RESISTANCE-MEDIATING POLYMORPHISMS OF *PLASMODIUM FALCIPARUM* AMONG ISOLATES FROM CHILDREN WITH SEVERE MALARIA IN KUMASI, GHANA.

H.H. ABRUQUAH[1], F.Y. BIO[1], S.C.K. TAY[2], AND B.W.L. LAWSON[3]
[1]University Health Services, Kwame Nkrumah University of Science & Technology, Kumasi, Ghana
[2]Department of Clinical Microbiology, School of Medical Sciences, KNUST, Kumasi, Ghana
[3]Faculty of Applied and Theoretical Biology, KNUST, Kumasi, Ghana

Corresponding Author: *Dr. Fred Yaw Bio* ***E-mail:*** *bioy97@yahoo.co.uk*

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SUMMARY

Background: Antimalarial drug resistance has been a major contributor to the failure of the battle against malaria in many developing countries. The *P. falciparum* genes, *pfcrt* and *pfmdr*-1, have been implicated in chloroquine resistance. The objective of this study was to determine the presence of mutant alleles of these chloroquine resistance genes among isolates of *P. falciparum* from children presenting with severe malaria in Ghana.

Methods: Venous blood samples were taken from patients, and plasma chloroquine levels measured. *P. falciparum* chromosomal DNA was isolated from the blood samples, and subjected to PCR, restriction digestion and sequencing. Resulting data were analysed using the STATA statistical software.

Results: Of 140 children recruited into the study, 109 (77.9%) had detectable pre-treatment chloroquine levels. PCR and restriction digestion analysis of the *pfcrt* gene indicated that 124 (88.6%) had the mutant T76 gene, and that this correlated with higher chloroquine levels. Sequence analysis of these showed consistent genetic sequences for chloroquine resistant and sensitive parasites with respect to Pfcrt codons 72 through 76.The Pfcrt T76 mutation was found in 88.4% of isolates having the Pfmdr-1Y86 mutation. The Pfmdr-1 Y86 mutation was found in 67.6% of isolates having the Pfcrt T76 mutation.

Conclusion: The study affirms Pfcrt as a better chloroquine resistance marker. Both mutations are independently selected by chloroquine levels and that one mutation (Y86) might modify/increase the effect of the other (T76). This study also depicts the much-overlooked antimalarial drug resistance situation in the area and emphasizes the need for a proper treatment strategy.

Keywords: *Plasmodium falciparum*, chloroquine (CHQ), *pfcrt*, *pfmdr*-1, mutation.

INTRODUCTION

Malaria affects approximately 40% of the world's population especially those living in the world's poorest nations.[1] Ninety per cent of deaths due to malaria occur in Africa, south of the Sahara, with majority of the cases being young children, with an African child dying of the disease every 30 seconds.[2]

Shortages of resources undeniably remain the significant obstacles to malaria control in several developing countries, but drug resistance has been a major additional contributor to the failure of the battle against the disease in many of these countries.[3] As a matter of fact, the continuous changing patterns of drug resistance necessitate the use of drugs that are more expensive and may have dangerous side effects. Artemisinin and its derivatives have, in the past decade, become the first line of treatment in some parts of the world.[4] However, their indiscriminate use for self-treatment of suspected uncomplicated malaria may also be ringing the alarm bell for the development of resistance to them in the ensuing decade.[5]

Resistance of *Plasmodium falciparum* to chloroquine (CHQ), the best known of the 4-aminoquinolones, was first noted in the late 1950s from Colombia and Thailand.[6] Reports of similar resistance patterns quickly followed from other countries in South America and South East Asia, with the first well-documented case of chloroquine-resistant *P. falciparum* in Africa, reported from Kenya in 1979 by Kean in a tourist.[6] Soon afterwards chloroquine resistance was seen in other countries in East Africa and later spread with increasing frequency throughout most of Africa.[6,7] Chloroquine resistance has since spread across Africa and now only a few countries in the tropics are unaffected. In Ghana, chloroquine resistance has been reported.[8]

