IN VITRO SUSCEPTIBILITY OF PLASMODIUM FALCI PARUM ISOLATES TO CHLOROQUINE AND OTHER ANTIMALARIAL DRUGS IN GHANA

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SUMMARY
The in vitro susceptibilities of Plasmodium falciparum to chloroquine, mefloquine and quinine were investigated in three distinct eco-epidemiological zones in Ghana using a modification of the World Health Organisation (WHO) micro-test technique. In vitro drug-sensitivity-tests were based on the measurement of the effect of the drugs on the growth and development of malaria parasites. Parasites were cultured in the presence of a range of concentration of antimalarial drugs for one life cycle or part thereof. Sensitivity was then assessed by measuring the quantity of radio-labelled hypoxanthine incorporated into the parasites in drug containing wells compared to drug-free wells. The concentrations of the various drugs achieving 50% inhibition were determined. Of the 64 P. falciparum isolates tested, overall chloroquine resistance of 37.5% was observed. Though all the isolates were fully sensitive to mefloquine and quinine, four showed reduced sensitivity to quinine. The overall mean IC₅₀ determined for these antimalarial drugs were 1.1 x 10⁻⁶ mol/litre, 1.02 x 10⁻⁶ mol/litre and 2.3 x 10⁻⁶ mol/litre for chloroquine, mefloquine and quinine respectively. Findings from this study indicate increasing levels of P. falciparum resistance to chloroquine compared to levels that were seen about a decade ago in the country.

Keywords: Chloroquine, resistance, susceptibility, antimalarial drug, in vitro test

INTRODUCTION
Plasmodium falciparum malaria has become a global menace infecting a significant number of people worldwide. In Ghana, the disease accounts for up to 40% of all outpatient attendance and remains one of the leading causes of mortality and morbidity especially among children. It follows climatic and ecological patterns in the country and occurs all year round with increase in incidence during the wet season.

Chemotherapy is the mainstay of malaria control in Ghana with chloroquine as the first line antimalarial drug. In recent times, however, there has been the emergence in the country of strains of P. falciparum resistant to chloroquine. Since the first report of chloroquine resistance in Ghana by Nkengue and her colleagues, others have reported increasing chloroquine resistance in various studies across the country. The issue of chloroquine resistance in Ghana has not only complicated the treatment and prevention of the disease but has also threatened its use as the first choice antimalarial drug. Should the level of resistance become significantly high, there will be the need to replace it. Such an action must be justified with field-based evidence showing significant reduction in susceptibility of the parasites to chloroquine in the country.

The in vitro assay is based on culturing P. falciparum isolates in the presence of a range of concentrations of antimalarial drug for one life cycle or part thereof. The effect of antimalarial drugs is generally characterised by the inhibition of parasite growth and, consequently their multiplication. In this study inhibition of the incorporation of tritium-labelled hypoxanthine (a nucleic acid precursor) by the parasite served as the indicator of antimalarial activity.

Generally, the in vitro method allows for almost complete exclusion of host-related factors, such as drug failure or host immunity and it also provides a more objective insight into inherent drug sensitivity than do the in vivo test. However it must be stressed that results from the in vitro tests complement the outcome of patient’s clinical response to antimalarial drug.
This study was carried out as part of a health facility based chloroquine efficacy study undertaken in Ghana with the aim of gathering data for informed, evidence based decision on recommendations for drug treatment of uncomplicated malaria.

MATERIALS AND METHODS

Study Area
Three study sites were selected from 6 existing sentinel sites being used for the monitoring of treatment efficacy in the country. These represent 3 eco-epidemiological zones in the country; namely the forest, the middle semi deciduous forest and the northern savannah. The sites were Tarkwa, Holeo and Navrongo. Tarkwa is a gold mining town in the forest zone of the western region and it is considered an urban setting with easy access to antimalarial drugs. Due to the method of mining in the town, there are numerous open trenches containing stagnant water, which serve as breeding grounds for mosquitoes throughout the year. Malaria transmission in this area is therefore perennial with a slight increase during the main rainy seasons in April-July and September- November. Holeo lies in the middle belt of the country with semi-deciduous forest vegetation. It is an urban community where malaria is hyperendemic. Malaria transmission in this area is perennial with peaks occurring after the major rains in June. Navrongo is classified as a rural area, lying in the guinean savannah zone in the upper east region of Ghana with a dominant population of peasant farmers. The area receives all of its rains between May and September. The rest of the year is dry. However, due to the large water reservoir in the town meant to provide water for irrigation, mosquito breeding occurs throughout the year. Malaria is thus hyperendemic with the peak occurring between June and November.

