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Chloroquine Resistance and Host Genetic Factors among Nigerian Children with Uncomplicated P. falciparum Infection

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Abstract

Chloroquine resistance and host genetic factors among Nigerian children with uncomplicated *P.falciparum* infection.

Background: Chloroquine resistance is widespread. Host factors play a role in drug resistance. The study aims to determine the association between hosts red cell factors namely G6PD status, ABO blood group and haemoglobin genotype with *Pfmdr1* mutations and treatment outcome.

Methods: One hundred and twenty patients (aged 1-15years) with acute symptoms of *P.falciparum* malaria were treated with chloroquine (CQ). Blood sample was obtained for haematological parameters and analysis of parasite DNA. Nested PCR followed by restriction fragment length polymorphism (RFLP) at codon 86 of the *Pfmdr1* gene was determined in the 120 isolates. Relationship between *Pfmdr1* alleles, CQ resistance, G6PD deficient, sickle cell trait and parasite diversity was evaluated.

Results: Seventy of the 120 patients enrolled into this study completed the 14-day follow-up, aged 7.8 ± 4.6 years, 62 (52%) were females with parasites geometric mean 21,861 (range 1000-354,667) asexual parasites per microlitre of blood. Thirty-seven of the 70 (53%) were cured while 47% failed chloroquine treatment; mean parasite clearance time (PCT) was 7.1 days, whereas the mean fever clearance time was 3.4 days. Mutation revealed wild type N86 (56), mutant type Y86 (50) and mixed genotype N86+Y86 (14). No significant correlation was found between *Pfmdr1* mutations and *in-vivo* outcome (p=0.21). About 51% (36/70) of the *in-vivo* pre-treatment samples carried the *Pfmdr1* Y86 mutation and its prevalence varied with age (p=0.017). The *Pfmdr1*Y86 mutation was detected in majority (19/33) of patients with clinical failure OR=1.711; 95% CI: 0.517-5.668; p=0.378 and was more in younger OR=1.200; 95% CI: 0.782-1.840 than in older children OR=0.818; 95% CI: 0.507-1.321.

Conclusion: There is an association between *Pfmdr1*Y86 mutation and CQ treatment failure which tends to be stronger in younger children. These suggest the role of age and host immunity in modifying the relationship between molecular markers and resistance.

Introduction

Malaria remains a significant health problem in Nigeria with *Plasmodium falciparum* being the predominant species. It is responsible for 25% of infant mortality, 30% of childhood mortality and about 50% of outpatient visits [1]. Chloroquine which was once the mainstay of treatment of uncomplicated falciparum malaria is now threatened by resistance. Chloroquine-resistant parasites are present in most areas where malaria is endemic [2]. Consequently, the decline in efficacy of chloroquine has led to the use of alternative antimalarials, such as antifolates, mefloquine and artemisinin derivatives. However, parasite resistance to these drugs except artemisinin, is also becoming a real problem

[3]. The present reality is that many African countries with high rates of chloroquine-resistant malaria continue to use chloroquine for the treatment of malaria [4]. This is largely due to concerns that alternative drugs are either too expensive, toxic or relatively unavailable. Partial immunity in older children and adults in endemic areas enhances its antiparasitic effect [5, 6]. Chloroquine, despite the shift to artemisinin based therapy still remains a reference antimalarial drug and is still effective in the treatment of vivax malaria [7,8].

Several studies have suggested among other factors that mutations in two genes - *P. falciparum* chloroquine-resistant transporter (*Pfcrt)* and *P.falciparum* multidrug resistant (*Pfmdr1)* genes are associated with chloroquine resistance [9-11]. *Pfcrt* was identified and reported by Fidock et al. [12]. The resistance was associated with a reduced accumulation of chloroquine in the parasite digestive vacuole [13] but how the *pfcrt* gene exerts such an effect on the digestive vacuole is largely unclear. An acquired efflux system of chloroquine [14] and/or an increased acidity in the digestive vacuole [15] is assumed to play a role for the reduced accumulation. A lysine to threonine change at position 76 (K76T) has been found in every *in-vitro* chloroquine-resistant parasite from around the world [12, 16, 17]. Many studies have confirmed the presence of K76T mutations in the *in-vivo* chloroquine resistance but this mutation was not the sole requirement [11, 16, 18-20] suggesting that host factors affect the clearance of chloroquine-resistant parasites [11, 21].

