THE IN VITRO ASSESSMENT OF DRUG RESISTANT MALARIA IN MAKURDI, NORTH CENTRAL NIGERIA

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ABSTRACT
Multi-drug resistant malaria parasite strains have spread to new areas that were once free of such strains. This study evaluated the specific in vitro sensitivities of some standard antimalarial drugs, against Plasmodium falciparum isolates in Makurdi, North Central Nigeria. The standard schizonts growth inhibition assays was used to study the in vitro activities of quinine, artesunate, and amodiaquine against 146 isolates in children aged 2-14 years. 100 % of isolates were in vitro sensitive to quinine, geometric mean effective concentration (EC50) = 241.55 nM, EC90 = 676.08 nM, and EC99 = 993.12 nM; and artesunate, EC50 = 1.05 nM, EC90 = 2.42 nM, and EC99 = 3.16 nM. 1.37 % of isolates were resistant to amodiaquine, EC50 = 22.08 nM, EC90 = 66.22 nM and EC99 = 100.23 nM. Significant in vitro cross resistance was found at EC90 values of quinine-amodiaquine drug pair (r = + 0.342, P < 0.05), but not quinine – artesunate (r = + 0.057, P > 0.05) or artesunate-amodiaquine (r = + 0.088, P > 0.05). These results call for constant surveillance, to curb the spread of P. falciparum resistance to amodiaquine in Nigeria.

Keywords: Drug resistance, Plasmodium falciparum, Malaria, Nigeria.

INTRODUCTION
Malaria continues to be of great public health concern to many countries in the world. Recent estimates by the World Health Organization (WHO, 2009) revealed that half of the world population is at the risk of malaria and that 243 million people experienced clinical malaria in 2008, resulting in 863,000 deaths worldwide out of which 89 % occurred in Africa. In areas where Plasmodium falciparum is the most dominant causative agent of the disease, the impact of malaria, on the resident population has been very huge (Cooper et al., 1998). The worsening impact of falciparum malaria has continued mainly because of the rising wave of drug resistant malaria (Oliaro, 2005). In Nigeria, resistance to chloroquine and sulfadoxine/pyrimethamine by P. falciparum has been reported (Salako & Adeyonumu, 1987; Olorinola, 1989; Abdullahi et al., 2003; Oguche et al., 2004; Pitman et al., 2005). Worldwide, drug resistance has thwarted efforts at malaria control (Olliaro, 2005). This has led to the need for constant surveillance and monitoring for changes in malaria parasites sensitivity to different antimalarial drugs and the rapid switch to artemisinin combination therapies (ACTs), for the treatment of malaria (Kremsnner & Krishna, 2004; Olliaro & Taylor, 2004). Even though the use of ACTs together with insecticide treated bed nets has seen the reduction in malaria morbidity (Barnes et al., 2009), in vivo resistance to artemisins that were thought be immuned is still being reported (Dondorp et al., 2009).

The in vitro drug susceptibility test is an invaluable tool for the surveillance of drug resistant malaria worldwide (WHO, 2001; Plowe, 2003). It provides an alternative to clinical studies in malaria endemic areas, where drug susceptibility of parasite isolates can be obscured by different levels of acquired immunity in patients treated with antimalarial drugs (Russel et al., 2003). In Nigeria, data on the susceptibility profile of P. falciparum isolates to antimalarial drugs are sparse. However, it is imaginable that resistant P. falciparum, may have contributed reasonably to the malaria burden in the country. The aim of this study was to determine the baseline level of in vitro sensitivities of P. falciparum isolates to selected antimalarial drugs used in north central Nigeria, and to generate baseline data for future monitoring of parasite responses to those antimalarial drugs in the region.

MATERIALS AND METHODS
Study site: The study was conducted at the Bishop Murray Medical Centre, and the Federal Medical Centre, all in Makurdi, north central Nigeria. The study protocol was approved by the local ethics committee of each hospital, and lasted from October 2005 to December 2006.

