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Original Research Article

Plasmodium falciparum merozoite surface protein-1 genotypes among the children in Akure, Ondo State

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ABSTRACT

Background: Five species of *Plasmodium* known to cause human malaria are *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*, they contribute to the morbidity and mortality in malaria endemic regions of the world. *P. falciparum* infection is however the commonest in Nigeria and the strategy to control and eventually eliminate malaria requires an understanding of the genetic composition of *P. falciparum* isolates from symptomatic and asymptomatic individuals infected with the parasite.
Methods: Blood samples from 500 children under 12 years of age were screened for malaria parasite by microscopy method and confirmed by molecular methods. The extracted *P. falciparum* DNA were then genotyped, determined by nested polymerase chain reaction (PCR) using gene-specific forward and reverse primers. These were loaded unto 2% agarose gel for electrophoresis and fragment analyses.
Results: There were 321/500 (64.2%) children who tested positive for malaria parasite. Of all the positive specimens, 99.4% were identified as *P. falciparum* species and 0.6% were identified as *P. ovale*. The genotypes of the PfMSP1 identified in the study population were MAD 20, RO33 and K1. MAD 20 was the predominant allele with 84.11% of total identifiable alleles, RO33 represented 10.9% and K1 was 4.99% of the total alleles.
Conclusions: Three genotypes were identified in the present study population. MAD20 was the predominant allele. This can help to plan control strategy for the malaria infection in the study area.

Keywords: Akure, Children, Genotypes, Ondo State, PflMSP-1

INTRODUCTION

Malaria caused by *Plasmodium falciparum* in the tropics, remains an important global public health concern. Globally, it caused approximately 263 million cases in year 2023, taking its greatest toll on pregnant women and children killing over 597,000 people in 83 countries of the world.[1] An estimated 246 million cases (94% of all malaria cases) and 569,000 deaths were reported in the African sub-region with children under the age of 5 years alone accounting for about 80% of these malaria deaths in the region.[1] The scourge of malaria can only be reduced by the control, elimination and total eradication of the infection and these processes need an in-depth knowledge of the disease pathogenesis and vaccinology.

Plasmodium falciparum merozoite surface proteins (*Pf*MSP1 and *Pf*MSP2) are key targets for malaria research and vaccine development due to their high genetic diversity and role in parasite invasion of red blood cells that lead to the disease entity. Genotyping these proteins, particularly MSP1 and MSP2, helps characterize parasite populations and understand the genetic diversity within a population. The merozoite surface protein-1 (MSP-1) exhibits an extensive genetic polymorphism, with numerous alleles found in field isolates.[2] It is the most abundant of all GPI-anchored surface proteins in terms of copy number.[3] MSP1 is expressed as a high molecular weight protein (MW 190 kDa) that undergoes extensive proteolytic processing prior to egress of the merozoite from the schizont.[4] MSP-1 is encoded by the *msp-1* gene located on chromosome 9 which contains 17 blocks of sequences flanked by conserved regions, block 2, which is the most polymorphic part of MSP-1 and these are grouped into several allelic families.[5] The most widely used techniques for genotyping malaria infections are based on amplification by polymerase chain reaction of the polymorphic genes encoding the merozoite surface proteins.

The study aimed to determine the genetic composition of the *PfMSP-1* among the isolates of *P. falciparum* from symptomatic individuals in the study population. In this study, therefore, *Pf*MSP-1 genotypes were analyzed from patients who attended public health facilities in Akure, Ondo State, Nigeria.

METHODS

Study area

The study was carried out in Akure, Ondo State using eleven health facilities where children access health care apart from the routine immunization services. The hospitals included in the study were: comprehensive health centre Arakale, comprehensive health centre Oke-Ijebu/ Ijomu/Oba Nla, comprehensive health centre Isolo, basic health centre Adegbola, basic health centre Oke-Aro, basic health centre Orita-Obele, basic health centre Sagari, Esure primary health centre, Federal Medical Centre Akure Annex, Mother and Child Hospital, Oke-Aro and the University of Medical Sciences Teaching Hospital, Akure Complex (UNIMEDTH).

Ethical clearance

Ethical approval was obtained from the research and ethics committee of the Ondo State Ministry of Health with protocol number OSHREC 10/01/23/500. Also, informed consent was obtained from the parents of study subjects after the advantages of the research had been explained to them.

