Multiple insecticide resistance mechanisms in *Anopheles gambiae* and *Culex quinquefasciatus* from Benin, West Africa

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Abstract

Because free-insecticide treated net distribution is planned in Benin (West Africa) during the next few years, we investigated the type, frequency and distribution of insecticide resistance mechanisms in *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes in four localities selected on the basis of contrasting agricultural practices, use of insecticides and environment. Bioassays with WHO diagnostic test kits were carried out using pyrethroid, carbamate, organophosphate and organochlorine insecticides. *An.* *gambiae* mosquitoes were identified to species and to M or S molecular forms using PCR techniques. Molecular and biochemical assays were carried out to identify *kdr* and *Ace.1* mutations in individual mosquitoes and to detect any increase in the activity of enzymes typically involved in insecticide metabolism (oxidase, esterase and glutathion-S-transférases). WHO diagnostic tests showed high frequency of resistance in *An. gambiae* and *Cx. quinquefasciatus* to permethrin and DDT in three areas. This was consistent with the presence of target site insensitivity due to *kdr* mutation and to increased metabolism through enzymatic activity. *Kdr* was expressed in both M and S forms. However, less than 1% of *An. gambiae* or *Cx. quinquefasciatus* showed the presence of the *Ace.1*R mutation. Carbamate/OP resistance was present at higher frequency in *Culex* than in *An. gambiae*. Dieldrin resistance was present in both species at all four localities. A higher frequency of pyrethroid-resistance was found in *An. gambiae* mosquitoes collected in urban areas compared to those collected in rice growing areas. The expansion of vegetable growing within urban areas probably contributed to selection pressure on mosquitoes. The detection of multiple resistance mechanisms in both *An. gambiae* and *Cx. quinquefasciatus* in Benin may represent a threat for the efficacy of ITNs and other forms of vector control such as indoor residual spraying in the future.

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1. Introduction

Malaria is a major public health problem in Benin, particularly in the high transmission coastal and lagunar areas of the country (Velema et al., 1991). The strategy of the national malaria control programme is based on effective case management and the use of insecticide
treated nets (ITNs) among vulnerable groups. In the last World Malaria Report published in 2005, WHO reported that less than 10% of children under five slept under ITNs in Benin, far below the 60% target of the Abuja declaration (WHO, 2000). The proportion of pregnant women sleeping under ITN (3.8%) is equally low (Kinde-Gazard et al., 2004).

National campaigns of free or highly-subsidized ITN distributed in Mali (2003), Zambia (2003) and Togo (2004) achieved scaling-up of ITN (>60%) coverage (WHO, 2005). Pyrethroids are currently the only insecticides advocated by WHOPES for ITNs (Zaim et al., 2000). However the development of pyrethroid-resistance in the primary malaria vectors, Anopheles gambiae s.l. and An. funestus (Chandre et al., 1999; Hargreaves et al., 2000), is of grave concern. Pyrethroid-resistance has been reported in An. gambiae in many African countries including Kenya (Vulule et al., 1999), Côte d’Ivoire (Elissa et al., 1993), Benin (Akogbêto and Yakoubou, 1999), Burkina faso (Diabate et al., 2002a), Mali (Fanello et al., 2003), Nigeria (Awolola et al., 2002) and Cameroun (Etang et al., 2003). The strong level of pyrethroid-resistance in Culex quinquefasciatus in Africa (Chandre et al., 1998) also represents an obstacle to malaria prevention as people may not perceive the personal protective effect of ITNs if Culex fails to be killed. There are two main mechanisms involved in pyrethroid-resistance: an increase of detoxification and/or metabolism through high levels of multi-function oxidase (MFO) and non-specific esterase (NSE) (Vulule et al., 1999; Etang et al., 2003) and alterations at site of action in the sodium channel, i.e. the kdr mutation (Chandre et al., 1999; Ranson et al., 2000a).