Subjects: Enrolled subjects were febrile symptomatic children aged 2-14 years, who reported to the hospital with a history of fever, and whose guardian gave written informed consent. Prior to treatment, 2.5ml of venous blood was collected into heparin treated tubes, for microscopic detection of P. falciparum mono infections with Giemsa stain, and in vitro drug susceptibility test. Subjects with symptoms of severe malaria infections, a recent history of malaria pre-treatment with antimalarial drugs, and confirmed severe anaemia (PCV ≤ 21%) were excluded from the study. Confirmed P. falciparum mono infections with parasite density of 2,000 to 80,000 asexual forms per μl of blood were included in the in vitro test (WHO, 2001).
Antimalarial drug dilutions and plates preparation: Stock solutions, 1mg/ml of antimalarial drugs, amodiaquine, artemisun and quinine (Sigma Aldrich) were each prepared in an appropriate solvent. The solutions were sonicated and filtered with 0.22 µm membrane filter (Millipore). A further dilution of each stock was made to yield a working solution of desired concentration. From the working solutions, two fold serial dilutions were performed on a 96-well flat bottom micro culture plate, and the plates dried in an incubator at 37 °C (Russel et al., 2003). The range of the final drug concentrations were, amodiaquine: 6.25 – 400 nM; artemisun: 0.34 – 22 nM; and quinine: 50 – 3200 nM.

In vitro cultivation of *Plasmodium falciparum* isolates and drug susceptibility test: The in vitro cultivation of *P. falciparum* isolates followed a modification of the standard culture techniques (Trager & Jensen, 1976; Haynes et al., 1976), while drug susceptibility test followed the standard procedure for schizonts inhibition (WHO, 2001). The culture medium consisted of 10.43g RPMI 1640 (Invitrogen), 5.96g HEPES, and 25mM NaHCO₃ (Sigma Aldrich), per litre of double distilled water, supplemented with 5% albuamax II (Gibco) (Cranmer et al., 1997). The medium was sterilized by filtration through 0.22 µm membrane filter, and addition of 0.5ml of 50mg/ml gentamicin. 200µl of a 1:20 dilution of parasites were placed in a candle jar, and incubated at 37°C, for 26 hrs (WHO 2001), at the end of the incubation period, thick films were made, and stained with 2.5% Giemsa stain for 35 mins. The mean number of schizonts formed per 200 asexual parasites were counted and recorded.

Determination of *in vitro* effective concentration (EC) values of the antimalarial drugs: The mean number of schizonts counts per well, were fed directly into non linear regression software, HN-NonLin, specific for malaria *in vitro* drug sensitivity test. Individual dose response curves were generated, and their EC₅₀, EC₉₀, and EC₉₉ values determined. Standard drug resistant clones were not included in the assay. However, drug resistant *P. falciparum* parasites were identified as isolates with EC₅₀ or EC₉₀ values greater than published threshold values for sensitive parasite isolates. The threshold of resistance were; quinine: EC₅₀ >500 nM or EC₉₀ > 2560 nM; amodiaquine: EC₅₀ > 80 nM, or EC₉₀ > 400 nM (Pradines et al., 1998; WHO, 2001; Pradines et al., 2006). For Artesunate, threshold EC₅₀ and EC₉₀ values for *in vitro* resistance have not yet been determined (Noedl et al., 2003), therefore the estimated EC values were reported as a baseline data for future comparison.

Data Analysis: The geometric means and 95 % confidence intervals (CI) of EC values were estimated. Estimated EC values were reported as a baseline data for future comparison. In each case the level of significance was set at P ≤ 0.05.

RESULTS
Out of 174 parasite isolates tested *in vitro*, 146 (83.91%) yielded complete data for the determination of EC values for the three drugs tested. The geometric mean EC values for each drug and their 95% confidence intervals (CI) are shown in Table 1. *In vitro* resistance to amodiaquine - isolates with EC₅₀ > 80 nM among the cultured isolates was 1.37% or (2/146). No resistant isolates were found against quinine, and artemisun (Figs 1, 2, and 3).

