Molecular detection of *Plasmodium* Species in Four Southern States of Nigeria

Tolulope Alade¹, Dibo Pughikumor², Ebidor Lawani³, Bukola Fabunni⁴, Mirabeau Tatfeng⁵

Abstract: This study investigated the *Plasmodium* species polymorphism in the four Southern states of Nigeria (Bayelsa, Rivers, Edo and Delta). Eight hundred and forty six (846) subjects participated in the study. Malaria parasites identification was carried out using standard parasitological techniques. Genotyping of *Plasmodium* species was carried out by Polymerase Chain reaction. Species polymorphism was determined by nested PCR. Results showed that the prevalences of malaria infection by states were 78.1%, 68.8%, 62.8% and 58.8% in Bayelsa, Rivers, Edo and Delta respectively. Children below the age of 5 years were more susceptible to infection (P<0.01). Species polymorphism were *Plasmodium falciparum* (Pf) (39.2%), *Plasmodium vivax* (Pv) (8.8%), and *Plasmodium ovale* (Po) (17.0%). In the four Southern states, Bayelsa had the highest prevalence of *Plasmodium falciparum* (62.5%), followed by Delta (47.4%), Rivers (40.5%) and the least prevalence from Edo state, also Edo state had the highest prevalence of *Plasmodium vivax* (11.3%) followed by Delta 9.3%, Rivers 9.2% while Bayelsa had the least prevalence 0%, also Bayelsa had the highest prevalence of *Plasmodium ovale* (22.5%) followed by Rivers 18.3%, Delta 15.5% and Edo state had the least prevalence. Co-infection existed between Pf and Pv was (7.1%) Pf and Po was (15.4%), Pv and Po was (6.0%). Multiple infection of the species Pf, Pv and Po was (6.0%). This calls for urgent intervention to maintain drug policy in treatment of each of the *Plasmodium* species not only falciparum.

Keywords: *Plasmodium* species, polymorphism, Southern states, Polymerase Chain Reaction

1. Introduction

Malaria is an intense disease which was triggered by the protozoan parasites of genus *Plasmodium* by which *falciparum* species being the most harmful. In 2015, 212 million malaria incidence was assessed universally and 429 thousand demises. The findings about the infection were not relied on only medical indications but also on some non-detailed signs presented by other diseases. In view of this, malaria causes repeated problems for diagnosis and treatment in malaria endemic region in the Southern states. Malaria symptoms were collectively described as headache, fever, fatigue, loss of appetite, body pains, nausea, vomiting, dry cough, confusion and respiratory distress (Mary et al., 2017). The occurrence of febrile periods can be determined by the parasite species, *P. vivax* and *P. ovale* occurring every 48 hours, *P. malariae* every 72 hours and *P. falciparum* every 24 hours. In an immunocompromised patient, malaria occurs rapidly with some sensation of sickness and fever up to 39°C or more. The typical technique for malaria findings is relied on the Giemsa-stained thin and thick blood films by light microscopy. This microscopy is fast, easy to accomplish and sensitive, but it needs high technical know-how. Rapid diagnostic tests (RDTs) for antigen discovery were used alongside with microscopy or especially where there was restricted contact with decent microscopy facilities. RDTs are immuno chromatographic tests directing toward antigens of one or more *Plasmodium* species (Ansa et al., 2010) although this analysis was not specific and sensitive enough because of false positive reaction which may occur after treatment due to the parasite antigen which may still be in blood circulation. Furthermore, *Plasmodium* genus and species-specific nested Polymerase chain reaction (PCR) assays were established for malaria diagnosis which had more advantage over microscopy and RDT in that PCR is extremely specific and sensitive.

2. Materials and Methods

Study area

The study area was the five Niger Delta states, Bayelsa located at Latitude (04° 15′ North) (05 023° South), Longitude 05 022° West and 06 045 East. Delta State located at 5°30′ North 6° East 5/5000N 6,000E, Edo state located on Longitude 06.04E and 06.43E and Latitude 05.44N and 7.34N, River state located on Latitude 26°25 and 27°40 North, Longitude 73°10 and 75°15E, Cross River state located at 04°15 and 5°N and Longitude 8°25E

Ethical approval

Ethical approval was sought for and received from the Ethical committee of the teaching hospitals,

Sample collection/ Sample size

Eight hundred and forty six whole blood samples were collected, 2mls of venous blood was collected into EDTA bottle from each of the teaching hospitals across the states.

