Risk Factors For The Presence Of Artemisinin Antibodies Amongst Patients Undergoing Treatment For Malaria In Benin City, Nigeria

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Abstract—Artemisinin have being used for the control of malaria worldwide for over a decade and its listing by WHO as the first-line drug for treatment of both severe and uncomplicated malaria and the observed treatment failure have warranted the need to screening malaria patients for the presence of antibodies to malaria. In our locality where there is unregulated use of antimicrobials, the need to ascertain the prevalence of antibodies to artemisinin and evaluate the potential risk factors cannot be overemphasised, hence this study. Blood specimens were collected from 400 randomised patients undergoing treatment for malaria in Benin City, Nigeria. Data on socio-demography were collected with the aid of a well-structured questionnaire. Artemisinin antibodies were detected by drug absorption mechanism (DAM) and immune complex reaction (IMC) methods. ABO, rhesus blood group, and haemoglobin (Hb) phenotype were determined by using standard technique. A total of 112(28.00%) out of the 400 participants had artemisinin antibodies. Gender, marital status, level of education, residential area and living arrangement did not significantly affect the prevalence of artemisinin antibodies whereas age and ethnicity significantly affected the prevalence of artemisinin antibodies ($p=0.0244$ and 0.0001 respectively). Duration of the last artemisinin used and the mostly used brand of artemisinin as well as the ABO and rhesus blood groups and haemoglobin phenotypes did not significantly affect the production of artemisinin antibodies. Although of all the risk factors age and ethnicity were identified as the only risk factors for the development of artemisinin, we therefore advocate the prudent use of artemisinin-containing antimalarial and concerted efforts in combating self-medication with this drugs to avoid the development of resistance.

Index Terms—Antibodies; Artemisinin; Prevalence; Risk factors.

I. INTRODUCTION

Malaria is an infectious disease caused by a microscopic parasite known as *Plasmodium* spp. which is transmitted between humans by mosquitoes [1]. It is known to be the cause of 216 million reported cases of illness and 445,000 deaths in 2016 with about 90% of this morbidity and mortality cases domiciled in Sub-Sahara Africa [2]. Malaria is a risk for 97% of Nigeria's population. The remaining 3%

of the population live in the malaria free highlands [3]. Malaria can be a severe, potentially fatal disease (especially when caused by *Plasmodium falciparum*) with symptoms ranging from high fever, chills, flu-like illness etc [4].

Anaemia is a known complication of malaria and over half of malaria related deaths are attributable to severe anaemia [5], [6]. The goal of most current National Malaria Control Programs and most malaria activities is to reduce the number of malaria-related cases and deaths. The fight to reduce malaria transmission to a level where it is no longer a public health problem have led to the use of anti-malaria drugs worldwide [4]. Due to high resistance to most anti-malaria drugs, artemisinin based combination therapy are recommended as first line treatment for *falciparum* malaria [7]. Resistance to artemisinin has been reported in South-East Asia [8], [9]. In Nigeria, antimicrobial use is unregulated and over the counter sales of anti-microbial agents without prescription are common [10]-[12]. This selective pressure can result in drug resistance and antibody formation. Indeed, autoimmune haemolytic anaemia resulting from artesunate administration have been reported [13]-[16]. A recent report has demonstrated the presence of artemisinin resistant *P. falciparum* in Africa [17]. Another recent study demonstrated the presence of antibodies to sulphadoxine in our environment [18]. However, to the best of our knowledge no study has been done to ascertain the risk factors and determine the presence of artemisinin antibodies in our locality.

Therefore, this study aimed to determine the prevalence of artemisinin antibodies among patients with malaria and also determine the impact of demography and some possible risk factors on the prevalence of artemisinin antibodies.