Another extensively investigated gene is the *P. falciparum* multi-drug resistance 1 (pfmdr1), a homologue of the mammalian multiple drug resistance gene encoding a P-glycoprotein on the chromosome 5 of the *P.falciparum*. It is a typical member of the ATP-binding cassette transporter superfamily localized to the parasite vacuole, where it may regulate intracellular drug concentrations [22]. Mutation was observed at the 86, 184, 1034, 1042, and 1246 positions, which were strongly linked to the chloroquine resistance in laboratory clones obtained from various regions [9]. Several field studies indicated the positive association between the asparagine to tyrosine change at position 86 (N86Y) and the chloroquine resistance both *in-vitro* [10, 23-25] and *in-vivo* [26-28]. However, other studies have cast doubts on this association [18, 29]. Currently, *Pfmdr1* mutations are said to assist the chloroquine-resistant parasites by augmenting the level of resistance [30]. Although mutations in the *Pfcrt* gene appear to be necessary for chloroquine resistance *in-vitro* and for chloroquine treatment failure *in-vivo*, other factors such as host immunity and other parasite genes, including *Pfmdr1* are important in determining clinical outcome.

Certain genetically determined red cell factors which include sickle cell trait, ABO blood group antigens and glucose-6-phosphate dehydrogenase (G6PD) deficiency have been reported to confer some degree of resistance against malaria parasite infection [31-33] and chloroquine uptake in red cells [34]. Host genetic factors account for about one quarter to one third of the total variation in susceptibility in the populations to malaria, and household-related factors (i.e. environmental factors) contribute to a similar level. Genetic factors also appear to underlie some striking differences in resistance to malaria that have been observed between ethnic groups who live in the same area. For example, the Fulani of Burkina Faso, were found to have a lower prevalence of malaria parasitaemia and fewer clinical attacks of malaria than other ethnic groups who lived in closely neighboring villages [35]. Although the genetic makeup of the parasite is almost certainly a determining factor, there may be a wide variety of subtle immunological adaptations that occur after birth, which affect how an individual will respond when first exposed to infection [36]. Chloroquine resistance phenotype may therefore not be sufficiently based on allelic polymorphisms of one gene alone but multigenic in nature. Furthermore, interplay of several mutations and some co-factors as well as host genetic alterations should be considered.

We hereby present reports on the relative association between host's red cell factors namely G6PD status, ABO blood group and haemoglobin genotype with Pfmdr1 mutations and treatment outcome.

Subjects and Methods

Study location

The main recruitment centers were University College Hospital and Abanla Health Centre located 20 km south of Ibadan city. The study protocol was reviewed and approved by the Joint University of Ibadan/University College Hospital ethical committee. Informed consent was obtained from parents or legal guardians of children enrolled into the study.

One hundred and twenty patients (aged 1-15 years) presenting with acute symptoms of *P.falciparum* malaria were enrolled into the study. Inclusion criteria included fever or a history of fever (axillary temperature ≥37.5°C) within the past 24 - 48 hours preceding presentation, microscopically proven *P.falciparum* malaria, parasite density ≥1000 parasites per microlitre of blood, residence within an accessible address and agreement to return to the clinic for follow-up, uncomplicated malaria with absence of symptoms of severe malaria or danger signs, no history of antimalarial drug ingestion in the two weeks preceding presentation and negative urine test result for 4-aminoquinolines (Dill-Glazko). Patients were excluded if they had history of antimalarial drug ingestion in the two weeks preceding presentation, had concomitant illness at presentation or during the follow-up period, transfusion within the last 3 months and withdrawal of consent by parent or guardian. While 120 patients were enrolled into this study, 70 completed the 14-day follow-up.

