Plasmodium falciparum Merozoite Surface Protein-1 Polymorphisms among Asymptomatic Sickle Cell Anemia Patients in Nigeria

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Abstract- Asymptomatic malaria (ASM) has been implicated in the development of hemolytic crisis in infected sickle cell anemia (SCA) patients worldwide. This study surveyed steady state SCA Nigerian patients for ASM to investigate the influence of malaria prevention behaviors and age on parasitaemia and multiplicity of infection (MOI). A total of 78 steady SCA patients aged 5 – 27 years on routine care at three health facilities in Lagos were investigated for ASM by light microscopy and PCR with a multiplicity of infection determined by genotyping block 2 of merozoite surface protein 1 (msp1) gene of *Plasmodium falciparum* (*P. falciparum*). Use of malaria prevention measures was captured using a semi-structured questionnaire. The prevalence rates of ASM (due to Pf only) by microscopy and PCR were found to be 27.3% and 47.4% respectively ($P < 0.05$) with a Mean + SEM parasite density of 2238.4 + 464.3 parasites/uL. Five distinct msp1 genotypes [K1 (2), MAD20 (2), RO33 (1)] were detected and significant ($P<0.05$) disparity in allele frequencies (K1, 91.8%, MAD20, 32.4%; RO33, 18.9%) was found. The overall MOI was 1.43 and 37.8% of infections were polyclonal ($P<0.05$). ASM was associated with non-use of preventive measures and occurred in 62.1% of SCA patients aged < 10y with lower MOI of 1.3 compared to 38.1% in older patients with a higher MOI of 1.5 ($P<0.05$). We conclude that PCR improved the diagnosis of ASM among Nigerian SCA patients with infections being of low complexity and associated with non-use of preventive interventions and R033 msp1 allele selection.
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Keywords: Asymptomatic malaria; Plasmodium falciparum; Parasite diversity; Multiplicity of infection; Chemoprophylaxis

Introduction

Despite the yearly improvement in the global malaria prevention and control efforts since 2000, malaria remains a major scourge in Sub-Saharan Africa, causing morbidity and mortality in children under 5 years, pregnant women and immunosuppressed populations like sickle cell anemia (SCA) patients (1). In 2010, an estimated 305,800 newborns with SCA were reported globally with 75% of these births occurring in Africa (2). Due to its perennial transmission in many African countries, particularly in the West Africa sub-region, where SCA is more prevalent, malaria remains a major cause of severe anemia, crisis and deaths among SCA patients (1). It has been reported that between 50% and 80% of SCA patients die annually and malaria remains a major cause of these deaths (2). Asymptomatic malaria (ASM) is well known as a common manifestation of plasmodium infection in malaria endemic countries in Africa irrespective of the level of transmission (3,4). The concerning aspect of ASM is the role it plays in the development of clinical malaria and other consequences such as anemia, thrombocytopenia, low birth weight and cognitive impairment as well as making carriers serve as hotpops of malaria transmission (5,6,7,8). The passive surveillance system of national control programmes and

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post-treatment gametocytaemia induced by anti-malarial drugs have been implicated as potential causes of many ASM cases that are often undetected and untreated in many malaria endemic countries (8). Meanwhile, in consideration of its importance, ASM is seen as reflecting a state of immunological tolerance that is mediated by two types of anti-malarial immunity: clinical immunity, which prevent symptoms and anti-parasitic immunity, which conditions parasitaemia to a lower density, thereby providing protection against uncomplicated and severe malaria (9). The multiplicity of infection (MOI) defined as the number of parasite clones per infection has been used to explain partial immunity conferred by ASM in African children living in areas of high and low or unstable malaria transmission (4,9,10). Many investigators have linked MOI with protection in high transmission areas and clinical malaria susceptibility in low transmission areas; age and exposure in children have important roles in the acquisition of malarial immunity (9,10). Generally, the prevalence of ASM has been shown to vary in different geographical areas and high-risk population in Africa. Bousemma *et al.,* (11), Nkoghe *et al.,* (12), and Kimbi *et al*., (13) reported ASM

