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PLASMODIUM PARASITAEMIA IN SICKLERS AND NON-SICKLERS ATTENDING THE GENERAL HOSPITAL CALABAR, SOUTH- SOUTH NIGERIA: A CROSS-SECTIONAL STUDY

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ABSTRACT

Malaria remains a public health problem in countries of the world where the disease is prevalent. In 2015 alone, it accounted for 429,000 deaths globally according to World Health Organization report. 92% of these deaths occurred in the Africa region. Sickle cell disease is common in people of African descent and in other areas of the world where malaria is prevalent. Epidemiological studies have established an association between *Plasmodium falciparum* and sickle cell states. Studies have shown that the heterozygous state (HbAS) confers a protective advantage against malaria attacks and its lethal complications which is not seen in the homozygous state (HbSS) and in subjects with normal haemoglobin (HbAA). The mechanism that underpins this phenomenon has been the subject of scientific enquiry for over six decades. 100 subjects with malaria symptoms attending the outpatient's clinic of the General Hospital Calabar were purposively selected and enlisted for the study which was done between December 2015 and February, 2016. Finger- pricked blood sample was obtained for slide smears, PCV and genotype of participants which comprised 34 persons with normal haemoglobin (HbAA), 34 with HbAS while 30 had HbSS. Only10 out of the 34 carriers (29%) had parasitaemia as against 83% each of those with HbAA and HbSS. The mean parasite count for HbAA subjects (2126, SD1319) was twice as high as that for HbSS (1083, SD729) and seven times higher than in subjects with HbAS (286, SD55). Parasitaemia was significantly associated with genotype with AS being the least likely to be infected (Chi Square 28.68, df 2, and p-value 0.000). We concluded that the carrier state AS protects both against parasitaemia and heavy parasite density.

KEYWORDS: *Plasmodium falciparum*, Sicklers, Non-Sicklers, General Hospital Calabar.

INTRODUCTION

Malaria remains a huge public health challenge in countries of the world where the disease is prevalent. According to the World Health Organization (WHO), malaria accounted for 429,000 deaths in 2015, 92% of which occurred in the Africa region (WHO, 2015). Under-5 children are particularly vulnerable to Malaria and in 2015 alone, an estimated 303,000 mortalities occurred in that age group, 292,000 (96%) of which occurred in the Africa region (WHO, 2015).

Malaria is caused by plasmodium species – one of the most important Protozoan parasites of man. Four species of the parasite have been identified namely:-*Plasmodium ovale*, *Plasmodium vivax*, *Plasmodium falciparum* and *Plasmodium malariae*. Of these, *P. falciparum* causes the most severe illness in Nigeria while infection from the other species is rare. Malaria is an acute febrile illness which presents with fever, headache, body pains, vomiting and lassitude. If not promptly treated, it could be complicated by severe anaemia, kidney and cerebral manifestations. These complications could be fatal in vulnerable groups like under-5 children and pregnant women. The WHO recommends prompt treatment with Artemisinine-based combination therapy and prevention through vector control by use of Long Lasting Insecticide Treated bed Nets (LLITNs) and Indoor Residual Spraying (IRS) with insecticides. Plasmodium infectivity is seasonal with peak levels in the rainy season, which coincides with the period of heightened agricultural activities. These provide the arthropod vector – the female anopheles mosquito with a favourable breeding ground.

Sickle cell disease is an inherited condition caused by Haemoglobin S – an oxygen carrying protein in red blood cells. Individuals who inherit one copy of the abnormal haemoglobin gene (HbS) are said to be carriers while those who inherit two copies of the abnormal haemoglobin gene (HbSS) are referred to as sicklers. In

conditions of low oxygen tension, the Red Blood Cells (RBCs) in the homozygous state (HBSS) assume a sickle shape from which the disease derives its name. The sickled RBCs clog the microvessels leading to ischaemia in the tissues. This causes a chronic debilitating ailment with clinical features such as anaemia, enlarged spleen and jaundice. Sicklers are prone to exacerbations of the illness called crises. The commonest of this is the vaso-occlusive or bone pain crisis which causes severe pain in the long bones of the body. The other crises – haemolytic, aplastic and sequestration are relatively rare among sicklers in Nigeria. Remarkably, individuals heterogenous for the trait (HbAS) do not have these crises, yet they protect the carrier from malaria. This could be the reason why the sickle cell alleles are most common in people of African descent as well as in other areas of the world where malaria is prevalent (Ashley-Koch et.al., 2000).

