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Co-existence of Dihydrofolate Reductase (*dhfr108*) Gene with *Plasmodium falciparum* Chloroquine Resistance Transporter Gene (Pfcrt T76) in *P. falciparum* Isolates from Gezira State, Central Sudan

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Abstract: Malaria parasite multi-drug resistance poses serious health problems in tropical countries. The aim of this study was to assess the Sulfadoxine-pyrimethamine (S/P) resistance of *Plasmodium falciparum* parasite in central Sudan, using the molecular markers.

The genotying of *P. falciparum* parasite from forty-four patients using RFLP and PCR showed that the polymorphism of *dhfr* gene was in codons 51, 59 and 108. In codon 51; two strains (4.5%) were mutant type; 3 (6.8%) were found as mixed infection (both mutant and wild types) and 28 (63.6%) were found as wild type. One sample (2.2 %) was *dhfr* 59 mutant and 31 (70.4. %) were wild type, while 14 (31.8%) were *dhfr* 108 mutant; three (6.8%) were found as mixed infection and 24 (54.5%) were wild types. The Screening of *dhps* 540 polymorphisms of the gene revealed that 2 (4.5%) were found as mixed infection, and 42 (95.5%) as wild type. Fifteen samples were analyzed for *Pfcrt* T76, and *Pfmdr-1* Y 86 for CQ resistant polymorphisms from the current study, the result showed that 33.3% were found to be mutant at *dhfr* 108 and *Pfcrt*T*76* genes reflecting the link between *dhfr*108 and *Pfcrt*76 genes.

In conclusion, the polymorphism in the *dhfr* and *dhps* genes in central Sudan are increasing, but less abundant compared to the neighboring countries. However, the current studies indicate the link between *dhfr*108 and *Pfcrt*76 genes. Therefore, further study is need for using the S/P in areas that confirmed with chloroquine resistant strains.

Keywords: Malaria, drug resistant, dhfr, dhps, Pfcrt, Pfmdr-1, Sudan.

INTRODUCTION

Malaria is one of the most common infectious diseases in the world. There were an estimated 247 million malaria cases among 3.3 billion people at risk in 2006, causing nearly a million deaths, mostly of children under 5 years. 109 countries were endemic for malaria in 2008, 45 within the WHO African region [1,2].

Plasmodium falciparum a multi-drug resistant parasite is an increasing threat to the malaria control and accelerated the morbidity and mortality rate in malaria endemic regions. Resistance to pyrimethamine has been associated with point mutations in the dihydrofolate reductase gene (*dhfr- gene*) and resistance to sulphadoxine with mutations in the dihydropeteroate synthase gene (*dhps*-gene) [3,4]. Asn 108 mutant is considered essential for pyrimethamine resistance and the degree of resistance increases with additional mutations at Ileu 51, Arg 59, or triple mutations. To date, more than 25 different combinations of *dhfr* and *dhps* alleles have been observed in field isolates [4]. Study in Malawi showed that patients infected with parasites carrying the *dhps* Gly- 437, Glu- 540 double mutant and the *dhfr* triple Asn 108/I leu 51-Arg 59 mutant had a specifically high relative risk of treatment failure than did those infected with parasites carrying the *dhfr* triple mutant alone. Such a quintuple mutant (3*dhfr* and 2*dhps* mutation) was suggested as relevant molecular marker for failure of Sulfadoxine-pyrimethamine (S/P) treatment of uncomplicated *P. falciparum* [4]. The presence of a single *dhfr* mutation (Arg59) with a single *dhps* mutation (Glu540) might be a useful predictor of high-degree of S/P resistance [5]. The aim of this study was to identify the frequency of dhfr 51, 59,108 and dhps 540 polymorphism in *P. falciparum* and to identify if there is any association between the different polymorphisms of *P. falciparum* isolates (*Pfcrt* T76, *Pfmdr-1* Y 86 and *dhf*, *dhps*) in the Gezira state-central Sudan.

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MATERIALS AND METHODS

Study Site and Population

The study was cross sectional study conducted at Marengan clinical center, Gezira state, central Sudan during

Sept. 2002 - March 2003. One hundred and seventy six consecutive patients with symptoms or signs suggesting malaria infection were screened for the presence of malaria parasites in their peripheral blood using microscopy.

