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ORIGINAL RESEARCH

Unraveling Key Chloroquine Resistance-Associated Alleles Among *Plasmodium falciparum* Isolates in South Darfur State, Sudan Twelve Years After Drug Withdrawal

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Background: Due to the increasing resistance of *Plasmodium falciparum* to chloroquine (CQ) in Sudan, a shift from CQ to artesunate combined with sulfadoxine/pyrimethamine as a first-line treatment for uncomplicated falciparum malaria was adopted in 2004. This study aimed to determine the frequency distribution of K76T and N86Y mutations in *P. falciparum* chloroquine resistance transporter (*pfcrt*) and *P. falciparum* multidrug resistance 1 (*pfmdr1*) genes as key markers of resistance to CQ among *P. falciparum* isolates from patients in Nyala district of South Darfur state, west of Sudan.

Methods: A descriptive, cross-sectional study was conducted among 75 P. falciparum isolates from Sudanese patients diagnosed with falciparum malaria mono-infection. Parasite DNA was extracted from dried blood spots and amplified using a nested polymerase chain reaction (PCR). Then, restriction fragment length polymorphism (RFLP) was used to detect the genetic polymorphisms in codons 76 of *pfcrt* and 86 of *pfmdr1*. PCR-RFLP products were analyzed using 1.5% gel electrophoresis to identify the genetic polymorphisms in the studied codons. The wild-type (pfcrt K76 and pfmdr1 N86), mutant (*pfcrt* 76T and *pfmdr1* 86Y) and mixed-type (*pfcrt* K76T and *pfmdr1* N86Y) alleles were expressed as frequencies and proportions.

Results: The wild-type *pfcrt* K76 allele was observed among 34.7% of isolates and the mutant 76T allele among 20% of isolates, while the mixed-type K76T allele was observed among 45.3% of isolates. On the other hand, 54.7% of isolates harbored the wild-type *pfmdr1* N86 allele and 5.3% of isolates had the mutant 86Y allele, while the mixed-type N86Y allele was observed among 40% of isolates.

Conclusion: The key molecular markers associated with CQ resistance (*pfcrt* 76T and *pfmdr1* 86Y) are still circulating in high frequency among *P. falciparum* isolates in South Darfur state, about twelve years after the official withdrawal of the drug as a treatment for uncomplicated falciparum malaria.

Keywords: chloroquine, drug resistance, molecular markers, *pfcrt*, *pfmdr1*, Sudan

Introduction

Sudan accounted for an estimated 41% of the malaria cases in the Eastern Mediterranean Region (EMR) of the World Health Organization (WHO) in 2022,[1] with about 8 million suspected cases and 1.4 million confirmed cases. Malaria in the country is predominantly caused by *Plasmodium falciparum* (80.5%), followed by *Plasmodium vivax* (9.2%).[1] The entire population is at risk of malaria, with approximately 87% being at high risk.[1] Malaria endemicity is primarily low to moderate in the country's northern, eastern and western states.[2] According to the last Malaria Indicator Survey (MIS) in 2016, the overall malaria prevalence in Sudan is 5.9%.[3] The prevalence varies across different regions of the country. In

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the states of Khartoum, Red Sea, Northern and River Nile, it is less than 1%, while it exceeds 20% in Central Darfur state.[3] The prevalence is around or above 10% in the states of South and West Darfur, Blue Nile and South Kordofan.[3] In South Darfur state, malaria transmission is largely mesoendemic, with pockets of hyperendemicity.[4]

Chloroquine (CQ) has been used for treating falciparum malaria for more than four decades.[5] In the late 1970s, CQ sensitivity was reported among more than 99% of Sudanese patients infected with *P. falciparum*.[6] However, *P. falciparum* was resistant to CQ in 42% of patients in an in-vivo study in eastern Sudan in the late 1980s.[7] Since then, CQ resistance has been reported from different parts of Sudan.[8–13] In response to the growing problem of *P. falciparum* resistance to CQ, the National Malaria Control Programme in Sudan transitioned from CQ monotherapy to artemisinin-based combination therapy (ACT) for treating uncomplicated falciparum malaria in 2004.[5,14]

Resistance of *P. falciparum* to CQ is a complex trait involving multiple genes, with initial resistance often linked to mutations occurring in a specific gene called *P. falciparum* CQ resistance transporter (*pfcrt*) that encodes the PfCRT in the membrane of its digestive vacuole.[15–17] The mutation at codon 76 in PfCRT (K76T), which leads to the substitution of lysine (K) with threonine (T), is critical for CQ resistance, serving as a key marker of resistance in vitro and treatment failure in vivo.[18–20] However, mutations in other codons of *pfcrt* can be associated with CQ resistance.[15,21]

