First Report of the L1014F kdr Mutation in Wild Populations of *Anopheles Arabiensis* in Cabo Verde, West Africa

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Short report

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Abstract

Background

Due to the lack of vaccines, malaria control mainly involves the control of the incriminated vector, *Anopheles* spp, using chemical insecticides. However, the prolonged and indiscriminate use of these compounds has led to the emergence of resistance in *Anopheles* populations from African continent. The surveillance of this resistance is more frequent in other African countries; however, Cabo Verde does not carry out this surveillance in the same way. Thus, this study aimed to investigate the circulation of the L1014F and L1014S alleles in natural populations of *Anopheles arabiensis* from the city of Praia, Cabo Verde.

Methods

Larvae of *Anopheles* genus were collected in two areas of Praia and reared in the laboratory until maturity. Mosquitoes were morphologically identified by classical taxonomy and then, subjected to molecular species identification using molecular markers. All *Anopheles arabiensis* were subjected to PCR to screen for mutations associated to resistance in the *Na_v* gene.

Results

A total of 105 mosquitoes, belonging to the *Anopheles gambiae* complex, were identified by classical taxonomy were also identified by molecular taxonomy. The molecular identification showed that 100% of the *An. gambiae* s.l. analyzed were *An. arabiensis*. Analysis of the *Na_v* gene revealed the presence of L1014S and L1014F alleles with frequencies of 0.14 and 0.23, respectively.

Conclusions

Our data demonstrate, for the first time, the presence of the L1014F allele in the *An. arabiensis* population from Cabo Verde and an increase of the previously found Kdr L1014S allele. The results presented in this study demonstrate the need to establish new approaches in vector control programs in Cabo Verde.

Background

Malaria is considered one of the most important diseases caused by protozoan parasites *Plasmodium* spp. These parasites are transmitted by mosquitos, such as *Anopheles* sp., and the highest world incidence is found in the African continent [1], where *Plasmodium* is mainly transmitted by mosquitoes of the *Anopheles gambiae* complex [2–4]. Currently, it is known that this complex consists of at least seven cryptic species [5,6], of which the most efficient are *Anopheles gambiae* s.s. (or simply *Anopheles gambiae*) and *An. arabiensis* [7–9]. In Cabo Verde, an archipelago located in West Africa, *An. arabiensis* is the only species from the complex associated with the disease transmission [10–14].

Malaria prevention relies mostly on vector control interventions based on the use of chemical approaches, such as long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) [15,16]. In Cabo Verde, since 1999, the vector control program has implemented the use of IRS with deltamethrin (an insecticide of the pyrethroid class) to control adult mosquitoes [17].

The extensive and indiscriminate use of chemical compounds has resulted in the emergence of an wide-range resistance in wild *Anopheles* populations, mainly in African countries [18]. Two mechanisms have been reported underlying resistance to xenobiotics in these populations: target site insensitivity and metabolic resistance [19,20]. Both mechanisms have already been documented in *An. arabiensis* in West Africa [21]. Target site insensitivity in *Anopheles* is caused mainly by mutations present in the voltage-gated sodium channel (Na_v) (domains II-IV) and they are commonly known as Knockdown resistance (*kdr*). This particular mechanism has been reported in several studies conducted throughout the African continent [22]. *Kdr* mutations are among the best characterized point mutations and they are often associated with resistance to pyrethroid (PYR) and organochlorine (OC) insecticides [23]. Two *kdr* mutations have been detected in the Na_v gene (at position 1014 of the encoded protein) of *Anopheles* populations: the replacement of leucine for phenylalanine (L1014F), found mainly in West Africa (the “*kdr-west*”) and leucine for serine (L1014S), found mainly in East Africa (the “*kdr-east*”) [19,24–26]. These mutations have been frequently detected in mosquitoes from African countries [27–32] and they have been used as a molecular marker for PYR resistance in *Anopheles* populations [33–35].

Information over molecular mechanisms underlying resistance to xenobiotics in *Anopheles* populations is limited in Cabo Verde, thus, poorly understood in the archipelago. So far, a single molecular study has been carried out in Cabo Verde, reporting the L1014S mutation circulation in local *An. arabiensis* [13].

The historical use of insecticides to control malaria in Cabo Verde unveil the necessity of finding molecular markers that may aid the detection and management of insecticide resistance in *An. arabiensis*. Thus, the present study aimed to investigate the circulation of the L1014F and L1014S alleles, often associated with resistance to pyrethroids, in natural *Anopheles arabiensis* populations collected from Santiago island, in Cabo Verde.

