

Pro- and anti-inflammatory immune response profiling prevents severe malaria among Nigerians infected with *Plasmodium falciparum*: the future for malaria vaccines and therapeutics.

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Abstract

Background Available evidence indicates that the various stages of the malaria parasite life cycle have specific immune responses. The pro-inflammatory cytokines tend to play an important role in preventing malaria and killing the parasites. Furthermore, the relative levels of pro-and anti-inflammatory cytokines are essential mediators of malaria anemia production and outcomes. Natural human immune responses to malaria recognize extracellular sporozoites and merozoites, both of which have surface-exposed antigens, and which are currently being developed for various vaccines. **Methods** A total of four hundred sixty-two (462) participants were tested for *Plasmodium falciparum*. The procedure employed were parasite staining using World Health Organization parasitology laboratory protocol [Microscopy] of Giemsa staining and Enzyme linked immunosorbent assay [ELISA]. **Results** The subjects in this study showed high levels of INF- γ and TNF- α which decreases with increased malaria severity and high parasite density. These results suggest that INF- γ cytokine and TNF- α may contribute to protection against severe malaria anaemia and parasite clearance. Conversely, infected participants showed higher levels of IL-10, which decreases with severe malaria parasite, furthermore IL-10 levels correlated with parasite density. These findings suggest that higher levels of anti-inflammatory cytokines, especially IL-10 levels may contribute to pathogenesis of complicated malaria by inhibiting the INF- γ and TNF- α production. **Conclusion** Molecular biological and other serological analysis are needed to elucidate the implication of these cytokines and other pro-inflammatory cytokines as IL-17, IL-21 and IL-22 in the responses to malaria and consequently their involvement in malaria vaccine construct/development as well as other therapeutics for the treatment and elimination of the malaria parasite in our environment.

Introduction

Available evidence indicates that the various stages of the malaria parasite life cycle have specific immune responses [1, 2]. The pro-inflammatory cytokines tend to play an important role in preventing malaria and killing the parasites. Furthermore, the relative levels of pro-and anti-inflammatory cytokines are essential mediators of malaria anemia production and outcomes [26, 4]. Early production of pro-inflammatory T-helper 1 (Th1) cytokines such as tumor necrosis factor (TNF)-alpha, interleukin (IL)-12 and interferon (IFN)-gamma may limit progression to severe complications from uncomplicated malaria [10, 11]. They actually limit the growth of parasites and stimulate monocyte phagocytosis to improve clearance of parasitized erythrocytes. While, cytokine IL-6 is a major mediator of the acute phase response [3, 26]. Often involved in the immune response to *Plasmodium* are other pro-inflammatory cytokines such as IL-17 and IL-22, developed by other cell subtypes including Th17 cells [26]. An increase in peripheral blood IL-17-producing CD4⁺ T cells has been reported during *P. vivax* infection, along with the production of the pro-inflammatory cytokines IFN- γ , IL-10 and the transforming growth factor (TGF)-beta [12]. During murine infection IL-22 activation defends against liver damage [13]. However, if these pro-inflammatory responses during the acute infection are not properly regulated, severe malaria complications may arise [14, 15]. Hence, the need for anti-inflammatory responses to regulate the development of pro-inflammatory cytokines and subsequent cytopathic consequences. Regulatory cytokines such as IL-10

play a major role in infection with *Plasmodium*, neutralizing excessive development of inflammatory Th1 cytokines [16, 17]. Anti-inflammatory Th2 cytokines including IL-4 and IL-13 control the humoral immune response, leading to the clearance of parasites and inhibiting the development of Th1 cytokines [18, 19].

Given the significance of the development of pro-and anti-inflammatory cytokines in the human immune response to infection with *Plasmodium falciparum* malaria, this is not well established in Nigerians. Accordingly, in this research we examine pro-and anti-inflammatory responses in western Nigerians subjects by evaluating collected plasma cytokines from participants in Baiyeku community according to their malaria status [3, 9, 26].

