Frequency Distribution of Antimalarial Drug Resistance Alleles among *Plasmodium falciparum* Isolates from Gezira State, Central Sudan, and Gedarif State, Eastern Sudan

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Abstract. In 2004, Sudan adopted artesunate + sulfadoxine/pyrimethamine (SP) combination as the first-line drug, in response to the high level of *falciparum* resistance to antimalarials. In 2007, a molecular study on antimalarial resistance linked genes, *pfprt*, *pfmdr1*, *pfdhfr*, *pfdhps*, and *pfATPase6*, was conducted on 198 isolates from central and eastern Sudan. We observed a high frequency of point mutations at almost all loci analyzed, mainly of *pfprt* 76T (72.7%), *pfdhfr* 51I (75.3%), and *pfdhfr* 108N (72.7%) alleles. The MARK III *in vitro* test for chloroquine sensitivity in 45 *P. falciparum* isolates showed that 37.8% of the isolates were low resistant and 6.7% were fully resistant. This study represents the most recent molecular investigation on antimalarial resistance in this area after the adoption of artemisinin-based combination therapy (ACT), and underlines the importance of the analysis of SP resistance evolution to monitor the efficacy of ACT therapy in endemic areas.

INTRODUCTION

Malaria is still one of the most threatening diseases for people living in tropical and subtropical areas and its control is seriously hampered by the quick development of antimalarial drug resistance by *Plasmodium falciparum*.

During the second half of the 20th century, chloroquine (CQ) was the antimalarial treatment of choice, because it was safe, cheap, and highly effective against susceptible malaria parasites. The CQ resistance arose more than 40 years ago in southeast Asia and South America, and increasing rates of CQ resistance contribute to rising morbidity and mortality from malaria in Africa.

The CQ resistance is determined by the major point mutation at codon 76 of the *P. falciparum* CQ resistance transporter (*pfprt*) gene,¹ which is highly correlated with increased clinical CQ tolerance and treatment failure.^{1–3} In addition, point mutations in *P. falciparum* multi-drug resistance gene 1 (*pfmdr1*) (e.g., N86Y, Y183F, S1034C, N1042D, and D1246Y) have been shown to modulate CQ resistance⁴ and possibly lumefantrine resistance.⁵

Similarly, resistance of malaria parasites to antifolates is conferred by mutation in dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), two enzymes involved in the parasite's folate synthesis.^{6–10} In particular, a combination of five point mutations, *pfdhfr* N51I, C59R, S108N, and *pfdhps* A437G and K540E, have been shown to be strongly associated with sulfadoxine/pyrimethamine (SP) resistance in Africa.^{11–13}

Adding artemisinin derivatives (i.e., artesunate) to standard antimalarial drugs (i.e., SP) is a strategy to reduce treatment failure and transmission potential, because artemisinin deriva-

tives act rapidly and kill malaria parasites that are resistant to other drugs.¹⁴ In fact, many malaria endemic countries have lately adopted artemisinin-based combination therapy (ACT) as the first-line drug for treating uncomplicated malaria, as recommended by the World Health Organization (WHO).¹⁵

Although ACT should represent the best option available to prevent the induction of resistance parasites, the risk of development of resistance in *P. falciparum* to artemisinin compounds is serious, given the recent observation of two cases of clinical resistance in Cambodia¹⁶ and cases of diminished *P. falciparum* susceptibility to arthemeter.^{17,18}

Recently, it was observed that variations in susceptibility profiles of malaria parasites to artemisinin derivatives have been associated with single nucleotide polymorphisms (SNPs) in sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA)-type of *P. falciparum* ATPase6 (*pfATPase6*) gene, the putative target for artemisinins,^{17,19,20} suggesting a possible role of *pfATPase6* gene as a molecular marker of artemisinin resistance.

Sudan is the largest country in Africa, comprising more than 8% of the entire continent. The total population is estimated to be 39.2 million inhabitants, of whom 75% live in rural areas. Malaria is a leading cause of morbidity and mortality, resulting in 3.1 million cases and 2,500 deaths annually.²¹ The entire country is at risk of malaria, although with different levels of endemicity. In the northern, eastern, and western states malaria endemicity is mainly low to moderate with predominantly seasonal transmission and epidemic outbreaks. In southern Sudan, malaria is moderate to high or highly intense, generally with perennial transmission. *Plasmodium falciparum* is by far the predominant parasite species.

