Am. J. Trop. Med. Hyg., 88(1), 2013, pp. 43–47
doi:10.4269/ajtmh.2012.12-0364

Characterization of the *Plasmodium falciparum* Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase Gene in Samples from Equatorial Guinea before Implementation of Artemisinin-Based Combination Therapy

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Abstract. *Plasmodium falciparum* resistance to the primary drugs used for treatment of malaria has become the main obstacle to malaria control. Artemisinin combination therapies are the current treatment strategy, and it has been suggested that resistance to artemisinin derivatives may be related to mutations in the *Plasmodium falciparum* sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase ortholog of the mammalian sarco-endoplasmic reticulum Ca^{2+} ATPase gene, known as the *pfatp6* gene. Thus, the purpose of this study was to determine the prevalence of single-nucleotide polymorphisms (SNPs) in *pfatp6.* The presence of different SNPs was detected by polymerase chain reaction amplification of the *pfatp6* gene, and then sequencing to identify all possible alleles of the gene. A total of 20 SNPs were detected, including eight SNPs that have not been previously described: K481R in Malabo; R801H on Annobon Island; and the synonymous SNPs *a*141*t*, *c*1788*t*, *a*2211*g*, *t*2739*g*, *a*2760*c*, and *g*2836*a*. The genotypic profile of *pfatp6* in samples from Equatorial Guinea, may be a useful epidemiologic tool for monitoring local efficacy of artemisinin combination therapies.

INTRODUCTION

Half the world's population is at risk for malaria. The region most affected by this disease is Africa, accounting for 78% of the estimated 225 million malaria episodes and 91% of all malaria-related deaths worldwide in 2009.[1] Children less than five years of age in stable transmission areas, who have not yet developed protective immunity against the most severe forms of the disease, constitute a special risk group and have the most malaria deaths worldwide.[2] Equatorial Guinea is an area of stable malaria transmission, with *Plasmodium falciparum* being the most prevalent species: it is not only implicated in more than 85% of reported cases, but is the main cause of death in children less than five years of age, accounting for 28% of total deaths in 2008.[3]

The spread of antimalarial drug resistance over the past few decades has led to efficacy monitoring being intensified to enable early detection of resistance. Since 2006, most countries to which *P. falciparum* is endemic have progressively updated their treatment policies, changing from chloroquine and sulfadoxine-pyrimethamine (SP) treatments to artemisinin combination therapies (ACTs), which are recommended as being the best current treatment for uncomplicated *P. falciparum* malaria.

Unfortunately, there has been a lag in the implementation of these treatment policies because of various factors, such as high costs.[4] Despite observed changes in parasite sensitivity to artemisinins (ARTs) in western Cambodia and along the Thailand-Myanmar border,[5–8] the clinical and parasitologic efficacy of ACTs has not been compromised. However, both components of the drug combination are currently at risk for resistance, and using an ART with an ineffective partner medicine can increase the risk of development and spread of artemisinin resistance.[1]

Since 2007, artesunate/amodiaquine has been the first-line treatment for uncomplicated *P. falciparum* malaria in Equatorial Guinea (National Malaria Control Program). It is proposed that ARTs lead to parasite death by binding and inhibiting the *P. falciparum* sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase ortholog of mammalian sarco-endoplasmic reticulum Ca^{2+} ATPase gene, known as PfATP6.[9] Recent studies have focused on PfATP6 structure, and docking simulations suggest a direct interaction between ARTs and PfATP6, possibly by hydrophobic interactions.[10,11] Additional studies in *Xenopus laevis* oocytes with amino acid variants at residue 263 (L263, 263S, and 263A) have shown that Leu263 may modulate sensitivity to ARTs.[12]

Additional support for PfATP6 as a possible target of ART compounds in *P. falciparum* has emerged from a field study, which reported an association between an S769N substitution in PfATP6 and elevated *in vitro* artemether 50% inhibitory concentrations (IC_{50s}) from French Guiana isolates, suggesting a potential role for polymorphisms at *pfatp6* as candidate markers for ART resistance emergence.[13] This same study reported one case in Senegal of greatly increased artemether IC_{50}, which was a PfATPase6 E431K A623E double mutant, although each mutation was found alone in other isolates with no altered parameters. This result is why several studies have focused on the analysis of known or unknown single-nucleotide polymorphisms (SNPs) in *pfatp6*, reporting on the high diversity of this gene.[5,14–24] However, it is believed that the variability at a single SNP of this gene is insufficient to induce resistance to ARTs,[25] and that any alteration in the parasite response to artemisinins is probably multigenic and more complex than it seems, with another candidate being the multidrug resistance 1 gene.[26,27] However, most studies agree on the importance of continuous screening of *pfatp6*, with a view to detecting possible changes in its genotypic pattern as a response to increasing ACT pressure.