Methods

Description of Study Area

This was a community surveyed cross-sectional study for *Plasmodium falciparum* malaria, conducted in Baiyeku Community in the Ikorodu Local Government Area of Lagos State, Nigeria [Figure 1]. Baiyeku is a rural settlement with favorable ecological features for all year round transmission of malaria. It is an ancient town in the Ikorodu Local Government Area located along the Lagos Lagoon on Latitude 6° 37' 0"N and Longitude 3° 36' 30". Baiyeku shares boundary with Ogun State and is inhabited by about 800, 000 people [From 2006 census]. Baiyeku has a variety of ethnic groups. The main/predominant languages spoken are Yoruba and English. The majority of the people are subsistence farmers, petty traders or fishermen. The population consists of Muslims, Christians and traditionalists [Fig 1].

Figure 1. Map of Lagos State showing Baiyeku in Ikorodu LGA.

(Source: Remote Sensing & GIS Lab; Department of Geography, University of Lagos, 2019)

Inclusion criteria

Informed consent was obtained from the participants prior to blood sample collection for smear microscopy and Enzyme Linked Immunosorbent Assay [ELISA].

- a) Participants, adults 18-60 years who have been exposed to malaria and who have lived in endemic areas of malaria for at least six months, each giving informed consent.
- b) Children 10-17 years of age who have lived in endemic areas of malaria for at least six months, each giving informed consent.
- c) Children 1-9 years of age who have lived in endemic areas of malaria for at least six months with informed consent from parents / guardians.

Exclusion criteria

Persons who were unwilling or unable to provide informed consent.

Research eligibility evaluation

Persons fulfilling inclusion criteria were asked if they wished to participate in the study and the participants were given full disclosure of the necessary information alongside the consent form. They were asked to read or have read to them, the consent form and to sign the form. Individuals not providing informed consent or who chose not to consent their minor were not enrolled.

Subject Registration

Individuals who met and consented to the eligibility criteria were registered as study participants. The study involved voluntary and informed consent (participants 18 years of age) or informed parental consent (children 1-9 years of age) or consent (children 10-17 years of age) prior to blood sample collection. A personal identification number (PID #) was assigned to each participant and their blood samples for easy of identification and avoidance mix-up.

Blood Collection Techniques

The study recruited four hundred sixty-two (462) participants. 5 ml of venous blood were collected into plain EDTA tube using standard phlebotomy practice and aseptic techniques. The blood samples were stored at -80 ° C for downstream applications as described by Kiechle et al [2].

Ethical Consideration

Ethical approval (Ref no: IRB/17/021) was obtained from the Institutional Review Board of the Nigerian Institute of Medical Research (NIMR), Lagos to conduct the study. Advocacy and necessary permission was obtained from the head of the community as well.

Microscopy

Detection /Examination of blood for parasitemia

Procedure

A total of four hundred sixty- two (462) participants were tested for infection with *Plasmodium falciparum* and the procedure for parasite staining using Giemsa staining was adopted from World Health Organization parasitology laboratory protocol [WHO, 2003].

In the center of a labeled frosted slides, small drop of blood were placed and spread, and the smears were allowed to dry. The slides were then stained with a 10% Giemsa solution for 8-10 minutes. The slides were viewed under the microscope at X100 (Olympus CX21, UK). Parasitemia was measured by counting the number of malaria parasites against a number of leucocytes in the thick blood film, based on a putative mean count of white blood cells density of 8000 white blood cells per μl . The malaria parasite

density MPD (Parasite per μl of blood) was calculated [*Parasites per μl = number of parasites x 8000/number of leucocytes*].

Evaluation of Cytokines by Enzyme Linked Immunosorbent Assay (ELISA)

Levels of Tumor Necrosis Factor Alpha (TNF- α), Interferon Gamma (IFN- γ) and Interleukin-10 (IL-10) were assessed in sera of the study participants including the controls, using antigen capture ELISA (Enzyme Linked Immunosorbent Assay) kits based on antigen-antibody reaction (Maxisorb; NUNC Denmark) employing the manufacturer’s protocol. Samples and standards were added using appropriate diluents into ELISA pre-coated plates. After incubation and washing step (Washer:BioTek Instrumentals, USA), goat anti-human IgG HRP conjugate reagent (Sigma A9544) were added followed by Chromogen solution A. The Optical Density (OD) was read within 15 minutes at 450 nm, on an ELISA reader (BioTek EL 800, BioTek Instrumentals, USA) and the concentration from the standard curve obtained using values for the standards (Voller *et al.*, 1978).

