Molecular Markers Assessment of Chloroquine Resistance to *Plasmodium falciparum* Isolates in Wad Medani District, Gezira State, Sudan

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Abstract:

Background:

Molecular surveillance of antimalarials resistance is of significant importance for the endemic countries, the introduction of polymerase chain reaction (PCR) into malaria research becomes an essential tool to investigate several genetic mutations to predict or monitor antimalarial drugs resistance .

Method:

In Wad Medani district central Sudan, 51 blood isolates were collected in filter paper from individuals infected with *P. falciparum*, DNA was extracted from each sample and then subjected to molecular analysis f**or** chloroquine transporter (*pfcrt)* and multi-drugs resistance1 (*Pfmdr1)*, using the polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) method.

Result:

The results showed that 30/40 (75%) were *pfcrt* at codon 76 mutant and 10/40(25%) were wild type while the results of *pfmdr1* at codon 86 demonstrated that 22/37(59.5%) were mutant and 15/37(40.5%) wild type, both mutations were abundant in 19 of the isolates while each mutaion alone was abundant in 11 and 3 for *pfcrt* and *pfmdr1* respectively, indicating that the frequency of CQ resistant mutations was 33/40(82.5%).

Conclusion;

This study concluded that the mutations in *pfcrt* and *pfmdr* genes were abundant with high frequency among the *P. falciparum* population

Keywords: PCR-RFLP, Antimalarial drugs resistant, malaria, Sudan

Introduction:

In Africa Sub-Sahara, the population prefers antimalarial drugs which are affordable , easy adminstered and safe for all age groups[1]. One of the important factors limiting success in the treatment of malaria, whether for prevention or for cure, is the varying response of individual parasites to the drugs used. Parasite populations may adapt to the introduced chemical environment and thereby enter the state of drug resistance, and become capable of passing on their genetic information to future generations of drug-resistant malaria parasites[2].

The emergence of resistance in *Plasmodium falciparum* to these inexpensive drugs, CQ and SP have been severely compromised the efforts to control malaria[3],

There are basically two approaches to the assessment of the antimalarial drug susceptibility of *Plasmodium falciparum*: *in vivo* and *in vitro* assays including several methods[4]. The introduction of polymerase chain reaction (PCR) into malaria research offered an interesting perspectives for the investigation of several genetic mutations that confer antimalarial drug resistance[5]. Molecular markers for antimalarial resistance identification, including *pfmdr1* and *pfcrt* polymorphisms that associated with chloroquine resistance. Polymorphisms in *pfmdr-1* may also be associated with resistance to mefloquine, quinine and artemisinin. The genetic information for the early detection of resistance foci and future monitoring of drug resistant malaria has been applied as epidemiological tool in conjunction with the conventional *in vitro* and *in vivo* drug sensitivity assessments [6,7].

In different regions of Africa, the prevalence of chloroquine resistant polymorphisms found to be 83.3% in Madagascar [8] , 90.7% in Mozampique [9], 100% in Mali [10], 82% in Burcina Faso[11], 100% in Uganda[12] and 82% in Kenya [13]. In Sudan, malaria is a leading cause of morbidity and mortality [14]. Chloroquine had been the most frequently used drug in Sudan as a first line for years in last decades [15]. Increased *in vivo* chloroquine resistance more than 25% had been documentd in different sentinal sites in Sudan[16]. *In vitro* study were performed in 42 *Plasmodium falciparum* isolates in Gezira-central Sudan yielded high degree of *in vitro* resistance to CQ (69%) [17]. In eastern Sudan, Polymorphisms were examined in two *Plasmodium falciparum* genes, indicated that there was a significant association between *pfmdr1*-Y86 and *pfcrt*-T76 genes and both high frequency of *in vitro* and *in vivo* resistance[18]. In upper Nile in south Sudan, association was reported between the *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene and the *P. falciparum* multiple drug resistance 1 (*pfmdr1*) with chloroquine and amodiaquine *in vivo* rsistance [19]. In Akuem South Sudan, the chloroquine resistance were assessed in a Sudanese parasite population, mutations in *pfcrt* and *pfmdrI* were genotyped. The result revealed that 63% of the parasites carried mutation at *pfcrt* for CQ resistance, while 31% carried the mutation at *pfmdr1* [20]. Surveillance by molecular techniques become a useful epidemiological tool and extremely essential for the detection of antimalarial drugs resistance. In addition, genetic data might provide valuable information for the identification of new drug or vaccine targets [21]. In Gezira – central Sudan, this study was perrfomed to estimate the prevalence of *Pfcrt* and *Pfmdr1* mutations that linked to chloroquine resistance.

