

**GENETIC POLYMORPHISMS ASSOCIATED WITH CHLOROQUINE RESISTANCE  
AMONG PLASMODIUM FALCIPARUM ISOLATES FROM ALJUNAYNAH DISTRICT,  
WEST DARFUR STATE, SUDAN****Abdalmoneim M. Magboul<sup>1\*</sup>, Hanan Babiker Eltahir<sup>2</sup>, Ibrahim M. Hassan<sup>1</sup>, Hafiz Y. Mohammed<sup>1</sup>, Mohammed A. Suliman<sup>1</sup>, Rabah M. Ibrahim<sup>1</sup> and Ammar A. Abdalla<sup>1</sup>**<sup>1</sup>Department of Parasitology and Medical Entomology, Faculty of Medical Laboratory Sciences, Elimam Elmahdi University, Kosti City, Sudan.<sup>2</sup>Department of Biochemistry, Faculty of Medicine and Health Science, Elimam Elmahdi University, Kosti City, Sudan.**\*Corresponding Author: Abdalmoneim M. Magboul**

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**ABSTRACT**

Chloroquine CQ resistance has been shown to be associated with point mutations in *Pfcr* and *Pfmdr1*. These genes encode for digestive vacuole transmembrane proteins *Pfcr* and *Pgh1* respectively. Polymorphisms were examined in these genes in 25 isolates from Aljunaynah, west Darfur state, Sudan. The isolates were subjected to PCR-RFLP analysis for the *Pfmdr1* N86Y and *Pfcr* K76T mutations. The frequency of mutant allele for CQ *pfcr* T 76 was 19 (38%) and *pfmdr1* Y 86 was 7 (14%). The study concluded that the frequency of chloroquine drug resistance molecular markers were higher in the study area and lower than that reported in other Sudan states. The result necessitates the evaluation of CQ *in vivo* therapeutic efficacy in endemic areas for more effective malaria control strategies

**KEYWORDS:** Chloroquine drug resistance, PCR-RFLP analysis, Molecular markers, Polymorphisms, Mutations, West Darfur.**INTRODUCTION**

Malaria infected human for over 50,000 year and *Plasmodium* may have been a human pathogen for the entire history of the species.<sup>[1]</sup> Globally, it is a major health problem affecting 130 countries with about 3.4 billion (half of world's population) at risk. Exact numbers are unknown, but an estimated 300 - 500 million clinical cases of malaria worldwide each year resulting in 1.5- 2.7 million deaths, 77% occur in children under five.<sup>[2,3,4,5]</sup> In Sudan, it is estimated that about 16.6% of all the inpatient attendance in both public and private health services are due to malaria, resulting in an estimated 7.5 million cases and 35,000 deaths annually.<sup>[6,7]</sup> In Darfur a high mortality may occur among vulnerable displaced populations because of the concentration of people, the lack of adequate housing and preventive measures resulting in increased exposure to mosquito bites and reduced access to effective treatment.<sup>[8]</sup>

The tremendous success of chloroquine and its heavy use through the decades eventually led to chloroquine resistance in *P. falciparum* and *P. vivax*, the two parasite species responsible for most human malaria cases. Foci of resistant *P. falciparum* were detected in Colombia and at the Cambodia-Thailand border during the late 1950s.

Resistant strains from these foci spread steadily through South America, Southeast Asia and almost all in Asia and Oceania by 1989. Africa was spared until the late 1970s, when resistance was detected in Kenya and Tanzania. In 2000, chloroquine resistance was present in almost all countries with falciparum malaria.<sup>[9,10, 11]</sup> The possible occurrence of chloroquine resistant forms of falciparum malaria in Sudan was initially suggested by Omer, in central Sudan.<sup>[12]</sup> Recently, a high level of chloroquine resistance falciparum malaria was rapid increase in resistance in many parts of the Sudan (table 1) may be partially due to social and environmental changes occurring in the area.

