Susceptibility to three pyrethroids and detection of knockdown resistance mutation in Ghanaian *Anopheles gambiae sensu stricto*

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**ABSTRACT:** Although insecticides play a crucial role in malaria control programs, this strategy is threatened by the development of resistance in the vectors to commonly used public health insecticides. Due to their known efficacy and lower toxicity to humans and non-target organisms, pyrethroids have been used in many vector control projects. In West Africa, pyrethroid resistance in the major malaria vector, *Anopheles gambiae*, had been reported in several countries. It was, therefore, imperative to investigate the susceptibility of this very important malaria vector in Ghana and characterize the resistance mechanism. Larvae of *Anopheles gambiae sensu lato* were collected from rural and urban sites and reared to adults. Species identification was by morphological characteristics, PCR, and *Hha*I restriction digest. Female mosquitoes that were two to three days old were selected and exposed to World Health Organization (WHO) diagnostic doses of three pyrethroids. A susceptible laboratory strain of *An. gambiae sensu stricto* was used as a reference. Both survivors and dead mosquitoes from bioassays were screened for the knockdown resistance mutation. *An. gambiae sensu stricto* was the only sibling species of the complex present in these localities with the molecular S form being predominant (>95%). Resistance to pyrethroids up to 8.5 fold was observed, with very high *kdr* frequency. The relative ease in using molecular techniques has resulted in rapid detection of ostensible insecticide resistance genes in malaria vectors. However, it is even more important to complement these molecular tools with routine insecticide testing in the field, especially if the insecticides are earmarked for public health use. *Journal of Vector Ecology* 33 (2): 255-262. 2008.

**Keyword Index:** *Anopheles gambiae*, pyrethroid resistance, south-eastern Ghana.

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**INTRODUCTION**

Targeting of adult female *Anopheles* with insecticides has been the most successful vector control method since the 1940s and was a key factor in the eradication of malaria in the U.S.A., U.S.S.R., southern Europe, and most Caribbean islands (Macdonald 1957, Bruce-Chwatt 1985, Spielman et al. 2001, Curtis 2002). During the World Health Organization’s Global Malaria Eradication Program between 1955 and 1969, insecticide spraying led to enormous, and in some instances, sustained reductions in the disease burden in many endemic regions, including countries on the Indian sub-continent and parts of South America (Trigg and Kondrachine 1998, Cueto 2007). Failure of vector control programs could largely be attributed to emergence of insecticide resistance. In 1975 the WHO reported that dichloro-diphenyl-trichloroethane (DDT) and benzene hexachloride (BHC) resistance was undermining malaria control efforts (Hemingway and Ranson 2000).

Agricultural activities are believed to have contributed to the level of insecticide resistance observed in disease vectors (see reviews in WHO 1986a, 1986b, WHO/FAO/UNEP 1987). Indeed, resistance to insecticides has appeared in the major arthropod vectors from almost every genus. The first case of DDT resistance was reported in *Aedes* mosquitoes in 1947 (Brown 1986). The controversy surrounding the use of DDT has switched attention to the use of pyrethroids which are considered to be less toxic to humans and other non-target organisms (Curtis 1994, Taverne 1999, Bouwman 2000).

Currently, pyrethroids are the only class of insecticide recommended by the WHO for the treatment of bed nets in the framework of the WHO’s Pesticide Evaluation Scheme (Zaim et al. 2000, Hougard et al. 2002a, Zaim and Guillet 2002). However, resistance to pyrethroids has emerged and it is spreading rapidly (Corbel et al. 2004, Etang et al. 2006, Nauen 2007). Pyrethroid resistance in *Anopheles*, therefore constitutes a serious threat to malaria control initiatives (Hemingsway et al. 2002, Etang et al. 2004, WHO 2005).

Pyrethroid resistance due to *kdr* mutation is well characterized in *Anopheles gambiae s. s.* in West Africa (Elissa et al. 1994, Chandre et al. 1999). This resistance mechanism is due to a single nucleotide polymorphism in the gene encoding sub-unit II of the sodium channel gene and leads to the substitution of leucine for phenylalanine (Leu/Phe) (Martinez-Torres et al. 1998). In east Africa, a second *kdr* mutation in the same amino acid results in leucine-serine (Leu/Ser) substitution (Ranson et al. 2000).

