Science World Journal Vol 4 (No 4) 2009
www.scienceworldjournal.org
ISSN 1597-6343

Full length Research Article

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 $(EC)_{50}$ = 241.55 nM, EC_{90} = 676.08 nM, and EC_{99} = 993.12 nM; and artesunate, EC_{50} = 1.05 nM, EC_{90} = 2.42 nM, and EC_{99} = 3.16 nM. 1.37 % of isolates were resistant to amodiaquine, EC_{50} = 22.08 nM, EC_{90} = 66.22 nM and EC_{99} = 100.23 nM. Significant *in vitro* cross resistance was found at EC_{50} 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 (Olliaro, 2005). In Nigeria, resistance to chloroquine and sulfadoxine/pyrimethamine by *P. falciparum* has been reported (Salako & Aderounmu, 1987; Olurinola, 1989; Abdullahi *et al*., 2003; Oguche *et al*., 2004; Pitmang *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 (Kremsner & 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 artemisinins 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, artesunate 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; artesunate: 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 $NaHCO_3$ (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 malaria positive blood, from each patient was transferred to wells on the micro culture plates, pre-dosed with varying concentrations of antimalarial drugs. The plates were placed in a candle jar, and incubated at 37°C, for 26-30 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 in duplicate wells 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 EC_{50}, EC_{90}, and EC_{99} values determined. Standard drug resistant clones were not included in the assay. However, drug resistant *P. falciparum* parasites were identified as isolates with EC_{50} or EC_{99} values greater than published threshold values for sensitive parasite isolates. The threshold of resistance were; quinine: EC_{50} >500 nM or EC_{99} > 2560 nM; amodiaquine: EC_{50} > 80 nM, or EC_{99} > 400 nM (Pradines *et al*., 1998; WHO, 2001; Pradines *et al*., 2006). For Artesunate, threshold EC_{50} and EC_{99} 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. Pearson correlation coefficient was used to determine correlation between drug pairs. In each case the level of significance was set at $P \leq 0.05$.

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 EC_{50} > 80 nM among the cultured isolates was 1.37% or (2/146). No resistant isolates were found against quinine, and artesunate (Figs 1, 2, and 3).

TABLE 1. GEOMETRIC MEAN EC_{50} EC_{90} AND EC_{99}, 95% CONFIDENCE INTERVAL (CI) OF ANTIMALARIAL DRUGS AGAINST *Plasmodium falciparum* ISOLATES, N = 146.

Drugs	Geometric Mean EC, (95% CI) nanomolar (nM)		
	EC_{50}	EC_{90}	EC_{99}
Artesunate	1.05 (1.03 – 1.07)	2.42 (2.37 – 2.47)	3.16 (3.07 – 3.26)
Amodiaquine	22.08 (20.75 – 23.44)	66.22 (61.38 – 71.45)	100.23 (93.11 - 107.89)
Quinine	241.55 (232.81 – 250.61)	676.08 (652.08 – 700.97)	993.12 (959.84 – 1027.54)

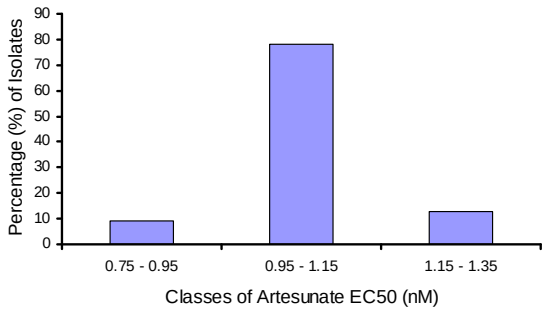

FIG. 1. RELATIVE EC_{50} (nM) DISTRIBUTION PATTERNS OF ARTESUNATE AMONG *P. falciparum* ISOLATES AT MAKURDI

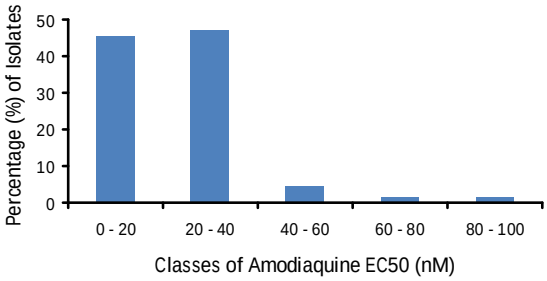

FIG. 2. RELATIVE EC_{50} (nM) DISTRIBUTION PATTERNS OF AMODIAQUINE AMONG *P. falciparum* ISOLATES AT MAKURDI

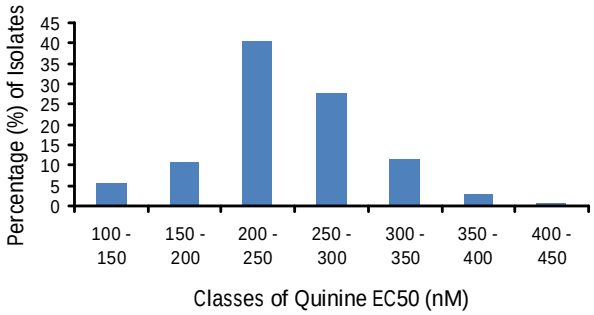

FIG. 3. RELATIVE EC_{50} (nM) DISTRIBUTION PATTERNS OF QUININE AMONG *P. falciparum* ISOLATES AT MAKURDI

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 EC_{50} 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* EC_{50} 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 EC_{50}. (R = PEARSON CORRELATION COEFFICIENT)

Drug pair	n	r	R^2%	P
Quinine-amodiaquine	146	+ 0.342	11.70	< 0.05
Quinine-artesunate	146	+ 0.057	0.32	> 0.05
Artesunate-amodiaquine	146	+ 0.088	0.77	> 0.05

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 EC_{50}, EC_{90}, and EC_{99} 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 EC_{50} and EC_{99} values of quinine are comparably close to the corresponding inhibitory concentration - IC_{50} and IC_{99} values of 0.25 µM and 0.80 µM respectively, reported almost two decades

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 being 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 subjected 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.

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