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In vitro EFFICACY OF ACT DRUGS ON *Plasmodium falciparum* CLINICAL ISOLATES FROM KANO AND KATSINA STATES, NIGERIA

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ABSTRACT
In vitro drug susceptibility profile of Plasmodium falciparum collected between 2012 and 2014 from malaria patients attending some hospitals in Kano and Katsina States was evaluated in this study. This is to provide base line information on current effectiveness of commonly prescribed ACT drugs (Artemether-lumefantrine AL, Dihydroartemisinin-piperaquine DHP, Artesunate-amodiaquine AA). Plasmodium falciparum isolates were identified by rapid diagnostic test (malaria HRP2 kit) and microscopic examination of Giemsa stained blood films. One hundred and fifty (150) isolates (90 from kano and 60 collected from katsina) were successfully cultivated. In vitro antimalarial drug sensitivity test was carried out using Schizont growth inhibition assay. Fifty percent inhibitory concentrations (IC_{50}) were calculated using Hn nonlin software and dose response curves. The results revealed that all the P. falciparum isolates were 100% sensitive to the three ACTs. The geometric mean IC_{50} values of the isolates were found to be below the resistance thresholds cut off values as 2.04±0.42nM, 3.67±0.4nM and 4.70±0.43nM for AL, DHP and AA respectively. This suggests that all the ACTs are still efficacious in the treatment of malaria in the two states. Further research is therefore, recommended to confirm these findings using in vivo and molecular approach.
Key words: ACTs, malaria, in vitro, Plasmodium falciparum

INTRODUCTION

Resistance of *P. falciparum* to the common and cheap antimalarial drugs chloroquine and sulpadoxyne-pyrimethamine has a profound public health impact in malaria endemic areas like Nigeria. This increasing drug resistance has necessitated change in antimalarial therapy in Africa. In view of this, the World Health Organization recommends the use of artemisinin combination therapy (ACT) to improve treatment efficacy and slow the development of resistance (WHO, 2010). ACT has been accepted as an effective strategy to combat the emergence and spread of malaria drug resistance. However, some studies provide evidence of declining susceptibility of *P. falciparum* to ACTs (Jambou *et al.,* 2005; Pradines *et al.,* 2006). Decreased sensitivity to ACTs is alarming since there is no new class of antimalarial drugs that are ready to replace these derivatives. Thus, the World Health Organization (WHO) has launched an intensive campaign to monitor the emergence of ACT resistance (WHO, 2010).

Four different ways of monitoring and reporting parasite susceptibility or resistance to antimalaria drugs exist. These include the *in vivo* tests, the *in vitro* test, the use of animal models and molecular characterization (Kaira *et al.*, 2006).

Several types of *in vitro* antimalarial drug sensitivity assays are available but very few Nigerian laboratories have the capacity to conduct these assays. This includes measuring parasitaemia, schizont maturation, protein production, DNA nucleotide incorporation, DNA fluorescence staining, and ELISA (Noedl *et al.*, 2003). Procedure of schizont maturation assay is use to evaluate the efficacy of ACTs in this study. This method is labor intensive but requires little technical equipments to perform. This paper provides base line information on *in vitro* susceptibility profile of *P. falciparum* clinical isolates to different ACTs (Artesunate-amodiaquine, Dihydroartemisinin-piperaquine, Arthemeter-lumefantrine) used in Kano and Katsina state Nigeria as recommended by World Health Organization.

MATERIALS AND METHODS

Sampling

The ethical committee of Kano and Katsina state ministry of Health, Nigeria approved the study protocol. *P. falciparum* infected blood was obtained from 652 malaria patients attending some hospitals in the states. The sampling hospitals include Murtala Muhammad Specialist Hospital, Hasiya Bayero Paediatric Hospital, Wudil, Gaya and Kura General Hospitals in Kano State and Kankiya, Daura, Funtua, Ingawa and Kusada General Hospitals in Katsina State.

Samples were also obtained from malaria positive volunteers who attended primary health care centers and local pharmacies in the randomly selected LGAs.

Malaria infection was diagnosed using rapid malaria test kit (care start HRP2) and confirmed by microscopic examination of Giemsa stained blood films according to the procedures of Cheesbrough (2000) and Arora and Arora (2009). Three milliliter (3mls) of venous blood samples collected from each of the subjects in EDTA coated tubes (Dickinson) were temporarily stored on wet ice in thermos flask with the tubes not making direct contact with the ice. When the infected blood sample was not immediately processed, it was cryopreserved using 25ml glycerol and 72ml of 4.2% sorbitol in normal saline. The sample was then refrigerated or kept in liquid Nitrogen (WHO, 2001).