Accordingly, the purpose of this study was to identify the allelic profile of the *PfATPase6* gene in Equatorial Guinea by using samples collected before the introduction of ACTs to collect baseline data that would be useful for inferring

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whether the frequency of particular *pfatp6* genotypes might be influenced by treatment in the future.

MATERIALS AND METHODS

Study area. Equatorial Guinea is a country in central Africa situated in the Gulf of Guinea. It is divided into two regions, the mainland area between Cameroon and Gabon, known as Río Muni, and the island area (Bioko, Annobon, Corisco, Elobey Chico and Elobey Grande) (Figure 1). Bioko, the largest island, is located 40 km from the coast of Cameroon and is where Malabo, the country's capital, is located. The climate is tropical, with a rainy season during May–October and a dry season during December–March. Bata is the largest city on the mainland, and has a tropical climate with two dry seasons (December–March and June–September) alternating with two rainy seasons (March–June and September–December). Annobon is a small volcanic island (17 km^2), which is located south of São Tomé and Príncipe, more than 300 miles (approximately 483 km) from the coastline of Gabon and more than 600 miles (approximately 967 km) from the country's capital city. The rainy season is from November through April–May and the dry season is from June through October.

Biological samples. *Plasmodium falciparum* field isolates were collected in 2005 as part of an *in vivo* study conducted at the Malabo and Bata Regional Hospitals,[28] and in 2004 during two cross-sectional studies undertaken on the small island of Annobon.[29] Blood samples were collected from children les than five years of age with uncomplicated *P. falciparum* infections. Diagnosis was carried out by microscopic examination of Giemsa-stained thick blood films. For each sample, approximately 20 μL of finger prick blood was spotted on a piece of 3 MM filter paper (Whatman, Maidstone, United Kingdom) and air-dried. The dried filter paper samples were stored until DNA extraction for subsequent molecular studies.

Extraction of DNA and polymerase chain reaction amplification. Parasite genomic DNA was extracted from the filter paper by using a SpeedTools Tissue DNA Extraction Kit (Biotools B&M Laboratories SA, Madrid, Spain) in accordance with the manufacturer's instructions. Amplification of the *pfatp6* gene was performed by means of five consecutive polymerase chain reactions (PCRs), which generated overlapping fragments, as described elsewhere.[17] Agarose gel electrophoresis was used to ascertain the quality and concentration of PCR products for each fragment. These fragments were subsequently purified, where necessary, by using a commercial kit (QIAquick PCR Purification Kit; QIAGEN, Hilden, Germany) and used as a template for sequencing.

DNA sequencing. DNA sequencing was performed directly from two independent PCR products for each fragment (forward and reverse) by using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and a 3730*xl* DNA Analyzer (Applied Biosystems). Subsequent steps were performed with Sequencing Analysis 5.3.1 software, and entailed comparing the sense and antisense results for each fragment to solve potential ambiguities, and joining overlapping fragments to obtain the complete gene, which was aligned with the 3D7 DNA sequence (GenBank accession no. PFA0310c) to check for genetic polymorphisms.

Ethics. This study was approved by the Ministry of Health and Welfare of Equatorial Guinea. Written informed consent was obtained from all adult participants and all parents or guardians of children.

RESULTS

Of the 55 isolates, a near-full length *pfatp6* sequence was successfully obtained for 48 samples (28–3,078 basepairs). Because of technical difficulties, we were only able to obtain the 28–3,078-basepair sequence for 10 of 17 isolates from Malabo. For the remaining 7 samples, the compiled sequence spanned nucleotides 28–2,467.

