Historical literature review and molecular analysis of malaria drug resistance markers of Plasmodium falciparum field-isolates from Sudan.

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Abstract

Background Malaria infection is still known to be a worldwide public health problem, especially in tropical and sub-tropical African countries like Sudan. The fight against malaria is still taking place due to many factors. One of these factors is the presence of *Plasmodium falciparum* drug resistant parasites. This study is aiming at studying the *P. falciparum* drug resistance markers and analyzing the historical literature on these markers in Sudan. Methods A descriptive cross-sectional healthcare-centers based study conducted in Khartoum state between December 2017 and July 2018. Febrile patients diagnosed with *P. falciparum* malaria infection were recruited. Two ml blood samples were collected prior to start treatment. Genotyping of the specific point mutations in the *P. falciparum* genome was done using Sanger sequencing method for the *Pfcr*t, *Pfmdr*-1, *Pfdhfr*, and *Pfdhps* genes. Data deposited by the worldwide antimalarial resistance network 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. One-way ANOVA test was used to calculate the least significance of frequency distribution in the molecular markers collected from the previous reports from Sudan in comparison to this study. Pearson correlation was used to investigate the association between the different drug resistance markers. Results Drug molecular markers analysis was successfully done on the 20 *P. falciparum* isolates. the *Pfcr*t K76 showed the highest frequency; 16 (80%). *Pfcr*t 76T was 4 (20%). For the *Pfmdr*-1 marker, 9 (45%) isolates were carrying the N86 allele and 11 (55%) were 86Y allele. While the Y184F of the *Pfmdr*-1 showed higher frequency of 184F compared to Y184; 16 (80%) and 4 (20%), respectively. Concerning the double *Pfmdr*-1 haplotype, NY haplotype was 2 (10%), NF was 7 (35%), YF was 9 (45%), and YY was 2 (10%). In the *Pfdhfr*, 51I allele showed higher frequency compared to N51; 18 (90%) and 2 (10%), respectively. Whereas for C59R, C59 was 18 (90%), and 59R was 2 (10%). For S108N, 18 (90%) for 108N and 2 (10%) for S108. The triplet haplotype ICN of the *Pfdhfr*; was the most frequent haplotype; 16 (80%). Concerning the *Pfdhps*, all the 20 (100%) isolates were carrying the mutant alleles; 437G and 540E. the *Pfdhps* haplotype present was the double GE haplotype only. No statistically significant correlation was found for the *Pfcr*t, *Pfmdr*-1, *Pfdhfr*, and *Pfdhps*. Historical reports on *P. falciparum* multidrug resistant collected from 1989 to 2016 showed extreme fluctuation. High prevalence of *Pfcr*t 76T allele was observed in Khartoum throughout all years of previous studies, while in Gedaref *Pfcr*t 76T showing increased prevalence each year. All studied genes were showing increase prevalence of the mutant alleles and reduction of the wildtype alleles. In this study, the GE mutant haplotype was prevalent in all the studied samples. Frequency distribution of the *Pfcr*t K76T and *Pfmdr*-1 N86Y alleles, *Pfmdr*-1; N86Y and Y184F, *Pfdhfr*; N51I and S108N, and *Pfdhps*; A437G and K540E double haplotypes was significantly different across the whole years in Sudan. Conclusion This study describes the distribution of *P. falciparum* multidrug resistance markers throughout Sudan providing a solid baseline data of the status of these markers which could be very useful for the malaria control program not only for establishing surveillance system that monitor the change in and/or the emergence of malaria drug resistance but it will also offer a guidance for the evidence-base decision-making regarding the treatment protocol national and regional wise.