***In vitro* assay**

Blood samples obtained before malaria treatment were tested for in *vitro* sensitivity to different artemisinins combination drugs using schizont growth inhibition assay described by WHO (2001)and Ikpa *et al.*(2009).

Stock solution 1mg/ml each of Artemether-lumefantrine (Novartis pharma Switzerland) in ethanol, Dihydroartemisinin-piperaquine (Novartis) and Artesunate-amodiaquine (Novartis) in sterile distilled water were prepared. From these stock solutions subsequent dilutions were made using incomplete RPMI 1640 medium (IRMI1640) as a diluents to yield a desired two fold concentration for each ACT. Fifty microlitre (50µl) of each concentration was distributed to 96 well microculture plate (Nunc) and dried in an incubator at 37^0C. The concentration for each well ranges from 0.62-39.68nM, 2.08-133.12nM and 3.29-210.56 nM for AL, DHP and AA respectively. The plates were stored at 4^0C in sterile plastic containers.

Plasmodium falciparum in vitro cultivation followed standard culture techniques by Trager and Jensen (1976). The culture media is made up of 10.43g RPMI 1640 (sigma), supplemented with 5% albumax II (Gibco) and buffered with 5.96gHepes and 25mM $NaHCO_3$ (sigma Aldrich) per liter of double distilled water. Gentamicin (0.5ml of 50mg/ml) was added to inhibit bacterial growth after sterilization by filtering through a millipore filter of 0.22µm porosity. Malaria parasites infected blood (2% parasitaemia level) from each subject was suspended in RPMI1640 medium in 1:20 dilution. Two hundred microliter (200µl) of the mixture was transferred to different wells of micro culture plates predosed with varying concentration of antimalarial drugs (ACTs), including drug free control wells. The plates were incubated for 30 hours in a candle jar (92% N_2, 5% CO_2 and 3% O_2) at 37^0C. Thick blood films were prepared from each well after incubation and stained with 2.5% Giemsa stain according to the procedure of (Cheesbrough) 2000. The IC_{50} of ACTs against the isolates was determined from dose response curves according to the procedure of Noedl *et al.* (2001). Mean percentage of the parasite inhibition *in vitro* was calculated as

$$\frac{A - B}{A} \times 100$$

where A is equal to mean number of schizonts that mature in free drug wells and B is equal to mean number of schizonts that mature in drug treated wells.

The results of *in vitro* assay are expressed as 50% inhibitory concentration ($1C_{50}$) defined as the concentration of antimalarial drug that inhibits 50% of schizont maturation as compared with the development in drug free control wells. *In vitro* resistance threshold was determined according to (Pradines *et al.,* 1998) as > 2 × standard deviation above the mean.

Data analysis

Statistical analysis was performed using SAS software general linear model version 9.3 and nonlinear regression software (HN-Nonlin) and the level of significance (p) was fixed at 0.05.

RESULTS AND DISCUSSION

The *in vitro* culture was performed on 652 isolates of *Plasmodium falciparum,* 436(67%) from Kano and 216 (33%) from Katsina. One hundred and fifty (23%) of the cultured isolates grew *in vitro* and yielded complete results for the *in vitro* drug susceptibility tests (Table 1). The proportions of successful assay were 21% (n=90) and 28% (n=60) for Kano and Katsina respectively. The *in vitro* culture of *P. falciparum* is a very delicate one whose success depends on several parameters that are difficult to control, including insufficient maturation of schizonts and contamination. This could be the possible reason for poor cultivation success observed in this study.

The percentage growth inhibition of each antimalarial was dose dependent and increased with increasing drug concentrations (Table 2). The three (3) ACTs (Artemether-lumefantrine, Dihydroartemisinin-piperaquine, and Artesunate-amodiaquine) each achieved 100% parasite growth inhibition at higher concentrations. However, there was no significant difference in the level of parasite growth inhibition *in vitro* among the three drugs, at the various concentrations used (X^2 = 15.63, df = 12 P>0.05). The mean IC_{50} values, Range and the resistance threshold for all the ACTs are presented in Table 3.