Microscopical Examination

Thick blood films were made on a clean glass slide and stained with 10% Giemsa stain (see appendix) for 30mins after which the stain was washed off using buffered water, air dried and examined under the microscope using X100 oil immersion objectives (WHO, 2010). The stained thick smear was used for detection of the presence of malaria parasite and estimation of malaria parasite density whereas the thin film was for species identification.

Rapid Diagnostic Test

SD BIOLINE, a rapid diagnostic test kit that detects histidine-rich protein 2 (HRP-2) specific for *P. falciparum* was used for screening alongside the microscopic technique. The kit has 99.7% sensitivity and 99.5% specificity for *P. falciparum* (Akindele et al., 2003)
DNA Extraction
DNA was extracted using the quick gDNA mini prep DNA extraction kit supplied by Inqaba Biotechnological, South Africa. One hundred microliter (100µl) of whole blood was pipetted into a microcentrifuge tube, and four hundred microlitre (400µl) of Genomic lysis buffer was added. The samples were mixed by vortexing for five seconds and were allowed to stand at room temperature for ten minutes. The mixture was transferred to a zymo-spin column in a collection tube. It was centrifuged at twelve thousand revolutions per minute (12,000rpm) for one minute. The flow through and the collection tubes were discarded. The zymo-spin column was transferred to a new collection tube and two hundred microlitres (200µl) of DNA pre-wash buffer was added and centrifuged at twelve thousand revolutions per minute for one minute. Five hundred microlitre (500µl) of g-DNA wash buffer was added to the spin column and centrifuged at the 12,000rpm for 1 minute. The spin column was transferred into a 1.5 micro centrifuge tube and one hundred microlitres (100µl) of DNA elution buffer was added to the spin column and incubated at room temperature for five minutes and centrifuged at 14,000rpm for 30secs to elute the DNA.

Plasmodium Species Polymorphism
Nested PCR was used for the Plasmodium falciparum genus of 0.4C of primers both forward rPLU6TTAAAAATTGTTGCAGTTAAAACG and reverse primers rPLUS5CGTGTGTGGTGCCTTAAACCTTC in a final volume of 25ul PCR conditions 94°C x3mins, 94°C x1min,60°C x2mins, 72°C x1:30mins, 72°C x5mins for 30cyclesand each of the species-specific primers were used for P. falciparum FAL1: TTTAAGTGGTGGGAAAACCAAAATATT CTCAATCATGACT and FAL 2: ACACAATGAACCTAATCATGACTACGTCGA, VIV1: CGCTCTAGCTTAACCACATAACTGATAC, VIV2:ACCTTCCAAGCCGAAGCAAAGAAAGTCCTTA, MAL1: ATAACATAGTTATCGTTAAGAATAACCGC, MAL 2: AAAATTCCTACGTCAAAAATTATACAA at PCR conditions of 94°C x 3mins, 94°C x 1min, 50°C x 1:45mins, 68°C x 1:30mins, 68°C x7mins OVACTGTCTTTGCAATCATTCTAC RVS COMMON GTATCTGATCGTCTTACATCCC 94°C x3mins, 94°C x1min, 57°C x1:45mins, 68°C x 1:30mins, 68°C x7mins for 45cycles.

Statistical analysis: Graph pad prism was used for the statistical analysis.

3. Results
Table 1: Prevalence of malaria by age across the states

<table>
<thead>
<tr>
<th>Age (yrs)</th>
<th>Bayelsa</th>
<th>Rivers</th>
<th>Edo</th>
<th>Delta</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE</td>
<td>NI</td>
<td>NE</td>
<td>NI</td>
<td>NE</td>
<td>NI</td>
</tr>
<tr>
<td>0-5</td>
<td>37</td>
<td>36</td>
<td>68</td>
<td>63</td>
<td>28</td>
</tr>
<tr>
<td>6-10</td>
<td>21</td>
<td>18</td>
<td>39</td>
<td>30</td>
<td>36</td>
</tr>
<tr>
<td>11-15</td>
<td>4</td>
<td>3</td>
<td>22</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>16-20</td>
<td>21</td>
<td>18</td>
<td>24</td>
<td>12</td>
<td>28</td>
</tr>
<tr>
<td>21-25</td>
<td>19</td>
<td>12</td>
<td>13</td>
<td>6</td>
<td>21</td>
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<tr>
<td>26-30</td>
<td>36</td>
<td>24</td>
<td>12</td>
<td>9</td>
<td>21</td>
</tr>
<tr>
<td>31-35</td>
<td>17</td>
<td>15</td>
<td>11</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>36+</td>
<td>37</td>
<td>24</td>
<td>29</td>
<td>12</td>
<td>39</td>
</tr>
<tr>
<td>Total</td>
<td>192</td>
<td>150</td>
<td>218</td>
<td>150</td>
<td>196</td>
</tr>
</tbody>
</table>

