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Sero-reactivity and Molecular Studies on Subjects with Malaria Immunity and Susceptibility in An Endemic Region

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Abstract

Nigeria is a malaria-endemic country, yet many inhabitants of the country rarely suffer from the disease in spite of regular exposures to the vector of Plasmodium and without administration of curative or prophylactic drug. The aim of the study was to identify the subjects with resistance against malaria and conduct serological and genomic investigations their unique blood samples. One thousand two hundred and seventy (1,270) apparently healthy volunteers, aged 16-55 years participated in the study. Malaria distribution among the subjects was assessed by microscopy and serology techniques using six millilitres of the blood samples withdrawn from the subjects and questionnaire administered. Subjects with resistance against malaria were identified, their blood sera collected, tested, titrated and graded. Molecular analysis of the blood samples with respect to Fc gamma receptor IIa was conducted on malaria resistant and susceptible subjects. . The study revealed 21.0% malaria infection rate by microscopy, 40.3% by serology method while 21.0% was assessed to develop natural resistance against malaria infection. Genomic analysis of blood samples from malaria resistant individuals depicted greater association with R/R131 (36.7%). The study revealed that some individuals in this locality are genetically and immunologically protected against malarial infection. Further studies on the specific genes and immunoglobulins responsible for their uniqueness are recommended.

Keywords: Malaria-immunity, Susceptibility, Sero-reactivity, Molecular, Endemic

1. Introduction

In Africa, malaria accounts for 10% of the total disease burden while transmission occurs in about 107 countries globally [1-4]. It also accounts for 30 to 50% in-patients and up to 50% out-patients in highly endemic areas and about 40% of public health expenditure is spent on malaria annually [5-7]. In Nigeria, malaria is a common disease and it posed a serious public health problem [8, 9].

Although the disease is preventable and curable, its related deaths account for up to 11% of maternal mortality, 25% of infant mortality and 30% of under-five mortality [10-12] resulting in about approximately one million deaths annually. In the regions where malaria is rampant, 85% of the infection usually effects children below 5 years and the prevalence is high amongst expectant mother [13, 14].

Malaria remains a serious global challenge in view of its high mortality rate and economic burden despite all efforts to roll it back by over 100 countries of the world [15, 16]. In Nigeria, malaria constitutes a major public health issue9 causing at least 300,000 deaths every year [17, 18]. However, the official estimate of malaria episodes in Nigeria documented an average of four episodes per person per year [6, 19].

The frequency of antibodies against malaria parasites has been used as a means of assessing malaria infectivity [20-24] and show potential to detect recent changes in plasmodium density. Several authors had earlier reported [25, 26] that it is more difficult to assess plasmodium density by the conventional malaria parasitemia determination during scanty infection or when mosquito numbers are low.

Previous authors also reported an association between decreased anti-MSP-1 antibodies and decreased risk of clinical malaria depending on the type of Fc γ R IIa allele involved [27, 28] while others reported MSP-1 as a potential antigen for malaria vaccine preparation in the nearest future [29, 30].

The alarming rate of drug resistance against malaria infection has posed a major threat against effective anti-malarial preparations in endemic region [31-33] therefore regular search for new anti-malaria preparations and vaccine remains a global priority.

The aim of this study was to determine the frequency of subjects with natural immunity against malaria and conduct serological and molecular analyses on their blood samples. It is expected that the findings in the study could serve as a useful guide for preparation of malaria vaccine or new anti-malaria preparations

2. Materials and Methods

2.1 Sample collection and processing

This study was conducted between August 2017 and September 2018 on one thousand two hundred and seventy (1,270) apparently healthy volunteered students and workers within Owo and Ilorin communities aged 16-55 years. Six millilitres of the blood sample from each subject was collected, 2ml of which was put into EDTA container and properly mixed. 2ml was dispensed into heparinized bottle while the remaining 2ml was put in a plain container.

The 2ml-blood sample in EDTA container was used for thick malaria films preparation in duplicates. All the thick films were stained by Giemsa technique according to Chessbrough [34]. Only subjects tested negative for malaria parasite were further screened by serology. The 2ml-blood in each of the heparinized containers were used for molecular analysis while sera extracted from the blood samples kept in the plain containers were investigated for anti-MSP-1 antibody. All the sera from subjects were titrated and graded as low, moderate and high titer anti-merozoite sera.

