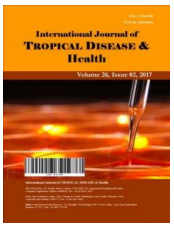

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Subclinical Malaria and Reticulocyte Count in Apparently Healthy Female Undergraduate Students in Rivers State University, Port Harcourt, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Author EME designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SGC and CCA managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The study was aimed at determining subclinical malaria and estimating reticulocyte count in apparently healthy female undergraduate students of Rivers State University, Port Harcourt.
Study Design: This is a non-randomized, comparative case-control study.
Place and Duration of Study: The study was conducted using female students residing at the hostels of Rivers State University, Port Harcourt. Analysis was carried out at the Haematology Laboratory, Department of Medical Laboratory Science, Rivers State University, Port Harcourt, Nigeria, between July and August, 2018.
Methodology: For the subjects used in this study, a total of 32 students (32%) that were diagnosed of having *Plasmodium falciparum* malaria infection were used as test subjects, while a total of 68 students (68%) that were diagnosed to be *Plasmodium falciparum* negative, and without

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malaria, were used as control. Thick and thin blood films examination using Giemsa staining technique was used to detect and calculate the malaria parasite density while a thin blood film examination using new methylene blue staining technique was used to evaluate the reticulocyte count in the blood.

Results: The reticulocyte count of test subjects (subjects with *Plasmodium falciparum* malaria) was 0.15 ± 0.04% and that of control subjects (subjects without any malaria parasite) was 0.31 ± 0.08%. The test subjects had significantly lower reticulocyte count ($p < 0.0001$) than the control subjects. The age range "15-19" years had the highest malaria parasite density of 0.52 ± 0.18%, while "25-29" years had the least parasite density of 0.33 ± 0.24. There was no statistical variation in malaria parasite density according to age ranges ($p = 0.13$; $p > 0.05$). However, the age range of "15-19" years had the lowest reticulocyte count as most of the female students within this age group were diagnosed to have been infected with malaria parasite.

Conclusion: This study revealed that reticulocyte counts of malaria (*Plasmodium falciparum*) infected individuals decreased when compared to those without malaria parasite and this decrease was statistically significant. There was no statistical significant variation in malaria parasite density irrespective of age ranges. Prophylaxis for malaria in such settings would be an efficient means of preventing infectious reservoirs and higher rates of subclinical malaria infection.

Keywords: Subclinical malaria; reticulocyte count; Plasmodium falciparum; female undergraduate.

1. INTRODUCTION

Malaria is a life-threatening disease of man caused by a parasite of the genus *Plasmodium*, which is transmitted through the bite of infected female Anopheles mosquitoes [1]. In tropical regions, malaria has been a common disease. An estimated 1.2 billion persons are at high risk of being infected with malaria and developing the disease, while about 3.3 billion people are at risk [2]. The second leading cause of death from infectious diseases in Africa is malaria after HIV/AIDS [3]. More than half of the world's population lives in areas where they are at risk of malarial infection [4]. Malaria is caused by *Plasmodium falciparum*, *Plasmodium malariae, Plasmodium ovale, Plasmodium vivax,* and *Plasmodium knowlesi* [5,6]. *Plasmodium falciparum* is the most common species identified (~75%) among those infected, followed by *Plasmodium vivax* (~20%) [7]. *Plasmodium falciparum* traditionally accounts for the majority of deaths [8]. Recent evidence suggests that *P. vivax* malaria is associated with potentially life-threatening conditions about as often as with a diagnosis of *P. falciparum* infection [9]. However, *P. vivax* proportionally is more common outside Africa [10]. It is estimated that about 132 billion Naira is lost to malaria annually in Nigeria in the form of treatment costs and prevention [11]. Malaria remains a major public health challenge where it estimates for more cases and death than any other country in the world [12]. In Nigeria, high prevalence of malaria parasitaemia has been reported [13,14,15]. The variation among other target groups is not much as prevalence of 28.0% was recorded among blood donors in Port Harcourt [16], and 26.0% prevalence was reported among pregnant women attending ante-natal clinic in Port Harcourt [17].