II. MATERIALS AND METHODS

A. Study Population

This study was conducted in Benin City, Nigeria with a total of 400 randomised participants (234 males and 166 females) clinically and laboratorily confirmed to have malaria parasite infection with evidence of artemisinin resistance attending out-patients clinics or admitted in the wards of major hospitals in Benin City, Nigeria. A structured questionnaire was used to collect data on socio-demography and informed consent was obtained from all participants or their parents/guardians in case of children prior to specimen collection. All malaria infected patients who have taken an artemisinin therapy at least once and

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presenting with both clinical symptoms and diagnostic features of malaria parasite infection were included in the study. While those not willing to give consent and/or had no history of previous exposure to artemisinin were excluded from the study. This study was approved by the Ethics and Research Committee of Edo State Ministry of Health, Benin City, Nigeria.

B. Specimen Collection

Blood (10mL) was collected from each study participant and dispensed in 5ml amount into plain and EDTA containers. The blood in the plain container was allowed to clot and the serum obtained was used to detect antibodies to artemisinin. The blood specimens in the EDTA container was used for ABO and Rhesus blood grouping as well as Haemoglobin phenotypes.

C. Malaria Microscopy

Malaria was diagnosed by examination of stained thick blood films as previously described by Akinbo et al [19]. Briefly, thick and thin film was made from each blood specimen and stained in 10% Giemsa stain for 30 minutes. The film was examined using oil immersion lens and a total of 200 fields per film was examined. The Plasmodium spp. present was identified and the parasite density was calculated from Giemsa stained thick blood film by multiplying the ratio of number of malaria parasite to 500 white blood cells by the patient white blood cells count to give malaria density in cells/ml.

D. ABO and Rhesus Blood Grouping

ABO and rhesus blood group were determined as previously described [20]. Briefly, a drop of each participant's blood was placed on three separate area of white tile. Each drop of blood mixed with a drop of commercially prepared antiserum A, B, and D respectively and observed for agglutination.

E. Haemoglobin Phenotype

The method described by Omoregie et al [21], was used for the determination of haemoglobin phenotype (Hb-phenotype). Cellulose acetate paper and tris- buffer, pH8.5 was used. Blood sample was applied on the paper after lysis in water. The strips then suspended in the genotype tank (Shandon 600X 100) with one end in each compartment filled with buffer. Standard Hb AA, AS, SS, SC and AC were spotted along with the test sample. The tank was then covered and electrophoresis carried out for 10 minutes at a potential difference of 220 volt. The strips were then removed and read macroscopically comparing the mobility of the test with standard Hb AA, Hb AS, Hb SS, Hb SC and Hb AC.

F. Detection of Antibodies to Artemisinin

Artemisinin antibodies were detected by both drug-adsorption method and immune complex reaction as modified and described by Ikuoyogie et al [18]. Briefly, 0.5g of pure artemisinin powder was dissolved in 10ml of normal saline.

Drug Absorption Method: Equal volumes of 10% Human group O red cells and artemisinin solution was placed inside a test tube and incubated at 37oC for 30minutes. After the

incubation period, the drug red cell suspension was washed 4 times with normal saline to remove excess drug. Equal volumes of the drug-red cell mixture and patients serum was placed in a test tube and incubated at 37oC for 1hr. This was followed by brief centrifugation and agglutination or haemolysis was watched out for. If negative, the entire mixture would be washed 4 times with normal saline. After washing, anti-human globulin (AHG) was added followed by brief centrifugation and agglutination or haemolysis was watched out for.

Immune Complex Method: Into a clean test tube was placed equal volumes of artemisinin, patient serum and 5% human group O red cells, this was incubated at 37oC for 1hr. This was be followed by brief centrifugation and agglutination or haemolysis was watched out for. If negative, the entire mixture would be washed 4 times with normal saline. After washing, anti-human globulin (AHG) was added followed by brief centrifugation and agglutination or haemolysis was watched out for.

Controls were performed in the same way for each of the methods with:-

1. Patients serum and group O red cells without drugs.
2. Drug-red cell suspension without patient's serum.

Both controls gave negative results.