Various genetic alterations have been shown to be associated with CQ resistance. Mainly, two genes known as *P. falciparum* multidrug resistance gene *Pfmdr1*, which codes for *Pgh1*, a P-glycoprotein homologue and the CQ resistance transporter gene *Pfcr*, which codes for CQ resistance transporter protein have been identified as potential genes of CQ resistance. Several point mutations in *Pfmdr1* gene at positions 754, 1049, 3598, 3622 and 4234 results in amino acid changes at codon N86Y, Y184F, S1034C, N1042D, and D1246Y have been shown to modulate CQ, mefloquine and possibly lumefantrine resistance. The mutation in codon 86 result

in an amino acid alteration an asparagine to tyrosine (N86Y) appear to be the most important as this may alter the transport activity of the protein<sup>[13,14]</sup>. However, a few studies have reported contrasting observations with regard to the role of *Pfmdr1* gene mutations in CQ resistance.<sup>[14]</sup>

CQ resistance is also associated with a mutation in the transporter gene *Pfcr1*. A K76T mutation appears to be necessary for the resistance phenotype, and is the most reliable molecular marker of resistance among the various *Pfcr1* mutations. In addition, other amino-acid substitutions at positions 72, 74, 75, 220, 271, 326, 356, and 371 are associated with chloroquine-resistant phenotype to a varying degree possibly in relation to the

geographic origin of the parasites.<sup>[11,15,16]</sup> One mutation, the substitution of threonine (T76) for lysine (K76) at position 76 (K76T), was present in all resistant isolates and absent from all sensitive isolates tested *in vitro*.<sup>[9,17]</sup> *Pfcr1* K76T mutation has not been observed in CQ responders, and therefore, has been accepted as a good molecular marker for CQ resistance in *P. falciparum*.<sup>[14]</sup>

In *P. falciparum* endemic areas, CQ was the recommended first line treatment for uncomplicated malaria. However, now days this has been changed to artesunate-based combination therapies.<sup>[18,19]</sup> Despite this, in many malaria-affected areas CQ is still used for non-complicated malaria.<sup>[14]</sup>

**Table 1: The frequency of *Pfcr1* K76T and *Pfmdr1* N86Y detected in the neighbouring states compared to the present study during the period 2001- 2015.**

Year	Location	Gene frequency	Reference
2015	Wad Medani, central Sudan	<i>Pfcr1</i> ; mutant type 30/40 (75%) wild type 10/40 (25%) <i>Pfmdr1</i> ; mutant type 22/37 (59.5%) wild type 15/37 (40.5%)	20
2013	Kassala, east Sudan	<i>Pfcr1</i> ; mutant type 44/63 (69.8%) wild type 19/63 (30.2%) <i>Pfmdr1</i> ; mutant type 37/63 (58.7%) wild type 26/63 (41.3%)	21
2010	Central and east Sudan	<i>Pfcr1</i> ; mutant type 144/198 (72.7%) wild type 54/198 (27.3%)	22
2010	Gezira and Gadarif	<i>Pfcr1</i> ; mutant type 63/100 (63%) wild type 37/100 (37%) <i>Pfmdr1</i> ; mutant type 46/100 (46%) wild type 54/100 (54%)	23
2001	Village in east Sudan	<i>Pfcr1</i> ; mutant type 50/50 (100%) wild type 0 <i>Pfmdr1</i> ; mutant type 50/50 (100%) wild type 0	24

## MATERIALS AND METHODS

### Sample collection

This study was carried out in Aljunaynah district, western Darfur state, western Sudan (figure -1). The blood samples were collected from 25 patients of both sexes with different ages that used ACTs as antimalarial drug. The sample individually spotted on Whatman 3MM filter paper, after microscopic and ICT confirmation of *P. falciparum* mono infection. Patient's informed consent (substitution) was obtained before inclusion of the blood samples in the study which was reviewed and approved by the Ethical Committee of Elimam Elmahdi University and health administration in the state.

