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Detection of *Plasmodium falciparum* Chloroquine Resistance Transporter Mutant Allele K76T in Three Regional Areas in Sudan

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Abstract

Background

Chloroquine resistance in Sudan lead the ministry of health to change the first line of malaria treatment from chloroquine to artemisinin, this study was designed to detect *Plasmdium falciparum* Chloroquine Transporter (*Pfcrt*) mutant allele K76T in three regional areas in Sudan.

Materials and Methods

Three hundred (300) *P.falciparum* positive samples were collected from three regional areas in Sudan,positivesamples were confirmed by usingdirect microscopical stained blood films, DNA was extracted usingChelix method and then were amplified using Nested RFLP-PCR method to detect the mutant allele of *Pfcrt* K76T. Data were analyzed using SPSS 16.5 by Chi-squire test.

Results

Mutant allele of (*Pfcrt*K76T) was detected in the study areasincluding Al-Dinder (12.3%) , Khartoum (10.3%) and Al-Damer (9.3%) respectively, *Pfcrt*K76T among the female (22%) was detected higher than male (10%) which found to be statistically significant at *P*.value=0.002, finally the *Pfcrt*K76T in association with age groups, showed (7.7%, 6.0%, 10,7% and 7,7%) in age groups (<10, 10-19, 20-50 and >50) respectively which found to be statistically insignificant at *P*.value=0.202.

Key words:*Pfcrt*K76T, Antimalarial resistance, Al- Dinder, Al-Damer.

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Introduction

Malaria has been one of the greatest afflictions, in the same ranks as human immunodeficiency virus (HIV), influenza, and tuberculosis [1]. The agent of malaria is an obligate intracellular sporozoan in the genus *Plasmodium*, which contains four species; *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium vivax*, and*Plasmodium ovale*[2].Malaria is transmitted by the blood feeding of infectious female*Anopheles*mosquitoes [3].Human malarial parasites develop through liver stage and a blood stage [2].Many African children suffer repeated clinical episodes of malaria due to *P. falciparum* and in the order of 1% of these episodes are fatal, and this may be due to environmental and genetic factors [4].The epidemiology of malaria was once distributed throughout most of the world, the control of mosquitoes in temperate areas has successfully restricted it mostly to a belt extending around the equator. Despite this achievement, approximately 300 million to 500 million new cases are still reported each year, about 90% of them in Africa. The most frequent victims are children and young adults as well as pregnant women, of whom at least 2 million die annually [5].

Anti-malarial drug resistance is a major challenge to the control of *falciparum* malaria, the leading cause of morbidity and mortality especially in Africa and Southern Asia [6]. The first *P. falciparum* chloroquine resistance (CQ) was reported in the late 1950 in Southeast Asia along the Thai-Cambodian border [6,7]. Further spread of CQ resistance was shown later to include neighboring countries in Asia, Africa and South America. Moreover, *P. falciparum* has been also reported resistant to other anti-malarial drugs including sulphadoxine/pyrimethamine drug combination, mefloquine, atovaquone and artemisinin [6,8].

Plasmodium falciparum chloroquine resistance transporter (*pfcrt*)is a gene found on chromosome 7, which is the main determinant of chloroquine resistance [9,10,11]. A single point mutation on the *pfcrt* gene resulting in replacement of an amino acid lysine by threonine in the *pfcrt*gene at codon 76 has been proven to be critical for chloroquine resistance by transfection experiment[12,13]. The K76T mutation has been linked to chloroquine resistance in parasite isolates collected worldwide[14,9].In addition, more than 15 mutations in the *pfcrt* gene resulting in amino acid changes have been reported[7]. Until now, a number of *pfcrt* haplotypes have been identified based on amino acids 72–76[13,15]. These haplotypes have been linked to the geographic origin of the isolates and their chloroquine susceptibility status[15]. There are two common haplotypes found, CVIET and SVMNT, were identified in chloroquine-resistant

isolates from Asia/Africa and South America, respectively, and a CVMNK haplotype is universally identified in chloroquine-sensitive parasites K76 [12,16]. Recent studies indicate the important role of amino acid polymorphisms in the *pfcrt* on the pattern and level of antimalarial drug resistance [13,17].Moreover, additional amino acid substitutes in the *pfcrt* altered the level of chloroquine resistance [12,13,18].