Methods

Study area

Cabo Verde is an archipelago of volcanic origin, composed of ten islands, located on the West African coast. The archipelago of dry tropical climate is inhabited by approximately 500.000 people [14,36]. Approximately 60% of the entire population resides on the island of Santiago, the largest island of them all (991 Km²) and where the city of Praia—capital of Cabo Verde—is located (Figure 1) [37,38]. This study was carried out in Achada Grande Trás (W23°29'12.22", N14°55'11.85") and Várzea (W23°30'44.32", N14°55'1.27") (Figure 1), two neighborhoods from Praia. Located on the southern coast of Santiago

island, the city is the economic and cultural center of Cabo Verde. Geographically, Praia may be described as a set of plateaus and respective surrounding valleys [39].

Mosquito collection and morphological identification

Larvae from the *Anopheles* genus were collected in May and October 2017 (a single collection was carried out per month) in natural mosquito breeding sites, using an adapted one-pint dipper. After each collection, larvae were sent to the insectary of Entomology Department from the Jean Piaget University of Cabo Verde and reared until adult stage, under standard laboratory conditions (25-30 °C, 65-75% relative humidity and a 12 h light/dark cycle) [25,40]. These specimen were then identified by classical taxonomy with the identification key described by Ribeiro et al. [10]. Only mosquitoes from the *Anopheles gambiae* complex were used for further molecular analysis. After morphological identification, these mosquitoes were preserved in 70% ethanol until DNA extraction and sent to the Entomology Department of the Aggeu Magalhães Institute (FIOCRUZ-PE) for molecular analysis.

Molecular identification of *Anopheles gambiae* complex species and *kdr* genotyping

Genomic DNA from individual mosquitoes was extracted following the protocol described by Ayres et al. [41] and then stored at -20°C. Molecular identification of species was performed by PCR, according to the protocol described by Scott et al. [42]. DNA samples from *Anopheles gambiae* s.s, kindly provided by Dr. Maria Helena Silva Filha (Entomology Department at FIOCRUZ-PE), were included in each reaction as positive controls. After species identification, DNA samples were screened for the L1014F and L1014S *kdr* mutations, using a PCR protocol described by da Cruz et al. [13].

Fragment sequencing and data analysis

PCR products were sequenced using both forward/reverse primers at the Nucleus of Technology Platforms (NPT) from FIOCRUZ-PE, using the Sanger method (ABI 3500xl automatic system - Applied Biosystems). CodonCode Aligner Program (version 3.7.1) was used to check the quality of both sequences, as well as editing and assembling the contigs (assembly criteria: sequences with ≥ 20 quality were used to generate consensus sequences, based on the PHRED program). Sequence alignment and mutation identification were performed using the BioEdit program (version 7.2.6) [43].

Table 1. L1014S/L1014F genotype and allele frequency from *An. arabiensis* collected in in Praia, Cabo Verde.

Localities	N	Susceptible genotype	L1014S genotypes		Allelic frequency		L1014F genotypes		Allelic frequency	
		SS	RR	RS	R	S	RR	RS	R	S
Várzea	26	17	1	1	0.08	0.92	6	1	0.27	0.73
AGT	65	35	3	9	0.16	0.84	4	14	0.21	0.79
Total	91	52	4	10	0.14	0.86	10	15	0.23	0.77

Note: AGT Achada Grande Trás, N number of *Anopheles arabiensis* analyzed, R resistant allele, S susceptible wild type allele, RR homozygous individuals for L1014F/S mutations, RS heterozygous individuals, SS susceptible homozygous individuals.

The table should come after the first paragraph of the section "kdr genotyping"

Results

Species identification and

A total of 105 *Anopheles* specimens belonging to the *An. gambiae* complex, were identified by classical taxonomy and submitted to molecular species identification. This molecular analysis identified 100% of the individuals as *An. arabiensis*. All these individuals were used to screening Kdr-mutations.

kdr genotyping

Out of 105 individuals submitted to *kdr-east/kdr-west* genotyping and sequencing, 91 resulted in informative sequences. Out of these 91 genotyped individuals, ten were heterozygous (RS) and four were homozygous (RR) for the *kdr-east* mutation (L1014S). For the *kdr-west* mutation (L1014F), 15 mosquitoes were heterozygous (RS) and 10 were homozygous (RR). The remaining (52) were homozygous for the susceptible genotype (SS) (Table 1).

The DNA sequences obtained here for each mutation were submitted to GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) with the following access codes: RR (MW577186) and RS (MW577187) for the L1014F allele; and RR (MW577183) and RS (MW577184) for the L1014S allele. For the susceptible genotype (SS) the access code is MW577185. The L1014S and L1014F allele frequencies were 0.14 and 0.23, respectively (Table 1). Although the two alleles were found in both collection areas, L1014S allele was found more frequently in individuals collected in Achada Grande Trás, while the L1014F allele was found more frequently in individuals collected in Várzea (Figure 2).

