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Incidence and *in vitro* susceptibility profile of *Plasmodium falciparum* isolates to antimalarial agents in Lafia, Nigeria

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ABSTRACT

Malaria burden has been reported to be wide spread worldwide. The level of *Plasmodium* species resistance to recommended antimalarial drugs has also been reported to be on the increase. Thus It is necessary to monitor antimalarial drugs' efficacy and the intensity of malaria in Nigeria. This study was designed to determine the incidence of malaria and the susceptibility of *Plasmodium falciparum* isolates from patients of Dalhatu Araf Specialist Hospital Lafia to reported prescribed Antimalarials in Lafia. A retrospective study was carried out to know the pattern of antimalarial drug prescriptions, age and gender of malaria patients for 2014, 2015 and 2016. The antimalarial susceptibility of Plasmodium falciparum was also carried out by the method used by Peletiri et al., 2012. The observed Malaria prevalence was 5.9%, 4.4% and 4.8% for 2014, 2015 and 2016 respectively. The incidences of malaria among different age groups were 19.07%, 15.86%, and 44.30% for 0-5 years, 6-18 years and 19 years above respectively. Malaria was observed higher in females 58.2%, than male 41.1%. Antimalarial drug prescription was basically Artemisinin combination therapy 88.67%. Out of the 91 *Plasmodium falciparum* isolates evaluated, 32 (35.16%) were resistant to piperaquine, 26 (28.57%) were resistant to artesunate, 25 (27.00%) were resistant to chloroquine, 16 (17.58%) were resistant to Lumefantrine and 10 (10.99%) were resistant to artemether. This study gives current information on the incidence of reported malaria and antimalarial susceptibility pattern of *Plasmodium falciparum* Isolates in Lafia Nigeria.

Keywords: *Plasmodium falciparum,* Artesunate, Artemether, Chloroquine, Lumefantrine, Piperaquine.

INTRODUCTION

Malaria is an ancient disease that has existed for several thousand years (as evidenced from Plasmodial DNA in mummies (Lalremruata et al., 2013). It has been reported as one of the most important parasitic disease of man and it is still a major health problem in tropical countries (W.H.O 2015a). Alongside the significant morbidity and mortality caused by malaria are socioeconomic burdens, health inequities, and drain on human resources and productivity, interrupting educational training and causing persistent economic disadvantages thereby (WHO 2011). *Plasmodium falciparum* and *Plasmodium vivax* account for the vast majority (90%) of human malarial infections worldwide. In addition, *Plasmodium ovale*, *Plasmodium malariae* and the recently included *Plasmodium knowlesi* are also implicated

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(Ramasamy, 2014). Malaria affects all population, however, greater risk groups are children under 5 years of age, nonimmune travellers, patients with HIV/Aids and pregnant women, all of which results from inadequate immunity from the infection (Ikpa et al., 2014; Ukibe et al., 2010). Artemisinin Combination Therapy is the current national treatment for malaria (WHO 2015a). Preventive measures involve the use of Insecticide-treated nets (ITNs) and indoor residual spraying (Wangrawa et al., 2015).

The fight on malaria has been prolonged or slowed down by several interconnected challenges ranging from lack of robust, predictable and sustained international and domestic challenges. Biological barriers resulting from emergence of parasite resistance to antimalarial medicines and mosquito resistance to insecticides has made malaria control a big problem (WHO; 2015b). The developing resistance to the artemisinin and partner drugs has led to further ethnobotanical research in search of more potent antiplasmodial compounds (MFOPA et al., 2017).

As in 2010, 85% of Nigerians lived in areas supporting mesoendemic transmission, 15% lived under conditions of hyper-holoendemicity and areas within FCT Abuja, Adamawa and Borno State support hypoendemicity (NMIS, 2015).