Treatment and classification Criteria

Patients were treated with CQ (Nivaquine™ from May & Baker, Lagos, Nigeria) (25 mg/kg body weight) in divided doses of 10, 10, and 5 mg/kg body weight for 3 consecutive days. 2 ml blood sample was withdrawn intravenously on day 0 into EDTA bottles for haematological parameters. Finger prick blood samples were collected on days 0, 3, 7, and 14 spotted onto 3mm filter paper (Whatmann International, Ltd., Maidstone, United Kingdom) for extraction and analysis of parasite DNA. The filter paper samples were stored in air-tight water proof bag at 4˚C until used.

Classification of response to treatment was based on

parasitological outcome:RIII: no reduction in parasitaemia, or reduction to >25% of day 0 level, by day 3.

- RII: reduction in parasitaemia to <25% of day 0 level without clearance leading to re-treatment or followed by persistent parasitaemia.
- RI: Initial clearance of parasites indicated by negative thick smear after day 0, with subsequent positive thick smear by day 14.
- Sensitive: clearance of parasites by day 14 with no recurrence of parasitaemia [3, 37].

Assessment of parasitaemia

Giemsa-stained blood films obtained from the patients were examined by light microscopy under an oil-immersion objective. Parasitaemia in thick films was estimated by counting the number of asexual forms of *P.falciparum* corresponding to 200 leucocytes. The parasite density was calculated by assuming a leucocyte count of 8000/µl of blood.

Determination of hematological parameters

Blood samples in EDTA bottles were used to determine haematocrit, leucocyte counts and mean corpuscular volume by coulter cell counter. ABO blood groups were determined using standard methods.

Determination of glucose-6-phosphate dehydrogenase status

Glucose-6-phosphate dehydrogenase (G6PD) status was assessed by fluorescent spot test as previously described [38]. The test is based on the principle that NADPH generated in red blood cells in the presence of G6PD fluoresces under long wavelength ultraviolet (UV) light.

Haemoglobin genotype was determined on cellulose acetate membrane at pH 8.6 as described by Kohn [39].

Preparation and amplification of DNA, RFLP

Parasite DNA was extracted from filter paper using the Chelex method as described by Djimde et al. [11]. The detection of the *Pfmdr1* N86Y mutations was done using nested PCR followed by restriction fragment length polymorphism (RFLP). The primary amplification was done using primers MDR1 5′- GCG CGC GTT GAA CAA AAA GAG TAC CC -3′ and MDR2 5′-GGG CCC TCG TAC CAA TTC CTG AAC TC -3′. 5 µl of the DNA was used as template in a 25 µl reaction mixture containing 1 µM of primers, 1X PCR buffer (Sigma), 2.5 mM $MgCl_2$ (Sigma), 0.2 mM dNTPs (PROMEGA) and1U of Taq DNA Polymerase (New England Biolabs) and made up with double-distilled water. The samples were incubated for 3 minutes at 95°C for denaturation prior to cycles (95°C for 30 secs, 45°C for 30 secs, and 65°C for 45 secs). After 44 cycles, primers extension was continued for 15 mins at 72°C. For the secondary amplification (nested PCR), 2 µl of the primary product was further amplified under same conditions as the primary amplification. After 34 cycles, primer extension was continued for 15 mins at 72°C. Ten microlitre (10 µl) of the PCR products were resolved by electrophoresis on a 2% agarose gel, stained with ethidium bromide and sized against a 100-base pair (bp) molecular weight marker (New England Biolabs, Beverly, MA). Bands were seen at 300 bp for successful amplifications.

Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism analysis was performed to detect single-base changes in the PCR products. The PCR product was digested by the mutation specific restriction enzyme AFL III according to the manufacturer's instructions (New England Biolabs, Beverly, MA, USA) with subsequent electrophoresis in an ethidium bromide-stained 2% agarose gel and sized against a 100 bp molecular weight marker. 3D7-clone DNA was used as the wild-type control and Dd2 as the mutant control. Bands were seen at 300bp for samples having the wild-type allele (N86) and 110 bp and 190 bp for the mutant (Y86).

Identification of recrudescent infections using the MSP1 marker

Pre- and post-treatment (smear-positive) samples were genotyped using the highly polymorphic loci of MSP1 using the PCR-based method described by Snounou et al. [40]. Briefly, 5 µl of the extracted DNA was amplified using 1 µM of primers O1 5′-CACATGAAAGTTATCAAGAACTTGTC-3′ and O2 5′- GTACGTCTAATTCATTTGCACG-3′ in a 25 µl reaction mixture containing 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1X PCR Buffer and 1U Taq Polymerase. An initial denaturation was done at 94°C for 3 mins, followed by another denaturation for 30 secs, primers were annealed at 50°C for 45 secs and extension was carried out at 68°C for 2mins. After 30 cycles, a final extension was done at 72°C for 3 mins. A second round PCR was done by using 2 µl of primary product in a 25 µl reaction mixture containing 1 µM of primers N1 5′-GCAGTATTGACAGGTTATGG-3′ and N2 5′-GATTGAAAGGTATTTGAC-3′ , 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1X PCR buffer and 1U Taq polymerase. The PCR conditions were same as used for the primary reaction. 10 µl of the secondary product was resolved in a 1.8% agarose gel, stained with ethidium bromide and sized against a 100-bp ladder. Bands seen for MSP1 ranged between 450-550 bp.

Results

One hundred and twenty patients were enrolled into the study with age range 1-15 years old. Sixty-two (52%) of the patients were females while the rest were males. Mean age of the patients was 7.8 ± 4.6 years, parasitaemia ranged from 1000 to 354,667 asexual parasites per microlitre of blood with a geometric mean of 21,861 parasites. Their mean weight was 17.9 ± 7.4 kg, mean axillary temperature was 38.3 ± 1.3°C, mean packed cell volume (PCV) was 30.29 ± 7.1%, and a mean white blood cell count (WBC) of 6973 ± 3633.

Patients and in vivo treatment outcome

Out of the 120 patients enrolled into the study, 70 were successfully followed up to day 14 after being treated with standard doses of CQ. 37 of the 70 (53%) were cured with CQ. The mean parasite clearance time (PCT) was 170.4 ± 102.7 hrs (7.1 days), whereas the mean fever clearance time was 81.6 ±

39.8 hrs (3.4 days). The 33 (47%) who failed treatment to CQ were classified accordingly as follows; RI (6), RII (21) and RIII (6).

Prevalence of Pfmdr 1 mutation in pre-treatment isolates of patients with *P.falciparum* infection

Nested PCR followed by restriction fragment length polymorphism (RFLP) was done to determine polymorphisms at codon 86 of the pfmdr1 gene. Polymorphism was successfully determined in the 120 isolates and classified thus: wild type N86 (56), mutant type Y86 (50) and mixed genotype N86+Y86 (14) as shown in

Pfmdr1 mutation and in vivo treatment outcome

For statistical analysis, mixed infections harboring both the wild and mutant alleles (N and Y) were classified as mutant. Relationship between pfmdr1 alleles and CQ resistance was determined by chi square. The risks of having resistant or sensitive infection alleles among G6PD deficient, sickle cell trait and parasite diversity was evaluated using odds ratio. No significant correlation was found between pfmdr1 mutations and *in vivo* outcome (p=0.21). About 51% (36/70) of the *in vivo* pre-treatment samples carried the Pfmdr1Y86 mutation and its prevalence varied with age (p=0.017). The pfmdr1Y86 mutation was detected in majority of patients (19/33) with clinical failure OR=1.711; 95% CI: 0.517-5.668; p=0.378 but did not reach statistical significance. The risk of having the pfmdr1 Y86 mutation was more in younger (OR=1.200; 95% CI: 0.782-1.840) than in older children (OR=0.818; 95% CI: 0.507-1.321).

