Available online at http://www.ifgdg.org

Int. J. Biol. Chem. Sci. 14(4): 1459-1469, May 2020

ISSN 1997-342X (Online), ISSN 1991-8631 (Print)

International Journal of Biological and Chemical Sciences

Original Paper http://ajol.info/index.php/ijbcs http://indexmedicus.afro.who.int

In vitro antimalarial susceptibility of *Plasmodium falciparum* isolates from patients in Jos, Plateau State, Nigeria

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ABSTRACT

Despite concerted efforts to eradicate malaria, it is still one of the most devastating infectious diseases in the tropics due largely to emergence and widespread *P. falciparum* drug resistance. This research investigated the *in vitro* antimalarial susceptibility of *Plasmodium falciparum* isolates from patients attending Plateau State Specialist Hospital, Jos, Plateau State, Nigeria, using the World Health Organization (WHO) standardized *in vitro* micro-test system. Foremost, the records of reported malaria cases in the hospital were collated for the prevalence of malaria and the frequency of commonly prescribed antimalarial drugs were ascertained. A total of 7936 persons comprising of 3659 males (46.11%) and 4277 females (53.89%), were reportedly infected with malaria parasite from January 2013 to December 2014 in the hospital studied. Blood samples were collected from 131 volunteered patients. Of these samples, 114 (87.02%) had malaria parasites, with 108 (94.74%) samples positive for *P. falciparum*. Of 88 *P. falciparum* isolates used for *in vitro* antimalarial susceptibility test, 33 (37.50%), 27 (30.68%), 22 (25.00%), 22 (25.00%), and 22 (25.00%) were resistant to chloroquine, artemether, amodiaquine, artesunate, and lumefantrine respectively. This study showed the presence of resistant *P. falciparum* in the study area. Adoption of efficient intervention strategies is warranted in order to curb increase in antimalarial drug resistance.

Keywords: Samples, parasite, resistance, study, positive, prescribed.

INTRODUCTION

Malaria is an acute infectious disease caused by *Plasmodium* species. Malaria in humans has been reportedly caused by five species of *Plasmodium* viz: *P. falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi* (William and Menon, 2014). The malaria parasites are known to be transmitted to people through the bites of infected female *Anopheles* mosquitoes, called "malaria vectors". Reported typical malaria symptoms include fever and headache, which in severe cases can progress to coma or death (WHO, 2013). According to Ukibe et al. (2010) anaemia is one of the commonest complications of malaria which results from the parasite's destruction of the red blood cells.

Malaria is a neglected tropical disease and still remains a public health concern around the world especially in Nigeria where the disease causes alarming cases of morbidity and mortality (Nanvyat et al., 2017; Akanni et al., 2019). Some of the economic impacts of malaria include costs of health care, loss of

DOI : https://doi.org/10.4314/ijbcs.v14i4.23

working days due to sickness, reduced school attendance, decreased productivity due to brain damage from cerebral malaria, and loss of investment and tourism (WHO, 2001).

The control or eradication of malaria has been very difficult to achieve majorly because the parasites have ability for resistance to antimalarial drugs (Anaemene, 2012). According to Wande and Babatunde (2017), the widespread resistance of *P. falciparum* to conventional antimalarial drugs is a key factor contributing to the increasing malaria mortality and morbidity in Africa.

The rapid spread of antimalarial drug resistance over the past few decades has necessitated increased monitoring for further resistance, to ensure proper management of clinical cases and early detection of changing patterns of resistance (WHO, 2005).

This study was designed to evaluate the prevalence of reported malaria cases and antimalarial drugs prescription. Furthermore, this study aimed at determining the *in vitro* susceptibility of *Plasmodium falciparum* isolated from volunteered patients attending Plateau State Specialist Hospital Jos, Plateau State, Nigeria to some selected antimalarial drugs commonly observed prescribed in this locality.

MATERIALS AND METHODS

Study site

The study samples were collected from Plateau State Specialist Hospital (PSSH). The hospital is located at Old Bukuru road Jos, Plateau State, and serves as a referral centre for other general and cottage hospitals within Plateau State (Ukaegbu et al., 2014). Jos is the capital of Plateau State. Jos city is located on the Jos Plateau at about 1,238 metres or 4,062 feet above sea level. The overall average relative humidity of Jos is 55% (Ita et al., 2017). Jos is located in the Northern guinea savannah between longitude 8 º 20 ´N and latitude 9 º 30 ´E. Average monthly temperatures in Jos range from 21–25 °C (70–77 °F), and night-time temperatures drop as low as 7 °C (45 °F) from mid-November to late January. The city experiences two seasons, the dry season (October to March) and the wet season (April to September). Jos receives an average of 1314.8 mm of rainfall yearly (Ishaya et al., 2018). Several surface water dams serve as source of water for domestic as well as industrial use, irrigation, and for generation of hydroelectric power. These dams include the Shen dam, the Laminga dam and the Bukuru dam. Several ponds were also created as a result of mining activities, which are presently used for household and irrigation purposes and also serve as breeding sites for mosquitoes.

