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

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Abstract: *This study investigated the Plasmodiumspecies 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 5years 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 indicationsbut also on some non-detailed signs presented by other diseases In view of this malariacauses 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 occurence of febrile periods can be determined by the parasite species, P. vivaxand P. ovaleoccurring every 48 hours, P. malariaeevery 72 hours and P. falciparumevery 24 hours. In an immunocompromised patient, malaria occurs rapidly with simple sensation of sickness and fever up to 39 °C ormore. The typical technique for malaria findings is relied on the Giemsa-stained thin and thick blood films by light microscopy. This microscopy is fast, easyto accomplish and sensitive, but r 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 Plasmodiumspecies(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 g*enus 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 (04^0 15^0 North) (05 023^0 South), Longitude 05 022^0 West and 06 045 East. Delta State located at 5^030^0North 6^0East 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 26^025 and 27^040 North, Longitude 73^010 and 75^015E, Cross River state located at 04^015 and 5^0N and Longitude 8^025E

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 microliter (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 minutes (12,000rpm) for one minute. The flow through and the collection tubes were discarded. The zymo-spin columns were 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 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 rPLU6TTAAAATTGTTGCAGTTAAAACG and reverse primers rPLU5CCTGTTGT TGCCTTAAACTTC in a final volume of 25ul PCR conditions 94^0c x3mins, 94^0c x1min,60^0c x2mins, 72^0c x1:30mins, 72^0c x5mins for 30cyclesand each of the species-specific primers were used for *P. falciparum* FAL1: TTAAACTGGTTTGGGAAAACCAAATATATT CTCAATCATGACTA and FAL 2: ACACAATGAACTCAATCATGACTACCCGTCCA, VIV1: CGCTTCTAGCTTAACCACATAACTGATAC, VIV2:ACCTTCCAAGCCGAAGCAAAGAAAGTCCTTA, MAL1: ATAACATAGTTGTACGTTAAGAATAACCGC, MAL 2: AAAATTCCCATGCATAAAAA ATTATACAAA at PCR conditions of 94^0c x 3mins, 94^0c x1min, 50^0c x 1:45mins, 68^0c x1:30mins, 68^0c x7mins OVACTGTTCTTTGCATTCCTTATGC RVS COMMON GTATCTGATCGTCTTCACTCCC 94^0c x3mins, 94^0c x1min, 57^0c x1:45mins, 68^0c x 1:30mins, 68^0c 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

Age (yrs)	Bayelsa		Rivers		Edo		Delta		Total	
	NE	NI	NE	NI	NE	NI	NE	NI	NE	N1
0-5	37	36	68	63	28	21	9	6	142	126
6-10	21	18	39	30	36	30	39	27	135	105
11-15	4	3	22	15	11	9	14	9	51	36
16-20	21	18	24	12	28	18	40	21	113	69
21-25	19	12	13	6	21	15	26	18	79	51
26-30	36	24	12	9	21	9	25	18	94	59
31-35	17	15	11	3	12	3	20	6	60	27
36+	37	24	29	12	39	18	67	36	172	90
Total	**192**	**150**	**218**	**150**	**196**	**123**	**240**	**141**	**846**	**563**

Key: NE-Nos examined
NI- Nos infected

Table 2: Distribution of Subjects by Gender and by States

Gender	Bayelsa	Rivers	Edo	Delta	Total
Male	107	120(25.9)	118(28.5)	118(28.5)	463(54.7)
Female	85(22.2)	98(25.6)	78(20.4)	122(20.4)	383(45.3)
Total	**192**	**218(25.8)**	**196(23.2)**	**240(28.4)**	**846**