Although the *kdr* mutation is predominantly associated with the molecular S form of *An. gambiae s. s.*, more...
recently it has been detected in both the M and S forms from Cameroon (Etang et al. 2006). Synthetic pyrethroids exhibit a certain degree of selective toxicity to insects and have therefore been considered to be relatively safe for public health use (Reigart and Roberts 1999). In view of the significant role pyrethroids play in malaria control, it was imperative to carry out this study to determine the pyrethroid susceptibility status and resistance mechanism in Ghanaian malaria vectors, compare the results with observations in neighboring countries, and evaluate the extent to which occurrence of resistance may affect control programs.

MATERIALS AND METHODS

Study sites
Dodowa, representing the rural setting, is located in the Dangme-West District of the Greater Accra Region. The town lies between longitude 0° 20’ E and latitude 5° 40’ N of the Equator. It has a population of 8,408, with the main occupation being subsistence farming. Mean annual temperature is approximately 27° C, and mean annual rainfall is 1,200 mm. Malaria transmission is stable with seasonal variation (Afari et al. 1993, Ofori et al. 2002, Asante 2005). Accra is the capital city of Ghana and situated in the driest south-east coastal plains of the country. It lies between longitude 0° 10’ E and 5° 36’ N of the Equator. The population is >1.6 million and represents a metropolis. Mean annual temperature is about the same as Dodowa, but with lower mean annual rainfall of <750 mm. Figure 1 is a map of the study sites.

Pyrethroid susceptibility tests
Anopheles larvae were collected from rural and urban southeastern Ghana and reared to adults in the laboratory. Two- to three-day-old female mosquitoes were selected and exposed to diagnostic doses of permethrin (0.75%), deltamethrin (0.05%), and lambdacyhalothrin (0.05%) using WHO standard assays. Each test of a batch of 20-25 mosquitoes was replicated five to seven times with different mosquitoes to account for inter-batch variability. Mosquitoes exposed to untreated papers impregnated with Dow Corning 556 silicone fluid served as controls. The number of mosquitoes knocked down was recorded at 10, 15, 20, 30, 40, 50, and 60 min and also at 80 min in the recovery tube if 50% knockdown was not achieved after 60 min. This allowed the determination of 50% and 90% knockdown time (KDT50 and KDT90, respectively). Mortality was scored at 24 h. The laboratory-maintained An. gambiae Kisumu strain, known to be susceptible to all the pyrethroids commonly used for malaria vector control, was used as a reference strain (Vulule et al. 1994). All specimens were individually preserved on silica gel in Eppendorf tubes for subsequent molecular analysis. Abbott's formula:

\[
\text{Susceptibility} = \frac{100 - \% \text{ test mortality} \times 100}{100 - \% \text{ control mortality}}
\]

was used to correct for mortalities at 24 h post-exposure in field-collected mosquito samples, where control mortalities were between 5% and 20%. The data from pyrethroid susceptibility tests were analyzed using Minitab and LDP Line software.
Morphological identification of mosquitoes
A total of 1,025 field-collected female mosquitoes was used in the susceptibility tests. Out of this number, 550 specimens were randomly selected for morphological identification using the keys of Gillies and de Meillon (Gillies and de Meillon 1968) and Gillies and Coetzee (Gillies and Coetzee 1987).

Molecular assays
Species identification by PCR and HhaI restriction digest
Genomic DNA was extracted from all specimens using the method described in (Collins et al. 1987). Species-specific ribosomal DNA (rDNA) oligo primers capable of distinguishing between five sibling species of the *An. gambiae* complex were used in a cocktail assay (Scott et al. 1993). *HhaI* restriction digest was used to characterize all *An. gambiae* s.s. DNA samples into the molecular M and S forms (Fanello et al. 2002).

Knockdown resistance (kdr) assay
The method of Martinez-Torres et al. (1998) was followed. *An. gambiae* Kisumu (homozygous susceptible for the *kdr* allele) and *An. gambiae* VKPR (homozygous resistance for the *kdr* allele) were used as reference strains.

