



Investigating the Presence of *Plasmodium falciparum* Chloroquine Resistant Transporter (Pfcr) Drug-resistance Alleles in Some Northern Nigerian States

M. H. Ruqayyah^{1*}, K. Nafisatu¹ and B. Balarabe Musa²

¹Department of Biological Sciences, Federal University Dutse, Jigawa State, Nigeria.

²Department of Biological sciences, University of Abuja, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author MHR designed the study, wrote the protocol and wrote the first draft of the manuscript. Author KN performed the statistical analysis. Author BBM managed the data collection of the study. Authors MHR and KN managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

This study was designed to investigate the presence of Pfcr drug- resistance alleles (CQ resistant biomarker) and attempted to analyse the outcome in some states of northern Nigeria. A total of four hundred and thirteen (413) *Plasmodium falciparum* positive blood samples were collected from Kaduna, Jigawa, Katsina and Kebbi states during the period of April-August 2013. The samples were genotyped at codon 76 using specific primers for Pfcr gene. The data was analysed using Chi-square to determine significance association. Four hundred and thirteen (413) *P. falciparum* positive samples were genotyped at codon 76 of pfcr gene. Sixty eight 68(16.5%) samples contained single K76 (Chloroquine sensitive) alleles, 49(11.9%) contained 76T, while 16(3.9%) contained mixed K76T alleles. K76 alleles were highest in Kaduna state 17(32.1%) and lowest in Kebbi state 10(7.4%), 76T was highest in Jigawa state 11 (25.6%) and lowest in Kebbi state

*Corresponding author: E-mail: ruqayyahmohd@yahoo.co.uk, ruqayyahmohd@gmail.com, ruqayyahmohd@fud.edu.ng;

7(5.2%) while K76T was highest in Jigawa state 5(11.5%) and lowest in Kebbi state 2(1.5%) with significant difference between the states $P < 0.05$. K76 was higher among females 43(17.6%), 76T was also higher females 30(12.2%) while K76T was higher among males 7 (4.2%). K76 was higher among age group of 16-25 years 17(22.4%) and least among 26-40years age group 13 (13.5%). 76T was also higher among 26-40 years age group 17(17.7%) and least among age group >40 years 1(2.0%) and K76T was higher among age group 16-25 years 6(7.9%) and least in >40 years of age 1(2.0%) with high significant difference $P < 0.05$ between the age groups. The results of this study genetically confirms the use of CQ for malaria treatment in the area and attributed the varied distribution across the states, to high irrigation activities, self medication leading to dosage non compliance and improper diagnosis due to use of low sensitive RDT in most government hospitals. The need for enlightenment of the populace cannot over emphasize.

Keywords: Chloroquine; drug- resistance alleles; Northern Nigeria; Pfcrt.

1. INTRODUCTION

Malaria parasites are micro-organisms that belong to the genus *Plasmodium* a unicellular protozoan infecting the erythrocytes. There are more than 170 species of *Plasmodium*, which can infect many animal species such as reptiles, birds, mammals and amphibians [1]. However only five species of *Plasmodium* infect humans; *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi* [2]. Although five parasite species infect human beings nearly all malaria deaths and the larger proportion of morbidity are caused by *P. falciparum* having been described as the most dangerous [3]. More than a third of the world's population (about 2 billion people) live in malaria endemic areas and 1 billion people are estimated to carry the parasites all the time. In Africa alone, there are an estimated 200-450 million cases of fever in children infected with malaria each year [4]. Malaria remains one of the major health problems in sub-Saharan Africa [5, 6]. Though there are encouraging reports that malaria morbidity and mortality are declining [7]. It is still an overwhelming public health problem, with an estimated 207 million cases and 627,000 deaths every year worldwide. Malaria accounts for 25% of all deaths of children under the age of five across Africa, it affects over 50 million pregnant women and is responsible for 10% of maternal mortalities every year [8]. The economic impact of malaria is enormous especially in African countries with lean resources. As much as 40% of health care spending in endemic countries goes on malaria costing the continent \$12 billion a year [8]. The disease also affect the socio-economic and development of the poor countries, population studies have shown that in Kenya, 11% of primary school days are lost to malaria. The disease also causes losses of 26% of the nation's Gross Domestic Production (GDP). In Nigeria 1 – 5% of the country's GDP is

lost due to malaria. It is one of the four most common causes of childhood mortality with 50% of the population having at least one episode of malaria each year, which the under five children have up to 2–4 attacks annually. *P. falciparum* is responsible for 98% of malaria cases with stable perennial transmission in all parts of the country [9].

