Chloroquine prophylaxis associated with high prevalence of Plasmodium falciparum Pfcrt K76T mutation in people with sickle-cell disease in Benin City, Nigeria


Introduction

Falciparum malaria (P. falciparum), transmitted by the female Anopheles mosquito is the most deadly form of the four types of the disease (the other three being P. vivax, P. ovale and P. malariae). Each year, malaria attacks about 400 million people, two to three million of whom succumb to the illness. Most malaria victims are children. In malarious areas, the high frequency of haemoglobinopathies, such as sickle-cell...
disease (SCD), support their protective role against *P. falciparum* malaria. However, in patients homozygous for sickle haemoglobin (SS), the persistence of unrecognizable *P. falciparum* infection could trigger acute hemolytic events and/or recurrent vaso-occlusive crises (VOCs).

The precise mechanism by which sickle-cell trait imparts resistance to malaria is unknown. A number of factors likely are involved and contribute in varying degrees to the defence against malaria. Sickle-cell trait red cells infected with the *P. falciparum* parasite deform, presumably because the parasite reduces the oxygen tension within the erythrocytes to very low levels as it carries out its metabolism. Deformation of sickle trait erythrocytes would mark these cells as abnormal and target them for destruction by phagocytes.

CQ resistance is associated with a T76 mutation of the *P. falciparum* chloroquine resistance transporter gene (*Pfcrt*) while a multidrug resistance analogue (*Pfmdr1*) Y86 variation may modulate its degree. The aim of this study was to compare the prevalence rate of the *Pfcrt* T76 mutation in *P. falciparum*-infected homozygote (SS), heterozygote (AS) and homozygote (AA) individuals in Benin City.

### Material & Methods

**Study area and sample collection:** A total of 229 capillary blood samples was collected for this study. These samples were collected from individuals acquainted to chloroquine prophylaxis, who presented with symptoms of uncomplicated malaria between the months of April and June 2006 in Medical Centres in the Benin metropolis. Benin City is a cosmopolitan town where malaria infection is endemic. The months of April and June in Benin City are characterized by high transmission rate as these months mark the beginning of the rainy season.

**Haemoglobin genotyping:** An aliquot of washed blood cells was transferred into a clean test tube and lysed by adding few drops of water. Each sample was spotted on a cellulose acetate paper along side with control with an applicator stick. Then the cellulose acetate paper was then placed in an electrophoretic tank. Electrophoresis was run at 60 volt for 10 min. The haemoglobin types were determined by distance comparing with standard.

**DNA extraction, PCR–restriction fragment length polymorphism (RFLP) of the Pfcrt genes:** The fresh venous or capillary blood was blotted onto a piece of filter paper. DNA of *P. falciparum* was extracted from the dried blood spots by a modified Saponin/Chlex® (Sigma-Aldrich Corp., St. Louis, MO) method.

Genotyping of the resistance markers *Pfcrt* K76T was carried out by PCR-RFLP. A fragment of the *Pfcrt* gene containing codon 76 was first obtained by PCR amplification using a novel “Nested” approach. Consequently, several primer pairs were designed and tested, of which only those consistently producing single PCR products were chosen for downstream analysis. The lower primer was 5’-AATAAAgTTgTgAgTTTCCggA-3’, hybridizing from positions 280 to 300. The upper primer was 5’-TgTgCTCATgTgTTTAAACTT-3’, hybridizing from positions 130 to 150 in the *Pfcrt* sequence. The PCR components, in a final volume of 25 μl, were 1.6 mM MgCl₂, 640 μM deoxynucleotide triphosphate, buffer 1x, 0.3 μM of each primer, 0.5 U of Ampli Taq polymerase (AmpliTaq Gold; Applied Biosystems, Foster City, CA, USA) and 4 μl of DNA samples. Cycling conditions for the semi-nested PCR were, 45 cycles: 94°C for 3 min, 94°C for 30 sec, 56°C for 30 sec, 60°C for 45 sec, 65°C for 15 min while that of nested PCR were, 35 cycles: 94°C for 3 min, 94°C for 30 sec, 56°C for 30 sec, 60°C for 45 sec, and finally 65°C for 10 min.

Following nested amplification of the *Pfcrt* fragments harbouring codon 76, the presence of triplets AAA, encoding lysine (K) or ACA, encoding threo-
nine (T), was detected by incubation of the corresponding PCR fragments with endonuclease \textit{Apo I} (New England Biolabs). \textit{Apo I} thus excised products containing AAA, generating three fragments of 137, 124 and 10 bps while PCR products harbouring the alternative allele ACA were cut only at the monomorphic site, resulting in two fragments of 261 and 10 bps.

The digestion products resulting from restriction digests were checked with 2% agarose gel on a horizontal electrophoretic apparatus (2006, Edvotek M12) together with a marker. Gels were stained with ethidium bromide and visualized under UV (ultraviolet) transillumination.

\textit{Statistical analysis:} Data generated from this study were analysed using the $\chi^2$-square test.

\section*{Results}

This study showed that among 424 subjects investigated, 207 (48.8\%) with haemoglobin type ‘AA’, 136 (32\%) with ‘AS’ and 81 (19.1\%) with ‘SS’, of which malaria parasites were detected in 189 (91.3\%) haemoglobin AA, 101 (74.2\%) haemoglobin AS, and 63 (77.7\%) in haemoglobin SS individuals. Malaria prevalence rate among the three haemoglobin genotypes was not statistically significant (p>0.05), also more haemoglobin AA individuals experience higher level of parasitaemia (>1000 parasites/μl) than AS and SS individuals (Table 1).