Statistical Analysis of data

Chi-square (χ^2) test was used to compare data sets generated, Students-t-test, ANOVA, Pearsons correlations and graphs were also used to analyze the data.

Results

Demographic Profile of Participants

Four hundred and sixty two (462) participants, consisting of 136 males and 326 females, were screened for *Plasmodium falciparum* malaria in this study. Of this population, 70 were microscopically positive for *Plasmodium falciparum* (Table 2), corresponding to a prevalence of 15.2%. The female participants were predominantly positive (70%) while males constituted 30% positives.

Of the 70 participants microscopically confirmed positive for *Plasmodium falciparum*, 65.7% were participants below 17 years of age while the adult participants aged 18 years and above constituted 34.3%. Overall, the median age of the participants was 21 years.

The seventy (70) age-matched controls on the other hand, were microscopically confirmed negative. The median age for the healthy controls was 21 years [Table 1].

Table 1: Demographic profile of study population.

93(20.1 %) subjects had a positive RDT test for diagnostic method and 70(15.2 %) had a positive malaria test for microscopic gold diagnostic standard [Figure 2].

Table 2. Malaria parasite density of participants

Malaria Density Distribution

Of the 462 participants tested, 70 (15.2%) were microscopically positive for malaria, 21(30%) were male and 49(70%) were female. There was a significant difference between male and female participants' ($p<0.01$) geometric mean parasite density (GMPD). Similarly, the results showed a statistical difference, $p<0.01$ between the age groups tested, with the highest GMPD in subjects under 5 years of age ($P<0.01$) compared with those over the age of 5 [Table 2].

Fig. 2 Showing parasite density (Trophozoites) in the blood smear [A] & [B].

Table 3: Prevalence of malaria in relation to BMI status of participants

BMI status of participants in relation to malaria prevalence

Subject with low body mass index [BMI] $<18 \text{ kg/m}^2$ showed a very high prevalence of (46.8%), compared to those with Normal [BMI] $18.5\text{-}24.9 \text{ (kg/m}^2\text{)}$ with a prevalence of (27.3%). The overweight and obese subjects with a Body mass index of [BMI] $25.0 - 29.9 \text{ [kg/m}^2\text{]}$ and [BMI] $>30 \text{ (kg/m}^2\text{)}$ showed a prevalence of (12.6%) and (13.4%). [Table 3] respectively.

Table 4: Relationship between BMI Status and malaria parasitemia.

$\chi^2 = 4.521$, $df = 6$, $p = 0.607$ ($p > 0.05$)

Relationship between BMI Status and Malaria Parasitemia (Microscopy)

In this study [Table 4], the underweight infected participants had a higher parasite load of 68.6% with a parasitemia level of $< 100/\mu\text{l}$, while those with normal BMI had a parasite density load of 31.4% with parasitemia levels of $1,000 < 100,000$. The overweight and obese had a parasitemia level of

$< 1,000 \geq 10,000 \geq 100,000$ with a parasite load of 2.9% and 8.6% [$p > 0.01$] respectively.

Table 5. Evaluation of malaria Parasite density in relation to cytokine levels of participants.

There were predominance cytokine immune responses among all screened and evaluated participants. TNF- α Showed ($1.98 \pm .015$) lower preponderance compared to IFN- γ ($2.46 \pm .05$) and IL-10 ($2.21 \pm .054$). [Table 6]. Subjects who were grossly infected with *Plasmodium falciparum* showed an elevated pro and anti-inflammatory immune responses compared to individuals who tested negative to malaria parasite. [Table 5]. There was a significance difference between infected and non-infected individuals ($p < 0.01$).

Fig. 3. Cytokine levels of participants infected with *Plasmodium falciparum* in comparison with the healthy controls.

