AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY MAY 2010 ISBN 1595-689X VOL 11(2)
AJCEM/200985/21015 -http://www.ajol.info/journals/ajcem

AFR. J. CLN. EXPER. MICROBIOL 11(2): 111-119

IN VITRO SURVEILLANCE OF DRUG RESISTANT FALCIPARUM MALARIA IN NORTH CENTRAL NIGERIA

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ABSTRACT

Background: drug resistant malaria is spreading inexorably to areas with drug sensitive malaria parasites. This study compared the *in vitro* sensitivities of *Plasmodium falciparum* fresh parasite isolates, to some standard antimalarial drugs, in Makurdi and Masaka located over 300 km apart, in north central Nigeria.

Methods: The *in vitro* responses of *P. falciparum* isolates; 43 and 39 in Makurdi and Masaka were evaluated by the standard schizonts growth inhibition assay in children aged 2-14 years.

Results: The geometric mean effective concentration-EC_{50}, EC_{90} and EC_{99} of quinine between Makurdi and Masaka differed significantly ($P < 0.05$). A similar difference ($P < 0.05$), was observed with the artesunate antimalarial at EC_{90} and EC_{99} levels, but not at EC_{50}. No significant difference ($P > 0.05$) was observed in the EC values of amodiaquine between the two locations. 5.13 % (2/39) of parasites at Masaka were *in vitro* resistant to amodiaquine with $EC_{50} > 80$ nM. The rest of the isolates were sensitive to the three antimalarial drugs at both locations.

Conclusion: The results demonstrated low *in vitro* resistance of *P. falciparum* to amodiaquine in the region. Constant monitoring and intervention is needed to curtail the spread of resistance to antimalarials in Nigeria.

KEY WORDS: *Plasmodium falciparum*, Resistance, Antimalarials, Nigeria.

INTRODUCTION

The changing patterns in the epidemiology of malaria worldwide have led inexorably to the spread of resistant strains of malaria parasites, and reduced efficacy to very vital antimalarial drugs such as chloroquine and sulfadoxine/pyrimethamine (1,2). The emergence of drug resistant malaria among countries in sub-Saharan Africa where the impact of malaria due to *P. falciparum* is most felt has created as dire necessity for constant surveillance and monitoring of *P. falciparum* responses to the available antimalarial chemotherapies used in the region, using appropriate measures(3). Constant monitoring is necessary in order to create a reliable national database which could be relied upon, to design and implement appropriate control measures aimed at preventing the wanton spread of multidrug resistance malaria within and across national borders.

In Nigeria, despite the importance of surveillance data to effective malaria control, the existing data on parasite susceptibility to several antimalarial drugs that are used in the country other than chloroquine are sparse. Only a few reports exist, particularly on the latter (4, 5, 6).

Malaria epidemiology, and indeed the spread of multidrug resistant parasite strains associated with it could vary even within the same border due to ecological, environmental, demographic, and cultural factors associated with a given population. Thus different malaria control strategies may be needed, such as the use of two or more different antimalarial drugs to combat malaria among areas with different levels of malaria parasite sensitivities to a specific antimalarial drug.

It was the aim of this surveillance study to determine and compare the level of *in vitro* sensitivities of *P. falciparum* isolates to some selected standard antimalarial drugs, used for malaria treatment in Makurdi and Masaka, located over 300 km apart within the same region of north central Nigeria, and to generate baseline data for future monitoring of parasite responses to those antimalarial drugs in the region.