Background

Malaria infection is still known to be a worldwide public health problem, especially in tropical and sub-tropical African countries [1]. Based on the WHO estimations in 2019, malaria infections reached 228 million cases with 405 thousand related-deaths [1]. In Sudan, anti-malarial resistance among *Plasmodium falciparum* had emerged since 1955 investigated by Abbot, who firstly reported the phenomenon of drug-resistant in Wad Madani when using Amodiaquine-hydrochloride (AQ) and Pyrimethamine (P) to treat *P. falciparum* infections [2]. During the late 70s of the last century, high proportions of drug resistance were reported when Chloroquine (CQ) was introduced as the first-line treatment [3]. In 1991, Babiker and his colleagues investigated the drug resistance against CQ, Mefloquine (MQ) and P. They reported that drug response varieties where some isolates in in-vitro testing were found to be resistant for both CQ and P, while others were resistant to either CQ or P, whereas, no resistance against MQ has been reported [4]. Also, a similar case resistance against P was published by Ibrahim et al., 1991 in Sennar state-Sudan [5]. Other studies reported malaria drug resistance throughout the country [6-10]. However, by 2004 the National Malaria Programme (NMP) changed the malaria management protocol and shifted from CQ to the artemisinin-based combination treatment (ACTs). The combination of Artesunate (AS) and Sulfadoxine-Pyrimethamine (SP) was then adopted as the first-line treatment against uncomplicated falciparum malaria, and artemether-lumefantrine (AL) was considered as a second-line treatment [11]. Therapeutic Efficacy Studies (TES) were conducted to monitor and detect the emergence of drug-resistant malaria parasites. Another approach for the early detection of the emergence of drug resistance was implemented by using molecular markers in order to investigate the efficacy of treatments in vitro. Once the deployment of AS+SP as a first-line treatment for *P. falciparum* malaria, TESs were conducted to evaluate SP in the form of monotherapy or combined with other anti-malarial drugs. These studies reported different efficacies of treatment and failure of SP monotherapy [12-16]. Between 2010 and 2015, Ahmed et al. conducted a study to evaluate the efficacy of AS+SP and AL and AL showed high efficacy with declining efficacy of AS+SP to treat uncomplicated malaria [17]. Altogether, studies conducted in Sudan between 2011 and 2015 detected high failure rates of treatment with AS-SP, ranging from 12.3% to 22.2%. The evidence prompted a decision to change the new first-line of treatment to AL [18]. In 2017, Mohamed et al. reported a high rate of treatment failure against AS+SP which has led to the adoption of Dihydroartemisinin-piperaquine (DHA-PPQ) as the first-line treatment [19]. A year later, Hamid et al. (2018), stated that AS+SP is still effective against falciparum malaria in New halfa and Gezira Slanj regions [20].

P. falciparum chloroquine resistance transporter (*Pfcr*t) and *P. falciparum* multidrug resistance gene 1 (*Pfmdr*-1) are previously known membrane transporters associated with resistance to the drug combination of CQ and AQ or MQ and Lumefantrine (L) [21]. Also, with the suggestion of previous studies, point mutations in *Pfmdr*-1 are associated with the resistant to quinoline-containing drugs and artemisinin derivatives [21, 22]. Also, the presence of *Pfmdr*-1 multicopy results in resistance to MQ and L [23, 24]. However, the K76T mutation in *Pfcr*t mostly associated with the *Pfcr*t 72C, 73V, 74I, 75E, and 76T. The CVIET haplotype of *Pfcr*t 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 [21, 24], and reduce susceptibility to MQ and L [24, 25].

Previous studies focused on the *P. falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthase (*Pfdhps*) which are known targets of pyrimethamine and sulfadoxine, respectively. Both drugs are inhibiting the folate pathway leading to the death of the parasite [19]. The emergence of resistant parasites has been documented in many parts of Africa [26]. Mutations in the *Pfdhfr* codons 51, 59, and 164 [16] and the substitutions at the *Pfdhps* in the codons 436, 437, 540, 581 and 613, suggested to be conferring resistance to sulfadoxine [19].