Table 6. Cytokine levels of participants infected with *Plasmodium falciparum* in comparison with the healthy controls

The parasite density of malaria showed different elevated levels of pro and anti-inflammatory cytokines among the infected individuals. TNF- α (pg/ml) levels [1.7668 ± 0.4412] were highest in individuals with $\geq 100,000$ malaria parasite density compared to $<1,000$ [1.3760 ± 0.858] and $\geq 10,000$ [1.5758 ± 0.6849] individuals. IFN- γ (ng/ml) levels were lowest in individuals with $<1,000$ [1.2500 ± 0.9029] parasite density and highest in participants with $\geq 10,000$ and $\geq 100,000$ [1.8500 ± 0.0764] and [1.933 ± 0.2108] parasite density. An elevated level of IL-10 (pg/ml) was observed in subjects with $\geq 100,000$ [2.000 ± 0.5774] parasite density compared to $\geq 10,000$ [1.5833 ± 0.1028] and $<1,000$ [1.5714 ± 0.1373], IL-10 cytokines elevation levels were highest compared to all levels of cytokines investigated. There was a significant difference [$p < 0.01$] between malaria parasite density and cytokine elevated levels in the infected subjects [Table 6].

Discussion

Although in many parts of Africa the burden of *Plasmodium falciparum* malaria is gradually decreasing, it is characterized by spatial and temporal variability, which presents new and evolving challenges for malaria control programmes. Nigeria presents the highest malaria prevalence in sub-Saharan Africa as a result new malaria treatment and therapeutics and possibly vaccines are needed to combat this scourge.

In this study, we assessed the demographic prevalence of malaria parasite, its density and severity among four hundred and sixty-two (462) subjects, and the pro- and anti-inflammatory cytokine profiles of tumor necrosis factor- α (TNF α), interferon gamma (IFN- γ) and interleukin-10 (IL-10) among western Nigerians for future vaccines and therapeutics arsenals in combating malaria infections. Of these subjects, 134 were males and 328 females. The study participants age groups ranged from 1- 9 years of age, 10- 17 years of age and 18- 60 years of age from the Beiyeku community of Lagos, western Nigeria. A prevalence of 15.2% was observed among the participants in all age groups and gender. One of the immediate presumed reason could be educational level and sample size. Some literatures attributed high malaria prevalence to education levels; for instances a population that is well informed about the use of long lasting treated nets (LLN), insecticide residual spraying (IRS) and intermittent preventive treatment (IPT) of pregnant women could help reduce the prevalence of the disease, it could also be attributed to the sample size involved in various studies, also environmental factors such as rainfall and climatic change plays prominence in biodiversity of the vector. In the present study we employed a larger sample size compared to many other studies conducted in Nigeria and other parts of Africa. Similarly, the malaria parasite geometric density was higher in females compared to males who were positive, the relationship between parasite density and disease incidence is complex and non-linear. A multitude of factors including the sample's age structure, sampling timing in relation to local malaria transmission seasons and the methodology and rigor applied to parasite detection density. Findings such as those reported here were in consonance to those of Irene et al, [7]. The age group less (<5 years) recorded a higher geometric

malaria density compared to those over 5 years old. The higher mean parasite density peaks observed in children were as a result of a less developed immune system that cannot effectively clear parasites load compared to adults. Increased in age or adulthood reduced the prevalence of infection and parasite density. The observed decrease in parasite density among adults is most likely due to the development of clinical immunity that is not sterile over time. This immunity controls infection and is usually pronounced in children over 15 years of age and in adults. These people have been exposed to mosquito bites over the years and, consequently, to malaria many times more. Such limited immunity allows people to tolerate serious malaria infection without getting ill even if they can get malaria. Parasite densities in children therefore appear to give a true picture of the intensity of an infection than in adults, which can be a useful indicator for monitoring the intensity of the disease. Similar findings were reported in the study of Odongo Aginya et al [5] in Uganda, Markell et al [6] and Molineaux [8].

Subject with lower body mass index (BMI) showed a very high prevalence of (46.8%), those with normal (BMI) had a prevalence of (27.3%). The overweight and obese with a Body mass index of [BMI] 25.0 – 29.9 [kg/m²] and [BMI] >30 (kg/m²) showed a prevalence of (12.6%) and (13.4%) respectively. This physiological factors had been shown to contribute to low/high level parasitemia in several studies [26]. Similar findings were observed in the current study. The reason could be attributed to nutritional status, pregnancy and diabetics. Monte *et al* [10] research findings however elucidated otherwise, it was observed in the study that high BMI, obesity and diabetes acts as protective factor against malaria. Katja *et al* [11] proved a total contrast to our finding, the study showed that BMI, obesity and diabetes are factors that increased malaria parasitemia. All of the forgoing studies are linked to physiological sociodemographic indices.

