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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.* 2005; 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 1988; 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

Intervention programme in Karuzi (2002–2005)

In the central highland Karuzi province (2°54′–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 km^2; 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 km^2, 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./m^2. 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 2005) 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 Anophelines 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 *Anopheles* was identified using a simplified morphological key adapted from Gillies and Coetzee (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.

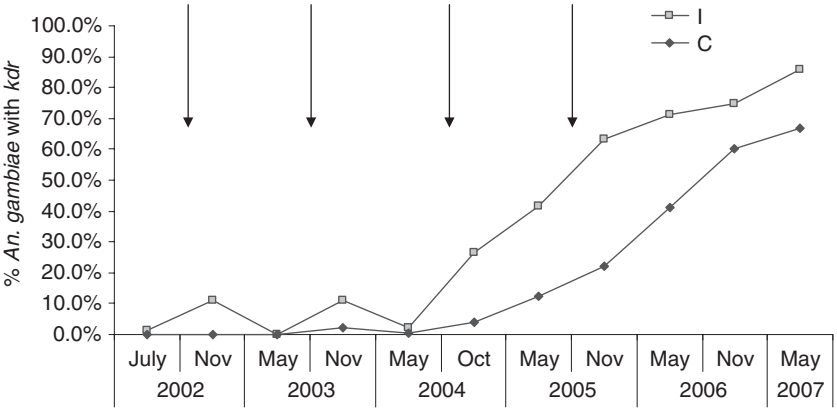

Figure 1 Distribution of the *kdr* genotypes of the wild caught *An. gambiae* s.l. collected in surveys (first three blocks) and in samples bioassayed (last block). The pie charts show the relative *kdr* genotypes proportion. Homozygotes for the *kdr* mutation (RR) are in black, heterozygotes (RS) in grey and susceptible homozygotes (SS) in white. Results were summed to reach atleast ten *Anopheles* tested (when fewer were tested, the numbers are displayed on the map).

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 assess the reliability of the morphological identification.

The East African *kdr* mutation in *An. gambiae* s.s. and *An. arabiensis* on specimens collected during the entomological surveys was detected using an adapted version of the allele-specific PCR developed by Ranson *et al.* (2000) 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 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

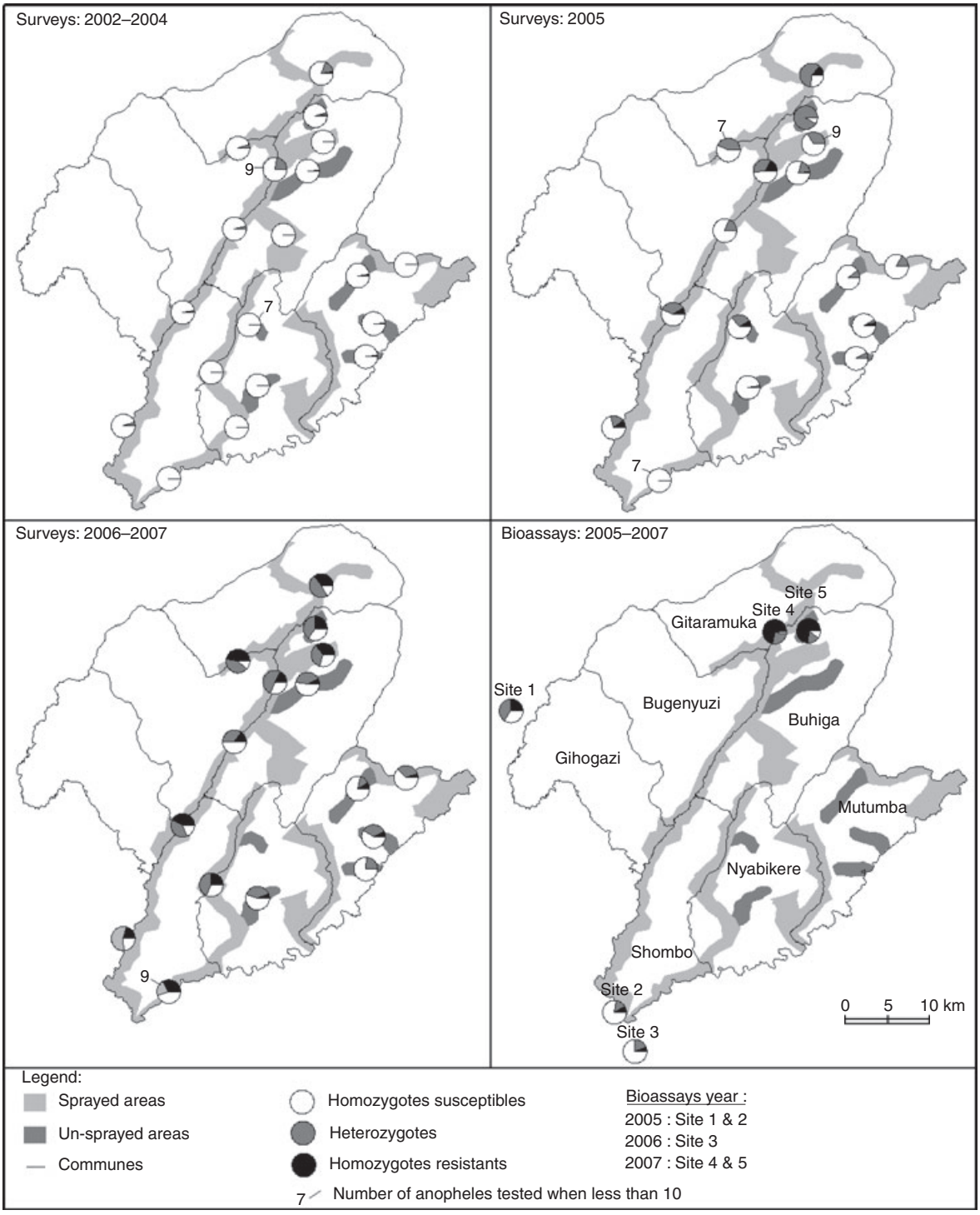

Figure 2 Occurrence of the East African Leu-Ser *kdr* mutation in *Anopheles gambiae* s.l. in intervention (I) and control (C) valleys between 2002 and 2007. Arrows represent the spraying times. The global estimation of the *kdr* occurrence in the *An. gambiae* s.l. population was obtained from a weighted average of the proportion of mosquitoes tested from each house.