Thus, routine assessment of data on malaria and monitoring of drug resistance in malaria-endemic countries like Nigeria, with research into key contributing factors to resistance will enable effective prevention of drug resistance from spreading. The *in vitro* micro-test is thus a more objective approach to parasite resistance as it is base on direct contact between the parasite and increasing antimalarial agent concentration. This study was designed to determine the prevalence of malaria as well as the susceptibility profile of *Plasmodium falciparum* isolated from patients of Dalhatu Araf Specialist Hospital Lafia to reported commonly prescribed Antimalarial drugs in this study facility in Lafia Nigeria.

MATERIALS AND METHODS

Study area

Lafia is a town in North central of Nigeria. It is the capital city of Nasarawa State and has a population of 330,712 inhabitants according to the 2006 census results. It is located at latitude 8.49 and longitude 8.52 and it is situated at elevation 179 meters above sea level. It has an average annual temperature and rainfall of 26 °C and 674 mm respectively.

Study Site

This study was carried out at Dalhatu Araf Specialist Hospital Lafia.

Ethical Consideration

Ethical clearance was obtained from Nassarawa State Ethical committee. Written informed consent was obtained from patients prior to recruitment into this study. Consent for the children was provided by the parents/guardians.

Retrospective Analysis

A retrospective study was carried out to know the pattern of antimalarial prescription, gender and age distribution of patients presenting with malaria at Dalhatu Araf Specialist Hospital Lafia.

Sample Collection

Blood samples were collected from Dalhatu Araf Specialist Hospital Lafia parasitology laboratory.

Microscopic examination

Thick and thin films were prepared and stained with 10% Giemsa for the microscopic examination of malaria parasite. Blood films were examined microscopically using 100 x (oil immersion) objectives. The thick films were used to determine the parasite densities while thin films were used to identify the parasite species.

Counting parasite numbers

Parasites were counted by estimating the parasite numbers/µl of blood from the thick film. This was carried out by

multiplying the average number of parasites per high power field (100 x objective) by 500.

Preparation of drugs

The antimalarial agents used for this study were; Artemether (Emzor), Lumefantrine (Sigma Aldrisch), Piperaquine (Sigma), Chloroquine (Sigma) and Artesunate (Emzor). Stock solutions of these agents were prepared in appropriate solvents; Artemether, Artesunate and chloroquine were dissolved in ethanol (100%), Piperaquine was dissolved in Sterile Distilled Water while Lumefantrine was dissolved in 100% DMSO. Further dilutions of each drug were made to obtain a working solution of desired concentration.

Performance of the *in vitro* micro test

The antimalarial susceptibility of Plasmodium falciparum was carried out by the method used by Peletiri et al. (2012).

From the working solutions of the antimalarial agents, 10 µl of each antimalarial concentration was used to dose a well of a 96-well flat bottom culture plates and left to dry in incubator at 37 $^{\circ}$C. The 96-well culture plate consisted of rows A-H; row was drug-free (control) while rows B-F represent the drug concentrations in an increasing order of doubling dilutions.

Ninety microlitres of a complete medium (RPMI 1640, blood serum and sodium bicarbonate) was added to each well, followed by the addition of standardized10 µl parasitized blood. The plates were incubated at 37 °c for 30 hours anaerobically in 5% CO_2.

After incubation, thick blood films were made from each well in duplicate. The blood smears were air-dried for 24 hours and stained with 10% Giemsa stain for 20 minutes. The stained thick films were examined with the oil immersion objective (100x) to observe schizont maturation. The percentage of schizont inhibition was computed and the inhibitory concentration at 50% determined for each test antimalarial agents.

Statistical analysis

Nonlinear regression analysis was used to determine the IC_{50} of individual plasmodium isolates susceptibility to each antimalarial agent (Excel 2010). Test for significance at 95% confidence limit was performed.

RESULTS

Retrospective study

The total number of reported malaria cases at Dalhatu Araf Specialist Hospital Lafia for 2014, 2015 and 2016 were 61,936, 95,287 and 53,026. The malaria prevalence rate for the three years was 5.90%, 4.40% and 4.80% respectively (Average 5.03%).