Sample size determination

The sample size for the study was calculated using the prevalence rate obtained from the retrospective study (7.95%) at a confidence level of 95%. The formula used for the calculation was adopted from Kadam and Bhalerao (2010):

$$n = \frac{Z_a^2 P Z_{1-ß}}{L^2}$$

Where n = Number of samples

Z_a *= Standard normal deviation at 95% confidence limit = 1.96.*

P = Prevalence or mean of incidence for the years under review = 0.0795

$Z_{1-ß}$ *= 1-P = 1 – 0.0795 = 0.9205*

L = allowable error of 5% = 0.05

From the formular above, the sample size was calculated as 112.4509 ≈ 113.

Eligibility criteria

Participation in this study was opened to persons of all ages and sexes who presented with clinical symptoms of malaria and who/whose guardian gave oral consent to be included in the study. Also, only patients who had mono-infections with *Plasmodium falciparum* and asexual parasitaemias in excess of 1,000 parasites but less than 80,000 parasites per micro-litre of blood were included in the *in vitro* test (WHO, 2001).

Ethical consideration

Ethical approval for the research (PSSH/ADM/ETH.CO/2015/004 and PSSH/ADM/ETH.CO/2015/07) was obtained from the Plateau State Specialist Hospital

Health Research Ethics Committee. Oral consent was obtained from the patients, or, in the case of children, from their parents.

Retrospective study

Records of reported malaria cases for 2013 and 2014 were obtained from the records department of the hospital. Data on age and gender distribution of patients diagnosed with malaria in the hospital in 2013 and 2014 were obtained as well as antimalarial drug prescriptions to patients in the hospital in 2013 and 2014.

Media preparation

The medium was prepared using the method recommended by Basco et al. (2007) for preparing 1 litre of medium. The sterile culture medium consisted of 10.4 g of sterile RPMI 1640 powder (Sigma Aldrich), 5.94 g HEPES powder (Sigma Aldrich), and 0.625 ml of 40 mg/mL gentamicin in sterile distilled water. Sodium bicarbonate ($NaHCO_3$) (0.05 g/mL) and serum from O^+ human blood (10% $^{vol}/_{vol}$) were added to prepare the complete RPMI medium immediately before use.

Preparation of drugs

Graded antimalarial drug solutions were prepared. Stock solutions of chloroquine diphosphate powder (Sigma Aldrich), amodiaquine dihydrochloride dehydrate (Sigma Aldrich), artesunate powder (Lincoln Pharmaceuticals, India) and artemether were dissolved in 70% ethanol to obtain highest concentrations of 178.8 µMol/L, 4.4 µMol/l, 3084.4 µMol/L, and 72.4 µMol/L respectively. The concentration of lumefantrine (2140.4 µMol/L) was prepared by dissolving lumefantrine powder (Sigma Aldrich) in 100% Dimethyl Sulfoxide (DMSO). From the working solutions, five two-fold dilutions of each drug was made and used to dose sterile 96-well flat bottom culture plates and left to dry. Rows B-F of the culture plate were dosed with the antimalarial drugs while well A was left as the control well without drug. The method outlined by Basco et al. (2007) was used with modification.

Sample collection and examination

Blood samples were collected with the assistance of a Medical Laboratory Technician in the hospital. Safety procedures were adopted in the collection of blood samples by swabbing the area to be sampled with ethanol (70% $^{vol}/_{vol}$) and allowing it to dry before collection. Two millilitres of venous blood was collected with a sterile disposable syringe into EDTA bottles and transported aseptically in a sterile cooler within 6 hours of collection to the Pharmaceutical Microbiology Laboratory of Ahmadu Bello University Zaria, Nigeria, for microscopic detection of *Plasmodium* species infections using Giemsa stain, and *in vitro* antimalarial drug susceptibility test.