Study Population
Children aged 6-59 months presenting to hospitals in the selected areas with fever or history of fever were screened for inclusion into the study. Inclusion and exclusion criteria for the study were in line with WHO protocol for the assessment of therapeutic efficacy in areas of high transmission (WHO, 1996)\(^5\). Briefly all patients had monoinfection of *P. falciparum* with densities ranging from 2,000 to 100,000 asexual parasites per microlitre of blood with no other cause for their fever. Patients with signs and symptoms of severe and complicated malaria were excluded from the studies. A history of recent intake of antimalarial drugs was not an exclusion criterion. The study was thoroughly explained to the parents/guardian of potential children and given the chance to ask questions. Children were only enrolled after parents or guardians had signed the informed consent form (approved by the Noguchi Memorial Institute for Medical Research IRB) for the study.

Drug Treatment
Patients were treated with 25mg chloroquine per kilogram body-weight orally over a three-day period in line with the WHO standardised 14 days test of therapeutic efficacy *in vitro*. Children were followed up for fourteen days with examination of blood films for malaria parasites on days 1, 2, 3, 7 and 14 respectively. Guardians of patients were advised to report to the clinical should there be persistence of symptoms or any danger sign of malaria at any time during the follow up period. Patients' response to treatment were classified in accordance with WHO criteria\(^6\).

**In Vitro Test**
An *in vitro* assessment of the susceptibilities of *P. falciparum* isolates was performed using a modification of the WHO micro-technique\(^6\). The modification involves the use of inhibition of radiolabelled hypoxanthine incorporation by parasite to demonstrate drug effect. Prior to treatment, in vivo venous blood was collected from each patients and processed by standard methods used to remove the leukocytes, platelets and any antimalarial drugs in the plasma\(^6\). The red blood cells were diluted with complete RPMI 1640 (Sigma, USA) parasite-growing medium supplemented with 25 mM HEPES, 25 mM NaHCO\(_3\) and 10% normal human serum.

The culture was carried out in a 96-well microtitre plate pre-dosed with various concentrations of the antimalarial drugs (namely, chloroquine, mefloquine and quinine). The antimalarial drugs used for plate coating were supplied by WRAIR Inventory Laboratory, USA. Fifty microliters of blood-media mixture were dispensed into each well containing an appropriate concentration of drug. The concentration per well of drugs ranged between 1 and 64 pmol (0.2 - 12.8 \(\mu\)mol/L blood) for chloroquine, 2 and 128 pmol (0.4 - 25.6 \(\mu\)mol/L blood) for mefloquine and 4 to 256 pmol (0.8 - 51.2 \(\mu\)mol/L blood) for quinine. Plates were incubated at 37°C in a candle jar placed in an incubator. One \(\mu\)Ci of \(^3\)H-hypoxanthine (supplied by NEN, Boston, USA) was added to each well after 18 hours and the culture incubated for an additional 24 hours.

At the end of the culture period, the plates were frozen to terminate the assay and later thawed to lyse the erythrocytes. The contents of each well was then harvested onto a glass fibre paper using
the Filtermate 196 cell harvester (Canberra Company, USA). The filters were dried and incorporation of \(^{3}H\)-hypoxanthine by the parasites measured with a Matrix scintillation counter (Canberra Company, USA).

**Assessment of Drug Susceptibility**

The quantity of \(^{3}H\)-hypoxanthine incorporated into the parasites in drug containing wells compared to drug-free wells was used as a measure of parasitic growth.

