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Comparison of *In Vivo* and *In Vitro* Tests of Resistance in *Plasmodium Falciparum* Positive Patients Treated With Artemisinin Based Combination Therapy in Northwestern Nigeria

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Abstract

Comparative studies on *in vitro* and *in vivo* drug response of *Plasmodium falciparum* isolates were carried out among malaria positive resident of Kano and Katsina states of Nigeria, to determine the effectiveness of *in vitro* antimalarial drug sensitivity test as a substitute for *in vivo* therapeutic response analysis. The *in vitro* test was carried out using Schizont growth inhibition assay which was evaluated by comparing its results with the *in vivo*/therapeutic response determined by 28 days follow-up of the *Plasmodium falciparum* positive patient treated with different Artemisinin combination therapy (Artemether-lumefantrine, Dihydroartemisinin-piperaquine, Artesunate-amodiaquine). Out of 652 patients enrolled, 227 (34.8%) completed the 28 days follow-up, and 120 isolates from subjects with complete follow-up data yielded an interpretable *in vitro* test. A total of 100 of 120 patients (83.3%) had adequate clinical and parasitological response. The geometric mean 50% inhibitory concentrations (IC_{50}) of the isolates obtained from these patients were 2.03nM, 3.65nM and 4.68nM for Artemether-lumefantrine (AL), Dihydroartemisinin-piperaquine (DHP) and Artesunate-amodiaquine (AA) respectively (*in vitro* and *in vivo* sensitive). Treatment failure was observed in 20 (16.7%) of 120 patients whose IC_{50} values were 2.11nM, 3.77nM and 4.80nM for AL, DHP and AA respectively. Moreover all the isolates of the patients responding with treatment failure yielded a discordant result (i.e. *in vivo* resistance and *in vitro* sensitive). Thus, the result of this study indicates poor agreement between the *in vitro* and *in vivo* test (Kappa value = 0) with regards to treatment failure. The *in vitro* assay cannot therefore be used as a substitute for *in vivo* therapeutic test for drug efficacy.

Keywords: Acts; Malaria; I*n Vitro*; *In Vivo*; *Plasmodium Falciparum*

Introduction

Malaria is a mosquito-borne infectious disease caused by an intracellular protozoan parasite of the genus plasmodium [25]. Five species of plasmodia namely; *plasmodium falciparum, P. vivax, P. ovale, P. malanae and P. Knowlesi,* cause the disease in humans. The most serious forms of the disease are caused by *Plasmodium falciparum* [1]. Resistance of *P. falciparum* to the traditional antimalarial drugs (Chloroquine and Sulpadoxyne-pyrimethamine) is a growing problem and is thought to have contributed to increased malaria mortality in recent years [2]. In Nigeria as well as other countries, malaria treatment and control are hindered by the spread of resistance to common antimalarial drugs. This increasing drug resistance has necessitated change in antimalarial therapy in Africa. This has led the World Health Organization to recommend the use of Artemisinin combination therapy (ACT), which is highly efficacious.Multiple highly effective ACT regimens are now available, but the optimal choice for malaria in most areas remains uncertain [3]. ACT has been promoted to be an effective strategy to combat the emergence and spread of resistance. However evidence of resistance has already emerged in some, parts of the world [3]. Decreased sensitivity to ACTs is alarming since there is no alternative class of antimalarial ready to replace the Artemisinin derivatives. Thus the world health organization (WHO) has launched an intensive campaign to monitor ACT resistance [3].

Four different ways of monitoring and reporting parasite susceptibility or resistance to antimalarial drugs exist. These include the *in vivo* tests, the *in vitro* test, the use of animal models and molecular characterization [4]. *In vivo* and *in vitro* test of resistance are the most commonly employed methods in the field [5].

Generally the *in vivo* test has been the standard test normally used in making policy decision, concerning the efficacy of a given drug in an area or region. However, the *in vivo* tests are time consuming and require that subjects participating in the test should

be under periodic treatment and examination for a minimum follow up period of between 14-28 days. The test is also affected by factors such as the patient's immunity, variation of drug absorption and metabolism, and possible misclassification of new infections as recrudescence [6]. These necessitate the need to determine the effectiveness of *in vitro* antimalarial drug sensitivity tests as a substitute for *in vivo* test.

This study was aimed at comparing the *in vivo* and *in vitro* test of resistance in *plasmodium falciparum* positive patients treated with different ACTs, with a view to evaluate the possible use of *in vitro* antimalarial drug sensitivity tests as a substitute for therapeutic response analysis.