This study reports the geometric mean IC_{50} values for Artemether-lumefantrine as 2.04nM, Dihydroartemisinin-piperaquine 3.67nM and Artesunate-amodiaquine 4.70nM. The definition of *in vitro* parasite resistance to antimalarial drugs applied to the present study was based on the threshold cut-off values of IC_{50} of various antimalarial drugs, which distinguish resistant from sensitive parasites. These critical values are established based on comparative analysis of *in vivo* and *in vitro* results collected around the world (Pradines *et al*. 2006). Because of the apparent lack of confirmation of *P. falciparum* resistance to artemisinins and its derivatives in humans, these values are not yet established for ACTs. Therefore, the threshold IC_{50} values were calculated from the formula (IC_{50}mean+2SD) (Pradines*et al*. 1998) as 2.88, 4.51 and 5.56nM for AL, DHP and AA respectively. However, in all the isolates tested the IC_{50}values are below the respective cut- off values for resistance. These results are consistent with other studies from Africa reporting IC_{50}values of artemisinin derivatives as 2.2 and 2.6nM from Senegal, and Rwanda respectively (Ferreira *et al*. 2007; Tinto *et al*. 2006). In contrast to the finding of this research, some surveys around the world provide evidence of reduced *in vitro* susceptibility of *Plasmodium falciparum* to artemisinin derivatives (Artemether and Dihydroartemisinin) (Jambou *et al*. 2005; Pradines *et al*. 2006).

All the isolates tested were 100% sensitive to both ACTs which makes these regimens of choice in the management of cases of malaria and in the control of antimalarial drug resistance. These results are consistent with the previous report that found prevalence of *in vitro* sensitivity of *P. falciparum* to Arthemether-lumefantrine (Coartem)of up to 96.29% in Cotonou, Benin Republic (Gouissi and Salifou, 2012).

The correlation between the *In vitro* responses of *Plasmodium falciparum* isolates to the ACT pairs is presented in Table 4. Significant positive correlation was obtained between AL and DHP ($r=0.6078$, $p<0.001$) AL and AA ($r=0.599$, $P<0.001$) and between DHP and AA ($r=0.966$ $p<0.001$). This indicates the possibility of cross-resistance, thus parasite resistance to any of these ACTs could eventually lead to resistance of others.

Table I: *Plasmodium falciparum* parasites isolated and cultured for *in vitro* Drug Susceptibility Test from some hospitals in kano and Katsina state.

Characteristics	Kano	Katsina	Total
Number of isolates cultured (%)	436 (67)	216 (33)	652 (100)
Number of isolates failed for *In vitro* culture (%)	346 (79)	156 (72)	502 (77)
Number of isolates successfully cultured (%)	90 (21)	60 (28)	150 (23)

Key: AL – Arthemeter - lumefantrine, DHP – Dihydroartemisin – piperaquine, AA – Artesunate Amodiaquine

Table 2: Mean Growth Inhibition of *Plasmodium falciparum in vitro* at different concentration of ACTs

	Drug conc. (nM)			Mean parasite growth inhibition ±SD%		
S/N	AL	AA	DHP	AL	AA	DHP
1	0.62	3.29	2.08	16.2±1.92	15.8±1.6	19.6±2.1
2	1.24	6.58	4.16	40.3±3.92	58.04±2.3	50.1±2.8
3	2.48	13.16	8.32	68.6±4.13	89.4±3.1	89±3.8
4	4.96	26.32	16.64	91.4±5.18	90.2±4.6	96±3.1
5	9.92	52.64	33.28	100±0.0	97.3±4.8	100±0.0
6	19.84	105.28	66.56	100±0.0	100±0.0	100±0.0
7	39.68	210.56	133.12	100±0.0	100±0.0	100±0.0

$X^2=15.63$, df =12, $p>0.05$; n =150

Key: AL – Arthemeter – lumefantrine; DHP – Dihydroartemisin – piperaquine; AA – Artesunate-Amodiaquine

Table 3: *In vitro* susceptibility of *Plasmodium falciparum* isolates to ACTs (IC_{50} values)

Drug (ACT)	IC_{50}mean±SD (nM)	Range (nM)	Resistance threshold (nM)
AL	2.04±0.42	1.23 – 2.67	>2.88
DHP	3.67±0.42	3.01 – 4.23	>4.51
AA	4.70±0.43	4.08 – 5.50	>5.56

Key: AL – Arthemeter – lumefantrine; DHP – Dihydroartemisin – piperaquine; AA – Artesunate-Amodiaquine n =150

Table 4: Correlation of *In vitro* responses of *Plasmodium falciparum* showing Pearson correlation coefficient between the three tested ACTs

Drug Pair	r	P-value
AL – DHP	0.6078	<0.001
AL – AA	0.5999	<0.001
DHP – AA	0.9677	<0.001

Key: AL – Arthemeter - lumefantrine; DHP – Dihydroartemisin- piperaquine; AA – Artesunate-Amodiaquine

CONCLUSION

The findings of this research provide no evidence of *in vitro* resistance of *P. falciparum* to ACTs. This suggest that all the ACTs tested (Artemether-lumefantrine, Dihydroartemisinin-piperaquine and Artesunate-amodiaquine) are still efficacious in the treatment of uncomplicated malaria in Kano and katsina State. Further research is therefore, recommended to confirm these findings using *in vivo* and molecular approach.

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