### Genetic characterization of the parasite

Parasite DNA was extracted from dried blood spots, using Chelex method.<sup>[17]</sup> and *P. falciparum* mutations associated with resistance to CQ was typed by nested PCR technique.<sup>[14]</sup>

### Detection of N86Y mutation in *Pfmdr1* gene

During nest1 reaction, primers 1F=5'ATGGGTAAAGAGCAGAAAGA3' and 1R=5' AACGC- AAGTAATACATAAA GTCA3' were used to amplify the region flanking codon 86. Nested primers 2F=5' TGGTAACCTCAGTATCAAA GAA3' and 2R=5' ATAAACCTAAAAAGGA- ACTGG3' were used to amplify the PCR product in nest2 reaction. In nest 1, PCR parameters were, initial denaturation at 94°C for 3 minutes, followed by 45 cycles, each of 30 sec at 92°C, 45 sec at 48°C, 1 min at 65°C followed by the final extension at 65°C for 5 min. In nest 2, only 20 cycles of PCR were run (Techne thermal cycler, UK).

### Restriction digestion with *Apo I*

The finally amplified product was subjected to restriction digestion with *Apo I* (wild type allele) (New England Biolabs, UK) by incubating at 37°C for one hour with the one unit of enzyme. The digests were resolved on 1.5% agarose gel stained with ethidium bromide and results

were recorded on the gel electrophoresis UV-Translaminator (MPSU, UK).

#### Detection of the K76T mutation in *Pfprt* gene

For the K76T mutation, during nest1, primers 1F=5'CCGTTAATAATAAATACACGCAG3' and 1R=5'GCATGTT ACAAAC TATAGTTACC3' were used and for nest2 2F=5'TGTGCT-CATGTGTTTAAACTT3' and 2R=5'CA AACTA TAGTTACCAATTTT3' were used. Nest1 PCR parameters were initial denaturation at 95°C for 5 minutes followed by 45 cycles, each of 30 sec at 92°C,

56 sec at 30°C, 1 min at 60°C followed by the final extension at 60°C for 3 min. In nest2 PCR, initial denaturation at 95°C for 5 minutes followed by 25 cycles, each of 30 sec at 92°C, 30 sec at 48°C, 30 sec at 65°C followed by the final extension at 65°C for 3 min were done. The nested PCR product was digested with *ApoI* as described above.

#### Statistical analysis

Associations between the different mutations were tested using Chi-square test. P values < 0.05 were considered significant for all statistical analysis.



Figure 1: Study area; Map of western Darfur state. Source: www.google.com. 2016.

## RESULTS

A total of 25 *P. falciparum* isolates were used in the study. To ascertain the genetic polymorphism leading to the CQ resistance, nested PCR for *Pfmdr1* and *Pfprt* genes was performed. As shown in table (2 and 3), the frequency of the mutant allele *Pfmdr1* Y86 was 14% and mixed *Pfmdr1* N and Y allele was detected in 7/25 of the isolates. The frequency of the mutant *Pfprt* T76 was 38% and mixed population KT allele was detected in 13/25 of the isolates. To confirm the status of the amplicon, the PCR product was digested with *ApoI* enzyme. On nested PCR for *Pfmdr1*, all the isolates showed the *Pfmdr1*-codon 86 region amplicon with the product size of 500 bp. On digestion with the *ApoI* in the case of CQ

sensitive isolates, *ApoI* digestion generated two fragments of 250 bp and 226 bp respectively (Figure 2 and 3). The nested PCR of *Pfprt* gene showed an amplicon of 145 bp in the case of CQ sensitive isolates, the digestion resulted in one fragment of 111 bp suggesting the presence of K76 allele at codon 76 (Figure 4 and 5).

To study the associations between the loci *Pfprt* K76T and *Pfmdr1* N86Y, it was observed that 4 (16%) samples have the two mutations. In these isolates, *Pfmdr1* N86Y mutation was found to be associated with *Pfprt* K76T mutation (Chi<sup>2</sup> test, P= 0.001).

Table 2: Result of mutation of *Pfmdr1* N86Y and *Pfprt* K76T in west Darfur state.

