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Malaria Treatment schedules and Socio- economic implications of mutation in the pfmdr1 and pfcrt genes of Plasmodium falciparum isolates in asymptomatic carriers in Nigeria

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Abstract

Mutation in the pfcrt and pfmdr-1, genes have been implicated both to be putative CQ resistance markers.

Blood samples from 130 volunteers were obtained and genotyped by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) for pfmdr1 and pfcrt genes.

A total of 40(30.8%) questionnaires were administered to the adults and 90(69.2%) were administered to the school children. 14(10.8%) had microscopically detectable parasites on day zero with a mean parasites counts of 40,231 parasites/µL. CQ was administered to all infected, higher parasite density was observed in the poorer population. PCR-RFLP analysis on 14 parasite positive samples for pfcrt and pfmdr genotypes showed polymorphism around pfmdr1 N86Y. The parasite count decreased progressively from day 0 to day 14 to negligible levels. Conversely, our subjects still harboured sensitive strains of the parasite. Our PCR analysis of the pfcrtK76T yielded no result. A significant relationship was observed between respondents' treatment behaviour and mutation in the pfmdr1 genes of Plasmodium falciparum.

Keywords: Polymorphism, *falciparum*, Genotyped, mutation, Chloroquine

1. Introduction

Malaria remains the major cause of disease and death in the world, especially among children and pregnant women [3, 13, 14, 18]. Malaria treatments depend on the type and severity of the disease. Emerging and the spread of CQ-resistant malaria have contributed to a resurgence of malaria, particularly in sub-Saharan Africa [8]. Chloroquine (CQ) although many countries of the world have changed their antimalarial policy, is safe, inexpensive, and used for the treatment of uncomplicated malaria [15]. Chloroquine (CQ) was withdrawn as the first-line antimalarial drug in Nigeria in 2005 because of a widespread and high-level clinical failure rate across the country [7]. The *P. falciparum* CQ resistance transporter mutation (*pfcrt*) K76T and mutation on the *pfmdr1*-76 (tyrosine) have been variously implicated as likely candidates for chloroquine resistance [1]. Single base *pfmdr1* changes have been associated with the exchange of amino acids in some chloroquine resistant isolates and may play a role in the determination of drug resistance [11]. The biochemical and genetic mechanism of CQ resistance underlying this phenomenon have been extensively studied [4, 9, 19, 20, 21]. Malaria is not just a commonly associated with poverty, but is also a cause of poverty and a major hindrance to economic development [18]. This study is based on assessing malaria treatment scheduled, the socio-economics of CQ resistant falciparum malaria and the implication of Chloroquine resistant markers in the study population.

2.0 Materials and Methods

A cross sectional survey was conducted among school children aged 11-18 and few selected teachers aged between 30-60 years at the Anglican grammar school (population 2,000) in Yemetu, Ibadan. The school has a mixed ethnic nationality composition but a Yoruba majority. One hundred and thirty subjects were recruited to participate in the study after which questionnaires were administered to all subjects recruited. Most of the volunteers that participated in the study had not taken antimalarial drugs in the last few days. Anyone who was experiencing clinical symptoms was given oral administration of chloroquine according to the recommendation of the manufacturer. Antimalarial treatments were based on microscopic examination for malaria and later any positive samples were subjected to sub microscopic examination using the polymerase chain reaction assay. The number of parasites per microscopic of blood was calculated by comparing the number of infected erythrocytes per 200 leucocytes with the average white blood cell (WBC). Samples were considered negative if no parasite was detected after thorough examination of the Giemsa stained thick smear. All subjects whether symptomatic or asymptomatic were finger pricked for malaria parasite for microscopy test, and a drop of blood was collected on filter paper for PCR analysis. The study protocol was earlier approved by the Joint Ethical review Committee of the University College hospital of Ibadan. Informed

consent was obtained from the Children guardian/ parents.

2.1.1 DNA Extraction

The parasite DNA was extracted from finger pricked blood samples blotted onto filter paper strips (3MM, Whatmann), by using methanol fixation and heat extraction technique. About 0.25-0.5 cm^2 piece of impregnated filter paper was cut off using scalpels, by wiping-off the scalpels on ethanol-treated cotton wool between cutting in order to prevent any carryover of blood and left at-20^0C overnight.