Forty-four *P. falciparum* patients (54.5% females and 45.5% male) above two years of age were further studied to identify DHPS 540 and DHFR 108, 59, 51 polymorphisms.

Ethical Approval

The Study protocol was reviewed and passed by the ethical and scientific committees of the Institute of Nuclear Medicine (INMO), University of Gezira and the Directorate of Research, MOH, Gezira, Sudan. Oral consent was obtained from each participant enrolled in the study.

Methodology

Blood sample from finger bricks were used to make thick and thin blood films. Blood films were stained with Giemsa and examined under the microscope.

DNA Extraction and Polymerase Chain Reaction (PCR)

DNA was extracted from filter paper blood sample using methanol extraction method [6].

Outer and Nested PCR

Outer PCR reactions were performed with 5 µl of DNA prepared in 30 µl volumes which contains 1X PCR Buffer II (Gene Amp® 10X Buffer II [100 mM Tris-HCl pH 8.3, 500 mM KCl], (Cinna Gen Inc.,Iran) 1.5 mM MgCL2 (MgCl2, Applied Bio system), 0.2 mM each of the 4 dNTP (Gene Amp® dNTPs, Applied Bio system), 1.0 µ mol of each sense and antisense 1 U of AmpliTaq Gold (Applied Bio system). The cycling parameters used were: [94°C for 10 min ; 94 °C for 1 min; 50 °C for 2 min and 72 °C for 2min. for 40 cycles and 72 °C for 10 min.].

The product of the outer reaction 2 µl was used as a template for the second inner reaction. Forty µl of each of the primers specific for *dhfr* & *dhps* genes were used. The cycles parameters were: [94 °C for 10 min; 94°C for1 min; 45 °C for 1 min and 72 °C for 2min. for 35 cycles and 72 °C for 10 min.].

The amplified DNA products were analyzed electrophoretically by size fraction on agrose gels (1.5%) stained with ethidium bromide, and the gels were visualized under ultraviolet trans-illumination, and documented.

Genotype of *dhfr* 51 and *dhfr* 59 by Restriction Fragment Length Polymorphism

The nested PCR 113 bp products (with outer 147bp and inner 113 bp) were split in two tubes with 12 µl PCR mix each and digested by using 0.2 µl EcoR1 enzyme for dhfr mutation at position 51 and Bsrg 1 enzyme for dhfr mutation at position 59**.** The conditions were 2 µl NEB U buffer10x in 6 µl H2O and 0, 2 µl of the appropriate enzyme (10 U/ µl). The mixtures were incubated over night at 37° C. Digested PCR products were loaded on 10 % non-denaturaning polyacrylamide gel (30% Protogel Acrylamide/Bis-acrylamide,37.5:1) provided by (FMC BioProducts) and electrophoresed at 90 V for two hours. The digested fragments were stained in 1µg/ml ethidium bromide solution for 10-15 minutes and visualized with UV light.

Genotype of *dhps* 108 and *dhps* 540 [7]

25 µl nested PCR 254 bp product (with outer 414 bp and inner 254 bp) of each sample was digested in PCR tube, by adding 3 µl NEB 2 buffer 10x, 0,2 µl Alu1 enzyme for dhfr mutation at positions 108 and Fok 1 enzyme for dhps mutation at position 540. (10 U/ µl) the mixtures were incubated over night at 37 °C. Digested PCR products were loaded on 10 % non-denaturaning polyacrylamide gel (30% Protogel [Acrylamide/Bis-acrylamide, 37.5:1]) provided by (FMC BioProducts) and electrophoresed at 90 V for two hours. Then the digested fragments were stained in 1µg/ml ethidium bromide solution for 10-15 minutes and visualized with UV light [7]. The association between the two genes (*dhfr* and *dhps*) polymorphisms was identified using the ANOVA test.