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

Methods

A descriptive cross-sectional healthcare-centers based study conducted in Khartoum, Sennar, River Nile, Ad Damazin and Gedaref states between December 2017 and July 2018. Febrile patients (axillary temperature < 37 C) who were microscopically diagnosed with *P. falciparum* malaria infection were recruited to participate in the study after obtaining informed consent to collect the blood sample. Participants diagnosed with *P. falciparum*/ *P. vivax* co-infection and *P. vivax* mono-infections were excluded from the study. Two ml blood samples were collected prior to start treatment. No follow up data were obtained from patients after treatment. Blood samples were preserved into lithium heparin anticoagulation blood containers and shipped to the biotechnology lab, faculty of pharmacy at The Ahfad University for women for DNA extraction and molecular genotyping of drug resistance markers.

DNA extraction

The genomic DNA was extracted using the Guanidine Chloride extraction method described previously by Ciulla et al. (1988), with minor modifications [27]. In brief, 1 ml from each blood sample was placed into 2.5 ml tube and washed using RBCs washing buffer; and then nucleated and parasite cells were lysed using cell lysis buffer. 1 ml of 6 M Guanidine chloride solution and 10 micro of proteinase K enzyme were added and incubated over night at 37°C. The following day, 2 ml Chloroform were added to the mixture and centrifuged at 14000 rpm for 10 min; 3 layers separation were formed. The upper layer containing the parasite and human DNA was transferred into new 2 ml tubes; and 1 ml absolute ethanol was added to precipitate the DNA. Precipitated DNA was then washed with 70% ethanol and allowed to dry for 1 hour before being dissolved with 200 micro distilled water. Purity and concentration of the extracted DNA was measured using Nanodrop 1000 spectrophotometer (USA). DNA was stored in -20°C until molecular examinations later.

Parasite genotyping and assessment of multiple infection

To confirm the microscopic diagnosis, the protocol and primers sets described previously by Snounou et al. (1993) were used [28]. Whereas, for assessment of multiple infection, primers and PCR conditions used were according to the previously described protocol [29].

Drug resistance markers assessment

Genotyping of the specific point mutations in the *P. falciparum* genome was done using Sanger sequencing method using the primers sets for the *Pfcr*t, *Pfmdr*-1, *Pfdhfr*, and *Pfdhps* genes as described previously [30]. Primers sets used in this study were described in table 1. PCR amplification conditions were set to 95°C for 5 min as initial denaturation followed by 35 cycles of 95°C for 1 min, 55°C for 1 min (for *Pfcr*t, *Pfmdr*-1) or 58°C for 1 min (for *Pfdhfr* and *Pfdhps*), and 72°C for 1 min, followed by final extension step at 72°C for 10 min. All thermal conditions were performed in MJ thermocycler PCR machine (USA). PCR amplicons were then sequenced in both directions using the forward and reverse primer 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 *Pfcr*t, 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) using the standard translation code in order to determine sequences mutations at the *Pfcr*t codons; 72, 73, 74, 75, and 76; *Pfmdr*-1 codons 86 and 184; *Pfdhfr* codons 51, 59, and 164; and the substitutions at the *Pfdhps* in the codons 436, 437, 540, 581 and 613.

Previous reports on drug resistance markers

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-surveyor>), and ACT partner drug molecular surveyors (<https://www.wwarn.org/tracking-resistance/act-partner-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 Supplementary 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 (LSD) of frequency distribution in the molecular markers collected from the previous reports from Sudan in comparison to this study. Pearson correlation was used to investigate the association between the different drug resistance markers. P value ≤ 0.05 was considered significant.

Results

The molecular genotyping of the field-isolated *P. falciparum* and multiclonal infections assessment

In this small-scale study, a total of 28 malaria parasite isolates were genotyped, of them, 2 and 6 isolates were excluded since were *P. falciparum*/*P. vivax* coinfections, *P. vivax* infection, respectively. The remaining 20 isolates were *P. falciparum* mono-infections. Multiclonal infections assessment for the 20 isolates were 16 (80%) MAD20, 2 (10%) K1, and 2 (10%) RO33, based on the merozoite surface protein 1 (MSP-1) gene.