Key: NE-Nos examined
NI- Nos infected

Table 2: Distribution of Subjects by Gender and by States

<table>
<thead>
<tr>
<th>Gender</th>
<th>Bayelsa</th>
<th>Rivers</th>
<th>Edo</th>
<th>Delta</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>107</td>
<td>120(25.9)</td>
<td>118(28.5)</td>
<td>118(28.5)</td>
<td>463(54.7)</td>
</tr>
<tr>
<td>Female</td>
<td>85(22.2)</td>
<td>98(25.6)</td>
<td>78(20.4)</td>
<td>122(20.4)</td>
<td>383(43.3)</td>
</tr>
</tbody>
</table>

In total, of the 463 males participant 303 (65.4%) were infected with malaria while of the 383 females participants, 261(68.1%) were infected with malaria given a total of 564 (66.7%) of the 846 participants that were infected with malaria within the four Southern States.
Figure 3: Chart showing co-infection of *Plasmodium* species in the four Southern states

Plate 1: Gel electrophoresis showing *Plasmodium falciparum* prevalence, Lane 1-15 represent the sample, lane M represent the quick load 100bp molecular ladder.

Plate 2: Gel electrophoresis showing *Plasmodium vivax* (120bp) Lane 1-11 represent the samples while Lane M represents the Quick-Load 100bp.
Males were more affected by malaria infection in the study which could be due to their nonchalant attitude in taking treatment on time.

Plasmodium is considered as one of the universal health difficulties in Nigeria particularly amid children aged below five years and pregnant women across the endemic region. In Africa region there was a prevalence of 91% and 86% of these were children below five years of age. Malaria is the 3rd important origin of death for children under five years worldwide. Malaria is also 2nd leading cause of death from infectious disease in Africa after HIV (AIDS) (WHO, 2010).

Species Polymorphisms in four Southern states

The species polymorphisms across four southern states showed that *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* distribution mono-infection in four Southern states (62.5%, 0%, 22.5%), (40.5%, 9.2%, 18.3%), (27.3%, 11.3%, 15.1%), (47.4%, 9.3%, 15.5%) for Bayelsa, Rivers, Edo and Delta respectively. Also co-infection existed among *P. falciparum* and *P. vivax*, *P. falciparum* and *P. ovale*. *P. vivax* was (0%, 15.0%, 0%), (4.5%, 13.7%, 6.9%), (11.3%, 16.4%, 8.8%), (6.2%, 15.5%, 3.1%) for Bayelsa, Rivers, Edo and Delta respectively and there was multiple infection of *P. falciparum*, *P. vivax* and *P. ovale* 0%, 6.9%, 8.8% and 3.1% for Bayelsa, Rivers, Edo and Delta states. There was no polymorphism for *Plasmodium malariae* in the course of this study. There existed a statistical difference among the species (P<0.0001).

4. Discussion

Malaria is considered as one of the universal health difficulties in Nigeria particularly amid children aged below five years and pregnant women across the endemic region. In Africa region there was a prevalence of 91% and 86% of these were children below five years of age. Malaria is the 3rd important origin of death for children under five years worldwide. Malaria is also 2nd leading cause of death from infectious disease in Africa after HIV (AIDS) (WHO, 2010).

PCR performed better than blood-slide expert microscopy and HRP- RDT in this study. This work is in agreement with the work done by (Mosha et al., 2013, Cordray et al., 2012) that Polymerase chain reaction (PCR) is observed as one of the few utmost sensitive molecular methods for identifying parasites as low as 0.01–0.2 parasites/μL of blood.