The CQ has been the most used drug as a first line for years in Sudan. Some studies showed that in five hospitals of central Sudan, 85.6% of patients admitted for severe malaria had received CQ before admission²² and that CQ was also the commonly prescribed drug by health care providers.²³ First cases of CQ resistance were reported in 1978,²⁴ and several *in vivo* and *in vitro* studies have subsequently documented a

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high presence of CQ resistance in different Sudanese areas (central Sudan²⁵⁻²⁷; eastern Sudan^{28,29}).

Sulfadoxine/pyrimethamine resistance was reported in Sudan since the early 1990s³⁰ and was documented by *in vivo* studies conducted in eastern Sudan just before ACT deployment.³¹⁻³³ The presence of highly resistant *pf dhfr/pf dhps* quintuple mutants have been previously reported in studies conducted in different areas of southern³⁴ and eastern Sudan.³¹

In 2004, because of CQ and SP resistance, the national antimalarial drug policy changed from monotherapy to artemisinin-based combination therapy. The National Malaria Control Program decided to replace CQ with artesunate + sulfadoxine/pyrimethamine as the first-line drug and artemether-lumefantrine has replaced SP as the second-line treatment.³⁵ The aim of this study was to assess the effect of the current antimalarial drug policy, i.e., ACT, on prevalence of mutations in target genes, in two malarious areas of Sudan where to the best of our knowledge no recent investigations on the same subject have been carried out.

MATERIALS AND METHODS

Study sites. The fieldwork was carried out in health facilities located in and around Wad Medani–Gezira State (central Sudan) and Elfaou locality–Gedaref State (eastern Sudan) (Figure 1) between June and December 2007, corresponding to the period of high malaria transmission. These study sites are located in an irrigated agricultural scheme, where transmission is seasonal as identified by epidemiological strata of malaria in Sudan and are inhabited by different ethnic groups and tribes from all regions of Sudan. Wad Medani is the main town of the irrigated area of the Gezira Agricultural Scheme where malaria is classified as mesoendemic to hyperendemic with an

unstable transmission pattern. Elfaou is located in the west part of Gedaref State neighboring Gezira State. It is the main town of the Erahad irrigation scheme.

Patients and blood samples. Participants were enrolled in the study after appropriate informed consent was obtained. Ethical approval for this study was obtained from the Ethical Committee of the Blue Nile National Institute for Communicable Disease/University of Gezira and from the State Health Authority in Gezira.

Blood samples were collected from 212 (central Sudan) and 63 (eastern Sudan) microscopically confirmed *P. falciparum*-infected patients. Of 275 patients screened, 235 patients (42% males, 58% females, age range: 4–45 years) met the criteria for inclusion in the analysis: patients had *P. falciparum* monoinfection, asexual parasitaemias in excess of 1,000 parasites, but less than 80,000 parasites (mean of 29,520 parasites) per μ L blood, and had not received quinine and artemisinin within the last 7 days, 4-aminoquinolines within the last 14 days, pyrimethamine and/or sulfonamide within the last 28 days, or mefloquine within the last 56 days.

For molecular analysis, infected blood was collected by finger prick, blotted *in triplicate* on a filter paper, and air dried. Filter papers were wrapped separately in a plastic bag and stored at room temperature. Total DNA was extracted from 149 samples using the phenol/chloroform method⁹ and from 86 by using PureLink Genomic DNA Kit (Invitrogen, Carlsbad, CA), following the manufacturer's instructions.

For the *in vitro* test, 2 mL of blood were collected from subjects into a sterile EDTA container after cleaning the site of puncture with 70% ethanol.