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 *Pfcr*t K76 showed the highest frequency; 16 (80%). *Pfcr*t 76T was 4 (20%). None of the isolates were carrying mixed *Pfcr*t allele infection. 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 higher frequency of 184F compared to Y184; 16 (80%) and 4 (20%), respectively. Concerning the double *Pfmdr*-1 haplotype, NY haplotype was 2 (10%), NF was 7 (35%), YF was 9 (45%), and YY was 2 (10%).

The *Pfdhfr* N51I showed higher frequency of 51I compared to N51; 18 (90%) and 2 (10%), respectively. Whereas for C59R, C59 was 18 (90%), and 59R was 2 (10%). For S108N, 18 (90%) for 108N and 2 (10%) for S108. For the triplet haplotype of the *Pfdhfr*, the haplotype ICN was the most frequent haplotype; 16 (80%). IRN and NCS were only present in two isolates; 2 (10%) for each.

Concerning the *Pfdhps* drug molecular marker, all the 20 (100%) isolates were carrying the mutant alleles; 437G and 540E. the *Pfdhps* haplotype present was the double GE haplotype only (Table 2).

No statistically significant correlation was found for the *Pfcr*t, *Pfmdr*-1, *Pfdhfr*, and *Pfdhps*. However, a statistically significantly positive correlation was observed for *Pfmdr*-1 and the combined *Pfdhfr* and *Pfdhps* alleles, Pearson $r' = 0.509$, P value = 0.035. While, for the *Pfcr*t and the combined *Pfdhfr* and *Pfdhps*, statistically insignificant negative correlation was found, Pearson $r' = -0.248$, P value = 0.291. *Pfdhps* was not correlated separately with any of the other mutations since present in all the studied samples.

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

Reports on *P. falciparum* multidrug resistant collected from 1989 to 2016 showed extreme fluctuation. High prevalence of *Pfcr*t 76T allele was observed in Khartoum throughout all years of previous studies. However, in the current study the *Pfcr*t K76 is very high compared to the previous years. While in Gedaref *Pfcr*t 76T showing increased prevalence each year, reaching 89% in the study conducted in 2007. The *Pfcr*t 76T allele during the previous studies is showing higher prevalence compared to *Pfcr*t K76. The situation of the increase in *P. falciparum* resistance markers among the previous studies was relatively similar. All studied genes showing increase prevalence of the mutant alleles and a reduction of the wildtype alleles. However, in Khartoum the *Pfdhfr* IN and *Pfdhps* GE haplotypes prevalence in 2016 and 2018 isolates constitute nearly all the studied samples comparing to other locations (Fig. 1).

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

For the *Pfcr*t, the T allele that confers CQ resistant was at 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. In this study, the frequency of the K76 wildtype allele was higher compared to all previous years; 80%. Whereas, the N86Y mutation of the *Pfmdr-1* was extremely flocculation during the past years (Fig. 2).

Concerning the *Pfdhfr* N51I and S108N, in 1996-1997 the NS wildtype haplotype was showing low frequency compared to the IN mutant haplotype; 18.6% and 74.3%. while in 1998-1999, frequency of the NS haplotype reached to 100%. The prevalence of the NS haplotype from 2002-2003 continued to decrease down to 10% in 2017-2018. On the other hand, the IN mutant haplotype increased to 85.5% in 2002-2003 reaching 92.7% in 2009-2012 and remained constant approximately at 90% in 2017-2018. However, for the *Pfdhps*, in 1998-1999 AK wildtype haplotype was 93.1%, but in 2002-2003 GE mutant haplotype increased to 75.1%. in 2007 the AK wildtype haplotype increased again to 77.8% and decreased to 36.1% in 2009-2012 and 51.1% in 2016. However, in this study in 2017-2018, the GE mutant haplotype was prevalent in all the studied samples 20 (100%) (Fig. 3).

