A snapshot of *Plasmodium falciparum* malaria drug resistance markers in Sudan: a pilot study

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Abstract

**Objectives:** Malaria infection is still known to be a worldwide public health problem, especially in tropical and sub-tropical African countries like Sudan. A pilot study conducted to describe the trend of *P. falciparum* drug resistance markers in 2017–2018 in comparison to CQ and AS/SP eras in Sudan. The *Pfcrt*, *Pfmdr-1*, *Pfdhfr*, and *Pfdhps* genes were investigated. Data deposited by the worldwide antimalarial resistance network was consulted, and the molecular markers previously reported from Sudan were analyzed.

**Results:** Drug molecular markers analysis was successfully done on 20 *P. falciparum* isolates. The *Pfcrt* K76 showed high frequency; 16 (80%). For the *Pfmdr-1*, 9 (45%) isolates were carrying the N86 allele, and 11 (55%) were 86Y allele. While the Y184F of the *Pfmdr-1* showed a higher frequency of 184F compared to Y184; 16 (80%) and 4 (20%), respectively. In the *Pfdhfr*, 51I allele showed higher frequency compared to N51; 18 (90%) and 2 (10%), respectively. For S108N, 18 (90%) were 108 N and 2 (10%) were S108. In the *Pfdhps*, all isolates were carrying the mutant alleles; 437G and 540E. The frequency distribution of the *Pfcrt*, *Pfmdr-1*, *Pfdhfr*, *Pfdhps* was significantly different across the whole years in Sudan.

**Keywords:** *Plasmodium falciparum*, Molecular markers, Multi drug resistance, Sudan

Introduction

Malaria infection is still known to be a worldwide public health problem, especially in tropical and sub-tropical African countries [1]. In Sudan, in the late 70s of the last century, a high proportion of drug resistance was reported when Chloroquine (CQ) was introduced as the first-line treatment for falciparum malaria [2]. By 2004 the malaria treatment protocol was shifted to the artemisinin-based combination treatment (ACTs); Artesunate (AS) and Sulfadoxine–Pyrimethamine (SP) as a first-line treatment against uncomplicated falciparum malaria, and arteether–lumefantrine (AL) as a second-line treatment [3]. In 2017, the malaria treatment protocol was shifted to AL for the treatment of uncomplicated falciparum malaria, and Quinine for treating the severe infections [4]. Subsequently, therapeutic efficacy studies were conducted to monitor and detect the emergence of drug-resistant malaria parasites [5–9]. However, another approach for the early detection of drug resistance emergence was implemented by using molecular markers to investigate the efficacy of treatments in-vitro. *P. falciparum* chloroquine resistance transporter (*Pfcrt*) and *P. falciparum* multidrug resistance gene 1 (*Pfmdr-1*)

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are previously known membrane transporters associated with resistance to the drug combination of CQ and AQ or MQ and Lumefantrine (L) [10, 11]. The CVIET haplotype of Pfcrtr is known as the most robust CQ resistance marker in Africa [12–15]. In vitro experiments showed that N86Y and Y184F mutations in the Pfmdr-1 gene increases the inhibitory concentrations of CQ and AQ [10, 12], and reduce susceptibility to MQ and L [12, 13].

Previous studies on the P. falciparum dihydrofolate reductase (Pfdhfr) and dihydropteroate synthase (Pfdhps) has been identified as known targets of SP antimalarial drugs [14]. The mutations in the Pfdhfr codons 51, 59, and 164; and the Pfdhps codons 436, 437, 540, 581, and 613 were conferring the resistance [14].

The need for updated molecular markers studies to investigate the frequency of falciparum malaria drug-resistant is extreme. This pilot study aims to describe the trend of P. falciparum drug resistance markers in 2017–2018 in comparison to CQ and AS/SP eras in Sudan.

Materials and methods
This pilot study was conducted in Khartoum state between December 2017 and July 2018. Febrile patients (axillary temperature < 37 °C) who were diagnosed microscopically by examining Giemsa stained blood films as falciparum malaria infection were recruited. Informed consent was taken from the patients before sample collection. Participants diagnosed with P. falciparum/P. vivax co-infection and P. vivax mono-infections were excluded.

Sample collection and DNA extraction
Two ml blood samples were collected before starting the treatment and preserved into lithium heparin blood containers for DNA extraction using the Guanidine Chloride extraction method as described previously [15]. DNA was stored in –20 °C until molecular examinations later.