G. Statistical Analysis

The data obtained were analyzed with chi-square (χ^2) test using the statistical software GraphPad InStat version 2.05 for Windows 7, GraphPad Software, La Jolla California USA, www.graphpad.com.

III. RESULTS

A total of 112(28.00%) out of the 400 participants had artemisinin antibodies. Although DAM detected more (12.00%) of the antibodies, there was no significant difference ($p=0.0562$) in the rate of detection of the artemisinin antibodies amongst the methods used (Table I).

TABLE I: PREVALENCE OF ARTEMISININ ANTIBODIES IN RELATION TO METHODS OF DETECTION

Artemisinin Antibody Detection Method	No. Positive	Percentage
n=400		
Drug Absorption Mechanism (DAM)	48	12.00
Immune complex reaction (IMC)	29	7.25
Drug Absorption Mechanism + Immune complex reaction	35	8.75
Total	112	28.00

n=number tested; $p=0.0562$

Gender, marital status, level of education, residential area and living arrangement did not significantly affect the prevalence of artemisinin antibodies. Participants aged 58-65years had significantly higher ($p=0.0244$) artemisinin antibodies production. Also, ethnicity significantly affected the prevalence of artemisinin antibodies production ($p=0.0001$) as Igbo ethnic group recorded 58.62% as compared to others (Table II).

TABLE II: PREVALENCE OF ARTEMISININ ANTIBODIES AND SOME ASSOCIATED RISK FACTORS.

Characteristics	Number of patients tested	Number Positive (%)	OR	95% CI	P-value
Gender			1.192	0.76 95, 1.85 8	0.4949
Male	234	62(26.50)			
Female	166	50(30.12)			
Age (years)					0.0244
1-7	55	9(16.36)			
8-14	68	22(32.35)			
15-21	79	22(27.85)			
22-28	88	27(30.68)			
29-36	38	11(28.95)			
37-43	24	2(8.33)			
44-50	19	7(36.84)			
51-57	14	7(50.00)			
58-65	9	5(55.56)			
≥66	6	0(0)			
Marital Status					0.7436
Divorced	5	2(40.00)			
Married	114	35(30.70)			
Single	271	73(26.93)			
Widowed	10	2(20.00)			
Education Level					0.2711
Non-formal	118	26(23.21)			
Primary	81	27(24.11)			
Secondary	130	36(9.00)			
Tertiary	71	23(20.54)			
Ethnicity					0.0001
Igbo	29	17(58.62)			
Bini	283	87(30.74)			
Esan	48	4(8.33)			
Estako	15	1(6.67)			
Ijaw	2	1(50.00)			
Ika	1	0(0)*			
Isoko	1	0(0)*			
Owan	7	1(14.29)			
Urhobo	9	0(0)*			
Yoruba	5	1(20.00)			
Residential Area					0.4976
Rural	18	3(16.67)			
Sub-Urban	28	9(32.14)			
Urban	354	100(28.25)			
Living Arrangement					0.3943
Flat	337	90(26.71)			
One room	20	8(40)			
Two rooms	30	11(36.67)			
Self-contain					
Two Rooms	13	3(23.08)			

OR: odd ratio; CI: confidence interval.

*- not included in statistical analysis.

Duration of the last artemisinin used and the mostly used brand of artemisinin did not significantly affect the production of artemisinin antibodies (Table III). The prevalence of artemisinin antibodies was not significantly affected by ABO blood group (0.3038), rhesus blood group

(0.8486) and haemoglobin phenotypes (0.6523) (Table IV).

TABLE III: PREVALENCE OF ARTEMISININ ANTIBODIES IN RELATION TO ARTEMISININ USAGE.