Recrudescence and clonality of infection

Twenty-five matched pre- and post-treatment samples from patients who failed treatment were successfully amplified using msp1. DNA in the pre-treatment isolates were compared with DNA from post treatment isolates at recrudescence of infection. Post-treatment and primary infection parasites showing identical DNA bands were considered as recrudescence and hence true treatment failure, while non-identical bands indicated a new infection. The sizes of msp1 allele observed ranged from 450-550 bp but the 500 bp allele was most common. Polyclonal infections were found in one-third (8) of the samples while the rest were monoclonal. Recrudescent infections were observed in 88% (22) of the samples while re-infection was found only in three samples. Relationship between *in vivo* treatment outcome (R) and parasite diversity was tested using the Pearson's chi square test and a highly significant association was found (p=0.000). This result shows that most *P.falciparum* infections after treatment with standard dose of chloroquine were true recrudescence.

The relationship between pfmdr1 alleles and ABO blood group, Hb genotype and G6PD status was determined using the Pearson chi-square and no significant association was found (p>0.05).

Pfmdr1 versus clinical parameters

Clinical parameters namely fever, anaemia, spleen and liver size were measured. Fever was defined as temperature greater or equal to 37.5°C, while anaemia was defined as PCV less than 33%.

From the results, there was no evidence of an association between *in vivo* treatment outcome and fever or anaemia. No correlation was also found between treatment outcome and host factors (p values >0.05).

Relationship between pfmdr1 mutation and parasite density on day 0

Relationship between pfmdr1 mutation and parasite density on day 0 was determined using student t-test. Higher parasite density (geometric mean=38,467) was observed more in isolates harbouring pfmdr1 Y86 mutation than in those with pfmdr1 N86 (geometric mean=17,268). However, this did not reach statistical significance (p=0.10).

Relationship between In vivo treatment outcome and parasite density on day 0

The relationship between treatment outcome and parasite density on day 0 was also determined using Student t-test. Patients who were resistant to CQ were found to have relatively higher parasite densities (geometric mean=30,229) than those sensitive to CQ (geometric mean=16,893) but this did not reach statistical significance (p=0.13).

A statistically significant correlation was observed between parasitaemia on day0 and *in vivo* outcome (r=-354; p=0.004) **(Tables 1-10).**

Discussion

Chloroquine which was once the most preferred antimalarial drug in terms of cost, availability, safety and efficacy is currently overwhelmed with the tragedy of drug resistance and this has rendered it unpopular. In spite of its reduced efficacy, CQ however is still widely available and some people in African countries continue to use it for the treatment of malaria.

In this study, the role of genetic polymorphisms in mediating the clinical and parasitological outcome of children treated with standard dose of CQ was investigated. The enrolled children were from the urban and rural (Abanla) areas of Ibadan, southwest Nigeria. Previous studies reported that the frequency of usage of antimalarials particularly CQ, was considerably lower in Abanla compared with Ibadan [41]. The increasing failure rates of several antimalarial drugs in the majority of malaria-affected areas means that close monitoring of the epidemiology and dynamics of drug resistance are necessary if we are to implement measures to circumvent the problem.

This study revealed a 47% failure rate of children to CQ treatment which is in agreement with previous report [42]. However, majority of those who failed CQ treatment had high grade (RII) resistance. Although a high (35%) frequency of CQ resistance among *P.falciparum* malaria cases in Ibadan was earlier reported by [43], the present results show a continuous deterioration in CQ efficacy in this area. This was also further confirmed by molecular genotyping.