<table>
<thead>
<tr>
<th>Drugs</th>
<th>EC₅₀ (Geometric Mean EC, (95% CI) nanomolar (nM))</th>
<th>EC₉₀</th>
<th>EC₉₉</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artesunate</td>
<td>1.05 (1.03 – 1.07)</td>
<td>2.42 (2.37 – 2.47)</td>
<td>3.16 (3.07 – 3.26)</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>22.08 (20.75 – 23.44)</td>
<td>66.22 (61.38 – 71.45)</td>
<td>100.23 (93.11 - 107.89)</td>
</tr>
<tr>
<td>Quinine</td>
<td>241.55 (232.81 – 250.61)</td>
<td>676.08 (652.08 – 700.97)</td>
<td>993.12 (959.84 – 1027.54)</td>
</tr>
</tbody>
</table>

**FIG. 1. RELATIVE EC₅₀ (nM) DISTRIBUTION PATTERNS OF ARTESUNATE AMONG P. falciparum ISOLATES AT MAKURDI**
Although no in vitro resistance cut off values have been reported for artesunate, the EC values of individual isolates against the drug were very low, and fell within the range of EC50 values previously reported for sensitive isolates to the drug, or its active metabolite, dihydroartemisinin (Noedl et al., 2003; Woodrow et al., 2005; Pradines et al., 2006; Tinto et al., 2006). Correlation of in vitro responses of *P. falciparum* EC50 values of antimalarial drug pairs showed significant positive in vitro cross resistance between quinine-amodiaquine ($r = +0.342; P < 0.05$), but not between quinine-artesunate ($r = +0.057$), or artesunate-amodiaquine ($r = +0.088; P > 0.05$) (Table 2).

**TABLE 2. CORRELATION OF IN VITRO RESPONSES OF P. falciparum ISOLATES BETWEEN ANTIMALARIAL DRUG PAIRS AT EC50. (R = PEARSON CORRELATION COEFFICIENT)**

<table>
<thead>
<tr>
<th>Drug pair</th>
<th>n</th>
<th>r</th>
<th>R%</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinine-amodiaquine</td>
<td>146</td>
<td>0.342</td>
<td>11.70</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Quinine-artesunate</td>
<td>146</td>
<td>0.057</td>
<td>0.32</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Artesunate-amodiaquine</td>
<td>146</td>
<td>0.088</td>
<td>0.77</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The present results show that there was a one hundred percent in vitro sensitivity of *P. falciparum* fresh parasite isolates to artesunate and quinine in the study area but very low in vitro parasite resistance (1.37%) against amodiaquine. However, the very low levels of in vitro *P. falciparum* resistance against the latter drug demonstrated that the over all in vitro sensitivities of amodiaquine, like quinine, and artesunate, were equally very high in this area. These findings, regarding the sensitivities of amodiaquine, represent a sharp contrast from the results obtained by Oyedeji et al., (2005) in south western Nigeria in which, 39% of 36 *P. falciparum* isolates were reported to be in vitro resistant to amodiaquine. The corresponding mean EC50, EC90, and EC99 values of amodiaquine against *P. falciparum* were 0.06 µM, 0.26µM, and 0.59µM respectively (Oyedeji et al., 2005). Compared to the present EC values, the potency ratio of amodiaquine in south west Nigeria was approximately 3 fold; 4 fold; and a 6 fold decrease over Makurdi, at corresponding EC levels. This suggests that *P. falciparum* isolates in Makurdi, north central Nigeria, were more sensitive to amodiaquine, than strains circulating in south western Nigeria.