The sickle cell trait is thought to have evolved due to the vital protection it provides (Taylor et. al., 2013). Epidemiological studies in the last six decades have established an association between *Plasmodium falciparum* and sickle cell states. It has also been shown in studies that the heterozygous state confers a protective advantage against malaria attacks and its fatal complications which is not found in the homozygous state or in those with the normal haemoglobin (Hb AA) (William et al, 2005; Bunn, 2013). It is also known that the presence of the sickle cell gene whether in carriers or sicklers tend to inhibit high density *P. falciparum* parasitaemia (Allison, 1954; Williams et. al., 2005; Oden, et. al., 2015).

Under normoxic conditions, the invasion, growth and multiplication of *P.falciparum* in HbAS cells are the same as in HbAA erythrocytes.Under hypoxic exposure however, there is a reduction in the proportion of parasites in AS cells along with a block of maturation of ring forms to trophozoites (Bunn, 2013). In the hypoxic situation as well, cells infected with the *Plasmodium* species sickle much more readily than do uninfected cells (Roth et. al., 1978). There is sequestration of infected RBCs by endothelial adherence deep within the post – capillary beds. Low oxygen tension in these sites is likely to induce sickling and stunting of parasite growth and development. The sickle cells are removed from the circulation and destroyed in the reticular endothelial system and this reduces the parasite load in individuals with the sickle cell trait (Ayi et. al., 2004). In summary therefore, the protective mechanism involves enhanced phagocytosis, impaired growth and maturation of the parasite in infected RBCs and decreased deposition of parasitized RBCs in deep post-capillary beds (Bunn, 2013).

The objective of this study was to assess the level of parastiaemia in the heterozygous (AS) state and the homozygous Haemoglobin states (SS and AA) in a cross-sectional study. Apparently, there has been little or no study assessing parasitaemia simultaneously in the three Haemoglobin states in this part of the country. Findings would provide knowledge on this subject and should also guide policy in allocation of commodities in control programmes in a resource-poor setting such as Nigeria. These reasons justify the study.

MATERIALS AND METHODS

The study was carried out in the General Hospital Calabar from December 2015 to February 2016. The hospital is a secondary care facility that receives patients from Primary Health Centres and some private health facilities in Calabar and environs. Calabar falls within the malaria holo-endemic zone and lies on latitude 8.3^0 North and longitude 10.5^0 East on an altitude of 230 above sea level. 100 adults attending the general outpatients clinic were selected for the study through a purposive sampling proceedure. Consent was duly obtained from all participantss after the aim of the study was explained to them. All the participants had symptoms of malaria like fever, chills, body pains, lassitude, etc. and axillary temperature of 37.5^0c and above.

Blood sample for the study was obtained by thumb-pick with a sterile lancet after cleaning with cotton wool dipped in alcohol. Blood for malaria parasites was put on a slide, smeared and appropriately labelled. The sample was then stained using Giemsa stain which comprises Azur II (2.8g), Azur II eosine (0.8g), glycerine (125g) and pure methyl alcohol (375ml) respectively. Thereafter, two to three drops of buffer was added to the stained slide and buffer and stain were then mixed by soaking in and off buffer pipette, and left for 4-6 minutes before floating off with distilled water. Oil immersion was added and the slides examined using a light microscope. Parasitaemia was estimated by counting the parasites in each field (Cheesbrough, 2006). The Packed Cell Volume (PCV) of each participant was done using the haematocrit centrifuge while the genotype was determined by Haemoglobin electrophoresis.

Frequency tables and charts are used to summarize and display results while associations between variables are tested using the Chi square test on (SPSS Version 18) and by Analysis Of Variance (ANOVA) setting the level of significance at 95%. Confidentiality was ensured by coding the samples and only the researchers had access to the data. Ethical approval for the study was duly obtained from the Cross River State Ethical Review Committee in Calabar.

RESULTS

Of the 100 participants in the study, males were 34 while females were 66.56 (56%) of the participants fell within the 21 – 30 years bracket followed by those in 31-40 years 38 (38%) and 6 (6%) participants above 40 years. Participants who were sicklers were 30 (36%) while carriers and those with normal Haemoglobin expression

were 34 (34%) and 36 (36%) respectively (Table 1, Figure 1).