RESULTS

TABLE 1. Scores for 24-h mortality of pyrethroid bioassays: Accra.

<table>
<thead>
<tr>
<th>Pyrethroid</th>
<th>Dead</th>
<th>Alive</th>
<th>Total</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permethrin</td>
<td>62</td>
<td>48</td>
<td>110</td>
<td>56.4</td>
</tr>
<tr>
<td>L-cyhalothrin</td>
<td>56</td>
<td>52</td>
<td>108</td>
<td>51.9</td>
</tr>
<tr>
<td>Deltamethrin</td>
<td>101</td>
<td>15</td>
<td>116</td>
<td>87.1</td>
</tr>
</tbody>
</table>

TABLE 2. Scores for 24-h mortality of pyrethroid bioassays: Dodowa.

<table>
<thead>
<tr>
<th>Pyrethroid</th>
<th>Dead</th>
<th>Alive</th>
<th>Total</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permethrin</td>
<td>112</td>
<td>124</td>
<td>236</td>
<td>47.5</td>
</tr>
<tr>
<td>L-cyhalothrin</td>
<td>123</td>
<td>112</td>
<td>235</td>
<td>52.3</td>
</tr>
<tr>
<td>Deltamethrin</td>
<td>195</td>
<td>25</td>
<td>220</td>
<td>88.6</td>
</tr>
</tbody>
</table>

Morphological identification of mosquitoes
All 550 specimens were morphologically identified as *An. gambiae* s.l., which facilitated the choice of molecular

<table>
<thead>
<tr>
<th>Sample</th>
<th>KDT$_{50}$</th>
<th>KDT$_{90}$</th>
<th>Resistance Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An. gambiae</em> Kisumu</td>
<td>18</td>
<td>37</td>
<td>1</td>
</tr>
<tr>
<td><em>An. gambiae</em> A$^1$</td>
<td>104</td>
<td>645</td>
<td>5.8</td>
</tr>
<tr>
<td><em>An. gambiae</em> D$^2$</td>
<td>152</td>
<td>1322</td>
<td>8.5</td>
</tr>
</tbody>
</table>

*Time in min required to knockdown 50% and 90% of mosquitoes.

Table 4. Comparative knockdown rates for deltamethrin between the laboratory susceptible and field-collected *An. gambiae*.

<table>
<thead>
<tr>
<th>Sample</th>
<th>KDT$_{50}$</th>
<th>KDT$_{90}$</th>
<th>Resistance Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An. gambiae</em> Kisumu</td>
<td>16</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td><em>An. gambiae</em> A</td>
<td>33</td>
<td>84</td>
<td>2</td>
</tr>
<tr>
<td><em>An. gambiae</em> D</td>
<td>38</td>
<td>91</td>
<td>2.4</td>
</tr>
</tbody>
</table>
tools for the identification of specific sibling species by PCR and subsequent use of HhaI restriction digest to distinguish between M and S molecular forms.

Species identification by PCR and HhaI restriction digest

The accuracy of the PCR technique, based on species-specific nucleotide sequence in the ribosomal DNA intergenic spacers was >98% (Scott et al. 1993). Only a few DNA samples showed no amplification. All successful amplicons were An. gambiae s.s. (Length of amplified sequence was 390 bp). No other member of the species complex was detected in the sampling sites. Further characterization by HhaI restriction digest to distinguish between the molecular M and S forms revealed that the population was predominantly S form (>95%). The M form represented 3.6%, while the frequency of M-S hybrids was about 1%. The distribution of the molecular forms in both Accra and Dodowa is shown in Table 6.

Pyrethroid susceptibility tests

The distribution of the 1,025 female Anopheles gambiae mosquitoes used in the pyrethroid susceptibility tests included 324 from Accra (urban) and 961 from Dodowa. The results for 24-h mortality for the two localities are shown in Tables 1 and 2.

