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Geographic Structuring of the *Plasmodium falciparum* Sarco(endo)plasmic Reticulum Ca2+ ATPase (PfSERCA) Gene Diversity

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Abstract

Artemisinin, a thapsigargin-like sesquiterpene has been shown to inhibit the *Plasmodium falciparum* sarco/endoplasmic reticulum calcium-ATPase PfSERCA. To collect baseline *pfserca* sequence information before field deployment of Artemisinin-based Combination therapies that may select mutant parasites, we conducted a sequence analysis of 100 isolates from multiple sites in Africa, Asia and South America. Coding sequence diversity was large, with 29 mutated codons, including 32 SNPs (average of one SNP/115 bp), of which 19 were novel mutations. Most SNP detected in this study were clustered within a region in the cytosolic head of the protein. The PfSERCA functional domains were very well conserved, with non synonymous mutations located outside the functional domains, except for the S769N mutation associated in French Guiana with elevated IC_{50} for artemether. The S769N mutation is located close to the hinge of the headpiece, which in other species modulates calcium affinity and in consequence efficacy of inhibitors, possibly linking calcium homeostasis to drug resistance. Genetic diversity was highest in Senegal, Brazil and French Guiana, and few mutations were identified in Asia. Population genetic analysis was conducted for a partial fragment of the gene encompassing nucleotide coordinates 87-2862 (unambiguous sequence available for 96 isolates). This supported a geographic clustering, with a separation between Old and New World samples and one dominant ancestral haplotype. Genetic drift alone cannot explain the observed polymorphism, suggesting that other evolutionary mechanisms are operating. One possible contributor could be the frequency of haemoglobinopathies that are associated with calcium dysregulation in the erythrocyte.

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Introduction

Plasmodium falciparum malaria claims close to one million deaths every year, mainly in sub-Saharan African children. To face increasing anti-malarial treatment failure, many countries have adopted artemisinin-based combination therapy (ACT) [1]. However there are concerns about emerging resistance to artemisinin derivatives, with *in vitro* drug resistance to artemether reported in French Guiana and northern Brazil [2–3] and high *in vivo* tolerance evidenced by delayed parasite clearance emerging in Cambodia [4–8]. The mechanism of action of artemisinin derivatives and the molecular basis of artemisinin resistance and/or tolerance are poorly understood. It has been recently proposed that artemisinins inhibit PfSERCA, the *P. falciparum* Sarco(endo)plasmic reticulum Ca2+ATPase [9–10], and thus possibly act by perturbing calcium homeostasis of the intracellular parasite.

SERCA, the calcium pump of sarcoplasmic reticulum responsible for refilling calcium in the ER stores is critically important for cellular homeostasis and calcium signalling functions [11–13]. Unlike vertebrates that possess three *serca* genes [14], *P. falciparum* has a single SERCA, originally described as PfATPase6 [15]. Other Apicomplexan parasites such as *Toxoplasma gondii* and *Cryptosporidium parvum* also have a single copy *serca* gene [16]. SERCA proteins are evolutionary well conserved and are composed of three cytoplasmic domains (A, actuator; N, nucleotide binding; P, phosphorylation), ten transmembrane (M1–M10) helices constituting the transmembrane gate and small lumenal loops. Together the A, N and P domains participate in regulating calcium binding and release into the ER lumen. The whole molecule undergoes complex

conformational changes during activation, involving a cooperative binding of two $Ca2^{+}$ and phosphorylation of the cytosolic head of the molecule leading to conformational transitions opening the transmembrane channel [11,17–18]. Thapisgargin, a highly cell-penetrant sesquiterpene-lactone that once inside cells potently and specifically inhibits mammalian SERCA, binds to a cavity at the interface of the M3, M5 and M7 helices [19–20]. Importantly, specific inhibition of SERCA by thapsigargin kills cancer cells by provoking the apoptotic cell death pathway [21].

The sesquiterpene-lactone structure of thapsigargin, which is a highly specific SERCA inhibitor, is structurally reminiscent of artemisinin. Interestingly, both thapsigargin and artemisinin were shown to inhibit the ATP hydrolysis activity of the PfSERCA produced in the heterologous *Xenopus laevis* oocyte expression system [9]. Consistent with the notion of PfSERCA inhibition, thapsigargin antagonized the parasiticidal activity by artemisinins [9]. In line with this, artemisinin-susceptibility of the PfSERCA protein produced in the *Xenopus laevis* oocyst heterologous expression system was abolished by introduction of a single L263E point mutation [22]. Remarkably, a few years after the introduction of artemether combination therapy in French Guiana, a S769N PfSERCA polymorphism was observed in several patient isolates presenting with a markedly increased 50% Inhibitory Concentration (IC_{50}) for arthemether and hence were classified as *in vitro*-resistant [2–3]. However, this S769N polymorphism was also reported in one African isolate, which was susceptible to dihydroartemisinin *in vitro* [23]. It is worth noting that artemisinin-resistant mutants of the murine malaria parasite *P. chabaudi chabaudi* [24] or the related Apicomplexan parasite *T. gondii* had an unaltered *serca* gene sequence though calcium homeostasis was altered in these artemisinin-resistant *T. gondii* parasites [25]. Thus, although the relationship between *Pfserca* polymorphism and *P. falciparum* susceptibility to artemisinin remains to be confirmed, it is conceivable that artemisinin-resistance involves species-specific mechanisms, as shown for e.g. chloroquine [26].

Insights into the relative impact of *pfserca* polymorphisms on the biological function of the protein on the one hand and its susceptibility to artemisinins on the other hand may be gained from an analysis of field polymorphisms. Apart from the above mentioned novel S769N mutation observed in French Guiana [2–3] and Africa [23], a number of other *pfserca* polymorphisms have been reported in laboratory lines [27] and in field isolates from Africa [2,28–29], although their relationship with artemisinin susceptibility remains unknown. An intriguing aspect is the relatively large *pfserca* polymorphisms that they show relatively large sequence diversity in comparison to other so-called house keeping genes, such as the lactate dehydrogenase locus studied in the same lines [27]. To further explore *Pfserca* sequence diversity and to look for possible causes and geographical structuring, we sequenced a large panel of field isolates from various malaria-endemic geographic areas before widespread deployment of ACT. Our data show clear evidence for geographic structuring and indicate that as yet unknown evolutionary mechanisms are contributing to the large field polymorphism of this locus in some areas.

Results

Pfserca Polymorphism in the Field

We sequenced *pfserca* in 100 isolates collected from South America, Asia and Africa (Table 1). Full length coding sequence was determined for 56 isolates, and partial (positions 87-2862 of the gene sequence) was obtained for 40 isolates. Overall, 29 codons carried one or more SNP. Codons 538, 569 and 987 harboured two mutations. A total of 32 distinct SNPs were observed, of which 19 were non synonymous (NS) and 13 synonymous mutations, resulting in an average of one SNP/115 bp (Table 2). 19 mutations had not been described previously. Haplotype reconstruction allowed grouping samples into 28 distinct haplotypes/alleles (Table 3).

The predicted domain structure of PfSERCA based on alignment of the reference PfSERCA protein sequence with other sequences selected across the phylogenetic tree and the identified functional and structural domains [30–35] (detailed in supplementary Figures S1 and S2 with Supporting Information File #1) is presented in Figure 1 A and B. As previously noted by other authors [27,29], PfSERCA has the largest sequence of all those deposited in NCBI as it contains specific inserts absent from all other SERCAs. These inserts are mainly located in the N domain facing the cytoplasmic side of the ER. The location of the various SNPs observed in this panel of isolates is shown in Figure 1C. Three SNPs were located within the A domain (codons 37, 89 and 141), all three were NS mutations. Twenty one SNPs were located within the N-domain, 11 of which within the *Plasmodium*-specific sequences (with 7 mutated codons –codons 569, 570, 621, 623, 630, 639 and 643 within a *falciparum/reichenowi*-specific insert). Of the 21 SNPs affecting the N domain, only six were synonymous. In contrast, seven of eight SNPs located in within the P-domain were synonymous mutations, including two SNPs within a transmembrane domain. 3Five SNPs (R37K, W141G, S538S, S538R, T539I) were located in the vicinity of residues essential for the

Table 1. Details of samples for which *pfserca* sequence was studied.