Age group of patients presenting with malaria in 2014, 2015 and 2016

Using the age groups of 0-5, 6-18 and 19 above, the highest number of cases in 2014 was in the older group; 19 above (62.21%), followed by the minors; 0-5years (24.22%) and the least was observed in those within the 6-18 years (13.57%) . In 2015, the highest number of cases was in the older group; 19 above (68.30%), followed by the minors; 0-5 years (16.29%) and the least was observed in those within the 6-18years (15.34%). The highest number of cases in 2016 was in the older group; 19 above (64.60%), followed by those within the 6-18years group (18.74%) and the least was observed in the minors; 0-5years (16.60%). This is represented in Table 1.

Gender profile of reported malaria patients in 2014, 2015 and 2016

Data from the retrospective study showed that malaria cases were higher in females (59.77%, 61%, and 53.83%) for 2014, 2015 and 2016 as compared to male (40.23%, 38.93% and 44.17%) for the three years respectively. Table 2 shows the Gender profile of malaria patients at Dalhatu Araf Specialist Hospital Lafia for the three years.

Antimalarial drug prescription for 2014, 2015 and 2016

Antimalarial drugs prescription reported for patients in the three years were basically Artemisinin combination therapy; 90%, 90% and 86% for 2014, 2015 and 2016. Fansider (Sulphadoxine/pyremethamine) usage was 10%, 10% and 14% respectively. The Artemisinin Combination reported in use was Arthemeter/Lumefantrine (Coartem or Lonart) for the three years while Dihydroxyartemisinin/Piperaquine (Artequick) was prescribed in 2014 only (Table 3).

Prospective study result

In vitro antimalarial susceptibility test

The sample size for the study was 73 as calculated from the formular of Kadam and Bhalero (2010). A total of 112 samples were collected from patients with uncomplicated malaria at Dalhatu Araf Specialist Hospital Lafia. Only 91 were analyzed due to contamination and lack of schizont maturation. The antimalarial susceptibility test was carried out using five antimalarial agents viz; Chloroquine, Piperaquine, Artemether, Artesunate and Lumefantrine. This is represented in Table 4.

In Vitro Susceptibility of Plasmodium falciparum Isolates to Chloroquine

Some test isolates of *Plasmodium falciparum* (25 (27%)) displayed *in vitro* resistance to chloroquine. Using a peak plasma concentration of 4.47 µM as break point, the geometric IC50 mean was 3.68 µM and the IC50 range was observed to be 1.29-12.23 µM.

In vitro susceptibility of plasmodium falciparum isolates to piperaquine

The result of *in vitro* antimalarial susceptibility showed that 32 (35.16%) of the 91 isolates were resistant to piperaquine. Using a peak plasma concentration of 1.4 µM as break point, the geometric IC50 mean was 1.12 µM and the IC50 range was 0.07- 6.48 µM.

In vitro susceptibility of plasmodium falciparum isolates to Artemether

The result showed that 10 (10.99%) of the 91 isolates were resistant to test Artemether. At a break point of 1.81 µM, the geometric IC50 mean was 1.05 µM and the IC50 range was 0.29- 3.62 µM.

In vitro susceptibility of Plasmodium falciparum isolates to Artesunate

Twenty-six (26 (28.57%)) of the isolates were resistant to Artesunate. At a break point of 77.11 µM, the geometric IC50 mean was 49.08 µM and the IC50 range was 10.04- 157.74 µM.

In vitro susceptibility of Plasmodium falciparum isolates to Lumefantrine

Observation from the *in vitro* susceptibility result indicated that 16 (17.58%) of the isolates displayed *in vitro* resistance to Lumefantrine. At a break point of 53.51µM, the geometric IC50 mean was 27.77µM and the IC50 range was 1.32-100.76µM.

