Differential Molecular Investigation Assay of Tumor Necrosis Factor –Alpha (TNF-α), Interleukin-1 Beta (IL-1β), Interleukin-6 (IL-6), and Interleukin 10 (IL-10) among Children Potentially Diagnosed of Falciparum-Malaria of Niger Delta Extract

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Abstract
This cross-sectional study determined absolute parasitaemia and evaluated some inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and interleukin-10 (IL-10) concentrations in children infected with Plasmodium falciparum parasites in Port Harcourt, Rivers State, Nigeria. A total of 160 blood samples were randomly and aseptically collected through convenient sampling technique research design and were analysed respectively. The plasma obtained from the blood samples after centrifugation at 1500 rpm for 5 minutes were subjected to cytokine evaluation analysis using, commercially purchased standard Enzyme Linked Immunosorbent Assay kit (ELISA) in line with manufactures instructions- (ELISA - Elabscience Biotechnology Inc, USA). Malaria diagnosis and blood parameter assays were carried out using standard diagnostic techniques of haematological and parasitological benchmark standard respectively. However, result from the study showed that children within the age group 9-11 and 6-8 years had the highest and lowest mean parasitaemia levels of 4813.09±1180.05 parasites/µl and 2324.62±546.63 parasites/µl respectively (P=0.048). The mean concentration levels of IL-6, and IL-10 (365.30±40.31 pg/ml, and 318.57±39.29 pg/ml respectively) were significantly higher in children with severe parasitaemia (P=0.0014, and 0.0347 respectively).The mean cytokine levels of TNF-α, IL-β, IL-6, and IL-10, were significantly elevated in children with P. falciparum infection when compared to their respective healthy matched controls (P<0.0001). Correlations between plasma levels of IL-6 and P. falciparum parasite density in children showed a relatively strong positive relationship (P=0.0001). Inflammatory cytokines are involved in immunopathogenesis and immunoregulation of Plasmodium falciparum infection. Nonetheless, cytokines as found in this study could be used as a promising evidence based prognostic and diagnostic biomarkers for falciparum malaria progression or regression in our health facilities in the region.

Keywords: Inflammatory cytokines; Plasmodium falciparum; Parasitaemia; Malaria; Children; Niger Delta; Biomarker

Introduction
Malaria is a life-threatening disease caused by obligate intracellular Plasmodium parasites such as Plasmodium falciaparum, Plasmodium ovale, Plasmodium malariae, Plasmodium vivax, and Plasmodium knowlesi [1] Sporozoites, the infective stage of malaria parasites are notably transmitted via the bites of infected female Anopheles mosquitoes. Children who reside in poverty endemic areas in Africa are more susceptible to Plasmodium falciaparum infection. Nonetheless, in 2016, about 285,000 African children less than 5 years of age died of malaria before their fifth birthdays [2]. Also, about 219 million clinical cases of malaria were reported in 87 countries globally in 2017, with an estimated 435,000 deaths [1,3]. In sub-Saharan Africa, Plasmodium falciaparum is the most prevalent and deadly malaria parasite which accounted for 99.7% cases of malaria-hospitalization in 2017 [4]. However, Nigeria is one of the malaria burdened countries in West Africa [4]. Infants and children under 5 years of age, naïve adults, and pregnant women are critically very much vulnerable to Plasmodium falciaparum malaria in endemic settings [1,5,6]. It is strongly believed that...
Malaria infection is severe and fatal in children leading to 1 in 5 cases of childhood death in sub-Saharan Africa, [7] possibly due to their relative poor or partial immunity to the infection [2, 8].