Continent	Collection area	No samples	Year of collection	Sequenced*	Reference of sample collection
AFRICA	Equatorial Guinea	9	2005–2006	Partial	unpublished
	Senegal	16	2003	Full-length	Jambou et al., 2005
AMERICA	Brazil (Amazonas)	10	2003–2006	Partial	unpublished
	Brazil (Pará)	13	2005	partial	Ferreira et al., 2007
	French Guiana	20	2003	Full-length	Jambou et al., 2005
ASIA	Cambodia	24	2005	Full-length	Jambou et al., 2005
	Thailand	8	2002	partial	Lopes et al., 2002

*gene region sequenced:
full-length refers to entire coding sequence, corresponding nucleotide 1-3687 (cordon 1-1228), partial refers to region encompassing nucleotide coordinates 87-2862 in the Pfserca 3D7 reference sequence (PFA0310c).
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Table 2. List of the *pfserca* mutations detected.

Nucleotide position	Codon No	Wild-type codon	Mutant Codon	Aminoacid (NS: bold; Syn: *italics*)	Country of origin
110	37*	AGA	AAA	**R – K**	Brazil
266	89	ATA	ACA	**I – T**	Thailand
421	141*	TGG	GGG	**W – V**	Senegal
1204	402	TTA	GTA	**L – V**	Brazil French Guiana Senegal
1262	421**	GGA	GAA	**G – E**	French Guiana
1291	431**	AGA	AAA	***E – K***	Senegal
1323	441**	TAT	TAC	*Y – Y*	French Guiana
1329	443**	GAT	GAC	*D – D*	French Guiana
1395	465	AAT	AAC	*N – N*	French Guiana
1612	538	AGT	GGT	***S – R***	French Guiana
1614	538	AGT	AGC	*S – S*	French Guiana
1616	539	ACC	ATC	**T – I**	French Guiana
1671	557	TCT	TCA	*S – S*	Equatorial Guinea
1706	569**	AAT	AGT	***N – S***	Equatorial Guinea
1707	569**	AAT	AAA	**N – K**	Equatorial Guinea
1710	570**	ACA	ACC	*T – T*	French Guiana
1721	574**	ACA	CCA	**Q – P**	French Guiana
1861	621**	GCT	TCT	**A – S**	French Guiana
1868	623**	GCA	GAA	**A – E**	Senegal
1888	630**	GCT	TCT	**A – S**	Brazil
1916	639**	GGC	GAC	**G – D**	Brazil
1927	643**	GAA	CAA	**E – Q**	Equatorial Guinea
2049	683	AAT	AAG	**N – K**	Senegal
2306	769	AGT	AAT	**S – N**	French Guiana
2656	885*	ATT	ATA	*I – I*	Senegal
2694	898*	ATA	AAT	*I – I*	Brazil French Guiana Equatorial Guinea Senegal
2877	959	ATT	ATA	*I – I*	Cambodia
2960	987	ATT	CTT	**I – T**	Senegal
2962	987	ATT	ATA	*I – I*	Cambodia
2974	998	AGT	AGC	*S – S*	Senegal
3090	1030*	AAG	AAA	*K – K*	Cambodia
3093	1031*	TGC	TGT	*C – C*	Cambodia, Senegal

Legend: NS: non synonymous. Syn: synonymous. All nucleotide, codon and corresponding amino acid positions in the table are adjusted to correspond to positions in the reference 3D7 sequence (accession n. PFA0310c) (http://www.plasmodb.org/plasmo/). Catalytic domains (*): cation atpase_N AA 3 – 81 and atpase_C, AA 1031–1215, E1_E2 atpase AA 99 – 348/AA 358–364, hydrolase AA 790–935; *Plasmodium* specific area (**): AA 407–486, 569–671, 852–860.
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catalytic activity of the protein. No mutation was identified in the amino acids described as of major importance for the SERCA function in animal species (ATP, phosphate and Ca++ binding, reviewed in [18]).

PfSERCA Polymorphism and Its Relationship with *In Vitro* Susceptibility to Artemisinins

In the set of 100 patient isolates studied here, 60 had their *in vitro* susceptibility profile assessed for an artemisinin derivative, either artemether or artesunate [2]. Each of the isolates presenting an IC50 greater than 20 nM had a distinct haplotype, but some commonalities were observed. In French Guiana three isolates had an IC_{50} for artemether greater than 100 nM; their haplotype was [L402V N569S S769N I898I], [E431K 769N I898I], [L402V S538S Q574P S769N I898I]. The haplotype of two isolates from French Guiana with an IC_{50} for artemether in the 50–100 nM range was [S538S S769N I898I] and [S569S I898I]. Five isolates from French Guiana, which presented IC_{50} for artemether between 20 and 50 nM had the following haplotype [S769N I898I], [L402V S539R Q574P S769N I898I], [L402V S538R I898I], [I898I], [D443D S769N I898I]. In Senegal there were three isolates with an IC_{50} for artesunate between 20 and 45 nM, their haplotypes were [E431K A623E], [E431K] and [L402V A623E]. Of these mutations, only S769N, which was associated with a high level of *in vitro* artemether resistance (IC_{50} above 30 nM) in French Guiana. S769N is located outside the *falciparum*-specific insert, in a position close to the C-terminal hinge between the N- and P- domains.

Table 3. Estimates of genetic diversity per sample and neutrality tests.

	S (η_s)	*h*	H_d	*K*	π	*D*	*D**	*F**
French Guiana	9 (5)	12	0.889	2.084	0.00075	−0.614^{ns}	−1.203 ^{ns}	−1.197 ^{ns}
Pará	4 (1)	3	0.564	1.538	0.00055	0.657 ^{ns}	0.335 ^{ns}	0.474 ^{ns}
Amazonas	2 (0)	3	0.689	0.822	0.00030	0.526 ^{ns}	1.026 ^{ns}	1.010 ^{ns}
Senegal	6 (2)	9	0.908	1.800	0.00065	−0.016 ^{ns}	−0.047 ^{ns}	−0.044 ^{ns}
Eq. Guinea	5 (4)	6	0.889	1.444	0.00052	−0.910 ^{ns}	−1.239 ^{ns}	−1.291 ^{ns}
Thailand	2 (1)	3	0.464	0.679	0.00024	−0.448 ^{ns}	−0.149 ^{ns}	−0.238 ^{ns}
Cambodia	0 (0)	1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
America	13 (5)	15	0.818	1.998	0.00072	−1.032 ^{ns}	−0.943 ^{ns}	−1.147 ^{ns}
Africa	10 (6)	13	0.910	1.727	0.00062	−1.150 ^{ns}	−1.654 ^{ns}	−1.751 ^{ns}
Asia	2 (1)	3	0.140	0.209	0.00008	−1.241 ^{ns}	−0.714 ^{ns}	−0.995 ^{ns}
All samples	21 (10)	28	0.742	1.500	0.00054	−1.855*	−2.501*	−2.698*

Legend: The extent of *pfserca* gene sequence analysed here correspond to nucleotide coordinates 87-2862 of the PFA0310c *pfserca* sequence of the reference clone 3D7 (Plasmodium genome database PlasmoDB; http://www.plasmodb.org/plasmo/). *N*: sample size. *S*: number of segregating sites, of which the number of singletons (η_s) is presented in parenthesis. *h*: number of haplotypes. H_d: haplotype diversity (*i.e.* expected heterozygosity based on haplotype frequency). *K*: average number of nucleotide differences between two sequences. π: average number of nucleotide differences between two sequences, per site. *D*: Tajima's (1989) values for *D*-test. *D** and *F** are Fu & Li's (1993) values for the respective neutrality tests. ^{ns}: not significant. *$P<0.05$. n.a.: not applicable (monomorphic) .