Materials and Methods

Study area

The study was conducted at some Hospitals in Kano and Katsina, Nigeria in 2014. Malaria is hyper endemic in the study area with high transmission intensity during rainy season (April to October).

The study protocol was approved by the ethical committee of Kano and Katsina state hospital management board. Informed consent was obtained from parents or guardian of the study children while assent was obtained from the adults.

Subjects

Patients of all ages with symptoms of uncomplicated malaria including a fever or history of fever within 48 hours and mono infected with *P. falciparum* of ≥ 2000 asexual parasite /µl of blood participated in this study. Subject with symptoms of severe malaria, a recent history of use of antimalarial drugs, presence of other diseases and reported allergies to the study drugs were excluded from the study.

Study drugs

The study drugs include Artesunate-amodiaquine (Novartis),Dihydroartemisinin-piperaquine (WAIPA, Novartis,) and Arthemeter-lumefantrine (Novartis).

In vivo assay

Subjects enrollment, blood sample collection, treatment, follow- up and other laboratory procedures were carried out according to the procedure of [3,7,8,26]. Recruited subjects with prescription of any of the above ACTs were treated and followed up for 28 days. A part from first dose drug administration was not supervised. The drugs were administered orally according to body weight for three days: Arthemeter-lumefantrine (20mg:120mg) administered as one tablet to subjects of 5-14kg, two tablets to 15-24kg, three tablets to 25-34kg and four tablets to subjects > 35kg given twice daily. Dihydroartemisinin-piperaquine (40mg dihydroartemisinin/320mg piperaquine) tablets were given as half to one tablet to subject 5-14kg, two tablets to 15-24kg, three tablets to 25-34kg and four tablets to subjects > 35kg once daily. Artesunate-amodiaquine (100mg/270mg) was also administered according to body weight as half tablet to 5kg to 8.9kg, one (9-17.9kg), 1½ (18-34kg) and 2 tablets to subjects >35kg daily.

Recruited subjects were asked to return to the health centers for clinical and parasitological response evaluation on day 3, 7, 14, 21 and 28 post treatments. They were also advised to return at any other day if the sickness persisted. Some of the patients who did not turn up for scheduled follow-ups were visited at home. Patients were excluded during follow up for use of another antimalarial drug, serious adverse events requiring a change in treatment and withdrawal of informed consent or loss of follow up. Blood samples were taken on each follow-up day via finger prick to identify parasite clearance through microscopic examination of thick and thin Giemsa stained blood films. Treatment responses were recorded as classified by [10], early treatment failure ETF (parasitaemia > 25% of day 0 count and temperature >37.5°C), late clinical failure LCF (Present of parasitaemia after day 3 with axillary temperature >37.5°C), Late parasitological failure LPF (Present of parasitaemia after day 3 with axillary temperature <37.5°C) and adequate clinical and parasitological response ACPR (absence of parasitaemia on day 14 irrespective of axillary temperature).

In vitro assay

Blood samples obtained before treatment were tested for *in vitro* sensitivity to different artemisinins combination drugs mentioned above using Schizont growth inhibition assay described by [9,10].

Different concentrations of ACTs in the appropriate solvent were distributed in 96 wells micro culture plates and dried in an incubator. The *in vitro* cultivation of the parasite was achieved using RPMI 1640 medium following modification of the standard culture techniques [11]. Briefly the culture media is made up of 10.43g RPMI 1640 (sigma), supplemented with 5% albumax II (Gibco) and buffered with 5.96g Hepes and 25mM NaHCO3 (sigma Aldrich) per liter of double distilled water. The medium was sterilized by filtration through 0.22µm filter and 0.5ml of 50mg/ml gentamicin was added to inhibit bacterial growth. Malaria infected blood from each subject was suspended in 1640 medium in 1:20 dilution. Two hundred microliter (200µl) of the mixture

was transferred to 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 at 37 °C under reduced oxygen. Thick blood films were prepared from each well after incubation and stained with 2.5% Giemsa stain according to the procedure of [26]. The number of schizonts formed in each well per 2000 a sexual parasite was counted and recorded. TheIC50 of ACTs against the isolates was determined from dose response curves according to the procedure of [12]. Mean % of the parasite inhibition *in vitro* was calculated as $\frac{A-B}{A} X\ 100$; where Ais 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 were expressed as 50% inhibitory concentration ($1C_{50}$). *In vitro* resistance threshold was determined according to [11] as > 2 X standard deviation above the mean.