The prevalence of the *Pfmdr-1* double haplotype N86Y and Y184F was only obtained from 4 previous studies. Results of *Pfmdr-1* double haplotype in comparison with this study showed an increase in the NY mutant haplotype in 2017-2018 compared to 2016; 45% and 31.5%, respectively. While noted a quite reduction of the NY and YY wildtype haplotypes throughout all years (Fig. 4).

Frequency distribution of the *Pfcr*t K76T and *Pfmdr-1* N86Y alleles, *Pfmdr-1*; N86Y and Y184F, *Pfdhfr*, N51I and S108N, and *Pfdhps*; A437G and K540E double haplotypes was significantly different across the whole years in Sudan. an illustrated statistical significance and insignificance of frequency distribution of *P. falciparum* multidrug resistance markers between the different years intervals is described in supplementary file 2; (tables S1-S5).

Discussion

Studying the molecular markers to monitor the prevalence of drug resistance is extremely beneficial especially for public health programs that aim to reduce the prevalence of malaria infections because drug-resistant parasites are the major threat to achieve success malaria control/elimination. In this study we aimed to investigate the fluctuation in the *P. falciparum* multidrug resistance markers in Sudan during CQ, AS/SP and ACT eras. In the current small-scale study, the reported frequency of *Pfcr*t K76 wildtype allele in the filed-isolates was very high compared to all previous years, especially during the years of CQ deployment in Sudan [16]. However, since AS/SP era, post-CQ in 2004, *Pfcr*t K76 allele tends to increase. This could be occurred due to limited exposure of the *P. falciparum* parasite to the CQ or the complete absence of the pressure since infections with *P. vivax* or *P. falciparum* / *P. vivax* coinfection is been treated with combination of AL and PQ [1]. However, this increase in *Pfcr*t K76 might be due to the increase of AL

pressure. Similar results supporting the reasons for the increase of *Pfcr*t K76 allele were also reported from China [31].

In 2003, molecular markers studies confirmed that CQ was no longer suitable for malaria treatment and SP treatment failure was only ranging between 1.9% and 7.8%, this led to the change of treatment guidelines in 2004 [7, 32-34]. At the same time, the increase in *Pfdhfr* and *Pfdhps* double mutant haplotypes was noted in the following years [16]. The *Pfdhfr* IN haplotype and the *Pfdhps* GE haplotype which both confers the resistant to SP, increased significantly by 2007 [35]. In 2017-2018, both haplotypes were showing higher frequency; 90.0% and 100%. This could be due to the continued encountered pressure at the *Pfdhfr* and *Pfdhps* genes in the parasite, in parallel to the reduced *Pfcr*t 76T allele since CQ-pressure is limited. This phenomenon is observed significantly in all countries in which AS/SP was used as first-line therapy for malaria infections [36].

The prevalence of the *Pfmdr-1* NF haplotype in 2017-2018 which could confer the resistant to AL 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; P values 0.000 for 2009 to 2012 and 2016, and 0.005 for 2017 to 2018, 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 [37].

Although, in this study the quintuple NFSND haplotype of the *Pfmdr-1* is not completely studied, but this is also considered alarming since the presence of NF haplotype could be accompanied with the SND haplotype, and consequently leading to AL resistance since parasites carrying the NFSND haplotype reported to survive 15-fold higher AL concentrations [22]. Previously, 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, but with the ability to predict with the aid of the Genome-wide Analytical Study (GWAS) [38], this gave the chance to investigate further genes that could develop mutations under treatments pressure such as kelch13 and plasmepsins 2-3 multicopy at the same time investigating the *Pfmdr-1* NFSND haplotype could be the role evolution for the developed mutations since allow longer survival rate of the parasite [22, 39].

