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Idayat Ajoke Oyetunji, Abimbola Joseph Amoo, Titilope Adenike Adeyemo, Oyesola Oyewole Ojewunmi

Corresponding author: Idayat Ajoke Oyetunji, Sickle Cell Foundation Nigeria, National Sickle Cell Centre, Opposite Lagos University Teaching Hospital, Lagos, Nigeria. ajoke2009@ymail.com

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Asymptomatic malaria infection among children with Sickle Cell Anaemia: The role of IL-10 and possible predisposing factors

Idayat Ajoke Oyetunji[1,&], Abimbola Joseph Amoo[2], Titilope Adenike Adeyemo[3], Oyesola Oyewole Ojewunmi[1]

[1]Sickle Cell Foundation Nigeria, National Sickle Cell Centre, Opposite Lagos University Teaching Hospital, Lagos, Nigeria, [2]Department of Medical Microbiology and Parasitology, Obafemi Awolowo College of Health sciences, Olabisi Onabanjo University, Ago-Iwoye, Nigeria, [3]Department of Haematology and Blood Transfusion, Faculty of Clinical Science, College of Medicine, University of Lagos, Lagos University Teaching Hospital, Idi-Araba, Lagos, Nigeria

[&]Corresponding author
Idayat Ajoke Oyetunji, Sickle Cell Foundation Nigeria, National Sickle Cell Centre, Opposite Lagos University Teaching Hospital, Lagos, Nigeria

Abstract

Introduction: *malaria is a major contributor to morbidity and mortality of under five children with Sickle cell anaemia (SCA) in malaria endemic regions. Asymptomatic malaria infection carriers constitute a reservoir for malaria transmission which could be detrimental in patients with SCA despite the use of malaria chemoprophylaxis. This study, therefore, determined the prevalence of asymptomatic malaria, levels of IL-10 and association between asymptomatic malaria infection, possible predisposing factors and levels of IL-10 in children living with Sickle cell anaemia.* ***Methods:*** *a case-control study was conducted involving children aged 2-16 years; 60 children with SCA in steady state and 40 control subjects with HbAA. Blood samples were collected by veni-puncture and asymptomatic malaria infection was detected using Rapid diagnostic test (RDT), Giemsa-stained thin and thick blood films and nested Polymerase Chain Reaction (PCR) technique. Serum IL-10 levels were determined using ELISA method.* ***Results:*** *among patients with SCA, two samples (3.3%) were positive for RDT, three samples (5%) were positive for microscopy, and four samples (6.7%) were positive for nested PCR technique. None of the patients in the control group was positive for Plasmodium falciparum detection using the three diagnostic methods. The mean ± (SD) of IL-10 levels in SCA patients with asymptomatic malaria (9.14(1.08) pg/ml) was significantly higher than those without asymptomatic malaria (4.8(2.62) pg/ml) (P<0.0001).* ***Conclusion:*** *this study demonstrated accurate diagnosis of asymptomatic malaria infection using PCR-based technique. We established that asymptomatic malaria is present in steady state SCA children and is associated with elevated IL-10 levels.*

Introduction

Malaria is one of the prevailing human parasitic diseases, ranking first in terms of its socio-economic and community health burden in tropical and subtropical areas, in sub-Saharan African and South-west Asian countries [1-3]. *Plasmodium falciparum* and *Plasmodium vivax* signify the majority of infections amongst the five *Plasmodium* species that are capable of causing human infection; the former is accountable for the greater part of malaria linked mortality [4]; *vivax* malaria which was regarded as a benign disease, has currently and obviously emerged as a potentially fatal state [5], predominantly in non-African endemic regions. *Plasmodium* infection has a broad range of signs that are grouped into three major clinical groups: asymptomatic (presence of malaria parasite devoid of symptoms), mild and severe [6, 7]. Individuals with asymptomatic *Plasmodium* infection can remain infected for lengthy episodes [8]; they eventually build up symptomatic disease if they have a dysregulated response to the immune system. Asymptomatic cases offer a basic reservoir of parasites and they may become gametocyte carriers contributing to the persistent spread of malaria; parasites from the asymptomatic carrier could be more infectious than the symptomatic ones [7,9]. In Human malaria, the relationship between TNF-α, IL-6, IL-10 and disease severity has been documented [10]. At diagnosis and even in cases of uncomplicated malaria infection, raised plasma levels of these cytokines have been established [11,12]. IL-10, an essential regulator of immune response during *Plasmodium falciparum* infection plays its role by reducing the effects of other cytokines formed by T-helper (TH) 1 and CD8 cells [13,14]. IL-10 has been found to be high and is linked with parasitaemia in individuals with asymptomatic malaria infection thus, signifying that this cytokine may possibly operate to decrease symptoms [6,11,15].