The concentrations of the various drugs achieving 50% \((IC_{50})\) inhibition were determined from a regression analysis of log-dose/response curve. The \(IC_{50}\) was defined as the drug concentration corresponding to 50% uptake of \(^{3}H\)-hypoxanthine measured in the drug-free control well. Resistance was considered present when there was evidence of parasitic growth in wells containing chloroquine, quinine or mefloquine at concentrations of 8 pmol (1.6 \(x\) \(10^{6}\) mol/L blood), 256 pmol (51.2 \(x\) \(10^{4}\) mol/L blood) and 64 pmol (12.8 \(x\) \(10^{5}\)) or more respectively.

**RESULTS**

A total of 64 out of 71 (90.1%) \(P. falciparum\) isolates from the three sites were successfully cultured. The results showed an overall, 37.5% \(P. falciparum\) resistance to chloroquine (Table 2).

**Table 1** Overall response in vitro of \(P. falciparum\) isolates from three sites in Ghana to chloroquine, mefloquine and quinine

<table>
<thead>
<tr>
<th>Drug conc. Pmol/well</th>
<th>No. of isolates with complete inhibition (%)</th>
<th>Percentage inhibition (average)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(C_{q}) n=6</td>
<td>(Mef) n=48</td>
</tr>
<tr>
<td>----------------------</td>
<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
<td>1</td>
<td>0(0)</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>2(3.1)</td>
<td>0(0)</td>
</tr>
<tr>
<td>4</td>
<td>17(26.6)</td>
<td>5(10.4)</td>
</tr>
<tr>
<td>8</td>
<td>*40(62.5)</td>
<td>23(47.9)</td>
</tr>
<tr>
<td>16</td>
<td>43(67.2)</td>
<td>39(81.2)</td>
</tr>
<tr>
<td>32</td>
<td>52(81.2)</td>
<td>48(100)</td>
</tr>
<tr>
<td>64</td>
<td>59(92.2)</td>
<td>*48(100)</td>
</tr>
<tr>
<td>128</td>
<td>48(100)</td>
<td>-</td>
</tr>
<tr>
<td>256</td>
<td>-</td>
<td>48(100)</td>
</tr>
</tbody>
</table>

\(C_{q}\)-chloroquine; \(Mef\)-mefloquine; \(Quin\)-quinine

*Concentration for which schizont growth is indicative of resistance.

Chloroquine resistance were found to be 47.6%, 36.4% and 31.3% in Navrongo, Tarkwa and Hohoe respectively and the \(IC_{50}\) values for chloroquine determined in these areas were; 1.75 \(x\) \(10^{6}\), 0.61 \(x\) \(10^{6}\) and 0.83 \(x\) \(10^{4}\) respectively (Table 3). None of the isolates showed resistance to mefloquine and quinine, though there was a slight reduction of chloroquine achieving 50% inhibition (\(IC_{50}\)) determined in this study was found to be above the World Health Organisation (WHO) recommended critical value (0.8 \(x\) \(10^{6}\)) needed for complete inhibition and closer to the threshold for chloroquine resistance in vitro which is 1.6 \(x\) \(10^{6}\).

**Table 2** Summary of resistance to antimalarial drugs (%)

<table>
<thead>
<tr>
<th>Site</th>
<th>Chloroquine</th>
<th>Mefloquine</th>
<th>Quinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hohoe</td>
<td>31.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Navrongo</td>
<td>47.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tarkwa</td>
<td>36.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Overall</td>
<td>37.5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 3** Inhibition concentration by regression analysis for 50% inhibition