Cytokine(s) profiling were conducted in the various participants, TNF- α Showed ($1.98 \pm .015$) lower preponderance compared to IFN- γ ($2.46 \pm .05$) and IL-10 ($2.21 \pm .054$). Subjects who were grossly infected with *Plasmodium falciparum* showed an elevated pro and anti-inflammatory immune responses compared to individuals who tested negative to malaria parasite. Several studies showed a markedly similar results. Pro-inflammatory cytokines are elevated during malaria infection. IFN- γ is a key molecule in the defense of human antimalarial host as demonstrated by Wikler et al [12] in uncomplicated *P. falciparum* malaria patients [14]. Interferon gamma is an active macrophage that contributes to the innate immune response to malaria. It is primarily produced by CD8 and CD4 T lymphocytes in a specific immune response and by NK cells in an unspecific response [16]. Interferon gamma is essential for the resolution of primary infection by limiting the initial phase of parasite replication, it also contributes to acute malaria symptoms such as fever, nausea and headache by inducing TNF alpha [17]. Th1 cytokines, namely IFN- α and IL-2, have a significant effect on the outcome of the disease and on the development of protection against *Plasmodium sp* infection. IL-10 modulates this pro- inflammatory response [18]. TFN- α have been shown to correlate with malaria severity in several studies [16, 20, 11], low levels of TNF- α levels were observed in our study. This could be explained by the negative action of IL-10 on pro-inflammatory responses. IL-10 completely abolishes TNF- α production in response to malarial antigens IL-10 levels increased significantly with the degree of parasitemia/malaria density in this study, it was

however, lower in asymptomatic individuals compared to infected subjects. IL-10 is a critical pro-inflammatory cytokine [37, 38]. There was also an increased IL-10 plasma levels in infected patients in this study compared to non-infected patients, particularly those who tested positive for malaria. IL-10 levels increased as parasitemia increases. These results are consistent with other studies in which the severity of malaria and increased parasitemia have been associated with increased IL-10 levels [19]. This suggests that *P. falciparum* invigorates IL-10 generation in dose-dependent manner. IL-10 is utilized by regulatory T cells to control immune responses. *P. falciparum*-infected erythrocytes actuate regulatory T cell expansion, followed by increased IL-10 and IL-6 production [19, 13]. In addition to a direct correlation between T regulatory cell numbers and IL-10 plasma levels in infected patients [15,18], it has been shown in various studies that CD4+CD25+Foxp3+ T cells induced during malaria upregulate the IL-10 expression [22,23]. Other authors also report that regulatory T cells are associated with higher rates of parasite development, suggesting that *P. falciparum*-mediated induction of regulatory T cells may well be a destructive factor [25]. In summary, this study revealed that the severity of malaria infection due to *P. falciparum* could be regulated and cleared in the blood systems by pro and anti-inflammatory arsenals and also be future valuable immune tools for vaccines constructs and therapeutics.

Conclusion

The subjects in this study showed high levels of INF- γ and TNF- α among positive malaria cases, which increases with increased malaria severity and high parasite density. These results suggest that INF- γ cytokine and TNF- α may contribute to protection against severe malaria anemia and parasite clearance. Conversely, infected participants were shown to have higher levels of IL-10, which decreases with severe malaria parasite, furthermore IL-10 levels correlated with parasite density. These findings suggest that higher levels of anti-inflammatory cytokines, especially IL-10 levels may contribute to pathogenesis of complicated malaria by inhibiting the INF- γ and TNF- α production. Further biological analysis and studies are needed to elucidate the implication of these cytokines and other cytokines such as IL-17, IL-21 and IL-22 among positive and healthy subjects in other parts of Nigeria, this could aid in the development of indigenous vaccines and other therapeutics for the treatment and elimination of the malaria parasite in our communities and environment.

Declarations

Ethical Approval and Consent to participate

Ethical approval (Ref no: IRB/17/021) was obtained from the Institutional Review Board of the Nigerian Institute of Medical Research (NIMR), Lagos to conduct the study. Due advocacy and necessary permission was obtained from the Head of the community as well and as written informed consent from study participants.