METHODS

Study site: the study was conducted at the Bishop Murray Medical Centre Makurdi. Samples were obtained from the Medical Centre as well as the Primary Health Care Centre Masaka, a sub urban area of the Federal Capital Territory (Abuja) in north central Nigeria. Samples collected from Masaka were transported on wet ice to Makurdi and processed within 48 hours. The study protocol was approved by the local ethics committee of the hospital. It lasted from the malaria transmission season between April to October 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.[7]

***In vitro* parasite culture and growth inhibition assay**: the *in vitro* cultivation of *P. falciparum* isolates followed a modification of the standard culture techniques (8,9). Malaria positive blood samples were first washed three times in RPMI 1640 medium, to remove the leukocytes and the buffy coat and the infected erythrocytes re-suspended in the culture medium at 50% haematocrit and stored briefly at 4^{o}C. The parasite growth inhibition assay followed the standard procedure for schizonts

inhibition.[7] A blood medium mixture (BMM) was prepared from a 1:20 dilution of the re-suspended infected erythrocytes, made in a sterile culture medium consisting of 10.43g RPMI 1640 (Invitrogen), 5.96g HEPES, and 25mM $NaHCO_3$ (Sigma Aldrich), per litre of double distilled water and 0.5ml of 50mg/ml gentamicin, and supplemented with 5% albuamax II (Gibco).[10] 200µl of the BMM per well was pipetted on to the wells of a sterile flat bottom 96 well micro culture plates, pre-dosed with a serial 2-fold varying concentrations of antimalarial drugs. The range of the final antimalarial drug concentrations on the plates were, amodiaquine: 6.25 – 400 nM; artesunate: 0.34 – 22 nM; and quinine: 50 – 3200 nM. The plates were covered, placed in a humid candle jar, and incubated at 37°C, for 26-30 hours,[7] at the end of the incubation period, thick films were made, and stained with 2.5% Giemsa stain for 35 minutes. The mean number of schizonts formed in duplicate wells per 200 asexual parasites were counted and recorded.

Determination of effective concentrations (EC) and Potency ratios (PR) of antimalarial drugs: the number of schizont counts was fed into a non linear regression software; HN-NonLin made available free of charge by H. Noedl, at http://malaria.farch.net 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. Potency ratios of each drug were estimated as EC_x A/EC_x B where x = EC values at 50%, 90% and 99%. Standard drug resistant clones were not included in the assay; however, drug resistant *P. falciparum* parasites were identified as isolates with EC_{50} values, greater than published threshold values for sensitive parasite isolates. The threshold of resistance were; amodiaquine: EC_{50} > 80 nM, quinine: EC_{50} > 800 nM (11,12). Artesunate: values not yet determined (13); estimated EC values were therefore reported as a baseline data for future comparison.

DATA ANALYSIS

Geometric mean EC values and 95 % confidence intervals (CI) of each antimalarial drug were estimated for each site; non paired t – test was used to compare EC values between locations, while potency ratios of drugs were compared by ANOVA. The level of significance was set at $P \leq 0.05$.

RESULTS

The percentages of *P. falciparum* parasite isolates that were successfully tested for *in vitro* drug susceptibility test against the three standard antimalarial drugs at the two sampled locations were 82.69 % (43/52) and 76.47 % (39/51) of the original numbers that were subjected to the tests at Makurdi and Masaka respectively. The geometric mean

EC values of quinine between Makurdi and Masaka differed significantly at EC_{50}, EC_{90}, and EC_{99} ($P < 0.05$, table 1). The EC values of the artesunate antimalarial drug, between the two sites were statistically different at EC_{90}, and EC_{99}, but not at EC_{50}. Comparable values for amodiaquine against *P. falciparum* parasite isolates at Makurdi and Masaka were not significantly different ($P > 0.05$).

Table 1. Geometric Mean EC_{50} EC_{90} and EC_{99}, 95% Confidence Interval (CI) of Antimalarial Drugs against *Plasmodium falciparum* Isolates at Makurdi and Masaka.