Data analysis

Statistical analysis was performed using SAS software general linear model version 9.3, OpenEpi version 2.3, and nonlinear regression software (HN-Nonlin). The level of significance (p) was fixed at 0.05; parameters were compared between patients using T-test, ANOVA and Chi-Square. The results of *in vivo* and *in vitro* tests of resistance was compared using kappa statistics to calculate the index of agreement interpreted as 0-0.2, slight agreement; 0.21-0.40, fair ; 0.41- 0.60 moderate; 0.61-0.80, good and > 0.81 very good agreement [5].

Results

A total of 1536 subjects presented to the study hospitals with symptoms of malaria were screened. Six hundred and fifty two subjects met the study criteria and were enrolled in the 28 days follow-up. Table 1 represents the summary of the enrollment and the outcomes.

Among the 652 subjects enrolled, 227 (34.8%) completed 28 days follow up and 425 (65.2%) lost to follow-up. Two hundred and one (88.5%) of the subjects who completed 28 – days follow-up had adequate clinical and parasitological response (ACPR) and 26 (11.5%) failed the treatment.

Location	No. of subject screened	No. of subjects enrolled	No. of subjects excluded/lost to (%) follow-up	No. of subjects who completed 28-days (%) follow-up	Subjects with (%) ACPR	Subjects with treatment (%) failure
Kano	1000	436	285 (65.4)	151 (34.6)	134 (88.7)	17 (11.3)
Katsina	536	216	140 (64.8)	76 (35.2)	67 (88.2)	09 (11.8)
Total	1536	652	425 (65.2)	227 (34.8)	201 (88.5)	26 (11.5)

Table 1: Subject enrollments and the outcome for *In vivo* study of ACTs in Kano and Katsina States

Therapeutic characteristics/clinical parameters of the subjects who completed 28 days clinical study were compared between patients with an adequate clinical and parasitological response and patients with treatment failure presented in Table 2. The mean age, duration of symptoms before treatment and parasitaemia values of subject with ACPR (10.8 years, 4.4 days, 15,600 parasites/µl) were found to be significantly different (P<0.05) from that of subject with treatment failure (4.54 years, 6.9 days and 19,980 parasites/µl) respectively.

S/N	Parameters	Subjects with susceptible malarial parasites (ACPR)	Subjects with treatment failures (TF)	P-VALUES
1	Number of subjects	201	26	
2	Mean age (years)	10.8±9.8	4.54±3	<0.05
3	Sex ratio (male: female)	109:92	16:10	
4	Mean duration of symptoms in days (range)	4.4 (1 – 10)	6.9 (4 – 14)	
5	Mean Temperature (°C)	38.6±1.1	38.9±1.2	>0.05
6	Geometric mean parasitaemia (asexual parasite/µl (Range)	15,600 (1,230 – 29,600)	19,980 (13,200 – 41, 600)	<0.05

Table 2: Therapeutic indices in malaria subject who completed 28- day's clinical study of ACTs

The *In vitro* drug susceptibility tests were 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 3). The proportions of successful assay were 21% (n=90) and 28% (n=60) for Kano and Katsina respectively.

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)

Table 3: *Plasmodium falciparum* parasites isolated and cultured for *in vitro* Drug Susceptibility Test

The percentage growth inhibition of each antimalarial was dose dependent and increased with increasing drug concentrations (Table 4). The three (3) ACTs (Artemther-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).

Artemether-lumefantrinehas the lowest IC_{50} value of 2.04nM, followed by DHP with IC_{50} value of 3.67nM and AA have the highest IC_{50} value of 4.70nM. The geometric mean IC_{50} value of all the ACTs against *P. falciparum* isolates of subjects from Kano and Katsina were not statistically different P>0.05.

	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

Table 4: Mean Growth Inhibition of *Plasmodium falciparum in vitro* at different concentration of ACTs n = 150

The *In vitro* geometric mean IC_{50} values for isolates obtained from 100 patients with an adequate clinical response was 2.04nM for AL, 3.67nM for DHP and 4.70nM for AA (Table 5). Among the isolates obtained from patients with treatment failure (n=20), the geometric mean IC_{50} values were 2.11, 3.77 and 4.80nM for AL, DHP and AA respectively. Thirty (30) isolates were from subject with unknown in vivo therapeutic status and their IC_{50} values were found to be 2.09nM (AL), 3.79nM (DHP) and 4.65nM (AA) respectively. There was no statistical difference in the IC_{50} values of all the ACTs against isolates from subject with ACPR, treatment failure and subject with unknown therapeutic response P>0.005. The IC_{50} values of all the ACTs tested against the *P. falciparum* isolates are below the resistant threshold.