Thick and thin films were prepared and allowed to dry. The thin films were fixed with methanol. Both films were stained with Giemsa (5% $^{vol}/_{vol}$) for 30 minutes, allowed to dry, and examined microscopically using X100 oil immersion objectives as described by WHO (2001). The thick films were used to determine the parasite densities while thin films were used to identify the parasite species and infective stages. Parasite density per micro-litre of blood (parasitemia) was estimated from the thick film by multiplying the average number of parasites per high power field (X100 objective) by 500. Ten fields were examined to determine the average number of parasites per high power field as recommended by Greenwood and Armstrong (1991).

***In vitro* micro-test**

The antimalarial drug activity against *Plasmodium falciparum* isolates was determined using the World Health Organization (WHO) standardized *in vitro* micro-test system (WHO, 2001). Each of the wells B-F of a sterile tissue culture plate was dosed with 10 µL of the antimalarial drugs diluted to various concentrations (well A was left as the control well without drug) and allowed to dry. Ninety micro-litre (90 µL) of complete culture medium (RPMI 1640 and serum) was pipetted into each well (A-F) of the pre-dosed plates followed by the addition of 10 µL of the standardized infected erythrocytes with *P. falciparum* isolates, aseptically into each of the wells. The plates were incubated

anaerobically at 37 °C for 30 hours with 5% carbon dioxide. After incubation, a thick blood film was made from each well. The blood smears were then air-dried for 24 hours and stained with 5% Giemsa stain for 30 minutes. The stained thick films were air-dried and examined with the oil immersion objective (X100). Parasites were counted from ten microscopic fields and their average was calculated. The counts were expressed as a percentage of the control.

Determination of IC_{50}

Percentage schizont inhibitions were calculated from the average counts for the drug concentrations, and plotted against the log of corresponding concentrations of drugs in the test wells. Regression lines were plotted using Microsoft excel 2010, and the IC_{50s} (50 percent inhibitory concentrations) of the drugs were calculated from the equations of the linear graphs. Antimalarial drug resistant *P. falciparum* isolates were identified as isolates with IC_{50} values greater than peak plasma concentration of chloroquine, amodiaquine, artesunate, artemether, and lumefantrine viz: 4.47 µMol/L, 0.11 µMol/L, 77.11 µMol/L, 1.81 µMol/L and 53.51 µMol/L.

RESULTS

Prevalence of malaria from retrospective study

The monthly distribution of the recorded cases of Malaria in Plateau State Specialist Hospital (PSSH) is presented in Table 1. The number of reported malaria cases increased from 3245 in 2013 to 4691 in 2014, which gives a total of 7936 cases. A total of 99,796 patients were reported attended PSSH from 1st January, 2013 to 31st December, 2014. The prevalence of malaria for the two years reviewed was calculated as 7.95%. Generally, high malaria cases were reported in the rainy season in the two years studied.

Prevalence of malaria among age groups (retrospective study)

Table 2 shows the age-related prevalence of malaria in PSSH. From 1 January, 2013 to 31 December, 2014, the highest prevalence of malaria in PSSH was reported among persons aged above 18 years (4311 (54.32%)). This is followed by children aged 0-5 years (2263 (28.52%)) and 6-18 years (1362 (17.16%)).

Prevalence of malaria based on sex (retrospective study)

Of the total positive cases of malaria reported in PSSH from 1 January, 2013 to 31 December, 2014, 4277 (53.89%) were females while 3659 (46.11%) were males (Table 3).

Pattern of antimalarial drugs administration (retrospective study)

Artemether/lumefantrine (34.3%) was the antimalarial drug mostly administered in PSSH in the year 2013 while quinine (5.8%) was least administered. In 2014, dihydroartemisinin/piperaquine (38.4%) was the antimalarial drug that was mostly administered in PSSH, while artemether (2.1%) was least administered (Figure 1). This result shows that ACTs were mostly administered to malaria patients in the study centre.

Profile of *Plasmodium* species

Of the total blood samples collected, 114 (87.0%) had malaria parasites, with 108 (94.7%) of the positive samples positive for *Plasmodium falciparum* and 6 (5.3%) samples positive for *Plasmodium malariae* (Table 4).

***In vitro* antimalarial sensitivity test**

Table 5 shows the range of antimalarial inhibitory concentrations at 50% (IC_{50}) gotten from the *P. falciparum* sensitivity test, and the percentage of resistant *P. falciparum* isolates observed. From the result obtained, 33 (37.50%), 27 (30.68%), 22 (25.00%), 22 (25.00%), and 22 (25.00%) were resistant to chloroquine, artemether, amodiaquine, artesunate, and lumefantrine respectively.