2.2 Selection criteria of subjects

In the context of this study, an individual was considered to develop resistance against malaria infection if such subject has no history of malaria for over six months of exposure to the vector of the parasite, not sleeping under mosquito net and without taking any curative or prophylactic medication. In addition, the subjects must be tested negative for malaria parasitemia in peripheral blood by microscopy and record low level of MSP-1 antibody against Pf MSP-1 antigen.

2.3 Typing and titration of sample sera against Pf MSP-1 antigen

The test cassette was removed from the foil pouch, and it was placed on a flat dry surface. Ten microliters of the serum was dropped in the hole on the cassette followed by 3 drops of the diluent. The reading was taken after 10 minutes and any positive result after ten minutes was disregarded. The test was recorded positive when both test and control produced pink lines within 10 minutes while the negative result was recorded when only the control line appeared within the same time. Serial dilution of sera was conducted with physiological saline followed by titration of the diluted sera with Pf MSP-1 antigen.

2.4 Molecular Analysis

Extraction of DNA from immune blood cells DNA was extracted from 2ml whole blood collected from each participant. The blood cell was washed in lysis buffer twice to exclude non-nucleated RBC, leaving nucleated WBC and platelets. The mixture was treated with RNase and proteinase K to digest RNA and protein impurities respectively form the cell lysis solution.

To the resultant mixture, protein precipitant was added to precipitate the protein impurities. The mixture was centrifuged and the supernatant decanted into propan-2-ol to precipitate DNA. The DNA pellet was washed in two changes of 70% alcohol, centrifuged and the DNA pellet dissolved in DNA hydration solution.

2.5 DNA amplification using Allele-Specific PCR Primer

The gene of interest was searched on Web-based Allele-Specific PCR Primer designing tool (WASP). This primer design tool was linked to the National centre for bioinformatics information (NCBI) where all genomic information were banked for research use. WASP automatically generates all existing alleles.

Parameters like primer length, melting temperature, product size range and self-complementarities were selected and the information was used to produce primer pairs for amplification of the Fc gamma receptor gene. A 96 - well plate was placed unto an ice bucket as the holder for the 0.2ml wall PCR tube. All PCR reagents were added unto cold 0.2ml thin-walled PCR tube to prevent nuclease activity and non-specific priming. PCR reagents were added in the following order: 12.5µl of 1X dream Taq PCR master mix (Thermo Fischer, Japan), 0.5µl 0f 10µmol of each primer and 1µl DNA. The mixture was made up to 25µl with nuclease-free water.

2.6 Gel electrophoresis

A total of 10 µL of PCR product was loaded in each well, with 100bp ladder occupying the first well. The gels were allowed to run for 30 minutes at 120V from negative to the positive electrode. After the period, the gel was placed on the UV trans-illuminator and observed for the bands of the amplicon. The negative control lane showed no visible bands. The picture of visible bands on the gel was finally taken and compared with respect to the ladder.

2.7 Questionnaire administration

Prior to blood sample collection, a questionnaire was administered to all the participants to assess their out-door exposure to mosquito, usage of mosquito nets, recent treatment with anti-malaria drugs and prophylaxis, frequency of malaria cases per participant per year and respondents' demographic factors. In some few cases of illiteracy, a structured interview was conducted in respondents' local languages. To ascertain the information supplied by the participants, their malaria status was regularly monitored by contact tracing over six months after providing the information.

2.8 Statistical analyses

Data obtained from the study were input into the computer and analysed with SPSS version 23 software (SPSS Inc, USA). Malaria distribution by microscopy and serology with respect to age and gender were subjected to analysis of variance (ANOVA). Odd ratio statistics was used to assess the distribution of Fc gamma receptor IIa alleles among malaria resistance individuals. The test for significance level was based on a P value less than 0.05.