Real-time PCR assay. All samples were genotyped at *pf crt* 76 codon, *pf dhfr* 51-59-108 codons, and *pf dhps* 436-437 codons using a 5'-nuclease real-time polymerase chain reaction (PCR) assay. Primers and probes sequences were

FIGURE 1. Map of Sudan. On the right are the sites of this study. The geographical coordinates are indicated in the figure.

previously described^{36–37} and were synthesized by Roche-Diagnostics (Mannheim, Germany). Wild-type and mutant-type probes were labeled at a FAM and HEX fluorescent reported at their 5' ends, respectively. Real-time PCR was performed with a LightCycler 480 System (Roche) according to the following steps: pre-incubation 95°C for 10 min; amplification 95°C for 10 sec, 55°C for 20 sec, and 72°C for 5 sec for 45 cycles. The PCR assays were optimized according to the LightCycler 480 Probes Master Kit, and 0.5 µM of each primer and 0.2 µM of each probe, used in combination, were used in this study. All reactions were done in duplicate, in a final volume of 20 µL. The DNAs from W2 and 3D7 laboratory strains and from characterized field isolates were used as reference standards.

PCR-restriction fragment length polymorphism (RFLP) analysis. Detection of *pfmdr1* 86 and *pfdhps* 540 polymorphisms was performed by the PCR-RFLP method. *Pfmdr1* amplification was achieved under conditions described in Duraisingh and others.³⁸ The PCR products were digested at 37°C with 1U of *AflIII* (New England Biolabs, Ipswich, MA) restriction enzyme. Detection of *pfdhps* 540 polymorphism was performed using primers described in Alker and others.³⁶ The PCR products were digested at 37°C with 1U of *FokI* (Biolabs) restriction enzyme, as described in Duraisingh and others.³⁹

Sequencing. Assessment of single nucleotide polymorphisms for the *pfATPase6* gene was based on PCR and the sequencing method, as described previously in Menegon and others.⁴⁰

Statistical analysis. Differences in mutation frequency of the studied genotypes by sampling sites or by sampling period were analyzed by the two-sample test of proportions (STATA Statistical Software: Release 8.1, 2003, Stata Corp., College Station, TX). The same statistical test was adopted to compare the 59R mutation frequency observed in 2003³¹ and in this study. Results are showed as *z*(test) and *p*(*z*) values.

In vitro test. The micro-technique test (Mark III; supplied by WHO Division of Control of Tropical Disease 2001, England) was used to evaluate *in vitro* sensitivity to CQ of *P. falciparum* malaria parasites. Sixty-nine malaria cases were selected for this study, according to the *in vitro* protocol described in Mark III-WHO.⁴¹ The test was performed in tissue culture plates pre-dosed with drugs in increasing concentrations; briefly, 0.9 mL of RPMI 1640 medium were taken into the sterile falcon tube and 100 µL from well-mixed blood mentioned previously were added. All wells of appropriate columns were dosed with 50 µL of blood-medium mixture using the 50 µL fixed volume Eppendorf pipette and a disposable sterile tip, as provided with the test kit. Plates were incubated at 37.5°C in a candle jar for 24 to 30 h. At the end of incubation, blood from each well was harvested and a thick film was prepared; thick films were stained for 30 min in a Giemsa stain at a dilution of 1% (vol/vol) in buffered water of pH 6.8. After drying, films were examined through a light microscope with oil immersion lens, and the number of schizonts with three or more nuclei of a total of 200 asexual parasites (i.e., schizonts and trophozoites) was counted. For an acceptable test, schizont maturation in control must be 10% or more (i.e., 20 schizonts with three or more nuclei per 200 asexual parasites). Average results for EC50, EC90, EC95, and EC99 (i.e., drug concentrations producing 50%, 90%, 95%, or 99% inhibition of schizont maturation, respectively) were calculated using the WHO

log probit program (www.who.int/csr/drugresist/malaria/en/probit.xls) in all isolates.

