

Detection of Single Point Mutation of Plasmodium Falciparum Multi-Drug Resistance 1 Gene in Three Different Areas in Sudan

*Ahmed Bakheet Abd Alla¹, Tayseer Elamin Mohamed Elfaki¹,
Mohamed Baha Eldin Ahmed Saad², Ali Elamin Nasir³ and
Ezzalarab Kamil Ahmed⁴

¹Department of Parasitology and Medical Entomology-College of Medical Laboratory Science- Sudan University of Science and Technology.

¹Head Department of Parasitology and Medical Entomology-College of Medical Laboratory Science- Sudan University of Science and Technology.

² College of Medical Laboratory Science- Omdurman Ahlia University.

³Department of Parasitology and Medical Entomology-College of Medical Laboratory Science- Sudan University of Science and Technology

⁴Department of Parasitology and Medical Entomology-College of Medical Laboratory Science- Sudan University of Science and Technology

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 single point mutation of Plasmodium falciparum multi-drug resistance-1 (Pfmdr-1) N86Y mutant allele in three different areas in Sudan.

Materials and Methods: Three hundred (300) P.falciparum positive samples were collected from three areas in Sudan, positive samples were confirmed by using direct microscopical stained blood films, DNA was extracted using Chelix method and then were amplified using Nested RFLP-PCR method to detect the Pfmdr-1 N86Y mutant allele. Data were analyzed using SPSS 16.5 by Chi-square test.

Results: Single point mutation of Pfmdr-1 N86Y mutant allele was detected in the study areas including Khartoum (15.7%) , River Nile (10.7%) and Sennar (9.7%) respectively, Pfmdr-1 N86Y among the males and females were equal (18 %) which found to be statistically insignificant at P. value=0.167, finally the Pfmdr-1 N86Y in association with age groups, showed (8.0%, 4.7%, 15.7% and 7.7%) among age groups (<10, 10-19, 20-50 and >50) respectively which found to be statistically insignificant at P.value=0.399.

Keywords: Pfmdr-1 N86Y, Anti-malarial resistance, Sennar, River Nile.

Date of Submission: 29 -07-2017

Date of acceptance: 30-09-2017

I. Introduction

Malaria has been one of the greatest afflictions, in the same ranks as human immunodeficiency virus (HIV), influenza, and tuberculosis¹. 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 ovale². Malaria is transmitted by the blood feeding of infectious female Anopheles mosquitoes, and understanding mosquito ecology and population dynamics can inform how best to defeat malaria³. 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 Asia⁴.

Chloroquine (CQ) and other quinoline-based drugs have been used for the prophylaxis and treatment of malaria for more than 50 years in all of the malarial countries because of its cost effectiveness, few side effects, and easy availability⁵. P. falciparum multi-drug resistance 1 (Pfmdr-1), a gene on chromosome 5 encoding a P-glycoprotein homolog 1 (Pgh1), also contributes to chloroquine resistance⁶. A few studies of resistant parasites from in vitro drug selection indicate that alteration of the Pfmdr-1 gene copy number contributes to changes in the level of chloroquine resistance^{7,8}. Pfmdr-1 an Asn→Tyr mutation at amino acid 86 (N86Y) and other mutations in this gene correlated with chloroquine resistance⁴. Also, Pfmdr-1 has been associated with altered in vitro and in vivo parasite response to arylaminoalcohols, including lumefantrine⁹, molfequine and artemisinin¹⁰. Multidrug resistant P. falciparum malaria is common in Southeast Asia, but difficult to identify and treat. Genes that encode parasite transport proteins maybe involved in export of drugs and so cause resistance. Increase in Pfmdr-1 copy number predicts failure even after chemotherapy with the highly effective combination of mefloquine and 3 days' artesunate. Monitoring of Pfmdr-1 copy number will be useful in epidemiological

surveys of drug resistance in *P. falciparum*, and potentially for predicting treatment failure in individual patients¹¹.

II. Materials And Methods

Study design

It's descriptive cross sectional study.

Study area

The study was carried in three areas in Sudan, Sennar state which are (holo malaria endemic area), Khartoum state (meso endemic area) and River Nile state (hypo endemic area).