Sample size

Sample size was determined using the online tool called the Raosoft sample size calculator software (Raosoft, 2024).[6] Based on the year 2022 estimated population size of Akure of 774,000 and with a margin error of 5%, 95% confidence level, and at least 50% response distribution, the calculated sample size was 384, but the sample size was rounded up to 500 to allow for any form of non-response. Therefore, forty-five consecutive children were recruited from ten centres and fifty children were recruited from UNIMEDTH being a reference hospital.

Recruitment of participants

The participants were 500 children aged 1 day to 12 years (Figure 1). The study was a cross-sectional survey and hospital-based. Recruitment was from various points of entry into the hospitals vis-à-vis emergency room, newborn unit, children's ward and the out-patient department (OPD) from April 1 to September, 30th 2023.

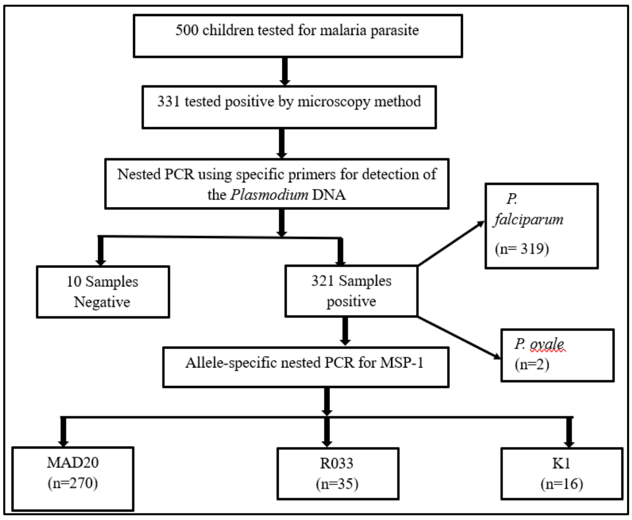

Figure 1: Algorithm for the methodology.
PCR = Polymerase chain reaction, n = number

Malaria parasite screening

Blood samples were collected from the children by venipuncture and kept in the ethylene di-amine tetra acetic acid (EDTA) bottles to prevent clotting. Thick and thin blood films were prepared and used for the screening of the blood samples for malaria parasites by microscopy method. Thick blood film was used to detect the presence of malaria parasites while thin blood films were used to identify the *Plasmodium* species. The films were made on a clean grease-free glass slide and stained with Giemsa stain for 15 minutes.[7] Thin film was fixed with methanol but the thick film was not fixed. The slides were allowed to dry after which oil immersion were added, and then viewed under the light microscope at x100 objective lens for the characteristics features of malaria parasite.[8]

Blood sample collection for molecular analysis

After microscopy screening of the blood samples, all positive blood samples for malaria parasite were identified and isolated, these were subsequently used for the nested polymerase chain reaction (PCR) test. For each blood sample, two to three drops of blood were spotted on the 3 mm Whatman filter paper with the use of a pasture pipette and the blood was allowed to dry properly under room temperature (28±2°C) and kept between cardboard paper on which proper labelling was done and kept in a container with desiccants to prevent air reaction.

Molecular analysis

Malaria Parasite Deoxyribonucleic Acid (DNA) Extraction from Dried Blood Spots (DBS) using ZymoBIOMICS™ DNA Extraction Miniprep kit

DNA was extracted from blood samples that were placed on the dry blood spot (DBS). DNA extraction for nested PCR assays was done using the ZymoBIOMICS™ DNA extraction miniprep kit according to the manufacturer's guide.[9] Two pieces of 3 mm disk from the Whatman filter paper dry blood spots were punched out using a sterile hole punch and dropped into appropriately labelled 1.5 mm micro-centrifuge tube. The punch was cleaned and sterilized each time in sequence with 5% bleach (sodium hypochlorite) followed by distilled water and then 70% ethanol. To lyse the sample, 4:1 volume of genomic lysis buffer was added and the tissue was homogenized, everything amounting to 200 µl of tissue and lysis buffer solution. The solution was incubated at 85°C for 10 minutes followed by addition of 20 µl of proteinase K stock solution.