Prevalence rates of 73.4%, 1.7-8.7% and 60 -100% among Kenyan, Gabonese and Cameroonian children. An ASM prevalence rate of 24% by microscopy was also recently reported by Douamba *et al*., among pregnant women from Burkina Faso. In Nigeria, ASM has also been reported by many investigators (14). Ojuroungbe *et al*., reported an ASM prevalence rate of 25.6% among children aged 4 – 15 years in Osogbo, south West Nigeria, linking its occurrence with helminthic co-infection (15). In another study, Uneke *et al.,* found a correlation between ASM, anemia and low birth weight among pregnant women in southeast Nigeria (16). However, despite a concern of its occurrence, there has been a dearth of information regarding the burden, risk factors and consequences of ASM in SCA patients in Nigeria. Kotila *et al*., reported a prevalence rate of 24% by microscopy among asymptomatic sickle cell adults in Ibadan, south West Nigeria (17). Although this study is of relevance as it validated SCA patients as potential sources of malaria transmission during the dry season in the study area coupled with a potential limitation of chemoprophylaxis in averting ASM, two important limitations were found. First, the study included patients with sickle cell haemoglobin C disease whose course of crisis is lower in frequency and magnitude compared to SCA patients (10% of cases) and the second is the inclusion of patients who received malaria treatment in the month preceding the study. The latter may lead to an overestimation of ASM among the patients studied. Apart from chemoprophylaxis, other malaria prevention methods have not been evaluated as risk factors for ASM in the SCA population and there is a lack of data on the genetic diversity of parasite population involved in ASM in this cohort. Information on risk factors and complexity of infection would be needed for designing appropriate preventive interventions against ASM and for generating baseline data for use in surveillance of ASM SCA patients at risk of clinical malaria in Nigeria. The purpose of this study was to determine the prevalence of ASM by microscopy and PCR and investigate its association with malaria prevention practices among a cohort of steady state SCA patients in Lagos. The complexity of ASM infection based on msp1 block 2 polymorphisms in this cohort was also investigated.

Materials and Methods

Study design and setting.

This was a cross-sectional study of steady state SCA patients aged 5 – 27 years on routine care at three health facilities in Lagos between February – April, 2009 in Lagos. The health facilities, which provide routine care services for SCA patients were Lagos State University Teaching Hospital (LASUTH), Maternal and Child Health Complex (MCHC), General Hospital Ikorodu and Massey Street Children hospital (MSCH). Lagos is located on latitude 6.50 north of the equator with a mean rainfall of 1538 mm and average minimum and maximum temperatures are put at 22.7oC and 30.7oC per annum (18). Malaria is transmitted throughout the year in the state with high intensity of transmission experienced during the rainy season from May – July, October and November and low transmission during the dry season from December – February and during the short period of no rain (August –September). An estimated 17.5 million people live in Lagos and all the inhabitants are at risk of malaria infection (18,19). Female anopheles mosquitoes belonging to the Anopheles gambie complex remains the main vector of transmission, while *P. falciparum* is responsible for over 95% of reported malaria cases in the state (19). Malaria prevalence survey of 2010 reported parasite rates of 6.9% and 3.5% in children and adults. Use of net the previous night was 26.5% in children and 17% of the general population. Post LLIN campaign survey in 2012 revealed a net retention was 88.1% in the state (18).

Sampling and Patients' selection

The studied SCA patients were selected by convenient sampling as they present for routine care at MCHC, LASUTH and MSCH during the study period. Informed consent was obtained from each patient prior to enrollment. For minors, consent was obtained from their caregivers. The purpose, procedures, benefits, risks, and confidentiality components of the study were explained to the patients in the course of seeking their consents. Patients excluded from the study included those who declined consent, patients with fever, history of fever or fever related symptoms such as a headache and vomiting in the previous 72 h at presentation, patients who received malaria treatment in the previous 8 weeks and patients in crises as previously described (18). The study protocol was approved by the ethics committee of Hospital Management Board, Lagos state. After enrollment, the SCA status of each patient was confirmed by a positive sickling test and s-haemoglobin genotyping at pH 8.6 by acetate cellulose electrophoresis compared with the control HbAA, HbAS, HbSS and HbSC (20). The patients' case files were reviewed for age, sex, and number of blood transfusion in the previous six months. This was followed by the administration of a pre-tested semi-structured questionnaire on each patient to obtain information on the intake of anti-malarial chemoprophylaxis (daily for proguanil or in the last 7 days for pyrimethamine) and sleeping under LLIN the previous night as well as using an insecticide spray. The minimum number of patients for this study was calculated to be 76 using the proportion method of randomization that was based on the recent mean parasite prevalence rate of 5.2% (p) in the state with 90% statistical power at 95% confidence interval (z=1.96) and an error rate (d) of 5%. Patients diagnosed with asymptomatic malaria by microscopy or PCR were treated with arthemether-lumefantrine according to the national malaria treatment guidelines for children and adults (21).