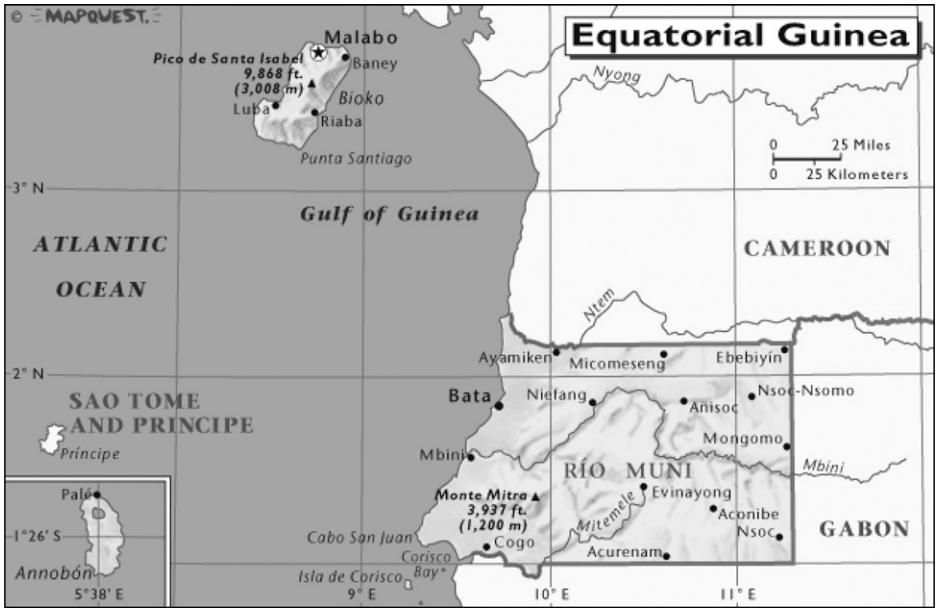

FIGURE 1. Map of Equatorial Guinea showing the country's two regions, i.e., the mainland area (Río Muni) and the island area. Map obtained from http://go.hrw.com/atlas/norm_htm/eqguinea.htm.

TABLE 1

Distribution of single-nucleotide polymorphisms, by geographic region, Equatorial Guinea

Mutations		No. mutations				Frequency (%)
Nucleotide	Amino acid	Malabo	Bata	Annobon	Total	Total
A141T	47 SYN	0	0	1	1	< 2
C727T	H243Y	0	0	1	1	< 2
A945T	315 SYN	0	1	0	1	< 2
T1204G	L402V	3	2	2	7	12.7
G1291A	E431K	4	3	2	9	16.4
A1320T	440 SYN	0	0	1	1	< 2
A1442G	K481R	1	0	0	1	< 2
T1671A	557 SYN	0	1	0	1	< 2
A1706G	N569S	1	0	0	1	< 2
T1707A	N569K	3	2	0	5	9
C1788T	596 SYN	0	1	0	1	< 2
G1888T	A630S	1	0	1	2	3.6
G1927C	643 SYN	0	1	0	1	< 2
A2211G	737 SYN	0	1	0	1	< 2
C2239T	H747Y	2	0	0	2	3.6
G2402A	R801H	0	0	1	1	< 2
T2694A	898 SYN	7*	12	14	33	66*
T2739G	913 SYN	0*	0	2	2	3.6*
A2760C	920 SYN	0*	1	0	1	< 2*
G2836A	946 SYN	0*	0	1	1	< 2*

*For these mutations and frequency calculations, the fact that in Malabo only 10 samples have been sequenced has been taken into account.

A total of 20 SNPs were detected in Equatorial Guinea, and 9 of these resulted in amino acid substitutions (Table 1). Most of these substitutions have been found in different locations throughout Africa,[14–16,18,19,24] including the most frequent and ubiquitous SNPs, such as *t*1204*g* (12.7%) and *g*1291*a* (16.4%), which encode the L402V and E431K mutations, respectively, and the synonymous mutation *t*2694*a* (66%). To our knowledge, however, the following eight SNPs have never been previously described: *a*1442*g* in Malabo and *g*2402*a* on Annobon Island, encoding the amino acid changes K481R and R801H respectively; and the synonymous *a*141*t*, *c*1788*t*, *a*2211*g*, *t*2739*g*, *a*2760*c*, and *g*2836*a* SNPs. These novel point mutations appear to be sporadic, and each was shown to be confined to a specific geographic region. Neither of the mutations reported as altering susceptibility to artemisinin derivatives, namely L263E and S769N, was found.[12,13,30]

The number of SNPs grouped by region of origin is shown in Table 2. On Annobon Island, 10 SNPs were detected, of which 6 were found at no other site in the country. The most common SNP was the ubiquitous *t*2694*a*, which was present in 14 of 15 samples (Table 1). Five of the polymorphisms were non-synonymous, and of these polymorphisms, two were found exclusively on Annobon Island, namely, H243Y, which had been reported in other parts of Africa,[14,18,19] and R801H, which, to our knowledge, was detected for the first time.