The diagnosis of asymptomatic malaria infection is not clear due to apparent lack of clinical manifestation and low level of parasites [16]. Although the gold standard for malaria diagnosis is microscopy, its detection limits, subjective nature of the diagnosis which depends on the training and experience of the microscopist, permit a variance in accurate diagnosis. However, malaria rapid diagnostic test (RDT) which is based on parasite

antigen detection using antibodies provides alternative to microscopy; this method also is not without its limitations in terms of its sensitivity and specificity in malaria infection detection. Polymerase Chain reaction (PCR)-based diagnostic method of malaria infection has been proven reliable and more accurate in malaria diagnosis. PCR-based method detected the prevalence of 19.2% of sub-microscopic *P. falciparum* carriage in RDT and microscopy negative samples indicating the sensitivity and specificity of PCR in malaria detection [17]. In malaria endemic areas, asymptomatic malaria infection is widespread and has become a serious concern as efforts are rising towards eliminating the parasite [18]. Despite the implication of socioeconomic conditions in the distribution of asymptomatic malaria infection as well as the susceptibility of children, few studies have explicitly focused on the need to further evaluate the burden of asymptomatic malaria infection using more sensitive method (PCR) as well as the role of IL-10 among children living with sickle cell disease (SCD) to scale up elimination and eradication in Nigeria. Globally, about 85% of all children with SCD occur in Africa in which about 150,000 children are born in Nigeria every year [19]. To this end, malaria is frequently observed as a major problem for African children living with SCD; hence, they are given appropriate malaria prophylaxis [20]. *Plasmodium falciparum* has also been reported as a characteristic or distinctive factor of painful crisis in people living with SCD residing in endemic countries [21,22]. Therefore, this study will explore the role of IL-10 levels and possible risk factors in asymptomatic malaria infection and make a definite diagnosis of *Plasmodium* infections.

Methods

Recruitment: this study comprised sixty children with SCD (in steady state) and forty children (the siblings living without SCD as well as other apparently healthy individuals from the community without SCD) as the control group, aged 2-16 years. The study was carried out at the Sickle Cell Foundation Nigeria between March and May, 2016. Fifty-three (83.3%) of sixty subjects with SCD were on daily use of proguanil as malaria prophylaxis while none of the control subjects was on routine use of any malaria prophylaxis and they had not been treated for malaria three months preceding recruitment for this study. Steady state Hb SS refers to subjects with no history of illness such as infection, inflammation during the previous 4 weeks, no history of hospital admission 2-3 days after the point in time in question, no treatment with medications such as antibiotics that may affect the blood counts during the previous 3 weeks, those without history of an acute painful episode that required hospitalisation for at least four successive weeks after a previous painful crisis [8]. Subjects with or without SCD who had blood transfusion three months prior to the study recruitment were excluded. Any child with no recent history of symptoms and/or signs of malaria, axillary temperature of < 37^{o}C and shows laboratory confirmation of malaria infection was regarded as being asymptomatic [6,23].

Ethical consideration: ethical approval was obtained from the Ethics and Research Committee of Lagos University Teaching Hospital (HREC registration no: NHREC: 19/12/2008a). Additionally, a written informed consent was obtained from the parents/guardians and verbal assent from children older than 7 years of the study participants before sample collection.