Nevertheless, given the huge public health implications posed by malaria burden in Africa, especially in the remote communities of Sub-Saharan Africa, with no visible functional health facilities, thus, there is an urgent need to explore complementary immune-diagnostic cum preventive strategies to curbing the menace of malaria, especially among children in sub-Saharan Africa. Nevertheless, evidence based research has shown that many attempts to develop effective malaria vaccine yielded unsatisfactory outcomes, probably due to the complexity of Plasmodium falciparum parasite [9]. However, recently the first documented malaria vaccine, RTS, S/AS01 (RTS, S), against Plasmodium falciparum was introduced in three pilot African countries, such as Malawi, Ghana, and Kenya respectively. This first malaria vaccine does not confer absolute immunity but offers partial resistance against falciparum malaria in young African children [1]. Hence, the urgent need to developing effective vaccine as a strategic intervention for global prevention of malaria in children remains saccosant and a task that must be done to save the lives of the weak and most vulnerable subjects in our communities across the malaria endemic regions. The complex pathogenesis of P. falciparum malaria appears to involve dysregulation of the immune system, therefore, a better knowledge of mechanisms of protective immunity and immunopathology of falciparum malaria would provide significant clues on how to explore the immune system to potentially actualize the goals of developing an improve and robust vaccines against malaria parasitemia.

Nevertheless, cytokines are glycoproteins secreted by variety of cells that regulate the host immune response to foreign antigens such as infectious agents, and to other stimuli such as inflammation, and trauma. Cytokines act as immune modulating agents, thus assist in the regulation and development of the effector phases of both innate and specific immune responses through the activities of neutrophil-rich inflammatory responses, and activated T lymphocytes respectively [10, 11]. Some inflammatory cytokines are implicated in the immunopathogenesis of falciparum malaria. Several studies have shown that glycosylphosphatidylinositol (GPIs) from Plasmodium falciparum has toxigenic property which can stimulate tumor necrosis factor- alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and interferon gamma (IFN-γ) in macrophages [12, 13]. Study has also shown, that the naturally acquired anti-glycosylphosphatidylinositol antibodies in the serum of malaria patients neutralize GPIs and thereby, may provide immune-resistance against clinical symptoms of severe malaria [13]. Furthermore, similar study suggested that changes in concentrations of some inflammatory cytokines and chemokines in plasmodiasis may prove useful in evaluating either the progression or the retrogression of uncomplicated to severe falciparum malaria [14]. It has been reported that excessive production of proinflammatory cytokine, tumor necrosis factor (TNF), can cause pathology while early production of proinflammatory T helper type 1 (Th1) cytokines, tumor necrosis factor (TNF), interleukin-12 (IL-12), and possibly interferon-gamma (IFN-γ) may limit the progression from uncomplicated malaria to severe, and other life-threatening complications [14]. Several studies agree that Th1 responses are important for clearance of falciparum malaria. Hence, elevated levels of interleukin-6 (IL-6), and interleukin-10 (IL-10), were observed in serum of patients with severe P. falciparum malaria [14, 15]. In many parts of Africa, interventions such as the use of long-lasting insecticidal nets (LLINs), and WHO-recommended chemotherapies have reduced the burden of malaria [16]. However, several studies incriminated such prevention strategies of enhancing the upsurge of drug-resistant Plasmodium falciparum, and insecticide-resistant female Anopheles mosquitoes [17, 18, 19]. This study measured absolute parasitaemia and variations in inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-10) among falciparum malaria-infected-children in Port Harcourt, Rivers State, Nigeria. Nonetheless, it is therefore strongly believed that data and robust recommendations that would be generated from this present study would provide enough leverage, background and massive workable practical impetus that would improve on prompt diagnosis, management and prevention of malaria parasitemia among children in the region.

Materials and Methods

Study area

This cross-sectional study was conducted in Health facilities in Port Harcourt (Braithwaite Memorial Hospital and College of Health and Technology Health Services Clinics) between the months of July, 2017 to October, 2018. Nonetheless, Port Harcourt is the largest city in Rivers State, located at 4° 45’ N 6° 50’ E / 4.750 ’ N 6.833° E of Nigeria within the oil-rich Niger Delta, South-South geopolitical zone of Nigeria. The climate of the area is characterized by rainy and dry seasons. The rainy season starts in May and last till October, with July and August as Months of peak rainfall. The dry season which starts from December to March are marked with periodic harmattan and very dusty. The vegetation of the study area reflects that of the mangrove rainforest zones. Anopheles gambiae complex is the main malaria vector in Nigeria while P. falciparum is the most prevalent malaria species [7, 20, 21].