Isolated gene	Mutant type	Wild type	Mutant/wild type
<i>Pfmdr1</i> N86Y	0/25	18/25	7/25
<i>Pfprt</i> K76T	3/25	9/25	13/25

Table 3: The allele frequency of *Pfmdr1* 86 and *Pfprt* K76T in west Darfur state.

Alleles frequency	<i>Pfmdr1</i> N86Y		<i>Pfprt</i> K76T	
	Alleles- N86	Alleles- 86Y	Alleles- K76	Alleles- 76T
	43 (86%)	7 (14%)	31 (62%)	19 (38%)

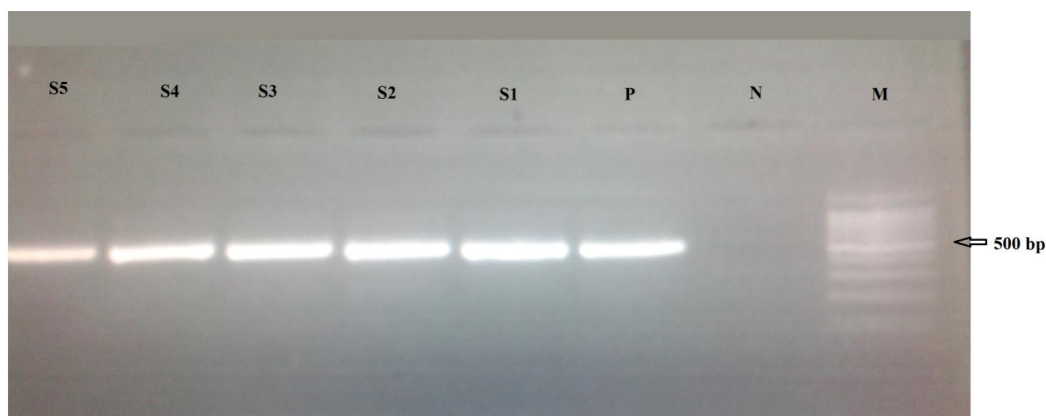


Figure 2: Representative photomicrograph showing the results of PCR amplified products for *Pfmdr1* gene. In photograph, (M) is 100 bp DNA ladder; (N) is empty well as negative control, (P) is positive sample as positive internal control and (S1- S5) are PCR products of *P. falciparum* isolates.

