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Full Length Research Article

INVITRO SENITIVITY PROFILE OF PLASMODIUM FALCIFARUM CLINICAL ISOLATES TO CHOLOROQUINE AND ARTEMISIN COMBINATION THERAPHY FROM KANO AND JIGAWA STATES, NIGERIA

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ABSTRACT
Malaria continues to cause unacceptably high level of disease and death especially in Africa, with Nigeria bearing the largest burden. Accumulated efforts have been put in place to manage transmission but emergence of *Plasmodium falcifarum* resistant species has been a major obstacle to the control efforts. This study aimed to evaluate the sensitivity of *Plasmodium falcifarum* isolate to previously used choloroquine and currently in use Artemisinin and Dihydroartemisinin- amodiaquine in Kano and Jigawa States, Nigeria. Sensitivity of parasite to the drugs was carried out using WHO invitro Micro test procedure. The results revealed high resistance to chloroquine in all the study sites, reduced susceptibility to artemisinin in Hadejia and reduced susceptibility to Dihydro-artemisinin- amodiaquine in Kura and Kano Municiapal. The geometric IC50 values for artemisinin and Dihyrodroartemisini-amodiaquine were found to be below the resistant threshold cut off values as 3.66nM and 1.29nM respectively, while that of chloroquine were far above the threshold value (504.66nM). These indicate that, the P. *falcifarum* is still resistant to choloroquine and Artemisin and its derivatives are still effective for malaria treatment in the states.

Keywords: *Plasmodium falcifarum*, ACT, Chloroquine, Sensitivity profile.

INTRODUCTION

Malaria continues to cause unacceptably high levels of disease and death, as documented in successive editions of the World malaria report. According to the latest report, there were an estimated 241 million cases and 627 000 deaths globally in 2020 and heavily concentrated in the WHO African Region (WHO, 2021). In 2018, Africa accounted for 93% of all cases and more than half of the cases were in six countries: Nigeria (25% of cases); Democratic Republic of the Congo (12%); Uganda (5%); Côte d'Ivoire, Mozambique and Niger (4% each) (WHO, 2019). In Nigeria, the burden was high (58.3%) in the Northwest zone of the country particularly in the rice producing states of Sokoto, Kebbi, Jigawa and Kano states (NMIS, 2015). *Plasmodium falcifarum* is the major specie responsible for malaria infection in humans and account for the majority of cases and death in Africa (Cibulski *et al*., 2016). Other minor species known to infect human include *P vivax, P ovale* and *P malariae* (*Cook and Zumla, 2009*). Accumulated efforts supported by several donor agencies and government and private institutions to reduce attributable morbidity and mortality focused mainly on improving access to effective treatment, preventing malaria during pregnancy, reducing mosquito–human contact by widespread use of insecticide-treated bed nets and ensuring timely and appropriate action during malaria epidemics (WHO, 2003). Several chemotherapeutic agents such as chloroquine and its derivatives, sulphadoxine- pyremethamine have been sequentially introduced to control transmission, but rapid development of resistance to these drugs endangered this control effort worldwide. Emergence of chloroquine resistance in 1980s, was considered responsible for the dramatic increase in child morbidity and mortality in Africa (Trape, 2001). Currently (ACTs) are frontline, fast acting drugs officially use for treatment of malaria but Pf resistance to ACTs has emerged in some part of the world, threatening global malaria control and elimination (Menard and Dondrop, 2017). Emergence and spread of ACT resistance in Pf has become a public health concern all over the malaria endemic countries of the globe (Olukosi *et al.,*2014; Ngassa *et al*., 2016). These resistant strains were believed to originate from Thai-Cambodian border (Dandrop *et al*., 2009) and dispersed to many other malaria endemic regions (Mishra *et al*., 2016). In Nigeria, chloroquine was officially withdrawn in 2005 and replaced with ACT in 2010 as first line treatment. Unfortunately, resistance to ACTs was later reported in areas of South-East Asia and there are fears that it may spread to Africa (Ariey *et al*., 2014; Ashley *et al*., 2014; Takala-Harrison *et al*., 2015). This could subsequently affect malaria eradication and elimination efforts.

Chloroquine resistance is widely distributed; however, reports from some African countries indicated a decline in the resistant parasite population after chloroquine discontinuation (Ndiaye *et al*., 2012), although this reduction in resistance varied between countries. Early detection of resistance is essentially important to minimize recurrence of 1980 experience when chloroquine resistance resulted in escalated malaria death in young African children (Murray *et al*., 2012). Researchers reported the decline in parasite resistance to chloroquine in some countries in Africa (Alexandra *et al*., 2021), suggesting the need to determine the current susceptibility of parasite to chloroquine after sixteen years withdrawal in Nigeria.