RESULTS

Between June and December 2007, a total of 235 blood samples from *P. falciparum*-infected individuals were examined for the presence of mutations in five genes related to antimalarial resistance: in particular, we investigated one codon of *pfcr1* gene (K76T), one codon of *pfmdr1* (N86Y), three codons of the *pfdhfr* gene (N51I, C59R, S108N), three codons of the *pfdhps* gene (S436A, A437G, K540E), and SNPs in two regions of the *pfATPase6* gene, from nucleotide (nt) 652 to 1422 and from nt 1744 to 2402 (nt number is according to the ATG start codon). Among the blood samples examined, 198 samples gave PCR-positive results for each of the genes included in our study, exactly 27 samples from Gedarif State (eastern Sudan), and 171 samples from Gezira State (central Sudan). Thirty-seven patients (16%) with PCR-negative results were excluded from analysis.

There were 14 samples (7%) that showed mixed infections of both wild-type and mutant alleles. These mixed wild/mutant infections were considered as mutants. Moreover, a total of 69 samples were analyzed *in vitro* for their susceptibility to CQ, however only 45 (65.2%) isolates with an adequate schizonts growth were successfully tested.

Polymorphism analysis. Our results showed that 185 samples (93.4%) out of 198 analyzed carried a mutant allele of at least one gene associated with resistance to antimalarial drugs chloroquine and sulfadoxine-pyrimethamine. The results are summarized in Table 1 and Table 2. The majority of the isolates were found to carry the mutant codons *pfdhfr*I₅₁ (75.3%) and *pfdhfr*N₁₀₈ (72.7%), whereas *pfdhfr*R₅₉ was found in 15 isolates (7.6%). With respect to *pfdhps* gene, *pfdhps*A₄₃₆, *pfdhps*G₄₃₇, and *pfdhps*E₅₄₀ were detected in 8.1%, 20.7%, and 16.7% of the isolates, respectively.

Prevalence of the CQ resistance-related mutant alleles was relatively high: *pfcr1*T₇₆ allele was present in 72.7% of the isolates, whereas 86Y mutation of the *pfmdr1* gene was detected in 55.5% of the isolates.

Isolates from eastern Sudan (Gedarif) showed a higher prevalence of all but one mutation when compared with isolates from central Sudan (Gezira). This difference has been confirmed as significant by statistical analysis for *pfcr1*76T, *pfmdr1*86Y, *pfdhfr*108N, and *pfdhps*436A mutated codons (Table 1A). None of the isolates from eastern Sudan had *pfdhfr*59R mutation.

Moreover, when we compared samples from central Sudan collected during June–July (*N* = 71) and those collected during November–December (*N* = 100), we observed that the rate of almost all mutations is lower in the isolates collected in November–December. However, statistical analysis showed that there were no significant differences between *Plasmodium* genotype frequencies from parasite populations collected in two different periods within the same sampling area (Table 1B).

By the analysis of allele combinations of these four resistance markers, we observed 41 different haplotypic conformations in our isolates (Table 2). In particular, we found 5 distinct haplotypes in isolates that carried a single point mutation (8.6% of the isolates analyzed), 8 haplotypes in double mutant isolates (14.7%), 12 haplotypes in triple mutant isolates (22.7%), 4 haplotypes in mutants isolates carrying quadruple mutations (25.3%), 8 haplotypes in quintuple mutant isolates (13%), and

TABLE 1
Frequency of specific point mutations in isolates analyzed in this study*