Consent for publication

Not applicable

Availability of supporting data

Data is not restricted

Competing interests

The authors declare that they have no competing interests

Funding

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Authors' contributions

DO: Design the study, collected the data and contributed to the analysis.

PAM: Design the study, wrote the manuscript and contributed to the analysis.

AO: Contributed to data analysis and manuscript writing

DAD: Supervised the entire project work and contribute to the manuscript and data analysis.

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Tables

Table 1
Demographic profile of study population.

Profile			Total
Number of participants tested for <i>P. falciparum</i> malaria			462
Number of Healthy Volunteers			70
Number of participants microscopically positive for <i>P. falciparum</i> malaria			70
Prevalence of malaria (%)			15.15%
Gender of participants	(positives)	Male	21
		Female	49
	(Negatives)	Male	441
		Female	413
Tests Age Groups			
Positive	<5 years		10
	5-9 years		26
	10-17 years		10
	18-60 years		24
Controls Age Groups			
	<5 years		14
			20
	5-9 years		9
			27
	10-17 years		
	18-60 years		

Table 2. Malaria parasite density of participants

GROUP	% (Frequency)	Geometric mean parasite density (GMPD) Parasite/uL	P-value
Male	30 (21)	1148.459974 ± 3920.9515077	0.001
Female	70 (49)	2032.656172 ± 7711.7352504	-
<5years	14.3 (10)	3074.974498 ± 8889.8975496	0.001
5-9years	37.1 (26)	1862.417209± 16966.2337278	
10-17years	14.3 (10)	1249.296051 ± 384.2184604	-
18-60years	34.3 (24)	1259.127428 ± 471.4340281	-

Table 3. Prevalence of malaria in relation to BMI status of participants

STATUS	Frequency	%
Underweight (BMI <18.5) (kg/m2)	216	46.8%
Normal (BMI 18.5-24.9) (kg/m2)	126	27.3%
Overweight (BMI 25.0 – 29.9) (kg/m2)	58	(12.6%)
Obese (BMI >= 30) (kg/m2)	62	(13.4%)
Total	462	(100%)

Table 4. Relationship between BMI Status and malaria parasitemia.

Malaria parasite/ul)	parasitaemia <i>n</i>	BMI Status			
		Underweight	Normal	overweight	obese
000	30 (42.9%)	18	8	0	2
,000 but 100,000	38	18	14	2	4
100,000	(54.3%)	12	0	0	0
	2 (2.9%)				
TOTAL	70	48	22	2	6

Table 5. Evaluation of malaria Parasite density in relation to cytokine levels of participants

CYTOKINE	Malaria Parasite Density Groups (parasite/μl)			P-value
	1,000 (n=30)	≥10,000 (n=38)	≥100,000 (n= 2)	
TNF-α (pg/ml)	1.5758 ± 0.6849	1.3760 ± 0.858	1.7668 ± 0.4412	0.033
IFN-γ (ng/ml)	1.2500 ± 0.9029	1.8500 ± 0.0764	1.333 ± 0.2108	0.084
IL-10 (pg/ml)	1.5714 ± 0.1373	1.5833 ± 0.1028	2.000 ± 0.5774	0.073

Values are Mean ± S.E.M

Table 6. Cytokine levels of participants infected with *Plasmodium falciparum* in comparison with the healthy controls

CYTOKINE	TEST			CONTROLS
	Asymptomatic + Symptomatic	Asymptomatic	Symptomatic	
TNF- α (pg/ml)	1.96 \pm 0.840	1.12 \pm 0.010	0.84 \pm 0.030	0.26 \pm 0.01
IFN- γ (ng/ml)	1.33 \pm 0.013	0.97 \pm 0.00	0.36 \pm 0.07	0.36 \pm 0.03
IL-10 (pg/ml)	1.30 \pm 0.09	1.00 \pm 0.02	0.30 \pm 0.07	0.86 \pm 0.07
P value	0.022	0.036	0.059	