Although, as previously described by Wang et al., 1997, established the association of *Pfdhfr* and *Pfdhps* haplotypes with SP resistance [40]. In the present study, *Pfmdr-1* 86Y allele, *Pfdhfr* IN haplotype and *Pfdhps* GE haplotype were consisting the majority of the studied samples;90%. This however, is also similar to previous study conducted in Sudan where all the parasite isolates carrying the *Pfcr*t 76T and *Pfmdr-1* 86Y mutant alleles were carrying the mutant *Pfdhfr* IN haplotype and *Pfdhps* 540E mutation [35]. But difference obtained in this study is that most of the isolates were carrying *Pfcr*t K76 wildtype allele. As this is in accordance to stop the use of CQ in malaria treatment. As well, the prevalence of *Pfdhps* double haplotype GE 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 scarce information about the use of SP during pregnancy in Sudan (Mohamed et al., unpublished data). And the presence of *Pfdhfr* IRN in combination with *Pfdhps* GE haplotypes forming the quintuple mutant haplotype confer a high risk for treatment failure in malaria-infected children and nonpregnant adults who receive SP as a seasonal malaria chemo-prevention treatment (SMC-SP) [41]. However, previous studies indicated that IPTp-SP is still efficacious in some areas with high prevalence of resistant *P. falciparum* parasite [42]. Nevertheless, the increased resistance rate might compromise the implication of IPTp-SP [43-45].

In this study, a small frequency of R59 mutation was observed, this could be attributed to the small sample size investigated particularly that the R59 mutation is quite known throughout most of the African countries. However, comparing to previously reported studies in Sudan, this frequency is similar, as suggested previously, this could be due to high frequency of *Pfdhfr* 51I and 108N, and *Pfdhps* 437G and 540E mutations lead to a genetical cost-effectiveness competitions between the multi-drugs-resistance mutations within the parasites for determination of the effective combination of mutations that increase the parasite survival rate [46].

Conclusions And Recommendations

In addition to studying the molecular markers of malaria multi-drug resistance in the field collected isolates of *P. falciparum* we reviewed and summarized the previously published reports about malaria drugs resistance in Sudan. This study describes the distribution of *P. falciparum* multidrug resistance markers throughout Sudan. It also provides a solid baseline data of the status of these markers which could be very useful for the malaria control program not only for establishing surveillance system that monitor the change in and/or the emergence of malaria drug resistance but it will also offer a guidance for the evidence-base decision-making regarding the treatment protocol national and regional wise. Nevertheless, a country-wide monitoring and evaluation program for the early detection of the drug-resistance is extremely needed for more effective treatment protocol and a successful control of the disease.

List Of Abbreviations

ACTs; Artemisinin-based Combination Treatment

AL; Artemether-Lumefantrine

AQ; Amodiaquine-Hydrochloride

AS; Artesunate

CQ; Chloroquine

DHA-PPQ; Dihydroartemisinin-Piperaquine

GWAS; Genome-Wide Analytical Study

IPTp; Intermittent Preventive Therapy during Pregnancy

L; Lumefantrine

MSP-1; Merozoite Surface Protein 1

MQ; Mefloquine

P; Pyrimethamine

Pfcr1, *P. falciparum* Chloroquine Resistance Transporter

Pfdhfr, *P. falciparum* Dihydrofolate Reductase

Pfdhps, *P. falciparum* Dihydropteroate Synthase

Pfmdr-1; *P. Falciparum* Multidrug Resistance Gene 1

SMC-SP; Seasonal Malaria Chemo-Prevention Treatment

SP; Sulfadoxine-Pyrimethamine

TES; Therapeutic Efficacy Studies

WWARN; Worldwide Antimalarial Resistance Network

Declarations

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

Availability of data and material

All datasets used and analysed in this study are available in the manuscript. Sequences analysed in this study were not submitted into the NCBI database.