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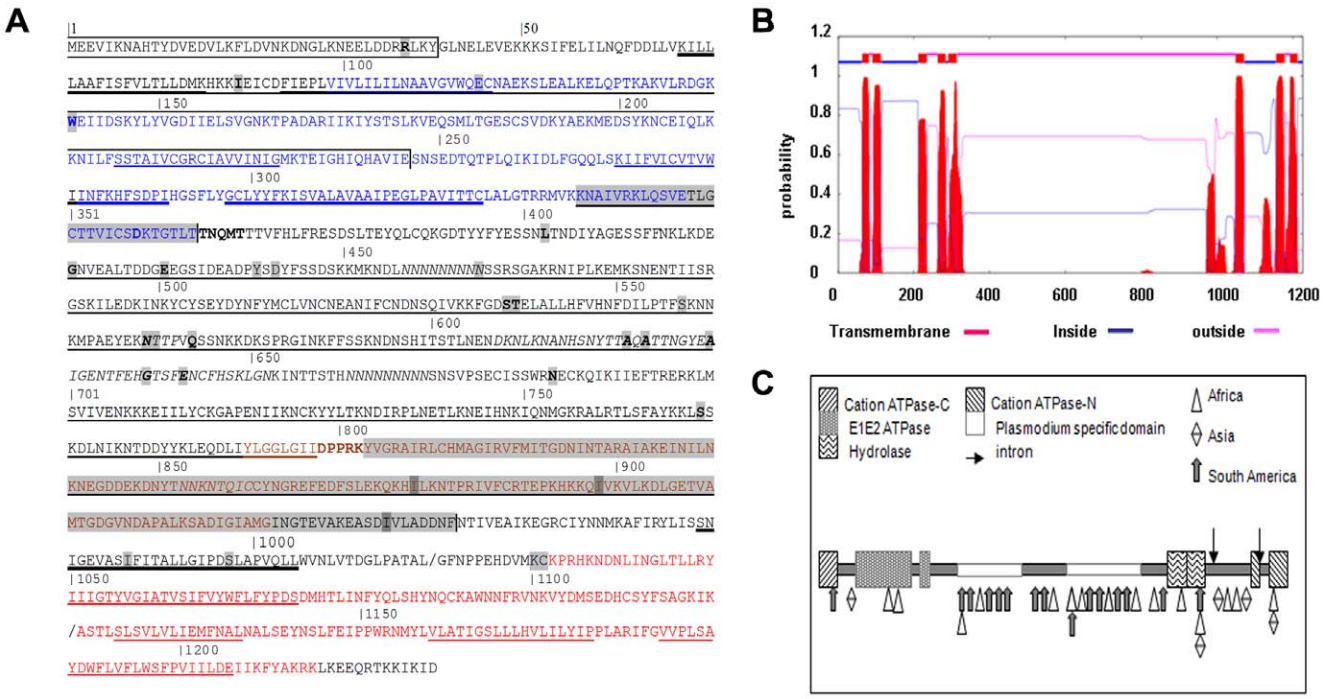


Figure 1. Predicted domains and localization of the mutations in Pf-SERCA protein sequence. A) Amino-Acid sequence of Pf-SERCA The functional domains of the protein are located according to the alignment with the rabbit ATPase: A (boxed), P (box-grey) and N (underlined), *Plasmodium*-specific domains are shown in italic. Hinge between P and N domains are bolded (TNQMT and DPPRK). Introns are located in the gene after L1019 and K1119 (noted by the sign/). Transmembrane domains are bold-underlined. Mutations are boxed in grey (non-synonymous mutation in bold). Three catalytic domains are predicted by PROSITE: cation atpase_N 3 – 81 and ATPase_C 1031–1215 (red), E1_E2 atpase 99 – 348/358-364 (blue), hydrolase 790–935 (braun). The position of the two introns is indicated with a/. B) Prediction of transmembrane domain by HMM (www.PROSITE.org) Ten transmembrane domains were predicted using TMpred with ''in to out'' (i-o) or ''out to in''(o-i) orientation: M1 AA67-85 (o-i), M2 AA94-116 (i-o), M3 AA216-233 (o-i), M4 AA271-290 (i-o), M5 AA298-325 (o-i), M6 AA979-1005 (i-o), M7 AA1053-1073 (o-i), M8 AA1124-1140 (i-o), M9 AA1158-1175 (o-I), M10 AA1183-1206 (i-o). C) Schematic representation of the catalytic domains of Pf-SERCA and localization of the mutations. Mutations are given symbols by continent of origin of the isolates (non synonymous mutations depicted with filled arrows, synonymous mutations depicted with open arrows).

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Genetic Diversity and Geographic Distribution

A total of 96 *pfserca* sequences were analysed for a fragment of 2785 bp encompassing nucleotides 87-2862. Nine positions corresponding to tri-nucleotide microsatellite repeats (AAT) starting at position 1292 were excluded from further analyses. Thus, we analysed a partial fragment of 2776 bp, which corresponded to nucleotide coordinates 87 – 2862 in the *pfserca* 3D7 reference sequence (accession n. PFA0310c) deposited at the Plasmodium genome database PlasmoDB. Overall, 21 variable sites were detected, of which 10 were singletons (positions 266, 421, 1262, 1329, 1612, 1671, 1707, 1710, 1868 and 1927) and 11 were parsimony informative (positions 110, 1204, 1291, 1614, 1706, 1721, 1888, 1916, 2049, 2306 and 2694). Of the 21 segregating sites, 5 were synonymous changes and 16 were non-synonymous mutations (Table 2).

Parasites from Africa and America presented the highest estimates of *pfserca* haplotype diversity, H_d (Table 3). Africa showed comparable estimates of variability among samples (*i.e.* countries) whereas in South America, the sample of French Guiana was the only showing values of H_d comparable to Africa. The two Brazilian samples had lower haplotype diversity, in particular Amazonas'. A similar trend was observed in terms of nucleotide diversity (Table 3), although in this case the South American sample from Pará presented an estimate of π intermediate to those obtained for African samples. Diversity for the *pfserca* gene from Asia was very low. The sample from Cambodia was monomorphic, while the sample from Thailand displayed the lowest estimates of haplotype and nucleotide diversities.

Tajima's [36] and Fu & Li's [37] (Table 3) tests showed no significant departures from neutrality, either within country or continents. Only when all sequences were pooled were significant departures ($P<0.05$) observed in all tests, but this probably results from ascertainment bias due to sampling of a non-randomly mating gene pool. The d_N/d_S ratios were below one, indicative of purifying selection, except for the sample of Amazonas in which no synonymous mutations were detected (Table 4). However, differences between d_N and d_S were non-significant in all samples. For the McDonald-Kreitman test, marginally significant ($P<0.05$) departures from neutrality associated with *NI* values above one (indicative of purifying selection) were detected in the samples of French Guiana and Senegal (Table 4). These tests were not considered significant after applying Bonferroni corrections for multiple tests (adjusted significance level: $\alpha' = 0.009$).

Overall, genetic differentiation of *pfserca* (global $F_{ST} = 0.214$, $P<0.001$) reflected the geographic origin of samples. Highly significant F_{ST} estimates ($P<0.001$) were obtained for the three pair wise comparisons: America vs. Africa ($F_{ST} = 0.097$), America vs. Asia ($F_{ST} = 0.088$), Africa vs. Asia ($F_{ST} = 0.117$). In Asia, all comparisons involving the monomorphic sample of Cambodia were significant, with the exception of the neighbour sample from Thailand (Table 5). In Africa, no significant genetic differentiation was observed between the two samples of this continent, ($F_{ST} = 0.066$). In contrast, American samples showed high pair wise F_{ST} values, particularly in comparisons involving Amazonas, which supports a high *P. falciparum* population subdivision in this area.