Material and Methods

Study sites and Patients recruitment

This study was conducted in Wad Medani area-Central Sudan. During the peak transmission of malaria, from September 2003 to March 2004. The patients who had mono infection of *P. falciparum* malaria, of all ages and sexes were selected from individuals presenting to Abosnoon, Eleribab and Banat health centers .

Samples collection and DNA extraction:

Blood infected with *P. falciparum* was collected by finger prick onto Whatman No. 3 filter paper from each enrolled case. DNA was extracted from each sample for molecular analysis of drug resistance polymorphisms in *P. falciparum,* as described by Plowe C.V.*et al,* [22].

***pfmdr1* and *pfcrt* genotypes:**

Point mutations in parasite *pfmdr1* at codion 86 and *pfcrt*at codon 76 genes which are associated with chloroquine resistance were assayed. Oligonucleotide primers are designed to flank amino acid codons 86 in the *Pfmdr*1 domain in nested PCR strategy. Point mutations were detected by RFLP analysis of resultant amplicons using methods adapted after Duraisingh *et al,*[23] and Frean J, [24]. Alleles at amino acid codon 76 of *Pfcrt* were detected by nested PCR and *Apo I* RFLP using the methods described by Fidock D. A. *et al,*[25].

Data analysis:

Data was analyzed by statistical program (SPSS) for windows. Allele proportions were calculated for codons of interest by dividing the number of samples with a particular allele to the number of samples with an identifiable allele at that position

Ethical approval:

The participants were only enrolled in the study after appropriate informed consent was obtained. Ethical approvel for this study was obtained from the ethical Committee of the Blue Nile National Institute for Communicable Diseases / University of Gezira (Wad Medani,– Sudan) and from the State Health Authority in Gezira.

Results:

51 patients were enrolled in this study, 32 males and 19 females, aged between 3 to 66 years and their determined parasitaemia were ranging from 1280 to 96550 parasites/µl of blood with a mean of 3920 parasites/µl.

DNA were extracted from 48 samples , 2 were lost during the DNA extraction, 40 of 46 were investigated for the prevalence of the targeted mutations (*pfrct* and *pfmdr1*) that confer CQ resistance. Table 1 and 2 showing the results of polymorphism mutations of CQ by PCR with RFLP, indicated that 30/40(75%) were *pfcrt* mutant and 10/40(25%) were wild type, while the results of *pfmdr1* demonstrated that 22/37(59.5%) were mutant and 15/37 (40.5%) were wild type. Both mutations were abundant in 19/40(47.5%) of the investigated isolates, while each mutaion alone was abundant in 11/40 (27.5%) for *pfcrt* and in 3/40 (7.5) for *pfmdr1* (isolate number 23, 38 and 41), revealed that the frequency of CQ

resistant markers was 33/40 (82.5%).The abundant of mutations among age < 13 years was 70.6% and mutations among age > 13 years were 72.4%. Also, a polymorphic *P. falciparum* strain were obsereved in 18 of the investigated isolates(figure1)

Table (1) The result of *P. falciparum* isoltes investigated for polymorphisms in *pfcrt and pfmdr1* genes (n = 46)

	PFRCTI	*PFMDR*
Sample No.	RFLP result	RFLP result
1	M	M
3	W	W
4	W	W
5	M	W
6	W	
7	W	W
8	M	M
9	W	
10	M	M
11	M	W
13	W	W
14	M	M
16	W	
17	W	W
18	W	
19	M	M
20	W	W
21	M	M

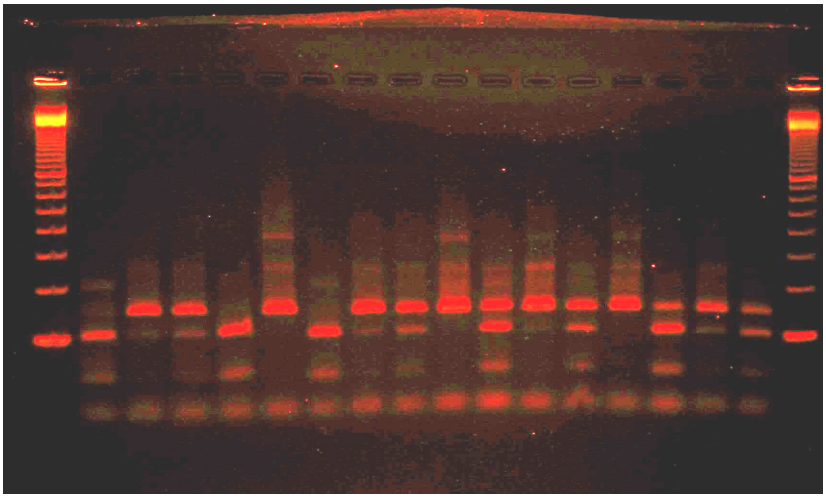
22	M	M
23	W	M
24	M	W
25	M	M
27	M	M
28	M	M
29	M	M
30	M	W
31	M	M
32	M	M
33	M	M
34	M	
35	M	W
36	W	W
37	M	M
38	W	M
39	M	M
40	M	M
41	W	M
42	M	
43	M	W
44	W	
45	M	W
47	M	M
48	M	M