Competing interests

The authors declare that they have no competing interests

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Not applicable

Author's 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 and approved the final manuscript.

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Tables

Table 1: Primers sets used for the amplification of the drug-target genes

Primer name	Primer sequence	Annealing temperature
Pfcr1 1 forward	5'-GTT CTT GTC TTG GTA AAT GT-3'	55°C
Pfcr1 1 reverse	5'- CGG ATG TTA CAA AAC TAT AGT T-3'	
Pfmdr-1 forward	5'-AGA GAA AAA AGA TGG TAA CCT CAG-3'	55°C
Pfmdr-1 reverse	5'-ACC ACA AAC ATA AAT TAA CGG-3'	
Pfdhfr forward	5'-TTC TCC TTT TTA TGA TGG AAC AAG T-3'	58°C
Pfdhfr reverse	5' ATA TTT GAA AAT CAT TTG GAT GTA TAG-3'	
Pfdhps forward	5'-TGC TTA AAT GAT ATG ATA CCC GAA TAT AAG-3'	58°C
Pfdhps reverse	5' TCC ACC TGA AAA GAA ATA CAT AAA T-3'	

Table 2: The distribution of multidrug resistance markers among the 2017-2018 study isolates.

Isolate ID	<i>Pfcr1</i>	<i>Pfmdr-1</i>	<i>Pfdhfr</i>	<i>Pfdhps</i>	Combined*
Isolate 1	K	YF	ICN	GE	KYFICNGE
Isolate 2	T	NF	ICN	GE	TNFICNGE
Isolate 3	K	YF	ICN	GE	KYFICNGE
Isolate 4	K	YY	ICN	GE	KYYICNGE
Isolate 5	T	NF	ICN	GE	TNFICNGE
Isolate 6	K	NY	ICN	GE	KNYICNGE
Isolate 7	K	YF	IRN	GE	KYFIRNGE
Isolate 8	K	YF	NCS	GE	KYFNCSGE
Isolate 9	K	NY	ICN	GE	KNYICNGE
Isolate 10	T	NF	ICN	GE	TNFICNGE
Isolate 11	K	NF	ICN	GE	KNFICNGE
Isolate 12	K	YF	NCS	GE	KYFNCSGE
Isolate 13	K	NF	ICN	GE	KNFICNGE
Isolate 14	K	YF	IRN	GE	KYFIRNGE
Isolate 15	T	NF	ICN	GE	TNFICNGE
Isolate 16	K	YF	ICN	GE	KYFICNGE
Isolate 17	K	YF	ICN	GE	KYFICNGE
Isolate 18	K	YY	ICN	GE	KYYICNGE
Isolate 19	K	YF	ICN	GE	KYFICNGE
Isolate 20	K	NF	ICN	GE	KNFICNGE

*Letters denotes the wildtype and mutant alleles of the *Pfcr*t K76T; *Pfmdr*-1 N86Y and Y184F; *Pfdhfr* N51I, C59R, and S108N; *Pfdhps* A437G and K540E.

Figures

Figure 1

Prevalence of *P. falciparum* drug resistance markers from 1989 to 2018 according to location. A: Prevalence of *Pfcr*t K76T alleles. B: Prevalence of *Pfmdr*-1 N86Y alleles. C: Prevalence of *Pfdhfr* double haplotypes N51I and S108N. D: Prevalence of *Pfdhps* double haplotypes A437G and K540E.

Figure 2

Frequency distribution of single Pfprt K76T and Pfmdr-1 N86Y genotypes in 2017-2018 samples compared with previously published reports.

Figure 3

Frequency distribution of double haplotypes of Pfdhfr N51I and S108N, and Pfdhps A437G and K540E in 2017-2018 samples compared with previously published reports.

Figure 4

Frequency distribution of double haplotypes of Pfmdr-1 N86Y and Y184F in 2017-2018 samples compared with previously published reports.

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