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Pfcrt K76T and Pfmdr 1 Resistance Genes in Post Chloroquine Era in South-South Region of Nigeria

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Authors' contributions

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

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ABSTRACT

Malaria, a global health problem especially in the sub-Sahara region has been posing a recurrent problem due to the resistance of the parasites to the available antimalarial drugs despite the preventive measures provided by WHO.

Aims: This study is aimed at determining the prevalence of resistance markers in four Niger Delta states of Nigeria, a decade after withdrawal of chloroquine.

Methods: Eight hundred and forty six (846) subjects participated in the study and were distributed as follows, 192(22.7%) Bayelsa; 218(25.8%) Rivers; 196(23.2%) Edo and 240(28.4%) Delta respectively. Malaria parasite identification was carried out using standard parasitological techniques. Genotyping of the resistance markers Pfcrt K76T and Pfmdr 1 was carried out by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP).

Results: Our findings revealed that the prevalence of malaria infection in the four Niger Delta states were 78.1%, 68.8%, 62.8% and 58.8% in Bayelsa, Rivers States, Edo and Delta respectively. There was no statistical difference in the prevalence of malaria within the four Niger

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Delta states. (P>0.05). Children below the age of 5 years recorded the highest infection rates when compared to subjects in other age groups (P< 0.01). Our findings also revealed that the distribution of mutant Pfcrt K76T and Pfmdr 1 genes across the four states were 12.0% and 28.6%, 4.0% and 22.0%, 14.6% and 29.3%, 10.6% and 25.0% in Bayelsa, Rivers, Edo and Delta state respectively. However, the prevalence of Pfcrt K76T in Rivers State was statistically lower when compared to other states (P < 0.01) while no statistical difference existed in the distribution of Pfmdr 1 mutant genes (P>0.01).

Conclusion: Prevalence of Pfcrt and Pfmdr 1 remained elevated in the Niger Delta states despite the withdrawal of chloroquine over a decade ago. Hence, Nigeria is far from an eventual re-introduction of chloroquine as its resistance markers still persist in our communities. Furthermore, the root cause of the persistence of these resistance markers needs to be investigated.

Keywords: Post chloroquine era; Pfcrt K76T; Pfmdr1; Niger Delta; Nigeria.

1. INTRODUCTION

Malaria is rated as third among the infectious diseases by WHO and is one of the major public health problems in malaria endemic region including south-south region of Nigeria. Chloroquine (CQ) has been the gold standard drug in the treatment of malaria, a sudden rise in *P. falciparum* cases resulted in the astronomical increase in the resistance of malaria against CQ. The import of this resistance is overwhelming resulting in the increase in morbidity and mortality. Chloroquine acts essentially by interfering with heme metabolism in the digestive vacuole of *P. falciparum* and CQ resistance results from reduced accumulation of drug by the parasites [1]. Genetic mutations have been shown to be associated with CQ resistance. There are two genes that were commonly involved which are *P. falciparum* multidrug resistance gene Pfmdr-1 which encodes Pgh-1, a glycoprotein homologue and the CQ resistance transporter gene Pfcrt which codes for CQ transporter protein. The two genes have been identified as the main cause of CQ resistance [1]. Chloroquine resistance has been correlated with mutations in a number of *P. falciparum* genes, although some results have been inconsistent [2]. The initial studies of the *P. falciparum* multidrug resistance gene (pfmdr-1), an AsnrTyr mutation at amino acid 86 (N86Y) and other mutations in the gene correlated with CQ resistance. However, in several fields of studies, associations between pfmdr-1 point mutations and *in- vivo* or *in -vitro* CQ resistance were not consistent. More recently, transfection studies showed that the replacement of mutant pfmdr-1 with the wild-type sequence in resistant parasites decreased CQ resistance from high to moderate levels [3]. Thus, it appears that although mutations in pfmdr-1 are not required for CQ resistance, polymorphisms may play a role in modulating this phenotype.

2. MATERIALS AND METHODS

2.1 Study Area and Sample Collection

The study was carried out in the four Niger Delta states, Bayelsa located at Latitude (04º 15º North) (05 023º South), Longitude 05 022º West and 06 045East. Delta state located at 5º30º North 6ºEast 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 26º25 and 27º40 North, Longitude 73º10 and 75º15E.

Two milliliters of venous blood was collected into EDTA bottle from each patient in each teaching hospital after observing the necessary aseptic measures and transported to the laboratory in cold box.