Extensive research has revealed that resistance to chloroquine occurs via an active energy-dependent process of efflux of chloroquine from the *P. falciparum* parasite cells, and that this involves a number of genes, namely, *P. falciparum* multi-drug resistance (*pfmdr*-1) and *P. falciparum* chloroquine resistance transporter (*pfcrt*) genes which are located on chromosomes 5 and 7 respectively.[6,9-12] A mutation of Asparagine to Tyrosine at amino acid position 86 (N86Y) of the *pfmdr*-1 with or without other mutations at other positions within this gene correlate well with chloroquine resistance, whereas within the *pfcrt* gene, a mutation of Lysine to Threonine at amino acid position 76 (K76T) with or without other mutations within this gene have also been implicated.[6,10-12]

In Ghana, malaria is the most frequent cause of morbidity and mortality and constitutes approximately 40% of all out-patient department diagnosis across the country, with 25% of all childhood deaths attributable to malaria.[13,14] Early treatment failure rates with chloroquine in five out of six districts studied in Ghana were above 25%, with three of them above 45%.[15,16]

The study was carried out to elucidate the presence of genetic markers of chloroquine resistance in *P. falciparum* strains in relation to plasma chloroquine levels among infants presenting to the Komfo Anokye Teaching Hospital with severe malaria.

MATERIALS AND METHODS

Study design

This study was carried out during the end of the high malaria season and the beginning of the low malaria season i.e. November to February, at the Komfo Anokye Teaching Hospital, a tertiary referral centre in Ghana's second largest city, Kumasi, with over 7,500 admissions per year and wards running at well over 150% bed occupancy (KATH Statistics, 2002). Paediatric patients, aged 4 to 120 months, were recruited into the study if they had clinical signs and symptoms suggestive of severe malaria based on World Health Organisation (WHO) criteria (17), and together with the presence of a blood film positive for asexual stages of *P. falciparum*. These included those with: unarousable coma, i.e. a Blantyre coma score of 2 or less; severe anaemia, of haemoglobin level of <5 g/dl or a haematocrit of <15%; severe respiratory distress, being the presence of intercostal and/ or sub-costal recession or deep acidotic breathing; prostration, being the inability to sit upright in a child normally able to do so, or if younger, an inability to drink or breastfeed.

Children with evidence of other diagnosis such as meningitis, pneumonia or renal failure were excluded from the study for ethical reasons. Detailed demographical data and clinical history were taken.

Blood samples, chloroquine levels and isolation of parasite DNA

In addition to the routine blood samples, 1 ml of venous blood was taken into lithium heparin tubes, and cellular and plasma components separated by centrifugation. The plasma was stored at –70°C, and an equivalent volume of 8 M urea/100 mM EDTA added to the red cell pellet and stored at 4°C.

Chloroquine levels in plasma were measured by an enzyme-linked immunosorbent assay (ELISA) method using monoclonal antibodies.[18] Chloroquine levels of >100 ng/ml indicated recent chloroquine use of therapeutic dosage. The lower limit of detection was 5 ng/ml. Chromosomal DNA of *P. falciparum* was isolated from the Urea/EDTA venous blood samples taken from patients recruited, using the DNeasy tissue kit (Qiagen) according to manufacturer's instructions.

PCR Amplification of *pfcrt* and *pfmdr*-1 genes

Polymerase chain reactions (PCRs) were done using Biometra® Thermocycler (*Biometra* - Göttingen, Germany). Seven (7) primer pairs were designed to amplify the whole of the *pfmdr*-1 gene at different points along its length. This was aimed at identifying new mutations that may exist in the Ghanaian strains of the *P. falciparum*. The segment of the *pfcrt* gene containing the polymorphisms at positions 72 through 76 was amplified through firstly an outer PCR, followed by a nested PCR with two primer pairs. All primers were designed using the 47573 base pair linear DNA sequence of the *P. falciparum* Chloroquine resistant strain Dd2/Indochina, as given by Pub Med accession number **AF030694**.