Ibadan has been reported to be holoendemic for malaria (an area of intense transmission) which poses a serious limitation to the *in vivo* test [44]. However, malaria researchers have overcome this limitation by taking advantage of current advances in

Table 1. Demographic and baseline data of Nigerian children with uncomplicated *P.falciparum* infection treated with chloroquine to assess chloroquine resistance and host genetic factors.

Parameter	Values
No of Patients	120
Sex	
Female	62
Male	58
Age (yrs)	
Range	Jan-15
Mean	7.8 ± 4.6
Parasite Density (Parasites/µl of blood)	
Range	1000 – 354,667
Geometric Mean	21,861
Proportion with parasitaemia ≤ 100,000	92%
Proportion with parasitaemia > 100,000	8%
Weight (kg)	
Range	8 – 38
Mean	.9 ± 7.4
Axillary Temperature (ºC)	
Mean	38.3 ± 1.3
Packed Cell Volume (%)	
Mean	30.3 ± 7.2
Range	14 - 48
White Blood Cell count	
Mean	6973 ± 3633
Range	1,600 – 21,300

Table 2. *In vivo* treatment outcome among Nigerian children with uncomplicated malaria treated with chloroquine to assess chloroquine resistance and host genetic factors.

Outcome	Frequency
Cured (S)	37 (53%)
RI	6 (8.5%)
RII	21 (30%)
RIII	6 (8.5%)
PCT (days)	7.1 ± 4.3
FCT (days)	3.4 ± 1.7

Table 3. Prevalence of *Pfmdr* 1 mutation in pre-treatment isolates among Nigerian children with uncomplicated *P.falciparum* infection treated with chloroquine to assess chloroquine resistance and host genetic factors.

Genotype	Frequency (%)
Pfmdr1 codon 86 Asn (N)	56 (46.7)
Tyr (Y)	50 (41.7)
Asn + Tyr (N+Y)	14 (11.6)
Total	120 (100)

$\chi 2=1.07$; p=0.30

Plasmodium biology and PCR technology to determine parasite genotypes in pre-treatment and post-treatment samples of patients clinically classified as treatment failures. In this study, the impact of chloroquine treatment on the dynamics and multiplicity of infection was evaluated in pre- and post-treatment *P.falciparum* isolates using PCR genotyping of MSP 1. The results showed that 88% of the infections in children who failed CQ

Table 4. Distribution of host red cell factors among Nigerian children with uncomplicated *P.falciparum* infection treated with chloroquine to assess chloroquine resistance and host genetic factors in children with *P. falciparum* infection.

Parameters Blood Group	Frequency (%)
A+	21 (17.5)
B+	27 (22.5)
O+	62 (51.7)
O-	5 (4.2)
AB+	1 (0.8)
AB-	1 (0.8)
B-	2 (1.7)
A-	1 (0.8)
Hb genotype	
AA	95 (79.1)
AS	20 (16.7)
AC	5 (4.2)
G6PD status	
Normal	109 (91%)
Deficient	11 (9%)

Table 5. *Pfmdr1* mutation and *in vivo* treatment outcome among Nigerian children with uncomplicated *P.falciparum* infection treated with chloroquine.

Treatment Outcome			
Pfmdr1	Cured	Resistant	Total
N86	20(59%)	14(41%)	34(100%)
Y86	17(47%)	19(53%)	36(100%)
Total	37	33	70

Table 6. Association between age stratification, *pfmdr1* mutation and *in vivo* outcome among Nigerian children with uncomplicated *P.falciparum* infection treated with chloroquine.