Materials and Methods:

Study design

It's descriptive cross-sectional study.

Study area

The study was carried out in three areas in Sudan, Aldinder(holo malaria endemic area), Khartoum(meso endemic area) and Adamer (hypo endemic area).

Study population

This study was carried out in patients suffering from malaria. Patients were recruited between September 2015to March 2016 at hospitals and medical centers in different places in Sudan, which they characterized by moderate perennial malaria transmission with a peak in December and January. Febrile patients (auxiliary temperature of ≥37.5°C) from all age groups were microscopically confirmed uncomplicated *P. falciparum* mono infection and a parasite count of a minimum of 1,000 a sexual parasites/µl.

Data collection

A structured questionnaire for socio demographic information and medical history was completed for each patient by a physician. The study was performed as per the WHO guidelines for antimalarial drug efficacy surveillance methods [19].

Sample size

The sample size was determined using the following equation:

$$N = \frac{t^2 \times P(P-1)}{M^2}$$

N = Sample size

t = 1.96

P = Prevalence of disease

M = 0.05

Based on the formula individuals was enrolled in the study (N = 272), the sample were completed to 300 sample and then the whole number of individuals were divided into three division, 100 sample from each area.

Methods

Collection of blood sample

Finger prick samples were taken from all participants, thick smears were prepared and stained with 10X Giemsa stain and slides were read under a 100X oil immersion field. Parasite density were obtained by counting a sexual parasites against 200 leucocytes and parasite density were calculated assuming an average of 8,000 leucocytes/µl. Slides were read by two experienced microscopists, a slide was considered negative if no parasites were detected in 100 high power fields. Blood spots were collected on Whatman® filter paper, were air dried and were stored in self-sealing bags for DNA analysis.

DNA extraction

Harris Uni-CoreTM puncher (Qiagen, Hilden, Germany) was used to punch out six of filter paper with dried blood sample 3 mm in diameter. The puncher was cleaned and blank filter paper pieces were punched out in the last step of the washing process was subjected to DNA extraction, and then was followed by PCR between random samples to ensure no transfer of parasite DNA between samples using this cleaning method. DNA was extracted from the dried blood sample using a method with Chelex-100® Molecular Biology Grade Resin (Bio-Rad Laboratories, Hercules, CA, USA) and was soaked in 0.5% saponin in phosphate buffered saline (PBS) solution overnight.

Molecular methods

Polymerase Chain Reaction/Restriction fragment Length Polymorphism (PCR/RFLP) was used to determine the resistant genes and study the genetic diversity/genetic variation of antimalarial resistant *P. falciparum.* DNA was extracted from patient blood spotted on the filter paper as mentioned above. The protocol for the extraction was carried out according to manufacturer's instruction.

Nested PCR and RFLP for *Pfcrt* mutation-specific detection

For amplification of the 1.6kb fragment of *Pfcrt*, a primary PCR was set up using the primers PfcrtF1 5'-CCGTTAATAATAAATACAGGC-3' and PfcrtR1 5'-CTTTTAAAATGGAAGGGT

GT-3'. Product from primary PCR (2µl of 10x dilution) was used in a follow-up, nested, allele-specific PCR amplifications to detect the codon for *pfcrt* 76K or 76T. These diagnostic PCR amplifications were used a common inner primer pair PfcrtF2 5'-GGCTCACGTTTAGGTGGA-3' and PfcrtR2 5'-TGAATTTCCCTTTTTATTTCCAAA-3' (detects the 76T codon) or PfcrtR3 5'-GTTCTTTTAGC AAAAATCT-3' (detects the 76K codon). The PCR stages for these diagnostic amplifications were at 94°C for 5 minutes, then were followed by 40 cycles at 94°C for 30 seconds, 52°C for 40 seconds, and 72°C for 45 seconds and a final extension of 72°C for 5 minutes. Purified genomic DNA from *P. falciparum* clones (chloroquine sensitive) and (chloroquine resistant) was used as positive controls, and water, extracted uninfected blood smears, and uninfected blood spots on filter paper was used as negative controls. The PCR products from the amplification reactions was evaluated by electrophoresis on 2% agarose gels containing ethidium bromide[20]. 10µl of the nested PCR product reaction mixture was treated directly with 3U of the restriction enzyme ApoI-HF for 1 to 2 hours at 50°C as recommended by the manufacturer (New England Biolabs). The enzyme ApoI-HF recognized and cut the 76K codon, releasing fragment from product[20].