Restriction Digest of *pfcrt* gene amplicons

Enzymatic digestion of the resulting 145 base pair fragment at codon 76 of the *pfcrt* amplicons was done using *Apo*-1 restriction enzyme (New England BioLabs®) according to manufacturer's instructions. This yielded two fragments of 31 and 114 base pairs in the case of the parasite strains containing the wild K76 variant, whereas the mutant T76 variant yielded one fragment since there was no digestion.

Nucleotide sequencing

Sequencing of the resulting DNA amplicons was performed with *Taq* polymerase-catalyzed cycle sequencing using fluorescent-labelled BigDye terminator kits, according to manufacturer's instructions, in an ABI 310 Genetic Analyser (Perkin-Elmer, UK).

The aforementioned primers were used to sequence the amplicons in both the forward and reverse directions of both the *pfcrt* and the *pfmdr*-1 genes.

Bio-Informatic Analyses

The Sequencing Analysis software for analysis of raw data produced by the ABI Prism genetic analyzer instruments was used. The software calculates the electropherograms from multicolour traces, which allow the data to be inspected visually for mutations.

Using the ABI Prism genetic analysis software, the resulting sequences were compared to the 47573 basepair linear DNA sequence of the *P. falciparum* Chloroquine resistant strain Dd2/Indochina, given by PubMed accession number **AF030694**.

Statistical Analyses

Resulting data were analysed using the STATA data analysis and statistical software.

Ethical Clearance

Ethical clearance for the study was obtained from the Research and Ethics Committee of the School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. Written informed consent to participate was obtained from the parent/guardian of each child after explaining to him/her the study and possible outcomes besides specific treatment.

RESULTS

Demographic characteristics

Blood samples of 140 children, all diagnosed with severe malaria, were available for analysis. They comprised 55% (77) males and 45% (63) females. Their ages ranged from 4 months to 120 months, with a median age of 21 months and a mean age of 28.40 months.

Pre-treatment plasma chloroquine levels

Of the 140 children presenting with severe malaria to the Komfo Anokye Teaching Hospital, 109 (77.9%) had detectable pre-treatment plasma chloroquine levels, whilst the remaining 31 (22.1%) had no detectable plasma chloroquine.

Only 45 (32.1%) of them had therapeutic chloroquine levels of above 100 ng/ml indicating recent chloroquine use of therapeutic dosage, with the remaining 95 (67.9%) having sub-therapeutic chloroquine levels. The median chloroquine level was 68 ng/ml, with no significant differences in chloroquine levels with respect to age groups (p=0.002).

Analysis of molecular markers of chloroquine resistance

Parasite DNA was successfully extracted from all the 140 blood samples and analyzed for *pfcrt* and *pfmdr*-1 genetic markers of chloroquine resistance.

Analysis of *pfcrt* gene

Two rounds of PCR namely, outer and nested PCR, yielded a 145bp PCR product for each of the 140 samples. These products contained the codons of interest, 72 through 76, which were analyzed both by enzymatic digestion and sequencing. A photo of agarose gel electrophoresis of 46 samples is shown in Figure 1.

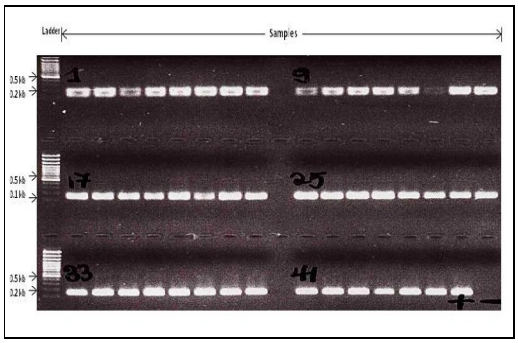

Figure 1 | Agarose gel electrophoresis photo of PCR amplification of the *pfcrt* genes of samples 1-46, positive and negative controls are indicated +/- respectively. All 46 samples shown in this photo yielded positive amplification of the *pfcrt* gene with a size of 145 bp. These PCR amplicons were later subjected to Apo-I enzymatic digestion.