Table 1: Demographic characteristics and Genotype distribution of participants.

Variable	Frequency (N=100)	Percentage (%)
Sex		
Male	34	34
Female	66	66
Total	100	
Age Group/years		
21-30	56	56
31-40	38	38
41-50	6	6
Total	100	
Genotype		
AA	36	36
AS	34	34
SS	30	30
Total	100	

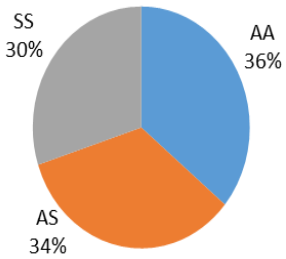

Figure 1: Genotype Distributionn of study participants

The mean age of participants was 31.37 years (SD 4.94). The mean PCV of participants in the study was AA 32% (SD 2.83), AS 33% (SD 2.11) and SS 27% (SD 4.05) while the mean parasite count was 2126.19 (SD 1319), 236.11 (SD 552) and 1085.73 (SD 729.31) for HB AA, HBAS and HBSS respectively. Parasitaemia was significantly associated with genotype with AS being the least likely to be infected Chi square (28.68 df 2) and p-value 0.000 (Table 2).

Table 2: Mean PCV and Mean Parasite Count of Participants by Genotype Category.

Genotype	Mean PCV	Mean Parasite Count
AA	32% (SD2.83)	2126.19 (SD1319)
AS	33% (SD2.11)	286.11 (SD552)
SS	27% SD4.05)	1083.73 (SD729)

There was no statistically significant association between parasitaemia and age-group (chi square 1.78, p-value 0.410) and none either with sex (Chi Square 0.864, p-value 0.353). Parasitaemia was however significantly associated with genotype (chi square 28.68 df2, p-value .000) (Table 3).

Table 3: Relationship between Age, Sex, Genotype and parasitaemia among study participants.

	Parasitaemia			
Variable	**Yes (n=65)**	**No (n=35)**	**Chi square(df 2)**	**p-value**
Age Group/years				
21-30	36	15	1.78	.410
31-40	25	16		
41-50	4	4		
Total	**65**	**35**		
Sex				
Male	20	14	0.864 (df1)	.353
Female	45	21		
Total	**65**	**35**		
Genotype				
AA	30	6		
AS	10	24	28.68(df2)	.000*
SS	25	5		
Total	**65**	**35**		
* Significant				

Parasitaemia was significantly higher in AA individuals than in those with AS (Chi Square 20.76, p-value 0.000) and significantly higher also in those with SS genotype (Chi Square 18.70; p-value 0.000). On the other hand, there was no significant difference in the proportion of those with parasitaemia between AA and SS genotypes.

A one-way between groups Analysis Of Variance was also conducted to explore the effect of genotype on parasite density (the genotype groups being AA, AS and SS). There was a statistically significant difference at the $p<0.05$ level in parasitaemia for the three categories- $F(2, 97) = 33.5$, $p=0.000$. (Table 4).

Table 4: One-way ANOVA showing the effect of Genotype on Parasitaemia.

	Sum of squares	df	Mean Square	F	Significance
Between Groups	5.967 E7	2	2.983 E7	33.5	.000
Within Groups	8.641 E7	97	890868.32		
Total	1.461 EB	99			

Post Hoc comparisons using the Tukey HSD test indicated that the mean score for AA (M=2126.2, SD1319.4) was significantly different from AS (M=286.1, SD552.2) and from SS (M=1083.7, SD729.3). The mean score for AS was also significantly different from that for SS. (Table 5)

Table 5: Post-Hoc Multiple comparisons using Tukey HSD Test.