2.2 Sample Analysis

2.2.1 Malaria parasite screening

Thick blood films were made on a clean glass slide and stained with 10% Giemsa stain for 30 mins after which the stain was washed off using buffered water, it was thereafter air dried and examined under the microscope using X100 oil immersion objectives [4]. The estimation of the malaria parasites and density was carried out using the stained thick smear while the malaria parasites identification was done with the stained thin smear.

2.2.2 Rapid diagnostic test

Five microlitres (5 µl) of whole blood was added into the sample well of the kit cassette, followed

by the addition of four drops(120 µl) of assay buffer vertically into the assay buffer well, the test result was then read after 15 minutes but not exceeding 30 minutes. The presence of two colour bands indicated a positive result, therefore only one band at the C line indicated a negative result while the test was considered invalid when the C line did not appear and was therefore repeated.

2.3 DNA Extraction

DNA was extracted using the quick gDNA mini prep DNA extraction kit supplied by Inqaba Biotechnological, South Africa. One hundred microliter (100 µl) of whole blood was pipetted into a microcentrifuge tube, and four hundred microliter (400 µl) of Genomic lysis buffer was added. The samples were mixed by vortexing for five seconds and then allowed to stand at room temperature for ten minutes. The mixture was transferred to a zymo-spin column in a collection tube and centrifuged at twelve thousand revolutions per minutes (12,000 rpm) for duration of 60 seconds. The flow through and the collection tubes were discarded while zymo-spin columns were transferred to a new collection tube and two hundred microlitre (200 µl) of DNA pre-wash buffer was added and centrifuged at twelve thousand revolutions per minute for a 60 second' duration. Five hundred microliters (500 µl) of g-DNA wash buffer was added to the spin column and centrifuged at 12,000 rpm for 60 seconds. The spin column was thereafter transferred into a 1.5 milliliter micro centrifuge tube and one hundred microlitres (100 µl) of DNA elution buffer was added to the spin column, incubated at room temperature for five minutes and centrifuged at 14,000 rpm for 30 seconds to elute the DNA.

2.4 DNA Quantification

DNA quantification and purity testing was carried out on a Nano-drop 1000 spectrophotometer by loading 2 µl of the extracted product on the lower pedestal. The concentration of the DNA was calculated by the nano drop software installed on a desktop computer.

2.4.1 Detection of the K76T mutations in Pfcrt gene

Nested PCR was used to amplify PFCRT gene, amplification was done at final volume of 20 µl. 1X master mix containing dNTPs, $MgCl_2$, buffer and Taq polymerase with 5 ul and 0.5 µl of DNA template and 0.5 C of both forward and reverse primers were used respectively for the PCR reaction Primers for primary amplification used were CRTP F: CCGTTAATAA TAAATACACGCAG CRTP R: GCATGTTA CAAAACTATAGTTACC and secondary amplification were ICRT/F: TGTGCTCATGTGTT TAAACTT ICRT/R: CAAAACTATAGTTACCAA TTTTG. PCR conditions for primary amplification were as follows: Initial denaturation temperature of 95ºC for 5 minutes, denaturation at 92ºC for 30 seconds, annealing at 56ºC for 40 seconds and initial extension at 65ºC for 1:30 seconds followed by final extension at 65ºC for 8 minutes after the 35th cycle. The PCR condition of the secondary are the same with the primary, but differ from that of the annealing temperature (47ºC for 40 seconds), initial extension (65ºC for 1.30 seconds), final extension (65ºC for 8 minutes), and the number of cycles was 30 (527 bp and145 bp).

2.4.2 Restriction digestion with Apo I

The amplified segment contained both a monomorphic and a polymorphic restriction site, APO1 thus excised products containing AAA, generating 2 fragments of 99 and 46bp. Appropriate control of *Plasmodium* genome was involved in the PCR-RFLP protocol, *P. falciparum* 3D7 (genotype Pfcrt 76K and Dd2 (genotype 76T) [5]. PCR products of 5 µl were digested overnight at 50ºC with the mutation-specific restriction enzymes APO1 to detect the PfcrtK76T. In PCR product, the DNA sequence was cleaved at the wild-type into two fragments 99 and 46 while the mutant allele was not cut. The digested products were resolved by electrophoresis in a 2% agarose gel tinted with ethidium bromide which was visualized by ultra violet trans-illuminator.