Table 1A	Prevalence of point mutations			Z†	p(z)†	Table 1B	Prevalence of point mutations			z‡	p(z)‡
	Total	Gedarif	Gezira				Gezira	Gezira			
	No. = 198	No. = 27	No. = 171				No. = 71	No. = 100			
	June–December	November–December	June–December				June–July	November–December			
	No. (%)	No. (%)	No. (%)				No. (%)	No. (%)			
<i>Pfcrtr</i> 76T	144 (72.7)	24 (88.8)	120 (70.1)	2.03	0.043	<i>Pfcrtr</i> 76T	54 (76)	66 (66)	1.41	0.159	
<i>Pfmdr</i> 186Y	110 (55.5)	23 (85.2)	87 (50.8)	3.34	0.001	<i>Pfmdr</i> 186Y	36 (50.7)	51 (51)	−0.04	0.969	
<i>Pfdhfr</i> 51I	149 (75.3)	21 (77.7)	128 (74.8)	0.32	0.0746	<i>Pfdhfr</i> 51I	58 (81.6)	70 (70)	1.72	0.085	
<i>Pfdhfr</i> 59R	15 (7.6)	0	15 (8.8)	−1.52	0.128	<i>Pfdhfr</i> 59R	7 (9.9)	8 (8)	0.43	0.665	
<i>Pfdhfr</i> 108N	144 (72.7)	24 (88.8)	120 (70.1)	2.03	0.043	<i>Pfdhfr</i> 108N	52 (73.3)	68 (68)	0.75	0.455	
<i>Pfdhps</i> 436A	16 (8.1)	5 (18.5)	11 (6.4)	2.15	0.032	<i>Pfdhps</i> 436A	4 (5.6)	7 (7)	−0.37	0.713	
<i>Pfdhps</i> 437G	41 (20.7)	8 (29.6)	33 (19.3)	1.24	0.214	<i>Pfdhps</i> 437G	16 (22.5)	17 (17)	0.9	0.369	
<i>Pfdhps</i> 540E	33 (16.7)	6 (22.2)	27 (15.7)	0.84	0.399	<i>Pfdhps</i> 540E	15 (21.2)	12 (12)	1.62	0.104	
<i>PfATPase6</i> -402V	8 (4)	1 (3.7)	7 (4)	−0.07	0.941	<i>PfATPase6</i> -402V	7 (9.8)	0	3.2	0.001	
<i>PfATPase6</i> -431K	35 (17.7)	12 (44.5)	23 (13.4)	3.94	< 0.001	<i>PfATPase6</i> -431K	13 (18.3)	10 (10)	1.57	0.117	

* 1A = allele frequencies and z test values among 198 *Plasmodium falciparum* isolates from Gedarf State (eastern Sudan) and Gezira State (central Sudan); 1B = allele frequencies and z test values among 171 *P. falciparum* isolates from Gedarf State (eastern Sudan) sampled in two different period of the year; z = two-sample test of proportion (z test); p(z): significance of z test.

† Comparison between Gedarf and Gezira.

‡ Comparison between Gezira isolates sampled in June–July and in November–December. Significance value p(z) < 0.05.

2 and 1 haplotypes in mutant isolates that carried 6 (7.6%) and 7 (1.5%) point mutations, respectively (Table 2, Figure 2). The wild-type sequence K₇₆N₈₆N₅₁C₅₉S₁₀₈S₄₃₆A₄₃₇K₅₄₀ was present in 6.6% of isolates (Table 2, Figure 2).

With regard to the haplotypes, isolates from eastern Sudan did not exhibit wild-type, single mutant type, and 7-fold mutant genotypes but they displayed a high prevalence of mutant alleles with four mutations (33.3%), whereas the majority of isolates from central Sudan were found to carry the triple mutant haplotype (25.1%) (Figure 2).

***P. falciparum* ATPase6 SNPs.** In this study, we analyzed the prevalence of single nucleotide polymorphisms in two regions of the *pfATPase6* gene. Most of the Sudanese parasite population harbored wild-type allele. Only two previously reported mutations were detected: 431K in 35 isolates (17.7%) and 402V in 8 isolates (4%) (Table 1).

Chloroquine susceptibility. *In vitro* activity of CQ was successfully evaluated in 45 isolates. The majority (55.6%) of tested isolates were fully sensitive to CQ, 37.8% had a low resistance level, and 6.7% were marked resistant (Table 3). The test results processed by log concentration–response probit analysis for determination of 50%, 90%, 95%, and 99% effective concentrations (EC₅₀, EC₉₀, EC₉₅, and EC₉₉) were: EC₅₀:312.1808 nmol/L, EC₉₀:930.0186 nmol/L, EC₉₅:1267.3088 nmol/L, and EC₉₉:3633.1583 nmol/L.