The heterogeneity of response by field-raised An. gambiae s.l. to the three pyrethroids was assessed by the LDP line program (http://www.ehabsoft.com/ldpline/). The response to deltamethrin showed greater departure from linearity compared with permethrin and lambda-cyhalothrin. There was no significant difference between sites. The standard deviations at 95% CI were as follows: 56.89 ± 6.77 for 0.75% permethrin, 28.27 ± 2.88 for 0.05% deltamethrin, and 32.67 ± 2.09 for 0.05% lambda-cyhalothrin. Comparative knockdown rates showing resistant ratios (RR) for permethrin, deltamethrin, and lambda-cyhalothrin of field-collected An. gambiae s.l. against pyrethroid susceptible laboratory strain An. gambiae Kisumu are given in Tables 3-5.

Frequency of the knockdown resistance (kdr) allele

The kdr homozygous genotype occurred with a very high frequency (RR=89.4%) at both Dodowa and Accra as shown in Table 7. Chi-square test analysis showed that there was no significant difference between the distributions of the kdr allele in rural (Dodowa) and urban (Accra) localities (p > 0.05). The M form was predominantly susceptible genotype (SS=89.5%) with heterozygous RS = 10.5%. None of the M form specimens was RR. The M-S hybrids were all heterozygous (RS) for the kdr alleles.

Table 5. Comparative knockdown rates for lambda-cyhalothrin between the laboratory susceptible and field-collected An. gambiae.

<table>
<thead>
<tr>
<th>Sample</th>
<th>KDT$_{50}$</th>
<th>KDT$_{90}$</th>
<th>Resistance Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>An. gambiae Kisumu</td>
<td>21</td>
<td>39</td>
<td>1</td>
</tr>
<tr>
<td>An. gambiae A</td>
<td>64</td>
<td>184</td>
<td>3</td>
</tr>
<tr>
<td>An. gambiae D</td>
<td>76</td>
<td>220</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Table 6. Distribution of the molecular forms of An. gambiae s. s. in Accra and Dodowa.

<table>
<thead>
<tr>
<th>Site</th>
<th>M (%)</th>
<th>M-S (%)</th>
<th>S (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accra</td>
<td>11 (5.85%)</td>
<td>2 (1.06%)</td>
<td>175 (93.08%)</td>
<td>188</td>
</tr>
<tr>
<td>Dodowa</td>
<td>8 (2.36%)</td>
<td>3 (0.88%)</td>
<td>328 (96.76%)</td>
<td>339</td>
</tr>
</tbody>
</table>

Table 7. Distribution of the kdr allele in Dodowa and Accra.

<table>
<thead>
<tr>
<th>Site</th>
<th>RR (%)</th>
<th>RS (%)</th>
<th>SS (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dodowa</td>
<td>299 (88.20%)</td>
<td>19 (5.60%)</td>
<td>21 (6.19%)</td>
<td>339</td>
</tr>
<tr>
<td>Accra</td>
<td>172 (91.49%)</td>
<td>3 (1.60%)</td>
<td>13 (6.91%)</td>
<td>188</td>
</tr>
</tbody>
</table>

R = Resistance allele; S = Susceptible allele.
DISCUSSION

*An. gambiae* exists as a species complex, with seven sibling species that are closely related and morphologically indistinguishable from each other by routine taxonomic methods, and yet distinctly different with respect to ecological and behavioral characteristics and vectorial competence (White 1974). In fact, certain complexes have only come to light during intensive control programs, being noted as having greater resistance to insecticides or apparently changed habitat (Hougard et al. 2002b). In West Africa, *An. gambiae* and *An. arabiensis* are the two main members of the species complex that transmit malaria, with the former being the more efficient vector due to its high degree of anthropophily (Pates et al. 2001, Besansky et al. 2004). The availability of molecular tools offers the opportunity to identify species accurately and to study their distribution in order to guide highly targeted vector control interventions. Thus, *An. gambiae* s. s. can be further characterized into S and M forms using these molecular techniques.