2.4.3 Detection of N86Y mutation in Pfmdr 1 gene

Nested PCR was used to amplify Pfmdr "86"gene, amplification was done at final volume of 20 µl. 1X master mix containing dNTPs, $MgCl_2$, buffer, and Taq polymerase with the DNA template of 5 µl and 0.5 µl for primary and secondary amplification respectively. Primers used for primary amplification were P1: ATGGGTAAAGAGCAGAAAGA P2: AACGC AAGTAATACATAAAGTCA and the secondary were P3: TGGTAACCTCAGTATCAAAGA P4: ATAAA CCTAAAAAGGAACTGG. Extracted

DNA samples for primary were amplified by an initial denaturation temperature of 94ºC for 3 min, denaturation at 94ºC for 30 seconds, annealing at 48ºC for 45 seconds and initial extension at 65ºC for 1min, followed by final extension at 65ºC for 7 minutes after the 40th cycle. The PCR condition of the secondary are the same but differ from that of the primary in the final extension temperature (65ºC for 5 minutes) and the run was for 25 cycles.

2.4.4 Restriction digestion with Afl III

The aliquot from nested amplification of Pfmdr 1 was also subjected to restriction digest with the enzymes Afl111 (New England Biolabs) at an incubation time of 37ºC for 3 hours. AFL111 cuts the PCR product after a mutation occurred at codon 86 try (TAT) to generate fragments 200 and 301 base pairs. The digested products were resolved in 2% agarose gel tinted with ethidium bromide and visualized under UV transilluminator [5].

3. RESULTS

Fig. 1 shows the distribution of malaria infection with the highest prevalence recorded in Bayelsa while Edo, the lowest.

Subjects between the ages of 0-5 had the highest burden of the malaria infection.

Females were the most frequently affected subjects by malaria infection.

The PCR presented the most reliable method of detecting malaria infection.

Bayelsa and Rivers had the highest rate of both the wild (WT) and mutant (MT) types genes with the lowest seen in Edo.

The female subjects in Bayelsa and Rivers had the highest rate of both the wild (WT) and mutant (MT) types genes with the lowest seen in Delta. Although, the male had the highest prevalence of malaria infection.

Children between the ages of 0-5 in Rivers had the highest rate of both the wild (WT) and mutant (MT) types genes with the lowest seen in Bayelsa.

The PFMDR gene was highest among the subjects in Rivers with the lowest occurrence in Bayelsa.