Polymorphism analysis of this group of isolates showed high prevalence of single and double mutations *pfcrtr*₇₆/*pfmdr*₁₈₆, related to CQ resistance (Table 3), however we found that isolates showed low correlation between molecular profile of both genes and *in vitro* susceptibility. In fact, two (66.7%) of three marked CQ-resistant isolates carried wild-type *pfcrtr*₇₆/*pfmdr*₁₈₆ allele, whereas double mutant *pfcrtr*₇₆/*pfmdr*₁₈₆ alleles were found in 40% of sensitive isolates. Concerning low-resistant isolates, they showed a high incidence of single (11.8%) or double (70.6%) point mutations at *pfcrtr*₇₆/*pfmdr*₁₈₆ codons; consequently, the correlation between *in vitro* outcome and mutation rate was relatively higher.

DISCUSSION

We believe that the current study is one of the few recent reports of drug resistance molecular markers in these

malarious areas of Sudan. In this view, it is interesting to compare our results with those obtained in studies previously carried out in this malaria endemic region.

In Sudan, malaria has a scattered distribution and varies a lot according to the different regions of the country: there are regions with high, stable malaria transmission (southern Sudan), others with a high seasonal transmission pattern (central/eastern Sudan), and finally, in the northern part of the country, spotted areas characterized by riverine and urban transmission. Furthermore, the level of *falciparum* resistance to the most used antimalarials differs from region to region,^{31,34,42} making a countrywide drug policy difficult.

In 2004, the National Control Malaria Program decided to adopt artesunate + sulfadoxine/pyrimethamine as the first-line drug and artemether-lumefantrine as the second line. However, the implementation of this decision and consequent real withdrawal of CQ from the local market were still in agenda in the year 2005. As reported by Malik and others,⁴² manufacturing and importation of CQ tablets and vials was stopped completely in January 2006 only. As a consequence, we might expect that CQ has been available in the local market for a long period after that date. This is an important consideration in evaluating the result of the molecular marker investigation.

In fact, frequency of *pfcrtr* 76T mutation observed in our study is still relatively high (72.7%, *N* = 144/198) and it is consistent with a current or recent presence of drug pressure, even if it is lower than previously reported from other Sudanese studies.^{31,43,44} A-Elbasit and others,³¹ for example, reported a *pfcrtr* 76T frequency close to fixation of mutated allele (*pfcrtr* 76T = 93%). In this scenario, the results of the *in vitro* tests are quite surprising and the observed high percentage of isolates showing full sensitivity to CQ is difficult to explain. Recent data from literature have already discussed the potential inaccuracies of *in vitro* drug susceptibility tests^{45,46} and also some conflicting results between the frequency of *pfcrtr* mutations and *in vitro/in vivo* tests.^{47–49} However, we can speculate that *in vitro* tests data obtained in our study could have resulted from the limited number of tests performed since, according to the WHO protocol, a correct MARK III outcome can be obtained by performing at least 100 tests. If we take into account the recognized strong link between presence of *pfcrtr* mutations

TABLE 2
Types and frequency of haplotypes identified in Sudanese isolates by analysis of four molecular markers of CQ and SP resistance*

Types and frequency of haplotypes identified in Sudanese isolates by analysis of four molecular markers of CQ and ST resistance									
No. of point mutations		Haplotype*		No. isolates from Gezira		No. isolates from Gedarf		Total %	
0		KNNCSSAK		13		0		6.6	
1		TNNCSSAK		8		0		4.1	
1		KYNCSSAK		2		0		1	
1		KNICSSAK		4		0		2	
1		KNNCSAAK		2		0		1	
1		KNNCSSGK		1		0		0.5	
2		TYNCSSAK		8		1		4.6	
2		TNICSSAK		2		0		1	
2		TNNCNSAK		0		1		0.5	
2		TNNCSAAK		0		1		0.5	
2		KYNCNSAK		1		1		1	
2		KYNCSSGK		0		1		0.5	
2		KNICNSAK		9		0		4.6	
2		KNNRNSAK		4		0		2	
3		TNICNSAK		22		2		12.2	
3		TYNCSSGK		1		0		0.5	
3		TYICSSAK		3		0		1.5	
3		TYNCSSAE		1		0		0.5	
3		TNIRSSAK		1		0		0.5	
3		TNNCSAAE		1		0		0.5	
3		TNNCSSGE		1		0		0.5	
3		KYICNSAK		5		0		2.5	
3		KNIRNSAK		3		0		1.5	
3		KNICNAAK		2		0		1	
3		KNICNSGK		2		0		1	
3		KNICSSGE		1		0		0.5	
4		TYICNSAK		38		9		23.8	
4		TNIRNSAK		1		0		0.5	
4		KYICNAAK		1		0		0.5	
4		KYICNSGK		1		0		0.5	
5		TYIRNSAK		2		0		1	
5		TYICNAAK		4		4		4	
5		TYICNSGK		3		1		2	
5		TYICNSAE		1		0		0.5	
5		TYICSSGE		1		0		0.5	
5		TYNCNSGE		0		1		0.5	
5		KYICNSGE		0		1		0.5	
5		TNICNSGE		8		0		4	
6		TYICNSGE		10		4		7.1	
6		TNIRNSGE		1		0		0.5	
7		TYIRNSGE		3		0		1.5	
Reference	Crt 76	Mdr1 86	Dhfr 51	Dhfr 59	Dhfr 108	Dhps 436	Dhps 437	Dhps 540	
Wild-type	K aaa	N aat	N aat	C tgt	S agc	S tct	A gct	K aaa	
Mutant	T aca	Y tat	I att	R cgt	N aac	A gct	G ggt	E gaa	