3. Results

Figures 1 and 2 show the respondents' perceived frequency of malaria infection per year in relation to age and gender. Out of total 1270 subjects examined, 383(30.2) have malaria every 1-3months, while 461(36.3) subjects have malaria every 4- 6 months and 426 (33.5) every 7- 12 months. By gender consideration, on the overall 156 (28.1%) male subjects and 465 (34.7%) female subjects had malaria frequency at interval of 1-3 months in a year; 158 (28.4%) males and 175 (24.5%) females have malaria frequency every 4-6 months, while 242 (43.5%) males and 74 (10.4%) females have a frequency between 7-12 months

Table 1 shows the distribution of Plasmodium infection by microscopy and serology in relation to age. On the overall, the highest rate of malaria (33.5%) by microscopy was recorded within the age group 26-35 years while the highest frequency of malaria immunity (45.3%) was recorded within the 16 – 25 years age group. The least rate of malaria infection was documented within the same age bracket 16 – 25 years by serology and microscopy recording 325(38.2%) and 140(16.4 %) respectively. Statistically by analysis of variance (ANOVA), the results showed no significant difference in malaria frequency by serology and microscopy methods with respect to age (Tcal = 0.603177; $P > 0.05$).

Distribution of serum titer grades among malaria immune subjects in relation to age and gender is as depicted in Table 2. Out of the total 490 sera that were collected from the subjects that were resistant to malaria infection, 267(54.5%), 48(9.8%) and 175(35.7%) recorded low, moderate and high titer values respectively. The highest frequency of low titer 215(16.9%) was recorded within the age bracket 16-25 years while the least was recorded among age group 46 – 55years. Similarly, more low titer subjects were documented among the males 190(14.9%) than their female counterparts with 77(6.0%). However when the serum grading was subjected to statistical analysis by chi-square, the result showed that there was no significant difference in the distribution of titer grades in relation to age (Tcal = 0.1957 ; $P > 0.05$) and gender (Tcal = 0.3654 ; $P > 0.05$).

Table 3 shows the distribution of the Fc gamma receptor IIa alleles in malaria resistant and susceptible subjects with respect to the odd ratio at 95% confidence interval. Using the Fisher Exact test, Odd ratio, result showed a significant difference in the distribution of the allele among the individuals. Homozygous allele R131 appears to confer higher protection on malaria resistant individuals than heterozygote allele R131H going by the values of Odd Ratio (OR=0.18, 95% CI= 0.07-0.44), while homozygote allele H131 was slightly protective (OR= 0.48, 95% CI= 0.19-1.19)

Table 4 depicts the frequencies of Fc γ R IIa allele among malaria resistant and susceptible subjects. Out of 1270 subjects examined, 225 had homozygous R131 genotype; comprising 180(36.7%) malaria resistant and 45(5.6%) susceptible subjects. 560 homozygous H131 genotype had 150 (30.6%)malaria resistant and 410(52.6%) susceptible while a total of 485 heterozygote allele R131H was recorded in the study of representing 160 (32.7%)resistant and 325 (41.7%) susceptible subjects. Statistically, no significant difference in the distribution of allele was recorded with respect to subjects' immunity against malaria (Tcal = 1.69, $P > 0.05$)

Table 1: Frequency of malaria immunity in relation to age

Age (years)	Number Examined	Microscopy positive (%)	Serology positive (%)	Subjects with malaria immunity (%)
16 – 25	850	140 (16.4)	325 (38.2)	385 (45.3)
26 –35	254	85 (33.5)	107 (42.1)	62(24.4)
36 – 45	120	31 (25.8)	57 (47.5)	32 (26.7)
46 – 55	46	11 (23.9)	24 (52.2)	11 (23.9)
TOTAL	1270	267 (21.0)	513(40.3)	490 (38.6)

Note: Immunity against malaria implies No signs, symptoms prophylaxis and treatment of malaria for at least six months, No malaria parasite in peripheral blood and positive for MSP-1 serology test.

Table 2: Distribution of serum grades collected from subjects with immunity against malaria in relation to age and gender

Age group	Number Examined	Frequency of malaria immune sera/Grade		
		Low (%)	(Moderate) (%)	High (%)
16 – 25	385	215(55.8)	20(5.1)	150(39.1)
26 – 35	62	26 (41.9)	16(25.8)	20(32.3)
36 – 45	32	17(53.1)	10(31.3)	5(15.6)
46 – 55	11	9(81.8)	2(18.2)	0(0.00)
Gender				
Female	156	77(49.)	12(7.7)	67(42.9)
Male	334	190(56.9)	36(10.8)	108(32.3)
Total	490	267(54.5)	48(9.8)	175(35.7)

Table 3: Distribution of FcγR IIa alleles in malaria immune and susceptible subjects with respect to Odd Ratio analysis

FcγR IIa genotype	Immune	Susceptible	95% CI	P-value	Odd Ratio
R131	266	120	0.07-0.44	0.001	0.18
H131	121	329	0.19-1.19	0.11	0.48
R131H	103	331	0.56-3.89	0.96	1.00