Table 1: Monthly prevalence of malaria in Plateau State Specialist Hospital in 2013 and 2014.

Months	2013	2014	2013-2014
January	30 (0.9%)	379 (8.1%)	409 (5.2%)
February	228 (7.0%)	281 (6.0%)	509 (6.4%)
March	301 (9.3%)	344 (7.3%)	645 (8.1%)
April	234 (7.2%)	496 (10.6%)	730 (9.2%)
May	227 (7.0%)	534 (11.4%)	761 (9.6%)
June	386 (11.9%)	513 (10.9%)	899 (11.3%)
July	231 (7.1%)	524 (11.2%)	755 (9.5%)
August	249 (7.7%)	445 (9.5%)	694 (8.7%)
September	278 (8.6%)	443 (9.4%)	721 (9.1%)
October	417 (12.9%)	352 (7.5%)	769 (9.7%)
November	413 (12.7%)	290 (6.2%)	703 (8.9%)
December	251 (7.7%)	90 (1.9%)	341 (4.3%)
Total	3245 (100.00%)	4691 (100.00%)	7936 (100.00%)
Average	270.4	390.9	661.3

Table 2: Age-related prevalence of malaria in Plateau State Specialist Hospital in 2013 and 2014.

Age Groups	2013-2014
0-5 Years	2263 (28.52%)
6-18 Years	1362 (17.16%)
>18 Years	4311 (54.32%)
Total	7936 (100.00%)

Table 3: Sex-related prevalence of malaria in Plateau State Specialist Hospital in 2013 and 2014.

Sex	2013-2014
Males	3659 (46.11%)
Females	4277 (53.89%)
Total	7936 (100.00%)

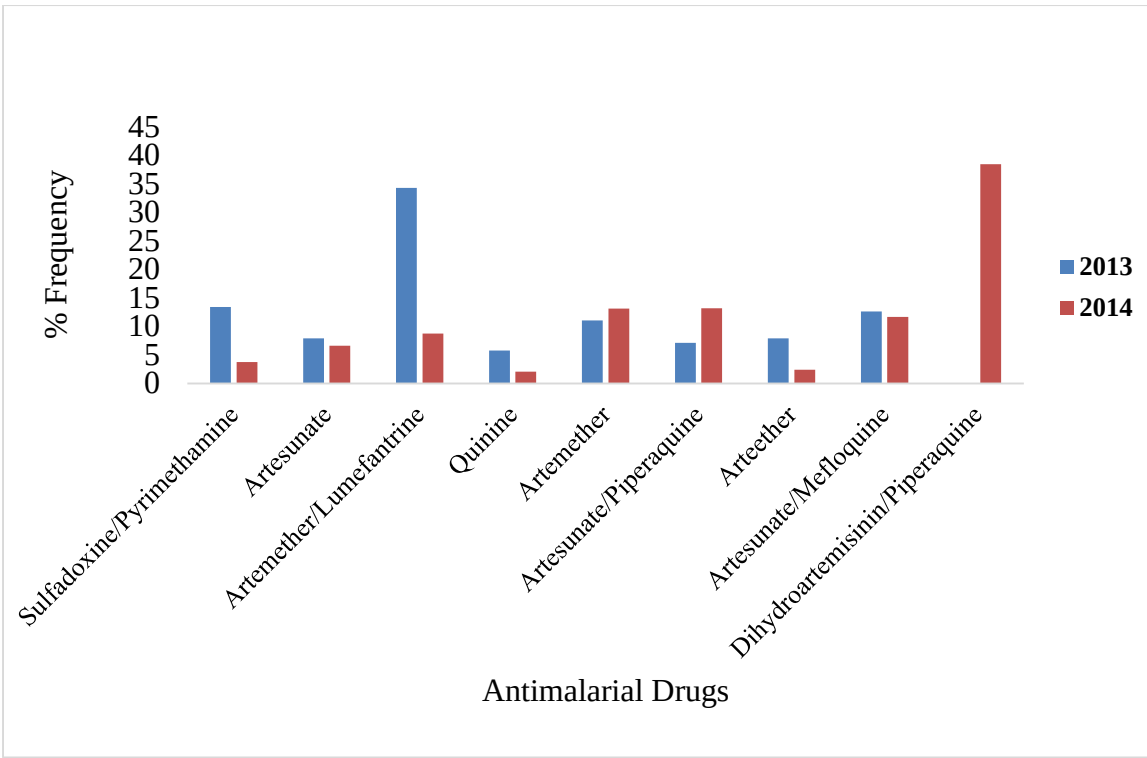

Figure 1: Antimalarial drugs administered in Plateau State Specialist Hospital in 2013 and 2014.