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

Table 5:I*n vitro* susceptibility of *Plasmodium falciparum* isolates to ACTs (IC_{50} values n=150)

The *in vitro* susceptibility patterns of the three ACTs against *P. falciparum* parasite isolates at both Kano and Katsina demonstrated that all the isolates were 100% sensitive (Table 6).

Drug (ACT)	ACPR (susceptible) n=100	Treatment failure (Resistant) n=20	Unknown *In vivo* status(n=30)
AL	2.03a+0.39	2.11a+0.43	2.09a+0.38
DHP	3.65b+0.44	3.77b+0.44	3.79b+0.48
AA	4.68c+0.42	4.80c+0.5	4.65c+0.49

*Means bearing same letters are not significantly different (P>0.05)

Table 6: MeanIC_{50} values (nM) of ACTs with respect to *In vivo* status of the infected subjects

The validity of the *in vitro* results determined using the threshold IC_{50} value for ACTs resistance (2.88nM for AL, 4.51nM for DHP and 5.56nM for AA respectively), as compared with the therapeutic response is shown in (Table 7). The *In vitro* and *in vivo* results were compared using the Kappa statistics. The overall Kappa statistics (Kappa coefficient) for agreement between *In vivo* and *In vitro* test was 0.0 in the present study, thus demonstrating poor /slight agreement between the two methods.

In vitro test result	ACPR	Treatment Failure	Total
AL IC_{50}<2.88	41	09	50
IC_{50}>2.88	00	00	00
DHP IC_{50}<4.51	37	03	40
IC_{50} >4.51	00	00	00
AA IC_{50}<5.56	22	08	30
IC_{50} >5.56	00	00	00
Total	**100**	**20**	**120**

IC_{50}< value = in vitro sensitive;IC_{50}> value =resistant; kappa coefficient = 0.0
Table 7: Validity of *In vitro* test of ACTs resistance to detect *In vivo* drug efficacy based on Therapeutic response

Discussion

Monitoring ACT treatment response for early detection of resistance is an important issue in malaria control. The *in vitro* and *in vivo* activities of different ACTs were evaluated and compared in this study. The *in vivo* aspect of this study revealed high ACT treatment failure (11.5%) compared to 7% failure rate reported in western Nigeria [13] and 5.2% in Tanzania [14] in a 42 days follow- up study. The failure rate (11.5%) observed in this study is similar to the failure rate observed in Tanzania of 11.2% during 28 days follow-up by [15]. The higher failure rate observed in this study could be explained in a number of ways. Firstly, molecular genotyping of pretreatment and post treatment parasite was not conducted to distinguish between treatment failure and new infections. Secondly, drug administration was not properly monitored apart from first dosage because the subjects were out patients and also blood drug levels were not determined in these patients in order to confirm these findings.

Of the total 652 parasite isolates of *Plasmodium falciparum* subjected to *in vitro* sensitivity tests, successful responses were seen in only 150 isolates, this could be due to the fact that *in vitro* culture of *P. falciparum* remains a very delicate test whose success depends on several parameters which are difficult to control, including insufficient maturation of schizonts and contamination.

All isolates tested were 100% sensitive to both ACTs (Arthemether-lumefantrine, Dihydroartemisinin- piperaquine and Artesunate-amodiaquine); which makes these regimens of choice not only in the management of cases of malaria but also in the treatment of remedies for common 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 [16].

The definition of *in vitro* parasite resistance to antimalarial drugs was based on the threshold cut-off values of IC_{50} of various antimalarial drugs, which distinguish resistant from sensitive parasites. Therefore the threshold IC_{50} values were calculated from the formula (IC_{50}mean+2SD) [17] as 2.88, 4.51 and 5.56nM for AL, DHP and AA respectively. However, in all the isolates tested the IC_{50} values were found to be below the respective cut- off values for resistance.

This study reports the geometric mean IC_{50} values for Artemether-lumefantrine as 2.04nM, Dihydroartemisinin-piperaquine3.67nM and Artesunate-amodiaquine 4.70nM. 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 [17,18]. 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 (Art emetherand Dihydroartemisinin) [16,20].