Data and sample collection: a structured questionnaire based on possible predisposing factors for asymptomatic malaria was developed. Five millilitres of venous blood sample was collected from each participant, 3ml was dispensed into ethylenediamine tetra acetic (EDTA) tubes and the remaining 2ml was poured into a plain tube with clot activator. The blood sample in each EDTA tube was used for the RDT for *Plasmodium falciparum* and preparation of thin and thick blood films immediately after collection. The remaining was then stored at -20^{o}C for the molecular diagnosis of asymptomatic parasitaemia for six

weeks. The blood sample in each plain tube was spun at 3000rpm for 10minutes immediately after collection. The serum was dispensed into cryovials and stored at -20°C for six weeks for the measurement of IL-10 levels by ELISA technique.

Detection of malaria infection: this was achieved using RDT for *Plasmodium falciparum* detection and microscopic techniques using 10 % Geimsa stained fixed thin and unfixed thick blood films [24].

Quality control of microscopy: the films were made in duplicates, stained with good quality Geimsa stain following Standard Operating Procedures. One of the duplicate was read by an experienced medical laboratory scientist and the other read by a WHO certified Microscopist.

Molecular diagnosis of malaria infection: DNA preparation from blood: this was prepared using DNA extraction kit from Jena Bioscience (Spin column based genomic DNA purification from whole blood) following the Manufacturer´s instructions. DNA amplification by nested PCR: The PCR assays were performed using Applied Biosystem thermal cycler, 9800. The first nested PCR was performed in a 15 &#956l total reaction volume containing 1 &#956l DNA template, genus-specific primers (rPLU1 and rPLU5), 5X Taq master mix and PCR grade water. The PCR conditions were: initial denaturation at 92°C for 2 min, 30 cycles of denaturation at 92°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min. The amplification process was completed with a final extension at 72°C for 2 min. Nested-2 PCR was performed in a 25 &#956l reaction volume using 1 &#956l of the nested-1 PCR product as a template DNA using the genus-specific (rPLU 3 and rPLU 4) and species-specific primer pairs (rFAL 1 + rFAL 2, rMAL 1 + rMAL 2, rOVA 1 + rOVA 2, rVIV 1 + rVIV 2). The cycling conditions were similar to the nested-1 PCR except that the annealing temperature was 62°C for the genus-specific primers (rPLU 3 and rPLU 4) and 58°C for the species-specific primers. The primers sequence and sizes of the PCR products have been previously described by Singh *et al*. (1999) [25]. The amplified products were run through 2% agarose gel stained with ethidium bromide. A low molecular weight DNA ladder (from Jena Bioscience, Jena, Germany) was used. The gels were visualized in a gel documentation system (Alpha imager, Alpha Innotech, USA).

Data analysis: data were entered and analyzed using SPSS 15.0. Descriptive statistics, student´s t-test and Pearson correlation coefficient test were carried out. P-values less than 0.05 were taken as being statistically significant.

Results

Socio-demographic and clinical characteristics of the study participants: the mean± (SD) age of subjects with SCD was 6.76± (3.24) years (range 2-16) while the control group was 7.77±(3.85) years with P = 0.161. There was no significant difference in the level of education and monthly income between the study participants with SCD and the control group (P>0.05). However, the nature of the living facility of those with SCD differs significantly from those of the control group (P<0.0001). The mean± (SD) of body temperature of the study participants living with SCD was significantly lower compared to those without SCD (P = 0.037) (Table 1).

Asymptomatic malaria infection: among patients with SCD, two samples (3.3%) were positive for RDT, three samples (5%) were positive for microscopy, and four samples (6.7%) were positive for nested PCR technique. None of the patients in the control group was positive for *Plasmodium falciparum* detection using the three diagnostic methods. The frequency of asymptomatic malaria infection, however, using nPCR was not significant compared to the control group (P = 0.96) (Table 2). The two samples that were positive for RDT were also positive for microscopy but only one sample was positive for all the three methods examined in this study. It should be noted that none of the study subjects was positive for *Plasmodium* species other

than *Plasmodium falciparum* during the PCR malaria detection assay.

