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Determination of *Plasmodium* Specific Immunoglobulin G and M in Patients with Malaria in Ikorodu, Lagos, Nigeria

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Abstract

This study was conducted to determine the presence of *Plasmodium* specific IgG/IgM, its relationship with clinical symptoms, parasite density, age and sex in four primary health care facilities in Ikorodu Lagos, Nigeria. A total number of 1218 patients 450(36.9%) male and 768(63.1%) female were recruited for the study. Microscopy method was used to screen the patients of which 235 were positive for malaria parasite. A total of 167 sera was pooled out from the positive samples and tested for *Plasmodium* specific IgG/IgM using enzyme linked immunosorbent assay (ELISA). 135(83.9%) were positive for *Plasmodium* specific IgG/IgM and presenting with fever, while 26(16.1%) were negative with fever. The parasite density was 30671.71 ± 31809.3 with majority of participants that had moderate parasitaemia and presented with fever, having higher positivity for *Plasmodium* specific IgG/IgM 41(89.15%) while 5(10.9%) with fever and moderate parasitaemia were negative for *Plasmodium* specific IgG/IgM. There was no significant correlation between IgG/IgM and clinical signs/symptom, parasite density, age and sex ($P > 0.05$). These findings demonstrated that individuals living in this community mount a strong antibody response against *P. falciparum* parasites and these antibodies persist as a result of infection and re-infection irrespective of clinical presentation, age and sex differences.

Keywords

Malaria, *Plasmodium,* Immunoglobulin, Primary Health

1. Introduction

Malaria is the most devastating parasitic disease in the world, and it is estimated that around 200 million clinical cases occur yearly, with about 600,000 deaths. Africa is the most affected continent with about 90% of all malaria deaths occurring here, while Nigeria and Democratic Republic of the Congo account for over 40% of the estimated total of malaria deaths globally [1]. In holo-endemic areas of *Plasmodium falciparum* malaria, the presentation of the disease is heterogeneous, ranging from asymptomatic to severe malaria. Geographical, biological, clinical, immunological and genetic factors are responsible for these differences [2-4].

Malaria infection generates strong immune responses brought about by the production of mainly Immunoglobulin M (IgM) and Immunoglobulin G (IgG) but includes other immunoglobulins as well. Even though most immunoglobulins carry no specificity to malaria, species-specific antibodies react with various types of parasite antigens. The significance is that this enables parasites to use this variability to evade immune response [5]. Therefore, the poor and slowly developing immune responses to malaria are

partly due to immune evasion strategies of the parasite. Malaria symptoms and clinical complications of are caused by the erythrocytic stage of the infection, and majority of the acquired immune response is against these blood-stage parasites [6-7]. The capacity for immune evasion enables *P. falciparum* to cause repeated and chronic infections; after repeated exposure to malaria, individuals eventually develop effective immunity that controls parasitaemia and prevents severe and life threatening complications. Antibodies are an important component of acquired protective immunity, and the passive transfer of immunoglobulin from immune donors to individuals with *P. falciparum* infection has been shown to reduce parasitaemia and clinical symptoms [8]. Also protective immunity has been seen in neonate which the mother transferred IgG. Antibodies to malaria can be targeted to sporozoites and merozoites (both of these are exposed briefly to the immune system before they invade their respective cells), or to parasite antigens that are inserted into the erythrocyte cell membrane (parasite-infected erythrocyte surface antigens, PIESAs). The predominance of Immunoglobulin G sub-group 1 (IgG1) and Immunoglobulin G sub-group 3 (IgG3) cytophilic antibodies (Ab) in endemic areas has been associated with either lower parasitaemia or a lower risk of malaria attack [9]. Otherwise, noncytophilic antibodies, such as IgG4, may inhibit effector mechanisms by competing with cytophilic antibodies and are considered non protective [10]. IgG2 is non cytophilic, but could be correlated with protection in individuals carrying a specific allelic variant of monocytes FcγRIIA receptor that can bind IgG2 [11]. IgE levels and IgE anti-plasmodial antibodies are elevated in human and experimental malaria infections, but their role in protection and/or pathogenesis is not well-established in malaria [12]. In fact, a negative correlation between the IgE level and placental parasitaemia [13-14] and levels of haemoglobin and platelets [15] has been found. The IgE level was higher in cerebral *P. falciparum* malaria than in uncomplicated malaria [16-17], while among patients with severe malaria, the increase in IgE levels was related to the deepness of the coma [14]. Data showed higher IgE functional activity in asymptomatic and uncomplicated malaria patients than in severe or cerebral malaria groups [18]. Contradictory results have also been found in relation to IgM Ab. Even though there are indirect epidemiological data suggesting that IgM antibodies does not participate in protection [19-20], some evidence suggests a protective role in cases such as: (a) mice with an X-linked recessive B-cell deficiency do not produce IgM and are susceptible to *Plasmodium yoelii* [19]; (b) the addition of monoclonal IgM antibodies to a malaria vaccine, raising its protective properties (c) the IgM level, which correlated with a decrease of the parasitaemia in individuals living in an area of hyper endemic malaria [19]. In terms of IgA antibodies, to the best of our knowledge no specific function in malaria has been ascribed to these antibodies to date.