The molecular S form of *An. gambiae* s. s. was predominant and constituted >95% of all samples analyzed. The role of habitat selection by species preferentially choosing and occupying a non-random set of available habitats greatly influences species interactions and is of major importance for the interpretation of spatial and temporal distributions of populations and a variety of other ecological phenomena such as species divergence and diversification (Morris 2003). This invariably influences the spatio-temporal distribution of malaria vectors in sub-Saharan Africa where different eco-geographical zones exist. The frequency of M-S hybrids observed in southeastern Ghana was about 1%. Although M-S hybrids are rare in nature, heterogamous insemination of ~1% has been observed (Tripet et al. 2001), demonstrating the existence of incomplete premating barriers (della Torre et al. 1997). Further ecological study of African malaria vectors is warranted in view of changing land use pattern and urbanization (Donnelly et al. 2005). This will inform vector control strategies involving insecticide application, particularly in the form of treated bed nets.

Insecticide-treated bed nets are known to reduce child mortality by up to 60% or more in malaria endemic countries in sub-Saharan Africa (Alonso et al. 1991, D’Alessandro et al. 1995, Neville et al. 1996, Binka et al. 1998). There is thus strong advocacy for their use to reduce the malaria burden (WHOPES 2005, Roberts 2007). Being the major synthetic organic insecticides the WHO recommends for the treatment of bed nets, pyrethroids play a crucial role in vector-based malaria intervention strategies (WHOPES 2005). However, vector resistance to this class of insecticides could militate against their usability. High kdr frequencies of >90% have been detected in *An. gambiae* in Cote d’Ivoire Burkina Faso and Ghana (Chandre et al. 2000, Diabate et al. 2002, Yawson et al. 2004). However, the Ghanaian study did not include susceptibility tests and could therefore not associate kdr mutation with pyrethroid resistance. Further, Yawson et al. (2004) detected kdr mutation in both the M and S forms of *An. gambiae* s. s., and this mutation was not associated with the M form as reported in the present study. If the presence of kdr in the M form occurs by introgression as posited, then it is difficult to explain the complete absence of M-S hybrids, while reporting kdr in M form in a region where both M and S occur in sympatry (Weill et al. 2000, Diabate et al. 2003). The Leu/Ser mutation was not detected in *An. gambiae* samples from southeastern Ghana, although both the Leu/Phe and Leu/Ser mutations have been reported to occur simultaneously in *An. gambiae* s. s. from Uganda (Verhaeghen et al. 2006). Presently, the kdr mutation has also been reported from other sub-Saharan African countries such as Nigeria, Equatorial Guinea, and Cameroon (Awolola et al. 2005, Reimer et al. 2005, Etang et al. 2006).

Overall, more than 85% of the mosquitoes that were killed by the WHO diagnostic doses of the pyrethroids tested (i.e., permethrin, deltamethrin, and lambda-cyhalothrin) showed homozygous resistance (RR) by kdr PCR assays. This may be explained by the fact that the WHO diagnostic dose was twice the lethal dose that will normally kill 99% (LD99) of a susceptible mosquito population. At such a high dosage, some resistance mosquitoes were likely to be killed. However, since these doubled LD99 values remain below the acute ranges of mammalian oral and dermal toxicities for these pyrethroids, they could be recommended for bed net treatment (Scherb and Weigelt 1994).

At comparable toxicities, deltamethrin was the most efficacious and had the least resistance ratio (RR = 2.4). Lambda-cyhalothrin (RR = 3.5) performed better than permethrin (RR = 8.5). One reason for the poor performance of permethrin is probably due to the fact that permethrin has been used much longer for agricultural and public health purposes than lambda-cyhalothrin and deltamethrin. Also, deltamethrin and lambda-cyhalothrin belong to the subgroup of pyrethroids containing an alpha-cyano group in their chemical structure and are extremely potent against insects even at much lower concentrations (Burr and Ray 2004, WHOPES 2005).

The level of pyrethroid resistance observed in *An. gambiae* s. s. populations in southeastern Ghana has implications for the ITN component of the nation’s malaria control program. This awareness could guide the choice of appropriate doses of pyrethroids for bed net treatment. Further, it is imperative to monitor pyrethroid resistance/susceptibility levels elsewhere in the country in order to deploy timely interventions and delay or mitigate the onset of resistance in the malaria vectors.

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