The mixture was vortexed for 4-6 seconds and allowed to stand for 5-10 minutes at room temperature. The mixture was transferred to a zymo-spin column in a collection tube and centrifuged for one minute. The collection tube was discarded with the flow through. The zymo-spin was transferred to new collection tube, 200 µl of DNA pre-wash buffer was added to the spin column and centrifuged for one minute.

Five hundred (500 µl) of g-DNA wash buffer was added to the spin column and centrifuged for one minute before transferring the column to a clean microcentrifuge tube. Approximately 50 µl DNA elution buffer was added to the spin column. It was incubated for 2-5 minutes at room temperature; this was then centrifuged at top speed for 30 seconds to elute the DNA. The eluted DNA was examined for purity in nano drop to ensure its purity before storing in refrigerator at -20°C for further molecular analysis.

Parasite DNA yield and quality determination

The quality and quantity of extracted DNA were determined using the spectrophotometric method with NANODROP 1000^{R} (Thermo Fisher Scientific, USA), which quantified the amount of extracted DNA in nanogramme per microlitre (ng/µl) and assessed the quality (purity) based on the ratio of absorbance at 260 nm:280 nm for all the samples.[10]

Plasmodium falciparum MSP1 genotyping

In the process of genotyping for the MSP1 gene in extracted DNA from the blood samples, 2 µl of the primary amplified products in final volume of 15 µl were used (Table 1). The genotyping was determined by nested polymerase chain reaction (PCR) using gene-specific forward and reverse primers having similar annealing temperature of 48°C (Table 2).

Nested PCRs were performed to amplify the polymorphic sequence block 2 of *P. falciparum MSP-1* as previously described.[10] Amplifications were performed in a final volume of 15 µl and cloned or monomorphic parasite lines were used as positive template controls. The first round PCR amplification was performed using two microliters of DNA and the primers were designed on the conserved regions in sequence blocks 1 and 3 on either side of block 2. The second round nested PCR amplification was also carried out using 2 µl of the primary amplified products. The amplifications were performed in a DNA Engine Tetrad PTC-225 thermal cycler (MJ Research, USA), the products were then loaded onto a 2% agarose gel (Peqlab Erlagen, Germany), electrophoresed and stained with ethidium bromide. The DNA was visualized under a gel documentation system and the fragment size polymorphism for each allelic family determined. It was assumed that each PCR fragment represented at least one parasite genotype. The methods and PCR primers used were adopted from previously published study.[11]

Table 1: Composition for the primary and secondary PCR reaction (*Pf*MSP1).

Components	Stock conc.	Final conc.	Volume/reaction
PCR water	-	-	8.8µl
Buffer	10x	1x	1.5µl
MgCl	25 mM	1.5 mM	0.9µl
dNTPs	25 mM	0.2 mM	0.48 µl
Primer (forward)	10 µm	0.4 µm	0.6 µl
Primer (Reverse)	10 µm	0.4 µm	0.6 µl
Taq Polymerase	5 U/µl	0.04U	0.12 µl
DNA sample	160 ng/µl	160 ng/µl	2 µl
Total volume	-	-	15 µl

conc: concentration, mM: millimole, µl: micro-litre, ng: nanogram, PCR: polymerase chain reaction, MgCl: Magnesium chloride, dNTPs: DeoxyNucleotide TriphosphateS, DNA: Deoxyribonucleic nucleic acid.

Table 2: PCR amplification of *Plasmodium falciparum* merozoite surface protein (PfMSP-1) primer sequences.

Genes	Primer	Sequence (5_–3_)	PCR conditions	Size (base pair, bp)	References
Primary					
MSP-1	Forward	CTTAACCTGCTAATT AGCGAT	Initial denaturation: 94°C for 03:00 minutes, denaturation: 94°C for 30 s, annealing: 48°C for 45 seconds, extension: 65°C for 1 minute, final extension: 65°C for 5 minutes	675	Grabias et al, 2019[11]
	Reverse	CCTCGTTCAAGATTA ATAATT			
Secondary					
MSP-1	Forward	AAGAAAACGAATTA TTTGGG	Initial denaturation: 94°C for 03:00 minutes, denaturation: 94°C for 30 s, annealing: 48°C for 45seconds, extension: 65°C for 1 minute, final extension: 65°C for 5 minutes	675	Grabias et al, 2019[11]
	Reverse	AGAAACATCAGTAT TCAACG			