Study population

This study was carried in patients with malaria in Sudan. Patients were recruited between September 2015 and 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 $\geq 37.5^{\circ}\text{C}$) from all age groups were microscopically confirmed uncomplicated *P. falciparum* mono infection and a parasite count of a minimum of 1,000 asexual parasites/ μl . 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 methods¹².

Sample size

The sample size was determined using the following equation:

$$N = (t^2 \times P(P-1))/M^2$$

N = Sample size

t = 1.96

P = Prevalence of disease (3.6 %)

M = 0.05

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

III. Methods

Collection of blood sample

Blood samples were collected for malaria screening from both finger prick and venipuncture. This is to check the presence of healthy asexual parasites in the peripheral smear of patients. Safety procedures were adopted in the collection of finger-prick blood samples by swabbing the area to be sampled with 70% alcohol and allowing it to dry before collection. About 2-5 ml of blood was then drawn (venepuncture) with a sterile disposable syringe in E.D.T.A container. The blood samples were transported to the laboratory at 40°C. Drops of peripheral blood were placed on Whatman (Qiagen, Hilden, Germany) 3MM filter paper and air-dried and kept in plastic bags until use. 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.

DNA extraction

Harris Uni-Core™ 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 anti-malarial 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.

PCR and RFLP for detection of Pfmdr-1 gene

Gene segments spanning codon 86 of the Pfmdr-1 gene was amplified in 20 μl of standard PCR mixture containing 3 μl of extracted DNA and 1 μl of the primers MDR1 5'-ATG GGT AAA GAG CAG AAA GA-3' and MDR2 5'-AAC GCA AGT AAT ACA TAA AGT CA-3'. The PCR amplification stages were at 94°C for 2 minutes, followed by 35 cycles at 94°C for 20 seconds, 52°C for 10 seconds, 48°C for 10 seconds, and 60°C for

1.5 minutes. A second, nested amplification from this segment was then performed under the same PCR conditions using 3µl of the product solution and primers MDR3 5'-TGG TAA CCT CAG TAT CAA AGAA-3' and MDR4 5'-ATA AAC CTA AAA AGG AAC TGG-3'. Presence of the mutant 86Y codon was detected by digestion of 10 µl of the second amplification product solution 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 products of restriction digestion was separated by electrophoresis on a 2% agarose gel and detected by staining with ethidium bromide.

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 was presented in tables and graphs using excel¹⁴.

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.

IV. Results

From the 300 positive malaria samples, all of them are successfully typed by Nested PCR (figure 1) then followed by RFLP for detection of mutation in loci N86Y (figure 2), the frequency of mutant allele Pfmdr-1 N86Y was found (36%). The investigation revealed that the highest mutant allele Pfmdr-1 N86Y (15.7%) was detected in Khartoum while the lowest mutant allele (9.7%) was detected in Sennar (table 1, figure 3). The differences in mutant allele Pfmdr-1 N86Y between all areas were found to be statistically insignificant at P.value=0.018. The results showed that the Pfmdr-1 N86Y mutant allele was reported among females, and males are equal (18%) with mixed wild and mutant allele (0.7 %) which only was reported among females (table 2, figure 4). The difference in mutant allele Pfmdr-1 N86Y among sex was found to be detected statistically insignificant at P.value=0.167. The highest Pfmdr-1 N86Y mutant allele (15.7%) was detected among age group between 20 -50 years old with mixed wild and mutant allele (0.7 %), and the lowest mutant allele (4.7%) was detected among the 10 -19 years old (table 3, figure 5). The differences in mutant allele Pfmdr-1 N86Y between all age groups were found to be statistically insignificant at P.value=0.399.