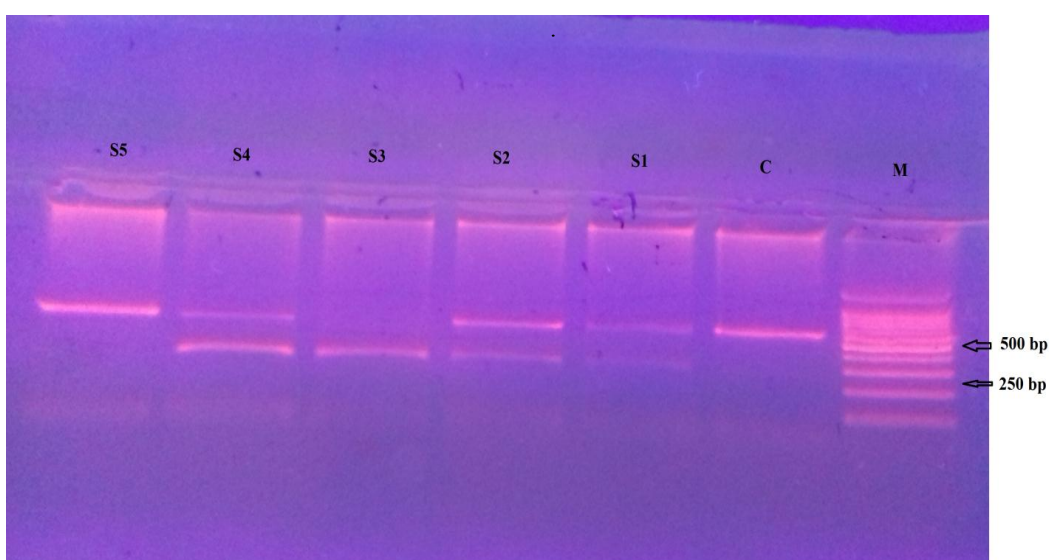


Figure 3: Representative photomicrograph showing the results of RFLP after digestion of PCR amplified product with endonuclease, *ApoI* for *Pfmdr1* gene (N86) in chloroquine sensitive *Plasmodium falciparum* isolates. In photograph, (M) is 100 bp DNA ladder; (C) is undigested product as control, (S3) is chloroquine sensitive *P. falciparum* isolates, (S1-S2-S4) have both sensitive and resist alleles and (S5) is chloroquine resistant *P. falciparum* isolates.

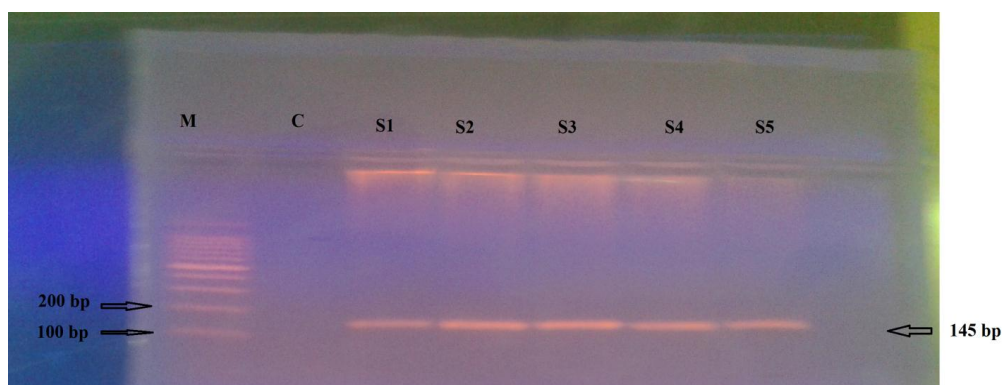
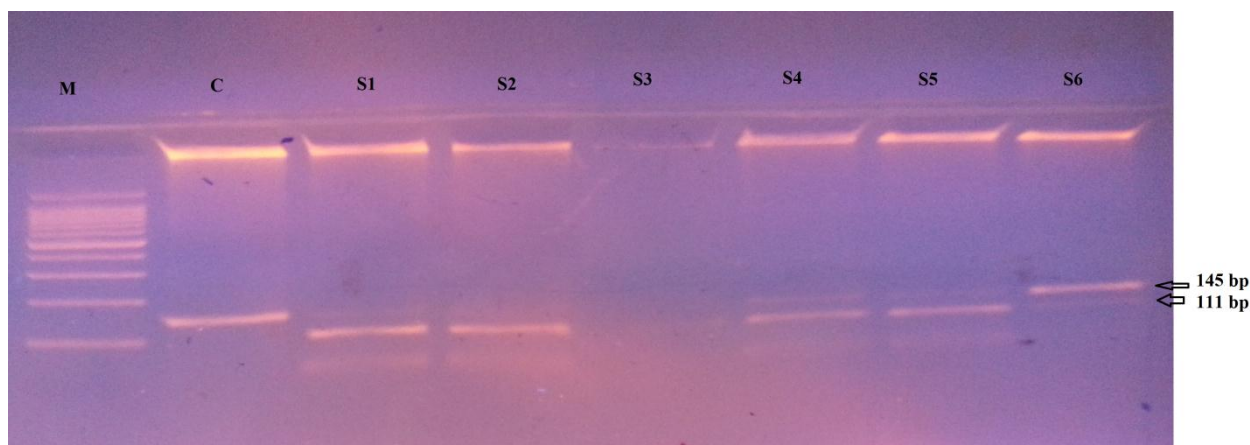


Figure 4: Representative photomicrograph showing the results of PCR amplified products for *PfCRT* gene. In photograph, (M) is 100 bp DNA ladder; (C) is empty well as negative control, (S1) is positive sample as positive internal control and (S2- S5) are PCR products of *P. falciparum* isolates.