The primer sequence adopted

The amplification reaction of the *MSP1* involved the use of the primer pair for the forward and the reverse reaction was 5’-CTTAACCTGCTAATTAGCGAT-3’, and 5’-CCTCGTTCAAGATTAATAATT -3’ and 5’-AAGAAAACGAATTATTTGGG -3’, and 5’-AGAAACATCAGTATTCAACG -3’ respectively for a 675 base pair product (Table 2). The PCR reactions were also carried out in a final volume of 15 µl. The PCR reactions were carried out using a DNA Engine Tetrad PTC-225 thermal cycler (MJ Research, USA) with cycling parameters of an initial denaturation at 94°C for 3 minutes followed by 25 cycles of 92°C for 30 seconds, annealing at 48°C for 45 seconds, extension at 65°C for 1 minute and a final cycle of extension at 65°C for 5 minutes.[11]

RESULTS

Prevalence of malaria and Plasmodium species identified among the children

Table 3 showed the overall prevalence of malaria infection in the children and the Plasmodium species identified. There were 321 (64.2%) children who tested positive for malaria parasite. Of all the positive specimens, 99.4% were identified as *P. falciparum* species and 0.6% were identified as *P. ovale*.

Table 3: Overall prevalence of malaria and *Plasmodium* species identified among the children.

Variables	Total number examined (N=500)	Prevalence (%)
Malaria parasite		
Positive	321	64.2
Negative	179	35.8
Malaria species		
Plasmodium falciparum	319	99.4
Plasmodium ovale	2	0.6

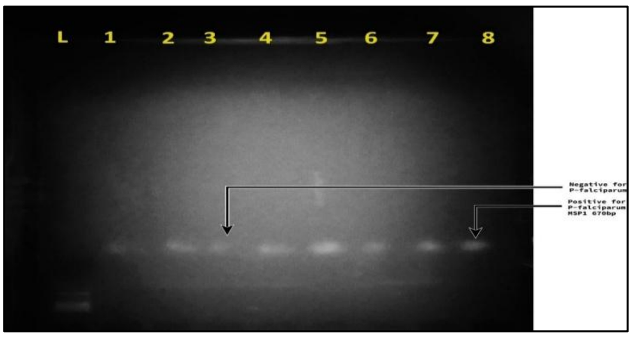

Figure 2: Plate 1 showing electrophoregram of *Pf*MSP1 resolved on 2% agarose gel to confirm the presence of *P. falciparum*.

Lanes 1 and 3 are negative for *P. falciparum*; lanes 2, 4, 5, 6, 7 and 8 are positive for *P. falciparum.*

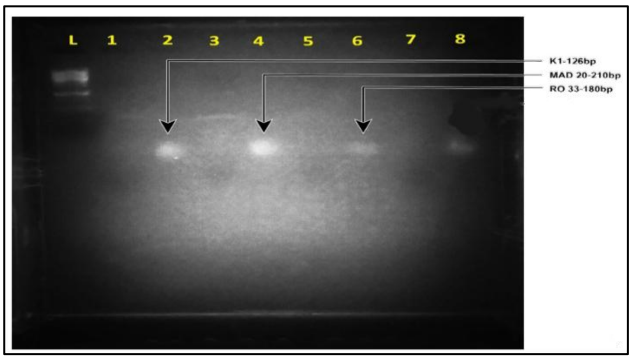

Figure 3: Plate 2 showing electrophoregram of *Pf*MSP1 resolved on 2% agarose gel showing the *Pf*MSP-1 genotypes.

Lane 1 = 670kb bp DNA ladder; lanes 2, 4, 6 = *Pf*MSP-1 genotypes.

Confirmation of P. falciparum by molecular test

Plate 1 showed the electropherogram of PfMSP1 resolved on 2% agarose gel to confirm the presence of *P. falciparum* in the samples. Lanes 1 and 3 were negative for *P. falciparum* while lanes 2, 4, 5, 6, 7 and 8 were positive for *P. falciparum*.

Genotyping of the MSP-1

Plate 2 showed the electropherogram of PfMSP1 resolved on 2% Agarose gel showing the PfMSP1 genotypes. Lane 1 (670 kb) was the DNA ladder while lanes 2, 4, and 6 showed the alleles of PfMSP1 genotypes in the samples.