Laboratory investigations

Haematology and microscopy

Venous blood sample (2 mL) was collected from each patient into sequestrin bottle and used for haemoglobin determination and total leukocyte count using standard methods (22). Diagnosis of asymptomatic falciparum malaria was performed by light microscopy and PCR as described by Snounou *et al.,* (23). For the microscopic test, two grease-free slides, each comprising thin (3 uL of whole blood) and thick (15 uL of whole blood) were prepared per sample. The thin film was fixed in methanol, and both films were stained with 3% Giemsa stain for 45 min after drying. Both films were then examined under oil immersion (x 1000 magnification) for the detection of malaria parasite by species using the thin film and counting of the asexual erythrocytic stage of *P. falciparum* using the thick film. The slides were also examined for the presence of gametocytes. This was done by two trained microscopists who worked independently. Parasites were counted against 300 leukocytes by two and parasitaemia was measured as an average of two counts. Parasitaemia was measured as a number of parasites per uL of blood-based on the number leukocytes counted per uL of blood for each patient. A slide was considered to be negative after examining 100 high power fields without parasites. No *P. falciparum* positive slides with discordant in count > 20% was observed and no gametocytes were seen. All the asexual stage parasites detected were those of Plasmodium falciparum.

Molecular methods

For PCR, parasite DNA was first extracted from 400 uL of whole blood samples using the Qiagen blood DNA extraction kit (Qiagen Hilden, Germany) and following the protocol provided by the manufacturer. The prepared DNA samples were assessed for yield and purity and then stored at -20oC until use. Each extracted parasite DNA was then used to genotype block 2 allele families of msp1 by nested PCR using primers designed by Snounou *et al.,* (23) (Table 1). The primary PCR reaction was a 25 uL reaction mixture comprising 1X of PCR buffer, 1.5mM MgCl2, 125uM of dNTPs, 30 picomoles of each primer, 75 – 100 ng of DNA and 1.25 units of Taq polymerase. All reagents were from Biomers (Biomers, Germany). Amplification was done in a Techne thermocycler (TC 312) under the following conditions: initial denaturation at 940C then 3 cycles of 940C for 1 min, 580C for 2 min and 720C for 2 min, followed by 25 cycles of denaturation at 940C for 1 min, primer annealing at 580C for 2 min and extension at 720C for 2 min and finally a single annealing step at 580C for 2 min and an extension step at 720C for 5 min. The secondary PCR reaction was also a 25 uL reaction, using 3 uL of the primary PCR product as the DNA template with 2 mM of MgCl2, 30 picomole each of allele specific primers for K1, MAD20 and R033 (Table 1), 200 uM of dNTPs and 1.25 units of Taq polymerase in 1 X PCR buffer. The cycling conditions for the secondary PCR were an initial denaturation at 950C for 10 min, followed by 40 cycles of denaturation at 940C

for 30 sec, primer annealing at 580C for 30 sec and extension at 720C for 1 min and, a final extension at 720C for 10 min. The resulting PCR products were electrophoresed on 3% agarose gel pre-stained with ethidium bromide (0.5 ug/mL), and DNA bands were visualized under a UV transilluminator. The molecular sizes of the visualized DNA bands were extrapolated based on the mobility of 50 bp DNA markers (Fermentas, Germany).

Table 1. Sequences of primers used to amplify the MSP-1 genes of *P. falciparum* isolates recovered from the asymptomatic sickle cell anemia patients.