2.1.2 Amplification of *pfmdr1* genes

A nested mutation specific polymerase chain reaction (PCR) was used for the *pfmdr1* and/ or mutation specific PCR for *pfcrt* genes were done in 25 µl reaction mixture containing 2µl of template DNA (primary amplicon), 0.2µM dNTPs, 2.5µM $MgCl_2$ 1x Buffer, 2.5 Units of Taq polymerase (Ampli Taq Gold, Applied Biosystems, Foster city, CA) , and 1.0µM of each primer. In the initial PCR, the forward primer used for the amplification of *pfmdr1* codon 86 was *mdr*-1GC and the reverse primer used was *mdr*-2GC. In the Nested PCR, the forward primer was *mdr*3B and the reverse primer was *mdr*4B. The initial and nested amplification condition was described elsewhere [12]. A known positive DNA (parasite DNA from cell culture) HB3, a mutant type Dd2 and negative controls (blanks) were run with each set of PCR reaction.

After the secondary Nested amplification, 10-15µl of each reaction was analyzed in 2% agarose gel and visualized under ultraviolet (UV) transillummination after staining with Ethidium Bromide. Amplification of K76T gene mutation was performed using both nested and mutation specific PCR containing 2µl of template DNA, 1x buffer, 30µM $MgCl_2$, 0.2µM dNTPs, 2.5 units Taq polymerase and 1.0 µM of each primer. The amplification of *pfcrt*K76T was carried out using either the conventional nested PCR as in *pfmdr1*N86Y or mutation specific PCR. In the mutation specific PCR for *pfcrt*K76T, F_1= CRT_1 and R_1 =CRT_2 were forward and reverse primer respectively for initial primary amplification around codon 76 of the *pfcrt* genes. PCR assay was set-up for the primary amplification; 5µl amplicon was used for the initial primary amplification. For the secondary amplification, a common primer: $TCRP_3$ was used for both K76 (wild type) and 76T (mutant) after which specific primers were used for K76 ($TCRP_4$) and 76T ($TCRP_4$) respectively. PCR assay was then set up for the secondary amplification around codon 76 for about two hours based on the standardized condition of the laboratory for *pfcrt* PCR conditions.

2.1.3 Restriction Enzymes Length Polymorphisms (RFLP)

Following amplification of the fragments concerned, polymorphism in the *pfcrt* and *pfmdr1* were assessed as follow: *pfcrt* 76 K and *pfmdr1* 86 N were detected by incubation of the corresponding PCR fragments with restriction enzymes APOI and AFIII (ACPuPyGT) respectively. The RFLP digest was done in a 15µl reaction mixture containing 5µl of template DNA, 1.5µl of 10x buffer 3.0, 15µl of 100x BSA and 2.5 units of 5µl AFI III. The mixture was incubated at 37^0C in water baths for 4-6 hours or overnight. The electrophoresis of the enzyme digest product was run and visualized under ultraviolet (UV) transillumination after staining with Ethidium Bromide using the controls: Dd2- negative (mutant), HB3-positive (wild type) and B-blank.

2.1.4 Statistical analysis

Statistical significance was assessed using the Z-test scores (Snow et al; 1991, Andersen 1997) using analytical procedures (SPSS and Excel window) for data analysis. Children with Z-scores <-1.0 SD (CI=99%) were classified as underweight (Poor) and >-1.0 SD were well fed (Rich). The World Health Organization (WHO) and national center for Health Statistics (NCHS) maintain a global database on child growth and malnutrition among children aged 2-18 years. Here, weight for age was used to assess underweight as an indicator of under nutrition because of its availability and its ability to capture both stunting (generally associated with long-term under nutrition and wasting of recent and acute under nutrition) [16]. In adults, mean per capital income (MPPCI) was used as poverty index. An adult was classified as a low income- earner and high income earner according to World Bank MPPCI ≥ ₦ 5,445.11.

3.0 Results

From table 1 above, we observed that only RY 69 aged 13 years belong to low socio economic status harbored 560 parasites/µL of blood on recruitment day with no amplification on RFLP analysis but yielded no reaction with PCR. Other children who are from high income class harboured both resistant strains (Y) and mixed infection (N/Y). For example, RY 032 aged 18 years are of high income status based on weight-for-age classification hraboured resistant strains (Y) on day 0 and whose parasitemia status was 105 cells/µL of blood. It was observed that the parasites' total count decreased progressively from table 1 above progressively from a total of 1579 cells/µL, 335 cells/µL to 126 cells/µL on day 0, 3 and 7 respectively.