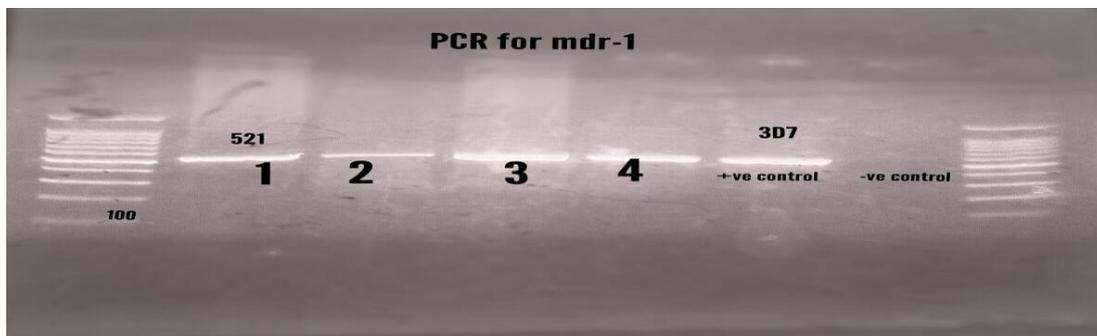


FIGURE (1): PCR for Pfmdr-1

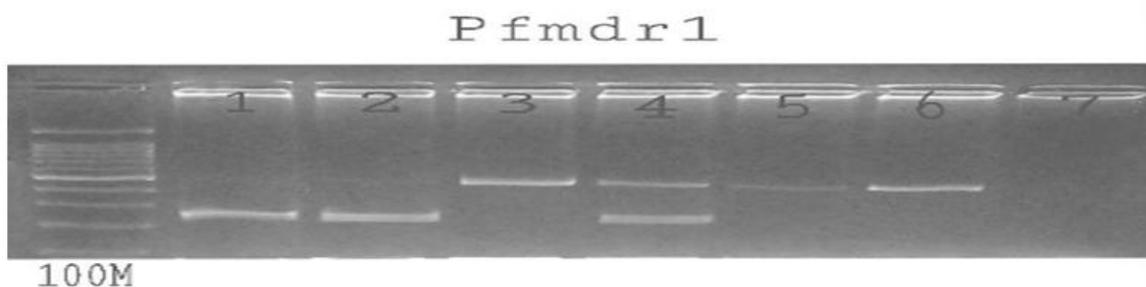


Figure (2): RFLP for Pfmdr-1 digestion with Apo 1-HF

TABLE (1): The Wild, Mutant And Mixed Allele In Each Area

Area	No. examined	Wild allele (%)	Mutant allele (%)	Mixed allele (%)
Sennar	100	71 (23.7%)	29 (9.7%)	0 (0.0%)
Khartoum	100	53 (17.7%)	47 (15.7%)	0 (0.0%)
River Nile	100	66 (22.0%)	32 (10.7%)	2 (0.7%)

P. value=0.018

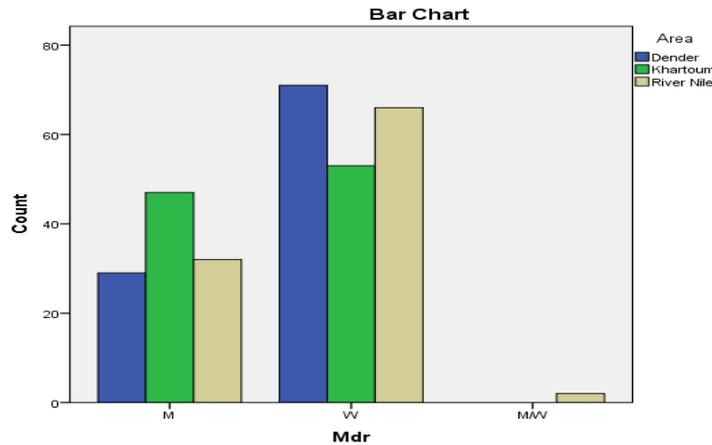


Figure (3): The wild, mutant and mixed allele in each area

Table (2): The wild, mutant and mixed allele according to sex

Gender	No. examined	Wild allele (%)	Mutant allele (%)	Mixed allele (%)
Male	133	79 (26.3%)	54 (18.0%)	0 (0.0%)
Female	167	111 (37.0%)	54 (18.0%)	2 (0.7%)