MATERIALS AND METHODS

Description of the Study area

The study was conducted at Hadejia, Kura and Kano municipal Local Government areas of Jigawa and Kano states, Nigeria. Kano

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State is located within the Sudan Savannah zone of West Africa about 840 kilometers from the edge of the Sahara desert. The vegetation of Kano State is semi-arid Savannah sandwiched by the Sahel Savannah in the north and the Guinea Savannah in the south. The state has the largest irrigation projects in Nigeria, with six irrigation projects and more than twenty earth dams of various sizes (Sulaiman et al., 2014). The project was precisely conducted at Kura which is located at Kadawa irrigation project within the catchment area of Tiga dam and non-rice producing Kano Municipal LGAs. (11° 42'N, 8°33'E). Jigawa state is situated within the Sudan savannah vegetation zone, but there are traces of Guinea Savannah in the southern part of the state. The study was conducted in rice producing Hadejia LGA situated at Hadejia-Jama'are River Basin area.

Inclusion and exclusion criteria
Study population are adults and children Pf malaria positive patients attending Kano States public Health facilities (Kura General Hospital, Murtala Muhammad Specialist Hospital and Hasiya Bayero Paediatric Hospital) and Jigawa state Public Health facilities (Hadejia General Hospitals). Patients not on anti-malaria therapy and resident of Kano and Jigawa States were included in the study. Individuals with mixed infection, pregnant women and who are in transit within the state and on anti-malaria therapy were excluded from the study.

Ethical Clearance.
The study protocol was independently reviewed and approved by ethics committees of Kano and Jigawa states Ministry of Health with Protocol number NHREC/17/03/2018 and MOH/SEC/1.5/235/009 respectively. A written informed consent was read and signed by all participants or their parents or guardians before any study procedure was performed. Three hundred (300) patients aged 1 to 45 years were recruited for the study.

Sample Collection and microscopic examination
Blood samples (3.0mL) from participants were collected and transported in ice to Department of Microbiology, Bayero University Kano and Aminu Kano Teaching Hospital (AKTH). Slides positivity were confirmed by two WHO certified laboratory Scientist using thin and thick films. The films were fixed with methanol and stained with 3% Giemsa stain of pH 7.0 for 30 min as recommended by WHO as recommended by (WHO ,2000), and examined microscopically using oil immersion objectives lens as described by (cheesebrough 2000). The thick films were used to determine the parasite densities while thin films were used to identify the parasite species and infective stages. Sample with minimum of 2% parasitemial count were selected for in vitro study.

Sample preparation
Samples with 2% and above percentage parasitemia were centrifuged at 800g for 5mins. The supernatant were discarded and RPMI Media were added to the pellet and centrifuge again at 800g for 5mins.

Preparation of culture media.
Stock of 1L culture solution was prepared by mixing 25mL of 1M Hepes, 2mL of 10mg/mL gentamicin, 20mL of 10Mm hypoxanthine, 5g albumax and 25mL heat inactivated human serum to 928mL RPMI-1640 culture media. Magnetic stirrer was used to ensure complete dissolution of the components and filtered using 0.22µm pore size filter and stored at 4°C.

Preparation of drug solution
Drug solution was prepared according to WHO invitro microtest procedure with little modification (WHO, 1990; 2001). Stock solution of 1mg /ml of chloroquine sulphate (company), Artemisisnin (Novartis) and Dihydro artemisinin- amodiaquine (Novartis) were all prepared in 1% DMSO dissolved in sterile distilled water according to WHO standard operating procedure. From these stock solutions, subsequent dilution was made with 1% DMSO to yield desired concentrations for each drug. A quantity of 50µL of each concentration was distributed in 96 well microculture plates and dried in an incubator at 37°C.The desired concentration of the drugs were CQ: 200 to 12,800 nM, ART:3.0 to 192nM and DAA: 2.08 to 133nM. The plates were stored at 4°C in a sterile plastic container.