Drug resistance emergence and spread of clones of *P. falciparum* to most available anti-malarial drugs makes the control of the disease difficult to achieve. Chloroquine (CQ) was the first antimalarial widely used in endemic areas including Nigeria, but CQ resistance was first documented in Thailand in the late 1950's, spread to African in 1974 [10], and subsequently came Nigeria in the early 1980's and continued over a period of time until 2005 when CQ was completely banned. Based on this the Nigerian government changed its first line drug to the more expensive artemisinin based combination therapy (ACT) and recommended that all fevers be treated presumptively with ACTs where confirmation cannot be made [11]. However lately *P. falciparum* resistant to artemisinin derivatives (ACT) was recorded in Western Cambodia, threatening the entire world's malaria control and elimination effort [12,13].

Despite the established resistance and national policy of ACT of as the first-line treatment of uncomplicated malaria, Malaria Indicator Survey (MIS) 2010 indicates that over 70% of children treated for malaria in Nigeria received chloroquine (CQ) or sulfadoxine pyrimethamine (SP), mainly on account of cost and effectiveness in uncomplicated cases of malaria [13]. Although the resistance to chloroquine by *P. falciparum* has prompted many studies within the last decade in different parts of Nigeria [14; 15]. For instance while a high resistance to Chloroquine by malaria parasite has also been

reported in southeastern Nigeria [16], a study carried out in Northeastern Nigeria, showed most strains of *P. falciparum* were found to be fully sensitive to chloroquine [16]. Patric et al., [17] showed chloroquine is still effective in the treatment of uncomplicated malaria in Delta state.

Chloroquine (CQ) resistant *falciparum* malaria is caused by mutations on the *P. falciparum* CQ resistance transporter (Pfcr) which is a strong predictor of CQ resistance.

Mutations on the Pfcr-K76T are directly linked with both in-vitro and clinical resistance and are thus used as a biomarker of CQ resistance [18].

This study was designed therefore to investigate the distribution of pfcr resistant alleles and analyse the possible factors responsible for the outcome.

2. MATERIALS AND METHODS

2.1 Area of Study

The study was conducted in randomly selected states of northern Nigeria. These were Kaduna, Katsina, Kebbi and Jigawa States, between the periods of April-August 2013. The states lie within the Savannah region of Nigeria. Where the rainy season is usually from the months of April to October and the cold and dry season is within the months of November to March. Malaria is meso to hyper-endemic in the whole states and it is seasonally transmitted, with the main peak of transmission from early June to late August and second/minor peak from early October to mid-November. These transmission periods corresponds to the rainy and dry season when the mosquito population is high [19]. *Plasmodium falciparum* is the most common species [20].

2.2 Sampling Procedure

Four states were randomly selected from the north-western region of Nigeria. One hospital each was also randomly selected from the states, thus; Kaduna, Jigawa, Katsina and Kebbi states. Outpatients individuals whose samples were presented with uncomplicated malaria from the visited health facilities were collected.

2.3 Ethical Consideration

Scientific and Ethical permit/clearance were obtained from Kano, Kaduna, Katsina, Kebbi, and Jigawa State Ministries of Health/Hospital

Management Board (MOH/HMB) before commencement of the research.

2.4 Sample Storage and Transportation

Plasmodium falciparum positive samples were blotted on Whitman filter paper (24 cm) in quadruplets. It was allowed to dry and stored in a separate clean envelope. The samples were then taken to University of Abertay, Dundee in Scotland for the molecular analysis.

2.5 Molecular Analysis

Real time Polymerase Chain Reaction (RT-PCR) was used to determine the susceptible and resistant alleles of Pfcr gene of the *Plasmodium falciparum* positive samples.