Malaria transmission in humans occurs through indirect, vector transmission and natural living reservoirs. In the transmission of malaria, 30 of the 400 different species of Anopheles mosquitoes are of significant importance [18]. Transmission begins when a female Anopheles mosquito bites an infected human and ingests protozoan gametocytes. The parasite incubates in the mosquito for 8-35 days before the infectious sporozoites are formed. The disease is transferred when the mosquito bites a human host and introduces the malarial sporozoites [19]. Malaria can be classified as uncomplicated or severe (complicated). Uncomplicated malaria have symptoms that include tiredness, fever, vomiting and headache while in severe or complicated malaria cases it can cause yellowing of the skin, coma, seizures or even death [20]. Ten to fifteen days after being bitten by infected mosquito, symptoms ensue. If not properly treated, people may have recurrences of the disease months later and in those who have recently survived an infection. Re-infection usually causes milder symptoms if not properly treated in those who have recently survived an infection [21]. This partial resistance disappears over months to years if the person has no continuing exposure to malaria parasites [20].

There is no standard definition for "subclinical" malaria infections but it is generally accepted to be malaria parasitaemia of any density, in the absence of fever or other acute symptoms, in individuals who have not received recent anti-malarial treatment [22]. The vast majority of individuals with detectible malaria parasitaemia can be categorized as asymptomatic based on this definition, regardless of the level of malaria transmission. This definition includes: Early detection of rising parasitaemia that has yet to reach the pyrogenic threshold (that is, the density of parasitized erythrocytes that is sufficient to trigger innate immune responses and fever) [23]; infections that are intermittently symptomatic but not severe enough to cause the person to seek health care; and long-standing infections imperfectly controlled by the immune response [24]. Asymptomatic parasitaemia is prevalent in highly endemic areas of Africa, It is generally assumed that asymptomatic parasitaemia is involved in the development of partial immunity in endemic areas and may protect against clinical disease from new infections [25]. Notwithstanding, asymptomatic parasitaemia provides a reservoir for transmission and may be a precursor in the progression to symptomatic disease [26]. In low-transmission areas, sub-microscopic carriers can become the source of approximately 20-50% of all transmission [27]. The prevalence of asymptomatic parasitaemia depends upon the high or low transmission area, period of residency in the endemic area, age, development of partial immunity by the previous repeated exposures to malaria, gender, use of insecticide treated mosquito bed nets, and the genetic back-ground [28,29]. Malaria transmission depends on two primary factors: location of mosquito breeding sites, and clustering of human habitations where people serving as reservoirs of parasites for mosquito infection lives [30].

Anaemia is a common complication in malaria infection and it causes death of the patients. The pathophysiology of malarial anaemia is said to be multi-factorial and complex [31]. Different malaria parasites have preference for the type of red cells they attack. *Plasmodium falciparum* has the capability of invading all red blood cell age classes, while *Plasmodium vivax* and *Plasmodium ovale* exhibit a strong preference for the youngest red blood cells (reticulocytes) and *Plasmodium malariae invades* the mature red blood cells [32]. The red blood cell invasion preferences for *Plasmodium knowlesi* are still yet to be identified [33]. There is obvious loss of infected erythrocytes through parasite maturation during infection but many uninfected cells are also destroyed in the process of clearing due to antibody sensitization or red cell membrane changes and increased reticulo-endothelial activity in spleen. Additional factor contributing to worsening the condition has been said to be the suppression of erythropoiesis [34]. Anaemia may either develop rapidly with severe haemolysis, or present as a relatively slow rate of red-cell destruction in the presence of persistent bone-marrow suppression [35]. Reticulocytes which are non-nucleated immature red cells are delayed in release during acute malaria infection, indicating transient suppression of the normal erythropoietin (Epo) response [36]. The effect is probably controlled by an autologous serum factor that suppresses the growth of an early precursor red cell [37].