Preliminary surveys in Benin indicated that the kdr mechanism confers cross-resistance to DDT and pyrethroids in An. gambiae in the coastal areas (Corbel et al., 2004) whereas in the northern part of the country, vectors are susceptible to deltamethrin and lambdacyhalothrin but resistant to permethrin (Akogbêto and Yakoubou, 1999). High levels of resistance to deltamethrin, permethrin and DDT are also reported from the coastal and lagunar southern part of the country (i.e. mainly in Cotonou, the capital and largest city in Benin). The intense use of DDT in agricultural settings and during the WHO malaria eradication programme in the 1950s and 1960s were suspected to be the main factors selecting for pyrethroid and DDT resistance in An. gambiae populations (Akogbêto et al., 2005). Various insecticidal products (organophosphates, pyrethroids, etc.) are used to control agricultural pests and the amount applied is generally far higher than that consumed in public health against malaria vectors (Chandre et al., 1999). Benin is one of the biggest producers of cotton in West Africa and 90% of pesticide products are directed against cotton pests (Anon., 2002; IFDC, 2005). Small-scale vegetable farming is an important source of livelihood in urban and peri-urban environments (Tiamiyou, 1995) and provides income and food for tens of thousands of families (PADAP, 2003). Intensive pesticide use in urban vegetable areas (Dinham, 2003) may induce strong selection of resistance in mosquito larvae, thereby impeding malaria vector control operations.

With support of American Red Cross, UNICEF and WHO, a large-scale programme based on free-ITN distribution in combination with measles immunization shall be implemented in Benin in the next years (Guillet, personal communication). To attain a better understanding of the resistance situation in Benin it is important to characterise the spatial distribution of resistance in An. gambiae and Cx. quinquefasciatus in a variety of ecological settings and then attempt to correlate this resistance with pesticide usage. Through a combination of insecticide bioassay, biochemical and molecular techniques, we investigated the type and frequency of resistance to carbamates, pyrethroids and OPs and assessed the implications for vector control strategy.

2. Materials and methods

2.1. Study area

Four contrasting localities of Benin were selected for mosquito collection on the basis of variation in agricultural production, use of insecticides and/or ecological settings (Fig. 1). The localities were: (i) a 100 ha rice growing area (Malanville, 11°52N–3°23E) in the far north of Benin near the Niger River, (ii) an urban vegetable growing area (Parakou, 9°21N–2°37E) located
in the central area of the country, (iii) a conurbation (Cotonou) with two sampling sites located on the edges of the city at Ladji (6°23′N–2°25′), a crop growing area close to the Nokoué lake, and Aséncia (6°21′N, 2°23′E), an important vegetable growing area involved in cultivation of cabbages, lettuce, tomatoes, etc., and where farmers apply significant amounts of pesticide for crop protection (Akogbeto et al., 2005). The southern zone (Cotonou) is characterized by a tropical coastal Guinean climate with 2 rainy seasons (April–July and September–November). The main annual rainfall is more than 1300 mm. The middle part of the country (Parakou) is tropical Sudano-Guinean climate with an average rainfall of 1100 mm per year. The northern part (Malanville) is characterized by a Sudanian climate (semiarid) with only one rainy season per year (main annual rainfall = 900 mm). Experimental huts belonging to the Centre de Recherches Entomologiques de Cotonou (CREC) are located in Malanville and Ladji.

2.2. Mosquito collection

From June to September 2005 (rainy season), An. gambiae larvae were sampled from puddles, shallow wells or rice fields and Cx. quinquefasciatus larvae were sampled from polluted drains, septic tanks, or gutters within urban areas. Larvae were brought back to the laboratory for emergence and testing of adults. Two strains of An. gambiae (kisumu) and Cx. quinquefasciatus (SLAB) were used as reference strains to compare the susceptibility levels of the field populations.

2.3. Identification of sibling species and M and S molecular forms of An. gambiae

All An. gambiae s.l. were identified to species using PCR (Scott et al., 1993) and as M and S forms by PCR-RFLP (Favia et al., 1997).