Apo-1 enzymatic digestion of the resulting 145 bp fragment at codon 76 yielded two fragments of 31 and 114 base pairs in the case of the parasite strains containing the wild K76 variant, whereas the mutant T76 variant yielded only one fragment since there was no digestion. Multi-clonal variants yielded three fragments (145, 114, and 31 bp products). Restriction digest of the 140 samples gave the following results: 123 (87.9%) mutant types, 14 (10%) wild types and 3 (2.1%) mixed infections, which were also confirmed by sequencing [see Figures 2 and 3].

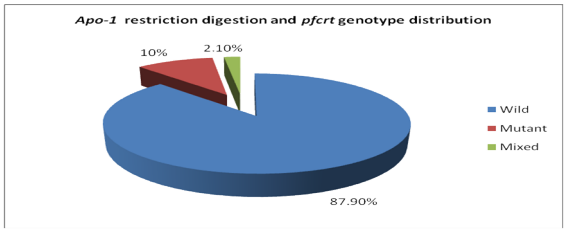

Figure 2 | *Apo*-1 restriction digestion and *pfcrt* genotype distribution among the 140 *P. falciparum* samples. 123 (87.9%) mutant types, 14 (10%) wild types and 3 (2.1%) mixed infections, which were also confirmed by sequencing.

Genetic sequences of the isolates from each of the 140 blood samples were determined using the ABI Prism genetic sequencer. Visual inspection of the sequence along the length of the gene was done. No new mutations were observed in the *pfcrt* gene, except for the known mutant codons of S72, M74, N75, and T76.

All the 123 mutant samples that showed single bands with *Apo*-1 digestion (mutants) had a nucleotide change of adenine (A) to cytosine (C) at nucleotide position 227, indicating an amino acid substitution of lysine with a threonine at amino acid position 76 of the *pfcrt* gene [see Figure 3]. The 14 wild types did not show any nucleotide change, whilst the 3 mixed isolates showed the presence of both adenine (A) and cytosine (C) peaks at nucleotide position 227.

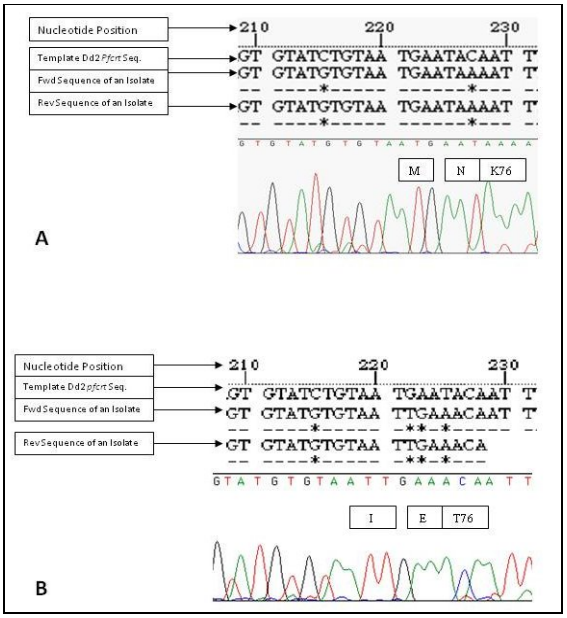

Figure 3 | Sequence chromatographs of a wild type (A) and a mutant type (B) *pfcrt* genes. Each of the coloured peaks indicates a nucleotide, duly translated directly above each peak, and aligned with the *pfcrt* template sequence of the Dd2 mutant isolate in the upper window. Both the forward and the reverse sequences are represented, with a dash (-) beneath a nucleotide indicating conformity and an asterix (*) indicating non-conformity. The nucleotide positions are indicated in figures as 210 through 230. The amino acids at positions 74, 75 and 76 are indicated as M, N and K76 respectively on the wild type chromatograph, and as I, E and T76 on the mutant type chromatograph.