Invitro culture of *Plasmodium falcifarum* isolate and drug susceptibility test.
The invitro culture cultivation of Pf isolate was done following a modification of standard culture technique (Sandra and Vicky, 2017). The dug susceptibility test was carried out using standard procedure of schizonts maturation inhibition technique (WHO, 2001). 100µl of 1:10 dilution of Pf positive blood pellet and culture media were transferred to wells of microculture plates, predisposed with varying concentration of chloroquine (CQ), artemisinin (ART), Dihydroartemisinin-amodiaquine (DAA) and control wells. The plates were placed in a candle jar and incubated at 5% CO_2 and 37°C for 26-30h. At the end of incubation period, thick films were made and stained with 2.5% Giemsa stain for 35mins. The mean number of schizonts formed in duplicate wells per 200 asexual parasite relative to control wells were counted and recorded as no of schizonts per 200 parasites in the test well after incubation/no of schizonts per 200 parasites in the control well after incubation. The IC_{50} and IC_{90} of the drugs against the isolates was determined from dose response curves according to the procedure of Noedl *et al.* (2001). Mean percentage of the parasite inhibition was calculated as

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

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% and 90% inhibitory concentration ($1C_{50}$ and IC_{90}) 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 Mean + 2 × standard deviation.

Statistical analysis.
Schizonts maturation inhibition were anlaysed using univariate analysis of variance statistical test by SPSS version 23. Inhibitory concentration was analyzed using ANOVA statistical test by Graphpad instat statistical software. All the tests were performed at 95% confidence interval.

RESULTS AND DISCUSSION
The results of Pf isolate culture collected from Hadejia, Kura and KMC are presented in Table 1. The Number of isolates collected from HDJ and KUR and KMC were 100 each. The percentage of isolate cultured were 80%, 76% and 60% and percentage of successful culture were 25%, 19.7% and 30% respectively.

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Table 1: Invitro culture of Pf isolates collected from Hadejia, Kura and Kano municipal.

	HDJ	KUR	KMC
No of isolate collected	100	100	100
No of isolate cultured	80 (80%)	76 (76%)	60(60%)
No of isolate successfully cultured	20 (25%)	15 (19.7%)	18(30%)
No of isolate failed to culture	60 (75%)	61 (80.3%)	42(70%)

Key: HDJ: Hadejia KUR: Kura KMC: Kano Municipal

The sensitivity pattern of the isolates to antimalarial compounds in the study sites are presented in Figure 1. The percentage of resistant isolates to CQ were 80%, 80% and 88.8%. The percentage resistance to ART were 6.6%.11.1% and 11.1% , the percentage resistance to DAA were 0.0%, 11.1% and 11.1% in HDJ, KUR and KMC respectively.

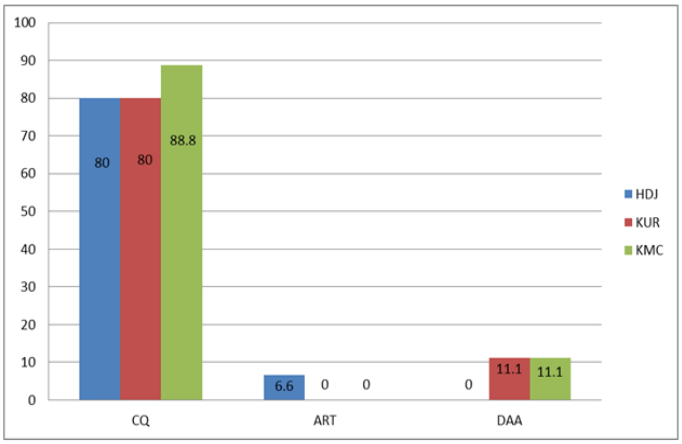

Figure 1: Resistance pattern of Pf isolates to antimalarial compounds.

Key: HDJ: Hadejia KUR: Kura KMC: Kano Municipal CQ: Chloroquine ART: Artemisinin DAA: Dihydroartemisinin-Amodiaquine

The Results of schizonts maturation inhibition is presented in Table2. The inhibition shown to be dose dependent proportional to any drug concentration and increase with increasing drug concentration. Growth was observed at high dose of CO (12,800nM) in all the study sites but completely inhibited at high dose of ART (192nM) in all study sites, partial growth was observed at high dose of DAA (133nM). There is no significant variation in growth inhibition in all study sites in all drugs at each concentration. However, significant variation was observed with respect to CQ at (3200nM) in Kura. Similarly high schizonts maturation was observed due to CQ exposure compared to ART and DAA in all the sites.

Table 2: Schizonts maturation inhibition of P. falcifarum isolate with respect to different antimalarial drugs at different concentration.