2.4. Insecticide susceptibility test

Mortality and knock-down resulting from tarsal contact with treated filter paper (Whatman 1 CH) were measured using WHO test kits (WHO, 1998) against An. gambiae and Cx. quinquefasciatus females reared from larval collections. Mosquitoes were assayed using WHO discriminating dosages of six insecticides belonging to different chemical classes: permethrin (25/75) 1%, DDT 4%, chlorpyrifos methyl (CM) 0.4%, malathion 5%, carbosulfan 0.4% and dieldrin 0.4 and 4%.

Four batches of 25 unfed females, aged 2–5 days, were exposed to impregnated papers for 1 h (the same exposure time was used for Anopheles and Culex mosquitoes). With dieldrin, adult mosquitoes were consecutively exposed to 0.4% dieldrin for 1 h to kill susceptible individuals (SS) and the survivors exposed for 24 h later to 4% for 2 h to discriminate heterozygotes (RS) according to Rowland (1991). The number knocked down were recorded every 10 min for permethrin and DDT and mortality rate was recorded after 24 h. Tests with untreated papers were run in parallel and served as control. All specimens (including the susceptible reference mosquitoes) were kept at −20 °C for biochemical and molecular analysis.

2.5. Biochemical analysis

Mixed function oxidase (MFO), non-specific esterase (NSE) and glutathione-S-transferases (GST) activity were assayed in individual 2–5 days old adult An. gambiae and Cx. quinquefasciatus that had been reared from larvae and not previously exposed to insecticides, using 47 specimens per microtitre plate according to the method described by Hemingway (1998).

2.6. PCR detection of the Kdr and Ace.1 mutations

Polymerase chain reaction diagnostic test for detection of kdr “Leu-phe” mutations was carried out on An. gambiae and Cx. quinquefasciatus mosquitoes as described by Martinez-Torres et al. (1998, 1999). The PCR-RFLP diagnostic test was used to detect the presence of G119S mutation (Ace.1 gene) as described by Weill et al. (2004).

2.7. Statistical analysis

Biochemical assay data (enzymatic activity per mg protein) were compared between groups using Kruskall–Wallis non-parametric test (Statistica software). Conformity of kdr, Rdl and Ace.1 frequency with Hardy Weinberg expectations was tested for each population using the exact probability test (Rousset and Raymond, 1995).

3. Results

3.1. Resistance status

Figs. 2 and 3 show the insecticide resistance status of four An. gambiae s.l. and Cx. quinquefasciatus populations from Benin, compared with the susceptible reference strains kisumu and SLAB.
All insecticides tested against the susceptible strain (kisumu) of \textit{An. gambiae} at the WHO diagnostic dosage killed between 92 and 100% of mosquitoes, except dieldrin which induced 68 and 73% mortality at 0.4 and 4%, respectively. At Ladj and Asocna (vegetable growing areas), the absence of KD effect coupled with low mortality rates with DDT and permethrin suggested the presence of the $kdr$ mutation at high frequency. Mosquitoes collected in Ladj and Asocna were, however, fully susceptible to carbamates and OPs (Fig. 2). In Parakou, \textit{An. gambiae} was susceptible to organophosphates but resistant to DDT, and showed reduced susceptibility to permethrin and carbosulfan (88 and 85% mortality, respectively). In the northern rice field area (Malanville), \textit{An. gambiae} was susceptible to permethrin, CM and malathion but slightly resistant to DDT and carbosulfan (79% and 75% mortality, respectively). A high frequency of resistance to dieldrin was recorded in all populations of \textit{An. gambiae} (Table 1).