The *P. falciparum* multidrug resistance 1 (*pfmdr1*) gene encodes the PfMDR1 transporter protein, which is likely to regulate drug accumulation in its digestive vacuole.[22] In vitro studies have shown that the mutation at codon 86 in PfMDR1 (N86Y), which leads to the substitution of asparagine (N) with tyrosine (Y), was found to be partially associated with resistance to CQ among field isolates and laboratory lines of *P. falciparum*.[20,23–26] The role of *pfmdr1* N86Y in CQ treatment failure is supported by a meta-analysis, though the association was weak.[27]

Molecular markers of resistance provide an effective means of monitoring resistance, often serving as reliable alternatives to in vitro and in vivo studies in many instances. These markers are particularly useful in detecting the emergence of resistance before treatment failure and monitoring resistance patterns following drug withdrawal.[28,29] As the intensity of CQ pressure has diminished compared to previous decades, mutations in these genes can offer valuable insights into the genetic trends of CQ resistance. After the withdrawal of CQ, several reports have shown a decline in resistant genotypes.[30–36] However, there are reports on the persistence of high resistance-associated mutations in some countries after the withdrawal of CQ as a treatment for falciparum malaria.[37,38]

Given that CQ has been officially discontinued as a treatment for falciparum malaria for the past decade, this study aimed to determine the frequency distribution of the genetic polymorphisms in *pfcrt* K76T and *pfmdr1* N86Y as key markers of CQ resistance among *P. falciparum* isolates from patients in South Darfur state, Sudan.

Methods

Study Design and Sample Collection

A cross-sectional study was conducted among *P. falciparum* isolates from 75 conveniently selected Sudanese patients (41 males and 34 females) in Nyala district, South Darfur state, west of Sudan (Figure 1) in 2016. The median age (interquartile range) of patients was 22 years (11), with an age range of 2–65). These patients were diagnosed with *P. falciparum* mono-infection using microscopy by two expert microscopists or rapid diagnostic tests (RDTs) (SD Bioline® Malaria Antigen *Pf*/Pan test (Standard Diagnostics, Inc., Kyonggi, Korea) in the Malaria Laboratory in South Darfur state. Patients of both sexes and aged 2–65 years were included after obtaining written informed consent. Patients who were negative for falciparum malaria by microscopy or RDTs or who were co-infected with other *Plasmodium* species were excluded. Finger-prick blood was collected and spotted onto Whatman 3MM filter papers (Whatman International, Ltd., Maidstone, UK) for the collection of dried blood spots (DBS) for molecular study.

Detection of *Pfcrt* K76T and *pfmdr1* N86Y Polymorphisms

The Chelex® 100 technique was used to extract parasite DNA from DBS, following the protocol described by Warhurst et al.[39] To identify the *pfcrt* K76T and *pfmdr1* N86Y polymorphisms in *P. falciparum* isolates, nested polymerase chain reaction (PCR) was performed using the primer sequences and amplification cycles outlined by Shrivastava et al.[20] PCR amplifications were performed in Techne thermocycler (Techne, Staffordshire, UK) in the Molecular Biology Laboratory,