49	M	
50	W	
51	M	W

NB: M = mutant, W = wild type

Table 2:- Mollecular assessment outcome:

Characteristics	CQ (n=40)
-*pfcrt* at codon 76 alone	11 of 40
- *pmdr1*at codon 86 alone	3 of 40
--Both *pfcrt* & *pmdr1*	19 of 40
-Molecular Markers frequency:-	33 of 40 (82.5%)



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure (1) PCR gel image showing the PCR-RFLP genotyping of Pfcrt mutation at codon 76. Lane 1 and 18 DNA size marker. Isolates in lanes 2, 5, and 7 were wild type (Sensitive). Isolates in lanes 3, 6, 10, 12 and 14 were mutant type (Resistant). Isolates in lane 4, 8, 9, 11, 13, 15, 16 and 17 were polymorphic *P.falciparum* population (Resistant and sensitive).

Discussion:

This study revealed that the polymorphism mutations of *pfcrt* at codon 76 and *pfmdr*1at codon 86 were 82.5% among the investigated isolates. This high finding of molecular markers when compared to other studies in Afrrica, it is similar to that reported from Comoros Union in Madagascar[8], Burcina Faso[11] and Kenya [13], while the frquency o CQ resistant markers found to be less than that reported from Mozambique[9,] Mali [10] and that reported from Uganda[11], conveying that the CQ resistant markers are well established and highly prevalent in south Sub- Sahara Africa. In Sudan,the genotyping level obtained by this study is similar to that reported from Gedarif in east Sudan[18], while it is less than that reported from east upper Nile[19]and more than that reported from Bahr Elgazal in South Sudan[20] , and this high level of mutations resistant to chloroquine were in consistancy with the high frequency of resistance documented by *in vivo* and *in vitro* studies in the same study area[16,17]. As conveyed by this study, there was no difference between the prevalence of chloroquine resistant mutations among the ages < 13 or > 13 years of the population, and this high mutations (82.5%) was much higher than that reported by *in vivo* study (25%)[16], indicating that the acquired immunity in the population.is a part of malaria curing and control as documented before by other studies [26,27]. In figure 1, some individuals among the population had a mixed infection of strains explaining that they were lacking for personal protection as a control measure to stop transmission of genetic alleles and this polymorphic of the plasmodium could be a factor of spread of drug resistance. The molecular data obtained by this study showed that the allele of *pfcrt* at codon 76 is strongly associated with CQ resistance rather than *pfmdr1* gene, as documented by previous studies[18,28] .

In 2004, the National Malaria Control Program in Sudanb decided to replace CQ with artesunate + sulfadox-ine/pyrimethamine as the first-line drug and artemether-lumefantrine has replaced SP as the second-line treatment[29] . So, molecular markers for antimalarial resistance identification, including *pfcrt* polymorphisms that associated with chloroquine resistance and Polymorphisms in *pfmdr-1* associated with resistance to chloroquine, quinine, mefloquine and artemisinin can be applied for predicting resistance in thsese antimalarial drugs in endemic areas including the ACT and quinine[7]. However, the molecular surveillance is a useful epidemiological tool, and in parallel with the *in vitro*

and *in vivo* drug sensitivity assessments enable the stakeholders to establish an investigation tools for monitoring the efficacy of antimalaria drugs.

ist of abbreviations:

PFCRT *Plasmodium falciparum* chloroquine resistant transporter

Pfmdr1 *Plasmodium falciparum* multi-drugs resistance1

DNA deoxyreboneuclic acid

PCR polymerase chain reaction

RFLP restriction fragment length polymorphism

CQ chloroquine

SP sulphadoxine/pyrimethamine

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Conflict of interest: Authors have declared that no competing interests exist.

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