Immunity is an important factor, especially among adults in areas of moderate or intense transmission conditions. Immunity is developed over years of exposure, and while it never gives complete protection, it does reduce the risk that malaria infection will cause severe disease. For this reason, most malaria deaths in sub-Saharan Africa where Nigeria belongs occur in young children, whereas in areas with less transmission and low immunity, all age groups are at risk. The level of antimalaria immunity influences the clinical outcome of the disease in different locations and age group with antibody responses known to persist much longer than the parasite infection and this can be used to estimate the level of malaria exposure. IgG and IgM are major antibodies released in malaria infection or disease, and assessment of the antibody levels could help ascertain protection/severity of the disease. Therefore, the study of antibody distributions among malaria positive patients with different clinical presentations and age group could serve as a pointer in determining the protective role of antibody among individuals in Ikorodu, Lagos State.

2. Materials and Methods

2.1. Study Area/Study Site

The field study was conducted at the following Primary Health Care Centers (PHC); Imota, Oreta, Agura and Ijede, all located in Ikorodu Local Government Area of Lagos State Nigeria. The health centers are open to patients for 24hr services. Ikorodu is a peri urban and Local Government Area in Lagos State, Nigeria. It is located along the Lagos Lagoon and shares a boundary with Ogun State. Ikorodu is at latitude 6° 37' 0" N and longitude 3° 31' 0" E. The study sites are as follows: Imota Primary Health Care (PHC) Center, Imota; Oreta PHC, Oreta; Agura PHC, Agura and Ijede General Hospital, Ijede. The Laboratory study was conducted at ANDI Center of Excellence for Malaria Diagnosis, College of Medicine, University of Lagos, Idi-Araba, Lagos. The population of Ikorodu is estimated to be 535,619 [21]. The major occupation of the people of Ikorodu is farming, fishing, business and some in civil service. Most of the people in this community that are engaged in farming and fishing do so as result of the large mass of water surrounding them. The major language of these communities in Ikorodu is Yoruba and an average of sixty patients visits the various health facilities every day. Children and women make up a larger number of patients that make use of the health facilities.

2.2. Study Design

The study was cross sectional, involving parasitologic and immunologic studies of malaria antigen/antibodies with plasmodium, exploring the IgG/IgM antibody presence in different patients, age group, presenting different clinical malaria.

2.3. Patient Recruitment

Patients were asked about their age, signs and symptoms with their sex recorded in the case report form. Patients with history of malaria symptoms such as fever (axillary

temperature ≥37.5°C), patients who had not taken anti-malaria drugs two weeks prior to presenting to the health centre were recruited for the study, while those who refused to give their consent whether qualified for the study or not were excluded from the study. All patients that presented with malaria symptoms were screened after microscopic examination. It was from this number that the sub-set for the IgG/IgM studies was selected.