Table 4: Profile of *Plasmodium* species among malaria positive patients in Plateau State Specialist Hospital in 2018.

Parasite species	Frequency (%)
Plasmodium falciparum	108 (94.7%)
Plasmodium malariae	6 (5.3%)
Total	114 (100%)

Table 5: Range of test antimalarial inhibitory concentrations at 50% (IC_{50}) against *Plasmodium falciparum* isolates from 88 volunteered malaria patients in Plateau State Specialist Hospital in 2018.

Antimalarial agents	Peak plasma concentrations	Range of IC_{50} (n=88)	Resistant *Plasmodium falciparum* isolates (%)
Chloroquine	4.47 µMol/L	0.13-286.01 µMol/L	33(37.50%)
Amodiaquine	0.11 µMol/L	0.00-0.31 µMol/L	22(25.00%)
Artesunate	77.11 µMol/L	14.41-578.88 µMol/L	22(25.00%)
Artemether	1.81 µMol/L	0.50-6.82 µMol/L	27(30.68%)
Lumefantrine	53.51 µMol/L	6.18-954.58 µMol/L	22(25.00%)

DISCUSSION

The result from the retrospective study showed a low overall prevalence of 7.95%. Similar study carried out from October, 2012 to March, 2013 in Jos by Ita et al. (2017) reported overall prevalence of 15%. However, Nanvyat et al. (2017), Afolabi et al. (2015), and Idoko et al. (2015), reported higher prevalence of 48.1% in Plateau State, 45.79% in Akure, Ondo State, and 46.5% in Kaduna State, respectively. It is worthy to note that the prevalence rate reported in this study was obtained from the number of malaria cases reported in the hospital from 1 January, 2013 to 31 December, 2014. This period includes both the rainy season, when it is reported that malaria transmission is higher, and the dry season, when transmission is low (Olayemi et al., 2011). High precipitation during the rainy season results in the proliferation of anopheline breeding habitats, in addition to providing favourable humidity for the survival and dispersal of adult mosquitoes (Devi and Jauhari, 2005; Olayemi et al., 2011). The higher prevalence rates obtained by Afolabi et al. (2015) and Idoko et al. (2015) were conducted during the rainy season only.

Observation from the retrospective study showed a higher overall prevalence of malaria in females (53.89%) than in males (46.11%). This agrees with the work of Kalu et al. (2012), who reported prevalence of 91.20% in females in Aba, Nigeria. Olasehinde et al. (2015) also reported a higher prevalence in females (51.8%) than in males (48.2%) in South-western Nigeria. Another research conducted in Kano, North-western Nigeria by Nas et al. (2017) also showed more female malaria patients. According to WHO (2007), given equal exposure, adult men and women are equally vulnerable to malaria infection, except for pregnant women who are at greater risk of severe malaria in most endemic areas, due to their reported decreased immunity (WHO, 2007).

This study showed a higher prevalence of 54.32% among persons aged above 18 years, followed by children aged 0-5 years with 28.52% and 6-18 years who had prevalence of 17.16%. According to WHO (2017), children under 5 years of age (including infants) are the most vulnerable group in high transmission areas of the world, to have malaria. However, some researchers have reported a higher prevalence of malaria among persons older than 5 years of age, as found in this work. A higher prevalence among persons aged 21-30 years (92.31%) was reported by Kalu et al. (2012) in Aba, Abia State, Nigeria. The observed high infection rate in persons aged above 18 years could be due to their inability to access medical care. This observation could also be due to negligence. The malaria control programme in Nigeria also targets mostly children aged 0-5 years and pregnant women only.

Dihydroartemisinin/piperaquine, artemether/lumefantrine, and artemether were observed mostly prescribed for malaria patients in the study area. The use of the first two drugs could be in compliance to the current antimalarial treatment policy which encourages the use of a fast-acting artemisinin-based compound in combination with a drug from a different class in order to improve cure rate, and reduce the development of resistance (WHO, 2015).