Our RFLP yielded amplification at *pfmdr* 1 in only 3(42.9%) of the parasitemic group. According to the World Bank

classification, the poverty index of and adult was determined as the ratio of their income to the house hold size. Based on these, we observed that 4(57.1%) adults were high income earner while 3(42.9%) were low income earner. More parasites were isolated from the recurrent malaria in low income earner than in their high income counterparts. On day 0 we observed that 2(28.6%) were infected with resistant strains; one (14.3%) had mixed infection. On day 3 of the follow-up schedule, 2(28.6%) had resistant strains, 1(14.3%) had wild type while and mixed infection. However, we observed that only 1(14.3%) harbored mixed infection up till day 7. All infection did not exceed day 7. Our study here showed a 3:1 amplification in the *pfmdr1* genes on day 0, fifty-fifty in both the high income earner and low income on day 3, and only (14.3%) amplifications on day 7. On day 14 we noticed total clearance of parasite in all the groups. Generally, we observed progressive clearance of parasites on day 0 (38,653) to (1800) in adult population.

Table 3 showed that respondents who were of high economic status 9(22.5%) seek appropriate medical attention at Hospital when they noticed symptoms of malaria, 4(10.0%) visit drug vendors (chemist shop) when they are sick. Most of the low income earners 19(47.5%) do not buy another antimalarial drug if treatment fails, rather they combine different western drugs or western drugs and herbs as unlike the high income group that go to hospital 11(27.5%) if treatment fails. Most of the drugs familiar with the respondents were over-the-counter antimalarial drugs that are cheap one-dosed drugs and mode of prescription of adults was majorly self medication.

Discussion

This study is consistent with previous work of [12, 20] where 48% and 35.1% frequency of polymorphism (point mutation) in the *pfmdr1* genes were reported in Ibadan. We noticed that 5/11 (45.5%) subjects harbored Y86 (mutant) and 5/11 (36.4%) N86Y (mixed) *pfmdr1* alleles on day 0 and follow-up days. This is consistent with the work of [5,6] where *pfmdr1* mutation Y86 was significantly selected for by chloroquine treatment which is attributable to patients *falciparum* malaria infection that recurred or persisted after treatment with standard oral chloroquine therapy indicating the high selection for this mutation in parasite capable of surviving in the presence of chloroquine examined amplification in post chloroquine treatment samples whereas such amplification was not detected in the pre-treatment samples. Although re-infection or recrudescence might be suspected here, re-infection should be rare during a fourteen day follow up period and sub-therapeutic chloroquine therapy. A more likely explanation was that these were mixed infection consisting predominantly of sensitive (N86) parasite whose levels were below the threshold of initial detection by PCR or RFLP methods. During exposure to chloroquine, sensitive (N86) parasite would have been cleared as the resistant (N86) strain population increases [5]. We noticed a general difficulty in clearance of chloroquine resistant strains in low income earners than in the high income adults. Although in areas such as the area under study, cases of resistance are not unexpected because the socioeconomic status of the population is low and a large percentage of the population self treat malaria (data not shown). We attempted the social implication of the chloroquine resistant marker: *pfmdr1* N86Y and *pfcrt* K76T or whether the prevalence of one or both of these markers could be a predicative index of the socio economic status of the bearer.

The study revealed that individuals of high social economic class are less likely to have *pfmdr1* N86K alleles which further corroborate Filmer's work who observed a positive correlation between socioeconomic status [10] and CQ resistance, although other studies contradicted this assertion. It is often expected that this work should be consistent with previous works on the overall prevalence of the N86Y mutation in the *pfmdr1* and K76T mutation in ratio 2:3 respectively [2]. We observed that adults were more vulnerable to malaria parasites and the ability to clear resistant strains is high reduced in adults, the reason for this was yet understood.

In previous studies, *pfcrt* K76T gene has been identified as the primary genetic marker in chloroquine resistance, which has been show to be predictive of rate s of chloroquine resistance, among different setting when adjusted for age. This study however failed to establish the relationship predictive of modulative role of *pfmdr1* Y86 in chloroquine resistance. Efforts to verify the overall prevalence of the K76T mutation in *pfcrt* was impossible, as there seem to be carryover of the primary product when visualized in gel electrophoresis under UV illumination using both specific PCR and conventional PCR techniques. More resistant parasites was isolated from our adult respondents compared to isolates from young adults the health seeking behaviour of adults might be responsible for this, however this study could not establish this assertion in children respondents.