2.4. Sampling

Collection of Blood Sample and Separation of Serum

Blood sample was collected from patients that met all the inclusion criteria. Blood was collected from venipunture and 3-5mls of blood was collected into EDTA container/plane bottle. The EDTA tubes were mixed by gentle inversion of the tubes. Each tube was labeled with patient ID number and date of blood collection (DD/MM/YY), using a marker pen. The blood was used to prepare smears, make blood spots on filter paper and aliquot of serum into cryovials. The sera were stored immediately at -86°C.

Whole blood samples collected in plain container/bottles were allowed to coagulate/clot. The serum samples were then separated from the whole blood with the aid of a Pasteur pipette into cryo-vials with patient ID number. The separated serum was then stored in the freezer at -86°C.

2.5. Preparation of Blood Films for Parasite Diagnosis/Quantifications

2.5.1. Thin Smear Preparation

The thin blood film was prepared with 2µL of blood dropped near the frosted end of a grease free slide using an automated micropipette. Another 'spreader' slide was held with polished edges at a 45° angle and then drawn into the 2ml of blood. The blood was allowed to spread almost to the width of the slide. The 'spreader' slide was rapidly and smoothly pushed to the opposite end of site. The smear was allowed to dry at room temperature. The smear had feathered edge-the area where the microscopic examination was performed on the stained smear. The thin smear was then fixed with methanol.

2.5.2. Thick Smear Preparation

The thick blood film was made with 12µL of blood was needed with a diameter of 12mm the edge of another slide was used to spread the blood using a circular motion until a uniform spread and a proper density is achieved. The smear was allowed to dry for 8hrs on a flat surface at room temperature. Both smears (thick and thin) were done on the same slide and each patient had two slides- one labeled as read 'R' while the other labeled archive 'A'. Both smears were protected from flies, dust and extreme heat.

2.6. Staining

The films were stained with 3% Giemsa stain for 45mins. Then flooded with distilled water carefully and gently to avoid scum sticking to the film, the films were then allowed to dry, thereafter, viewed under the microscope using x100 magnification (oil immersion objective) for malaria parasites.

2.7. Determination of Total White Blood Cell (WBC)

2.7.1. Dilution

Using an automated micro pipette, 20µL of the whole blood collected in EDTA container was diluted in a plain container containing 380µL of turks solution. It was then mixed properly and allowed to stand for about 10mins.

2.7.2. Charging of the Neubauer Chamber and Loading

A clean Neubauer counting chamber was placed on a horizontal surface and using a firm pressure, the cover-slip was slide into position on the counting chamber until a rainbow effect was seen on both sides (Newton's ring). The diluted blood was mixed thoroughly and a quantity was withdrawn into a capillary tube. The chamber was then filled with the dilution mixture by holding the capillary tube at an angle of 45° and lightly, touches the tip against the edge of the cover-glass. The filled haematocytometer was then placed in a Petri dish that contains a piece of moist filter paper and the cells allowed to settle for 20mins. Thereafter, the chamber was then placed on the microscope stage and the number of cells counted using ×10 objective magnification. All four chambers counted and multiplied by 50 (factor).

2.7.3. Determination of Parasite Density

With two tally counters, one for the asexual stage of the parasite and the other for the WBC count. The parasites observed were counted simultaneously with WBCs up to a total of 500 WBCs. Malaria blood film was considered negative after 200 High Power Field was scanned systematically and no parasites seen. The parasite density (parasitaemia) was computed using the formula below.

$$\text{Parasite Density (Parasite/µL of blood)} = \frac{\text{Parasite count x leukocyte count}}{\text{WBC count}}$$