S/N	PCR REACTION	PRIMER	PRIMER SEQUENCE
1	Primary PCR	MSP1-OF	5- -CTAGAAGCTTTAGAAGATGCAGTATTG-3-
		MSP1-OR	5- -CTTAAATAGTATTCTAATTCAAGTGGATCA-3-
2	Secondary PCR	MSP1-IKF	5- -AAATGAAGAAGAAATTACTACAAAAGGTGC-3-
		MSP1-IKR	5- -GCTTGCATCAGCTGGAGGGCTTGCACCAGA-3-
		MSP1-IMF	5- -AAATGAAGGAACAAGTGGAACAGCTGTTAC-3-
		MSP1-IMR	5- -ATCTGAAGGATTTGTACGTCTTGAATTACC-3-
		MSP1-IROF	5- -TAAAGGATGGAGCAAATACTCAAGTTGTTG-3-
		MSP1-IROR	5- -CATCTGAAGGATTTGCAGCACCTGGAGATC-3-

Definitions and Data management

Asymptomatic malaria (ASM) was defined as the presence of asexual stage of *P. falciparum* in the whole blood (microscopy) or detection of one or more msp1 allele bands from DNA samples by electrophoresis after PCR amplification. Isolates with one msp1 alleles were considered to elicit monoclonal infections while those with two or more msp1 alleles were considered as polyclonal infections. The multiplicity of infection (MOI) was determined by calculation of the total number of msp1 alleles seen divided by the number of msp1 positive samples. Msp1 polymorphism was determined as the number of distinct K1, MAD20 and R033 alleles recovered on the basis of size differences. The frequency of a msp1 allele (i.e. allele frequency) was expressed as a percentage of the total number of allele DNA bands detected in msp1 positive samples.

The number of msp1 genotypes was defined as the numbers of distinct types of msp1 block 2 alleles detected. Infection type was defined by clonality (i.e. monoclonal vs. polyclonal) or by allele type. For each infection type, parasite density was computed only for cases with parasitaemia by microscopy since quantitative PCR was not done. Data were double entered into Microsoft excel and Microsoft access worksheets, cleaned and validated before analyses. Continuous variables such as haemoglobin (Hb), total leukocytes (WBC) and parasitaemia were computed as a mean $\pm$ standard error of mean (SEM) while categorical variables such as sex, residence and use of chemoprophylaxis were computed as numbers and percentages (%). Parasite infection variables were compared between $\leq$ 10 years and 11 and above age groups. Univariate analysis was performed to evaluate the association between ASM or *P. falciparum* infection parameters and other covariates such as age, sex and malaria prevention practice indicators (i.e. chemoprophylaxis, LLIN and insecticide spray use rates). This was done using student's t-test and chi-square test or Fischer exact test for mean and proportion. Multiple allele group comparison for parasitaemia was done using two-tailed one-way analysis of variance (ANOVA) and Turkey-Kramer posthoc test. Outcomes of analyses with *P*-value < 0.05 was regarded as significant. Analyses were done using Statistical package for social science version 15.0 (SPSS 15.0).

Results

Data presented in Table 2 summarizes the general characteristics of the studied steady state SCA patients. A total of 78 steady-state SCA patients with a mean age of 14.2 years and comprising 59% females were enrolled. Among the patients screened for ASM, 37 (47.4%) were positive by PCR while 17 (21.8%) were positive by microscopy (P<0.05) (Tables 2&3). Although no significant (P>0.05) disparity in mean age was observed between ASM positive and ASM negative patients, age<10 years, rural residence, and not sleeping under LLIN or spraying insecticide spray the previous night were significantly (P = 0.0026 – 0.046) associated with ASM. Mean haemoglobin level of 8.4 $\pm$0.1 g/dl among ASM patients was also significantly (P<0.05) lower than 9.2 $\pm$ 0.3 g/dl in ASM negative patients. The disparity in parasite density between age<10 y and 11 years and above was not significant (P>0.05). On the whole, 14.1% of the SCA with ASM elicited parasitaemia in the range of 500 – 5000 parasites/uL.