To analyse this geographic subdivision, a genealogical network was produced using the TCS software with correction of the reticulations [38] (Figure 2). The ancestral haplotype (A1) was present in all samples and it was the predominant haplotype in the Asian ones. The second most represented haplotype (A2) was present only in Africa and Asia. All remaining haplotypes were region or country-specific. Samples from French Guiana revealed a complex genealogical pattern derived from A1 and formed by unique branches separated from A1 by up to six mutational steps with low-frequency internal clades.

Discussion

The large sequence diversity observed in this analysis confirms findings from previous *pfserca* molecular surveys. For instance, in Niger six different mutations were found [28] while Dahlstrom *et al.* [29] found 31 mutations in 302 samples from Zanzibar and three mutations in 39 Tanzanian samples. Similarly Menegon *et al.* [39] found seven mutations in 71 specimens. The synthesis of all reported polymorphisms is provided in Table S1 (with Figure - References S1). The overall degree of *pfserca* polymorphism in our study was one SNP/115 bp and when including all published sequences (some of which being quite partial) this figure was one SNP/55 bp. This somehow puts *pfserca* at odds compared to genes

Table 4. d_N/d_S ratio and McDonald-Kreitman test of neutrality for the *pfserca* gene.

	d_N/d_S ratio		McDonald-Kreitman test					
			Interspecific fixed nucleotide differences		Intraspecific nucleotide polymorphisms			
	d_N/d_S	*P*-value[1]	*Syn*	*Nsyn*	*Syn*	*Nsyn*	*NI*	*P*-value[2]
French Guiana	0.53	0.511	43	15	3	6	5.7	0.022
Pará	0.59	0.707	43	15	1	3	8.6	0.070
Amazonas	∞	0.169	43	15	0	2	n.a.	0.077
Senegal	0.75	0.824	43	15	1	5	14.3	0.010
Eq. Guinea	0.22	0.318	43	15	2	3	4.3	0.136
Thailand	0.15	0.415	43	15	1	1	2.9	0.475
Cambodia	n.a.	n.a.	43	15	0	0	n.c.	n.c.

Legend *P*-value[1]: two-tailed Z-test based on 1000 bootstraps with an alternative hypothesis of $d_N \neq d_S$. *Syn*: synonymous. *Nsyn*: non-synonymous. *NI*: neutrality index. *P*-value[2]: *P-value* of Fisher's Exact test. ∞: infinite (no synonymous polymorphisms). n.a.: not applicable (no intraspecific synonymous polymorphisms). n.c.: not computed (monomorphic sample).

The extent of *pfserca* gene sequence analysed here correspond to nucleotide coordinates 87-2862 of the PFA0310c *pfserca* sequence of the reference clone 3D7 (Plasmodium genome database PlasmoDB; http://www.plasmodb.org/plasmo/).

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Table 5. Genetic differentiation between samples, measured by F_{ST}.

	French Guiana	Pará	Amazonas	Senegal	Equatorial Guinea	Thailand
French Guiana	-					
Pará	**0.213**	-				
Amazonas	**0.308**	0.341	-			
Senegal	**0.179**	0.136	**0.336**	-		
Equato Guinea	0.190	0.077	**0.367**	0.066	-	
Thailand	0.175	0.092	0.412	0.078	−0.019	-
Cambodia	**0.198**	0.231	**0.543**	**0.200**	**0.188**	0.095

Legend: Underlined: $P<0.05$; In bold: significant estimate after Bonferroni correction. The extent of *pfserca* gene sequence analysed here correspond to nucleotide coordinates 87-2862 of the PFA0310c *pfserca* sequence of the reference clone 3D7 (Plasmodium genome database PlasmoDB; http://www.plasmodb.org/plasmo/).
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presenting similar GO classification. These figures are indeed much higher than estimated average of one SNP/910 bp in coding regions [40], one common SNP per 842 bp [41], one SNP/780 bp across three fully sequenced genomes and one every 446 bases across certain large genomic regions [42]. Considering that intronic sequences that evolve more rapidly than coding sequences were excluded from our analysis, our SNP rates are if anything going to have been underestimated. Kidgell et al reported that genes located in the central regions of chromosomes (as is *pfserca*) usually present minimal sequence diversity [43]. Interestingly however, as noted in chromosome or genome wide studies [41–42] most SNP were rare, with 15 SNPs observed once and only 6 SNPs presenting a>5% frequency in the samples analysed. As a result, in the set of samples studied, a minority of SNPs (3 of 32) were observed in multiple settings, and a similar conclusion can be drawn when analysing the compiled list of SNPs reported to date, with only 14 of 68 mutations observed in multiple settings.

Parasites from Africa and America were the most genetically diverse. Within the African continent samples showed comparable estimates of variability among the different countries sampled. Samples from Asia presented the lowest genetic diversity. These observations are in line with a previous report indicating reduced diversity in Asian *P. falciparum* parasites inferred by typing of 183 SNPs across chromosome 3 [40], but contrast with other studies showing lower genetic diversity in South America than in Asia [44–46]. For instance, Mehlotra et al report a much lower *Pfcrt* and *Pfmdr* haplotype diversity in samples from South America, including Brazil and Guyana – which borders French Guiana- compared to parasites from African areas [45], contrasting with our observation of similar, large diversity in Africa and South America for *pfserca*. Neafsey et al studied a large panel of SNPs across the 14 chromosomes in a set of isolates including isolates from Brazil, Senegal and Thailand –three geographic areas sampled here as well- and found that the lowest diversity was in Brazil [46]. In our study, the lowest diversity of the three areas was Thailand. The reason of our discrepancy with the latter studies is unclear but this may be due to differences in technical approach used to study polymorphism or differences in sampling or in the genomic regions analysed, since the degree of genetic differentiation in *P. falciparum* has been shown to vary across the genome. Moreover we did not include the non coding regions in our analysis and did not survey intronic or flanking 5′ or 3′ microsatellite diversity, known to be much larger than coding sequences.

Importantly, genetic differentiation at the *pfserca* gene reflected the geographic origin of samples. When samples were pooled by continent, highly significant ($P<0.001$) F_{ST} estimates were obtained for the three pair wise comparisons ($0.088 \leq F_{ST} \leq 0.117$). This is in line with numerous studies of chromosomal or mitochondrial genes [40–43,46]. Within continents, the monomorphic sample of Cambodia was not significantly different from the neighbour sample from Thailand, and virtually no population substructure was observed in Africa. In contrast, samples from the American continent showed significantly high pair wise F_{ST} values, particularly in comparisons involving the sample from Amazonas. This suggests a much higher *P. falciparum* population substructure is detected by this gene in South America, a conclusion in agreement with the notion of a higher *P. falciparum* population differentiation in regions of lower malaria transmission [44]. Interestingly, estimates of genetic variation in America were greatly influenced by the French Guiana cohort, in which diversity was comparable to Africa. This may be due to the particular micro-epidemiological scenario in French Guiana, with patchy foci, each with a small number of inhabitants having easy access to antimalarial drugs, a situation that favours outgrowth of variant parasites. This was further confirmed by a genealogy displaying multiple tip haplotypes suggesting a local recent expansion of *pfserca* diversity. The peculiar epidemiology of the French Guiana area was also outlined by a previous study of the mitochondrial *cytb* gene, which displayed a particularly elevated polymorphism with specific SNPs, some reaching very high frequency [47].