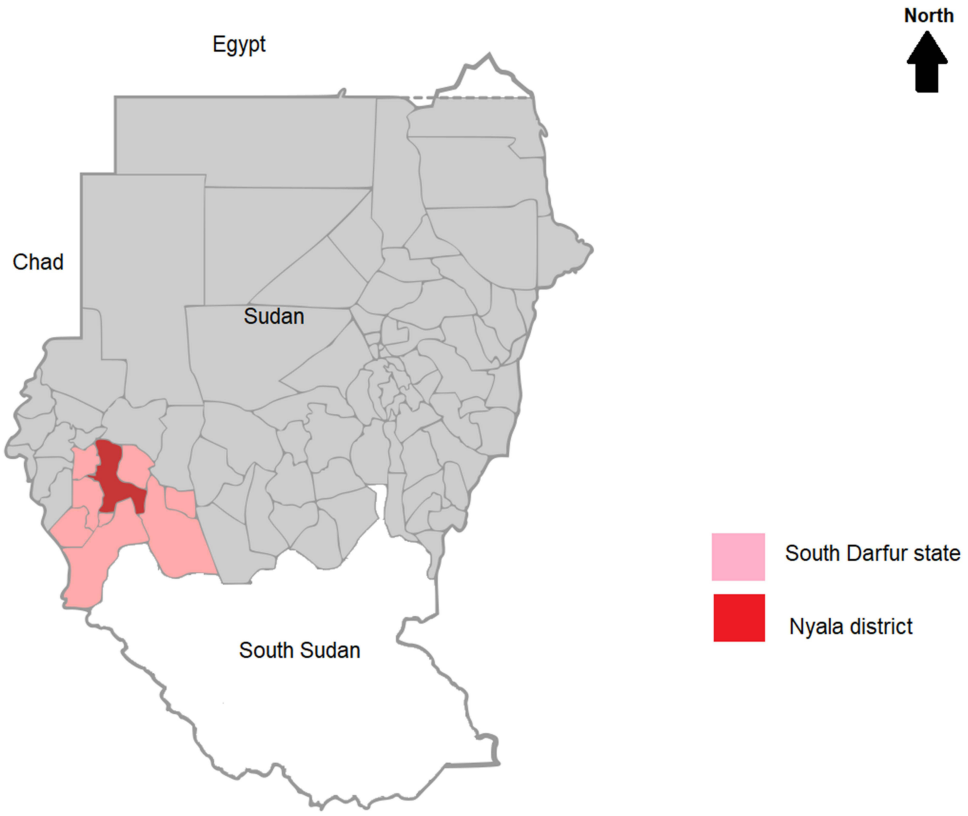

Figure 1 Map of Sudan showing the locations of Nyala district and South Darfur state.

Faculty of Medical Laboratory Sciences, Sudan University of Science and Technology in Khartoum. The amplified products of nested PCR were subsequently subjected to digestion with the Apo I restriction enzyme (New England Biolabs, UK) for restriction fragment length polymorphism (RFLP) analysis, as described by Shrivastava et al.[20] Amplicons and RFLP patterns were analyzed on 1.5% agarose gels, which were stained with ethidium bromide and visualized using an ultraviolet illuminator.

Ethical Considerations

The study protocol was reviewed and approved by the Ethics Committee of the Faculty of Medical Laboratory Sciences, University of Gezira, Wad Madani, Sudan. Written informed consent was obtained from patients or their parents. This study was conducted in compliance with the Declaration of Helsinki for medical studies involving human subjects.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics, Version 21 (IBM Corp., Armonk, NY, USA). Genetic polymorphisms were expressed as the frequencies and proportions of wild-type, mutant and mixed-type alleles of *pfcrt* and *pfmdr1*.

Results

All 75 *P. falciparum* isolates investigated in this study were successfully characterized for codons 76 of *pfcrt* and 86 of *pfmdr1*. Table 1 shows that 34.7% of isolates harbored the wild-type *pfcrt* K76 allele and 20% of isolates had the mutant 76T allele, while the mixed-type K76T allele was observed among 45.3% of isolates. On the other hand, Table 2 shows that 54.7% of isolates harbored the wild-type *pfmdr1* N86 allele and 5.3% of isolates had the mutant 86Y allele, while the mixed-type N86Y allele was observed among 40% of isolates.

Table 1 Frequency Distribution of *Pfcrt* K76T Alleles Among *P. Falciparum* Isolates from Patients in Nyala District of South Darfur State, Sudan (2016)*

Alleles of *pfcrt*	n (%)
Wild type (K76)	26 (34.7)
Mutant type (76T)	15 (20)
Mixed type (K76T)	34 (45.3)

Note: *The number of isolates was 75.

Table 2 Frequency Distribution of *pfmdr1* N86Y Alleles Among *P. Falciparum* Isolates from Patients in Nyala District of South Darfur State, Sudan (2016)*

Alleles of *pfmdr1*	n (%)
Wild type (N86)	41 (54.7)
Mutant type (86Y)	4 (5.3)
Mixed type (N86Y)	30 (40)

Note: *The number of isolates was 75.

Discussion

Following the widespread and intense global use of CQ starting in the late 1940s, the first documentation of chloroquine-resistant *P. falciparum* emerged in Cambodia and at the Cambodia-Thailand border approximately a decade later.[40] During the 1960s and 1970s, drug-resistant strains originating from these regions began to disseminate progressively across South America, Southeast Asia and India.[40] In the late 1970s, resistance to CQ was introduced into East Africa through a selective sweep originating from Southeast Asia.[17,41] Subsequently, within a decade, resistant strains of *P. falciparum* rapidly spread across the entire African continent.[40] Sudan was one of the African countries where the emergence of CQ-resistant falciparum malaria was first reported in the late 1970s.[6]