OUTCOME					
Age Grp	Pfmdr1	R (%)	S%	Total	P-Value
(<5yrs)	N86	3 (25)	9 (75)	12 (100)	
	Y86	15 (75)	5 (25)	20 (100)	0.017
	Total	18 (100)	14 (100)	32	
≥5yrs	N86	9 (41)	13 (59)	22 (100)	
	Y86	6(38)	10 (62)	16 (10)	0.271
	Total	15(100)	23 (100)	38	

treatment were truly resistant during a 14-day follow up period with similarity in the parasite genotypes from pre- and post-treatment samples. Three different groups of parasite population were observed among patients that failed CQ treatment. The first group had identical MSP1 genotypes present in both pre- and post-treatment isolates, suggesting that all these were resistant parasites. The second group of parasite populations showed the presence of only a subset of the pre-treatment parasite population in the post-treatment in addition to new ones. This indicates that a mixed population of malaria parasite strains (sensitive and resistant) exists in pre-treatment isolates in this group. While the antimalarial drug on administration would kill the sensitive strains, the resistant strains become more virulent and multiply later, which is observed in the post-treatment isolates in the MSP1 genotyping. The third group of parasite

Table 7. Distribution of *Pfmdr1* mutation and host red cell factors among Nigerian children with uncomplicated *P.falciparum* infection treated with chloroquine.

Parameter	Y86 (%)	N86 (%)	Total
Blood group*A+	10(48)	11 (52)	21 (100)
B+	16 (59)	11 (41)	27 (100)
O+	34 (55)	28 (45)	62 (100)
O-	3 (60)	2 (40)	5 (100)
AB+	0 (0)	1 (100)	1 (100)
AB-	0 (0)	1 (100)	1 (100)
B-	0(0)	2 (100)	2 (100)
A-	1 (100)	0 (0)	1 (100)
Hb genotype**		47	
AA	48 (75)	(83.9)	95
AS	12 (18.8)	8 (14.3)	20
AC	4 (6.2)	1 (1.8)	5
G6PD*** `			
Normal	57 (89.1)	52 (92.9)	109
Deficient	7 (10.9)	4 (7.1)	11

*p=0.66; **p=0.63; ***p=0.69

Table 8. Distribution of *Pfmdr1* mutation and clinical parameters at enrollment among Nigerian children with uncomplicated *P.falciparum* infection treated with chloroquine.

		Pfmdr1		
Parameter	Yes/No	N86 (%)	Y86 (%)	Total
Fever	Yes	40(71.4)	45(70.3)	85
	No	16(28.6)	19(29.7)	35
Anaemia	Yes	44(78.6)	41(64)	85
	No	12(21.4)	23(36)	35

population is infections that could be attributed to new infections that were not observed in the pre-treatment isolates. However, an alternative explanation for this is that some of the infections detected in the post-treatment samples were not detected in the pre-treatment isolates as they may have been released into the circulatory system from the liver stage after the pre-treatment sample was collected. An explanation for this could be the fact that infection in this area is polyclonal. It is also possible that the new infection was a minor population of *P.falciparum* that was not detected by PCR in the analysis of pre-treatment isolates and later became a major parasite population in the post-treatment isolates, thus became detectable by PCR.

Reports on the association between pfmdr1 genotypes and quinoline resistance have not been very convincing. Some

Table 9. Treatment outcome versus Host red cell factors among Nigerian children with uncomplicated *P.falciparum* infection treated with chloroquine.

	OUTCOME		
Parameter	R (%)	S (%)	Total
Blood group* A+	8 (24.2)	10 (27)	18
B+	6 (18.2)	7 (18.9)	13
O+	16 (48.5)	18 (48.7)	34
O-	2 (6.1)	2 (5.4)	4
AB+	1 (3.0)	0 (0)	1
Hb Genotype ** AA	29 (87.9)	30 (81.1)	59
AS	3 (9.1)	7 (18.9)	10
AC	1 (3.0)	0 (0)	1
G6PD*** Normal	33(100)	36 (97.3)	69
Deficient	0 (0)	1 (2.7)	1

*p=0.99; **p=0.45; ***p=0.57

Table 10. Treatment outcome versus clinical parameters among Nigerian children with uncomplicated *P.falciparum* infection treated with chloroquine to assess chloroquine resistance and host genetic factors.