Figures

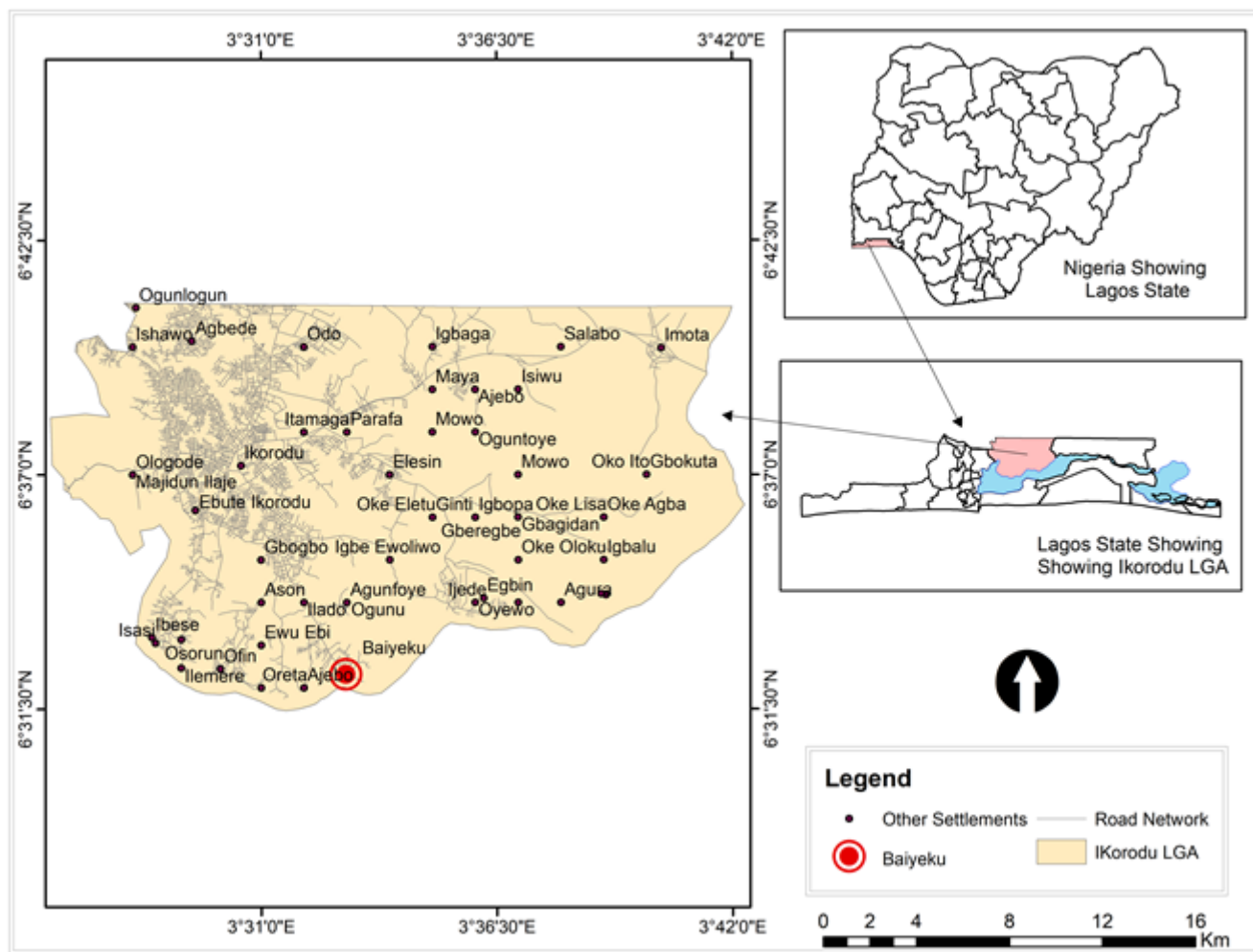
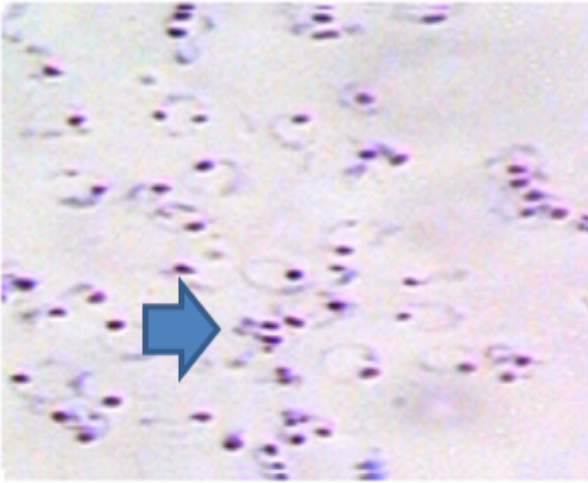
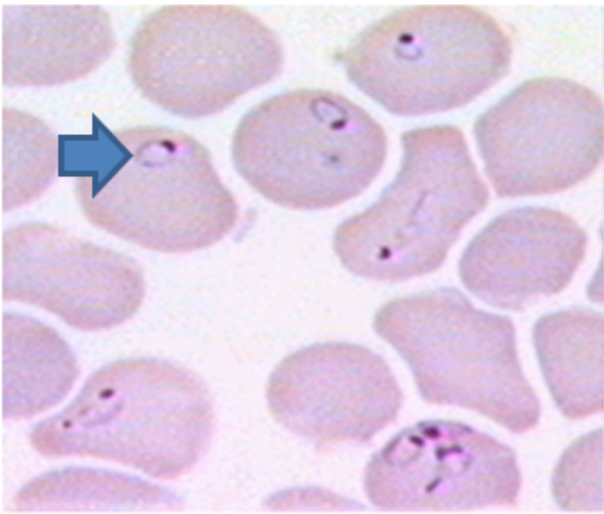