The result from microscopy showed that *Plasmodium falciparum* infections were more prevalent in the study area. This agrees with the results of related studies carried out in Ondo State, Nigeria, by Afolabi et al. (2015), where 57.3% prevalence of *P. falciparum* infections was reported. According to Shapiro et al. (2017), the temperature optimum for *P. falciparum* transmission is 26 °C, with a minimum and maximum of 17 °C and 35 °C, respectively. The fact that the temperature in the study area falls within this suitable range for *P. falciparum* development and

transmission possibly justifies the high *P. falciparum* prevalence found in this study.

The result from the *in vitro* susceptibility test showed that 37.50% of the *P. falciparum* isolates were resistant to chloroquine. Umar et al. (2017) reported 94.9% resistance to chloroquine in Kaduna State, Northern Nigeria. Chloroquine resistance of 69.9% was also reported by Soniran et al. (2017) in Ogun State, Nigeria. Oyedeji et al. (2005), also reported 80% *P. falciparum* resistance to chloroquine in Ibadan, Nigeria. A research conducted by Zatra et al. (2012) in Gabon also showed that 43.5% of the *P. falciparum* isolates tested were resistant to chloroquine. It is worth noting that despite the non-usage of chloroquine in the study area, a considerable level of resistance was observed. This probably points to the possibility of the existence and circulation of genes responsible for chloroquine resistance in the study area.

Test *P. falciparum* was observed to be 30.68% resistant to artemether in this study. Umar et al. (2017), similarly, reported 38.0% of *P. falciparum* isolates to be resistant to artemether in Kaduna, Nigeria. Another research conducted in Bauchi, North-eastern Nigeria by Atang et al. (2019) also reported 14.12% resistance of *P. falciparum* to artemether. Artemisinin derivatives were generally reported to be very active against malaria parasites until some few years ago when *P. falciparum* resistance to artemisinin was detected in five countries of the Greater Mekong sub-region namely Cambodia, Lao People's Democratic Republic, Myanmar, Thailand and Vietnam (WHO, 2016). The considerable level of resistance to artemether found in this study may not be unconnected with the use of artemether singly as observed from the result of the retrospective study in this work. The use of artemether in combination with another slow acting antimalarial drug such as lumefantrine is therefore in danger of a serious threat of development of resistance.

Seventy-five percent (75%) of the *P. falciparum* isolates tested were sensitive to amodiaquine. The result from this study slightly differs from that of Balogun et al. (2016) who reported 98.8% sensitivity of *P. falciparum* to amodiaquine in North-eastern Nigeria. Ikpa et al. (2010) also reported 94.87% and 100% sensitivity to amodiaquine in Masaka and Makurdi, respectively, in North-central Nigeria. In contrast, Zatra et al. (2012) found 52.2% resistance to monodesethylamodiaquine, a principal metabolite of amodiaquine, in Gabon. The decreased susceptibility recorded in this study suggests the possibility of future decline in sensitivity of the drug and therefore calls for more research on the drug in order to detect and combat the possible change in pattern of resistance.

This study showed that 25% of test *P. falciparum* were resistant to artesunate. Umar et al. (2017), similarly, reported 35.4% *P. falciparum* resistance to artesunate in Kaduna, Northern Nigeria. With the considerable level of resistance against artesunate found in this study, it is expedient to suggest that the use of artesunate as a single drug should be carefully monitored.

From this study, a total of 22 isolates (25%) were resistant to lumefantrine. This level of resistance to lumefantrine also calls for more caution since the drug is commonly used in combination with artemether as ACT. More research needs to be done in order to further monitor the pattern of resistance to the currently prescribed antimalarial drugs so as to ensure their effectiveness.

Conclusion

In conclusion, this study has shown a considerable presence of *in vitro* resistant *P. falciparum* isolates to chloroquine, artemether, amodiaquine, artesunate, and lumefantrine in the study area. It is therefore necessary to constantly monitor the susceptibility of malaria parasites to commonly prescribed antimalarial

drugs for better treatment policies and to also adopt more effective control measures in order to limit the spread of resistance.

COMPETING INTERESTS

The authors declare that there are no competing interests with respect to this study.

AUTHORS' CONTRIBUTIONS

Experimental design: JOE. Sample Collection and analysis: PM, JYA, ADA and FOS. Data analysis: JYA and JOE. Supervision: JOE and ARO.

ACKNOWLEDGEMENTS

I acknowledge the Health Research Ethics Committee of Plateau State Specialist Hospital Jos for the ethical clearance to carry out this research and Patricia Mclean of the Medical Laboratory Department of the hospital, for the sample collection. I also thank the laboratory staff of the Department of Pharmaceutical Microbiology, Ahmadu Bello University Zaria for their assistance in various ways.

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