The present EC50 and EC90 values of quinine are comparably close to the corresponding inhibitory concentration - IC50 and IC90 values of 0.25 µM and 0.80 µM respectively, reported almost two decades earlier.
ago also in south western Nigeria (Salako et al., 1988). These values compared to the present EC values approximately yielded a potency ratio of 1 at corresponding EC levels. Implying that, the in vitro sensitivities of circulating strain of P. falciparum isolates to quinine, between the south west, and north central regions in the country, have remained relatively very stable; despite nearly two decades of use, since the re-introduction of quinine antimalarial drug, in the country. Like in many other countries, quinine has mainly been used in Nigeria for the treatment of severe and drug resistant falciparum malaria, usually under medical supervision (Abdullahi et al., 2003). Relevant data suggests that quinine is also very effective else where in West Africa (Agnamey et al., 2006). The absence of in vitro P. falciparum resistance to quinine, in this study, and the apparent stability of in vitro responses of P. falciparum to quinine in Nigeria over a long period of time, suggests its continued relevance for resolving malaria infections in the country.

Amodiaquine has quickly assumed the position of its sister analogue, chloroquine as an alternative drug for the treatment of uncomplicated malaria in the country with higher efficacies, due to high levels of parasite resistance to the latter (Molta et al., 2003). It is readily available across the counter in this country for self treatment of malaria, and could therefore be subject to irrational use, during self treatment. The low levels of in vitro resistance observed against the drug in the present study, and elsewhere in the country (Oyedeji et al., 2005) suggests an imminent threat of wide scale parasite resistance to the drug. It also calls for the need for constant surveillance and vigilance to curb the imminent spread of drug resistant strains of P. falciparum in the country.

The artesunate antimalarial is a relatively new entrant on the scene in Nigeria with little information about the drug. The drug is also readily available in major cities in the country, but its average cost makes it prohibitive for wanton self purchase and self treatment for an average Nigerian. Currently, artesunate plus amodiaquine, is also used for the treatment of uncomplicated malaria in the country. Emerging in vivo data else where, and in Nigeria suggests some encouraging development in this regard (Mutabingwa et al., 2005; Agnamey et al., 2006; Meremikwu et al., 2006; Djimde et al., 2008). There is also the fear that full scale deployment of artesunate plus amodiaquine for the home management of malaria as it is being considered may result in complete failure of the combination in the near future, particularly due to non compliance with the treatment regimen as a result of adverse reactions to amodiaquine. This may fuel the early loss of both drugs, thus the deployment of artesunate plus amodiaquine combination for the self home management of malaria should proceed with caution. The combination should not be prescribed and administered as non fixed combination without supervision. It should be routinely administered otherwise, only when a reliable fixed dose combination with minimum contraindications, produced in blister pack with a short treatment period has been produced.

The present data did not observe significant in vitro cross resistance between artesunate and quinine as has been reported by others (Noedl et al., 2003) but cross resistance did exist between quinine and amodiaquine in agreement with previous findings (Pradines et al., 1998). Considering the high in vitro sensitivities of quinine, and artesunate observed, and the lack of in vitro cross resistance between the two drugs, it could be possible to combine quinine and artesunate for the management of severe malaria. This would parallel the combination between amodiaquine and artesunate, being used for the management of acute malaria (Kremsner and Krishna 2004; Mutabingwa et al., 2005; Meremikwu et al., 2006; Djimde et al., 2008; Oyakhirome et al., 2007) Already, synergy between artesunate and quinine has been reported in vitro (Fivelman et al., 1999). Thus, artesunate plus quinine combination in areas with high P. falciparum parasite sensitivities to these drugs as in the present instance would engender two plausible possibilities. Namely, to achieve effective and high cure rates for both severe and acute clinical malaria, and to prolong the vital therapeutic life of both antimalarial agents.

In conclusion, the present data has demonstrated high in vitro sensitivities of artesunate, amodiaquine and quinine against P. falciparum isolates in this area, albeit low levels of in vitro resistance to amodiaquine. Subsequent surveys should in addition to the present procedure integrate both in vivo and molecular surveillance, in order to characterize the true nature of P. falciparum isolates in this area. Such efforts are necessary in order to evolve appropriate controls measures against the spread of drug resistant parasite strains.

REFERENCES


Ikpa et al., (SWJ):16-21

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