<table>
<thead>
<tr>
<th>Site</th>
<th>Chloroquine (IC_{50}) (mol/litre)</th>
<th>Mefloquine (IC_{50}) (mol/litre)</th>
<th>Quinine (IC_{50}) (mol/litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Navrongo</td>
<td>1.75 (x) (10^{6})</td>
<td>0.78 (x) (10^{6})</td>
<td>3.05 (x) (10^{6})</td>
</tr>
<tr>
<td>Tarkwa</td>
<td>0.61 (x) (10^{6})</td>
<td>0.88 (x) (10^{6})</td>
<td>1.53 (x) (10^{6})</td>
</tr>
<tr>
<td>Hohoe</td>
<td>0.83 (x) (10^{6})</td>
<td>1.36 (x) (10^{6})</td>
<td>2.46 (x) (10^{6})</td>
</tr>
<tr>
<td>Overall</td>
<td>1.1 (x) (10^{6})</td>
<td>1.02 (x) (10^{6})</td>
<td>2.3 (x) (10^{6})</td>
</tr>
</tbody>
</table>

The overall mean \(IC_{50}\) calculated for the antimalarial drugs were 1.1 \(x\) \(10^{6}\) mol/litre, 1.02 \(x\) \(10^{6}\) mol/litre and 2.3 \(x\) \(10^{6}\) mol/litre for chloroquine, mefloquine and quinine respectively. The concentra-
Even at a concentration of 4.8\times10^6, which is thrice the threshold for chloroquine resistance in vitro, there was evidence of schizont maturation in 12(16.9\%) of the isolates indicating the presence of strains of *P. falciparum* that are highly resistant to chloroquine.

**DISCUSSION**

It is evident from observations made in this study that *P. falciparum* isolates from different parts of Ghana have become resistant to the first line antimalarial drug, chloroquine. The study showed an overall chloroquine resistance of 37.5\%.

An interesting and important observation made in this study is the higher chloroquine resistance found in isolates in Navrongo, a rural setting which we expect to have the least rate of resistance. This situation contrasts earlier findings in Ghana which showed an increase in drug resistance from the rural to urban areas. The assumption is that the emergence and spread of chloroquine resistance is precipitated by drug pressure which is more common in the urban areas where medical facilities are concentrated and where there is greater accessibility and more frequent use of chloroquine and other antimalarial drugs.

A possible reason for the observed increased in chloroquine resistance even in the rural areas could be the recently introduced home based treatment for malaria under the Roll Back Malaria program in the country. The intensive advert in the media might have led to an upsurge in the use of chloroquine with a consequent increase in parasite resistance to the drug due to drug pressure.

The level of chloroquine resistance observed in this study compares well with results from the in vivo study done concurrently which showed treatment failure rate of about 15-30\% in the country (Koram et al – unpublished). Previous reports had shown 45\% chloroquine resistance in *P. falciparum* in the country.

Resistance of *P. falciparum* to chloroquine is common in most malaria endemic countries of Africa especially in eastern Africa. Thus Malawi and Kenya in 1993 and 1996 respectively changed their recommendation for first-line treatment of uncomplicated malaria from chloroquine to sulfadoxine/pyrimethamine, and Botswana and South Africa revised their treatment guidelines in 1997.

Evidence of a reduced susceptibility of *P. falciparum* to chloroquine obtained in this study as well as that from in vivo treatment outcome is strong enough to form the basis for a review of the national antimalarial drug treatment policy in Ghana.

This study demonstrates the absence of strains of *P. falciparum* resistant to quinine in the three study sites (overall IC50 was 2.3 \times 10^6 mol/litre). This should be a welcome piece of news for the health authorities in Ghana. This is because quinine remains the drug of choice for the treatment of severe and complicated malaria in the country. Hence the presence of parasites resistant to quinine would have been of great concern to the National Malaria Control Program. However, the observed delay in susceptibility of 4 of the isolates to quinine calls for a closer monitoring of this drug.

All the isolates tested were sensitive to mefloquine with an overall IC50 of 1.02x10^6 mol/litre. This finding contrasts report of cross-resistance with chloroquine observed in Tanzania but agrees with observation of full mefloquine sensitivity in an area of Brazil with a high degree and frequency of chloroquine resistance.

This study further confirms the usefulness of the in vitro technique to establish the susceptibility of malaria parasites to antimalarial drugs.

In conclusion, this study reveals the presence in the Ghana of *P. falciparum* isolates, which are highly resistant to chloroquine.

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**REFERENCE**