The percentages of SCA aged > 10 years and > 11 years with ASM at this density range were 17.2% and 12.2%, respectively (*P* >0.05), while the overall MOI observed for the ASM was 1.43 (Table 3), one to three distinct parasite infections per sample were seen with age 11 years and above associated with higher MOI compared with infection in infection in the < 10 y age group. (1.5 vs. 1.3; *P*<0.05) (Table 3).

Further analysis showed that 37.8% of the ASM infections were polyclonal (*P*<0.05) and was associated with higher parasite density compared to monoclonal infections (3850 vs. 1480 parasites/uL; *P*<0.05) (Figure 1). Allele pattern of infection revealed the occurrence of the 200 bp KI allele in 78.4% of ASM cases seen, followed by MAD20 180 bp (24.3%), RO33 180 bp (18.9%) and MAD20 220 bp (8.1%). No parasites were seen by microscopy for the KI 250 msp1 allele. The observed disparity in the occurrence of these alleles was also significant (*P*<0.05) but density disparity was not significant (Figure 1). The non-usage of chemoprophylaxis, LLIN and insecticide spray was also associated with significant (*P*<0.05) increase in MOI (1.1 – 1.3 vs. 1.3 – 1.6) with further selection of RO33 P. falciparum strains due to chemoprophylaxis non-use (Table 4).

Table 2. General characteristics of the SCA patients: comparison of variables based on ASM positivity by PCR

Variable	Description	Total N = 78	ASM positive N = 37	ASM negative N = 41	*P*-Value
Mean age	Mean ± SEM, years	14.2 ± 0.6	13.3 ± 0.9	15 ± 0.8	> 0.05
Age ≤ 10 years	n (%)	29 (37.2)	18 (23.1)	11 (14.1)	0.046
Sex	Female, n(%)	46 (59)	21 (56.7)	25 (61)	0.71
Residence	Rural, n (%)	19 (24.4)	14 (37.8)	5 (12.2)	0.009
Sleep under LLIN the previous night	No, n (%)	43 (55.1)	27 (73)	16 (39)	0.0026
Use of chemoprophylaxis	No, n (%)	10 (12.8)	7 (18.9)	3 (7.3)	0.13
Use of insecticide spray the previous night	No, n (%)	36 (46.2)	22 (59.5)	14 (34.1)	0.025
Blood transfusion in the previous 6 months	Yes, n (%)	6 (7.7)	3 (8.1)	3 (7.3)	0.9
Crisis in the previous 6 months	Yes, n (%)	10 (12.8)	6 (16.2)	4 (9.7)	0.39
Haemoglobin	Mean ± SEM, g/dl	8.8 ± 0.08	8.4 ± 0.1	9.2 ± 0.3	< 0.001
Leukocyte count	Mean ± SEM, Cells x 10^3/mm^3	11.6 ± 0.1	11.8 ± 0.2	11.4 ± 1.0	>0.05

Bolded *P*-Values are significant.

Table 3. Characteristics and pattern of *Plasmodium falciparum* parasitaemia according to age among the asymptomatic SCA patients

P. falciparum infection variables		Total N = 78	≤ 10 y N = 29	11 y and above N = 49	*P*-Value
Parasite rate by microscopy, n (%)		17 (21.8)	8 (27.6)	9 (18.4)	0.34
Parasite rate by PCR, n (%)		37 (47.4)*	18 (62.1)	19 (38.8)	0.046
Parasite density, parasites/uL, mean ± SD		2238.8 ± 464.3	1820 ± 558.5	2611.1 ± 731.4	0.41
@Parasite density, parasites/uL, range, n (%)	**< 500**	4 (5.1)	3 (10.3)	1 (2.0)	
	500 – 5000	11 (14.1)	5 (17.2)	6 (12.2)	0.21
	> 5000	2 (2.6)	0 (0)	2 (4.1)	
	MOI	1.43	1.33	1.53	0.01
	Msp1 genotrypes	5	3	5	ND
Allele frequency, n(%)	**K1**	34 (91.8)**	16 (88.8)	18 (84.7)	
	MAD20	12 (32.4)	6 (33.3)	6 (31.6)	0.24
	RO33	7 (18.9)	1 (5.6)	6 (31.6)	