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.
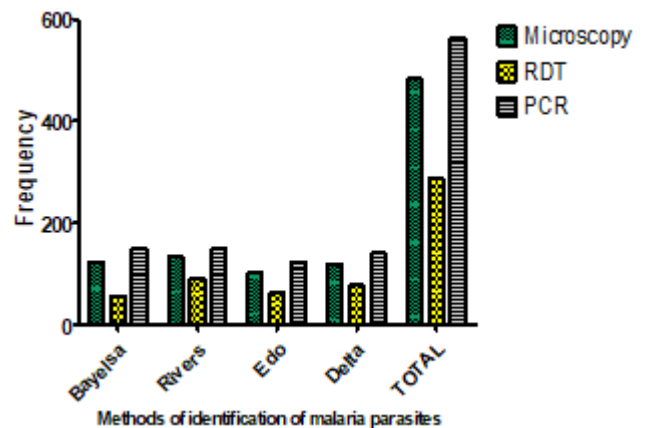

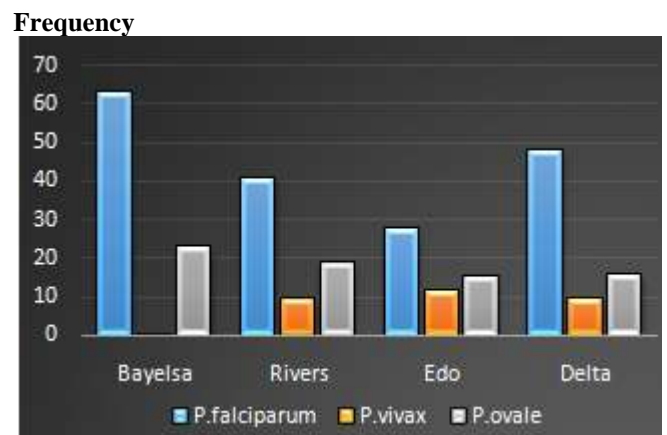**Figure 1:** Methods of identification of malaria parasites

Frequency

Figure 2: Chart showing *Plasmodium species* polymorphism in the four Southern states.
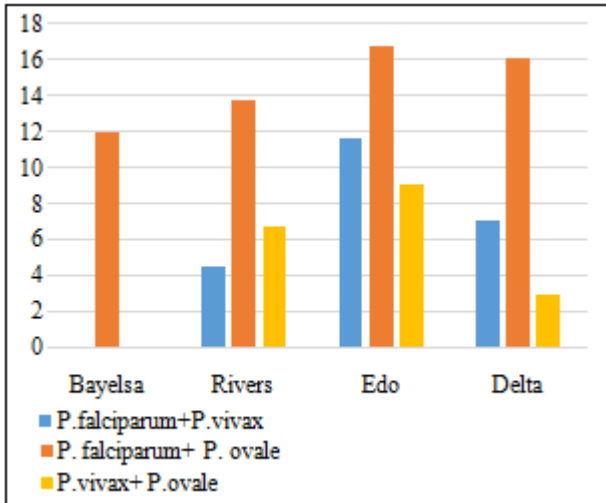
Frequency

Figure 3: Chart showing co-infection of *Plasmodium* species in the four Southern states
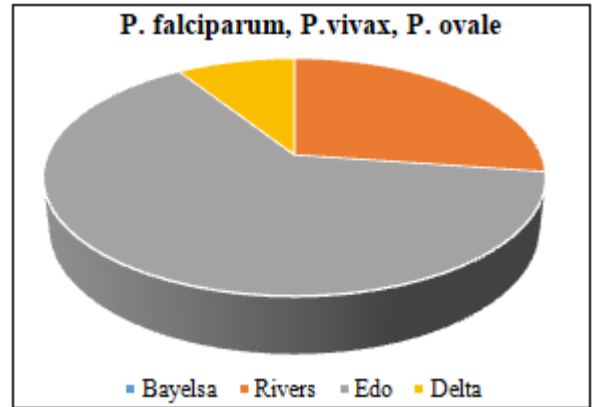