Characteristics	Number of patients tested	Number Positive (%)	P-value
Duration of the last artemisinin use (months)			0.7175
≤1	167	42(10.5)	
2	106	33(8.3)	
3	26	7(1.8)	
≥4	101	30(7.5)	
Mostly used Artemisinin combination Therapy of Participants			0.2808
Amaterm	22	8(36.36)	
Artequin	15	1(6.67)	
Artesunate	16	7(43.75)	
Camosunate	13	2(15.38)	
Coartem	52	17(32.69)	
Klaritem	6	1(16.67)	
Lonart	123	35(28.46)	
Lumartem	48	9(18.75)	
P-Alaxin	92	27(29.35)	
D-Artep	13	5(38.46)	

TABLE IV: EFFECT OF ABO, RHESUS BLOOD GROUPS AND HAEMOGLOBIN PHENOTYPES ON THE PREVALENCE OF ARTEMISININ ANTIBODIES.

Characteristics	Number of patients tested	Number Positive (%)	p-value
ABO blood group			0.3028
A	19	5 (26.32)	
AB	9	0(0)	
B	10	3(30)	
O	362	104(28.73)	
Rhesus blood group			0.8486
Positive	392	110 (28.06)	
Negative	8	2 (25)	
Haemoglobin phenotypes			0.6523
AA	367	105(28.61)	
AS	29	6(20.69)	
SS	4	1(25.00)	

IV. DISCUSSION

The emergence of *Plasmodium falciparum* resistant to chloroquine and some other antimalarial therapies led to the World Health Organisation recommendation of the use of artemisinin combination therapy (ACT) as a first line treatment for uncomplicated malaria [22]. Hence, early diagnosis of malaria with a corresponding treatment with ACT has been seen as key components of global malaria elimination programme [23]-[25]. This is mainly due to the fact that ACT effect rapid and sustained parasitological cure in patients with *Plasmodium falciparum* malaria and have shown to reduce transmission of this species in areas with moderate and low endemicity [26]-[28]. There is a significant increasing use of ACT in the treatment of malaria in hospitals in Nigeria [29]. Although ACTs have been

reported to have mild and well tolerated adverse reaction on patients, there are recent reports on its capacity to cause haemolysis with a resultant severe autoimmune haemolytic anaemia when used in treating both complicated and uncomplicated malaria [14], [30]-[32]. There is no report on the prevalence of artemisinin antibodies in our environment; therefore, this study was conducted to fill this knowledge gap.

Our study revealed that 28.00% of the participants had artemisinin antibodies in their system. The presence of these drug-induced antibodies has an increased risk of haemolytic anaemia as there are several reports of delayed autoimmune haemolytic anaemia following the administration of artemisinin containing drug [14], [16], [30]-[35]. Although, there are no published data on the prevalence of artemisinin antibodies to compare our findings, the high prevalence observed in this study could be attributed to the WHO guideline for the use of artemisinin as the first line drug for the treatment of malaria and its consequential increased use in hospitals in Nigeria coupled with the poor regulation of use and misuse of antimicrobial agents in Nigeria [10], [22], [25], [29].

There was no significant difference ($p=0.0562$) in the rate of detection of the artemisinin antibodies by the different methods used, although DAM detected 12% of the participants while DAM and IMC detected 8.75% of the participants positive for artemisinin antibodies. Although the mechanism of the drug-induced haemolysis of artemisinin antibodies is unclear [14], [16]. The findings of this research indicate that antibodies to artemisinin can be detected by both methods with some cases being detected by only one method which is similar to the findings of Ikuoyogie et al [18], which though examined sulphadoxine antibodies. We therefore recommend that both methods be used for detection of artemisinin antibodies as there were instances where DAM method detected artemisinin antibodies and IMC method did not, and vice versa.