With the susceptible strain (SLAB) of \textit{Cx. quinquefasciatus}, high mortality rates were recorded with all insecticides (from 98 to 100%) except with dieldrin (Fig. 3). Cross-resistance between DDT and permethrin

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**Fig. 2. Insecticide susceptibility status of \textit{Anopheles gambiae s.l.} at four sites in Benin compared with the susceptible reference strain kisumu.**
was found in Ladji, Asecna and Parakou whereas DDT but not permethrin resistance was found in Malanville. Survival with carbosulfan ranged from 25 to 70% between locations. Culex from Asecna showed resistance to chlorpyrifos-methyl. Dieldrin resistance was reported in the four localities tested (Table 2).

3.2. Biochemical assays

Figs. 4 and 5 shows the mean level of enzymatic activity (MFO, NSE and GST) of four An. gambiae and Cx. quinquefasciatus populations from Benin, compared with the susceptible reference strains kisumu and SLAB. An. gambiae s.l. mosquitoes from Malanville and Ladji showed significantly higher MFO content ($P<0.001$) than the susceptible reference strain kisumu. The level of esterase activity (using α-naphtyl acetate as a substrate) in Ladji population was significantly higher than that measured for kisumu and other field populations ($P<0.001$). Nevertheless, assays using β-naphtyl acetate as a substrate did not reveal any significant differences between Kisumu and Ladji ($P=0.65$). Overall, the mean level of α- and β-esterase activity in Malanville and Parakou was significantly lower than that of the susceptible strain ($P<0.05$). Differences in the levels of GST activity between populations were also observed,
Table 1
Species identification, molecular forms and frequency of the kdr, Ace.1 and Rdl alleles and genotypes in Anopheles gambiae s.l. from Benin

<table>
<thead>
<tr>
<th>Locality</th>
<th>Species a</th>
<th>Mol. Form.</th>
<th>Kdr mutation</th>
<th>Ace.1 mutation</th>
<th>Rdl mutation b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aa</td>
<td>Ag</td>
<td>M</td>
<td>S</td>
<td>K</td>
</tr>
<tr>
<td>Ladji</td>
<td>0</td>
<td>47</td>
<td>47</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Malanville</td>
<td>2a</td>
<td>42</td>
<td>42</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Asecna</td>
<td>0</td>
<td>45</td>
<td>45</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Parakou a</td>
<td>10</td>
<td>35</td>
<td>1</td>
<td>34</td>
<td>29</td>
</tr>
</tbody>
</table>

a Aa, An. arabiensis; Ag, An. gambiae s.s.
b Estimated genotypes according to the sequential test method of Rowland (1991). SS, RS and RR genotypes correspond to mosquitoes which die after exposure to 0.4%, die after exposure to 4% and survive after exposure to 4% dieldrin, respectively. Owing to partial dominance of Rdl in An. gambiae some heterozygotes may be misclassified as homozygotes for resistance and then lead to overestimation of Rdl frequency.

Malanville and Asecna displaying the highest enzymatic activity compared with Kisumu, Ladji and Parakou (P < 0.05).

In Cx. quinquefasciatus, mosquitoes from Ladji showed higher MFO content than those of the SLAB strain (P < 0.05). Higher levels of esterases (using either α- or β-naphyl acetate) and GST activity were detected in all field populations compared with the SLAB strain (P < 0.05). Levels of esterases and GST activities in Malanville was however lower than those measured for Ladji, Parakou and Asecna (P < 0.05).

3.3. Species and molecular forms of Anopheles gambiae

Mosquitoes from the bioassay control batches were analyzed by PCR for identification of species and to molecular forms of An. gambiae s.s. (Table 1). Only An. gambiae s.s. was found in Ladji and Asecna (City of Cotonou) while An. arabiensis was detected in Malanville (4.5%) and Parakou (22.2%). Both M and S forms of the An. gambiae s.s. species were present in Benin. The M form was found in the Coastal areas (Ladji and Asecna) and the northern rice field area (Malanville), while the S form was only detected in sudano-guinean ecotype (Parakou) at very high frequency (97%).