Genotype of *Pfcrt* and *Pfmdr*-1

The mutations in the two genes Pfcrt and Pf mdr-1 have been abolished out the restriction site for Apo 1 enzyme. Digestion with this enzyme was used for typing these polymorphisms. In a total volume of 15 µl, 2 µl PCR product were digested overnight at 50°C with 1 U Apo I, 1.5 µl 10x NE Buffer 3 (100 mM Nacl, 50 mM Tris-Hcl, 10 mM magnesium chloride and 1mM dithiothreitol (pH 7.9 @ 25 °C), 0.15 µl of 100X BSA (200µg/ml) and deionized water. Using the Genomic DNA from strain 3D7 and Dd_2 were Amplified and digested in the same way serving as control for complete digestion, and undigested fragment respectively. The mutations in the two genes Pfcrt and Pf mdr-1 have abolished the restriction site for Apo 1 enzyme. Digestion with this enzyme was used for typing of this polymorphism. In a total volume of 15 µl, 2 µl PCR product were digested overnight at 50°C with 1 U Apo I, 1.5 µl 10x NE Buffer 3 (100 mM Nacl, 50 mM Tris-Hcl,10 mM magnesium chloride and 1mM dithiothreitol (pH 7.9 @ 25 °C), 0.15 µl of 100X BSA (200µg/ml) and deionized water.

RESULTS

The study shows that the majority (94%) of infections were *P. falciparum* while the remainders were mixed infection *P. falciparum* and *P. vivax* or *P. vivax* only.

Table **1** shows the occurrences of mutant genes in the study sample. Only 2/44 were *dhfr* 51 mutant and 3/44 were found as mixed infection; mutant and wild type in the same

Table 1. Occurrence of Resistant Gene Types in the Study Samples

	Mutant	mixed	wild	N.D.*
DHFR108	14 (31.8%)	3(6.8%)	24(54.5%)	3 (6.8%)
DHFR59	1(2.25%)	0	31(70.4%)	12 (27.2%)
DHFR51	2 (4.5%)	3 (6.8%)	28(63.6%)	11 (25%)
DHPS540	0	2 (4.5%)	42(95.4%)	-

n: 44
*N.D.: Not done

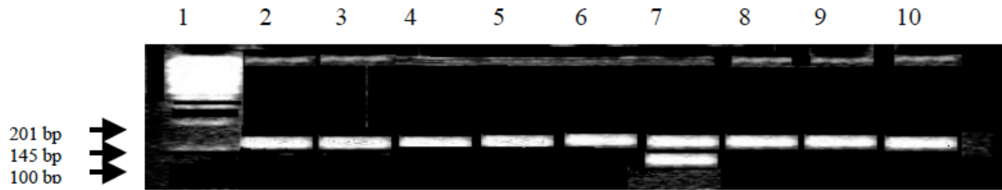

Fig. (1). PCR-RFLP screening of *P. falciparum* isolate for codon dhfr 51 polymorphism.

Lane **1** DNA marker, Lanes **2**-**6** and **8**-**10** are (uncutted full length 113 bp). PCR product (sensitive). Lane **7**: 51 polymorphism (cutted by EcoR1 restriction enzyme to 78 and 35 bp).

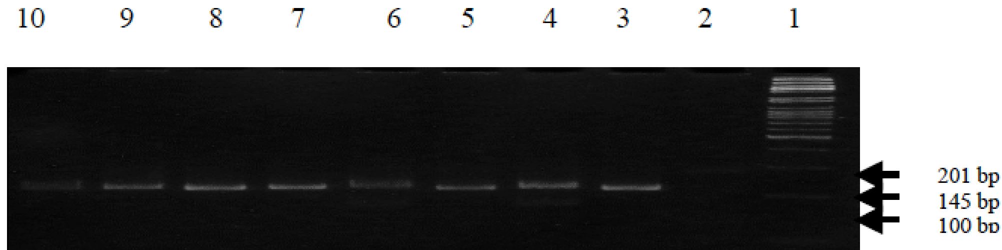

Fig. (2). PCR-RFLP screening of *P. falciparum* isolate for *dhps* codon 540 polymorphism.

Lane **1**: DNA marker, Lane **2**: digested PCR product (positive control). Lane **4** and **6**; 540 polymorphism (mixed type). Lane **3**, **5** and **7**- **10**; 540 polymorphism (wild type).

sample, while (63.6%) n=26 were undigested and considered as wild type Fig. (**1**). For *dhfr* 59, only one sample (2.2 %) was mutant and 31 (70.4. %) were wild type. Fourteen sample (31.8%) were *dhfr* 108 mutant and 24 (54.5%) were wild type, 3 (6.8%) were found as mix infection represented by mutant and wild type in the same sample. The prevalence of gene mutation at position 540 indicate that 4.5% were found as mix infection represented by mutant and wild in the same sample and 95.5% was found as wild type Fig. (**2**). It has been found that there was significant difference between *dhfr*108 and *dhps* 540 (P = 0.024).

