

**Malaria vectors in Bioko Island (Equatorial Guinea):
PCR determination of the members of *Anopheles gambiae* Giles
complex (Diptera: Culicidae) and pyrethroid knockdown resistance (*kdr*)
in *An. gambiae* sensu stricto**

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ABSTRACT: *Anopheles gambiae* sensu lato Giles, 1902 and *Anopheles funestus* Giles, 1900 are the main malaria vectors on the island of Bioko (Equatorial Guinea). This study was carried out to determine: a) members of the *An. gambiae* complex that may be present on the island of Bioko and, b) the sensitivity of *An. gambiae* sensu stricto to pyrethroids. The analysis by PCR detected the presence of *An. gambiae* s.s. as the major vector of the complex and the "forest chromosomal form" was demonstrated by cytogenetic analysis. The presence of *Anopheles melas* in the southwest, north and southeast of the island justifies its study as a vector. The molecular characterization of pyrethroid knockdown resistance (*kdr*) showed that the populations of *An. gambiae* s.s. were sensitive and no mutations were found. This fact justifies the implementation on a large scale of pyrethroid-impregnated bednets within the framework of the Malaria Control Program of Equatorial Guinea. *Journal of Vector Ecology* 27(1): 102-106. 2002.

Keyword Index: *Anopheles* mosquitoes, Equatorial Guinea (West-Central Africa), *An. gambiae* complex, pyrethroid resistance (*kdr*).

INTRODUCTION

Anopheles gambiae sensu lato Giles and *Anopheles funestus* Giles are considered the main malaria vectors in the African-equatorial region (Hamon et al. 1956, White 1974). Both species are also involved in malaria transmission in Equatorial Guinea (Molina et al. 1993). Malaria is hyperendemic throughout Equatorial Guinea and is one of the three main causes of morbidity and mortality. Previous taxonomic surveys carried out in Equatorial Guinea (Bioko, 3° 43' N, 8° 43' E; and Bata, mainland region, 1° 51' N, 9° 46' E) revealed the presence of five anopheline species: *An. gambiae* s.l., *An. funestus*, *An. lloreti* Gil-Collado, *An. cinctus* Newstead & Carter

and *An. smithi* Theobald (Gil-Collado 1936, 1953). Baseline entomological data in Bioko showed low density rates for both *An. gambiae* s.l. and *An. funestus* compared with the results reported in the surrounding volcanic islands of Sao Tomé and Príncipe (Pinto et al. 2000) and additionally, densities of *An. gambiae* s.l. increased during the rainy season while *An. funestus* populations remained stable during both dry and wet seasons (Molina et al. 1993). The *An. gambiae* complex in Africa includes two species that breed in salt water (*An. melas* in West Africa and *An. merus* in East Africa) and three species that breed in freshwater (*An. gambiae* Giles, *An. arabiensis* Patton and *An. quadriannulatus* Theobald) (White 1973). A sixth member, *An. bwambae*,

was described in the Semliki forest of Uganda associated with water with high mineral content (White 1973). The *An. gambiae* complex now includes 7 species with the recent description of *An. quadriannulatus* B from Ethiopia (Hunt et al. 1998). These seven sibling species can be distinguished by banding patterns of their polytene chromosomes (Coluzzi et al. 1985), enzyme electrophoresis (Miles 1978), PCR (Paskewitz and Collins 1990), *in situ* hybridization of polytene chromosomes with DNA probes (Gale and Crampton 1987), random amplified polymorphic DNA (RAPD) (Wilkerson et al. 1993) or differences in cuticular hydrocarbons (Carlson and Service 1980).

The objectives of this study were to identify which of the members of the *An. gambiae* complex are involved in malaria transmission in the island of Bioko and to examine the pattern of pyrethroid knockdown resistance (*kdr*) of the members of the *An. gambiae* complex on this island.

MATERIALS AND METHODS

Study area and stations of capture

Equatorial Guinea lies in Central-Africa (Gulf of Guinea) between 1°-3° 47' N and 5° 30'-11° 30' E and its overall area of 28,068 km² is divided into an insular (Bioko, Annobon, Corisco, Elobey Grande and Elobey Chico Islands) and a continental part (Rio Muni, between Cameroon and Gabon). The surveys were carried out on Bioko, an island on a large volcanic fracture zone originating to the South of Lake Chad and extending to Mount Cameroon (4 km) on the continent.

Indoor resting collections, light trap (miniature CDC), exit window trap, tent trap and indoor collection using human bait (provided with antimalarial chemoprophylaxis), were the trapping methods used in this work. We trapped a total of 1,662 adult anophelines that were differentiated as 1,309 *An. funestus* and 353 *An. gambiae* s.l. using the key of Diagne et al. (1994). In neighboring breeding areas (next to capture stations) we trapped 80 larvae from each station (10 from each station were used for molecular studies). Eight stations were chosen on the island of Bioko, designated as S1, S2, S3 and S4 in the north region, S5 and S6 in the eastern region of the island, and S7 and S8 in the southwestern region. Capture stations were mapped with three handheld global positioning systems (GPS). For human bait collections, mosquitoes were collected by two people with mouth aspirators who alternated bait and capture operations every two hours. Captures started at 18:00 h and finished at 06:30 h, grouping specimens according to the hour at which they were collected.