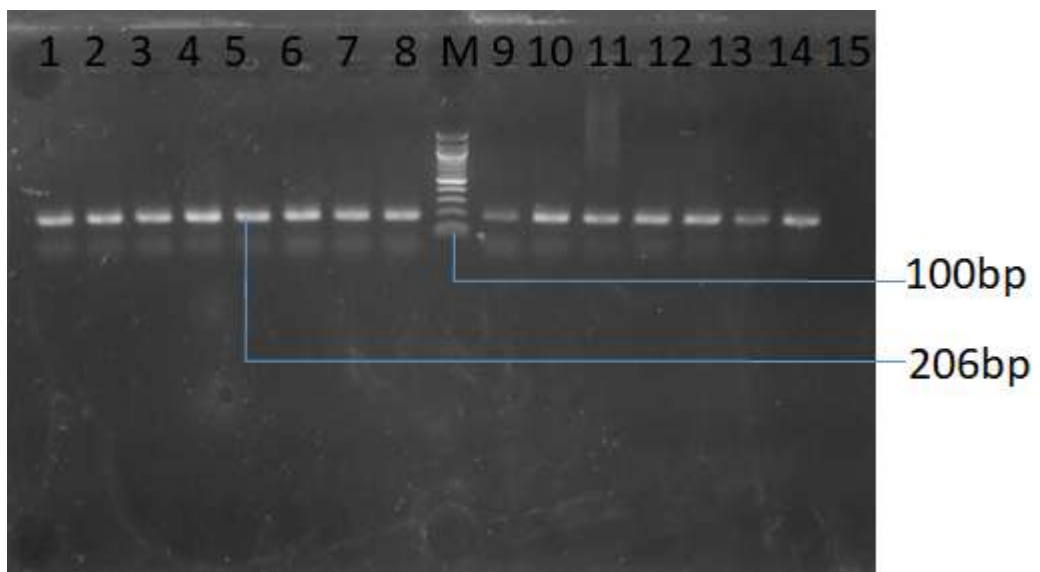**Figure 4:** Chart showing multiple infection of *Plasmodium* species in the four Southern states

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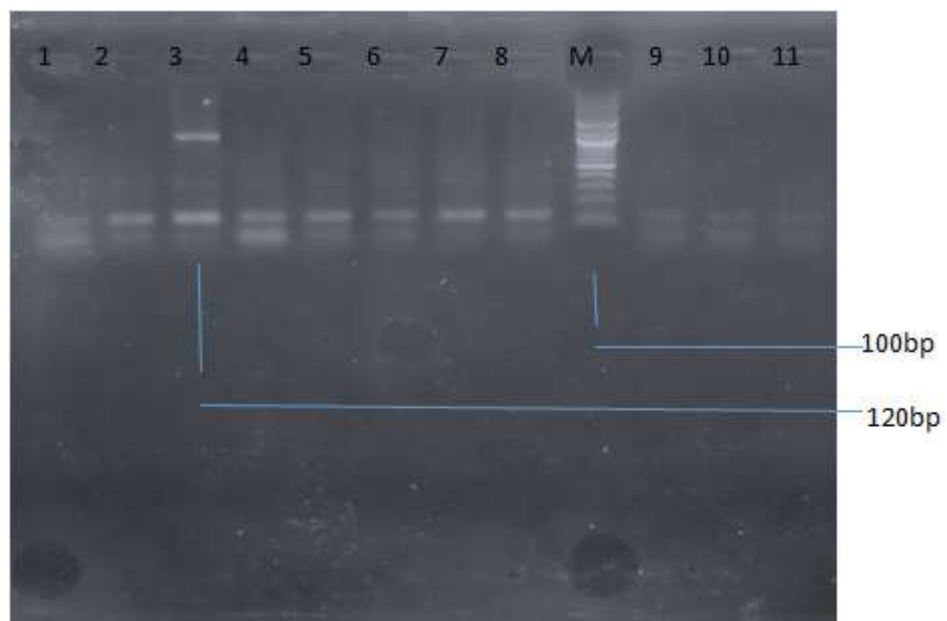