2.6 DNA Extraction Protocol

A modified Qiagen protocol for DNA extraction was used. Sterile paper punch was used to cut out the dry blood spots (DBS). DNA was extracted from using a modified Qiagen protocol (QIAamp) DNA blood kit Blood Protocol (Qiagen, Hilden, Germany).

2.6.1 RT- PCR for detection of mutation on *Plasmodium falciparum* chloroquine resistance transporter (Pfcr) gene

The oligonucleotides and probes were adopted from the work of Ojorongbe et al., [21] and designed by Sysmex UK Ltd. The sensor probe labeled with fluorescein at the 3' end is designed to be perfectly complementary to the mutation site. An amplification primer iLC labeled with Cy5 on the third base from the 3'end is used as a reverse primer which is extended during amplification. During FRET, fluorescein which is excited by the light source of the Rotor Gene instrument transfers its energy to the Cy5 incorporated into the PCR product working as anchor probe [22,23]. A specific melting temperature is then obtained for each genotype: a sensor probe spanning one mismatch could still hybridise to the target sequence but will melt off at lower temperature than a sensor probe with a perfect match [21].

2.6.2 Primers and probe

The primers and probe used were
F: 5'-CTTGTCTTGGTAAATGTGCTCA-3'
R: 5-GTTACCAATTTTGTAAAGTTCT-3'
Sensor Probe:
SPTGTGTAATTGAAACAATTTTGTCTAA-3

With a melting temperature of 65.3 ± 0.4 for the wild type and 46.5 ± 0.2 for the mutant type.

2.7 Statistical Analysis

The data was analysed using statistical package for social sciences (SPSS) version 21. Chi-square was used to determine if there were significance association in prevalence between the states gender and age at $P < 0.05$. Odd ratio was used to determine difference in prevalence between males and females.

3. RESULTS

3.1 Prevalence of Pfcrt in the Study Area

A total of four hundred and thirteen (413) *plasmodium falciparum* positive samples were genotyped at codon 76 of pfcrt gene. Eighty one 68(16.5%) samples contained single K76 (chloroquine sensitive) alleles, 49(11.9%) contained 76T, while 16(3.9%) contained mixed K76T alleles. K76 alleles were highest in Kaduna state 17(32.1%) and lowest in Kebbi state 10(7.4%), 76T was highest in Jigawa state 11 (25.6%) and lowest in Kebbi state 7(5.2%) while K76T was highest in Jigawa state 5(11.5%) and lowest in Kebbi state 2(1.5%). K76 was higher among females 43(17.6%), 76T was also higher

females 30(12.2%) while K76T was higher among males 7 (4.2%). K76 was higher among age group of 16-25 years 17(22.4%) and least among 26-40years age group 13 (13.5%). 76T was also higher among 26-40years age group 17(17.7%) and least among age group >40years 1(2.0%) and K76T was higher among age group 16-25 years 6(7.9%) and least in >40 years of age 1(2.0%). The results are shown on Tables 1-3.

4. DISCUSSION

The study showed that the CQ sensitive alleles of Pfcrt (K76) were found to be higher 68 (16.5%) in the study population, when compared with the resistant alleles 76T 49 (11.9%) and 16 (3.9%), the results showed a possible re emergence of CQ sensitive genes, although at a slower rate when compared to the reports obtained by [24], from Malawi that showed a recovery of the susceptible pfcrt K76 from <15% to 100% within 13 years of CQ withdrawal. The results may further indicate that either CQ remained widely in use at the community level even after it's banned or is showing a mounting pressure on Artemether-lumefantrine (AL), or both [25]. It further suggests that CQ can be used to a certain level for malaria treatment as (Pfcrt, K76) do not have resistance to chloroquine [26,27]. However the presence of the mutant parasites with pfcrt

Table 1. Distribution of Pfcrt alleles in the study population

States	Number examined	Pfcrt alleles		
		K76 (%)	76T (%)	K76T (%)
Kaduna State	53	17 (32.08)	10 (18.87)	3(5.66)
Jigawa State	43	8 (18.60)	11 (25.58)	5(11.63)
Kebbi State	135	10 (7.4)	7 (5.2)	2 (1.5)
Katsina State	182	33 (18.1)	21 (11.5)	6 (3.3)
Total	413	68 (16.5)	49(11.9)	16(3.9)
	Chi square	19.322	16.483	6.256
	Df	4	4	4