2.7.4. Elisa Procedure

The kits for used (Malaria IgG/IgM ELISA, manufactured by Standard Diagnostic (SD) INC. Korea with LOT No: 230001 and EXP. Date 14/01/2014) was brought out from the refrigerator to attain room temperature following other manufacturer instructions. The serum samples were also brought out of the freezer to thaw. The test strip wells for both negative (3 wells) and positive (2 wells) controls were prepared. Aliquot of 10µL of controls and patient's sample to each well of the 96wells then, 100µL of sample diluents put into each well. Micro plate was covered with a plate sealer, mixed well and incubated at 37°C for 60mins. The plate was washed 5times with diluted washing solution of 350µL for 10secs soak time for each wash. Also 100µL of diluted enzyme conjugate was added to each well. It was covered with an adhesive plate, mixed well and incubated for 37°C

for 30mins. The wells were washed five times again with 350µL of diluted washing solution. 100µL of mixed TMB solution was added and incubated in the dark for 10mins. 100µL of stopping solution was pipette into each well after incubation, mixing well. The blue color changed to yellow, with the absorbance of the well read within 30mins of adding the stop solution at 450nm wavelength. The result after a short while was displayed on the screen and then printed out. All precautions were observed to the later.

2.8. Ethical Considerations

Approval to carry out this research was obtained from College of Medicine Ethics Committee of the University of Lagos and Lagos State Health Management Board for the study to be done in the health facilities. Assent/informed consent by parents/guardians was obtained from children less than 18 years and adults. The entire research was conducted in line with good clinical laboratory practice and patients who were positive for malaria parasite were treated adequately.

2.9. Data Analysis

The data was analyzed using Epi info 3.5.3 statistical software. Univariate analysis involved calculations of percentage, mean, geometric mean, median and interquartile range. Differences between means were compared using one way analysis of variance or Kruskal-Wallis test which ever was applicable. Chi square test, Fisher's exact test was used to test for associations between variants.

3. Results

A total of 235 patients were positive by microscopy from the total population recruited for the study (1218) giving a prevalence of 19.3%.

3.1. Sub Set of Research Participants Used in the IgG/IgM ELISA Study

Out of the 235 positive patients, samples from 167 participants were tested for *Plasmodium* specific IgG/IgM using ELISA. The 167 patients' samples that were analyzed were all positive by microscopy of, these 74(44.3%) were male and 93(55.7%) were female. Four age groupings were done with their age and temperature ranges recorded respectively. The mean age ± SD of the ELISA sub-group was 15.2 ± 12.1 (range 3 – 63) while the mean temperature ± SD was 37.4 ± 1.34 (range 37.4 – 40.4). A total number of 84 (50.3%) presented with temperature less than 37.5°C while 83 (49.7%) presented with temperature equal to or greater than 37.5°C (Table 1).

Table 1. *Demographic Characteristics of participants in the ELISA subset.*

DESCRIPTION	No. (%)
No.. of participants	167
Sex	
Male	74(44.3)
Female	93(55.7)
AGE(YEARS)	
MEAN±SD	15.2±12.1
RANGE	3-63
1–5	20(80.0)
6–10	46(85.2)
11–15	29(87.9)
<15	43(78.2)

3.2. Relationship Between Clinical Symptoms with Plasmodium Specific IgG/IgM of the ELISA Sub-set

Among the 167 participants 138(82.60%) were positive for malaria specific IgG/IgM by ELISA while 29(17.40%) were negative by ELISA. The results also showed that all the subjects presented with one clinical symptom or the other but majority had history of fever 135(83.9%) coupled with other symptoms and were positive by ELISA while 26(16.1%) did not present with history of fever and were negative by ELISA. Body pains was also common among participants 98(81.7%) and were positive by ELISA while 22(18.7%) were negative by ELISA. A large proportion amongst participants also presented with weakness of the body and positive by ELISA while one presented with weakness and was negative by ELISA.

Table 2. *Relationship between clinical signs and symptoms of patients with Plasmodium specific IgG/IgM.*

SIGNS/SYMPTOMS	No. of persons	No. of Positive by ELISA(%)	No. of Negative by ELISA(%)	X^2	P
FEVER	161	135(83.9%)	26(16.1%)		0.066*
CHILLS	138	115(83.3%)	23(16.7%)	0.2703	0.603
BODYPAINS	120	98(81.7%)	22(18.7%)	5.032	0.598
HEADACHE	141	119(84.4%)	22(15.6%)	1.9604	0.161
LOSSOFAPPETITE	33	26(78.8%)	7(21.2%)	0.85	0.515
COUGH	14	11(78.6%)	3(21.4%)	0.1758	0.675
WEAKNESS	8	7(87.5%)	1(12.5%)	0.1386	0.710
CATARRH	10	9(90.0%)	1(10%)	0.4021	0.526
SWEATING	1	1(100.0%)	0	0.2114	0.646
VOMITING	46	38(82.6%)	8(17.4%)	<0.001	0.996
STOMACHEACHE	26	23(88.5%)	3(11.5%)	0.7286	0.393