Drug	Geometric Mean EC, (95% CI) nM		t-test	df	P-values
	Makurdi	Masaka			
AMQ: EC_{50}	21.64 nM (16.22 – 27.06)	24.89 nM (20.78 – 29.00)	0.68	80	> 0.05
EC_{90}	61.86 nM (51.46 – 72.26)	76.54 nM (62.43 – 90.65)	1.89	80	> 0.05
EC_{99}	91.51 nM (74.14 – 108.88)	115.32 nM (94.53 – 136.11)	1.89	80	> 0.05
ART: EC_{50}	1.06 nM (1.02 – 1.10)	1.05nM (1.05 - 1.08)	0.39	80	> 0.05
EC_{90}	2.33 nM (2.24 – 2.41)	2.47 nM (2.38 – 2.56)	2.14	80	< 0.05
EC_{99}	2.97 nM (2.81 – 3.12)	3.25 nM (3.06 – 3.44)	2.37	80	< 0.05
QNN: EC_{50}	232.63 nM (219.33 – 245.93)	286.64 nM (270.08 – 303.20)	5.07	80	< 0.05
EC_{90}	650.82 nM (614.96 – 686.68)	791.80 nM (751.89 – 831.73)	5.22	80	< 0.05
EC_{99}	953.63 nM (911.58 – 1005.68)	1125.79 nM (1083.78 – 1167.80)	4.82	80	< 0.05

AMQ = amodiaquine, ART = artesunate, QNN = quinine.

The potency ratio of the individual antimalarial drugs which measured the different *in vitro* activities of amodiaquine, artesunate, and quinine against *P. falciparum* isolates at the two locations shows that there was no significant difference in the sensitivities of each antimalarial drug between Masaka and Makurdi, ANOVA ($F_{2, 8,}$ df) = 3.39 $P > 0.05$, table 2.

Table 2. Potency Ratios of the Antimalarial Drugs at Masaka and Makurdi

EC_x	Potency ratios of drugs		
	Amodiaquine	Artesunate	Quinine
50%	1.15	0.99	1.23
90%	1.24	1.06	1.22
99%	1.30	1.22	1.18

ANOVA ($F_{2, 8,}$ df) = 3.39, P > 0.05

All the parasite isolates (100%) were *in vitro* sensitive to quinine and artesunate, at Makurdi and Masaka as determined by their individual EC responses to the drugs. In contrast, 5.13 % (2/39) of *P. falciparum* isolates at Masaka had values of EC_{50} > 80 nM and were classified as *in vitro* resistant to amodiaquine. No isolate at Makurdi exhibited a similar disposition towards the amodiaquine antimalarial drug, table 3.

Table 3. Percentage (%) of *in vitro* Sensitive and Resistant Isolates of *P. falciparum*

Antimalarial Drug	**location**	***n***	***in vitro* sensitive (%)**	***in vitro* resistant (%)**
Amodiaquine	**Makurdi**	**43**	**43 (100.00%)**	**-**
	Masaka	**39**	**37 (94.87 %)**	**2 (5.13 %)**
Artesunate	**Makurdi**	**43**	**43 (100.00%)**	**-**
	Masaka	**39**	**39 (100.00%)**	**-**
Quinine	**Makurdi**	**43**	**43 (100.00%)**	**-**
	Masaka	**39**	**39 (100.00%)**	**-**

DISCUSSION

The findings from the present data indicate that, wide differences exist in the effective concentration values of *P. falciparum* parasite isolates to quinine in Makurdi and Masaka. This was in spite of the fact that all the parasite isolates obtained from these locations were *in vitro* sensitive to quinine; isolates at Masaka had consistently higher geometric mean EC_{50}, EC_{90}, and EC_{99} values compared to those at Makurdi. This demonstrates that the continued use of quinine in these areas, and possibly, future development of parasite resistance to the drug may evolve at different intervals between these sites. Quinine has remained very useful for the treatment of chloroquine resistant falciparum malaria in Nigeria;[5] and most isolates are highly *in vitro* sensitive to the drug in the country compared to other regions in the world (13, 14). The present

EC_{50} values of quinine in north central Nigeria are very close to 50% inhibitory concentration (IC_{50}) = 0.25µM previously reported in western Nigeria nearly two decades ago (15), suggesting that the sensitivity of quinine in the country has remained very stable over the years, at least between the west and north central parts of Nigeria.