**Figure 5: Representative photomicrograph showing the results of RFLP after digestion of PCR amplified product with endonuclease, *ApoI* for *Pfert* gene (K76) in chloroquine sensitive *Plasmodium falciparum* isolates. In photograph, (M) is 100 bp DNA ladder; (C) is undigested product as control, (S1-S2-S5) are chloroquine sensitive *P. falciparum* isolates, (S4) have both sensitive and resist alleles and (S6) is chloroquine resistant *P. falciparum* isolates.**

## DISCUSSIONS

*P. falciparum* resistance to chloroquine first emerged in the late 1950's in South East Asia and spread to other areas in Asia and then to Africa. It is determined by the major point mutation at codon 76 of the *P. falciparum* CQ resistance transporter (*Pfert*) gene and *P. falciparum* multi-drug resistance gene 1 (*Pfmdr1*) 86 codon which are highly correlated with increased clinical CQ tolerance and treatment failure.<sup>[22]</sup> In Sudan the first cases of CQ resistance were reported by Omer,<sup>[12]</sup> and several *in vivo* and *in vitro* studies have subsequently documented a high presence of CQ resistance in different Sudanese areas. The fast rate of emergence of CQ resistance has hampered the control of malaria. The development of molecular techniques for the rapid identification of drug resistant parasites is of immense importance for the epidemiology and information on the choice of antimalarial treatment regimens.<sup>[2,25]</sup> This is the first molecular study carried out in this geographical area focused on the mutations of *pfmdr1* and *Pfert* genes strongly associated to CQ resistance. The purpose of the study was to estimate the prevalence of CQ resistance molecular marker genes in western Darfur state. For these 25 samples were analysed for polymorphism mutation in *Pfmdr1* at codon 86 and *Pfert* at codon 76. The study indicated that 0/25 and 3/25 were mutant, 18/25 and 9/25 were wild and 7/25 and 13/25 of the isolates had mutant/wild type infection respectively. The frequency of mutant allele for CQ *Pfert* T76 was 19 (38%) and *Pfmdr1* Y86 was 7 (14%).

This degree of resistance was found to be lower than that reported in studies conducted in the neighbouring states of Sudan by some others as shown in table 1.<sup>[20,21,22,23,24]</sup>

In comparison with that reported in Africa, it was found to be lower than that reported in Akuem, Bahr El Gazal province and Upper Nile, South Sudan,<sup>[26,27]</sup> in Bonoua and Samo, Côte-d'Ivoire<sup>[28]</sup>, in Tiwi and Mbita towns of

Kenya,<sup>[17]</sup> in Papua New Guinea,<sup>[29]</sup> Angola in paediatric hospital Dr. David Bernardino,<sup>[30]</sup> and in the area of Agogo, Ghana.<sup>[31]</sup> In Korogwe district, Tanzania, Alifrangis, *et- al*<sup>[32]</sup> was found that the frequency of *Pfert* is higher than that reported in our study.

In the other parts of the world, it was found to be lower than that reported in Thailand,<sup>[33]</sup> in Northeast Indian,<sup>[14]</sup> and In a Brazilian.<sup>[34]</sup>