Of the eight SNPs found in Malabo, three were exclusive to this location and all were non-synonymous (Table 2). The most common variants were the *t*2694*a* SNP, present in 7 of 10 samples; L402V and N569K, both present in 3 of 17 samples; and E431K, present in 4 of 17 samples. Seven of the polymorphisms were non-synonymous, and three were location-specific: K481R, never previously detected; N569S, previously reported by our group in Equatorial Guinea[24]; and H747Y, previously reported in Africa[18] (Table 1).

TABLE 2

Number and type of SNPs according to location, Equatorial Guinea*

		No. SNPs			
Location	No. sequences	SNPs	Location-specific	Non-synonymous	Non-synonymous location-specific
Malabo	17	8	3	7	3
Bata	23	10	6	3	0
Annobon	15	10	6	5	2

*SNPs = single-nucleotide polymorphisms.

In Bata, the main city on the mainland, 10 SNPs were identified, 6 of which (all synonymous) were found nowhere else in the country (Table 2). Although less prevalent than in Malabo and Annobon overall, the most common SNP was the synonymous *t*2694*a*, which was present in 12 of 23 samples. Only three of the polymorphisms were non-synonymous and none was location-specific. Three synonymous SNPs, located at residues 596, 737, and 920, had never been previously described (Table 1).

In addition to the SNPs described above, we also found two additional genetic variants, in which one or two insertions of the AAT codon (coding for asparagine: N) adjacent to residue 465, occurred as follows: two samples, one from Malabo and another from Bata, displayed the insertion of a single N at this locus, and one sample from Bata had two N insertions (Table 3).

The different haplotypes present in the parasite population were reconstructed by taking all non-synonymous substitutions into account. In doing so, 14 haplotypes were identified, each occurring at different frequencies (Table 3). Haplotype number 1, which is the same as that of the reference sequence of the 3D7 strain of *P. falciparum*, was the most prevalent across the study area. Two other haplotypes (2 and 7) that differing from haplotype 1 in only one SNP, were the next most frequent, and were found in all regions. Five haplotypes (3–6 and 8) were exclusive to Malabo, three haplotypes (9–11) were found exclusively in Bata, and three haplotypes (12–14) were specific to Annobon.

DISCUSSION

Because there are currently no alternative drugs to replace artemisinin derivatives in the treatment of uncomplicated malaria, the emergence and spread of drug-resistant parasite populations may have disastrous consequences in malaria-endemic areas. Several studies have reported cases of decreased *in vivo* sensitivity to artemisinin derivatives in western Cambodia and on the Thailand-Myanmar border, where uncontrolled use of such derivatives has subjected the *Plasmodium* populations to strong drug pressure.[5–8] Increased *in vitro* IC_{50s} to the artemisinin derivative artemether have also been reported in *P. falciparum* parasites from French Guiana.[13] One interesting common genetic feature of the French Guiana parasites that displayed artemether IC_{50} values > 30 nM was the presence of an S769N mutation in the *pfatp6* gene.[13] Interestingly this S769N mutation is not commonly found in Africa, with the only exception of an isolate from a patient who referred have traveled to Africa.[19] Consequently, this study suggested that *pfatp6* genetic variants among natural populations of *P. falciparum* may display different responses to artemisinins.

The purpose of this study was to identify genetic variation in the *PfATPase6* gene in three differentiated geographic areas of Equatorial Guinea and establish a basis for monitoring changes, which may occur over time and may result from

TABLE 3

Amino acid haplotypes of the *Plasmodium falciparum* sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase gene and number of samples at each location, Equatorial Guinea*