Socio-economic risk factors of malaria infection among SCD group: the socio-economic risk factors of subjects with SCD who had asymptomatic malaria infection was not significantly different from those that were negative (P>0.05) (Table 3).

IL-10 levels and asymptomatic malaria infection in patients with SCD: IL-10 levels in subjects with SCD with asymptomatic malaria infection was significantly higher than those that were negative for malaria infection (9.14±1.08 vs 4.80±2.62). IL-10 values in pg/ml was significantly associated with asymptomatic malaria parasitaemia (P<0.0001). However, the mean±SD values among the control group that were not infected was higher than that of the SCD group that were negative for asymptomatic malaria parasitaemia (Table 4).

Discussion

Sickle cell disease (SCD) contributes significantly to under five mortality in sub-Saharan Africa and malaria is commonly regarded as a major contributor in these children [20, 26-28]. Global distribution of HbS reflects the selective advantage of sickle gene which offers protection against *Plasmodium falciparum* for sickle cell carriers (Hb AS) in malaria endemic countries. The protective effect of HbAS against malaria infection differs significantly from HbSS, although prevalence of malaria parasitaemia is lower in patients with Sickle cell anaemia (SCA) than in patients without SCA, severe anaemia and mortality are remarkable in patients with SCA hospitalised with malaria [29, 30]. Hence, malaria chemoprophylaxis is offered routinely to persons living with SCD to prevent malarial infection and sickle cell crises. Substantial evidence has revealed that prophylaxis improves haemoglobin concentrations and reduces severe anaemia, the number of clinical malaria attacks, and mortality [31]. Asymptomatic malaria parasitaemia is suggestive of low densities of *Plasmodium* parasites (asexual or gametocytes) below the limit of microscopic detection and the carriers constitute a reservoir for malaria transmission. Use of insecticide treated-bed nets (ITN) for the control and prevention of malaria is remarkable with the decline in malaria related morbidity and mortality. The statistical significance in the frequency of patients with SCA who use ITN compared to the control in this study could results from the knowledge about malaria prevention strategies gained during genetic counseling. This is in agreement with the finding of Lengeler (2004) which reported effectiveness of ITNs in malaria prevention program in reducing childhood mortality and morbidity [32]. In malaria endemic regions, diagnosis of asymptomatic malaria infection is quite challenging. PCR assay, a DNA-based molecular diagnostic technique, has been found to be more sensitive and specific than RDT and microscopy [33]. The sensitivity, specificity, and positive predictive values of nPCR in comparison with microscopy were 100, 97.2, and 73.3% respectively [34]. Meanwhile, Golassa *et al*. (2013) reported sensitivity of PCR relative to microscopy and RDT as 90.7% and 80%, respectively with the overall PCR-based prevalence of *Plasmodium falciparum* infection of 5.6 and 3.3 fold higher than that detected by microscopy and RDT respectively [17]. They further showed the prevalence of *Plasmodium falciparum* infection of 3.7%, 6.9% and 20.6% using Microscopy, RDT and PCR-based detection methods among the Ethiopians with sub-microscopic *Plasmodium* infection.

Microscopy-based prevalence of asymptomatic malaria parasitaemia in apparently healthy children was 6.8% and 7.4% in Ethiopia and Zambia respectively but 25.6% was reported among primary school children in Osun state, Nigeria. No asymptomatic malaria case was detected among our control subjects indicating that they did not harbour asexual parasites and gametocytes in keeping with zero prevalence of asymptomatic malaria parasitaemia found among apparently healthy children in India and Tanzania [33, 35]. Although majority of our control subjects were not