Statistical analysis

All information and data was analyzed by using Statistical Package of Social Science (SPSS) (version 16; Corp., College station, Tax), using Chi-square test, then data were presented in tables using excel.

Ethical consideration

The approval was taken from Research Committee of College of Medical Laboratory Science, Sudan University of Science and Technology. Written informed consent was obtained from all study participants or from their guardians after explaining the study purpose.

Results

From the 300 positive malaria samples, all of them are successfully typed by Nested PCR then followed by RFLP for detection of mutation in lociK76T, the frequency of mutant allele *Pfcrt*K76T was 32%.

The results showed that the highly mutant allele *Pfcrt*K76T (22%) was reported among female, while male reported (10%) mutant allele (table 1). The difference in mutant allele *Pfcrt* K76T among sex was found to be detected statistically significant at *P*.value=0.002. .

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The highest mutant allele *Pfcrt* K76T (40%) was detected among age group between 20 -50 years old and the lowest mutant allele (15%) was detected among the 10 -20 years old (table 2). The differences in mutant allele *Pfcrt*K76T between all age groups were found to be statistically insignificant at *P*.value=0.202.

The investigation revealed that the highest mutant allele *Pfcrt*K76T (37%) was detected in Al-Dinder while the lowest mutant allele (28%) was detected in Al-Damer (table 3). The differences in mutant allele *Pfcrt* K76T between all areas were found to be statistically insignificant at *P*.value=0.381.

Table 1:

Detection of mutant allele *Pfcrt*K76T in the study areas according to gender.

Gender	No. examined	Mutant allele (%)	Wild type (%)
Male	133	30 (10%)	101 (33.7)
Female	167	66 (22%)	103 (34.4%)

P.value=0.002.

Table 2:

Detection of mutant allele *Pfcrt*K76T in the study areas according to age groups.

Age groups (year)	No. examined	Mutant allele (%)	Wild type (%)
Less than 10	58	23 (7.7%)	35 (11.7%)
10 – 19	45	18 (6.0%)	27 (9.0%)
20 – 50	120	32 (10.7%)	88 (29.3%)
More than 50	77	23 (7.7%)	54 (18.0%)

Table 3:

The mutant allele *Pfcrt*K76T in each area.

Area	No. examined	Mutant allele (%)	Wilde type (%)
Al- Damer	100	28 (9.3%)	72 (24%)
Khartoum	100	33 (10.3%)	69 (23%)
Al- Dinder	100	37 (12.3%)	63 (21%)

P.value= 0.381.

Discussion

Our study aimed to detect the *Pfcrt* mutant allele in three areas in Sudan which comprise the hyper, holo, meso and hypoendemic malarial transmission area in Sudan, we focusing in differences in detection of *Pfcrt* mutant allele in the study areas and we used different age groups in our study.

From the results, it was obvious that the overall of *Pfcrt*K76T mutant allele in the study areas was relatively high (32%). This rate was found to be closer to the rate (34.1 %), reported by Schönfeld*et al.* (2007)[21] in Tanzania,however it was lower than the rate (93.3 %)reported by Figueiredo*et al.* (2010) [22] in Angola.

The investigation revealed that, the highest *Pfcrt* mutant allele (10.7%) was found in age group from 20-30 years old, thisfinding was disagreedwith the study done in Yemen by Al- Mekhlafi *et al.*(2011) [23]who reported(71%) in the same age group.Also our finding disagreed with study done in Nigeria by Muhammed *et al.* (2017)[24]who reported (14.9%).

Our results also showed that, Al-Dinder area was the highest *Pfcrt* mutant allele K76T (12.3%) followed by Khartoum (10.3%) and Al-Damer (9.3%), in our opinion this not a major differences because the malaria endemic in all over Sudan, so there are no obvious differences.

Conclusion

The *Pfcrt* mutant allele K76T ispersist in Sudan after eleven years from the Ministry of Health in Sudan changed the first line of malaria treatment from chloroquine to artemisinin. However the relation between the chloroquine resistant marker *Pfcrt*and other malaria drug resistance markers had strong prevalence simultaneously.

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