In the present study, the mutation in codon 86 results in an amino acid alteration an asparagine to tyrosine (N86Y) appear to be important as this may alter the transport activity of the protein confirming the role of this mutation in the CQ resistance. Southeast Asian CQ resistant isolates have shown N86Y mutation while CQ resistant South American isolates were negative for N86Y, and showed mutations at positions 184, 1034, 1042 and 1246.<sup>[13,14]</sup> However, contrasting observations is now becoming evident that victoria transport by *Pfmdr1* is therefore inwardly directed into and not out of the digestive vacuole,<sup>[22,33,34,35]</sup> Similarly, due to mutations in *Pfert* gene, involving the substitution from lysine (K) to threonine (T) at position 76 (K76T) has also been observed in the CQ resistant strains.<sup>[11,34,35]</sup> Both wild-type and resistant forms of *Pfert* demonstrated that chloroquine resistance is due to the direct transport of a protonated form of the drug out of the parasite vacuole via the K76T *Pfert* mutant revealed its 100% association with the *in vitro* CQ resistance.<sup>[9,33,36]</sup> The result of the present study showed significant association ( $P= 0.001$ ) between *Pfmdr1* N86Y and *Pfert* K76T so it is in accordance with the earlier studies conducted by others.<sup>[14,24,30]</sup> These results further suggest that *Pfmdr1* (N86Y) and *Pfert* (K76T) are potentially useful markers of the assessment of *in vitro* CQ resistance.

From the data on prevalence of mutation in *Pfert* and *Pfmdr1* genes, the study can infer that western Darfur state indicates relatively higher transmission rate

although it sites is low transmission zone and exhibit epidemic malaria since there is a more polyclonal population structure (high mixed infection rates 7/25 and 13/25 of *Pfmdr 1* and *Pfcr1* respectively) owing to exclusive recombination among local *P. falciparum*. Ako, *et- al*<sup>[28]</sup> suggested that that genetic diversity is more intense in urban areas due to an influx of people from surrounding rural areas bringing parasites to new locations hence higher mixed-infection rate suggests higher malaria transmission in this a area. These observations support the idea that the heterogeneity of the human population could be an important element in the greater parasite diversity as well. Thus a determinant is not necessarily whether a site is urban or rural but rather the heterogeneity of the parasites brought by people as they migrate from other areas into the site being studied.

Due to displaced populations in the study area with the concentration of people, the lack of adequate housing and preventive measures resulting in increased exposure to mosquito bites and reduced access to effective treatment, most people can get the medication without medical advice since it is available in green grocers and tea sellers which lead to inadequate drug intake (poor compliance or inappropriate dosing regimens) and reliance on presumptive treatment; all these factors can facilitate the development of antimalarial drug resistance. The use of presumptive treatment for malaria has the potential for facilitating resistance by greatly increasing the number of people who are treated unnecessarily but will still be exerting selective pressure on the circulating parasite population.<sup>[37,38,39]</sup> Drug quality has also been implicated in ineffective treatment and possibly drug resistance, either through deterioration due to inadequate handling and storage or drugs may not contain sufficient quantities of the active ingredients. Also factor such as malnutrition and concurrent infections (including HIV) that decrease the effectiveness of the immune system in clearing parasite residuum after treatment also appear to increase survivorship of parasites and facilitate development and intensification of resistance. This mechanism has been suggested as a significant contributor to resistance in South-East Asia.<sup>[8,40]</sup>

The low prevalence of CQ resistance revealed by this study and high prevalence by the previous studies conducted in the other states indicated that the *in vitro* CQ resistance level is decrease possibly it is attributed to the CQ withdrawn from malaria treatment policy in Sudan ten years ago. In Asia and West Africa, the return of the efficacy of CQ in treating falciparum malaria has been demonstrated in China, Malawi and Tanzania where it found that chloroquine is again an efficacious treatment for malaria.<sup>[32,41,42]</sup> It has been suggested that reducing the use of CQ in a region could result in the re-emergence of CQ sensitive *P. falciparum* parasites, so permitting the reintroduction of this safe and well tolerate drug.

It recommend continuing monitoring antimalarial drugs resistance markers aiming to keep effective drugs and to overcome emerging of resistant mutations also the development of simple molecular tests to help the analysis of antimalarial drug resistance in field settings remains an important goal.

## CONCLUSION

This is the first molecular study carried out in this geographical area focused on the mutations of *Pfmdr1* and *Pfcr1* genes strongly associated to CQ resistance. The results of the study showed that the frequency of chloroquine drug resistance molecular markers were higher in west Darfur state and lower than that conducted in the other Sudan states.

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