Table 4: Distribution of FcγR IIa allele among malaria resistant and susceptible subjects

	SUBJECTS EXAMINED WITH		
FcγR IIa genotype	Mp Immunty (%)	No immunity (%)	Total
R131	180 (36.7)	45 (5.6)	225
H131	150 (30.6)	410 (52.6)	560
R131H	160 (32.7)	325 (41.7)	485
Total	490 (38.6)	780(61.4)	1270

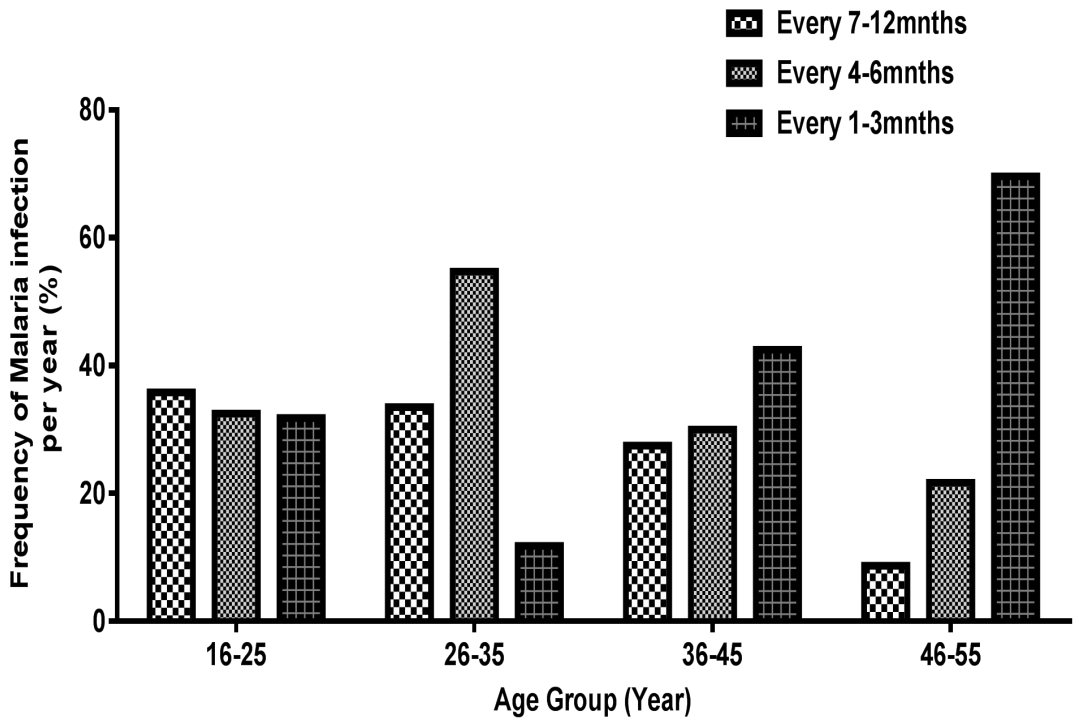

Figure 1: Respondents' perceived frequency of malaria infection in relation to age