**Table 2:** Species polymorphisms of Plasmodium species in the four Southern states

<table>
<thead>
<tr>
<th>States</th>
<th>Species %</th>
<th>Co-Infection</th>
<th>Multiple infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bayelsa</td>
<td>P. fal (62.5) 0(0.0) 18(22.5)</td>
<td>0</td>
<td>12(15.0) 0</td>
</tr>
<tr>
<td>Rivers</td>
<td>53(40.5) 12(9.2) 24(18.3)</td>
<td>6(4.5) 18(13.7)</td>
<td>9(6.9) 9(6.9) 131</td>
</tr>
<tr>
<td>Edo</td>
<td>65(27.3) 27(11.3) 36(15.1)</td>
<td>27(11.3) 39(16.4)</td>
<td>21(8.8) 21(8.8) 238</td>
</tr>
<tr>
<td>Delta</td>
<td>46(47.4) 9(9.3) 15(15.5)</td>
<td>6(6.2) 15(15.5)</td>
<td>3(3.1) 3(3.1) 97</td>
</tr>
<tr>
<td>Total</td>
<td>214(39.2) 48(8.8) 93(17.0)</td>
<td>39(7.1) 84(15.4)</td>
<td>33(6.0) 33(6.0) 546</td>
</tr>
</tbody>
</table>

X²=52.11, df=30, p value < 0.5

Plate 3: Gel electrophoresis showing *Plasmodium ovale* prevalence, Lane 1, 2, 6, 9-13 were successful and lane PCRs performed better than blood-slide expert microscopy and HRP- RDT in this study. This work is in agreement with the work done by (Mosha et al., 2013, Cordray et al., 2012) that Polymerase chain reaction (PCR) is observed as one of the few utmost sensitive molecular methods for identifying parasites as low as 0.01–0.2 parasites/μL of blood.

**Plasmodium falciparum** mono-infection (figure 2, plate 1) accounts for highest prevalence in Bayelsa, (62.5%) followed by Delta (47.4%), Rivers(40.5%) and Edo had the least (27.3%) *P. vivax* mono-infection (figure 2, plate 2) was found in three states but Edo had the highest prevalence of 11.3% followed by Delta (9.3%) and Rivers (9.2%). *P. ovale* (figure 2, plate 3) was found across the four Southern states with Bayelsa of the highest prevalence (22.5%), Rivers (18.3%), Delta (15.5%) while Edo had the least prevalence of 15.1%. Overall prevalence showed that *P. falciparum* had the highest prevalence (60.8%) followed by *P. ovale* (26.4%) and *P. vivax* had the lowest prevalence of (12.8%) this agrees with the study carried out by WHO that *P. falciparum* 98.8%, 1% of *P. malariae* and 0.2% mixed infection of *P. falciparum* with *P. ovale*. *P. falciparum* is the most virulent and also has the greatest propensity for developing resistance. This study also agrees with the report by WHO (2012) that 64% for *Plasmodium vivax*, 36% for *Plasmodium falciparum* also disagrees with the study carried out in Pakistan, Yasinzai et al., 2013 who had increasing number of *Plasmodium falciparum* infection ranging from 25 to 90%. Similar studies in North–Central by Nwandu et al., (2014) reported 60.6% of *Plasmodium falciparum* also in South-East by Mbanu et al., (2015) reported 58%. A study by Kalu et al., (2012) reported 19.5% of *Plasmodium vivax*. In South
America, P. vivax was prevalent (70-90%). Also there was existence of co-infection among the Plasmodium species co-infection among P. falciparum and P. vivax shows (24.5%), P. falciparum and P. ovale shows (29.1%), P. vivax and P. ovale shows 13.1%. Also a study by Rosalyn et al., 2015 in Cameroon recorded 42% and 10.5% for P. vivax and P. ovale respectively. In contrast to the study conducted by Khatoo et al., (2010) that P. falciparum and P. vivax was the most prevalent. Also, in this study multiple infections of P. falciparum, P. ovale and P. vivax were observed. The statistical analysis showed that there was a significant difference among the species found across the states (p value < 0.0001) the rise of P. falciparum may be partially contributable to failed treatment of Chloroquine resistant infections (Nizamani et al., 2006).

5. Conclusion

Molecular detection of Plasmodium species is highly recommended to assist in maintaining drug policy in treating malaria not only caused by falciparum.

References