In the TCS genealogical network, the most frequent haplotype was represented in samples from all continents surveyed and it is also likely to be the ancestral haplotype of *pfserca*. No clear overrepresentation of samples from a particular continent occurred within this haplotype and all internal haplotypes originating from the ancestor were distributed equally between South America and Africa. The network revealed a high number of low frequency external clades, which may be indicative that the gene is evolving rapidly. The particular reasons why this happens are difficult to disentangle from epidemiological or gene-specific function scenarios. *Pfserca* is the only serca gene of *Plasmodium*, making it highly specialized and unique for the parasite's calcium homeostasis. Interestingly, it has been recently suggested that highly specialized genes in the *Plasmodium* genome are more likely to undergo accelerated selective processes [48–49]. However, neither Tajima's nor Fu & Li's tests provided evidence of departures from neutrality and calculations of the McDonald-Kreitman test identified only marginally significant departures in French Guiana ($P=0.022$) and in Senegal ($P=0.010$). These were associated with NI values >1 indicative of purifying selection. Although the P-values were not considered significant after Bonferroni corrections for multiple tests, it was interesting to

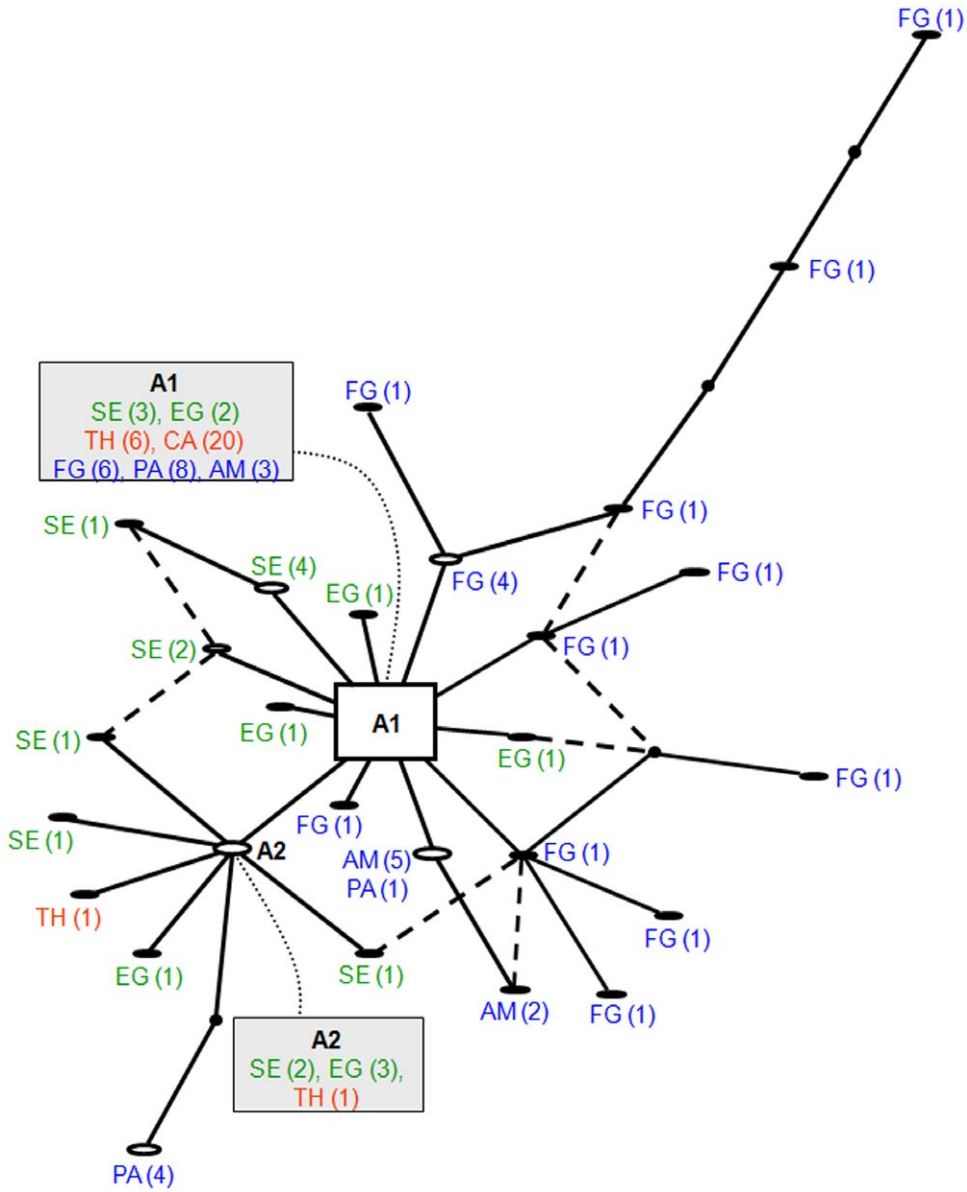


Figure 2. TCS network for the pfserca gene of Plasmodium falciparum. Each distinct haplotype is represented by an oval with size proportional to its frequency (in parenthesis). Mutational steps are represented by lines. Dots connecting lines represent putative haplotypes that were not sampled. Dashed lines refer to reticulations corrected according to Crandall et al. [38]. Colours correspond to each continent sampled: blue - America (AM: Amazonas; FG: French Guiana; PA: Para), green - Africa (EQ: Equatorial Guinea; SE: Senegal), red - Asia (CA: Cambodia; TH: Thailand).
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notice that these were the two countries where decreased *in vitro* susceptibility of *P. falciparum* to an artemisinin derivative was reported at the time of sample collection [2]. Purifying selection acts to prevent divergence of structure and function by selecting against extreme values of a trait, in contrast to directional selection, where a single phenotype is selected and therefore allele frequency shifts in one direction. The latter type of natural selection is more likely to occur on drug resistant determinants but probably does not apply for the present set of samples collected before substantial artemisinin pressure was in place in all the areas surveyed. Furthermore, the two most frequent SNPs were synonymous and their frequency varied greatly with the area/continent surveyed. The I898I mutation reached very high local frequency in Asia (0.78 in Cambodia, 0.85 in Thailand), but also in other areas such as Brazil (local frequency 0.85) or Equatorial Guinea (local frequency 0.50), while they were not detected at all in Senegal or French Guiana. Although we cannot exclude that such synonymous mutations may not be phenotypically silent as some synonymous *mdr* mutations were shown to influence the rate of translation, protein folding and substrate specificity [50], we tentatively conclude that it is likely that neutral evolution operating on possible multiple colonising events is currently shaping polymorphism at the *pfserca* gene of *P. falciparum* at this stage in history i.e. before massive worldwide deployment of artemisinin derivatives. Multiple colonisation events by ancestral parasites spreading out of Africa to the other continents have been suggested by the analysis of mitochondrial DNA [51].

The most obvious and statistically significant association regarding artemisinin susceptibility was that of the 769N mutation with an increased IC_{50} for artemether [2]. This was only observed

in the set of samples from French Guiana. The mutation is located in the vicinity of the predicted hinge domain of the protein, which influences binding of the calcium ion. As the inhibitory effect of thapsigargin depends on the engagement of the first calcium ion in the binding site [17,30,52,53], it is possible that the 769N mutation decreases the capacity of artemisinin to impair this process. This mutation was not detected in Asia and in the vast majority of African isolates studied so far [28–29], except in one dihydroartemisinin-susceptible isolate whose *in vitro* susceptibility to artemether remains unknown [23]. The marked geographical clustering of isolates from Asia, Africa and America observed here may account for the particular artemether resistance/*pfserca* mutations relationship found in America, especially as high levels of *in vitro* resistance to artemether have only been observed in America so far and as the genetic background of parasites from South America differs from Asia or Africa [40–44,46]. The association of 769N with artemether susceptibility and its relationship to the *in vitro* susceptibility to dihydroartemisinin certainly needs to be further studied. To ascertain the functional role of this mutation, biochemical studies with the mutant enzyme are needed along with genetic exchange studies in *P. falciparum* parasites. It is however possible that some mutations such as 769N have a heavy fitness cost, as parasites harbouring the mutation could not be propagated *in vitro* under standard culture conditions.

An important question for future studies is whether or not *pfserca* genetic characteristics change over time and in particular under the pressure by artemisinin derivatives delivered as combination therapies. In this regard, a recent survey of haplotype diversity for two chloroquine resistance candidate genes, *pfcrt* and *pfmdr1*, provides important clues for interpreting genetic variation of drug resistance candidate genes in light of drug pressure. This study showed that overall genetic variation in the *pfcrt* gene was lower than in the *pfmdr1* gene because chloroquine has exerted a stronger selective pressure on *pfcrt* favouring the occurrence of selective sweeps around the locus, resulting in reduced genetic diversity with time [45]. Because *pfmdr1* is a putative modulator of responses to artemisinin combination therapies as well as of chloroquine, future patterns of *pfmdr1* variation may help understand the specific selective pressures of the newly implemented combination therapy policy. The present data will serve as a useful snapshot to infer how *pfserca* polymorphisms is affected by drug pressure and whether some SNPs/haplotypes are selected by artemisinin combination therapies.