using insecticide treated bed nets, use of screened windows and doors with mosquito nets and indoor spraying of insecticide largely practised by the subjects could be responsible for the zero prevalence observed in our study. Microscopic malaria infection detection which showed 5% was much lower compared to 24% reported a decade ago in Ibadan, Nigeria [20]. The widespread and effective practice of the malaria control and prevention strategies of the patients may account for the lower frequency reported in this study. It has been shown that adolescents and adults are more likely to develop asymptomatic malaria due to a certain level of age-dependent immunity which develops [17,36]. Therefore, younger age of patients with SCD in our study (mean± (SD): 7.2±(3.5) years) compared to young adults (mean age: 22 years) could also account for the low prevalence we reported. Among the patients with SCA, PCR-based prevalence of asymptomatic *Plasmodium falciparum* infection was 6.7% demonstrating superior diagnostic performance of PCR assay compared to RDT and microscopy. This prevalence reported among patients with SCA in spite of the fact that they were on malaria chemoprophylaxis (Proguanil) suggests that malaria infection is worth considering in outpatient clinic and during hospitalisation. IL-10 is an anti-inflammatory cytokine; its high levels coupled with tumor necrosis factor-alpha and lower levels of IL-12 suppress anti-parasitic cellular immune responses leading to severe malaria [37]. Higher IL-10 levels observed in patients with SCA who were positive for PCR-based asymptomatic *Plasmodium falciparum* detection is similar to other studies that reported relationship between clinical protection to malaria and high plasma IL-10 levels [6, 38]. Hugosson *et al*. (2004) observed that in children with asymptomatic malaria, clinical protection to malaria is connected with an increased in self-reactive response, low levels of Plasmodium falciparum-specific IgG, high plasma levels of interferon-gamma and IL-10. He further demonstrated that high IL-10 levels were associated with less-effective *Plasmodium falciparum* parasites clearance. High IL-10 levels in our patients are suggestive that those patients are more prone to slower parasite clearance [39]. Thus, proposing a hypothesis that there is an association between asymptomatic malaria and IL-10 levels in children living with SCD.

Conclusion

This study demonstrated accurate diagnosis of asymptomatic malaria infection using PCR-based technique. We established that asymptomatic malaria is present in steady state SCA children and is associated with elevated IL-10 levels.

What is known about this topic

- *The diagnosis of asymptomatic malaria infection is not clear due to lack of clinical manifestation or parasites at low levels;*
- *Plasmodium falciparum infection has also been reported as a distinctive factor of painful crisis in people living with SCD residing in endemic countries; hence they are given appropriate malaria prophylaxis;*
- *In human malaria, relationship between IL-10 and disease severity has been established.*

What this study adds

- *Asymptomatic Plasmodium falciparum infection is present in children with SCA in steady state that is on daily malaria prophylaxis;*
- *PCR technique is an important tool in diagnosing asymptomatic Plasmodium infection in children with SCA in steady state;*
- *Asymptomatic Plasmodium falciparum infection is associated with elevated IL-10 levels in children with SCA in steady state.*

Competing interests

The authors declare no competing interests.

Authors' contributions

Idayat Ajoke Oyetunji: conceptualization; Oyetunji, Adeyemo, Amoo and Ojewunmi: design; Oyetunji and Ojewunmi: acquisition of data; Oyetunji and Ojewunmi: data analysis and interpretation; Oyetunji: article draft; Oyetunji, Adeyemo, Amoo and Ojewunmi: article review. All the authors have read and agreed to the final manuscript.

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Tables

Table 1: socio-demographic and clinical characteristics of the study participants
Table 2: prevalence of Asymptomatic malaria infection among the study participants
Table 3: socio-economic risk factors of malaria infection among SCD group
Table 4: IL-10 level and asymptomatic malaria infection