Table 4: Genotyping PfMSP1 alleles.

Name of allele	MAD 20	RO 33	K1
Number positive	270	35	16
Number negative	51	286	305
Total	321	321	321
Percentage positive	84.11	10.9	4.99

Table 4 showed the genotypes of the PfMSP1 present in the study population. Three genotypes were identifiable in the study population. These were MAD 20, RO33 and K1. MAD 20 was the predominant allele with 84.11% of total identifiable alleles, RO33 represented 10.9% of the total allelic family and K1 was 4.99% of total.

DISCUSSION

The Plasmodium species identified among the children

Plasmodium falciparum constituted 99.4% of the malaria infection in the current study, similar to earlier reports from Akure which was 99.2%, it is also comparable to report of 99.7% from Kaduna and 96.0% from Cameroon.[12-14] The 99.4% *P. falciparum* infection reported in this study is however, slightly lower than report from Jos, Ilorin, Kwara State and Nnewi, Anambra where infecting species was 100% *P. falciparum*.[15-17] The finding in this study is higher than 51.8% *P. falciparum* reported in Awka, higher than the 62.5% reported from Abuja and 65.5% for *P. falciparum* in India.[18-20] Five species of *Plasmodium* known to cause human malaria are *P. falciparum, P, vivax, P. ovale, P. malariae and P. knowlesi,* they contribute to the morbidity and mortality in malaria endemic regions of the world. However, *P. falciparum* is the predominant species of malaria parasite responsible for more than 98% percent of all malaria infections in Nigeria, up to 15% are caused by *P. malariae* and less than 5% are caused by *P. ovale* infections.[21,22] Mixed infections with *P. falciparum* are also common, while our study reported *P. ovale* as the infecting species in 0.6% of the study population, the report from Awka did not find any *P. vivax* or *P. ovale* in their study.[18]

Genotype of the PfMSP-1 in the study population

Three genotypes were identified in the present study population. These were MAD20, RO33 and K1. MAD20 was the predominant allele with 84.11% of total identifiable alleles, RO33 represented 10.9% of the total allelic family and K1 was 4.99% of total. This result differs from earlier report from Igbo-Ora, southwest, Nigeria where RO33 was the predominant genotype which was 57% of their total sample, while K1 and MAD20 were 26% and 17% respectively.[23] The *Plasmodium falciparum* merozoite surface protein 1 (*PfMSP*-1) gene is highly polymorphic, with its block 2 region being the most variable. This region is classified into three main allelic types: MAD20, RO33 and K1. Some studies have indicated that K1 family is the predominant allelic type of the msp-1 gene, often carried alone or in association with RO33 and MAD20 types.[23,24] The report in this study is also similar to reports from Niger State, North-Central Nigeria, where it was reported that msp1 was the most common allele among symptomatic patients, with predominance of MAD20 allele.[25] Other findings in the North-West and North-Central regions contradicted this because their study also incorporated MSP-2 and glutamate-rich protein (GLURP) genes and so they reported more alleles.[26,27] Furthermore, earlier study in Kosofe and Ikorodu local government of Lagos State, Nigeria and Senegal reported K1 as the most predominant allele and according to the report, K1 was 48% while RO33 was 15% in Lagos and K1 allele was 44% while RO33 was 6% in Senegal.[28] It is pertinent to genotype the MSP alleles because this has implication on malaria control programme. Having multiplicity of infection (MOI) and diverse *falciparum* strains could pose problems in employing the conventional control methods. For example, in Nigeria, the detection of low-moderate level of MOI in Nigeria shows that though transmission rate of malaria is high, but same clones of parasites are circulating in the area. Hence, similar control strategy can be planned and implemented in the areas. When the alleles are multiple, control interventions for malaria parasite will continue to face multiples hurdles ranging from development of resistance to drugs, insecticide resistance by the vectors, and difficulty in getting reliable vaccine to confer the necessary protection against the malaria parasite.

CONCLUSION

The three genotypes of the PfMSP-1 identified in this study were MAD20, K1 and RO33. This genetic composition of *P. falciparum* in malaria cases in this study population can therefore form a baseline information on the strategy to control and eventually eliminate malaria in the region.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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