Figure 1

Map of Lagos State showing Baiyeku in Ikorodu LGA. (Source: Remote Sensing & GIS Lab; Department of Geography, University of Lagos, 2019)



A



B

Figure 2

Photomicrograph of trophozoite stages of *Plasmodium falciparum* in (A) thick film; (B) thin blood film.

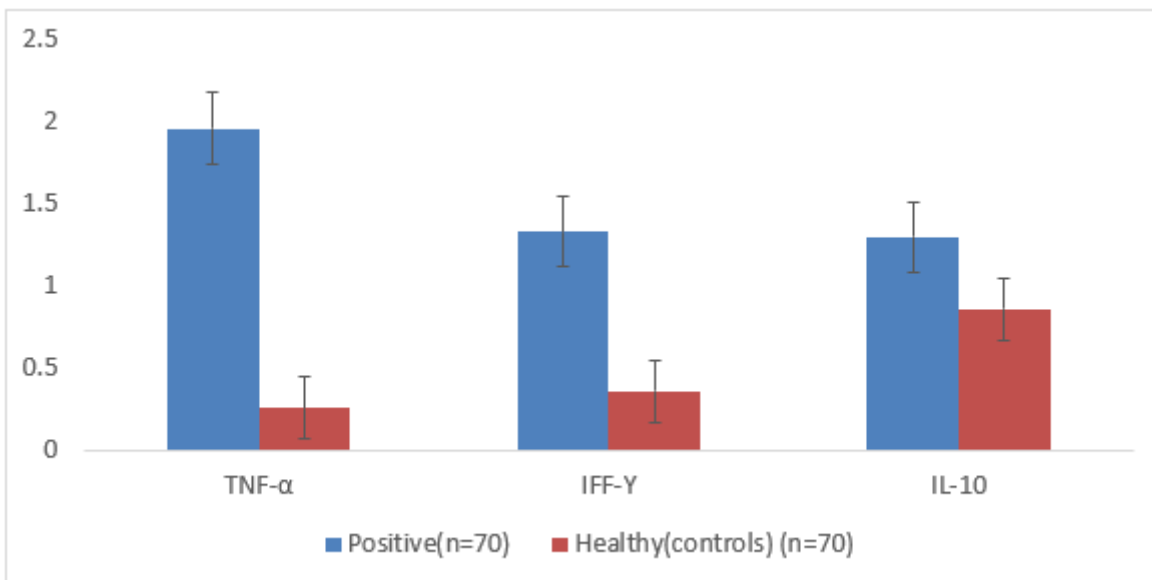


Figure 3

Cytokine levels of participants infected with *Plasmodium falciparum* in comparison with the healthy controls