@Parasite density was determined only for 18 samples that showed parasitaemia by microscopy. **P*<0.05 (Parasite rate by PCR vs. parasite rate by microscopy); ***P*<0.05 (K1 alleles vs. other alleles)

Table 4. Association between *P. falciparum* infection variables and malaria prevention practice indicators among the SCA patients

Parameter		Parasitedensity,parasites/uL Mean + SEM	MOI	Msp1 block2 alleles K1	MAD20	RO33
Residence	Rural (N = 19)	2850 + 819.7[b]	1.4	17 (89.5)	6 (31.6)	5 (26.3)
	Urban (N = 18)	1820 + 907.6	1.5	13 (72.2)	6 (33.3)	1 (5.6)
Chemoprophylaxis	Yes (n = 29)	1604.6 + 387.9[a]	1.3	27 (93.1)	10 (34.5)	3 (10.3)
	No (n = 8)	3300 + 1038	1.6*	7 (87.5)	2 (25)	4 (50)[a]
LLIN	Yes (n = 10)	1480 + 445.2[a]	1.1	9 (90)	2 (20)	0 (0)
	No (n = 27)	3630 + 791.1	1.6**	13 (48.1)	5 (18.5)	2 (7.4)
Insecticide spray	Yes (n = 15)	1570 + 365.1[a]	1.2	13 (86.7)	5 (33.3)	2 (13.3)
	No (n = 22)	3580 + 425.7	1.5*	21 (95.5)	7 (31.8)	5 (22.7)

Data are mean + SEM and number (%). The disparity between mean values was evaluated by student's t-test and proportions by Chi-square (2) or Fischer Exact test. *P*-value < 0.05 was considered to be significant. *P<0.01; **P<0.001; [a]P<0.05

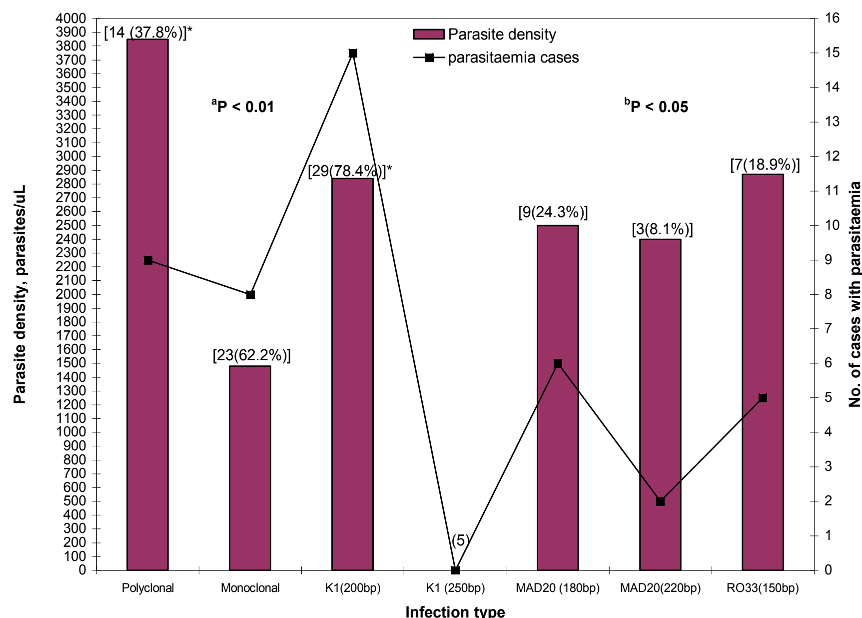

Figure 1. Plasmodium falciparum parsitaemia and infection dynamics among the asymptomatic sickle cell araemia patient

Each bar represents mean parasite density of cases with parasitaemia by light microscopy. Figures in parasitaemia represent total number of microscopic and sub-microscopic asymptomatic infections detected. *P<0.01 (polyclonal vs. monoctonal infections). Student's t-test; *P<0.05 (allele infection parasitaemia comparison); *P<0.05 (proportion between alleles and between polyclonal and monoclonal).