P. value= 0.167

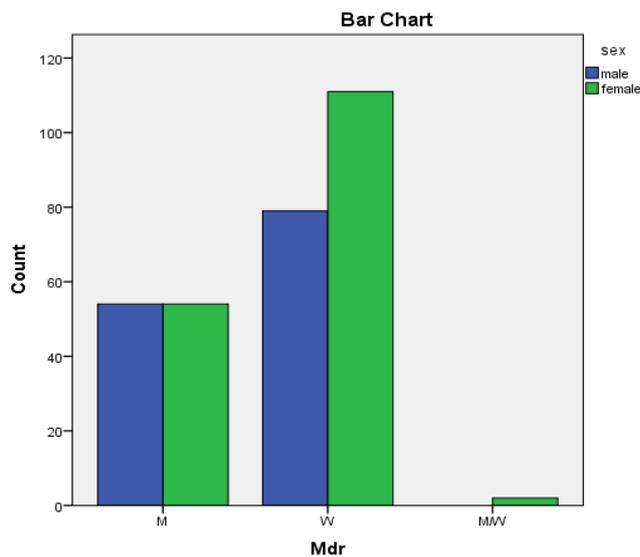


Figure (4): The wild, mutant and mixed allele according to sex

Table (3): The wild, mutant and mixed allele according to age groups

Age groups	No. examined	Wild allele (%)	Mutant allele (%)	Mixed allele (%)
less than 10	58	34 (11.3%)	24 (8.0%)	0 (0.0%)
10-19	45	31 (10.3%)	14 (4.7%)	0 (0.0%)
20-50	120	71 (23.7%)	47 (15.7%)	2 (0.7%)
more than 50	77	54 (18.0%)	23 (7.7%)	0 (0.0%)

P. value= 0.399

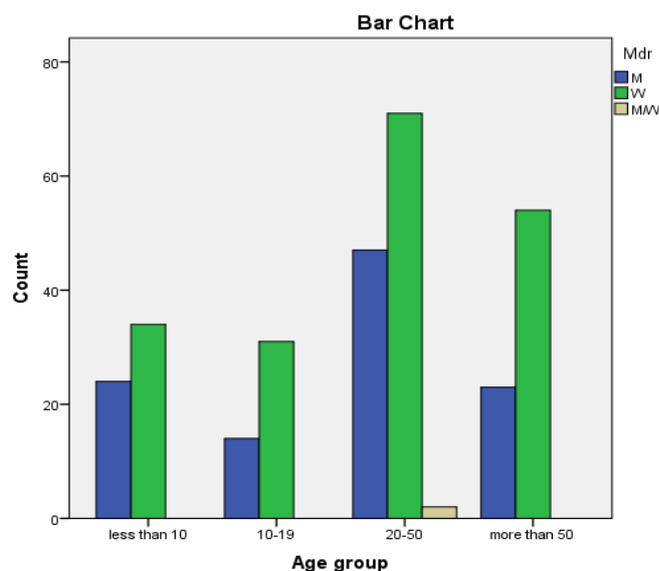


Figure (5): The wild, mutant and mixed allele according to age groups

V. Discussion

Our study aimed to detect the Pfmdr-1 N86Y mutant allele in three areas in Sudan which comprise the hyper, holo, meso and hypo endemic malarial transmission area in Sudan, we focusing in differences in detection of Pfmdr-1 N86Y mutant allele in the study areas and we used different age groups in our study. From study is obvious the prevalence of Pfmdr-1 N86Y mutant allele (36%), which means the Chloroquine and other anti-malarial drug were found in Sudan although the ministry of health in Sudan was changed the first line of malaria treatment from Chloroquine to artemisinin in 2005. Our results showed that, Khartoum was the highest Pfmdr-1 N86Y (15.7%) followed by River Nile (10.7%) and Sennar (9.7 %), in our opinion this normal distribution although Khartoum had the highest prevalence due to high number of population in Khartoum, so there are no obvious differences.

The results obtained from this study revealed that, the results, it was obvious that the overall of Pfmdr-1 N86Y mutant allele in the study areas was relatively high (36%) it was lower than the rate (55.5%) reported by Menegon et al., (2010) in Sudan¹³.

In our opinion the equal prevalence of Pfmdr-1 N86Y mutant allele (18.0%) among males and females in our study was due drug resistance is not affected by gender. The investigation revealed that, the highest Pfmdr-1 N86Y mutant allele (15.7%) was found in age groups from (20 – 50) years old, this finding was lower than study done in Nigeria by Muhammad et al., (2017) who reported (27.6 %) in the same age group¹⁴.