The result of *in vitro* studies, which shows 100% sensitive isolates contradicts the *in vivo* report of this study, this suggest poor agreement of the two methods of assessing effectiveness of ACT in northwestern Nigeria. *Plasmodium falciparum* isolates from subjects with adequate clinical and parasitological response (*in vivo* sensitive) were found to be 100% *in vitro* sensitive to all ACTs. The discordant results were only observed in subject with treatment failure in which all the isolates of the subjects were *in vitro* sensitive. This could be due to limitation of this study that fails to establish the relationship between treatment failure and *in vivo* drug resistance due to some factors. The factors may include patient immunity, variation in pharmacodynamics and pharmacokinetics, host genetic factors, late emergence of secondary or tertiary broods of parasite from liver and possibly misclassification of new infection as recrudescence. These factors can account for the reason of discordant results. Both *in vitro* and *in vivo* tests of resistance have their limitation and in any case do not measure the same biological phenomenon. The i*n vitro* assays exclude several host factors that influence the results of the *in vivo* test. However, in view of some discordant results, the *in vitro* test cannot substitute for *in vivo* data on therapeutic efficacy [5].

According to the [21] the uses of *in vitro* tests provide information on the qualitative response of *P. falciparum* irrespective of the patients' immune status unlike *in vivo* tests. Thus the *in vitro* tests serve as one of the useful epidemiological tools for assessing the baseline sensitivity of drugs, and for monitoring drug response of *P. falciparum* over time and place. The test provides the necessary background information for the development and evolution of drug policies in a given malaria control program. This is because; noticeable changes in the parasites drug sensitivity profile *in vitro* in follow up studies could be an indicator of a future therapeutic failure [22]. Unlike the *in vivo* tests, the results of the *in vitro* test are not equally affected by on-going malaria transmission. The

tests could be conducted simultaneously with many drugs, and independent of the patient clinical condition. *In vitro* tests also permit the monitoring of drug response with compounds for which protocols for *in vivo* tests, has not yet being developed [23-25].

Conclusion

The IC_{50}values of all the ACTs tested (Artemether – lumefantrine, Dihydroartemisinin-piperaquine, Artesunate-amodiaquine) against *P. falciparum* isolates are below the resistance threshold (2.88nM, 4.51nM and 5.56nM for AL, DHP and AA respectively). This revealed that all the ACTs are still efficacious in the treatment of uncomplicated malaria in Northwestern Nigeria. All the isolates of the patients responding with treatment failure yielded a discordant result (i.e. *in vivo* resistance and *in vitro* sensitive). Thus, the result of this study indicates poor agreement between the *in vitro* and *in vivo* test of resistance (Kappa value=0) with regards to treatment failure. The *in vitro* assay cannot therefore be used as a substitute for *in vivo* therapeutic test for drug efficacy.

References

1. Perandin F (2003) Identification of Plasmodium falciparum P. vivax P. ovale and P. malariae and detection of mixed infection in patients with imported malaria in Italy. New Microbiology 26: 91-100.

2. Greenwood BM, Bojang K, Whitty CJ, and Target GA (2005) "Malaria" Lancet 365:1487-98.

3. World Health Organization (2006) WHO guidelines for the treatment of malaria. Technical document WHO/HTM/MAL/2006.1108.

4. Kaira BS, Chawla S, Gupta P, and Valencha N (2006) Screening of antimalarial drugs: an overview. Indian J Pharmacol 38: 5-12.

5. Ringwald P, Basco LK (1999) Comparison of in vivo and in vitro tests of resistance in patients treated with chloroquine in Yaounde Cameroon. Bull World Health Organ 77: 34-43.

6. Adjuik M, Agnamey P, Babiker A, Borrmann S, Brasseur P (2002) Amodiaquine-artesunate versus Amodiaquine for uncomplicated Plasmodium falciparum malaria in African children: a randomised multicentre trial. Lancet 359: 1365-72.

7. Yeka A, Dorsey G, Kamya MR, Talisuna A, Lugemwa M (2008) Artemether-Lumefantrine versus Dihydroartemisinin-Piperaquine for Treating Uncomplicated Malaria: A Randomized Trial to Guide Policy in Uganda. PLoS ONE 3: e2390.

8. Gbotosho GO, Sowunmi A, Okuboyejo TM, Happi CT, Folarin OA (2007) Therapeutic efficacy and effects of Arthemeterlumefantrine andamodiaquine sulfa phyrimethamine on gametocyte carriage in children with uncomplicated plasmodium falciparum in south western Nigeria. Am J Trop Med Hyg 77: 235-41.