The differences observed in the artesunate antimalarial drug at EC_{90}, and EC_{99} levels, at the two sites may reflect inherent differences prevalent in the nature of *P. falciparum* parasites in Makurdi and Masaka. However the geometric mean EC_{50} values of the drug against parasite isolates at each site were low, not significantly different, and comparably similar to values generally reported for parasite isolates that were susceptible to the drug in other parts of the world.[13,14] The global importance of the artemisinin derivatives and by extension the artesunate antimalarial drug in the current scheme of malaria treatment is predicated on the ability of the drugs to rapidly kill and eliminate the metabolically actively destructive stages of the human malaria parasites (16), and save lives. At the moment there is little evidence of parasite resistance to these drugs as has recently been found with other key antimalarial drugs (12,17). Yet, the danger still looms large as *P. falciparum* has often in the past found a way round to acquire resistance against other antimalarial drugs, the artemisinin derivatives may not be an exception in the future.

Thus to delay parasite resistance and sustain the prolong use of these useful drugs, their combination with other antimalarial drugs have often been advocated (18). In areas with high sensitivity to artesunate, amodiaquine, and quinine as found in the present survey, a combination of artesunate/quinine for the treatment of severe malaria, and artesunate/amodiaquine for the treatment of acute malaria could prove very useful. Such combinations have been reported to produce high cure rates in the treatment of uncomplicated malaria elsewhere (19, 20), and reduced the high expenditure associated with the treatment of malaria, with ineffective antimalarials (21).

Despite the lack of significance difference in the EC values of amodiaquine in Makurdi and Masaka at all the levels of the effective concentrations analysed, 5.13 % of isolates at Masaka were *in vitro* resistance to the drug, unlike their counterpart at Makurdi. Compare to a recent study in western Nigeria, *in vitro* EC values of amodiaquine against *P. falciparum* parasites were EC_{50} = 0.06 µM, EC_{90} = 0.26µM, and EC_{99} = 0.59µM and were higher than the present values in north central Nigeria (22). Moreover, 39% of a mere 36 parasite isolates observed in that study were *in vitro* resistant to amodiaquine,

which highlights wide spread *in vitro* resistance of *P. falciparum* isolates to amodiaquine in western Nigeria, and a noticeable gap in the *in vitro* susceptibility profiles of *P. falciparum* isolates to amodiaquine between the west and north central Nigeria. Thus isolates in north central Nigeria appear to be more *in vitro* sensitive to amodiaquine than their counterparts in the western part of the country. The present and the previous data also suggest that resistance to amodiaquine has emerged in the country, and may be spreading at an undetermined level. Increased parasite resistance to the drug *in vivo* would progressively limit the relevance of amodiaquine, as a viable option for combination therapy with artesunate in the treatment of uncomplicated malaria in Nigeria The usefulness of the artesunate/amodiaquine combination in producing high cure rates in clinical malaria has been demonstrated in Nigeria and other countries (23,24,25). Generally, *in vitro* studies provide a clue which may be detectable in the clinical treatment outcome of parasite responses to antimalarial drugs *in vivo*. Although the potency ratios of the three drugs used in the current survey did not suggest any significant *in vitro* differences among the activities of these antimalarial drugs, relying solely on *in vitro* assessment to define possible activity similarities or otherwise of antimalarial drugs may not be sufficient. This is because *in vitro* studies do not take cognizance of acquired immunity against malaria parasites, which is promoted by certain antimalarial interventions (26). Further studies combining *in vitro* and *in vivo* as well as molecular characterization may be necessary to determine the actual activities of these antimalarial drugs in the north central region of Nigeria.

In conclusion, the present study has provided evidence of *in vitro* resistance of *P. falciparum* to amodiaquine in north central Nigeria. It might be necessary to constantly monitor and initiate critical control measures to limit the spread of resistance to amodiaquine and other antimalarial drugs in the country, which might develop as a result of cross resistance between drugs.

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