Parasite genotyping and drug resistance markers assessment
The microscopic diagnosis was confirmed using the primers described previously [16]. Genotyping of the specific point mutations in the P. falciparum genome was done using the Sanger sequencing method by using the primers sets for Pfcrtr, Pfmdr-1, Pfdhfr, and Pfdhps genes as described previously [17]. PCR amplicons were sequenced in both directions using the forward and reverse primers for each gene to exclude any base-calling errors that could be obtained during sequencing. Sequences were validated using GENtle software (v1.9.4) and aligned in comparison with the wildtype P. falciparum 3D7 strain reference sequences (PF3D7_0709000 for Pfcrtr, PF3D7_0523000 for Pfmdr-1, PF3D7_1324800 for Pfdhfr, and PF3D7_0810800 for Pfdhps). The deduced amino acids were translated from nucleotide sequences using MEGA7 software (v7.0.26) to determine sequences mutations at the Pfcrtr codon 76; Pfmdr-1 codons 86 and 184; Pfdhfr codons 51, 59, and 108; and the substitutions at the Pfdhps in codons 437 and 540. The nucleotide sequences used in this study have been deposited in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/) under the accession numbers MT995200–MT995259.

Previous reports on drug resistance markers in Sudan
Data deposited by the worldwide antimalarial resistance network (WWARN) (https://www.wwarn.org/) was consulted, and the molecular markers previously reported from Sudan were identified, collected, and analyzed to compare between past and present frequency of malaria drug resistance mutations. Data sets included SP molecular surveyors (https://www.wwarn.org/sp-molecular-surve yor) and ACT partner drug molecular surveyors (https://www.wwarn.org/tracking-resistance/act-partn er-drug-molecular-surveyor). Numbers and drug molecular marker genotypes of P. falciparum isolates included in the historical literature review data set analyzed in this study are presented in Additional file 1.

Statistical analysis
The statistical analysis was done using the statistical Package for Social Sciences (SPSS, v20.0). One-way ANOVA test was used to calculate the least significance difference of frequency distribution in the molecular markers. Pearson correlation was used to investigate the association between the different drug resistance markers. P value ≤ 0.05 was considered statistically significant.

Results
Molecular genotyping results
In this pilot study, a total of 28 malaria parasite isolates were genotyped, of them, 2 and 6 isolates were excluded since were P. falciparum/P. vivax co-infections and P. vivax infections, respectively. The remaining 20 isolates were confirmed by PCR as P. falciparum mono-infections.

Frequency of P. falciparum drug resistance markers
Drug molecular markers analysis was successfully done on the 20 P. falciparum isolates. Out of the 20 isolates, Pfcrtr K76 showed the highest frequency; 16 (80%). Pfcrtr 76 T was 4 (20%). None of the isolates was carrying mixed Pfcrtr allele infection; K/T. For the Pfmdr-1 marker, 9 (45%) isolates were carrying the N86 allele and 11 (55%) were carrying the 86Y allele. While the Y184F of the Pfmdr-1 showed a higher frequency of 184F compared to Y184; 16 (80%) and 4 (20%), respectively. Concerning the
double Pfmdr-1 haplotypes; NY haplotype was 2 (10%), NF was 7 (35%), YF was 9 (45%), and YY was 2 (10%).

The Pfâ¶fr N51I showed a higher frequency of 51I compared to N51; 18 (90%) and 2 (10%), respectively. Whereas for Pfâ¶fr C59R, C59 was 18 (90%), and 59R was 2 (10%). For Pfâ¶fr S108N, 18 (90%) were 108N and 2 (10%) were S108. For the triplet haplotype of the Pfâ¶fr, the haplotype ICN was the most frequent; 16 (80%). IRN and NCS were only present in two isolates; 2 (10%) for each. For the Pfâ¶ps, all the 20 (100%) isolates were carrying the mutant alleles; 437G and 540E (Table 1). A statistically significant positive correlation was observed for Pfmdr-1 and the combined Pfâ¶fr and Pfâ¶ps alleles, Pearson \( r' = 0.509 \), \( P \) value = 0.035. While, for the Pfâ¶rt and the combined Pfâ¶fr and Pfâ¶ps, a statistically insignificant negative correlation was found, Pearson's \( r = -0.248 \), \( P \) value = 0.291.

The trend in *P. falciparum* multidrug resistance from 1989 to 2018

The T allele of the Pfâ¶rt was at a higher frequency during 2000–2001 (89.6%) however, T mutant allele frequency started to dropdown reaching up to 43.9% in 2016 and bottomed at 20% in 2018. Frequency of Pfâ¶rt K76 allele was higher compared to all previous years; 80%, while the N86Y mutation of the Pfmdr-1 was extremely flocculation during the past years (Fig. 1).