		OUTCOME		
		R (%)	S (%)	Total
Fever*	Yes	22 (66.7)	28 (75.7)	50
	No	11 (33.3)	9 (24.3)	20
Anaemia**	Yes	18 (54.5)	28 (75.7)	46
	No	15 (45.5)	9 (24.3)	24

researchers suggested that pfmdr1Y86 can be correlated with increased CQ resistance in parasites which originated from different areas of the world [9, 10, 23, 45], while other field studies have not corroborated these findings [24, 46, 47]. However, the results of a *P.falciparum* genetic cross indicated that CQ resistance did not depend on the pfmdr1 gene [48]. Based on gene knock out and transfection studies, it has been proposed that pfmdr1 alone is not sufficient to provide chloroquine resistance but it may help in the developmental process of drug resistance [49, 50].

In this study, there appears to be an association between pfmdr1 Y86 mutation and CQ treatment failure but this did not reach statistical significance (p>0.05). The strength of this association of Y86 with CQ treatment failure was found to be higher in children <5years of age than in older children. The stronger association observed in younger children confirms the role of host immunity in modifying the relation between molecular markers and resistance [11, 51]. Indeed, the study of the association of *Pfmdr1* and *Pfcrt* alleles showed that CQ resistance is more dependent on the *Pfcrt* T76 but not on that of *Pfmdr1* Y86 [11]. Findings from this study suggest slight but not complete implication of *Pfmdr1* Y86 in CQ resistance in Nigeria, since the presence of both N and Y alleles was slightly largely dependent on their CQ response, indicating that CQ appears to exert selective pressure on this area of the gene. The isolates also having been drawn from both the urban and rural settings could be a better representative of the general situation in this part of Nigeria. The results are consistent with other studies of pfmdr1 mutations among *P. falciparum* CQ-

resistant strains conducted in Malaysia [52], Guinea-Bissau [53], Nigeria [54] and sub-Saharan Africa [22]. These data, however, are in conflict with reports of lack of association between *Pfmdr1* mutations and CQ resistance in *P. falciparum* using field isolates from Sudan [47], Thailand [55], Indonesia [27] and laboratory strains [56]. The highest frequency of the host genetic factors were found in O+(52%) among the ABO blood groups, AA (79%) among the Hb genotypes and G6PD normal (91%) for G6PD status. The prevalence of the various blood group antigens is in agreement with previous reports [57, 58]. Chi-square test for an association between *Pfmdr1* alleles (N86 and Y86) and G6PD status, ABO blood group or Hb genotype showed no significant association (p values >0.05). Although no significant association was found between treatment outcome and G6PD status, ABO blood group or Hb genotype, there was a greater risk (odds ratio 2.3) of developing resistance to chloroquine in the HbAA individuals than those having HbAS. Because nearly all CQ treatment failures were recrudescent, statistical comparisons between recrudescence and reinfection were not possible.

When the association between resistant infections and clinical parameters were tested independently using chi square, there was no trend towards an association between them (p>0.05). A strong association was however found between treatment outcome (resistance) and age ($\chi 2$=8.96; p=0.003). This result is in agreement with previous reports [11].

In conclusion**,** there appears to be an association between pfmdr1Y86 mutation and CQ treatment failure which tends to be stronger in younger children, thus suggesting the role of age and host immunity in modifying the relationship between molecular markers and resistance. ABO blood group, Hb genotype and G6PD status do not have any significant influence on treatment outcome and pfmdr1 polymorphisms but there is greater risk of developing resistance to CQ in HbAA than HbAS individuals. Parasite density at enrollment is associated with chloroquine treatment failure. Host red cell genetic factors known to be specific markers for malaria infection were not directly associated with Pfmdr1 Y86 allele.

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