Table 2. Distribution of pfcrt alleles based on gender

Sex	Number examined	Pfcrt alleles		
		K76(%)	76T(%)	K76T(%)
Male	168	24 (14.3)	19 (11.3)	7 (4.2)
Female	245	43 (17.6)	30 (12.2)	9 (3.3)
Total	413	68 (16.5)	49 (11.9)	16 (3.9)
	Chi square	1.003	0.221	0.289
	df	1	1	1
	P value	0.317ns	0.638ns	0.591ns
	Odd ratio	0.776	0.873	1.304
	C.I.	0.472 - 1.276	0.496 - 1.537	0.494 - 3.443

Table 3. Distribution of Pfcrt alleles based on age

Age	Number examined	Pfcrt alleles		
		K76 (%)	76T (%)	K76T (%)
1 - 5 years	127	20 (15.7)	10 (15.6)	4 (3.1)
6 - 15 years	64	9 (14.1)	8 (12.5)	2 (3.1)
16 - 25 years	76	17 (22.4)	13 (17.1)	6 (7.9)
26 - 40 years	96	13 (13.5)	17 (17.7)	3 (3.1)
>40 years	50	9 (18.0)	1 (2.0)	1 (2.0)
Total	413	68 (16.5)	49 (11.9)	16 (3.9)
	Chi square	2.708	8.383	3.584
	df	4	4	4
	P value	0.608ns	0.079ns	0.465ns

76T (28.3%) is a threat to some of the ACT in use. As report from an *in-vitro* study conducted in Nigeria showed an association between the 76T mutation and decreased susceptibility to artemether [28]. In addition an increasing trend for K76 will create a future problem for ACT use because it has been seen in recrudescence samples after AL use [29]. One of the mutations, the Pfcrt-K76T, is directly linked with both *in-vitro* and clinical resistance and is thus used as a biomarker of CQ resistance [18]. Also another report from Ibadan Nigeria a neighbouring town to Osogbo had suggested an association and linkage disequilibrium between the Pfcrt T76 alleles in Chloroquine-resistant isolates [30]. It had also been shown that a strong association was reported between K76T and chloroquine resistance [26,27].

Moreover high prevalence of malaria in most parts of Nigeria coupled with a solitary reliance on ACT treatment and the banned in the use of chloroquine since 2005 poses a situation where selection and propagation of ACT resistance and re emergence of CQ sensitive genes is highly possible [31]. Finally, factors such as farmland activities, especially irrigation farming, might also have contributed to increased transmission [31], and the higher prevalence of resistance alleles obtained in Jigawa and Kaduna States [25].

5. CONCLUSION

Pfcrt (33.4%) have been found to be prevalent in the study area. Both sensitive and resistance alleles were mapped out in the study area as follows: 76T (12.4%), K76 (17.4%) and K76T (3.6%).

6. RECOMMENDATIONS

In summary, the results of this study give evidence to the presence of the 76T pfcrt point

mutations in the study area. The observations made in this study with the prevalence of the molecular markers are in line with what is expected after the change of the malaria treatment policy. As the chloroquine resistant genotypes are not too high, the drug can be introduced as prophylaxis for malaria risk groups, such as children and pregnant women. The findings cannot be compared to previous researchers as there are non but there is need to continually monitor molecular markers of all the anti-malarial drugs currently in use in Nigeria to allow for early detection of reduced or increased parasite susceptibility to the drugs. As this trend has been observed in other countries and provides evidence that removal of drug pressure can result into full recovery of efficacy to drugs that were previous rendered ineffective due to resistance.

It must also be stressed that findings from this study have given some insight into the genetic background of the parasites in circulation in North West Nigeria. However the study should be repeated every after 2-4 years to be able to clearly see the trend. Above all continual education of the populace is highly recommended.

CONSENT

Written informed consent was obtained from patients prior using their samples. Consent for the children was provided by the parents/guardians.

ETHICAL APPROVAL

Scientific and Ethical permit/clearance were obtained from Kano, Kaduna, Katsina, Kebbi, and Jigawa State Ministries of Health/Hospital Management Board (MOH/HMB) before commencement of the research.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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