Investigating the presence of *Plasmodium falciparum* Chloroquine resistant transporter (*Pfcrt*) drug-resistance alleles in some Northern Nigerian states

Abstract

In Nigeria *Plasmodium falciparum* is responsible for 98% of malaria cases with stable perennial transmission in all parts of the country. Resistance to Chloroquine (CQ) in the 80's lead to change to more expensive drug Artemisinin Combination Therapy (ACT). The high cost of ACT resulted in persistent use of CQ for malaria treatment due to its low cost in most parts of Nigeria. Therefore due to unofficial reintroduction of CQ for malaria treatment, this study was designed to investigate the presence of *Pfcrt* drug- resistance alleles (CQ resistant biomarker) and attempted to analyze 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 *Pfcrt* gene. The data was analyzed using Chi-square to determine significance association. Four hundred and thirteen (413) *plasmodium falciparum* positive samples were genotyped at codon 76 of *pfcrt* 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 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 26-40years 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) 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.

Key words: Chloroquine, Drug- resistance Alleles, Northern Nigeria, *Pfcrt*

INTRODUCTION

Malaria parasites are micro-organisms that belong to the genus *Plasmodium* a unicellular protozoan infecting the erythrocytes. There are more than 100 species of *Plasmodium*, which can infect many animal species such as reptiles, birds and mammals. However only five species of *Plasmodium* infect humans; *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale* and *Plasmodium knowlesi* (Cox-Singh and Singh, 2008). 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 (Snow *et al*., 2003).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 (Breman *et al.,*2001). Malaria remains one of the major health problems in sub-Saharan Africa (Kebbe *et al*., 2014; Nevil, 1990). Though there are encouraging reports that malaria morbidity and mortality are declining (O'Meara, 2010). 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 (Kabore, 2001).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 (Kabore, 2001). 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 (TDR, 2000).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. *Plasmodium falciparum* is responsible for 98% of malaria cases with stable perennial transmission in all parts of the country (FMOH, 2005).

Drug resistance emergence and spread of clones of *Plasmodium falciparum* to most available anti-malarial drugs makes the control of the disease difficult to achieve. Chloroquine (CQ) was the first antimalarial to be widely usedin endemic areas including Nigeria, but CQ resistance was first documented in Thailand in the late 1950's, spread to African in 1974 (Olatunde,1997), 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 arthemisinin based combination therapy (ACT) and recommended that all fevers be treated presumptively with ACTs where confirmation cannot be made (FMOH,2005). However lately *P.falciparum* resistant to artimisinin derivatives (ACT) was recorded in Western Combodia, threatening the entire world's malaria control and elimination effort (Dondorp *et al.,* 2009; WHO, 2010).

Despite the established resistance and national policy of ACT of as the first-line treatment of uncomplicated malaria, Malaria Indicative 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 (NMFS,2011). Although the resistance to chloroquine by *P. falciparum* has prompted many studies within the last decade in different parts of Nigeria (Molta, 1995; Umotong *et al.,* 1991; Salako *et al.,* 1990; Sowunmi *et al.,* 1990; Sowunmi and Salako, 1992).For instance while a high resistance to Chloroquine by malaria parasite has also been reported in southeastern Nigeria (Umotong *et al.,* 1991), a study carried out in Northeastern Nigeria, showed most strains of *P. falciparum* were found to be fully sensitive to chloroquine (Molta, 1995). Patrict *et al*., 2003) 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 (Pfcrt) which is a strong predictor of CQ resistance.

Mutations on the Pfcrt-K76T are directly linked with both in-vitro and clinical resistance and are thus used as a biomarker of CQ resistance (Wallens and Plowe 2001).

This study was designed therefore to investigates the distribution of *pfcrt* resistant alleles and analyze the possible factors responsible for the outcome.

MATERIALS AND METHODS

3.1 Area of study:

The study was conducted in some 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 (WHO, 1992). *Plasmodium falciparum* is the most common species (WHO, 2013).

3.2 Sampling Procedure

Four states were randomly selected from the north-western region of Nigeria. One (1) hospital each was also ramdomly selected from the states, thus; Kaduna, Jigawa, Katsina and Kebbi states. Outpatients individuals whose samples where presented with uncomplicated malaria from the visited health facilities were collected.

3.6 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.

3.7 Participation Consent

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

Sample storage and Transportation

Plasmodiu falciparum positive samples were blotted on Whitman filter paper (24cm) 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.

Molecular Analysis

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

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).

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

The oligonucleotides and probes were adopted from the work of Ojurongbe *et al.,*2007 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 (Kearns *et al*., 2001; de Monbrison *et al*., 2003). A specific melting temperature is then obtained for each genotype: a sensor probe spanning one mismatch could still hybridize to the target sequence but will melt off at lower temperature than a sensor probe with a perfect match (Ojurongbe *et al.,* 2007).

Primers and Probe

The primers and probe used were

F: 5’-CTTGTCTTGGTAAATGTGCTCA-3’

R: 5-GTTACCAATTTTGTTTAAAGTTCT-3’

Sensor Probe:

SPTGTGTAATTGAAACAATTTTTGCTAA-3

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

Statistical Analysis

The data was analyzed 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.

4.1.3 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.

Table 1: Distribution of *Pfcrt* alleles in the study population

States	**Number Examined**	***Pfcrt* 76T**		
		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

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166 Table 2: Distribution of *pfcrt* alleles based on gender

Sex	**Number Examined**	***Pfcrt* 76T**		
		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

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168 Table 3: Distribution of *Pfcrt* alleles based on age

Age	**Number Examined**	***Pfcrt* K76**	***Pfcrt* 76T**	***Pfcrt* K76T**
		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

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170 **Discussion**

171 The study showed that the CQ sensitive alleles of *Pfcrt* were predomantly prevalent (47.9%
172 and 17.4%) respectively in the study population, this suggests that CQ can be used to a
173 certain level for malaria treatment as (*Pfcrt*, K76) do not have resistance to chloroquine
174 (Sutar *et al.,*2011; Bin-Dajem *et al*.,2011). However the presence of the mutant parasites with
175 *pfcrt* T76 (28.3%) is a threat to some of the ACT in use. As report from an in vitro study
176 conducted in Nigeria showed an association between the T76 mutation and decreased
177 susceptibility to artemether (Bustamante *et al.,*2012). In addition an increasing trend for K76
178 will create a future problem for ACT use because it has been seen in recrudescent samples
179 after AL use (Sisowath *et al*.,2005). One of the mutations, the Pfcrt-K76T, is directly linked
180 with both in-vitro and clinical resistance and is thus used as a biomarker of CQ resistance
181 (Wellens and Plowe 2001). 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 (Happi *et al.,* 2003).

It had also been shown that a strong association was reported between K76T for chloroquine resistance (Sutar *et al.,* 2011; Bin-Dajem *et al*.,2011). On the other hand, when *Pfcrt*-K76Toccurs with *Pfmdr*1 mutant type (86Y), the parasite shows resistance to chloroquine, but (*Pfcrt*, K76) and (*Pfmd1*, N86) do not have resistance to chloroquine. This implies that the presence of (*Pfcrt*, K76T) mutation is pre-condition for the *Pfmdr*1 parasite to develop multi-drug resistance property against chloroquine and also *Pfmdr*1 increases the level of chloroquine resistance having synergetic effect with (*Pfcrt*, K76T). High parasite population with (*Pfcrt*, K76T) mutation favours the emergence of (*Pfmdr1*, N86Y) mutation (Mittra *et al*., 2006).

CONCLUSSIONS AND RECOMMENDATION

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%)

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 can not 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.

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