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Table 1: socio-demographic and clinical characteristics of the study participants					
Characteristics		**SCD and Control n(%)**	**SCD n (%)**	**Control n(%)**	**P-value**
Sex					
	Male	62 (62.0)	43 (71.7)	17 (28.3)	0.015
	Female	38 (38.0)	17 (28.3)	21 (52.5)	
Age					
	Mean±SD	7.17±3.5	6.76±3.24	7.77±3.85	0.161
Level of Education					
	None		7 (11.7)	2 (5.0)	
	Primary		9 (15.0)	7 (17.5)	0.656
	Secondary		20 (33.3)	16 (40.0)	
	Tertiary		24 (40.0)	15 (37.5)	
Monthly income in Naira					
	<20,000		27 (45.0)	11 (27.5)	
	<50,000		18 (30.0)	21(52.5)	
	50,000-99,999		9 (15.0)	3 (7.5)	0.202
	100,000-199,999		2(3.3)	2 (5.0)	
	200,000-299,999		1(1.7)	0(0.0)	
	>300,000		3(5.0)	3(7.5)	
Nature of Living facility					
	A room		23 (38.3)	16 (40.0)	
	Miniflat		15 (25.0)	4 (10.0)	
	Standard flat		21 (35.0)	2 (5.0)	<0.0001
	Duplex		0 (0.0)	3 (7.5)	
	Others		1 (1.7)	15(37.5)	
Body Mass Index (BMI)					
	Mean±SD		15.63±2.27	15.98±2.23	0.447
Body Temperature in oC					
	Mean±SD		36.57±0.28	36.69±0.23	0.037
Sleeping under treated bed nets					
	Yes		32 (53.3)	8 (20.0)	<0.0001
	No		28 (46.7)	32 (80.0)	
Frequency of use of treated bed nets					
	Always		18 (56.3)	4 (50.0)	0.751
	Occasionally		14 (43.7)	4 (50.0)	
Presence of screened windows and doors with mosquito nets					
	Yes		55 (91.7)	39 (97.5)	0.229
	No		5 (8.3)	1 (2.5)	
Indoor residual spraying of insecticide					
	Yes		60 (100.0)	40 (100.0)	
	No		0 (0.0)	0 (0.0)	

Table 2: prevalence of asymptomatic malaria infection among the study participants					
Parasite		**PCR**			**P-value**
		Positive n (%)	**Negative n (%)**	**Total n (%)**	
Plasmodium falciparum					
	SCD	4 (6.7)	56 (93.3)	60 (100)	0.96
	Control	0 (0.0)	40 (100)	40 100)	

Table 3: socio-economic risk factors of malaria infection among SCD group				
Risk factors		**Malaria infection (PCR)**		
		Positive n (%)	**Negative n (%)**	**P-value**
Nature of drainage				
	Opened	3 (5.0)	33 (55.0)	
	Closed	0 (0.0)	10 (16.6)	0.644
	None	1 (1.7)	13 (21.7)	
Nature of living facility				
	A room	4 (6.7)	19 (31.7)	
	Miniflat	0 (0.0)	15 (25.0)	0.075
	Standard flat	0 (0.0)	21 (35.0)	
	Others	0 (0.0)	1 (1.6)	
Sleeping under treated bed nets				
	Yes	2 (3.3)	30 (50.0)	0.641
	No	2 (3.3)	26 (43.3)	
Frequency of use of treated bed nets				
	Always	2 (3.3)	17 (28.3)	
	Occasionally	0 (0.0)	14 (23.3)	0.477
	Never	2 (3.3)	25 (41.7)	
Presence of screened windows and doors with mosquito nets				
	Yes	3 (5.0)	52 (86.7)	0.301
	No	1 (1.5)	4 (6.7)	
Indoor residual spraying of insecticide				
	Yes	4 (6.7)	56 (93.3)	
	No	0 (0.0)	0 (0.0)	
Daily use of malaria prophylaxis				
	Yes	4 (6.7)	56 (93.3)	
	No	0 (0.0)	0 (0.0)	

Table 4: IL-10 level and asymptomatic malaria infection				
Characteristics		**Malaria infection by PCR**		**P-value**
		Positive	**Negative**	
IL-10 in pg/ml				
SCD				
	Mean±SD	9.14±1.08	4.80±2.62	<0.0001
Control				
	Mean±SD	---	4.96 ±7.03	