Plate 2: Gel electrophoresis showing *Plasmodium vivax* (120bp) *Lane* 1 -11 represent the samples while Lane M represents the Quick-Load 100bp
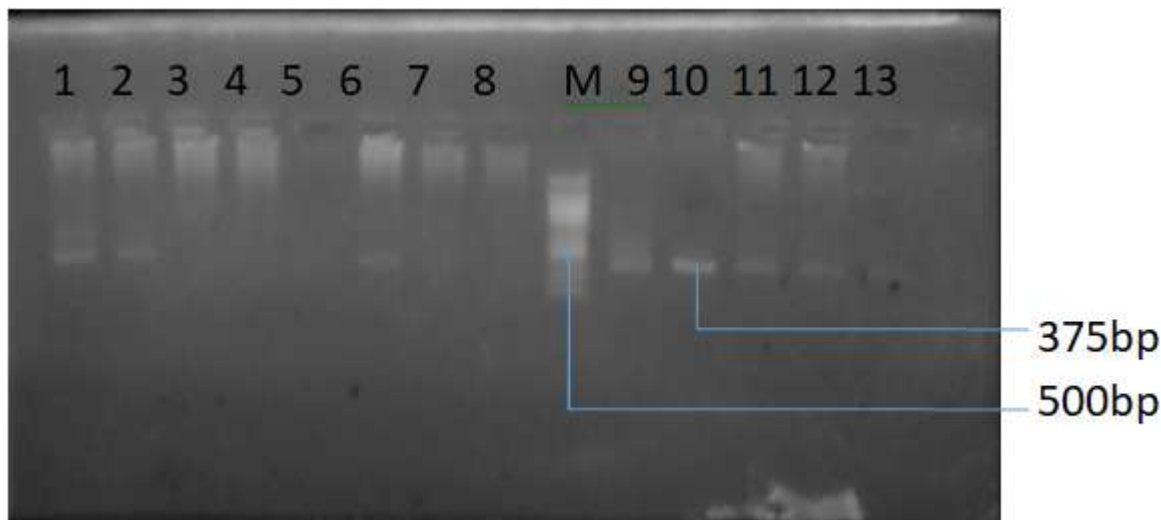
Plate 3: Gel electrophoresis showing *Plasmodium ovale* prevalence, Lane 1, 2, 6, 9-13 were successful and lane

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

States	Species %			Co-Infection		Multiple infection		Total
	P. fal	*P. vivax*	*P. ovale*	*P. fal + P. viv*	*P. fal + P. ova*	*P. viv+ P. ova*	*P.fal + P. viv +P. ova*	
Bayelsa	50(62.5)	0 (0.0)	18(22.5)	0	12(15.0)	0	0	80
Rivers	53(40.5)	12 (9.2)	24(18.3)	6(4.5)	18(13.7)	9(6.9)	9(6.9)	131
Edo	65(27.3)	27(11.3)	36(15.1)	27(11.3	39(16.4)	21(8.8)	21(8.8)	238
Delta	46(47.4)	9(9.3)	15(15.5)	6 (6.2)	15(15.5)	3(3.1)	3(3.1)	97
Total	214(39.2)	48(8.8)	93(17.0)	39(7.1)	84(15.4)	33(6.0)	33(6.0)	546
X^2=52.11, df=30 p value < 0.5								

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 healthdifficulties 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).

Males were more affected by malaria infection in the study which could be due to their nonchalant attitude in taking treatment on time.

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 thefew utmost sensitive molecular methods for identifying parasites as low as 0.01–0.2 parasites/µL of blood.

Plasmodium falciparum monoinfection (figue 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 disagrees 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 disagrees 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 Khatoon *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 asssit in maintaining drug policy in treating malaria not only caused by*falciparum.*

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