In sub-Saharan Africa over 90 % of all deaths are caused by malaria [38]. According to Chukwura et al. [39], *Plasmodium falciparum* malaria is the most prevalent and virulent in Nigeria and it is capable of causing mental apathy, weakness and generally retard economic development; accounting for up to 98% of severe cases with significant mortality and morbidity [38]. For the treatment of uncomplicated malaria caused by *Plasmodium falciparum*, Artemisinin-based combination therapy (ACT) can be used for the treatment and another partner drug is recommended [40]. Chloroquine is recommended for infections caused by *Plasmodium vivax*, injectible artesunate is administered in the case of severe malaria [41]. Treatment of these patients with anti-malarial drugs however increases reticulocyte numbers, as *Plasmodium falciparum* is one of the causes of dyserythropoiesis and ineffective erythropoiesis [35].

Since asymptomatic parasitaemia provides a reservoir for transmission and may be a precursor in the progression to symptomatic disease, it is pertinent to ascertain the level of subclinical malaria among female undergraduate students living in the hostels with a view to proffering a better control and management strategies for malaria, hence this research. This study was aimed at determining subclinical malaria and estimating reticulocyte count in apparently healthy female undergraduate students of Rivers State University, Port Harcourt.

2. MATERIALS AND METHODS

2.1 Study Area

The study area was the female hostels in Rivers State University (RSU), Nkpolu-Orowurokwo, Port-Harcourt, Nigeria, from the month of July through August, 2018. The University which was formerly called Rivers State University of Science and Technology is a University located in Diobu area of Nkpolu-Orowurokwo. The University has a GPS coordinate of 4.7958^0N, 7.0246^0E, and female undergraduate student population in the hostel is about 1,000.

2.2 Study Population

This study exercise was carried out among female undergraduate hostel students of the Rivers State University. A total of 100 students were sampled, 40 students from Niger Delta Development Commission (NDDC) hostel, 40 students from hostel C and 20 students from hostel H. Only Rivers State University undergraduate female students within the age of 15-29 years were included in this study population. Malaria symptomatic individuals experiencing fever, malaise and nausea; individuals that are not female undergraduate students of Rivers state university; individuals that are not within 15-29 years; individuals having health challenges whether genetically or pathological; those that have been on malaria medication within three weeks prior to sample collection; and those that declined consent were excluded from this study.

2.3 Collection of Blood Samples

Venous blood sample was collected with the use of vacutainer needle from each participant, of which 3.0 ml of collected blood from each participant was added into individualized vacutainer tube containing 0.5 ml of 1.2 mg/ml dipotassium ethylene diamine tetra-acetic acid (EDTA) and promptly analyzed for malaria parasite and reticulocyte count.

2.4 Identification of Malaria Parasite

2.4.1 Principle of Giemsa's stain

Giemsa solution is composed of eosin and methylene blue (azure). The eosin component stains the parasite nucleus red, while the methylene blue component stains the cytoplasm blue.

2.4.2 Procedure for thin blood film preparation

One micro litre of blood was dropped near the end of a slide. The edge of the spreader was placed in front of the blood at an angle of 30-45. The spreader was drawn back until it touched the drop of blood and the drop spread along the line of contact between the spreader and the slide on which the film was to be made. The spreader was pushed along the slide with a smooth movement. The film was allowed to air-dry at room temperature. The participant's identification number was directly written on the frosted end, using a lead pencil.

2.4.3 Procedure for thick blood film preparation

Two micro litres of fresh blood were placed on a clean glass slide, and the blood mixed with a corner of another slide in a circular motion over an area about two centimeters in diameter. The blood film was allowed to air-dry at room temperature.

2.4.4 Procedure for thin blood film staining using Giemsa's stain

One in 10 dilution of Giemsa stain was made by mixing 1ml of stain and 9ml of buffered water. The film was prepared and fixed in absolute methanol for 1 minute. The film was allowed to air-dry. It was flooded with diluted Giemsa stain for 10 minutes and washed in buffered water at pH 7.2. The film was dried on a rack in a vertical position. The stained film was examined microscopically using oil immersion objective (100 x), (Olympus Microscope).