Differentiation of *An. gambiae* complex by PCR

The study was carried out with two groups containing larvae and adults from the capture stations in Bioko. A total of 80 larvae (ten per station) and 264 adult mosquitoes (33 selected at random per station) were collected for PCR by personnel of the National Malaria Control Program from all stations.

Mosquitoes from Bioko were previously classified using the key described by Diagne et al. (1994) and stored below 0° C. Samples were preserved in 80% ethanol and sent from Equatorial Guinea to the National Institute of Health Carlos III in Madrid where they were analyzed by PCR.

DNA extraction for larvae and adults was carried out individually, adding 100 ml of lysis buffer (0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl, 0.05 M EDTA, pH 9.1 and 0.5% SDS) and homogenizing into a microcentrifuge tube with a pellet pestle. The homogenate was incubated at 65° C for 1 h, and 15 µl of 8 M potassium acetate solution was then added and centrifuged for 10 min at 14,000 rpm. The supernatant was recovered and two volumes of ethanol 0° C were added and centrifuged at 14,000 rpm for 15 min. Finally, the pellet was air-dried and suspended in 20 ml of distilled water.

PCR was performed by following a slightly modified version (in the "master mix" and the times of the program of amplification) of the protocol described by Scott et al. (1993). DNAs of *An. melas*, *An. gambiae* s.s., *An. arabiensis*, *An. quadriannulatus* and *An. funestus* were used as positive controls. DNA from a Spanish colony of *An. atroparvus*, provided by the National Center for Tropical Medicine, and distilled water in the master mix, were used as negative controls.

Sixteen partially gravid females of *An. gambiae* s.l. containing polytene chromosomes were fixed with ethanol and acetic acid glacial 100% (3:1) and analyzed in Rome following the technique described by Coluzzi et al. (1985).

Molecular characterization of pyrethroid knockdown resistance (*kdr*)

Ten *An. gambiae* s.s. per capture station (selected at random) were analyzed to evaluate pyrethroid resistance. DNA extraction was performed as described above. The primers used and protocols followed were described by Martínez-Torres et al. (1998). Genotyping of susceptible and resistant individuals was possible after amplifying the DNA template from mosquitoes (35 cycles -94° C 1', 52° C 1', 72° C 2" and final 72° C 10'). This method provided an internal control band (293 bp) and a band of different size in susceptible (137 bp) and *kdr* (195 bp) mosquitoes. Three bands (susceptible x

resistant cross) indicated that heterozygotes were also distinguished. DNA provided by the National Center for Tropical Medicine in Madrid from resistant and sensitive *An. gambiae* s.s. was used as positive and negative controls.

RESULTS

Eighty larvae (ten per station) and 264 adults were selected from a total of 650. A majority of *An. gambiae* s.l. analyzed by PCR were determined to be *An. gambiae* s.s. with the exception of two larvae from S7 and six from S4. Seventeen adult mosquitoes were identified as *An. melas* (2 from S7, 5 from S4, and 10 from S6). Figure 1 shows the map of Bioko Island with the location of the capture stations and the results (differentiation of *An. gambiae* complex and susceptibility to pyrethroids). Indoor human bait collection was the most effective trapping method for adult anophelines (55% of all trapped specimens).

Cytogenetic analysis of the polytene chromosomes of all the partially gravid females of *An. gambiae* s.s. from Bioko showed the typical pattern of "Forest cytotype" or "Forest chromosomal form" according to nomenclature of Coluzzi et al. (1985) and were monomorphic for the standard arrangements of both

chromosomal arms 2R and 2L.

DNA from heads and abdomens of adult mosquitoes were analyzed in the study of *kdr* resistance and all mosquitoes (10 randomly selected *An. gambiae* s.s. adults per capture station) exhibited the susceptible *kdr* genotype. Common bands of 239 bp and the susceptible band (137 pb) indicated the susceptibility of all mosquitoes studied including *An. melas*.

DISCUSSION

Results presented here corroborate previous reports concerning the anopheline distribution in the island of Bioko (Molina 1993). *An. gambiae* s.s. (confirmed by PCR) showed high or pronounced endophily, as several females were captured resting indoors engorged with fresh blood from the previous night. Some gravid females probably rested in vegetation and in the surrounding forest, although we were unable to collect *Anopheles* resting outdoors. Moreover, the endophily in *An. funestus* seems to be an acquired and well-established character, as was corroborated by the high proportion of specimens of this species captured by indoor collection using human bait, which was the most effective method of capture. The intense and perennial malaria transmission could be a consequence of the high