Discussion

The surveillance of alleles associated with insecticide resistance in *Anopheles arabiensis* populations is more frequent in other African countries, however, Cabo Verde does not carry out surveillance in the same

way as other African countries[44]. This study provides an update on the presence and frequency of *kdr* alleles in natural populations of *Anopheles arabiensis* in Cabo Verde.

Our results demonstrate, for the first time, the presence of the 1014F allele in the *An. arabiensis* population from Cabo Verde. Although the sampling periods and localities are different, our data also showed that the 1014S allele (*Kdr-east*) increased in frequency from 0.073 to 0.14, in comparison with the study conducted by da Cruz et al. [13] with samples collected in Cabo Verde in 2015, showing that this allele is being gradually selected.

The *Kdr-west* and *Kdr-east* mutations have been found in several countries of the African continent such as Burkina Faso, Côte d'Ivoire, Togo, Tanzania, Kenya, Senegal, Uganda, Ethiopia and Democratic Republic of Congo [23,27,50,51,29,31,32,45–49], which indicate a dissemination of these two alleles across the continent, as a consequence of the gene flow between the different *Anopheles* populations and biogeographic regions [52].

In the present study, both mutations were found in the two locations (Achada Grande Trás and Várzea), indicating that these mutations may be well distributed in Praia, where vector control based on chemical compounds is more frequent. However, a broader collection must be performed in other neighborhoods in the city of Praia, to estimate the allele frequency and their geographical distribution. As mentioned by da Cruz et al. [13], it is possible that high frequency of alleles is associated with frequent use of pyrethroids in certain locations. More recently, temephos resistance was reported in *An. arabiensis* from the City of Praia [53], collected at the same time and at the same locations, which suggest a possible selective pressure for resistance to insecticides due to their greater use (frequency and quantity). Unfortunately, there is currently no public data on the use of pyrethroids by location to estimate the possible association between the use of chemical compounds in the island and the reported resistance data.

It is important to note that this is the second study related to mutations associated with molecular resistance in *An. arabiensis* carried out in Cabo Verde. These *kdr* mutations found in our study may be responsible for the resistance to deltamethrin 0.05% reported by DePina et al. [54] in the *An. arabiensis* population from city of Praia. Those samples were collected in the same period that our samples were collected. This suggests that the vector is adapting to the insecticides used in the local vector control program. The presence of these mutations represents a threat to vector control in Cabo Verde, since nets impregnated with long-lasting insecticides, recently implemented by the Ministry of Health, contain deltamethrin, which could contribute to an increase in resistance to pyrethroids in the wild *Anopheles* population.

The results found in this study emphasize the need for frequent monitoring of the anopheline's susceptibility to insecticides used in the vector control program in Cabo Verde. WHO [22] recommends periodical monitoring of insecticide resistance in vector populations and that the monitoring process of insecticide resistance in malaria vector mosquitoes include tests to determine the phenotypic frequency as bioassays and the mechanisms of resistance (e.g. molecular tests that determine the allele and genotype frequency). Unfortunately, in this study, it was not possible to perform bioassays to determine

the phenotype of resistance to pyrethroids, therefore, we strongly recommend that in further studies, phenotypic analysis be performed followed by the determination of molecular mechanisms of resistance.

Conclusions

This study revealed the increase of the Kdr 1014S allele, when compared with previous findings, as well the presence of the Kdr 1014F allele (identified for the first time) in *An. arabiensis* individuals, collected on the island of Santiago. These results highlight the urgent need to create new vector surveillance strategies to establish new approaches in the vector control program in Cabo Verde. Molecular monitoring of resistance to chemical insecticides should continue to be carried out in order to guide the competent authorities in making decisions such as the implementation of new insecticides in the malaria vector control in Cabo Verde.

Abbreviations

AGT: Achada Grande Trás; *An. arabiensis*: *Anopheles arabiensis*; IRS: indoor residual spraying; kdr: knockdown resistance; LLINs: long-lasting insecticide nets; PCR: polymerase chain reaction; Na_v : voltage-gated sodium channel; RR: homozygous individuals for L1014F/S mutations; RS: heterozygous individuals; SS: homozygous individuals; WHO: World Health Organization.

Declarations

Author's contributions

DC made substantial contributions to conception and design, or acquisition of data. DC made all the experiments and was a major contributor in writing the manuscript. MP, DG, EG, LG, SP and CA were involved in drafting the manuscript and revising it critically for important intellectual content. CA and MP supervised the project. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interest

The authors declare that they have no competing interests.

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Figures

Figure 1

Geographic location of the Cabo Verde archipelago and collection sites. Source: edited in ArcGis, 2020.

Figure 2

Allelic frequency distribution of L1014S and L1014F of the Nav gene at the collection sites, in Praia, Cabo Verde Note: n is the total number of individuals used for detection of L1014S and L1014F mutations in the Nav gene.

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