(I) Patient Genotype	(J) Patient Genotype	Mean difference (I-J)	Standard Error	Significance
Tukey HSD	AS	1840.077*	225.717	.000
AA	SS	1042.461*	233.328	.000
	AA	-1840.077*	225.717	.000
AS	SS	-797.616*	236.427	.003
	AA	-1042.461*	233.328	.000
SS	AS	797.616*	236.427	.003

***The mean difference is significant at the 0.05 level**

The means plot of parasitaemia among the Genotypes is shown in Figure 2

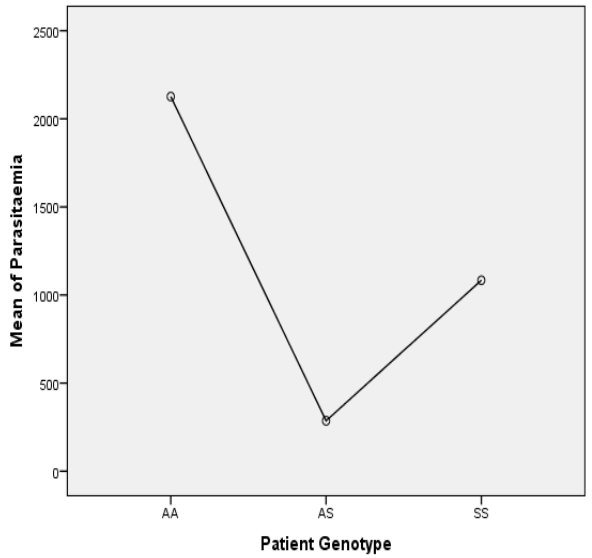

Figure 2: Means plot of parasitaemia among the genotypes.

DISCUSSION

This study established a clear reduction in the proportion of subjects with parasitaemia in carriers (HbAS) than in the homozygous states with only 10 out of 34 (29%) being infected compared with 83% infectivity in HbAA and HbSS. Although the proportion of those infected was about the same in the homozygous states, other studies have demonstrated a higher proportion of infectivity among AA subjects than SS.(Oden et. al., 2015). The parasite load was however far less in both AS and SS participants compared with AA with the mean parasite count being seven times higher in AA individuals than AS subjects and twice as high as in the SS subjects (Table 3). As has been shown in studies, the presence of the sickle cell gene whether in carriers or sicklers tend to inhibit high density falciparum parasitaemia (Williams et al., 2005; Allison, 1954). Start next paragraph with "Of great curiosity is the fact that sickle cell carriers (HbAS) enjoy a protective advantage against falciparum malaria attacks and its lethal complications which has not been demonstrated in the homozygous subjects (HbSS) (Bunn, 2013; William et al, 2005). They do not as well suffer from the debilitating crises which is the lot of sicklers. These observations have provoked intense research over the past six decades to explain the mechanism of the protective edge possessed by carriers.Under hypoxic conditions, sickling occurs as much as six times more readily in cells that harbour parasites than in the uninfected ones (Roth et.al., 1978, Luzzato et. al.,1990). This may result in intra cellular parasite death (Friedman, 1979). Enhanced sickling was limited to AS erythrocytes, containing ring forms of plasmodium. There is rapid clearance and destruction of circulating parasitized RBCs, and this tends to protect AS individuals from severe infections (Luzzato, 1980). AS erythrocytes, ß-thal and G6PD-deficient RBCs containing ring forms (but not trophozoites) are more prone to uptake by macrophages than normal RBCs (Ayi et. al., 2004). A dominant feature of the protection is the sequestration of ring-containing RBCs by adherence to vascular endothelium in the post capillary beds. Low oxygen tension prevailing in these sites is likely to induce "sickling polymer formation" in AS RBCs thereby impeding parasite growth and maturation

(Bunns, 2013). Research continues in a bid to fully explain the protective mechanism.

CONCLUSION

It has been clearly established, that the sickle cell trait confers high resistance against both heavy parasite density and severe malaria and its attendant sequale by a mechanism not yet fully understood. So far, the explanation is by enhanced phagocytocis of infected and sickled RBCs, impaired growth and maturation of the parasites in sickled RBCs, as well as decreased deposition of parasitized RBCs in deep post-capillary beds (Bunns, 2013). Immunity from repeated infections has also been adduced as working in synergy with the mechanisms stated earlier.

LIMITATION OF STUDY

This being a cross-sectional study with potential for selection bias, findings should be generalized with caution. The study did not investigate the protective mechanism of AS genotype which should inform future studies in this direction.

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E.M. conceptualized and supervised the study**,** E.E. did the laboratory work, and O.A did the statistical analysis whileE.M, O.A, E.O. and J.A. wrote up and type-set the manuscript. All the authors have read and made inputs to the final draft. We acknowledge the assistance of the Laboratory scientists who helped out with the bench work.

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