2.4.5 Procedure for staining of thick blood film using Giemsa stain

One in 10 dilutions of Giemsa stain was prepared by mixing 1ml of stain and 9 ml of buffered water. The film was dried for five hours and then covered with diluted Giemsa stain for 10 minutes. The film was washed in buffered water at pH 7.2 and dried in a vertical position. The stained film was examined microscopically using an Olympus microscope with oil immersion objective (100 x).

2.5 Reticulocyte Count

2.5.1 Principle of reticulocyte count

Reticulocytes are immature red blood cells that contain remnant cytoplasmic ribonucleic acid (RNA) and organelles such as mitochondria and

ribosomes. Reticulocytes are visualized by staining with supravital stains (methylene blue or brilliant cresyl blue) that precipitate the RNA and organelles.

2.5.2 Procedure for reticulocyte count

Two micro litres of blood and two micro litres of the staining solution was mixed in a test tube and allowed to stand at room temperature for 20 minutes. The tube was gently tapped to remix the content and with a drop of the mixture, a blood film was prepared in the same manner as for peripheral smear. The film was allowed to air-dry completely. The stained film was examined microscopically using the oil immersion objective (100 x), (Olympus Microscope).

2.5.3 Calculation of percentage reticulocyte count

The percentage reticulocyte count was calculated using the formula:

$$\% \text{ Reticulocyte count} = \frac{\text{Number of reticulocytes counted}}{\text{Number of red blood cells+reticulocyte counted}} \times 100$$

Normal reference range (healthy range) for reticulocyte count = 0.2 - 2.0% [42].

2.6 Reading of Slides and Counting of Malaria Parasites Density

The slides were microscopically examined using the low magnification (10 x, 40 x objective lens of an Olympus Microscope) to ascertain a definite field with even distribution of red blood cells before finally examining with 100 x or (oil immersion) objective lens of the same microscope. For malaria, the thick blood film was used to indicate the presence of malaria parasites and thin film was used for the specie identification and quantification. Malaria parasite density was then calculated using the formula:

$$\text{Malaria parasite density (\%)} = \frac{\text{Number of parasitized red blood cells}}{\text{Red blood cells observed}} \times 100$$

Levels of parasitaemia as described by Manas et al, [43] are:

1. High parasitaemia (> 10 parasite/1 oil field)
2. Moderate parasitaemia (1-10 parasite/1 oil field)
3. Low parasitaemia (1-100 parasite/100 oil field)

2.7 Statistical Analysis

The data generated were analyzed using special package for social science (SPSS) version 22.0. Comparison of reticulocyte count between malaria positive and malaria negative subjects was analysed with student independent t-test. Comparison of malaria parasite density according to age ranges was analysed with single factor analysis of variance (ANOVA). Correlation between malaria parasite density and reticulocyte count was analysed using the t-test. Values for t-test and correlation value below or equal 0.05 were considered statistically significant.

3. RESULTS

3.1 Comparison of Reticulocyte Count between Malaria Parasite Positive Subjects and Malaria Parasite Negative Subjects

The mean reticulocyte count for malaria parasite positive subjects was 0.15 ± 0.04% while that of the malaria parasite negative subjects was 0.31 ± 0.08%. The malaria parasite positive subjects had significantly lower mean reticulocyte count ($p < 0.0001$) than the malaria parasite negative subjects. Details are shown in Table 1.

3.2 Comparison between Reticulocyte Count and Malaria Parasite Density

The reticulocyte count has a significant negative correlation with malaria parasite density ($r = -0.39$, $p<0.0001$) as shown in Fig. 1.

3.3 Comparison of Reticulocyte Count between Malaria Parasite Densities According to Age Ranges

The malaria parasite density for subjects in age range A: “15-19” years were 0.52 ± 0.18%, B: “20-24” years were 0.40 ± 0.17% while that of C: “25-29” years were 0.33 ± 0.24%. There was no significant difference in malaria parasite density according to age ranges ($p = 0.13$). Details are shown in Table 2.