The transmission of malaria in Nigeria cuts across barriers posed by gender, age, marital status, educational status, ethnicity, residential area and living arrangement which makes the need for optimal care and control of malaria a task for both government and the populace [36]. The poor regulation of use and misuse of antimicrobial agents in Nigeria with large room for antimalarial use without prescription [37], [38] may attribute to the reason for the observed non-significant effect of gender ($p=0.4949$), marital status ($p=0.7436$), educational status ($p=0.2711$), residential area ($p=0.4976$) and living arrangement ($p=0.3943$) on the production of artemisinin antibodies amongst the study participants as most individuals indulge in self-medication regardless of their status on the aforementioned demographic factors. However, age and ethnicity significantly affected the production of artemisinin antibodies with the prevalence of artemisinin antibodies being significantly higher ($p=0.0244$) in subjects within the age range of 58-65 years (55.56%) and amongst subjects of Igbo ethnic extraction (58.62%). Although there are limited data on the relationship between the use of anti-malaria and age as well as ethnicity, the study of Esan et al., (2018) [38] revealed that the Igbos were the second most involved ethnic group in self-medication in Nigeria after Hausa. Since

Hausa were not involved in our study, it follows that the high prevalence of artemisinin antibodies amongst the Igbo people in our study is due to the high indulgence in self-medication by this ethnic group. Moreover that artemisinin had been a very effective first line anti-malaria drug in our locality and the Igbos being capitalist in nature [39]. The Igbos in order to maintain good health and not breakdown, they therefore may engage in over use of artemisinin thereby leading to the high prevalence of artemisinin antibodies. Artemisinin being a drug with a low adverse outcome is a drug of choice for individuals of all age groups but a recent study have shown that individuals between the ages of 25-54 years have high preference for the use of artemisinin based combination therapy [40]. This can be attributed to the reason for the high prevalence of artemisinin antibodies observed within these age groups in our study.

The development of artemisinin antibodies may be due to the frequency of use of artemisinin and not the last time of use as there was no significant difference in the duration of the last time of artemisinin use in this study ($p=0.7175$). Although there are no data on the prevalence of artemisinin antibodies, the method used in this study detected IgG and IgM antibodies that take part in type II hypersensitivity reaction hence, it follows that those who took artemisinin-containing drugs within one month prior to specimen collection will have a higher dose of the drug in their system, thereby have higher titres of the antibodies in their system, this phenomenon was observed when individuals who took the drug in less than one month (10.5%) were compared with those who took it within the last two months (8.3%) and three months (1.8%) before samples were obtained from them. This finding in the group of ≤ 1 month to 3 months of the last use of artemisinin therapy agrees with the findings of Ikuoyogie et al [18], which though examined for sulphadoxine antibodies. The inconsistency in the prevalence for the antibodies in the group of those who took artemisinin within four months and beyond may be due to the broad nature of the group and physiological difference between the participants.

To delay or prevent emergence of resistance, artemisinins are combined with one of several longer-acting drugs — amodiaquine, mefloquine, sulfadoxine/pyrimethamine or lumefantrine — which permit elimination of the residual malarial parasites [41]. This gave rise to different artemisinin combination therapies (ACT) with a complex clinical pharmacology with a suspected drug-drug interaction. However, there was no significant effect of the various combination therapies on the prevalence of artemisinin antibodies production in this study ($p=0.2808$). This may be due to the fact that artemisinin combination therapies are recommended to patients in most cases because of the presence of artemisinin and not because of the combined long-acting drug since ACT is recommended by WHO without a specification of a particular combination therapy [42].

In this study, it was observed that the prevalence of artemisinin antibodies was not significantly different amongst individuals of different ABO blood group ($p=0.3028$), rhesus blood group ($p=0.8486$) and haemoglobin phenotypes ($p=0.6523$). This implies artemisinin binds to red blood cells of individuals

irrespective of their haemoglobin constituents or their ABO or rhesus blood group to activate antibody production using drug absorption mechanism. These findings are in line with the findings in previous study that have reported that there was no association of haemoglobin phenotypes, ABO and rhesus blood groups with the efficacy of artemisinin [43].

V. CONCLUSION

An overall prevalence of 28.00% artemisinin antibodies among patients with malaria was observed in this study. Age and ethnicity were identified as risk factors for the development of artemisinin antibodies; we therefore advocate the prudent use of artemisinin-containing antimalarial and concerted effort in combating self-medication with ACT to avoid the development of resistance.

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