*P-valueby Fisher's Exact Test

3.3. Relationship Between Parasite Density with ELISA Sub-set

The geometric mean parasite density was 11,560 with range 63 - 245647. The parasite densities for all the patients were grouped as low, moderate and high parasitaemia respectively. The mean ± SD parasite density was 30671.71 ± 31809.3. Participants with moderate parasitaemia (1001 – 10000) had the higher positivity by ELISA 42(87.5%) while those with low parasitaemia (1 – 1000) had the lowest 14(77.8%), (Table 3).

Table 3. *Relationship between parasite densities of patients with Plasmodium specific IgG/IgM ELISA.*

Parasite density grouping (P/µl)		NEG	POS	TOTAL
LOW	1-1000	4(22.2%)	14(77.8%)	18(100%)
MODERATE	1001-10000	6(12.5%)	42(87.5%)	48(100%)
HIGH	>10000	19(18.8%)	82(81.2%)	101(100%)
TOTAL		29(17.4%)	138(82.6%)	167(100%)

Kruskal-wallis P=0.8212

3.4. Parasite Density and Sex in the ELISA Sub-set

Of the 167 microscopy positive patients enrolled, 76(81.7%) female were positive by ELISA while 62(83.8%) male were positive by ELISA. Male patients with moderate parasitaemia (1001 – 10000) had the highest percentage positivity by ELISA 18(90%) with two patients negative by ELISA 2(10%) while female with low parasitaemia (1 – 1000) had the lowest percentage positivity by ELISA 6(66.7%) with three patients negative by ELISA 3(33.3%). The difference between the male and female group was not significant (P = 0.4264), (Table 4).

Table 4. *Relationship between parasite densities of participants with Plasmodium specific IgG/IgM based on sex.*

Parasitedensity(P/µl)IgG/IgMSex	1–1000(low)		1001–10000(moderate)		>10000(high)		TOTAL No. (%)
	Pos	Neg	Pos	Neg	Pos	Neg	
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	
MALE	8(88.9)	1(11.1)	18(90)	2(10)	36(80)	9(20)	74(100)
FEMALE	6(66.7)	3(33.3)	24(85.7)	4(24.3)	46(82.1)	10(17.9)	93(100)
TOTAL	14(8.4)	4(2.4)	42(25.1)	6(3.6)	82(49.1)	19(11.4)	167(100)

P–Value by Fisher Exact Test=0.4264

3.5. Age and Symptoms in the ELISA Sub-set

The mean age ± SD of the total number of patients positive by ELISA was 14.89±11.85 while those negative was 16.80±13.50 with age ranges 3 – 55years (ELISA negative) and 3 – 63years (ELISA positive) respectively. Age group 11 – 15years had the highest percentage positivity by ELISA with high percentage presenting with fever 29(87.9%) while age groups > 15years had the lowest percentage positive by ELISA 43(78.2%). (Table 5).

Table 5. *Relationship between participants 'age group and Plasmodium specific IgG/IgM.*

ELISA			
Age group(yrs)	NEG	POS	TOTAL
1-5	5(20.0%)	20(80.0%)	25(100.0%)
6-10	8(14.8%)	46(85.2%)	54(100.0%)
11-15	4(12.1%)	29(87.9%)	33(100.0%)
>15	12(21.8%)	43(78.2%)	55(100.0%)
TOTAL	29(17.4%)	138(82.6%)	167(100.0%)