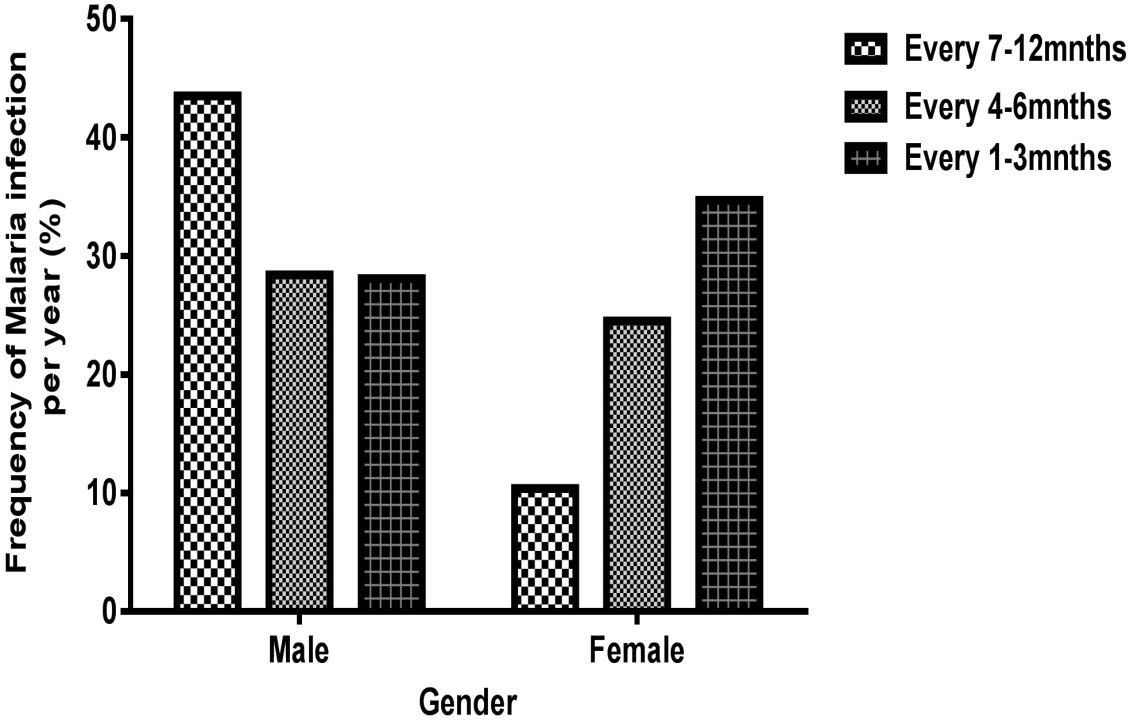

Figure 2: Respondents' perceived frequency of malaria infection in relation to gender

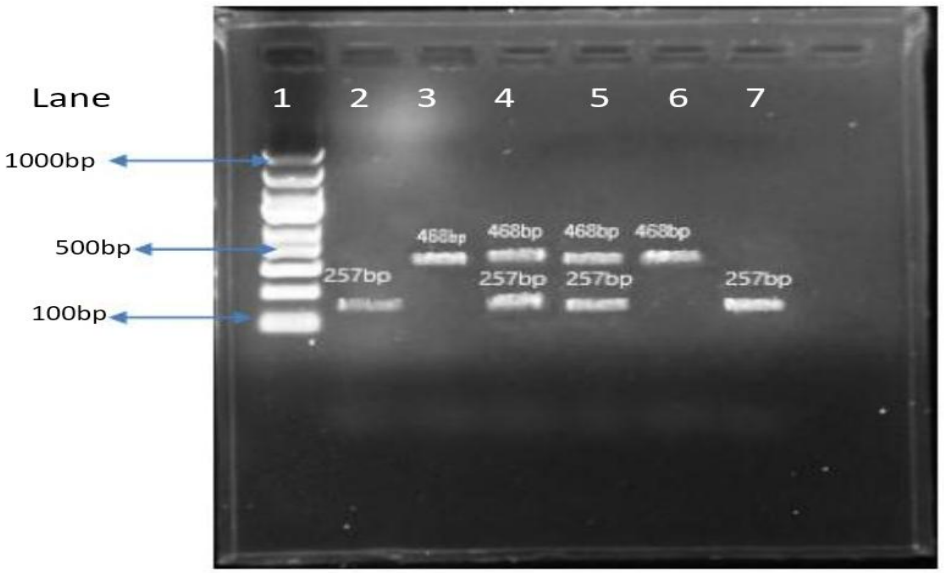

Figure 3: The Agarose gel picture showing gel bands of Gamma Receptor IIa alleles
Lane 1 represents the molecular weight marker, lanes 2 and lane 7 are bands for H/H131 (257bp), lanes 3 and 6 represent R/R131 band (468bp) while lanes 4 and 5 are the bands for Heterozygote allele R131H.

4. Discussion

On the overall, this study revealed 21.0% positive cases of malaria by microscopy while serology method reported it as 40.3%. Although several studies [20-26] have alluded to the efficacy of plasmodium antibody detection as an acceptable method of assessing the frequency of malaria infection, however the disparity between the gold standard microscopy and serology in the present study is worrisome. The reason for the higher result by serology could probably be attributed to the fact that antibody against an antigen could still be detectable long time after total clearance of the infection thereby producing false positive result [35, 38].