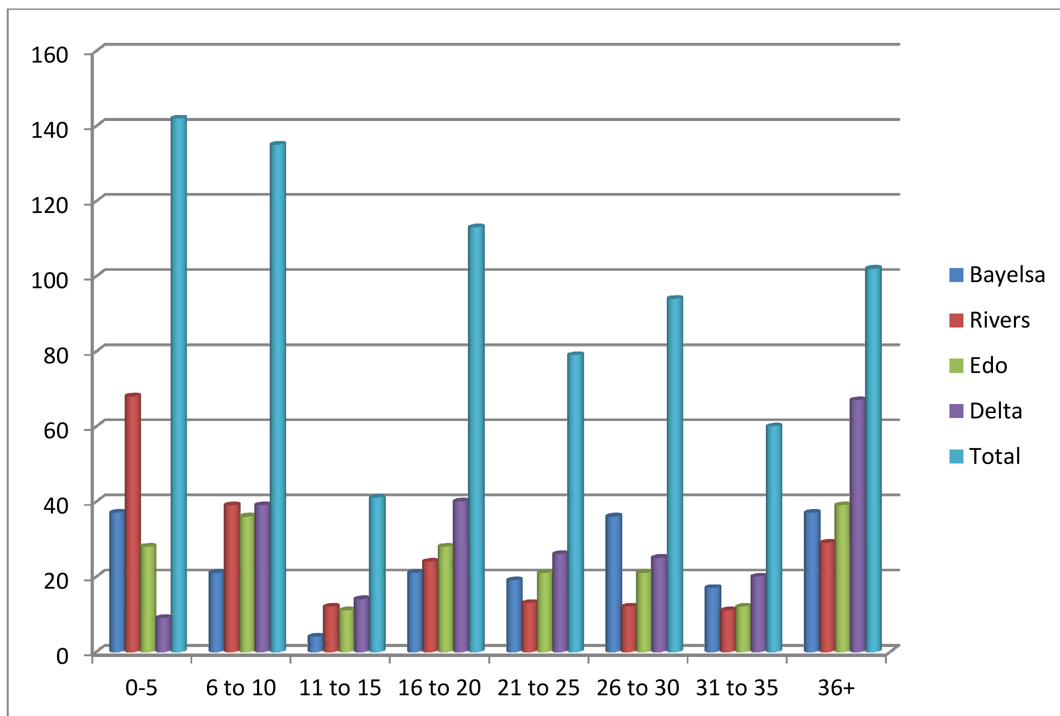

Fig. 1. Distribution of malaria infection according to states

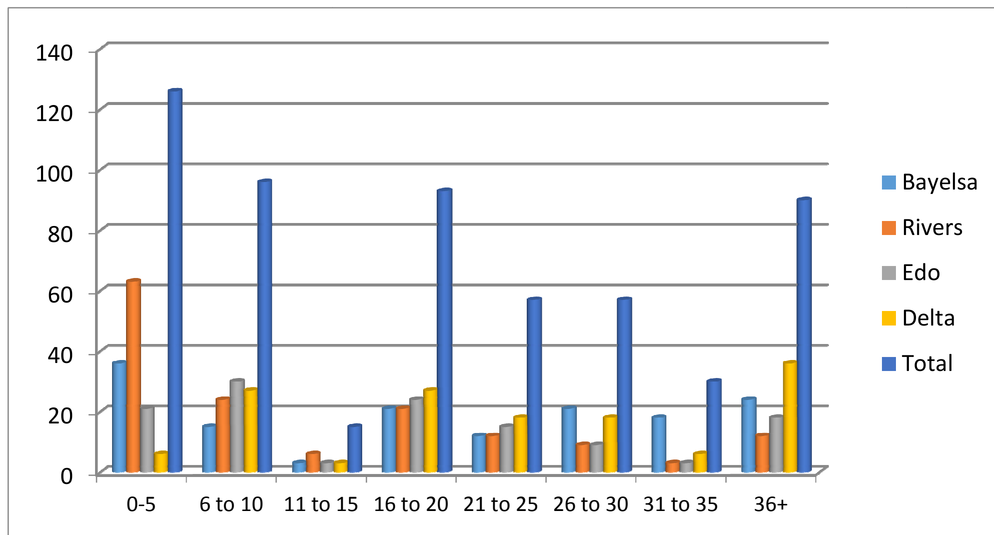

Fig. 2. Distribution of malaria infection according to age

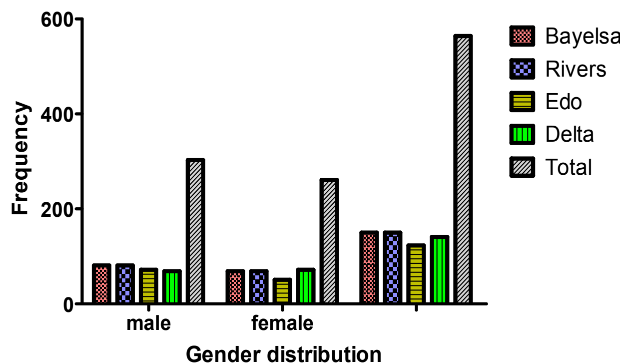

Fig. 3. Distribution of malaria infected subjects according to gender

4. DISCUSSION

In South- south areas with high transmission of malaria, children under 5years are particularly susceptible to infection, illness and deaths (70%) of all malaria occurs in this group. (Between 2010 and 2015; malaria death rate fell by 29% globally, but it still remains a major killer of children under 5 years old [4]. Malaria is holoendemic in South-South States, transmission occurs throughout the year, the long rainy season produces intense transmissions (March – August). Older children were also at risk of having malaria which is similar to studies carried out by [6-8].

Also from this study, males in (Rivers, Bayelsa and Edo) had higher prevalence of malaria infection than females; this is in agreement with Mahende et al. 2016 in a study carried out in Tanzania that males had (52.9%) while females had 47.1% of malaria infection. In Delta females had the highest prevalence of malaria infection which also agrees with reports by that women and children are more vulnerable to malaria infection due to women's high work burden in areas where mosquitoes are prevalent, men are less likely to view themselves as at risk of infection less likely to seek timely treatment when they are ill and more to likely to practice self-medication to save time and resources.

This study evaluated the performance of different diagnostic tools in detecting malaria parasite infection in South-South geo-political zone of Nigeria. PCR performed better than blood-slide expert microscopy and HRP-RDT in this present study. Also, Polymerase chain reaction (PCR) is regarded as one of few most sensitive molecular techniques for detecting parasites at limits of 0.01–0.2 parasites/μL of blood.