Table 1: Age groups of patients presenting with Malaria at Dalhatu Araf Specialist Hospital Lafia for the years 2014, 2015 and 2016**.**

AGE GROUPS	2014 (%)	2015 (%)	2016 (%)	AVERAGE %
0-5	885 (24.2)	683 (16.3)	424 (16.7)	19.07
6-18	496 (13.57)	643 (15.3)	477 (18.7)	15.86
19above	2273 (62.2)	2863(68.3)	1644 (64.6)	65.03

Table 2: Profile of gender of malaria patients in 2014, 2015 and 2016.

GENDER	2014(%)	2015 (%)	2016(%)	AVERAGE %
MALE	1470 (40.2)	1632 (38.9)	1124 (44.2)	41.10
FEMALE	2184 (59.8)	2557 (61)	1421 (55.8)	58.87

Table 3: Antimalarial drug prescription at Dalhatu Araf Specialist Hospital Lafia for the years 2014, 2015 and 2016**.**

Prescribed agent	2014 (%)	2015 (%)	2016 (%)	Average %
Artemisinin Combination Therapy	2667 (90)	3770 (90)	2191 (86)	88.67
Sulfadoxine/ Pyrimethamine	366 (10)	419 (10)	357 (14)	11.33

Table 4: Resistance Profile and Inhibitory Concentrations of Test Antimalarial Agents (50%) to Plasmodium falciparum Isolates.

Antimalarial Agents	Peakplasma Concentration	% Resistance	IC50 Range	Geometric IC50 Mean
Artesunate	77.11uM	28.57	10.04 - 157.74uM	49.08uM
Artemether	1.81uM	10.99	0.29 – 3.62uM	1.05uM
Chloroquine	4.47uM	27	1.29 – 12.23uM	3.68uM
Piperaquine	1.4uM	35.16	0.07 – 6.48uM	1.12uM
Lumefantrine	53.51uM	17.58	1.32 - 100.76uM	27.77uM

DISCUSSION

Malaria has been reported to be one of the world's most infectious diseases and threatens the lives and wellbeing of people particularly in the tropics. To overcome the widespread and increasing level of parasite resistance to antimalarial drugs, the World Health Organization (WHO) recommended the use of artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated P. falciparum malaria. Base on this recommendation, most malaria endemic countries worldwide changed their antimalarial treatment protocols to the use of artemether (ATH) lumefantrine (LUM) and artesunate (AS)-amodiaquine (AQ) as first-line treatment of uncomplicated malaria (WHO 2010).

Observation from the data obtained on age showed that malaria was higher among the adults (62.2%, 68.3% and 64.6%) for 2014, 2015 and 2016 respectively followed by the minors (13.57% and 16.3%) for 2014 and 2015. The least was observed in the 6-18 years group (13.57% and 15.3%) for 2014 and 2015 respectively. In 2016, cases were higher

in the 6-18 age group (18.7%) than the minors (16.7%). This is similar to the work of Nas et al. (2017) who reported 34.1% cases in adults as compared to 28.3% among the 10-18years. This however contradicts the results of Singh et al. (2014) and Nmadu et al. (2015) who reported higher cases in children 60% and 58% respectively.

Gender wise, Malaria was observed higher in female patients (59.8%, 61% and 53.8%) than the male (40.2%, 38.9% and 47.2%) for the three years respectively. This agrees with the work of Nas et al. (2017) who reported 54% cases in female and 46% in male. The result does not agree with the work of Nwaorgu and Orajaka (2011) who reported that male are more prone to malaria than females. The antimalarial drugs observed prescribed at the Dalhatu Araf Specialist Hospital Lafia were basically Artemisinin combination therapy. This result agrees with the survey of Shorinwa and Ebong (2012) who revealed that Artemisinin combination therapy drugs were the most frequently purchased antimalarial drugs and the study of Okoro and Jamiu (2018) who reported high usage of Artemisinin Combination Therapy (95.8%) for malaria treatment.

Result on monthly distribution on report malaria cases showed the highest occurrence in the month of October. There was report of malaria cases throughout the year. The highest reported malaria cases were in October corresponding to the rainy season when condition for the multiplication of malaria vector is favorable.