The distribution of these *pfcrt* genotypes with respect to chloroquine levels are represented in Table 1. It was observed that plasma chloroquine levels were significantly higher (p=0.001) among patients with the mutant variant of the *pfcrt* gene [median CHQ level-76.0 ng/L, mean CHQ level-95.1 ng/L] than among those with the wild type [median CHQ level-0.0ng/L, mean CHQ level-51.0ng/L] or the mixed or multi-clonal isolates [median CHQ level-0.0 ng/L, mean CHQ level-0.0 ng/L].

Table 1 | *pfcrt* genotype distribution and plasma chloroquine levels. plasma chloroquine levels were significantly higher (p=0.001) among patients with the mutant variant of the *pfcrt* gene [median CHQ level-76.0 ng/L, mean CHQ level-95.1 ng/L] than among those with the wild type [median CHQ level-0.0ng/L, mean CHQ level-51.0ng/L] or the mixed or multi-clonal isolates [median CHQ level-0.0 ng/L, mean CHQ level-0.0 ng/L].

CHQ Conc (ng/L)	*pfcrt* Genotype Distribution		
	Wild Type	Mutant Type	Mixed Type
>300	1	5	0
201-300	0	13	0
101-200	1	25	0
5-100	4	60	0
<5	8	20	3
Total	14	123	3

Analysis of *pfmdr* gene

Seven (7) primer pairs were designed to amplify the whole of the *Pfmdr*-1 gene at different points along its length. This was aimed at identifying new mutations that may exist in the Ghanaian strains of the *P. falciparum*. Mutations were observed at three codon positions, namely, 1034, 1042, and 1246 of the *pfmdr*-1 gene in forty-seven (47) isolates analyzed.

The 47 samples were subsequently analyzed for the N86Y and Y184F codons. Twenty six (26) of the 47 samples carried the Y86 mutant codon, whereas 32 carried the F184 mutant codon. Both mutations involved nucleotide substitution of adenine (A) with thymine (T) at positions 256 and 551 respectively, resulting in substitution of tyrosine (Tyr) for Asparagine (Asn) and Phenylalanine (Phe) for tyrosine (Tyr) respectively.

Table 2 a and b | Correlation between the *pfmdr*-1Y86 mutation and the *pfcrt* T76 mutation.

A	Sequence	N86Y		Total
		TAT	AAT	
K76 T	ACA (123)	**23 (88.5%)**	11	34
	AAA (14)	**3 (11.5%)**	1	4
	ACA/AAA (3)	**0 (0.0%)**	9	9
	Total	26	21	47*

Analysis *pfcrt* and *pfmdr* genes

The relation between the Pfmdr-1 Y86 mutation and the Pfcrt T76 mutation was assessed. The presence of the T76 mutant gene in the Y86 mutant gene was stronger (p=0.002) than the reverse (see Tables 2a & b).

Presence of the Pfcrt T76 mutation in samples with the Pfmdr-1 Y86 mutation.

The presence of the T76 mutant gene in the Y86 mutant gene was stronger (p=0.002) than the reverse

B	Sequence	K76T			Total
		ACA (123)	AAA (14)	ACA/AAA (3)	
N86Y	TAT (26)	**23 (67.6%)**	3	0	26
	AAT (21)	**11 (32.4)**	9	1	21
	Total	34	12	1	47*

Presence of the Pfmdr-1Y86 mutation in samples with the Pfcrt T76 mutation.

The presence of the Y86 mutant gene in the T76 mutant gene was not strong.