Table 1. Age distribution of Pfcrt gene across south-south states (four Niger Delta states)

Age	Bayelsa			Rivers			Edo			Delta		
	WT (%)	MT (%)	Total	WT (%)	MT (%)	Total	WT (%)	MT (%)	Total	WT (%)	MT (%)	Total
<5	33(22.0)	3(2.0)	36	63(42.0	0(0.0)	63	15(12.2)	6(4.9)	21	3(2.1)	3(2.1)	6
5-15	15(10.0)	3(2.0)	18	27(18.0)	3(2.0)	30	30(24.3)	3(2.4)	33	24(17.0)	6(4.3)	30
16-25	27(18.0	6(4.0)	33	30(20.0)	3(2.0)	33	36(29.3)	3(2.4)	39	42(29.8)	3(2.1)	45
26-35	33(22.0)	6(4.0)	39	12(8.0)	0(0.0)	12	12(9.8)	0(0.0)	12	21(14.9)	3(2.1)	24
36	24(16.0)	0(0.0)	24	12(8.0)	0(0.0)	12	12(9.8)	6(4.9)	18	36(25.5)	0(0.0)	36
Total	132(88.0)	18(12.0)	150	144(96.0)	6(4.0)	150	105(85.4)	18(14.6)	123	126(89.4)	15(10.6)	141

*X^2 = 30.63, df=5, p value 0.0099(s) ** X^2=16.24, df=5 p value <0.0062** X^2= 19.58, df=5, pvalue 0.0015(s) ** X^2= 17.66, df=5, pvalue 0.0034***

Table 2. Gender distribution of Pfcrt gene across south-south states (four niger delta states)

Gender	Bayelsa			Edo					Delta		
	WT (%)	MT (%)	Total	WT (%)	Total	WT (%)	MT (%)	Total	WT (%)	MT (%)	Total
Male	69(46.0)	12(8.0)	81	78(52.0	81	63(51.2)	9(7.3)	72	60(42.6	9(6.4)	69
Female	63(42.0)	6(4.0)	69	66(44.0)	69	42(34.2)	9(7.3)	51	66(46.8)	6(4.3)	72
Total	132(88.0)	18(12.0)	150	144(96.0)	150	105(85.4)	18(14.6)	123	126(89.4)	15(10.6)	141

The female subjects in Bayelsa and Rivers had the highest rate of both the wild (WT) and mutant (MT) types genes with the lowest seen in Delta. Although, the male had the highest prevalence of malaria infection

Table 3. Age distribution of Pfmdr gene across south-south states (four Niger Delta states)

Age	Bayelsa			Rivers			Edo			Delta		
	WT (%)	MT (%)	Total	WT (%)	MT (%)	Total	WT (%)	MT (%)	Total	WT (%)	MT (%)	Total
<5	27(25.7)	3(2.9)	30	51(34.0)	12(8.0)	63	15(12.2)	6(4.9)	21	6(5.6)	0(0.0)	6
5-15	9(8.6)	3(2.9)	12	24(16.0)	6(4.0)	30	21(24.1)	12(9.7)	33	21(22.2)	9(8.3)	30
16-25	12(11.4)	9(8.6)	21	27(18.0)	6(4.0)	33	27(31.0)	12(9.7)	39	27(25.0)	6(5.6)	33
26-35	21(20.0)	9(8.6)	30	9(6.0)	3(2.0)	12	9(10.3)	3(2.4)	12	9(8.3)	6(5.6)	15
36	6(5.7)	6(5.7)	12	6(4.0)	6(4.0)	12	15(17.2)	3(2.4)	18	18(16.7)	6(5.6)	24
TOTAL	75(71.4)	30(28.6)	105	117(78.0)	33(22.0)	150	87(70.7)	36(29.3)	123	81(75.0)	27(25.0)	108

Children between the ages of 0-5 in Rivers had the highest rate of both the wild (WT) and mutant (MT) types genes with the lowest seen in Bayelsa
*X^2= 100.1, df=15, pvalue <0.0001***, X^2= 113.8, df=15, pvalue <0.0001*** X^2= 186.2, df= 15, pvalue <0.0001***, X^2= 203.2, df=15, pvalue <0.0001****