The significance of the large *pfserca* polymorphism and the consequences of the mutations described here on the protein's function and regulation are unclear. As indicated above, the dominant allele in all settings was the 3D7-type supposedly coding for a wild type sequence. In Cambodia, all mutations were synonymous, and likewise all but one sample from Thailand had only synonymous SNPs. This suggests substantial functional constraints. In line with the bioinformatic modelling [35] alignment of the PfSERCA sequence with other proteins outlined high conservation of all functional domains and transmembrane helices of the protein. PfSERCA is the largest SERCA described so far due to the presence of large *falciparum*-specific inserts most of which map to a region of the extra-membranous head that in other SERCAs is associated with closing the calcium channel. This suggests that these inserts could influence the channels properties by altering the conformational changes associated with calcium transport, and/or cope with specific requirements associated with intracellular calcium homeostasis in the human erythrocyte or hepatocyte host cell. As artemisinin was shown to inhibit *T. gondii* SERCA and trigger calcium release [54], the link between PfSERCA and calcium homeostasis deserves detailed investigation. This could furthermore offer new insights on factors possibly contributing to the high level of polymorphism of PfSERCA. Interestingly, the areas where the highest PfSERCA diversity was observed in this work and in previous studies are those with an elevated prevalence of haemoglobinopathies such as HbS, namely African populations [55] and South American countries with a substantial population of African origin (e.g. Belize, French Guiana and north Brazil). Sickle cell anaemia and beta-thalassaemia are associated with an elevation of total red cell calcium content [56–59]. This abnormally high cytosolic calcium content could be a selective force for mutant PfSERCA as an enhanced activity of this pump is needed in such environments to restore physiological intracellular calcium stores [60–61]. This possibility is currently under investigation.

Materials and Methods

For more details see also Materials and Methods S1.

Ethics Statement

This program received ethical clearance from all the National Ethical Committees of the countries involved in the study (NEC of Senegal, Cambodia, Brazil, Thailand, and the CCPRB of French Guiana). All the patients enrolled (or their family) gave a written informed consent for the protocol.

Sample Collection

Blood samples were obtained from consenting patients attending dispensaries with uncomplicated *P. falciparum* malaria (Table 1). The following isolates were used as characteristic haplotypes: F126 (GU456693), F128 (GU456694), F119 (GU456695), F91 (GU456696), F94 (GU456697), F64 (GU456698), F139 (GU456699), F132 (GU456700), F122 (GU456701), F108 (GU456702), F41 (GU456703), AM54 (GU456704), AM46 (GU456705), BR67 (GU456706), 8A (GU456707), 66A (GU456708), 22A (GU456709), 22G (GU456710), 24G (GU456711), 7A (GU456712), 58A (GU456713), 80A (GU456714), 9A (GU456715), EQG66 (GU456716), EQG41 (GU456717), EQG72 (GU456718), EQG28 (GU456719), RC17 (GU456720).

Sequencing of Field Isolates

Parasite DNA was extracted from red blood cells by standard phenol/chloroform method. Primers used were synthesized based on the published sequence of *Pfatp6* (Genbank, AN AJ532679). Samples from Thailand, Equatorial Guinea, Brazil 5amazonas and Parà) were sequenced as described [62]. Samples from French Guiana, Senegal and Cambodia were amplified using the following primers pairs (numbering refers to indicates the position of the first nucleotide of the primer in the PFA0310c sequence): 20 5′ATGCTCATACATACGATGTTGAG/1607 5′ AATATGAACAGTGATCCTCAGAC; 1268 5′ TTGAAGCTTTAACGGATGATGGA/3039 5′ TACCTAGTGCTGTTGCTGGTAA; 3207 5′ AATCCACCAGAACATGACGTAAT/3457 5′AATATGAACAGTGATCCTCAGAC; 3335 5′ TAGGCAAGCACCTTATCTTTATC/3952 5′ TTTTCTTGGTTCTTTGCTCTTCC. PCR reactions were done in 50 µL in the presence of 2.5 mM MgCl2, 2.5 U AmpliTaq Gold (Applied Biosystems). An initial denaturation at 94°C for 9 min, was followed by 38 cycles at 94°C for 1 min, 60°C for 2.5 min (for primers 20/1607 and 1268/3039, and 3 min at 56°C for primers 3207/3457 and 3335/3952) 72°C for 2 min followed by one cycle were the last elongation was for 10 min. PCR products were sequenced on both strands using internal primers essentially as described [47]. Potential sequence ambiguities were resolved either by re-sequencing or through close

inspection of the corresponding chromatogram, using PhredPhrap (www.phrap.org).

Bioinformatic Analysis

Sequences have been aligned using CLUSTALX software (version1.81) [63] and BOXSHADE (www.pasteur.fr). Functional areas were predicted using PROSITE. Prediction of the PfSERCA domains was performed by alignment with a set of other eukaryotic serca (*P. yoelii*, *P. gallinaceum*, *P. knowlesi*, *P. berghei*, *P. reichenowi*, *P. vivax*, *Oryctolagus cuniculus*, *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Caenorhabditis elegans*, *Leishmania donovani*, *Trypanosoma cruzi*). Helix prediction was performed using TMpred software (www.expasy.ch).

Phylogenetic Analysis of the Coding Sequence

Sequence polymorphism analysis and neutrality tests were performed using the software DnaSP 4.50.3 [64]. Neutrality was tested using Tajima's [36] and Fu & Li's [37] tests. Departures from selective neutrality at the protein level were assessed by the d_N/d_S ratio and the McDonald-Kreitman test. For between-species comparisons, a sequence of the *ATPase6* gene from the closely related *P. reichenowi* (GenBank n° AB122148.1) was used. For d_N/d_S ratios, significance was determined by a two-tailed Z-test available in MEGA 4.0.2 [65]. Fisher's exact tests on 2×2 contingency tables were used for McDonald-Kreitman tests, as implemented in DnaSP [66].

Levels of genetic differentiation were measured by the fixation index F_{ST}, based on nucleotide frequency differences [64]. To assess if F_{ST} values significantly differed from zero, permutation tests were performed based on the K_{ST}* estimator of pair wise nucleotide differences [64]. Calculations were performed with DnaSP. A genealogical network was produced using the TCS software [67], with correction of the reticulations following Crandall and Templeton [38].

Whenever multiple tests were performed, the nominal significance level ($\alpha = 0.05$) was adjusted by Bonferroni corrections to avoid type I error (i.e. false rejection).