3.4. Detection of resistance genes by PCR

Allele and genotype frequencies at the kdr and Ace.1 loci of An. gambiae and Cx. quinquefasciatus from Benin are shown in Tables 1 and 2. In An. gambiae kdr showed a cline from south to north, with PCR indicating the presence of kdr at high frequency (80%) in the coastal belt (Ladji and Asecna), intermediate frequency in the central vegetable region of Parakou (20%) and low frequency in the rice growing region of Malanville (6%). The Ace.1 mutation was detected in a single specimen collected in Parakou. No Kdr and Ace.1 alleles were found in An. arabiensis.

With Cx. quinquefasciatus, the kdr frequency was also less common in the central and northern areas (Table 2). Ace.1 was detected in two heterozygous specimens collected in Parakou. Within both mosquito species, the distribution of the kdr genotypes did not significantly differ from the H-W equilibrium (P > 0.05, Table 3), except in the An. gambiae population collected at Malanville (FIS = 0.792, P = 0.002). Wright index was not calculated for the Rdl gene because of the partial dominance of the gene which may have led to an over-estimation of RR genotypes in the population (genotype identification by PCR would be required for such analysis).

Table 2
Allele and genotype frequencies of the kdr, Ace.1 and Rdl locus of Culex quinquefasciatus from Benin

<table>
<thead>
<tr>
<th>Locality</th>
<th>Kdr mutation</th>
<th>Ace.1 mutation</th>
<th>Rdl mutation a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS</td>
<td>RS</td>
<td>RR</td>
</tr>
<tr>
<td>Ladji</td>
<td>4</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Malanville</td>
<td>10</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Asecna</td>
<td>5</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>Parakou</td>
<td>14</td>
<td>14</td>
<td>2</td>
</tr>
</tbody>
</table>

a Estimated genotypes according to the sequential test method of Rowland (1991). SS, RS and RR genotypes correspond to mosquitoes which die after exposure to 0.4%, die after exposure to 4% and survive after exposure to 4% dieldrin, respectively.
4. Discussion

A high frequency of insecticide resistance was found in *An. gambiae* and *Cx. quinquefasciatus* in Benin and this resistance was associated with the presence of target site modification and increased metabolic detoxification.

Within the *An. gambiae* complex, the S form was only found in Parakou (Sudano-guinean ecotype) while the M form was identified in the coastal area of Cotonou (guinean ecotype) and the northern ricefield area of Malanville (Sudano ecotype). Geographic distribution seems correlated with ecological or climatic factors as the M form is more adapted to dryer environment and breeds along irrigated fields, while the S form is normally found in humid forested areas and temporary pools (Wondji et al., 2002). Both M and S expressed pyrethroid-resistance with the involvement of the *kdr*
neighbouring countries (N’Guessan et al., 2003) under-
With the expansion of agricultural practises within urban areas, the amount of pesticides being applied to the environment is greatly increasing. This may favour the development of multiple resistance mechanisms. With the recent reporting of both East and West African Kdr variants in An. gambiae from Uganda (Verhaeghen et al., 2006), Gabon (Pinto et al., 2006) and Cameroon (Etang et al., 2006), there is a need to pursue the monitoring of pyrethroid-resistance within the An. gambiae complex as one can expect also a rapid spread of the Leu-Ser mutation in West Africa. Until recently field trials of ITN implemented in West Africa showed that ITNs still achieve a good control of resistant An. gambiae mosquitoes displaying the kdr mutation (Darriet et al., 2000; Henry et al., 2005). However, the impact of increase metabolism coupled with the kdr mutation on vector control operations has not been fully investigated. An experimental hut study carried out at Ladj in 2004 showed a rather low efficacy of permethrin treated nets and indoor residual spraying has been reported in Ladj but continued efficacy in Malanville (N’Guessan et al., 2007). These results underline the need to investigate the role of enzymes in pyrethroid-resistance through the use of classical synergists.

The presence of multiple resistance mechanisms in An. gambiae and Cx. quinquefasciatus in Benin may be an obstacle for the future success of malaria control programmes based on ITNs or indoor residual spraying with pyrethroids or DDT.

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References