Table 4. Gender distribution of Pfmdr gene in four niger delta states

Gender	Bayelsa			Rivers			Edo			Delta		
	WT (%)	MT (%)	Total	WT (%)	MT (%)	Total	WT (%)	MT (%)	Total	WT (%)	MT (%)	Total
Male	45(42.9)	18(17.1)	63	57(38.0)	24(16.0)	81	57(46.3)	15(12.2)	72	48(44.4)	18(16.7)	66
Female	30(28.6)	12(11.5)	42	60(40.0)	9(6.0)	69	30(24.4)	21(17.1)	51	33(30.6)	9(8.3)	42
Total	75(71.4)	30(28.6)	105	117(78.0)	33(22.0)	150	87(70.7)	36(29.3)	123	81(75.0)	27(25.0)	108

The PFMDR gene was highest among the subjects in Rivers with the lowest occurrence in Bayelsa

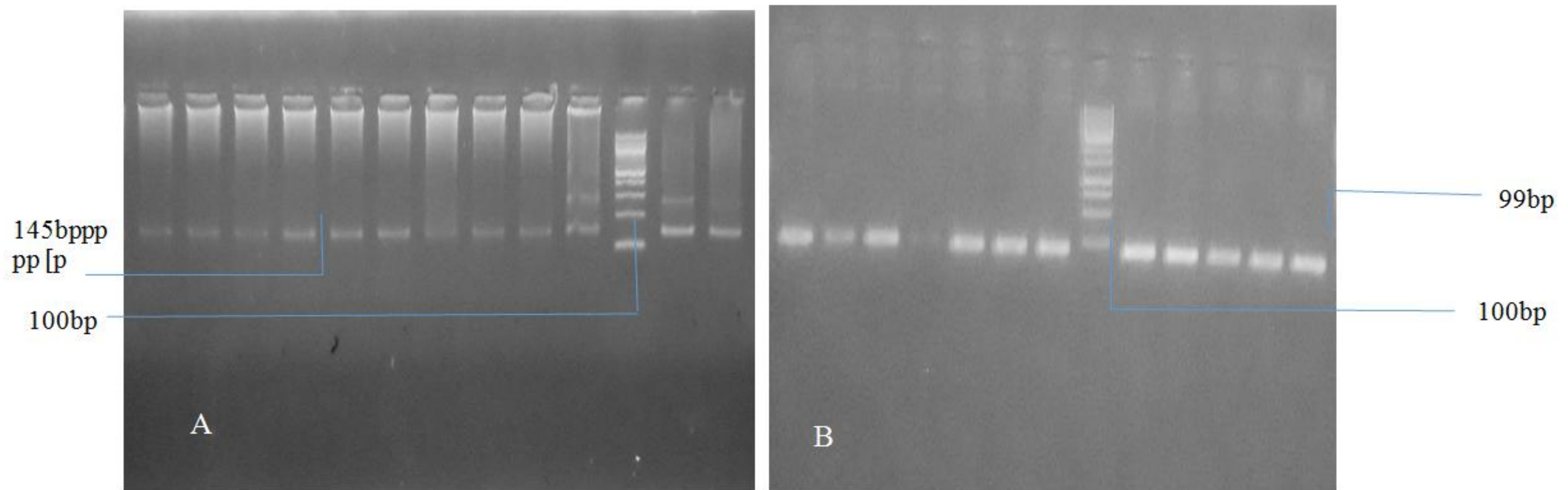

Plate 1. A: Undigested PCR product of Pfcrt gene (145bp) B: Digested product of Pfcrt gene by APO1 (99 and 46bp)