Discussion

The steady state of SCA has been described as a state of sub-clinical chronic inflammation in which affected patients remains susceptible to infectious diseases, including malaria (24,25). In a malaria endemic Lagos, this study found 47.4% of the steady SCA patients study to harbor 5 distinct clones of *P. falciparum* strains asymptomatically when PCR was used for diagnosis and 21.8% ASM rate when light microscopy was employed. Association of ASM with further reduction in haemoglobin level and its greater occurrence among patients of rural residence and who were not sleeping under LLIN and using insecticide spray. This study confirms the greater sensitivity of PCR over microscopy in diagnosing ASM and reveals a higher rate of ASM

among Nigerian patients with SCA than previously reported. The observed 21.8% ASM rate among our SCA patients is similar to the 25.6% rate reported by Ojuroungbe *et al*., (15) among children aged 4 – 15 years and 24% reported by Kotila *et al*., (17) among asymptomatic adults, though both investigators did not provide information on parasite diversity associated with the ASM cases seen. Although in the work of Ojurougbe *et al*., (15), haemoglobin S status of the infected children was provided, African children heterozygous for the haemoglobin S gene has been shown to habor *P. falciparum* strains asymptomatically at an equal frequency to children homozygous for the sickle gene (26). However, our finding implies that 25.9% of more cases of ASM will be detected if PCR instead of microscopy is used for diagnosing ASM, giving a better picture of ASM burden in SCA population. This is also applicable to non-SCA patients with ASM irrespective of age in Nigeria. Therefore, in the context of malaria elimination as recently adopted by the national malaria control programme, using PCR for malaria diagnosis would enable identification and treatment of a higher number of people, including SCA patients. This would provide tremendous benefits in reducing transmission to a significant level and enhance the feasibility of eliminating malaria from Nigeria. The relevance of PCR in ASM diagnosis has also been demonstrated by Ntounmi *et al*., (26), who reported P falciparum ASM prevalence rate of 61% among Gabonese children with different haemoglobin variants. In terms of species composition, only *P. falciparum* trophozoites were detected in this study. This is contrary to the findings of Danquah *et al.,* (27).

The workers detected *P. falciparum*, P malariae and P. ovale by PCR at 74.5%, 9.7% and 5.5% in 56% of 2108 asymptomatic Ghanaian children. This disparity can be attributed to the differences in sample size and malaria transmission patterns of the study areas. In this study, sampling was done between the end of dry season and in the middle of rainy when the transmission is not at its peak in Lagos. Studies conducted during this period have consistently demonstrated the predominance of *P. falciparum* (28, 29). However, in this study, gametocytes were not seen by microscopy to inform the infectiousness of SCA subjects who harbored parasites. This may be because the molecular approach of detecting sub-microscopic gametocytes (30) was not adopted in this study. Anemia has been reported as a common consequence of ASM, our observed lower Hb among parasite carriers compared to non-carriers alluded to this fact. Our finding seems to align with work of Olaniyi and Arinola (25) who reported elevation in C-reactive protein but the reduction in transferrin and haptoglobin levels in steady state SCA patients with *P. falciparum* parasitaemia, suggesting enhanced inflammatory response and decreased erythropoietic activity. In this study, the occurrence of ASM was associated with non-use of LLIN and insecticide spray. Several studies have identified the non-use of these preventive measures as risk factors for clinical malaria in Nigerian patients like other malaria endemic areas outside Nigeria. Therefore, our finding indicates that risk factors associated with clinical malaria also promote the occurrence of ASM among SCA patients in the country. Although evidence from the clinical trial has revealed preventive benefits of chemoprophylaxis in reducing the frequency of crisis and blood transfusion and conferring protection against clinical malaria, concerns are mounting regarding protection against asymptomatic malaria (31). This study also did not find an association between the use of chemoprophylaxis with pyrimethamine or proguanil and the occurrence of ASM in our SCA cohort and thus agrees with the finding of Kotila *et al*., (17). Meanwhile, mutations occurring in *P. falciparum* dihydrofolate reductase and cytochrome oxidase genes have been linked to the therapeutic failure of pyrimethamine and sulphadoxine-pyrimethamine in several epidemiological studies, including those done in Nigeria (32-34). These markers were not investigated in this study.