9. Ikpa TF, Ajayi JA, Imandeh GN, Usar JI (2009) The in vitro assessment of drug resistant malaria in Makurdi North central Nigeria. Science world journal 4: 16-21

10. World Health Organization (1996) Assessment of the therapeutic efficacy of antimalarial drugs for uncomplicated falciparum malaria in areas with intense transmission. WHO/MAL96. 1077 Geneva.

11. Trager W, Jensen JB (1976) Human malarial parasites in continuous culture. Science 193: 673-5.

12. Noedl H, Wernsdorfer WH, Krudsood S, wailairatana P, Viriyavejakul P, et al. (2001). In vivo and in vitro model for the assessment of clinically relevant antimalarial cross-resistance. Am J Trop Med Hyg 65: 696-9.

13. Pradines B, Tall A, Parzy D, Spiegel A, Fusai T, et al. (1998). In vitro activity of pyronaridine and amodiaquine against African isolates (Senegal) of Plasmodium falciparum in comparison with standard antimalarial agents. J Antimicrob Chemother 42: 333-9.

14. Happi CT, Gbotosho G, Folarin OA, Sowunmi A, Hudson T, et al. (2008) Selection of plasmodium falciparum mdr1 in asexual stages and gametocytes by artemetherlumefantrine in Nigerian children with uncomplicated falciparum malaria. J Antimicrobial Agents and Chemotherapy 53: 885-95.

15. Sisowath CJ, Stromberg A, Martensson M, Msellem C, Obondo A, et al. (2005) In vivo selection of Plasmodium falciparum Pfmdr186N coding alleles by artemetherlumefantrine (co-artem). J Infect Dis 191: 1014-7.

16. Mutabingwa TK, Anthony D, Heller A, Hallet R, Ahmed J. et al. (2005) Amodiaquine alone amodiaquine/sulfadoxine-pyrimethamine amodiaquine/artesunate and arthemether/lumefantrine for out-patient treatment of malaria in Tanzanian children: a four arm randomized effectiveness trial. Lancet 365: 1474-80.

17. Gouissi FM and Salifou S (2012) In vitro antimalarial activity of COARTEM Adv Agric Sci Eng Research 2:110-4.

18. Pradines B, Hovette P, Fusai T, Atanda HL, Baret E, et al. (2006) Prevalence of in vitro resistance to eleven standard or new antimalarial drugs among P. falciparum from pointe-Noire Republic of Congo. J Clin Microbiol 44: 2404-08.

19. Ferreira ID, Lopes D, Martinelli AM, Ferreira C, do Rosario VC, et al. (2007) In vitro assessment of artesunate artemether and amodiaquine susceptibility and molecular analysis of putative resistance associated mutations of P. falciparum from Sao Tome and Principe. Trop Med Int Health 12: 353-62.

20. Tinto H, Rwagacondo C, Karema C, Mupfasoni D, Vandoren W, et al. (2006) In vitro susceptibility of Plasmodium falciparum to monodesethyamodiaquine dihydroartemisinin and quinine in an area of high chloroquine resistance in Rwanda. Afr Health Sci : 111-6.

21. World Health Organization (2001) In Vitro Micro-test (Mark III) for the Assessment of the response of Plasmodium falciparum to chloroquine mefloquine quinine amodiaquine sulfadoxine/pyrimethamine and artemisinin. Instruction for use of the in vitromicrotest kit (Mark III).CTD/MAL/97.2.0 Rev. 2001 Geneva World Health Organization.

22. Jambou R, Legrand E, Niang M, Khim N, Lim P, et al. (2005) Resistance of Plasmodium falciparum field isolates to in vitroartemether and point mutations of the SERCA-type PfATPase6. Lancet 366: 1960-3.

23. Brasseur P, Kouamouo J, Moyou-somo R, Druilhe P (1992) Multi- drug resistant falciparum malaria in Cameroon in 1987_1988. Stable figures of prevalence of chloroquineand quinine resistant isolates in the original foci. Am J Trop Med and Hyg 46:1-7.

24. World Health Organization (2010) Guidelines for the treatment of malaria. WHO Press Geneva Switzerland.

25. Holding PA, and Snow RW (2001) Impacts of P. falciparum malaria on performance and learning: Review of the evidence. Med Hyg 64.1

26. Cheesbrough M (2000) District Laboratory Practice Manual in Tropical Countries. Cambridge University press 235-45.