Table 1: **The Socio-epidemiological data and molecular analysis of pfmdr 1 N86Y in children**

Id no	age	household Size	sex	SES	Parasite count/ul D_o D_3 D_7 D_{14}	PCR (CQ marker amplified D_o D_3 D_7 D_{14}	D_o D_3 D_7 D_{14}
RY 05	13	4	M	high	120 115 AB AB	NR - - -	A - - -
RY 32	18	6	F	high	105 140 56 AB	Y - - -	A - - -
RY 33	18	6	F	high	80 80 70 AB	N/Y - - -	A - - -
RY 55	11	3	M	high	74 AB AB AB	NR - - -	NA - - -
RY 69	13	6	F	low	560 420 AB AB	NR - - -	NA - - -
RY 72	19	6	F	high	560 AB AB AB	NR - - -	NA - - -
RY 74	18	1	F	high	80 AB AB AB	NR - - -	A - - -
TOTAL					1579 335 126	1:2	

AB= Absent, NA= No amplification, NR=no result, SES=Socio economic status, Y=resistance strain, N= wild type, N/Y= mixed infection

Table 2: **The Socio-epidemiological data and molecular analysis of pfmdr 1 N86Y in Adults**

Id no	I	HS	Sex	SES	D_o	D_3	D_7	D_{14}	Parasite cells/μL blood D_o	D_3	D_7	D_{14}	RFLP result PCR (CQ marker amplified D_o	D_3	D_7	D_{14}
AG 07	26,000	4	M	H	1920	1400	AB	AB	NR	NR	NR	NR	NA	A	-	-
AG 20	21,000	F	L	3040	4080	2080	1800	Y	NY	NR	NR	A	A	NA	NA	
AG 26	23,000	6	F	L	1920	1160	AB	AB	NR	Y	-	-	NA	A	-	-
AG 28	28,980	3	M	L	1000	520	A	AB	Y	N	-	-	A	NA	-	-
AG 29	25,600	6	F	L	320	560	520	AB	NR	Y	NY	-	A	A	A	-
AG 37	32,000	6	F	L	29,120	AB	AB	AB	NY	-	-	-	A	-	-	-
AG 38	19,000	2	F	H	1333	680	AB	AB	NR	NR	-	-	NA	A	-	-
Total	38,653	8,400	2,600	1,800					2:1	2:1	100%		1:1	2:1	100%	

AB= Absent, NA= No amplification, NR=no result, SES=Socio economic status, Y=resistance strain, N= wild type, N/Y= mixed infection, Adult= MPPCI mean Population Per capital Income= N 5445.11,<- N5445.11=Poor, >= N5445.11 = Rich H= high=Low, I=Income per month

Table 3: Malaria treatment behavior of the adults by Monthly Income Status

Choice of treatment	Monthly Income Status		
	High	Low	Total
Hospital/Clinic	9(22.5)	4(10.0)	13(32.5)
Maternity Home	0(0)	0(0)	0(0)
Chemist	4(10.0)	6(15.0)	10(25.0)
Drug store/tray	1(2.5)	2(5.0)	3(7.5)
Self medication	4(10.0)	9(22.4)	13(32.5)
Traditional healer	-	1(2.5)	1(2.5)
Total	18(45.0)	22(55.0)	40(100.0)