However, for the first time and similar to other malaria prevention measures studied, not using chemoprophylaxis was found to significantly correlate with a higher multiplicity of infection (MOI) coupled with a specific selection of RO33 allele among Nigerian SCA patients with ASM. In this study, the observed MOI of 1.43 among our ASM cases that was characterized by 1-3 distinct parasite infection per sample implies the low complexity of infection. This can be explained by the higher rate of monoclonal infections seen in 62.2% of ASM cases detected by PCR. Our MOI of 1.43 is similar to the 1.1 reported by Olaseinde *et al*., (35) for malaria cases detected recently in Ogun states, south west Nigeria but lower than the MOI range of 3 – 4 reported by Happi *et al*., (36) prior to treatment policy change of 2005 in Nigeria. Whether or not the MOI observed in this study reflects a changing epidemiology of malaria in Lagos or other factors such asymptomatic manifestation of malaria and the higher pyrogenic threshold for fever during the rainy season will require an investigation of malaria transmission and mosquito infectivity or in-breeding in the study area. MOI has also

been found useful in understanding parasite diversity and mechanisms of anti-malarial immunity, both of which have values in planning control intervention and designing malaria vaccines. In this study, block 2 domain of msp1 was genotyped being the domain under the strongest diversifying selection and involving apart from K1 and MAD20, an additional RO33 allele family absent in the other 16 domains of the erythrocyte invasion protein coding gene (37). In this study, 5 distinct msp1 genotypes, comprising 2 K1 alleles of sizes 200 bp and 250 bp, 2MAD20 alleles of sizes 180 bp and 220 bp and 1 RO33 allele of size 150 bp. Despite the similar low complexity of infection reported by Olaseinde *et al.,* (35), 8 distinct msp1 genotypes, comprising 4K1, 3 MAD20 and 1RO33 were found. This again suggests that the selection of msp1 alleles for *P. falciparum* infections vary between geographical areas and may be due to the difference in malaria transmission pattern and impact of control interventions. However, this study reveals a consistency in the predominance and presence of several polymorphisms of the K1 alleles among field isolates of P falciparum in south West Nigeria (35 – 37). The specific selection of the RO33 allele due to non-use of chemoprophylaxis will require further studies for a meaningful interpretation. This is because results have been inconsistent, regarding the influence of the RO33 allele on clinical malaria susceptibility. In a Cameroonian study, RO33/K1 co-infection was found to be strongly associated with fever and anemia irrespective of age in infected patients (38).

On the contrary, al-Yaman *et al*., (39) found asymptomatic infection due to the RO33 allele to be associated with reduced risk of clinical infection in a highly endemic area of Papua New Guinea. However, contrary to the significant allele-dependent variations in parasitaemia observed by these workers (38,39), we found an in-significant difference in parasitaemia elicited by parasite population belonging to different alleles (singly or in combination) among our SCA cohort with ASM. This disparity may be attributed to the differences in sample size, geographical location and study designs between the two studies.

However, it is important to note that this study has some limitations that will be addressed in future studies. First is the need for quantitative PCR to enable quantitation of sub-microscopic parasitaemia and molecular approach for detecting and quantitating sub-microscopic gametocytaemia to enable conclusion on the infectiousness of steady state SCA patients with ASM in Lagos and other regions of the country. Lastly, the small sample size and cross-sectional design of this study prevent adjustment of variables such as rural residence and sex for multivariate analysis to reveal independent determinants of ASM in the current study cohort. We were also unable to monitor the evolution of the observed parasite diversity as the course of infection becomes symptomatic. Such information has value in malaria vaccine design. Therefore, longitudinal and large sample size studies in Lagos and other regions of the country is sought.

Nevertheless, despite the above limitations, this study has revealed commonality in risk factors associated with clinical and asymptomatic malaria in the country and has thus highlighted the need to scale up behavioral change communication intervention in the country in the context of scaling up for impact (SUFI) by targeting universal access to information, education and communication (IEC) materials in Nigeria as a whole.

In conclusion, the findings from this study indicate that ASM infection appears to be more common than previously reported among Nigerian SCA patients with infection characterized by low complexity, K1 allele dominance and potential selection of the RO33 allele in the absence of chemoprophylaxis.

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