X^2=1.758;P=0.624

4. Discussion

There was no statistical significant difference between *Plasmodium* specific IgG/IgM and clinical symptoms of malaria at (P > 0.05). This possibly could be as a result of these study participants living in malaria endemic area, where malaria transmission is stable and where they have a greater breadth and magnitude of antibody responses. In Ikorodu Local Government Area (LGA) where malaria is mesoendemic, adults would likely have developed potent but nonsterile immunity against malaria in which individuals chronically harbor low-grade parasitaemia and only occasionally suffer from mild clinical malaria, a clinical state known as premonition as supported by *Perignon and Druilhe* [22], therefore, irrespective of the presence or absence of malaria IgG/IgM, there are clinical symptoms in malaria infection as a result of infection and re-infection. In some studies, researchers have found that high levels of antibodies are associated with lower parasitaemia as reported by Shi *et al.* [23] and protection against malaria attacks and its associated symptoms by Egan *et al.* [24] which is in disagreement with this study, whereas in other studies the researchers failed to observe such an association [25]. This study also corresponds with study carried out by Soe Soe *et al.* [26] where none of the IgG subclass responses against MSP1-19 were associated with protection against clinical malaria when specific antibody responses were corrected for the confounding effect of age-dependent exposure to *P. falciparum* malaria in Ghana. However, individuals living in these endemic areas, with increased exposure which expectedly could have provided clinical benefits from these

responses with the presence of malaria antibodies, surprisingly seem to have no effect with the symptoms they present and increase their risk of malaria disease. This is in accordance with Bejon *et al.* [27] who reported Stable and unstable malaria hotspots in longitudinal cohort studies in Kenya.

There was no correlation between the presence and absence of malaria specific IgG/IgM and malaria parasitaemia too, possibly because individuals with malaria in Ikorodu which is an endemic area have rapid immunological response to malaria antigen ($P > 0.05$). At low, moderate and high parasite density, there is presence of substantial antibody which as a result of having continuous exposure to mosquito bite, thereby making them have 'always at alert' plasmodium specific IgG/IgM which are major antibodies in malaria infection [27]. Other studies that showed levels of malaria-specific IgG_1 and IgG_3 responses have been associated with lower parasite density and reduced risk of clinical malaria [28-29], presumably because IgG_1 and IgG_3 are key components of Fcγ receptor (FcγR)–mediated effector responses, including antibody-dependent cell-mediated cytotoxicity and antibody-mediated phagocytosis [23]. However, this study did not show any relationship most probably because of the generality of the malaria antibody detection but not the isotypes or subtype that could be responsible for lower parasite density and reduced clinical symptoms.

There was also no association between plasmodium specific IgG/IgM, age ($P > 0.05$) and sex ($P > 0.05$). Majority of the people are females who engage in going to stream and farm with their children for daily upkeep. Their community is surrounded by river that support the breeding of anopheles mosquito which transmits malaria, therefore, the transmission of malaria is without season. The participants in this community therefore, irrespective of age and sex always have the malaria antibodies, as they are always exposed to malaria infection that also builds up there immunity. Although antibody response to *P. falciparum* antigens have previously been shown to decline within a few weeks after the clearance of parasites in many individuals as reported by Cavanagh *et al*. [30], Boutlis *et al*. [31], and Kinyanjui *et al.* [32]. Another study carried out in Kenya on children with clinical malaria showed that the estimated time for a 50% decline of antibodies to recombinant proteins was less than 10 days for both IgG1 and IgG3 [32]. However, the study carried out here was not with specific subtype of IgG1 and IgG3, but specific IgG/IgM (no subtypes). The individuals used for this study may have had persistent antibodies against the various strains of *plasmodium falciparum* much longer than the catabolic half-lives of antibodies would allow. These could be the reason why there was no association as compared to other studies. Although declines in specific antibody do occur in children compared to adult, a substantial majority were positive to malaria by ELISA in this study, suggesting steady-state antibody production in these individuals not withstanding their sex and age as against the study done by McCallum *et al.* [33] who recorded that antibodies that are reactive in an ELISA assay tend to increase with age.

5. Conclusion

In conclusion therefore, this study demonstrates that individuals living in these communities mount a strong antibody response against *P. falciparum* parasites and that these antibodies persist irrespective of the presence or absence of clinical symptoms of malaria, level of parasitaemia, sex, and age differences, that are evident already at an early age.

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