Ethics statement

Ethical permission was obtained from the State Ministry of Health and Rivers State University Teaching Hospital, Port Harcourt ethical committee respectively. Also, informed consent was obtained from participants or their respective parents or guardians before sample collection process was activated.
Exclusion criteria

Children with mixed infection of malaria parasites and those with overt chronic infections were excluded. Also children whom their parents could not consent to be included in the study was out rightly removed from the study population, even as adults were removed from the study.

Inclusion criteria

Those who their parents consented were included in the study. Only children were recruited for the study, even as those with chronic illness were carefully excluded from the study.

Experimental

A total of 160 blood samples were selected for the study out of which, eighty (80) samples (n=80) were positive for *P. falciparum* parasites while another 80 samples were (n=80) negative for *P. falciparum* parasites from children, thus those negative samples were used as healthy matched controls settings for the study.

About 5mls of venous blood were collected aseptically into EDTA container, labelled with barcode and taken to laboratory for analysis. Thick and thin malaria blood films were made as described by Cheesbrough [22]. The slides were stained with 3% solution of Giemsa stain as described by World Health Organisation procedure protocol [23]. The presence of *P. falciparum* parasitaemia was determined by microscopic examination of thick and thin smears using x100 oil immersion objective field respectively.

Determination of absolute parasite density

The absolute parasite density of the children infected with *P. falciparum* parasites was determined and calculated as described by Agomo et al. [24]; the total leucocyte count determination for each sample was done using Sysmex XP-300 automated haematology analyzer according to the manufacturers’ instructions.

Determination of degree of Parasitaemia

The degree of parasitaemia was graded as described by Adesina et al. [25] Parasitaemia with absolute parasite density of less than 1000 per micro litre of blood (<1000 parasites/µl) was graded as low parasitaemia; parasitaemia with absolute parasite density of greater than 1000 per micro litre of blood but less than 10,000 per micro litre of blood (1000 to 9,999 parasites/µl) was graded as moderate parasitaemia while parasitaemia with absolute parasite density of greater than 10,000 per micro litre of blood (>10,000 parasites/µl) was graded as high parasitaemia.

Determination of cytokine concentration in plasma

The *in vitro* quantitative determination of cytokine concentrations in plasma such as human IL-6, IL-1β, TNF-α, and IL-10 were measured using the sandwich enzyme-linked immunosorbent assays (ELISAs) technique with Human IL-6, IL-1β, TNF-α, and IL-10 Enzyme Linked Immunosorbent Assay Kits according the manufacturers’ instructions (Catalog Numbers: E-EL-H0102; E-EL-H0149; E-EL-H0109; and E-EL-H0103 Elabscience® , USA). After securing the desired number of pre-coated micro ELISA plate wells in the micro-well holder, 100 µl of standards of various concentrations, samples and controls were dispensed into appropriate wells. The micro-plate wells were covered with the sealer provided in the kit and incubated for 90 minutes at temperature of 37 degree centigrade (37 °C). This was followed by removal of the liquid out of each well into the sink, then the plate was placed face down on absorbent paper with some force to remove any remaining liquid from the wells. Immediately 100µl of biotinylated detection antibody was added to each well, covered with the plate sealer, gently mixed up and incubated for 60 minutes at 37 °C. After incubation, the solution was aspirated from each well, 350µl of wash buffer was added to each well, soaked for one to two minutes and was further aspirated from each well. Aspiration and washing was repeated thrice, while the remaining liquid from the wells were removed by striking the wells sharply with the plate face down on absorbent paper at the end of washing. Then, 100µl of HRP conjugate working solution was added into each well. It was covered with the plate sealer and gently mixed and incubated for 30 minutes at 37 °C. Then, the plate was aspirated and washed thoroughly for five times. Ninety microlitres (90µl) of substrate reagent was added to each well, was covered with new plate sealer, and incubated for 15 minutes at 37 °C in the dark.