In spite of the high rate of malaria infection in this study, 21.0% of the subjects investigated still developed resistance to the infection even with regular exposure to the causative vector of the infection. A subject was considered to develop resistance against malaria infection if the subject has no history of malaria for over six months of exposure to the vector of the parasite, not sleeping under mosquito net and without taking any curative or prophylactic medication. In addition, the subjects were tested negative for malaria parasitemia in peripheral blood by microscopy but recorded low level antibody against Pf MSP-1 antigen. Our finding therefore agrees with the previous studies [29, 30] that documented MSP-1 as a potential antigen for malarial vaccine

According to findings from some previous studies [27, 28], the lower the titer level of ant-MSP-1 in a subject, the less the risk of clinical malaria. Therefore, the reactivity of the sera of the study subjects were categorized into high, moderate and low grades of antibody titers against MSP-1 antigen. Assessing the distribution of low titer of anti-MSP-1 (Titers=2-4) in relation to age and gender, the highest frequency 215/1270 (16.9%) was recorded within the age bracket 16-25 years. Our study also observes that as the age increases, the occurrence of low titer reduces implying that malaria immunity reduces with age. Similarly, low titer sera were more frequently documented among the males (14.9%) than their female counterparts with 6.0%.

Although a slightly higher prevalence of R131 was recorded in the study, there was no significant difference in the distribution of Fc γ R IIa allele with respect to subject immunity to malaria. The finding is in agreement with those previously reported [39, 40]. However, our finding differs from the results of research conducted in Mali by Maiga and his Associates [41, 42] in which H/H131 allele recorded a significantly higher frequency. The variation could be attributed to the difference in the age of the sampled populations in both studies. While we investigated apparently healthy adults, both children and adults were included in the study in Mali. Also, the variation could be attributed to the fact that our study was carried out on the general population and not on a selected ethnic group like the Dogo and Fulani that may be genetically homogeneous in nature. The study revealed that individuals with resistance against malaria recorded a higher frequency (36.7%) of homozygous allele R131 while the predominant allele in subjects susceptible to malaria was R131H with the frequency of 67.3%. Analysis of variance showed a significant variation in the distribution of Fc γ R IIa allele between malaria resistant subjects and those that were susceptible to malaria. (Tcal = 3.431; P <0.05). The malaria susceptible subjects also recorded a higher frequency of the heterozygote allele R131H of 75.8%.

Judging the relationship between the distribution of allele among subjects with immunity against malaria and calculated odds ratio, significantly higher level was recorded in genotype R131 than other alleles. This implies that homozygous allele R131 is capable of conferring higher immunity on individuals against malaria better than heterozygote allele R131H going by the values of Odd Ratio (OR=0.18, 95% CI= 0.07-0.44), while homozygote allele H131 was slightly protective (OR= 0.48, 95% CI= 0.19-1.19). Our present findings are in consonance with the results documented by previous authors [43, 44]. However other researchers observed that the 131 R/R genotype was associated with susceptibility to severe malarial anaemia and various grades of malaria [45, 46].

5. Conclusion and recommendation

Although the study was conducted in the malaria-endemic area, surprisingly the finding showed that 21.0% of the sampled population were resistant to malaria infection. The clinical implication of these findings is that some individuals in this locality are genetically and immunologically protected against malarial infection. Therefore, further studies on the specific genes and immunoglobulin responsible for the uniqueness in such individuals are recommended, as these can serve as baseline information for the development of malaria vaccine candidate or new anti-malaria preparations in the nearest future.

Authors contributions

The research was conceptualized and designed by AAA. Co-researchers SOJ, TDA and SAN reviewed the research proposal, conducted the research, collated, organized and analysed the data. AAA supervised the research methodology and data analysis. The initial draft of the article was jointly written by SOJ, TDA and SAN while the corrected version was prepared by AAA. Final manuscript was collectively reviewed and approved by all authors

Ethical approval and statement

We affirm that this work was approved by Ethical and Animal Care Committee of School of Basic Medical Sciences, Kwara State University Malete, Nigeria with Reference Number KW/SBMS/ERC/115 and that the study was conducted on the human subjects in accordance with code of ethics of World Medical Association (Helsinki declaration of 1975 and revised in 2008).

Conflict of interest and funding

All authors of this manuscript have unanimously agreed to publish it in American Journal of Biomedical Sciences if ultimately accepted. We also affirm that the work has not been presented in any form or sent to any journal for publication. This study is self-sponsored and has not

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