4. DISCUSSION

This study was done to evaluate subclinical malaria and reticulocyte count of apparently healthy female undergraduate students in Rivers State University, Port Harcourt. Malaria is indeed by far the most important tropical parasitic disease causing great suffering and loss of lives.

Table 1. Comparison of mean ± standard deviation of reticulocyte count between malaria parasite positive subjects and malaria parasite negative subjects

Subjects	Mean reticulocyte count (%)
Malaria Parasite Positive (32)	0.15 ± 0.04
Malaria Parasite Negative (68)	0.31 ± 0.08
p-value	<0.0001

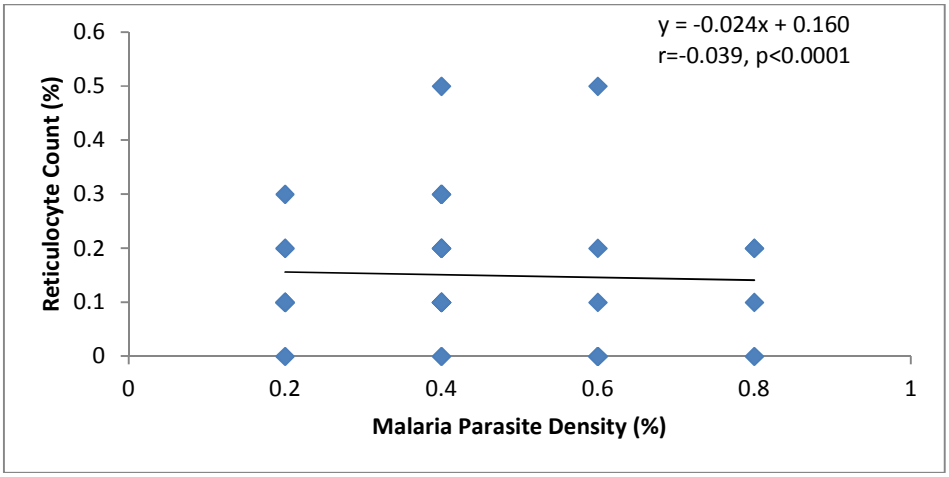

Fig. 1. Scatter diagram of reticulocyte count and malaria parasite density

Table 2. Comparison of reticulocyte count between malaria parasite densities according to age ranges

Age range (Years)	Malaria parasite density (%)
A (15-19)	0.52 ± 0.18
B (20-24)	0.40 ± 0.17
C (25-29)	0.33 ± 0.24
p-value	0.13

From this study, 32% of participants were *Plasmodium falciparum* positive. This result is similar to the work done by Ezeigbo and Ezeigbo [44], who reported cases of 35.3% malaria parasitic infection at the Abia Polythechnic, South Eastern Nigeria. The percentage positivity of *Plasmodium falciparum* malaria obtained from this study was at variance with work done in Southern Nigeria by Omolade and his co-researchers in 2010 who reported malaria parasitic infection rate of 83.3% and by Fernado and his colleagues [45] who reported parasitic infection rate of 77% in Senegal. This study was conducted in a University environment among individuals of higher learning and understanding and may have contributed to the low level of asymptomatic or subclinical estimation obtained. High standard of education usually affect health awareness and therefore has a positive impact on health since they are probably better informed about vector control measures.

Plasmodium falciparum was the only *plasmodium species* encountered in this study which represents a major problem in Nigeria. This collaborates with other researches carried out in Lagos state South West Nigeria [46], and in Port Harcourt metropolis [47], where only infections of *Plasmodium falciparum* were reported. On the other hand, Matur and his colleagues [48], reported cases of *Plasmodium falciparum* and *Plasmodium malariae* while Sam and his co-researchers [49], reported cases of mixed infections of *Plasmodium falciparum* (95.6%), *Plasmodium malariae* (3.3%), *Plasmodium ovale* (0.7%) and *Plasmodium vivax* (0.4%) in Ogun state, Nigeria.