Supporting Information

Figure S1 Mapping of the three main domains in Pf-SERCA Legend Three major motifs were detected using PROSITE: A) pfam00122.11 (E1-E2 ATPase) (residues 107-357 of PfSERCA); B) pfam00689.11 (Cation_ATPase_C) (residues 1032-1215 of PfSERCA), and C) pfam00702.11 (Hydrolase) (residues 790-931 of PfSERCA). Domains are mapped with sequences characteristic of the functions (indicated by accession number). In red conserved amino acid of the motifs.
Found at: doi:10.1371/journal.pone.0009424.s001 (0.47 MB PPT)

Figure S2 Alignment of Pf-SERCA with other known SERCA to point out amino acids of interest in the falciparum protein. Alignment of the SERCA protein sequences from five Plasmodium species with ten sequences selected across the phylogenetic tree. PfSERCA has the largest sequence of all those deposited in NCBI. The global SERCA structure is easily identifiable, with a cytosolic head composed of three domains homologous to the rabbit A, P, and N domains. Note that the PfSERCA and the P.reichenowi SERCA sequence contain specific inserts absent from all other SERCAs. These inserts, mainly located in the N domain located on the cytoplasmic side of the ER contain no identifiable motif. The region was predicted to be part of the extra-membranous head responsible for closing the Ca++ channel during the E1-E2 conformational change. The integral membrane domain of the protein is composed of ten helix domains, in line with other SERCAs. The two calcium pockets are completely conserved with only a shift in the position in the sequence due to a larger head. The key P-phosphorylation residue [1] was predicted to be D357, in line with the analysis of Nagamune et al [2]. In other SERCAs, phosphorylation of this aspartate in the presence of calcium induces a major conformational E1-E2 transition in the enzyme, associated with the release of the cation into the luminal side of the membrane (reviewed in [3]). This movement involves two well conserved hinges [4–6] that are also present in PfSERCA: i) at the interface of N and A domains (motif 601DPPRK in rabbit SERCA, corresponding to motif 798DPPRK in PfSERCA), and ii) between N and P domains (308TNQM in rabbit, 405TNDI in PfSERCA). Along the same line, C674 recently identified as a key residue in the NO-induced glutathione regulation of the rabbit SERCA, was identified as C887 in the PfSERCA sequence [7–8]. The thapsigargin binding site involving (F256-I765-Y837) in the human SERCA can putatively be located in the PfSERCA (F264-I976-Y1048, Figure S2), and is juxtaposed to the putative artemisinin site (L263, I272, F273, I1041) as proposed by in silico docking after deleting falciparum-specific inserts [9]. Three major domains are mapped: A domain (in blue), N domain (in green), P domain (in red). Conserved amino acids are bolded. Three numbering of amino acids are shown, P.falciparum, rabbit and human.
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Materials and Methods S1
Found at: doi:10.1371/journal.pone.0009424.s003 (0.04 MB DOC)

Table S1
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References S1
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Author Contributions

Conceived and designed the experiments: RJ. Performed the experiments: RJ AM JP EL MN NK LP BV MTE CB TF PB AB IDF CF MGA. Analyzed the data: RJ AM SG MN PC. Contributed reagents/materials/analysis tools: AM JP EL LP TF PB AB IDF CF PPV MGA. Wrote the paper: RJ OMP PC.

References

1. World Malaria Report (2008) WHO. www.who.int.
2. Jambou R, Legrand E, Niang M, Khim N, Lim P, et al. (2005) Resistance of Plasmodium falciparum field isolates to in-vitro artemether and point mutations of the SERCA-type PfATPase6. Lancet 366: 1960–1963.
3. Legrand E, Volney B, Meynard JB, Esterre P, Mercereau-Puijalon O (2007) Resistance to Dihydroartemisinin. Emerging Infectious Diseases 13: 808–9.
4. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM (2008) Artemisinin Resistance in Cambodia 1 (ARC1) Study Consortium Evidence