The prevalence rate of mutant genes showed that, out of 189 positive samples from haemoglobin AA genotyped individuals, 38 (20.2\%) individuals harboured the mutant \textit{Pfcrt} gene. Of the 101 samples from haemoglobin AS individuals examined, 83 (82.1\%) carried the wild type of the \textit{Pfcrt} gene and 18 (17.9\%) had the mutant gene while 24 (38.1\%) mutant \textit{Pfcrt} were identified from the 63 haemoglobin SS individuals studied. The difference in the prevalence rate of mutant \textit{Pfcrt} genes between haemoglobin SS, AA and AS was highly significant (p <0.05) (Fig. 1 and Table 2).

\begin{table}[h]
\centering
\caption{Prevalence rate of malaria infection by genotype using thick film}
\begin{tabular}{lcccccc}
\hline
\hline
AA (n=207) & 189 (91.3) & 82 (43.3) & 61 (32.2) & 19 (10) & 13 (6.8) & 9 (4.7) & 5 (2.6) \\
AS (n=136) & 101 (74.2) & 57 (56.4) & 32 (31.6) & 5 (4.9) & 5 (4.9) & 2 (1.9) & – \\
SS (n=81) & 63 (77.7) & 30 (47.6) & 19 (30.1) & 7 (11.1) & 4 (6.3) & 2 (3.1) & 1 (1.5) \\
Total (n=424) & 353 (83.2) & 169 (47.8) & 112 (31.7) & 31 (8.7) & 22 (6.2) & 6 (1.6) & 2 (0.5) \\
\hline
\end{tabular}
\end{table}

Figures in parentheses indicate percentages; $\chi^2 = 2.3321$, df = 2, Asymp sig (2-sided) = 0.123; p >0.05.
Findings from this study revealed that there was no statistical difference in the prevalence rate of malaria infection in individuals with haemoglobin ‘AA’, ‘AS’ and ‘SS’ genotyped subjects. Malaria parasites were detected in 189 (91.3%) hemoglobin AA, 101 (74.2%) haemoglobin AS, and 63 (77.7%) in haemoglobin SS individuals. Malaria prevalence rate among the three haemoglobin genotypes was not statistically significant (p >0.05), however, higher parasitaemia level was found in the AA and SS individuals but not in the AS. This may indicate a kind of resistance in term of proliferation of parasites occurred in the haemoglobin AS individuals. Cheesbrough\textsuperscript{9}, however reported that genes which select against “severe” falciparum malaria include: haemoglobin S gene, Thalassemia genes, Glucose-6-phosphate dehydrogenase (G6PD) deficiency genes, Ovalocytosis and Duffy genes. Haemoglobin ‘AA’ genotyped individuals are believed to suffer severe malaria compared to haemoglobin ‘AS’ genotyped individuals while the ‘SS’ genotyped individuals are also known to suffer severe malaria infection. In the light of this, an important question is raised. If the haemoglobin S gene selects against malaria infection, why are the ‘SS’ genotyped individuals then suffer severe form of malaria while the ‘AS’ genotyped individuals are exempted? Cheesbrough\textsuperscript{9} further explained that protection is afforded only to heterozygous individuals (haemoglobin AS) against severe malaria. The protective mechanism is not fully understood. Cells containing HbAS sickle more quickly when they contain parasites than when they are non-parasitized, probably because the parasites lower the pH which leads to damage and sickling. The parasites in these cells are then thought to be rapidly phagocytosed. The parasites are therefore, destroyed before they develop into schizonts. In homozygous persons (HbSS), the phagocytic action of the spleen is frequently inadequate and the removal of parasitized cells will also need to compete with the removal of non-parasitized irreversibly sickles-cells. Homozygous (HbSS) persons may die from severe malaria.

A high prevalence \textit{Pfcrt} mutant gene was also recorded in the haemoglobin “SS” genotyped individuals when compared to the haemoglobin “AA” and “AS” individuals. Several factors have been earmarked to contribute to the emergence of these resistant genes notably environmental factors, host factors and drug pressure. This high prevalence in the haemoglobin “SS” genotyped individuals could be as a result of uncontrolled usage of chloroquine as prophylaxis. Chloroquine prophylaxis is responsible for increased drug consumption and increased drug pressure that may lead to the selection of drug-resistant parasites\textsuperscript{10}.

In conclusion, rapid intervention measures are needed as a matter of urgency to curb the up rise in the prevalence of the chloroquine resistance gene in our communities.

Table 2. Prevalence rate of \textit{Pfcrt} mutant genes by genotype

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>\textit{Pfcrt}</th>
<th>Wild type</th>
<th>Mutant</th>
</tr>
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<tbody>
<tr>
<td>AA (n = 189)</td>
<td>151 (79.8)</td>
<td>38 (20.2)</td>
<td></td>
</tr>
<tr>
<td>AS (n = 101)</td>
<td>83 (82.1)</td>
<td>18 (17.9)</td>
<td></td>
</tr>
<tr>
<td>SS (n = 63)</td>
<td>39 (61.9)</td>
<td>24 (38.1)</td>
<td></td>
</tr>
<tr>
<td>Total (n = 353)</td>
<td>273 (77.3)</td>
<td>80 (23.7)</td>
<td></td>
</tr>
</tbody>
</table>

Figures in parentheses indicate percentages; $\chi^2 = 23.576$, df = 2, Asymp sig (2-sided) = 0.005; p <0.05.

References

3. Martin TW, Weisman IM, Zeballos RJ, Stephenson SR. Exercise and hypoxia increase sickling in venous blood from an exercising limb in individuals with sickle-cell trait. \textit{Am
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