Following incubation, the reaction was stopped by adding 50µl of stop solution to each well. A qualitative result was observed with respect to sample colour changed from blue to yellow completely. The optical density value of the coloured solution in each well at once, was determined using a micro-plate reader (Rayto Microplate Reader RT-2100C, China) at 450nm wavelength. Analysis was performed in duplicates. Cytokines concentrations (pg/µL) were determined using the standard curve and multiplied by the dilution factor. The detection range of cytokine assayed was 7.81-500 pg/µL for IL-1β, IL-6, TNF-α, and IL-10.

Statistical Analysis

The data generated from this study were analysed using tables and statistical tools. Statistical analysis was carried out on the various groups of data generated from this study with the aid of Microsoft Excel version 2007 and statistical package for social sciences (SPSS) version 23, using analysis of variance (ANOVA), Pearson correlation tests, and student t-tests. Results were presented in graphs and tables. The level of statistical significance was set as *P* < 0.05.

Results

Table 1 shows the gender-related distribution of children infected with *P. falciparum* parasites. The male had higher prevalence 51.3%, compared with the female children who had a prevalence of 48.7%, however, the difference between them was not statistically significant (χ²=0.0499; *P* > 0.05).
Table 1: Distribution of Children Infected with *P. falciparum* Malaria by Gender.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Infected (%)</th>
<th>Uninfected (%)</th>
<th>(\chi^2)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>41 (51.3)</td>
<td>40 (50.0)</td>
<td>0.0246</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Female</td>
<td>39 (48.7)</td>
<td>40 (50.0)</td>
<td>0.0253</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Total</td>
<td>80</td>
<td>80</td>
<td>0.0499</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

Table 2: Comparisons of Mean SEM Parasitaemia of *P. falciparum* Malaria-infected Children by Age.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>No. Infected (%)</th>
<th>Mean ± SE Parasitaemia (Parasites/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-5</td>
<td>43 (53.75)</td>
<td>4213.95±434.67</td>
</tr>
<tr>
<td>6-8</td>
<td>23 (28.75)</td>
<td>2324.62±546.63</td>
</tr>
<tr>
<td>9-11</td>
<td>9 (11.25)</td>
<td>4813.09±1180.05</td>
</tr>
<tr>
<td>12-14</td>
<td>5 (6.25)</td>
<td>3379.60±962.80</td>
</tr>
<tr>
<td>F</td>
<td>2.725</td>
<td>2.758</td>
</tr>
</tbody>
</table>

Table 3 shows comparisons of the mean concentrations of cytokines and degree of parasitaemia among *P. falciparum* malaria-infected children. Tumor necrosis factor alpha (TNF-α) had a mean concentration of 88.64 ± 20.42 pg/ml in severe parasitaemia, 81.79 ± 6.36 pg/ml in moderate parasitaemia, and 72.77±10.55 pg/ml in mild parasitaemia. Comparisons of the mean concentrations of TNF-α in various degree of parasitaemia was not statistically significant (\(P=0.6857\)). The mean concentration of IL-1β was higher in severe parasitaemia (24.67±2.00 pg/ml) compared with moderate parasitaemia (20.39±1.00 pg/ml) and mild parasitaemia (18.45±1.38 pg/ml). However, comparisons of their means showed no statistically significant difference (\(P=0.1816\)). Interleukin-6 (IL-6) had a higher mean concentration in severe parasitaemia (365.30±40.31 pg/ml), 172.48±19.61 pg/ml in moderate parasitaemia and 111.44±26.96 pg/ml in mild parasitaemia. Comparisons of their mean concentrations showed a statistical significant difference (\(P=0.0014\)). Interleukin-10 (IL-10) had mean concentrations of 318.57±39.29 pg/ml in severe parasitaemia, 230.26±16.08 pg/ml in moderate parasitaemia and 178.51±23.85 pg/ml in mild parasitaemia. Comparisons of the mean concentrations showed a statistically significant difference (\(P=0.0347\)).