Drugs	Concentration (nM)	Percentage Inhibition		
		KMC	KUR	HDJ
CQ [a]	200	40.05±8.6	44.37±8.3	42.35±4.1
	800	47.38±8.7	50.74±8.5	50.18±8.9
	3200	53.61±13.3[c]	69.48±11.4[d]	58.78±12.3
	12800	86.89±6.1[e]	91.67±5.4	87.70±6.8
ART [b]	03	71.81±14.6	68.52±11.8	68.62±15.9
	12	97.20±3.9	96.59±9.1	98.71±2.7
	48	99.10±2.1	99.52±1.8	100.00±0.0
	192	100.00±0.0[f]	100.00±0.0	100.00±0.0
DAA [b]	2.08	74.68±13.3	70.45±13.6	69.07±14.4
	8.32	96.7±3.2	98.32±3.3	97.05±8.1
	33.2	99.48±1.4	99.69±1.2	99.64±1.6
	133	99.78±0.9[f]	99..69±1.2	99.52±0.8

Key:
HDJ: Hadejia KUR: Kura KMC: Kano Municipal CQ: Chloroquine ART: Artemisinin DAA: Dihydroartemisinin-Amodiaquine. Values are presented as Mean±SD. Values with different superscript indicate significant difference at p≤0.05

The inhibitory concentration (IC_{50} and IC_{90}) of CQ, ART and DAA are respectively presented in Figure 2, 3 and 4. The IC_{50} values for CQ is higher in KMC (609nM) than in HDJ (251.2nM). With no significant variation, the IC50 with respect to ART are 3.38nm, 3.11nM and 0.98nM respectively. With respect to DAA, the IC50 were 1.98nM, 0.34 and 0.24nM respectively.

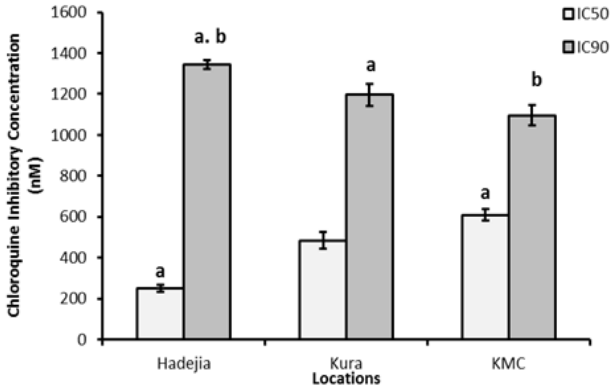

Figure 2: Chloroquine inhibitory concentration to Pf clinical isolates in Hadejia, Kura and Kano Municipal

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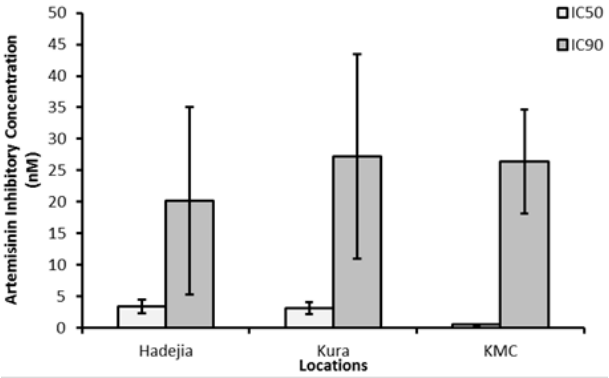

Figure 3: Artemisinin inhibitory concentration to Pf clinical isolates in Hadejia, Kura and Kano Municipal

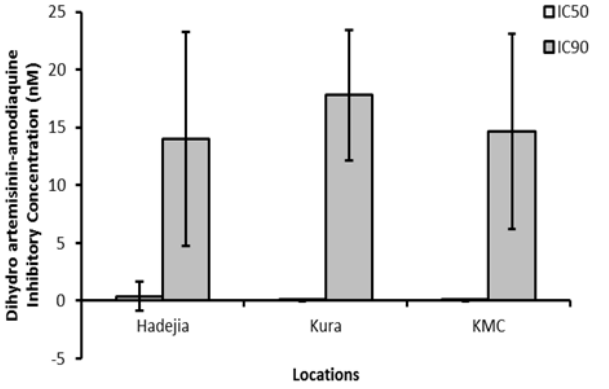

Figure 4: Dihydroartemisinin-amodiaquine inhibitory concentration to Pf clinical isolates in Hadejia, Kura and Kano Municipal

The correlation between the *In vitro* responses of *Pf* isolates to the drug pairs is presented in Table 3. Significant positive correlation was obtained between ART and DAA with respect to IC50 and IC90 (r=0.84 and 0.78) respectively. Negative correlation was obtained between CQ and ART and DAA (r=-0.07 and -0.03) with respect to IC50.