From the result of this study, 32% of female students that were positive for malaria parasite had lower reticulocyte count of 0.15 ± 0.04% when compared to 68% of female students that were negative for malaria parasite with

reticulocyte count of 0.31 ± 0.08%. This indicates that there was a significant difference in the reticulocyte counts of the positive and negative subjects. Also, in comparing the reticulocyte count with malaria parasite density, the reticulocyte count had a significant negative correlation with malaria parasite density ($r = -0.39$, $p < 0.0001$). In other words, when there is an increase in malaria parasite density, reticulocyte count decreases.

The significant difference in the reticulocyte counts of the positive and negative subjects and the significant negative correlation of reticulocyte count with malaria parasite density could be due to ineffective erythropoiesis. This collaborated with the findings of Thawani and his co-worker [50], who reported that dyserythropoiesis occurs in the bone marrow of patients with low parasitaemia. Roberts and his colleagues [51] also reported that during acute malaria infection reticulocyte release is delayed, depicting transient suppression of normal erythropoietin (epo) response, which is probably mediated by an autologous serum factor that suppresses the growth of an early-precursor red cell. Another researcher also collaborated by reporting that there could be reduced reticulocyte as a result of inhibition of haemopoiesis arising from the release of interferon gamma and tissue necrotizing factor (tnf) and bone marrow hypoxia, dysfunction of haemopoiesis as a result of sinusoidal obstruction of parasitized red cells. This depressed erythropoietic response may contribute to the development of anaemia, which is the primary clinical manifestation of malarial infections especially in severe cases causing mortality and morbidity.

Also from this study, the results of female undergraduate students of the age range A:(15-19 years) were mostly affected, with malaria parasite density of 0.52 ± 0.18%, followed by the age range of B:(20- 24 years) with 0.40 ± 0.17% and least affected age range C:(25-29 years) with 0.33 ± 0.24%. However, the result contrasted the report of Ebenezer and Eekpa [52], who observed more infected rate in the age range of 29-31 years (25.0%)| followed by age range of 17- 19 years (17.65%) and least in the age range of 23-25 years (9.09%), carried out among new in-takes in Isaac Boro College of Education, Sagbama, Bayelsa State, Nigeria. The variation of the malaria parasite density according to the age ranges from the study was not statistically significant ($p = 0.13$, $p > 0.05$). This insignificance in statistical variation could arise due to an equal chance of being infected irrespective of ages as long as they are confined to the same environmental conditions and also the clustering of human habitations in the hostels where people are serving as reservoirs of malaria parasites.

The results obtained showed that that the age range of 15-19 years had the lowest reticulocyte count when compared to the other age ranges of 20-24 years and 25-29 years which had approximately equal reticulocyte counts. This decreased reticulocyte count seen in the age range of 15-19 years could be as a result of the increased effect of malaria parasitaemia on this age group seen from the study. Esan [53], reported that the haematopoietic system is modestly affected by aging and these effects become particularly noticeable after the age of 65years, where there is a continuous decrease in the volume of the haematopoietic marrow, while it is markedly increased in children as their bone marrow is fully active and extremely cellular.

5. CONCLUSION

There is a moderately high estimation of subclinical malaria amongst the female undergraduates boarding in the hostel in Rivers State University as reflected in the study. This study has indicated that there is reduced reticulocyte count with malaria parasitic infection due to the suppression of the bone marrow by the malaria parasites. This could impact negatively on the health of the population as individuals gradually go into anaemic condition. Therefore, improving hygienic conditions, and periodic insecticide sprays in and around the hostels can go a long way in reducing the mosquito population, and treating of asymptomatic individuals with malaria in order to reduce transmission of malaria parasite.

CONSENT AND ETHICAL APPROVAL

Informed consent was obtained from apparently healthy subjects prior to enrolment upon ethical clearance by the Ethics Committee of the Department of Medical Laboratory Science, Rivers State University.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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