Citation: Azuonwu O, Wokem GN, Dimkpa FB. Differential Molecular Investigation Assay of Tumor Necrosis Factor –Alpha (TNF-α), Interleukin-1 Beta (IL-1β), Interleukin-6 (IL-6), and Interleukin 10 (IL-10) among Children Potentially Diagnosed of *Falciparum*-Malaria of Niger Delta Extract. LOJ Med Sci 4(2)-2019. LOJMS.MS.ID.000181. DOI: 10.32474/LOJMS.2019.04.000181.
Table 3: Comparisons of Mean SEM of Cytokine and degree of Parasitaemia among Infected Children.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mild (1-999 Parasites/µl)</th>
<th>Moderate (1000-9999 Parasites/µl)</th>
<th>Severe (&gt;10,000 Parasites/µl)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-Iβ (pg/ml)</td>
<td>5.23 ± 0.56</td>
<td>20.12 ± 0.78</td>
<td>218.90 ± 12.82</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>17.85 ± 23.85</td>
<td>230.26 ± 16.08</td>
<td>318.57 ± 39.29</td>
<td>0.0347</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>72.77 ± 10.55</td>
<td>81.79 ± 6.36</td>
<td>88.64 ± 20.42</td>
<td>0.6857</td>
</tr>
</tbody>
</table>

Table 4: Comparisons of Mean SEM Cytokines Levels of P. falciparum Malaria Infected Children and Un-infected Children.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Un-infected n=80</th>
<th>Infected n=80</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (pg/ml)</td>
<td>30.46 ± 1.74</td>
<td>79.74 ± 5.23</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-Iβ (pg/ml)</td>
<td>6.46 ± 0.56</td>
<td>20.12 ± 0.78</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>25.69 ± 2.54</td>
<td>167.80 ± 16.37</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>20.12 ± 0.78</td>
<td>218.90 ± 12.82</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 5.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TNF-α (pg/ml)</th>
<th>IL-Iβ (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un-infected</td>
<td>30.46±1.74</td>
<td>6.46±0.56</td>
<td>25.69±2.54</td>
<td>20.12±0.78</td>
</tr>
<tr>
<td>Infected</td>
<td>79.74±5.23</td>
<td>20.12±0.78</td>
<td>167.80±16.37</td>
<td>218.90±12.82</td>
</tr>
</tbody>
</table>

Figure 4: Positive Correlation between Parasite Density and IL-10 Plasma Levels in Children Infected with P. falciparum (r=0.28; P=0.013).

Table 4 shows the mean concentrations of cytokines such as TNF-α, IL-Iβ, IL-6 and IL-10 evaluated in P. falciparum malaria-infected children by treatment. The concentration of TNF-α was 30.46 ± 1.74 pg/ml in uninfected children and 79.74 ± 5.23 pg/ml in infected children. Comparisons of their mean concentrations showed a statistically significant difference (P<0.0001). Interleukin-1 beta (IL-Iβ) had a mean concentration of 6.46±0.56 pg/ml in uninfected children and 20.12±0.78 pg/ml in infected children (P=0.0001). Interleukin-6 (IL-6) had a mean concentration of 25.69±2.54 pg/ml in uninfected children and 167.80±16.37 pg/ml in infected children (P<0.0001). The mean concentrations of IL-10 was 26.64±6.47 pg/ml and 218.90±12.82 pg/ml in uninfected and infected children respectively, and the comparisons of the mean concentrations of IL-10 was statistically significant (P<0.0001).

References


Citation: Azuonwu O, Wokem GN, Dimkpa FB. Differential Molecular Investigation Assay of Tumor Necrosis Factor –Alpha (TNF-α), Interleukin-1 Beta (IL-1β), Interleukin-6 (IL-6), and Interleukin 10 (IL-10) among Children Potentially Diagnosed of Falciparum-Malaria of Niger Delta Extract. LOJ Med Sci 4(2)-2019. LOJMS.MS.ID.000181. DOI: 10.32474/LOJMS.2019.04.000181.