Fifteen samples were tested for detection of chloroquine genes *Pfcrt* and *Pfmdr-1* polymorphisms Fig. (**3**). The result shows that 5/15 (33.3%) were found with both polymorphisms for *dhfr*108 and *Pfcrt*T76, this result may suggest that there was association between the mutations in the *dhfr*108 and *Pfcrt*T 76 genes.

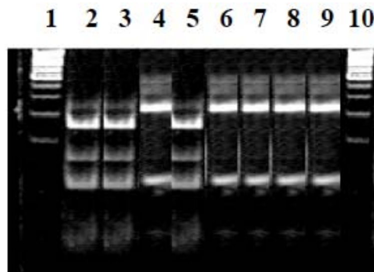

Fig. (3). PCR-RFLP screening of *P. falciparum* isolate for Pfcrt codon 76 polymorphism.

Lane **1** and **10**: DNA markers, Lane **2**: digested PCR product (positive control). Lane **3** and **5**: K76 polymorphism (cutted by Apo1 restriction enzyme to145 and 64 bp). Lane **4**, **6**, **7**, **8** and **9**: T76 polymorphism (uncutted full length 209 pb).

Only two samples 13.3% harboured both polymorphisms for the *dhfr*108 and *Pfmdr1* at the same time. While there was no relationship between the *dhfr* 51, 59 and *dhps* 540 with *Pfcrt*T76 and *Pfmdr1*.

DISCUSSION

The prevalence of *dhfr* and *dhps* polymorphisms in different *P. falciparum* isolates from central Sudan was determined by using PCR-RFLP in this study. Previous studies carried out in this country showed emergence of chloroquine and S/P resistance [8-10]. Chloroquine resistance (CQ) in a *P. falciparum* genetic and *fcrt*T76, having point mutations is associated completely with CQ in parasite lines from Asia, Africa, and South America [11]. *P. falciparum* resistance to S/P is conferred by point mutations in parasite *dhfr* and *dhps* genes, which encoding the enzymes targeted by these drugs, *in vitro* anti-folate resistance is associated with point mutations in the *dhfr* domain of the *dhfr*-thymidylate synthetase gene. Among the point mutations in the *dhfr* gene, a Ser to Asn-108 polymorphism is considered the key mutation that confers resistance to anti-folate drugs. The PCR-RFLP genotypes result from the present study revealed that target mutations conferring S/P are 9.6% (mutant type). In addition more than 71% of the cases were characterized as (wild type). While 4.5% of the cases were found as mixed infection represented by mutant and wild in the same sample. When this result compared with other areas in Sudan it is less than that reported in Khartoum where the Pyrimethamine-Sulfadoxine efficacy was assessed before treatment and the point mutations were detected only at codons 51 and 108 of *dhfr* and codon 436 of *dhps* and the frequency of *dhfr* 51/108 and *dhps* 436 mutations was 79% and 8%, respectively [12]. This is probably due to the fact that there are more malaria programmes in Gezira state which gives more information about drug uses strategies and in areas where drug usage decreased, the spread of resistance has also decreased. Genetic studies show that resistance to S/P may have arisen in southern and eastern Africa on only a few occasions, so

these resistant strains have spread extensively and rapidly [13]. S/P seems to have an unpromising future as a first line anti-malarial in Africa. Despite those results from Africa and the gametocytaemia follows treatment, S/P is still clinically effective as proved by this study and can have an impact in malaria control programs, especially in combination with other drugs like artensunate to prevent the increasing mutations and to sustain its low in vivo resistance. Interestingly we found 33.3% of the study samples were found to be mutant for *dhfr*108 and *Pfcrt*T76 and only 13.3% harboured mutant type for the *dhfr*108 and *Pfmdr* [10].

We conclude that S/P seems to be a suitable drug for the study area. However, the study identified the percentage of parasites carrying drug resistance polymorphism conferring resistance against S/P and chloroquine, therefore, it might be more suitable to switch to combination therapy with artemisinin derivatives to prevent the spread of multidrug resistance.

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