Table 3: Correlation of invitro response of Pf showing Pearson correlation coefficient between three antimalarial drugs.

Drug pair	IC50	IC90	Threshold values (nM)
CQ-ART	-0.07	0.11	CQ = 504.66
CQ-DAA	-0.03	0.23	ART= 3.66
ART-DAA	0.84	0.78	DAA= 1.29

CQ: Chloroquine **ART**: Artemisinin **DAA**: Dihydroartemisinin- Amodiaquine.

The low success rate of the culture obtained in this study is not surprising considering the fact that, the study relies on several hard to control parameters such as poor schizonts maturation and contamination. A number of studies have reported low or high culture success rate. Awute *et al.* (2019) successfully investigated 85 out 122 samples for invitro susceptibility response to four antimalarial agents. Similarly, Aminu and Mukhtar (2017) reported 21% and 28% success in culture grew to investigate antimalarial drug susceptibility in Kano and Katsina respectively.

High resistance status to CQ and reduced susceptibility to ART and DAA were obtained in all the study sites except in HDJ were full susceptibility to DAA was achieved. Susceptibility status to CQ in this study is an indication that Pf is still resistant to CQ despite several years of official withdrawal. The level of CQ resistance varies within and outside the country and may be attributed to previous exposure to improper therapeutic regimes, over-the-counter availability of drug, and high drug pressure by improper prescribing habits of private practitioners. Awute *et al*. (2019) found that 27.06% of Pf isolates were resistant to Chloroquine. Similar finding was reported by Shujatullah *et al*. (2012) that 24.07% from Aligarh were resistant. Umar *et al.* (2017) observed 94.9% resistance to Chloroquine among pregnant women in Kaduna, Nigeria while Peletri *et al*. (2012) reported slightly lower resistance of *P. falciparum* isolates of 88.9% to Chloroquine in Abuja, Nigeria. Moderate resistance of 68.9% was revealed in studies conducted by Olasehinde *et al*. (2014) in Ogun State, Nigeria. Reduced and full suceptibilty to Artemisinin and ACTs have been reported by number of studies. Awute *et al*. (2019) reported 18.8% resistance to (Artemether-Lumefantrine) and 14.1% to Artemether. Falade *et al*. (2005) has reported 14% reduction in susceptibility to Artemether-Lumefantrine among African Children.

Conversely. Aminu and Mukhtar (2017) reported 100% sensitivity to tested ACTs. The dose dependent schizonts maturation inhibition and insignificant variation in growth inhibition at various drug concentration obtained in this study corroborate the finding of Aminu and Mukhatr (2017). However, on contrary, they reported high (100%) growth inhibition at high dose concentration in the three tested ACTs. IC50 implies drug concentration at which 50% of the parasite could not mature to schizont stage while IC90 implies that the anti-malarial drugs concentration that could inhibit 90% of the parasites from maturing to schizont stage. The inhibitory concentration (IC50) obtained in this study is within the limit of established resistance threshold value defined by (WHO, 2001). However, the geometric threshold value for ART (3.66nM) obtained in this study is higher than that of DAA (1.29nM). This support the fact that, ideal approach to anti-malarial treatment is the use of a combination of two or more drugs, rather than a single antimalarial drug, preferably with an artemisinin derivative as one of the drugs (WHO, 2001; Ashley *et al*., 2007). The result is similar to what was reported earlier by Aminu and Mukhtar (2017) that reported geometric mean IC50 values of 2.04+0.42nM, 3.67+0.4nM and 4.70+0.43nM for AL, DHP and AA respectively from Kano and neighboring Katsina State, Nigeria. These results are consistent with other studies from Africa reporting IC50values of artemisinin derivatives as 2.2 and 2.6nM from Senegal, and Rwanda respectively (Ferreira *et al.,* 2007; Tinto *et al*., 2006). Similarly, Number of studies provide evidence of reduced *in vitro* susceptibility of *Pf* to artemisinin derivatives (Artemether and Dihydroartemisinin) (Jambou *et al.,* 2005; Pradines *et al.,* 2006). It is important to note that reduced in vitro susceptibility does not directly translate to diminished therapeutic efficacy, but it signal and quest for increased vigilance and rapid development of new drug combinations. Significant correlation (r=0.84) was obtained between ART and DAA. This indicates the possibility of cross-

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resistance, thus parasite resistance to any of these two drugs could eventually lead to resistance in others.

Conclusion

Baesd on this findings, it may be concluded that the current malaria treatment policy in the states is not under immediate threat of resistance development due to ACT.

Conflict of interest

There is no conflict of interest.

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