CQ was officially withdrawn from Sudan malaria treatment policies about twelve years before the implementation of the present study. However, the key molecular markers associated with its resistance are still circulating among *P. falciparum* isolates from patients in Nyala district of South Darfur state in the west of the country. The present study revealed the presence of wild-type alleles of *pfcrt* K76 and *pfmdr1* N86 among approximately one-third and one-half of parasite isolates, respectively. However, the remaining isolates harbored either the mutant alleles (76T and 86Y) or mixed-type alleles (K76T and N86Y). The *pfcrt* 76T and *pfmdr1* 86Y mutations were reported to be associated with high-level CQ resistance among *P. falciparum* isolates from Asar village in eastern Sudan in the early 2000s, when CQ was recommended for treating uncomplicated falciparum malaria.[25] In 2003, the *pfcrt* 76T but not the pfmdr1 86Y was reported to be associated with the in-vivo efficacy of CQ against *P. falciparum* in the Upper Nile state of South Sudan.[42]

Compared to the frequency of the mutant alleles in the present study, the mutant *pfcrt* 76T and *pfmdr1* 86Y alleles were detected among 63% (63/100) and 31% (31/100) of *P. falciparum* isolates, respectively, collected in 2002 before CQ withdrawal from Akuem village of Bahr El Gazal province in South Sudan.[43] A study conducted three years after official CQ withdrawal showed that the mutant types *pfcrt* 76T and *pfmdr1* 86Y were present among 72.7% (144/198) and 55.5% (110/198) of *P. falciparum* isolates, respectively, in central and eastern Sudan.[2] In 2015, *pfcrt* 76T and *pfmdr1* 86Y mutant alleles were detected among 75% (30/40) and 59.5% (22/37) of *P. falciparum* isolates, respectively, in Wad Madani district of Gezira State in central Sudan.[44] During 2015–2017, *pfcrt* 76T and *pfmdr1* 86Y mutant alleles were detected among 71.8% (153/213) and 53.6% (104/194) of *P. falciparum* isolates, respectively, in Khartoum, New Halfa, Gezira and North Kordofan.[45] A higher frequency of 80% (16/20) was reported for the mutant pfcrt 76T allele among

P. falciparum isolates from Khartoum in 2018.[46] Lower frequencies of *pfcrt* 76T (25.8%; 31/120) and *pfmdr1* 86Y (21.7%; 25/115) have been recently reported among parasite isolates from Blue Nile State, Southeast Sudan.[47]

After the withdrawal of chloroquine and the subsequent absence of its selective pressure, susceptible strains tend to dominate, allowing for the potential recovery of chloroquine efficacy.[33,48] Nevertheless, it seems that this restorative outcome has not been attained in Sudan. Despite the withdrawal of CQ from Sudan's malaria treatment policies more than ten years ago, the persistence of high frequencies of *pfcrt* 76T and *pfmdr1* 86Y mutant alleles among *P. falciparum isolates* in the present study is concerning. This finding highlights the ongoing presence and potential impact of drug-resistant strains in this region in western Sudan, emphasizing the need for continued vigilance and the implementation of effective alternative antimalarial strategies. The high frequency of *pfcrt* 76T and *pfmdr1* 86Y mutant alleles in the present study indicates that CQ is still prescribed in the country for treating uncomplicated falciparum malaria. For instance, a survey in 15 states of the country showed that CQ is available at 5% of 244 public health facilities.[49] Moreover, the impact of self-medication and the irrational prescription of antimalarial drugs by community pharmacists could not be ruled out. In line with the present study, the persistence of high resistance-associated mutations after withdrawal of CQ as a treatment for falciparum malaria has been reported from India and elsewhere.[37,38] However, several reports have shown a decrease in resistant genotypes after CQ withdrawal, suggesting the likelihood of its efficacy reversal, including in Malawi, Kenya, Tanzania, Senegal and China.[30–36,50]

The present study is limited by the small sample size of isolates investigated for the molecular markers of resistance to CQ and the convenience sampling of study subjects because of financial and logistic constraints. However, the findings of the study provide insights into the high circulation of parasite isolates harboring mutant alleles associated with CQ resistance years after its official discontinuation for treating falciparum malaria. Therefore, large-scale studies are recommended to investigate the molecular markers associated with CQ resistance. Furthermore, antimalarial drug prescribing practices need to be studied to assess the use of CQ monotherapy for treating falciparum malaria and the level of adherence of physicians and other healthcare providers to the guidelines of the national treatment policy.

Conclusion

The key molecular markers associated with CQ resistance (*pfcrt* 76T and *pfmdr1* 86Y) are still circulating in high frequency among *P. falciparum* isolates in Nyala district of South Darfur state, west of Sudan, about twelve years after the official withdrawal of the drug as a treatment for uncomplicated falciparum malaria. The sustained circulation of these resistant alleles indicates that the local malaria parasite population is still under the selective pressure that favors the survival and spread of CQ-resistant parasites. Consequently, reintroducing CQ as a treatment option for falciparum malaria in the near future is not feasible.

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Disclosure

The authors report no conflicts of interest in this work.

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