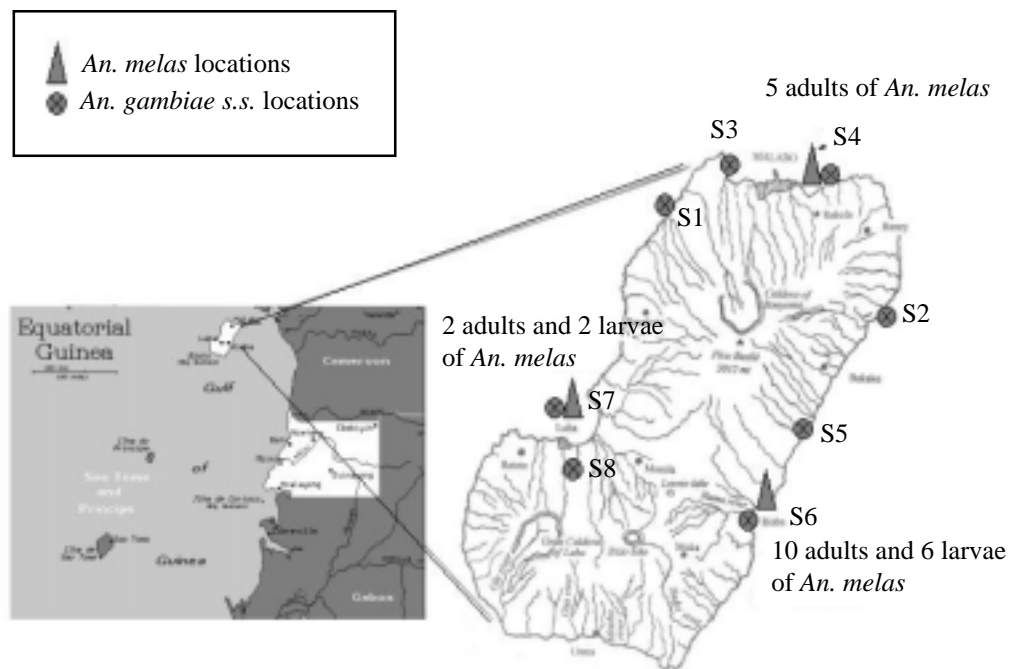


Figure 1. Samples, capture stations, location of the members of *An. gambiae* complex and pyrethroid knockdown resistance (*kdr*) in the island of Bioko, Equatorial Guinea. In these locations, 80 *An. gambiae* randomly selected from each capture station were *kdr* sensitive.

sporozoite rates found in Bioko, in spite of the low anopheline density (Molina et al. 1993).

In accordance with the first objective of the present study, the presence of *An. melas* on the island of Bioko was confirmed with adults and larvae from the three health areas of Bioko (Bioko is divided in three health areas and *An. melas* was collected in each of these areas). This result suggests the presence of this member of the *An. gambiae* complex on Bioko as a vector of malaria. The possible implications of *An. melas* on the transmission of human malaria in this region requires further study. *An. melas* has a wide geographical distribution in Sub-Saharan areas, mainly in the countries of west African coast with some exceptions, but only detected in Cameroon, Angola and now in Equatorial Guinea from the central Africa region (Coetzee et al. 2000). The implication of *An. melas* as a malaria vector had been reported though its infestation rate is significantly lower than that of *An. gambiae* s.s. in areas where the two species are sympatric (Akogbeto and Romano 1999).

The results of PCR confirm the hypothesis that *An. gambiae* s.s. is the main member of the *An. gambiae* complex on the island, which is expected for the equatorial African area. Analysis of polytene chromosomes showed the "forest cytotype" of monomorphic *An. gambiae* s.s. for the standard arrangements of 2R and 2L chromosomal arms. *An. gambiae* s.s. was detected by PCR from all regions of the island of Bioko while *An. melas* was located near the bay of Luba (the bigger bay of the island of Bioko), in Riaba and in Cacahual, two villages located near the coast. PCR was a useful tool for determining the species within the complex from DNA of adults and larvae, although some PCR reactions were inhibited (these inhibitions were due to some not well digested cuticular components that inhibited the reaction of amplification).

kdr is the major mechanism of pyrethroid resistance in field populations. In accordance with this fact, our second objective was to study the sensitivity pattern of anopheline populations to pyrethroids in Bioko. In our previous study (Molina et al. 1993) we analyzed the sensitivity of *An. gambiae* and *An. funestus* populations to different concentrations of insecticide in accordance with the standard protocols designed by WHO. After this previous study, the National Malaria Control Program of Equatorial Guinea decided to initiate a large trial on the island of Bioko based on the use of bednets impregnated with pyrethroids. In the present study we have analyzed the pyrethroid knockdown resistance (*kdr*) four years after the beginning of the bednet-based program. All anophelines studied on the island were sensitive to pyrethroids using this molecular marker,

which corroborates the results obtained for decreasing malaria mortality rates in children under ten years old during the last two years (data not published).

In conclusion, we have determined the presence of *An. melas* in the southwest, north and southeast of the island and characterized the *An. gambiae* s.s. using a sensitive molecular method for pyrethroid knockdown resistance (*kdr*). This increases the justification for implementating a large-scale study of pyrethroid-impregnated bednets within the framework of the Malaria Control Program of Equatorial Guinea. The main objective of these studies is to implement a more selective approach for vector control programs in Equatorial Guinea in relation to the incriminated species, their ecology and their role in malaria transmission. Improved efficiency and selectivity of vector control is becoming a major goal in order to make the best out of the available tools and control the impact of interventions on the environment.

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