*The construction of haplotype involved *pfcr76*, *pfmdr1-86*, *pfdhfr51-59-108*, and *pfdhps 436-437-540*. Mutant codon is in **boldface**.

and CQ resistance and we look at the frequency of *pfcr76* T mutation from this study, we can assume that CQ is definitely an unsuitable drug for this area. These data suggested that prevalence of molecular markers for CQ should be monitored (while this drug is being replaced by combination therapies) to verify if the level of CQ resistance is decreasing in this area. In fact, as previously reported,^{50,51} recession of chloroquine-resistant parasites preceded the complete withdrawal of CQ in areas with high malaria transmission.

As far as ACT implementation is concerned, unfortunately falciparum tolerance/resistance to artemisinin derivatives has been recently reported in Cambodia.¹⁶ This recent report increases the interest in monitoring *pfATPase6* point mutations that have been suggested to be involved in modulating artemisinins sensitivity, even if their role is still under debate.

In our investigation on *pfATPase6* gene polymorphism, we found two mutated codons (431K and 402V) only and at low frequencies (17.7% and 4%, respectively). In particular, E431K substitution was previously detected at higher frequencies both in Western (Cameroon, 37%⁵²) and Eastern (Zanzibar

31%, Tanzania 39%⁵³) Africa. Moreover, we previously identified 431K substitution in 17% of 73 isolates from nine African countries, as described in Menegon and others.⁴⁰ Taking into consideration that the first-line treatment currently adopted in Sudan is AS + SP, it is important to look carefully at the *dhfr/dhps* mutation prevalence observed in our study. An important issue is the very low frequency of mutant IRN-GE (2%, Table 2), haplotype that is commonly indicated as a good predictor of treatment failure, therefore, the SP combination is expected to be still effective in this region. However, it is necessary to note the frequency of *pfdhfr51/59/108* point mutations singularly (Table 1), in fact, while prevalence of the 51 and 108 mutations, observed in our study, are substantially comparable to the one reported in previous studies carried out in the same Sudanese regions (characterized by a seasonal malaria transmission and dated 2002/2003^{31,33,54}), frequency of 59R mutations significantly increased from 0.6%³¹ to 7.6% ($z = 3.255$, $p(z) = 0.001$). This increase of *pfdhfr59R* frequency in these last 3–4 years could be considered a signal of spread of SP resistance in this area. In view of this, we believe that

FIGURE 2. Overall prevalence of Sudanese isolates carrying point mutations observed in a total of eight codons in the *pfcr*, *pfmdr*, *pfdfhr*, and *pfdhps* genes.

in vivo tests assessing falciparum sensitivity to pyrimetamine and sulfadoxine drug combination are needed, because the lack of efficacy of SP will adversely affect the outcome of ACT treatment.