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

	n	Occurrence of *kdr* mutation (%)	Multivariate analysis		
			OR	CI 95%	*P*-value
Valleys					
Untreated	1233	13.3	1.0		0.003
Treated	566	53.3	2.7	1.4–5.2	
Net used					
0	1664	19.0	1.0		0.357
≤1	135	35.0	0.7	0.4–1.4	
Communes					
Mutumba	554	5.7	1.0		<0.001
Shombo	428	20.5	2.4	1.1–5.4	
Buhiga	415	23.1	2.7	1.3–5.8	
Nyabikere	169	35.0	3.3	1.8–5.9	
Bugenyuzi	93	52.8	3.7	1.8–7.6	
Gitaramuka	140	70.5	6.3	2.5–15.8	
Years					
2002	395	1.1	1.0		<0.001
2003	220	1.0	1.2	0.3–4.2	
2004	377	1.8	2.3	0.9–5.7	
2005	411	23.5	22.5	9.6–53.1	
2006	268	52.8	62.6	28.9–135.8	
2007	128	82.6	168.6	70.2–405.1	

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

			DDT 4%		Permethrin 0.75%		Deltamethrin 0.05%	
Species	Locations	Sites no.* (years)	*n*	% Mortality (no.)	*n*	% Mortality (no.)	*n*	% Mortality (no.)
An. funestus	Mutaho	1 (2005)	99	98 (97)	94	99 (93)	104	100 (104)
	Gitega	2 (2005)	–	–	60	100 (60)	–	–
		3 (2006)	92	98 (90)	86	97 (83)	–	–
	Karuzi	4 (2007)	96	97 (93)	94	99 (93)	86	100 (86)
		5 (2007)	81	95 (77)	83	100 (83)	101	100 (101)
An. gambiae	Mutaho	1 (2005)	102	98 (100)	153	87 (133)	80	99 (79)
	Gitega	2 (2005)	31	100 (31)	83	93 (77)	–	–
		3 (2006)	101	96 (99)	107	84 (90)	101	100 (101)
	Karuzi	4 (2007)	98	58 (57)	189	57 (108)	177	94 (167)
		5 (2007)	19	79 (15)	–	–	20	100 (20)

*Site location can be found on Figure 2 part bioassays, *n* = sample size.

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

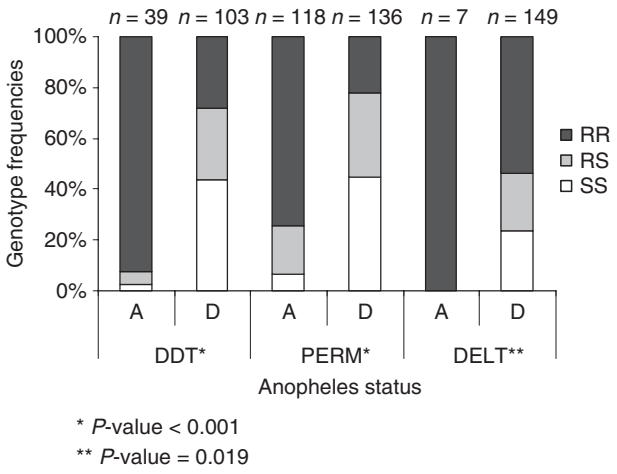

Figure 3 *Kdr* genotypes frequency found in live (A) and dead (D) *An. gambiae* s.l. 24 h after exposure to discriminative dose of insecticides. The *P*-value indicates significance in the difference in genotypes frequency between dead and alive mosquitoes.

while the extremely poor population did not have access to insecticide for treating subsistence crops. Moreover, although the number of coffee stalks has always been more important in the northern part of the province, this does not correlates with the occurrence of the *kdr* mutation.

In bioassays, the homozygous resistant genotype dominates among survivors, but does not entirely explain the bioassay results. For other mosquito species as well as, no clear correlation was described between the presence of a *kdr* mutation and the resistance phenotype (McAbee *et al.* 2004; Xu *et al.* 2006). In *Culex quinquefasciatus*, a high correlation was only found between *kdr* allelic expression and levels of insecticide resistance via transcriptional regulation (Xu *et al.* 2006). However, in our study, it cannot be ruled out that in addition to the *kdr*, metabolic-based resistance mechanisms may also be involved. Bioassays in Mutaho and Gitega show a possible association of the *kdr* mutation with permethrin resistance but not with DDT resistance, and in Karuzi high level of *kdr* mutation coincides with a similar level of resistance for DDT and permethrin. This contradicts Ranson *et al.* (2000) who found that the East African *kdr* mutation conferred DDT resistance, and to a lesser extent permethrin resistance.

Indoor residual spraying efficacy changed during the study period. Whereas the *Anopheles* density during the first 3 years of the spraying campaign was reduced to less than 0.5/house, it was higher than 1/house in the three surveys in 2005 and 2006, although still significantly lower than the untreated valleys (Protopopoff *et al.* 2007). 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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