease efficacy of IRS against An. gambiae in Equatorial Guinea (Sharp et al. 2007) and Benin (N’Guessan et al. 2007) and the East African kdr mutation could have a similar effect in Burundi. It is therefore remarkable that in Karuzi, after intensive use of type II (α-cyano-) synthetic pyrethroids in the IRS campaign during 5 years, mosquitoes were still extremely susceptible to deltamethrin, as shown by the bioassays 2 years after stopping the intervention, despite the presence of the East African kdr mutation in 97% of the An. gambiae s.l. This confirms the observations of Reimer et al. (2008) that in populations with high kdr frequency, type II pyrethroids would be more efficacious than type I (e.g. permethrin) or DDT. Therefore, using the East African kdr mutation as a marker of pyrethroid resistance must be employed with caution. Probably, the lower-than-expected efficacy observed has several, non-mutually exclusive explanations, i.e. a general increase of the Anopheles population due to meteorological factors and/or the decrease of the LNs coverage, LNs use having an additional impact on Anopheles reduction in sprayed houses (Protopopoff et al. 2007). Because this increase was observed in all intervention areas, a lower quality of spraying was excluded.

In Burundi, the national malaria prevention programme is based on LN distribution to children and pregnant women and on IRS (pyrethroids) in the high-risk areas. Assessing and monitoring insecticide resistance in the malaria vectors should be a priority for the sustainability of the current malaria preventive activities in Burundi. Moreover, resistance management strategies should be implemented to delay emergence or expansion of insecticide resistance. Pyrethroids should be reserved only for net treatment, while non-pyrethroids such as carbamates or organophosphates should be used for IRS. Rotation, mixtures or mosaics of different classes of insecticide with different target sites should also be further evaluated for resistance management in the future.

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