of artemisinin-resistant malaria in western Cambodia. N Engl J Med 359: 2619–20.
5. Lim P, Alker AP, Khim N, Shah NK, Incardona S, et al. (2009) Pfmdr1 copy number and arteminisin derivatives combination therapy failure in falciparum malaria in Cambodia. Malar J 8: 11.
6. Rogers WO, Sem R, Tero T, Chim P, Lim P, et al. (2009) Failure of artesunate-mefloquine combination therapy for uncomplicated Plasmodium falciparum malaria in southern Cambodia. Malar J 8: 10.
7. Carrara VI, Zwang J, Ashley EA, Price RN, Stepniewska K, et al. (2009) Changes in the treatment responses to artesunate-mefloquine on the northwestern border of Thailand during 13 years of continuous deployment. PLoS ONE 4: e4551.
8. Maude RJ, Pontavornpinyo W, Saralamba S, Aguas R, Yeung S, et al. (2009) The last man standing is the most resistant: eliminating artemisinin-resistant malaria in Cambodia. Malar J 8: 31.
9. Eckstein-Ludwig U, Webb RJ, Van Goethem ID, East JM, Lee AG, et al. (2003) Artemisinins target the SERCA of Plasmodium falciparum. Nature 424: 957–61.
10. Krishna S, Woodrow CJ, Staines HM, Haynes RK, Mercereau-Puijalon O (2006) Re-evaluation of how artemisinins work in light of emerging evidence of in vitro resistance. Trends in Molecular Medicine 12: 200–5.
11. Toyoshima C, Inesi G (2004) Structural basis of ion pumping by Ca2+-ATPase of the sarcoplasmic reticulum. Annu Rev Biochem 73: 269–92.
12. Toyoshima C (2009) How Ca(2+)-ATPase pumps ions across the sarcoplasmic reticulum membrane. Biochim Biophys Acta 1793: 941–6.
13. Berridge MJ, Lipp P, Bootman MD (2000) The versatility and universality of calcium signaling. Nat Rev Mol Cell Biol 1: 11–21.
14. Wuytack F, Raeymaekers L, Missiaen L (2002) Molecular physiology of the SERCA and SPCA pumps. Cell Calcium 32: 279–305.
15. Kimura M, Yamaguchi Y, Takada S, Tanabe K (1993) Cloning of a Ca(2+)-ATPase gene of Plasmodium falciparum and comparison with vertebrate Ca(2+)-ATPases. J Cell Sci 104: 1129–36.
16. Nagamune K, Sibley LD (2006) Comparative genomic and phylogenetic analyses of calcium ATPases and calcium-regulated proteins in the apicomplexa. Mol Biol Evol 23: 1613–27.
17. Toyoshima C, Nomura H, Sugita Y (2003) Structural basis of ion pumping by Ca^{2+}-ATPase of sarcoplasmic reticulum. FEBS Letters 555: 106–10.
18. Toyoshima C (2008) Structural aspects of ion pumping by Ca2+-ATPase of sarcoplasmic reticulum. Arch Biochem Biophys 476: 3–11.
19. Xu C, Ma H, Inesi G, Al-Shawi MK, Toyoshima C (2004) Specific structural requirements for the inhibitory effect of Thapsigargin on CA++ATPase SERCA. J Biol Chem 279: 17973–9.
20. Inesi G, Lewis D, Ma H, Prasad A, Toyoshima C (2006) Concerted conformational effects of Ca2+ and ATP are required for activation of sequential reactions in the Ca2+ ATPase (SERCA) catalytic cycle. Biochemistry 45: 13769–78.
21. Christensen SB, Skytte DM, Denmeade SR, Dionne C, Møller JV, et al. (2009) A Trojan horse in drug development: targeting of thapsigargins towards prostate cancer cells. Anticancer Agents Med Chem 9: 276–94.
22. Uhlemann AC, Cameron A, Eckstein-Ludwig U, Fischbarg J, Iserovich P, et al. (2005) A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. Nat Struct Mol Biol 12: 628–9.
23. Cojean S, Hubert V, Le Bras J, Durand R (2006) Resistance to dihydroartemisinin. Emerg Infect Dis 12: 1798–9.
24. Afonso A, Hunt P, Cheesman S, Alves A, Cravo P (2006) Malaria parasites can develop stable resistance to artemisinin but lack mutations in candidate gene ATPase 6, tctp, mdr1 and cg10. Antimicrob Agents Chemother 50: 480–489.
25. Nagamune K, Beatty WL, Sibley LD (2007) Artemisinin induces calcium-dependent protein secretion in the protozoan parasite Toxoplasma gondii. Eukaryot Cell 6: 2147–56.
26. Hunt P, Cravo PV, Donleavy P, Carlton JM, Walliker D (2004) Chloroquine resistance in Plasmodium chabaudi: are chloroquine-resistance transporter (crt) and multi-drug resistance (mdr1) orthologues involved? Mol Biochem Parasitol 133: 27–35.
27. Tanabe K, Sakihama N, Hattori T, Ranford-Cartwright L, Goldman I, et al. (2004) Genetic distance in housekeeping genes between Plasmodium falciparum and Plasmodium reichenowi and within P. falciparum. J Mol Evol 59: 687–94.
28. Ibrahim ML, Khim N, Adam HH, Ariey F, Duchemin JB (2009) Polymorphism of PfATPase in Niger: detection of three new point mutations. Malar J 8: 28.
29. Dahlström S, Veiga MI, Ferreira P, Mårtensson A, Kaneko A, et al. (2008) Diversity of the sarco/endoplasmic reticulum Ca(2+)-ATPase orthologue of Plasmodium falciparum (PfATP6). Infect Genet Evol 8: 340–5.
30. Ma H, Lewis D, Xu C, Inesi G, Toyoshima C (2005) Functional and structural roles of critical amino acids within the"N", "P", and "A" domains of the Ca2+ ATPase (SERCA) headpiece. Biochemistry 44: 8090–100.
31. Toyoshima C, Nomura H (2002) Structural changes in the calcium pump accompanying the dissociation of calcium. Nature 418: 605–11.
32. Montigny C, Picard M, Lenoir G, Gauron C, Toyoshima C, et al. (2007) Inhibitors bound to Ca(2+)-free sarcoplasmic reticulum Ca(2+)-ATPase lock its transmembrane region but not necessarily its cytosolic region, revealing the flexibility of the loops connecting transmembrane and cytosolic domains. Biochemistry 46: 15162–74.
33. Adachi T, Weisbrod RM, Pimentel DR, Ying J, Sharov VS, et al. (2004) S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. Nat Med 10: 1200–7.
34. Cohen RA, Adachi T (2006) Nitric-Oxide-Induced Vasodilatation: Regulation by Physiologic S-Glutathiolation and Pathologic Oxidation of the Sarcoplasmic Endoplasmic Reticulum Calcium ATPase. Trends Cardiovasc Med 16: 109–114.
35. Jung M, Kim H, Nam KY, No KT (2005) Three-dimensional structure of Plasmodium falciparum Ca2+ -ATPase(PfATP6) and docking of artemisinin derivatives to PfATP6. Bioorg Med Chem Lett 15: 2994–2997.
36. Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123: 585–595.
37. Fu YX, Li WH (1993) Statistical tests of neutrality of mutations. Genetics 133: 693–709.
38. Crandall KA, Templeton AR (1993) Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. Genetics 134: 959–969.
39. Menegon M, Sannela AR, Majori G, Severini C (2008) Detection of novel point mutation in the Plasmodium falciparum ATPase candidate gene for resistance to artemisinin. Parasitol Int 57: 233–235.
40. Mu J, Awadalla P, Duan J, McGee KM, Joy DA, et al. (2005) Recombination hotspots and population structure in Plasmodium falciparum. PLoS Biol 3: e335.
41. Mu J, Awadalla P, Duan J, McGee KM, Keebler J, et al. (2007) Genome-wide variation and identification of vaccine targets in the *Plasmodium falciparum* genome. Nat Genet 39: 126–130.
42. Volkman SK, Sabeti PC, DeCaprio D, Neafsey DE, Schaffner SF, et al. (2007) A genomewide map of diversity in *Plasmodium falciparum*. Nat Genet 39: 113–119.
43. Kidgell C, Volkman SK, Daily J, Borevitz JO, Plouffe D, et al. (2006) A systematic map of genetic variation in *Plasmodium falciparum*. PLoS Pathog 2: e57.
44. Anderson TJ, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, et al. (2001) Microsatellite markers reveal a spectrum of population structures in the malaria parasite Plasmodium falciparum. Mol Biol Evol 17: 1467–82.
45. Mehlotra RK, Mattera G, Bockarie MJ, Maguire JD, Baird JK, et al. (2008) Discordant patterns of genetic variation at two chloroquine resistance loci in worldwide populations of the malaria parasite Plasmodium falciparum. Antimicrob Agents Chemother 52: 2212–22.
46. Neafsey DE, Schaffner SF, Volkman SK, Park D, Montgomery P, et al. (2008) Genome-wide SNP genotyping highlights the role of natural selection in Plasmodium falciparum population divergence. Genome Biol 9: R171.
47. Ekala MT, Khim N, Legrand E, Randrianarivelojosia M, Jambou R, et al. (2007) Sequence analysis of Plasmodium falciparum cytochrome b in multiple geographic sites. Malar J 6: 164.
48. Gardner MJ, Tettelin H, Carucci DJ, Cummings LM, Aravind L, et al. (1998) Chromosome 2 sequence of the human malaria parasite Plasmodium falciparum. Science 282: 1126–32.
49. Jeffares DC, Pain A, Berry A, Cox AV, Stalker J, et al. (2007) Genome variation and evolution of the malaria parasite Plasmodium falciparum. Nat Genet 39: 120–5.
50. Kimchi-Sarfaty C, Oh JM, Kim IW, Sauna ZE, Calcagno AM, et al. (2007) A "silent" polymorphism in the MDR1 gene changes substrate specificity. Science 315: 525–8.
51. Joy DA, Feng X, Mu J, Furuya T, Chotivanich K, et al. (2003) Early origin and recent expansion of Plasmodium falciparum. Science 300: 318–21.
52. Toshihiko T, Kurzydlowski K, Lyttonll J, MacLennan DH (1992) The Nucleotide Binding/Hinge DomainP lays a Crucial Role in Determining Isoform-specific Ca2′ dependence of Organellar Ca2′-ATPases. J Biol Chem 267: 14490–14436.
53. Yu M, Lin J, Khadeer M, Yeh Y, Inesi G, Hussain A (1999) Effects of various amino acid 256 mutations on sarcoplasmic/endoplasmic reticulum Ca2+ ATPase function and their role in the cellular adaptive response to thapsigargin. Arch Biochem Biophys 362: 225–32.
54. Nagamune K, Moreno SN, Sibley LD (2007) Artemisinin-resistant mutants of Toxoplasma gondii have altered calcium homeostasis. Antimicrob Agents Chemother 51: 3816–23.
55. Modell B, Darlison M (2008) Global epidemiology of haemoglobin disorders and derived service indicators. WHO bulletin 86: 480–487.
56. Hebbel RP, Steinberg MH, Eaton JW (1981) Erythrocyte calcium abnormalities in sickle cell disease. Prog Clin Biol Res 51: 321–32.
57. Mentzer WC, Clark MR (1983) Disorders of erythrocyte cation permeability and water content associated with hemolytic anemia. Biomembranes 11: 79–118.
58. Wu SN (2003) Large-conductance Ca2+- activated K+ channels:physiological role and pharmacology. Curr Med Chem 10: 649–61.
59. Föller M, Huber SM, Lang F (2008) Erythrocyte programmed cell death. IUBMB Life 60: 661–8.
60. Kawazoe U, Fang J, Miranda S, de Souza W, Plattner H, et al. (2008) Acidocalcisomes in Apicomplexan parasites. Experimental Parasitology 118: 2–9.
61. Inesi G, Lewis D, Toyoshima C, Hirata A, de Meis L (2008) Conformational fluctuations of the Ca2+-ATPase in the native membrane environment: Effects of pH, temperature, catalytic substrates, and thapsigargin. J Biol Chem 283: 1189–96.
62. Ferreira ID, Lopes D, Martinelli A, Ferreira C, do Rosário VE, et al. (2007) In vitro assessment of artesunate, artemether and amodiaquine susceptibility and molecular analysis of putative resistance-associated mutations of Plasmodium falciparum from São Tomé and Príncipe. Trop Med Int Health 12: 353–62.

63. Thompson JD, Gibson TJ, Higgins DG (2002) Multiple sequence alignment using ClustalW and ClustalX. Curr Protoc Bioinformatics Chapter 2: Unit 2.3.
64. Rozas J, Sánchez-Delbarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19: 2496–2497.
65. Tamura K, Dudley J, Nei M, Kumar S (2007) *MEGA4*: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.
66. Hudson RR, Slatkin M, Maddison WP (1992) Estimation of levels of gene flow from DNA sequence data. Genetics 132: 583–589.
67. Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation Genetics 132: 619–633.