Concerning the Pfâ¶fr N51I and S108N, in 1996–1997 the NS wildtype haplotype showed low frequency compared to the IN mutant haplotype; 18.6% and 74.3%, respectively. While in 1998–1999 the frequency of the NS haplotype reached to 100%. Whereas the prevalence of the NS haplotype from 2002–2003 continued to decrease to 10% in 2017–2018. On the other hand, the IN mutant haplotype increased to 85.5% in 2002–2003, and reaching 92.7% in 2009–2012; and remained constant approximately 90% in 2017–2018. Also, for the Pfâ¶ps, in 1998–1999 AK wildtype haplotype was 93.1%, but in 2002–2003 GE mutant haplotype increased to 75.1%. In 2017, the AK wildtype haplotype increased again to 77.8% and decreased to 36.1% in 2009–2012. And, in 2016 reached 51.1%. However, in this study in 2017–2018, the GE mutant haplotype was prevalent in all the studied samples 20 (100%) (Fig. 2).

The frequency distribution of the Pfâ¶rt and Pfmdr-1, Pfâ¶fr, and Pfâ¶ps mutations was significantly different across the whole years in Sudan. An illustrated statistical significance and insignificance of the frequency distribution of *P. falciparum* multidrug resistance markers between the different years’ intervals are described in Additional file 2 Tables S1–S5.

### Table 1. The distribution of multidrug resistance markers among the 2017–2018 study isolates

| Isolate ID | Pfâ¶rt | Pfmdr-1 | Pfâ¶fr | Pfâ¶ps |
|------------|--------|---------|--------|--------|
| Isolate 1  | K      | YF      | ICN    | GE     |
| Isolate 2  | T      | NF      | ICN    | GE     |
| Isolate 3  | K      | YF      | ICN    | GE     |
| Isolate 4  | K      | YY      | ICN    | GE     |
| Isolate 5  | T      | NF      | ICN    | GE     |
| Isolate 6  | K      | NY      | ICN    | GE     |
| Isolate 7  | K      | YF      | IRN    | GE     |
| Isolate 8  | K      | YF      | NCS    | GE     |
| Isolate 9  | K      | NY      | ICN    | GE     |
| Isolate 10 | T      | NF      | ICN    | GE     |
| Isolate 11 | K      | NF      | ICN    | GE     |
| Isolate 12 | K      | YF      | NCS    | GE     |
| Isolate 13 | K      | NF      | ICN    | GE     |
| Isolate 14 | K      | YF      | IRN    | GE     |
| Isolate 15 | T      | NF      | ICN    | GE     |
| Isolate 16 | K      | YF      | ICN    | GE     |
| Isolate 17 | K      | YF      | ICN    | GE     |
| Isolate 18 | K      | YY      | ICN    | GE     |
| Isolate 19 | K      | YF      | ICN    | GE     |
| Isolate 20 | K      | NF      | ICN    | GE     |

*Letters denotes the wildtype and mutant alleles of the Pfâ¶rt K76T; Pfmdr-1 N86Y and Y184F; Pfâ¶fr N51I, C59R, and S108N; Pfâ¶ps A437G and K540E. Mutant alleles were written in bold.

### Discussion

The reported frequency of Pfâ¶rt K76 allele in this study was significantly high compared to previous years, especially during the CQ deployment years in Sudan [9]. During the AS/SP era; since 2004, Pfâ¶rt K76 allele had increased. This increase can be suggested due to limited or ambient exposure of *P. falciparum* parasite to CQ or might be due to an increase in AL pressure. Similar results were reported from Malawi, and Tanzania supporting the increase of Pfâ¶rt K76 allele [18–21]. While, in Zambia, complete disappearance of Pfâ¶rt 76 T allele from the examined isolates was reported [22].

The Pfâ¶fr IN haplotype and Pfâ¶ps GE haplotype significantly increased in 2017–2018 where both haplotypes were showing high frequency; 90% and 100%, respectively. This increase could be due to the enduring pressure of AS/SP since 2007 [23]. This phenomenon was observed in all countries where AS/SP was used to treat uncomplicated malaria such as Nigeria and Equatorial Guinea; where the prevalence of mutant haplotypes reached more than 90% [24–26].