References

- [1]. Petersen, I., Estman, R., and Lanzer, M. Drug resistant malaria: Molecular mechanisms and implication for public health. FEBS LETTERS. 2011. 10, 1551-1556.
- [2]. Najera, J, T., Gonzalez, M., and Alonso, P, L. Some lessons for the future from the global malaria programm (1955 -1969). PLoS. Med. Hyg. 2001. 64, 12 - 17.
- [3]. Eckhoff, P, A. A malaria transmission-directed model of mosquito life cycle and ecology. Coinfection with Lethal and Nonlethal Murine Malaria Parasites, Malaria Journal, 2011. 10. 303.
- [4]. Atroosh et al.: The detection of pfcr and pfmdr1point mutations as molecular markers of chloroquine drug resistance, Pahang, Malaysia. Malaria Journal 2012 11:251.
- [5]. Rastaghi AE, Nateghpou M, Assmar M, Razavi MR, Kanbara H, Uemura H, Naddaf SR, Keshavarz H, Raeisi A, Mohebbali M. Detection of K76T Mutation in pfcr Gene as an Applicable Ge-netic Marker for Prediction of Chloroquine Resistant falciparum Malaria in Isolates from an Endemic District of Iran. Iranian Journal of Parasitology. 2008;3(2):48-56.
- [6]. Duraisingh MT, Cowman AF. Contribution of the pfmdr1 gene to antimalarial drug-resistance. Actatropica. 2005 Jun 30;94(3):181-90.
- [7]. Setthaudom C, Tan-ariya P, Sitthichot N, Khositnithikul R, Suwandittakul N, Leelayoova S, Mungthin M. Role of Plasmodium falciparum chloroquine resistance transporter and multidrug resistance 1 genes on in vitro chloroquine resistance in isolates of Plasmodium falciparum from Thailand. The American journal of tropical medicine and hygiene. 2011 Oct 1;85(4):606-11.
- [8]. Sidhu AB, Uhlemann AC, Valderramos SG, Valderramos JC, Krishna S, Fidock DA. Decreasing pfmdr1 copy number in Plasmodium falciparum malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. The Journal of infectious diseases. 2006 Aug 15;194(4):528-35.
- [9]. Sisowath C, Ferreira PE, Bustamante LY, Dahlström S, Mårtensson A, Björkman A, Krishna S, Gil JP. The role of pfmdr1 in Plasmodium falciparum tolerance to artemether-lumefantrine in Africa. Tropical Medicine & International Health. 2007 Jun 1;12(6):736-42.

- [10]. Sidhu AB, Valderramos SG, Fidock DA. pfm_{dr1} mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in Plasmodium falciparum. Molecular microbiology. 2005 Aug 1;57(4):913-26.
- [11]. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ, Nosten F. Mefloquine resistance in Plasmodium falciparum and increased pfm_{dr1} gene copy number. The Lancet. 2004 Aug 6;364(9432):438-47.
- [12]. Gadalla, N, B., Elzaki, S, E., Mukhtar, E., Warhurst, D, C., El-Sayed, B., Sutherland, C, J. Dynamics of pf_{cr}t alleles CVMNK and CVIET in chloroquine-treated Sudanese patients infected with Plasmodium falciparum. Malar J, 9, 74. Muhammad, R. H., Nock ,I. H., Ndams, I. S., George, J. B., and Deen, Y. Distribution of pfm_{dr1} and pf_{cr}t chloroquine drug resistance alleles in north-western Nigeria. MWJ. 2010. 8:15.
- [13]. Menegon M, Talha AA, Severini C, Elbushra SM, Mohamedani AA, Malik EM, Mohamed TA, Wernsdorfer WH, Majori G, Nour BY. Frequency distribution of antimalarial drug resistance alleles among Plasmodium falciparum isolates from Gezira State, central Sudan, and Gedarif State, eastern Sudan. The American journal of tropical medicine and hygiene. 2010 Aug 5;83(2):250-7.
- [14]. Muhammad, R. H., Nock ,I. H., Ndams, I. S., George, J. B., and Deen, Y. (2017). Distribution of pfm_{dr1} and pf_{cr}t chloroquine drug resistance alleles in north-western Nigeria. MWJ , 8:15.

*Ahmed Bakheet Abd Alla. "Detection of Single Point Mutation of Plasmodium Falciparum Multi-Drug Resistance 1 Gene in Three Different Areas in Sudan." IOSR Journal of Dental and Medical Sciences (IOSR-JDMS) 16.11 (2017): 89-94