We have also taken into consideration the frequency of each mutated codon by sampling areas (Gedarif versus Gezira) and by sampling periods (June/July versus November/December, Gezira). The results of statistical analysis showed that the differences in mutations frequencies between the two sampling periods are not significant, thus indicating that probably the sampling period encompassed the same malaria transmission season. Conversely, when comparing the two sampling areas, there are significant differences in frequency for four mutated codons (*pfcr*T76T, *pfmdr*186Y, *pfdfhr*108N, and *pfdhps*436A). However, we have to be cautious in drawing conclusions if we consider that there are some differences between the two batches of samples (samples size and sampling period) and that information about the malaria treatment implementation in the two study areas is lacking. Therefore, we can only speculate that probably falciparum populations in Gedarif are exposed to a higher drug pressure than parasite populations from Gezira.

Finally, interesting issues arise from analysis of the different haplotypes generated by the association of all identified mutations in four analyzed genes. In fact, the most prevalent haplotype is quadruple mutant $T_{cr76}Y_{mdr186}I_{dhfr51}N_{dhfr108}$ (23.8%), but also haplotypes showing five or six mutations are well represented in the falciparum isolates circulating in this study area, in particular sextuple mutant $T_{cr76}Y_{mdr186}I_{dhfr51}N_{dhfr108}G_{dhps437}E_{dhps540}$ that accounts for 7.1%. Moreover,

we found also three isolates showing seven mutations $T_{cr76}Y_{mdr186}I_{dhfr51}R_{dhfr59}N_{dhfr108}G_{dhps437}E_{dhps540}$. The presence of multiple haplotypes in African falciparum isolates has been already described and discussed in previous studies.^{43,55,56} The reason for these observed patterns of mutations in specific genes scattered on four different chromosomes and linked to the resistance to a different class of antimalarials is still to be elucidated. We can speculate that this association could occur in endemic areas where the probably high fitness cost of multiple mutations is balanced by simultaneous pressure of different drugs. The current situation in Sudan could represent an example: as stated previously, because in this country CQ is probably still available and used (or has been used until sometime ago) especially in rural setting and, as a part of ACT, SP combination is used as well. This could explain the detection in the isolates analyzed in our study of the TYIN, TYINGE, and TYIRNGE haplotypes. The circulation of these isolates harboring multiple CQ and SP resistance mutations in an endemic area is not to be underestimated, because, as previously reported,⁵⁷ selection of a high degree of resistance toward a given antimalarial, in this case the pyrimethamine, could be accompanied by increase of a total number of mutations in *P. falciparum*. Hence, the Sudanese isolates of *P. falciparum* carrying multiple mutations are expected to be somewhat resistant and in case of accumulation of further mutations could become really difficult to be treated.

In conclusion, this study supports the usefulness of molecular marker screening to be carried out in the frame of country surveillance activities. Monitoring evolution of *P. falciparum*

TABLE 3
Patterns of *pfcr* and *pfmdr* point mutations from the 45 isolates tested for CQ *in vitro* susceptibility*

<i>In vitro</i> test result	<i>pfcr</i> codons		<i>pfmdr</i> codons		<i>pfcr/pfmdr</i> codons			
	Wt K76	Mut T76	Wt N86	Mut Y86	Wt/wt	Wt/mut	Mut/wt	Mut/mut
	K76/N86	K76/Y86	T76/N86	T76/Y86				
Sensitive (<i>N</i> = 25)	12 (48%)	13 (52%)	14 (56%)	11 (44%)	11 (44%)	1 (4%)	3 (12%)	10 (40%)
Low resistant (<i>N</i> = 17)	3 (17%)	14 (82.4%)	3 (17.6%)	14 (82.4%)	1 (5.9%)	2 (11.8%)	2 (11.8%)	12 (70.6%)
Marked resistant (<i>N</i> = 3)	2 (66.7%)	1 (33.3%)	3 (100%)	0	2 (66.7%)	0	1 (33.3%)	0

*N = no. isolates; wt = wild-type codon; mut = mutant codon.

drug resistance by analyzing the prevalence of drug resistance mutations in an endemic area as Sudan, where both logistical and political problems make the implementation of the Efficacy Therapeutic Tests difficult, could provide key information for the deployment of an effective countrywide drug policy.

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