The prevalence of Pfmdr-1 NF haplotype in 2017–2018 is noted among 35% of the study samples. Comparison between previous years for the frequency distribution of Pfmdr-1 double haplotypes showed a statistically significant difference between all years and 2008 when at
that time the recommended malaria treatment was AS/SP, and since 2009 the use of AL is significantly increased due to malpractice in drug use, such as usage of incorrect dosage and insufficient information stated to patients about the prescribed treatment which may lead to the increase in resistance and recurrent infections rates [27].
Previously, the development of molecular markers for AL resistance was thought to be difficult because there were no known resistant lab lines can be used as resistance controls. Meanwhile, investigating the \textit{Pfmdr-1} N86S haplotype could be the role evolution for the developed mutations because it allows a longer survival rate of the parasite [11, 28].

In the present study, \textit{Pfmdr-1} 86Y allele, \textit{Pfdhfr} IN haplotype, and \textit{Pfdhps} GE haplotype were constituting the majority of the studied samples; 90%, which is also similar to a previous study conducted in Sudan; where all the investigated parasite isolates were carrying the \textit{Pfmdr-1} 86Y allele, \textit{Pfdhfr} IN haplotype, and \textit{Pfdhps} 540E [23].

The prevalence of \textit{Pfdhps} double haplotype GE detected in this study could hinder the effect of SP if used as intermittent preventive therapy during pregnancy (IPTp). Although, SP as IPTp was not implemented and there is no information about the use of SP during pregnancy
in Sudan [29]. The presence of PfHfr IRN in combination with PfDhps GE haplotypes forming the quintuple mutant haplotype confers a high risk for treatment failure in malaria-infected children and nonpregnant adults who receive SP as a seasonal malaria chemoprevention treatment (SMC-SP) [30]. However, previous studies indicated that IPTp-SP is still efficacious in areas with a high prevalence of resistant *P. falciparum* parasite [31]. Nevertheless, the increased resistance rate might compromise the implication of IPTp-SP [32–34].

**Conclusion**

This study describes the distribution of *P. falciparum* multidrug resistance markers throughout Sudan. The study provides a baseline data of the status of these markers which could be very useful for the malaria control program for establishing surveillance system to monitor the emergence of malaria drug resistance for more effective treatment protocol and successful control of the disease.

**Limitations**

- The lack of clinical and background information, particularly, the previously used drug before the blood sampling substantially can affect the prevalence of the alleles. Therefore, complete clinical history information is needed and can be very useful in future studies.

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s13104-020-05363-0.

**Additional file 1:** Table S1. Numbers and drug molecular markers genotypes of *P. falciparum* isolates from Sudan 1989 – 2018.

**Additional file 2:** Table S2. Statistical significance of Pfcrt K76T frequency distribution across the different previous studies conducted in Sudan.

**Table S3:** Statistical frequency distribution of Pfmdr-1 N86Y frequency across the different previous studies conducted in Sudan.

**Table S4:** Statistical frequency distribution of PfDhps A437G and K540E double haplotype frequency distribution across the different previous studies conducted in Sudan.

**Table S5:** Statistical frequency distribution of Pfmdr-1 N86Y and Y184F double haplotype frequency distribution across the different previous studies conducted in Sudan.

**Abbreviations**

ACTs: Artemisinin-based combination therapies; AL: Artemether–Lumefantrine; AQ: Armodiaquine-hydrochloride; AS: Artesunate; CQ: Chloroquine; IPTp: Intermittent preventive therapy during pregnancy; L: Lumefantrine; MQ: Mefloquine; Pfcrt: *P. falciparum* Chloroquine resistance transporter; PfDhps: *P. falciparum* Dihydrofolate reductase; PfDhps: *P. falciparum* Dihydropteroate synthase; Pfmdr-1: *P. falciparum* Multidrug resistance gene 1; SP: Sulfadoxine–Pyrimethamine; WWARN: Worldwide Antimalarial Resistance Network.

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**Authors’ contributions**

NSM and CHS conceived, designed the study protocol, HA, HAO, AEA, AMY, YBE, EYO, ARE, MMO and EES carried out the parasitological and molecular examinations: MM, AAM, YA, AA, MMO, MSA, and RAO conducted the genetic and molecular markers analysis. NSM, HA, HAO, AEA and EES analysed and interpreted the data. NSM, AA, MSA, and RAO designed and supervised the experiments. NSM, AA, and CHS drafted the manuscript. All authors read, revised, and approved the final manuscript.

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**Availability of data and material**

All datasets used and analysed in this study are available in the manuscript. DNA sequences analysed during the current study are available at the NCBI GenBank database under the accession numbers MT995200–MT995259.

**Ethics approval and consent to participate**

This study was reviewed and approved by the National University Biomedical Research Ethics Committee, National University-Sudan. Oral and written informed consents were obtained from participants’ or parents or guardians in case of children.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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