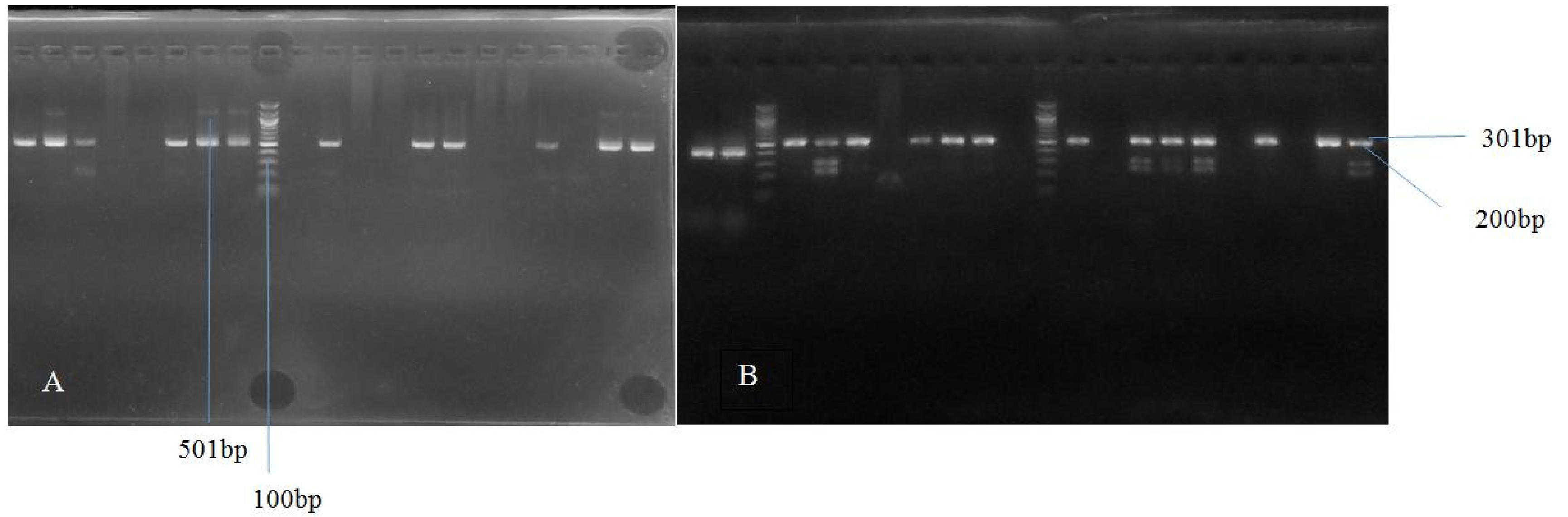

Plate 2 A. Undigested PCR product of Pfmdr gene (501bp) B: Digested product of Pfmdr gene by Afl111 (200 and 301bp)

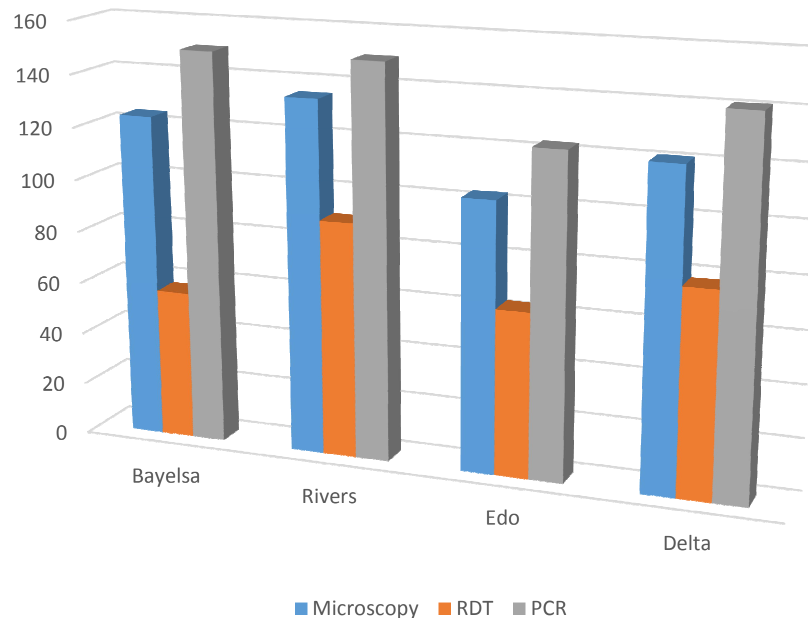

Fig. 4. Distribution of subjects based on methods of identification (microscopy, RDT and PCR)