The *in vitro* micro-test provides information on the quantitative drug response of *Plasmodium* parasites irrespective of the patient's immune status. It is an epidemiological tool for assessing baseline sensitivity and for monitoring the drug response of *Plasmodium* parasites over time and place.

The *in vitro* test result showed that P. *falciparum* isolates were susceptible to artemether (89%). This agrees with the work of Bustamante et al. (2012) who also reported a high susceptibility of test *P. falciparum* to Artemether. Result on Artesunate activity showed that 71% of the test P. falciparum were Susceptible. Aminu and Mukhtar (2012) and Olasehinde et al. (2014) reported that 100% of test P. *falciparum* isolates were susceptible to artesunate in vitro.

The in vitro results from this study showed reduced susceptibility of test *P. falciparum* against the Artemisinin derivatives tested such as Artesunate 71% and Artemether 89.01%. The reduced *in vitro* Susceptibility of test *P. falciparum* to investigated antimalarial agents may not be synonymous with diminished therapeutic effectiveness but a possible alarm for emerging resistance thus providing early warning of impending resistance before it becomes clinically apparent. The reduced susceptibility to artemisinin derivatives observed in this study may be due to the misuse or substandard Artemisinin monotherapy for the treatment of uncomplicated malaria. The report of Uzochukwu et al. (2010) in Nigeria showed that the prescription of Artemisinin monotherapy was 18.2% and 15.8% in the southwest and southeast part of Nigeria. The misuse or substandard Artemisinin monotherapy for the treatment of uncomplicated malaria might result to emergence of resistant P. *falciparum* (Bustamante et al., 2012). Declining artemisinin effectiveness has been reported to increases the emergence of resistant P. falciparum (White, 2012).

The *in vitro* antimalarial activity of Lumefantrine and Piperaquine was evaluated and observations from the results showed that test *P. falciparum* was 82% and 65% susceptible respectively. The reduced in vitro susceptibility of test *P. falciparum* to Lumefantrine and Piperaquine might be because of the previous use of chloroquine.

In Vitro test has been reported useful for monitoring changes overtime in susceptibility of *P. falciparum* to antimalarial drug that has been withdrawn. This study reveals a 73% susceptibility of test *P. falciparum* to chloroquine. The low resistance observed might be an evidence of returning effectiveness of chloroquine. This study agrees with the work of Olasehinde et al.

(2014) who reported 80% in vitro susceptibility of *Plasmodium falciparum* to chloroquine at Ijebu. This, however, does not agree with the work of Balogun et al. (2016) who reported that 67% of test P. *falciparum* were observed to be resistant in Azare and 84% were resistant in Maiduguri. However, it was observed that chloroquine was still in use among malarial patients in other parts of Nigeria. The continuous prescription of chloroquine might have increased the pressure for emergence of chloroquine resistant *P. falciparum* in Azare and Maiduguri. It was observed in the retrospective survey in Lafia that chloroquine was not prescribed. This might have accounted for the low level of chloroquine resistant P. falciparum isolates observed.

Conclusion

This study showed that malaria cases were reported throughout the year in Lafia, Nigeria with the highest incidence in October every year. Observations from the study also showed that antimalarial monotherapy treatments were not common and that ACT was widely priscribed. *In vitro* susceptibility study also displayed emergence of multiple antimalarial resistant *P. falciparum* isolates. The order of *In vitro* effectiveness of the test antimalarial agent was observed to be Artemether (89%) > Lumefantrine (82%) > Chloroquine (73%) > Artesunate (71%) > Piperaquine (65%). More research should be carried out to provide evidence for the stratification of the country's malaria epidemiology. Evidence of drug activity should be provided at regular intervals to guide policy and interventions.

COMPETING INTERESTS

Authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

JOE: Designed and supervised the study; ROB: Supervised the Study; FOS: Sample collection, experiment and Data analysis; ADA: Sample collection and Experiment; JYA: Sample collection and Experiment.

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