Sequences of remaining samples were not analyzed.

DISCUSSION

The results demonstrated the extent of pre-hospital treatment among the study population and the widespread use of chloroquine. Although 50% of these babies had visited other health facilities prior to the onset of the severe malaria and their subsequent admission at the Komfo Anokye Teaching Hospital, the remaining 50% reported home treatment with chloroquine, which was consistent with similar report from Kenya.[19]

Home management of suspected malaria among infants has been promoted all across malaria endemic areas by the WHO.[20-24] Thus 77.9% of these children with detectable chloroquine levels was a good indicator of the success in the fight against malaria deaths through home management among infants. However, with as high as 67.9% of them having sub therapeutic levels (i.e. 87.2% of those with detectable chloroquine levels), there is the need for increased awareness in proper home treatment of malaria among infants. A previous study indicated that only 3.7% of febrile infants were given appropriate treatment at home prior to hospitalization.[24]

Interviewing mothers in this study revealed that, in practice, sub-therapeutic pre-hospitalization chloroquine levels could be attributed to either mothers not measuring medicines (syrups) accurately for their babies leading to improper dosing and under-dosage or most babies not swallowing all the medicines properly measured and administered to them orally by their mothers resulting in under-dosage.

There was no significant difference in chloroquine levels with respect to age of patient, which is inconsistent with previous reports from populations with uncomplicated malaria.[25] However, as indicated in Table 1, the Pfcrt T76 mutant variant correlated with higher plasma chloroquine levels (p=0.001) which is consistent with the findings of May and Meyer.[25]

The two main methods, namely *Apo*-1 enzymatic digestion and the sequencing, employed here to analyze the *pfcrt* gene status of the parasite strains from the study participants served to confirm or complement findings from each of the methods. Sensitive strains, mutant strains and multi-clonal strains characterized using *Apo*-1 enzymatic digestion were duly confirmed by sequencing as shown in Figure 3.

The finding of 87.9% of patients harbouring the T76 mutant strain far exceeds the documented chloroquine treatment failure of ≥25% of central Ghana.[15] Although there is not much historical data and this finding is among only severe malaria patients, the pattern may well permit the hypothesis that chloroquine resistance contributes to the incidence of severe malaria. This is even so since the Pfcrt T76 strain correlated with higher plasma chloroquine levels.

The occurrence of mutations at three codons 1034, 1042, and 1246 of the *pfmdr*-1 gene in the 47 isolates downplayed the role of these codons in the determination of chloroquine resistance status. This could be the normal genetic code of the parasite strain of Kumasi. Emphasis was therefore laid only on the two codons of N86Y and Y184F.

It could be observed in Tables 2a & b that the presence of the *Pfcrt* T76 mutation confers a strong likelihood for the mutant Y86 of the *pfmdr*-1 to be present than the reverse (p=0.002); 88.5% of the *Pfcrt* T76 mutant strains also had the Pfmdr-1 Y86 point mutation as opposed to 67.6% of the Pfmdr-1 Y86 mutant strain harbouring the Pfcrt T76 point mutation. It is therefore

likely that the Y86 mutation modifies or increases the effect of T76.

CONCLUSION

The above data may indicate that inappropriate home treatment of malaria is a common thing within the Kumasi metropolis, which calls for improved education to ensure that patients are given the appropriate treatment when malaria is suspected. Again, the data have shown that chloroquine resistance among *P. falciparum* isolates in Kumasi is mediated by the *pfcrt* and the *pfmdr*-1 genes. The two mutations, T76 and Y86, occurring on two different genes on two different chromosomes, are all true markers of chloroquine resistance, which are likely to be independently selected by plasma chloroquine levels. We can, however, conclude from this study that one mutation (Y86) might modify or increase the effect of the other (T76) as indicated by the strong presence of Pfcrt T76 in Pfmdr-1 Y86 (p=0.002).

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