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References

1. Al-Mekhlafi AM, Mahdy MAK, Al-Mekhlafi HM, Azazy AA, Fong MY (2011). High frequency of *Plasmodium falciparum* chloroquine resistance marker (*pfcrt* T76 mutation) in Yemen: an urgent need to re-examine malaria drug policy. Parasit Vectors 4:94
2. Annie-Claude L, Samir PC and Kelvin CK (2003) Molecular surveillance system for global pattern of drug resistance in imported malaria Emerging Infectious Disease Vol 9, No. 1 33-36
3. Corbel V, Henry MC (2011). Prevention and control of malaria and sleeping sickness in Africa: Where are we and were we are we going? Parasitology & Vectors
4. Cremer G, Basco L.K, Le Bras J, Camus D, Slominanny C, (1995) *Plasmodium falciparum*: detection of P-glycoprotein in chloroquine-susceptible and chloroquine resistant clones and isolates. Experimental Parasitology 81: 1-8
5. Djimde A, Doumbo O.K, Cortese JF, Kayentao K, Doumbo S, Diourte Y, Dicko A, Su X-Z, Noruma T, Fidock DA, Wellems TE, Plowe CV (2001) a molecular marker for *Chloroquine* resistance *falciparum* malaria. New England Journal of Medicine 344: 257-263
6. Durasingh MT, Jones P, Sambou I, Von Seidlein L, Pinder M, Washurt DC, Doumbo T, Fidock S, Diourte Y, Dicko AD, Su X, Nomura TE, Plowe CV, (1997). The tyrosine 86 allele of the *pfmdr1* gene of *Plasmodium falciparum* is associated with increased sensitivity to the antimalarials
7. Efunshile M, Abiodun TR, Ghebremedhin B, Konig W, Konig B (2011). Prevalence of the molecular marker of chloroquine resistance (pfcrt 76) in Nigeria 5 years after withdrawal of the drug as first-line antimalarial: A cross sectional study. South Africa Journal of Child Health Vol. 5, No 2
8. Farcas GA, Soeller R, Zhong K, Zahirieh and Kain KC (2006) Real time polymerase Chain reaction assay for the rapid detection and characterization of Chloroquine –resistant *Plasmodium falciparum* malaria in returned travelers Clinical infectious Diseases 42:622-7
9. Fidock, DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos L.M, Sidhu AB, Naude B, Deitsch KW, Su X-Z, Wotton JC, Roepe PD and Wellems TE, (2000) mutation in the *Plasmodium falciparum* digestive vacuole transmembrane protein *pfcrt* and evidence of their role in *Chloroquine* resistance. Molecular & cell 6:861-871.
10. Filmer D (2002) Fever and its treatment in the more and less poor in sub-Saharan Africa Washington DC: World Bank
11. Foote SJ, Kyle DE, Martin Rk, Oduola A.M, Forsyth K, Kemp DJ, Cowman A.F, (1990) several alleles of the multidrug resistance genes are closely linked to Chloroquine resistance in *Plasmodium falciparum.* Nature 345:255-258
12. Happi TC, Thomas SM, Gbotosho GO, Falade CO, Akinboye DO, Gerena L, Hudson T, Sowunmi A, Kyle

DE, Milhous W, Wirth DF, Odula AMJ (2003) point mutation in the *pfcrt* and *pfmdr1* genes of *Plasmodium falciparum* and clinical response to Chloroquine, among malaria patients from Nigeria Annual Tropical Medicine & Parasitology Vol.5. 439-451

13. Hviid L (2007): Adhesion specificities of *Plasmodium falciparum*-infected erythrocytes in the pathogenesis of pregnancy associated malaria. American Journal of Pathology 170:1817-1819
14. Jourbert F, Harrison CM, Koegelenberg RJ, Odendaal CJ, de Beer TA (2009). Discovery: an interactive resource for the rational selection and comparison of putative drug target proteins in malaria. Malaria Journal, 8:178
15. Ketema T, Getahun K, Bacha K (2011). Therapeutic efficacy of chloroquine for treatment of Plasmodium vivax malaria cases in Halaba district, South Ethiopia. Parasit Vector
16. Pelletier DL, (1994). The relationship between child anthropometry and mortality in developing countries implication for policy, programmes and future research Am J Clin Nutri 52:391
17. Plowe CV, Roper C, Barnwell JW, Happi CT, Joshi HH, Mbacham W, Meshnick SR, Mugittu K, Naidoo T, Price RN, Shafer RW, Sibley CH, Sutherland CJ, Zimmerman PA, Rosenthal PJ (2007). World Antimalarial Resistance Network (WARN) III: Molecular markers for drug resistant malaria. Malaria Journal 6:121
18. Sachs J, Malaney P (2002). The economic and social burden of malaria. Nature 415:680-685
19. Slater AFG (1993). Chloroquine: mechanism of drug action and resistance in *Plasmodium falciparum.* Pharmacology and Therapeutics. 57:203-235 (Cross ref.) (Medline)
20. *Sowunmi CO, Adedeji AA, Falade CO, Happi TC, and Oduola Am (2001) randomized comparison of Chloroquine and amodiaqiune in the treatment of acute, uncomplicated Plasmodium falciparum malaria in Children Ann Trop Med & Para. 95:549-558.*
21. Ward SA, Bray PG, Mungthin M and Hawley (1995). Current views on the mechanisms of resistance to quinoline-containing drug in *Plasmodium falciparum* Ann. Trop Med Parasitol 89:121-124 (Medline)
22. Wilson CM. Serrano AE, Wasley A, Bogenschutz MP, Shankar AH, Wirth DF (1998) amplification of a gene related to mammalian *mdr-1* genes in drug resistant *Plasmodium falciparum*. Science 24: 1184-1186.