	Amino acid positions									No. samples			
Haplotype	243	402	431	–	481	569	630	747	801	Malabo	Bata	Annobon	Total
1	H	L	E	–	K	N	A	H	R	8	15	9	32
2	.	V	.	–	.	.	.	.	.	1	1	2	4
3	.	.	.	–	.	.	.	Y	.	1			1
4	.	.	K	–	.	K	.	.	.	2			2
5	.	.	.	N-	.	S	.	.	.	1			1
6	.	V	.	–	R	K		Y	.	1			1
7	.	.	K	–	.	.	.	.	.	2	3	1	6
8	.	V	.	–	.	.	S	.	.	1			1
9	.	V	.	N-	.	.	.	.	.		1		1
10	.	.	.	–	.	K	.	.	.		2		2
11	.	.	.	NN	.	.	.	.	.		1		1
12	.	.	K	–	.	.	S	.	.			1	1
13	Y	.	.	–	.	.	.	.	.			1	1
14	.	.	.	–	.	.	.	.	H			1	1
Total	NA	NA	NA	NA	NA	NA	NA	NA	NA	17	23	15	55

*Haplotype number 1 corresponds to sequence 3D7 (GenBank accession no. PFA0310c). – = point of insertion of asparagine residues adjacent to position 465; · = sequence identity; NA = not applicable.

ACT pressure. In addition to *Pfatp6* mutations already described in other areas, eight new point mutations were identified. These were K481R, R801H and the synonymous SNPs *a*141*t*, *c*1788*t*, *a*2211*g*, *t*2739*g*, *a*2760*c*, and *g*2836*a*. The phenotypic significance of the novel non-synonymous mutations K481R and R801H is difficult to predict in the absence of functional studies. Nonetheless, according to a previous predictive model of PfATP6 protein domain architecture,[24] the R801H mutation is in a hydrolase catalytic domain of the peptide, located from residues 790–935. However, whether this mutation may alter the protein's function and, consequently, parasite susceptibility to artemisinins, is unknown.

The PfATP6 E431K variant has been associated with increased artemether IC_{50} when found in combination with mutation A623E in *P. falciparum* isolates from Senegal.[13] In our study, this mutation was the second most prevalent (16.4%) because it occurred at every site sampled and was present in three distinct haplotypes. Howeveer, because the A623E mutation was not identified in our sample cohort, none of the haplotypes contained the E431K and A623E mutations simultaneously. Further analysis will be undertaken to ascertain the influence of mutations on structural variations of protein, and to relate these changes to *in vivo* parasite resistance to artemisinin derivatives.

Before ACT implementation, an *in vivo* study that had been conducted locally to evaluate the efficacy of combination drugs such as AS/SP and AQ/SP, showed that both combinations were safe and efficacious as first-line treatments for treatment for uncomplicated *P. falciparum* malaria, with a success rate > 96%.[28] Because the samples used for our study were collected during the same period as for the above *in vivo* study, the *pfatp6* genotypes identified must be presumed to represent baseline genetic variation in the gene in the absence of ART pressure. After five years of using AS/AQ as the first-line treatment for uncomplicated malaria in Equatorial Guinea, a new *in vivo* study is now being conducted to assess the effectiveness of treatment and, at a molecular level, to determine the potential effect of ACT treatment on the evolution of PfATP6. It will shortly be possible to verify whether current treatment pressure has favored the selection of some of the mutations or whether new SNPs have emerged.

Overall, 20 SNPs and 14 distinct haplotypes were identified in a cohort of 55 samples. Although these results confirm this gene's high genetic diversity, a feature already reported in earlier studies,[5,14–21,24] some SNPs were found at low frequencies and at only one location in each case. At present, the significance of this high gene diversity in *pfatp6* and its potential influence on the future efficacy of ACTs is largely unknown. However, in the event that peptide variants of PfATP6 protein might be able to modulate the parasite's response to artemisinin derivatives, the gene's high genetic diversity would establish a highly favorable basis for the selection of rare mutants under drug pressure. Consequently, constant monitoring of *pfatp6* genetic variation and assessment of its potential correlation with treatment would provide an essential means of assessing the importance of specific mutations to the evolution of ACT efficacy.

Received June 7, 2012. Accepted for publication October 1, 2012.

Published online November 26, 2012.

Financial support: This study was supported by the Spanish Agency for International Development Cooperation and the Collaborative Research Network in Tropical Diseases.

Disclaimer: The views expressed in this article are those of the authors and do not necessarily reflect the views of the authors' institutions or the sponsors.

Disclosure: None of the authors has any financial interest in any product discussed in this article. The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all of the data in the study and had final responsibility for the decision to submit for publication.

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