Chloroquine kills the parasite by accumulating in the digestive vacuole and inhibiting the heme detoxification pathway CQ resistance occurs due to the reduced accumulation of the drug in the digestive vacuole of the parasite. There are two molecular markers available for determining the CQ resistance: *P. Falciparum* chloro quine resistance transporter (*pfcrt*) and*. Falciparum* multidrug resistance (*pfmdr1*). The *pfcrt* gene localized on chromosome 7 has 13 exons and ten putative transmembrane domains spanning the digestive vacuole of the parasite [9]. *Pfmdr1* gene located on chromosome 5 encodes for P-glycoprotein homologue 1 (Pgh1) protein present in the digestive vacuole of the parasite. Mutation in the PfCRT protein at K76T is the primary determinant for CQ resistance, whereas N86Y mutation in PfMDR1 protein may enhance the degree of resistance, Mutation in other regions of these PfCRT and PfMDR1 protein also confers resistance to CQ, but exclusively in the presence of K76T mutation [10]. Considering the potential ramifications of the establishment of endemicity of drug-resistant malaria parasites, several surveys have assessed the presence of CQR parasites. These studies have predominantly relied upon detection of mutations in the *P. falciparum* Chloroquine resistance transporter (*pfcrt*) gene as a proxy for possible drug resistance. Although the presence of a single point mutation does not prove clinical resistance, the substitution at position 76 from lysine (K76) to threonine (T76) is a useful surrogate marker. Conventionally, the mutation has been detected by mutation-specific restriction–endonuclease digestion of the *pfcrt*-nested PCR fragments with *Apo*I; the gene sequence containing the T76 threonine substitution associated with chloroquine resistance is resistant to digestion and that containing the wild-type lysine (K76) associated with drug sensitivity reveals 99-bp and 46-bp fragments on agarose gel electrophoresis because DNA sequencing has become more widely available and routinely practical, direct sequencing of the nested *pfcrt* PCR product for the presence of the mutation has been used.

Gender distribution of Pfcrt across the four Niger Delta states revealed that there is no statistical difference between male and female (P.> 0.5) this is in agreement with the previous studies by WHO declared that Pfcrt mutation happened irrespective of sex.

The lower distribution of Pfmdr 1 86Y across the states (28.6%, 22.0%, 29.3% and 25.0%) for Bayelsa, Rivers, Edo and Delta respectively suggests recovery of the wild type allele which is in agreement with the study conducted by (Dual et al. 2007) where the prevalence of wild type strains increased from 77% to 86%. Overall

prevalence of Y86 PFMDR was 26.2% which disagrees with a study carried out in Ghana in 2010 where the prevalence of Y86 was 86% on the mutant type and the wild 28% (Snonnou et al., 1999) also agrees with the study carried out in India by Goswami et al., 2014 who had 41.79% also in Gabon 2009, the prevalence of Y86 increased to 31% [1]. The PFMDR1 gene expression levels have been considered in the etiology of the parasite resistance to some anti-malarial drugs and it is being explored in epidemic logical studies increase in PFMDR gene copy number has been linked to *P. falciparum* diminished susceptibility to anti-malaria drugs, such as mefloquine and AL (Artemether Lumenfantrine) [11]. The apparent disappearance of the chloroquine-resistant parasites with PFCRT T76 and PFMDR 86 from most of the sub-Saharan African endemic areas will help in the re-introduction of the cheapest antimalarial in combination with an artemisinin derivative. Also this work is in agreement with the study carried out by [12-14] where they found higher prevalence of wild type 86% but there was a decrease in T76 in Ghana (2010) which is an indicator that chloroquine is still effective even after it has been withdrawn. Also a study in Nigeria by (Happi et al., 2006) shows a relationship with PFCRT T76 and PFMDR Y86 allele associated with AQ resistance. In this study there was no relationship with T76 and Y86 because there was a lower prevalence of 12.8% of T76 and 26.2% of Y86.

In this study there was no association of N86T mutation of the PFMDR gene with CQ resistance and this non-association was in agreement with previous findings by [15-17] PFMDR gene mutation has poor correlation with CQ resistance [18]. When PFMDR gene product was digested with AFL 111enzyme, it cleaved into two fragments (300 and 201bp) these isolates were considered as CQ resistant with mutant Y allele at 86th position.

The lower distribution of *pfmdr1* drug resistance alleles 86Y across the states, 33.0% in Katsina, 32.56% in Jigawa, 31.1% in Kebbi, 18.9% in Kaduna and 11.32% in Kano suggests recovery of the wild type alleles as the same scenario was observed for Pfmdr 1 N86Y, where there was a decline in the prevalence of the mutant from 46% to 28% from 2005 to 2010 and an increase in the prevalence of the wild strains N86 from 77% to 86% in Ghana.

Similar observations were made in other studies in Africa where K76T were investigated concurrently. This is also an indication of a gradual gain of stability of these genotypes in the population similar to what was observed by Nzila's group in Kenya.

5. CONCLUSION

Prevalences of Pfcrt and Pfmdr 1 remained elevated in the Niger Delta states despite the withdrawal of chloroquine over a decade ago. Hence, Nigeria is far from an eventual re-introduction of chloroquine as its resistance markers still persist in our communities. Furthermore, the root cause of the persistence of these resistance markers needs to be investigated.

CONSENT

It is not applicable.

ETHICAL APPROVAL

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

ACKNOWLEDGEMENT

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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